Viral Gene Techniques

Edited by

Kenneth W. Adolph

Methods in Molecular Genetics

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Volume 7

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Volume 7

Viral Gene Techniques

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Front cover photograph: Primary human astroglioma cells infected with LacZ AAV at approximately one virus per cell. The blue nucleus within certain cells indicates infection and expression of the recombinant AAV virus. Courtesy of A. W. Skulimowski and R. J. Samulski, The Gene Therapy Center, The University of North Carolina at Chapel Hill.

This book is printed on acid-free paper. ♾

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Preface

The new series *Methods in Molecular Genetics* provides practical experimental procedures for use in the laboratory. Because the introduction of molecular genetic techniques and related methodology has revolutionized biological research, a wide range of methods are covered. The power and applicability of these techniques have led to detailed molecular answers to important biological questions and have changed the emphasis of biological research, including medical research, from the isolation and characterization of cellular material to studies of genes and their protein products.

Molecular genetics and related fields are concerned with genes: DNA sequences of genes, regulation of gene expression, and the proteins encoded by genes. The consequences of gene activity at the cellular and developmental levels are also investigated. In medical research, knowledge of the causes of human disease is reaching an increasingly sophisticated level now that disease genes and their products can be studied. The techniques of molecular genetics are also being widely applied to other biological systems, including viruses, bacteria, and plants, and the utilization of gene cloning methodology for the commercial production of proteins for medicine and industry is the foundation of biotechnology. The revolution in biology that began with the introduction of DNA sequencing and cloning techniques will continue as new procedures of increasing usefulness and convenience are developed.

In addition to the basic DNA methods, instrumentation and cell biology innovations are contributing to the advances in molecular genetics. Important examples include gel electrophoresis and DNA sequencing instrumentation, *in situ* hybridization, and transgenic animal technology. Such related methodology must be considered along with the DNA procedures.

The chapters of *Viral Gene Techniques,* are organized into four sections: Viral Vectors; DNA Viruses; Human Immunodeficiency Virus/Retroviruses; and RNA Viruses. Procedures are given for laboratory studies of the fundamental molecular processes of virus infection. In addition, methods related to human gene therapy are presented. The gene therapy vectors that are described include adenovirus and adeno-associated virus. Among other DNA viruses discussed in the volume are herpes simplex virus and hepatitis virus. Methods for HIV research are described, as well as protocols for investigating RNA viruses such as poliovirus and influenza virus.

Methods in Molecular Genetics will be of value to researchers, as well as to students and technicians, in a number of biological disciplines because of the wide applicability of the procedures and the range of topics covered.

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Section I

Viral Vectors

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[1] Adeno-Associated Virus: Integrating Vectors for Human Gene Therapy

A. W. Skulimowski and R. J. Samulski

Introduction

The treatment of human disease by introducing genetic material into specific cells of the affected patient is considered the next major advance in medical technology. Gene therapy has enormous potential to treat both heritable and infectious diseases (reviewed in Ref. 1). Successful implementation of genetic transfer can occur only after substantial development of new technologies, including methods of delivery and persistence of novel gene expression. Viral research has revealed a number of viruses that can be modified to introduce novel genes efficiently into a variety of cell types, including adenovirus, retrovirus, vaccinia virus, herpesvirus, and adeno-associated virus (AAV) (reviewed in Refs. 2 and 3).

Most of these viral vectors express the introduced gene transiently, with the exception of retroviruses and AAVs, which have the ability to integrate into the cellular chromosome. Research in our laboratory at the University of North Carolina at Chapel Hill has centered around the study of the molecular biology of AAV in order to exploit the unique features of this virus to develop an efficient viral vector for human gene therapy. Some of the unique features that make this virus attractive for gene therapy include the facts that AAV is prevalent in humans, it has never been identified as a causative agent of human disease, and it is able to insert its genome locus-specifically into human chromosomes (reviewed in Ref. 4).

This article describes the generation of a recombinant AAV (rAAV) that expresses the *Escherichia coli* β-galactosidase (*lacZ*) gene and its use in identifying cell types permissive to AAV infection.

AAV Structure

AAVs are members of the family Parvoviridiae, in the genus *dependovirus,* appropriately named for their complete dependence on coinfection with a helper virus [adenovirus, vaccinia, herpes simplex virus, or human cytomegalovirus (CMV)] for productive infection (5–9). In the absence of helper virus, the wild-type (wt) AAV genome integrates efficiently in a locus-specific manner into the cellular genome and can exist as a provirus for many cellular generations until rescue with a helper virus (reviewed in Ref. 4).

AAVs are among the smallest DNA animal viruses. The single-stranded linear

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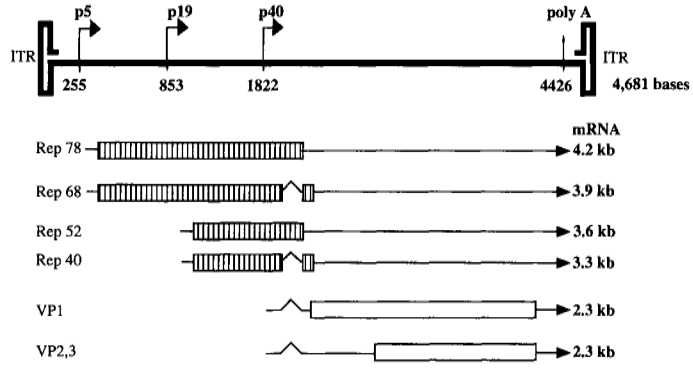

FIG. 1 Organization of the AAV genome. AAV ITRs flank the genome. Short arrows depict promoter positions. The left ORF (striped boxes) encodes the replication proteins Rep78, Rep68, Rep52, and Rep40. The right ORF (open boxes) encodes the capsid proteins VP1, VP2, and VP3.

AAVs are among the smallest DNA animal viruses. The single-stranded linear DNA genome, of 4.7 kb, is encapsidated in an icosahedral structure, approximately 20 nm in diameter (10). The organization of the genome is shown in Fig. 1. There are two open reading frames (ORFs). The ORF that extends over the left half of the genome codes for replication (Rep) proteins, and the right ORF codes for structural (Cap) proteins. There are three promoters, located at 5, 19, and 40 map units, respectively. At the ends of the genome, there are inverted terminal repeats (ITRs) of 145 bp (11,12). The terminal 125 bases are palindromic, but the overall palindrome is interrupted by two shorter palindromes in the center of the larger palindrome, forming a T-shaped structure after maximum base pairing. This hairpin structure is used as a primer for DNA replication. The ITRs do not appear to contain dominant promoter or enhancer activity. It has been shown that the ITRs are the only elements required *in cis* for integration, rescue, replication, and encapsidation of the AAV genome (13). Since the Rep and Cap proteins can be provided *in trans,* this has allowed the generation of viable rAAV vectors that can transduce a foreign gene into a target cell's chromosome in a stable manner.

Construction of rAAV Vectors

Cloning of the entire AAV2 genome into pBR plasmids that were infectious facilitated the initial manipulations of the genome in order to replace AAV functions with foreign genes and generate infectious rAAV vectors (14–17). In these studies Rep or Cap functions were provided by cotransfection with plasmids carrying the rep or cap genes. However, it became clear that homology between the vector and the helper

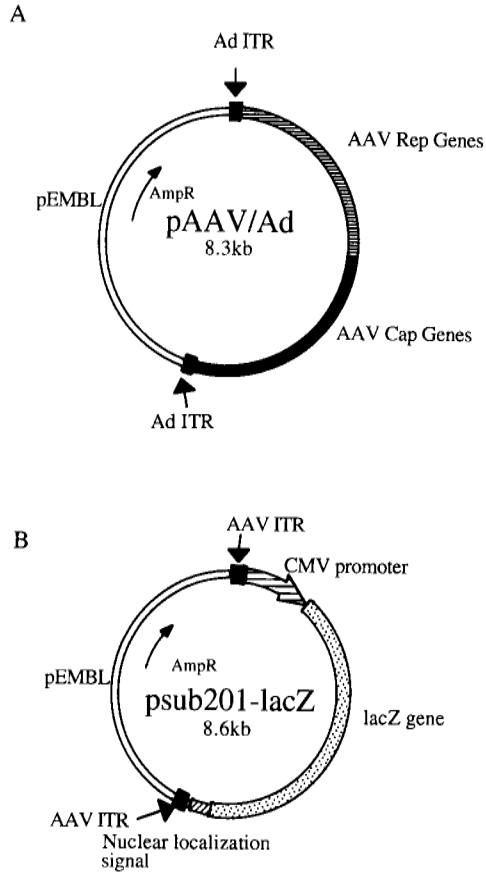

FIG. 2 Plasmid maps of (A) the helper plasmid, pAAV/Ad, and (B) the rAAV plasmid, psub201–lacZ.

plasmid resulted in recombination and the generation of wt AAV. To eliminate the production of wt AAV, a helper plasmid was constructed that did not share any sequences with the recombinant AAV plasmid. pAAV/Ad contains the entire AAV genome, except for 190 bp of each AAV ITR, which were replaced with 107 bp of the adenovirus terminal sequences in pEMBL as shown in Fig. 2A. When pAAV/Ad helper plasmid is cotransfected with an rAAV plasmid, which contains the gene of interest between the terminal 191-bp AAV ITRs, in Ad-infected cells high levels of Rep and Cap proteins are expressed and only the rAAV DNAs are excised, replicated, and packaged into AAV virions (13). The rAAV plasmid that is most commonly used as a vector backbone to insert nonviral genes into AAV particles in our laboratory is psub201 (18). This plasmid contains the AAV genome, in pEMBL8(+).

Two *Xba*I sites located at 19b and 4484 nucleotides flank the AAV ORFs. Digestion at these sites leaves a fragment containing only the plasmid sequences and the AAV terminal repeats. This vector makes it possible to replace almost the entire AAV genome with any promoter and gene of interest. Currently, inserts up to 4.7 kb will be efficiently packaged into rAAV virions. Following this strategy, the *lacZ* gene for β-galactosidase, controlled by the CMV promoter, was cloned into the rAAV plasmid, producing psub201–lacZ, as shown in Fig. 2B. Using *lacZ* as a reporter gene in AAV vectors has enabled the accurate titration of recombinant viral stocks as well as monitoring the permissibility of different cell types to AAV infection.

Generation of Recombinant *lacZ* AAV Virions

The production of recombinant *lacZ* AAV involves cotransfection of the AAV vector and helper plasmids into permissive cells, followed by adenovirus infection. A summary is shown in Fig. 3. We routinely use 293 cells, maintained with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 0.1 mg/ml streptomycin for the transfection of plasmids. The 293 cell line is a transformed human embryonic kidney cell line, which constitutively produces adenovirus E1A and E1B proteins (19) and efficiently expresses transfected DNA (20). During routine maintenance of 293 cells at 37° C, 5% CO_2, the cells are passaged before reaching confluence, and permissiveness to adenovirus is constantly monitored.

Transfection Method

For transfection, 293 cells are plated on 100-mm tissue culture dishes to obtain 80% confluence at the time of transfection. Recombinant virus titers are dependent on transfection efficiency, and are highest when using liposome agents. Typically, a 1 : 5 ratio of the recombinant plasmid, psub201–lacZ (5 μg), and helper plasmid, pAAV/Ad (28 μg), are cotransfected for each 100-mm dish of 293 cells, using *N*–[1–(2, 3–Dioleoyloxy)propyl]–N,N,N–trimethylammonium methyl sulfate (DOTAP) (Boehringer-Mannheim, Indianapolis, IN) as the transfection reagent according to the manufacturer's instructions.

1. Dilute 70 μl of DOTAP (1 mg/ml) to 250 μl with HEPES-buffered saline (HBS) (20 m*M* HEPES and 150 m*M* NaCl, pH 7.4) in a polystyrene tube. In a separate polystyrene tube dilute 5 μg of psub201–lacZ and 28 μg of pAAV/Ad to 250 μl with HBS.
2. Gently add the DNA/HBS mixture to the DOTAP/HBS solution, mix gently, and incubate for 10 min at room temperature.

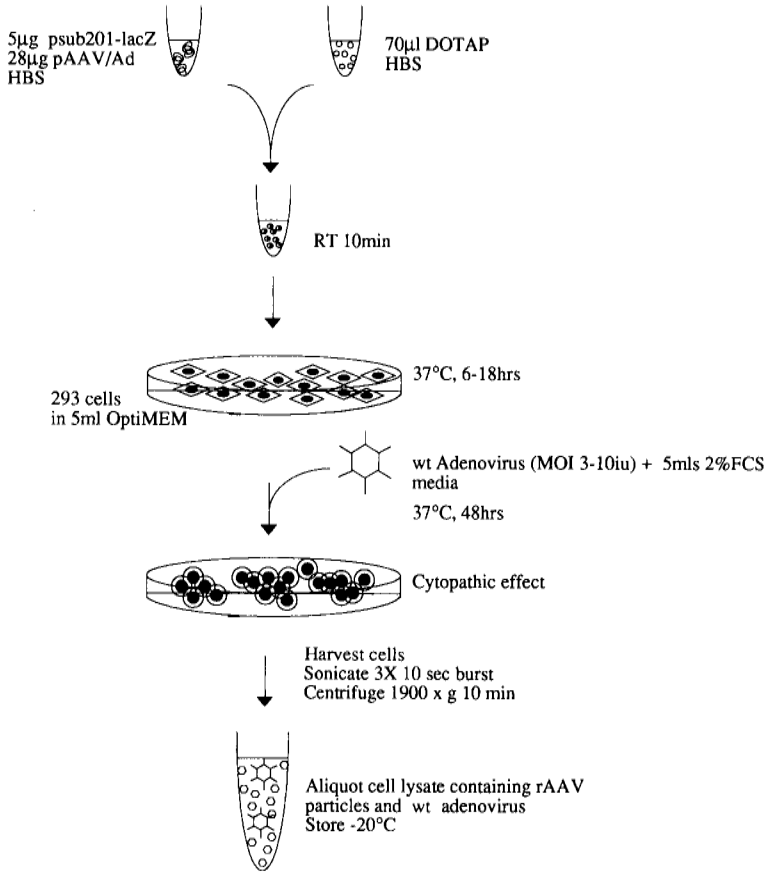

FIG. 3 Schematic representation of production of recombinant *lacZ* AAV. RT, Room temperature; MOI, multiplicity of infection; iu, infectious units.

3. During this incubation wash the 293 cells with OptiMEM reduced serum medium (GIBCO–Bethesda Research Laboratories Gaithersburg, MD), and add 5 ml of OptiMEM to the plate.

4. Gently add the DOTAP/DNA/HBS mixture to the plate and incubate the cells from 6 to 18 hr at 37° C, 5% CO_2. We have tested a range of incubation times from 6 to 18 hr and noted no difference in transfection efficiency.

5. Replace the medium with 5 ml of DMEM plus 2% FCS and adenovirus type 5 at a multiplicity of infection of 3–10 infectious units per cell. As a control for monitoring transfection efficiency, adenovirus is not added to one of the transfected plates.

6. Incubate at 37° C, 5% CO_2, for a minimum of 48 hr. Infected cells will appear clumped and rounded, exhibiting a classic cytopathic effect.

7. Harvest the virus (described below) when a full cytopathic effect is observed. The plate of transfected cells without adenovirus is stained for *lacZ* activity (21) to estimate the transfection efficiency.

Assaying Excision and Replication of Viral DNA

Since the primary step in generating AAV vector involves rescue and replication from the plasmid backbone, these functions are routinely assayed. Low-molecular-weight DNA can be extracted by a modification of the Hirt method (22) to monitor whether the rAAV DNA successfully excised from the plasmid and replicated.

1. Collect cells from a 100-mm, transfected, adenovirus-coinfected plate, centrifuge at 500 *g* for 5 min, and resuspend the cell pellet in 0.765 ml of 10 m*M* Tris, pH 7.4, 100 m*M* EDTA.
2. Add 85 μl of 10% SDS to a final concentration of 1%, mix gently until the lysate clears and becomes viscous. Then add 0.25 ml of 5 *M* NaCl, mix thoroughly by inversion, and incubate 16 hr at 4° C.
3. Transfer the lysate to an Eppendorf centrifuge tube and centrifuge at 15,000 *g* for 30 min.
4. With equal volumes of phenol and chloroform, extract the supernatant and precipitate the DNA with an equal volume of 2-propanol.
5. Wash the DNA pellet with 70% ethanol, dry briefly, and resuspend in 100 μl of Tris–EDTA plus RNase (20 μg/ml).
6. On a 0.8% agarose Tris–acetate–EDTA gel, electrophorese 10-μl aliquots of the Hirt DNA solution before and after digestion with 10 U of *Dpn*I (37° C, 2 hr; Boehringer-Mannheim) (23).
7. Stain the gel with ethidium bromide (1 μg/ml) for 30 min. The monomer and dimer replicative intermediates of the AAV vector (24) should be visible (approximately 4.7 and 9.4 kb, respectively). *Dpn*I will digest methylated DNA of prokaryotic origin, so the monomer and dimer replicative intermediates should be completely resistant to digestion, while any residual plasmid DNA will be degraded. If the vector DNA replication is not observed, the following assays should be checked.

a. *Transfection procedure.* Suboptimal transfection efficiency may be responsible for poor yields of replicating vector DNA. It is important that the transfection efficiency is at least 50%.

b. *Integrity of AAV ITRs.* AAV DNA replication requires the presence of complete AAV ITRs. Integrity of the ITRs may be assessed by digesting psub201–lacZ with *Pvu*II, *Sma*I, and *Xba*I. *Pvu*II sites are situated at the plasmid/ITR junctions, *Sma*I sites are situated within the terminal repeat sequences, and *Xba*I sites are at the junctions between the ITR and the *lacZ* cassette.

c. *Southern Blot.* Southern transfer of Hirt DNA to a membrane followed by hybridization with a recombinant gene-specific probe (23) is a more sensitive method for detecting replicating DNA. This step can substitute for direct visualization of replicating vector DNA after ethidium bromide staining. However, when all steps are optimized (transfection, adenovirus coinfection, and virus harvesting), direct visualization of replicative intermediates should be possible.

Harvesting rAAV

AAV accumulates in the nuclei of infected cells, so it is necessary to use techniques that disrupt the nucleus and liberate the virus from within the cell.

1. Transfer the cells and the media to centrifuge tubes and pellet at 500 *g* for 5 min.
2. Resuspend the pellet in phosphate-buffered saline (PBS; 0.137 *M* NaCl, 6.75 m*M* Na_2HPO_4, 2.5 m*M* NaH_2PO_4) at 1 ml per plate.
3. Sonicate the cells in three 10-sec bursts on ice (output control, 5; duty cycle, 50%).
4. Centrifuge at 1800 *g* for 10 min to remove cellular debris.
5. Aliquot the crude cell lysate, containing both recombinant *lacZ* and wt adenovirus, and store at $-20°$C.

Prior to using the crude cell lysate to transduce cells, it may be desirable to inactivate the adenovirus by heating the cell lysate at 56°C for 1 hr. Since 293 cells constitutively express E1 proteins, adenovirus E1A mutants can be utilized as helper virus. These mutants are not permissive in non-293 cells and heat inactivation is not necessary. For the purpose of investigating the permissibility of different cell lines, or titration of *lacZ* AAV, the presence of adenovirus is not deleterious and heat inactivation can be omitted.

Infection with lacZ AAV

The number of infectious *lacZ* AAV particles is determined by infection of 293 cells with aliquots of virus, ranging from 10 to 100 μl.

1. Wash 60-mm tissue culture dishes of 85% confluent 293 cells twice with PBS.
2. Add the viral aliquot in 1 ml of serum-free DMEM and incubate at 37°C, 5% CO_2, for 1 hr, occasionally swirling the plates to prevent them from drying out.
3. Add 4 ml of DMEM plus 2% FCS and incubate for a further 24–36 hr before staining for β-galactosidase expression.

4. Assay for β-galactosidase expression (21).

a. Wash the cells with PBS and fix with 2% formaldehyde, 0.2% glutaraldehyde for 5 min at 4°C.

b. Incubate the cells with freshly prepared 1 mg/ml X-Gal, 1.64 mg/ml potassium ferricyanide, 2.12 mg/ml potassium ferrocyanide, 2 m*M* $MgCl_2$, at 37°C for 2–16 hr.

c. Titers are calculated by counting the number of cells with blue nuclei.

Using routine procedures, titers of 10^5–10^6 transducing particles per milliliter can be obtained.

Testing Cell Types for AAV Infectability

The *lacZ* AAV prepared using this protocol is suitable for monitoring the permissiveness of various cell types for AAV. The cell type under investigation is plated in 60-mm tissue culture dishes or Lab-Tech slides (Nunc, Naperville, IL) and grown until 80% confluence is reached. Infection with rAAV is as described above, except that the cells are infected with 1–10 transducing units per cell in a minimal volume of the appropriate medium without serum. After incubation for 1 hr, medium supplemented with 2% FCS is added and infection is continued for 24 hr. Cells are stained as described previously. Blue cells are suggestive that the *lacZ* AAV virus was able to enter the cells, uncoat, and become double-stranded and transcriptionally active. It should be noted that these short-term assays do not monitor vector integration. Examples of permissive cells are shown in Fig. 4. *lacZ* AAV is transcriptionally active in primary rat hepatocytes and primary human astroglioma cells.

Conclusion

AAV is attractive as a gene therapy vector, primarily because of its classification as a nonpathogenic human virus that can stably integrate into the host cell chromosome. Wild–type AAV, which integrates to a region within the long arm of human chromosome 19, has never been shown to be pathogenic. Since AAV integration does not display the same specificity (reviewed in Ref. 6), the role of vector integration *in vivo* remains to be evaluated. The apparent lack of any pathogenicity associated with AAV and its ability to transduce a broad range of cell types appear advantageous for gene replacement. We have described a method of generating rAAV, using the example of the β-galactosidase AAV, which is a convenient vector for rapidly analyzing whether various cell types are susceptible to AAV infection. After indication of infectivity, it is possible to confidently utilize rAAV expressing therapeutic genes into these spe-

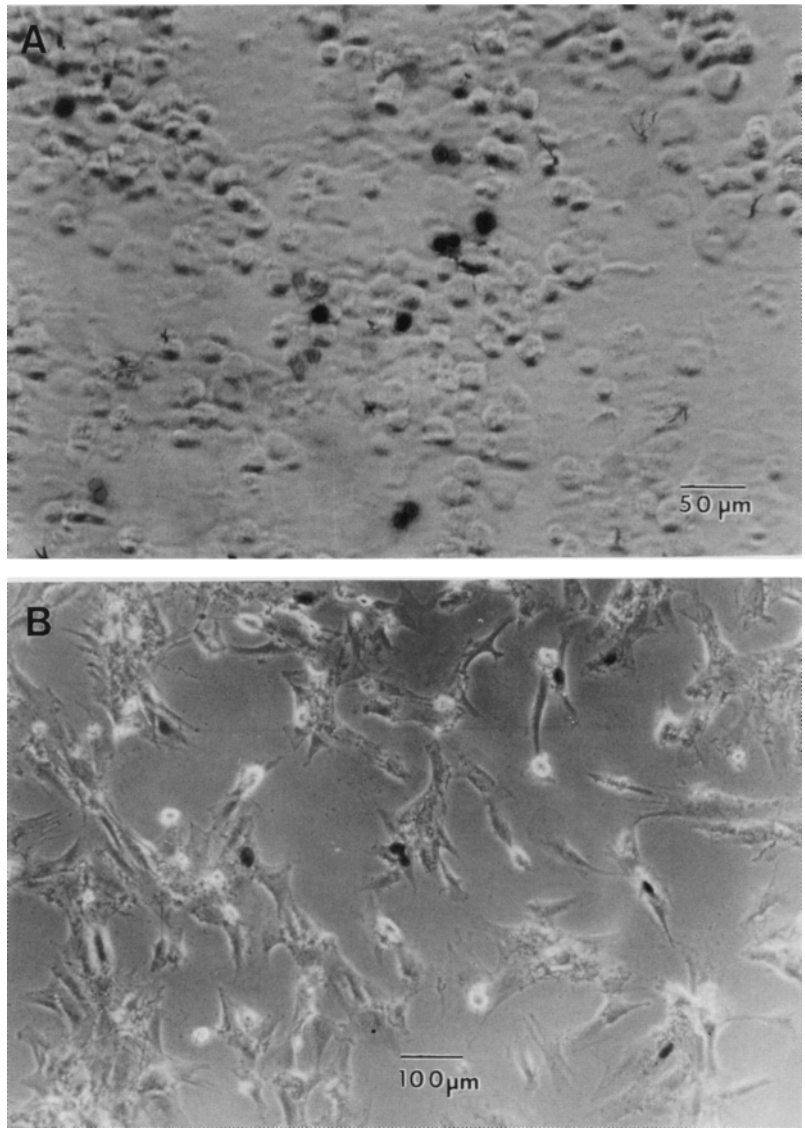

FIG. 4 Examples of cells infected with *lacZ* AAV. (A) Primary rat hepatocytes infected at a multiplicity of less than one virus per cell. (B) Primary human astroglioma cells infected at approximately one virus per cell. The dark nucleus within certain cells indicates infection and expression of the rAAV virus. See cover for a color photograph of part B.

cific cell types, as seen with the examples of the cystic fibrosis transmembrane regulator (*CFTR*) gene (25) and the human γ-globin gene (26) to infect specific cell types.

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References

1. K. Roemer and T. Friedmann, *Eur. J. Biochem.* **208,** 211 (1992).
2. R. C. Mulligan, *Science* **260,** 926 (1993).
3. P. W. Rigby, *J. Gen. Virol.* **64,** 255 (1983).
4. R. J. Samulski, *Curr. Opin. Genet. Dev.* **3,** 74 (1993).
5. R. W. Atchison, B. C. Casto, and W. M. Hammon, *Science* **149,** 754 (1965).
6. J. A. Rose and F. Koczot, *J. Virol.* **10,** 1 (1972).
7. J. R. Schlehofer, M. Ehrbar, and H. zur Hausen, *Virology* **152,** 110 (1986).
8. R. M. Buller, J. E. Janik, E. D. Sebring, and J. A. Rose, *J. Virol.* **40,** 241 (1981).
9. R. A. McPherson, L. J. Rosenthal, and J. A. Rose, *Virology* **147,** 217 (1985).
10. H. D. Mayor, R. M. Jamison, L. E. Jordan, and J. L. Melnick, *J. Bacteriol.* **90,** 235 (1965).
11. F. J. Koczot, B. J. Carter, C. F. Garon, and J. A. Rose, *Proc. Natl. Acad. Sci. U.S.A.* **70,** 215 (1973).
12. E. Lusby, K. H. Fife, and K. I. Berns, *J. Virol.* **34,** 402 (1980).
13. R. J. Samulski, L.-S. Chang, and T. Shenk, *J. Virol.* **63,** 3822 (1989).
14. R. J. Samulski, K. I. Bern, M. Tan, and N. Muzyczka, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2077 (1982).
15. P. L. Hermanot and N. Muzyczka, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 6466 (1984).
16. J.-D. Tratschin, M. H. P. West, T. Sandbank, and B. J. Carter, *Mol. Cell. Biol.* **4,** 2072 (1984).
17. S. K. McLaughlin, P. Collis, P. L. Hermonat, and N. Muzyczka, *J. Virol.* **62,** 1963 (1988).
18. R. J. Samulski, L.-S. Chang, and T. Shenk, *J. Virol.* **61,** 3096 (1987).
19. F. L. Graham, J. Smiley, W. C. Russell, and R. Nairn, *J. Gen. Virol.* **36,** 59 (1977).
20. J. C. Alwine, *Mol. Cell. Biol.* **5,** 1034 (1985).
21. J. R. Sanes, J. L. Rubenstein, and J. F. Nicolas, *EMBO J.* **5,** 3133 (1986).
22. B. Hirt, *J. Mol. Biol.* **26,** 365 (1967).
23. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
24. K. I. Berns, *Microbiol. Rev.* **54,** 316 (1990).
25. T. R. Flotte, S. A. Afione, R. Solow, M. L. Drumm, D. Markakis, W. B. Guggino, P. L. Zeitlin, and B. J. Carter, *J. Biol. Chem.* **268,** 3781 (1993).
26. C. E. Walsh, J. M. Liu, X. Xiao, N. S. Young, A. W. Nienhuis, and R. J. Samulski, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7257 (1992).

[2] Techniques for Human Adenovirus Vector Construction and Characterization

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Introduction

Human adenoviruses (Ads) have attracted considerable attention lately for their potential use as vectors for gene therapy, for recombinant vaccines, and for high-level protein expression in mammalian cells (1). Among the reasons for this interest are: (1) the 36-kilobase pair (kbp) double-stranded DNA genome of Ad is relatively easy to manipulate by recombinant DNA techniques, and (2) Ad can infect and direct high levels of protein expression in both proliferating and quiescent cells, an important feature for vectors requiring *in vivo* expression. In addition, Ad infects a variety of cell types, including fibroblasts, epithelial cells, stromal cells, endothelial cells, and hepatocytes of human, primate, canine, and rodent origin, although the latter two cell types are infected to a lesser extent. In permissive cells the virus replicates efficiently, generally producing about 1000 plaque-forming units (pfu) per infected cell, thus enabling the production of high-titer viral stocks. Late in infection, most of the infected cell protein is virally encoded, potentiating the use of replication-proficient recombinant Ads as vectors for recombinant protein production. In nondividing nonpermissive cells the viral genome may persist as an episome and may continue to express for long periods, which is desirable for most gene therapy applications.

In this article we describe several plasmid-based systems for inserting foreign genes into the Ad genome, and the methods used to purify, grow, and titrate recombinant viruses. Our vectors are based on the human Ad5 genome, the structure of which is shown in Fig. 1. In a normal infection early genes (*E1A, E1B, E2, E3,* and *E4*) are expressed prior to DNA replication. Late gene expression, driven predominantly by the major late promoter at 16 map units, occurs after DNA replication. The products of the *E1A* gene are required for the expression of all the other Ad genes. Thus, E1 deletion viruses are defective for replication in all cell types except the E1-complementing 293 cell line (2). The E3 region, which may be important for virus persistence *in vivo,* is dispensible for growth of the virus *in vitro.* Both the E1 and E3 regions, therefore, provide convenient sites for insertions of foreign sequences to generate helper-independent recombinant viruses.

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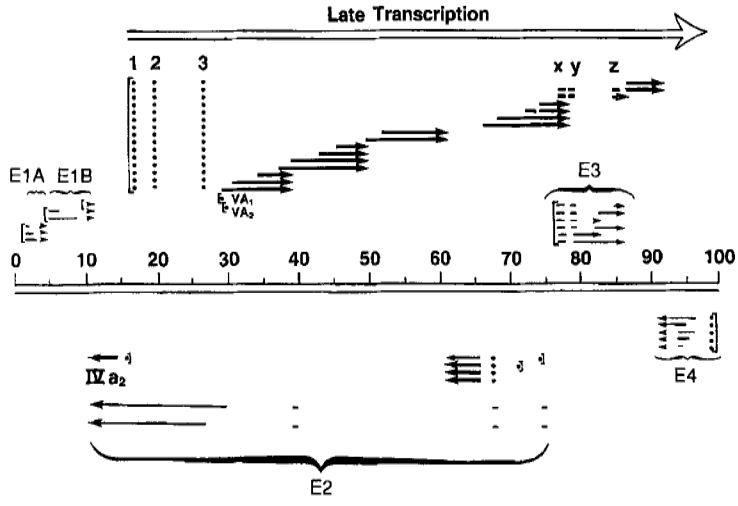


FIG. 1 Structure of the Ad5 genome. Early region transcripts are indicated as thin arrows, and late transcripts are indicated as bold arrows. The primary transcript originating from the major late promoter at 16 map units and terminating near the right end of the genome is indicated by a large open arrow. This transcript is processed into five families of late mRNAs spliced to a common tripartite leader (1, 2, and 3 at map units 16.5, 19.5, and 26.5, respectively), although some mRNA species contain additional leaders. (For more details see H. S. Ginsberg, "The Adenoviruses." Plenum, New York, 1984.)

Selection of Ad Vectors and Orientation of the Insert

By deleting nonessential Ad sequences in E1 and E3, we have developed vector systems for inserting foreign genes up to 8.3 kbp in length. In general, these systems rely on *in vivo* recombination between shuttle plasmids containing the gene of interest flanked by Ad E1 or E3 sequences and a second plasmid containing essentially the entire Ad genome in a circular form (Fig. 2). A large number of shuttle plasmids have been generated that differ in the size and location of the Ad deletion, the regulatory sequences present, and the unique restriction sites available for insertion of the foreign gene (Table I). The Ad genome plasmids (Table II) have all been designed to give low or no background of nonrecombinant infectious progeny upon cotransfection: pJM17 (3) is based on Ad5 *dl*309 (4) and has a deletion/substitution in E3 and an insertion of pBR322 DNA in E1, which exceeds the packaging constraints of Ad; the pBHG plasmids (5) are missing the Ad sequences from nucleotides (nt) 188–1339, which includes the essential viral packaging signal; and pFG173 (6) carries a lethal deletion in E3 (from about nt 27,320 to 30,560).

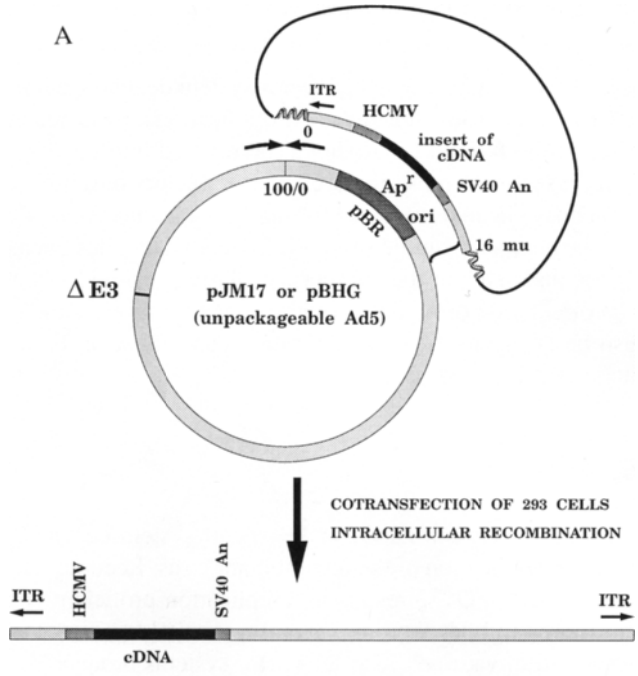
The selection of a suitable vector system depends mainly on the following conditions or properties: the intended application of the recombinant virus determines whether the foreign DNA should be rescued into E1 or E3; the size of the foreign DNA insert may prescribe a choice of vectors based on cloning capacity; and the regulatory elements required for the desired expression of the foreign DNA will dictate the choice of shuttle plasmid. Once a system has been chosen, the foreign DNA can be inserted into the appropriate shuttle plasmid by standard cloning techniques. The orientation of the insert with respect to the E1 and E3 transcription units must also be determined, since orientation can influence the level of protein expression attained in infected cells.

E1 versus E3 Insertion

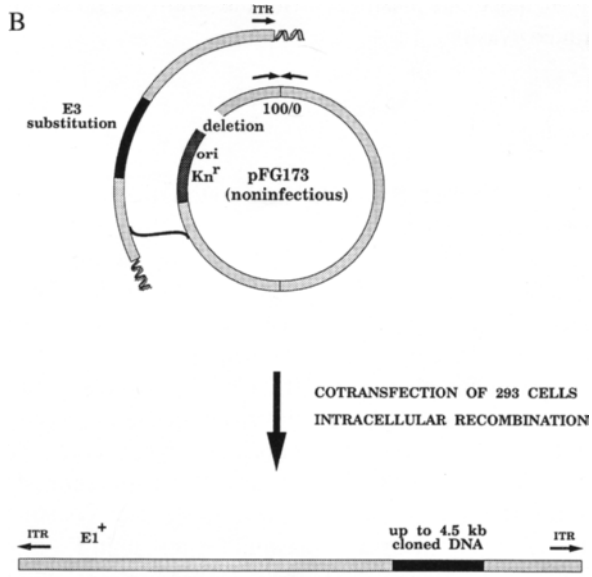
The choice between an E1 or E3 vector system depends, to a large extent, on the intended application of the recombinant virus. Recombination between an E3 shuttle plasmid and pFG173 results in a replication-proficient virus that potentially directs short-term high-level protein expression suitable for recombinant protein production or for use in vaccines. The E1 vector systems produce recombinant virus defective for replication, which generally results in a greater duration—but, in some cases, lower levels—of expression, which may be most useful for some gene therapy applications. The Ad genome plasmid pBHGE3 may provide for the longest expression *in vivo,* since this plasmid carries the wild-type E3 region, which is involved in host immune evasion.

Cloning Capacity

The choice of vectors also depends on the size of the gene to be rescued. Because the packaging constraints of Ad are somewhat flexible, up to 2 kb of foreign DNA can be introduced into the viral genome without necessitating any deletion of wild-type Ad sequences. However, it appears that recombinant viruses, the sizes of which approach the upper limit of packaging, may be less stable than slightly smaller recombinants (7); therefore, we recommend using one of the following deletion vectors. The Ad genome plasmid pBHG11 has, in addition to the deletion of the packaging signal described above, a *Pac*I restriction site in place of the E3 sequences from nt 27,865 to 30,995. This plasmid has the highest potential cloning capacity (theoretically, up to 8.3 kbp) (Table II) of any Ad vector reported to date, when cotransfected with a pΔE1 shuttle plasmid (5). In this vector system the foreign gene can be inserted either into the pΔE1 plasmid for rescue in E1 or into the unique *Pac*I site of pBHG11 for rescue in E3. In the latter case cotransfection with a plasmid containing



DEFECTIVE VIRAL VECTOR



NONDEFECTIVE VIRAL VECTOR

left-end viral sequences is still required to rescue the viral packaging signal for recombinant virus replication. Foreign DNA can be rescued into either E1 or E3 with pBHG10 in the same manner as with pBHG11, but the capacity of pBHG10 is slightly smaller (the E3 deletion in this case extends from nt 28,133 to 30,818).

The choice of shuttle plasmid can also affect the cloning capacity, but to a lesser extent than do the Ad genome plasmids. The pXC plasmids (8,9) have a slightly smaller E1 deletion (nt 452–3328) than that of the other E1 shuttle plasmids (nt 342–3523) (5,10) listed in Table IA. The deletion (nt 28,133–30,818) in the pAB E3 shuttle plasmids (Table IB) is slightly larger than that in pFG144K3 (nt 28,592–30,470), resulting in a slightly higher capacity for inserts of foreign DNA (7).

Regulatory Elements Required for Expression

The choice of shuttle plasmid is largely determined by the regulatory elements required for proper expression of the foreign insert. If the foreign sequence carries its own promoter and polyadenylation signals, then the most suitable E1 shuttle plasmids would be the pXC or pΔE1 plasmids. The other E1 shuttle plasmids (Table IA) contain strong enhancer/promoters derived from (*a*) −299 to +76 of the human cytomegalovirus immediate-early gene (*HCMV*) (11), (*b*) −450 to +910 of the human β-actin gene (12), or (*c*) the Ad2 major late promoter (MLP) at 16 map units (13). The level of expression obtained with E1 insertion vectors depends mainly on the strength of the promoter immediately upstream of the coding sequence for the foreign gene. In some plasmids the simian virus 40 (SV40) polyadenylation signal has been inserted downstream of the polycloning site, for the cloning of inserts which do not already carry this regulatory element.

FIG. 2 Rescue of foreign DNA sequences into Ad vectors. (A) The Ad E1 insertion vector system. The general strategy used to introduce foreign DNA inserts into the E1 region for rescue into virus is illustrated. 293 cells are cotransfected with a circular Ad genome plasmid (pJM17, pBHG10, pBHG11, pBHGE3, or a derivative containing an insert at the *Pac*I site in E3) plus an E1 shuttle plasmid containing the foreign DNA insert (black bar) flanked by regulatory sequences (dark shaded bar), if desired, and left-end viral sequences (light shaded bar) including the packaging signals. *In vivo* recombination (∫) within a 2-kb sequence common to both plasmids generates the E1 insertion virus depicted below. This virus is defective for replication in all cells except 293 cells. (B) The Ad E3 insertion vector system. A general strategy used to introduce foreign DNA inserts into the E3 region for rescue into $E1^+$ virus is illustrated. 293 cells are cotransfected with pFG173 and an E3 shuttle plasmid containing right-end Ad viral sequences (shaded bar) with foreign DNA sequences (black bar) in place of a portion of the E3 region. Intracellular recombination (∫) generates the replication-proficient virus depicted below. ITR, inverted terminal repeat; mu, map units; ori, origin.

TABLE I Shuttle Plasmids

Plasmid	Regulatory sequences[a]	Net deletion (kb)[b]	Cloning sites[c]
A. E1 shuttle plasmids for cotransfection with pBHG10, pBHG11, pBHGE3, and pJM17			
pXCX2	—	2.88	X
pXCJL1	—	2.88	X–B–Xh–S–C
pXCLJ2	—	2.88	C–S–Xh–B–X
pΔE1sp1A	—	3.19	C–B–Xh–X–EV–E–H–S–Bg
pΔE1sp1B	—	3.19	C–S–H–E–EV–X–Xh–B–Bg
pHCMVsp1A	HCMV (L)	2.81	C–B–Xh–X–EV–E–H–S
pHCMVsp1B	HCMV (L)	2.81	C–S–H–E–EV–X–Xh–B
pHCMVsp1C	HCMV (L)/SV40 pA	2.66	C*–B–Xh–X–EV–E–H–S
pHCMVsp1D	HCMV (L)/SV40 pA	2.66	C*–S–H–E–EV–X–Xh–B
pCA3	HCMV (L)/SV40 pA	2.66	B–Xh–X–EV–E–H–S
pCA4	HCMV (L)/SV40 pA	2.66	S–H–E–EV–X–Xh–B
pCA13	HCMV (R)/SV40 pA	2.66	S–H–E–EV–X–Xh–B
pCA14	HCMV (R)/SV40 pA	2.66	B–Xh–X–EV–E–H–S
pβActsp1A	β-Actin (L)	1.74	C–B–X–EV–E–H–S
pβActsp1B	β-Actin (L)	1.74	C–S–H–E–EV–X–B
pCA1	β-Actin (L)/SV40 pA	1.58	S–H–E–EV–X–B
pCA2	β-Actin (L)/SV40 pA	1.58	B–X–EV–E–H–S
pMLPsp1A	MLP (R)	2.23	B–X–EV–E–H–S–Bg
B. E3 shuttle plasmids for cotransfection with pFG173			
pFG144K3	—	1.88	X
pAB14	—	2.69	X
pAB16	—	2.69	X–B–Xh–S–C
pAB17	—	2.69	C–S–Xh–B–X
pAB21	—	2.69	S–Xh–B–X
pAB26	—	2.69	X–B–Sc–Xh–S–C
pAB27	—	2.69	C–S–Xh–Sc–B–X

[a] Heterologous regulatory sequences present in the shuttle plasmids to control expression of the inserted foreign gene: HCMV, human cytomegalovirus immediate-early gene promoter (L for leftward, R for rightward orientation); SV40 pA, SV40 polyadenylation signal; β-actin, human β-actin promoter; and MLP, Ad2 major late promoter.

[b] The E1 deletion in the pXC series of shuttle plasmids extends from nt 452 to 3328. All of the other E1 shuttle plasmids contain a deletion from nt 342 to 3523. The E3 deletion present in pFG144K3 extends from nt 28,592 to 30,470, and the deletion in the pAB series of plasmids extends from nt 28,133 to 30,818. *Net deletion* refers to the size of the deletion minus the size of the regulatory sequences.

[c] Restriction sites available for cloning foreign gene inserts. X, *Xba*I; B, *Bam*HI; Xh, *Xho*I; S, *Sal*I; C, *Cla*I; EV, *Eco*RV; E, *Eco*RI; H, *Hin*dIII; Bg, *Bgl*II; and Sc, *Sca*I. *Cla*I sites indicated with an asterisk are methylated, unless the plasmid is grown in bacteria defective for *dam* methylase.

None of the E3 shuttle plasmids have heterologous promoters or polyadenylation signals. Although insertion of a promoter upstream of a gene in E3 may drive transcription, this is often not efficient: for example, the SV40 early promoter fails to

TABLE II Circular Ad Genome Plasmids

Plasmid	Predicted maximum insert size (kb)[a]	Location of rescued genes	Recombinant progeny
pJM17	5.4	E1	Defective[b]
pBHG10	7.9	E1 or E3 or both	Defective
pBHG11	8.3	E1 or E3 or both	Defective
pBHGE3	5.2	E1	Defective
pFG173	4.7	E3	Nondefective

[a] Based on recombination between a pΔE1 shuttle plasmid and pJM17, or one of the pBHG plasmids or a pAB shuttle plasmid and pFG173.
[b] Defective for replication in all cells except 293 cells.

direct significant levels of expression in some E3 recombinant viruses. In contrast, some promoterless constructs express relatively high levels of recombinant proteins, presumably driven by the endogenous MLP or E3 promoter. Furthermore, the kinetics of expression (early versus late after infection), in some cases, appears to be dependent more on the gene being expressed than on the promoter inserted immediately upstream of the gene. It is not, at this time, possible to predict which E3 constructs will utilize an inserted promoter; consequently, care must be taken in analyzing E3 insertion recombinants for appropriate expression, particularly, for example, when tissue-specific expression is desired.

Orientation of the Insert

Foreign genes, including their own or heterologous promoters, can be inserted into the shuttle plasmids in an orientation either parallel or antiparallel to the E1 or E3 transcription unit. For all E1 vectors we have examined to date, higher expression levels have been obtained with inserts in the parallel orientation. Figure 3 presents data from an experiment in which human fibroblast MRC5 cells were infected with Ad (in a pJM17 background) carrying the *lacZ* reporter gene in E1 in either the rightward (E1-parallel) (AdCA17lacZ) or the leftward (E1-antiparallel) (AdCA12-lacZ) orientation. When *lacZ* is transcribed in the same direction as the E1 transcription unit (i.e., rightward), expression was up to 7-fold greater than that with the gene in the opposite orientation. To allow foreign gene insertion in either orientation, the E1 shuttle plasmids have been constructed with regulatory elements in either the same (R, for rightward) or opposite (L, for leftward) orientation as E1 (Table I). In general, inserts in E3 also express higher in the E3-parallel orientation, probably, at least with most vectors constructed to date, due to regulation by the endogenous E3 promoter or MLP rather than by the promoter inserted with the foreign gene.

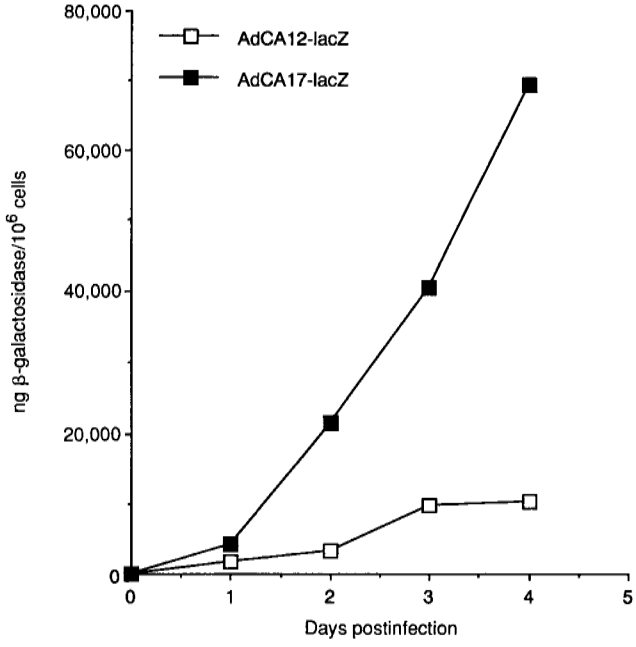


FIG. 3 Expression of β-galactosidase in cells infected with virus carrying *lacZ* inserted in E1 in either a parallel or antiparallel orientation. MRC5 cells (a human fibroblast cell strain) were infected with either AdCA12lacZ, which expresses the gene encoding β-galactosidase under the HCMV immediate-early gene promoter in the leftward orientation, or AdCA17lacZ, which expresses the gene in the rightward orientation, at an MOI of 10 pfu per cell. Cells were harvested daily for 4 days and cell pellets were assayed for β-galactosidase activity as described by J. Sambrook, E. F. Fritsch, and T. Maniatis, *in* "Molecular Cloning: A Laboratory Manual," 2nd ed., p. 16.66. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). The values were normalized to 10^6 cells.

Overview of the Technique

In this article we describe the procedures used to transfer a foreign gene from an Ad E1 or E3 shuttle plasmid to an infectious Ad5 vector, and to amplify and purify that vector. First, we present a protocol for growth of plasmid-bearing bacteria which minimizes rearrangements of the large circular Ad genome plasmids. Plasmid DNA is then extracted from these cultures by alkaline lysis followed by CsCl banding, as described in a number of cloning manuals (e.g., Ref. 14). Next, the foreign gene is rescued into the virus by cotransfecting the shuttle plasmid with a noninfectious "genomic" plasmid and selecting recombinants capable of forming plaques on the E1-complementing cell line 293. After expansion in 293 cultures, these plaques are screened by isolation and restriction of the viral DNA. Restricted viral DNA frag-

ments are analyzed by agarose gel electrophoresis, for which protocols can be found in many cloning manuals (14). The correct recombinants are then plaque-purified and amplified in 293 monolayer cultures. Because most of the virus remains associated with the infected cells until very late in infection, high-titer stocks can be prepared easily by collecting infected cells (e.g., by low-speed centrifugation) followed by disruption of the concentrated cell suspension to release the virus. After plaque titration this crude lysate can be used for most *in vitro* experiments with little interference from 293 cellular proteins and DNA, which are invariably present. For animal experiments, or in cases in which interference has been a problem, further purification of the virus by CsCl banding, as described here, is required.

Materials and Special Equipment

Becton Dickinson BBL Lennox L broth (LB) base can be obtained from Fisher Scientific. All tissue culture reagents [including minimum essential medium (MEM) F11: Cat. No. 61100; Joklik's modified MEM: Cat. No. 22300] can be obtained from GIBCO–Bethesda Research Laboratories. All sera are heat-inactivated at 56° C for 30 min prior to use. Beckman 50Ti and SW50.1 rotors are required.

Solutions

LB: 20 g of dehydrated BBL LB broth base and 1 g of glucose per 1 liter of water. Autoclave.

LB–agar: 2% Bacto-agar in LB. Autoclave. Cool LB–agar to about 50° C, then add antibiotics and pour into sterile petri dishes. Store at 4° C.

Complete MEMF11 (or Joklik's modified MEM): MEMF11 or Joklik's modified MEM containing 2 m*M* L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 μg/ml Fungizone. Store at 4° C for up to 2 weeks. Add heat-inactivated newborn bovine serum (NBS) or horse serum (HS) prior to use.

10× citric saline: 1.35 *M* KCl and 0.15 *M* sodium citrate. Autoclave and store at 4° C. Dilute 1 : 10 in sterile water to prepare 1 × citric saline.

HEPES-buffered saline (HBS): 21 m*M* HEPES, 0.137 *M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, and 5.5 m*M* glucose, pH 71. Aliquot into glass bottles, autoclave, and store at 4° C.

10× SSC: 1.5 *M* NaCl and 0.15 *M* Na citrate, pH 7.0. Autoclave. Prepare 0.1 × sodium chloride, sodium citrate, (SSC) by diluting 10× SSC followed by autoclaving.

2 mg/ml salmon sperm DNA: Dissolve 100 mg of salmon sperm DNA in 50 ml of sterile 0.1 × SSC by stirring overnight at room temperature. Determine the concentration by reading the optical density at 260 nm. Store in small aliquots at −20° C.

2.5 *M* $CaCl_2$: Sterilize by filtration and store in small plastic tubes at 4° C.

2× MEMF11 for overlay: 0.2% yeast extract, 10% HS, 4 m*M* glutamine, 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 5 μg/ml Fungizone in 2× MEMF11. Store at 4° C.

MEMF11–agarose overlay: Warm 2× MEMF11 for overlay to 37°C. Autoclave 1 g of agarose per 100 ml of water, then cool to 44°C. Combine equal volumes of agarose and 2× MEMF11 immediately before use.

10× Phosphate-buffered saline (PBS): 1.37 *M* NaCl, 82 m*M* Na_2HPO_4, 15 m*M* KH_2PO_4, and 27 m*M* KCl. Autoclave. Dilute 1:10 in sterile water to prepare 1× PBS.

PBS^{2+}: To sterile 1× PBS, add 0.01 vol of sterile 68 m*M* $CaCl_2$ and 0.01 vol of 50 m*M* sterile $MgCl_2$.

Pronase stock solution: 0.5% (w/v) pronase in 10 m*M* Tris-HCl, pH 7.5. Heat at 56°C for 15 min, then incubate at 37°C for 1 hr. Aliquot and store at −20°C. Working pronase–sodium dodecyl sulfate (SDS) solution: 0.05% pronase in 10 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, and 0.5% (w/v) SDS.

Carnoy's fixative: Methanol:glacial acetic acid (3:1).

Orcein: 2% (w/v) orcein dye in 50% (v/v) acetic acid. Filter through Whatman No. 1 paper.

DNase I solution: Dissolve 100 mg of pancreatic DNase I in 10 ml of 20 m*M* Tris-HCl, pH 7.4, 50 m*M* NaCl, 1 m*M* dithiothreitol, 0.1 mg/ml bovine serum albumin, and 50% glycerol. Store in small aliquots at −20°C.

RNase A solution: Dissolve 100 mg of RNase A in 10 ml of 10 m*M* Tris-HCl, pH 7.4, and 15 m*M* NaCl. Store in small aliquots at −20°C.

Saturated CsCl solution: At room temperature add sufficient CsCl to 10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA to saturate the buffer. Store at 4°C, but bring to room temperature before use.

Preparation of Plasmid DNA for Cotransfections

1. Streak out plasmid-bearing bacteria on LB–agar plates containing appropriate antibiotics and use the next day.

2. Pick two or more colonies off the plate, resuspend each in 5 ml of LB plus antibiotics, and shake at 37°C for several hours.

3. Add each 5-ml culture to 500 ml of LB plus antibiotics and continue shaking overnight.

4. Purify the plasmid DNA by alkaline lysis of the bacteria and CsCl banding as described in standard cloning manuals (14). Plasmid DNA that has not undergone any detectable rearrangement (as determined by restriction and agarose gel electrophoresis) is suitable for use in cotransfections.

Cotransfection for Rescue of Recombinant Ad Vectors

1. Monolayer cultures of 293 cells are maintained in 150-mm dishes in complete MEMF11 medium plus 10% NBS. Split cells 1:2 or 1:3 at 90% confluence by re-

moving the medium, washing each dish twice with 10 ml of 1 × citric saline, then incubating for a maximum of 15 min at room temperature in 2 ml of 1 × citric saline to detach cells. 293 cells should be refed with fresh medium every 3 days if they are not ready to passage.

2. Set up low-passage (<p40) 293 cells in 60-mm dishes to be about 70–80% confluent at the time of use. As a rule of thumb, one 150-mm dish of nearly confluent 293 cells can be split into eight 60-mm dishes for use the following day. Use of dishes after 2 days is also satisfactory, provided that the cells are not confluent.

3. Add 0.005 vol of 2mg/ml salmon sperm carrier DNA to 1 × HBS (prepare 6 ml per virus to be rescued), and shear by vortexing for 1 min.

4. For each virus to be rescued, aliquot 2 ml of HBS plus carrier DNA (enough for four dishes) into each of three sterile clear plastic tubes.

5. To these tubes add shuttle plasmid DNA and the circular Ad genome plasmid with which it will recombine, in the following amounts, respectively: 20 μg plus 20 μg, 20 μg plus 40 μg, and 40 μg plus 40 μg. A useful positive control for transfection efficiency is the infectious plasmid pFG140 (15).

6. Shake gently, then slowly add 100 μl of 2.5 *M* $CaCl_2$ dropwise to each tube with gentle mixing.

7. Let stand at room temperature for 15–30 min. (A fine precipitate should form within a few minutes.) Without removing the growth medium, add 0.5 ml of DNA suspension to each dish of cells, then incubate at 37° C for 4–5 hr or overnight.

8. Remove the medium and add to each dish 10 ml of MEMF11–agarose overlay. After the agarose solidifies, incubate at 37° C. Plaques should appear after about 1 week and are readily visible by the eye as a consequence of light scattering by dead and rounded cells in an otherwise smooth cell monolayer.

Screening Ad Plaque Isolates

The following protocol describes the amplification of plaque isolates by growth in 293 cells. The conditioned medium containing released virus can be stored for further purification, while the cell monolayer, with which most of the virus remains, is harvested for viral DNA analysis.

1. Pick well-isolated plaques from transfected cultures (see above) by punching out agar plugs with a sterile Pasteur pipette. Store agar plugs in 0.5 ml of sterile PBS^{2+} plus 10% glycerol at −70° C until use.

2. Set up 60-mm dishes of 293 cells (one dish per plaque) to be 80–90% confluent at the time of infection.

3. Remove the medium from the cells and add 0.2 ml of virus (agar plug suspension). Rock dishes once and adsorb at room temperature for 30 min. Add 5 ml of complete MEMF11 plus 5% HS and incubate at 37° C.

4. Viruses are ready to harvest when all cells are rounded [due to the viral "cytopathic effect" (CPE)] and most have detached from the dish (usually 3–4 days).

5. To permit collection of the medium while retaining the majority of the infected cells, leave the dishes undisturbed in the tissue culture hood for 30 min. Gently remove 4 ml of medium and add to a sterile vial containing 0.5 ml of sterile glycerol. Store these candidate viruses at −70° C. Slowly aspirate any remaining medium from the plate. If this is done carefully, the majority of the cells will remain in the dish.

6. To extract DNA from the infected cells, add 0.5 ml of pronase–SDS solution to each dish and incubate at 37° C for 4–18 hr.

7. Transfer the viscous lysate to a microfuge tube, and extract once with buffer-saturated phenol. Spin for 10 min. Collect the aqueous phase (top) and transfer it to a fresh tube.

8. Add 1 ml of 96% ethanol to precipitate the DNA. Mix by inversion; a fibrous precipitate should be easily visible. Spin for 10 min at 14,000 rpm. Wash the pellet twice with 96% ethanol and air-dry.

9. Dissolve the DNA pellet in 100 μl of 0.1 × SSC (complete solubilization may take several hours), and digest 10 μl with *Hin*dIII (1 U overnight is usually sufficient for complete digestion).

10. Analyze the digests on a 1% agarose gel with appropriate markers (a *Hin*dIII digest of wild-type Ad5 being one convenient marker) as described in standard cloning manuals (14). If the CPE was complete, viral DNA bands should be easily visible above a background smear of cellular DNA. Note that in *Hin*dIII digests of human DNA, there will be a band of repetitive cellular DNA at 1.8 kb, not to be confused with viral DNA. Figure 4 shows the *Hin*dIII restriction maps of recombinant viruses obtained by cotransfection of E1 or E3 shuttle plasmids with pBHGE3, pJM17, pBHG10, pBHG11, or pFG173.

11. Verify candidate recombinants using other diagnostic restriction enzymes. Correct recombinants should be further purified by at least one round of plaque purification, as described below, and screening, prior to the preparation of high-titer stocks.

Plaque Assays for Purification and Titration of Ad

1. Set up 60-mm dishes of 293 cells to be confluent at the time of infection.

2. Remove the medium from the dishes. Spread 0.2 ml of virus dilution (dilute agar plug suspension appropriately in PBS^{2+} if you wish to plaque-purify, or dilute stock for titration) evenly over each dish. We typically assay dilutions ranging from 10^{-2} to 10^{-6} for plaque purification or 10^{-3} to 10^{-10} for virus titration of concentrated stocks. Titrations are done in duplicate or triplicate. Adsorb the virus for 30

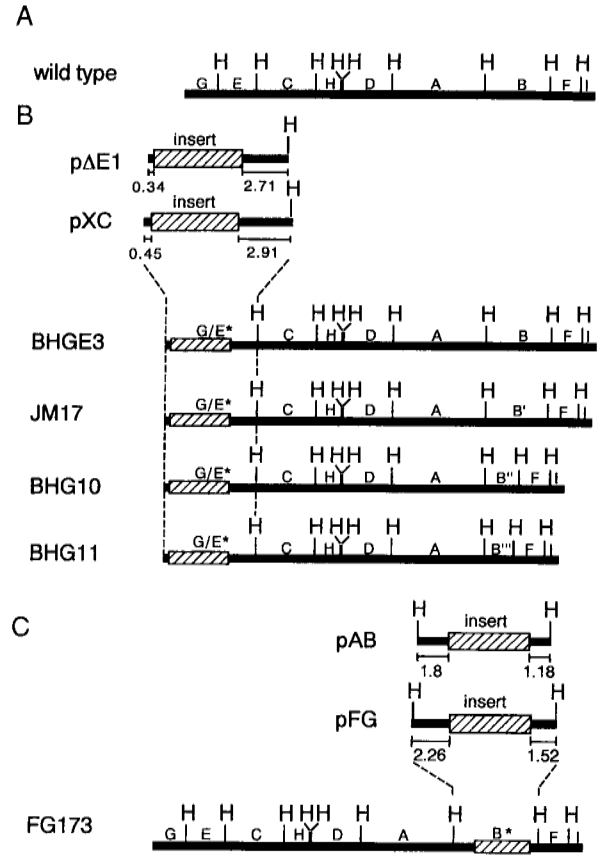


FIG. 4 *Hin*dIII restriction maps of recombinant vectors generated by cotransfecting 293 cells with Ad5 vector systems. Wild-type viral sequences are indicated by black bars and foreign DNA sequences (including any regulatory elements) are represented by hatched bars. The positions of *Hin*dIII sites are indicated by the letter H. Wild-type viral *Hin*dIII fragments are lettered in order of decreasing size, as follows: A, 8.01 kb; B, 5.66 kb; C, 5.32 kb; D, 4.60 kb; E, 3.44 kb; F, 2.94 kb; G, 2.80 kb; H, 2.08 kb; and I, 1.00 kb (the 75-bp J fragment is not shown). The sizes of the E3 *Hin*dIII fragments that carry deletions are: B′, 5.4 kb; B″, 2.98 kb; and B‴, 2.53 kb. The G/E* and B* fragments vary in size, depending on the insert size and the presence and location of *Hin*dIII sites in the insert. The sizes of the Ad sequences retained in *Hin*dIII fragments G/E* and B* are indicated just below the Ad5 DNA flanking the inserts. Except for the pXC plasmids, all of the shuttle plasmids listed in Table IA have the same Ad sequences as the pΔE1 series shown here. (A) *Hin*dIII restriction map of wild-type virus. (B) *Hin*dIII restriction map of recombinant E1 insertion vectors. (C) *Hin*dIII restriction map of recombinant E3 insertion vectors.

min at room temperature. Add 10 ml of MEMF11–agarose overlay, cool, then incubate at 37° C.

3. Plaques should be visible within 4–5 days, and should be counted for titration at 7 days and again at 10 days. For plaque purification proceed as for isolation of plaques following transfections.

Preparation of High-Titer Viral Stocks (Crude Lysates) from Cells in Monolayer

1. Set up 150-mm dishes of 293 cells to be 90% confluent at the time of infection. We generally use eight or more dishes for each virus.

2. To prepare high-titer stocks, remove the medium from the 293 cells and infect at a multiplicity of infection (MOI) of 1–10 pfu per cell (1 ml of virus suspension per 150-mm dish). For the initial stock preparation we dilute virus (from the untitrated 4-ml sample stored at −70° C after the last round of viral screening) 1 : 8 with PBS^{2+}. To minimize the probability of rearrangement of the recombinant virus, prepare subsequent high-titer stocks from the same viral screening sample.

3. Adsorb for 30 min, then add 25 ml of complete MEMF11 plus 5% HS to each dish. Incubate at 37° C and examine daily for signs of CPE.

4. When CPE is nearly complete, i.e., most cells are rounded but not all are detached, harvest the cells by scraping them off the dish, combining the cells with spent medium, and centrifuging at 800 *g* for 15 min. Remove the medium, and resuspend the cell pellet in 2 ml of PBS^{2+} plus 10% glycerol per 150-mm dish. Freeze (at −70° C) and thaw (at 37° C) the crude virus stock three times prior to titration. Store aliquots at −70° C.

Preparation of High-Titer Viral Stocks (Purified) from Cells in Suspension

Recombinant Ads can be purified from crude lysates of either monolayer or suspension cultures. Due to the greater ease of handling suspension cultures (one 4-liter suspension culture is equivalent in cell number to approximately 80 of the 150-mm dishes), this source is preferable for the preparation of purified high-titer viral stocks, as described here.

1. For infection with replication-defective viruses (E1 insertion recombinants), grow 293N3S (16) cells in spinner culture to a density of $2–4 \times 10^5$ cells per milliliter in 4 liters of complete Joklik's modified MEM plus 10% HS. For infection with replication-proficient viruses (recombinants with the E1 region intact), 4 liters of KB cells are grown to a density of $5–6 \times 10^5$ cells per milliliter in complete Joklik's

modified MEM plus 10% NBS. Centrifuge the cell suspension at 750 *g* for 20 min, saving 50% of the conditioned medium. Resuspend the cell pellet in 0.1 vol of fresh medium.

2. Add the virus at a MOI of 10–20 pfu per cell and stir gently at 37° C for 1 hr. Add 2 liters of conditioned medium and 2 liters of fresh medium, and continue stirring at 37° C.

3. Monitor the infection twice daily by inclusion body staining as follows:

a. Remove a 5-ml aliquot from the infected spinner culture. Spin for 10 min at 750 *g*. Resuspend the pellet in 0.5 ml of 1% sodium citrate.

b. Incubate at room temperature for 10 min, then add 0.5 ml of Carnoy's fixative and let stand for 10 min at room temperature.

c. Add 1 ml of 1% sodium citrate and spin for 10 min at 750 *g*. Resuspend the pellet in a few drops of 1% sodium citrate.

d. Add one drop of fixed cells to a slide and let air-dry for at least 1 hr; then add one drop of orcein and a coverglass. Under the microscope inclusion bodies appear as densely staining nuclear structures resulting from an accumulation of large amounts of virus and viral products at late times in infection. A negative control (uninfected cells) should be included in the initial tests.

4. When inclusion bodies are visible in 80–90% of the cells (36–72 hr), harvest by centrifugation at 750 *g* for 20 min in sterile 1-liter bottles. Combine the pellets in a small volume of medium, transfer to sterile 50-ml tubes, and spin again. Resuspend the pellet in 20–30 ml of 0.1 *M* Tris-HCl, pH 8.0 Store at −70° C until use.

5. Thaw the frozen crude stock and add 0.1 vol of 5% sodium deoxycholate. Mix well and incubate at room temperature for 30 min. This disrupts the cells without disrupting the virions, resulting in a relatively clear, highly viscous suspension.

6. Add 0.01 vol of 2 *M* $MgCl_2$, 0.005 vol of DNase I solution, and 0.005 vol of RNase A solution, then mix thoroughly. Incubate at 37° C for 30–45 min, mixing well every 10 min.

7. Add 1.8 ml of saturated CsCl solution (equilibrated to room temperature) for each 3.1 ml of virus suspension. Note that the CsCl concentration is critical, so volumes must be accurately determined. The final density should be 1.35 g/ml.

8. Transfer the virus to Beckman 50Ti Ultraclear Quickseal tubes and spin in a precooled Beckman 50Ti rotor for 16–20 hr at 4° C and 35,000 rpm.

9. Collect the viral bands in a small volume and pool. (One can collect them by puncturing the top of the tube with a hot needle, then puncturing the bottom, and controlling the flow of solution out the bottom with a gloved finger over the top hole.)

10. Centrifuge the pooled virus in a precooled Beckman SW50.1 rotor at 35,000 rpm, 4° C, for 16–20 hr.

11. Collect the virus band in the smallest volume possible and dialyze at 4° C against two changes of 500 vol of PBS^{2+} plus 10% glycerol for at least 4 hr each change. It is important, especially for *in vivo* work, to remove all traces of CsCl.

Precipitation of the virus is occasionally observed when the virus concentration is very high. It is quite difficult to resuspend precipitated virus.

Analysis of the Constructs

After the desired recombinant Ad virus has been constructed, it should be examined for its ability to express the foreign gene. The most suitable procedure for detecting expression would depend on the reagents available for that particular protein. If antibodies to the denatured protein are available, then Western blotting analysis of infected cell extracts (or infected cell supernatants, if the protein is secreted) is the simplest method to quantitate protein expression. If antibodies to the native protein are available, then enzyme-linked immunosorbent assay (ELISA) or immunoprecipitation of extracts and/or supernatants can be used to assess protein levels. See the work of Graham and Prevec (17) for additional details. If at all possible, the biological activity of the recombinant protein should also be tested to ensure that the expressed protein is functional. In our laboratory at McMaster University (Hamilton, Ontario, Canada), to date, all proteins expressed by Ad vectors are fully functional, in contrast to some recombinant proteins produced by other methods, such as by overexpression in bacteria. Because cells from different tissues and species vary in their ability to take up Ad, it may be desirable to analyze expression levels *in vitro* in a variety of cell types before proceeding to *in vivo* experiments.

It is important to confirm the replication deficiency of E1 deletion Ad vectors. Even a very low level of contamination with wild-type virus in nonreplicating vector stocks could lead to spurious results, since the wild-type virus could act as the helper for replication of the recombinant vector, thus increasing the copy number of the foreign gene and at the same time leading to lysis of the infected cells. The replication proficiency of a viral stock can be tested by infecting, at an MOI of 10 pfu per cell, three 60-mm dishes of 293 cells (permissive for the replication of E1 deletion Ad viruses) and four dishes of MRC5 cells (human fibroblasts nonpermissive for $E1^-$ viruses), then harvesting 293 cells at 0, 1, and 2 days and MRC5 cells at 0, 2, 4, and 6 days. Spin down the cells, resuspend the pellet in 0.5 ml of PBS^{2+}, freeze–thaw at least once, and titrate on 293 cells. Wild-type virus and an E1 deletion virus (e.g., *dl*312; see Ref. 15) should be included as positive and negative controls, respectively. The titer of recombinant virus-infected 293 cell extracts should increase by about 2 orders of magnitude by the second day, whereas the titer of recombinant virus-infected MRC5 extracts should not increase with time. Contamination with wild-type virus may arise by insufficient plaque purification of the recombinant vector when recombinant virus construction has used pJM17, but it can also be caused by improper handling of wild-type virus stocks or infections (care must be taken to prevent cross-contamination of the viral stocks). Replication-proficient virus can also arise

by recombination of the vector with E1 sequences in the 293 cell genome. The frequency with which this occurs is not known at present.

Safety Considerations

Because Ads with inserts in E3 are infectious in humans, it is important to use caution when handling recombinant Ads. Experimentation with Ad vectors should be carried out in at least a P2 containment facility in accordance with relevant regulations. If inadvertantly exposed, individuals without previous immunity to Ad5 may seroconvert not only against Ad5, but also against the foreign gene product expressed. This should be avoided, especially if the development of antibody may cause misdiagnosis of a particular disease. Finally, no known toxic or potentially toxic gene product should be expressed from nondefective Ad vectors.

Pitfalls

There can be a number of different causes for failure to obtain the proper recombinant virus following cotransfection. First, the transfection efficiency may be low (which can be assessed by transfection with infectious plasmid DNA such as pFG140). In our experience the 293 cells used in transfections must be at low passage, growing slowly, and be slightly subconfluent at the time of use. In addition, the plasmid DNA must be very pure: we routinely use CsCl-banded DNA in cotransfections. Finally, although infrequently, the desired recombinant might not be obtained because the foreign gene insert or product is toxic to the cells or virus, in which case it would not be possible to rescue a high-expression level recombinant Ad. Use of inducible promoters may circumvent such problems.

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References

1. K. L. Berkner, *BioTechniques* **6,** 616 (1988).
2. F. L. Graham, J. Smiley, W. C. Russell, and R. Nairn, *J. Gen. Virol.* **36,** 59 (1977).

3. J. McGrory, D. Bautista, and F. L. Graham, *Virology* **163,** 614 (1988).
4. N. Jones and T. Shenk, *Cell (Cambridge, Mass)* **17,** 683 (1979).
5. A. J. Bett, W. Haddara, L. Prevec, and F. L. Graham, *Proc. Natl. Acad. Sci.* **91,** 8802 (1994).
6. S. K. Mittal, M. R. McDermott, D. C. Johnson, L. Prevec, and F. L. Graham, *Virus Res.* **28,** 67 (1993).
7. A. J. Bett, L. Prevec, and F. L. Graham, *J. Virol.* **67,** 5911 (1993).
8. R. Spessot, K. Inchley, T. M. Hupel, and S. Bacchetti, *Virology* **168,** 378 (1989).
9. F. L. Graham and J. Lu, unpublished observations (1992).
10. C. L. Addison, A. J. Bett, and F. L. Graham, unpublished observations (1992).
11. M. Boshart, F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, and W. Schaffner, *Cell (Cambridge, Mass.)* **41,** 521 (1985).
12. P. Gunning, J. Leavitt, G. Muscat, S.-Y. Ng, and L. Kedes, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 4831 (1987).
13. D. Davidson and J. A. Hassell, *J. Virol.* **61,** 1226 (1987).
14. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
15. F. L. Graham, *EMBO J.* **3,** 2917 (1984).
16. F. L. Graham, *J. Gen. Virol.* **68,** 937 (1987).
17. F. L. Graham and L. Prevec, *in* "Methods in Molecular Biology," Vol. 7. (E. J. Murray, ed.), p. 109. Humana, Clifton, New Jersey, 1991.

[3] Construction and Isolation of Recombinant Adenoviruses with Gene Replacements

David J. Spector and Lorna A. Samaniego

Introduction

Human adenovirus has become a vector of choice, not only for introducing foreign genes into cell culture or animals for transient expression studies, but also for the rapidly developing field of human gene therapy. The advantages of the system have been widely advertised (1): the virus is easy to manipulate and store, grows to high titers, has a broad host range, and is capable of mediating high levels of expression of the transduced gene. For gene therapy applications, additional advantages include its apparent safety and the low probability of stable integration.

The most common vectors are derived from adenovirus type 5, the preferred strain for genetic analysis and manipulation. The sequence of the genome is known (2) and the genetic organization and protein functions are well defined (see Fig. 1). A brief summary of the replication cycle of the virus provides a context for understanding the principles of recombinant construction. Adenovirus proteins exhibit cascade regulation with the onset of DNA replication (about 6–8 hr postinfection in permissive cells), providing a separation between prereplicative (early) and postreplicative (late) events. Within the early phase there are two distinct regulatory classes of gene products. The *E1a* gene (by convention located at the left end of the linear viral DNA) is expressed in the absence of prior viral protein synthesis. *E1a* encodes regulatory proteins that provide functions necessary for the expression of the other early genetic units, *E1b, E2* (*a* and *b*), *E3,* and *E4. E1a* function is also responsible for early activation of the major late promoter, so named because its high level of activity in the late-infected cell mediates the abundant expression of most of the viral proteins. In addition, *E1a* also activates transcription from the class III unit encoding viral-associated RNAs.

The extensive knowledge of the genetic organization and the viral life cycle has allowed the identification of regions of the genome that either are nonessential for growth in laboratory cell culture or, for essential functions, can be provided in *trans* by stable cell lines. In particular, two regions have been targeted for foreign gene replacement (3). The *E3* region is nonessential for growth of the virus in cell culture (4). Therefore, *E3* may be deleted and replaced with a foreign gene without compromising the infectivity of the virus, at least *in vitro* (3, 5). For gene delivery applications in which replication of the virus is undesirable, *E3*-deleted vectors are not appropriate. Region *E1* (including *E1a* and *E1b*) is essential for virus replication.

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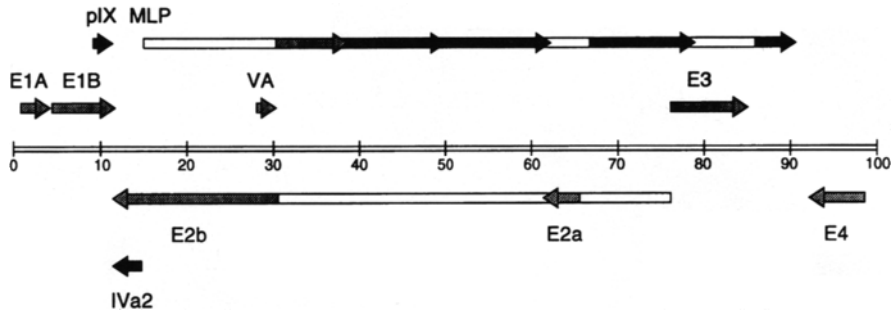

FIG. 1 Genetic organization of the adenovirus 5 genome. The regions that encode early (crosshatched arrows) or late (solid arrows) gene products are indicated; the arrows indicate the direction of transcription and the open bars delineate those regions that are transcribed but, except for small exons, are not expressed. MLP, Major late promoter.

However, *E1* gene products can be supplied in *trans* in cell line 293 (6), thus allowing efficient propagation of defective viruses carrying replacements of *E1* DNA by foreign genes (3). These defective viruses, although capable of high-level expression of the foreign gene, cannot replicate in animals or humans and thus avoid potential complications, such as cell destruction, which might accompany replication. Here, we consider *E1* gene replacements only.

Strategies for Gene Replacement in Region E1

There are two standard strategies for producing adenoviruses with *E1* gene replacements (Fig. 2). Both take advantage of the fact that adenovirus DNA is infectious, and they rely on a transfected permissive cell to regenerate viable adenovirus genomes by homologous recombination of sequences from two separate DNA molecules (7). In one strategy both DNA molecules are propagated as *Escherichia coli* plasmids (8). One serves as the cloning vector for the gene replacement and the other provides any required viral genomic sequences not present in the first. The obvious advantage of this system is that one need not isolate DNA from virions to produce recombinants. Also, a clever selection scheme, based on the DNA encapsidation limit of the virion, has been developed for recombinant isolation (9). A potential disadvantage is the difficulty in propagating plasmids with large insertions of adenovirus DNA.

The second strategy uses one DNA molecule that is plasmid derived and one that is derived from a viral genome (10, 11). The viral DNA fragment includes most of the genome, but lacks two *cis* elements required for viability: an encapsidation signal and origin of replication, both located within 355 bp of the left terminus. Convenient unique restriction endonuclease sites in the *E1* region of the genome of several viral

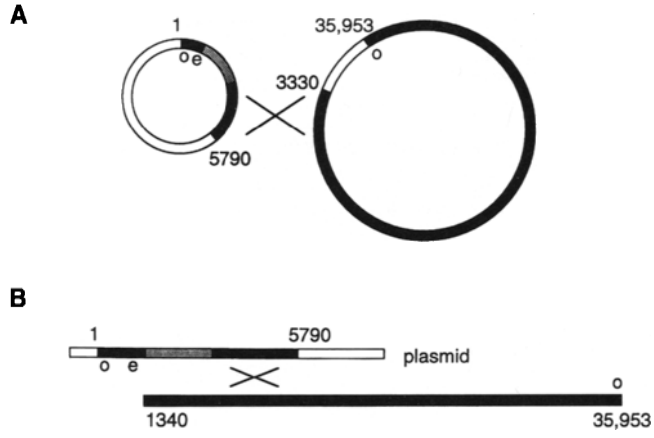

FIG. 2 Strategies for recombinant adenovirus isolation. (A) Plasmid based. The open regions are plasmid derived, the shaded region contains the foreign gene product, and the black region represents adenovirus DNA. o, Origin of viral DNA replication; e, enhancer–encapsidation signals. The numbers shown are adenovirus 5 sequence positions at the boundaries of viral DNA, whereas the X indicates the region in which homologous recombination will regenerate viable viral DNAs. (B) Using purified viral DNA. A linearized plasmid DNA and viral DNA segment are indicated. Designations are as in part (A).

strains allow for the generation of this substrate. Gene replacement manipulations are performed in the plasmid, which provides the missing origin of replication and DNA encapsidation signal, powerful biological selections for the acquisition of plasmid sequences, as well as a substrate for homologous recombination with the viral DNA segment (see Fig. 2). The protocol provided below includes methods for viral DNA isolation, since our laboratory routinely uses this strategy. Also, the method of virus purification can be used to produce high-titer stocks [10^{12} plaque-forming units (pfu)/ml or more] for administration into humans or animal models.

Plasmid Cloning Vector

The standard arrangement for a plasmid-derived substrate for recombinant adenovirus isolation consists of the following elements, in linear order (a model vector is shown in Fig. 3).

1. *Left-end adenovirus 5 sequences from nucleotides (nt) 1 to 354 (the SacII site).* This region includes an origin of replication, contained in the terminal 36–45 bp (12), redundant DNA encapsidation signals, located between nt 238 and 358 (13),

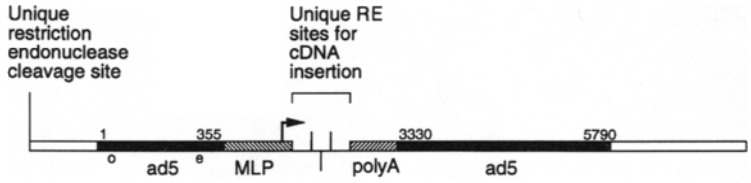

FIG. 3 Generic vector for replacement of the *E1* gene region in adenovirus (ad). A linear form of the vector is shown. For details see the text. MLP, Adenovirus major late promoter/tripartite leader; poly(A) is the simian virus 40 polyadenylation signal; RE, restriction endonuclease; other designations are as in Fig. 2.

and the enhancer portion of the *E1a* transcription control region (14). The extreme left end need not extend exactly to nt 1, as following recombination a second copy of the replication origin, which is repeated at the right end, will regenerate a complete left-end copy during DNA replication (15). Some vectors contain sequences extended to nt 452 (the *Pvu*II site), still deleting the *E1a* promoter, but including all of the rightmost encapsidation sequence.

2. *Transcription control elements for the gene to be insert.* There are two common expression systems. One uses the *E1a* enhancer to drive the major late promoter and the 5′ untranslated region (known as the tripartite leader sequence, since it is derived from a three-exon structure). The 5′ untranslated region enhances the translational efficiency of downstream open reading frames (16). The second uses a completely foreign transcription control region, such as the human cytomegalovirus major immediate-early enhancer promoter, which mediates high levels of expression in a wide variety of cell types.

3. *Unique restriction enzyme sites for insertion of the foreign gene (usually as cDNA).*

4. *mRNA 3′ end-forming element.* Most expression systems use a polyadenylation site derived from the simian virus 40 early transcription unit to ensure 3′ end formation. However, the 3′ adenovirus sequences present in most vectors (see item 5) will provide both 3′ end formation and splicing signals for the genetic insert.

5. *Substrate for homologous recombination.* After the foreign gene sequences and their transcription control elements, vectors usually contain adenovirus sequences from nt 3327 (the *Bgl*II site) to 5788 (the *Xho*I site) to serve as the substrate for homologous recombination. In our experience regions as small as 1000 bp serve just as well.

6. *Unique restriction enzyme site at the boundary of or within plasmid sequences for linearizing the vector without interrupting the viral gene replacement unit.* Such sites need not be adjacent to the left end of adenovirus DNA. The left end will be regenerated during DNA replication (10).

Virus Strains

For replacement of genes in the *E1* region of the viral genome, the commonly used strains are *dl*309 and *dl*327. *dl*309 is a biologically selected, restriction enzyme–site-loss variant of wild-type adenovirus 5 that retains only a single *Xba*I site at nt 1339 (17, 18). *dl*309 also has a single *Cla*I site at nt 917 (adenovirus 5 is 35,953 bp). Digestion of the viral DNA with these two enzymes produces 920- and 420-bp fragments, as well as the large segment containing most of the genome. As it lacks an origin of replication and a DNA encapsidation signal, the large segment is noninfectious, even in 293 cells, which supply the missing *E1a* gene functions. However, after recombination with homologous sequences in a plasmid containing the missing *cis*-acting elements, viable genomes are regenerated. The small fragments present in the digestion mixture can also ligate to the large segment to restore viability; in fact, ligation of the 920-bp fragment, which contains both *cis*-acting signals, is sufficient to restore viability in 293 cells. However, in practice, it is not necessary to separate the large segment from the small viral DNA fragments; by providing an excess of plasmid DNA, the recombination reaction occurs at a frequency sufficiently high that 20–60% of the restored *cis*-acting signals are plasmid derived.

The virus encapsidates no more than 105% of the wild-type length DNA (8, 19). If the foreign sequences including the inserted gene contain more than 1800 bp of extra DNA, after accounting for the removal of *E1* sequences, then the virion packages deletion variants only. In this case extra space can be obtained by using strain *dl*327, which lacks an additional 1878 bp in region *E3* (20). *dl*327 is nondefective for growth in tissue culture, although, in our experience, plaque formation on 293 cells takes 2–3 additional days (see below). *dl*327 contains multiple *Xba*I sites but only the single *Cla*I site. Therefore, substrate for recombination should be generated with *Cla*I only. Despite the single digestion site, the background of undigested (or religated) genomes with *dl*327 generally is no higher than that with *dl*309.

293 Cells

In our experience the most important variable in recombinant virus isolation is the condition of the 293 cells, which are used to propagate adenoviruses that lack *E1a* and *E1b* genes. This cell line was originally isolated by Graham and co-workers as a single transformant of human embryonic kidney cells by sheared adenovirus type 5 DNA (6). The cells express E1a and E1b proteins but not polypeptide IX, which is expressed from a late gene nested within the 3′ part of the *E1b* transcription unit. For efficient growth and plaquing of the virus, 293 cells should have a flat morphology and a doubling time of about 36 hr. Although the cells are not overly adherent, they remain attached to plastic surfaces during the transfection or infection adsorption

period. In 1981 we obtained a lot of cells from the American Type Culture Collection (ATCC, Rockville, MD) (CRL 1573, no batch designation) at passage: 28 that retain the desired properties for about 20–25 subcultures at 1:3 or 1:4. Subsequently, the cells increase their doubling time, change their morphology, and become less adherent. More recently, we obtained another lot of cells (batch F-10062) at passage: 31 that grow in islands rather than as a more dispersed monolayer and do not adhere to the plastic sufficiently for the manipulations. We do not know the conditions responsible for the differences in these cells. Lower passage cells with the desired properties may be purchased from Microbix Biosystems, Inc., Ontario, Canada.

Methods

Purification of Adenovirus DNA

Solutions

Tris-buffered saline (TBS): 8 g/liter NaCl, 3 g/liter KCl, 1 g/liter Na_2HPO_4, 10 g/liter dextrose, 30 g/liter tris(hydroxymethyl)aminoethane, 17 ml/liter HCl, 1 g/liter $MgCl_2 \cdot 6H_2O$, and 1 g/liter $CaCl_2$, adjusted to pH 7.4 and filtered to sterilize

0.01 *M* Tris, pH 8.1: 12.1 g/liter tris(hydroxymethyl)aminoethane, adjusted to pH 8.1 with HCl and filtered to sterilize

TCTFE: 1,1,2-Trichloro-1,2,2-trifluoroethane (Mallinckrodt Inc., St. Louis, MO)

CsCl ($\rho = 1.43$): Dissolve 43 g of CsCl in 60 ml of 0.01 *M* Tris, pH 8.1; adjust with solid CsCl or buffer to a refractive index of 1.3745 ± 0.002.

CsCl ($\rho = 1.23$): Dilute CsCl ($\rho = 1.43$) with an equal volume of 0.01 *M* Tris, pH 8.1; adjust with solid CsCl or buffer to a refractive index of 1.3561 ± 0.002.

CsCl ($\rho = 1.34$): Dissolve 57 g of CsCl in 112 ml of 0.01 *M*Tris, pH 8.1; adjust with solid CsCl or buffer to a refractive index of 1.3665 ± 0.002.

Storage solution: 0.01 *M* Tris–HCl (pH 8.1), 0.15 *M* NaCl, 0.1% (w/v) bovine serum albumin (crystalline, fraction V), and 50% (v/v) glycerol 0.02 *M* sodium phosphate (pH 7.2) and 0.5% (w/v) sodium dodecyl sulfate (SDS)

Tris–EDTA: 0.1 *M* Tris–HCl (pH 8.5) and 0.01 *M* EDTA

Phenol–Tris–EDTA: Distilled phenol saturated with Tris–EDTA

Chloroform–isoamyl alcohol (IAA): 24 parts choloroform and 1 part isoamyl alcohol

TE: 0.01 *M* Tris–HCl (pH 7.5) and 0.001 *M* EDTA

Procedure

The amount of viral DNA desired will determine the volume of cell culture to be infected. The theoretical yield of viral DNA from 1 OD_{260} (optical density at 260 nm)

of disrupted virions (about 10^{12} particles) is about 40 μg. One liter of infected culture (3×10^8 cells) should yield about 13 OD units. Therefore, the theoretical yield of viral DNA from 1 liter of culture is about 520 μg. The actual recovery of DNA from extracted virions is about 60%.

HeLa or KB cells are maintained in suspension cultures between 3×10^5 and 8×10^5 cells per milliliter in Eagle's minimum essential medium for suspension supplemented with 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.075% (w/v) sodium bicarbonate, 0.1% (w/v) glucose, and 5% (v/v) fetal bovine serum (FBS). Prior to infection (21) collect the cells by centrifugation for 15 min at 600 *g* at room temperature (or 37° C, if a warm room is available) and resuspend in warm medium without serum at a concentration of 10^7 cells per milliliter (but in no case less than 10 ml) in a suspension flask. Add adenovirus strain *dl*309 or *dl*327 at a multiplicity of 15 pfu per cell and incubate the culture at 37° C for 1 hr. Adjust the cell concentration to 3×10^5 cells per milliliter by adding the infected cells to a suspension culture flask containing warm medium supplemented with 2% (v/v) FBS. After incubation for 36–40 hr at 37° C, collect the cells by centrifugation at 600 *g* at 4° C for 10 min and resuspend in ice-cold TBS at 0.04 vol of the cell suspension. Again, collect the cells by centrifugation at 600 *g* at 4° C for 5 min. The pellets may be stored frozen indefinitely at −70° C.

For virus purification (22, 23) thaw the pellets and resuspend them in 8 ml of 0.01 *M* Tris (pH 8.1) per 3×10^8 cells. Lyse the cells by three cycles of freezing and thawing and add an equal volume of TCTFE to the lysate. After vigorous shaking for about 1 min, separate the phases by centrifugation for 5 min at 1500 *g* at 4° C. Carefully remove the virus suspension (top phase) from the interphase and the organic (bottom) phase. The suspension may be reextracted with TCTFE.

For every 8 ml of virus suspension, prepare a 14 × 89-mm ultracentrifuge tube containing 2 ml of CsCl ($\rho = 1.23$), layered carefully over 2 ml of CsCl ($\rho = 1.43$). Layer the virus suspension over the step gradient and centrifuge in an SW41 rotor (Beckman Instruments, Inc., Fullerton, CA) at 30,000 rpm for 2–4 hr at 4° C. The virus band at the interface of the two CsCl concentrations appears very sharp. Empty capsids band slightly higher than the virions, and other diffuse or flocculent material may be distributed in the gradient. Collect the virions in as small a volume as possible. The virions are purified further by equilibrium density centrifugation. Adjust the volume of the virion suspension to 5 ml with CsCl ($\rho = 1.34$); add it to a preweighed 13 × 51-mm ultracentrifuge tube. The net weight of the contents should be 6.8 g. If the weight is less, add solid CsCl to adjust to 6.8 g; if the weight is more, add 0.01 *M* Tris (pH 8.1) to adjust the density to 1.34 g/ml. Centrifuge to equilibrium in an SW55 rotor at 30,000 rpm for 18–20 hr at 4° C and collect the virus band. The virus can be rebanded if additional purity is required. If the virus is to be used for DNA preparation, it may be stored indefinitely at 4° C. If the virus is to be used as a stock for reinfection, it should be diluted 10-fold with storage solution and kept at −20° C.

To determine the virion concentration, dilute virus suspensions in CsCl 1:50 in 0.02 *M* sodium phosphate (pH 7.2) plus 0.5% (w/v) SDS. One OD_{260} unit per milliliter is equivalent to 1.1×10^{12} particles (24). To prepare viral DNA, dialyze the virus suspension for 2 hr at 4° C against 500 ml of Tris–EDTA (precipitation may occur) and transfer the virus suspension to a 15-ml glass high-speed centrifuge tube. Add SDS to a final concentration of 1% and proteinase K to a final concentration of 1 mg/ml. Incubate at 37° C for 2 hr. Add 1 vol of phenol–Tris–EDTA and 0.2 vol of chloroform–IAA and mix gently by inversion for 3 min. Centrifuge at 15,000 *g* for 5 min at 4° C to separate the phases. If an interphase (white) is present, remove the top phase (which contains DNA) with a large-bore pipette. If no interphase is present, remove the bottom phase (organic) from below the aqueous phase and discard the organic phase. Repeat the extraction twice. After the last extraction remove the aqueous phase containing the DNA. Dialyze the viral DNA for 24 hr at 4° C against three changes of 500 ml of 0.1× TE. The viral DNA may be frozen at −20° C for long-term storage (i.e., more than a few months) or kept at 4° C.

Preparation of DNA Substrates for Transfection

Strain *dl*309 DNA contains a single ClaI site and a single *Xba*I site. Strain *dl*327 contains multiple *Xba*I sites, and so should be digested only with *Cla*I. We generally prepare at least 50 μg of substrate at a time in a total volume of about 0.5 ml. Once the digestion is verified as complete, add EDTA to a final concentration of 20 m*M* and store the digestion mixture at 4° C or −20° C until use. Make the plasmid DNA linear by restriction enzyme digestion (we usually prepare 10 μg), adjust the solution to 20 m*M* EDTA, and store as described above for the viral DNA.

293 Cells

293 cells are maintained in Dulbecco's modified Eagle's medium (MEM) supplemented with 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.075% (w/v) sodium bicarbonate, and 10% (v/v) FBS (GIBCO Laboratories, Grand Island, NY). Subculture the cells using only versene (0.4 g/liter EDTA, 8 g/liter NaCl, 1.1 g/liter Na_2HPO_4, 0.2 g/liter NaH_2PO_4, and 0.2 g/liter KCl, adjusted to pH 7.4 and autoclaved). Note that the cells are sensitive to the versene and plating efficiency is increased by performing all manipulations as soon as the cells detach from the dish. Although 293 cells in a confluent monolayer will continue to divide and crowd, for best results subculture cells immediately upon reaching confluence (about 2×10^7 cells per 150-cm^2 flask). Subculture the cells routinely at 1:3 or 1:4 twice a week. For transfection or plaque assays seed 60-mm dishes at 1:2 (1.5×10^6 cells per dish) and use them 2 days after subculture.

Transfection and Virus Isolation

Solutions

10× HEPES-buffered saline (HBS): 50 g/liter HEPES, 80 g/liter NaCl, 3.7 g/liter KCl, 1.25 g/liter Na_2HPO_4, and 10 g/liter dextrose, filtered to sterilize and stored at 4° C

2× HBS: Take 10× HBS and add 3 vol of sterile distilled water. Adjust the pH to 7.10 ± 0.05 with NaOH and adjust the final concentration to 2× HBS. Filter to sterilize and store at 4° C for up to 6 months.

2.5 *M* $CaCl_2$: Filter to sterilize and store in 0.5- to 1.0-ml aliquots at −70° C.

Glycerol medium: 50% 2× Dulbecco's MEM, 20% (v/v) glycerol, 2 m*M* glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.075% (w/v) sodium bicarbonate.

Overlay medium: Mix 50 ml of 2× Dulbecco's MEM, 1 ml of 0.2 *M* glutamine, 1 ml of 10,000 U/ml penicillin, 1 ml of 10 mg/ml streptomycin, 3 ml of 7.5% (w/v) sodium bicarbonate, 1 ml of 10% (w/v) yeast extract, and 5 ml of FBS and keep at 45–50° C. Melt 50 ml of sterile 2% agar (Bacto-Agar, Difco Laboratories, Detroit, MI) and cool to 45–50° C. Mix with medium and keep at 45–50° C until use. The medium should be held at this temperature for no longer than 45 min.

Phosphate-buffered saline A (PBS-A): 8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na_2HPO_4, and 0.2 g/liter NaH_2PO_4, adjusted to pH 7.2 and autoclaved, then adjusted to 0.1 g/liter $CaCl_2$, 0.1 g/liter $MgCl_2 \cdot H_2O$, and 0.1% (w/v) bovine serum albumin with filtered stock solutions (20× for the salts, 50× for the albumin)

Procedure

For the transfection (25) all manipulations should be performed in a laminar flow hood with sterile materials (except for the DNA solutions). In a 1.5-ml microcentrifuge tube mix 2 μg of viral DNA, 4 μg of plasmid DNA, and 0.05 ml of $CaCl_2$, and adjust the volume to 0.5 ml with sterile distilled water. Add 0.5 ml of 2× HBS to a 15-ml polypropylene centrifuge tube. Connect a disposable 1-ml pipette to a filtered air supply and insert the pipette into the centrifuge tube so that the tip is below the meniscus of the HBS. Bubble air at about two to three bubbles per second. Add the DNA solution dropwise to the side of the centrifuge tube and allow it to run down the tube into the bubbled HBS. Remove the 1-ml pipette, cap the tubes, and let stand at room temperature for 30–45 min. The precipitate should be milky but not flocculent. Failure to form a precipitate is most often due to incorrect pH of the HBS.

Remove the medium from the 293 cells. Gently tap the centrifuge tube to resuspend the precipitate and add 0.5 ml dropwise to each of two dishes. Keep at 37° C for

20 min and then add growth medium containing 0.1× HBS and 12.5 m*M* $CaCl_2$. Return the dishes to the incubator for 4–5 hr.

DNA uptake is promoted by a short exposure of transfected cells to medium containing 20% glycerol (26). Remove the medium and add 3 ml of glycerol medium for 45 sec. Since the time of exposure to glycerol medium is critical, expose only two to four dishes at a time. Remove the glycerol medium and immediately wash the dishes twice with 3 ml of growth medium without serum. Do not remove the last wash until all of the dishes have been treated. Then remove the last wash and add 6 ml of overlay medium to each dish by running the medium down the inside of the edge of the dish. When the overlay medium solidifies, return the dishes to the incubator.

These amounts of DNA generally produce five to 30 plaques per plate. We often run a control without plasmid, and it is not unusual to see backgrounds at an equivalent level, possibly from end-to-end ligation of the DNA fragments in the viral DNA digestion mixture. Do not be discouraged if this should occur. The presence of the homologous viral DNA sequences in the plasmid seems to favor recombination over ligation, so that a significant proportion of the plaques obtained in the presence of plasmid DNA are recombinant.

With a strain *dl*309 genetic background plaques are usually visible within 4–5 days and can be picked on day 6 or 7. With a strain *dl*327 background plaques may not be visible until 6–7 days and can be picked on day 8 or 9. Plaques should be visible without staining. Mark the location of the plaque on the underside of the dish. Using a cotton-plugged sterile Pasteur pipette, stab the agar over the plaque and aspirate the agar plug into the pipette. Expel the agar plug into 1 ml of PBS-A in a 5-ml polyethylene culture tube, and wash out the pipette several times with the same 1 ml of PBS-A. Vortex the tubes briefly to resuspend the virus particles in the agar plug and store at −20° C.

Identification of Recombinant Viruses

Solutions

Hirt buffer 1: 0.01 *M* tris(hydroxymethyl)aminoethane, 0.01 *M* EDTA, 0.01 *M* NaCl, and 0.5% (w/v) Nonidet P-40, adjusted to pH 7.2
Hirt buffer 2: Same as buffer 1 but without detergent
5 *M* NaCl
TE–phenol: Distilled phenol saturated with TE
Chloroform–IAA (as above)
TE (as above)
1 *M* sodium acetate (pH 5.0)
Ethanol
RNase A: 2 mg/ml DNase-free RNase

Procedure

The following protocol is used to screen for the predicted genomic structure of the desired recombinant. Other screening methods may be used to identify viruses containing a particular genetic insertion or producing a desired protein. The protocols described here will assist in manipulating the infected cells.

Remove the medium from a 60-mm dish containing a subconfluent monolayer of 293 cells and wash once with 2 ml of PBS-A. Remove the wash and add 0.5 ml of the virus plaque suspension. After a 1-hr adsorption period at 37° C, during which the dish is tilted every 15 min to keep the monolayer moist, add 4.5 ml of growth medium. A cytopathic effect should be evident within 1–2 days and rampant by 3–4 days. The cells round up and aggregate into characteristic "grapelike clusters" and float in the medium. When all of the cells are rounded or detached, remove the cells and the medium into a 15-ml polyethylene centrifuge tube. Freeze and thaw the suspension three times and remove the debris by centrifugation at 1500 *g* for 15 min at 4° C. Transfer the suspension to a clean sterile culture tube and store at −70° C. This preparation is referred to here as a "plaque stock" and generally has a titer of about 10^8 pfu/ml.

Screening for genomic structure is carried out according to the method of Volkert and Young (27). Infect a subconfluent monolayer of 293 cells in a 60-mm dish as above, with 0.25–0.5 ml of the plaque stock. After 20–24 hr, if the cells are still attached to the dishes, remove the medium and add 1 ml of TBS. Remove the cells from the surface by pipetting and transfer them to a 1.5-ml microcentrifuge tube. (If the cells are floating, collect the cells and the medium in a 15-ml polypropylene centrifuge tube and pellet the cells at 600 *g* for 10 min at 4°. C. Resuspend the cells in 1 ml of TBS and transfer them to a 1.5-ml microcentrifuge tube.) Pellet the cells at 4° C in a microcentrifuge for 15 sec and resuspend in 1 ml of Hirt buffer 1. The cells should be disrupted, but the nuclei containing the viral DNA will remain intact. Pellet the nuclei at 4° C in a microcentrifuge for 30 sec. Carefully remove the supernatant and discard. Thoroughly disperse the nuclei in 0.5 ml of Hirt buffer 2 and incubate at 37° C for 2hr. Add 0.25 ml of 5 *M* NaCl and keep on ice for 30 min (or overnight).

Remove the precipitate by centrifugation at 27,000 *g* for 30 min at 4° C. In our experience a microcentrifuge is not sufficient for this separation. Carefully transfer the supernatant (pouring works best, although some viscous material may be left behind) to a fresh microcentrifuge tube. Extract the supernatant with 1 vol of TE–phenol and 0.25 vol of chloroform–IAA. Mix for 5 min and separate the phases in the microcentrifuge for 1 min. Collect the aqueous phase containing the nucleic acid and repeat the extraction with 1 vol of chloroform–IAA. Precipitate the nucleic acid with 2 vol of ethanol.

Centrifuge the nucleic acid for 10 min in a microcentrifuge at 4° C. Carefully remove the supernatant. (*NOTE: The pellet is easy to disturb and may be hard to see, so be very careful not to lose it.*) If some supernatant is still present, centrifuge the

tube for a few seconds and remove the remaining supernatant. Resuspend the pellet in 0.2 ml of 0.1× TE. Add 0.02 ml of 1 *M* sodium acetate and 0.5 ml of ethanol to reprecipitate the nucleic acid. Collect the precipitate as before, making sure to remove as much of the supernatant as possible. Thorough removal of the supernatant will increase the efficiency of subsequent restriction endonuclease digestion.

Resuspend the nucleic acid in 0.05 ml of 0.1× TE. Add 2 μl of RNase A and incubate at 37° C for 10 min. These preparations can be stored indefinitely at 4° C. An 8-μl aliquot (in a 10-μl reaction mixture) is sufficient for restriction endonuclease analysis.

Figure 4 shows an example of the data generated from restriction endonuclease digestion of "rapid" DNA preparations. The background arises from digestion of the variable amount of cellular DNA that contaminates the viral DNA preparations. The *Hin*dIII site in region *E1* of the parental strain (*dl*327) produces DNA fragments E and G. These fragments are predicted to be lost, with the gain of a high-molecular-weight fragment when *E1* is replaced by a gene insert (in this case, the human cyto-

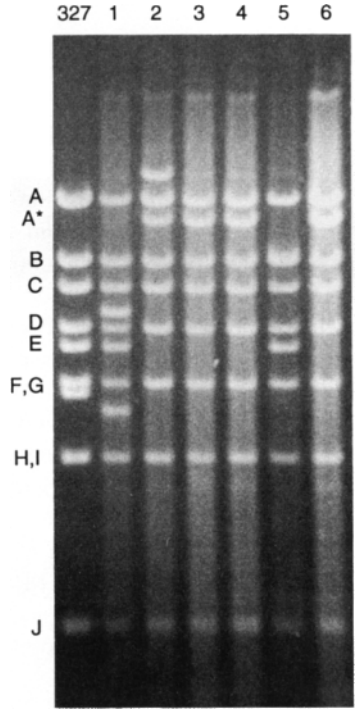

FIG. 4 Restriction endonuclease site screening for recombinant virus genomes. DNA preparations isolated as described in the text were cut with *Hin*dIII and the patterns are displayed in a 1% (w/v) agarose gel. For details see text.

megalovirus *IE2* gene) lacking a *Hin*dIII site. Candidate genomes containing the predicted structure (new fragment A*) were identified in lanes 3, 4, and 6, with a possibly incompletely digested DNA in lane 2. Lanes 1 and 5 contained viral DNAs resembling neither the parent nor the predicted recombinant.

Once a sample with the desired genomic structure is identified, the stock may be characterized further by protein production or polymerase chain reaction-based sequencing. Generally, about 20–60% of the samples screened will have the desired structure. Because of diffusion under the agar overlay, plaque stocks often will be cross-contaminated with the progeny of other plaques. Therefore, we routinely perform two additional rounds of plaque purification. Titer either the original agar plug sample (about 10^5/ml) or plaque stock (about 10^8/ml) on 293 cells. Dilute the virus serially in PBS-A and add to dishes exactly as described for the preparation of plaque stocks, except that after the 1-hr adsorption period, add 6 ml of agar overlay rather than growth medium. Pick plaques from plates containing the least number of plaques (three to 10). Picked plaques suspended in PBS-A should be either rescreened as described above or immediately replaqued. Always rescreen plaques after the final purification for the correct genetic structure or expression. To ensure purity, verify that all plaques on the dish containing the chosen isolate have identical structures.

Growth of High-Titer Virus Stocks in 293 Cells

For routine stock preparation infect 293 cells growing in large flasks or roller bottles of 293 cells with 5 pfu per cell as described above for 60-mm dishes and incubate in growth medium for 48–52 hr at 37° C. The infected cells usually disperse into the medium with only mild physical agitation of the culture flask or bottle. Prepare cell pellets containing virus exactly as described above for growth of wild-type virus strains in KB or HeLa cells. If the virus is to be used as a stock for repassage, resuspend the pellet in 1 ml of 0.01 *M* Tris (pH 8.1) per 150 cm^2 flask (about 2×10^7 cells), freeze and thaw the suspension three times, and remove the debris by centrifugation at 1500 *g* for 30 min at 4° C. Aliquot the virus-containing supernatant and store at −70° C. Stocks prepared in this way usually have titers of 10^{10} or greater and can be frozen and thawed multiple times.

If more concentrated stocks are needed, resuspend the infected cell pellets in 8 ml of 0.01 *M* Tris–MCl per 3×10^8 cells and purify the recombinant as described above for wild-type virus. It may be necessary to remove the CsCl by dialysis prior to administration into animals. Viral yields per cell from 293 cells generally are about one fifth of those from KB or HeLa cells. 293 cells can be established in suspension culture, and there have been reports using such cultures to facilitate virus isolation. As with 293 cell monolayers, these cultures vary in their properties, especially with respect to virus propagation, and lines should be evaluated and monitored carefully. Virus stocks should be titered on 293 cells using the procedures described, except

that a second overlay of 3 ml per dish is added on day 5 (*dl*309-derived viruses) or day 6 (*dl*327-derived viruses). The medium used for the second overlay contains 0.005% (w/v) neutral red to assist in the visualization of plaques.

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References

1. M. A. Rosenfeld, W. Siegfried, K. Yoshimura, K. Yoneyama, M. Fukayama, L. E. Stier, P. K. Paakko, P. Gilardi, L. D. Stratford–Perricaudet, M. Perricaudet, S. Jallat, A. Pavirani, J. P. Lecocq, and R. G. Crystal, *Science* **252,** 431 (1991).
2. J. Chroboczek, F. Bieber, and B. Jacrot, *Virology* **186,** 280 (1992).
3. K. L. Berkner and P. A. Sharp, *Nucleic Acids Res.* **11,** 6003 (1993).
4. T. J. Kelly and A. M. Lewis, *J. Virol.* **12,** 643 (1973).
5. Y. Haj-Ahmad and F. L. Graham, *J. Virol.* **57,** 267 (1986).
6. F. L. Graham, J. Smiley, W. C. Russell, and R. Nairn, *J. Gen. Virol.* **36,** 59 (1977).
7. G. Chinnadurai, S. Chinnadurai, and J. Brusca, *J. Virol.* **32,** 623 (1979).
8. Ghosh-Choudury, Y. Haj-Ahmad, and F. L. Graham, *EMBO J.* **6,** 1733 (1987).
9. W. J. McGrory, D. S. Bautista, and F. L. Graham, *Virology* **163,** 614 (1988).
10. N. D. Stow, *J. Virol.* **37,** 171 (1981).
11. Y.-S. Ho, R. Galos, and J. Williams, *Virology* **122,** 109 (1982).
12. R. T. Hay and I. M. McDougall, *J. Gen. Virol.* **67,** 321 (1986).
13. M. Grable and P. Hearing, *J. Virol.* **64,** 2047 (1990).
14. P. Hearing and T. Shenk, *Cell (Cambridge, Mass.)* **33,** 695 (1983).
15. N. D. Stow, *Nucleic Acids Res.* **10,** 5105 (1982).
16. J. Logan and T. Shenk, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3655 (1984).
17. N. Jones and T. Shenk, *Cell (Cambridge, Mass.)* **17,** 683 (1979).
18. B. Thimmappaya, N. Jones, and T. Shenk, *Cell (Cambridge, Mass.)* **18,** 947 (1979).
19. A. J. Bett, L. Prevec, and F. L. Graham, *J. Virol.* **67,** 5911 (1993).
20. B. Thimmappaya, C. Weinberger, R. J. Schneider, and T. Shenk, *Cell (Cambridge, Mass.)* **31,** 543 (1982).
21. E. Everitt, B. Sundquist, and L. Philipson, *J. Virol.* **8,** 742 (1971).
22. M. Green and M. Pina, *Virology* **20,** 199 (1963).
23. B. Sundquist, E. Everitt, L. Philipson, and S. Hoglund, *J. Virol.* **11,** 449 (1973).
24. J. V. Maizel, D. O. White, and M. D. Scharff, *Virology* **6,** 115 (1968).
25. F. L. Graham and A. J. van der Eb, *Virology* **52,** 456 (1973).
26. E. Frost and J. Williams, *Virology* **91,** 39 (1978).
27. F. C. Volkert and C. S. H. Young, *Virology* **125,** 175 (1983).

[4] Construction and Use of Recombinant Vaccinia Virus Vectors

Douglas Grosenbach and Dennis E. Hruby

Introduction

Elucidation of the mechanisms governing the expression and regulation of eukaryotic genes has been, and continues to be, the focus of a great deal of research interest. Recently, it has become possible to isolate individual components of these systems and express them within the context of a heterologous system in order to assess their biological activity. Likewise, a central approach to producing subunit vaccines involves the expression of cloned bacterial or viral genes in eukaryotic systems in order to induce protective immunity without exposure to the pathogen itself. New techniques in genetic engineering and molecular biology have provided several novel approaches for the construction and use of recombinant eukaryotic vector systems. These include a variety of mammalian expression plasmids (1), and a number of different animal virus vectors (2–6), of which vaccinia virus (VV) is the most well known (7).

VV has been used as a eukaryotic cloning and expression vector for the study of gene systems and protein expression as well as for the development of new vaccines. The development of VV as a vector has been fueled by basic research into the replication of poxviruses (Fig. 1), which has identified many attributes contributing to its use as recombinant vectors or vaccines (8). These attributes include a completely sequenced, large, double-stranded DNA genome that accepts large and/or multiple foreign DNA inserts. DNA replication and transcription take place entirely within the cytoplasm of infected cells and are mediated by virus-encoded enzymes and cofactors that act on VV-specific sequences. This eliminates the necessity of maintaining many of the contextual requirements needed for eukaryotic gene expression, such as promoters, enhancers, splice junctions, polyadenylation sites, and RNA transport signals. Ideally, an open reading frame or cDNA may be directly abutted to a VV promoter, resulting in expression levels characteristic of that promoter and independent of host regulatory or enzymatic functions. In addition to the ease of construction, the cytoplasmic replication of VV nullifies many safety concerns inherent to other recombinant viral vaccines that replicate in the nuclear compartment in close proximity to cellular genes. Mammalian proteins expressed from VV are processed and modified in a manner comparable to that when expressed in their native environment, while the broad host range of VV allows for expression in numerous species and cell types (8). Recombinant VV-based vaccines are inexpensive to produce,

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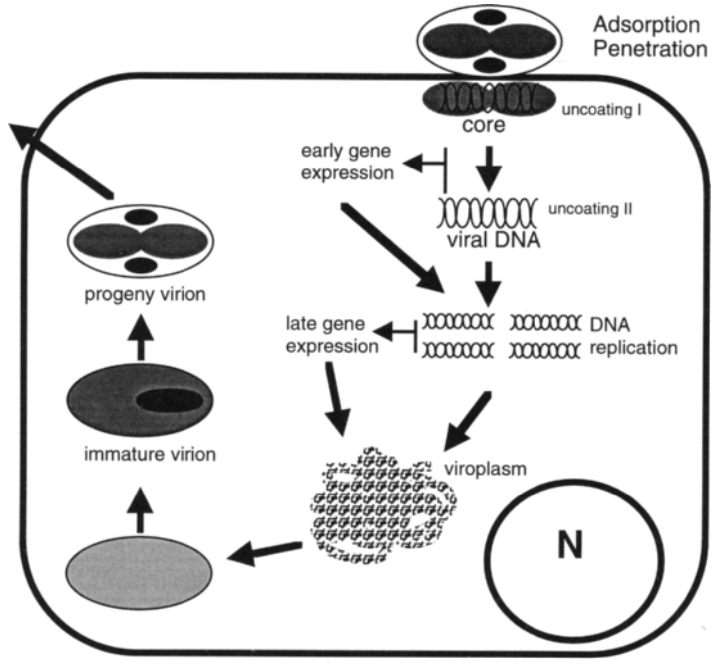

FIG. 1 Vaccinia virus replication cycle. N, nucleus.

stable for years after lyophilization, and safe in immunocompetent hosts, inducing both humoral and cellular responses.

Our laboratory at Oregon State University has had considerable experience with constructing recombinant VV for a variety of purposes. These include recombinants used for the study of VV itself, utilizing recombinant DNA technology to insertionally inactivate genes (9) or introduce foreign transcriptional regulatory elements (10). These recombinants have been useful in identifying the function of gene products and the importance of temporal regulation of expression as determined by resulting phenotypic differences between recombinant and wild-type viruses. Other recombinant viruses have been constructed for the purpose of overexpression of heterologous or fusion proteins. Proteins expressed in this fashion include chloramphenicol acetyltransferase, neomycin phosphotransferase, human proenkephalin, Sindbis virus structural proteins, tobacco etch virus polyproteins, murine nerve growth factor, yeast *KEX2* endopeptidase, murine preproopiomelanocortin, and streptococcal M protein. The expressed proteins have been analyzed for correct proteolytic processing, immunogenicity and recognition by antisera, incorporation into virions, cell-type dependent differential expression, enzymatic activity, and ability to confer antibiotic resistance on recombinant viruses.

In this article we draw on our experience to outline the rationale for the construction and use of recombinant VV-based expression systems as well as comment on the specific techniques and methodology required to use this system.

Safety Precautions

VV has been used as a live vaccine against smallpox for over 200 years resulting in the eradication of this disease (11). Vaccinia virus is related to variola virus, the causative agent of smallpox, but does not itself cause disease. It is not related to varicella virus, a herpesvirus that causes chickenpox. Although older adults have been exposed to VV through vaccination, the younger generation is immunologically naive. Therefore, the Centers for Disease Control and Prevention and the National Institutes of Health recommend that those working with VV are vaccinated against it on a 10-year basis. However, the benefit and necessity of this have recently been questioned (12). Exposure of those with skin disorders or immunodeficient persons to VV is contraindicated, and even healthy individuals should avoid exposure of the mucous membranes to droplets or aerosols that might contain VV.

Class I or II biological safety cabinets and institution of biosafety level 2 practices are appropriate for the use, handling, and storage of VV (13). Organizational safety management should be consulted for additional requirements involving the handling of pathogenic organisms. Additionally, the safety precautions specific for the organism from which the foreign DNA is derived are suggested for that DNA and its product(s).

Principles of the System

The general premise of the VV expression system is as follows (see Fig. 2): (1) A gene or cDNA of interest is cloned into a plasmid behind a VV promoter with little or no 5′ untranslated sequence intervening and little or no extraneous 3′ terminal sequence included. (2) This expression cassette is flanked by sequence homologous to a nonessential gene in VV, which serves as a target for recombination into the VV genome. (3) Cells are infected with VV followed by transfection with the recombination plasmid. (4) Total virus yields are isolated and subjected to plaque assay under selective or screening conditions, depending on the type of construct. (5) Viral plaques presumed to contain the gene of interest are amplified and screened for the appropriate genotype. (6) Potential recombinants should then be tested to determine transcription and translation kinetics before proceeding with experiments for which the recombinant was designed and constructed. High-titer stocks may be made and stored at $-70°$ C, at which they are stable for many years.

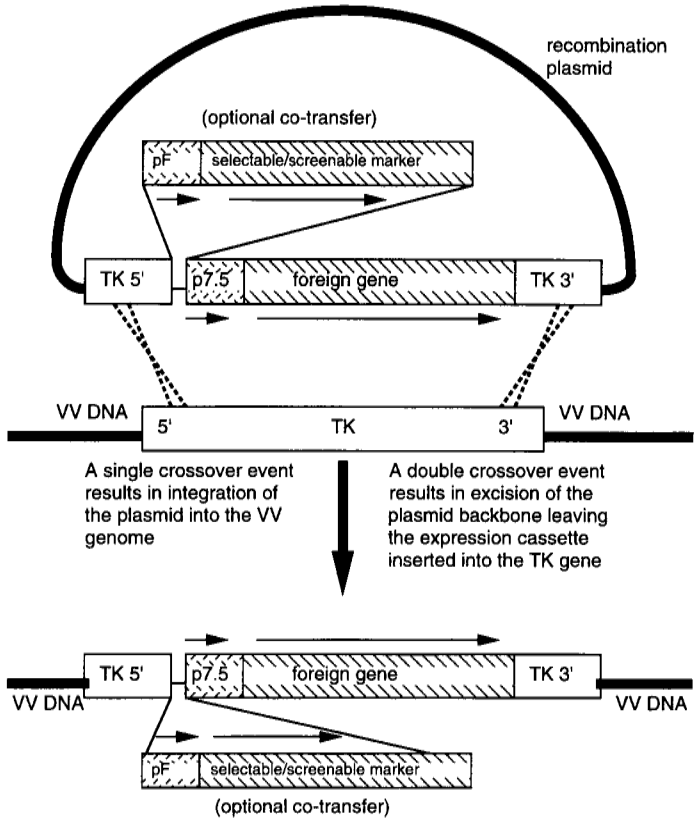

FIG. 2 Schematic representation of events leading to the production of recombinant vaccinia virus (VV). pF, F promoter; TK, thymidine kinase gene.

Following the general guidelines presented here, it is possible to have a purified stock of recombinant VV within 3–4 weeks from the date of the initial transfection with the recombination plasmid. The time required for construction of the plasmid vector will vary depending on the ease with which the gene or cDNA may be isolated and cloned. Likewise, the time involved in the further characterization of expressed products is contingent on the specific design of the system and the techniques utilized.

Materials and Methods

Standard molecular biological techniques and manipulations of DNA are carried out essentially as described by Maniatis and co-workers (14). Various strains of VV,

including the WR, Copenhagen, and IHD-J strains, have been used in the construction of recombinant vaccinia viruses. These may be obtained from the American Type Culture Collection (ATCC, Rockville, MD) or individual research laboratories and should be propagated, purified, titered, and maintained as previously described (15). Cell lines to be utilized are dependent on the specific needs of the system (see "Optimization of the System") and are maintained and propagated in accordance with ATCC specification, unless otherwise noted.

Choosing the Recombination Plasmid

Many plasmids already exist whose intended use is for the construction of recombinant VV. These are available either commercially or on request from individual research laboratories and are suitable for most routine needs in the construction of recombinant VV. In choosing from the available plasmid vectors, certain variables must be considered, including expression levels needed, expression kinetics desired (or required, if interaction with host gene products is necessary), availability of unique restriction sites, and selection or screening for recombinants.

The level of protein produced and the time frame in which it will be made by recombinant VV during infection are dependent on which promoter the gene or cDNA has been abutted to. Several natural VV promoters, as well as synthetic promoters recognized by the VV transcriptional machinery, have been isolated and characterized with respect to the expression of gene products (8). The 7.5K promoter (p7.5) is a natural promoter for an approximately 7.5-kDa protein of unknown function in VV. Production of this protein commences at early times during a VV infection, tapers off slightly, then recommences at late times, resulting in nearly constitutive expression (16). A promoter active only at early times during infection is the F promoter (pF). Time of expression and protein expression levels from this promoter at early times are similar to those characteristic of p7.5, and it is often used in conjunction with p7.5. The pF is used to direct expression of a co-recombined selectable or screenable marker, while p7.5 directs expression of the gene of interest. This allows early expression of both gene products, while preventing the loss of genetic information due to recombination between the two promoters. A chief concern when using VV early promoters is premature termination of transcription. Message initiated from an early promoter or early/late promoter at early times during infection will be discretely terminated approximately 50 nucleotides downstream of a TTTTTNT sequence (17), where *N* is any nucleotide. If a cryptic termination sequence is present in the gene to be recombined and early expression is desired, then site-directed mutagenesis of one or more nucleotides within this sequence will be necessary to obtain full-length transcripts.

There are also a number of strong, natural or synthetic, late promoters that may be used. Generally, late promoters are used to achieve high levels of expression. The

most widely used late promoters are p11 (18) and pCAE (19), the natural promoters for an 11-kDa protein and the cowpox A-type inclusion protein, respectively. The TTTTTNT sequence is not recognized by VV late transcriptional machinery. Therefore, message initiated from early/late and late promoters at late times during infection does not terminate discretely and will result in mRNAs of heterogeneous length. Hence, analysis of recombinants constructed with early/late or late promoters by Northern blot is of little use, but due to high expression levels of protein, analyses by other methods are feasible (see below). Several recombination plasmids have been constructed with synthetic late promoters recognized by VV transcriptional machinery (20). The expression level observed for these promoters appears to be roughly twice that observed for VV strong late promoters.

The highest expression levels obtained, though, have come from the hybrid VV/bacteriophage T7 system (21). In this system the bacteriophage T7 promoter is used instead of a promoter recognized by the VV transcriptional machinery. After recombination into the VV genome, transcription is by the T7 polymerase, which is either transiently expressed from a transfected plasmid or expressed from a coinfecting virus that has the gene encoding T7 polymerase recombined into its genome previously, such as vTF7-3. The expression levels obtained from this system are approximately 10-fold that observed for expression directed by p7.5. Expression kinetics are regulated only by the promoter directing expression of the T7 polymerase (e.g., p7.5 in vTF7-3).

While expression kinetics and levels are determined by the choice of promoter, the DNA flanking the promoter:gene of interest expression cassette will allow the recombinational event between the plasmid and VV genomic DNA. It is essential that the flanking DNA be nonessential to VV for replication in tissue culture cells. Otherwise, recombination will result in the insertional inactivation of a gene necessary for the survival of VV and no recombinants will be isolated. A number of genes or regions of the VV genome have been identified as nonessential and would serve as appropriate targets for recombination. The likelihood of a recombinational event occurring between the plasmid and the *Hin*dIII C, F, and K regions, the hemagglutinin (*HA*) gene, or the thymidine kinase (*TK*) gene—all of which are nonessential—is the same (approximately 1:1000). However, only recombination into the *HA* and *TK* loci will result in screenable and/or selectable progeny virions without the cotransfer of another marker.

The *TK* locus is most often the target for recombination, because recombination results in insertional inactivation of the *TK* gene, allowing biochemical selection for and amplification of recombinant virions (22). The product expressed from an intact *TK* gene will phosphorylate 5-bromo-2-deoxyuridine (BrdU) if it is added to the medium. Phosphorylation of BrdU allows for its lethal incorporation into the DNA of developing virions. Thymidine kinase-deficient (tk^-) virus will be selected for tk^- cell lines while in the presence of BrdU—permitting the identification, isolation, and amplification of putative recombinants.

The tk^- VV arises at a frequency of 10^{-3} to 10^{-4} and by BrdU selection alone will

be indistinguishable from tk^- virions resulting from inactivation of *TK* via recombination. Thus, BrdU selection alone is not sufficiently stringent for identification of recombinants. Rather, subsequent brute force screening methods must be applied. One can circumvent this latter effort by the cotransfer of a selectable or screenable marker. The marker is placed under the direction of a VV promoter different from that directing the expression of the gene of interest to prevent recombination between them. The expression cassettes for the resistance marker as well as for the gene of interest are placed within the flanking VV DNA, allowing for the cotransfer of the gene of interest and the marker. By dual selection for recombinants, there will be a significant reduction in the amount of work (and time) necessary to isolate recombinant VV.

A number of recombination plasmids are available that will mediate the cotransfer of selectable or screenable markers—some which target the *TK* locus for recombination, others which target various other nonessential genes or regions. Recombinant VV resulting from the transfer of genetic information from the plasmid pSC11 (18) are viable in the presence of BrdU and can be distinguished from spontaneous tk^- mutants by their ability to form blue plaques when grown in the presence of the lactose analog X-gal, due to cotransfer of the *lacZ* gene that codes for β-galactosidase. Progeny virions arising from recombination mediated by the plasmid pTK–gpt–F1s (23) are viable in the presence of BrdU and are able to overcome lethal mycophenolic acid poisoning of guanosine monophosphate (GMP) synthesis. The resistance to mycophenolic acid is due to the cotransfer of the *gpt* gene, which codes for xanthine–guanosine phosphoribosyltranferase and allows utilization of xanthine and hypoxanthine in the GMP salvage pathway.

Our laboratory has shown that the cotransfer of the *neo* gene, which codes for neomycin phosphotransferase II (NEO), confers resistance to the synthetic kanamycin analog, G418, to recombinant viruses (24). G418 selection is exceptionally stringent, resulting in complete abrogation of wild-type plaque formation. Those recombinants expressing NEO, though, retain their ability to form plaques in the presence of G418. An added advantage to using *neo* as a selectable marker is its stability once it is recombined into the VV genome. Due to an inherent instability of the *gpt* gene when it is recombined into VV, mycophenolic acid selection must be maintained if *gpt* is to be preserved. Once selection is removed, it is likely that a nonhomologous recombinational event will occur, resulting in excision of *gpt* from the VV genome. While loss of *gpt* itself is of no concern, excision of *gpt* may disrupt neighboring loci, the most proximal being the gene of interest.

Construction of the Recombination Plasmid

To introduce genes or cDNAs into the VV genome, they must first be mobilized into a plasmid able to mediate recombination. Most plasmids constructed for these purposes contain multiple unique restriction sites just downstream of the VV promoter

to facilitate cloning of the gene or cDNA proximal to the promoter. After restriction with an appropriate restriction endonuclease(s), treatment with T4 DNA polymerase (if a blunt cloning procedure is to be used), and alkaline phosphatase treatment of the plasmid vector, the DNAs are isolated on a low-melting-point agarose gel. While visualizing the ethidium bromide (EtBr)-stained DNA bands with long-wavelength ultraviolet light, the bands corresponding to the appropriate DNAs are excised and transferred to microfuge tubes using a sterile technique. The DNA-containing agarose is melted by incubation at 65°C for 10 min and then frozen at −70°C for 30 min. After a brief thawing period the agarose is pelleted by centrifugation at 12,000 *g* for 10 min. Estimating DNA concentration by fluorescence in the gel, the insert (gene or cDNA) and the plasmid vector are mixed in a 10:1 ratio. T4 DNA ligase and appropriate buffer are added and the reaction is incubated at temperatures and times dependent on the nature of the DNA ends (i.e., sticky versus blunt). The ligation reactions are then transformed into competent *Escherichia coli* and plated onto antibiotic-containing agar. Small-scale preparations of plasmid DNA are made from cultures inoculated with single colonies from the plates. Restriction enzyme analysis is usually sufficient to determine the presence and orientation of the insert with respect to the promoter, but sequencing to ensure the integrity of insert and promoter is the best way to determine that no mutations have been introduced. After identification of clones, large-scale preparations of high-purity DNA must be made. We have found that plasmid DNA purified by either CsCl or anion-exchange methods are transfected efficiently into mammalian cells (25).

Liposome-Mediated Transfection of VV-Infected Ltk⁻ Cells

Ltk⁻ cells are plated onto 35-mm dishes at a density of 5×10^5 cells per dish in normal media. On the following day they are infected with VV at a multiplicity of 0.05 plaque-forming units (pfu) per cell. After a 30-min to 1-hr adsorption at room temperature, the inoculum is removed and replaced with the transfecting media. In the past DNA transfections were by the calcium phosphate technique (26), but recent efforts by our laboratory have adopted the liposome-mediated transfection technique of Rose *et al.* (27) for our own uses, resulting in much higher transfection efficiencies.

Briefly, liposomes are prepared by mixing 100 μl of a 10-mg/ml solution (in $CHCl_3$) of dioleoyl-L-α-phosphatidylethanolamine (Sigma, St. Louis, MO) with 4 μl of a 100-mg/ml solution (in $CHCl_3$) of dimethyldioctadecylammonium bromide (Sigma) and drying by nitrogen overflow for 5 min. The lipids are then resuspended in 1 ml of sterile, distilled, deionized water (ddH_2O) and probe-sonicated on ice using a Branson Sonifier 250 (output control setting, 1.5; duty cycle, 90%) for 8–10 min or until the solution is no longer cloudy.

The transfection media should be prepared 10 min prior to the end of the virus adsorption period. The liposome transfection media should be mixed in a polystyrene

as opposed to polypropylene tube to prevent loss of the liposome–DNA complex due to adherence to the walls of the tube. It is prepared as follows: 30 μl of the liposome preparation and 5 μg of the DNA to be transfected are added to 1 ml of serum-free Eagle's minimum essential media (MEM-E) (Sigma) and mixed gently by inverting the tube. The transfection media should sit at room temperature for 10 min to allow association of the DNA with the liposomes. If transfecting more than one plate, the transfecting media should be prepared as individual 1-ml aliquots—one for each plate—as opposed to batch preparation. Due to the hydrophobic nature of lipids, they will aggregate, as aliquots taken from large batches will have differing transfection efficiencies resulting from varied lipid concentrations.

After adsorption for the appropriate length of time, the viral inoculum is removed and replaced with 1 ml of transfection media containing the recombination plasmid. After rocking the plates gently to ensure even distribution of the liposomes, the plates are placed at 37° C, 5% CO_2, for 3 hr to allow the liposome-mediated transfection of the recombination plasmid. After 3 hr the transfection media should be completely removed to prevent further toxicity to the cells, and replaced with 2 ml of MEM-E containing 5% (v/v) fetal calf serum (FCS), 2 m*M* L-glutamine, and 10 μg/ml gentamicin sulfate. The plates are incubated an additional 48–72 hr or until all cells exhibit the cytopathic effect (CPE) of an ongoing VV infection, which is observed as cell rounding under the light microscope. At this time total progeny should be harvested and crude stocks should be made and titered by plaque assay, utilizing methods previously described (15). The tk^- progeny are amplified by low-multiplicity (0.01–0.05 pfu per cell) passages through Ltk^- cells in the presence of 25 μg/ml BrdU. "Crude stocks" are made and titered by plaque assay.

Identification of Recombinant VV by Plaque Hybridization

The following procedure is an adaptation of the method described by Villarreal *et al.* (28), in which tk^- progeny plaques are directly transferred to nitrocellulose filters and probed with DNA specific for the gene of interest. Putative recombinants are identified by probe hybridization, and then subjected to further analyses and purification.

Previously titered crude stocks that have been passaged through Ltk^- cells in the presence of BrdU are inoculated onto monolayers of BSC_{40} cells grown to confluence in 100-mm dishes. The viral inoculum should be sufficiently diluted to allow the formation of 10^2–10^3 plaques per plate. Plaques may be observed by light microscopy and should be approximately 1 mm in size before staining the monolayer and lifting the plaques. The monolayer is stained by the addition of 1 ml of 1% (w/v) neutral red stain in phosphate-buffered saline plus 1 m*M* $MgCl_2$ (PBS-M) to the growth medium and placement at 30° C for 3 hr to overnight. For the plaque lift the following materials are needed per dish: two additional 100-mm dishes, two S & S Nytran 82-mm circular filters (Schleicher & Schuel, St. Louis), one Whatman No. 1

9-cm filter (VWR Scientific), a sterile hole punch, forceps, PBS-M, 50 m*M* Tris–Cl (pH 7.6) plus 150 m*M* NaCl, Kimwipes, and a piece of Parafilm cut approximately $\frac{1}{2}$ inch wide.

After removal of the growth media, an S & S Nytran filter is placed over the stained cells to transfer the monolayer. Using a Kimwipe soaked in 50 m*M* Tris–Cl (pH 7.6) plus 150 m*M* NaCl, the back of the filter is blotted firmly and evenly. The filter is transferred from the dish to a new dish and placed face up on a PBS-M–soaked Whatman 9-cm filter. A replica filter is made by placing another 82-mm S & S Nytran filter over the first and pressing them together using a soaked Kimwipe just as when lifting the monolayer. While the filters are together, orientation holes are made using the sterile hole punch. The original filter is set aside to air-dry and the duplicate is replaced on the PBS-M–soaked filter in the 100-mm dish, which is then sealed with Parafilm and stored at −70° C.

The DNA on the original filter is denatured by placing it on a 0.5 *M* NaOH-soaked Whatman 3MM filter for 5 min, followed by blotting on paper towels. This procedure is to be performed three times, after which the filters are neutralized by repeating the above with 1 *M* Tris–Cl (pH 7.6) three times. The filter is then washed by soaking it in 100 m*M* Tris–Cl (pH 7.6) plus 150 m*M* NaCl for 5–10 min with gentle agitation at 2-min intervals. After air-drying for 15 min, the filter is subjected to baking in a vacuum oven at 80° C and 25 psi for 2 hr, prehybridization with sheared salmon sperm DNA, hybridization with nick-translated radiolabeled probe specific for the gene of interest, and fluorography following standard procedures for Southern analysis of DNA (14). Prior to placing the filter against film, orientation marks should be highlighted with a fluorescent dye.

The putative recombinants are recovered from the duplicate filter that has been stored at −70° C. The filter is thawed and placed against the developed film so that the orientation holes are aligned. The plaques that were labeled by the probe are punched out of the duplicate filter and placed in a sterile tube containing 200 μl of PBS. The tubes are frozen and thawed twice and subjected to indirect sonication six times for 10 sec each time to free the recombinant VV from the filter. The entire contents of each tube are inoculated onto individual wells of BSC_{40} cells grown to confluence in 24-well tissue culture dishes. After harvest these are maintained as crude stocks (see below).

Dot Blot Analysis of Crude Recombinant VV Stocks

The cells that have been infected by a single plaque punched out of the duplicate filter are harvested when the entire monolayer of an individual well is exhibiting the CPE of an ongoing VV infection. The period in which this happens may differ from well to well, depending on the amount of virus in the inoculum. To harvest the cells, the growth media are removed and replaced with 0.5 ml of PBS-M. The cells are then subjected to three cycles of freezing and thawing to lyse the cells and free them from

the bottom of the well. The remaining adherent cells may be freed from the bottom of the well by the addition of 0.5 ml of a solution of 0.25% (w/v) trypsin plus 1 m*M* EDTA (pH 8.0) and incubation at 37° C for 15 min. After tritration with a 1-ml pipettor until the contents of the well are homogeneous, 0.5 ml of the cell extract is transferred to a 1.5-ml microfuge tube containing 25 μl of FCS to inactivate the trypsin. This is to be stored at −70° C as a crude stock. The remaining 0.5-ml cell extract is transferred to a nylon filter via a dot blot apparatus, using a different slot for each individual well of infected cell extracts. The filter is processed in a manner identical to that used in the plaque lift/hybridization technique described above. After exposure of the filter to film, the crude stocks with the highest relative purity are identified by hybridization with the radioactively labeled probe. Those stocks that have been identified as being the most pure are further purified to ensure that 100% of the population is recombinant.

Plaque Purification of VV Recombinants

It is likely that a significant percentage of the population arising from the virus punched out of the filter from the original plaque lift may not be the recombinant of interest. It will be necessary to isolate individual virions from this population and screen their progeny for the recombined gene. This will ensure that the final preparations of recombinant virus are 100% pure. The most efficient method to achieve this by low-multiplicity infections of cell monolayers overlaid with agarose to prevent contamination of one population with another. The subpopulations (plaques) are amplified and screened to detect the recombinants. This is repeated until 100% of the population is recombinant.

BSC_{40} cells grown to confluence in 100-mm dishes are inoculated with the crude recombinant stocks that appear to be purest by dot blot analysis, sufficiently diluted so as to allow the formation of approximately 100 plaques per plate. After adsorption for 30–60 min, the inoculum is removed and replaced by 8 ml of agarose-containing growth media prepared as follows: 1.2 g of SeaPlaque agarose (FMC BioProducts) in 50 ml of ddH_2O is autoclave-sterilized and kept at 37° C until needed. Meanwhile, 50 ml of 2× MEM-E containing 10% (v/v) FCS, 4 m*M* L-glutamine, and 20 μg/ml of gentamicin sulfate is warmed to 37° C. Just prior to removal of the viral inoculum from the cells, the agarose solution and the 2× MEM-E are mixed 1 : 1. The agarose-containing growth media should be delivered using sterile disposable pipettes. After overlaying the infected cells, the plates are incubated at room temperature for 15 min to allow the agarose to solidify, then incubated at 37° C for 36–48 hr. The monolayers may be stained in the following manner: 1 g of SeaPlaque agarose in 100 ml of PBS is autoclave-sterilized and allowed to cool to 50° C, after which 2 ml of a 1% (w/v) neutral red solution is added. After the addition of 5 ml of this solution to each dish, they are allowed to cool to room temperature and are then placed at 30° C for 16–24 hr.

After incubation for the appropriate length of time, plaques will be visible to the

naked eye if the dish is placed against a light box. For every plaque that is to be harvested, a sterile tube containing 250 μl of PBS-M is prepared. To harvest the plaques, approximately 50 μl of the PBS-M from one of the tubes is drawn into a sterile Pasteur pipette, which is then inserted into the agarose over a plaque with gentle suction applied to draw up the agarose plug. Extreme care should be taken to avoid contamination with other plaques when performing this technique. When all of the agarose down to the cell monolayer has been drawn into the pipette, it is then blown into one of the sterile tubes containing PBS-M. The tubes are vortexed to release the virus from the agarose and disperse it throughout the medium. The entire contents of the tube are inoculated onto single wells of 24-well plates seeded with BSC_{40} cells that have grown to confluence. When the entire monolayer is exhibiting a CPE from the infection, it is harvested and crude stocks are made and analyzed by dot blot and Southern hybridization of a labeled probe in a manner identical to that described above. Again, the relative purity of a virus population is determined by the efficiency of the hybridization with the labeled probe. Individual plaques are again isolated, amplified, and subjected to dot blot analysis. This is to be repeated until all plaques arising from a crude stock are demonstrated to contain the gene of interest. High-titer purified stocks of the recombinant are made and stored in aliquots at $-70°$ C.

Identification of Recombinant VV by Neomycin Resistance

The time and effort involved in the techniques detailed above, which outline the necessary steps in the identification and isolation of recombinant VV by tk^- phenotype alone, may be substantially reduced by the cotransfer of the *neo* gene to the VV genome. As mentioned previously, plaque formation by VV in the presence of G418 is completely abrogated unless a resistance marker (*neo*) behind a suitable VV promoter is present. There is no eukaryotic equivalent to the enzymatic activity encoded by the *neo* gene; therefore, spontaneous occurence of G418 resistance is unlikely. By maintaining the dual selective pressure of BrdU *and* G418, virtually all of the viable virus recovered will be recombinant. The following is an adaptation of the method described by Franke and Hruby (29) for the identification of G418-resistant VV mediated by the transfer of the *neo* gene to the VV genome.

Our laboratory has demonstrated the necessity of pretreatment of cells in order to obtain maximal G418-mediated inhibition of VV replication. Mammalian cell lines differ widely in their susceptibility to G418, but exposure to 2 mg/ml of G418 in the growth media for 24–48 hr will inhibit the replication of mammalian cells utilized in these techniques. BSC_{40} cells on 100-mm tissue culture dishes are grown to semiconfluence in normal growth media. At 48 hr prior to infection, treatment with G418 is initiated and is maintained throughout the remainder of the procedure. G418-containing media are prepared by addition of the drug to a final concentration of

2 mg/ml in normal growth media containing 5% (v/v) FCS. The media is sterilized by passage through a 0.22-μm pore size filter. The normal media are removed from the cells and replaced with the G418-containing media. The cells are incubated at 37° C, 5% CO_2, until they are to be infected.

Previously titered crude stocks of putative recombinant VV that have been passaged through Ltk^- cells in the presence of BrdU are inoculated onto the pretreated confluent BSC_{40} cells. The viral inoculum should be sufficiently diluted to allow the formation of 10^2-10^3 plaques per plate. After an appropriate incubation period the inoculum is removed and replaced with a 2 mg/ml G418-containing agarose overlay. The presence of G418 will inhibit any viral replication other than recombinant VV. After incubation at 37° C for 36–48 hr, the monolayer is stained by a neutral red-containing agarose overlay, as described above, and further incubation at 30° C for 3–16 hr. For every plaque that is to be harvested, a sterile tube containing 250 μl of PBS-M is prepared. The plaques are transferred to the sterile tubes as described, and after vortexing the tubes the entire contents are inoculated onto one well of BSC_{40} cells grown to confluence on a 24-well tissue culture dish. When the entire monolayer exhibits a CPE, it is harvested and crude stocks are made and assayed for the presence of the gene of interest by dot blot and Southern analysis. Recombinants should be plaque-purified twice under selective conditions. After each round of purification, crude stocks should be screened by dot blot and Southern hybridization of a nick-translated radiolabeled probe specific for the gene of interest to identify purely recombinant populations.

Characterization of the Genomic Structure of Recombinant VV

To ensure the integrity of the expression cassette and subsequent expression of the gene of interest, it is essential to demonstrate that recombination occurred as anticipated. The genomic structure of VV is well characterized (8), and comparison of wild-type and recombinant genomes should yield observable differences in structure, allowing definitive proof of recombination as intended. The nomenclature for VV open reading frames (ORFs) is derived from their position relative to restriction endonuclease *Hin*dIII sites within the genome and the direction in which they are transcribed. The *TK* ORF is located within the 4.8-kilobase pair *Hin*dIII J fragment and is referred to as ORF J2R. If the *TK* locus was the target site, then recombination would result in a shift in the electrophoretic mobility of the *Hin*dIII J fragment equivalent to the size of the expression cassette, unless the inserted gene cassette contains one or more additional *Hin*dIII cleavage sites, in which case a series of smaller fragments will be obtained. The shift in mobility may be demonstrated to result from insertion of the expression cassette by Southern blot analysis. The following briefly describes the techniques utilized by Hruby *et al.* (25) to isolate and analyze VV genomic DNA.

Isolation of VV DNA must be from highly pure stocks of recombinant virus, or the results obtained may be ambiguous. Virus stocks isolated and amplified from a single plaque demonstrated to be comprised of only recombinant VV are used to infect BSC_{40} cells grown to 90% confluence in 100-mm dishes at a multiplicity of 0.1 pfu per cell. After incubation for 30–60 min, the inoculum is removed and replaced with 8 ml of normal growth media. The incubation is at 37° C, 5% CO_2, for 48–72 hr or until all cells are exhibiting a CPE. Taking care to avoid lysing the cells, they are harvested by either scraping the dish with a rubber policeman or washing them free with a pipette and transferring them to a 15-ml conical tube. The cells are pelleted by low-speed centrifugation at 4° C and washed once with PBS-M. After pelleting the cells again, they are resuspended in 600 μl of PBS-M and transferred to a 1.5-ml microfuge tube. The cells are lysed by the addition of 30 μl of 10% (v/v) Triton X-100, 1.5 μl of 2-mercaptoethanol, and 48 μl of 250 m*M* EDTA (pH 8.0), vortexing, and incubation on ice for 10 min. The cellular debris is pelleted by centrifugation in a microcentrifuge at 700 *g* for 2.5 min at 4° C. The supernatant is transferred to a new 1.5-ml microfuge tube and the viral cores are pelleted by centrifugation at 12,000 *g* for 10 min. The supernatant is discarded and the cores are resuspended in 100 μl of 10 m*M* Tris plus 1 m*M* EDTA (pH 7.6) (TE). From this step in the procedure, it is important that resuspension of pellets or mixing is by gentle pipetting or tapping with a finger instead of vortexing, to prevent shearing of the DNA. The cores are disrupted by the addition of 1.5 μl of 10 mg/ml proteinase K, 6.7 μl of 3 *M* NaCl, 0.3 μl of 2-mercaptoethanol, and 10 μl of 10% (w/v) sodium dodecyl sulfate, mixing by tapping with a finger, and incubation at 55° C for 30 min. The DNA is extracted twice with phenol:chloroform:isoamyl alcohol (25 : 24 : 1) and three to five times with water-saturated ether. After the last extraction the remaining traces of ether are evaporated by incubation at 65° C for 10 min. The DNA is ethanol-precipitated twice: the first time by the addition of 4 μl of 3 *M* NaCl and 2.5 vol of ethanol, gentle mixing, placement at −70° C for 30 min, and centrifugation at 12,000 *g* for 7 min. The pellet is resuspended in 100 μl of TE and the DNA is precipitated the second time by the addition of 5 μl of 3 *M* NaCl plus 2.5 vol of ethanol, placement at −70° C for 30 min, and centrifugation at 12,000 *g* for 10 min. After rinsing with 70% (v/v) ethanol and drying *in vacuo,* the pellet is resuspended in 20 μl of TE. Resuspension of the pellet is by incubation at 4° C for 12–16 hr rather than mixing or pipetting to prevent shearing of the DNA. The concentration of the DNA is determined by measurement of its optical density at 260 nm, at which one absorbance unit equals 50 μg of DNA per milliliter of solution.

Southern Analysis of VV Genomic DNA

Approximately 2 μg of VV DNA is needed to allow visualization of EtBr-stained *Hin*dIII fragments isolated on an agarose gel. The DNA from the recombinant virus, as well as wild-type VV DNA as a reference or control, is restricted with 10 U of

restriction endonuclease *Hin*dIII, 2 μl of appropriate buffer, and water, to a total volume of 20 μl, and incubation at 37° C for 2 hr. The digested DNA is then subjected to electrophoresis through a 0.7% (w/v) agarose gel at 25 V for 12–16 hr—recirculating the running buffer (Tris–acetate–EDTA) for the duration of the run. After staining with EtBr and observation of the shifted mobility of the recombinant *Hin*dIII J fragment relative to the wild-type VV *Hin*dIII J fragment, the gel is photographed, then transferred to a nitrocellulose filter and probed, using labeled nick-translated DNA specific for the gene of interest, by Southern blot analysis. Hybridization of the probe with the recombinant virus DNA should occur within the *Hin*dIII J fragment or its fragments exhibiting a shifted mobility equivalent to the size of the expression cassette.

Polymerase Chain Reaction Analysis of VV Genomic DNA

If appropriate primers are available, then polymerase chain reaction amplification of the target sequence (*TK*) is an efficient method for detecting the insert (14). Identical amplification reactions using primers specific for the *TK* ORF will yield distinctly different products when wild-type and recombinant genomes serve as the template DNAs. The method presented here is an adaptation of that outlined by O'Reilly *et al.* (30) for use with baculovirus vectors, and uses a three-primer system: the first, specific for the 5′ end of the *TK* gene; the second, specific for the 3′ end of the *TK* gene; and the third, specific for either the gene of interest or the VV promoter directing its expression.

Recombinant VV DNA as well as wild-type VV DNA is purified and the concentration is determined as outlined above. The reaction is prepared as follows: 1 μg of VV DNA, 100 pmol of each primer, 2.5 μl of a 10 m*M* stock of deoxyribose nucleoside triphosphates, 10 μl of 10× *Taq* DNA polymerase buffer (Mg^{2+} free), 6 μl of a 25.0 m*M* stock of $MgCl_2$, and sterile ddH_2O, to a total volume of 90 μl, are mixed in a 0.5-ml microfuge tube. After a 5-min "hot start" at 94° C, 2.5 U of *Taq* DNA polymerase in 10 μl of ddH_2O is added to the reaction mixture, which is then subjected to 25–35 cycles of denaturing at 94° C for 2 min, annealing of the primers at 50° C for 2 min, and primer extension at 72° C for 3 min in a thermocycler. The products may be observed by gel electrophoresis of 10–20 μl of the reaction through a 0.8% (w/v) agarose gel, staining with EtBr, and illumination with ultraviolet light.

The products of such a reaction will vary depending on whether wild-type or recombinant DNA was used as the template. If wild-type DNA was used as the template, the only product amplified will be from the *TK* ORF. If recombinant DNA is used, however, it is possible to amplify two products. The most likely product will be amplified from the promoter/gene of interest primer and the 3′ *TK* primer. The second is the amplification product from the 5′ and 3′ *TK* primers. If the gene of interest is lengthy (i.e., over 1000 bp) or an additional marker has been cotransferred, then the efficiency of this amplification is reduced and may not occur. Nevertheless,

the purity of the recombinants may be determined by the presence or absence of contaminating bands that would be the products of an intact *TK* locus or a single crossover recombination (see Fig. 2).

Analysis of Gene Expression from Recombinant VV

Transcription

Transcription originating from a VV late promoter will result in mRNAs of heterogeneous length due to the lack of recognition of stop signals by the VV late transcription machinery. Therefore, little information is gathered via Northern blot when late promoters have been utilized. If a VV early or early/late promoter has been used, then transcription will be discretely terminated approximately 50 nucleotides downstream of the appropriate sequence. It is not necessary to include this sequence in the gene to be recombined when recombination is targeted to the *TK* locus in the VV genome, for an early transcription stop sequence is located near the 3′ end of the *TK* ORF.

The following method (26) may be used to detect and analyze early mRNAs transcribed from VV: Confluent monolayers of BSC_{40} cells are infected with the recombinant virus at a multiplicity of 10 pfu per cell in the presence of 200 μg/ml cycloheximide to amplify early viral mRNA. The total cytoplasmic RNA is extracted from infected cells using the CsCl–Sarkosyl method (31). After glyoxylating the RNAs, they are separated by agarose gel electrophoresis, then transferred to nitrocellulose. The filter is probed with labeled nick-translated DNA specific for the gene of interest and then placed against film to detect hybridization of the probe with the RNA. Hybridization should occur with a species of RNA of appropriate length, allowing for initiation from within the promoter region, transcription through the gene of interest and termination near the 3′ end of the *TK* ORF. Initiation of transcription from within the promoter sequence may be confirmed by nuclease S1 mapping.

Translation

The high protein expression levels from recombinant VV allow the utilization of standard techniques (14) in their detection. Immunoreactivity of the protein may be demonstrated via immunoblot analysis of infected cell lysates and/or immunofluorescent analysis of infected cells. Immunoprecipitation of radioactively labeled infected cell extracts is useful in determining expression kinetics, stability of the product, and co- or posttranslational modifications of the protein, such as fatty acylation, phosphorylation, glycosylation, or ADP ribosylation.

Enzymatic Activity

Recombinant VV is often used to express enzymes that, in their native systems, are present in such low quantities that analysis is extremely difficult. Due to high expres-

sion levels, enzymes expressed from recombinant VV may be detected and quantitated by functional assays. For example, the recombinant virus VV:mPOMC (32), which expresses murine preproopiomelanocortin, was used to infect mammalian cells incapable of proteolytic maturation of the prohormone precursor to biologically active products. Proteolytic processing was facilitated by coinfection with a recombinant vaccinia virus expressing the yeast *KEX2* endopeptidase, resulting in an increase in specific activity 16–33 times as great as that observed in mock-infected cells or cells infected with control viruses. In this situation the production of the *KEX2* endopeptidase was not directly observed. Only the resulting increase in enzymatic activity was assayed for and easily detected.

Optimization of the System

The expression of products from recombinant VV may be modulated by a number of variables. Specifically, promoters used, mRNA stability, protein stability, multiplicity of infection, time course of the infection, and cell type all affect product yield. After cloning behind a certain promoter, it is difficult to obtain expression that is not intrinsic to that promoter, and degradation of expressed products probably cannot be altered without further manipulations to the DNA sequence. Having stated this, however, we have noted marked differences in the expression of proteins from recombinant VV simply by variation in the time course of the infection, in the multiplicity of the infection, or by expression in different cell types. The conditions presented in this section are for example only and are not necessarily indicative of the optimum for all recombinant VV.

Viral Multiplicity and Time Course of Infection

In optimizing VV:hPE expression of proenkephalin, a neuropeptide that is matured by way of proteolytic processing, it was observed that manipulation of virus titer led to variation in the precursor–intermediate–product ratio. By infecting at a low multiplicity, the predominant immunoreactive peptide was the mature product, whereas infection at a high multiplicity resulted in the accumulation of processing intermediates. In concert with the determination of the optimum multiplicity of infection, the time postinfection at which the protein is collected was examined for optimum yield. The intermittent activity of p7.5 results in expression of protein from 1 to 3 hr postinfection and again at about 7 hr until cell death (16). If the multiplicity of infection is greater than 1 pfu per cell, then the highest yield would be obtained at 3 hr postinfection or after 7 hours postinfection. However, often the optimum multiplicity of infection is less than 1 pfu per cell and infection of neighboring cells may be by secondary infection, resulting in asynchronous expression of the gene of interest. In

the characterization of VV:hPE, cells were infected at a multiplicity of 0.5 pfu per cell. At specific times postinfection the cells were lysed and the amount of immunoreactive Met-enkephalin (the final proteolytic product) was determined by radioimmunoprecipitation assay. The observation was that the greatest absolute amounts of protein could be detected after 8 hr postinfection. Therefore, infections were conducted over a span of 8–24 hr in order to further characterize proenkephalin as expressed from recombinant VV.

Cell Type-Dependent Expression

As mentioned in the introduction, a major attribute of VV is its ability to infect a wide variety of cell types from numerous species. Most mammalian cell lines allow the replication and production of infectious VV, and expression of VV gene products has even been observed in insect cells. The recombinant VV:hPE was used to infect BSC_{40}, AtT-20, GH_4, L, and P388D cell lines at multiplicities of infection previously demonstrated to optimize protein expression. At 24 hr postinfection the infected cells were assayed for immunoreactive Met-enkephalin. The level of expression observed varied from 7 p*M* per 10^6 L cells to more than 40 p*M* per 10^6 GH_4 cells.

Concluding Remarks

VV has been, and continues to be, a powerful tool for research on gene systems and their expression and regulation. The system is not without limitations, though. Infection with VV ultimately leads to cell death, due to its lytic nature. Therefore, the expression of products from recombinant VV is transient. Preventing viral replication by carrying out the infections in the presence of inhibitory drugs or by cloning into conditional-lethal mutants reduces the CPE and prolongs expression of the recombinant gene, but total product yields may be adversely affected. Because host protein synthesis is blocked by VV, the interaction of recombinant gene products with host gene products may be difficult to observe. The window of opportunity for such observations will be open for only a short time immediately following infection. The expression level of the recombinant gene product in this period may not be sufficiently high to allow detection. When expression levels of the recombinant gene product are high enough to detect, the host gene product may no longer be detectable if it is degraded. In addition to the transient nature of gene product expression from recombinant VV, its cytoplasmic replication limits its uses as well. Many mammalian expression vectors are being developed for use in gene therapy, and gene products often require nuclear localization and function in order to obtain authentic expression and processing. Therefore, vector systems other than VV would probably be more effective for these purposes.

Its limitations aside, VV holds significant promise in the development of new vaccines. The treatment of microbial diseases with antibiotics has resulted in selection for and an increased occurrence of antibiotic-resistant strains. New vaccines are urgently needed, and the record of safety and success of VV, along with its well-characterized replication strategy, establish it as an exceptionally capable system for the expression of immunogenic substrates. When used as a vaccine, it is inoculated as a live virus without severe attenuation. Hence, propagation of the vaccine strains do not require fastidious growth conditions. Once infected, the host immune system does not immediately overcome the virus, allowing for prolonged expression of the recombinant gene product within the host and induction of both humoral and cellular responses.

VV is large and complex, yet much of its life cycle and many of its gene products have been characterized. However, further investigation is needed to decipher its intricacies before the full potential of VV as an expression vector is realized.

References

1. M. Rose and D. Botstein, *in* "Methods in Enzymology" (R. Wu, L. Grossman, and K. Moldave, eds.), Vol. 101, p. 167. Academic Press, New York, 1983.
2. R. D. Cone and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 6349 (1984).
3. S. Karlsson, R. K. Humphreys, Y. Gluzman, and A. W. Nienhuis, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 158 (1985).
4. M. Arsenakis and B. Roizman, *in* "High Technology Route to Virus Vaccines" (G. R. Dreesman, J. G. Bronson, and R. C. Kennedy, eds.), p. 75. American Society for Microbiology, Washington, D.C., 1985.
5. M. Yamada, J. A. Lewis, and T. Grodzicker, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3567 (1985).
6. G. E. Smith, G. Ju, B. L. Ericson, J. Moschera, H. W. Lahm, R. Chizzonite, and M. D. Summers, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8404 (1985).
7. G. L. Smith and B. Moss, *BioTechniques* **1,** 120 (1986).
8. B. Moss, *in* "Virology" (B. N. Fields, D. M. Knippe, R. M. Chanock, M. S. Hirsch, J. Melnick, T. P. Monath, and B. Roizman, eds.), Vol. 2, p. 2079. Raven, New York, 1990.
9. M. K. Spriggs, D. E. Hruby, C. M. Maliszewski, D. J. Pickup, J. E. Sims, R. M. L. Buller, and J. K. VanSlyke, *Cell (Cambridge, Mass.)* **71,** 145 (1992).
10. M. P. Ravanello and D. E. Hruby, *J. Virol.* **68,** 6401 (1994).
11. D. Baxby, *Lancet* **2,** 919 (1989).
12. R. P. Wenzel and M. D. Nettelman, *Lancet* **2,** 630 (1989).
13. J. H. Richardson and W. E. Barkley, "Biosafety in Microbiological and Biomedical Laboratories," Health and Human Services Publication No. (NIH)88-8395. U.S. Government Printing Office, Washington, D.C., 1988.
14. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
15. P. L. Earl, N. Cooper, and B. Moss, *in* "Current Protocols in Molecular Biology" (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, eds.), Vol. 2, p. 16.16.1. Greene and Wiley (Interscience), New York, 1991.

16. J. Garces, K. Masternak, B. Kunz, and R. Wittek, *J. Virol.* **67,** 5394 (1993).
17. P. L. Earl, A. W. Hugin, and B. Moss, *J. Virol.* **64,** 2448 (1990).
18. S. Chakrabarti, K. Brechling, and B. Moss, *Mol. Cell. Biol.* **5,** 3403 (1985).
19. D. D. Patel, C. A. Ray, R. P. Drucker, and D. J. Pickup, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 9431 (1988).
20. A. J. Davison and B. Moss, *Nucleic Acids Res.* **18,** 4285 (1990).
21. T. R. Fuerst, E. G. Niles, F. W. Studier, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8122 (1986).
22. M. Mackett, G. L. Smith, and B. Moss, *J. Virol.* **49,** 857 (1984).
23. F. G. Faulkner and B. Moss, *J. Virol.* **62,** 1849 (1988).
24. C. A. Franke, C. M. Rice, J. H. Strauss, and D. E. Hruby, *Mol. Cell. Biol.* **5,** 1918 (1985).
25. D. E. Hruby, G. Thomas, E. Herbert, and C. A. Franke, *in* "Methods in Enzymology" (M. Conn, ed.), Vol. 124, p. 20. Academic Press, Orlando, Florida, 1986.
26. F. L. Graham and A. J. Van der Eb, *Virology* **52,** 456 (1973).
27. J. K. Rose, L. Buonocore, and M. A. Whitt, *BioTechniques* **10,** 520 (1991).
28. L. P. Villarreal and P. Berg, *Science* **196,** 183 (1977).
29. C. A. Franke and D. E. Hruby, *Nucleic Acids Res.* **16,** 1634 (1988).
30. D. R. O'Reilly, L. K. Miller, and V. K. Luckow, "Baculovirus Expression Vectors: A Laboratory Manual," Vol. 1, p. 161. Freeman, New York, 1992.
31. V. Glisin, R. Crkuenjakev, and C. Byus, *Biochemistry* **12,** 2633 (1974).
32. G. Thomas, B. A. Thorne, L. Thomas, R. G. Allen, D. E. Hruby, R. Fuller, and J. Thorner, *Science* **241,** 226 (1988).

[5] Epstein–Barr Viral Plasmid Vectors and Their Amplifiable Derivatives

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Introduction

Epstein–Barr virus (EBV) is a human herpesvirus that replicates its genomic DNA via two disparate mechanisms. EBV infects human B lymphocytes and induces them to proliferate. In these cells, EBV DNA is usually replicated as a plasmid (Fig. 1). The viral plasmid DNA replicates once per cell cycle during S phase (1, 2), as does the cell DNA of the host. Only one viral protein, EBV nuclear antigen 1 (EBNA-1), is required in *trans* to mediate this replication (3, 4), which in *cis* requires only the plasmid origin of DNA replication, *oriP* (5). This plasmid mode of viral DNA replication is associated with the nonproductive, or latent, phase in the life cycle of EBV. During nonproductive infection, no progeny virions are synthesized and the infected proliferating cell can often divide *ad infinitum* and is said to be immortalized. Were this to be the only phase in the life cycle of this virus, EBV would be lost for lack of infectious progeny; but EBV does have a productive, or lytic, phase in its life cycle. Occasionally, individual B cells harboring EBV genomes undergo some ill-defined change(s) that allows them to support the lytic phase of EBV. During this phase, viral DNA is replicated via a mechanism distinct from that used during the latent phase. The virus uses a lytic origin of DNA replication, *oriLyt* (6), and requires seven or more viral proteins to support DNA replication (7). The DNA product of this replication is a polymer of viral genomes that may be generated via a rolling-circle mechanism (6). This replicative mode amplifies the viral DNA 100–1000 times over the course of 1–2 days and yields genomes that can be packaged into newly synthesized viral capsids (6). The encapsidated DNAs mature into virions that are the progeny in this productive cycle. The lytic phase of the viral life cycle leads to cell death and release of the progeny virus that can carry on the infectious cycle.

The identification and characterization of the two modes of replication of EBV DNA have led to the practical development of two classes of vectors derived from EBV. One class, containing the latent origin of replication, replicates extrachromosomally during S phase, segregates efficiently, and is maintained in a variety of mammalian cells. A second class, containing both the latent and lytic origins of replication, also replicates as a plasmid but can be induced to amplify itself. In this article we describe in detail what has been learned about the *cis*- and *trans*-acting elements that contribute to each mode of EBV's DNA replication. This information

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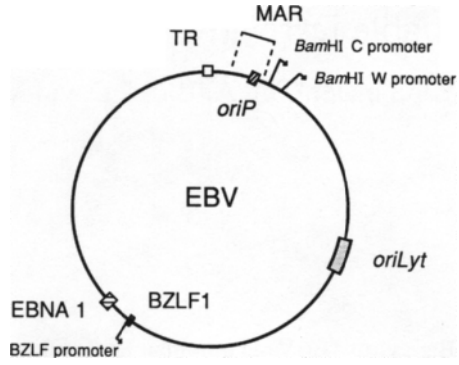

FIG. 1 Landmarks on the Epstein–Barr virus (EBV) genome considered in this article. The EBV genome contains approximately 165 kbp (79) of duplex DNA, which is linear in the virion and circularizes via the terminal repeats (TR; open box) upon entering the host cell during infection (82). EBV nuclear antigen 1 (EBNA-1; hatched box) is transcribed from either the *Bam*HI C or *Bam*HI W promoter to yield a primary transcript of approximately 100 kbp (83–85). EBNA-1 binds to the plasmid origin of replication (*oriP;* striped box) and is required for latent replication (3, 4). The nuclear matrix attachment region (MAR; brackets) maps within a domain that includes *oriP* (15). The transcription factor Bam Z leftward reading frame 1 (BZLF-1; solid box), which is transcribed from the BZLF promoter, binds to the lytic origin of replication (*oriLyt;* stippled box) and is required for the lytic phase of the EBV life cycle (7, 61, 62, 83).

will serve as a foundation for the development and application of vectors derived from EBV. We then describe several ways in which these vectors have been used successfully.

oriP

The latent origin of plasmid replication, *oriP,* has been defined in functional assays to consist of two *cis*-acting DNA sequences: a family of direct repeats and a region of dyad symmetry (Fig. 2) (8). The family of repeats includes 20 repeated sequences: 17 imperfect copies of a 30-bp repeat and three truncated copies of the repeat. The dyad symmetry element contains four partial copies of the repeat sequence; two are part of a 65-bp dyad that could theoretically form a secondary cruciform structure and two are at the base of this possible stem–loop sequence. EBNA-1 can bind to all of the related repeats within *oriP* and is the only viral protein required for plasmid replication (3, 4, 9).

The family of repeats of *oriP* has been shown to have a function independent of the dyad symmetry element. The family of repeats can act to enhance transcription

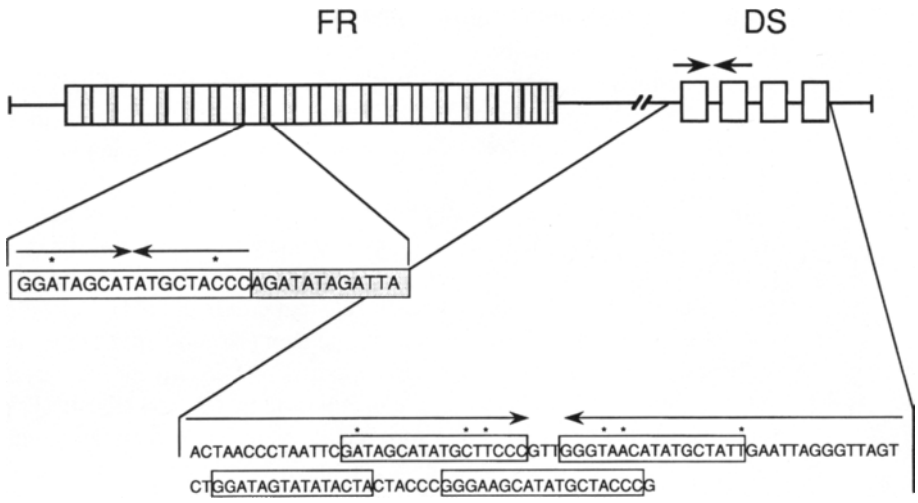

FIG. 2 The structure of the Epstein–Barr virus (EBV) latent origin of plasmid replication (*oriP*). *oriP* consists of two *cis*-acting sequences required for function: the family of repeats (FR) and the dyad symmetry element (DS) [7421–8042 and 9021–9133 bp, respectively, in the B95-8 strain (83)]. The family of repeats includes 20 repeated sequences, depicted as boxes, 17 of which are imperfect copies of a 30-bp repeat and three of which are truncated copies of the repeat. EBV nuclear antigen 1 (EBNA-1) binds specifically to this 30-bp repeat, which is expanded below on the left. The EBNA-1 binding consensus sequence contains a 9-bp inverted repeat (thin arrows) with a 1-bp mismatch (asterisk). The remaining 12 bp of the 30-bp repeat contain a 4-bp direct repeat that is separated by 2 bp (stippled area). Approximately 960 bp downstream lies the dyad symmetry element. The dyad symmetry element (expanded below on the right) contains four truncated copies of the core repeat sequence (boxes); two are part of a 65-bp dyad (thin arrows) containing three mismatches (asterisks) and two are located immediately downstream of the dyad (8).

from either the EBV *Bam*HI C promoter or the Latent Membrane Protein promoter, which are active during latent infection, or heterologous promoters when EBNA-1 is supplied in *trans* (10–12). The amount of transcriptional enhancement provided by the family of repeats and EBNA-1 varies with the promoter and the cell type but is independent of orientation and, to a large extent, of position (11). These observations indicate that vectors containing *oriP* have an intrinsic transcriptional enhancer that may positively affect nearby promoters.

In the presence of EBNA-1, plasmids containing the family of repeats and a gene encoding resistance to a drug can provide transient drug resistance to their host cell (8, 11, 13). This resistance is strikingly protracted; it can last for 10 cell generations. This prolonged resistance indicates that the family of repeats bound by EBNA-1 may

contribute to the retention of *oriP* plasmids in the nucleus (13, 14). This interpretation may reflect the action of a nuclear matrix attachment region within the EBV genome, which overlaps with *oriP* (15). Clearly, the transient drug resistance also reflects the enhancer activity of the family of repeats, which, in the presence of EBNA-1, supports an increased expression of the gene encoding the drug resistance (8, 11).

Both the family of repeats and the dyad symmetry element are necessary in wild-type *oriP* to support plasmid replication. Functions have been assigned to each of these elements in an active wild-type origin by analyzing replicative intermediates containing that origin. This study indicated that bidirectional DNA synthesis initiates within or quite near the dyad symmetry element (16); that is, it is the site at which a replication complex can assemble to support the DNA unwinding, primer synthesis, and elongation required to begin semiconservative DNA synthesis. This study also found that while one replication fork readily proceeds around the circular template, the other is slowed or stopped upon encountering the family of repeats (16). In the context of the viral genome, this barrier to fork migration leads to the direction of migration of the unimpeded replication fork being the same direction as most of the transcriptional units expressed during the latent phase of the EBV life cycle. The extent to which this ensured codirectionality of DNA replication and RNA synthesis affects the efficiency of either process for EBV is not known. However, in *Escherichia coli* and in replication reactions *in vitro* using T4 replication proteins, the migration of replication forks is slowed when these processes are opposed (17–19). Whether the ability of the family of repeats to act as a barrier to the migration of replication forks is a consequence of an as yet unidentified function it provides, or is simply an effect of the known roles of the family of repeats in transcription and replication, is not known.

Various derivatives of *oriP* have been used to investigate the functional constraints of this origin and the role of each of its elements in replication. Neither the family of repeats nor the dyad symmetry element alone usually supports replication (8, 20). Multiple copies of the dyad symmetry element do, however, support DNA replication in the presence of EBNA-1 (21). Thus, the dyad symmetry element can substitute for the family of repeats and must have a function distinct from that of the family of repeats.

The role of the family of repeats for replication is similar to that of a transcriptional enhancer in that each can act over variable distances and independently of its orientation. Derivatives of *oriP*, in which the intervening DNA between the family of repeats and the dyad symmetry element has been either reduced from 980 to 20 bp or increased to 2357 bp, are functional. Also, plasmids containing the family of repeats in either orientation with respect to the dyad symmetry element replicate (8).

In the presence of selection, plasmids with *oriP* are maintained at a constant average number per cell in EBNA-1–positive cells that support their replication. This

number appears to be determined by the amount of DNA initially taken up and replicated by the cell, and not by subsequent amplification (2). The number of *oriP* plasmids per cell in different clones ranges from one to 100 (3). In the absence of selection, approximately 2–6% of the cells lose wild-type *oriP* plasmids per cell generation, depending on the cell line and the type of selection used (8, 10, 22). In the absence of selection, derivatives of *oriP* that contain a second copy of the dyad symmetry element flanking the family of repeats are lost from cells at the same rate as plasmids containing wild-type *oriP* (22). In independent experiments, cells harboring either wild-type *oriP* plasmids or a derivative of *oriP* containing a second copy of the dyad symmetry element were propagated in the absence of selection for 20–30 cell generations; then selection was reapplied and subclones were isolated. The average number of copies of plasmids per surviving cell in each subclone was measured. This average copy number in the reselected subclones was similar to that of the parents for either the wild-type *oriP* plasmids or the replicons containing a second copy of the dyad symmetry element (22). These results indicate that even when two sites for the initiation of replication are on one plasmid, only one initiation event occurs per cell cycle; gross amplification of plasmid DNA containing multiple dyad symmetry elements does not occur (21–23). Derivates of *oriP* that contain a second copy of the family of repeats flanking the dyad symmetry element in one orientation are less efficiently maintained in the absence of selection than are plasmids that contain wild-type *oriP.* This effect is quite small. Either the ability of the family of repeats to act as a barrier to the migration of replication forks or some other function of the family of repeats can hinder replication or segregation (22). Plasmids containing two copies of wild-type *oriP* are maintained in cells in numbers similar to those of plasmids containing one copy of *oriP;* both types of plasmids are lost at approximately similar rates in the absence of selection (23). The latter observation indicates that *oriP* does not contain a centromere-like function (*cen*) akin to that of *Saccharomyces cerevisiae.* Two copies of the *cen* element of this yeast on one plasmid render that plasmid extraordinarily mitotically unstable (24, 25). How *oriP* plasmids contrive to maintain themselves efficiently in cells is not yet clear.

Studies with wild-type *oriP* and various derivatives do provide some insight into the process by which these plasmids are lost from cells when this infrequent event occurs. Experiments that have measured the rate of loss of *oriP* plasmids from cells, whether by measuring the loss of cells no longer resistant to a drug or by measuring the physical loss of *oriP* DNA, have arrived at similar rates (3, 11, 22, 23). This finding indicates that the loss of drug resistance by cells may primarily reflect a failure to segregate the plasmids encoding drug resistance, and not a failure to replicate the plasmids during each cell generation.

Plasmids with *oriP* replicate in a wide variety of host cells, including fibroblastic, epithelial, erythroid, and lymphoid cells of human origin as well as at least fibroblastic cells from monkeys and dogs (3). These plasmids do not replicate in rodent cell

lines, although the family of repeats does act as a transcriptional enhancer in the presence of EBNA-1 in rodent cells (21). What dictates the host range for replication of *oriP* plasmids is not understood.

EBNA-1

EBNA-1 activates the family of repeats as a transcriptional enhancer and mediates plasmid replication of *oriP*-containing plasmids. Several features and activities of this protein that are likely to contribute to these functions have been identified. EBNA-1 from the B95-8 strain of EBV is a protein of 641 amino acids that is found as a dimer in solution (26) (Fig. 3). The protein contains a repeated array of glycines and alanines between residues 89 and 328; however, the majority of the silklike repeats are dispensable for all of the known activities of EBNA-1 (3, 9, 27–30). Amino acids 39–86 and 329–410 are rich in basic residues, while amino acids 410–445 are rich in acidic residues. Small deletions throughout these regions can diminish the biological activities of EBNA-1 (28). The C-terminal amino acids 619–641 are also rich in acidic amino acids (13 of 22 residues are acidic). Removal of these 22 amino acids has little effect on most activities of the protein (28, 31–33), with the exception that this may impair the ability of EBNA-1 to support the stable maintenance of *oriP* plasmids (28).

Several activities of the EBNA-1 protein have been mapped to restricted domains

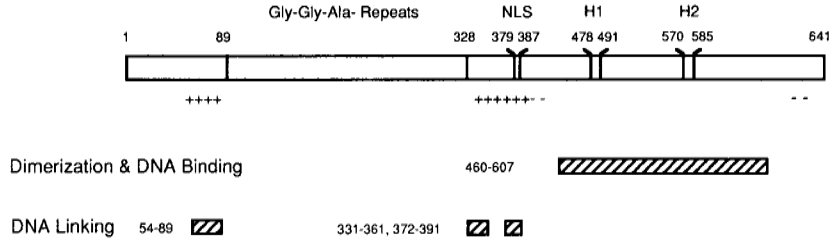

FIG. 3 The structure of Epstein–Barr virus nuclear antigen 1 (EBNA-1). The EBNA-1 protein consists of two domains flanking an internal repeated sequence. The internal repeated sequence consists entirely of glycine and alanine residues arranged in a repeated array. A region rich in basic residues (+) is located on each side of the repeats and the C terminus is rich in acidic residues (−). At least one nuclear localization sequences (NLS) has been defined for EBNA-1 (32). The two helices of the proposed basic helix–span–helix domain (bHSH) (37, 38) within the DNA binding and dimerization domain are denoted H1 and H2 (34). The hatched bars represent amino acids shown to be sufficient for the DNA binding and dimerization (34, 35) or looping (33, 40, 41) activities of EBNA-1.

within the protein. The ability of EBNA-1 to localize to the nucleus has been mapped to a nuclear localization sequence (NLS) found between amino acids 379 and 387 (32). This study identified one NLS in EBNA-1; however, variants of EBNA-1 in which mutations have deleted some or all of this NLS still activate transcription and support replication associated with *oriP* (28, 31). Therefore, additional NLSs may be present in wild-type EBNA-1.

EBNA-1 binds DNA as a dimer in a sequence-specific manner. One domain between amino acid 460 (34) and amino acid 607 (35) is sufficient for both protein dimerization and specific DNA binding. This domain superficially resembles the dimerization and DNA binding domains associated with the basic helix–loop–helix (bHLH) family of transcription factors (34). Recent crystallographic studies of one member of the bHLH family, Max, however, make it unlikely that EBNA-1 belongs to this structural family (36). Rather, the dimerization and DNA binding domain of EBNA-1 may be more closely related to those described as a basic helix–span–helix (bHSH) found in proteins such as AP-2 (37, 38). Characteristics shared by both EBNA-1 and members of the family of bHSH proteins include (1) the separation of the basic and first helical regions by a helix-breaking proline residue, (2) a span region greater than 50 amino acids in length, and (3) the ability of recognize specifically large regions of DNA, which can exceed 16 bp of deoxyribonucleotides in length (34, 36, 37). At least one small deletion within the putative span region does not affect the functions of EBNA-1 (28).

Upon binding to DNA specifically at multiple sites, EBNA-1 can cause the DNA to loop intramolecularly or it can link DNAs intermolecularly if each has specific binding sites (29, 30, 33, 39). This activity of the protein requires not only the dimerization and DNA binding domain of EBNA-1 but also residues N terminal to this domain. A derivative of EBNA-1 that consists of residues 350–641 loops DNA intramolecularly (40). In separate experiments a derivative of EBNA-1 that consists of residues 361–641 can link DNA intermolecularly, while one that consists of residues 372–641 cannot (41). The linking domain(s) of EBNA-1 appears to be complex; several regions of EBNA-1 that are rich in basic residues can mediate this function. Chimeric proteins, consisting of residues 54–89, 331–361, or 372–391 of EBNA-1 fused to the dimerization and DNA binding domain of GAL4 can link DNAs containing GAL4 binding sites (41). The acidic region from residues 620 to 641 has been shown not to be required for DNA looping (33). What role this looping or linking activity of EBNA-1 may contribute to the functions of EBNA-1 in cells is not known. However, the origin-binding protein of the prokaryotic plasmid R6K also has a capacity to loop together sites within the origins of replication of R6K (42), and this activity has been shown to be important in activating replication from one of the plasmid's origins (43) The origin-binding protein, UL9, of herpes simplex virus 1 (HSV-1) also induces a loop between two binding sites at the origin of that virus (44); this activity has not yet been shown to be involved in replication from this origin.

The activities mapped to restricted domains of EBNA-1 are unlikely to be sufficient to mediate the intact protein's functions *in vivo*. The dimerization and DNA binding domain of EBNA-1 alone, for example, does not activate transcription upon binding to the enhancer composed of the family of repeats (T. Middleton and B. Sugden, manuscript in preparation). It seems likely that EBNA-1 will activate transcription by binding to cellular proteins that are part of the transcription complex. A region outside of EBNA-1's dimerization and DNA binding domain is probably needed for this binding. Such specific protein interactions with EBNA-1 and cellular proteins have not yet been identified, though. Activities in addition to those already associated with EBNA-1 have been sought to explain the protein's contribution to DNA replication. By analogy with the origin-binding protein, T antigen, of simian virus 40 (SV40) (45) and the origin-binding protein, El, of bovine papilloma virus (BPV) (46), investigators originally thought that EBNA-1 would have intrinsic DNA-dependent ATPase and helicase activities with which to initiate the unwinding of DNA at the dyad symmetry element of *oriP*. However, EBNA-1 purified from either insect cells or monkey cells lacks these enzymatic activities (26, 39). One can therefore speculate that EBNA-1 will contribute, at least in part, to the DNA replication of *oriP* by recruiting cellular proteins to the origin to induce the formation of a replication initiation complex. No direct evidence for such a role for EBNA-1 has been established yet, however.

One caveat in these interpretations and conjectures is that EBNA-1 is known to be post-translationally modified by phosphorylation on serine residues (47). Whether or not this post-translational modification affects the function of EBNA-1 is not known. In the case of SV40, phosphorylation of T antigen can either augment or inhibit its capacity to support the initiation of SV 40 DNA synthesis, and these opposing effects are regulated by which residues of T antigen are phosphorylated (48). Until the form of EBNA-1 that is competent to activate the family of repeats enhancer and support replication of *oriP* is defined, it will be difficult to identify the activities of this protein that, together, mediate the functions of EBNA-1 in cells.

oriP Plus EBNA-1

The binding of the DNA binding domain of EBNA-1 to *oriP* has been studied in detail. High-resolution footprinting indicates that 16 bp in each of the repeats is protected from chemical modification when bound by this domain of EBNA-1 (49). The sequences of the repeats that compose the family of repeats and the dyad symmetry element do vary. Their relative affinities for the DNA binding domain of EBNA-1 have been measured in competitive binding studies (35). An array of synthetic 30-bp oligonucleotides in which a 20-bp core has been systematically varied while embedded in a constant flanking sequence has been tested for the ability to be bound by the DNA binding domain of EBNA-1 (35). This study also indicates that EBNA-1 rec-

ognizes a 16-bp palindrome that can vary in sequence only in its central 4 bp (35). Repeats in the dyad symmetry element [the sight at which DNA synthesis initiates (16)] bind EBNA-1 with a lower affinity than do those having the consensus sequence within the family of repeats (26, 35). This difference in binding affinities between these two regions has fostered a model in which the family of repeats serves to concentrate EBNA-1 locally in order to facilitate EBNA-1 binding to the dyad symmetry element (29, 40).

Competitive binding studies have identified a consensus DNA binding site for EBNA-1 that is found occasionally within the family of repeats (35). The dissociation constant for the DNA binding domain from a single consensus binding site has been estimated to be 10^{-11} *M* (35). Therefore, this binding is as least as avid as binding of the lac repressor to its operator DNA.

Upon binding to *oriP,* EBNA-1 distorts the DNA. EBNA-1 binding exposes two thymine nucleotides in the dyad symmetery element so that they are sensitive to being oxidized by $KMnO_4$ (50, 51). This distortion is subtle, though, and is consistent with neither the dyad symmetry element being extensively unwound nor this region of DNA being stabilized as a cruciform upon being bound by EBNA-1. Binding to specific DNA does affect the conformation of the DNA binding domain of EBNA-1. The C-terminal domain of EBNA-1 becomes strikingly resistant to protease digestion when it is bound specifically to DNA (37).

Although both the family of repeats and the dyad symmetry have multiple binding sites for EBNA-1, this protein does not appear to bind cooperatively to these elements of *oriP* (26, 52). That is, there is no evidence that occupancy of one binding site by EBNA-1 increases the affinity of an adjacent binding site for EBNA-1 in DNA filter-binding assays. However, in functional assays *in vivo,* multiple binding sites for EBNA-1 do appear to act cooperatively (21). For example, in the presence of EBNA-1, when increasing numbers of EBNA-1 binding sites derived from the family of repeats are positioned next to the dyad symmetry element, the efficiency of transient and stable replication increases nonlinearly with increasing numbers of repeats (21, 53). One possible explanation for this apparent contradiction between the binding affinity assays and the functional assays is that the cooperativity observed *in vivo* reflects a higher-order interaction than simply that of EBNA-1 binding to DNA. For example, the linking of EBNA-1 bound to DNA to another moiety of EBNA-1 bound to DNA may behave cooperatively.

The number of molecules of EBNA-1 in a cell does not correlate with the number of EBV genomes in a cell (54). This and other observations indicate that the average number of *oriP* plasmids per cell is not likely to be regulated by the number of EBNA-1 molecules per cell. Again, this assertion must be tempered with our not knowing which post-translationally modified forms of EBNA-1 carry out the various functions of this protein. The posssibility remains that particular modified forms of EBNA-1 could regulate the copy number of *oriP* plasmids and that this form of EBNA-1 may not be present in different cells as a constant percentage of the total

EBNA-1 found in those cells. If this notion were correct, amplification of the viral genome during latent replication could be obtained only when the initial level of EBV plasmid genomes is established upon their first being introduced into a cell. Thereafter, the latent replication rate of viral genomes would remain constant in the cells.

oriLyt

Induction of the lytic phase of the EBV life cycle occurs spontaneously and at different rates among clones of EBV-immortalized B cells. The lytic phase can also be induced in a fraction of cells (less than 1% to over 20% of the cells in different clones) by treating them with active phorbol esters (55), sodium butyrate (56), or, in some cases, anti-immunoglobulin antibodies (57). Finally, the lytic phase of the viral life cycle can also be induced by transfecting an expression vector for the EBV gene, *BamZ* leftward reading frame-1, BZLF-1, into cells (58). BZLF-1 is a transcription factor that is a member of the b-zip family, sharing dimerization and DNA-binding motifs with cellular proteins such as c-jun and c-fos (59). BZLF-1 stimulates the transcription of viral genes that initiate a regulatory cascade committing the virus and the host cell to the lytic phase of the EBV life cycle (60). BZLF-1 also contributes directly to regulating viral lytic DNA replication (61). It appears to be the origin-binding protein of *oriLyt* (61).

Early after induction of the lytic phase of the EBV life cycle, the mode of viral DNA replication changes. Neither *oriP* nor EBNA-1 appears to participate in the lytic mode of replication. Rather, the origin of lytic replication, *oriLyt,* and at least seven viral proteins act to amplify viral DNA rapidly (6, 7) (Fig. 4). *oriLyt* has been defined functionally and is found to be larger and more complex than *oriP* (6, 62). Wild-type *oriLyt* spans approximately 6 kilobase pairs (kbp) and is composed of two small domains absolutely required for replication and a variety of auxiliary regions that can augment the efficiency of replication. An extensive deletional analysis of wild-type *oriLyt* has demonstrated that one of the two required domains contains two binding sites for BZLF-1 (62). The second, downstream, domain contains binding sites for several cellular proteins. One protein that binds to this second core domain of *oriLyt* has been tentatively identified as SP1 or a related protein. It is not yet understood what role these cellular proteins play in regulating replicating from *oriLyt* (63). Three additional binding sites lie within the auxiliary regions of *oriLyt* (62). Lytic replication is not preserved in *oriLyt* mutants in which these binding sites have been replaced collectively by either binding sites for the BPV transactivator E2 or the yeast transactivator GAL4 in the presence of E2 or a chimeric GAL4:VP16, respectively (61). *oriLyt*-mediated replication requires BZLF-1, and this requirement cannot be substituted by the specific binding of other potent transcriptional activators. BZLF-1 must provide to *oriLyt* a distinctive but as yet unknown replication function.

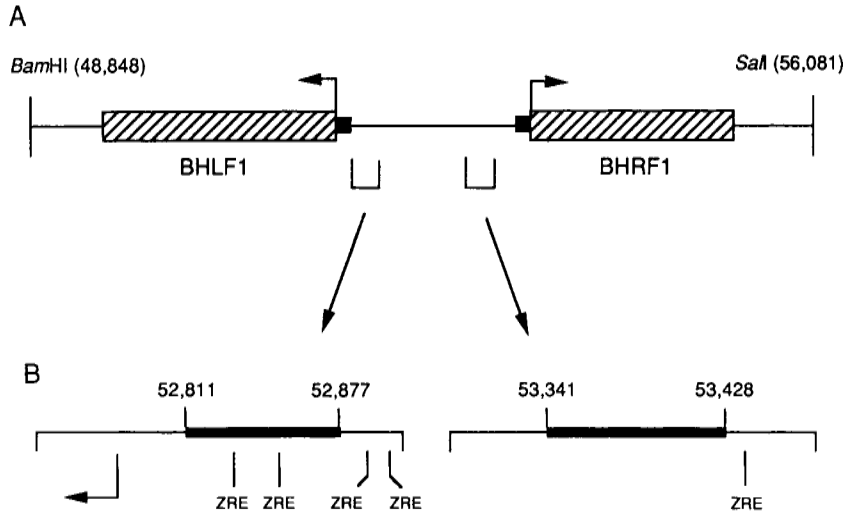

FIG. 4 The structure of the lytic origin of replication (*oriLyt*) of Epstein–Barr virus (EBV). (A) The *Bam*HI to *Sal*I fragment that encompasses the lytic origin of replication. The coordinates that encompass *oriLyt* (48,848–56,081 bp) represent the position of the fragment in the B95-8 strain of EBV. The genes that encode *Bam*H leftward reading frame-1 and *Bam*H rightward reading frame-1 (BHLF-1 and BHRF-1, striped box) are located in this region, and the promoter regions (black box) and the 5′ RNA start sites (arrows) of these genes are shown. The brackets represent essential and auxiliary *oriLyt* elements, enlarged in (B). (B) The enlarged view of the minimal *oriLyt* elements. The bold black lines represent sequences of *oriLyt* found to be essential for replication. The regions proximal to the essential regions are considered the auxiliary elements of *oriLyt,* and the leftmost auxiliary element contains the open reading frame of the BHLF-1 gene (62). *Bam*Z leftward reading frame-1 (BZLF-1) binding sites are denoted ZRE (BZLF-1 *r*esponse *e*lement). Contained in the leftmost essential element are two BZLF-1 binding sites. The coordinates represent the position of the essential elements as they are found in B95-8.

The mechanism by which the auxiliary regions contributes to *oriLyt*-mediated replication is not known. These regions do contain many binding sites for both cellular and viral transcription factors. These sites constitute both the *Bam*H leftward reading frame-1 (BHLF-1) and *Bam*H rightward reading frame-1 (BHRF-1) promoters of EBV, which are embedded within *oriLyt* (62). Part of the auxiliary region can be functionally substituted by the human cytomegalovirus immediate-early promoter/enhancer (6). These observations indicate that the cellular and/or viral transcriptional apparatus contributes to *oriLyt*-mediated replication. An intimate relationship between origins of replication and binding sites for transcription factors has been noted for several other viral origins (64). How viral and cellular transcriptional machinery contributes to the function of origins of replication remains uncertain, though.

Products of *oriLyt*-mediated replication have been identified and partially analyzed. The first form of amplified DNA detected after induction is a covalently closed, circular molecule with the same length as the parental molecule, but apparently with a different superhelical density (6). Subsequent replication produces a polymer of the parental *oriLyt* plasmid in induced cells (6). Although the structure of this polymer has not been elucidated, it has been assumed, by analogy to HSV-1 replicative intermediates, to be composed of head-to-tail concatemers (65). Such molecules are thought to be the immediate precursors to the DNA products formed by the cleavage and packaging of these concatemers into viral capsids.

The viral genes required in *trans* to support *oriLyt*-mediated replication have been identified through the use of complementation assays similar to those used to identify the HSV-1 genes required to support replication from its lytic origins. Specific fragments of EBV DNA were introduced into an EBV-negative cell along with an *oriLyt* plasmid, and replication of the *oriLyt* plasmid was monitored. Increasingly smaller derivatives of these specific viral fragments were tested in combination until a minimal set was identified that supports replication of *oriLyt* (7). These EBV genes include ones that encode the enzymatic activities required to unwind DNA, initiate, and elongate DNA synthesis. Also included in this required set of genes is BZLF-1. As noted previously, BZLF-1 binds specifically to sites within *oriLyt,* and may provide the origin binding function that promotes the formation of a replication initiation complex at *oriLyt* (7, 61).

Vectors Derived from *oriP*

A variety of vectors have been developed with *oriP* and used successfully as shuttle vectors, to express exogenous genes, and to isolate cDNAs functionally. The requirement of these vectors for EBNA-1 has been met either by expressing EBNA-1 on the vector or by using recipient cells, such as all EBV-immortalized cells, that express EBNA-1 constitutively (3). The latter approach yields a 10 to 100-fold increased efficiency of cells supporting *oriP* vectors (3). Apparently, expression of EBNA-1 in a cell at the time of introduction of an *oriP* vector dramatically increases the likelihood that the *oriP* vector will replicate in that cell. One model to explain this finding is that EBNA-1 binds to *oriP* in order to support its initial replication, but once the transfected DNA is bound as nucleosomes, EBNA-1 no longer can bind DNA. If this model were correct, EBNA-1 expressed from an incoming vector might not be expressed prior to nucleosomes forming on that vector DNA. The above consideration is important only if an experimental design requires a high percentage (over 1%) of the initially transfected cells to support *oriP* vectors. When such a requirement exists, as with cDNA cloning vectors, for example, it is particularly desirable to use cell recipients that express EBNA-1 constitutively.

One general use of *oriP* plasmids has been as shuttle vectors to enumerate and analyze mutational lesions in mammalian cells. In addition to *oriP* and a gene conferring drug resistance in the mammalian cell, these shuttle vectors usually contain a ColE1 origin for replication in bacteria, a gene conferring drug resistance in bacteria, and a target gene for mutagenesis whose product can be selected in bacteria. *oriP* shuttle vectors generally have been the most satisfying ones used to study mutagenesis in mammalian cells because they are free of the high rate of "spontaneous" mutations (66) associated with other replicons, particularly those based on SV40. In fact, the rate of spontaneous mutation in *oriP* plasmids resident in a given cell is identical to that of the hypoxanthine–guanosine phosphoribosyltransferase (HGPRT) locus in that cell (66). A virtue of *oriP* vectors gleaned from these studies is that genes expressed stably in them are no more prone to accumulate mutations than are the genes of the host cell.

A second use of *oriP* plasmids has been to express genes added to them in recipient cells. This use has the advantage over integration into cellular DNA of the genes to be expressed in that the context of DNA into which the gene is inserted is known. The possible disadvantage of these vectors is that the enhancer function of *oriP* may affect expression of the inserted gene. Large plasmids that contain *oriP* and λ *cos* sites have been constructed and do replicate in human cells, so that large genes can be inserted into *oriP* plasmids and expressed (67). Small genes and cDNAs have also been expressed efficiently on *oriP*-containing plasmids (68, 69). Finally, *oriP* plasmids have been used successfully to express antisense RNA to cellular mRNAs and thereby inhibit the expression of a particular cellular gene (70).

A third use of *oriP* plasmids has been envisioned for a decade, but has only recently been successful. *oriP* plasmids can be used to isolate cDNAs that encode proteins which complement defects in a recipient cell. Various *oriP* plasmids have been designed as vectors for insertion and expression of libraries of cDNAs and been shown to be useful in reconstruction experiments (71–73). These plasmids have been used to isolate specific cDNAs by selection or screening in characterized recipient cells (74, 75). In general, the usefulness of *oriP* plasmids for cDNA cloning and isolation depends on the efficiency of uptake, expression, and selection of those plasmids. For efficient cloning and isolation of cDNAs, it is important that the recipient cells express EBNA-1 constitutively, that efficient levels of transfection be attained (electroporation is usually the method of choice), and that expression of the inserted cDNA be compatible with both expression of any other plasmid genes required and cell survival. Although *oriP* vectors often support high levels of expression of inserted genes, this level of expression is often limited by the plasmid copy number and the promoter used. Repeated screening for transfected cells that express high levels of the Tumor Necrosis Factor receptor, for example, led to isolation of cells in which the *oriP* vector was integrated (76), perhaps because the number of *oriP* plasmids initially introduced into the cells and their stable copy number were incompatible with the high level of expression sought.

Vectors Derived from *oriP* and *oriLyt*

Vectors that contain both *oriP* and *oriLyt* are dependent in *trans* on EBNA-1 for plasmid replication and on several EBV genes for their amplification when the productive phase of the viral life cycle is induced in a recipient cell (61, 77). So far, such vectors have been used primarily to study EBV. They permit the efficient insertion via recombination of genes into EBV genomes (77, 78), and when they also contain the terminal repeats of EBV, they can be packaged into EBV virions (77). Ths latter finding may provide a particularly useful application for such vectors. Introducing DNAs into primary human B lymphocytes by transfection is difficult. EBV, however, infects human B lymphocytes efficiently. Packaging of *oriP* + *oriLyt* vectors into EBV virions permits these vectors to be introduced efficiently by infection into primary human B cells. This approach can accommodate large inserts; *oriP* + *oriLyt* vectors containing the terminal repeats of EBV are efficiently packaged when they range in length from 160 to 180 kbp (79). Only a portion of EBV DNA is essential for the transformation of primary B cells (80, 81). F plasmids containing the essential DNA can be manipulated in *E. coli,* and, when introduced into a cell with a helper virus, they can be packaged. The packaged plasmids can infect primary B cells and can be used for mutational analyses of EBV or for the introduction of heterologous DNA into primary B cells (81).

Summary

EBV replicates its DNA via two modes: During the latent phase of the EBV life cycle, the virus replicates its DNA as a multicopy plasmid once per cell cycle in S phase (1, 2); during the lytic phase of EBV's life cycle the virus amplifies its DNA 100- to 1000- fold within one or two cell generations (6). These two modes of DNA replication require distinct *cis*- and *trans*-acting viral genetic elements, which have been identified and used to construct different vectors that mimic the two modes of the DNA replication of EBV.

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References

1. A. Adams, *J. Virol.* **61**, 1743 (1987).
2. J. L. Yates and N. Guan, *J. Virol.* **65**, 483 (1991).

3. J. L. Yates, N. Warren, and B. Sugden, *Nature (London)* **313**, 812 (1985).
4. S. Lupton and A. J. Levine, *Mol. Cell. Biol.* **5**, 2533 (1985).
5. J. Yates, N. Warren, D. Reisman, and B. Sugden, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3806 (1984).
6. W. Hammerschmidt and B. Sugden, *Cell (Cambridge, Mass.)* **55**, 427 (1988).
7. E. D. Fixman, G. S. Hayward, and S. D. Hayward, *J. Virol.*, **66**, 5030 (1992).
8. D. Reisman, J. Yates, and B. Sugden, *Mol. Cell. Biol.* **5**, 1822 (1985).
9. D. R. Rawlins, G. Milman, S. D. Hayward, and G. S. Hayward, *Cell (Cambridge, Mass.)* **42**, 859 (1985).
10. B. Sugden and N. Warren, *J. Virol.* **63**, 2644 (1989).
11. D. Reisman and B. Sugden, *Mol. Cell. Biol.* **6**, 3838 (1986).
12. T. A. Gahn and B. Sugden, *J. Virol.* **69**, 2633 (1995).
13. P. J. Krysan, S. B. Haase, and M. P. Calos, *Mol. Cell. Biol.* **9**, 1026 (1989).
14. T. Middleton and B. Sugden, *J. Virol.* **68**, 4067 (1994).
15. S. Jankelevich, J. L. Kolman, J. W. Bodnar, and G. Miller, *EMBO J.* **11**, 1165 (1992).
16. T. A. Gahn and C. L. Schildkraut, *Cell (Cambridge, Mass.)* **58**, 527 (1989).
17. S. French, *Science* **258**, 1362 (1992).
18. B. Liu, L. M. Wong, R. L. Tinker, E. P. Geiduschek, and B. M. Alberts, *Nature (London)* **366**, 33 (1993).
19. B. Liu and B. M. Alberts, *Science* **267**, 1131 (1995).
20. S. Harrison, K. Fisenne, and J. Hearing, *J. Virol.* **68**, 1913 (1994).
21. D. A. Wysokenski and J. L. Yates, *J. Virol.* **63**, 2657 (1989).
22. A. L. Kirchmaier and B. Sugden, *J. Virol.* **69**, 1280 (1995).
23. B. Sugden and N. Warren, *Mol. Biol. Med.* **5**, 85 (1988).
24. C. Mann and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 228 (1983).
25. D. Koshland, L. Rutledge, M. Fitzgerald-Hayes, and L. H. Hartwell, *Cell (Cambridge, Mass.)* **48**, 801 (1987).
26. L. Frappier and M. O'Donnell, *J. Biol. Chem.* **266**, 7819 (1991).
27. M. Polvino-Bodnar, J. Kiso, and P. A. Schaffer, *Nucleic Acids Res.* **16**, 3415 (1988).
28. J. L. Yates and S. M. Camiolo, *in* "Cancer Cells, Vol. 6: Eukaryotic DNA Replication," p. 197. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988.
29. W. Su, T. Middleton, B. Sugden, and H. Echols, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10870 (1991).
30. L. Frappier and M. O'Donnell, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10875 (1991).
31. M. Polvino-Bodnar and P. A. Schaffer, *Virology* **187**, 591 (1992).
32. R. F. Ambinder, M. Mullen, Y.-N. Chang, G. S. Hayward, and S. D. Hayward, *J. Virol.* **65**, 1466 (1991).
33. K. Goldsmith, L. Bendell, and L. Frappier, *J. Virol.* **67**, 3418 (1993).
34. N. Inoue, S. Harada, T. Honma, T. Kitamura, and K. Yanagi, *Virology* **182**, 84 (1991).
35. R. F. Ambinder, W. A. Shah, D. R. Rawlins, G. S. Hayward, and S. D. Hayward, *J. Virol.* **64**, 2369 (1990).
36. A. R. Ferré-D'Amaré, G. C. Prendergast, E. B. Ziff, and S. K. Burley, *Nature (London)* **363**, 38 (1993).
37. W. A. Shah, R. A. Ambinder, G. S. Hayward, and S. D. Hayward, *J. Virol.* **66**, 3355 (1992).
38. T. Williams and R. Tjian, *Science* **251**, 1067 (1991).
39. T. Middleton and B. Sugden, *J. Virol.* **66**, 489 (1992).

40. L. Frappier, K. Goldsmith, and L. Bendell, *J. Biol. Chem.* **269**, 1057 (1994).
41. D. Mackey and B. Sugden, submitted.
42. S. Mukherjee, H. Erickson, and D. Bastia, *Cell (Cambridge, Mass.)* **52**, 375 (1988).
43. A. Miron, S. Mukherjee, and S. Bastia, *EMBO J.* **11**, 1205 (1992).
44. A. Koff, J. F. Schwedes, and P. Tegtmeyer, *J. Virol.* **65**, 3284 (1991).
45. H. Stahl, P. Drage, and R. Knippers, *EMBO J.* **5**, 1939 (1986).
46. M. R. Lentz, D. Pak, I. Mohr, and M. Botchan, *J. Virol.* **67**, 1414 (1993).
47. J. C. Hearing and A. J. Levine, *Virology* **145**, 105 (1985).
48. E. Fanning and R. Knippers, *Annu. Rev. Biochem.* **61**, 55 (1992).
49. A. S. Kimball, G. Milman, and T. D. Tullius, *Mol. Cell. Biol.* **9**, 2738 (1989).
50. L. Frappier and M. O'Donnell, *J. Virol.* **66**, 1786 (1992).
51. J. Hearing, Y. Mülhaupt, and S. Harper, *J. Virol.* **66**, 694, (1992).
52. C. H. Jones, D. Hayward, and D. R. Rawlins, *J. Virol.* **63**, 101 (1989).
53. T. Chittenden, S. Lupton, and A. J. Levine, *J. Virol.* **63**, 3016 (1989).
54. L. Sternås, T. Middleton, and B. Sugden, *J. Virol.* **64**, 2407 (1990).
55. H. Zur Hausen, F. J. O'Neill, U. K. Freese, and E. Hecker, *Nature (London)* **272**, 373 (1978).
56. J. Luka, B. Kallin, and G. Klein, *Virology* **94**, 228 (1979).
57. K. Takada and Y. Ono, *J. Virol.* **63**, 445 (1989).
58. G. Miller, *J. Infect. Dis.* **161**, 833 (1990).
59. P. J. Farrell, D. T. Rowe, C. M. Rooney, and T. Kouzarides, *EMBO J.* **8**, 127 (1989).
60. A. J. Sinclair, M. Brimmel, F. Shanahan, and P. J. Farrell, *J. Virol.* **65**, 2237 (1991).
61. A. Schepers, D. Pich, and W. Hammerschmidt, *EMBO J.* **12**, 3921 (1993).
62. A. Schepers, D. Pich, J. Mankertz, and W. Hammerschmidt, *J. Virol.* **67**, 4237 (1993).
63. H. Gruffat, O. Renner, D. Pich, and W. Hammerschmidt, *J. Virol.* **69**, 1878 (1995).
64. M. L. DePamphilis, *Annu. Rev. Biochem.* **62**, 29 (1993).
65. R. J. Jacob, L. S. Morse, and B. Roizman, *J. Virol.* **29**, 448 (1979).
66. N. R. Drinkwater and D. K. Klinedinst, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3402 (1986).
67. D. Kioussis, F. Wilson, C. Daniels, C. Leveton, J. Taverne, and J. H. L. Playfair, *EMBO J.* **6**, 355 (1987).
68. A. Jalanko, A. Kallio, M. Rouhonen-Lehto, H. Söderlund, and I. Ulmanen, *Biochim. Biophys. Acta* **949**, 206 (1988).
69. T. G. Jensen, B. S. Andresen, P. Bross, U. B. Jensen, E. Holme, S. Kølvraa, N. Gregersen, and L. Bolund, *Biochim. Biophys. Acta* **1180**, 65 (1992).
70. J. E. Hambor, C. A. Hauer, H.-K. Shu, R. K. Groger, D. R. Kaplan, and M. L. Tykocinski, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4010 (1988).
71. R. F. Margolskee, P. Kavathas, and P. Berg, *Mol. Cell. Biol.* **8**, 2837 (1988).
72. P. B. G. M. Belt, H. Groeneveld, W. J. Teubel, P. van de Putte, and C. Backendorf, *Gene* **84**, 407 (1989).
73. C. Peterson and R. Legerski, *Gene* **107**, 279 (1991).
74. P. B. G. M. Belt, W. Jongmans, J. de Wit, J. H. J. Hoeijmakers, P. van de Putte, and C. Backendorf, *Nucleic Acids Res.* **19**, 4861 (1991).
75. L. C. Pan, R. F. Margolskee, and H. M. Blau, *Somat. Cell Mol. Genet.* **18**, 163 (1992).
76. R. A. Heller, K. Song, D. Villaret, R. Margolskee, J. Dunne, H. Hayakawa, and G. M. Ringold, *J. Biol. Chem.* **265**, 5708 (1990).
77. W. Hammerschmidt and B. Sugden, *Nature (London)* **340**, 393 (1989).
78. M.-A. Lee, O.-J. Kim, and J. L. Yates, *Virology* **189**, 253 (1992).

79. T. A. Bloss and B. Sugden, *J. Virol.* **68**, 8217 (1994).
80. E. Robertson and E. Kieff, *J. Virol.* **69**, 983 (1995).
81. B. Kempkes, D. Pich, R. Zeidler, B. Sugden, and W. Hammerschmidt, *J. Virol.* **69**, 231 (1995).
82. C. R. Kintner and B. Sugden, *Cell (Cambridge, Mass.)* **17**, 661 (1979).
83. R. Baer, A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Séguin, P. S. Tuffnell, and B. G. Barrell, *Nature (London)* **310**, 207 (1984).
84. M. Bodescot, O. Brison, and M. Perricaudet, *Nucleic Acids Res.* **14**, 2611 (1986).
85. M. Bodescot, M. Perricaudet, and P. J. Farrell, *J. Virol.* **61**, 3424 (1987).

Appendix

Here, we describe general procedures for working with EBV plasmid vectors and their amplifiable derivatives that have been used in experiments described in the text.

Selection of Cells to Maintain Vectors That Express Resistance to Hygromycin B or G418

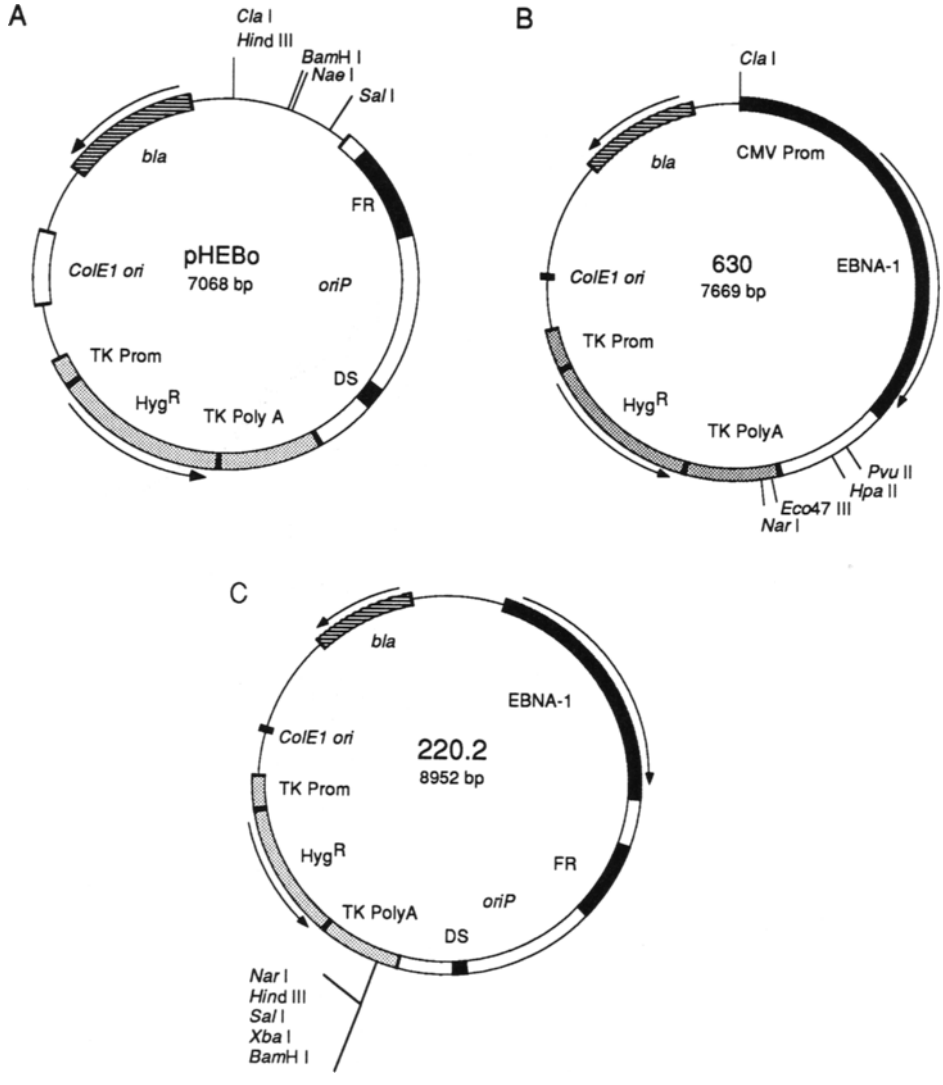
1. Plate nonadherent cells to be tested at 10^4 cells per milliliter in complete medium and distribute 1 ml into each of 24 wells in a 24-well cluster dish. Plate adherent cells to be tested such that they will be 1–5% confluent upon adhering in 1 ml of complete medium in each well of a 24-well cluster dish.

2. Add a selective agent at different concentrations to duplicate wells of each plate. For hygromycin B use 0, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, and 1000 μg/ml final concentration. For G418 use 0, 50, 100, 200, 300, 400, 500, 750, 1000, 1500, 2000, and 2500 μg/ml media final concentration. The selective agents must be at neutral pH in isotonic medium before they are added to the cells (G418 solutions are often acidic).

3. The treated cells should be incubated for 14–21 days and inspected visually every second day. Cells that are unaffected because they receive no drug or concentrations of drug to which they are spontaneously resistant will proliferate and may eventually die at high cell numbers before the end of the experiment. These instances must be distinguished from cell death that results from drug toxicity.

4. At the end of the incubation period, the lowest concentration of drug that leads to the death of all cells must be identified. The next higher concentration should be used to select cells that maintain introduced vectors that encode resistance to that drug.

5. To generate cell lines that maintain EBV plasmid vectors, transfect plasmid DNA into cells by electroporation. A procedure for the optimization of electroporation parameters has been described by Knutson and Yee (1). For stable assays, select



APPENDIX FIG. 1 Epstein–Barr viral (EBV) plasmid vectors. (A) Plasmid pHEBo (10) encodes the ColE1 origin (*ColE1 ori;* white box) and the β-lactamase gene encoding resistance to ampicillin (*bla;* hatched box) in a pBR322 background (13) for propagation in *Escherichia coli.* pHEBo also contains *oriP* [family of repeats (FR) and dyad symmetry element (DS); black box] (14) for latent replication in mammalian cells expressing EBNA-1, and the hygromycin B phosphotransferase gene encoding resistance to hygromycin (*Hyg*^{R}*;* light stippled box) (15) driven by the herpes simplex virus 1 (HSV-1) thymidine kinase (TK) promoter (9) and containing a simian virus 40 polyadenylation signal. Unique restriction sites are noted for the introduction of additional sequences. A strong promoter within the β-lactamase sequences

for cells that maintain vectors by propagating them for 14–21 days in complete medium at the optimum concentration of drug.

Constructing an Effective oriP Plasmid for Foreign Gene Expression

EBV plasmid vectors may be used for either short-term or stable expression of proteins in a variety of host cells (2–4). The level of expression of a foreign gene of interest will depend on several factors: the cell line used (see text), the number of *oriP* plasmids per cell, the plasmid or integrated state of the vector, the promoter driving the foreign gene, and the placement of the foreign gene within the plasmid. The number of copies of plasmids per cell and the plasmid/integrated state of the vector may be determined by Southern analysis (5) of total cellular DNA from cells containing the EBV-based vector that has been digested with the appropriate restriction enzymes. The use of an inducible promoter such as the mouse mammary tumor virus long terminal repeat (6) can allow modulation of the expression of the gene of interest. An efficient promoter such as the SV40 early promoter (7) or the human cytomegalovirus immediate-early promoter (8) can allow a high level of gene expression, whereas inefficient promoters such as the HSV-1 thymidine kinase promoter (9) usually supports a low level of expression of genes in these vectors. Appendix Fig. 1 depicts three EBV-based plasmids available for the latent expression of foreign genes. Plasmid pHEBo (10) contains *oriP* and a selectable marker in a pBR322 background. Genes to be expressed in pHEBo can be inserted in the unique sites between *Cla*I and *Sal*I along with the *cis*-acting signals required to express them appropriately. pHEBo must be used in either EBV$^+$ cell lines or cell lines that express EBNA-1 constitutively. Plasmid 630 contains the gene for EBNA-1 and a selectable marker in a pBR322 background. Cell lines containing integrated copies of 630 will provide EBNA-1 for the maintenance of pHEBo in the absence of the viral genome (11). Plasmid 220.2 contains *oriP*, EBNA-1, and a selectable marker in a pBR322 background. Plasmid 220.2 can be used to express foreign genes; in these cases transcription of the gene inserted in the polylinker is efficient when it is directed

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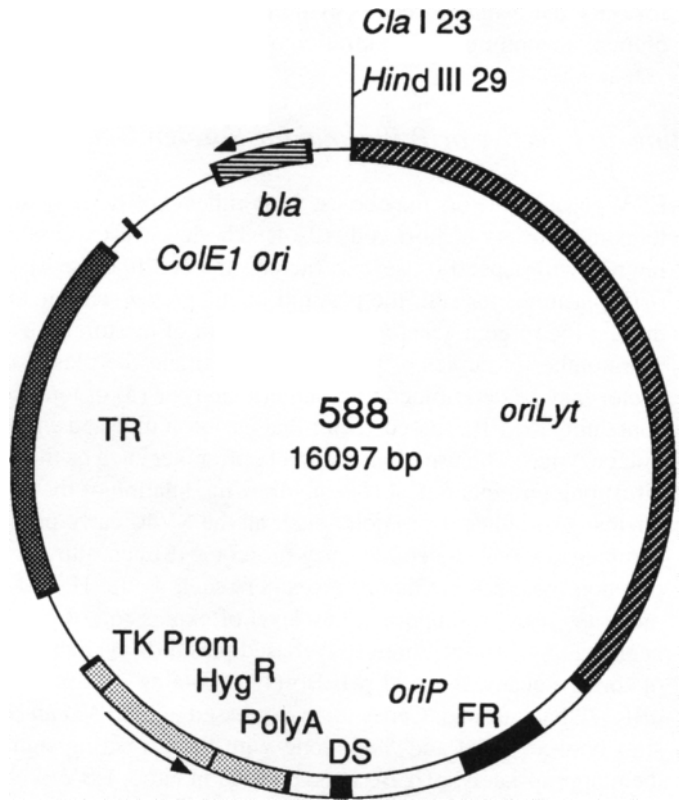
will allow the expression of genes inserted at this site. (B) Plasmid 630 contains the gene for EBV nuclear antigen 1 (EBNA-1) (16) expressed from the cytomegalovirus immediate-early promoter (8), *Hyg*^{R} (light stippled box) driven by the HSV-1 TK promoter and containing a TK polyadenylation signal, and the ColE1 origin (*ColE1 ori;* open box) and the β-lactamase gene encoding resistance to ampicillin (*bla;* hatched box) in a pBR322 background for propagation in *E. coli.* (C) Plasmid 220.2 contains sequences included in pHEBo as well as the gene encoding EBNA-1 (dark stippled box), which is driven from a fortuitous promoter within the β-lactamase sequences. 220.2 also contains a polylinker (unique restriction sites noted) for cloning genes of interest into this plasmid. The arrows beside the genes indicate the direction of transcription. Prom, Promoter.



APPENDIX FIG. 2 Epstein–Barr viral (EBV) amplifiable plasmid 588. Plasmid 588 contains the ColE1 origin (*ColE1 ori;* solid box) and the gene encoding resistance to β-lactamase (*bla;* hatched box) in a pBR322 background (13) for propagation in *Escherichia coli.* Plasmid 588 also contains *oriP* [family of repeats (FR) and dyad symmetry element (DS); black box] (14) for latent replication in mammalian cells expressing EBV nuclear antigen 1 (EBNA-1), and the gene encoding resistance to hygromycin (*Hyg*^{R}*;* light stippled box) (15) for selection of mammalian cells maintaining these plasmids. The terminal repeats (TR; dark stippled box) and *oriLyt* (striped box) sequences (16) are included for amplification in EBV$^+$ cells. Unique restriction sites are noted for the introduction of additional sequences. A promoter within the β-lactamase sequences will allow the expression of genes inserted at this site. The arrows beside the genes indicate the direction of transcription. TK Prom, Thymidine kinase promoter.

clockwise with the appropriate *cis*-acting signals (12). Derivatives of pHEBo, 630, and 220.2 have been constructed that express resistance to G418 rather than to hygromycin B.

Induction of Lytic Phase in EBV and Subsequent Isolation of Viral Particles

Plasmid 588, which is shown in Appendix Fig. 2, contains *oriP, oriLyt,* the terminal repeats, and a selectable marker in a pBR322 background. Upon induction of the lytic phase of EBV in EBV^+ cells that also contain 588, concatemers of this plasmid will be generated, packaged in viral particles, and released along with the endogenous EBV helper virus. The following protocol describes how to induce the lytic phase of EBV.

1. Propagate EBV^+ cells (e.g., B95-8) in complete medium (e.g., RPMI 1640 and 10% (v/v) calf serum) to $0.5-1 \times 10^6$ cells per milliliter 37° C.
2. Induce the lytic cycle of EBV by adding 20 ng/ml 12-0-tetradecanoylphorbol 13-acetate and 3 m*M* sodium butyrate (final concentrations).
3. Incubate the cells at 37° C for 4–5 days. At this point most of the cells will be dead, as monitored by dye exclusion assays.
4. Harvest the virus by pelleting the cell debris at 580 *g* for 20 min. Remove the supernatant and leave the pellet; the supernatant contains the released virus.
5. Filter the resulting supernatant through a 0.8-μm filter. The filtering guarantees removal of residual viable cells and cell debris. The virus is stable in complete medium (with 10% (v/v) serum) at pH 7.4 at 4° C for several months.
6. If products of genes expressed from derivatives of 588 are to be isolated from cells in which the plasmid has been amplified by induction of the lytic phase of the EBV life cycle, then the cells must be harvested approximately 48 hr after induction before they have begun to lyse.

Appendix References

1. J. C. Knutson and D. Yee, *Anal. Biochem.* **164**, 44 (1987).
2. J. L. Yates and N. Guan, *J. Virol.* **65**, 483 (1991).
3. D. A. Wysokenski and J. L. Yates, *J. Virol.* **63**, 2657 (1989).
4. G. Cachianes, C. Ho, R. F. Weber, S. R. Williams, D. V. Goeddel, and D. W. Leung, *BioTechniques* **15**, 255 (1993).
5. E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
6. H. Diggelmann, A. L. Vessaz, and E. Buetti, *Virology* **122**, 332 (1982).
7. V. B. Reddy, P. K. Ghosh, P. Lebowitz, M. Piatak, and S. M. Weissman, *J. Virol.* **30**, 279 (1979).
8. M. Stinski and T. J. Roehr, *J. Virol.* **55**, 431 (1985).
9. S. L. McKnight, *Nucleic Acids Res.* **8**, 5949 (1980).
10. B. Sugden, K. Marsh, and J. Yates, *Mol. Cell. Biol.* **5**, 410 (1985).
11. T. Middleton and B. Sugden, *J. Virol.* **66**, 1795 (1992).
12. J. M. Young, C. Cheadle, J. S. Foulke, Jr., W. N. Drohan, and N. Sarver, *Gene* **62**, 171 (1988).

13. M. Lusky and M. Botchan, *Nature (London)* **293**, 79 (1981).
14. D. Reisman, J. Yates, and B. Sugden, *Mol. Cell. Biol.* **5**, 1822 (1985).
15. L. Gritz and J. Davies, *Gene* **25**, 179 (1983).
16. R. Baer, A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell, *Nature (London)* **310**, 207 (1984).

Section II

DNA Viruses

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[6] Methods to Study Epstein–Barr Virus and p53 Status in Human Cells

Alison J. Sinclair and Paul J. Farrell

Introduction

Epstein–Barr virus (EBV) immortalization of primary B lymphocytes results in the establishment of permanently growing lymphoblastoid cell lines (LCLs). Several published procedures describe how to use EBV to generate LCLs using crudely fractionated leukocytes from small samples of human blood (see, e.g., Ref. 1); this is a very convenient method for establishing cell lines derived from individuals, and such LCLs are routinely used as a permanent source of material for genetic investigations. EBV is also a significant human pathogen, causing most infectious mononucleosis and contributing to several types of cancer, including Burkitt's lymphoma (BL), nasopharyngeal carcinoma, and lymphomas in those who are immunosuppressed either following transplant surgery or because of the acquired immunodeficiency syndrome (AIDS).

We have investigated further the molecular events that lead to immortalization by EBV, and for this it is necessary to produce a high-titer virus and to infect pure primary B lymphocytes. In this article we describe the production of high-titer virus from the B95-8 cell line and describe a procedure to isolate large quantities of primary B lymphocytes. We have also studied the gene expression and genetic map of EBV, and convenient procedures for preparing probes for analyzing RNA expression and for transfecting cell lines with EBV *oriP*-based vectors are described. The status of *p53* in LCLs and in human tumor cells has been a topic of considerable interest, and a simple polymerase chain reaction (PCR)-based approach to the isolation of the coding region of the entire human *p53* gene for subsequent characterization is also reported.

Growth and Preparation of EBV

EBV^+ cell lines derived from biopsies of the EBV-associated disease BL or from LCLs can be cultured *in vitro*. The viral gene expression in the great majority of the cells in these lines is restricted to the latency genes, and virus production is only detectable in a small proportion of the cells. In some cell lines the viral replicative cycle can be activated by various stimuli to increase the amount of EBV shed into the culture medium (2–4), and protocols have been established to obtain high-titer virus from cell lines. For example, wild-type immortalizing EBV has been produced

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from the BL cell line Akata (4) and from the LCL strain B95-8 (5). Nonimmortalizing defective virus, which is useful for investigating the molecular mechanisms of virus infection, is produced from the BL cell line P3HR1 (6). B95-8, a monkey B-cell line infected with EBV from an infectious mononucleosis patient in the United States, routinely produces high titers of immortalizing virus and is the most frequently used source of laboratory EBV (5). B95-8 is a type I or A-type strain of EBV and is the prototype strain from which the EBV DNA sequence was derived (7) and for which the genetic map has been studied in closest detail. Its preparation is described in detail below.

1. The B95-8 cell line (available from ECACC, Porton Down, UK; Cat. No. 85011419) is cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), penicillin, and streptomycin at a density of $2-8 \times 10^5$ cells per milliliter until the volume reaches 500 ml.
2. The cells are allowed to saturate (at a density of about 10^6 cells per milliliter) and phorbol-12-myristate 13-acetate is added to a final concentration of 30 ng/ml.
3. After 6 days the medium containing the virus is separated from the cells by centrifuging at 1300 rpm for 5 min at 4°C in a Sorvall (Newtown, CT) RC3C centrifuge.
4. Any remaining cells are removed by passing the tissue culture medium through a 0.8-μm filter.
5. The medium is transferred to 16×30-ml polyallomer tubes and the virus particles are pelleted by centrifugation at 25,000 rpm for 60 min in an SW27 rotor (Beckman, Palo Alto, CA) at 4°C.
6. The medium is carefully removed with an aspirator, and the virus (visible as an opaque pellet on the side of the tube) is resuspended by repeated pipetting in 100 μl of fresh tissue culture medium.
7. The virus is pooled and diluted with tissue culture medium to 10 ml (1/50th of the original volume).
8. Any contaminants are removed by passing the virus through a 0.8-μm filter.
9. The virus stock is dispensed in 1-ml aliquots into cryogenic vials and stored in liquid nitrogen.

This procedure yields sufficient virus to infect about 5×10^8 lymphocytes. The virus stocks can be tested for infectivity of B lymphocytes by a number of assays, depending on future requirements. These include expression of EBV genes, stimulation of DNA synthesis (see below), and outgrowth of LCLs.

Isolation of Quiescent Primary B Lymphocytes from Buffy Coat Residues

We have modified a procedure described earlier (8) to isolate large quantities of quiescent primary B lymphocytes from peripheral blood products in a cost-effective manner (Fig. 1). The first stage in this process uses a relatively crude density gradient to remove the majority of the erythrocytes and to enrich mononuclear lymphocytes.

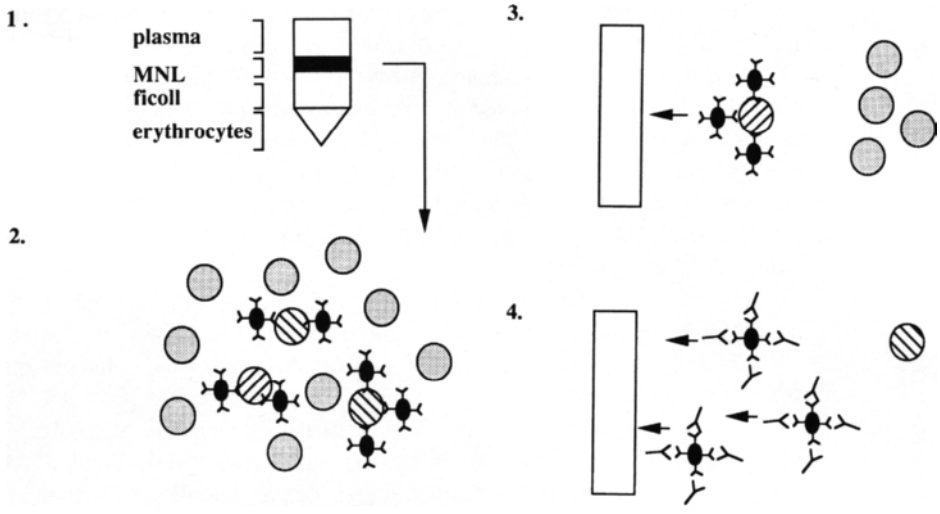

FIG. 1 The four stages in the purification of primary B lymphocytes. Step 1: Enrichment of mononuclear lymphocytes (MNL). Step 2: Incubation with CD19 beads. Step 3: Isolation of selected cells. Step 4: Removal of selected cells. B and T lymphocytes are represented as hatched and stippled spheres, respectively, and the Dynabeads are represented as solid spheres.

The B lymphocytes are then readily isolated by positive selection using lineage-specific antibody-coated magnetic beads. Under these conditions the optimum ratio of beads to target cells is 2:1, which efficiently isolates the B lymphocytes from the mononuclear lymphocyte fraction. The final stage in the purification involves removing the beads from the cells by competition with an antibody directed against mouse Fab (9). The yields and characteristics of the isolated cells are described below.

1. Platelet-depleted buffy coats obtained from the National Blood Transfusion Service, UK, are used as a source of human B lymphocytes. Buffy coats from 30 U of blood (approximately 1200 ml) are pooled.
2. Falcon tubes (50 × 50 ml) are prepared with 25 ml of Ficoll-paque (Pharmacia, Uppsula, Sweden) in each.
3. Twenty-five milliliters of buffy coat is carefully layered over the Ficoll-paque and the gradients are centrifuged at 1300 rpm for 30 min at 20° C in a Sorvall RC3C centrifuge and halted without the brake.
4. After centrifugation the gradients contain a 5- to 10-ml layer of plasma at the top, a white layer of mononuclear lymphocytes at the interface, a 25-ml layer of Ficoll-paque and a 10-ml pellet of erythrocytes at the bottom of the gradient. The layer of plasma is carefully removed by aspiration.
5. The mononuclear lymphocyte fractions (5–10 ml each gradient) are removed with a 10-ml pipette, transferred to a 250-ml tube, and diluted with 4 vol of phosphate-buffered saline (PBS) supplemented with 1% (v/v) FCS (PBSS).

6. The lymphocytes are pelleted by centrifugation at 1300 rpm for 5 min at 20° C in a Sorvall RC3C centrifuge, pooled, and resuspended in 30 ml of PBSS at 4° C.

7. Anti-CD19–coated magnetic beads (1.2×10^9 beads, 3 ml of Dynabeads M-450 pan-B, Dynal, Oslo, Norway) are incubated with 30 ml of cold PBSS in a 50-ml plastic tube for 5 min. The tube is placed in a magnetic particle concentrator (Dynal) for 5 min, which collects the Dynabeads on the side of the tube. The PBSS is decanted and the Dynabeads are resuspended in a 3 ml of PBSS.

8. The washed Dynabeads are added to the lymphocytes and incubated with gentle rolling for 30 min at 4° C.

9. The magnetic particle concentrator is used to collect the rosettes of Dynabeads and $CD19^+$ lymphocytes. The PBSS and the nonselected cells are discarded.

10. The rosettes are washed with 30 ml of PBSS at 4° C and collected again. This step is repeated five times.

11. The rosettes are resuspended in 9 ml of PBSS, and 1 ml of a polyclonal antibody raised against mouse Fab fragments (Detachabead, Dynal) is added. After a 60-min incubation at room temperature, the Dynabeads are collected with the magnetic particle concentrator.

12. The residual Dynabeads are washed with 10 ml of PBSS to recover any free lymphocytes, then the Dynabeads are collected with the magnetic particle concentrator.

13. The lymphocytes are left in suspension and transferred to a fresh tube. Any remaining Dynabeads are collected by repeated incubation with the magnetic particle concentrator.

14. The lymphocytes are pelleted by centrifugation at 1300 rpm for 5 min and washed in 10 ml of PBSS. After a further round of centrifugation the lymphocytes are counted and resuspended in tissue culture medium at 1×10^6 cells per milliliter.

15. The cells are usually cultured at 37° C for 20 hr before use.

This protocol generally yields $1-2 \times 10^7$ B lymphocytes per unit of peripheral blood. Lineage-specific antigen expression suggests that the cells are pure, and a lack of DNA synthesis suggests that they are quiescent (8, 10). Infection of the cells with EBV results in expression of EBV latency genes, outgrowth of immortal LCLs, and stimulation of DNA synthesis (10) (Fig. 2).

Transfection of Lymphoid Cell Lines Using *oriP* Vectors

Transfection of lymphoid cell lines and selection of transfected cell lines is often a problem because of the low transfection efficiencies achieved, combined with the inherent difficulties associated with culturing these cells at low densities. The *oriP* element of EBV has been shown to confer episomal replication and maintenance of plasmids containing this element in cell lines that contain the EBV nuclear antigen 1 (EBNA-1) protein (11). The *oriP* element gives a substantial increase in transfection

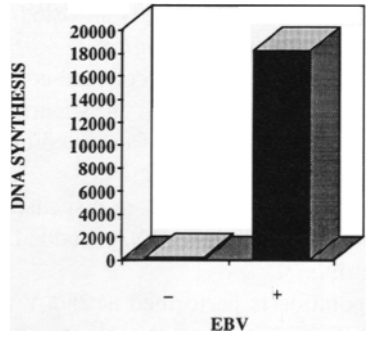

FIG. 2 The stimulation in DNA synthesis resulting from infection of pure B lymphocytes by B95-8 EBV. Primary B lymphocytes were purified and cultured in 1-ml aliquots as described. Two hundred microliters of stock B95-8 Epstein–Barr virus (EBV) was added where indicated (− or +). [^{3}H]Thymidine (1 μCi) was added to the tissue culture medium and the cells were incubated for 6 days. The amount of [^{3}H]thymidine incorporated into DNA was determined [S. Funderud, B. Erikstein, H. C. Asheim, K. Nustad, T. Stokke, H. K. Blomhoff, H. Holte, and E. B. Smeland, *Eur. J. Immunol.* **20,** 201 (1990)] and plotted as a measure of DNA synthesis.

efficiency, making plasmids containing this element useful vectors for transfection of cell lines expressing EBNA-1. In our experience the transfection efficiencies without oriP are usually so low that the inclusion of oriP is virtually essential for routine transfection of EBV$^+$ BL cell lines and human LCLs. Versions of the *oriP* vectors containing the *EBNA-1* gene have also been developed; these can, in principle, be used in cells lacking EBNA-1, but we have found the *oriP* vectors to be mose useful when transfecting cell lines that already express EBNA-1. The most satisfactory drug selection is for hygromycin resistance, although G418 resistance also works well. Hygromycin is significantly less expensive and is used at lower concentrations than G418.

1. Cells are grown carefully with daily dilution in the range of $2-8 \times 10^5$ cells per milliliter for several days prior to the experiment to ensure that they are growing well. The growth medium is generally RPMI 1640 with 10% (v/v) heat-inactivated FCS, penicillin, and streptomycin. With some BL cell lines and some sera it is necessary to use 15% (v/v) serum to achieve optimal growth and transfection.

2. The day before the transfection the cells are either diluted in fresh medium or collected by centrifugation and resuspended in fresh medium at about 3×10^5 cells per milliliter and then grown overnight.

3. The DNA to be transfected is sterilized by ethanol precipitation. Usually, 10 μg of plasmid DNA is used for each electroporation. The appropriate volume of each DNA is placed in a 1.5-ml microfuge tube, made up to 0.2 *M* sodium acetate (pH 5.2), and 2.5 vol of ethanol is added. After mixing and chilling briefly on dry ice, the DNA is microfuged for 10 min. The supernatant is removed with a drawn-

out Pasteur pipette and the pellet is air-dried in a sterile cabinet. The DNA is then redissolved in sterile water at 1 mg/ml.

4. Just before transfection the cells are counted and then collected by centrifugation at 1300 rpm for 5 min at 4° C. The supernatant (conditioned medium) is retained and the cells are resuspended in fresh medium at a concentration of 3×10^8 cells per milliliter.

5. Aliquots (0.25 ml) of cells are dispensed to Bio-Rad electroporation cuvettes (0.4-cm electrode gap) and the DNA is added. The cells and the DNA are mixed by repeated pipetting.

6. Electroporation is performed at 250 V and 960 μF using a Bio-Rad (Hemel Hempstead, UK) Genepulser with a capacitance extender. Under these conditions the time constant usually measures between 38 and 42. After electroporation the samples are left at room temperature for 5 min.

7. The contents of the cuvette are then transferred to 10 ml of medium (1 : 1 fresh medium and conditioned medium) in a 25cm^2 tissue culture flask and incubated at 37° C overnight. Normally, 50–90% of the cells appear to be dying at this point.

8. The next day selective drug is added (usually hygromycin at 0.3 mg/ml, but it is important to test the sensitivity of each target cell line first) and the contents of the flask are distributed into six wells of a standard 24-well plate. Every 3–4 days most of the medium is aspirated off, avoiding the cells, and replaced with fresh medium containing selective drug. Foci of growing cells are apparent within 2–3 weeks, and the wells with drug-resistant cells should start to appear yellow, indicating cell growth, between media changes after about 3 weeks.

9. The contents from one well can usually be transferred to a 25cm^2 flask with 5 ml of medium 4 weeks after electroporation.

We have generated many cell lines transfected with oriP vectors, using the Akata (4) and Mutu (12) BL cell lines and a variety of LCLs (13–17). The Mep4 vector (Invitrogen, San Diego, CA) controls expression from the metallothionein promoter, which is effectively silent in most cell lines until induced by the addition of cadmium or zinc. This feature has proved useful for investigating the effects of a growth-suppressing gene in BL cells (16) and also for directing the transient expression of viral and cell genes (14, 15, 17). We have also used vectors with the cytomegalovirus immediate-early promoter, for example, pSNOC (13) or pCEP4 (Invitrogen), to direct the constitutive expression of both cell and viral genes (13, 15).

Prime-Cut Probes from M13 Clones for Northern Blots and S1 Mapping

Single-stranded probes for Northern blots and RNA protection can be made conveniently by *in vitro* transcription, but as large-scale DNA sequencing of complex genomes becomes more common, the M13 clones characterized during the sequencing program can be very useful resources for making probes for the analysis of gene expression from the sequenced region. One of the first and clearest examples of this structural approach to genetic analysis was for EBV (18). The prime-cut method (19)

is a quick, simple, and reliable technique for making short, defined, single-stranded DNA probes starting from M13 DNA, which is not a suitable template for the *in vitro* transcription approach. Because the resulting probes are single stranded and have been gel-purified, they give clean results with low background both on Northern blots and in S1 nuclease mapping experiments.

M13 DNA Prepared as for DNA Sequencing

1. Grow an overnight culture of TG1, JM109, or an equivalent *Escherichia coli* strain in a standard rich medium such as 2xTY (6g Tryptone, 10g yeast extract, 5g NaCl per liter) by inoculating the medium with a bacterial colony maintained on a minimal glucose plate.
2. Dilute the bacterial overnight culture 1:100 in 2xTY and dispense 1.5-ml aliquots into sterile tubes. Inoculate each with one recombinant M13 plaque by piercing the plaque with a sterile cocktail stick and shaking briefly in the diluted bacterial cultures.
3. Grow with fast shaking at 37° C for 5 hr.
4. Pour each culture into a 1.5-ml plastic microfuge tube; spin for 5 min in the microfuge (12,000 rpm at room temperature).
5. Pour the supernatant into a new tube, avoiding any of the bacterial pellet.
6. Add 200 μl of 2.5 *M* NaCl and 20% (w/v) PEG (polyethylene glycol 6000, BDH Biochemicals, Lutterworth, UK). Mix and leave at room temperature for 10 min.
7. Pellet the M13 by microfuging for 5 min.
8. Carefully remove all of the supernatant (tip out liquid, recentrifuge briefly, and remove any remaining liquid with a drawn-out Pasteur pipette). Failure to remove all of the PEG at this stage is a common problem.
9. Resuspend the pellet in 100 μl of TE buffer [10 m*M* Tris–HCl (pH 7.5) and 1 m*M* EDTA] by vortexing.
10. Extract with 100 μl of water-saturated phenol:chloroform (1:1 v/v). Vortex well, microfuge for 2 min, and take the upper layer into a fresh tube, avoiding any material from the interface.
11. Ethanol-precipitate DNA by adding 10 μl of 2 *M* sodium acetate (pH 5.2) and 250 μl of ethanol.
12. Chill briefly on dry ice, then microfuge for 10 min.
13. Remove the supernatant and add 1 ml of -20° C ethanol. Microfuge for 5 min.
14. Remove all of the ethanol supernatant, using a drawn-out Pasteur pipette.
15. Air-dry briefly and redissolve in 30 μl of TE buffer.

Prime-Cut Probe

Single-stranded recombinant M13 DNA is annealed with the M13 sequencing primer and a complementary radiolabeled strand is synthesized using Klenow DNA poly-

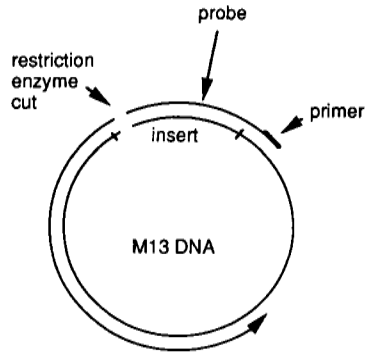

FIG. 3 A prime-cut probe is made by annealing an oligonucleotide to single-stranded M13 DNA containing an appropriate cloned insert, followed by extending the 3′ end of the oligonucleotide with Klenow DNA polymerase. Subsequent restriction digestion cleaves the double-stranded DNA, and the probe fragment can be isolated by electrophoresis on a denaturing gel.

merase; thus, the 5′ end of the probe is defined by the sequencing primer (Fig. 3). Digestion at a restriction enzyme site located either within the insert or in the downstream polylinker is used to define the 3′ end of the probe (Fig. 3). The linear radioactive single strand is then efficiently purified from the complementary unlabeled M13 linear DNA by size separation on a denaturing gel. It is convenient to prepare up to six different probes at once, and the procedure takes only 1 working day. It is usually convenient to premix the reagents marked here with an asterisk.

1. Mix in a 0.5-ml plastic microfuge tube
   3 μl of M13 single-stranded DNA
   *1 μl of 54 m*M* Tris–Cl (pH7.6), 0.27 *M* NaCl, 54 m*M* $MgCl_2$, and 6 m*M* dithiothreitol
   *1 μl of primer (equivalent to 5 ng; normally, use GTTTTCCCAGTCACGAC primer)
   5 μl (total)

   Incubate the closed tube in a 65° C oven for 30 min (make the gel described below during this time if you have not done so earlier).

2. Cool the tube to room temperature and add
   * 2.0 μl of [α^{32}P]dCTP (10 mCi/ml, 3000 Ci/mmol)
   * 1.3 μl of distilled water
   * 2.5 μl of 0.5 m*M* each dATP, dGTP, and dTTP (pH 7.5)
   * 0.2 μl of Klenow DNA polymerase
   11.0 μl (total)

   Incubate at room temperature for 10 min.

3. Add
   1.0 μl of 0.5 m*M* dCTP
   12.0 μl (total)

   Incubate at room temperature for 5 min.

4. Heat at 65° C for 5 min, then cool to room temperature.
5. Make the final volume up to 20 μl for restriction digest, adding appropriate buffer for the enzyme selected. Typically, use 1 μl of enzyme and digest at 37° C for 20 min.

6. To stop the reaction, add
   3 μl of 0.25 *M* EDTA (pH 7.5)
   20 μl of formamide/dyes for electrophoresis

   Heat at 95° C for 5 min, then load each reaction onto a single wide slot in 6% (w/v) polyacrylamide gel containing 8 *M* urea. The exact size of the gel is not too important; typically, use short sequencing plates with a recipe of

   24 g of urea
   10 ml of 30% (w/v) acrylamide
   10 ml of 1% (w/v) bisacrylamide
   10 ml of distilled water
   2.5 ml of 20× TBE [242·3g Tris, 61g boric acid, 14·9g EDTA (ethylene diamine tetraacetic acid) per liter]
   50 ml (total)

   Dissolve the urea at 37° C and add 70 μl of TEMED (tetramethylenediamine) and 70 μl of 25% ammonium persulfate just before pouring. Use a slot former with about six wide slots.

7. Electrophoresis at 1300 V for about 1 hr is sufficient to separate the probe from the M13 DNA. Separate the glass plates, leaving the gel attached to one plate, and cover with Saran wrap. Take extra care to use appropriate shielding to avoid exposing yourself to the radioactive gel during this section. Autoradiograph for 2–5 min, taping the film lightly to the Saran wrap and marking across the edges of the film onto the Saran wrap. Develop the film and use the marks to locate the probes on the gel.
8. Excise the radioactive probe band from the gel and transfer it to 0.85 ml of 100 m*M* NaCl, 1 m*M* EDTA, and 10 m*M* Tris–Cl (pH 7.5) with 10 μg/ml tRNA in a 1.5-ml plastic microfuge tube. Take care not to fragment the gel slice. Incubate for 2 hr at 65° C with occasional mixing, then transfer two 0.4-ml aliquots of supernatant, avoiding the gel slice, to new 1.5-ml microfuge tubes and add 1 ml of ethanol to each. Chill on dry ice and then microfuge for 10 min to pellet the

nucleic acids. Remove the supernatant and resuspend the pellet in 50 μl of TE buffer. The typical yield is about 3×10^6 cpm (Cerenkov) of probe.

PCR Amplification Primers for Complete Human *p53* Gene

Mutations in the *p53* tumor suppressor gene are the most common genetic change detected so far in human cancers (20). These are distributed throughout the *p53* coding sequence, yet most are located in regions that have been described as the conserved boxes 2–5 (20, 21). Not all *p53* mutations have the same consequences for the function of the p53 protein, and it is frequently important to establish precisely the status of p53 in tumor biopsies. Primers that will amplify a region spanning exons 5–8 (where 85% of the reported mutations are found) have been reported (22), but 15% of the mutations occur in the other coding exons and so are not detected by this method. The PCR primers shown here will efficiently amplify the entire genomic coding region of the human *p53* gene in four segments (Fig. 4), each of which is a convenient size for subsequent DNA sequencing or single-strand conformation polymorphism (SSCP) analysis.

Using a human genomic *p53* clone (pATp53π, kindly supplied by L. Crawford, Dept. of Pathology, Cambridge, UK) and sequencing primers deduced from the published *p53* cDNA sequence (23), short stretches of DNA sequence were established spanning some of the intron–exon boundaries of *p53* and extending 50–100 nucleotides into the introns. This generated sufficient sequence to permit the selection of primers suitable for PCR. The primers chosen are listed below. Four fragments may thus be amplified from genomic DNA that encompass the entire *p53* coding sequence (Fig. 4A). PCR conditions were established empirically that give amplification of a single major product for each primer combination, and the products are illustrated using DNA extracted from Raji cells as template (Fig. 4B).

411N	AGACTGCCTTCCGGGTCACT	5′
410N	ACGGCCAGGCATTGAAGTCT	intron 4
409N	CTGTTCACTTGTGCCCTGAC	intron 4
408N	CTGACAACCACCCTTAACCC	intron 6
407N	ACTGGCCTCATCTTGGGCCT	intron 6
406N	CCCCTGATGCAAATGCCCCA	intron 9
413N	CTTCTCCCCCTCCTCTGTTG	intron 9
412N	GGCTGTCAGTGGGGAACAAG	3′

1. For a 100-μl PCR reaction mix add
   59.5 μl of distilled water
   10 μl of 500 m*M* KCl, 100 m*M* Tris–Cl (pH 8.3), and 15 m*M* $MgCl_2$

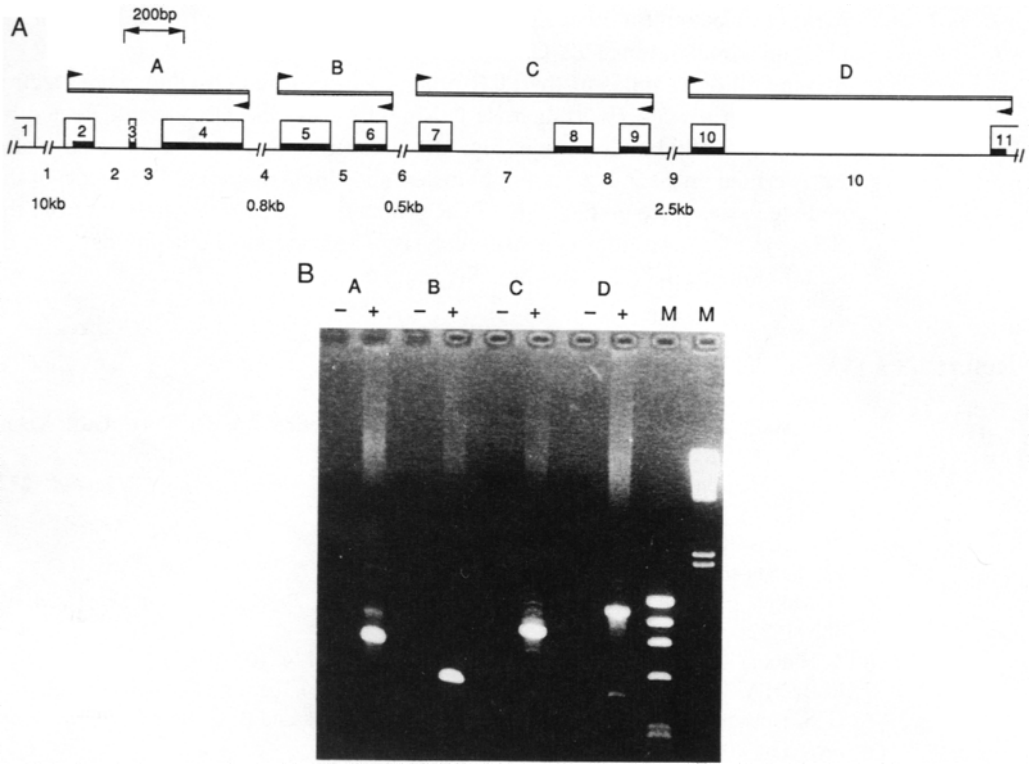

FIG. 4 (A) Polymerase chain reaction (PCR) products covering the entire *p53* coding sequence. The exon structure of the human *p53* gene is shown to scale; the exons are shown as numbered boxes, while the filled segments indicate the extent of the *p53* reading frame. Only parts of exons 1 and 11 are shown. The introns (numbered below) are also shown to scale, except where breaks are marked; the numbers below the breaks show the approximate length of the intron. The four primer pairs used for PCR are indicated by arrows and the resulting PCR products (A, B, C, and D) are illustrated by double lines. (B) PCR reactions contain primer pairs A (411N, 410N) B (409N, 408N), C (407N, 406N), and D (413N, 412N) and (+) Raji DNA as template. Tracks marked − contain no DNA template. Marker tracks (M) contain a *Hae*III digest of ϕX174 DNA and a *Hin*dIII digest of phage λ DNA.

10	μl of 2 m*M* each dATP, dGTP, dCTP, and dTTP
5	μl of one primer (50 pmol)
5	μl of another primer (50 pmol)
10	μl of template DNA (equivalent to 1 μg)
0.5	μl of *Taq* DNA polymerase (AmpliTaq, Perkin–Elmer, Norwalk, CT)
100	μl (total)

2. PCR is carried out for 30 cycles of 94° C for 1 min, 56° C for 1 min, 72° C for 2.5 min, and a final 8 min at 72° C.
3. Five-microliter samples of the PCR products from Raji cell DNA were electrophoresed on a 1.5% (w/v) agarose gel in TBE, and the DNA was detected by staining with ethidium bromide (Fig. 4B). The amplification conditions have also been verified on a series of tumor biopsies, and single major PCR products of the predicted sizes were obtained; the PCR products were also shown to be from the *p53* gene by nucleotide sequence analysis. The resulting PCR products can be used for either SSCP analysis or DNA sequencing.

References

1. E. V. Walls and D. Crawford, *in* "Lymphocytes: A Practical Approach" (G. G. B. Klaus, ed.), p. 149. IRL Press, Oxford, 1987.
2. H. zur Hausen, F. J. O'Neill, U. K. Freese, and E. Hecker, *Nature (London)* **272,** 373 (1978).
3. J. Luka, B. Kallin, and G. Klein, *Virology* **94,** 228 (1979).
4. K. Takada and Y. Ono, *J. Virol.* **63,** 445 (1989).
5. G. Miller, T. Shope, H. Lisco, D. Stitt, and M. Lipman, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 383 (1972).
6. Y. Hinuma and J. T. Grace, *Proc. Soc. Exp. Biol. Med.* **124,** 107 (1967).
7. R. Baer, A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell, *Nature (London)* **310,** 207 (1984).
8. S. Funderud, B. Erikstein, H. C. Asheim, K. Nustad, T. Stokke, H. K. Blomhoff, H. Holte, and E. B. Smeland, *Eur. J. Immunol.* **20,** 201 (1990).
9. A.-M. Rasmussen, E. B. Smeland, B. K. Erikstein, L. Caignault, and S. Funderud, *J. Immunol. Methods* **146,** 195 (1992).
10. A. J. Sinclair, I. Palmero, G. Peters, and P. J. Farrell, *EMBO J.* **13,** 3321 (1994).
11. J. L. Yates, N. Warren, and B. Sugden, *Nature (London)* **313,** 812 (1985).
12. C. D. Gregory, M. Rowe, and A. B. Rickinson, *J. Gen. Virol.* **171,** 1481 (1990).
13. G. Allan, G. J. Inman, B. D. Parker, D. T. Rowe, and P. J. Farrell, *J. Gen. Virol.* **73,** 1547 (1992).
14. R. Lau, G. Packham, and P. J. Farrell, *J. Virol.* **66,** 6233 (1992).
15. A. J. Sinclair, M. G. Jacquemin, L. Brooks, F. Shanahan, M. Brimmell, M. Rowe, and P. J. Farrell, *Virology* **199,** 339 (1994).
16. K. H. Vousden, T. Crook, and P. J. Farrell, *J. Gen. Virol.* **74,** 803 (1993).
17. I. D. Cook, F. Shanahan, and P. J. Farrell, *Virology* **205,** 217 (1994).
18. P. J. Farrell, *in* "Genetic Maps" (S. O'Brien, ed.), 6th ed., p. 1.120. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1993.
19. P. J. Farrell, A. Bankier, C. Seguin, P. Deininger, and B. G. Barrell, *EMBO J.* **2,** 1331 (1983).
20. M. Hollstein, D. Sidransky, B. Vogelstein, and C. C. Harris, *Science* **253,** 49 (1991).
21. C. C. de Fromentel and T. Soussi, *Genes Chromosomes Cancer* **4,** 1 (1992).
22. R. Iggo, K. Gatter, J. Bartek, D. Lane, and A. L. Harris, *Lancet* **335,** 675 (1990).
23. P. Lamb and L. Crawford, *Mol. Cell. Biol.* **6,** 1379 (1986).

[7] The SV40 Minichromosome

Claudia Gruss and Rolf Knippers

Introduction

Simian virus 40 (SV40) has served as a useful model system for numerous studies in the fields of eukaryotic gene transcription, DNA replication, and cell transformation (for reviews see Refs. 1 and 2). A reason for the popularity of SV40 as a model system is that the viral genome is small enough to be handled in standard biochemical procedures without much danger of damage or breakage, and yet it contains several interesting sequence elements that make it a eukaryotic genome in a nutshell. Thus, the viral genome contains promoters with complex arrays of regulatory elements as typically found in regulated cellular genes. As with most cellular genes, the viral genes are transcribed as primary transcripts that are processed and spliced to give functional mRNAs. The viral genome also contains an origin that functions well as a replication start site in biochemical assay systems (3, 4).

In addition, the viral genome is organized as chromatin, which has many structural features in common with cellular chromatin. Therefore, the viral chromatin complex is frequently referred to as the SV40 minichromosome.

It has become abundantly clear in recent years that the genetic function of the eukaryotic genome is determined not only by specific DNA sequences, but also by structural features of chromatin (5). For studies on the effects of chromatin structure on genetic activities, the viral minichromosome serves as a useful model. We summarize here current methods to isolate and characterize minichromosomes from infected cells.

Background

The SV40 genome is a circular, covalently closed, double-stranded DNA molecule of 5243 bp. It contains two functionally distinct genetic regions of approximately equal size. The "early" region, expressed soon after infection, is transcribed as one primary transcription product, which is differentially spliced to give two mRNAs encoding two regulatory proteins. The "late" region is also transcribed as a pre-mRNA that is subsequently spliced, producing mRNAs that primarily code for the protein components of the virus shell [Fig. 1 (6)].

Depending on the host cell, SV40 initiates distinctly different infection cycles (1). In cells of its natural host, the African green monkey, SV40 multiplies manifold, produces thousands of progeny virus particles, and eventually causes the death of the infected cell.

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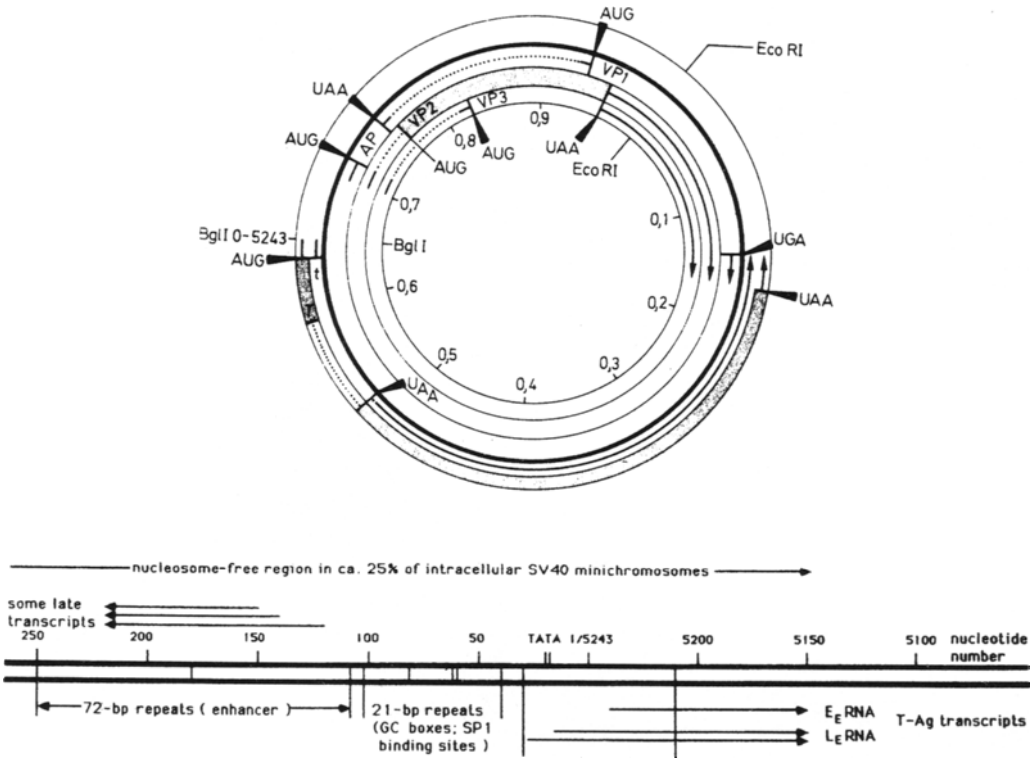

FIG. 1 Gene map of SV40. (Top) The numbering of the 5234 bp of the circular genome begins at the unique *BglI* restriction site and continues counterclockwise. An alternative system is indicated on the inner circle: The DNA is subdivided by decimal numbers, starting at the unique *Eco*RI site. Early transcription is counterclockwise and produces a primary transcript that is differently spliced by excision of intronic sequences (dotted). The mature mRNAs code for the large T antigen (T) or for the small t antigen (t). The open reading frames are indicated by the start and stop codons. Note that the two early mRNAs share sections at their 5′ ends. Late transcription proceeds in the clockwise direction. The primary transcript is processed to give mRNAs for the viral shell proteins VP1, VP2, and VP3. AP indicates the open reading frame for the viral agnoprotein, whose role in the infection cycle is not well defined. The open reading frames are indicated by start and stop codons. (From Ref. 6. Reprinted with permission.) (Bottom) The genomic control region. This region is free of nucleosomes in a fraction of intracellular minichromosomes. The sequence of the "minimal origin" is essential for replication, while adjacent regions increase the efficiency of replication. The TATA box, the GC boxes, and the enhancer of the early promoter are shown. During the early time of infection, transcription starts at the sites marked E_ERNA; later during infection transcription start sites are shifted as indicated by L_ERNA. Late in infection transcription in the clockwise direction is initiated at many closely spaced sites.

SV40 also infects other mammalian cells, such as mouse or hamster cells. In these nonpermissive cells, however, the viral genome replicates poorly, if at all, and is integrated into the genome of the host. If the early viral genes remain active after integration, the infected cells acquire the growth characteristics of established or immortalized cell lines. Subsets of the immortalized cells are further transformed to tumorigenicity and grow as tumors in susceptible animals. Animals with SV40-induced tumors produce antibodies against the early viral proteins, hence, their traditional designation as tumor, or T, antigens (7).

Small t antigen appears to regulate protein phosphatase 2A. It is dispensable for virus multiplication, at least under laboratory conditions, but positively affects the ability of SV40 to transform nonpermissive cells (7).

The multifunctional large T antigen, a polypeptide of 708 amino acids, plays essential roles in productive and nonpermissive infection cycles. It is required for the initiation of viral DNA replication and serves as a DNA helicase at replication forks. It also functions as a regulator of viral and cellular gene expression. In addition, intact large T antigen is essential for the induction and maintenance of the transformed state (for a review see Ref. 7).

The Viral Minichromosome

SV40 chromatin is a densely packed nucleoprotein particle when extracted from nuclei of infected cells under low-salt conditions. These particles contain the four core histones H2A, H2B, H3, and H4 in stoichiometric amounts as well as the outer histone H1 and a variety of nonhistone chromatin proteins. Treatment with 0.5 *M* salt removes the outer histone H1 and most of the nonhistone chromatin proteins and converts the dense nucleoprotein particle into an extended "string-of-beads" structure. Isolated histone H1, added to these extended complexes, reestablishes the compact conformation, showing that histone H1 maintains the closely packed state of native SV40 minichromosomes (8).

Micrococcal nuclease digestion experiments and electron microscopic examinations reveal 26–28 evenly spaced nucleosomes for most minichromosomes (9–11). This is consistent with the number of superhelical turns in deproteinized minichromosomal DNA. These superhelical turns are introduced by the wrapping of DNA around histone octamers, whereby one nucleosome is known to induce one superhelical turn (12). With 26–28 nucleosomes per 5243 bp of DNA, the nucleosomal repeat length is 185–200 bp.

However, about one quarter of all intracellular minichromosomes contain an average of only about 25 nucleosomes. The nucleosomes in these minichromosomes are not evenly distributed, but leave a nucleosome-free region of about 400 bp. This gap corresponds to the viral genomic control region with the two divergent promoters

and the origin of replication (see Fig. 1) (11, 13, 14). The nucleosome-free gap is probably caused by bound cellular transcription factors and/or the bound viral regulator protein, T antigen.

SV40 minichromosomes have served as excellent models for the study of chromatin assembly during replication. According to electron microscopic observations, the advancing replication fork moves up to, and even into, nucleosomes on the unreplicated DNA stem (11). Parental nucleosomes, or, more precisely, the tetramers of two H3 histones and two H4 histones (15), are distributed to the two emerging branches of replicated DNA (11, 16, 17), where they are completed to full nucleosomes at distances of 220–280 bp behind the replication point (11). Another set of nucleosomes is assembled from newly synthesized histones with the assistance of a chromatin assembly factor, CAF-1 (18, 19).

Isolated SV40 minichromosomes have also served as templates for *in vitro* transcription studies (see, e.g., Ref. 20), but the full potential of this transcription system has yet to be exploited.

Isolation and Purification of Viral DNA and Minichromosomes

Cells

The monkey kidney cell line CV-1 [American Type Culture Collection (ATCC), Rockville, MD; Cat. No. CCL 70] is used for infection with SV40. Cells are grown on 145-cm tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS) at 37° C in a 5% CO_2 atmosphere.

Preparation of Virus Lysate

Subconfluent CV-1 cells (80–90% confluent) are infected with SV40 (ATCC; Cat. No. VR 305) at a multiplicity of 0.05–0.1 plaque-forming units (pfu) per cell (see below). For this purpose

1. Remove the medium from the plates.
2. Add 2 ml of virus lysate.
3. Incubate for 1 hr at 37° C, with shaking every 15 min.
4. Add 18 ml of DMEM plus 5% (v/v) FCS.
5. Incubate for 6–8 days until most of the cells are floating.
6. Collect the cells together with the medium.
7. To break the collected cells, freeze and thaw the virus lysate three times.
8. Store in appropriate aliquots at −20° C.

Determination of Virus Titer

For optimal yields of DNA or minichromosomes, it is useful to titrate each virus lysate. This is achieved by infection with increasing amounts of virus lysate, followed by the preparation and quantitation of the viral DNA according to the Hirt procedure, described below.

Another possibility is the determination of viable virus particles by counting the number of pfu:

1. Plate 10^6 CV-1 cells on 6-cm cell culture dishes and allow the cells to settle for 24 hr.
2. Prepare 2-ml serial dilutions of the virus lysate from 1×10^{-2} through 1×10^{-8}.
3. Remove the medium from each plate and replace it with 2 ml of the virus dilution; incubate for 1 hr at 37° C, gently shaking every 15 min.
4. Remove the inoculum from each plate and add 10 ml of plaquing overlay [60 ml of 2% Bacto-agar (Roth, Karlsruhe, Germany) (melt at 100° C, cool to 55° C), 84 ml 2× DMEM, 7.2 ml of FCS, and 24 ml of water].
5. Allow overlays to solidify (10–30 min) at room temperature.
6. Incubate at 37° C in a 5% CO_2 atmosphere for 6 days, then add 3 ml of fresh plaquing overlay.
7. Twelve to 15 days after infection, add 3 ml of plaquing overlay, containing 0.35 ml of 0.01% neutral red, incubate overnight, and count the plaques.

Infection for DNA or Minichromosome Preparation

Subconfluent cell layers (80–90% confluent) are infected with the SV40 virus lysate at a multiplicity of 5–10 pfu per cell, as described above.

For many purposes it is useful to radioactively label the viral DNA, since this allows simple identification of the viral minichromosomes during the various purification steps. Add 10 μCi/ml [^{3}H]thymidine at 36 hr after infection for 2–4 hr, followed by a chase with 10 m*M* nonradioactive thymidine for an additional 1–2 hr.

Infected cells can be used for the preparation of either protein-free viral DNA or SV40 minichromosomes.

Preparation of SV40 DNA

Viral DNA can be isolated from infected cells 40–60 hr after infection. The preparation method was originally described by Hirt (21) and is therfore known as the Hirt procedure.

1. Wash the plates twice with phosphate-buffered saline [137 m*M* NaCl, 2.7 m*M* KCl, 8 m*M* Na_2HPO_4, and 1.8 m*M* KH_2PO_4 (pH 7)].
2. Add 2 ml per plate of Hirt solution [10 m*M* Tris–Cl (pH 8), 1 m*M* EDTA, and 0.6% sodium dodecyl sulfate (SDS)].
3. Leave the plates for 20 min at room temperature in a horizontal position to avoid shearing, then carefully pour the lysate into a wide centrifuge tube (e.g., an SS34 tube of the Sorvall centrifuge).
4. Add 0.25 vol of 5 *M* NaCl.
5. Mix by very carefully turning the tube 10 times; leave overnight at 4° C for the precipitation of cellular DNA and proteins.
6. Remove the high-molecular-weight cellular DNA by pelleting at 17,000*g* (e.g., in an SS34 rotor) for 1 hr at 4° C.
7. Remove the clear supernatant (Hirt supernatant) containing the SV40 DNA, and precipitate the DNA with 0.6 vol of isopropanol.
8. Dissolve the wet pellet in 50 m*M* MOPS solution [3-(*N*-morpholino)propanesulfonic acid] pH 8) plus 0.1 *M* sodium acetate; add 1 g/ml CsCl and 1 mg/ml ethidium bromide.
9. Separate the superhelical form I DNA from the nicked circular form II DNA and RNA by equilibrium centrifugation in a fixed-angle (e.g., Beckman 50 Ti) rotor for 36 hr at 120,000 *g* at 18° C.
10. Collect the superhelical DNA (lower band) with a syringe, and remove the ethidium bromide by three extractions with CsCl-saturated isopropanol.
11. Dialyze overnight against 1 liter of TEN solution [10 m*M* Tris–Cl (pH 8), 1m*M* EDTA, and 1 *M* NaCl] at 4° C, and for 4 hr against 1 liter of TE buffer [10 m*M* Tris–Cl (pH 8) plus 1 m*M* EDTA.
12. The typical yield is 40–50 μg of SV40 DNA per plate.

Extraction of Native SV40 Minichromosomes

The principle of this method was originally established for the extraction of nucleoprotein particles from the nucleoplasm. It involves the incubation of isolated nuclei in buffers of low ionic strength (22). The method was first applied to the preparation of viral nucleoprotein complexes by Su and DePamphilis (23).

1. At 38 hr after infection, remove the culture medium.
2. Wash the cells on the plates twice with ice-cold isotonic buffer [0.25 *M* sucrose, 20 m*M* Tris–Cl (pH 7.4), 137 m*M* NaCl, 5 m*M* KCl, 1 m*M* $CaCl_2$, and 0.5 m*M* $MgCl_2$].
3. Wash twice with cold hypotonic buffer [20 m*M* HEPES–KOH (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8), 5 m*M* potassium acetate, 0.5 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol]; remove any excess liquid.

4. Scrape off the cells with a piece of stiff rubber and disrupt with six strokes in a type S Dounce homogenizer; it is important to work as quickly as possible.

5. Pellet the nuclei by centrifugation at 4° C for 5 min at 2600 *g* and resuspend in 0.2 ml of hypotonic buffer per plate.

6. Keep the isolated nuclei on ice for 2 hr, with occasional shaking for the elution of SV40 nucleoprotein complexes [30–50% of the SV40 minichromosomes are extracted from the nuclei by this procedure (24)].

7. Remove the nuclei by centrifugation at 2600 *g* for 5 min at 4° C.

8. Pellet the cellular debris from the supernatant by centrifugation at 20,000 *g* for 10 min at 4° C .

9. Concentrate the eluted nucleoprotein complexes by centrifugation for 1 hr at 200,000 *g* in a swing-out (e.g., Beckman SW55) rotor at 4° C.

10. Wash the pelleted nucleoprotein in hypotonic buffer and gently resuspend in 40 μl per plate of hypotonic buffer (incubate for at least 1 hr on ice).

11. Remove insoluble material by centrifugation at 20,000 *g* for 10 min at 4° C.

12. Store the preparation in aliquots at − 70° C; thaw only once.

13. DNA concentrations, determined after phenol–chloroform extraction and RNase digestion, are usually in the range of 0.8–1 μg of DNA per microliter.

Preparation of Salt-Treated Minichromosomes

The native minichromosomes are densely packed particles that contain the four core histones and histone H1 in addition to an unspecified number of associated nonhistone proteins. Efficient and reversible dissociation of histone H1 (and most, if not all, nonhistone proteins) is achieved by eluting the minichromosomes from nuclei in ice-cold hypotonic buffer (0.2 ml per plate), containing 0.5 *M* potassium acetate. Otherwise, use the same procedure as described above.

It is important to note that not all viral nucleoprotein is in the form of free SV40 minichromosomes. A substantial and variable fraction consists of complexes, composed of SV40 DNA, organized as viral chromatin, and the viral shell proteins VP1, VP2, and VP3. These are most likely intermediates of the assembly pathway leading to mature virions (25). The relative amounts of these encapsidated virion precursors vary between experiments and may depend on cell culture conditions and times of infection. In addition, a large variety of cellular proteins and cellular nucleoprotein complexes elute from the nuclei together with viral nucleoprotein. In fact, the method described above is being successfully used to prepare heterogenous nuclear ribonucleoprotein (hnRNP) particles (22). Most of these eluted components coprecipitate by the purification scheme described above.

For a number of purposes, these crude viral minichromosome preparations may be sufficient. They serve, for example, as substrates for *in vitro* chromatin replication (17) (but minichromosomes in partially assembled virions are not accessible for replica-

tion factors and are therefore not replicated). In many cases, however, further purification of minichromosomes is necessary (15). This is achieved by sucrose gradient centrifugation.

Purification of SV40 Minichromosomes on Sucrose Gradients

For this purpose use linear 5–30% (w/v) sucrose or glycerol gradients, adjusted to the following buffer conditions.

Native minichromosomes	Salt-treated minichromosomes
10 m*M* NaCl	500 m*M* NaCl
10 m*M* Tris–Cl (pH 7.8)	10 m*M* Tris–Cl (pH 7.8)
1 m*M* EDTA	1 m*M* EDTA
1 m*M* dithiothreitol	1 m*M* dithiothreitol

1. Load 1 ml of the eluted minichromosomes (20,000 *g* supernatant, described above) on the corresponding 5–30% (w/v) sucrose gradient.
2. Centrifuge at 4°C for 165 min at 230,000 *g* in a swing-out (e.g., Beckman SW40) rotor.
3. SV40 minichromsomes containing fractions are identified by radioactive label or by absorption of ultraviolet light at 260 nm.

An additional method that we sometimes prefer is the determination of viral DNA by agarose gel electrophoresis. For this purpose aliquots from each gradient fraction (20 μl of a 600-μl fraction) are mixed with 4 μl of 6× agarose loading buffer containing 6% (w/v) SDS and then run on 0.8% (w/v) agarose gels. SV40 minichromosome-containing fractions are identified by comparison with the mobility of SV40 DNA, run in a parallel slot.

Under the sucrose gradient conditions described, partially assembled SV40 virions sediment at about 200 S and are well separated from free native minichromosomes, which sediment at 75 S (25). Sedimenting hnRNP particles partially overlap with the peak of 75 S minichromosomes. Salt-treated free minichromosomes have a reduced sedimentation rate of 50 S, mainly due to their lack of histone H1 and their more extended configuration (8).

For a further purification of minichromosomes, gradient fractions containing minichromosomes are pooled and concentrated on a 30% (w/v) sucrose cushion. This is accomplished by the following protocol.

1. Load the pooled fractions on a 1-ml 30% (w/v) sucrose cushion containing the corresponding salt concentration.
2. Centrifuge at 4°C for 16 hr at 120,000 *g* in a swing-out (e.g., Beckman SW55) rotor.

3. Wash the pelleted minichromosomes twice in hypotonic buffer [20 m*M* HEPES–KOH (pH 7.8), 5 m*M* potassium acetate, 0.5 m*M* $MgCl_2$, and 0,5 m*M* dithiothreitol].
4. Resuspend the pellet in 20 μl per plate of hypotonic buffer.
5. Remove insoluble material by centrifugation at 20,000 *g* for 10 min at 4° C.
6. Store the preparation in aliquots at −70°C.

Analysis of SV40 Minichromosomes

The chromatin structure of intact or transcribing and replicating SV40 minichromosomes can be analyzed either biochemically or by electron microscopic techniques.

Topology of SV40 DNA

The number of negative supercoils in deproteinized minichromosomal DNA corresponds to the number of nucleosomes originally present in chromatin (12). Determination of the number of topoisomers is used to check the quality of the prepared chromatin. Electrophoresis on standard 0.8% (w/v) agarose gels separates highly supercoiled form I DNA from a minor fraction of nicked relaxed form II DNA

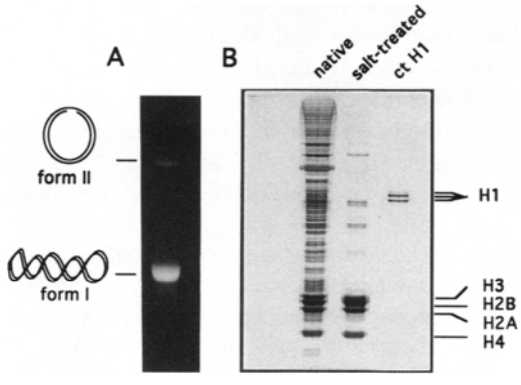

FIG. 2 Characterization of minichromosome preparations. (A) Structure of DNA. Native SV40 minichromosomes (1 μg) were deproteinized and run on a 0.8% (w/v) agarose gel. Covalently closed circular (form I) and open circular (form II) DNA were visualized by ethidium bromide staining. (B) Protein composition of salt-treated and native SV40 minichromosomes. Two micrograms each of salt-treated and native SV40 minichromosomes were resolved on a 15% (w/v) denaturing sodium dodecyl sulfate–polyacrylamide gel (26). Five hundred nanograms of calf thymus histone H1 (ct H1) were used as marker. Proteins were visualized by silver staining (27). The four core histones H3, H2B, H2A, and H4 are indicated.

(Fig. 2A). A fraction of 10% or more form II DNA indicates substantial endonucleolytic damage during preparation.

A quantitative estimation of topoisomer distribution can be achieved by two-dimensional agarose gel electrophoresis. The first dimension is standard gel electrophoresis, while electrophoresis in the second dimension is performed in the presence of the intercalating drug chloroquine (0.35 μM) (17, 19).

Protein Analysis

The distribution of DNA topoisomers and the nucleosomal repeat (as determined by micrococcal nuclease digestion; see below) are identical for salt-treated and native minichromosomes. However, protein analysis reveals clear differences with respect to the associated proteins in the two minichromosome preparations [Fig. 2B (26, 27)].

Micrococcal Nuclease Digestion

Micrococcal nuclease preferentially attacks the linker DNA between nucleosomes. A repeating pattern of mono-, di-, tri-, tetranucleosomes, etc., is created using increasing amounts of micrococcal nuclease. Alternatively, a given amount of micrococcal nuclease is used, and aliquots are withdrawn from the reaction mixture at increasing times after the start of the incubation.

As determined by this method, the repeating unit of intact nucleosomes consists of 180–200 nucleotides. Histone H3/H4 tetramers create a repeating pattern of approximately 80 nucleotides (28). This technique is used to check the quality of the chromatin. In addition and more importantly, dynamic processes such as the replication of chromatin are analyzed by micrococcal nuclease digestion. The following protocol has been successfuly used for the digestion of minichromosomes.

1. Add 0.1 M $CaCl_2$ to give a final concentration of 3 mM.
2. Remove one aliquot as the control ("zero time") sample and add to SE buffer [0.5% (w/v) Sarcosyl (Sigma, Diesenhofen, Germany), 20 mM EDTA].
3. Add micrococcal nuclease at 70 enzyme units per microgram of DNA in chromatin. (NOTE: The enzyme units as given by the suppliers are frequently not reliable; we therefore titrate each batch in a pilot experiment.)
4. Incubate at 20°C, remove samples at incubation times of 2, 8, and 32 min, and pipette into SE buffer.
5. Deproteinize each sample with proteinase K (1 mg/ml) in 0.2% (w/v) SDS for 3 hr.
6. Add 0.1 vol of 3 M sodium acetate and 2.5 vol of 100% (v/v) ethanol; keep at −70°C for 30 min and precipitate the DNA.

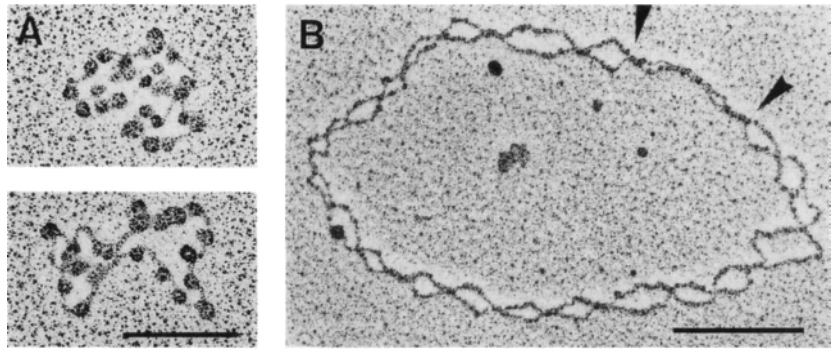

FIG. 3 Examination by electron microscopy. (A) Direction visualization of SV40 nucleoprotein complexes. Minichromosomes were fixed with 0.1% glutaraldehyde and spread with benzylalkyldimethyl ammonium chloride (29, 30). Individual nucleosomes, separated by small pieces of linker DNA, are seen as small spheres. Bar, 0.1 μm. (B) Psoralen–cross-linked minichromosomes. SV40 minichromosomes were treated with 4, 5′,8-trimethylpsoralen, deproteinized, relaxed with DNase I, and spread for electron microscopy under denaturing conditions (31). The locations of individual nucleosomes are seen as arrays of single-stranded bubbles with a size of 140–160 nucleotides. The nucleosome-free region appears as double-stranded DNA (between the arrowheads). Bar, 500 nucleotides. (From Ref. 11. Reprinted with permission.)

7. Investigate the reaction products by agarose gel [1.5% (w/v)] electrophoresis in 50 m*M* Tris, 380 m*M* glycine.

Electron Microscopy

Minichromosomes can be visualized by electron microscopy. Complexes [in 20 m*M* triethanolamine–HCl (pH 7.5), 1 m*M* EDTA] are fixed in 0.1% (w/v) glutaraldehyde for 20 min at 37°C. Fixed samples are spread by using benzylalkyldimethylammonium chloride (29, 30). The disadvantage of direct electron microscopic visualization is the poor spreading of individual nucleosomes, which are frequently superimposed and difficult to quantitate (Fig. 3A).

A powerful alternative is the psoralen cross-linking technique, which has provided valuable insights into dynamic processes such as replication or transcription. Treatment of chromatin with trimethylpsoralen results in the intercalation of psoralen in the linker region between nucleosomes, but not in the nucleosomal DNA (31). Chromatin is then deproteinized. Spreading of the deproteinized DNA under denaturing conditions allows the visualization of well-spread individual nucleosomes as arrays of single-stranded bubbles with a size of about 140–180 nucleotides (Fig. 3B). H3/

H4 tetramers protect roughly 80 nucleotides of DNA against psoralen cross-linking (15). The nucleosome density of a given piece of chromatin can be calculated by the so-called *R* value (11). This is the ration of the sum of the length of all single-stranded sections and the total contour length of a given molecule. For psoralen cross-linking of chromatin and preparation of the DNA, proceed as follows:

1. Add 0.05 vol of 4, 5′, 8-trimethylpsoralen (200 μg/ml [supplied, e.g., by Sigma (St. Louis, MO) as Trioxasalen] in 100% (v/v) EtOH) to 200 ng of SV40 minichromosomes.
2. Incubate for 5 min in the dark.
3. Irradiate for 1 hr 20 min on ice with 7-cm distance to an ultraviolet lamp (366 nm).
4. Repeat the procedure twice with addition of new psoralen.
5. Deproteinize for 2 hr at 50°C with proteinase K (500 μg/ml).
6. Extract twice with phenol and precipitate the DNA with ethanol.
7. Nick the covalently closed circular DNA molecules with DNase I (0.5 μg/100μl) for 1 hr at 37°C in the presence of 45 μg/ml ethidium bromide [DNase I buffer: 50 m*M* Tris–Cl (pH 8), 15 m*M* $MgCl_2$, and 100 m*M* NaCl].
8. Treat with proteinase K (500 μg/ml) for 30 min at 50°C.
9. Extract twice with phenol and precipitate the DNA with ethanol.
10. DNA is now ready for spreading for the electron microscope.

Acknowledgments

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References

1. J. Tooze (ed.), "Molecular Biology of Tumor Viruses. Part 2: DNA Tumor Viruses." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1981.
2. M. L. DePamphilis and M. K. Bradley, *in* "The Papovaviridae" (N. P. Saltzman, ed., p. 99.), Plenum, New York, 1986.
3. B. W. Stillman, *Annu. Rev. Cell Biol.* **5,** 671 (1989).
4. M. D. Challberg and T. J. Kelly, *Annu. Rev. Biochem.* **58,** 671 (1989).
5. G. Felsenfeld, *Nature (London)* **355,** 219 (1992).
6. R. Knippers, "Molekulare Genetik," 4th ed. Thieme-Verlage, Stuttgart, 1985.
7. E. Fanning and R. Knippers, *Annu. Rev. Biochem.* **61,** 55 (1992).
8. U. Müller, H. Zentgraf, I. Eicken, and W. Keller, *Science* **201,** 406 (1978).
9. M. Bellard, P. Oudet, J. E. Germond, and P. Chambon, *Eur. J. Biochem.* **70,** 543 (1976).

10. E. R. Shelton, P. M. Wassarman, and M. L. DePamphilis, *J. Mol. Biol.* **125,** 491 (1978).
11. J. M. Sogo, H. Stahl, T. Koller, and R. Knippers, *J. Mol. Biol.* **189,** 189 (1986).
12. W. Keller, *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4876 (1975).
13. E. G. Jakobovits, S. Bratosin, and Y. Aloni, *Nature (London)* **285,** 263 (1980).
14. S. Saragosti, G. Moyne, and M. Yaniv, *Cell (Cambridge, Mass.)* **20,** 65 (1980).
15. C. Gruss, J. Wu, T. Koller, and J. Sogo, *EMBO J.* **12,** 4533 (1993).
16. M. E. Cusick, M. L. DePamphilis, and P. M. Wassarman, *J. Mol. Biol.* **178,** 249 (1984).
17. T. Krude and R. Knippers, *Mol. Cell. Biol.* **11,** 6257 (1991).
18. S. Smith and B. W. Stillman, *Cell (Cambridge, Mass.)* **58,** 15 (1989).
19. T. Krude, C. de Maddalena, and R. Knippers, *Mol. Cell. Biol.* **13,** 1059 (1992).
20. L. C. Tack and P. Beard, *J. Virol.* **54,** 207 (1985).
21. B. Hirt, *J. Mol. Biol.* **26,** 365 (1967).
22. A. L. Beyer, M. E. Christensen, B. W. Walker, and W. M. Le Stourgeon, *Cell (Cambridge, Mass.)* **11,** 127 (1977).
23. R. Su and M. L. DePamphilis, *J. Virol.* **28,** 53 (1978).
24. K. H. Klempnauer, E. Fanning, B. Otto, and R. Knippers, *J. Mol. Biol.* **136,** 359 (1980).
25. I. Baumgartner, C. Kuhn, and E. Fanning, *Virology* **96,** 54 (1979).
26. U. Laemmli, *Nature (London)* **227,** 680 (1970).
27. W. Wray, T. Boulikas, V. Wray, and R. Hancock, *Anal. Biochem.* **118,** 197 (1981).
28. K. Zucker and A. Worcel, *J. Biol. Chem.* **265,** 14487 (1990).
29. H. Vollenweider, J. Sogo, and T. Koller, *Proc. Natl. Acad. Sci. U.S.A.* **72,** 83 (1975).
30. F. Thoma, T. Koller, and A. Klug, *J. Cell Biol.* **83,** 403 (1979).
31. J. M. Sogo, P. J. Ness, R. M. Widmer, R. W. Parish, and T. Koller, *J. Mol. Biol.* **178,** 897 (1984).

[8] Site-Specific Integration of Multigenic Shuttle Plasmids into the Herpes Simplex Virus Type 1 Genome Using a Cell-Free Cre–*lox* Recombination System

Siyamak Rasty, William F. Goins, and Joseph C. Glorioso

Introduction

Herpes simplex virus type 1 (HSV-1) is a neurotropic human herpesvirus. The viral genome is a large double-stranded DNA molecule, 152 kb in length, which encodes at least 75 gene products (1). During natural infection the virus infects fibroblasts and epithelial cells of the skin and mucosal surfaces (for a review see Ref. 2). Progeny virus particles produced at the site of primary infection encounter sensory nerve terminals which innervate that area. Retrograde axonal transport brings the viral nucleocapsid to the nerve cell body, wherein the viral DNA molecule circularizes. At this stage the viral genome can enter the lytic cascade of viral gene regulation or, more frequently, establish a latent or quiescent infection. During latency the viral genome remains transcriptionally silent, with the exception of the expression of a cluster of latency-associated transcripts (3).

The molecular biology of HSV-1 replication, pathogenesis, and latency has been extensively studied using ultrastructural, biochemical, and genetic approaches. The availability of the entire HSV-1 DNA sequence (4, 5) has greatly facilitated the use of genetic approaches for defining the role of individual functions in the viral life cycle. These genetic studies often require the evaluation of extensive panels of mutations introduced into the viral genome in *cis* for determining the effect of these changes on particular aspects of the biology of the virus. These evaluations include studies of viral genome structure, DNA synthesis, promoter activities, and the role of gene products involved in the many different aspects of virus–host cell interactions. The feasibility of these studies generally depends on the ability to recombine mutant sequences into the genome in such a manner as to replace wild-type sequences.

The construction of recombinant HSV-1 genomes has traditionally involved substantial sequential cloning steps in order to flank the gene cassette with HSV-1 DNA sequences for homologous recombination, using standard marker transfer techniques (6–8). In addition to the laborious cloning steps required for homologous recombination into the viral genome, these techinques have proved to be relatively inefficient (1–2%) in generating recombinant progeny viruses. While standard marker transfer procedures are adequate, genetic analysis of HSV-1 functions should be greatly fa-

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cilitated by the development of methods for the rapid production of recombinants, especially for studies of random nondirected mutations in which interesting phenotypes can be detected without prior knowledge of the responsible mutation. This rapid method requires that the mutant sequences be recoverable from the genome of interesting recombinants, suggesting the potential utility of a shuttle vector method for studying HSV-1 genetics.

We have adapted the site-specific Cre–*lox* recombination system of bacteriophage P1 (9) for introducing isolated mutant sequences into the HSV-1 genome. The Cre–*lox* method had previously been shown to be effective in recombining plasmid DNA with a frequency of 8% into the genome of pseudorabies virus (10), a swine herpesvirus that is an important pathogen in pigs. This reaction can be carried out in a cell-free environment and is reversible by treatment of recombinant virus DNA with the Cre recombinase. Thus, the shuttle vector can be recovered from viral recombinants that display unique phenotypes and can be used to transform bacteria in order to provide sufficient DNA for direct sequence analysis.

The bacteriophage P1 recombination system is relatively simple, consisting of a *cis*-acting 34-bp DNA element, designated *lox*P, at which recombination occurs, and a recombinase, Cre, which mediates site-specific recombination events between two DNA molecules, each possessing *lox*P sites (11–14). The recombination event can be intramolecular between two *lox*P sites within the same plasmid molecule, or it can be intermolecular between two plasmids, each containing a *lox*P site, or between a plasmid and a linear DNA molecule such as a viral genome. For use in generating recombinant HSV-1 genomes, this simple cell-free system requires only a shuttle plasmid with a *lox*P site, a recipient HSV-1 vector genome containing a *lox*P site, and purified Cre recombinase. Furthermore, we have introduced a *lac*Z reporter gene expression cassette into the *lox*P shuttle plasmid to enable the rapid identification of positive recombinants on the basis of their blue plaque phenotype following overlay of the infected cell monolayer with agarose containing a chromogenic substrate for β-galactosidase (9). We have previously used this system to analyze the role of specific amino acids in the cytoplasmic domain of HSV-1 glycoprotein B (gB) in fusion events mediated by gB (9, 15).

In the studies described here we have attempted to ascertain the ideal conditions for routinely performing the Cre–*lox* recombination reaction, using a variety of plasmid DNAs and specific target HSV-1 vector genomes. The first series of experiments describes the construction of a recipient viral genome and a shuttle plasmid, each containing a *lox*P *cis*-acting recombination site. The next set of assays details the proper conditions for overlaying the infected cell monolayer with the chromogenic material for detection of viral recombinants displaying expression of the β-galactosidase (*lac*Z) reporter gene present on the inserted shuttle plasmid. The final set of experiments identifies the best molar ratio of plasmid DNA to target viral genomic DNA to obtain the highest number of recombinants using the Cre–*lox* recombination procedure. Following these optimized conditions, we have routinely inserted a variety of

wild-type and mutant HSV-1 genes, as well as foreign gene cassettes, into replication-compromised and -defective HSV-1 genomes. This system can be similarly applied to other large viruses for the creation of recombinant viral genomes.

Construction of a *lox*P-Containing Recipient Viral Genome

In order to perform successful insertion of gene cassettes into the HSV-1 genome using the Cre–*lox* recombination system, a target viral genome containing a *lox*P recombination site must be constructed. To accomplish this goal, the thymidine kinase (*tk*) gene locus of HSV-1 was chosen as the site for insertion of the *lox*P element due to its ability to select for viral recombinants that are unable to synthesize TK. Standard marker transfer procedures (6–8, 16) were used to insert the *lox*P site into the *tk* gene of wild-type HSV-1, thereby disrupting TK synthesis (Fig. 1). We have designed the *lox*P site to contain translational stop codons in all three reading frames in order to disrupt synthesis of the gene product into which the *lox*P site has been inserted. The resulting recombinant Ktk :: *lox* can then be used to introduce mutant HSV-1 genes or foreign gene cassettes into the HSV-1 genome. By interrupting the *tk* gene, it also provides the opportunity to introduce the *tk* gene into the shuttle vector in order to use hypoxanthine–aminopterin–thymidine (HAT) medium in the selection of TK^+ recombinants.

Methods

Marker rescue procedures were used to transfer a *lox*P site into the *tk* gene locus of wild-type HSV-1. A 354-bp fragment from pBS64 (10) containing the 34-bp *lox*P site was inserted into the unique *Sna*BI site of the plasmid pUCX1, which contains the 3.5-kb HSV-1 *Bam*HI P fragment inserted into the *Bam*HI site of pUC19, producing the plasmid pJG101. The *lox*P site insert in pJG101 is in the same orientation as the *tk* gene and interrupts the *tk* coding sequence (see Fig. 1). The plasmid pJG101 (5 μg) was linearized by restriction enzyme digest, and co-transfected along with infectious wild-type KOS viral DNA (2 μg) into 60-mm plates containing subconfluent monolayers of Vero (African green monkey kidney) cells, using the calcium phosphate transfection procedure (16, 17). Positive recombinants were identified from the mixture of progeny virus particles arising from the transfections using three rounds of limiting dilution in 96-well microtiter plates (9). TK^- recombinants were selected in 1 × minimum essential medium (MEM)–10% fetal calf serum (FCS) containing 100 μg/ml thymidine 1-β-arabinofuranoside (araT; Sigma, St. Louis, MO), since insertion of the *lox*P site into *tk* blocks its expression. Dot blot assays using a ^{32}P-labeled *lox*P-specific probe were performed on cell lysates from the microtiter plate wells displaying single plaques (9, 18) in order to identify all TK^- recombi-

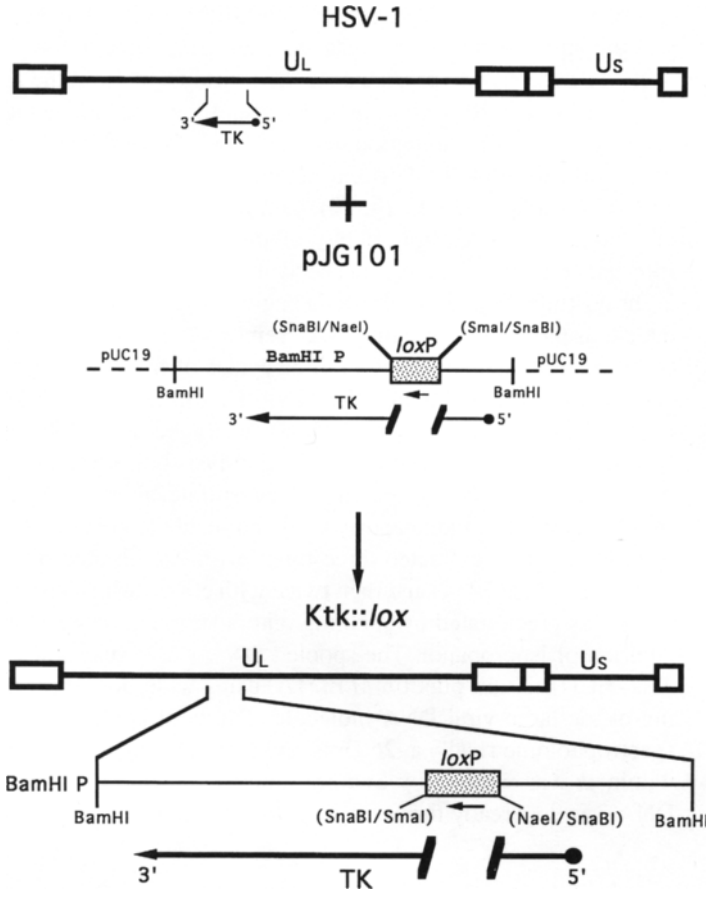

FIG. 1 Construction of a *lox*P-containing recombinant virus. Ktk :: *lox* was engineered by homologous recombination of the linearized plasmid pJG101 (9), containing a *lox*P site within the coding sequence of the thymidine kinase gene (*tk*), into the *tk* locus of wild-type KOS virus. Since the *lox*P site interrupts *tk*, TK$^-$ recombinants can be selected in the presence of 1-β-arabinofuranoside. Recombinants can be identified by dot blot hybridization using a radioactively labeled *lox*P fragment as probe. Following three rounds of virus purification by limiting dilution (9), a viral DNA stock was prepared and analyzed by Southern blotting (21) to confirm the presence of the *lox*P site within the *tk* locus of the newly generated recombinant. This preparation of viral DNA can now serve as a stock of target viral DNA for Cre–*lox* reactions. U_L, unique long sequence; U_S, unique short sequence.

nants that contained a *lox*P site insertion. This process was necessary since TK$^-$ viral recombinants can arise by spontaneous point mutations within the gene. Following three rounds of limiting dilution analysis in the presence of araT and dot blot hybrid-

ization with a *lox*P-specific probe, a high-titer stock of the purified TK$^-$ :: *lox*P virus was prepared for further analysis and future experiments.

It was necessary to prepare a stock of viral DNA from the *lox*P-containing recipient virus in order to confirm the presence of the *lox*P site in the *tk* gene locus and for use in Cre–*lox* recombination reactions. TK$^-$ recombinant viral DNA was prepared by modification of the Hirt extraction procedure for isolation of high-molecular-weight eukaryotic DNA (19, 20). Confluent monolayers were infected with the recombinant virus at a multiplicity of infection of 10 to ensure that every cell was infected and that infection proceeded at approximately the same rate in every cell. At 24 hr postinfection, when the cells begin to round up yet the monolayer still remains attached, the culture medium was removed and the cell monolayer was scraped from the flask and subjected to three cycles of freezing and thawing to release cell-associated virions. This mixture of virus particles and cell debris was added to the medium containing free virions and centrifuged at 20,000 rpm for 40 min at 4° C. The pelleted material was resuspended in lysis buffer [10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, 0.6% (w/v) sodium dodecyl sulfate, and 250 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN)] and incubated overnight at 42° C. The samples were then gently extracted three times with equilibrated phenol–chloroform–isoamyl alcohol (25 : 24 : 1) and then twice with chloroform–isoamyl alcohol (24 : 1). The DNA was precipitated by spooling onto a sterile glass Pasteur pipette following the addition of isopropanol. The spooled DNA was resuspended in TE buffer [10 m*M* Tris–HCl (pH 7.5), plus 1 m*M* EDTA) using wide-bore Pipetman tips to avoid shearing of the large viral DNA molecule. The viral DNA concentration was measured spectrophotometrically at 260 nm, and the insertion of the *lox*P site at the *Sna*BI site within *tk* was verified by Southern blot analysis (21). This purified stock of viral DNA was then ready for use in Cre–*lox* reactions.

Results

The use of selection in media containing either acyclovir or araT greatly improves the frequency of generating a recombinant with a *lox*P site inserted into the *tk* locus. In combination with dot blot hybridization analysis, the limiting dilution procedure was effective in purifying the recombinant. Because the infections are performed in 96-well microtiter plates, it is possible to screen considerable numbers of plaques. The media from each of the wells can be readily transferred to a new 96-well plate to serve as a stock after identification of positive recombinants by dot blot hybridization to cell lysates from each well. Since standard marker transfer techniques are used, it is imperative to flank the insert DNA with at least 500 bp of HSV-1 *tk* sequence. Increasing amounts of flanking sequence (1–3 kb) lead to even greater recombination frequencies.

The time at which cells are harvested for the production of viral DNA is crucial

not only to the yield obtained, but also the generation of viral DNA that is highly infectious. The ability of the viral DNA to produce infectious particles is important to the overall Cre–*lox* recombination frequency, since noninfectious viral genomes still possess a functional *lox*P site and can recombine with the shuttle plasmid, yet will not be able to produce plaques following DNA transfection of cell monolayers (see below). Another factor that influences the integrity of the viral DNA is the manner in which the sample is handled during the isolation procedure. Gentle but effective extractions with the use of wide-bore pipettes and Eppendorf tips has increased the yield of infectious viral DNA. Southern blot analysis of the viral DNA will show whether the DNA is intact or degraded, although it cannot be used to evaluate the infectivity of the viral DNA preparation. Thus, transfection of 1 μg of viral DNA into cell monolayers is required to determine the number of plaques resulting from 1 μg of viral DNA. Typically, the yield should be between 100 and 500 plaques per microgram of viral DNA. If the plaque count is lower than this range, the number of plaques resulting from the corresponding Cre–*lox* recombination reaction will decrease accordingly.

Construction of a Shuttle Plasmid Containing a *lox*P Site

In addition to engineering a recipient virus with a *lox*P site, a shuttle plasmid containing a *lox*P element must also be constructed. This will allow for insertion of the entire plasmid into the *lox*P-containing recipient viral genome at that site in the cell-free recombination reaction using purified Cre. To ease the identification of positive recombinants following insertion of the shuttle plasmid, a *lac*Z reporter gene expression cassette was included in the shuttle plasmid (Fig. 2). Cre–*lox*–mediated recombination of this plasmid into the recipient viral genome yields a virus that can produce blue plaques following X-gal agarose overlay.

Methods

The *lox*P and *lac*Z-containing shuttle plasmid pJG116 (Fig. 2) was constructed by several cloning steps. First, a 252-bp *Bam*HI/*Pvu*II *lox*P-containing fragment from pBS64 (10) was inserted into the *Bam*HI/*Sma*I sites of pBS$^-$ (Stratagene, La Jolla, CA) to produce the clone pBS*lox*P. Second, the human cytomegalovirus major immediate-early promoter–*lac*Z reporter gene cassette (HCMV IEp–*lac*Z), present on a 4.54-kb *Bam*HI fragment from pIEP–*lac*Z (22, 23), was introduced into the *Bam*HI site of pBS*lox*P, producing pJG103. Since few single-cutting cloning sites were available within the newly generated plasmid (pJG103), the *Hin*dIII site present at the junction of the HCMV promoter with *lac*Z, as well as the *Bam*HI site at the 3′ end of *lac*Z, was destroyed to produce pJG116.

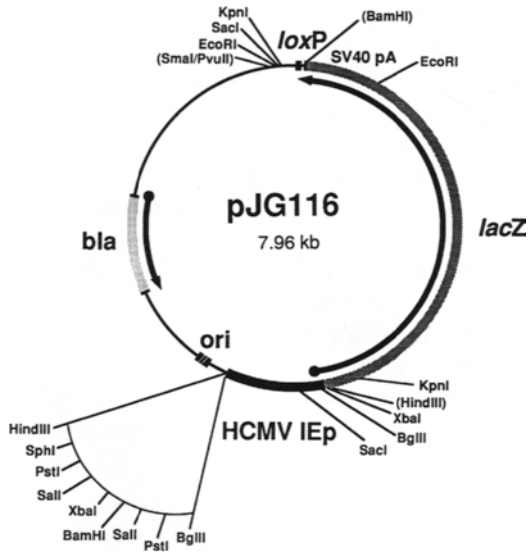

FIG. 2 Construction of a shuttle plasmid containing the *lac*Z reporter gene and a *lox*P recombination site. The shuttle plasmid pJG116 was produced by first inserting a *lox*P-containing fragment into the pBS$^-$ cloning vehicle followed by the introduction of the reporter gene cassette containing the strong human cytomegalovirus immediate-early gene promoter (HCMV IEp) driving *lac*Z. The *lac*Z gene contains the simian virus 40 (SV40) early gene polyadenylation and cleavage signals [SV40 poly(A)] for proper message processing. The 7.96-kb plasmid possesses unique *Hin*dIII and *Bam*HI sites, in addition to multiple *Pst*I and *Sal*I restriction sites, for insertion of foreign gene cassettes into the shuttle plasmid. ori, ColE1 origin of replication; bla, ampicillin resistance.

Results

The pJG116 shuttle plasmid was created to contain a functional *lox*P site for Cre-mediated recombination as well as a reporter gene cassette for rapid identification of recombinants possessing the shuttle plasmid. The strong HCMV IEp results in the production of high levels of β-galactosidase early in HSV-1 infection, which enables the easy identification of blue plaque recombinants. Several potential cloning sites exist within pJG116 for insertion of foreign gene cassettes. Unique *Hin*dIII and *Bam*HI sites are present upstream of the HCMV IEp, and two sites exist for both *Sal*I and *Pst*I within pJG116. Although the presence of the reporter gene cassette within the shuttle plasmid eases the identification of positive recombinants, we have used shuttle plasmids that contain only a *lox*P site and the gene of interest. However, recombinants produced using this modification can be detected only by dot blot hybridization.

Overlay Procedure for Detection of the Recombinant Blue Plaque Phenotype

Prior to the evaluation of the maximal conditions for generating the greatest number of recombinants by Cre–*lox,* it was necessary to ascertain the proper conditions used in overlaying the infected cell monolayer with the chromogenic substrate for optimal detection of recombinants displaying the blue plaque phenotype. A variety of parameters were considered, such as (1) the choice of the chromogenic substrate, X-gal (Boehringer-Mannheim) or Bluo-gal [GIBCO–Bethesda Research Laboratories (BRL), Bethesda, MD]; (2) the solvent used to dissolve the chromogenic substrate, dimethyl formamide (DMF; Sigma) or dimethyl sulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA), for both solubility of the chromogen and toxicity of the solvent to the cell monolayer; (3) the time following infection at which the infected cell monolayer is overlaid; and (4) the semisolid medium used in the overlay procedure. Since the goal of these experiments was to optimize the method of blue color plaque detection, a previously constructed HSV-1 recombinant (US3$^-$ :: pgC–*lac*Z) containing the glycoprotein C late gene promoter driving the *lac*Z gene cassette in the US3 locus of the viral genome (24) was used to infect confluent Vero cell monolayers prior to staining with the chromogenic substance.

Methods

Vero cells, grown to confluence in 60-mm dishes, were infected with 1×10^2 plaque-forming units (pfu) of the US3$^-$ :: pgC–*lac*Z recombinant (24). The virus was allowed to adsorb to the cell monolayer for 1 hr, and the plates were rocked back and forth every 15 min to evenly distribute the inoculum over the entire monolayer. The 1× MEM–Bluo-gal–agarose solution was prepared by adding 100× PSN (10,000 U/ml penicillin G plus 10,000 μg/ml streptomycin sulfate in 0.85% (w/v) saline; GIBCO–BRL), 100× glutamine (200 m*M;* GIBCO–BRL), and 100% FCS (GIBCO–BRL) to final concentrations of 2×, 2×, and 10%, respectively, in 2× MEM (GIBCO–BRL) medium. To this final volume was added an equal volume of 1.5% (w/v) low-melting-point (LMP) agarose (GIBCO–BRL) in double-distilled water that was autoclaved and cooled to 37° C, and to which was added Bluo-gal at a final concentration of 300 μg/ml. Bluo-gal is added from a 30 mg/ml stock prepared in DMF or DMSO. Bluo-gal is recommended over X-gal since (1) it produces a darker blue reaction product, allowing for easier isolation of a blue plaque in a background of white plaques; (2) it produces little or no background staining of the cell monolayer, as is seen with X-gal; and (3) it does not precipitate out upon addition of LMP agarose to the 2× MEM mixture (i.e., it appears to have a greater solubility than X-gal in either DMF or DMSO).

As an example, for the following experiment 70.0 ml of 1× MEM–Bluo-gal–agarose (12 plates at 5 ml per plate, or 60 ml total plus 10 ml extra volume as a safety margin) was prepared as follows: 31 ml of 2× MEM, 0.7 ml of 100× PSN, 0.7 ml

of 100× glutamine, 3.5 ml of 100% FCS, and 35.0 ml of 37° C-warmed 1.5% (w/v) LMP agarose were mixed together in a 150-ml bottle. This mixture was then kept in the 37° C CO_2 incubator and remained liquid and still usable after several days. In each case below 5-ml aliquots were removed, the media mixture bottle was immediately returned to 37° C to prevent solidification of the melted agarose, and 100 μl of 30 mg/ml Bluo-gal, dissolved in either DMF or DMSO, was added to each aliquot. At this point the aliquots were gently mixed to prevent bubble formation and were directly pipetted over the cell monolayer. Plates were kept at room temperature for about 30 min (or sometimes were transferred to the cold room after only a 10-min incubation at room temperature) to allow the agarose to solidify, and were then placed in a 37° C CO_2 incubator and observed periodically for the presence of blue color reaction in and around viral plaques.

Following virus adsorption 10 separate 1× MEM–Bluo-gal–agarose overlay procedures were tried. The monolayer was overlaid immediately with

1. 5 ml of 1× MEM–Bluo-gal–DMF–agarose
2. 5 ml of 1× MEM: At 24 hr postinfection the medium was replaced with 5 ml of 1× MEM–Bluo-gal–DMF–agarose.
3. 5 ml of 1× MEM: Upon the appearance of plaques, the medium was replaced with 5 ml of 1× MEM–Bluo-gal–DMF–agarose.
4. 5 ml of 1× MEM–1% (w/v) methylcellulose (Aldrich, Milwaukee, WI): Upon the appearance of plaques, the medium was replaced with 5 ml of 1× MEM–Bluo-gal–DMF–agarose.
5. 4 ml of 1× MEM–agarose without Bluo-gal: Upon the appearance of plaques, 1 ml of 1× MEM–Bluo-gal–DMF–agarose was added on top of the solidified 1× MEM–agarose.

6–10. Same as reactions 1–5, except that Bluo-gal was dissolved in DMSO instead of DMF. This was done to see whether there was a difference in the intensity of blue color development and also to test whether DMSO is less cytotoxic than DMF.

11 and 12. Control plates: Mock-infected cell monolayers were overlaid with 1× MEM–Bluo-gal–DMF–agarose (reaction 11) or 1× MEM–Bluo-gal–DMSO–agarose (reaction 12). This was done to determine the background blue color in a plate of uninfected cells not expressing the *lac*Z reporter gene.

Results

Overall, blue plaques appearing in plates containing Bluo-gal dissolved in DMF were a darker color than those that had the chromogen dissolved in DMSO, and thus were more easily detected. Also, DMSO and DMF appeared to exhibit equivalent levels of cytotoxicity. Therefore, our recommendation is to dissolve Bluo-gal in DMF at a concentration of 30 mg/ml. Also, no blue color was detected in plates 11 and 12, although the monolayer covered with DMSO-containing media looked slightly better than that covered with DMF-containing media. This confirmed that Bluo-gal is su-

perior to X-gal, since Bluo-gal did not display background staining of the cell monolayer, thereby enabling easier identification of positive recombinants.

The following details the results for each specific set of conditions described above:

1. Overlaying the monolayer with 1 × MEM–Bluo-gal–agarose immediately following virus adsorption resulted in at least a log reduction in the number of plaques. It seems that the presence of DMF (or DMSO) or the chromagen in the overlay medium prior to the appearance of viral plaques may be inhibiting plaque formation. This method is not recommended.
2. Five blue plaques appeared about 48 hr postinfection. By the next day a number of other plaques (totaling about 50) that had been white at 48 hr had now turned blue. Overall, the plaques looked pinpoint, were not too spread out, and could easily be picked.
3. Blue plaques appeared at about 6 hr after overlay with 1 × MEM–Bluo-gal–agarose. However, by about 48 hr postinfection, when the maximum number of blue plaques had developed, they appeared to be too spread out and were not as pinpoint as the plaques produced by the other methods (i.e., 2, 4, and 5). It seems that plaque formation is best accomplished when virus spread is retarded by methylcellulose or LMP agarose. This method is also not recommended.
4. This method seemed to be one of the better ones used. Again, blue plaques appeared at about 6 hr after overlay with 1 × MEM–Bluo-gal–agarose. Upon further incubation more pinpoint blue plaques were detected, totaling about 50 or 60.
5. This may represent the best method for detection and isolation of pinpoint blue recombinant HSV-1 plaques. First, the cells are not initially exposed to the toxic effects of DMF, and plaque formation is not hindered due to the presence of DMF or chromagen. By 48–72 hr postinfection plaques should be detectable, and 3–4 hr after overlay with 1 ml of 1 × MEM–Bluo-gal–agarose blue plaques can be detected. As with some of the other procedures, further incubation of the plates results in detection of more blue plaques.

6–10. As mentioned above, DMF proved somewhat superior to DMSO, but the methods produced similar results regardless of whether DMF or DMSO was used (i.e., reactions 1 and 6 produced similar results, except that the blue plaques detected in the former were darker).

The method of choice, at least in our laboratory, is to overlay the monolayer with 4 ml of 1 × MEM–agarose following adsorption of the virus inoculum. After the appearance of plaques (48–72 hr postinfection), 1 ml of 1 × MEM–Bluo-gal–DMF–agarose is overlaid on the monolayer to detect blue plaques. Blue plaques can be detected 1–6 hr following overlay when incubated at 37° C. The blue color can be enhanced by incubation of the plates briefly at 4° C. Individual well-isolated blue plaques can be picked using either a Pasteur pipette or Pipetman with a wide-bore tip, and the agarose plug can be resuspended into 0.5 ml of 1 × MEM–10% FCS. Three cycles of freezing and thawing followed by sonication helps to release virus

from the infected cells contained within the agarose plug. This virus can be either titered by standard methods or used to prepare a higher-titer virus stock for further purification steps.

We have recently optimized the limiting dilution procedure for detection of recombinants expressing the *lac*Z gene product. Individual blue plaques isolated by the above procedure are used in a modified limiting dilution assay. Briefly, 30 pfu of virus isolated from the agarose plug is added to 3×10^6 Vero cells in suspension within a 15-ml conical tube and the mixture is placed on a rocker platform at 37° C for 1 hr. Following virus adsorption the cells are plated in a 96-well plate and incubated at 37° C in a CO_2 incubator until the appearance of plaques (usually 2–3 days). Theoretically, this should produce approximately 30 wells of 96 that contain single plaques. At this point the medium in each well is transferred to a new 96-well plate and stored at −80° C for use as a virus stock. Each well is then overlaid with 0.1 ml of 1× MEM–Bluo-gal–agarose, the agarose is allowed to solidify at room temperature, and the plate is transferred to a 37° C CO_2 incubator. Wells possessing single blue plaques are readily detectable, thus allowing rapid identification of *lac*Z$^+$ recombinants. The advantage of this system is that it does not require the picking of a hopefully well-isolated single blue plaque from the agarose overlay, which can frequently lead to contamination of the single blue plaque with a neighboring clear plaque, particularly if the plaques are too numerous in the vicinity of the blue plaque recombinant. Instead, with this modified method, once wells with single blue plaques are identified, one can then go back to the frozen stock for use in subsequent rounds of limiting dilution. If the virus recombinant lacks a *lac*Z expression cassette, it is still possible to use this approach to isolate positive recombinants. Instead of overlaying the infected cells monolayer with 1× MEM–Bluo-gal–agarose, DNA from cell lysates of individual wells containing single plaques can be used in dot blot hybridization experiments with specific probes for identification of positive recombinants.

Optimization of the Cre–*lox* Reaction Conditions

An important variable in Cre–*lox* recombination reactions concerns the ratio of insert plasmid DNA to target viral DNA in the reaction mixture. When the ratio is too low, the recombination frequency drops due to the limited number of molecular interactions between the plasmid to be inserted and the target viral genome. When the ratio is too high, the number of positive recombinants also drops, but this is due to the higher prevalence of plasmid molecules interacting and recombining with one another instead of recombining into the large viral DNA molecule. Thus, we developed an assay to evaluate this parameter of the Cre–*lox* reaction, using Ktk :: *lox* viral DNA (Fig. 1) and pJG116 plasmid DNA (Fig. 2). Various molar ratios of pJG116 and Ktk :: *lox* DNA were used to set up the Cre–*lox* recombination reactions. The ratios selected were 5, 15, 30, 50, 100, 150, and 250 molar equivalents of plasmid DNA to the molar equivalent of 1 μg of viral DNA. Figure 3 depicts the molecules

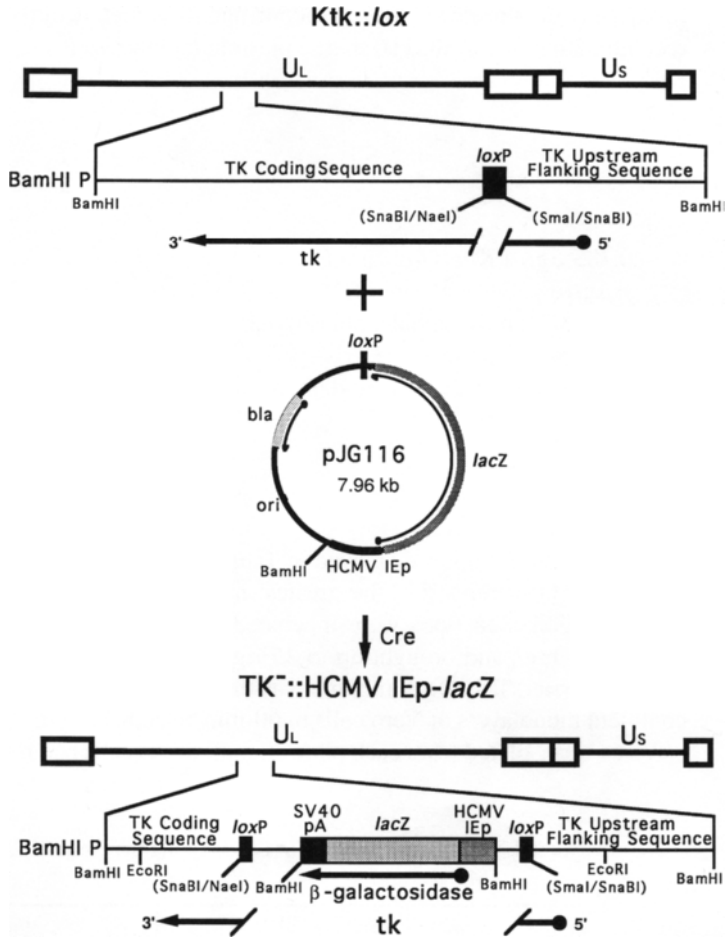

FIG. 3 Cre–*lox* recombination of a *lac*Z-expressing plasmid (pJG116) into the *tk* locus of the Ktk :: *lox* target viral genome. The plasmid pJG116 was constructed to contain a *lox*P element for site-specific recombination, as well as a reporter gene cassette with the human cytomegalovirus major immediate-early gene promoter (HCMV IEp) driving expression of the gene encoding β-galactosidase (*lac*Z) for rapid identification of recombinant viruses. When pJG116 DNA is mixed with Ktk :: *lox* viral DNA in conjunction with the Cre recombinase, the plasmid DNA molecule is incorporated into the *tk* locus of the Ktk :: *lox* target virus genome generating the TK⁻ :: HCMV IEp–*lac*Z recombinant. Treatment of recombinant viral genomic DNA, containing the inserted plasmid, with Cre will lead to excision of the sequences between the two *lox*P sites within the viral genome and the generation of the original plasmid and the recipient genome. U_L, unique long sequence; U_S, unique short sequence.

involved in this specific Cre–*lox* reaction and the structure of the virus resulting from recombination of the pJG116 shuttle plasmid into the *lox*P site within the *tk* locus of Ktk :: *lox*.

Reagents

0.385 μg/μl Ktk :: *lox* viral DNA
1 μg/μl pJG116 plasmid DNA
17% (w/v) polyvinylalcohol (Sigma)
3 × Cre buffer [150 m*M* Tris–HCl (pH 7.5), 100 m*M* NaCl, and 30 m*M* $MgCl_2$]
Cre recombinase (New England Nuclear–DuPont, Wilmington, DE)

Methods

Reactions were set up as displayed in Table I to ascertain which molar ratio was the most efficient in rendering the greatest number of recombinants by Cre-mediated recombination. Reactions were incubated at 30° C for 30 min, heat-inactivated at 70° C for 5 min, and brought up to 25 μg total DNA by addition of salmon sperm DNA as carrier. This DNA mixture was then used directly for transfection onto subconfluent monolayers of Vero cells in 60-mm petri dishes (16, 17).

Virus was isolated from each plate after all of the cells had rounded up. From each

TABLE I Reaction Mixtures for Determining the Optimal Cre–*lox* Molar Ratio[a]

Molar ratio	Viral DNA	Plasmid DNA	PVA	3 × Cre buffer	Cre	QH_2O
5	2.6	0.25	3.0	10.0	1.0	13.15
15	2.6	0.75	3.0	10.0	1.0	12.65
30	2.6	1.50	3.0	10.0	1.0	11.90
50	2.6	2.50	3.0	10.0	1.0	10.90
100	2.6	5.00	3.0	10.0	1.0	8.40
150	2.6	7.50	3.0	10.0	1.0	5.90
250	2.6	12.50	3.0	10.0	1.0	0.90
0	2.6	0.00	3.0	10.0	1.0	13.40
—	2.6	0.25	3.0	10.0	0.0	14.15

[a] All numbers (except for molar ratio) are expressed as volume in microliters. PVA, Polyvinylalcohol; QH_2O, double-distilled water; 0, no plasmid DNA; —, no Cre enzyme.

virus stock 10^{-4} and 10^{-6} dilutions were used to infect confluent monolayers of Vero cells in 60-mm plates. To infect the cells, medium was carefully aspirated off the cell monolayer and 0.2 ml of the appropriate dilution of virus (prepared in 1× MEM–10% FCS) was added to the cells. The plates were incubated at 37°C for 1 hr and rocked back and forth 15, 30, and 45 min after addition of virus to help distribute the inoculum over the entire surface of the monolayer. Recombinant viruses were identified by their blue plaque phenotype in the presence of Bluo-gal, as described in detail in the previous section, using procedure 5.

Results

The numbers of blue plaques produced using the various ratios of plasmid to virus DNA were determined for both the 10^{-4} and 10^{-6} dilutions. The overall percentages of blue plaques were calculated and displayed in graphic form versus the molar ratio of plasmid DNA to viral DNA (Fig. 4). As can be seen, a 50:1 molar ratio yielded the greatest number of blue plaque recombinants. In this assay the recombination frequency approached 20%. This number is much higher than the 1–2% recombination frequency obtained using standard homologous recombination techniques. In other Cre–*lox* reactions using the 50:1 molar ratio, we have obtained recombination

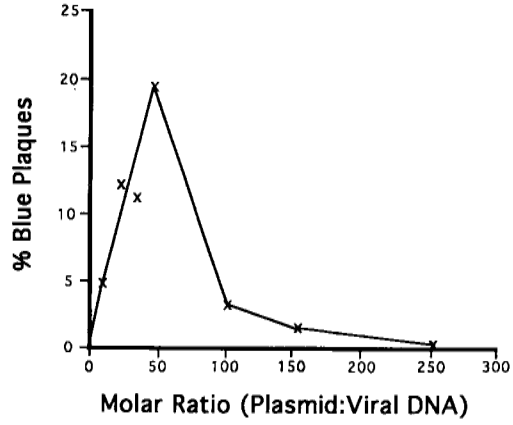

FIG. 4 Graph displaying the percentage of blue plaque recombinants versus the molar ratio of plasmid DNA to target viral DNA. Various molar equivalents of pJG116 DNA to Ktk :: *lox* viral DNA were used in Cre–*lox* recombination assays for determining the optimal ratio to obtain the greatest recombination frequency. The numbers of blue and clear plaques were determined as well as the percentage of recombinants generated. The data are plotted for the 10^{-4} dilution of the virus stock.

frequencies between 50% and 90%, as determined by both blue plaque color assays and dot blot hybridization techniques (data not shown).

Conclusions

Overall, the modified Cre–*lox* recombination procedure represents a dramatic improvement over standard marker transfer procedures used previously (6–8, 16) for applications requiring insertion of multiple genetic constructs into the viral genome. Marker transfer procedures require that considerable effort go into engineering the gene cassette with homologous viral flanking sequences in order to generate each recombinant virus. Efficient marker transfer procedures also require the plasmid DNA molecule to be linearized by digestion at a unique restriction enzyme site prior to homologous recombination. This can be difficult due to the lack of single restriction sites within a large DNA fragment. Finally, once a modified or novel gene has been recombined into the viral genome by marker transfer, it cannot be easily removed or modified.

These difficulties can be largely circumvented by use of the Cre–*lox* system. With the Cre–*lox* method the same *lox* plasmid can be used as a carrier for a large number of different gene cassettes, including those that have no homology with HSV DNA. The plasmid is integrated in linear form within the viral genome as part of the recombinational mechanism and is targeted to the same site each time without the need for flanking sequence homology. Importantly, the Cre–*lox* method uses a shuttle plasmid that can be introduced and removed from the viral genome for analysis in the virus and/or in a bacterial system.

We are currently exploiting this procedure for generating mutant viruses containing altered forms of the HSV-1 glycoproteins involved in virus attachment and penetration in order to identify residues important to specific stages and molecular events in these processes. This technique has worked well in identifying amino acid residues within the cytoplasmic tail of gB that are essential to cell membrane fusion and syncytial plaque formation (15). These types of experiments require the construction of viral genomes containing *lox*P sites in which the viral gene of interest is deleted from the viral genomes (9). If the deleted gene is essential for virus growth, introduction of the essential gene by Cre–*lox* recombination will rescue the virus and select for a recombinant capable of productive infection, provided that the inserted gene does not carry a lethal mutation. Prior to insertion of the essential gene, viral mutants deleted for genes essential to replication in cell culture require the construction and use of complementing cell lines that express the gene product in *trans* in order to propagate the *lox*P-containing deletion virus.

Specific site-directed or random mutagenesis techniques may be used to generate mutations in HSV-1 genes of interest. When employing random mutagenesis strate-

gies, the Cre–*lox* method possesses an additional advantage for rapidly engineering and identifying mutants. Due to the reversible nature of the Cre–*lox* recombination reaction, it is possible to mutagenize a segment of DNA encoding an HSV-1 gene product within a population of *lox*P-containing plasmids and introduce the entire mixture of mutated genes into the viral genome. Using limiting dilution procedures (9) to plaque-purify individual recombinants displaying a particular phenotype, one can identify the specific mutation(s) that contributes to the phenotype by recovery of the mutant plasmid for sequence analysis. That is, purified viral DNA from plaque-purified recombinants can subsequently be reacted with Cre enzyme to excise the original *lox*P shuttle plasmid containing the mutated gene of interest. This reverse Cre–*lox* reaction releases the plasmid for subsequent transformation and propagation in bacterial cells. The clone can then be analyzed by DNA sequencing to determine which specific base pair changes resulted in the mutant phenotype. This method avoids the need to identify the mutation prior to analysis of its possible effects on the biology of the gene product, and thereby can be used for phenotypic selection of interesting mutants.

We are also currently applying the Cre–*lox* system for testing the activity of a variety of eukaryotic promoter elements in the viral vector genome, emphasizing their function during latency within neurons of the brain. This method will allow us to evaluate a considerable number of promoters in recombinant viruses in a relatively short period, a task that could not be accomplished easily using standard marker transfer techniques. In a manner similar to analyzing mutations within specific HSV-1 gene products, Cre–*lox* can be used to characterize and map *cis* elements within promoters that play a role in their activity in specific cell types. For example, the phenylethanolamine *N*-methyltransferase (PNMT) promoter is highly active in primary chromaffin cells (25), a cell type that remains difficult to transfect using the variety of transfection techniques and protocols currently available. However, since HSV-1 can readily infect these cells, it should be possible to identify elements within the PNMT promoter that regulate its expression following Cre–*lox* recombination of promoter–reporter gene cassettes into the HSV-1 vector backbone. It should be noted that this kind of nonviral promoter study requires the use of nonreplicating viral mutants deleted for the essential immediate-early genes, since these products can affect the activity of foreign promoters in the viral genome.

We have already used the Cre–*lox* procedure for inserting foreign gene cassettes into HSV-1 vectors to evalute the expression of genes such as tyrosine hydroxylase and nerve growth factor in gene therapeutic approaches for the treatment of Parkinson's disease and other central nervous system disorders involving neural degeneration. Strategies have been developed to use the Cre–*lox* system to "recombine out" large regions of the HSV-1 genome to generate a virus capable of accommodating large foreign genes such as the 17-kb dystrophin cDNA, genomic genes with introns required for high-level tissue-specific expression, or multiple genes such as cytokines

for the treatment of brain gliomas. Overall, the Cre–*lox* recombination system represents a highly efficient process for genetic studies of HSV-1 proteins, and may also prove useful for introducing foreign gene cassettes into HSV-1.

References

1. B. Roizman and A. E. Sears, "Virology," p. 1795. Raven, New York, 1990.
2. J. G. Stevens, *Microbiol. Rev.* **53,** 318 (1989).
3. J. G. Stevens, E. K. Wagner, G. B. Devi-Rao, M. L. Cook, and L. T. Feldman, *Science* **235,** 1056 (1987).
4. D. J. McGeoch, M. A. Dalrymple, A. J. Davidson, A. Dolan, M. C. Frame, D. M. McNab, L. Perry, J. E. Scott, and P. Taylor, *J. Gen. Virol.* **69,** 1531 (1988).
5. D. J. McGeoch, A. Dolan, S. Donald, and D. H. Brauer, *Nucleic Acids Res.* **14,** 1727 (1986).
6. M. R. Capecchi and E. A. Wong, *Mol. Cell. Biol.* **7,** 2294 (1987).
7. R. S. Kucherlapati, E. M. Evans, K.-Y. Song, B. S. Morse, and O. Smithies, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3153 (1984).
8. K. R. Thomas, K. R. Folger, and M. R. Capecchi, *Cell (Cambridge, Mass.)* **44,** 419 (1986).
9. P. J. Gage, B. Sauer, M. Levine, and J. C. Glorioso, *J. Virol.* **66,** 5509 (1992).
10. B. Sauer, M. Whealy, A. Robbins, and L. Enquist, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 9108 (1987).
11. N. Sternberg and D. Hamilton, *J. Mol. Biol.* **150,** 467 (1981).
12. K. Abremski, R. Hoess, and N. Sternberg, *Cell (Cambridge, Mass.)* **32,** 1301 (1983).
13. K. Abremski, R. Hoess, *J. Biol. Chem.* **259,** 1509 (1984).
14. R. Hoess and K. Abremski, *J. Mol. Biol.* **181,** 351 (1985).
15. P. J. Gage, M. Levine, and J. C. Glorioso, *J. Virol.* **67,** 2191 (1993).
16. F. L. Homa, T. M. Otal, J. C. Glorioso, and M. Levine, *Mol. Cell. Biol.* **6,** 3652 (1986).
17. F. L. Graham and A. J. van der Eb, *Virology* **52,** 456 (1973).
18. B. A. White and F. C. Bancroft, *J. Biol. Chem.* **257,** 8569 (1982).
19. N. Blin and D. W. Stafford, *Nucleic Acids Res.* **3,** 2303 (1976).
20. M. Gross-Bellard, P. Oudet, and P. Chambon, *Eur. J. Biochem.* **36,** 32 (1973).
21. E. M. Southern, *J. Mol. Biol.* **98,** 503 (1975).
22. M. F. Stinski and T. J. Roehr, *J. Virol.* **55,** 431 (1985).
23. D. R. Thomsen, R. M. Stenberg, W. F. Goins, and M. F. Stinski, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 659 (1984).
24. D. J. Fink, L. R. Sternberg, P. C. Weber, M. Mata, W. F. Goins, and J. C. Glorioso, *Hum. Gene Ther.* **3,** 11 (1992).
25. J. M. Carroll, M. J. Evinger, H. M. Goodman, and T. H. Joh, *J. Mol. Neurosci.* **3,** 75 (1991).

[9] Strategies for Studying Transcription Regulation of Neurotropic Viruses in the Central Nervous System: Lesson from JC Virus

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Introduction

The study of eukaryotic viruses has provided key insights into cellular processes involved in gene expression. In this respect DNA viruses serve as ideal models to investigate gene regulation, since, in most cases, they are dependent on the host transcriptional and translational machinery for expressing their genes. Adenoviruses, papovaviruses, and herpes simplex virus, for example, have been particularly interesting, since they encode proteins that modify the transcriptional capabilities of the host cells and may function as transactivators of the viral promoter. Further, these proteins may exhibit oncogenic activity in which, by interacting with tumor suppressor proteins, they modulate cellular gene expression and cell proliferation.

DNA viruses are particularly amenable to molecular characterization in that the economic considerations of the virus limit the size of the regulatory region and distribution of *cis*-acting regulatory elements. Promoter elements can easily be isolated from viral DNA, mutated, and introduced into cells. The function of viral *cis*-acting elements can be assayed independently, once separated from the virus, in the form of a promoter–indicator construct. Identification of positive and negative factors influencing gene regulation is critical to the understanding of the mechanism of viral gene expression and regulation. Once the critical control elements are identified, a variety of approaches can be used to identify the respective host cell-derived *trans*-acting protein factors and clone the gene encoding these factors for detailed mechanistic studies. Understanding of the cellular and viral factors that regulate gene expression is critical for the development of rational strategies for antiviral therapy.

Here, we utilized the human neurotropic JC virus (JCV) as a model to present a detailed description of the methodologies that can be used to dissect viral gene regulation and identify *cis*-regulatory elements and the participant *trans*-regulatory factors for the central nervous system (CNS). Various approaches are discussed to determine functional regulatory elements and to isolate genes encoding cellular regulatory factors that are employed in viral gene transcription. These approaches should allow for further detailed analysis and understanding of viral and cellular regulation in the CNS.

Methods in Molecular Genetics, Volume 7

Strategies for Studying Transcription Regulation of Viral Promoters

Identification of cis-Acting Regulatory Sequence

Deletion Constructs Using Exonucleases

Studies on viral gene transcription have revealed complex and interrelated sets of positive and negative regulatory elements. Usually positioned upstream of the transcription site, these elements regulate viral RNA synthesis throughout the lytic cycle. Once viral transcripts and transcription initiation sites are localized, identification and mapping of viral promoter elements require construction of 5′ and 3′ deletion mutants and examination of the transcriptional activity of these deletion mutants in permissive cells. Wild-type and deleted promoters linked to an indicator gene [i.e., chloramphenicol acetyltransferase (*CAT*) or lucerifase genes] can be generated by simple restriction enzyme digestion and ligation to remove a desired fragment from the promoter. However, the availability of unique restriction enzyme sites severely limits the application of this strategy for generation of a useful panel of mutations. Restriction enzyme digestion followed by *Bal*31 deletion can be used to generate nested sets of deletion mutants at unique restriction enzyme sites within the promoter. By treatment with various concentrations of *Bal*31 and/or various times, the desired size deletions can be generated. A set of unidirectional 5′ and 3′ deletions can be regenerated using this technique by ligating with the digested products the appropriate DNA fragment that contains the site used for *Bal*31 digestions at one end of the fragment. A simpler approach to generating unidirectional 5′ and 3′ deletion constructs can be performed using exonuclease III (*Exo*III) followed by mung bean nuclease digestion. Exonuclease III progressively digests blunt 3′ ends or 3′ ends with 5′ overhangs. A fragment with both 5′ and 3′ overhangs will be digested only at the side of the 5′ overhang by ExoIII. The 5′ overhangs can also be made resistant to *Exo*III digestion by filling in the end (Klenow fragment of *Escherichia coli* DNA polymerase I) with a-thiol nucleoside triphosphates. After generating a protected end, a second restriction enzyme digestion can be used to generate an *Exo*III-susceptible end. Exonuclease digestion followed by digestion with mung bean nuclease to digest single-stranded DNA results in a unidirectionally deleted blunt-ended fragment that can be ligated or reinserted into a promoter–indicator construct. By performing the exonuclease reaction for various times, unidirectional deletions of various sizes can be generated by this technique with common end points. Such deletion kits are now available commercially (e.g., Strategene, La Jolla, CA).

In addition to the *Bal*31, *Exo*III, and mung bean nuclease deletion strategies, defined panels of deletion mutations can be generated by the polymerase chain reaction (PCR) (1, 2). To generate a promoter construct by PCR, oligonucleotides are designed as forward and reverse primers complimentary to sequences upstream and downstream of the promoter region. By amplifying for a number of cycles, promoter fragments can be generated from viral DNA or plasmids containing cloned frag-

ments. By adding sequences to the 5′ ends of the oligonucleotide primers, restriction enzyme sites can be added to the PCR products, facilitating the insertion of the promoter fragments into indicator plasmids. Many restriction enzymes function poorly at fragment ends, requiring the addition of irrelevant bases to the ends of the primers. The addition of three or four G or C residues at the ends of the primers generally alleviates this problem. Primers for this purpose, therefore, are usually 27–32 bases and include three or four G or C residues, then 6 bases for the restriction enzyme site, followed by 18–22 bases with sequence complementarity to the DNA of interest. Multiple forward or reverse primers can be designed such that PCR products generate the desired panel of unidirectional deletion mutants. The use of PCR to generate unidirectional deletion mutants is advantageous in that the desired deleted fragments are generated depending on the design of the primers. Restriction enzyme sites incorporated into the primers also permit directional cloning, further simplifying the construction and analysis. Deletion mutants within promoters can also be generated by the four-primer PCR approach (recombinant PCR) or, alternatively, the inverse PCR mutagenesis technique. In the latter procedure two PCR primers are designed such that they hybridize to either side of the desired deletion and extend in diverging orientations. In this way the circular plasmid is amplified, generating a linear product. The PCR product is then digested with the unique restriction enzyme contained in both PCR primers and the plasmid is self-ligated to recircularize and is transformed into *E. coli.* The inverse primers can be designed such that the terminal restriction enzyme site replaces a sequence of identical length. By performing a series of inverse PCR reactions with the appropriately engineered primers, one could rapidly generate an entire panel of linker-scanning mutations across a promoter region. This technique was previously laborious and essentially involved generating panels of 5′ and 3′ unidirectional deletions circularized with linker, followed by combining the appropriate promoter segments at the linker restriction site. Below, we describe the experimental procedures that were used to define the *cis*-acting regulatory elements of the JCV genome that confer glial specificity for viral gene transcription.

The genome of JCV consists of 5130 nucleotides: double-stranded covalently circular DNA with at least six distinct open reading frames (3). This virus utilizes the host cell transcription machinery to transcribe the viral promoter bidirectionally during lytic cycle infection (4). The control region of JCV contains two 98-bp tandem repeats, each of which contains an A/T-rich region (TATA box-like sequence). The tropism of JCV for glial cells of the CNS is determined, at least in part, by the tissue specificity of viral gene transcription (5–8). To analyze the regulatory elements of the JCV promoter, the 286-bp *Hin*dIII/*Pvu*II DNA fragment representing the central region of JCV was inserted into the promoterless reporter plasmid $pCAT_3M$ at the 5′ position of the reporter *CAT* gene (7). In order to define the regulatory elements of the promoter, a panel of *Bal*31 deletion mutants was generated.

Deletion mutants were generated by first linearizing this plasmid at the unique *Xba*I site in the polylinker upstream of the JCV promoter. Linear DNA was treated

with *Bal*31 nuclease for various times (1–15 min), after which the reaction was terminated by the addition of chelating agent. The *Bal*31 deletion reaction mixture routinely contains 2–3 μg of linearized DNA and 50 m*M* Tris (pH 7.5), 600 m*M* NaCl, 10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$, 50 μg/ml bovine serum albumin, and approximately 1 U of *Bal*31. Reactions are incubated at 37° C for 1, 3, 5, 10, and 15 min and terminated by the addition of EDTA and EGTA to a final concentration of 10 m*M* (9). Note that the amount of *Bal*31 and the length of the incubation time can be varied according to the supplier's recommendations. After *Bal*31 treatment the terminus of the DNA fragment was modified with T4 DNA polymerase. Commonly, this reaction is carried out in a 20- to 50-μl reaction mixture containing 1–2 μg of DNA, 5 U of enzymes in a buffer containing 50 m*M* Tris (pH 8.0), 5 m*M* $MgCl_2$, 5 m*M* dithiothreitol (DTT), 50 μg/ml bovine serum albumin, and a 300-μ*M* mixture of dNTPs at 37° C for 30 min (9). The JCV DNA was subsequently treated with a restriction enzyme, *Nru*I, which cleaves at the unique site in the vector in order to obtain a uniform 5′ terminus for all of the constructs (10). The extent of the deletion within each construct was determined by direct DNA sequences (Fig. 1).

To compare the transcriptional activity of the intact JCV promoter with that of its deleted variants, each construct was introduced into the glial cell line U-87MG by the calcium phosphate precipitation method (11), alone or together with a recombinant construct expressing the viral early protein T antigen pJC–T. In this construct the T antigen gene is constitutively expressed by a heterologous promoter derived

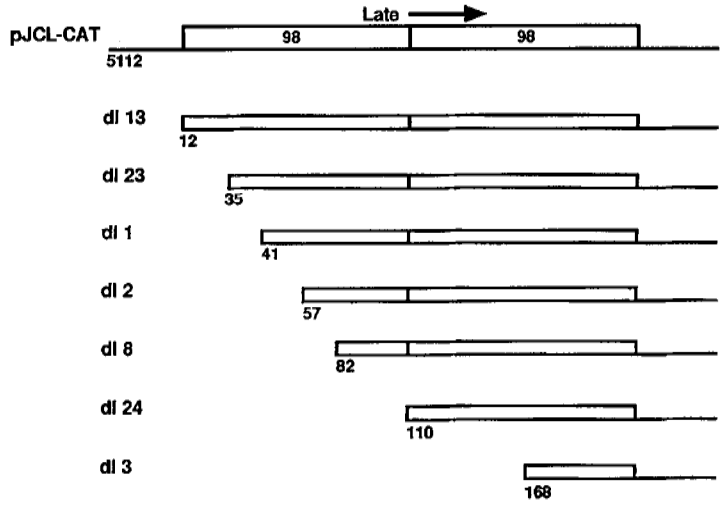

FIG. 1 Structure of the JCV_L promoter and the location of the derivative deletions. The arrow marked "Late" represents the direction of RNA transcription from the late promoter. The numbers below each line represent the first nucleotide of the JCV promoter in each deletion construct.

from cytomegalovirus. Use of pJC–T in transfection experiments has allowed us to study induction of the JVC late promoter by the viral early protein in glial cells. In this experiment cells were plated 12 hr before transfection at 5×10^5 cells per 60-mm dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were refed 3 hr before transfection. A transfection cocktail of 0.5 ml containing 50 m*M* HEPES (pH 7.1), 280 m*M* NaCl, and 1.5 m*M* $NaHPO_4$ was gently mixed with 0.5 ml of a solution containing 15 μg of DNA (mixture) and 124 m*M* $CaCl_2$. The DNA precipitates were allowed to stand for 15 min and were then applied to the cells. The cells were incubated for 4 hr in the presence of the calcium precipitate and then subjected to a 2-min glycerol shock. The CAT assay was conducted as described elsewhere (12). Briefly, cultures were harvested 48 hr posttransfection by scraping and resuspended in 0.25 *M* Tris (pH 7.8), and cells were lysed by five successive cycles of freezing and thawing. After extraction cell pellets were removed by centrifugation in a microcentrifuge. Equal amounts of cell extracts were incubated in the presence of a 4 m*M* acetyl coenzyme A, 2 μl of [^{14}C]chloramphenicol, and 0.25 *M* Tris (pH 7.8) at 37° C for 60–120 min. The reaction was terminated by the addition of 1.5 ml of ethyl acetate to extract the chloramphenicol. The ethyl acetate was dried and the chloramphenicol was taken up in 25 μl of ethyl acetate, spotted onto a thin-layer chromatography plate, and developed by ascending chromatography in chloroform–methanol (95:5). The percentage of conversion of chloramphenicol to the acetylated form was measured by localizing the substrate and acetylated spots by autoradiography and counting them in a liquid scintillation counter. As shown in Fig. 2 (11, 12), removal of the various regions of JCV sequence differentially influences the T-induced transcriptional activity of the viral late promoter, as determined by the level of CAT enzyme activity produced in cells transfected with the reporter constructs, alone or along with pJC–T plasmid

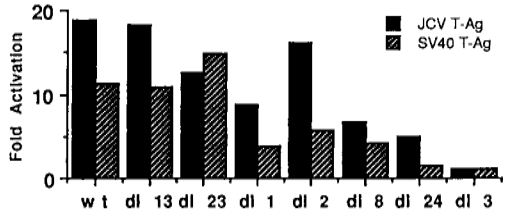

FIG. 2 Transient transfection experiments for *in vivo* functional analysis of JCV promoter in glial cells. CAT enzyme activity expressed from each mutant promoter was used to determine the relative contribution of each deleted sequence to the T antigen (T-ag)-induced transcription of the late promoter. The glial cell line U-87MG was transfected with the reporter JCV–CAT constructs, alone or with plasmids expressing JCV T antigen or simian virus 40 (SV40) T antigen by the calcium phosphate coprecipitation method (11) in a 60-mm dish, and after 48 hr the CAT assay was performed (12). The percentage of acetylation of chloramphenicol by CAT was determined by scintillation counting, and the levels of induction of the viral promoter by JCV and SV40 T antigen are presented on a scale of 0–20.

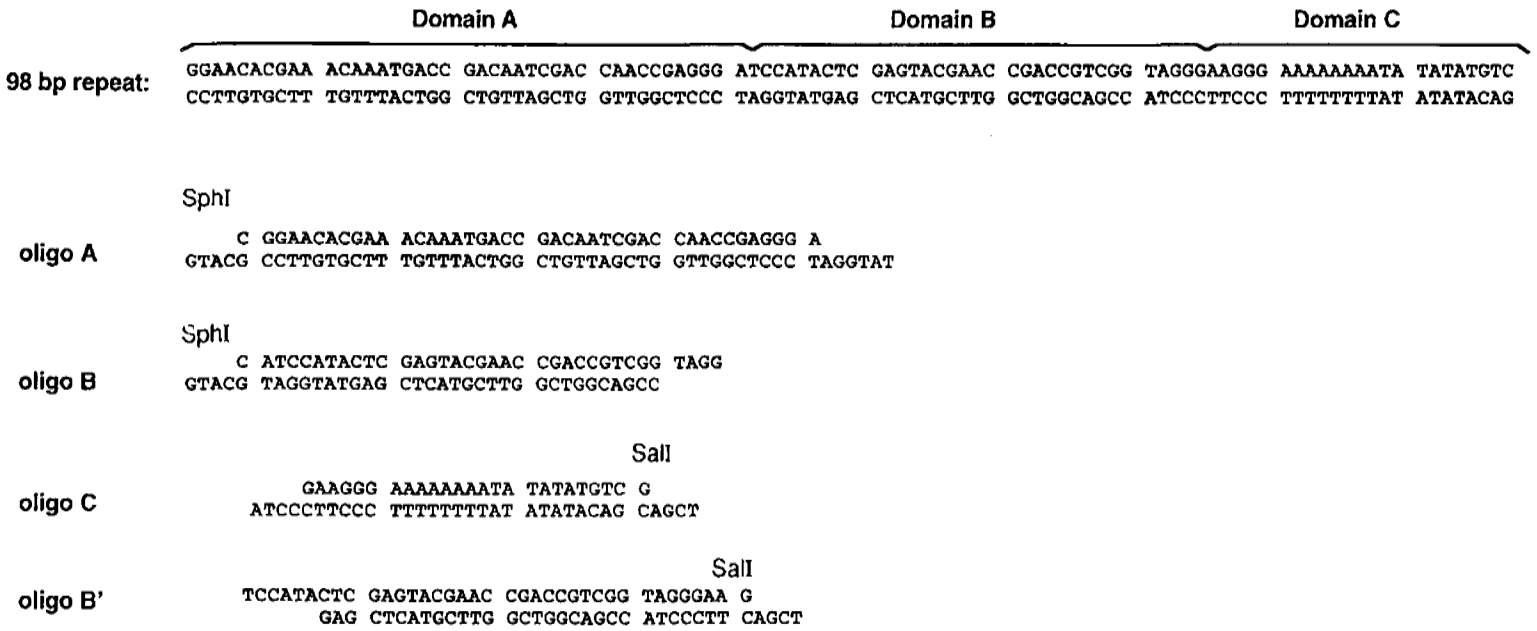

FIG. 3 Primary structure of the JCV 98-bp enhancer region. The three domains, A, B, and C, were designed according to their ability to bind to nuclear proteins from human fetal brain (13).

Synthetic Promoter and Hybrid Promoter Strategy

Here, we utilized the JCV regulatory region as a model to detail the strategy for building a synthetic viral promoter for functional analysis. The 98-bp enhancer–promoter of JCV was divided into three nonoverlapping domains, A, B, and C, for examination of their transcriptional activity in glial cells (13). The use of synthetic oligonucleotides A, B, and C, each containing approximately 4- to 6-bp overhangs, provides the means for construction of the viral promoter and their insertion into the specific site within the polylinker of the reporter plasmid. Two plasmids—pBL–CAT_3, which lacks any promoter–enhancer (14), and pA_{10}–CAT_2, which carries the simian virus 40 (SV40) basal promoter (15)—are used to constitute the JCV control domains upstream of the *CAT* gene. These vectors contain an array of unique restriction sites 5′ of the *CAT* gene. Six chimeric plasmids, pJC–CAT_A, pJC–CAT_B, and pJC–CAT_C, each carrying a single copy of the A, B, and C regions of the JCV, respectively, were generated in pBL–CAT_3 and pA_{10}–CAT_2 backgrounds. The position and the nucleotide composition of the A, B, and C regions of the 98-bp enhancer of JCV are shown in Fig. 3.

To construct pJC–CAT_A, oligonucleotide A with an *Sph*I site on one end and 6 bp complementary to domain B at the other end were synthesized. All oligonucleotides were phosphorylated with T4 polynucleotide kinase and ATP for the purposes of ligation. The *Sph*I-digested vector was treated with calf intestinal alkaline phosphatase to remove the phosphate group from the 5′ terminus of the linearized plasmid DNA and ligated with double-stranded oligonucleotide under appropriate conditions. To circularize the recombinant pJC–CAT_A, the sticky ends of the constructs were filled in by using T4 DNA polymerase and dNTPs prior to religation. After transformation of competent *E. coli* HB101, recombinants were identified by direct sequencing.

A similar approach was taken in constructing pJC–CAT_B. Again, an *Sph*I-compatible end was designed at the end of oligonucleotide B with a 4-bp sequence complementary to domain C at the other end of oligonucleotide B, as shown above. The plasmid pJC–CAT_C contains DNA fragment C with a *Sal*I restriction enzyme recognition site on one end and a 6-bp sequence complementary to domain B at the other. To construct this plasmid, pBL–CAT_3 was cleaved with *Sal*I instead of *Sph*I and treated as described above.

Three additional constructs, each containing two domains, AB, BC, and AC, were prepared as follows. To prepare the plasmid carrying domains A and B, pJC–CAT_{AB} double-stranded oligonucleotides A and B are ligated with linearized *Sph*I/*Sal*I-digested fragments. To construct pJC–CAT_{AC} and pJC–CAT_{BC}, the vector is cleaved with *Sph*I and *Sal*I, mixed with oligonucleotides A and C or B and C, ligated with T4 DNA ligase, treated with S1 to trim the ends, and recircularized by treatment with T4 DNA ligase. Use of these recombinants in transient transfection assay will allow us to examine the activity of each domain, alone or in the context of the neighboring region, in viral gene transcription. Figure 4 illustrates results from the transient trans-

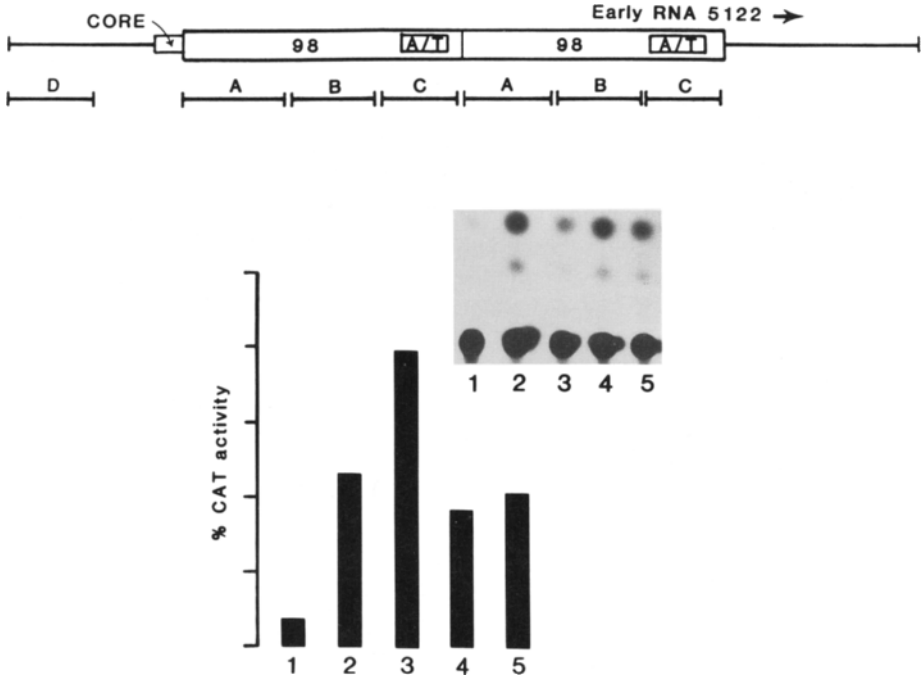

FIG. 4 Transcriptional activity of the JCV *cis*-regulatory elements in the hybrid promoter constructs. (Top) Structural organization of the JCV control region. The A/T box within each 98-bp repeat contains a 5′–$(TA)_4A_7$–3′ sequence. The core segment indicates the location of an octameric motif, the 5′–GTGCAAAG–3′ simian virus 40 core sequence. (Bottom) Role of the JCV DNA sequence in glial-specific transcription of the viral genome. Oligonucleotides spanning C (lane 2), A (lane 3), D (lane 4), and B (lane 5) of JCV were inserted at the *Bgl*II site of the pA_{10}–CAT_2 plasmid. Each construct was then introduced into the glial cells by the calcium phosphate precipitation procedure (11), and the production of CAT enzyme was measured after 48 hr. Lane 1 represents activity of the parent vector (pA_{10}–CAT_2), which contains no JCV sequence.

fection of glial and nonglial cells with the reporter CAT construct containing only one copy of JCV A, B, and C domains fused to the heterologous SV40 promoter in a pA_{10}–CAT_2 background. It is evident that each region of JCV differentially affects the basal transcription of the SV40 promoter in glial and nonglial cells.

Site-Directed Mutagenesis

More detailed analysis of the viral promoter elements usually requires utilization of a promoter–indicator construct with fine mutations of specific nucleotide residues within the regions of interest. DNA fragments comprising promoters or promoter elements can be subcloned into the replicative-form (double-stranded) M13 phage.

Single-stranded phage is purified by polyethylene glycol precipitation from supernatant and is used as template for mutagenesis. Oligonucleotides (18–20 bases) are designed to hybridize to the single-stranded DNA and introduce a small mutation (deletion or base pair alteration) in the region of interest. After annealing of the oligonucleotide with the single-stranded DNA, duplex DNA is generated using the oligonucleotide primer, deoxynucleotide triphosphates, and Klenow enzyme. Products of the mutagenesis reaction are transformed into an appropriate host for M13 phage infection (i.e., JM101) and plated with log phase cells, generating plaques in the top agar (9). The plaques are transferred to nitrocellulose membrane, and bound phage DNA is screened by hybridization with the ^{32}P-labeled mutagenic oligonucleotide primer. Hybridization is temperature- and sequence-dependent, and mutant phages can be distinguished from the wild-type phage by the washing temperature. Generally, the probe could be denatured at 65° C for 15 min, then incubated with filters at sliding temperatures to 37° C. Washing is carried out first at room temperature with 6× SSC buffer, followed by one wash at 37° C. A final wash is carried out for 10 min at 2–4° C below the theoretical melting temperature (T_m) for the oligonucleotide. For oligonucleotides of approximately 20 bases, the T_m is calculated as $T_m = [2(AT) + 4(GC)]$, where AT and GC are the number of AT and GC base pairs in the duplex. Positive plaques are further purified, mixed with JM bacterial cells, and plated for secondary and tertiary screening until a pure mutant phage preparation is generated. In the initial plating of the mutagenesis reaction, the majority of phages are of the wild type, with few positive mutants per plate of 10,000–100,000 plaques. While this low frequency is sufficient for generating the desired mutants, several strategies have been designed to increase the efficiency of mutagenesis. Improved strategies have been developed, increasing the mutagenesis frequency to the point that potential mutants can be directly sequenced without hybridization screening. Uridine-containing template phage is prepared in uracil-containing medium in a uracil *N*-glycosylase–negative strain of bacteria (ung^-/dut^-) (16). The template is used for mutagenesis and then introduced into ung-containing bacteria. The template strand is replaced by excision repair of the uridine-containing DNA and the duplex is restored by repair. This technique selects against the wild-type phage, dramatically increasing the efficiency of mutagenesis.

An alternative approach to site-directed mutagenesis is the four-primer PCR approach (17). Complementary primers (i.e., 36-mer) are generated containing the mutation of interest internally (deletions or substitutions), and PCR reactions are performed with mutagenic primers in both directions. The mutagenic primers (inside primers) serve as partners for PCR reactions with template and outside primers. In this method the DNA fragment containing the promoter element is amplified in two separate reactions generating right and left segments with a region of overlap. The desired fragments are purified from nondenaturing acrylamide gels by passive elution. The fragments are precipitated, mixed in approximately equimolar amounts, and subjected to a second round of PCR using only the outside primers. The region of overlap (36 bases) between the two PCR products allows partial duplex formation,

which is converted to a complete duplex by *Taq* polymerase. In subsequent rounds of PCR, the full-length DNA fragment containing the mutation is amplified. The PCR product from the secondary PCR can be digested with the restriction enzymes recognizing sites in the outside primers and the fragment cloned upstream of the desired indicator gene. A recombinant PCR approach has been designed requiring only three primers (1). Recombinant PCR can be used to introduce mutations and deletions, and can further be used to generate chimeric promoters or chimeric genes without the need to find compatible restriction enzyme sites.

Identification of a Nuclear Protein(s) Derived from CNS Cells That Interacts with the Viral Regulatory Elements

In general, it is known that the action of *cis*-regulatory enhancer–promoter elements is mediated by *trans*-acting cellular proteins. Thus, once the regulatory elements of the viral promoter are fully characterized, complementary studies, which include identification and characterization of the participant regulatory proteins, are generally performed. The initial phase of these studies includes preparation of nuclear extracts from the infected or uninfected permissive cells, and their use in DNA–protein interaction analysis. Similarly to the method in the previous section, we chose to utilize JCV and a glial cell system to describe the common strategies and methodologies used in the identification of nuclear regulatory proteins.

Preparation of Nuclear Extracts

Traditional procedure

Nuclear extract is prepared from glial cells (U-87MG) according to the procedure described by Dignam *et al.* (18). Confluent cells 10–100 × 225-cm^2 flasks (10^8–10^9) are harvested and resuspended in four packed cell volumes of buffer A containing 10 m*M* HEPES (pH 7.9), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* DTT, and 0.5 m*M* PMSF. After a 20-min incubation on ice, cells are Dounce-homogenized with a B pestle and nuclei are collected by centrifugation at 10,000 rpm. Nuclei are resuspended in four packed cell volumes of buffer B containing 20 m*M* HEPES (pH 7.9), 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.5 m*M* PMSF, and 25% glycerol and incubated for 20 min on ice. After centrifugation at 10,000 rpm for 30 min, nuclear proteins are collected and precipitated by ammonium sulfate (33% final concentration). The concentration of nuclear protein in buffer C containing 20 m*M* HEPES (pH 7.9), 20 m*M* KCl, 1 m*M* DTT, and 17% glycerol is adjusted to 10 mg/ml.

Rapid Procedure

This procedure is quick, is convenient for a large number of samples, and can be used to prepare extract from a small quantity of cells (19). Approximately 1 × 10^6 cells

are washed with 3 ml of Tris-buffered saline and pelleted by centrifugation at 1500 *g* for 10 min. The pellet is resuspended in 250 μl of ice-cold buffer A containing 10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* DTT, and 0.5 m*M* PMSF. After gentle pipetting with a pipette tip, the cells are allowed to swell for 15 min, then 25 μl of a 10% solution of NonidetP40 is added to the cell lysate and the tube is vigorously vortexed for 10–30 sec. The homogenate is centrifuged for 30 sec in a microcentrifuge. The nuclear pellet is resuspended in 50 μl of ice-cold buffer C containing 20 m*M* HEPES (pH 7.9), 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* DTT, and 1 m*M* PMSF, and the tube is vigorously rocked at 4° C for 15 min on a shaking platform. The nuclear extract is centrifuged for 5 min in a microcentrifuge at 4° C, and the supernatant is frozen in aliquots at − 70° C.

DNA–Protein Interaction Techniques

Once the extract is prepared, the integrity of the protein contents is examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) prior to their use in the DNA–protein interaction studies. The two common techniques, band-shift assay and photoaffinity labeling of proteins, are employed to assess binding of the nuclear protein to the *cis*-acting sequence of the viral promoter.

Band Shift

Band shift has been used extensively in the past decade for the identification of DNA-binding factors from eukaryotic cells. The procedure offers several advantages over other assays: (1) It is remarkably sensitive and detects specific DNA-binding protein factors to less than 100 molecules per cell; (2) it provides the resolving power of electrophoresis, which is lacking in some other assays; and (3) binding occurs under conditions in which the protein has not been denatured and subsequently renatured, as in some other assays. In this assay, to minimize binding of nonspecific proteins to the DNA probe, poly(dI–dC) is added to the binding reactions. After incubation samples are run on low-ionic-strength polyacrylamide gels. If DNA binding (slower migrating bands) is detected, several control studies are performed: (*a*) binding reactions are treated with protease (50 μg/ml) to ensure that the observed bands are due to the DNA–protein interactions, and (*b*) competition using homologous DNAs is performed to demonstrate the specificity of this interaction. This is usually done by adding increasing amounts of unlabeled competing DNA from related and unrelated DNA sequences.

Through the use of the band-shift technique, several nuclear proteins from glial cells that bind to the JCV regulatory sequence have been identified. Synthetic oligonucleotides representing the regulatory domains of JCV are labeled by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Approximately 20,000 cpm of the end-labeled double-stranded oligonucleotides are incubated with nuclear proteins in the presence of 600 μg/ml poly(dI–dC) in a final volume of 30 μl. Incubation is carried out at 0° C for 30 min in 10% glycerol, 12 m*M* HEPES (pH 7.9), 50 m*M* NaCl,

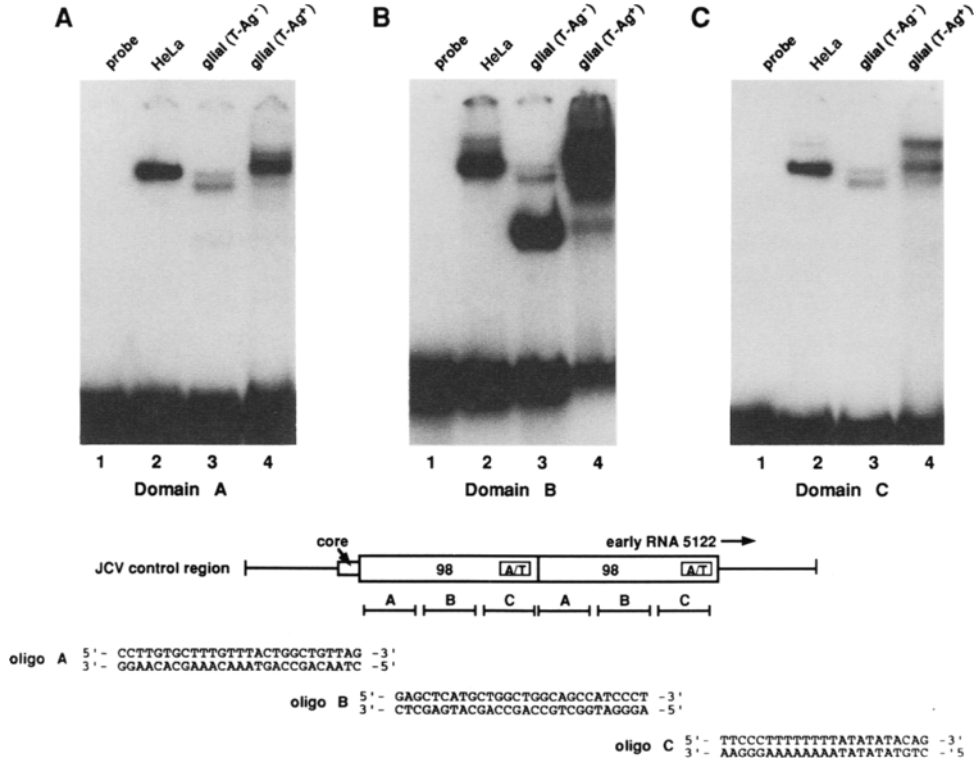

FIG. 5 Band-shift analysis of the JCV *cis*-acting regulatory sequence in extracts from HeLa, glial, and T antigen-producing glial extracts. End-labeled double-stranded oligonucleotide fragments were incubated with equal amounts of nuclear extracts (10 μg) from HeLa and glial cells, and the resulting complexes were separated by electrophoresis by native PAGE. Lane 1, no extract added; lane 2, nuclear extract from HeLa cells; lane 3, nuclear extract from glial cells with no T antigen (T-Ag$^-$); and lane 4, nuclear extract from T antigen-producing glial cells. The positions of each domain within the JCV 98-bp enhancer and their nucleotide compositions are shown below.

50 m*M* $MgCl_2$, 4 m*M* Tris (pH 8.0), and 0.8 m*M* DTT. Samples are layered on top of low-ionic-strength 9% polyacrylamide 19:1 gels. Electrophoresis is carried out for 2–3 hr at 180 V in 0.3–0.5× TBE solution at 4°C (Fig. 5).

Photoaffinity Labeling

Photoaffinity labeling identifies cellular proteins that interact with responsive DNA sequences. This approach was first used by Lin and Riggs (20) in a study in which they demonstrated that the lac repressor can be specifically cross-linked by ultraviolet

(UV) irradiation to a 5-bromo-2-deoxyuridine (BrdU)-incorporated DNA fragment containing lac operon. The UV light displaces bromide from BrdU and produces a free radical, a vicinal-reactive group of bound proteins cross-linked to the DNA. The presence of the nucleoprotein complexes which have slower electrophoretic mobilities are clearly detectable by this assay in SDS–PAGE. A parallel binding reaction with native probe should be carried out to ensure that BrdU substitution does not change the specific binding activity of the DNA. This technique became popular after its use to identify the regulatory proteins from HeLa cells that interact with the adenovirus major late promoter element (21). The UV–cross-linking technique has been used to measure the size of the DNA–protein complexes that formed by the association of various regions of JCV to nuclear proteins from glial cells (Fig. 6). Toward this end, the control domains of JCV were ^{32}P-labeled by nick translation in the presence of [^{32}P]dCTP, BrdU, dATP, dGTP, and DNA polymerase (Kornberg enzyme). Labeled oligonucleotides were separated from free gel filtration and mixed with extract under conditions described in the band-shift assay. Reaction mixtures were first

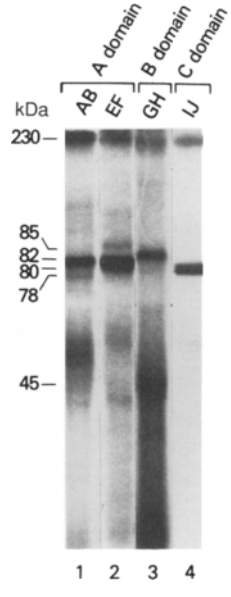

FIG. 6 Identification of JCV enhancer-binding proteins by ultraviolet (UV) cross-linking. 5-Bromo-2-deoxyuridine (BrdU)-incorporated ^{32}P-labeled oligonucleotides were mixed with brain extract. After a 30-min incubation samples were UV-irradiated for 2–3 min and treated with DNase I (20 μg/ml). The molecular size of the bound proteins were subsequently determined by SDS–PAGE. In lanes 1 and 2 oligonucleotides AB and EF spanning domain A were used as probes. In lanes 3 and 4 oligonucleotides GH (domain B) and IJ (domain C) were examined, respectively.

treated with UV light of 302-nm wavelength and then with micrococcal nuclease or DNase I (10 μg/ml) before resolving by SDS–PAGE. Perhaps it should be pointed out that the covalent attachment of a short (10- to 15-bp) DNA does not significantly alter the electrophoretic mobility of the protein (13, 20).

Alternative Method

An alternative method for the detection of the protein which interacts with the DNA target sequence is the Southwestern blot technique. In this assay crude or fractionated brain extract (80–100 μg in sample buffer) is loaded onto a 10% polyacrylamide gel, and after electrophoresis at 100 V, the proteins are transferred to nitrocellulose and then denatured in a buffer containing 50 m*M* Tris (pH 8.0), 7 *M* guanidine HCl, 50 m*M* DTT, 2 m*M* EDTA, and 0.25% Blotto [5% nonfat dry milk, 50 m*M* Tris (pH 7.5), 50 m*M* NaCl, 1 m*M* EDTA, and 1 m*M* DTT] for 1 hr at room temperature. The denaturing buffer is discarded and the proteins are renatured by immersing the nitrocellulose filter in renaturing buffer containing 50 m*M* Tris (pH 8.0), 100 m*M* NaCl, 2 m*M* DTT, 2 m*M* EDTA, 0.1% NonidetP40, and 0.25% Blotto for 24 hr at 4° C. The proteins are incubated for 2 hr at room temperature in TNE buffer containing 20 m*M* Tris (pH 8.0), 1 m*M* EDTA, and 100 m*M* NaCl before adding 1×10^6 cpm/ml ^{32}P–end-labeled double-stranded oligonucleotide probe. After a 1-hr incubation filters are washed three times in TNE solution to remove nonspecific bound materials prior to autoradiography.

Identification of Residues

For precise identification of the residues in DNA that are in close contact with the protein, the DMS protection assay, utilizing the chemical alkylation agent dimethyl sulfate (Me_2SO_4), is commonly used. This technique was used to identify the purine nucleotides in the B domain of JCV which are in close contact with the protein derived from glial cells. In this experiment one strand of B oligonucleotide was ^{32}P–end-labeled, annealed to the complementary strand, chemically modified with DMS (9), and used in band-shift reactions. Free and bound proteins were eluted from the gel and treated with piperidine to cleave the DNA backbone adjacent to N_7-methylated guanosyl and N_3-methylated adenosyl. The results shown in Fig. 7 illustrate that the free guanosyl residue in either strand, which comprises the NF-1 binding site, interferes with protein binding when methylated.

Isolation and Characterization of Genes Encoding the DNA-Binding Regulatory Protein(s)

Identification and characterization of regulatory proteins from host cells that modulate transcription of the viral genes by the analytical approach, as described above,

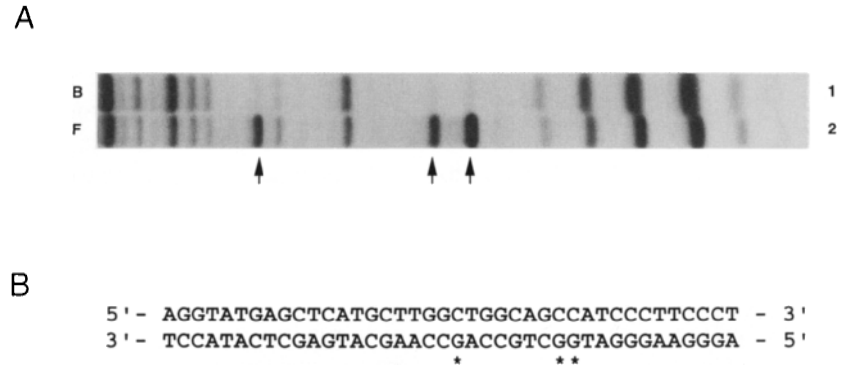

FIG. 7 Methylation interference analysis of the complexes formed in extract derived from glial cells. (A) Double-stranded free (F) and bound (B) oligonucleotides representing the B domain (labeled at the 5′ end of the late strand) were premodified by dimethyl sulfate, isolated from native gels, cleaved with piperidine, and analyzed on 12% polyacrylamide gels containing urea. (B) The sequence of the B domain is shown. G residues that interfere with complex formation in glial cell extract are shown with asterisks.

will provide important clues for isolating genes encoding these proteins. Two strategies have been adapted for the cloning of genes encoding activator proteins: (1) purification of the nuclear protein(s) to homogeneity in order to develop an antibody to be used as a probe in the screening of a cDNA expression library or obtaining the amino acid sequence for developing an oligonucleotide probe for library screening and (2) screening of a cDNA expression library made from the host cell, using a double-stranded synthetic oligonucleotide containing the target elements for binding as a probe.

Purification of the DNA-Binding Protein by Phosphocellulose and DNA Affinity Columns

The purification of the regulatory protein(s) is an important step in the characterization and cloning of host genes that regulate viral and host gene transcription. One important feature of any protein purification scheme is the rapidity of the assay. The gel mobility-shift assay can be used to detect virtually any protein interacting directly with a DNA sequence. DNA-binding regulatory factors can generally be purified in two steps. The first step is ion exchange, serving to eliminate the majority of the proteins in the mixture. DNA-binding proteins generally bind phosphocellulose at 0.1 *M* NaCl and can be step-eluted at higher salt fractions (0.3, 0.5, and 1 *M*). The fractions with the desired DNA-binding activity can be desalted and applied to an oligonucleotide affinity column. Oligonucleotides corresponding to the binding site

are linked to cyanogen bromide (CNBr)-activated Sepharose by a procedure previously described by Kadonaga (22). Stepwise salt elution is again used to determine the concentration at which the factor is released from DNA. This may be approximated from gel mobility-shift experiments using various amounts of DNA. Generally, one phosphocellulose column and two oligonucleotide affinity columns are sufficient for purification to near-homogeneity. Various matrices are available for coupling DNA. We have successfully used CNBr Sepharose for oligonucleotide coupling. Milligram quantities of DNA can be coupled to milliliter quantities of resin. In general, transcription factors are present in cells in small amounts and kilogram quantities of starting material are necessary to purify milligram quantities of pure protein. Since large quantities of starting material are required, it is usually more economical to start purification from tissue. In our purification of B-domain factor(s) recognizing the JCV promoter, we have successfully utilized calf brain and monkey brain as starting material (23).

In this respect nuclear extracts prepared from 1 kg of monkey brain tissue was fractionated by 500 ml of phosphocellulose P11 column, and after step elution with buffer A containing 10 m*M* HEPES (pH 7.9), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.5 m*M* DTT, and 0.5 m*M* PMSF, and 0.3, 0.5, or 1 *M* KCl, active fractions with B-binding activity were determined by band-shift assay (Fig. 8). The fractions 15, 16, and 17 were pooled, precipitated with 50% ammonium sulfate, and applied to a 3-ml DNA affinity column. The column was prepared by coupling CNBr-activated Sepharose CL-2B with a double-stranded oligonucleotide representing the B domain of the JCV promoter (23). The affinity column was equilibrated with 25 m*M* HEPES (pH 7.8), 20% (v/v) glycerol, 1 m*M* DTT, and 0.1 *M* KCl. The column was washed with 15 ml of DNA affinity column buffer and eluted with the same buffer containing 0.1, 0.5, and 1 *M* KCl. The fractions were examined for their DNA-binding activities by the band-shift assay and the size of the proteins bound to B oligonucleotide was determined by SDS–PAGE (23).

The biological activity of the purified brain-derived protein in transcription of the specific promoter could be examined by *in vitro* transcription combined with affinity absorption, precipitation, and partitioning techniques. In this approach specific DNA-binding factors are absorbed from the *in vitro* transcription extract derived from neural cells by preincubation with a plasmid containing multiple copies of the responsive regulatory sequence with the binding site for the protein, and subsequent removal of protein–DNA complexes by centrifugation. The pretreated extract is programmed subsequently to transcribe the viral promoter in the absence and/or presence of purified factor. To ensure depletion deficiencies, multiple preincubations are performed, and after final preincubation the extracts are tested by band-shift assay. In parallel, nonspecific DNA is mixed with the extract and treated similarly for use as a control in the *in vitro* assay. If a DNA-binding factor plays a role in regulation of viral promoter, depleting the extract of this protein is anticipated to specifically inactivate

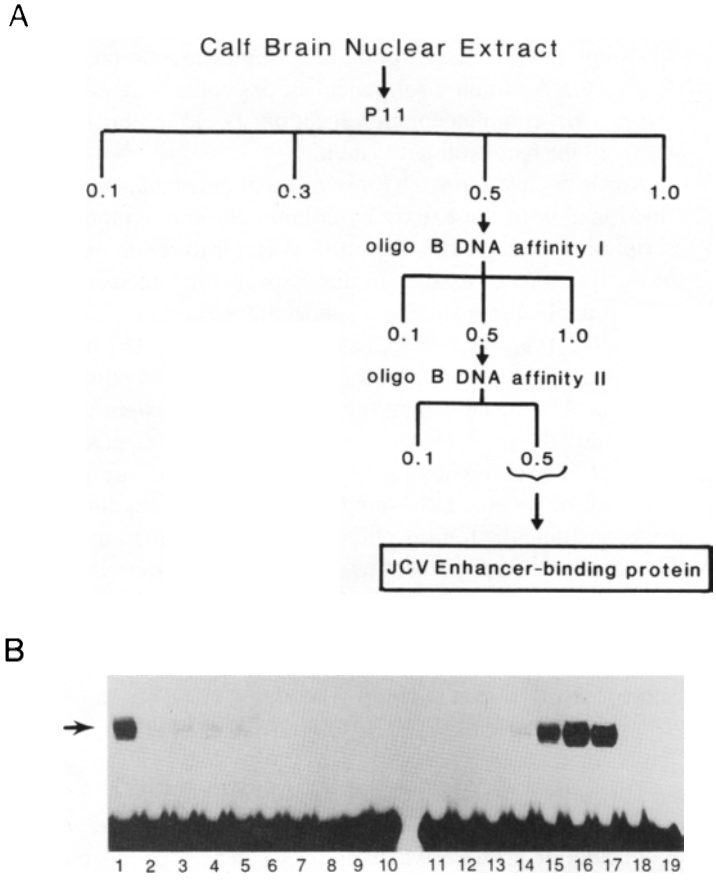

FIG. 8 Scheme for purification of B-binding factor. (A) Protocol for the purification of the protein from calf brain nuclear extract that recognizes the B domain of the JCV control region. Extract prepared from 1 kg of frozen calf brain, which contains approximately 12 g of total protein, was fractionated by phosphocellulose (P11) chromatography. Approximately 200 mg of protein off the column containing the specific B domain-binding protein was pooled after washing with 0.5 *M* KCl elution buffer. Approximately 40- to 60-fold purity over the crude extract was estimated at this stage. This determination was made by measuring the total protein concentration (Bio-Rad protein assay) in the fractions containing the binding activity and by performing the band-shift assay in the presence of carrier poly(dI–dC). (B) A representative band-shift assay illustrates purification of the DNA-binding protein. Binding of the protein components from the crude calf brain protein extract and from fractions eluted from the phosphocellulose column are shown. Lane 1, crude extract; lanes 2–8, fractions from 0.1 *M* KCl; lanes 9–12, fractions from 0.3 *M* KCl; lanes 13–18, fractions from 0.5 *M* KCl; and lane 19, fraction from 1.0 *M* KCl.

transcriptional activity of the promoter. Moreover, the extract should regain its ability to stimulate viral transcription after complementation with the corresponding purified factor. Addition of oligonucleotides containing factor recognition sequences to the *in vitro* complementation reaction should inhibit transcription of the viral promoter in the reconstituted system.

An alternative approach for removal of the regulatory protein from extract involves fractionation of the extract by column chromotography and reconstitution of transcription extract lacking the purified factor to reestablish the transcriptional activity of the fractionated extract. In this respect crude nuclear extract prepared from neural cells is applied to a small phosphocellulose column and fractionated by step elution of 0.1, 0.3, 0.5, and 1 *M* KCl as described above. The transcriptional activity of each chromatography fraction, alone or in combination with other fractions, is examined *in vitro*. After establishing the reconstitution system the chromatography fractions containing the specific DNA-binding protein are selected and further fractionated by DNA affinity chromatography. Unbound proteins are pooled and tested for the absence of the specific DNA-binding protein. This fraction could be subsequently used to reconstitute the transcription extract in combination with the other phosphocellulose chromatography fractions under conditions determined earlier. Again, depletion of a factor from the chromatography fraction is anticipated to reduce transcription of the viral promoter in the reconstitution system. Complementation of the reconstitution system with the corresponding purified DNA-binding factor is expected to restore transcription of the viral promoter *in vitro*. This approach has been used in order to evaluate transcriptional activity of the B-binding protein of JCV promoter from brain tissue in the *in vitro* system (23).

Once the protein is purified and its activity is verified by *in vitro* assay, limited amino acid sequencing of purified protein allows the generation of degenerate oligonucleotide primers, which, in turn, can be used to isolate the respective gene from a DNA library prepared from brain cells.

Screening of an Expression Library Using a Double-Stranded Synthetic Oligonucleotide

An alternative approach to obtain the cDNA encoding for the protein of interest includes the use of double-stranded oligonucleotide containing the binding sequence for the protein as a probe to screen an expression library. This approach, however, is useful only for factor subunits that bind DNA. Using this approach, a cDNA that encodes a protein with binding activity to the B oligonucleotide of JCV was isolated from a human brain cDNA library (24).

Biological Activity of the Cloned Gene Product in Prokaryotic and Eukaryotic Expression Systems

Once the gene for a host regulatory protein is cloned, the protein can be expressed in bacteria for functional analysis. Expression in *E. coli* is advantageous in that the level

of protein expression is high, facilitating purification. Several commonly used bacterial expression systems are available commercially, and milligram quantities of pure protein can be obtained from a liter of culture. In one system, available from Pharmacia, the protein of interest is expressed as a fusion protein with glutathione *S*-transferase. The fusion protein can be purified from extracts over glutathione Sepharose columns and eluted with solutions containing reduced glutathiones. The pRSET system available from Invitrogen and a similar vector system from Qiagen (Chatsworth, CA) utilize a short fusion with a six-histidine T antigen, enabling purification of proteins from bacteria over a nickel chelate (nitrilotriacetic acid) resin. Elution can be performed with mildly acid conditions (i.e., pH 5.9) or by elution with imidazole. This procedure is advantageous in that purification can be performed under denaturing conditions in 6 *M* guanidine-containing buffer. This eliminates the need for solubility of the protein during the initial stages of the purification. Proteins overexpressed in *E. coli* are often localized in inclusion bodies, requiring denaturing conditions for extraction. The activity of the overproduced protein in binding to its target DNA sequence and enhancing transcription of the respective promoter could be examined by parallel band-shift assay using specific oligonucleotide probe and an *in vitro* transcription/supplementation technique utilizing wild-type and specific mutant templates.

An alternative approach to investigate transcriptional activity of the cloned cDNA includes cotransfection of glial and nonglial cells with the promoter–CAT construct, alone or together with constructs containing the cDNA under the control of eukaryotic promoter elements. Production of the protein encoded by the cDNA will, in turn, regulate expression of the promoter-linked *CAT* gene in the transfected cells. Further experiments should also be performed to test the effect of cloned viral regulatory proteins on virus replication in permissive and nonpermissive cells. Such studies may be performed by transient transfection assay or in cell lines stably expressing the cloned genes (by infection with virus or transfection with viral DNA). These approaches will allow the investigation of the role of cloned regulatory factors in the life cycle of the intact virus.

Conclusion

In our studies with human papovavirus JC, we have identified, purified, and cloned transcription factors that contribute to gene regulation in glial cells. These factors obviously are present in cells in order to regulate the basal and/or tissue-specific expression of cellular genes. The strategies we describe should provide an example for studying viral and host transcriptional regulation in the CNS. The understanding of mechanisms that regulate gene expression in the CNS will be critical in order to effectively address neuropathogenic mechanisms. Gene therapy directed toward viral and/or host genes will require knowledge of virus–host interactions that contribute

to latency or, alternatively, result in gene activation. While antiviral gene therapy strategies are usually directed toward viral regulation, it may be possible to use strategies directed at cellular processes that are critical to the virus life cycle. Gene therapy in the CNS will be challenging in that, as in the case of myelin basic protein, myelinization occurs primarily at a specific stage of brain development. Therapeutic protocols that lead to an unprogrammed increase in the expression of the myelin basic protein in demyelinating diseases may result in toxicity if synthesis is temporally inappropriate and poorly controlled. Regulation of promoter elements in response to relevant cytokines will also be critical in controlling autocrine and paracrine mechanisms involved in CNS pathogenesis. Carefully constructed promoters containing various regulatory elements may be generated by some of the approaches described here. We are now rapidly approaching a level of understanding of gene regulation in the CNS such that we can address viral pathogenesis therapeutically via modification of cellular and viral gene regulation strategies. The methods and strategies described here will serve as an initial roadmap leading toward the elucidation of virus–host gene regulation in the CNS.

Acknowledgments

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References

1. G. Pont-Kingdom, *BioTechniques* **16,** 110 (1994).
2. B. R. Wren, J. Henderson, and J. M. Ketley, *BioTechniques* **16,** 94 (1994).
3. R. J. Frisque, G. L. Bream, and M. T. Cannella, *J. Virol.* **51,** 458 (1984).
4. E. O. Major, K. Ameniya, C. Tornatore, S. Houff, and J. Berger, *Clin. Microbiol. Rev.* **5,** 49 (1992).
5. H. Tada, M. Lashgari, J. Rappaport, and K. Khalili, *J. Virol.* **63,** 463 (1989).
6. L. Feigenbaum, K. Khalili, E. O. Major, and G. Khoury, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 3695 (1987).
7. S. Kenney, V. Natarajan, V. Strika, G. Khoury, and N. P. Salzman, *Science* **226,** 1337 (1984).
8. M. Lashgari, H. Tada, S. Amini, and K. Khalili, *Virology* **170,** 292 (1989).
9. F. M. Ausubel, "Current Protocols in Molecular Biology." Harvard Medical School, Boston, Massachusetts, 1987.
10. H. Tada, M. Lashgari, and K. Khalili, *Virology* **180,** 327 (1991).
11. F. L. Graham and A. J. van der Eb, *Virology* **52,** 456 (1973).

12. C. M. Gorman, L. F. Moffat, and B. H. Howard, *Mol. Cell. Biol.* **2,** 1044 (1982).
13. K. Khalili, J. Rappaport, and G. Khoury, *EMBO J.* **7,** 1205 (1988).
14. L. des Groseillers, E. Rassart, and P. Jolicoeur, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 4203 (1983).
15. L. A. Laimins, P. Gruss, R. Pozzatti, and G. Khoury, *J. Virol.* **49,** 183 (1984).
16. T. A. Kunkel, K. Bebenek, and J. McClary, *in* "Methods in Enzymology" (J. H. Miller, ed.), Vol. 204, p. 125. Academic Press, San Diego, 1991.
17. R. Higuchi, *in* "PCR Protocols: A Guide to Methods and Applications," p. 177. Academic Press, San Diego, 1990.
18. J. D. Dignam, R. M. Lebowitz, and R. G. Roeder, *Nucleic Acids Res.* **11,** 1475 (1983).
19. E. Schreiber, P. Matthias, M. M. Müller, and W. Schaffner, *Nucleic Acids Res.* **17,** 6419 (1989).
20. S. Y. Lin and A. D. Riggs, *Proc. Natl. Acad. Sci. U.S.A.* **71,** 947 (1974).
21. L. A. Chodosh, A. S. Baldwin, R. W. Carther, and P. A. Sharp, *Cell (Cambridge, Mass.)* **53,** 11 (1988).
22. J. T. Kadonaga, *in* "Methods in Enzymology" (R. T. Sauer, ed.), Vol. 208, p. 18. Academic Press, San Diego, 1991.
23. S. Ahmed, J. Rappaport, H. Tada, D. Kerr, and K. Khalili, *J. Biol. Chem.* **265,** 3899 (1990).
24. D. Kerr and K. Khalili, *J. Biol. Chem.* **266,** 15876 (1991).

[10] Analysis of the Nuclear DNA-Binding Activity in Cells Overexpressing Hepatitis B Viral X and 3′ Truncated S Transactivators

Volker Schlüter and Wolfgang H. Caselmann

Introduction

More than 300 million people are chronically infected with the hepatitis B virus (HBV) worldwide, 2.3 million of whom die annually from the sequelae of HBV-associated liver disease (1). Several lines of evidence suggest a causal relationship between chronic HBV infection and the development of hepatocellular carcinoma (HCC): There is a good geographic correlation between the HBV surface antigen (HBsAg) prevalence and the HCC incidence (1). In addition, HBsAg-seropositive patients are at an approximately 100-fold higher risk to develop HCC than HBsAg-negative controls (2). In the animal model immortalized fetal mouse hepatocytes stably transfected with HBV DNA give rise to clones of transformed cells that are tumorigenic in nude mice (3). In nearly all HBsAg-positive HCCs, HBV DNA sequences are chromosomally integrated in the hepatocyte genome. Therefore, genomic integration of HBV DNA is considered a crucial event in hepatocarcinogenesis. Since no preferred genomic site of HBV DNA integration has been identified so far, activation or inactivation of distinct cellular genes by insertion or deletion of viral DNA is not considered a general mechanism of HBV-associated hepatocarcinogenesis (4).

The recent identification of two HBV transactivators, X and S gene products, that stimulate gene expression from a variety of homologous and heterologous regulatory elements provides a fascinating new concept of HBV-associated hepatocyte transformation (5–9). The X protein (pX) comprising at least two functionally important domains (10, 11), was shown to activate indicator gene expression from the β-interferon promoter (5), the c-*myc* promoter (12), the major histocompatibility complex I regulatory elements (13), and different viral long terminal repeats (6, 14). So far, there is no evidence that pX is able to bind to DNA directly (15). Moreover, it was shown that it indirectly exerts its effects via cellular transcription factors such as AP-1, AP-2 (12), and NF-κB (14, 15) as well as ATF/CREB (16). There is experimental evidence that the protein kinase C signaling pathway can be used by the pX transactivator (17). A possible role of the pX transactivator in hepatocarcinogenesis is favored by recent experiments showing that NIH 3T3 cells synthesizing pX are tumorigenic in nude mice (18). This is supported by one report on transgenic mice expressing the *X* gene that developed liver carcinoma with high frequency (19).

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An additional transactivating function has been attributed to viral proteins translated from 3′ truncated HBV pre-S/S sequences (8, 9) that are frequently found to persist in human HCCs (20). Deletion and mutation analysis of the wild-type HBs protein revealed that the truncation of at least 87 C-terminal amino acids is required to generate the transactivating function (21, 22). C-terminal truncation of 115 amino acids of the middle hepatitis B surface protein (MHBs) gives rise to $MHBs^{t167}$, which is used as an example of transactivating HBs proteins. $MHBs^{t167}$ displays a different subcellular localization in comparison to MHBs and was shown to be retained in the endoplasmic reticulum (23). C-terminally truncated MHBs molecules have been reported to transactivate the simian virus 40 early enhancer (9) and the c-*myc* P2 promoter (8), AP-1 (17), and NF-κB–dependent gene expression (23). There is evidence that transactivation is dependent on a prooxidant state of the cell. NF-κB can mediate the transactivating effect, and transactivation by NF-κB can be blocked by the addition of antioxidants. The production of the $MHBs^{t167}$ transactivator may increase the intracellular concentration of reactive oxygen intermediates, which induce oxidative damage and mitogenesis of the cell and play a crucial role in the multistep process of carcinogenesis (23).

In this article we describe a practical approach to further investigate the *trans*-acting potential of pX and $MHBs^{t167}$ that mediate their effects via ubiquitous transcription factors. Since both transactivators are hardly detectable after transient cell transfection, we describe here the construction of recombinant vaccinia viruses producing large amounts of HBV transactivator proteins. The DNA–protein interaction induced by pX and $MHBs^{t167}$ transactivators is analyzed by electrophoretic mobility-shift assays using the binding motifs of specific cellular transcription factors.

Vaccinia Viruses as an Efficient Eukaryotic Expression System

The vaccinia virus expression system has been used for a wide range of applications in the last 10 years. The vaccinia virus that persists within the cytoplasm has a number of useful characteristics: (*a*) The large insertional capacity of up to 20 kb allows the introduction of almost all genes in vaccinia vectors. (*b*) The viruses infect most mammalian and avian cell lines, indicating the broad host range. (*c*) Depending on the promoter used, lower or very high levels of protein synthesis (up to 10% of the total proteins) are possible. (*d*) The complete eukaryotic protein processing and translocation system of the host cells (transport, secretion, glycosylation, myristylation, and phosphorylation) can be used for the study of the features of secreted proteins (24).

Because of these attributes, the vaccinia virus vector system is a favorable system for the synthesis of biologically active proteins and allows the analysis of protein transport and processing inside the cell. In addition, the failure to detect the transactivator proteins after transient cell transfection prompted us to adopt a highly effec-

tive infection strategy for gene expression. Therefore, we decided to use this system for the production and further analysis of the two HBV transactivator proteins MHBs^{t167} and pX.

Construction of Recombinant Vaccinia Viruses

For gene expression in the vaccina virus system, the gene of interest was usually inserted behind a homologous viral promoter such as the early/late 7.5 promoter or the late K11 promoter (24). In addition, a heterologous system for transcription of the gene of interest has been established (25). In this system the expression of the gene of interest is directed by the bacteriophage T7 promoter. The required T7 polymerase is supplied by a T7-recombinant vaccinia virus, vTF7-3, that is used for target cell infection.

For our experiments we used the heterologous expression system. The flow chart in Fig. 1 summarizes the process to generate recombinant vaccinia viruses using the

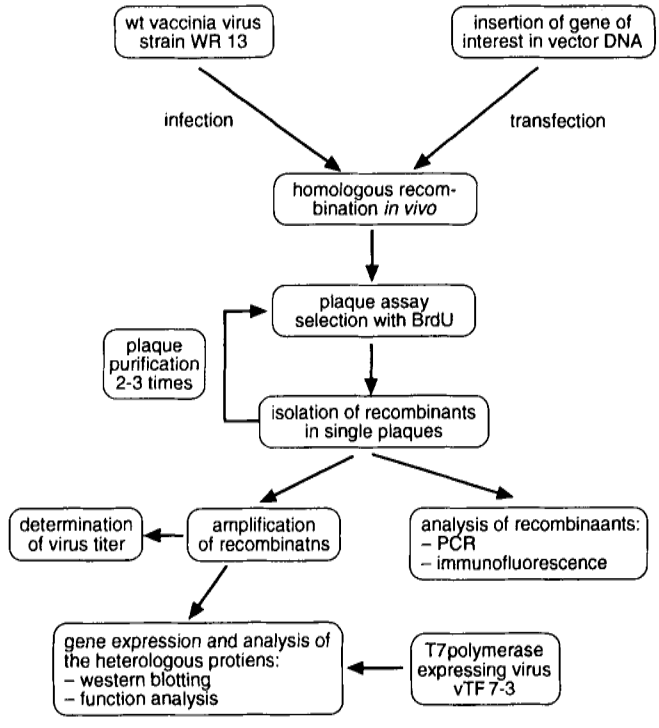

FIG. 1 Flow chart to demonstrate the generation of recombinant vaccinia viruses. wt, Wild-type; BrdU, 5-bromo-2-deoxyuridine; PCR, polymerase chain reaction. See text for details.

wild-type virus strain WR 13 (24) to introduce the gene of interest. The different steps shown in the figure are described below in detail.

Construction of Vaccinia Virus Expression Plasmids Encoding MHBs, MHBs^{t167}, and pX

For the expression using the vaccinina virus/T7 polymerase expression system, full-length pre-S2/S sequences ending at their natural stop codon at position 830, 3′ truncated pre-S2/S sequences lacking all nucleotides downstream of position 486, as well as full-length HBX sequences (nucleotides 1372–1825) were inserted in pTM-1 (26) for gene expression under control of the T7 promoter. The T7 termination sequence the 5′ nontranslated region of the encephalomyocarditis virus, containing a ribosomal binding site that allows a cap-independent translation (27), are contained for an efficient synthesis of the desired protein. The viral thymidine kinase (TK) sequences flanking the expression cassettes are used for homologous recombination *in vivo,* resulting in a viral TK$^-$ phenotype that is helpful for selecting recombinants with 5-bromo-2-deoxyuridine (BrdU; see below). The newly generated plasmids were called pT7S830, pT7S486, and pTMX, respectively (Fig. 2).

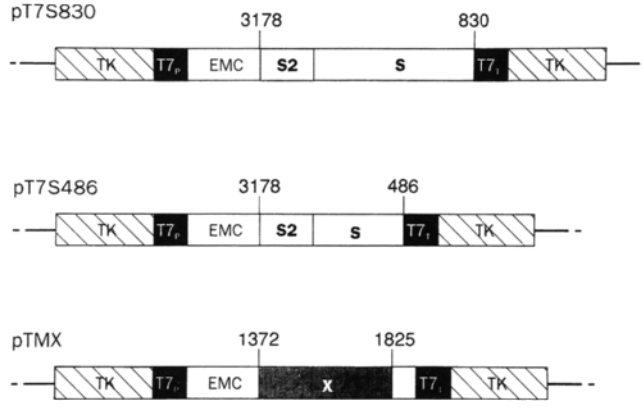

FIG. 2 Linearized plasmid maps of HBV-recombinant vaccinia vectors pT7S830, containing full-length HBV pre-S2/S sequences; pT7S486, comprising the C-terminally truncated HBS gene (nucleotide position 486); and pTMX, covering the complete HBX gene. TK, Thymidine kinase gene; $T7_P$, bacteriophage T7 promoter; EMC, 5′ nontranslated region of the encephalomyocarditis virus, comprising a ribosomal binding site; S2, S, and X, HBV sequences to be expressed; $T7_T$, bacteriophage T7 termination sequence. Numbers reflect the nucleotide position of the C terminus.

Generation of Recombinants

In principle, two different methods can be used to generate recombinant vaccinia viruses: (*a*) Cotransfection of cells with wild-type viral DNA and recombinant plasmid DNA or (*b*) infection with wild-type virus WR 13 followed by a transfection with plasmid DNA. We used the second method for the generation of recombinants.

HeLa [American Type Culture Collection, Rockville, MD (ATCC); Cat. No. CCL 2] or CV-1 (ATCC; Cat. No. CCL 70) cells (1×10^6), seeded in 60-mm (diameter) dishes, were infected with 0.1 plaque-forming units (pfu) of wild-type virus WR 13 in 0.5 ml of Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) fetal calf serum (FCS) and incubated for 2 hr at 37° C. Approximately 20 min before the end of the infection period, 5 μg of plasmid DNA was mixed with 10 μl of Lipofectin (GIBCO–Bethesda Research Laboratories, Heidelberg, Germany) in 0.5 ml of serum-free DMEM. After the incubation the medium containing WR 13 viruses was removed. Cells were intensively washed with phosphate-buffered saline (PBS) three times to remove the FCS. Afterward, cells were covered with the liposome–DNA solution and placed in a CO_2 incubator at 37° C for additional 3 hr. After removal of the liposome–DNA solution, cells were washed again with PBS three times and incubated for at least 2 days. Following the incubation period cells were harvested with PBS plus 10 m*M* EDTA, collected by centrifugation (room temperature, 2000 rpm) and resuspended in DMEM. Cells were lysed with three freeze–thaw cycles in liquid nitrogen, followed by incubation in a 37° C water bath. Cell debris was removed by centrifugation (Biofuge A, Heraens Sepatech, Osterode, Germany; 6000 rpm for 10 min at room temperature). Recombinant viruses contained in the cell lysate were then accessible for purification.

Selection and Purification of Recombinant Viruses

Several different methods for selecting recombinant viruses are available. For example, the coexpression of a bacterial antibiotic resistance gene such as guanine phosphoribosyltransferase (*gpt*) could be used for the selection of recombinants. Commonly used is selection via the viral TK^- phenotype. In pTM-1 the insertion of foreign DNA in sequences of the *TK* gene results in mutation and inactivation of the viral TK. However, an active TK enzyme is necessary to phosphorylate BrdU before it is incorporated into the viral DNA. This causes lethal mutations because of DNA–DNA mismatches and an inhibition of replication. Only the viruses with a TK^- phenotype are able to survive the BrdU addition. For the selection process the use of TK^- cells such as the 143 TK^- cell line (ATCC; Cat. No. CRL 8303) is necessary. 143 TK^- cells (5×10^5) per well were seeded in six-well tissue culture dishes and grown in DMEM plus 2.5% (v/v) FCS overnight. Following the aspiration of the medium, cells were washed with PBS and infected with 10-fold serial dilutions of the cell lysate obtained after the homologous recombination. Useful dilutions are

between 10^{-2} and 10^{-4}. Cells were incubated for 2–3 hr in a 5% CO_2 atmosphere at 37° C. Before the end of the infection period, 2% (w/v) low-melting-point (LMP) agarose was heated in a microwave and then cooled to 45° C. LMP agarose was mixed 1 : 1 (v/v) with 2 × DMEM plus 5% (v/v) FCS incubated in a 45° C water bath. For the selection of recombinants, BrdU was added at a final concentration of 100–200 μg/ml. DMEM was removed and the cells were covered with 3 ml of the LMP agarose–DMEM solution. After the agarose had gelled at room temperature, the dish was incubated for 2 days at 37° C. For visualization of the viral plaques, a second agarose overlay was prepared containing 100 μg of neutral red and equal volumes of 2% (w/v) agarose and 2 × DMEM. Each well was covered with 1 ml of this agarose overlay, and the plates were incubated at 37° C for at least 4 hr or overnight. Distinct well-separated plaques were isolated by picking with a sterile cotton-plugged Pasteur pipette. In order to collect a useful number of virus-containing cells, it is necessary to scrape the monolayer on the bottom of the well. Agarose plaques are aspirated inside the pipette to collect a useful number of virus-containing cells. The agarose plug is transferred in 0.5% (v/v) DMEM plus 2.5% (v/v) FCS and the cells are broken by three cycles of freezing and thawing in liquid nitrogen at 37° C as already described. To exclude the possibility that a mutation results in the viral TK^- phenotype, it is necessary to screen the obtained viruses with polymerase chain reaction or immunofluorescence methods. To obtain a clonal purified recombinant virus, two or three cycles of BrdU selection are necessary.

Virus Amplification and Determination of Virus Titers

The best choice for growing the newly isolated recombinant viruses is to use monolayer cultures such as CV-1 or HeLa cells. These were infected with viruses contained in 0.5 ml of DMEM (titer 1–2 pfu). Two hours after infection, DMEM plus 5% (v/v) FCS was added and the cells were incubated for 2 days in an incubator. After washing with PBS, the cells were harvested with PBS plus 10 m*M* EDTA and spun down. The cells were resuspended in serum-free medium and viruses were harvested by three cycles of freezing and thawing. Concentration of the viral solution is possible by spinning the lysate in a Sorvall SS34 rotor (Sorvall Inc., Newtown, Connecticut, USA) at 15,000 rpm for 30 min. One infected cell will produce 20–200 infectious viruses.

If large amounts of viruses are used for the experiments, spinner cultures (e.g., HeLa S3 cells) may be useful. HeLa spinner cells were collected by centrifugation and were resuspended at a concentration of 5×10^6/ml. Cells were infected with the virus for 1 hr at room temperature. Afterward, cells were diluted with medium to a final concentration of 2×10^6 and incubated for 2 days at 37° C. As described earlier, cells were collected by centrifugation and lysed by several cycles of freezing and thawing in order to obtain high virus titers.

To determine the titer, 5×10^5 CV-1 cells were seeded in six-well tissue culture

dishes. Cells were infected in duplicates of 10^{-6}, 10^{-7}, and 10^{-8} serial dilutions of the virus stock in 0.5 ml of DMEM plus 5% (v/v) FCS. The virus inoculum was removed after 3 hr and the cells were incubated at 37° C for 2 days. After removal of the medium, the cells were washed with PBS and then covered with 0.3% (w/v) crystal violet–0.4% (v/v) ethanol–PBS and incubated for 5 min. The crystal violet solution was removed and the cells were washed twice with PBS. The virus plaques were then visible and easy to count. Multiplication with the dilution factor allows determination of the virus titer.

Infection with Newly Generated Viruses and Protein Analysis

HeLa or liver-derived HepG2 cells (ATCC; Cat. No. HB 8065) were coinfected with 10 pfu of recombinant vaccinia virus vTF7-3, encoding the bacteriophage T7 polymerase (25), and an equal amount of the newly generated virus vT7S830, expressing full-length pre-S2/S sequences, or vT7S486, expressing C-terminal truncated pre-S2/S sequences under the control of the T7 promoter. The virus inoculum was removed 2 hr later, and the cells were washed with PBS and grown overnight in an incubator at 37° C. Using the T7 polymerase system, the highest amount of the heterologous proteins will be obtained if the cells are harvested 16–24 hr after infection. The cells were lysed with 0.5 ml of ice-cold RIPA buffer [150 m*M* NaCl, 1% (v/v) Nonidet P-40 (Sigma, St. Louis, MO), 0.5% (w/v) sodium dodecyl sulfate (SDS), and 50 m*M* Tris–HCl (pH 7.5)] at the dish. After a 10-min incubation at 4° C, the cells were scraped with a rubber policeman and the cell debris was removed by centrifugation (room temperature, 6000 rps). The resulting total cellular protein extracts were separated on a 15% (w/v) SDS–polyacrylamide gel and analyzed by Western blotting (Fig. 3) using the pre-S2–specific monoclonal antibody F124 (28).

As expected from previous results (23), three forms of MHBs, with sizes of 30, 33, and 36 kDa (Fig. 3, lane 4), and two forms of MHBs^{t167}, with sizes of 18.5 and 22 kD (Fig. 3, lane 2), were detectable. The different forms represent the respective unglycosylated and glycosylated forms. To show the correct intracellular protein processing, we added 1 μg/ml *N*-glucosidase inhibitor tunicamycin to the cell culture medium immediately after infection. After this treatment only single bands reflecting the unglycosylated MHBs and MHBs^{t167} proteins (Fig. 3, lanes 3 and 5) were detectable.

Electrophoretic Mobility-Shift Assays as a Test System for DNA–Protein Interaction

Since polymerase II-driven transcription is suppressed 2 hr after infection with vaccinia viruses (29), commonly used chloramphenicol aceyltransferase (CAT) or luci-

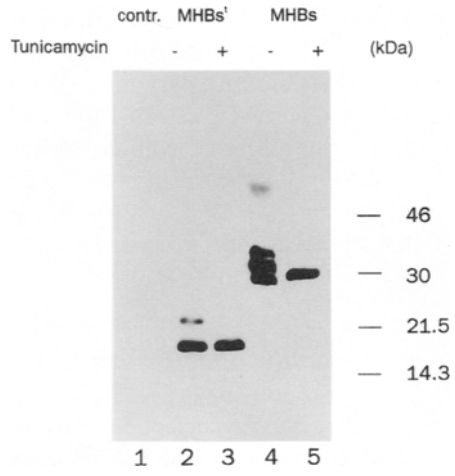

FIG. 3 Western blot analysis of glycosylated (lanes 2 and 4) and unglycosylated (lanes 3 and 5) MHBs and MHBs^{t167} proteins synthesized by recombinant vaccinia viruses using the pre-S2–specific monoclonal antibody F124. + or −, With or without tunicamycin treatment; Contr., protein extract prepared from cells infected exclusively with T7-recombinant vaccinia virus vTF7-3. On the right side, size markers are shown (in kilodaltons).

ferase assays cannot be applied to test the functionality of vaccinia virus-derived HBV transactivators. Therefore, we established electrophoretic mobility-shift analysis (EMSA) as an alternative system to show the effect of the transactivator proteins that are known to exert their *trans*-acting effects via cellular transcription factors such as AP-1 and NF-κB. In EMSA analysis synthetic oligonucleotides encompassing defined transcription factor-binding motifs are incubated with nuclear protein extracts. The attachment of a transcription factor to the corresponding binding sequence results in a retardation of the electrophoretic mobility of the complex as compared to the uncomplexed sample. The comparability of an increased nuclear DNA-binding activity in EMSA induced by pX and MHBs^{t167} and the stimulation of *CAT* reporter gene expression in CAT assays has recently been shown in COS 7 cells (23). In principle, the DNA-binding activity of repressing proteins could be analyzed in the same way.

The experimental conditions for performing EMSA are important. For example, the choice of the cell line influences the results. It depends on the cellular concentration of the analyzed transcription factors. The basal level of AP-1, for example, is very high in HepG2 cells, whereas its concentration is much lower in HeLa cells. Therefore, a further stimulation of AP-1 DNA-binding activity by HBV transactivators is hardly possible, but can be markedly achieved in HeLa cells. If a cell line is used that synthesizes the transcription factor only in low quantities, cotransfection of these cells with an expression vector expressing the desired transcription factor is

necessary to observe an effect. Another critical parameter is the salt condition. Depending on the transcription factor, cofactors such as magnesium (AP-1), potassium (SP1), and zinc (NF-1) are necessary for an excellent DNA binding. For unknown binding factors it is important to establish the best conditions.

Here, we present the EMSA conditions for satisfactory binding of transcription factors AP-1 and NF-κB to their respective recognition sequences. The influence of the HBV transactivators MHBs^{t167} and pX on the nuclear DNA-binding efficiency after infection with recombinant vaccinia viruses or transfection with plasmid DNA is shown.

Generation of Labeled Oligonucleotide Probes

Generally, the labeling of oligonucleotides can be performed either by using the large fragment of DNA polymerase I (the Klenow enzyme) or T4 polynucleotide kinase (PNK). For labeling with the Klenow enzyme, sticky ends of the oligonucleotide are necessary, whereas end labeling with PNK is practicable for all probes. Therefore, we discuss only the latter labeling protocol, using standard methods. For oligonucleotide labeling with PNK, the following components are mixed.

10–30 pmol of double-stranded oligonucleotide
1 μl of 10× kinase buffer
20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Braunschweig, Germany)
1 U of T4 PNK (Pharmacia, Freiburg, Germany)
Water up to a volume of 10 μl

After 1 hr the reaction is stopped by adding 30 μl of stopping buffer (0.1% (w/v) SDS, 0.1% (w/v) bromphenol blue, and 10 m*M* EDTA). The labeled oligonucleotide probe is purified by gel filtration chromatography using Sephadex G-50 (Pharmacia). Normally, we use self-made spin columns, but commonly available purification systems can be used with identical results. The specific activity of the oligonucleotide probes labeled with PNK ranges between 30,000 and 50,000 cpm/pmol. Purification by ethanol precipitation is not useful, because a large amount of non-incorporated nucleotides contaminates the labeled oligonucleotide.

Preparation of Nuclear Extracts from Cell Culture

Nuclear extracts are prepared following a modified protocol described by Dignam *et al.* (30). All steps should be performed on ice and the addition of protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) or aprotinin before each experimental step is important.

HeLa or HepG2 cells are infected with 5–10 pfu of recombinant vaccinia virus

vTF7-3 encoding the T7 bacteriophage polymerase and an equal amount of the respective recombinant virus vT7S486 encoding MHBs^{t167} (see above). The cells are detached with PBS plus 10 m*M* EDTA, collected by centrifugation (Heraeus Omnifuge 2.0RS 10′, 4° C, 1000 rpm) and resuspended in hypotonic buffer A (see below). The cells are incubated on ice for 15–20 min and then lysed with 15–20 strokes using a Dounce homogenizer (Wheaton Scientific, Millsville, New Jersey, USA). Nuclei are spun down by a 3-sec centrifugation (Heraeus Biofuge 13, 4° C, 4000 rpm) and resuspended in 50 μl of hypertonic buffer B and incubated on ice for an additional 15 min. The nuclear extract is separated from the cellular debris and contaminating viruses by centrifugation at 150,000 *g* for 30 min at 4° C. After the addition of 50 μl of buffer C, which results in a decreased NaCl concentration, the nuclear extracts can then be used for the EMSA. For later analysis freezing in liquid nitrogen and storage at −80° C are possible.

Buffer A

10 m*M* HEPES (pH 7.9)
10 m*M* KCl
1.5 m*M* $MgCl_2$
1 m*M* dithiothreitol (DTT)
0.1 m*M* PMSF
2 μg/μl aprotinin

Buffer B

20 m*M* HEPES (pH 7.9)
400 m*M* NaCl
0.2 m*M* EDTA
25% (v/v) glycerol
1 m*M* DTT
0.1 m*M* PMSF
2 μg/μl aprotinin

Buffer C

20 m*M* HEPES (pH 7.9)
0.2 m*M* EDTA
20% (v/v) glycerol
0.25% Nonidet P-40
1 m*M* DTT
0.1 m*M* PMSF
2 μg/μl aprotinin

EMSA

The reactions are performed in a total volume of 20 μl. The following components are mixed for a single reaction.

2 μl of 10× binding buffer [100 m*M* Tris–HCl (pH 7.5), 0.5 m*M* EDTA, and 43% (v/v) glycerol]
1 μg of acetylated bovine serum albumin (Stratagene, Heidelberg, Germany)
1–2 μg of poly(IC) (Boehringer-Mannheim, Mannheim, Germany)
1–5 μg of nuclear extract (cell line dependent)
100–200 m*M* NaCl
50,000 cpm of labeled oligonucleotide
10 m*M* $MgCl_2$ (only for AP-1 shifts)
Water up to a volume of 20 μl

The samples are incubated for 15–20 min at room temperature in the presence of the proteinase inhibitors PMSF and aprotinin. EMSA is performed on a 4.5% (w/v) polyacrylamide gel with an acrylamide–bisacrylamide ratio of 30:0.8 containing 1× running buffer. Electrophoresis is carried out at 20–25 mA for at least 4 hr.

50× Running Buffer

330 m*M* Tris–HCl (pH 7.5)
170 m*M* Sodium acetate (unbuffered)
50 m*M* EDTA (pH 7.5)

As shown in Fig. 4, MHBs^{t167} induces an increased DNA binding of the transcription factors NF-κB (Fig. 4A, lane 5) and AP-1 (Fig. 4B, lane 5) to its recognition sequences as compared to the negative control, in which cells were infected with vTF7-3 alone (Fig. 4A and B, lane 4). As a positive control, we used a nuclear extract prepared from cells that were induced with phorbol-12-myristate 13-acetate (PMA) (Fig. 4A and B, lane 1).

The synthetic oligonucleotides harboring the AP-1 or NF-κB binding motifs (underscored) were

AP-1: 5′–TTCCGGCTGACTCATCAAGCG–3′
3′–AAGGCCGACTGAGTAGTTCGC–5′

NF-κB: 5′–AGTTGAGGGACTTTCCCAGGC–3′
3′–TCAACTCCCCTGAAAGGGTCCG–5′

Specificity of EMSA Experiments

Two different methods can be used to show the specificity of the analyzed protein–DNA interaction. First, EMSA in the presence of a 50- to 100-fold molar excess of unlabeled competitor DNA compared to the labeled oligonucleotide results in the disappearance of the shifted specific band. This competition is shown in Fig. 4, where transcription factor–oligonucleotide complexes induced by MHBs^{t167} or PMA were

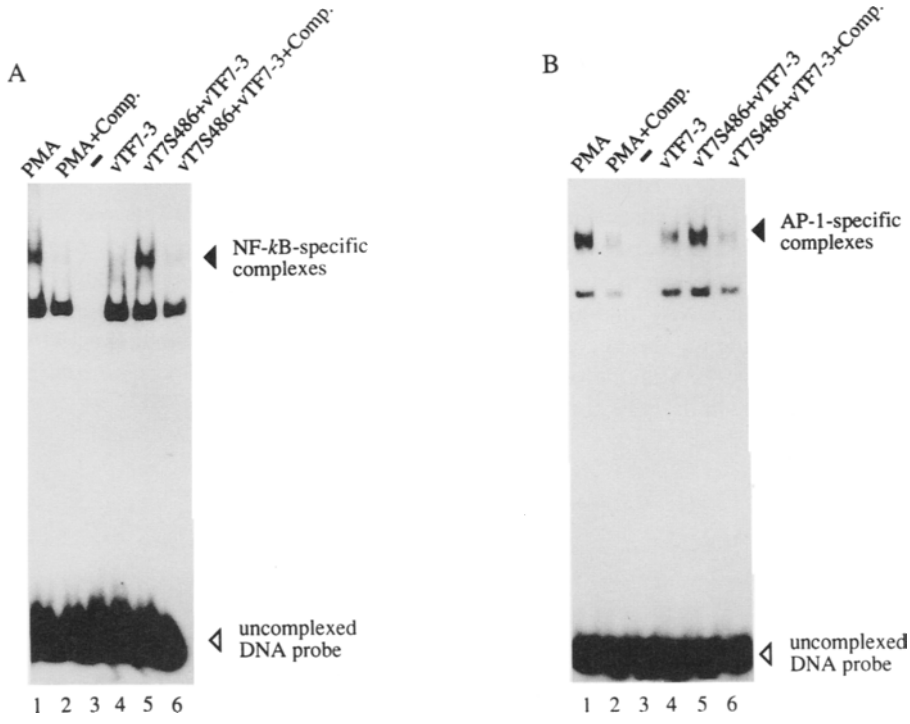

FIG. 4 The effect of MHBs^{t167} on the DNA–binding activity of NF-κB and AP-1. (A) Nuclear extracts prepared from HepG2 cells infected with the indicated recombinant vaccinia viruses vTF7-3 and vT7S486 (lanes 4–6) were analyzed by electrophoretic mobility-shift assays (EMSAs) with a ^{32}P-labeled probe comprising the NF-κB motif. In lanes 1 and 2 cells were treated with 100 ng/ml PMA for 90 min prior to harvesting. A 100-fold molar excess of unlabeled NF-κB oligonucleotide (Comp.) was added to the reactions in lanes 2 and 6. (B) Corresponding EMSA using an oligonucleotide comprising the AP-1 motif. Sections of fluorographs from native gels are shown. Solid arrowheads indicate the positions of specific complexes; open arrowheads, the positions of uncomplexed DNA probe.

investigated (Fig. 4A and B, lanes 2 and 6). Second, the addition of a specific antibody will generate a so-called supershift, since the DNA–protein–antibody complex migrates slower than the DNA–protein complex missing the antibody.

EMSA Using Nuclear Extracts Prepared after Transfection of Recombinant Plasmid DNA

In addition to the described method of double infection, the vaccinia virus system can be used for analysis of the protein–DNA interaction after transient transfection. In our laboratory transfection with liposomes has the best efficiencies.

HepG2 cells (2.5×10^6 cells per 100-mm dish) were infected with 10 pfu of the recombinant virus vTF7-3 encoding the T7 bacteriophage polymerase. After 30 min the cells were washed three times with serum-free DMEM and then incubated with 1 ml of serum-free DMEM containing 20 μl of Lipofectin and 10 μl of plasmid DNA. After incubation for 3–4 hr, the cells were washed twice with PBS and grown at 37° C in 10% (v/v) FCS plus DMEM overnight. Afterward, the cells were collected with PBS plus 10 m*M* EDTA and the mobility-shift analysis was performed essentially as described before.

As shown in Fig. 5, pTMX producing pX protein increases the AP-1–specific nuclear DNA–binding activity (lane 3) as compared to the vector control (lane 2). Using unlabeled competitor DNA, no enhanced DNA–binding activity is detectable (lane 4). Stimulation of the cells with PMA served as a positive control (lane 5) and can similarly be competed out with unlabeled DNA (lane 6).

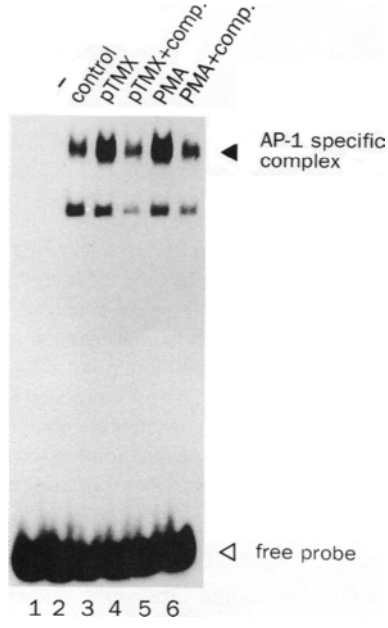

FIG. 5 The effect of pX on the DNA–binding activity of AP-1. Nuclear extracts prepared from HepG2 cells infected with the T7-recombinant vaccinia virus vTF7-3 (lane 2) and subsequently transfected with pTMX (lane 3) were analyzed by electrophoretic mobility-shift assays with a ^{32}P-labeled probe comprising the AP-1 motif. In lanes 5 and 6 cells were treated with 100 ng/ml PMA for 90 min prior to harvesting. A 100-fold molar excess of unlabeled AP-1 oligonucleotide (comp.) was added to the reactions in lanes 4 and 6. –, Free probe alone (lane 1). Sections of fluorographs from native gels are shown. Solid arrowheads indicate the positions of specific complexes and free probe.

Conclusion

The vaccinia virus expression system is a powerful tool to produce large protein amounts by cell infection with the recombinant viruses. The synthesized proteins contain the usual modifications (e.g., glycosyl residues) and are biologically active.

We have used HBV-recombinant vaccinia viruses to establish EMSA as a system to show the functionality of the HBV transactivators MHBs^{t167} and pX. This allows the study of specific DNA–protein interactions between the cellular transcription factors AP-1 and NF-κB, which are known to be induced by HBV transactivators, and their corresponding binding motifs. Both methods, double infection of the cells with HBV- and T7-recombinant vaccinia viruses as well as infection with T7-recombinant virus and transfection with HBV-containing vaccinia vectors, can effectively be used. The detailed experimental conditions have been given.

Acknowledgments

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References

1. W. Szmuness, *Prog. Med. Virol.* **24,** 40 (1978).
2. R. P. Beasley, *Cancer* **61,** 1942 (1988).
3. M. Höhne, S. Schäfer, M. A. Feitelson, D. Paul, and W. H. Gerlich, *EMBO J.* **9,** 1137 (1990).
4. T. Tokino and K. Matsubara, *J. Virol.* **65,** 6761 (1991).
5. J. S. Twu and R. Schloemer, *J. Virol.* **61,** 3448 (1987).
6. P. Zahm, P. H. Hofschneider, and R. Koshy, *Oncogene* **3,** 169 (1988).
7. M. Wollersheim, U. Debelka, and P. H. Hofschneider, *Oncogene* **3,** 545 (1988).
8. A. S. Kekulé, U. Lauer, M. Meyer, W. H. Caselmann, P. H. Hofschneider, and R. Koshy, *Nature (London)* **343,** 457 (1990).
9. W. H. Caselmann, M. Meyer, A. S. Kekulé, U. Lauer, P. H. Hofschneider, and R. Koshy, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2970 (1990).
10. M. Arii, S. Takada, and K. Koike, *Oncogene* **7,** 397 (1992).
11. S. E. Ritter, T. M. Whitten, A. T. Quets, and R. H. Schloemer, *Virology* **182,** 841 (1991).
12. E. Seto, P. J. Mitchell, and T. S. B. Yen, *Nature (London)* **344,** 72 (1990).
13. D.-X Zhou, A. Taraboulos, J.-H. Ou, and T. S. B. Yen, *J. Virol.* **64,** 4025 (1990).
14. J.-S. Twu and W. S. Robinson, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 2046 (1989).
15. A. Siddiqui, S. Jameel, and J. Mapoles, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 2513 (1987).
16. H. F. Maguire, J. P. Hoeffler, and A. Siddiqui, *Science* **252,** 842 (1991).

17. A. S. Kekulé, U. Lauer, L. Weiss, B. Luber, and P. H. Hofschneider, *Nature (London)* **361,** 742 (1993).
18. Y. Shirakata, M. Kawada, Y. Fujiki, H. Sano, M. Oda, K. Yaginuma, M. Kobayashi, and K. Koike, *Jpn. J. Cancer Res.* **80,** 617 (1989).
19. C.-M. Kim, K. Koike, I. Saito, T. Miyamura, and G. Jay, *Nature (London)* **351,** 317 (1991).
20. V. Schlüter, M. Meyer, P. H. Hofschneider, R. Koshy, and W. H. Caselmann, *Oncogene* **9,** 3335 (1994).
21. G. Natoli, M. L. Avantaggiati, C. Balsano, E. de Marizo, D. Collepardo, E. Elfassi, and M. Levrero, *Virology* **187,** 663 (1992).
22. U. Lauer, L. Weiss, P. H. Hofschneider, and A. S. Kekulé, *J. Virol.* **66,** 5284 (1992).
23. M. Meyer, W. H. Caselmann, V. Schlüter, R. Schreck, P. H. Hofschneider, and P. A. Baeuerle, *EMBO J.* **11,** 2991 (1992).
24. M. Macket, L. Smith, and B. Moss, *J. Virol.* **49,** 857 (1984).
25. T. R. Fuerst, E. G. Niles, W. Studier, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8122 (1986).
26. B. Moss, O. Elroy-Stein, T. Mizukami, W. A. Alexander, and T. R. Fuerst, *Nature (London)* **348,** 91 (1990).
27. O. Elroy-Stein, T. R. Fuerst, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6126 (1989).
28. A. Budkowska, M. M. Riottot, P. Dubreuil, Y. Lazizi, C. Brechot, E. Sobczak, M. A. Petiti, and J. Pillot, *J. Med. Virol.* **20,** 111 (1986).
29. C. Puckett and B. Moss, *Cell (Cambridge, Mass.)* **35,** 441 (1983).
30. D. Dignam, R. M. Lebovitz, and R. G. Roeder, *Nucleic Acids Res.* **11,** 1475 (1983).

[11] Investigations on Virus–Host Interactions: An Abortive System

Christiane Zock and Walter Doerfler

Introduction

Viruses are obligatory intracellular parasites with longstanding biological experience and have evolved to propagate efficiently in their host cells. In the course of infection, viral genomes have constantly been subjected to varying demands and selective pressures. Preconditions for a successful viral infection are suitable adaptations of the viruses to their hosts; for example, the viral genomes can incorporate foreign genetic information. These adaptations may lead to limited but optimized virus–host interactions that enable the virus to multiply only in a certain range of hosts.

The outcome of a viral infection depends on a number of factors, including both the characteristics of the virus and the susceptibility of the cells. At opposite ends of a scale of different possibilities lie interactions that eventually cause a productive or abortive infection or the malignant transformation of the host cells. DNA-containing viruses preferentially transform nonpermissive cells when at least a portion of the viral genome persists and continues to function constitutively.

In order to investigate aspects of the molecular mechanisms that are involved in the development of cancer, oncogenic viruses and their interactions with cells have been studied extensively from different points of view.

In general, oncogenesis can be schematically divided into two events. First, a "normal" cell must be converted to a tumor cell; second, this tumor cell must be able to propagate until a tumor is formed in the intact organism. Viral oncologists consider DNA tumor viruses suitable tools to investigate malignant transformation, since the fate of the viral genome and its interaction with the host cell can be analyzed in detail.

The discovery that some serotypes of adenoviruses are able to induce tumors in newborn hamsters (1, 2) or transform hamster cells in culture (3, 4) has highlighted the human adenoviruses as models to investigate DNA viral oncology, particularly because the molecular biology of these viruses has been studied in considerable detail (for recent review see Ref. 5).

Viral oncogenesis is characterized by a cascade of events and complex interactions of virus-encoded or host factors. Accordingly, it has been mandatory to determine the virus–host interactions at early and late stages of viral infection to elucidate the mechanisms involved in malignant transformation.

Our model system to investigate viral transformation is the abortive infection of baby hamster kidney (BHK21) cells with human adenovirus type 12 (Ad12). This system has been analyzed in detail (6–13).

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Abortive Infection of Hamster Cells with Ad12

Ad12 infects human cells productively, and new virions are generated. In contrast, the infection of hamster cells with Ad12 leads to an abortive infection. The Ad12 virions enter the cells, and the viral DNA reaches the nucleus, where it can become integrated into the cellular genome (6, 8, 14–17). Integrated Ad12 sequences can be found as early as 6–16 hr after infection, and the amount of intracellular viral DNA associated with high-molecular-mass cellular DNA can amount to 30% of this intranuclear viral DNA.

Integration of Viral DNA

In adenovirus-transformed or Ad12-induced tumor cells the viral genome is found exclusively in a chromosomally integrated form. It is important to analyze whether and how the integration of viral DNA into the cellular genome is directly or indirectly involved in oncogenesis. Hence, major emphasis has been placed on studying the integration of viral DNA into the host genome and on the analysis of the sites of junction between viral and cellular DNAs (6, 8, 15, 16, 18–30; for reviews see Ref. 17, 31, and 32). The results of the analyses of several junction sites between viral and cellular DNAs in the cells from different species (hamsters, mice, or humans) provide no evidence for a nucleotide sequence-specific integration of viral DNA (17, 32, 33). These data pertain to investigations on the state of Ad12 DNA in cell lines established and maintained for a long time in culture. The possibility, therefore, cannot be ruled out that differences exist between the sites of junction in the initial integration event and the analyzed sites of junction in clonal cell lines. Rearrangements of integrated viral genomes could have occurred between the initial virus infection and the development of a tumor or the establishment and maintenance of stable cell lines. Moreover, we have made the observation that the cellular DNA sequences at the junction sites (preinsertion sequences) in normal cells, prior to contact with viral DNA, have often been transcriptionally active (25, 26, 28, 34). Hence, we propose that recombination between viral (foreign) and cellular DNA occurs frequently at regions with an altered chromatin structure, possibly due to transcriptional activity. Under certain, so far poorly understood, conditions, Ad12-induced tumor cell lines can arise in which the patterns of Ad12 DNA integration appear to be identical (35).

Ad12 genomes are stably integrated into the host genome, and the integration pattern can remain constant over many cell generations (15). Nevertheless, revertant cells have been discovered that have totally or partially lost the integrated adenoviral genomes (36–40). Interestingly, cells subcultured from Ad12-induced hamster tumors that have lost almost all integrated Ad12 sequences upon passage in cell culture remain tumorigenic after injection into newborn hamsters (40). Apparently, integration and continued expression of Ad12 genes in the hamster genome are somehow in-

volved in oncogenesis but are not necessary to maintain the oncogenic phenotype. In this sense events during the initial virus–host interaction, namely, the abortive infection of hamster cells with Ad12, may be one of the preconditions for transformation.

Differential Expression of the Ad12 Genome

In studies to further characterize the abortive interaction of Ad12 with hamster cells, it has been found that in these nonpermissive cells only early viral genes are expressed (41–44). These early Ad12-specific mRNAs have been isolated and can be translated *in vitro* into viral proteins, as evidenced by size comparisons upon electrophoresis of the *in vitro*-translated proteins with those isolated from human cells productively infected with Ad12 (44–46). However, *in vitro* translations programmed with mRNAs isolated from Ad12-infected BHK21 cells lacked a 34-kDa protein, which is evident when mRNAs from productively infected KB cells have been translated (44).

By using antipeptide antibodies in immunoprecipitation analyses of cellular extracts from Ad12-infected human KB cells or hamster BHK21 cells, Ad12 E1A-specific proteins are detectable at 6 hr postinfection in KB cell extracts and not before 12 hr postinfection in BHK21 cell extracts. The levels of E1A proteins detectable in BHK21 cell extracts are only 10–15% of those in KB cell extracts. Moreover, the amount of the E1B 19-kDa protein in BHK21 cell extracts reaches 5–10% of that detected in KB cell extracts (47). In immunoprecipitates of infected KB cell extracts, three forms of the Ad12 E1A proteins are evident after electrophoresis: the 266R, 235R, and 235R* polypeptides (48). In contrast to KB cell extracts, the E1A protein designated 235R* is not demonstrable in BHK21 cell extracts (47) or in an Ad12-transformed hamster cell line (49).

In contrast to the early Ad12 genes, transcription of the late Ad12 genes cannot be detected in nonpermissive hamster BHK21 cells (43, 50, 51), and Ad12 DNA replication is completely blocked in hamster cells (7, 50–53). The inability of Ad12 to proliferate in hamster cells is not a common deficiency of all human adenoviruses, but rather is specific for Ad12, since human Ad2 can efficiently replicate in both human and hamster cells (7, 54). Furthermore, Ad2 is not oncogenic in animals. Accordingly, Ad2 and Ad12 exhibit distinct virus–host interactions in hamster and human cells, which eventually are responsible for the species specificity of the Ad12 genome. Hence, the differential expression of the Ad12 genome in hamster cells may be related to its oncogenic potential. Therefore, it is important to elucidate the regulatory mechanisms that are causative for the species specificity of Ad12.

Absence of Ad12 DNA Replication in Hamster Cells

The adenovirus genome possesses two structural features that are functionally important in viral DNA replication. First, the two ends of the linear genome carry iden-

tical inverted terminal repeats, which vary in length between different serotypes and contain the origin of DNA replication (55–58). Second, a terminal protein (pTP) is covalently attached to each 5′ terminus (59, 60). This protein is essential in the initiation of viral DNA replication (for reviews see Refs. 61 and 62).

Studies with an *in vitro* replication system demonstrated that the initiation of adenoviral DNA replication is dependent on a protein-priming mechanism. This initiation reaction requires pTP to be covalently bound to dCMP at the 5′ terminus and pTP must bind to the DNA polymerase (Ad Pol). After this initiation reaction at either terminus, elongation proceeds from one or each terminus to the opposite one by the addition of nucleotides to the free 3′-OH group of the covalently bound dCMP. Elongation requires Ad Pol and the viral DNA-binding protein (for review see Ref. 62). In addition to viral proteins, efficient initiation and elongation of viral DNA replication require the addition of cellular proteins. These include the transcription factors nuclear factor I (NF-I) (63, 64), which binds to the origin of replication during initiation, and NF-III, which enhances the replication process (65, 66). Moreover, NF-II, which has similarities to topoisomerase I, is necessary for elongation (67). Topoisomerase II also appears to be required for replication in intact cells, but not in cell extracts (68).

It has been demonstrated that crude extracts of BHK21 cells abortively infected with Ad12 support *in vitro* the synthesis of the Ad12 pTP–dCMP initiation complex, but this reaction is 2- to 5-fold less efficient than in extracts of productively infected KB cells (69). As documented earlier (7, 50, 51), there is no evidence that abortively infected BHK21 cells support the elongation reactions of Ad12 DNA replication, and this has also been shown in cell extracts (69). The activity and accumulation of Ad12 Pol are equivalent in infected human and hamster cell extracts. However, the amount of Ad12 DNA-binding protein is markedly decreased in extracts from infected hamster cells compared to infected permissive human cells (70).

Virus-Associated RNA and the Major Late Promoter

Further analyses of the abortive interaction of hamster cells with Ad12 have demonstrated that the Ad12 virus-associated (VA) RNA is not transcribed (71). The Ad2 genome encodes two low-molecular-mass RNAs, designated VA RNAs I and II, at around 30 map units on the viral genome [Fig. 1 (72)] (73–77). Low levels of VA RNAs I and II are expressed during the early phase, while at late times after infection, transcription of VA RNA I is dramatically increased. VA RNA I is required for the efficient translation of viral mRNAs at late times after infection (78–80). Thereby, VA RNA I is involved in the selective translation of viral mRNAs in comparison to cellular mRNAs late after infection (81). By binding to it, VA RNA I inactivates the cellular protein kinase double-stranded RNA-activated inhibitor (DAI), which is induced after viral infection (78–80, 82–86). DAI inhibits the translation by phosphorylation of the translation initiation factor E1F-2 (for reviews see Refs. 85 and 86).

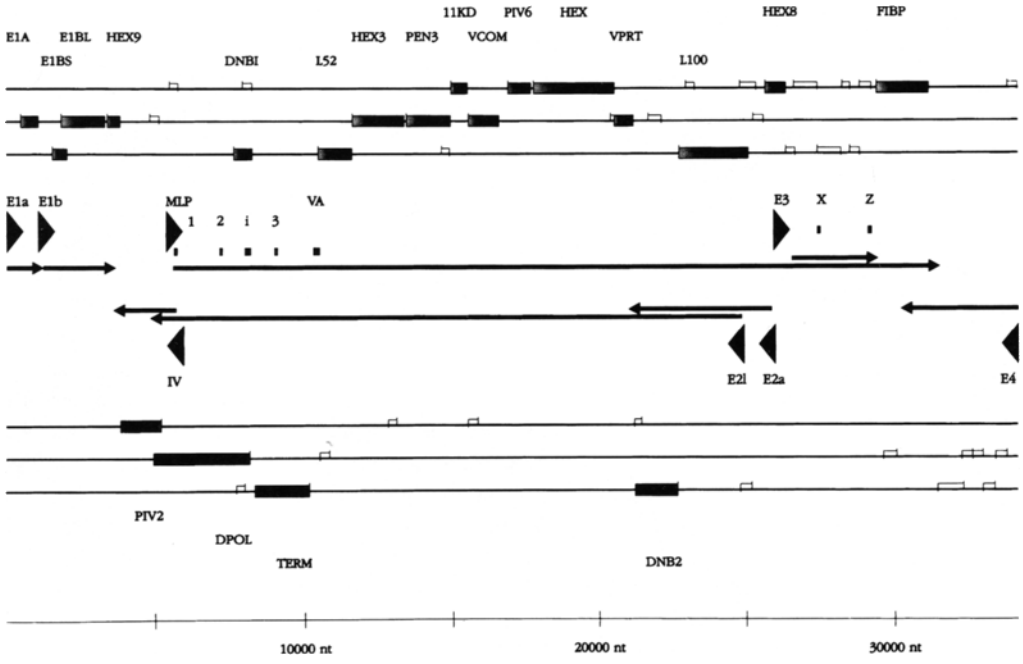


FIG. 1 Organization and expression of the Ad12 genome. Ad12 DNA transcription utilizes promoters conventionally assigned to early (E) and late (L) stages of transcription. Transcription units are depicted as bold arrows and known promoters are represented by triangles. Known open reading frames (ORFs) are presented as shaded boxes, and unknown ORFs are shown as open boxes. For details see the work of Sprengel *et al.* (72).

Another mechanism, which adenoviruses utilize to selectively translate viral mRNAs at late times after infection, involves the tripartite leader sequence, which is present in all late mRNAs originating from the major later promoter (MLP) (87–89) (Fig. 1). This tripartite leader at the 5′ terminus of all late adenoviral mRNAs initiated at the MLP supports their translation (90–93) and confers on mRNAs the ability to be translated without the initiation factor cap-binding protein complex (94–96). Translation of most capped mRNAs requires this complex, which binds cap structures and stimulates protein synthesis (for review see Ref. 97). Adenoviral infection leads to viral-mediated underphosphorylation and subsequent inactivation of cap-binding protein (98). This functional inactivation, therefore, contributes to the discrimination between cellular and MLP-derived late mRNAs.

Among the MLP-derived late mRNAs, the L1 family is of particular interest because the Ad2 L1 segment is the only late transcription unit active at both early and late times of infection (99–101). Moreover, the L1 region encodes several proteins important for the assembly of infectious virus particles (102, 103). The L1 52- or 55-

kDa protein species are present in all known virus assembly structures. This finding is consistent with a role in virus assembly. These proteins are found within empty capsids, assembly intermediates, and young virions, but not within mature virions (102, 103). Accordingly, it is thought that the L1 52- or 55-kDa proteins perform a scaffolding function during virion assembly. In this sense it is interesting that the Ad12 L1 region is transcribed neither at early nor at late times after the infection of hamster cells (71).

Partial Complementation of Ad12 Defects in Hamster Cells by Ad2 or Ad5 Functions

Since Ad2 and Ad5, which are both nononcogenic in newborn hamsters, can efficiently replicate in hamster cells (7, 54, 104), the question arises as to whether Ad2 or Ad5 (Ad2/5) functions can complement some of the deficiences of the Ad12 genome in hamster cells. It is conceivable that some of the Ad12-specific functions are insufficient to support viral replication in hamster cells. Additionally, cellular functions essential for Ad12 replication may be missing. In contrast, Ad2/5-specific functions are able to interact properly with the hamster cellular factors in support of viral DNA replication.

To identify factors necessary for Ad12 DNA replication in hamster cells, the cells have been doubly infected with Ad2/5 and Ad12. Subsequently, viral DNA replication and gene expression have been examined. In double-infected BHK21 cells Ad2/5 and Ad12 DNAs replicate, and late Ad12 genes are transcribed (50, 51). Ad2/5 virions are assembled, but the synthesis of Ad12 virions cannot be detected (50, 51).

In further experiments the Ad2/5 functions sufficient to rescue Ad12 DNA replication and late Ad12 gene transcription in hamster cells have been localized to the Ad2/5 E1 region (50, 51). In these experiments the Ad2-transformed hamster cell lines HE1, HE3, HE5, and HE7 (105, 106) or the Ad5-transformed hamster cell line BHK297–C131 (107) have been superinfected with Ad12. The BHK297–C131 cell line contains the left ~18% of the Ad5 genome, the E1 region, integrated into the cellular genome and constitutively expresses this region. Upon Ad12 infection of these cell lines, Ad12 DNA replicates and late Ad12 functions are transcribed (50, 51). Moreover, Ad12-infected BHK297–C131 cells elicit the transcription of the VA and L1 regions of Ad12 DNA (71). Again, newly assembled Ad12 virions have not been detected in these systems (50, 51).

The results of double-infection experiments using Ad12 and E1A (*dl312*) or E1B (*dl313*) deletion mutants of Ad5 (108–110) have demonstrated that the complementing functions in the Ad5 genome reside predominantly, but not exclusively, in the adenoviral E1B region. Nevertheless, a supporting role of the E1A region cannot be excluded (50, 51). BHK21 cell lines that have been stably transformed with either the E1A or E1B segment of Ad2 DNA and constitutively express E1A or E1B func-

tions are not capable of complementing the defects of Ad12 DNA replication or late gene transcription (R. Jüttermann, T. Klimkait, and W. Doerfler, unpublished observations). Conceivably, the combined action of early Ad2/5 functions is essential for the complementation of Ad12 defects in hamster cells. The complementation functions between Ad2/5 and Ad12 appear, to some extent, to be mutual. As mentioned already, Ad2/5 functions are able to rescue some of the Ad12 defects in hamster cells. Conversely, coinfection of hamster cells with Ad12 and the Ad5 deletion mutant *dl312* (E1A) or *dl313* (E1B) enabled the Ad5 deletion mutants to replicate in spite of their defects (51). In the absence of Ad12 functions, these mutants cannot replicate in hamster cells. Somehow, the Ad12 early functions, although by themselves incapable of supporting the replication and late gene transcription of Ad12 DNA in hamster cells, can substitute for the deletion in Ad5 DNA, which is then capable of replication and the complete transcriptional/translational program.

In a comparable experiment a recombinant adenovirus has been constructed that contained mainly Ad5 DNA sequences, except for the E1 region, which was derived from Ad12 DNA (111). This recombinant virus has been able to replicate in BHK21 cells but has failed to induce tumors upon injection into newborn hamsters. These results render it likely that the Ad12 defects in hamster cells are due to a failure of Ad12 functions to interact correctly with the hamster cellular environment in promoting Ad12 multiplication. However, the E1 functions of Ad12 DNA are capable of substituting for the E1 functions of Ad2/5 DNA in hamster cells to support Ad2/5 multiplication.

The failure of Ad12 virions to assemble in Ad12-infected hamster cells complemented by Ad2/5 functions can be explained by the finding that, although late Ad12 functions are transcribed, late Ad12 structural proteins are not synthesized. However, late Ad2/5 structural proteins are synthesized at normal levels (51, 111a). Obviously, the late Ad12 RNAs, which can be transcribed in the presence of complementing Ad2/5 functions in hamster cells, cannot be translated into proteins. The late Ad12 RNAs synthesized in the complementing system are polyadenylated but are produced in reduced amounts in comparison to the productive system. For example, the late Ad12 RNA derived from the Ad12 fiber gene has been investigated for its structural properties. This RNA exhibits the authentic nucleotide sequence of the Ad12 fiber gene and the complete tripartite leader sequence. Nevertheless, this RNA is not translated into fiber protein, even in the complementing hamster cell system. Cellular factors needed to promote late Ad12 RNA translation might be lacking or inhibitory factors might be present in hamster cells (111a).

It is likely that the interaction of late Ad2/5 and late Ad12 transcripts with hamster cell factors is quite specific and can somehow lead to the selective translation of the Ad2/5 late viral RNAs, but fails to support translation of the late Ad12 RNAs. It is tempting to speculate that cellular structures also participate in the selective translation of Ad2/5 late RNAs, in contrast to late Ad12 RNAs. It has been shown that in Ad2 and Ad12 doubly infected BHK21 cells or in Ad12-infected BHK297–C131

cells, the Ad12 VA RNA is transcribed (71). It remains to be determined whether this transcribed Ad12 VA RNA is biologically active. The biological function of the transcribed Ad12 VA RNA in the complemented system could be tested with an Ad5 recombinant virus, which carries a deletion in the Ad5 VA RNAs. It is unknown whether the Ad12 VA RNA would be capable of complementing the deletion in the VA RNA of the Ad5 genome.

Obviously, the deficiency of the Ad12 genome in hamster cells is complex and involves a multistage mechanism in which only some processes can be rescued by Ad2/5 functions. It will be interesting to elucidate the additional defects that cannot be complemented by Ad2/5 functions, since they might be intimately related to the transforming ability of Ad12.

Species Specificity of the MLP of Ad12 DNA

The adenovirus infectious cycle proceeds in a well-defined temporally regulated manner. At the time of onset of viral DNA replication, there is a switch from the early to the late phase. This transition affects many aspects of adenovirus gene expression, and the early and late expression patterns are clearly different. It is unknown how the early-to-late switch is controlled or by what mechanism DNA replication is linked to late gene expression. Attempts to elucidate this linkage have shown that the early-to-late transition is dependent on the state of the viral genome (112, 113). Virus genomes that have not undergone DNA replication cannot serve as templates for late gene expression, not even when superinfected into virus-infected cells that are already in the late phase. Continued DNA synthesis is not required, since production of late mRNAs can proceed in cells with DNA replication blocked after it has been initiated. Hence, DNA replication seems to lead to some kind of alteration in the structure of the viral templates.

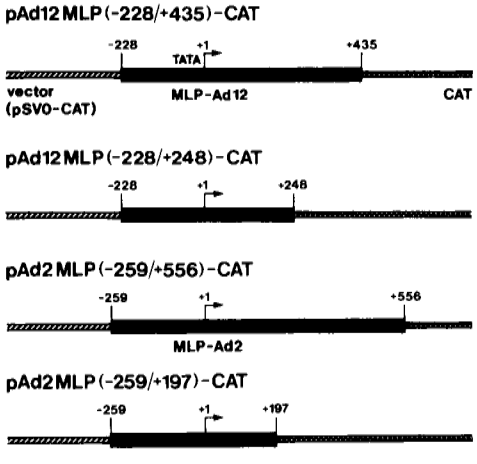
In the regulation of the early-to-late transition of the viral life cycle, several early viral gene products seem to be involved in the accumulation of late cytoplasmic messages derived from the MLP. Possible candidates as regulatory factors are the E1B 55-kDa and the E4 34-kDa [open reading frame (ORF) 6] proteins, which form a physical complex, and the E4 14-kDa (ORF3) protein (114–122).

The finding that the Ad2 L1 region, which is initiated at the MLP, is expressed during the productive infection at early and late times of infection suggests that the MLP might also be involved in the early-to-late transition (100, 101, 123, 124).

Since the early-to-late transition cannot proceed in Ad12-infected hamster cells, and late Ad12 functions that are nearly exclusively initiated at the MLP are therefore not transcribed, a detailed analysis of the Ad12 MLP has been initiated in our laboratory. The activity of the Ad12 MLP has been investigated in human and hamster cells. Studies on the Ad2 MLP have served as a positive control, since Ad2 can efficiently propagate in both human and hamster cells.

In these experiments the block of late Ad12 gene transcription in hamster cells can be explained by the species-specific insufficiency of the Ad12 MLP in hamster cells (12, 13, 125). In order to determine the Ad12 MLP sequences responsible for its species-specific expression, we have fused various segments of the Ad2 or Ad12 MLP to the prokaryotic gene for chloramphenicol acetyltransferase (CAT) as an indicator of gene activity and determined the activities of these promoter–CAT constructs in transient expression studies in uninfected or in Ad2- or Ad12-infected human HeLa or hamster BHK21 cells (Table I). Each two Ad2 or Ad12 MLP constructs have been used that carry either Ad2 MLP sequences comprising nucleotides −259 to +556 or −259 to +197, or Ad12 sequences between nucleotides −228 to +435 or −228 to +248 (Table I). The nucleotide numbers refer to MLP sequences counting from the transcription initiation site at +1. The shorter MLP constructs carry a deletion in the downstream promoter region corresponding to the first intron of the late viral genes in the Ad2 genome. The two Ad2 MLP constructs are transcribed with comparable efficiencies in both human and hamster cells, although the levels of MLP expression are enhanced in Ad2- or Ad12-infected HeLa or BHK21 cells. This finding is not surprising, since the MLP is a late promoter that attains maximal ac-

TABLE I Activities of the Ad2 or Ad12 Major Late Promoter ins Uninfected or Infected BHK21 or HeLa Cells[a]



BHK21			HeLa		
uninf.	Ad2 inf.	Ad12 inf.	uninf.	Ad2 inf.	Ad12 inf.
1.7 1.4 0.9 0.7 0.6	2.9 2.5 4.2 3.8	1.7 0.8 1.6 1 1.1 0.5	1.4 1.2 1.2	1.3 0.4 3.1	7.6 5.2 3.5
52 41.3 5.9 4.3 5.7	62 38.7 83.8 49.7	30 27 42.5 26.7 39.3 35.3	6.8 5.7 5	59.7 36.1 47.5	87.1 37.5 47.1
14 2.8 2.1 4.5 6.7	8.1 7.7 16.7 15	13.2 4 8.5 10.1 38.8 3.8	1.9 2.8 7.7	7 6 1	7.9 11.6 18.8
19 7.1 1.9 2.1 2.9	18 23.4 23.8 7.3	3.3 7.7 8.8 7.1 14.1 3.7	2.3 1.6 3	20.8 2.4 12	29.8 11.9 10.9

[a] Biological activities of two Ad12 major late promoter–chloramphenicol acetyltransferase (MLP–CAT) and two Ad2 MLP–CAT constructs in uninfected or Ad2- or Ad12-infected BHK21 or HeLa cells are presented. Numbers refer to the percentages of conversion of ^{14}C-labeled chloramphenicol (CAM) to acetylated forms of CAM by the CAT activities in extracts from cells transfected with the different constructs. The results of several independent experiments are included.

tivity late after infection and requires additional viral and cellular factors for activity (Table I). Since the Ad2 MLP is active in Ad12-infected human or hamster cells, early Ad12 functions are capable of transactivating this promoter in both cell types (Table I). Moreover, the deletion introduced into the downstream region of the Ad2 MLP has no effect on its expression in either host. In transfection experiments using the two Ad12 MLP constructs, the pAd12MLP(−228/+435)–CAT plasmid is silent in uninfected HeLa or BHK21 cells. This construct can be activated in hamster cells by Ad2 functions but not by Ad12 functions, whereas in human cells it can be activated by both of these functions (Table I) (Refs. 12 and 125). In these transfection experiments the pAd12MLP(−228/+435)–CAT construct thus seems to mimic the deficiency of the whole Ad12 genome in hamster cells.

It has been shown (125) that E1 functions of Ad2 are sufficient to provide auxiliary functions for Ad12 MLP activity even in hamster cells and help to overcome the species-specific suppression of the Ad12 MLP in hamster cells. In contrast, Ad12-infected hamster cells are able to promote only Ad2 MLP activity, but cannot activate the Ad12 MLP. Obviously, the Ad12 MLP can differentiate between auxiliary cellular functions of different species.

Transfection experiments with the pAd12MLP(−228/+248)–CAT plasmid, in which Ad12 sequences between +249 and +435, presumably in the first intron of the late Ad12 functions, have been deleted, lead to a very different result. This MLP–CAT construct does have activity in uninfected hamster and human cells. The species-specific suppression of the Ad12 MLP in hamster cells is thus lost when Ad12 sequences between +249 and +435 are deleted. For this Ad12 MLP construct, additional Ad2 factors are not required to rescue Ad12 MLP activity in hamster cells. Even in human cells, the deletion of the downstream Ad12 MLP segment leads to a drastic increase in MLP expression when compared to the pAd12MLP(−228/+435)–CAT construct (12). Again, expression of the MLP is enhanced in Ad2- or Ad12-infected cells.

The results of these transfection experiments argue for the existence of two modes in which the silent Ad12 MLP can be activated in hamster cells. One possibility is the transactivation of the Ad12 MLP by the Ad2 E1A protein (13, 125). This transactivation can occur in the presence of the Ad12 sequence between nucleotides +249 and +435. The target for the transactivating E1A protein in the Ad12 MLP is not known. The second mechanism to restore Ad12 MLP activity in hamster cells depends on the deletion of the Ad12 segment between nucleotides +249 and +435. It is unknown whether both mechanisms to activate the Ad12 MLP in hamster cells are interrelated.

Ad12 MLP Contains a Mitigator Element

It has been shown that Ad12 sequences in the MLP between nucleotides +249 and +435 are involved in the transcriptional block of this promoter in hamster cells. The

late transcriptional defect of Ad12 DNA in hamster cells has thus, at least partially, been localized to this late promoter segment. We have set out to characterize this inhibitory sequence in the Ad12 MLP in more detail. Several constructs have been generated with the *Bal*31 exonuclease that carry various deletions in the downstream region of the MLP of Ad12 DNA. The results of transient expression experiments with these MLP assemblies suggest that the minimal promoter element sufficient to mitigate or inhibit promoter function is located between nucleotides +320 and +352 in the Ad12 MLP. In contrast, the Ad2 MLP lacks a comparable mitigator element (12). These results provide evidence for the existence of a mitigator element in the first intron following the Ad12 MLP. In silencing the late viral genes, this mitigator contributes to the abortive infection of Ad12 in nonpermissive hamster cells.

Molecular Mechanism of Mitigator Function

The mitigator element in the downstream region of the Ad12 MLP between nucleotides +320 and +352 confers species-specific expression on this promoter. The mitigator shuts off the expression of this promoter in hamster cells and down-regulates it in permissive human cells (12). We have been interested in the molecular mechanism by which this DNA sequence differentiates between the species-specific milieu of human and hamster cells.

The transcriptional regulation of genes can be affected at different levels. It has therefore been necessary to determine, first, where in the cascade of transcriptionally activating elements in the Ad12 MLP the mitigator acts. Two methods with different sensitivities have been applied. The results of nuclear run-on and RNase protection experiments have provided no evidence for the presence of any transcripts initiated at the Ad12 MLP in Ad12-infected or Ad12-transformed hamster cells. Accordingly, transcription initiation and elongation of late Ad12 genes are completely blocked in Ad12-infected hamster cells (13). Apparently, the mitigator in the Ad12 MLP acts at the level of transcription initiation. As in the transient expression studies, the activity of the Ad12 MLP can be restored in nuclei isolated from hamster cells by the E1 functions of Ad2/5. These E1 functions can overcome the mitigator-mediated block in transcription initiation of the Ad12 MLP (13).

Addressing the question of how the mitigator in the downstream region of the Ad12 MLP inhibits the initiation of transcription, we have first investigated the possibility that proteins might be involved. Nucleotide sequence comparisons between the Ad12 mitigator between nucleotides +320 and +352 and the corresponding sequence in the Ad2 MLP have revealed a difference in 10 positions (underscored in Fig. 2). Nevertheless, nuclear proteins from human HeLa or hamster BHK21 cells are able to interact with either sequence and show similar migration behavior in electrophoretic mobility-shift assays (EMSAs) (13). Of course, this assay is not very sensitive and may not reveal minor functionally significant differences in protein–mitigator interactions.

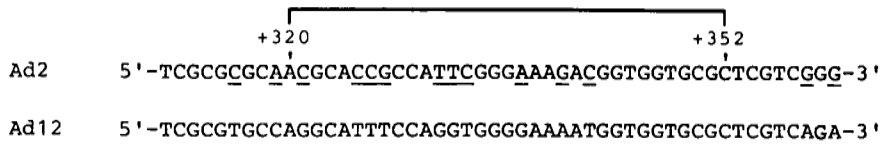


FIG. 2 Nucleotide sequences in the Ad12 mitigator (between nucleotides +320 and +352), the surrounding region, and the corresponding Ad2 major late promoter sequence. Nucleotides different between the Ad2 and Ad12 promoters are underscored in the Ad2 sequence.

In a gene bank search we noted that the two viral MLP sequences bear a certain similarity to the protein-binding sequence of the amphifunctional YY1 protein (Fig. 3). This cellular protein binds to the P5 promoter in the adeno-associated virus (AAV) genome and was originally isolated from HeLa cells (126, 127). The binding of YY1 protein to its cognate sites in the P5 promoter in AAV DNA mediates the transcriptional repression of this promoter. Interestingly, the adenovirus E1A protein relieves this repression by the YY1 protein and, beyond this, stimulates transcription through the YY1 binding site. Thus, the YY1 protein is thought to be a transcriptional repressor that can be converted to a transcriptional activator in the presence of the Ad5 E1A protein (126–128). YY1-related proteins are not only involved in the regulation of the AAV P5 promoter but also in a number of other viral and cellular promoters (129–134). Because of their widespread occurrence and functional pliability, YY1-related proteins may play a role in the expression of many genes and may be involved in the maintenance of viral latency.

In nonpermissive hamster cells the authentic mitigator-containing the Ad12 MLP can only be activated in the presence of E1 functions of Ad2/5. In this case the activity of the repressed Ad12 promoter can be restored by Ad2/5 E1A functions. Since the YY1 protein is also responsive to the adenoviral E1A protein, we have reasoned that the YY1 protein may be involved in the regulation of the Ad12 MLP.

By testing the binding ability of purified YY1 protein in EMSAs, we have demonstrated that YY1 protein binds not only to the originally found YY1-binding sequence in the AAV P5 promoter, but also to the Ad12 mitigator and the correspond-

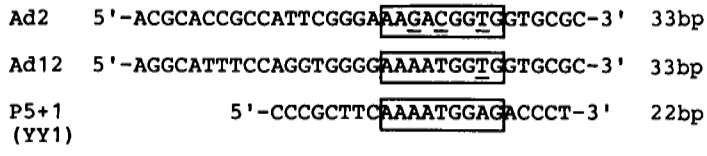


FIG. 3 Comparison of the YY1 protein binding sequence from the P5 promoter of AAV DNA, the Ad12 mitigator sequence, and the corresponding sequence in the Ad2 MLP. The YY1 protein binding site is boxed, and nucleotide sequence differences are underscored in the Ad2 and Ad12 DNA sequences.

ing sequence in the Ad2 MLP. When the same experiments have been performed with crude nuclear extracts, these nucleoprotein complexes have shown migration behavior similar to that of the nucleoprotein complexes formed with the purified YY1 protein. Perhaps YY1-related cellular proteins can bind in a similar fashion to both the Ad12 mitigator and the corresponding Ad2 sequences (13).

Cell-Free Transcription System Proves Unresponsive to the Ad12 Mitigator

As mentioned previously, the authentic Ad12 MLP containing the mitigator sequence is not functional in hamster cells or in isolated hamster nuclei. Moreover, differences in protein binding between the Ad12 mitigator and the corresponding Ad2 sequence with nuclear extracts from human or hamster cells have not been detected. Are the proteins interacting with the two viral sequences functionally distinct? We have performed cell-free transcription experiments using nuclear extracts from HeLa or BHK21 cells. In the course of these experiments, we have tried to transcribe the mitigator-containing Ad12 MLP or the Ad12 MLP devoid of the mitigator element. Surprisingly, either Ad12 MLP–CAT construct is transcribed with comparable efficiencies in extracts both from permissive HeLa and from nonpermissive BHK21 cells (13). Apparently, the Ad12 mitigator element can be recognized only in intact cells or in isolated nuclei, but not after the disruption of the nuclear topology. It is conceivable that additional nuclear components necessary for recognizing the mitigator element in hamster cells have remained inside the nucleus or have been lost during extraction with 0.42 *M* NaCl. On the other hand, it is also possible that nuclear structures and/or the arrangement of Ad12 DNA in the hamster cell nuclei is essential in eliciting the species-specific mitigator function of the Ad12 MLP.

Mitigator Element Works in Conjunction with Additional Signals

Since the mitigator element does not function in cell-free transcription, we have tried to dissect the mechanism of mitigator activity in transient expression experiments with several different Ad12 and Ad2 and promoter constructs.

By using the method of site-directed mutagenesis, the mitigator element in the Ad12 MLP has been converted to the equivalent sequence in the Ad2 MLP. This mutant Ad12 MLP construct is still inactive in hamster cells (13), although the authentic Ad2 MLP sequence, from which the mutant sequence in the Ad12 MLP has been copied, is active in these cells. Apparently, the authentic Ad2 MLP sequence can functionally substitute for the mitigator element in the Ad12 MLP.

Additionally, we have placed the Ad12 mitigator element downstream of the Ad2 MLP, which itself does not contain an inhibitory element. With this experiment we have addressed the question of whether the Ad12 mitigator was sufficient to affect

the function of the Ad2 MLP. However, the hybrid promoter is unaffected by the Ad12 mitigator in its activity in hamster cells compared to the expression of the authentic Ad2 MLP. The results of these experiments demonstrate that the mitigator itself is not sufficient to account for the deficient Ad12 MLP, but seems to depend on additional promoter sites in the Ad12 MLP. These additional promoter sites may not be present or may not be used in the Ad2 MLP, since the Ad12 mitigator does not function in an Ad2 MLP environment. Nevertheless, the mitigator element appears to be the crucial site in the Ad12 MLP to effect the inhibition in hamster cells, since its deletion results in a very strong increase in promoter function (Table I).

We speculate that the mitigator may function through the combined action of several sites in the Ad12 MLP. The possibility has been tested whether upstream sequences in the Ad12 MLP are involved in the mitigation process. Accordingly, we have used several Ad12 MLP–CAT constructs with deletions of varying lengths in their 5′ upstream promoter regions in transient expression experiments. These constructs are marginally activated in BHK21 cells, but can function well in the Ad12-transformed hamster cell line T637 (13). This cell line has been described earlier (135). These results suggest that the 5′ region in the Ad12 MLP contains sequences whose removal permits the Ad12 MLP to function in T637 hamster cells to a very limited extent in the presence of the mitigator element. Since this activation is most apparent in the Ad12-transformed cell line T637, it is conceivable that cellular functions, which are turned on in T637 but not in BHK21 cells, are responsible for the activation of the Ad12 MLP.

The results of transient expression experiments using the modified Ad2 or Ad12 MLP–CAT constructs permit one to conclude that upstream and downstream promoter sequences in the Ad12 MLP must act jointly in turning this promoter off in hamster cells. The possibility that proteins are involved in the concerted action of upstream and downstream promoter sites has been tested. We have shown that the two promoter regions in the Ad12 MLP bind similar cellular proteins in EMSAs. The cellular YY1 protein seems to bind both to downstream (+216 to +435) and upstream (− 228 to − 16) sequences in the Ad12 promoter. The results of competition experiments indicate that the cellular proteins from crude nuclear extracts binding to these Ad12 sequences might be related to the YY1 protein (13). The question remains as to whether the cellular YY1 protein exerts a crucial function in the regulation of the Ad12 MLP.

Conclusions

The authentic MLP of Ad12 DNA containing nucleotides − 228 to +435 relative to its cap site does not function in uninfected or Ad12-infected hamster BHK21 cells (12, 13, 125). We have also demonstrated that the transcription initiation of the Ad12 MLP is inhibited in BHK21 cells (13). Accordingly, the transcriptional defect in the late genes of Ad12 DNA can be explained in part by the deficiency of the viral MLP

in hamster cells. Furthermore, we have been able to show that the sequence between nucleotides +249 and +435 in the Ad12 MLP, which comprises a mitigator element in the sequence between nucleotides +320 and +352, is somehow responsible for the late transcriptional block of this promoter in hamster cells. Because the Ad2 MLP does not contain such an element (12), the mitigator seems to be specific for the Ad12 MLP and essentially contributes to the abortive infection of Ad12 in hamster cells. The mitigator is thus involved in the silencing of a major part of the viral genome, that is, the ensemble of all the late viral genes, which encode the viral structural proteins necessary for the assembly of new virions. This shutdown of major viral replicative functions may be a precondition for the malignant transformation of hamster cells by Ad12.

In attempts to elucidate the molecular mechanism of the mitigator element, we have observed that the authentic Ad12 MLP can function in cell-free transcription experiments with hamster cell nuclear extracts. In these experiments Ad12 MLP–CAT constructs devoid of the mitigator element are equally well transcribed in hamster cell extracts as Ad12 MLP–CAT constructs containing the mitigator. Apparently, intact nuclear topology and/or nuclear factors that cannot be extracted at moderate salt conditions are essential for successful interactions of cellular mechanisms with several Ad12 promoter sites. Conceivably, a certain state of Ad12 DNA in the nucleus may be necessary to facilitate late Ad12 gene transcription. Since the mitigator-containing Ad12 MLP–CAT construct, which is silent in intact hamster cells, can be transcribed under cell-free conditions, separation of the Ad12 genome from the constraints of its interactions with nuclear structures and/or with factors tightly bound in the nucleus abolishes the block of late Ad12 genome transcription. In a cell-free system with nuclear extracts, the relevant signals in the Ad12 MLP might not be recognized, and thus, the late Ad12 genes can be transcribed in nuclear extracts of hamster cells. Clearly, conditions existing in intact cells and the multitude of possible promoter interactions cannot be appropriately imitated by *in vitro* experiments in cell-free extracts.

Our current model to explain the function of the mitigator element implies the concerted action of several Ad12 MLP sites mediated through cellular factors in the inhibition of the Ad12 MLP in hamster cells. Even for permissive human cells, our results argue for a reduced activity of the Ad12 MLP containing the mitigator compared to the promoter construct devoid of this sequence (Table I). Perhaps the mitigator also contributes to the relatively low efficiency of Ad12 in productively infected human cells.

We submit that the Ad12 MLP, and perhaps major parts of the Ad12 genome, must assume a particular configuration in the nuclei of cells in order to attain optimal functionality, particularly of its late genes. This conformational requirement for Ad12 DNA is met in human cells but not in hamster cells. How does the Ad12 mitigator and other elements in the Ad12 MLP interfere with this conformational precondition that can be met by the Ad2 MLP?

This model would also account for the involvement of cellular proteins including

those similar or related to the cellular YY1 protein (126, 127). This cellular YY1 protein is involved in the regulation of the P5 promoter of the AAV (127) and is thought to be involved in the maintenance of viral latency. The efficient replication of human AAV can be achieved only in the presence of a helper virus (136), such as adenovirus or herpesvirus (137). One of the helper functions provided by adenovirus is encoded in the E1A region (138–141). The finding that the AAV P5 promoter is repressed by the cellular YY1 protein in the absence of adenoviral protein and activated by YY1 in the presence of the E1A protein (126–128) alludes to an involvement of the YY1 protein in the latency of AAV. Because we have evidence that YY1-related proteins participate in the repression of the Ad12 MLP in hamster cells and that this repression can be overcome by the adenoviral E1A protein, it will be necessary to elucidate the role of the YY1 protein in the regulation of the Ad12 MLP as well as its role in the abortive infection of hamster cells with Ad12.

Appendix: Techniques Used in These Investigations

Cells, Viruses, and Virus Infections

Human HeLa, hamster BHK21 (142), hamster T637 (135), and hamster BHK297–C131 cells (107) were grown in monolayer cultures in Dulbecco medium (143) supplemented with 10% (v/v) fetal bovine serum. Human Ad2 or Ad12 was propagated and purified as described previously (7). Extraction of viral DNA from CsCl-purified virions was performed with the sodium dodecyl sulfate (SDS)–proteinase K–phenol method (144). For infections, BHK21 cells were inoculated at 80–100 plaque-forming units (pfu) of Ad12 per cell or with 30–50 pfu of Ad2 per cell. HeLa cells were inoculated with Ad2 or Ad12 at 20–25 pfu.

Transfection of Cells with Plasmid Constructs and Assay for CAT Activity

Cells, either uninfected or Ad2 or Ad12 infected, were transfected at 16–18 hr post-infection with 10 μg of promoter–CAT gene constructs and 5 μg of the plasmid pCH110 by the calcium precipitation technique (145). At 4 hr or as late as 22 hr after the addition of the DNA–Ca^{2+} precipitates, the cells were treated with 15% (v/v) glycerol in HEPES-buffered saline for 2 min. The cells were then washed, fresh medium was added, and incubation was continued at 37° C. Extracts of transfected cells were prepared 44–48 hr after transfection, and CAT activity was determined according to standard procedures (146–148).

The plasmid pCH110 carried the β-galactosidase gene under the control of the early simian virus 40 promoter (149). Expression of β-galactosidase was analyzed as an internal standard to monitor comparable transfection in different experiments

(150). The enzyme activity of β-galactosidase was determined by measuring the optical density at 420 nm after incubation of 30 μl of cellular extracts with 1 ml of 60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, 10 m*M* KCl, 1 m*M* $MgCl_2$, 50 m*M* 2-mercaptoethanol, and 100 μl of *O*-nitrophenyl-β-D-galactopyranoside at 37° C for 30 min. The reaction was terminated after the addition of 0.5 ml of 1 *M* Na_2CO_3. CAT activity values were expressed as percentages of ^{14}C-labeled chloramphenicol converted to the acetylated forms and were normalized to β-galactosidase activities.

Preparation of Nuclear Extracts from BHK21 and HeLa Cells

Preparation of nuclear extracts was performed according to the method of Dignam *et al.* (151). HeLa and BHK21 cells were grown in suspension cultures to a density of 4×10^5 to 6×10^5 cells per milliliter. For harvest, the cells were pelleted at 1800 rpm and 4° C for 15 min and then washed with phosphate-buffered saline deficient in Mg^{2+} and Ca^{2+} (PBS-d) (152). Subsequently, the cells were incubated in 5 vol of lysis buffer [10 m*M* HEPES (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, and 0.5 m*M* dithiothreitol (DTT)] for 10–20 min at 0° C. The cells were carefully sedimented and resuspended in 2 vol of lysis buffer. To separate the cytoplasm and the nucleus, cytoplasmic membranes were ruptured mechanically by 10–15 (HeLa cells) or 25–30 (BHK21 cells) strokes in a tight-fitting Dounce homogenizer. After centrifugation at 4500 and 14,500 rpm (Beckman JA-20 centrifuge) for 20–25 min at 4° C, the nuclei were resuspended in 3 ml of 20 m*M* HEPES (pH 7.9), 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.5 m*M* DTT, 0.5 m*M* phenylmethylsulfonic acid (PMSF), 2.5 U/ml aprotinin, and 25% (v/v) glycerol for 10^9 nuclei and swirled in an ice bath for 30 min. Nuclear proteins and nuclear debris were separated by centrifugation at 14,500 rpm for 25 min at 4° C. The proteins in the supernatant were precipitated with 52% (w/v) $(NH_4)_2SO_4$ (4° C, pH 8.0) and sedimented at 17,000 rpm for 20–25 min at 4° C. The proteins were then resuspended in dialysis buffer [20 m*M* HEPES (pH 7.9), 20 m*M* KCl, 1 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.5 m*M* each of DTT and PMSF, 2.5 U/ml aprotinin, and 20% (v/v) glycerol] and dialyzed with three changes of buffer for 6 hr against the dialysis buffer. The dialysate was clarified by centrifugation, frozen in liquid nitrogen, and stored at −80° C.

Analysis of Protein–DNA Interactions by EMSA

Protein–DNA interactions were monitored according to published procedures (153–155). Synthetic oligodeoxyribonucleotides were synthesized in an Applied Biosystems 381A DNA synthesizer (Forster City, CA) and were 5′-terminally labeled using [$\gamma - ^{32}$P]ATP and T4 polynucleotide kinase. Subsequently, labeled DNA corresponding to 20,000 cpm (Cerenkov) was incubated for 30 min at 25° C with 0.5 μg of

poly(dA)–poly(dT) and 0.5 μg of poly(dI)–poly(dC) alternating copolymers (Pharmacia Uppsala, Sweden) as unspecific competitors and different amounts of nuclear proteins in a total volume of 20 μl of 12% (v/v) glycerol, 12 m*M* HEPES (pH 7.9), 80 m*M* KCl, 5 m*M* $MgCl_2$, 4 m*M* Tris–HCl, 0.5 m*M* EDTA, 0.6 m*M* DTT, and 1 m*M* $ZnCl_2$. The obtained nucleoprotein complexes were resolved by electrophoresis on native 4–6% (w/v) polyacrylamide gels (acrylamide–bisacrylamide, 38:2) in 0.5× TEB solution [50 m*M* Tris–HCl (pH 8.3), 38 m*M* sodium borate, and 12.5 m*M* EDTA]. After electrophoresis the gels were dried on Whatman (Clifton, NJ) 3MM paper and autoradiographed at −80° C on Kodak (Rochester, NY) XAR-5 films with an intensifying screen (156).

Isolation of Cytoplasmic RNA

Cytoplasmic RNA was isolated according to Scott *et al.* (157). The cells were harvested when 80–90% confluent by washing the monolayer (75 cm^2) twice with Tris–saline [13.7 m*M* NaCl, 5 m*M* KCl, 0.3 m*M* Na_2HPO_4, 25 m*M* Tris–HCl (ph 7.4), 0.1% (w/v) glucose, 0.03% (w/v) penicillin, and 0.015% (w/v) streptomycin] and subsequent incubation in 2 ml of 0.025% (w/v) trypsin in Tris–saline. After sedimenting the cells for 5 min at 4° C and 5700 *g*, the pellet was washed with 2 ml of ice-cold Tris–saline and resuspended in 1 ml of TSE buffer [100 m*M* NaCl, 10 m*M* Tris–HCl (pH 8.0), and 1 m*M* EDTA].

To separate the cytoplasm and the nucleus, the $MgCl_2$ and Nonidet P-40 (NP40; (Sigma, St. Louis, MO) concentrations in the buffer were adjusted to 5 m*M* and 0.5% (v/v), respectively. Subsequently, the solution was shaken vigorously and centrifuged for 5 min at 4° C and 5700 *g*. The cytoplasmic supernatant was adjusted to 25 m*M* EDTA (pH 8.0) and 0.5% (w/v) SDS. This solution was extracted with two volumes of hot (60° C) phenol, followed by extractions with phenol, phenol–chloroform, and chloroform. Finally, the RNA was ethanol-precipitated.

Isolation of Total RNA

Total RNA was prepared according to the technique of Chirgwin *et al.* (158) or Chomczynski and Sacchi (159). For the RNA preparation, according to Chirgwin *et al.* (158), the cells were washed twice with PBS-d (152) and lysed in 6.5 ml of 4 *M* guanidinium isothiocyanate, 20 m*M* sodium acetate (pH 5.2), and 0.5% (w/v) *N*-lauryl sarcosine (Sarkosyl, Sigma, St. Louis MO). The RNA was purified by layering the 6.5 ml of cell lysate on top of a CsCl cushion. The RNA was then pelleted by centrifugation for 12–20 hr at 30,000 rpm and 20° C and subsequently resuspended in 360 μl of 1 m*M* EDTA, 0.1% (w/v) SDS, and 20 m*M* Tris–HCl (pH 7.5), extracted with phenol–chloroform, and ethanol-precipitated. The RNA was dis-

solved in 360 μl of 10 m*M* $MgCl_2$, 10 m*M* Tris–HCl (pH 7.5), and 50 m*M* NaCl, treated with RNase-free DNase I for 15 min at 37° C, extracted with phenol–chloroform, and ethanol-precipitated.

For the isolation of total RNA according to Chomczynski and Sacchi (159), cells were washed twice with PBS-d and lysed in 3.5 ml of 4 *M* guanidinium isothiocyanate, 25 m*M* sodium citrate (pH 7.0), 0.5% (w/v) Sarkosyl, and 0.1 *M* 2-mercaptoethanol (buffer A). Subsequently, 0.35 ml of 2 *M* sodium acetate (pH 4.0), 3.5 ml of phenol, and 0.7 ml of chloroform were added to the homogenate. This suspension was shaken vigorously for 30 sec and cooled on ice for 20 min. After centrifugation at 10,000 *g* for 20 min at 4° C, the RNA was precipitated. The RNA pellet was resuspended in 500 μl of buffer A and was again precipitated. The RNA pellet was redissolved in 20 m*M* HEPES (pH 7.9), 5 m*M* $MgCl_2$, and 1 m*M* $CaCl_2$ and treated with DNase I. The RNA was again phenol–chloroform-extracted and ethanol-precipitated.

Nuclear Run-on Analyses

Techniques for nuclear run-on analyses were modified according to published procedures (160–162). For the preparation of nuclei, the cells (2×10^7 to 5×10^7) were washed with PBS-d, scraped, and pelleted. Subsequently, the cells were lysed by adding 4 ml of NP40 lysis buffer [0.5% (v/v) NP40, 10 m*M* Tris–HCl (pH 7.4), 10 m*M* NaCl, 3 m*M* $MgCl_2$, and 20 U/ml RNase inhibitor from human placenta (Sigma, St. Louis, MO)]. After centrifugation for 5 min at 500 *g* and 4° C, the pelleted nuclei were resuspended in 200 μl of glycerol storage buffer [50 m*M* Tris–HCl (pH 8.3), 40% (v/v) glycerol, 5 m*M* $MgCl_2$, and 0.1 m*M* EDTA], frozen in liquid nitrogen, and stored at −80° C.

To measure transcription rates, the frozen nuclei were thawed on ice and mixed with 200 μl of 2× reaction buffer [10 m*M* Tris–HCl (pH 8.0); 5 m*M* $MgCl_2$; 0.3 *M* KCl; 1 m*M* (each) ATP, CTP, and GTP; 4.8 m*M* DTT; 200 U/ml RNase inhibitor (Sigma); and 100 μCi of [α-^{32}P]UTP (800 Ci/mmol; New England Nuclear, Boston, MA)]. After incubation at 30°C for 30 min, 70 U of RNase-free DNase I (Boehringer, Mannheim, Germany) in 0.6 ml of HSB buffer [0.5 *M* NaCl, 50 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 10 m*M* Tris–HCl (pH 7.4)] was added, and the reaction mixture was further incubated for 30 min at 30° C. Subsequently, 200 μl of SDS–Tris buffer [5% (w/v) SDS, 0.5 *M* Tris–HCl (pH 7.5), and 125 m*M* EDTA] and 10 μl of proteinase K (20 mg/ml) were added and the reaction mixture was incubated for 30 min at 42° C to lyse the nuclei, followed by extraction of the lysates with phenol–chloroform. The obtained aqueous phase was passed over a 7-ml Sephadex-G50 (Uppsala, Sweden) column, which was developed with 50 m*M* Tris–HCl (pH 7.4), 10 m*M* EDTA, and 0.5% (w/v) SDS to purify the radiolabeled RNA. The RNA was subsequently ethanol-precipitated and resuspended in distilled water. This nuclear radiolabeled RNA was hybridized to the appropriate DNA, which was immobilized

on nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) with a Minifold dot blot apparatus (Schleicher & Schuell) according to the method of Kafatos *et al.* (163). For blotting the DNA onto nitrocellulose filters, the filters were prewetted in water and soaked briefly in 1 *M* ammonium acetate (pH 7.0) followed by 4× SSC (1× SSC: 0.15 *M* NaCl and 0.015 *M* sodium citrate). The DNA preparations to be blotted were denatured in 100 μl of 0.3 *M* NaOH for 5 min at 100° C, chilled on ice, and then neutralized by adding 100 μl of 2 *M* ammonium acetate (pH 7.0). DNA samples were fixed to the nitrocellulose filters under gentle vacuum. Finally, the filters were baked for 2 hr at 80° C.

For the hybridization of the radiolabeled nuclear RNA (1×10^6 to 2×10^6 cpm, Cerenkov) to the immobilized DNA, nitrocellulose filters were incubated with 2 ml of the radiolabeled RNA solution and 2 ml of TES–NaCl [10 m*M* Tris–HCl (pH 7.4), 10 m*M* EDTA, 0.2% (w/v) SDS, and 0.6 *M* NaCl]. After incubating the hybridization mixture under shaking for 36–48 hr at 65° C, the filters were washed twice in 2× SSC for 1 hr at 65° C and then treated with 8 ml of RNase A (10 mg/ml) in 8 ml of 2× SSC at 37° C for 30 min. Subsequently, the filters were washed again in 2× SSC at 37° C for 1 hr, air-dried on Whatman 3MM filter paper, and autoradiographed at −80° C on Kodak XAR-5 films.

Preparation of RNA Probes and the RNase Protection Method

Appropriate RNA probes were obtained by transcribing the DNA, which had been cloned into the polylinker site of the vector pBluescript KS+ (Stratagene, La Jolla, CA), with bacteriophage T3 RNA polymerase (164–167). For the transcription reactions the DNA templates were linearized, and *in vitro* transcription reactions by T3 RNA polymerase were performed according to the recommendations of the manufacturer (Stratagene) in the presence of 50 μCi [α-^{32}P]UTP (800 Ci/mmol; New England Nuclear), followed by the purification of the RNA transcripts by electrophoresis on polyacrylamide gels.

RNase protection experiments (168–169) were carried out by coprecipitating 0.25–40 μg of cellular RNA with 1×10^5 to 2×10^5 cpm (Cerenkov) of *in vitro*-transcribed RNA probes. This RNA mixture was hybridized for 12–16 hr in 30 μl of 80% (v/v) formamide, 40 m*M* PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.4), 400 m*M* NaCl, and 1 m*M* EDTA at appropriate temperatures. The RNA–RNA hybrids were treated with 350 μl of RNase buffer [10 m*M* Tris–HCl (pH 7.5), 5 m*M* EDTA, 300 m*M* NaCl, and 20 μg/ml RNase A) for 45 min at 37° C. Subsequently, the samples were treated with proteinase K, extracted with phenol–chloroform, and ethanol-precipitated. Finally, the RNA hybrids were analyzed by electrophoresis on a 6% (w/v) polyacrylamide gel (acrylamide–bisacrylamide, 38:2) containing 7 *M* urea in 0.5× TEB solution, and the gels were then dried on Whatman 3MM paper and autoradiographed at −80° C on Kodak XAR-5 films.

Cell-Free in Vitro Transcription Experiments

For cell-free reactions (modified according to Ref. 170), nuclear proteins (30–100 μg) were incubated in a total volume of 10–20 μl of 250 or 500 ng of template DNA, 10% (v/v) glycerol, 80 m*M* KCl, 5.5 m*M* $MgCl_2$, 20 m*M* HEPES (pH 7.9), 1.25 m*M* DTT, 1.05 m*M* EDTA, 0.25 m*M* PMSF, 1.25 U/ml aprotinin, 0.5 m*M* (each) ATP, CTP, GTP, and UTP, and 5 m*M* creatine phosphate for 1 hr at 30° C. After degradation of the template DNA at 37° C for 15 min with RNase-free DNase I, the reactions were terminated by the addition of 100 μl of 0.3% (w/v) SDS, 0.13 *M* NaCl, 10 m*M* $MgCl_2$, and 30 μg/ml yeast tRNA. The newly synthesized transcripts were extracted with phenol–chloroform, ethanol-precipitated, and analyzed by RNase protection.

Expression and Purification of the HIS–YY1 Fusion Protein

HIS–YY1 (126, 127) was expressed as a fusion protein in the bacterial strain RR (courtesy of Y. Shi, Harvard University Medical School, Cambridge, MA). One liter of bacterial culture was grown to an optical density at 600 nm (OD_{600}) of 0.8–0.9. Synthesis of the fusion protein was induced by growing the bacteria for 4 hr at 30° C in the presence of 2 m*M* isopropyl-thiogalactoside. Subsequently, the bacteria were harvested by centrifugation and lysed by incubation overnight at 4° C in 20 ml of 6 *M* guanidine–HCl (pH 8.0) plus 50 m*M* NaH_2PO_4. After centrifugation the fusion protein was purified on a nickel chelating column. This column was first washed with 10 ml of distilled water, followed by 5 ml of 0.1 *M* $NiSO_4$ and another 2 ml of distilled water. The column was then equilibrated with 10 ml of 6 *M* guanidine–HCl (pH 8.0), 0.1 *M* NaH_2PO_4, and 10 m*M* Tris–HCl. The entire bacterial lysate was loaded on the column, and the column was washed first with 10 ml of 6 *M* guanidine–HCl (pH 8.0), 0.1 *M* NaH_2PO_4, and 10 m*M* Tris–HCl, followed by 10 ml of 6 *M* guanidine–HCl (pH 6.25), 0.1 *M* NaH_2PO_4, and 10 m*M* Tris–HCl. The fusion protein was eluted with 5–7 ml of 6 *M* guanidine–HCl (pH 5.0), 0.1 *M* NaH_2PO_4, and 10 m*M* Tris–HCl. Guanidine was removed to allow renaturation of the fusion protein by stepwise dialysis. The protein was then stored at −80° C.

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References

1. R. J. Huebner, W. P. Rowe, and W. T. Lane, *Proc. Natl. Acad. Sci. U.S.A.* **48,** 2051 (1962).
2. J. J. Trentin, Y. Yabe, and G. Taylor, *Science* **137,** 835 (1962).
3. J. H. Pope and W. P. Rowe, *J. Exp. Med.* **120,** 577 (1964).
4. A. E. Freeman, P. H. Black, H. E. A. Vanderpool, P. H. Henby, J. B. Austin, and R. J. Huebner, *Proc. Natl. Acad. Sci. U.S.A.* **58,** 1205 (1967).
5. W. Doerfler and P. Böhm (eds.), *Curr. Top. Microbiol. Immunol.* **199 I–III** (1995).
6. W. Doerfler, *Proc. Natl. Acad. Sci. U.S.A.* **60,** 636 (1968).
7. W. Doerfler, *Virology* **38,** 587 (1969).
8. W. Doerfler, *J. Virol.* **6,** 652 (1970).
9. W. Doerfler, *Curr. Top. Microbiol. Immunol.* **71,** 1 (1975).
10. W. Doerfler, *Adv. Virus Res.* **39,** 89 (1991).
11. W. Doerfler, *in* "Malignant Transformation by DNA Viruses. Molecular Mechanisms" (W. Doerfler and P. Böhm, eds.), p. 87. VCH, Weinheim, Germany, 1992.
12. C. Zock and W. Doerfler, *EMBO J.* **9,** 1615 (1990).
13. C. Zock, A. Iselt, and W. Doerfler, *J. Virol.* **67,** 682 (1993).
14. W. Doerfler, *Curr. Top. Microbiol. Immunol.* **101,** 127 (1982).
15. D. Sutter, M. Westphal, and W. Doerfler, *Cell (Cambridge, Mass.)* **14,** 569 (1978).
16. S. Stabel, W. Doerfler, and R. R. Friis, *J. Virol.* **36,** 22 (1980).
17. W. Doerfler, R. Gahlmann, S. Stabel, R. Deuring, U. Lichtenberg, M. Schulz, D. Eick, and R. Leisten, *Curr. Top. Microbiol. Immunol.* **109,** 193 (1983).
18. J. Schick, K. Baczko, E. Fanning, J. Groneberg, H. Burger, and W. Doerfler, *Proc. Natl. Acad. Sci. U.S.A.* **73,** 1043 (1976).
19. R. Deuring, U. Winterhoff, F. Tamanoi, S. Stabel, and W. Doerfler, *Nature (London)* **293,** 81 (1981).
20. R. Gahlmann, R. Leisten, L. Vardimon, and W. Doerfler, *EMBO J.* **1,** 1101 (1982).
21. S. Stabel and W. Doerfler, *Nucleic Acids Res.* **10,** 8007 (1982).
22. R. Deuring and W. Doerfler, *Gene* **26,** 283 (1983).
23. R. Gahlmann and W. Doerfler, *Nucleic Acids Res.* **11,** 7347 (1983).
24. M. Schulz and W. Doerfler, *Nucleic Acids Res.* **12,** 4959 (1984).
25. M. Schulz, U. Freisem-Rabien, R. Jessberger, and W. Doerfler, *J. Virol.* **61,** 344 (1987).
26. U. Lichtenberg, C. Zock, and W. Doerfler, *J. Virol.* **61,** 2719 (1987).
27. R. Jessberger, D. Heuss, and W. Doerfler, *EMBO J.* **8,** 869 (1989).
28. R. Jessberger, B. Weisshaar, S. Stabel, and W. Doerfler, *Virus Res.* **13,** 113 (1989).
29. J. Tatzelt, B. Scholz, K. Fechteler, R. Jessberger, and W. Doerfler, *J. Mol. Biol.* **226,** 117 (1992).
30. J. Tatzelt, K. Fechteler, P. Langenbach, and W. Doerfler, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7356 (1993).
31. W. Doerfler, R. Gahlmann, S. Stabel, R. Deuring, M. Schulz, U. Lichtenberg, D. Eick, R. Jessberger, and R. Leisten, *in* "Recombinant DNA Research and Viruses" (Y. Becker, ed.), p. 125. Nijhoff, Boston, 1985.
32. K. Fechteler, J. Tatzelt, S. Huppertz, P. Wilgenbus, and W. Doerfler, *Curr. Top. Microbiol. Immunol.* **199/II,** 109 (1995).
33. J. Schellner, K. Stüber, and W. Doerfler, *Biochim. Biophys. Acta* **867,** 114 (1986).
34. R. Gahlmann, M. Schulz, and W. Doerfler, *EMBO J.* **3,** 3263 (1984).

35. G. Orend, A. Linkwitz, and W. Doerfler, *J. Virol.* **68,** 187 (1994).
36. J. Groneberg, D. Sutter, H. Soboll, and W. Doerfler, *J. Gen. Virol.* **40,** 635 (1978).
37. J. Groneberg and W. Doerfler, *Int. J. Cancer* **24,** 67 (1979).
38. D. Eick, S. Stabel, and W. Doerfler, *J. Virol.* **36,** 41 (1980).
39. D. Eick and W. Doerfler, *J. Virol.* **42,** 317 (1982).
40. I. Kuhlmann, S. Achten, R. Rudolph, and W. Doerfler, *EMBO J.* **1,** 79 (1982).
41. K. Raška, Jr., and W. A. Strohl, *Virology* **47,** 734 (1972).
42. J. Ortin and W. Doerfler, *J. Virol.* **15,** 27 (1975).
43. J. Ortin, K. H. Scheidtmann, R. Greenberg, M. Westphal, and W. Doerfler, *J. Virol.* **20,** 355 (1976).
44. H. Esche, R. Schilling, and W. Doerfler, *J. Virol.* **30,** 21 (1979).
45. H. Esche, M. Reuther, and K. Schughart, *Curr. Top. Microbiol. Immunol.* **111,** 91 (1984).
46. H. Esche and B. Siegmann, *J. Gen. Virol.* **60,** 99 (1982).
47. L. A. Lucher, *J. Gen. Virol.* **71,** 579 (1990).
48. L. A. Lucher, K. H. Brackmann, J. S. Symington, and M. Green, *J. Virol.* **58,** 592 (1986).
49. L. A. Lucher, D. Kimelman, J. S. Symington, K. H. Brackmann, M. A. Cartas, H. Thornton, and M. Green, *J. Virol.* **52,** 136 (1984).
50. T. Klimkait and W. Doerfler, *J. Virol.* **55,** 466 (1985).
51. T. Klimkait and W. Doerfler, *Virology* **161,** 109 (1987).
52. W. Doerfler and U. Lundholm, *Virology* **40,** 754 (1970).
53. E. Fanning and W. Doerfler, *J. Virol.* **20,** 373 (1976).
54. W. A. Strohl, H. C. Rouse, and R. W. Schlesinger, *Virology* **28,** 645 (1966).
55. C. F. Garon, K. W. Berry, and J. A. Rose, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2391 (1972).
56. J. Wolfson and D. Dressler, *Proc. Natl. Acad. Sci. U.S.A.* **69,** 3054 (1972).
57. J. R. Arrand and R. J. Roberts, *J. Mol. Biol.* **128,** 577 (1979).
58. A. Tolun, P. Aleström, and U. Pettersson, *Cell (Cambridge, Mass.)* **17,** 705 (1979).
59. A. J. Robinson, H. B. Younghusband, and A. J. D. Bellet, *Virology* **56,** 54 (1973).
60. D. M. K. Rekosh, W. C. Russell, A. J. D. Bellet, and A. J. Robinson, *Cell (Cambridge, Mass.)* **11,** 283 (1977).
61. E.-L. Winnacker, *Cell (Cambridge, Mass.)* **14,** 761 (1978).
62. M. D. Challberg and T. J. Kelly, *Annu. Rev. Biochem.* **58,** 671 (1989).
63. K. Nagata, R. A. Guggenheimer, and J. Hurwitz, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 6177 (1983).
64. K. A. Jones, J. T. Kadonaga, P. J. Rosenfeld, T. J. Kelly, and R. Tjian, *Cell (Cambridge, Mass.)* **48,** 79 (1987).
65. G. J. M. Pruijn, W. van Driel, and P. C. van der Vliet, *Nature (London)* **322,** 656 (1986).
66. G. J. M. Pruijn, W. van Driel, R. T. van Miltenburg, and P. C. van der Vliet, *EMBO J.* **6,** 3771 (1987).
67. K. Nagata, R. A. Guggenheimer, and J. Hurwitz, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 4266 (1983).
68. J. Schaack, P. Schedl, and T. Shenk, *J. Virol.* **64,** 78 (1990).
69. B. M. Chowrira and L. A. Lucher, *Virology* **176,** 289 (1990).
70. L. A. Lucher, B. Khuntirat, J. Zhao, and P. C. Angeletti, *Virology* **189,** 187 (1992).
71. R. Jüttermann, U. Weyer, and W. Doerfler, *J. Virol.* **63,** 3535 (1989).
72. J. Sprengel, B. Schmitz, D. Heuss-Neitzel, C. Zock, and W. Doerfler, *J. Virol.* **68,** 379 (1994).

73. P. R. Reich, B. G. Forget, S. M. Weissman, and J. A. Rose, *J. Mol. Biol.* **17,** 428 (1966).
74. M. B. Mathews, *Cell (Cambridge, Mass.)* **6,** 223 (1975).
75. H. Söderlund, U. Pettersson, B. Vennström, L. Philipson, and M. B. Mathews, *Cell (Cambridge, Mass.)* **7,** 585 (1976).
76. R. Weinmann, T. G. Brendler, H. J. Raskas, and R. G. Roeder, *Cell (Cambridge, Mass.)* **7,** 557 (1976).
77. G. Akusjärvi, M. B. Mathews, P. Andersson, B. Vennström, and U. Pettersson, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2424 (1980).
78. B. Thimmappaya, C. Weinberger, R. J. Schneider, and T. Shenk, *Cell (Cambridge, Mass.)* **31,** 543 (1982).
79. R. J. Schneider, C. Weinberger, and T. Shenk, *Cell (Cambridge, Mass.)* **37,** 291 (1984).
80. R. J. Schneider, B. Safer, S. M. Munemitsu, C. E. Samuel, and T. Shenk, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 4321 (1985).
81. A Babich, L. T. Feldman, J. R. Nevins, J. E. Darnell, and C. Weinberger, *Mol. Cell. Biol.* **3,** 1212 (1983).
82. P. A. Reichel, W. C. Merrick, J. Siekierka, and M. B. Mathews, *Nature (London)* **313,** 196 (1985).
83. J. Siekierka, T. M. Mariano, P. A. Reichel, and M. B. Mathews, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1959 (1985).
84. M. G. Katze, D. DeCorato, B. Safer, J. Galabru, and A. G. Hovanessian, *EMBO J.* **6,** 689 (1987).
85. R. J. Schneider and T. Shenk, *Annu. Rev. Biochem.* **56,** 317 (1987).
86. M. Kostura and M. B. Mathews, *Mol. Cell. Biol.* **9,** 1576 (1989).
87. S. M. Berget, C. Moore, and P. A. Sharp, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3171 (1977).
88. T. R. Broker, L. T. Chow, A. R. Dunn, R. E. Gelinas, J. A. Hassell, D. F. Klessig, J. B. Lewis, R. J. Roberts, and B. S. Zain, *Cold Spring Harbor Symp. Quant. Biol.* **42,** 531 (1977).
89. S. Zain, J. Sambrook, R. J. Roberts, W. Keller, M. Fried, and A. R. Dunn, *Cell (Cambridge, Mass.)* **16,** 851 (1979).
90. J. Logan and T. Shenk, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 3655 (1984).
91. K. L. Berkner and P. A. Sharp, *Nucleic Acids Res.* **13,** 841 (1985).
92. R. J. Kaufman, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 689 (1985).
93. F. V. Alonso-Caplen, M. G. Katze, and R. M. Krug, *J. Virol.* **62,** 1606 (1988).
94. P. J. Dolph, V. Racaniello, A. Villamarin, F. Palladino, and R. J. Schneider, *J. Virol.* **62,** 2059 (1988).
95. P. J. Dolph, J. Huang, and R. J. Schneider, *J. Virol.* **64,** 2669 (1990).
96. S. K. Jang, M. V. Davies, R. J. Kaufman, and E. Wimmer, *J. Virol.* **63,** 1651 (1989).
97. N. Sonenberg, *Adv. Virus Res.* **33,** 175 (1987).
98. J. Huang and R. J. Schneider, *Cell (Cambridge, Mass.)* **65,** 271 (1991).
99. L. T. Chow, J. B. Lewis, and T. R. Broker, *Cold Spring Harbor Symp. Quant. Biol.* **44,** 401 (1979).
100. G. Akusjärvi and H. Persson, *Nature (London)* **292,** 420 (1981).
101. J. R. Nevins and M. C. Wilson, *Nature (London)* **290,** 113 (1981).
102. T. B. Hasson, P. D. Soloway, D. A. Ornelles, W. Doerfler, and T. Shenk, *J. Virol.* **63,** 3612 (1989).

103. T. B. Hasson, D. A. Ornelles, and T. Shenk, *J. Virol.* **66,** 6133 (1992).
104. H. C. Rouse, W. A. Strohl, and R. W. Schlesinger, *Virology* **28,** 633 (1966).
105. J. L. Cook and A. M. Lewis, Jr., *Cancer Res.* **39,** 1455 (1979).
106. L. Vardimon and W. Doerfler, *J. Mol. Biol.* **147,** 227 (1981).
107. L. Visser, M. W. van Maarschalkerweerd, T. H. Rozijn, A. D. C. Wassenaar, A. M. C. B. Reemst, and J. S. Sussenbach, *Cold Spring Harbor Symp. Quant. Biol.* **44,** 541 (1979).
108. N. Jones and T. Shenk, *Cell (Cambridge, Mass.)* **17,** 683 (1979).
109. T. Shenk, N. Jones, W. Colby, and D. Fowlkes, *Cold Spring Harbor Symp. Quant. Biol.* **44,** 367 (1979).
110. J. Logan, S. Pilder, and T. Shenk, *in* "Cancer Cells" (G. T. van de Woude, A. J. Levine, W. C. Topp, and J. D. Wastson, eds.), Vol. 2, p. 527. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1984.
111. R. Bernards, M. G. W. de Leeuw, M.-J. Vaessen, A. Houweling, and A. J. van der Eb, *J. Virol.* **50,** 847 (1984).
111a. G. Schiedner, B. Schmitz, and W. Doerfler, *J. Virol.* **68,** 5476 (1994).
112. G. P. Thomas and M. B. Mathews, *Cell (Cambridge, Mass.)* **22,** 523 (1980).
113. E. Falck-Pedersen and L. Logan, *J. Virol.* **63,** 532 (1989).
114. L. E. Babiss, H. S. Ginsberg, and J. E. Darnell, Jr., *Mol. Cell. Biol.* **5,** 2552 (1985).
115. D. N. Halbert, J. R. Cutt, and T. Shenk, *J. Virol.* **56,** 250 (1985).
116. S. Pilder, M. Moore, J. Logan, and T. Shenk, *Mol. Cell. Biol.* **6,** 470 (1986).
117. D. H. Weinberg and G. Ketner, *J. Virol.* **57,** 833 (1986).
118. C. Hemström, K. Nordqvist, U. Pettersson, and A. Virtanen, *J. Virol.* **62,** 3258 (1988).
119. C. Hemström, A. Virtanen, E. Bridge, G. Ketner, and U. Pettersson, *J. Virol.* **65,** 1440 (1991).
120. E. Bridge and G. Ketner, *J. Virol.* **63,** 631 (1989).
121. M.-M. Huang and P. Hearing, *J. Virol.* **63,** 2605 (1989).
122. E. Bridge, C. Hemström, and U. Pettersson, *Virology* **183,** 260 (1991).
123. R. Evans, J. Weber, E. Ziff, and J. E. Darnell, *Nature (London)* **278,** 367 (1979).
124. A. R. Shaw and E. B. Ziff, *Cell (Cambridge, Mass.)* **22,** 905 (1980).
125. U. Weyer and W. Doerfler, *EMBO J.* **4,** 3015 (1985).
126. E. Seto, Y. Shi, and T. Shenk, *Nature (London)* **354,** 241 (1991).
127. Y. Shi, E. Seto, L.-S. Chang, and T. Shenk, *Cell (Cambridge, Mass.)* **67,** 377 (1991).
128. L.-S. Chang, Y Shi, and T. Shenk, *J. Virol.* **63,** 3479 (1989).
129. J. R. Flanagan, M. Murata, P. A. Burke, Y. Shirayoshi, E. Appella, P. A. Sharp, and K. Ozato, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 3145 (1991).
130. J. R. Flanagan, K. G. Becker, D. L. Ennist, S. L. Gleason, P. H. Driggers, B.-Z. Levi, E. Apella, and K. Ozato, *Mol. Cell. Biol.* **12,** 38 (1992).
131. N. Hariharan, D. E. Kelley, and R. P. Perry, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 9799 (1991).
132. E. A. Montalvo, Y. Shi, T. E. Shenk, and A. J. Levine, *J. Virol.* **65,** 3647 (1991).
133. K. Park and M. L. Atchison, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 9804 (1991).
134. T. Bauknecht, P. Angel, H.-D. Royer, and H. zur Hausen, *EMBO J.* **11,** 4607 (1992).
135. W. A. Strohl, H. Rouse, K. Teets, and R. W. Schlesinger, *Arch. Gesamte Virusforsch.* **31,** 93 (1970).
136. B. Yakobson, T. Koch, and E. Winocour, *J. Virol.* **61,** 972 (1987).
137. K. I. Berns and R. A. Bohenzky, *Adv. Virus Res.* **32,** 243 (1987).

138. J. E. Janik, M. M. Huston, and J. A. Rose, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1925 (1981).
139. C. A. Laughlin, N. Jones, and B. J. Carter, *J. Virol.* **41,** 868 (1982).
140. W. D. Richardson and H. Westphal, *J. Virol.* **51,** 404 (1984).
141. J.-D. Tratschin, M. H. P. West, J. Sandbank, and B. J. Carter, *Mol. Cell. Biol.* **4,** 2072 (1984).
142. M. Stoker and I. Macpherson, *Nature (London)* **203,** 1355 (1964).
143. R. Bablanian, H. J. Eggers, and I. Tamm, *Virology* **26,** 100 (1965).
144. W. Doerfler, U. Lundholm, and M. Hirsch-Kauffmann, *J. Virol.* **9,** 297 (1972).
145. F. L. Graham and A. J. van der Eb, *Virology* **54,** 536 (1973).
146. C. M. Gorman, L. F. Moffat, and B. H. Howard, *Mol. Cell. Biol.* **2,** 1044 (1982).
147. I. Kruczek and W. Doerfler, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 7586 (1983).
148. C. M. Gorman, *in* "DNA Cloning" (D. M. Glover, ed.), Vol. 2, p. 143. IRL Press, Oxford, 1985.
149. C. V. Hall, P. E. Jacob, G. M. Ringold, and F. Lee, *J. Mol. Appl. Genet.* **2,** 101 (1983).
150. P. Herbomel, B. Bourachot, and M. Yaniv, *Cell (Cambridge, Mass.)* **39,** 653 (1984).
151. J. D. Dignam, R. M. Lebovitz, and R. G. Roeder, *Nucleic Acids Res.* **11,** 1475 (1983).
152. R. L. Dulbecco and M. Vogt, *J. Exp. Med.* **99,** 167 (1954).
153. M. Fried and D. M. Crothers, *Nucleic Acids Res.* **9,** 6505 (1981).
154. M. M. Garner and A. Revzin, *Nucleic Acids Res.* **9,** 3047 (1981).
155. R. W. Carthew, L. A. Chodosh, and P. A. Sharp, *Cell (Cambridge, Mass.)* **43,** 439 (1985).
156. R. A. Laskey, *in* "Methods in Enzymology" (L. Grossman and K. Moldave, eds.), Vol. 65, p. 363. Academic Press, New York, 1980.
157. M. R. D. Scott, K.-H. Westphal, and P. W. J. Rigby, *Cell (Cambridge, Mass)* **34,** 557 (1983).
158. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, and W. J. Rutter, *Biochemistry* **18,** 5294 (1979).
159. P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162,** 156 (1987).
160. M. E. Greenberg and E. B. Ziff, *Nature (London)* **311,** 433 (1984).
161. S. P. Weinheimer and S. L. McKnight, *J. Mol. Biol.* **195,** 819 (1987).
162. K. Leong, W. Lee, and A. J. Berk, *J. Virol.* **64,** 51 (1990).
163. F. C. Kafatos, C. W. Jones, and A. Efstratiadis, *Nucleic Acids Res.* **7,** 1541 (1979).
164. F. W. Studier and A. H. Rosenberg, *J. Mol. Biol.* **153,** 503 (1981).
165. M. Chamberlin and T. Ryan, *in* "The Enzymes" (P. D. Boyer, ed.), Vol. 15, p. 87. Academic Press, New York, 1982.
166. P. Davanloo, A. H. Rosenberg, J. J. Dunn, and F. W. Studier, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2035 (1984).
167. S. Tabor and C. C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.* **82,** 1074 (1985).
168. D. A. Melton, P. A. Krieg, M. R. Rebagliati, T. Maniatis, E. Zinn, and M. R. Green, *Nucleic Acids Res.* **12,** 7035 (1984).
169. K. Zinn, D. DiMaio, and T. Maniatis, *Cell (Cambridge, Mass.)* **34,** 865 (1983).
170. P. Dobrzanski, A. Hoeveler, and W. Doerfler, *J. Virol.* **62,** 3941 (1988).

Section III

Human Immunodeficiency Virus/Retroviruses

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[12] Quantitation of Virus Stocks Produced from Cloned Human Immunodeficiency Virus DNA

Carol A. Deminie and Michael Emerman

Introduction

The human immunodeficiency virus (HIV) is a human lentivirus that specifically infects $CD4^+$ cells and is the causative agent of the acquired immunodeficiency syndrome (AIDS). HIV is a complex retrovirus. In addition to *gag*, *pol*, and *env*, genes that are contained in all retroviruses, HIV-1 and HIV-2 also encode two regulatory genes called *tat* and *rev*. The Tat protein is necessary for accumulation of high levels of promoter-distal transcripts from the viral long terminal repeat (LTR). The Rev protein is necessary at a posttranscription step to allow HIV transcripts that normally contain splice sites to leave the nucleus without being spliced. HIV-1 also contains the accessory genes *vpr*, *vif*, *vpu*, and *nef*, which do not exist in the onco-retroviruses. In addition, some strains make alternately spliced products such as *tev* and *revD* (1, 2) as well as HIV-2 and some simian immunodeficiency virus (SIV) strains encode a protein called Vpx (3, 4). The function of these genes is the subject of much investigation (for a review see Ref. 5), and their importance to the viral life cycle is becoming more evident. For example, although the *nef* gene is dispensable in most tissue culture systems, it plays an important role in pathogenesis *in vivo* (6).

Although valuable information concerning the functions of the different viral proteins, or regions of the proteins, can be obtained by the study of subgenomic fragments, at some point the significance of studies on isolated genes must be evaluated in the context of an entire provirus. This article describes techniques currently used in our laboratory to introduce molecular clones of HIV into mammalian cells by transfection, to determine whether virus is released from the transfected cells, and to quantitatively measure infectivity in a single-step assay.

Mammalian Cell Transfection with HIV Proviral Plasmids

Preliminary Considerations

Choice of Plasmid

In order to study virus made from different HIV proviral vectors, the DNA must be introduced into cell lines by transfection. In this way noninfectious as well as infectious virus can be analyzed. Because DNA is directly introduced into cells, where it nonspecifically integrates into chromosomes, transfection bypasses the need in the

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viral life cycle for the virus receptor on recipient cells, the need for reverse transcription of the viral genome, and the requirement for the integration function of the viral integrase protein.

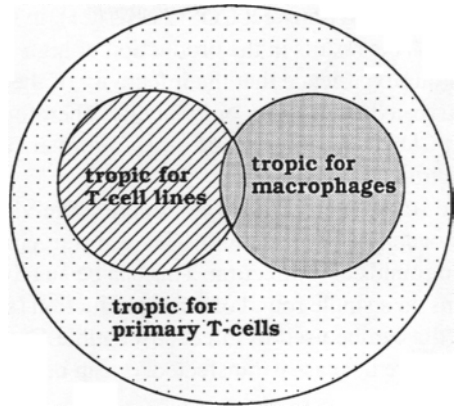
The advantage of making viral stocks from transfection over obtaining virus from a chronically infected cell line is that the chronically infected cell lines contain heterogenous mixtures of virus due to mutation and selection as the virus is passed. This problem can be more severe if the virus has been in continuous culture for some time. Therefore, virus isolated from transfected cells is more homogeneous than virus obtained from chronically infected cells [e.g., H9/IIIB (7)].

Plasmids containing many different HIV proviruses have been described in the literature and several are available from the National Institute of Allergy and Infectious Diseases (NIAID) AIDS Research and Reference Reagent Program. There can be specific advantages to using one plasmid over another to generate virus stocks. For example, virus tropism is an important consideration in certain experimental conditions. With a few exceptions, HIV will infect either tumor cell lines or macrophages, but generally will not infect both. Other virus stocks will infect primary T cells but will not infect tumor cell lines. Nearly all virus stocks, however, will infect primary T-cell lines. The specificity for tropism is determined, for the most part, by a small region in the *env* gene called the V3 loop, although other sequences in the *env* gene and other genes also play a role in determination of tropism (8–12). Only some of the virus strains will work in the MAGI assay described below, although all of them can be detected by reverse transcriptase (RT) assays, also described below. A simplified diagram of relative tropism and some representative proviruses that have been cloned into plasmids is shown in Fig. 1.

A second consideration in choosing the proviral DNA can be the genotype of the accessory proteins. Because the accessory proteins are not absolutely necessary for virus replication, passage of some strains in tissue culture before cloning has allowed them to accumulate mutations in these viral genes. For example, the widely used HXB2 clone has mutations in *vpr, vpu,* and *nef* (13). pNL4-3 (14) and pLAI (15), on the other hand, have full-length copies of all of these genes. The interactions between these auxiliary proteins are complex and not completely understood (16). A final consideration is that even though a provirus has the complete open reading frame, the function of that protein might be different in some strains from that in other strains. For example, the protein encoded by the *nef* gene of ELI is apparently different from the *nef* gene of the LAI-derived series of viruses (17). The catalog of sequences published by Los Alamos National Laboratory in *Human Retroviruses and AIDS, 1992* (18) contains a complete compilation of mutations of all of the sequences of proviruses.

Choice of Cells

The cells used for transfection of the HIV-1 proviral DNA must support efficient transcription from the HIV LTR. We have successfully used SW480 (14) and HeLa–



tropic for T-cell lines	pLai, pHXB2, pNL4-3, pSF-2
tropic for primary macrophages	pYU-2, pNLHXADA-SM
tropic for primary T-cells but not T-cell lines	pMAL, pJR-CSF

FIG. 1 Tropism of cloned HIV-1 isolates. A Venn diagram of HIV-1 tropism is shown at top. Nearly all HIV-1 isolates grow in primary T cells. A subset of these will grow in either macrophages or in T-cell lines, and there is only a small overlap of cloned isolates that grow in both macrophages and T-cell lines. Examples of each tropism are given, but readers are urged to consult the primary literature for exact details. References for the examples are as follows: pLAI (15), pHXB2 (13), pNL4.3 (14), and pSF-2 [J. A. Levy, M. C. Cheng, D. Dina, and P. A. Luciw, *Science* **232,** 998 (1986)], pYU-2 [Y. Li, H. Hui, C. J. Burgess, R. W. Price, P. M. Sharp, B. H. Hahn, and G. M. Shaw, *J. Virol.* **66,** 6587 (1992)], pNLHXADA-SM (pNLHXADA-SM is a hybrid between a macrophage-tropic virus and a pHXB2 derivative) [P. Westervelt, T. Henkel, D. Trowbridge, J. Orenstein, J. Heuser, H. Gendelman, and L. Ratner, *J. Virol* **66,** 3925 (1992)], pMAL (15), pJR-CSF [B. Chesebro, J. Nishio, S. Perryman, A. Cann, W. O'Brien, I. S. Chen, and K. Wehrly, *J. Virol.* **65,** 5782 (1991)].

Tat cells (19) to obtain high levels of secreted virus. We prefer the HeLa cells because they are easier to culture. While HeLa cells support the production of HIV, we have found that HeLa–Tat cells, which constitutively express the HIV LTR transactivator protein Tat from a integrated retroviral vector, give higher viral yields. This may be due to the fact that the Tat protein has been shown to be a limiting factor in efficient virus production (20). Thus, we usually use HeLa–Tat cells for virus production from transfection. More recently, we have switched to using 293 cells because of higher transfection efficiencies.

Proviral clones in vectors that contain the simian virus 40 (SV40) origin of repli-

cation can be used to transfect COS-7 cells (21), in which the plasmid sequences will be amplified. This results in the production of high levels of viral proteins. However, it has been our experience that the infectivity of the released virus is lower than that of virus harvested from HeLa or 293 cells. Therefore, it may be more advantageous to transfect COS-7 cells to harvest virus for protein analysis, but not if high levels of infectious virus are desired.

Since the transfected cells do not express the viral receptor CD4, the virus produced cannot spread in the culture. The resulting viral titers, then, are not affected by unregulated rounds of reinfection. This can be an advantage in determining the effect of mutations on a single round of virus production (see below). Virus harvested from the cell media can be used to infect susceptible $CD4^+$ cells that grow in suspension and therefore are not easily transfected, or can be titrated directly in the MAGI assay (see below).

Transfection Protocol

The calcium phosphate coprecipitation technique (22) is efficient enough to allow analysis of transient virus production. The best results are obtained with circular DNA that is free of bacterial RNA and proteins.

Reagents

2× (N, N-bis[2-Hydroxyethyl]-2-aminoethanesulfonic acid) (BES)-buffered saline (BBS): 50 m*M* BES, 280 m*M* NaCl, and 1.5 m*M* Na_2HPO_4; adjust pH to 6.95 with HCl at room temperature, filter-sterilize, and store at −20° C

0.25 *M* $CaCl_2$: Filter-sterilize and store at −20° C

Sterile phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, and 1.47 m*M* KH_2PO_4 (pH 7.4)

Dulbecco's modified Eagle's medium supplemented with 10% (v/v) newborn calf serum (DMEM-10)

Day 1

For production of large amounts of virus, plate the HeLa–Tat or 293 cells at 8×10^5 cells per T75 flask using 10 ml of DMEM-10. If only small amounts of virus are needed for infection of other cells, plate the cells at 2×10^5 cells per well in a six-well plate with 2 ml of medium. Quantities of virus needed for genetic studies (e.g., Ref. 23) are readily obtained by transfection of one or two wells of a six-well plate.

Day 2

Thaw the BBS and the $CaCl_2$. Both must be at room temperature before using. For each T75 flask, mix 0.5 ml of the 0.25 *M* $CaCl_2$ with 10–15 μg of the proviral DNA in a sterile 1.7-ml Eppendorf tube. Add 0.5 ml of the 2× BBS and vortex well. For

each well of a six-well plate, mix 2.5 μg of DNA with 150 μl of 0.25 *M* $CaCl_2$, then add 150 μl of 2× BBS. Incubate at room temperature for 15 min, then add the DNA mixture to the medium dropwise. The medium should turn yellow as each drop falls into the flask or well. Return the plates to the incubator. While the original protocol suggested was to incubate overnight at 35° C and in 3% CO_2 (22), we obtain satisfactory results using an incubator regulated at 37° C and 5% CO_2. **Note: After transfection with HIV proviral DNA, the cells must be moved to a BL2/3 facility. Work with infectious HIV stocks should be done in an approved BL2/3 laboratory by specially trained personnel.**

Day 3

A layer of fine precipitate will form overnight and should be visible in the cell culture medium under the microscope. Large coarse precipitate will result in lower transfection efficiency and may be due to impurities in the DNA preparation. Wash the cells twice with warm PBS and refeed with either 8 ml (75-mm flask) or 1.5 ml (six-well plate) of DMEM-10.

Days 4, 5, and 6

Medium can be collected on days 4, 5, and 6 (1–3 days after transfection) and pooled. Since efficient transfection with HIV often results in some cytopathic effect, cell debris should be pelleted by centrifugation at 5000 *g* for 5 min. The virus should be stored at −80° C in small aliquots, since repeated freezing and thawing dramatically decreases infectivity. If the virus is going to be used for an experiment in the next 48 hr, then it should be stored at 4° C rather than subjected to a freeze–thaw cycle.

Transfection Efficiency

Transfection efficiency varies greatly from cell line to cell line, ranging from 5% to 80% of the cells. Transfection efficiency can be quickly determined by using a plasmid encoding the β-galactosidase gene, such as pEQ176 (24). Twenty-four hours after washing the cells, they can be fixed and stained for expression of the β-galactosidase gene as described below. The pH of the 2× BBS is critical and must be exactly 6.95 or transfection efficiency will be low. We suggest making large batches of 2× BBS, testing the transfection efficiency, and then aliquoting for future use.

Isolation of clonal cell lines that express defective proviruses may also be done and may be necessary if only low levels of virus are secreted. In this case a plasmid encoding resistance to antibiotics such as G418 or hygromycin is cotransfected with the proviral vector. Use a six-well plate and add 0.5 μg of the plasmid encoding the gene for drug resistance to the $CaCl_2$–DNA mixture. Two days after transfection, the selection drug can be added to the cell medium, and about 10–14 days later clones can be isolated. Unfortunately, often expression of HIV proteins is toxic to cells, and

stable clones may be impossible to isolate. For example, expression of Vpr prevents cell proliferation.

RT Assay to Detect Virus Production

A quick and inexpensive way to detect HIV in the cell culture supernatant is to assay for RT activity. Retroviruses contain the virally encoded enzyme RT used to reverse-transcribe their RNA genome into DNA for subsequent integraton into host chromatin. The following protocol is an adaptation of an assay developed by Goff (25) that uses the RT enzyme in the virus to incorporate [32P]dTTP into a poly(A) template using oligo(dT) as a primer in an exogenous reaction, followed by retention of the elongated products on DE-81 paper (Whatman, Clifton, NJ). The assay was further modified for HIV (26), and we have changed it slightly since then.

RT activity can be measured 3–4 days after infection of susceptible cells with HIV or transfection of cells with a proviral vector. Concentration of viral particles in the cell culture medium by centrifugation may be required if a mutated proviral vector is used for transfection. Also, since mutation of murine leukemia virus and HIV Gag and Pol proteins have been shown to result in virions with little or no RT activity (27–29), an alternative method such as an enzyme-linked immunosorbent assay (ELISA) may be needed to detect secreted viral proteins. The $p24^{gag}$ ELISA is more sensitive than the RT assay but uses a monoclonal antibody to specifically detect the $p24^{gag}$ protein. If the provirus under study contains mutations in the capsid region of *gag* ($p24^{gag}$), the antibody may not recognize the protein efficiently. In addition, the antibody in some kits may only detect fully processed $p24^{gag}$ but not p24 in the context of the whole Gag protein. However, in general, perhaps the major advantage of the RT assay over the $p24^{gag}$ assay is the high cost of commercial ELISA kits, which can be prohibitive for routine use in many research laboratories.

Reagents

HIV RT Cocktail: Combine
- 50 m*M* Tris–HCl (pH 8.3)
- 2 m*M* dithiothreitol
- 75 m*M* KCl
- 0.05% (v/v) Nonidet P-40 (Sigma, St. Louis, MO)
- 10 μg/ml poly(A) (Boehringer-Mannheim, Indianapolis, IN)
- 1.5 μg/ml oligo(dT_{15}) (Boehringer-Mannheim)
- 5 m*M* $MgCl_2$

2× SSC: Make up from 20× SCC (30)

[^{32}P]dTTP (10 mCi/ml)

95% ethanol

DE-81 (Whatman) ion-exchange paper

The cocktail can be made in large (10- to 20-ml) batches without the [^{32}P]dTTP, and stored in 1- 2-ml aliquots at $-20°$C. Before using, thaw the amount of cocktail needed (50 μl per sample) and add 1 μl/ml [^{32}P]dTTP. It is not necessary for the [^{32}P]dTTP to be fresh.

1. Collect approximately 100 μl of medium from the transfected cells and centrifuge for 2 min at 3000 *g* in an Eppendorf tube to pellet cell debris, which can increase background signals.
2. Add 10 μl of the clarified supernatant to 50 μl of the cocktail and incubate for 1–2 hr at 37°C. Always include a negative control (medium from nontransfected cells) and, if possible, a positive control, using a known amount of wild-type HIV. Using a 96-well plate to incubate the samples is convenient if many samples are to be tested at once.
3. Cut one square of DEAE paper (DE-81) and one square of blotting paper to the size of the vacuum dot blotter. Place them in the dot blotter (DE-81 on top) and prewet them with 2× SSC. Apply the vacuum to remove any excess liquid. Turn off the vacuum and load 50 μl of each sample into a well. Samples can be quickly and easily loaded onto the vacuum dot blotter from the 96-well plate with a multiwell pipettor. Turn the vacuum on and using a squirt bottle to wash the wells five times with 2× SSC.
4. Carefully remove the DE-81 paper from the dot blotter and wash it twice with about 100 ml of 2× SSC for 5 min each with gentle agitation. Once wet, the DE-81 paper is very fragile. Rinse with 95% ethanol and air-dry. Expose to film with intensifying screens at $-80°$C. For virus with relatively high titers (10^4–10^5 infectious units per milliliter), a short exposure of 3–4 hr is adequate. To detect smaller amounts of virus, an overnight exposure is required.
5. Background problems: If high levels of background are detected in the mock samples, the cocktail can be filtered through a 0.45-μm filter to remove particulate matter. High levels of background in the virus samples, such as small nonspecific spots of radioactivity, may be due to cell debris and the medium should be spun longer at higher speeds before adding it to the cocktail. High amounts of background will interfere with accurate quantitation.
6. Quantitation of the assay: Several options are available for quantitation of the RT assay. After exposure of the filter to film, the filter can be cut and each individual dot can be subjected to liquid scintillation counting. Alternatively, we commonly rely on phosphorimaging of the DE-81 filter (31). Traditional film autoradiographic methods for quantitating the intensity of the "dot" of RT products are limited to a very small dynamic range. In our laboratory film autoradiography combined with densitometry is linear over less than a 25-fold range. The preferred method for quantitating radioactive products is storage phosphorimaging. This method uses a europium halide (Eu · BaFBr) crystal to store the energy liberated from ^{32}P decay. The Eu · BaFBr crystal can then be excited with red light (633 nm) to release the stored energy in the

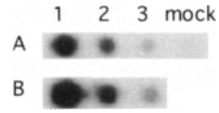


FIG. 2 Reverse transcriptase assay. Columns 1, 2, and 3 show 5-fold serial dilutions of virus. Row A is HIV with a known titer. The mock sample is, as expected, barely visible. Row B shows dilutions of virus collected from transfected cells. By using dilutions of a known virus to generate a standard curve, viral yields from a new infection or transfection can be determined after quantitation by phosphorimaging.

form of purple (390-nm) light. This light is collected and digitized to create a file with greater than 5 $\log_{10}$ (100,000-fold) of dynamic range. Specifically, we have used the Molecular Dynamics PhosphorImager 400A to acquire images and Molecular Dynamics ImageQuant version3.3 software to analyze the images. Microsoft Excel version4.0 is used for data reduction. Regions of interest are created around samples, and "volumes" are calculated as the sum of the intensities within the region minus the product of the area of the region times a background value. We have stored small aliquots of a control virus and use 5-fold dilutions to generate a standard curve for each assay (see Fig. 2).

Titration of HIV-1 Stocks with the MAGI Assay

The MAGI assay was developed as a means of rapidly and quantitatively determining the titer of laboratory-adapted HIV-1 stocks (32). We have also used the assay successfully with HIV-2 and with some SIV strains. The basis of the assay is the ability of the Tat protein of HIV-1 to specifically transactivate the HIV-1 LTR. In the absence of Tat, there is little expression of the LTR. Therefore, expression of the LTR can be used as a marker for the presence of Tat, and therefore, the presence of virus. In addition, expression of Env protein (gp120/gp41) on the cell surface will result in syncytia formation. The assay is represented schematically in Fig. 3.

A cell line called the HeLa–CD4 LTR/β-gal indicator cells was developed for this assay. First, the *CD4* gene was inserted into HeLa cells via a retrovirus vector that also encodes resistance to G418. G418-resistant cells were pooled and sorted for high expression of CD4. The cells were sorted on each of four successive passages by fluorescence-activated cell sorting with a fluorescein-labeled monoclonal antibody to human CD4 until stable expression of CD4 was achieved. The level of expression of CD4 in these cells is at least as high as on SupT1 cells, a human $CD4^+$ tumor cell line.

The HeLa–CD4 cells were then cotransfected with plasmid pJK2 and a plasmid that encodes resistance to hygromycin B (pCMV–hph). pJK2 is a plasmid that con-

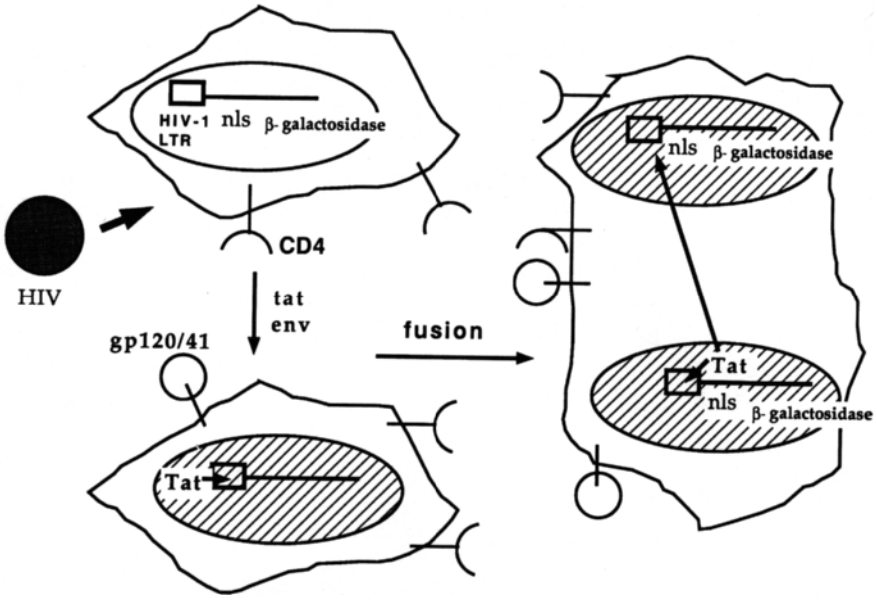


FIG. 3 MAGI assay for viral infectivity. The HeLa–CD4 LTR/β-gal cells are infected with HIV-1 (or HIV-2). Integration of the provirus is followed by production of Tat protein. Tat will then transactivate the LTR, which will lead to 100-fold increased expression of β-galactosidase. The β-galactosidase gene was modified by a nuclear localization signal (nls) at its 5′ end to concentrate the signal. Expression of nls–β-galactosidase will cause the nucleus to turn blue in the presence of X-Gal (see text for details). Expression of the Env protein of HIV-1 will also cause infected and uninfected cells to fuse. In this case all nuclei of the syncytia will turn blue.

tains HIV-1 LTR sequences from −138 to +80 (a minimal promoter and the Tat-responsive region, TAR) upstream of the β-galactosidase gene, followed by the poly(A) signal from SV40 and the enhancer region of SV40. Truncation of the LTR lowered the amount of expression of β-galactosidase in HeLa cells in the absence of Tat without affecting the level of expression in the presence of Tat. The β-galactosidase gene in pJK2 had been altered at its 5′ end so that the protein is targeted to the nucleus (Fig. 3). Nuclear localization of β-galactosidase allows easy visualization of expression of the introduced β-galactosidase gene, as opposed to the faint blue cytoplasmic stain sometimes seen with dead or dying cells.

Several clones were analyzed, and one clone (clone 8) with the lowest amount of background staining of β-galactosidase activity in the absence of Tat was chosen for development of the assay. However, there is some activity of the LTR in the absence of Tat. Nonetheless, the assay can be used to detect virus because the amount of time

that the cells are incubated with X-Gal is limited to the amount of time needed to see the Tat-mediated activation of the LTR, but not the basal level of expression of the LTR.

Reagents

1. *HeLa–CD4 LTR/β-gal indicator cells* can be obtained from the NIAID AIDS Research and Reference Reagent Program (685 Lofstand Lane, Rockville, MD 20850; Cat. No. 1470). The cells are sent for the cost of shipping. The cells are grown in DMEM-10. The cells are G418 and hygromycin resistant. Keep them in selection of 0.2 mg/ml G418 and 0.1 mg/ml hygromycin.

The cells should be split every 5–7 days, when they reach confluence at dilutions of 1:10 to 1:20 by trypsinization. The cells are very sensitive to trypsin and should not be overtreated. Many aliquots of the cells should be frozen after the first passage after receipt, and a new stock should be thawed every 20th passage. When the cells are mistreated (e.g., left overconfluent for the weekend), they express less CD4 and the titers are reduced.

2. *Fixing solution* is 1% formaldehyde and 0.2% glutaraldehyde in PBS. It can be made up in advance and stored in the cold room away from light. For 100 ml use 2.7 ml of formaldehyde (37% solution) and 0.8 ml of glutaraldehyde (25% solution, grade I; Sigma; Cat. No. G-6257).

3. *Staining solution* should be made up fresh from stocks for each use. For every 1 ml of staining solution to be used, combine

950 μl of PBS: See "Transfection Protocol" for the recipe
20 μl of 0.2 *M* potassium ferrocyanide: Keep stock at room temperature protected from light
20 μl of 0.2 *M* potassium ferricyanide: Keep stock at room temperature protected from light
1 μl of 2 *M* $MgCl_2$
10 μl of 40 mg/ml X-Gal: Make up X-Gal in dimethyl sulfoxide and store at −20° C in small aliquots protected from light with aluminum foil. The X-Gal stocks will turn yellow over time. This will not affect the assay. Discard the X-Gal solutions if they turn greenish brown. We have used X-Gal from many different vendors with equal success.

MAGI Assay

1. *Day 1: Plating the cells*—Plate the cells at 0.4×10^5 per 6-mm well (24-well plate) or 0.8×10^5 per 12-well plate. The amount plated will depend on the culture conditions. The cells should be 30% confluent the day after plating. Cells should be plated in DMEM-10. (Cells plated for infection do not require medium with G418 or hygromycin). It is important not to overtypsinize the cells.

2. *Day 2: Prepare the virus*—Virus that has been frozen at −80° C should be

allowed to thaw slowly by placing it at 4° C. Serial 10-fold dilutions of the virus stock should be made with cold DMEM-10. After the dilutions have been made, add DEAE–dextran (Pharmacia) to a final concentration of 30 μg/ml. A stock of 300 μg/ml of DEAE–dextran in Dulbecco's medium without serum can be made up in advance and stored at 4° C for months. (Some virus stocks may do better with higher concentratons of DEAE–dextran up to 100 μ/ml.)

3. *Day 2: Add the virus to the cells*—Remove the medium from the plate of HeLa–CD4 LTR/β-gal indicator cells and add 150 μl of each virus dilution per well for a 24-well plate or 300 μl of virus dilution per well for a 12-well plate. The small volume enhances the infection efficiency. Put the plate back into the 5% CO_2 incubator at 37° C for 2 hr. After the incubation add 1 ml of DMEM-10. It is not necessary to remove the initial inoculum if the virus stock was derived from transfection. However, if the virus stock was derived from infected cells that have been grown in RPMI, the initial inoculum must be removed because RPMI has an adverse effect on the growth of the HeLa–CD4 LTR/β-gal indicator cells.

4. *Day 4: Fix and stain the cells*—Incubate the cells for 40–48 hr. The cells should be just under confluence at the time they are stained. If they are overconfluent, it will be difficult to see the syncytia. To fix the cells, remove the medium and add 2 ml of fixing solution per well for *exactly* 5 min at room temperature. Note: Do *not* leave the fixing solution on the cells for more than 10 min. The β-gal activity decreases dramatically if the fixer is left on too long.

Remove the fixer by aspiration or with a pipette, and wash the cells twice with PBS. To save time, the washing steps can be done by pouring PBS from a bottle or by using a squirt bottle. Sterile technique is no longer required at this stage. Because formaldehyde–glutaraldehyde inactivates live HIV, the plates can be taken out of the BL2/3 facility at this time.

To each well add 350 μl (24-well plate) or 600 μl (12-well plate) of staining solution. Incubate the plate at 37° C for *exactly* 50 min. Extended incubations will result in background staining on noninfected cells. At the end of 50 min, remove the staining solution and wash the plates twice with PBS. The blue cells can now be counted. Alternatively, the plates can be stored at 4° C and counted up to several weeks later.

5. *Day 4 or later: Counting the blue cells*—Count the blue cells at 40× magnification using moderate light. With practice, an entire 12-well plate can be scanned in about 15 min. At least two dilutions of virus should be counted to make sure that the assay is linear. At very high multiplicities (over 1000 blue cells per well) the assay is nonlinear due to virus-induced cell death. The most useful information is derived from wells that contain 50–500 blue cells.

It is best to be conservative in counting blue cells. That is, a syncytium is counted as only one event, regardless of the number of nuclei in the syncytium. Likewise, two adjacent blue cells should be counted as only one event. Score only dark blue cells—very pale blue cells are more likely to be due to the basal level of activity. Always include several wells of uninfected cells to scan in parallel.

6. *Expected results*—The titer of virus (per milliliter) is derived by multiplying the dilution by the number of blue cells at that dilution. For example, if there are 30 blue cells in the well where 1 μl of virus was used for infection, then the titer is $30 \times 1000 = 3.0 \times 10^4$ infectious units per milliliter. Cultures of mock-infected cells should contain no blue cells, or at most one to three. Virus collected from a good transfection will have a titer of 5×10^4 to 1×10^6. Virus collected from acutely infected T cells will have a titer of 1×10^5 to 2×10^6. Virus with various mutations will have reduced titers.

The MAGI assay has been used to titer the commonly used HIV-1 laboratory strains LAI, IIIb, MN, and NL4-3. It is also used routinely to titer HIV-2 (ROD strain). Some patient isolates can also be titered with the MAGI assay. In general, if an isolate will infect a T-cell line (a syncytium-inducing strain), it can be titered with the MAGI assay. However, the majority of fresh patient isolates, and none of the macrophage-tropic isolates, will infect the HeLa–CD4 LTR/β-gal indicator cells. Therefore, this is not the method of choice to quantify virus with an unknown tropism. However, with laboratory-adapted isolates the assay is as sensitive as end point dilution in a T-cell line.

As an alternative protocol, instead of using cell-free virus, infected cells (1×10^5 and various dilutions) can be layered directly on the monolayer. In this case, do not use DEAE–dextran. It is not necessary to wash off the unattached cells before fixing.

Summary

We describe here a series of techniques used in our laboratory for determining the effects of mutations in proviral sequences on virus production and infectivity. By combining RT values and the MAGI assay, infectivities of wild-type and mutant viruses can be compared in single-step assays to discern the effects on individual parts of the virus life cycle.

Acknowledgments

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References

1. D. Benko, S. Schwartz, G. Pavlakis, and B. Felber, *J. Virol.* **64,** 2505 (1990).
2. J. Salfeld, H. G. Gottlinger, R. A. Sia, R. E. Park, J. G. Sodroski, and W. A. Haseltine, *EMBO J.* **9,** 965 (1990).
3. M. Guyader, M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon, *Nature (London)* **326,** 662 (1987).
4. L. Chakrabarti, M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo, *Nature (London)* **328,** 543 (1987).
5. B. R. Cullen and W. C. Greene, *Virology* **178,** 121 (1990).
6. H. W. Kestler, D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers, *Cell (Cambridge, Mass.)* **65,** 651 (1991).
7. M. Popovic, M. G. Sarngadharan, E. Reed, and R. C. Gallo, *Science* **224,** 497 (1984).
8. W. A. O'Brien, Y. Koyanagi, A. Namazie, J. Q. Zhao, A. Diagne, K. Idler, J. A. Zack, and I. S. Chen, *Nature (London)* **348,** 69 (1990).
9. S. S. Hwang, T. J. Boyle, H. K. Lyerly, and B. R. Cullen, *Science* **253,** 71 (1991).
10. T. Shioda, J. A. Levy, and M. C. Cheng, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 9434 (1992).
11. P. Westervelt, D. B. Trowbridge, L. G. Epstein, B. M. Blumberg, Y. Li, B. H. Hahn, G. M. Shaw, R. W. Price, and L. Ratner, *J. Virol.* **66,** 2577 (1992).
12. M. J. De, B. Brichacek, D. Salaun, J. C. Chermann, and I. Hirsch, *J. Virol.* **66,** 6797 (1992).
13. L. Ratner, A. Fisher, L. L. Jagodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and S. F. Wong, *AIDS Res. Hum. Retroviruses* **3,** 57 (1987).
14. A. Adachi, H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin, *J. Virol.* **59,** 284 (1986).
15. K. Peden, M. Emerman, and L. Montagnier, *Virology* **185,** 661 (1991).
16. F. Mufstafa and H. L. Robinson, *J. Virol.* **67,** 6909 (1993).
17. E. F. Terwilliger, E. Langhoff, D. Gabuzda, E. Zazopoulos, and W. A. Haseltine, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10971 (1991).
18. G. Myers, J. A. Berzofsky, B. Korber, R. F. Smith, and G. N. Pavlakis, "Human Retroviruses and AIDS, 1992" Los Alamos National Laboratory, Los Alamos, New Mexico, 1992.
19. J. V. Garcia and A. D. Miller, *AIDS Res. Hum. Retroviruses* **10,** 47 (1994).
20. M. Adams, L. Sharmeen, J. Kimplon, J. M. Romeo, J. V. Garcia, B. M. Peterlin, M. Broudine, and M. Emerman, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 3862 (1994).
21. K. A. Page, N. R. Landau, and D. R. Littman, *J. Virol.* **64,** 5270 (1990).
22. C. Chen and H. Okayama, *Mol. Cell. Biol.* **7,** 2745 (1987).
23. P. Lewis, M. Hensel, and M. Emerman, *EMBO J.* **11,** 3053 (1992).
24. B. J. Biegale and A. P. Geballe, *Virology* **183,** 381 (1991).
25. S. Goff, P. Traktman, and D. Baltimore, *J. Virol.* **38,** 239 (1981).
26. R. L. Willey, D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martin, *J. Virol.* **62,** 139 (1988).
27. P. Schwartzberg, J. Colicelli, M. L. Gordon, and S. P. Goff, *J. Virol.* **49,** 918 (1984).
28. K. Mergener, M. Facke, R. Welker, V. Brinkmann, H. R. Gelderblom, and H. Ktausslich, *Virology* **186,** 25 (1992).

29. J. Smith, M. Cho, M. Hammarskjold, and D. Rekosh, *J. Virol.* **64,** 2743 (1990).
30. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
31. R. F. Johnston, S. C. Pickett, and D. L. Barker, *Electrophoresis* **11,** 355 (1990).
32. J. Kimpton and M. Emerman, *J. Virol.* **66,** 2232 (1992).

[13] Quantitation of HIV-1 RNA in Plasma or Serum Samples

V. Natarajan and N. P. Salzman

Introduction

The polymerase chain reaction (PCR) has been used to quantitate levels of RNA and DNA from a variety of samples. However, it is only under ideal conditions that a direct correlation can be demonstrated between the amount of starting target material and the amount of PCR product (1, 2). With clinical samples the presence of inhibitors of PCR in samples and small variations in amplification efficiency between different samples make it difficult to accurately estimate the amount of the specific sequence present in the starting material (3–7). To avoid these problems, internal standards that share the same primers as the target RNA, but that contain either a deletion or an insertion, have been included in the reactions so that the products obtained from the standard and the target could be distinguished (8–13). This method, however, does not control for the variable loss of RNA during purification steps. It has been estimated that, on average, 36% of the RNA sample can be lost due to the extraction procedures used (14). To account for this variable loss, the PCR product from RNA that is specified by the gene under study is often compared to another RNA species that is constitutively expressed (15, 16). While such a control can be used with cellular RNA, it is not available for plasma or serum samples.

A variation in the use of an internal control is the competitive PCR procedure (17). In this method varying known amounts of an internal standard are added to equal aliquots of the sample containing the unknown target sequence. The internal standard and the target sequence compete equally for primer binding and amplification in the PCR. Variables such as the efficiency of amplification and the number of cycles will have the same effect on both templates. Equal amounts of products will be formed when the initial concentrations of the templates are equal. Experimentally, the ratio of products formed can be determined and the equivalence point can be calculated. This method has been successfully used to quantify the amount of human immunodeficiency virus type 1 (HIV-1) RNAs in clinical samples (18, 19). However, this method does not control the variations in RNA recovery from sample to sample.

We have developed a method to measure the HIV-1 RNA in patients' plasma or sera using an infectious mutant virus as an internal control (20). The mutant virus, VX-46, has a 25-bp insert in a conserved region between the primer binding and major splice donor sites. To utilize this virus as an internal control, different dilutions of this virus were added to aliquots of the plasma sample to be measured and RNA was isolated and reverse-transcribed to cDNA. PCR was performed with primers

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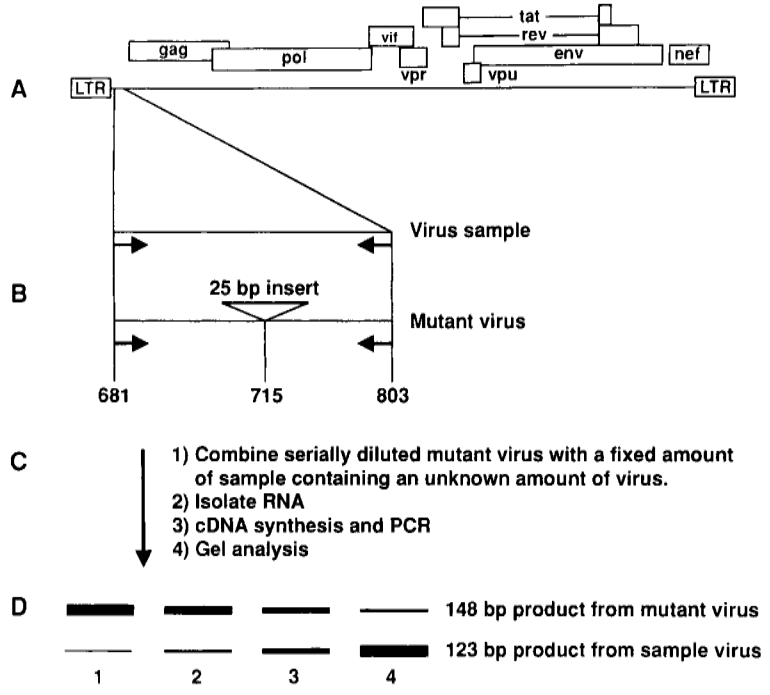

FIG. 1 Outline of the ICVPCR protocol. (A) Genome of HIV-1 with various gene products. (B) Location of the insert present in the mutant virus. The arrows represent the primers used in PCR. (C and D) Pattern of the expected PCR products from a typical ICVPCR experiment. LTR, Long terminal repeat.

selected to include the sequences on either side of the insert contained in the externally added virus. The DNA product from the control virus is 25 bp longer that that from the virus present in plasma. The amount of viral RNA present in a plasma sample is calculated after the PCR-amplified products are separated by gel electrophoresis (Fig. 1). Unlike other quantitative PCR assays, this internally controlled virion PCR (ICVPCR) assay eliminates errors introduced by variable recovery during the RNA purification step and enhances the accuracy of the assay. This method can also be applied to other DNA or RNA viruses.

Methods

Generation of Mutant HIV-1 Virus

A mutant virus (VX-46) was constructed by standard procedures (21) with a 25-bp DNA insert (5′–AGACATTAGACGCGTCTAGACGCG–3′) at nucleotide position

715 of pNL4.3 (22). This is a well-conserved region between the primer binding and Gag initiation sites of HIV-1 (23). The HIV-1 nucleotide numbering used is according to HIVNL4.3 (Genbank accession No. M19921). The mutant DNA was transfected into 293 cells, and the culture fluid at 48 hr after transfection was used to infect MT-2 cells (24). The mutant virus (VX-46) grew with kinetics similar to that of the wild-type virus (V. Natarajan, unpublished observations, 1993), and the cell-free virus was harvested, aliquotted, and stored at −70°C. Each aliquot was used once in ICVPCR experiments. The amount of virus in the culture supernatant was estimated by a p24 antigen enzyme-linked immunosorbent assay (ELISA) using Coulter (Hialeah, FL) reagents following the manufacturer's recommended protocol.

PCR Primers

The following ICVPCR primers were synthesized with an Applied Biosystems (Foster City, CA) DNA synthesizer. The position of the primer in the HIVNL4.3 sequence is given in parentheses.

ICVPCR-9: 5′-TCCCTGCTTGCCCATACTA-3′ (908 to 890, − strand)
ICVPCR-16: 5′-ATCTCTCGACGCAGGACT-3′ (681 to 698, + strand)
ICVPCR-17: 5′-GCTCTCGCACCCATCTCT-3′ (803 to 786, − strand)
ICVPCR-18: 5′-ACTAGCGGAGGCTAGAAGGA-3′ (765 to 784, + strand)

RNA Isolation and cDNA Synthesis

The sample volume was adjusted to 200 μl with cell culture medium and the following reagents were added in the order shown.

Sample	200 μl
6 *M* guanidium thiocyanate	400 μl
2 *M* sodium acetate (pH 4.0)	60 μl
Phenol–chloroform–isoamyl alcohol (25:24:1)	600 μl

The samples were mixed after each addition, incubated on ice for 20 min, and centrifuged at room temperature for 20 min. The aqueous phase was aspirated and mixed with 10 μg of carrier RNA (Sigma, St. Louis, MO; Cat. No. R-8508) and precipitated with an equal volume of isopropanol. The guanidium thiocyanate was prepared in 42 m*M* sodium citrate buffer (pH 4.0) containing 0.83% (v/v) sodium lauroylsarcosine and 0.2 m*M* 2-mercaptoethanol.

RNA recovered by centrifugation was washed with cold 70% ethanol and dissolved in 8 μl of DNase buffer containing 50 m*M* Tris (pH 8.0), 5 m*M* $MgCl_2$, and 10 U of RNase-free DNase (Boehringer-Mannheim, Indianapolis, IN; Cat. No. 776785) and incubated at 37°C for 15 min. The DNase was inactivated at 80°C

for 10 min and the RNA was used for cDNA synthesis in a 20-μl reaction using a cDNA cycle kit obtained from Invitrogen (San Diego, CA; Cat. No. K131001). Eight microliters of DNase-treated RNA was denatured by the addition of 2 μl of 100 m*M* methyl mercury hydroxide, and after a 5-min incubation at room temperature, 2.5 μl of 0.7 *M* 2-mercaptoethanol was added and the reaction was kept on ice. The cDNA synthesis was carried out in a 20-μl reaction mixture containing 100 m*M* Tris (pH 8.3), 40 m*M* KCl, 10 m*M* $MgCl_2$, 10 U of RNase inhibitor, 1.25 m*M* deoxynucleotide triphosphates (dNTPs), 40 pmol of ICVPCR-9 primer, and 5 U of avian myeloblastosis virus, reverse transcriptase and was incubated at 42°C for 1 hr. The reaction mixture was then heated to 95°C for 3 min and cooled on ice for 2 min, and 5 additional units of reverse transcriptase were added and the incubation was continued for an additional 1 hr at 42°C. Finally, the reaction was heated to 95°C for 3 min and the cDNA was either used for the PCR or stored at −20°C. The cDNA synthesis can also be carried out without the methyl mercury hydroxide denaturation step, but 4 m*M* of sodium pyrophosphate was added in the reaction.

PCR

Five microliters of cDNA was used in a reaction containing 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% (w/v) gelatin, 0.2 m*M* dNTPs, 25 pmol of primers ICVPCR-16 and -17, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a final volume of 50 μl. The samples were amplified in a Perkin–Elmer Cetus thermocycler (9600) with the following PCR cycle program: one cycle, 94°C for 60 sec, 55°C for 10 sec, and 72°C for 30 sec; 30 cycles, 94°C for 15 sec, 55°C for 30 sec, and 72°C for 60 sec; and a final incubation at 72°C for 10 min. The samples were then stored at 4°C until analysis. The competitor plasmid (pA1) DNA used to estimate the amount of cDNA from VX-46 virus contained pNL4.3 nucleotide sequences from 501 to 1448.

Analysis of the PCR Product

Fifteen microliters of PCR product was added to 5 μl of buffer containing 60 m*M* NaCl, 40 m*M* EDTA, and 0.5 pmol of 5′-^{32}P-labeled ICVPCR-18. The hybridization of the ^{32}P-labeled oligonucleotide to PCR-generated DNA was carried out in a thermocycler with the following PCR cycle program: one cycle, 94°C for 5 min, 55°C for 5 min, and 25°C for 2 min. Two microliters of 10× dye was added to the sample and 10 μl was separated in a 10% polyacrylamide gel, and autoradiographs were developed by exposing the gel to X-Omat AR film (Eastman-Kodak, Rochester, NY) with an intensifying screen (Cronex Lightning Plus, DuPont, Wilmington, DE) (25). The amount of radioactivity present in each band was quantitated using a Fuji Medical Systems (Stamford, CT) BAS1000 Bio-Imaging device by exposing the gel to a Fuji film Imaging Plate as described by Johnson *et al.* (26).

Estimation of RNA Present in a VX-46 Virus Preparation

RNA was isolated from VX-46 virus containing 100 pg of p24 antigen, and cDNA was synthesized using standard conditions. To establish and standardize the assay, cDNA from VX-46 virus was PCR-amplified in the presence of different amounts of plasmid DNA (pA1) containing pNL4.3 nucleotide sequences from 501 to 1448. The PCR product was hybridized with 5′-^{32}P-labeled ICVPCR-18 and separated in an acrylamide gel. As shown in Fig. 2A, the selected primers gave the predicted 123- and 148-bp DNA PCR products from the wild-type plasmid and mutant viral sequences, respectively. In some experiments additional minor bands can be seen. Most likely, these are heteroduplexes formed between the two expected bands. Also, the unused excess primer from the cDNA synthesis step can participate in the PCR reaction and generate a longer product. These bands do not interfere with accurate quantitation, because the quantitation by this method is based on relative levels of bands from target and competitive templates, not on the absolute amounts (18, 19). The amounts of radioactivity in the specific bands were determined and the ratios between the amounts of radioactivity present in wild-type and mutant DNA bands were plotted against the amount of input wild-type competitor plasmid DNA (Fig. 2B). The copy number of the mutant viral RNA was calculated from the plot for the ratio of 1 between the amount of radioactivity present in wild-type and mutant DNA bands. We calculate that the RNA isolated from the mutant virus with 100 pg of p24 antigen has 285,000 copies of viral RNA. The copy numbers derived from the RNAs isolated from mutant virus containing 25 pg and 500 fg of p24 antigen confirm that the mutant virus with 100 pg of p24 antigen has, on average, 300,000 copies of RNA.

A virus preparation with 100 pg of p24 would be calculated to contain about 1 million HIV particles, or 2 million copies of RNA (27, 28). Thus, the copy numbers determined in our experiment represent approximately 15% of the theoretical value. This reflects a loss of RNA during extraction and lower than the theoretical yields in the preparation of cDNA. However, the quantitation by competitive PCR relies on the relative levels of wild-type and mutant templates, rather than the absolute amount. Therefore, once the amount of RNA in a mutant virus preparation is determined, the recovery of RNA in each ICVPCR experiment does not affect the outcome of the results.

ICVPCR to Determine the Level of HIV-1 RNA in Plasma

Using this standardized VX-46 virus as the competitor during the RNA isolation, the amount of HIV-1 viral RNA in a patient's plasma was estimated. The whole blood samples were collected from HIV-1–seropositive patients in the presence of acid–citrate–dextrose or EDTA as an anticoagulant. The plasma was separated by centrifugation and stored at −70°C until use. Serum samples can also be used in this assay. Aliquots (100 μl) of serial dilutions of the mutant virus, VX-46, were added to 100 μl

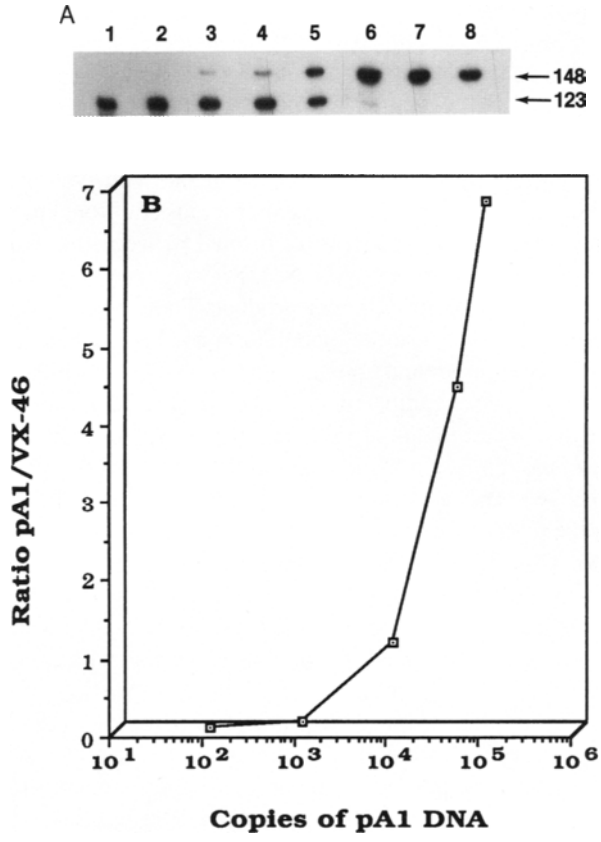

FIG. 2 Estimation of the RNA isolated from the mutant virus VX-46. (A) RNA isolated from the mutant virus containing 100 pg of p24 antigen was used to synthesize cDNA in a 60-μl reaction. Two microliters of the cDNA were used in the PCR containing various amounts of competitor wild-type DNA (pA1) containing HIVNL4.3 sequences from nucleotides 501 to 1448. The PCR products were hybridized with a ^{32}P-labeled probe and autoradiographed as described in "Methods." The copies of competitor DNA in the reactions were 10^6 (lane 1), 5×10^5 (lane 2), 10^5 (lane 3), 5×10^4 (lane 4), 10^4 (lane 5), 10^3 (lane 6), 10^2 (lane 7), and 0 (lane 8). The sizes shown on the right were the PCR products (in base pairs) from mutant and wild-type sequences, respectively. (B) The amounts of radioactivity present in bands corresponding to wild-type and mutant PCR products were estimated by exposing the gel to a storage Phosphor screen and quantitated using a Fuji Medical Systems BAS1000 device. The ratio between the amount of radioactivity present in wild-type (pA1) and mutant (VX-46) DNA bands was plotted against the amount of wild-type DNA used in the PCR. Based on the plot, 2 μl of cDNA has 9500 copies, which means that the mutant virus containing 100 pg of p24 antigen has 285,000 copies of RNA.

of patient's plasma or serum. Commonly, about four dilutions are sufficient to determine the equivalence point between the mutant and sample viral RNAs in PCR. If the equivalence point cannot be determined by these dilutions, the amount of the added mutant virus was adjusted.

RNA was extracted from the samples containing patient serum and the added mutant virus and was used for cDNA synthesis and for PCR. The PCR products were hybridized with the ^{32}P-labeled ICVPCR-18 and separated in a polyacrylamide gel. The amount of radioactivity present in each band was estimated and the ratio of radioactivity present in the mutant and wild-type (patient) DNA bands was plotted against the input mutant viral RNA. The results of such an analysis are shown in Fig. 3. To determine the reproducibility of the procedure, the amount of viral RNA

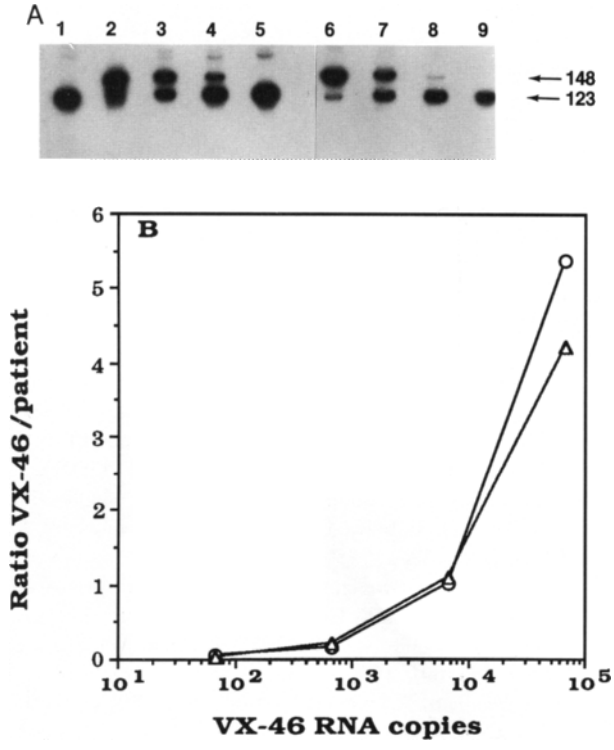

FIG. 3 ICVPCR for the estimation of HIV-1 RNA in a patient's plasma. (A) To 100-μl aliquots of a patient's plasma, different dilutions of mutant virus (VX-46) containing 0 (lane 1), 24 (lanes 2 and 6), 2.4 (lanes 3 and 7), 0.24 (lanes 4 and 8), and 0.024 (lanes 5 and 9) pg of p24 antigen were added. RNA isolation, cDNA synthesis, PCR, and gel analysis for lanes 1–5 and 6–9 were carried out by two separated experiments on different days. The size of DNA products from mutant (148) and wild-type (123) virus are shown on the right. (B) The radioactivity present in each band was quantitated and plotted as described in "Methods." The data for Δ———Δ were from lanes 2–5 and the data for O———O were from lanes 6–9.

in a plasma sample was estimated by performing the entire assay on two different days. We obtained RNA values of 60,000 and 68,000 copies per milliliter of plasma, demonstrating that the ICVPCR assay is a reliable and reproducible method.

Conclusion

By adopting the modified method described here, the mutant virion can serve the role of both a control for the RNA extraction procedure as well as a source of competitive RNA template in the PCR. Any inhibitors present in the sample which will affect the cDNA synthesis and/or PCR will affect equally the product formed from the RNA present in the sample and the mutant viral RNA, thereby increasing the accuracy of the PCR quantitation. This method could also provide a guide for determining whether an inhibitor of reverse transcriptase PCR is present in the sample, thus suggesting the use of a procedure to remove the inhibitor.

References

1. F. Ferre, *PCR Methods Appl.* **2,** 1 (1992).
2. M. A. Innis and D. H. Gelfand, *in* "PCR Protocols. A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 3. Academic Press, San Diego, 1990.
3. M. Holodniy, S. Kim, D. Katzenstein, M. Konrad, E. Groves, and T. C. Merigan, *J. Clin. Microbiol.* **29,** 676 (1991).
4. E. Beutler, T. Gelbart, and W. Kuhl, *BioTechniques* **9,** 166 (1990).
5. F. Coutlée, P. Saint Antoine, C. Olivier, A. Kessous-Elbaz, H. Voyer, F. Berrada, P. Bégin, L. Giroux, and R. Viscidi, *J. Infect. Dis.* **164,** 817 (1991).
6. S. F. An and K. A. Fleming, *J. Clin. Pathol.* **44,** 924 (1991).
7. C. T. Comey, B. Budowle, D. E. Adams, A. L. Baumstark, J. A. Lindsey, and L. A. Presley, *J. Forensic Sci.* **38,** 239 (1993).
8. A. M. Wang, M. V. Doyle, and D. F. Mark. *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9717 (1989).
9. M. Becker-André and K. Hahlbrock, *Nucleic Acids Res.* **17,** 9437 (1989).
10. J. Chelly, D. Montarras, C. Pinset, Y. Berwald-Netter, J.-C. Kaplan, and A. Kahn, *Eur. J. Biochem.* **187,** 691 (1990).
11. N. C. Bergenhem, P. J. Venta, P. J. Hopkins, H. J. Kim, and R. E. Tashian, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 8798 (1992).
12. S. Aoki-Sei, R. Yarchoan, S. Kageyama, D. T. Hoekzema, J. M. Pluda, K. M. Wyvill, S. Broder, and H. Mitsuya, *AIDS Res. Hum. Retroviruses* **8,** 1263 (1992).
13. P. D. Siebert and J. W. Larrick, *Nature (London)* **359,** 557 (1992).
14. S. Menzo, P. Bagnarelli, M. Giacca, A. Manzin, P. E. Varaldo, and M. Clementi, *J. Clin. Microbiol.* **30,** 1752 (1992).
15. M. R. Green, T. Treisman, and T. Maniatis, *Cell (Cambridge, Mass.)* **35,** 137 (1983).
16. S. H. Orkin, S. E. Antonarakis, and H. H. Kazazian, Jr., *J. Biol. Chem.* **259,** 8679 (1984).

17. G. Gilliland, S. Perrin, K. Blanchard, and H. F. Bunn, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2725 (1990).
18. M. Clementi, S. Menzo, P. Bagnarelli, A. Manzin, A. Valenza, and P. E. Varaldo, *PCR Methods Appl.* **2,** 191 (1993).
19. M. Piatak, Jr., M. S. Saag, L. C. Yang, S. J. Clark, J. C. Kappes, K.-C. Luk, B. H. Hahn, G. M. Shaw, and J. D. Lifson, *Science* **259,** 1749 (1993).
20. V. Natarajan, R. J. Plishka, E. W. Scott, H. C. Lane, and N. P. Salzman, *PCR Methods Appl.* **3,** 346-350 (1994).
21. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
22. A. Adachi, H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin, *J. Virol.* **59,** 284 (1986).
23. G. Myers, B. Korber, J. A. Berzofsky, R. F. Smith, and G. N. Pavlakis, "Human Retroviruses and AIDS: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences." Los Alamos National Laboratory, Los Alamos, New Mexico, 1992.
24. S. Harada, Y. Koyanagi, and N. Yamamoto, *Science* **229,** 563 (1985).
25. M. C. Psallidopoulos, S. M. Schnittman, L. M. Thompson III, M. Baseler, A. S. Fauci, H. C. Lane, and N. P. Salzman, *J. Virol.* **63,** 4626 (1989).
26. R. F. Johnston, S. C. Pickett, and D. L. Barker, *Electrophoresis* **11,** 355 (1990).
27. A. S. Bourinbaiar, *Nature (London)* **349,** 111 (1991).
28. A. S. Bourinbaiar, *AIDS Res. Hum. Retroviruses* **8,** 1545 (1992).

[14] Detection of Cell Fusion Mediated by the Envelopes of Human Retroviruses by Transactivation of a Reporter Gene

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Introduction

Retroviral Envelopes and Membrane Fusion

Membrane fusion allows retroviruses to enter cells and initiate their replication (reviewed in Refs. 1 and 2). This key step of the viral cycle is triggered by the interaction of the viral envelope glycoprotein with a cellular receptor, the CD4 molecule in the case of the human immunodeficiency virus (HIV). The subsequent events that eventually lead to the coalescence of the cellular and viral lipid bilayers are not fully understood. Conformational changes of the envelope and/or receptor are probably required as well as participation of other cellular proteins (2). Membrane fusion takes place either after endocytosis of the virus–receptor complex in the acidic context of the late endosome [e.g., murine leukemia virus (MLV)] or directly at the plasma membrane at neutral pH (e.g., HIV). In the latter case multinucleated giant cells—syncytia—can be detected in cocultures of infected cells with cells expressing the viral receptor, and arise from fusions between the two (3). The apparent differences in the syncytium-forming ability of HIV-permissive cell lines could reflect the involvement of accessory molecules, but the same fusion mechanism is probably involved in virus entry and syncytium formation (2).

The ability to mediate cell fusion is therefore a crucial property of envelope glycoproteins, and its blocking can be the mode of action of antiviral agents or neutralizing antibodies. Assays studying this step of the viral cycle are important with respect to many aspects of basic and applied research.

Assays to Detect Cell Fusion

Fusion induced by retroviral envelopes can be detected by direct microscopic observation of cell cultures. The visualization of syncytia can be eased by simple histological staining, such as the May–Grünwald–Giemsa treatment, which results in differential staining of the cytoplasm and the nucleus. Direct observation is simple and efficient, but limited in sensitivity and specificity. Adherent cell lines such as HeLa, COS, and NIH 3T3 often spontaneously form multinucleated structures, probably

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through incomplete mitosis. These events cannot be distinguished morphologically from specific cell–cell fusions. Also, the reading of such assays can be somewhat tedious, especially when fusion events are rare or if quantitative results are sought.

More recently, biophysical assays based on the redistribution of fluorescent dyes have been developed to detect cell fusion. While these techniques are extremely valuable for studying fusion mediated by many viruses (e.g., influenza), there seem to be technical difficulties in applying them to retroviruses. Also, these techniques require sophisticated apparatus and do not provide results at the individual cell level.

We have sought to improve the sensitivity and specificity of the direct fusion assays by introducing a silent reporter gene in one cell type, bearing the receptor, for example, and a transactivator in the potential fusion partner, bearing the viral envelope. Envelope/receptor-mediated fusion results in the transactivation of reporter gene expression detected by an appropriate assay.

Fusion Assay Based on the Transactivation of a Reporter Gene

Chronically HIV-infected lymphocytes represent a convenient source of envelope-expressing cells. Among other HIV proteins expressed by these effector cells is the Tat transactivator, an 86-amino-acid nuclear protein that exerts tight transcriptional control on the HIV promoter located in the long terminal repeat (LTR) (for a review see Ref. 4). The mode of action of Tat is not completely understood, but it must bind to a specific RNA sequence at the 5′ end of viral mRNAs, termed the transactivation-responsive (TAR) sequence, in order to cooperate with cellular transcription factors and increase the initiation and/or elongation of viral transcripts (5, 6). The basal level of expression of viral or reporter genes placed under the transcriptional control of the HIV-1 LTR is generally extremely low and can be induced over a 100-fold in the presence of Tat. This property of the HIV-1 LTR makes it an excellent inducible promoter.

The bacterial β-galactosidase gene (*lacZ*) was chosen as a reporter for the sensitivity and simplicity of the detection of its product (see below). We and others had previously established cell lines allowing the rapid titration of infectious HIV by an *in situ* β-galactosidase assay (7–9). These were derived by stable transfection of the *lacZ* gene placed under the transcriptional control of the HIV-1 LTR into the HeLa–CD4 cell line, which can be infected by laboratory-adapted strains of HIV (10). After virus entry the RNA genome is reverse-transcribed and integrated as a proviral DNA in the target cell chromosomes. Since Tat is expressed before the HIV structural proteins, transactivation of the LTR–lacZ reporter gene can precede the release of viral particles. An infected cell is detected by its high level of β-galactosidase activity and is stained blue after incubation with the X-Gal substrate (see below). The number of infected cells due to a single round of infection can be scored as early as 16 hr after contact with the virus.

We observed that LTR–lacZ transactivation was very efficient when target cells were fused by polyethylene glycol (PEG) treatment to cells expressing Tat (Fig. 1). The nuclear localization signal is able to direct a significant fraction of the Tat mole-

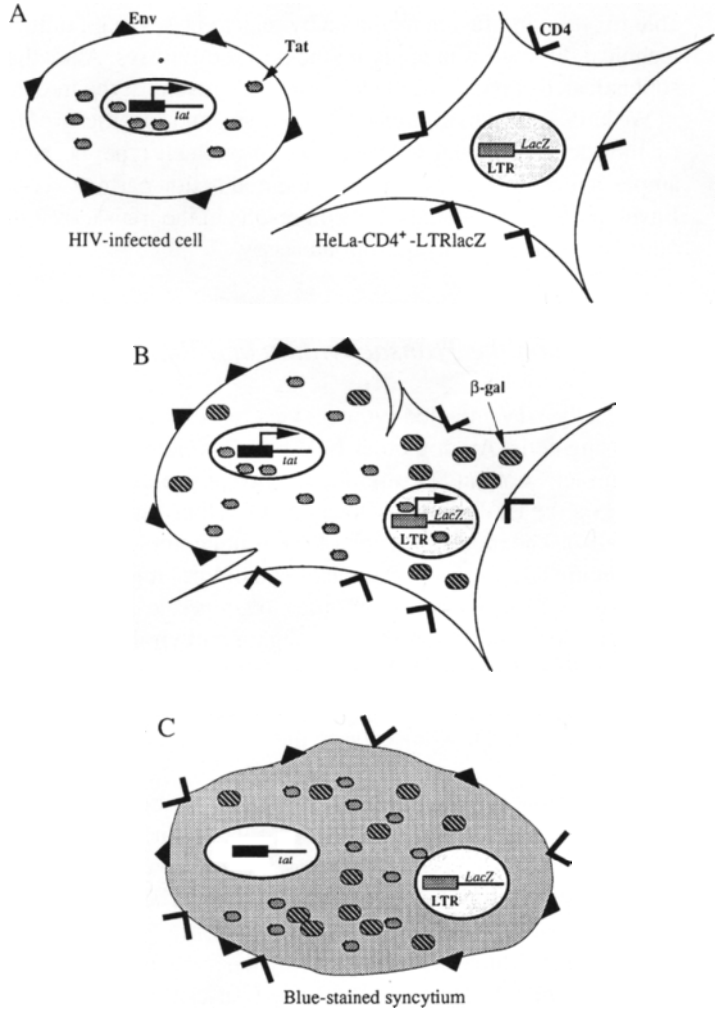

FIG. 1 Detection of cell fusion by transactivation of a LTR–lacZ reporter gene. Cells expressing HIV-1 Env and Tat (e.g., HIV-infected cells) are cocultured with HeLa–CD4$^+$ cells transfected with a HIV-1 LTR–lacZ plasmid (A). Upon fusion of the two cell types, Tat diffuses to the target cell nucleus and transactivates *lacZ* expression (B). This results in β-galactosidase (β-gal) production, and a blue-stained syncytium is detected on an *in situ* X-Gal assay (C).

cules to the nuclei bearing the LTR–lacZ transgene, which results in an increase in β-galactosidase acitivity in the syncytium, detectable by a blue staining upon an X-Gal assay (11). In contrast, the mere contact of Tat^+ and LTR–lacZ cells, where envelope/receptor-mediated fusion is not involved, does not lead to detectable transactivation (Fig. 2C and D).

This assay, based on complementation for the transactivation of a reporter gene, could be used to detect with good sensitivity and absolute specificity fusion events between $CD4^+$ and HIV-infected cells (11). Other types of LTR–lacZ target cells were developed to study HIV tropism, as well as techniques to test the fusogenic ability of mutant HIV envelopes. Finally, we showed that the same type of assay could be used to detect cell fusion events mediated by the envelope of another retrovirus, the human T-cell lymphotrophic virus type I (HTLV-I).

Establishment of LTR–lacZ Target Cell Lines

The sensitivity and specificity of the fusion assay depend on the target LTR–lacZ cell lines. The basal level of expression of *lacZ* must be as low as possible—at best, undetectable—and the LTR–lacZ transgene must be highly responsive to transactivation by Tat. Therefore, target cell lines must be carefully selected, cloned, and checked for these two parameters.

We have used the following procedure to derive LTR–lacZ targets from different cell types of human (HeLa and HeLa–CD4), simian (COS and COS–CD4), murine (NIH 3T3–CD4), and bovine (MDBK) origins. Since these cell lines differ widely in their medium and serum requirements, transfection, and plating efficiencies, only the main aspects of the selection process are outlined here.

1. Cells are cotransfected with the LTR–lacZ and drug resistance plasmids (ratio, 20:1) by a standard calcium phosphate precipitation technique. The LTR–lacZ plasmid we used (11) has a truncated version of the HIV-1 LTR retaining intact NF-κB and SP1 binding sites and TAR sequence (positions −139 to +82 of the LAI isolate, corresponding to a *Sca*I–*Hin*dIII fragment).
2. Forty-eight to 72 hr after transfection the cells are divided into 10-cm petri dishes at different dilutions (e.g., 10^{-1} to 10^{-3}) and the appropriate selection is applied until individual colonies can be picked. We have used neomycin, hygromycin B, or histidinol resistance to establish the different LTR–lacZ cell lines.
3. Ten to 30 colonies are picked with glass cylinders and expanded.
4. β-galactosidase background is tested on a subconfluent 35-mm plate (six-well) by an *in situ* X-Gal assay (see below). Ideally, no blue cells should be detected after overnight incubation at 37°C. However, cells can be used if a null background can be maintained for at least 1–2 hr of incubation.
5. Human or simian LTR–lacZ cells can be tested by transient transfection of the

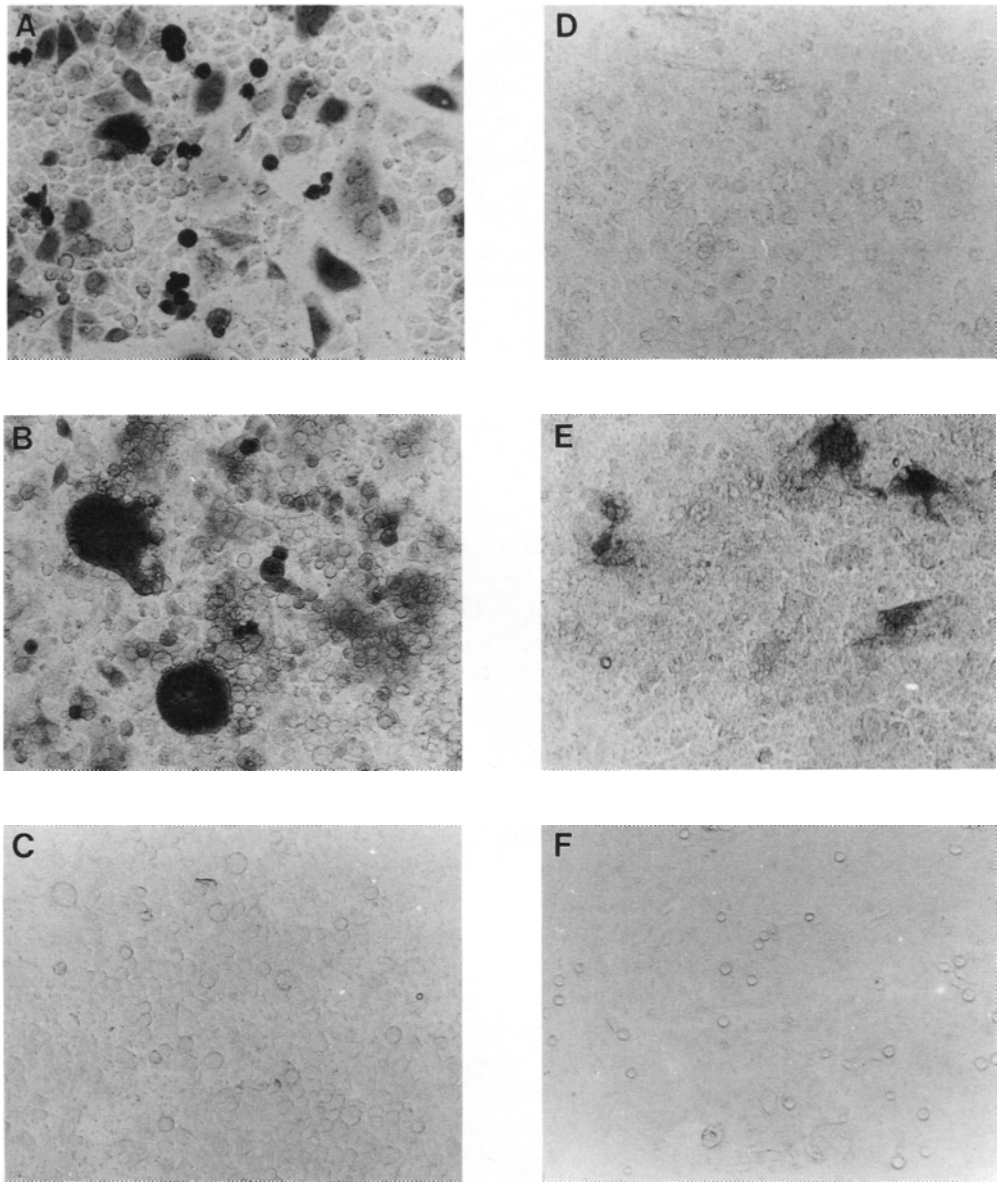

FIG. 2 Detection of HIV–env/CD4-dependent fusion. HeLa–CD4–LTR–lacZ were cocultured with cells expressing (A) the HIV-1 envelope (H9/IIIB, chronically infected T cells) or (B) HIV-2 envelope (CEM/HIV-2ROD, chronically infected T cells) or (C) uninfected T cells expressing Tat (Jurkat–Tat). (D) $CD4^-$ HeLa–LTR–lacZ cells were cocultured with H9/IIIB cells. Heterokaryons formed between 3T3–CD4 and (E) HeLa–LTR–lacZ or (F) COS–LTR–lacZ cells were cocultured with H9/IIIB cells. The X-Gal staining was carried out overnight at 4°C, and photographs were taken under 80× magnification.

tat gene driven by a strong constitutive promoter such as the cytomegalovirus immediate-early promoter [CMV–*tat* (12)]. A parallel transfection is performed with a CMV–lacZ plasmid as an indicator of transfection efficiency. The β-galactosidase activity measured 1–2 days after transfection of CMV–*tat* should be at least 10% of that of the CMV–lacZ control.

Target cells can also be derived from cells in which the HIV LTR transactivation is inefficient, such as murine cells (13). In these cells the basal *lacZ* expression is not significantly increased by *tat* transfection, but transactivation can be detected after PEG fusion with human cells expressing Tat (11). Tat transactivation probably requires cellular factors absent from murine cells which are supplemented by fusion with a human cell.

When selecting LTR–lacZ target cells, in a typical experiment only a minority of clones is highly responsive to Tat, and many must be rejected because of their high β-galactosidase background. This may be due to a position effect of the LTR–lacZ transgene or to inactivation of viral promoters in mammalian cells. If the only clones that are clearly Tat inducible show *lacZ* background, the situation can sometimes be improved after subcloning by limiting dilution.

6. Positive clones should be expanded and a large number of vials should be frozen under liquid nitrogen as soon as possible after selection.

7. Once selected and cloned, the LTR–lacZ cell lines should retain their characteristics. For some clones, cells must be maintained in selective media to prevent the appearance of background and/or loss of Tat transactivation. These parameters should be controlled in every experiment and long-term propagation (i.e., over 2 months) should be avoided.

Detection of Cell Fusion Mediated by the HIV Envelope

The product of the *lacZ* gene can be detected by simple enzymatic assays for β-galactosidase, either *in situ* on fixed cells, using the X-Gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or in cell extracts with the ONPG substrate (*o*-nitrophenyl-β-D-galactopyranoside).

Effector Cells

To detect fusion with $CD4^+$ LTR–lacZ cells, the HIV effector cells must express the HIV envelope on their surface and the Tat transactivator. Chronically infected cells, usually derived from T-lymphoid cell lines, are therefore a convenient source of effector cells. However, there is some variation in the level of surface envelope expression, and the cells must be handled in a biosafety facility (P3). Some cell lines, such as 8E5 (see Ref. 14), express HIV envelope and Tat from a defective HIV provirus.

Since these were derived from chronically HIV-infected cells and present a high risk of reversion to infectious HIV, they should also be handled in a P3 facility.

Effectors can also be obtained by transfection of an HIV envelope expression vector in cells already expressing Tat, or by transfection of a deleted HIV provirus expressing Tat and envelope but not the other structural proteins. Such transfected cells do not produce infectious virus and need not be handled under special safety conditions. Only a variable fraction of cells expresses the HIV envelope, depending on the transfection efficiency, and the sensitivity of the fusion assay can be lower than with a homogeneous population of envelope-expressing cells. It is nevertheless possible to rapidly compare the fusogenic properties of different envelopes.

We have succeeded in stably expressing a functional HIV-1 envelope in HeLa cells after transfection of a deleted HIV provirus carrying the *dhfr** gene contained in the same transcriptional unit as the *tat* and *env* genes and selection with methotrexate (15). These HeLa–env cells represent a stable and safe source of effector cells for fusion assays.

The envelope of HIV-1 and HIV-2 share only 50% amino acid identity (16), but both viruses use CD4 as their receptor. HIV-2–infected cells can be used to perform fusion assays with the HeLa–CD4–LTR–lacZ target cells (Fig. 2B). The induction of *lacZ* activity was comparable for HIV-1 and HIV-2 effectors (17), in spite of the lower efficiency of the Tat protein of HIV-2 on the HIV-1 LTR (18).

In Situ Detection of Fusion by β-Galactosidase Assay

1. Seed the HeLa–CD4–LTR–lacZ cells in six- to 96-well microtiter plates in order to obtain a 50–80% confluent monolayer the next day.
2. Add the HIV envelope-expressing effector cells. Suspension growing cells (HIV-infected cells) are added to a subconfluent HeLa–CD4 monolayer and can be in excess. Adherent effector cells (e.g., HeLa–env or transiently transfected COS or HeLa cells) are added to a 50% confluent monolayer, or alternatively can be plated at the same time as the CD4 cells, provided that the total number of cells does not exceed the plating capacity, which would counterselect the slowly adhering cells.
3. Coculture the target and effector cells for 8–24 hr. While cell fusion takes place in a matter of minutes, the assay requires a few hours for transactivation of *lacZ* expression to be completed. Blue syncytia can be detected as soon as 2 hr after initial contact of the effector and target cells, but maximum sensitivity is reached ~8 hr later. We usually incubate the cells overnight and fix them to perform the X-Gal assay the next day.
4. Remove the supernatant and the suspension growing cells, if any, and fix the cells in a 0.5% (v/v) glutaraldehyde solution (see appendix) for 5–10 min at room temperature (2 ml per well of a six-well plate).

5. Wash the fixed cells twice in phosphate-buffered saline (PBS).
6. Add the X-Gal solution (1–2 ml per well of a six-well plate; see appendix) and start incubation. Optimum incubation time and temperature for the best signal-to-background ratio should be determined for each LTR–lacZ cell line. Incubation time can range from 1 to 24 hr. The temperature is usually 37°C, but lowering it to 20°C or even 4°C, while extending the incubation time, can be a way to increase the signal-to-noise ratio.
7. Stop the X-Gal assay by washing the plates twice with PBS at room temperature. We read the plates with a 40× magnifying stereomicroscope, which gives a better contrast than tissue culture microscopes and facilitates the observation of rare blue cells within a monolayer. Plates can be stored under PBS at 4°C, but storage over 48 hr may cause some fading of the blue staining and the emergence of a diffuse β-galactosidase background. If needed, photographs should be taken in the 24 hr following the assay.

Detection of Fusion in Cell Extracts by ONPG Assay

After coculture the LTR–lacZ target cells and effector cells can by lysed and the total β-galactosidase activity can be measured in the extracts. This assay is workable, provided that the fusion efficiency is high and the target cells are well responsive to Tat.

Using cells stably expressing HIV envelope, the assay can be sensitive and reproducible enough to be performed in 96-well plates, which can be of interest in testing fusion inhibitors or neutralizing antibodies.

1. Perform the coculture of effector and target cells in 96-well plates as previously described.
2. Aspirate the medium and wash the cells in PBS (~200 μl per well).
3. Add 250 μl of the ONPG solution (see appendix) and incubate the plate at 37°C for 1–2 hr until a yellow color is clearly visible.
4. Read the absorbance at 450 nm.

Applications of the Fusion Assay to HIV Research

Testing Fusion Inhibitors

Compounds known to interfere with CD4 attachment and HIV entry, such as some anti-CD4 monoclonal antibodies (e.g., Leu3A), soluble CD4 and derived molecules, or anti-HIV envelope-neutralizing antibodies, block the formation of blue-staining syncytia in a dose–response-dependent manner (not shown). The specificity of the

assay and the possibility of performing it in 96-well microtiter plates and in the absence of infectious virus make it an attractive tool to screen for novel antiviral compounds, active in the early steps of the HIV cycle.

Studying HIV Tropism with LTR–lacZ/CD4 heterokaryons

Cells of murine origin transfected with human CD4 cannot be infected with HIV or fuse with HIV envelope-expressing cells because of their inability to support fusion (10). This restriction could have been due either to the presence of a dominant inhibitor in murine cells or to the lack of human-specific cofactors of the fusion process. To address this issue, we have tested heterokaryons formed by PEG treatment between fusion-resistant murine CD4 cells (NIH 3T3 cells transfected with human CD4) and human $CD4^-$ HeLa–LTR–lacZ cells. Heterokaryons are growth arrested and do not support HIV replication. Also, because they are already multinucleated, their ability to form syncytia with HIV envelope-expressing cells obviously cannot be tested by the direct observation of cocultures.

The specificity of the transactivation complementation assay enabled the detection of fusion events between murine or human LTR–lacZ/CD4 heterokaryons and HIV-infected cells (Fig. 2E). The lack of HIV envelope-mediated fusion in non-human cells can be complemented by human cell components and is not the consequence of a dominant-acting inhibitor (11). We could make the same observation in a human glioma cell line (U87MG–CD4) that cannot be infected by HIV-1 while being permissive to HIV-2 (19). These cells can complement murine and simian cells for fusion dependent on HIV-2 but not HIV-1 envelope. Complementation for HIV-1 fusion was observed in U87MG–CD4/HeLa–LTR–lacZ but not U87MG–CD4/COS–LTR–lacZ heterokaryons (17).

Therefore, testing heterokaryons formed between LTR–lacZ target cells, either permissive or resistant to envelope-mediated fusion, and various other cell types can be a general approach to the study of viral tropism.

The following experimental design can be used to form heterokaryons between adherent cells.

1. Seed the two cell types in a 1:1 ratio into a six-well plate in order to obtain a subconfluent monolayer the next day.
2. Aspirate the medium and wash the cells once with serum-free Dulbecco's modified Eagle's medium (DMEM) warmed to 37°C. Carefully aspirate all of the medium.
3. Add the 50% (w/v) PEG solution (see appendix) in serum-free DMEM warmed to 37°C (~1 ml per well), and leave it on the cells for 1 min or less. The resistance of cells to the PEG treatment may vary, and it may be necessary to shorten the contact with PEG or use a more dilute PEG solution if excessive mortality is observed.
4. Wash out the PEG extensively by rinsing the cells four times with warm serum-

free DMEM or PBS. Add the culture medium and incubate the cells at 37°C. Heterokaryons should be visible 1–2 hr after the PEG treatment.

5. Start cocultures with chronically infected cells 2–3 hr after PEG fusion and leave in contact for 12–14 hr.
6. Fix the cells and perform an X-Gal assay as previously described.

Heterokaryons can also be formed from cells in suspension, but with greatly reduced efficiency. The two types of cells are pelleted in a round-bottom tube, resuspended in 1 ml of 50% PEG solution, and 1 min later rapidly diluted in 10 ml of PBS or DMEM. The cells are then centrifuged and washed twice before being plated in culture medium. If one of the cell types (usually the LTR–lacZ cells) is able to adhere, the yield of heterokaryons can be sufficient to perform fusion experiments with HIV effector cells.

Fusogenic Properties of Mutant HIV Envelopes

Binding to the CD4 receptor and induction of membrane fusion are the principal functions of the HIV envelope. While CD4 binding can be accurately measured by biochemical assays, envelope-mediated fusion is difficult to score by classical syncytium formation assays. The transactivation complementation assay can provide quantitative information on the ability of mutant envelopes to mediate fusion and discriminate low-level from null fusogenic activities.

Application of the Assay to Cell Fusion Mediated by the HTLV-I Envelope

The presence of the *tat* gene in HIV-infected cells allowed us to detect fusion with target cells bearing *lacZ* driven by the HIV-1 LTR. We reasoned that other types of effector cells could be obtained by coexpression of Tat and any fusogenic protein. The transactivation assay could then be used to detect fusion with permissive cells bearing a Tat-inducible reporter gene. For example, we developed assays to detect fusion mediated by another human retrovirus, HTLV-I.

Its cellular receptor has not been identified, but there is genetic evidence that permissibility to HTLV-I is borne by the human chromosome 17 (see Ref. 20). The HTLV receptor seems to be a ubiquitous molecule, since all human cells tested are permissive, and well conserved in evolution, since very few cell types are found to be resistant to HTLV-I infection. Among these are the bovine MDBK and rat NRK cell lines. Interestingly, another rat cell line, XC, is fully permissive to HTLV-induced fusion (20).

HTLV-I is poorly infectious as a cell-free virus, which makes syncytium formation assays the principal source of information on envelope function. We have set up

different assays to study mutant HTLV envelopes in transient transfection assays or to test HTLV neutralization (Fig. 3).

Effector Cells Expressing HTLV-I Envelope and HIV-1 Tat

C91PL is a widely used cell line derived from human cord blood T lymphocytes immortalized by HTLV-I infection (21). It produces only low titers of virus, but the surface level and/or fusogenic activity of the HTLV-I envelope are high, since large syncytia (over 10 nuclei) are formed within hours of coculture of C91PL and permissive cells such as HeLa, COS, or XC cells. We handle C91PL cells and their derivatives under P3 conditions.

The HIV-1 *tat* gene was transduced into C91PL cells via an MLV-derived retrovirus vector also expressing the *neo* gene, and a G418-resistant population, designated C91–tat, was selected. When C91–tat cells were cocultured overnight with HeLa–LTR–lacZ cells, the X-Gal staining revealed up to 50% blue-stained syncytia (Fig. 4A). The specificity of the assay is indicated by the reduction of the number of blue syncytia in the presence of sera from HTLV-infected patients containing high titers of neutralizing antibodies (not shown). Also, the number of blue-stained syncytia correlates with the HTLV permissivity of the target cells: only a few blue-stained syncytia were detected in cocultures of C91–tat murine target cells (3T3–LTR–lacZ), while no evidence for fusion was seen with the bovine MDBK–LTR–lacZ cells. Using classical assays, NIH 3T3 and MDBK cell lines were both considered to be resistant to fusion with HTLV-I–infected cells (20). Therefore, the higher sensitivity of the *lacZ* transactivation assay allows detection of rare fusion events that cannot be unambiguously scored by direct observation.

In the C91–tat cells Tat is expressed from the MLV LTR, a relatively weak promoter in a human cell context, and there is some variation in the efficiency of transactivation. To circumvent this problem, it is possible to substitute the C91–tat effectors by a combination of cells expressing a high level of Tat (e.g., HIV-infected cells) and C91PL cells added sequentially or at the same time to the target LTR–lacZ cells (Fig. 3B). The formation of blue syncytia therefore requires the "bridging" of an LTR–lacZ target cell and a Tat-expressing cell by an HTLV envelope-expressing cell. This process seems to take place very efficiently, since the percentage of blue syncytia is higher in this "sandwich" assay than with C91–tat cells (Fig. 4B).

Testing Mutant HTLV-I Envelopes after Transient Expression

Since receptor binding cannot be measured, and the infectivity of cell-free HTLV is very low, syncytium formation assay is the simpler way to test the functionality of the HTLV envelope. Direct observation of cocultures of envelope-expressing cells

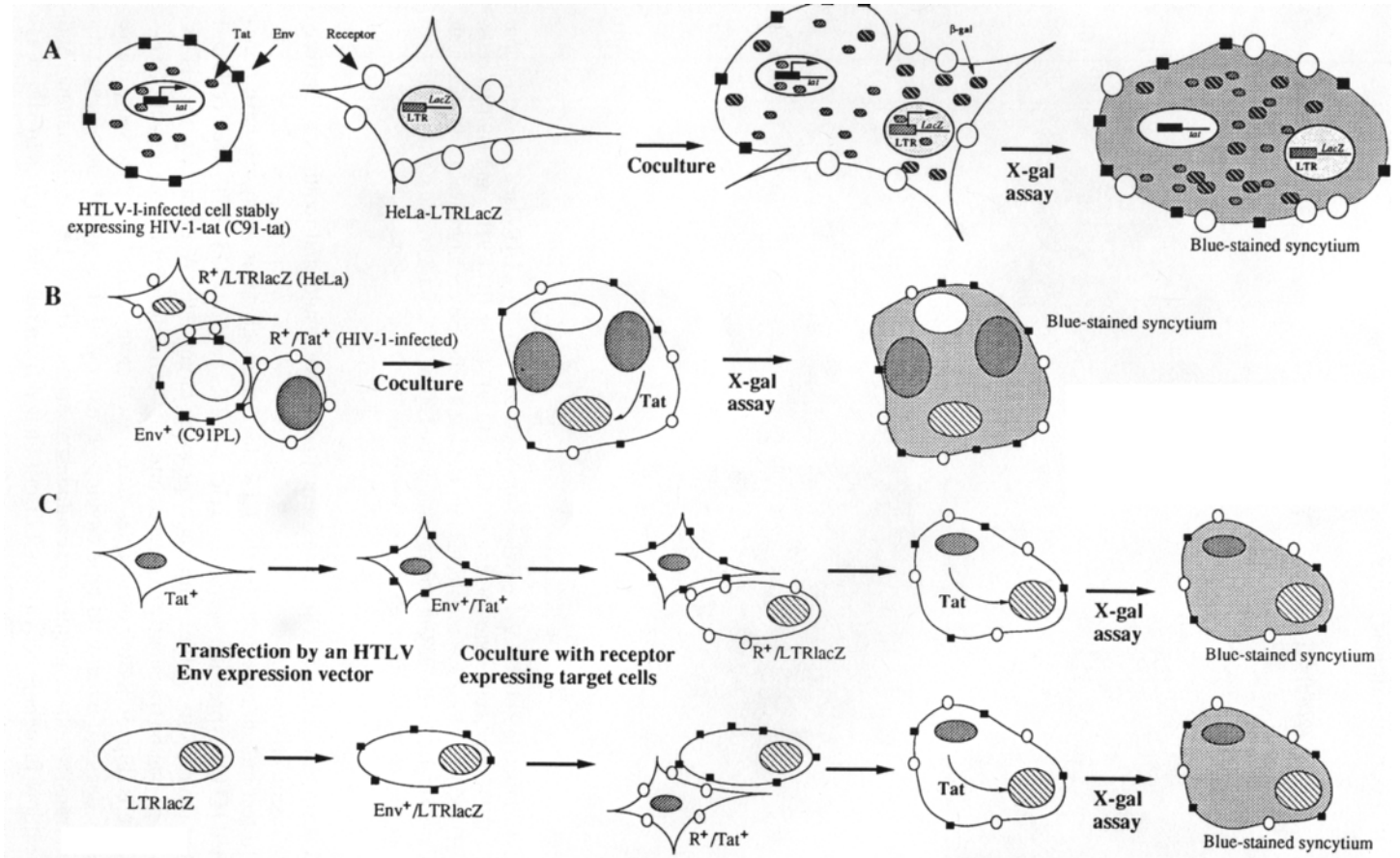

FIG. 3 Strategies to detect fusion of cells expressing HTLV-I envelope with LTR–lacZ target cells. C91–tat cells are chronically HTLV-I–infected C91PL cells transduced with a retroviral vector expressing HIV-1 Tat. (A) C91–tat cells are cocultured with permissive cells expressing the HTLV receptor (R) and bearing the HIV–LTR–lacZ reporter gene (e.g., HeLa–LTR–lacZ cells). (B) C91–tat can be replaced by the combination of C91PL and Tat-expressing cells permissive to HTLV (e.g., HIV-1–infected cells). (C) The HTLV envelope can be expressed by transient transfection of an expression vector in Tat^+ cells and fusion can be detected after coculture with HeLa–LTR–lacZ or COS–LTR–lacZ cells. Alternatively, the HTLV expression vector can be transfected into LTR–lacZ cells and fusion can be detected with Tat^+ cells.

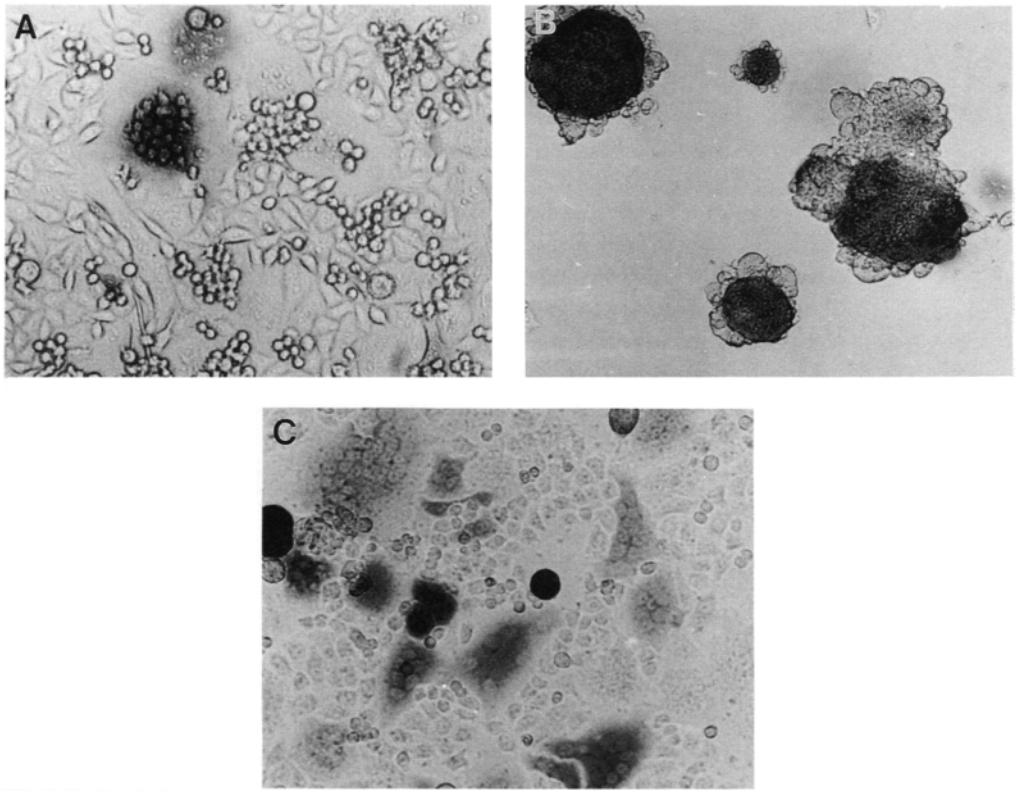

FIG. 4 Detection of HTLV-1 envelope-mediated cell fusion by LTR–lacZ transactivation. HeLa–LTR–lacZ cells were cocultured with (A) C91–tat or (B) C91PL and H9/IIIB cells. (C) HTLV-1 envelope was transfected into HeLa–tat cells and coculture was performed with HeLa–LTR–lacZ cells. X-Gal staining was carried out at 37°C for 2 hr, and photographs were taken under 80× magnification.

and permissive cells only provides semiquantitative results and requires a relatively high fusion efficiency.

To test mutant envelopes by the LTR–lacZ transactivation assay, we have transfected HTLV expression vectors into HeLa–LTR–lacZ or COS–LTR–lacZ cells and tested their ability to fuse with Tat-expressing target cells. Alternatively, the HTLV envelope can be transiently expressed into Tat^+ cells, then put in contact with LTR–lacZ cells (Fig. 3C). Both techniques are quite efficient, and blue-stained syncytia are readily detected when wild-type envelope is expressed (Fig. 4C). Using these transient transfection techniques, we could test a series of HTLV-I envelope mutants and reveal low fusogenic activities not discriminated from null phenotypes by direct assays (not shown).

Use of the Luciferase Reporter Gene to Detect Cell Fusion Mediated by the HTLV-I Envelope

The measure of the β-galactosidase activity in a whole-cell extract can reflect the extent of fusion between Tat^+ and LTR–lacZ cells, but this type of assay requires a high efficiency of fusion that is not always achieved. To improve the sensitivity of the fusion assay, we have sought to use the luciferase (*luc*) reporter gene (22) under the transcriptional control of the HIV-1 LTR. The assay was further simplified by using target cells transiently transfected with the LTR–luc plasmid (12) to circumvent the lengthy procedure of establishing and selecting target cell lines after stable transfection. The extreme sensitivity of the measurement of luciferase activity enabled the use of cells relatively resistant to transfection, such as C91PL cells.

The following protocol allows the detection of fusion events between C91PL and Tat-expressing cells, and provides rapid and quantitative results (Fig. 5).

1. C91PL cells are transfected using a modified diethylaminoethyl (DEAE)–dextran procedure (23). Briefly, $\sim 10^7$ cells are washed in PBS and resuspended in 1.5 ml of TS transfection buffer (see appendix) containing 10 μg of the pLTR–luc plasmid, and DEAE–dextran is added to a final concentration of 500 μg/ml. After a 20-min incubation at room temperature, the cells are diluted 10 times with PBS, washed, and resuspended in culture medium at $\sim 10^6$ cells per milliliter.
2. On the same day, $\sim 5 \times 10^5$ murine NiH 3T3, bovine MDBK, rat XC, and human HeLa cells and their Tat^+ derivatives are seeded in 35-mm plates (six-well plates) and allowed to adhere overnight.
3. Sixteen to 24 hr after transfection, C91PL cells are washed and resuspended in fresh medium, and $10^5 - 10^6$ cells are added to the adherent target cells. An equal number of C91PL cells must be dispensed into a separate well to measure the luciferase activity and estimate the transfection efficiency.
4. The luciferase activity is measured after 18–24 hr of coculture. For each well nonfused C91PL–LTR–luc cells are collected and centrifuged, and the pellet is resuspended and lysed in 300 μl of LB (see appendix). Adherent cells are washed twice with Ca^{2+}/Mg^{2+}-free PBS, trypsinized, and centrifuged, and the pellet is resuspended and lysed in 300 μl of LB. One hundred microliters of the ATP–luciferin mix (see appendix) is added to the lysate and the luciferase activity can be measured immediately using a luminometer. The luciferase activity standardized to the protein content of the extract is referred to as the luciferase index. The efficiency of the Tat-induced transactivation is estimated by comparing the luciferase index of the Tat^+ and parental Tat^- cell lines cocultured with C91PL–LTR–luc cells.

In a representative experiment (Fig. 5) we observed over a 100-fold difference in the luciferase index of HeLa and HeLa–tat cells cocultured with C91PL–LTR–luc cells, about a 40-fold difference between XC and XC–tat cells, and less than a 10-

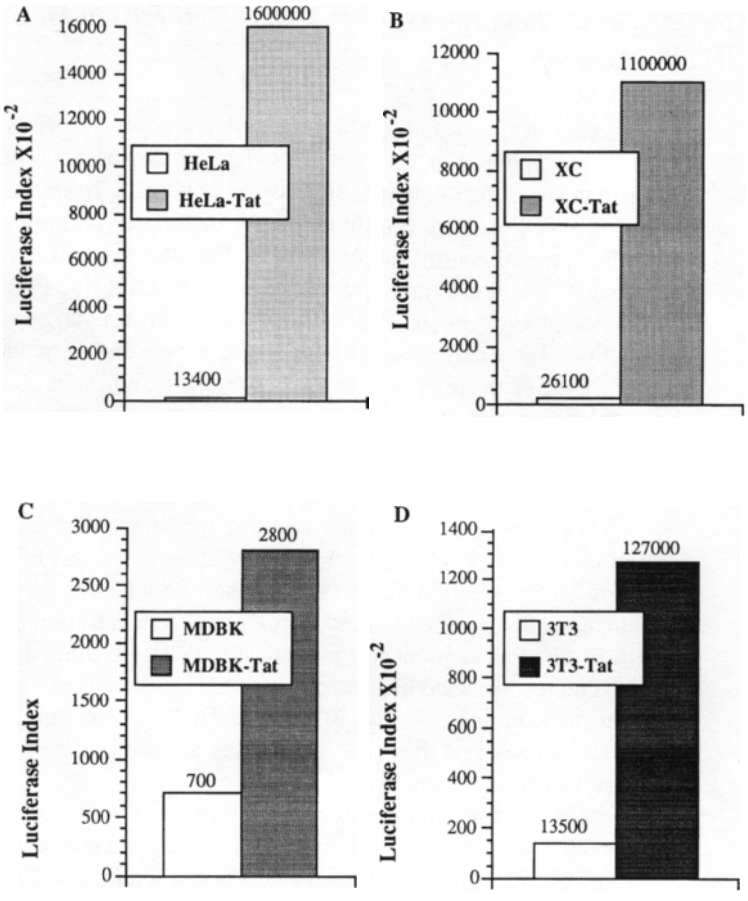

FIG. 5 Detection of HTLV-1 envelope-mediated cell fusion by LTR–luc transactivation. (A) Human HeLa cells, (B) rat XC cells, (C) bovine MDBK cells, and (D) murine NiH 3T3 cells, stably expressing the HIV-1 Tat protein or not, were cocultured for 24 hr with C91PL cells expressing the HTLV-1 envelope and transiently transfected with an (HIV-1) LTR–luc plasmid. Nonadherent cells (unfused C91PL) were washed out and the luciferase activity was measured in extracts from adherent cells. The luciferase index, representing the luciferase activity standardized to the protein content of the extract, is shown as arbitrary units. Note the difference in scale in (C).

fold difference between NIH 3T3 cells and their Tat^+ derivatives. The efficiency of fusion with the HTLV envelope-expressing cells seems well related to the measure of the luciferase activity in the coculture.

The level of luciferase activity in cocultures of C91PL–LTR–luc and the bovine MDBK–tat cells is extremely low (about 600-fold less than with HeLa–tat), consis-

tent with the apparent resistance of MDBK cells to HTLV-mediated fusion. However, there is a 4-fold difference in the luciferase index of MDBK and MDBK–tat. The extreme sensitivity of the assay could have allowed detection of very rare fusion events with C91PL cells not seen with the X-Gal assay. However, this difference could also be due to transcellular transactivation by Tat, a phenomenon previously reported between $CD4^+$ and HIV envelope-expressing cells in the absence of cell fusion (24). Such a low level of transactivation was unlikely to be detected by an *in situ* X-Gal assay.

Conclusion

Functional studies on retroviral envelopes are essential to understand the molecular mechanisms of virus entry. These are also probably responsible for fusion between envelope- and receptor-expressing cells, and syncytium formation assays represent a simple and rapid way to address envelope function.

The transactivation assay we have proposed is based on the induction of the expression of a reporter gene after fusion with another cell type that brings the HIV transactivator, Tat. In the case of the human retroviruses HIV and HTLV, it has already proved extremely valuable in studying viral tropism and test mutant envelopes and performing neutralization assays. We believe it could be applied to any fusogenic protein, provided that it can be expressed in the context of Tat^+ cells and LTR–lacZ target cells are obtained. Fusion can also be detected with target cells transiently transfected with a luciferase reporter gene, and quantitative results can be rapidly obtained.

Acknowledgments

We thank O. Schwartz (Institut Pasteur, Paris) for the C91–tat cell line and C. Denesvre (Institut Cochin de Génétique Moléculaire, Paris) for the XC–tat cell line.

Appendix

Buffers for β-Galactosidase Assay

Cell Fixation

The 0.5% (v/v) glutaraldehyde solution is prepared in PBS and can be stored for several weeks at 4°C. The 25% glutaraldehyde stock solution (Sigma, St. Louis, MO; Cat. No. G-5882) can be stored at 4°C for several months in a dark bottle.

X-Gal Solution (in PBS)

5 m*M* $K_3Fe(CN)_6$ plus 5 m*M* $K_4Fe(CN)_6$ (from 10× FeCN solution)
1 m*M* $MgCl_2$
0.4 mg/ml X-Gal (from 100× stock)

Store at 4°C.

10× FeCN Salts (in PBS)

50 m*M* $K_3Fe(CN)_6$
50 m*M* $K_4Fe(CN)_6 \cdot 3H_2O$

Store at 4°C.

100× X-Gal Stock

40 mg/ml X-Gal in dimethyl sulfoxide or dimethylformamide

Store aliquots at −20°C.

ONPG solution (freshly made in PBS)

3.5 m*M* ONPG (Sigma; Cat. No. N-1127)
0.5% (v/v) Nonidet P-40 (Sigma)
1 m*M* $MgCl_2$

Luciferase Assay

Luciferase Lysis Buffer (LB)

25 m*M* Tris–phosphate (pH 7.8)
8 m*M* $MgCl_2$
1 m*M* dithiothreitol
1% (v/v) Triton X-100
15% (v/v) glycerol

Store at 4°C.

Substrate Mix (in LB)

5 m*M* ATP (Sigma; Cat. No. A-0270), from 20 m*M* stock in PBS
0.25 m*M* luciferin (Sigma; Cat. No. L-6882), from 1 m*M* stock in PBS

Store at −20°C.

DEAE Transfection

Transfection Buffer (TS)

137 m*M* NaCl
5 m*M* KCl
0.7 m*M* $CaCl_2$
0.5 m*M* $MgCl_2$
0.6 m*M* $Na_2HPO_4 \cdot 12H_2O$
25 m*M* Tris–HCl (pH 7.4)

Store at room temperature or 4°C.

DEAE–Dextran Solution

20 mg/ml (Sigma; Cat. No. D-9885) in TS

Store at 4°C.

50% PEG Solution

1. Weigh 10–50 g of PEG 6000 (Merck; Cat. No. 807491) into a sterile glass bottle.
2. Add 1 ml of serile serum-free DMEM per gram of PEG.
3. Heat to 70°C in a water bath until the PEG is melted.
4. Pipette thoroughly until the solution is homogeneous. Store at room temperature. A precipitate may form but the PEG solution can be used for several weeks.

If long-term cultures are performed after PEG fusion, a totally sterile PEG solution can be made by autoclaving the PEG crystals (10 min at 120°C, liquid cycle) and cooling to 60–70°C before rapidly mixing with DMEM by pipetting.

References

1. E. Hunter and R. Swanstrom, *Curr. Top. Microbiol. Immunol.* **157,** 187 (1990).
2. R. A. Weiss, *in* "The Retroviridae" (J. A. Levy, ed.), Vol. 2, p. 1. Plenum, New York, 1993.
3. J. D. Lifson, M. B. Feinberg, J. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman, *Nature (London)* **323,** 725 (1986).
4. A. D. Frankel, *Curr. Opin. Genet. Dev.* **2,** 293 (1992).
5. B. R. Cullen, *Cell (Cambridge, Mass.)* **73,** 417 (1993).
6. K. A. Jones, *Curr. Opin. Cell Biol.* **5,** 461 (1993).

7. D. Rocancourt, C. Bonnerot, H. Jouin, M. Emerman, and J. F. Nicolas, *J. Virol.* **64,** 2660 (1990).
8. P. Charneau, A. Alizon, and F. Clavel, *J. Virol.* **66,** 2814 (1992).
9. J. Kimpton and M. Emerman, *J. Virol.* **66,** 2232 (1992).
10. P. J. Maddon, A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel, *Cell (Cambridge, Mass.)* **47,** 333 (1986).
11. T. Dragic, P. Charneau, F. Clavel, and M. Alizon, *J. Virol.* **66,** 4794 (1992).
12. O. Schwartz, J. L. Virelizier, L. Montagnier, and U. Hazan, *Gene* **88,** 197 (1990).
13. P. A Barry, E. Pratt-Lowe, R. E. Unger, and P. A. Luciw, *J. Virol.* **65,** 1392 (1991).
14. T. M. Folks, D. Powell, M. Lightfoote, S. Koenig, A. S. Fauci, S. Benn, A. Rabson, D. Daugherty, H. E. Gendelmann, M. D. Hoggan, S. Venkatesan, and M. A. Martin, *J. Exp. Med.* **164,** 280 (1986).
15. O. Schwartz, M. Alizon, J. M. Heard, and O. Danos, *Virology* **198,** 360 (1994).
16. M. Guyader, M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon, *Nature (London)* **326,** 662 (1987).
17. T. Dragic and M. Alizon, *J. Virol.* **67,** 2355 (1993).
18. M. Emerman, M. Guyader, L. Montagnier, D. Baltimore, and M. A. Muesing, *EMBO J.* **6,** 3755 (1987).
19. P. R. Clapham, D. Blanc, and R. A. Weiss, *Virology* **181,** 703 (1991).
20. M. A. Sommerfelt, B. P. Williams, P. R. Clapham, E. Solomon, P. N. Goodfellow, and R. A. Weiss, *Science* **242,** 1557 (1988).
21. M. Popovic, G. Lange-Wantzin, P. S. Sarin, D. Mann, and R. C. Gallo, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 5402 (1983).
22. J. R. De Wet, K. V. Wood, D. Helinski, and M. De Luca, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 7870 (1985).
23. Y. Wang, A. S. Larsen, and B. M. Peterlin, *J. Exp. Med.* **166,** 625 (1987).
24. A. Marcuzzi, I. Lowry, and O. K. Weinberger, *J. Virol.* **66,** 4536 (1992).

[15] Assembly of Recombinant Retroviral Gag Precursors into Pseudovirions in the Baculovirus–Insect Cell Expression System

Gregory J. Tobin, Jane K. Battles, Lynn Rasmussen, and Matthew A. Gonda

Introduction

The development of recombinant baculovirus–insect cell expression systems in the past decade (1, 2) has provided investigators with the capability of producing large amounts of recombinant proteins for a wide variety of applications. Recombinant baculoviruses have been used to synthesize immunogens for antibody production and vaccination, biologically active cytokines, viral proteins for structure–function studies, and toxins for use as insecticides (3–7). Baculovirus expression systems have many of the advantages found in other eukaryotic expression systems, for example, mRNA splicing and posttranslational modifications (8, 9), and generally provide greater yields of recombinant proteins than mammalian systems. Our laboratory and others (10–13) have utilized recombinant baculovirus technology to express retroviral Gag precursor proteins that can assemble into noninfectious viruslike particles (VLPs), also referred to as pseudovirions, in insect cells.

Baculovirus Biology and Recombinant Technology

Baculoviruses are relatively large complex insect viruses that possess a double-stranded DNA genome of 88–153 kilobase pairs and replicate in insect cells *in vitro* (4, 5). Baculoviruses have two morphologically distinct virion phenotypes: extracellular virus particles, which bud from the plasma membrane and facilitate virus spread within an insect or cell culture, and occlusion bodies, which form environmentally stable crystalline structures in the nuclei of infected cells. Occlusion body formation is a requisite for the dissemination of baculoviruses between hosts, but is not essential for virus replication and spread in cultured cells or within the host.

Most laboratories using recombinant baculovirus technology use the prototypical M-type nuclear polyhedrosis virus, *Autographa californica* (AcNMPV). The predominant viral proteins in the occlusion body are the 29-kDa polyhedrin protein, which forms a crystalline lattice, and a second protein, p10, of unknown function. Both polyhedrin and p10 proteins are nonessential for replication in cultured cells,

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and their promoters and loci in the genome can be exploited in the development of baculovirus vectors for the expression of recombinant proteins in insect cells. Since the initial expression of recombinant proteins using the polyhedrin (1, 2) and p10 promoters (14), a multitude of baculovirus expression systems, utilizing various baculovirus promoters, has been developed (15).

Expression of Retroviral Gag Precursor Proteins

Retroviruses are enveloped RNA viruses that replicate through a double-stranded DNA intermediate, the provirus. Native particles can be divided into two major components: the core and the viral envelope. The core in mature virions consists of viral genomic RNA in tight association with the *gag*-encoded proteins, capsid and nucleocapsid, as well as the *pol*-encoded proteins, protease, reverse transcriptase, and integrase. The *gag* and *pol* gene products are translated as Gag and Gag–Pol polyproteins that are processed by the virus-encoded protease into mature proteins after assembly of the virus into immature particles. Cleavage of the Gag precursor releases the matrix, capsid, and nucleocapsid proteins and, in addition, smaller intervening or C-terminal peptides of unknown function. The morphology and function of retroviral structural proteins have been extensively reviewed (16–20).

The *gag* coding region of the retrovirus genome can be cloned from the provirus for expression by heterologous promoters. Uncleaved Gag precursors expressed in eukaryotic cells can assemble and bud from cell membranes. Morphologically recognizable VLPs, or pseudovirions, are released. Excretion of retroviral pseudovirions has been observed in host cells that were infected with a variety of recombinant viruses engineered to express Gag precursor proteins. Examples of these viral vectors include baculoviruses (see below), vaccinia viruses (21, 22), fowlpox viruses (23), adenoviruses (24), and yeast Ty elements (25). These studies have demonstrated that the Gag precursor is the minimal particle-forming unit of a retrovirus. In addition, recombinant baculoviruses have been used to produce pseudovirions of other viruses, such as hepatitis B virus (26), poliovirus (27), human papillomavirus (28, 29), canine parvovirus (30), bovine rotavirus (31), and bluetongue virus (32). VLPs have become increasingly important for the experimental study of viral morphogenesis and as a vehicle for encapsidating viral antigen for ease of purification.

The expression of *gag* genes of the lentivirus genus of retroviruses in baculoviruses, in particular, has led to the production of pseudovirions of human immunodeficiency virus types 1 (HIV-1) (11) and 2 (HIV-2) (13), simian immunodeficiency virus (SIV) (12), feline immunodeficiency virus (FIV) (33), and bovine immunodeficiency virus (BIV) (10). Since the publication of these initial observations, the expression of pseudovirions has been used for a wide variety of studies, including particle assembly and secretion (33, 34), interaction between Gag and Env glycoproteins (35), packaging of additional viral epitopes (36, 37), and production of subunit vac-

cines (38, 39). Similar techniques have been used to generate and characterize lentivirus pseudovirions; these are described below.

Methods

Insect Cell Culture

Insect cell cultures for the propagation of baculoviruses and the production of recombinant gene products can be maintained conveniently at 22–27° C in either suspension or monolayer flasks. Although many insect cell lines support baculovirus replication, *Spodopteria frugiperda* 9 (Sf-9) is the most commonly utilized (40). Sf-9 cells can be propagated in either serum-supplemented media, such as Grace's (41), TNM-FH (42), TC100 (43), and IPL41 media (44), or in serum-free chemically defined media (45) such as EX-CELL400 (JRH Biosciences, Lenexa, KS) and SF900 (Life Technologies, Gaithersburg, MD). The low protein concentration of serum-free media is often advantageous and can facilitate the purification of secreted recombinant proteins. TNM-FH medium, based on Grace's formulation augmented with lactalbumin hydrolysate and yeastolate, is supplemented with 7–10% fetal bovine serum (FBS) and used for routine maintenance of cell cultures.

Construction of Baculovirus Transfer Vectors

Due to the large size of the baculovirus genome, foreign genes for expression are usually transferred into the AcNMPV genome via homologous recombination with a transfer vector plasmid. The transfer vector contains baculovirus DNA sequences flanking the region of foreign gene insertion. pVL941-based transfer vectors (46) containing the entire *gag* coding sequences can be constructed from restriction or polymerase chain reaction (PCR)-generated fragments, using conventional cloning techniques (47). To assist in the identification and isolation of recombinant baculoviruses, several transfer vectors that permit color selection can be used. Vectors pJVNhe1 (48), pAcDZ1 (49), and pBlueBacIII (Invitrogen, San Diego, CA) transfer the *Escherichia coli* β-galactosidase gene, as well as foreign DNA sequences, into the AcNMPV genome; the resulting recombinant baculoviruses make plaques that stain blue in the presence of X-Gal.

Purification of AcNMPV DNA

For the generation of recombinant baculoviruses, purified viral DNA is required for cotransfection with transfer vector plasmid containing the foreign DNA. Viral DNA

is isolated from sucrose gradient-purified extracellular baculovirus particles. Because of the large proportion of wild-type relative to recombinant baculoviruses produced from these cotransfections, it is useful to have a color selection for identification of recombinant plaques. DNA from the baculovirus AcNMPV–lacZ, which is functionally capable of making β-galactosidase, can be used as the target genome for recombination with a transfer vector lacking β-galactosidase expression. Prior to transfection, purified AcNMPV–lacZ DNA is linearized by digestion with *Bsu*36-I within the β-galactosidase sequences. Homologous recombination between linearized AcNMPV–lacZ DNA and the transfer vector results in recircularization of the viral DNA, exclusion of β-galactosidase sequences, and production of progeny virus that make clear plaques (50, 51). In addition to providing a colorimetric differentiation, the linearized vectors sharply reduce the number of nonrecombinant viruses produced by cotransfection.

Purification of Baculovirus Viral DNA

1. Seed 150-cm^2 flasks with 1.8×10^7 Sf-9 cells and infect with 10 plaque-forming units (pfu) of baculovirus per cell (see below). Harvest and clarify the medium 5 days postinfection by centrifugation at 5000 *g* for 20 min. Pellet the virions by centrifugation at 100,000 *g* for 1 hr at 4° C. Gently resuspend the pellets in 2–3 ml of 10 m*M* Tris–HCl (pH 8.0), 100 m*M* NaCl, and 1 m*M* EDTA (TNE buffer) and layer onto a 30–45% (w/w) sucrose step gradient made in TNE buffer. Centrifuge the gradients at 100,000 *g* for 3 hr at 12° C and harvest the virus from the 30–45% interface. Concentrate the virus by diluting in 5 vol of TNE buffer and pelleting again at 100,000 *g* for 1 hr at 4° C. Resuspend the virions in a small volume of TNE buffer and store in polypropylene tubes at 4° C.

2. Lyse the virus preparation in 0.5% (w/v) SDS and incubate for 30 min at 45° C. Add proteinase K to a concentration of 0.2 μg/ml and incubate for another 30 min at 45° C. Gently extract twice with phenol–chloroform–isoamyl alcohol (50:48:2) and twice with chloroform–isoamyl alcohol (24:1). Rock gently to mix, as vortexing will shear the DNA. Precipitate the virion DNA by the addition of 2 vol of 100% ethanol and pellet for 20 min at 2000 *g* at 25° C. Rinse the pellet with 70% ethanol and gently resuspend the DNA obtained from each 150-cm^2 culture flask in 100 μl of TNE buffer.

3. Aliquot and store the virion DNA in polypropylene tubes at 4° C. Large-bore pipette tips should be used to transfer the DNA to prevent shearing.

4. When AcNMPV–lacZ is used, digest 10 μg of AcNMPV–lacZ viral DNA for 2 hr with 100 U of *Bsu*36-I in a 100-μl volume. Extract with phenol–chloroform–isoamyl alcohol and chloroform–isoamyl alcohol, as above. Ethanol-precipitate, rinse the pellet with 70% ethanol, and gently resuspend in 100 μl of sterile water. Assess digestion by comparison with undigested AcNMPV–lacZ DNA in a nondenaturing 0.7% (w/v) agarose gel. Store the linearized DNA as above.

Cotransfection Procedures

Cotransfection can be accomplished by calcium phosphate coprecipitation, electroporation, or liposome-mediated gene transfer techniques (52, 53). The liposome-mediated transfection and calcium phosphate coprecipitation methods are commonly used.

Liposome-Mediated Transfection Method

1. Seed six-well culture plates with 10^6 cells per well. Once the cells attach, replace the medium with 3 ml of chemically defined serum-free media.
2. Mix 10 μl of Lipofectin (Life Technologies) with 10 μl of sterile water in a polystyrene tube. Combine 0.2 μg of baculovirus DNA (e.g., *Bsu*36-I–digested AcNMPV–lacZ) and 2 μg of transfer vector DNA (e.g., pVL1392 containing a foreign gene) in 20 μl of sterile water. Mix the DNA with diluted Lipofectin. Incubate at 22° C for 20 min.
3. Replace the medium with 1.5 ml of fresh serum-free medium. Slowly add Lipofectin–DNA solution to the cells. Swirl to mix. Incubate for 6–18 hr at 22° C.
4. Replace the medium with 3 ml of TNM-FH supplemented with 10% FBS and incubate for 3–5 days.
5. Clarify the medium by low-speed centrifugation (2000 *g* for 10 min at 4° C) prior to the isolation of recombinant baculovirus (see below).

Calcium Phosphate Coprecipitation Method

1. Allow 2×10^6 Sf-9 cells to attach to the surface of 25-cm^2 flasks.
2. Mix 1 μg of AcNMPV viral DNA and 2 μg of transfer vector DNA gently in 0.75 ml of transfection buffer that consists of 25 m*M* HEPES (pH 7.1), 140 m*M* NaCl, and 125 m*M* $CaCl_2$.
3. Replace the medium from the flasks with 0.75 ml of Grace's medium supplemented with 10% FBS and antibiotics.
4. While gently rocking the flasks, slowly add the DNA solution to the culture medium.
5. After a 4-hr incubation at 22° C, remove the DNA solution, rinse the cells with medium, and add 5 ml of TNM-FH medium supplemented with 10% FBS and antibiotics. Incubate for 3–5 days.
6. Clarify the medium by low-speed centrifugation (2000 *g* for 10 min at 4° C) and use immediately or store at 4° C prior to the isolation of recombinant baculoviruses (see below).

Isolation and Propagation of Recombinant Baculoviruses

Recombinant baculovirus can be isolated from wild-type AcNMPV by multiple rounds of limiting dilution hybridizations and/or by plaque purification methods (52,

54). Recombinant plaques can be distinguished microscopically from wild-type AcNMPV by the lack of occlusion bodies in the nuclei of infected cells. At a low frequency polyhedrin-negative nonrecombinant plaques arise spontaneously and can be excluded from consideration by further DNA or recombinant protein analysis. When AcNMPV–lacZ DNA is used to generate recombinant baculoviruses, the recombinant plaques can be distinguished by their lack of staining with X-Gal. In contrast, when a transfer vector that coexpresses β-galactosidase with the foreign gene is used with AcNMPV, recombinant plaques stain blue with X-Gal. Several independent recombinant baculoviruses should be purified initially, as the quantity of recombinant protein produced from different plaques can vary. Small-scale viral stocks should be generated from the recombinant plaques for characterization of recombinant protein expression. Larger virus stocks must be generated for experiments that require scale-up of recombinant protein expression.

Plaque Purification Method

1. Seed 1×10^6 cells in 2.5 ml of medium in six-well culture dishes. Allow the cells to attach.
2. Remove the medium from the culture dish, infect the cells with 10-fold dilutions of virus in 0.5 ml of medium, and rock gently for 2 hr to permit virus attachment. Note: The titer of baculoviruses in supernatants from infections versus transfections varies (i.e., supernatants from baculovirus infections contain approximately 10^8 pfu/ml, while the titer of progeny virus 3 days after cotransfection of the cells with viral DNA and transfer vector varies from 10^2 to 10^4 pfu/ml).
3. The plaque overlay is prepared as follows. Autoclave 2% (w/v) low-gelling-temperature SeaPlaque agarose (FMC Bio Products, Rockland, ME) in water and store at 22° C. Prepare 2× concentrated TNM-FH medium supplemented with 10% FBS and 2× antibiotics. Immediately prior to use, melt the agarose, cool to 37° C, and mix with an equal volume of 2× medium equilibrated at 37° C.
4. Aspirate the medium from the cells and chill the plates on ice to facilitate rapid gelling of the molten agar and to reduce the time at which the Sf-9 cells are subjected to an elevated temperature. Carefully overlay each culture with 2.5 ml of the 37° C agarose. After the overlay has set, incubate the culture dishes at 27° C in a humidified environment.
5. The plaques are examined and enumerated after 4–5 days to determine the virus titer (in pfu/ml). To facilitate visualization of the plaques, stain the monolayer with 5 ml of a solution of 0.01% (w/v) neutral red diluted in Grace's medium. X-Gal is added to a final concentration of 0.2% (w/v) to quantitate plaques derived from baculoviruses expressing β-galactosidase.
6. With practice, plaques arising from recombinant baculoviruses can be distinguished from those of wild-type AcNMPV by their lack of polyhedrin crystals (occlusion bodies) in the nuclei of infected cells. Recombinant plaques can be distinguished from nonrecombinant AcNMPV–lacZ plaques by their inability to stain with

X-Gal. Alternatively, recombinant plaques stain blue when using β-galactosidase–expressing transfer vectors.

7. Pick well-isolated plaques for additional rounds of purification or virus propagation by pipetting infected cells from under the agarose overlay. Mix the cell–agar plug with 200 μl of medium to recover virus. Recombinant plaques can be identified by PCR amplification of the foreign DNA sequences or dot blot hybridization.

8. Plaques can be amplified in 25-cm^2 culture flasks containing 3×10^6 cells immediately or further purified using successive plaquing steps prior to amplification. Seed stocks are harvested after 4–5 days, clarified by low-speed centrifugation (2000 *g* for 10 min at 4° C), and stored at 4° C.

Limiting Dilution Hybridization Method

1. Seed each well of a 96-well culture plate with 0.1 ml of medium containing 1.5×10^4 Sf-9 cells.

2. Make 10-fold serial dilutions of virus to be analyzed. Dilutions of 10^{-2} to 10^{-5} of supernatant from transfected cells are usually used in the initial screen. Subsequent rounds of baculovirus purification necessitate dilutions of 10^{-4} to 10^{-8} of supernatant from infected cells.

3. Add 100 μl of diluted virus to each well. Incubate the culture in a humidified environment for 4–5 days at 27° C.

4. Transfer the supernatant into a fresh 96-well plate and store at 4° C for further purification. Avoid cross-contamination between the wells, and leave the cells in the wells during supernatant transfer.

5. Lyse the cells for 15 min at 22° C in 100 μl of a solution containing 0.2 N NaOH and 0.2 *M* NaCl. Mix by agitating the plate or repeated pipetting of solution.

6. Neutralize by the addition of 25 μl of 5 *M* ammonium acetate.

7. Cut Whatman (Clifton, NJ) 3MM filter paper and either nylon or nitrocellulose membrane to fit into a 96-well dot blot apparatus (Schleicher & Schuell Keene, NH). Prewet the filter paper and the membrane with 1 *M* ammonium acetate and assemble the apparatus such that the 3MM paper supports the membrane. Attach the manifold to a liquid-trap flask connected to a vacuum source.

8. Transfer the lysates from the 96-well plate to the dot blot apparatus and apply negative pressure to remove the liquid.

9. When the wells have emptied, remove the membrane, rinse in a 4× SSC solution (0.6 *M* NaCl plus 0.06 *M* sodium citrate, pH 7.0), and air-dry.

10. Rehydrate the membrane in 2× SSC and hybridize using a labeled probe specific for the cloned DNA sequences.

11. Identify the wells containing recombinant baculovirus by autoradiographic or colorimetric methods.

12. Further purify by successive rounds of dot blot hybridization or plaque isolation, as described above.

Preparation of Virus Stock

1. Seed 150-cm^2 culture flasks with 1.8×10^7 cells. Allow the cells to attach. Remove the medium and add 0.1–0.2 pfu of recombinant baculovirus per cell in 5 ml of medium. Rock the flask gently for 1–2 hr to permit virus attachment.
2. Add 50 ml of antibiotic-free TNM-FH medium containing 10% FBS. Incubate for 4–5 days.
3. Harvest the medium and remove the debris by centrifugation (2000 *g* for 10 min at 4° C).
4. Determine the virus titer by plaque assay.
5. Aliquot the virus stock and store at 4° C, at which it is stable for 1 year. Alternatively, long-term stocks of cell-associated virus can be stored at −70° C in medium supplemented with 10% FBS and 10% dimethyl sulfoxide.

Production and Purification of Gag Pseudovirions

Infection of Sf-9 cells with recombinant AcNMPV containing a lentivirus *gag* gene results in the expression of Gag precursor protein. The Gag precursor assembles into pseudovirions at the plasma membrane, where they bud into the culture medium. Pseudovirions are purified from the supernatant on the basis of their particulate nature, using conventional virological methods (55).

1. Seed each 150-cm^2 flask with 1.8×10^7 Sf-9 cells and infect with 5 pfu per cell of recombinant baculovirus in a total volume of 10 ml. Gently rock the flask for 1–2 hr to permit virus attachment. Add 50 ml of TNM-FH medium supplemented with 10% FBS and antibiotics. Incubate for 3–4 days.
2. Harvest supernatant and clarify at 2000 *g* for 15 min at 4° C.
3. Precipitate the pseudovirions with the addition of NaCl and polyethylene glycol 6000 (EM Science, Gibbstown, NJ) to 2.3% and 8% (w/v), respectively. Stir overnight at 4° C.
4. Collect the precipitate by centrifugation at 5000 *g* for 15 min at 4° C. Resuspend the precipitate from each 150-cm^2 flask in 2 ml of TNE buffer.
5. Layer the samples onto discontinuous sucrose gradients consisting of 20 ml of 30% sucrose overlaying 7 ml of 45% (w/w) sucrose in Beckman (Fullerton, CA) SW28 ultracentrifuge tubes. Sediment for 3 hr at 85,000 *g*. Intact VLPs should be clearly visible as a blue–white band at the 30–45% sucrose interface. Use a pipette to carefully remove the VLP band from the gradient. VLPs retain their structure when stored as a sterile sucrose suspension at 4° C, but should be placed at −20° C for long-term storage.
6. To prepare concentrated VLPs, dilute the sucrose suspension in 4 vol of TNE buffer and sediment for 1 hr at 85,000 *g*. Resuspend the pelleted VLPs from each gradient in 1–2 ml of TNE buffer. A yield of 0.5–2 mg of Gag protein from each 150-cm^2 culture flask can be expected at this stage of purification.

7. For further purification, VLPs can be sedimented at 85,000 *g* for 3 hr through a 20–60% linear sucrose gradient. After centrifugation a blue–white band containing VLPs should be visible around the gradient midpoint (Fig. 1). Carefully remove the VLPs by either direct pipetting or fraction collection. The VLPs can be concentrated by dilution in TNE buffer and centrifugation as in step 6 above.

Characterization of Pseudovirion-Producing Cultures

Assays for Functional Gene Expression

Several assays are available for grossly assessing antigen expression by AcNMPV *gag*-infected cells. Because lentiviral Gag precursors assemble into VLPs and bud at the plasma membrane, both the supernatant and the infected cells can be analyzed for antigen production. Infected cells can be observed by immunofluorescence microscopy and electron microscopy. For example, BIV antigen is readily detected in over 90% of Sf-9 cells in a culture infected with AcNMPV–BIV *gag*, using indirect immunofluorescence with Gag-specific antiserum; the Gag antigen localizes both in the cytoplasm and as particulate matter on the surfaces of cells (Fig. 2). Electron microscopy has been used to examine the particulate nature of the expressed protein and the morphology of the lentivirus Gag-containing pseudovirions (Fig. 3) (10). Pseudovirions form at the plasma membrane and also accumulate in the cytoplasm. The ultrastructure and size (100–120 nm) of the extracellular pseudovirions are identical to those of budding and immature extracellular virus (Fig. 4) (10, 20). The lack of mature forms of VLPs is consistent with the exclusion of sequences encoding the viral protease during generation of recombinant baculoviruses. The overproduction of Gag precursors in insect cells also leads to the assembly of intracellular pseudovirions at the site of translation. Baculovirus particles are also produced by the recombinant baculovirus but are morphologically distinguishable from immature lentivirus pseudovirions. Immunoelectron microscopy of AcNMPV *gag*-infected insect cells can also be used to demonstrate the specific incorporation of Gag antigen into VLPs (10, 11).

Protein Analysis of Gag-Containing Particles

The particulate and secretory characteristics of recombinant Gag precursors enable the recovery and analysis of expressed antigen from the supernatants. Retroviruses and pseudovirions have a buoyant density of 1.16 g/ml and can be isolated as a compact band after sedimentation through a 20–60% sucrose gradient in the 37% region. The protein composition of the pseudovirions can be assessed by gel electrophoresis, Western blotting, radioimmunoprecipitation, and enzyme-linked immunosorbent assay (ELISA) (Fig. 1). Although the gradient fraction containing peak protein concentrations occurs in the top portion of the sucrose gradient, the highest antigen content

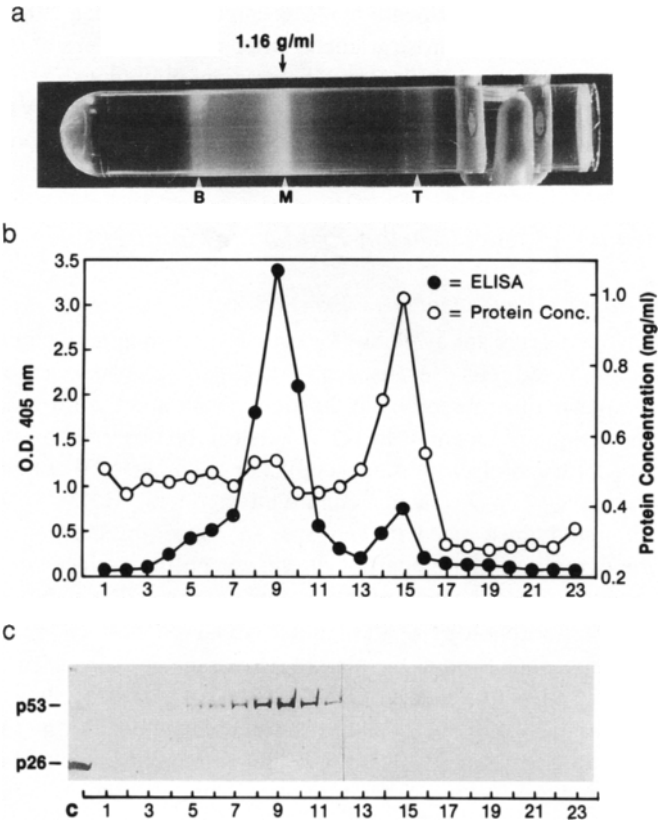

FIG. 1 Sucrose gradient purification and physical characterization of pseudovirions from supernatants of AcNMPV–BIV *gag*-infected insect cells. VLPs in the supernatants of Sf-9 cells infected by AcNMPV–BIV *gag* were harvested and layered onto a 20–60% (w/w) sucrose gradient, as described in "Methods." The pseudovirions were centrifuged at 100,000 *g* for 90 min at 12° C; 0.5-ml fractions were collected for analysis. (a) Photograph of a 20–60% (w/w) linear gradient after centrifugation at 100,000 *g* for 3 hr at 12° C. (b) Plots of protein concentration (○) and ELISA results using a rabbit anti-BIV serum (●) to locate fractions containing BIV Pr53 from pseudovirions in the gradient. (c) Western blot analysis of gradient fractions using rabbit anti-BIV serum. BIV virions were included in the Western blot as a positive control (○) for the rabbit antiserum. The locations of virion-derived BIV Gag precursor Pr53 (p53) and capsid (p26) on the control strip are indicated and serve as molecular weight markers for identifying the recombinant BIV Pr53 contained in the VLPs. Three major bands were resolved in the gradient and were identified as the top (T), middle (M), and bottom (B), as indicated. The middle band, which contained the majority of the VLPs and BIV Pr53, had a buoyant density of 1.16 g/ml. Electron microscopic examination of the material from the three bands confirmed that the majority of the VLPs migrated in the middle band. O.D., Optical density. [From L. Rasmussen, J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda, *Virology* **178,** 435 (1990) with permission of Academic Press.]

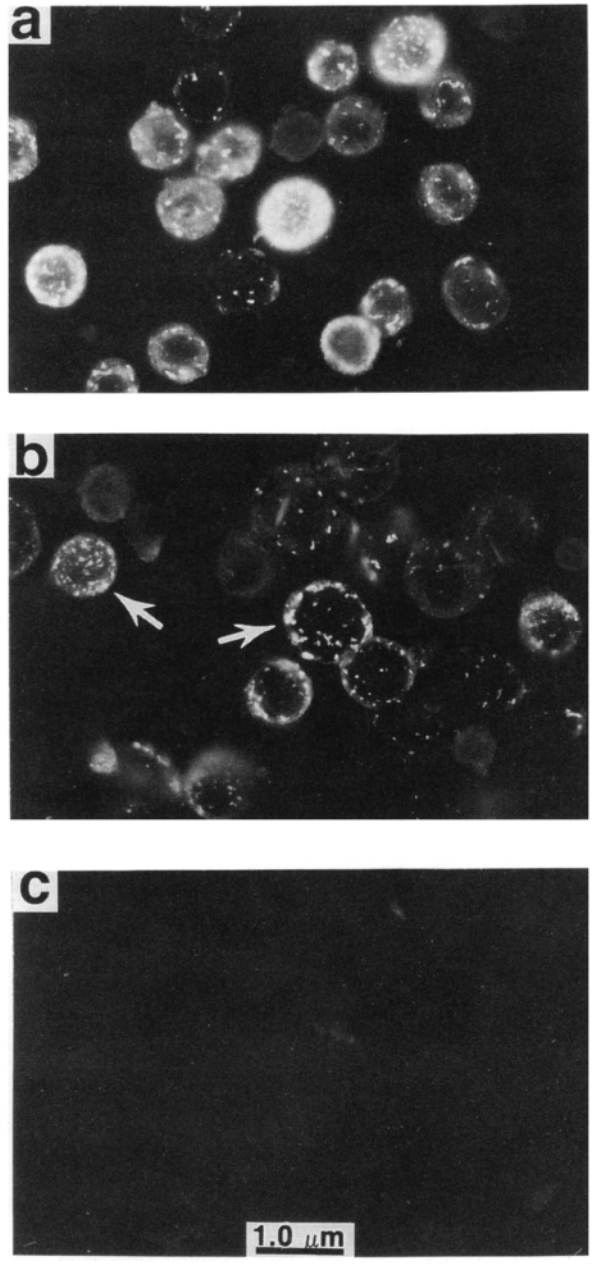

FIG. 2 Indirect immunofluorescence micrograph of AcNMPV–BIV *gag*-infected insect cells. Cells attached to glass slides were fixed in a 1:1 solution of acetone and methanol and rehydrated in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). A 1:30 dilution of rabbit anti-BIV serum in PBS and 1% BSA was used to detect BIV-specific antigen in recombinant baculovirus-infected cells. (a and b) Sf-9 cells infected with AcNMPV–BIV*gag*. (c) Uninfected Sf-9 cells. Note the diffuse intracellular staining in most cells in (a) and the speckled cell surface staining (arrows) in (b). Uninfected Sf-9 cells (c) were not reactive with BIV antiserum. [From L. Rasmussen, J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda, *Virology* **178,** 435 (1990) with permission of Academic Press.]

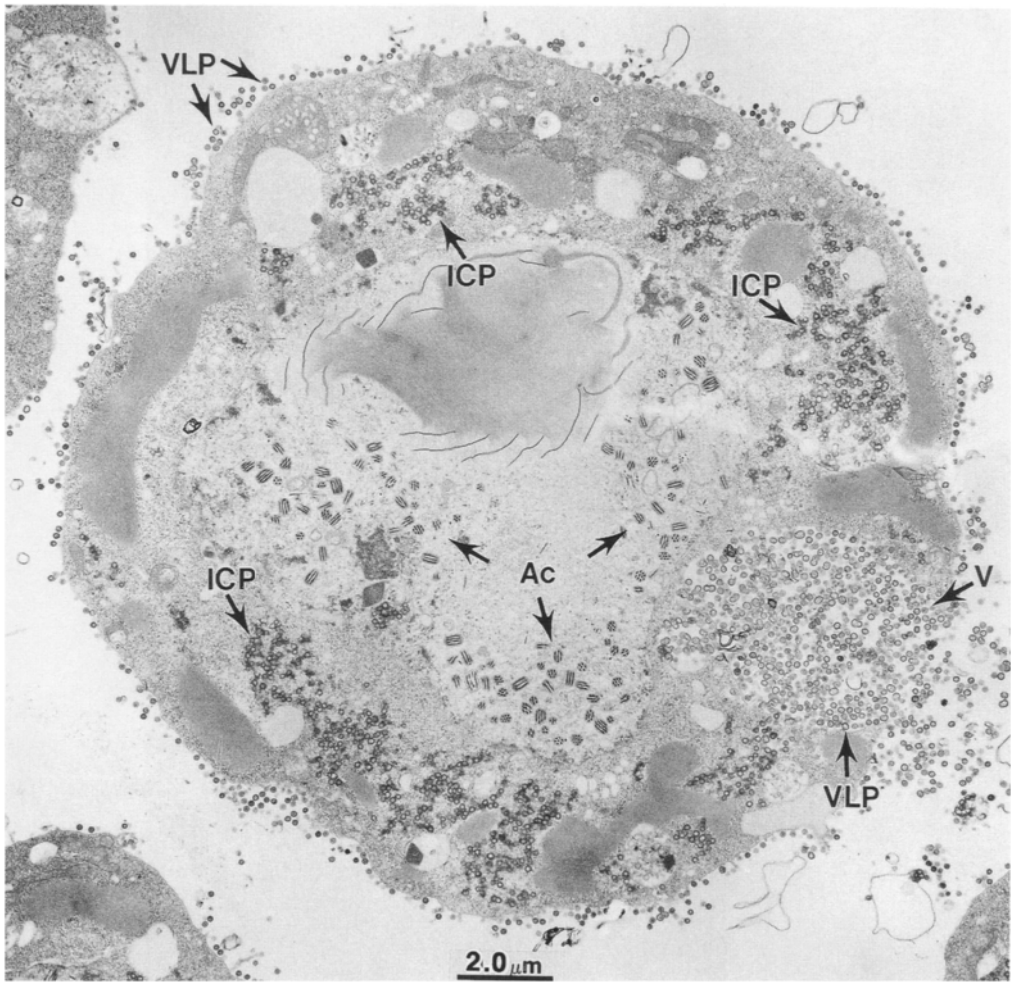

FIG. 3 Transmission electron micrograph of AcNMPV–BIV *gag*-infected Sf-9 cells. The micrograph shows AcNMPV–BIV *gag*-infected Sf-9 cells at low magnification. Many free extracellular VLPs (100–120 nm in diameter) can be seen on the perimeter of the cell and in a vacuole (V). The nuclear region has begun to degenerate and contains some nonoccluded baculoviruses (Ac). In addition to the extracellular VLPs, there are many intracellular particles (ICP) of the same size. [From L. Rasmussen, J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda, *Virology* **178,** 435 (1990) with permission of Academic Press.]

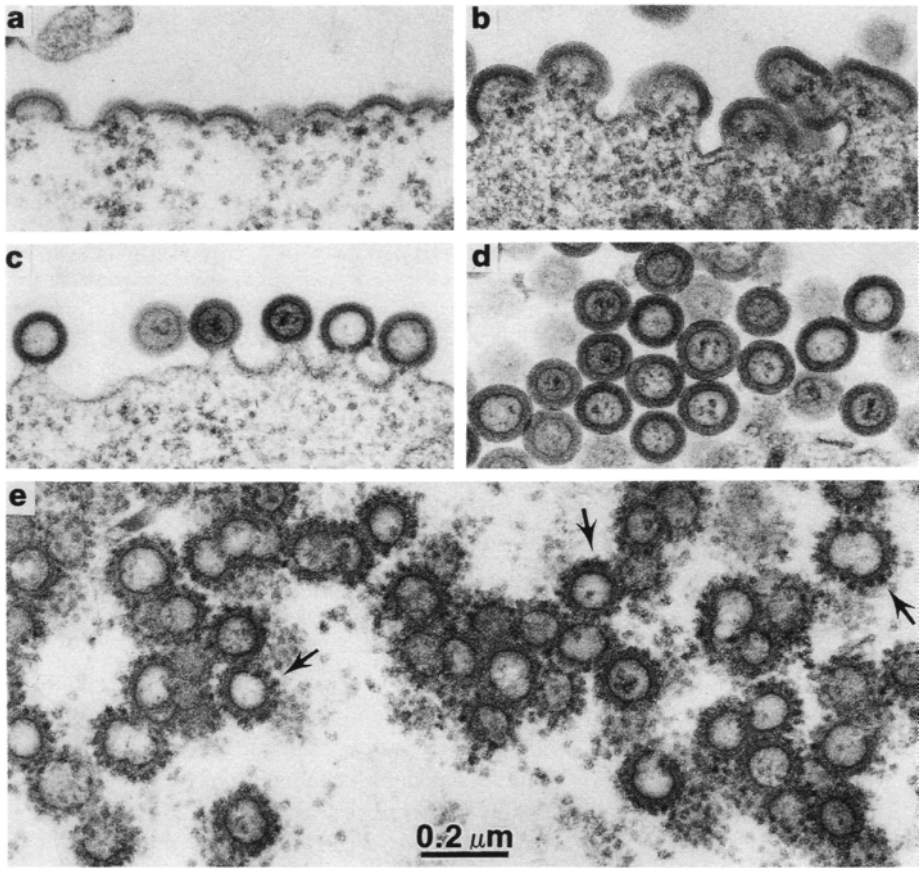

FIG. 4 Transmission electron micrograph of the morphogenesis of pseudovirions in AcNMPV–BIV *gag*-infected Sf-9 cells. VLPs and/or intracellular particles (ICPs) in AcNMPV–BIV *gag*-infected Sf-9 cells. (a) Early stage of budding. (b and c) Progressively later stages of budding. (d) Extracellular VLPs. (e) Accumulation of ICPs in Sf-9 cells infected with AcNMPV–BIV *gag*. Arrows in (e) indicate the position of ribosomes surrounding the ICPs. [From L. Rasmussen, J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda, *Virology* **178,** 435 (1990) with permission of Academic Pres.]

and pseudovirion concentration appear in the middle band, which has the same density as a retrovirus particle. The Gag precursor can be visualized by Coomassie staining of denaturing polyacrylamide gels and immunologically identified by Western blotting. For example, preparations of pseudovirions produced from recombinant baculoviruses containing the *gag* genes of HIV-1, SIV, and BIV are shown in Fig. 5.

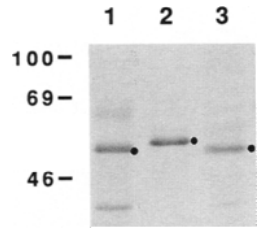

FIG. 5 Comparison of recombinant HIV, SIV, and BIV Gag precursor proteins obtained from pseudovirions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Sucrose gradient-purified pseudovirions were resolved by SDS–PAGE and visualized by staining with Coomassie Brilliant Blue G-250. Lanes 1, 2, and 3 contain HIV Pr55, SIV Pr57, and BIV Pr53, respectively. The dots emphasize the location of the protein of interest. The electrophoretic mobilities of the HIV-1 Pr55, SIV Pr57, and BIV Pr53 represent one of the few differences observed among these particles. Molecular weight markers are indicated on the left.

The recombinant Gag precursors migrate similarly to native Gag precursors and undergo correct posttranslational modification (e.g., myristic acid addition) as found in native proteins in mammalian cells (11).

Antigenic Properties of Gag Particles

Gag pseudovirions have been used as immunogens for the production of antisera and as antigen targets in ELISAs for naturally occurring antibodies, radioimmunoprecipitation, and Western blotting. The particulate nature of these VLPs may accentuate the immunogenicity of the Gag precursor, as ELISA titers exceeding 10^6 can be achieved when pseudovirions are introduced into animals with or without adjuvants. Antisera raised to the lentivirus Gag pseudovirions recognize the Gag precursor, Gag–Pol precursor, matrix, and capsid proteins in Western blots (Fig. 6) and radioimmunoprecipitation assays (10, 56).

Applications of Pseudovirion Technology

Retroviral Gag pseudovirions have been utilized primarily to study their antigenic and structural characteristics. They are especially useful as antigen sources for producing antibodies or analyzing serum samples. Future development of highly immunogenic Gag pseudovirions will no doubt also exploit their uses in vaccine development (36). The assembly of recombinant Gag precursors into physically stable particles offers an opportunity to package additional proteins or domains for antigenic presentation for immune stimulation. As long as the structural domains for Gag precursor interaction and particle assembly are not destroyed, the pseudovirions

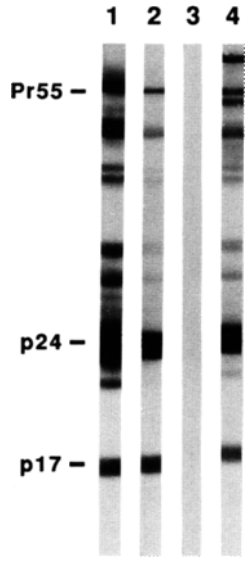

FIG. 6 Immunoblot analysis of rabbit antisera to HIV pseudovirions containing Pr55. Antisera were raised in rabbits immunized with 250 μg of HIV pseudovirions in either phosphate-buffered saline (lane 1) or Freund's adjuvant (lane 2). Sera from an unimmunized rabbit (lane 3) and an HIV-1–infected human (lane 4) were used as negative and positive controls, respectively. Commercially prepared HIV-1 whole virus Western blot strips were reacted with 1 : 100 and 1 : 1000 dilutions of primary and horseradish peroxidase-conjugated secondary antisera, respectively. Visualization of Gag proteins was by 4-chloro-1-naphthol.

should be capable of packaging additional immunoreactive domains (e.g., envelope proteins and cytokines). These domains can be expressed either in frame with the *gag* gene translation or through the *gag–pol* frameshifting mechanism. The delivery of cytokines, such as interleukins 2 or 12 or γ-interferon, which can be toxic when given systemically, may be more effective in modulating the immune response when released locally from pseudovirions.

Gag precursor proteins in pseudovirions can also be used as substrates in both *in vitro* and *in vivo* studies of viral protease action or inhibition. The Gag precursors can be released from purified pseudovirions by detergent solubilization for *in vitro* protease assays. Digestion with either recombinant or native viral protease results in the accurate cleavage of the Gag precursors (10, 11). Inclusion of a protease inhibitor, such as pepstatin A or a rationally designed inhibitor, can result in the reduction of Gag processing. The assembly of Gag pseudovirions in insect cells can be adapted to *in vivo* studies of protease inhibitors by the expression of lentivirus Gag–Pol precursors by recombinant baculoviruses. Inhibition of retroviral proteases expressed from

AcNMPV *gag–pol* can be measured by a reduction in Gag processing and an increase in pseudovirion production.

References

1. G. E. Smith, M. D. Summers, and M. J. Fraser, *Mol. Cell. Biol.* **3,** 2156 (1983).
2. G. D. Pennock, C. Shoemaker, and L. K. Miller, *Mol. Cell. Biol.* **4,** 399 (1984).
3. L. K. Miller, *Curr. Opin. Genet. Dev.* **3,** 97 (1993).
4. M. J. Fraser, *Curr. Top. Microbiol. Immunol.* **158,** 131 (1992).
5. H. A. Wood and R. R. Granados, *Annu. Rev. Microbiol.* **45,** 69 (1991).
6. J. M. Vlak and R. J. A. Keus, *in* "Viral Vaccines" (A. Mizrahi, ed.), p. 91. Wiley, New York, 1990.
7. S. Maeda, *Annu. Rev. Entomol.* **34,** 351 (1989).
8. K. Iatrou, R. G. Meidinger, and M. R. Goldsmith, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 9129 (1989).
9. K. T. Jeang, M. Holmgren-Konig, and G. Khoury, *J. Virol.* **61,** 1761 (1987).
10. L. Rasmussen, J. K. Battles, W. H. Ennis, K. Nagashima, and M. A. Gonda, *Virology* **178,** 435 (1990).
11. D. Gheysen, E. Jacobs, F. de Foresta, C. Thiriart, M. Francotte, D. Thines, and M. De Wilde, *Cell (Cambridge, Mass.)* **59,** 103 (1989).
12. M. Delchambre, D. Gheysen, D. Thines, C. Thiriart, E. Jacobs, E. Verdin, M. Horth, A. Burny, and F. Bex, *EMBO J.* **8,** 2653 (1989).
13. L. Luo, Y. Li, and C. Y. Kang, *Virology* **179,** 874 (1990).
14. J. M. Vlak, F. A. Klinkenberg, K. J. M. Zaal, M. Usmany, E. C. Klinge-Roode, J. B. F. Geervliet, J. Roosien, and J. W. M. Van Lent, *J. Gen. Virol.* **69,** 765 (1988).
15. D. H. L. Bishop, *Semin. Virol.* **3,** 253 (1992).
16. H. Varmus, *Science* **240,** 1427 (1988).
17. J. M. Coffin, *Curr. Top. Microbiol. Immunol.* **176,** 143 (1992).
18. J. A. Levy, *Microbiol. Rev.* **57,** 183 (1993).
19. M. A. Gonda, *AIDS* **6,** 759 (1992).
20. M. A. Gonda, *Ann. N.Y. Acad. Sci.* **724,** 22 (1994).
21. V. Karacostas, K. Nagashima, M. A. Gonda, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 8964 (1989).
22. V. Karacostas, E. J. Wolffe, K. Nagashima, M. A. Gonda, and B. Moss, *Virology* **193,** 661 (1993).
23. S. Jenkins, L. Gritz, C. H. Fedor, E. M. O'Neill, L. K. Cohen, and D. L. Panicali, *AIDS Res. Hum. Retroviruses* **7,** 991 (1991).
24. L. Prevec, B. S. Christie, K. E. Laurie, M. M. (Smith) Bailey, F. L. Graham, and K. L. Rosenthal, *J. Acquired Immune Deficiency Syndromes* **4,** 568 (1991).
25. J. E. Gilmour, J. M. Senior, N. R. Burns, M. P. Esnouf, K. Gull, S. M. Kingsman, A. J. Kingsman, and S. E. Adams, *AIDS* **3,** 717 (1989).
26. N. Higashihashi, Y. Arai, T. Enjo, T. Horiuchi, Y. Saeki, K. Sakano, Y. Sato, K. Takeda, S. Takashina, and T. Takahashi, *J. Virol. Methods* **35,** 159 (1991).
27. T. Urakawa, M. Ferguson, P. D. Minor, J. Cooper, M. Sullivan, J. W. Almond, and D. H. L. Bishop, *J. Gen. Virol.* **70,** 1453 (1989).

28. R. C. Rose, W. Bonnez, R. C. Reichman, and R. L. Garcea, *J. Virol.* **67,** 1936 (1993).
29. S.-Z. Xi and L. M. Banks, *J. Gen. Virol.* **72,** 2981 (1991).
30. J. A. López de Turiso, E. Cortés, C. Martínez, R. Ruiz de Ybáñez, I Simarro, C. Vela, and I. Casal, *J. Virol.* **66,** 2748 (1992).
31. M. Labbé, A. Charpilienne, S. E. Crawford, M. K. Estes, and J. Cohen, *J. Virol.* **65,** 2946 (1991).
32. A. S. Belyaev and P. Roy, *Nucleic Acids Res.* **21,** 1219 (1993).
33. S. Morikawa, T. F. Booth, and D. H. L. Bishop, *Virology* **183,** 288 (1991).
34. S. S. Hong and P. Boulanger, *J. Virol.* **67,** 2787 (1993).
35. D. R. Thomsen, A. L. Meyer, and L. E. Post, *J. Gen. Virol.* **73,** 1819 (1992).
36. L. Luo, Y. Li, P. M. Cannon, S. Kim, and C. Y. Kang, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10527 (1992).
37. J. C. Griffiths, S. J. Harris, G. T. Layton, E. L. Berrie, T. J. French, N. R. Burns, S. E. Adams, and A. J. Kingsman, *J. Virol.* **67,** 3191 (1993).
38. R. Wagner, H. Fliessbach, G. Wanner, M. Motz, M. Niedrig, G. Deby, A. von Brunn, and H. Wolf, *Arch. Virol.* **127,** 117 (1992).
39. L. Shen, G. P. Mazzara, S. O. DiSciullo, D. L. Panicali, and N. L. Letvin, *AIDS Res. Hum. Retroviruses* **9,** 129 (1993).
40. W. F. Hink, D. R. Thomsen, D. J. Davidson, A. L. Meyer, and F. J. Castellino, *Biotechnol. Prog.* **7,** 9 (1991).
41. T. D. C. Grace, *Nature (London)* **95,** 788 (1962).
42. W. F. Hink, *in* "Methods in Enzymology" (W. B. Jakoby and I. Pastan, eds.), Vol. 58, p. 450. Academic Press, New York, 1979.
43. G. R. Gardiner and H. Stockdale, *J. Invertebr. Pathol.* **25,** 363 (1975).
44. S. A. Weiss, G. C. Smith, S. S. Kalter, and J. L. Vaughn, *In Vitro* **17,** 495 (1981).
45. D. Inlow, A. Shauger, and B. Maiorella, *J. Tissue Cult. Methods* **12,** 13 (1989).
46. V. A. Luckow and M. D. Summers, *Virology* **167,** 56 (1988).
47. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
48. J. Vialard, M. Lalumière, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. Richardson, *J. Virol.* **64,** 37 (1990).
49. D. Zuidema, A. Schouten, M. Usmany, A. J. Maule, G. J. Belsham, J. Roosien, E. C. Klinge-Roode, J. W. M. van Lent, and J. M. Vlak, *J. Gen. Virol.* **71,** 2201 (1990).
50. P. A. Kitts, M. D. Ayres, and R. D. Possee, *Nucleic Acids Res.* **18,** 5667 (1990).
51. P. A. Kitts and R. D. Possee, *BioTechniques* **14,** 810 (1993).
52. M. D. Summers and G. E. Smith, *Tex. Agric. Exp. Stn., [Bull.]* **1555** (1987).
53. S. G. Mann and L. A. King, *J. Gen. Virol.* **70,** 3501 (1989).
54. D. L. Knudson, *Intervirology* **11,** 40 (1979).
55. C. V. Benton, H. M. Hodge, and D. L. Fine, *In Vitro* **14,** 192 (1978.).
56. J. K. Battles, M. Y. Hu, L. Rasmussen, G. J. Tobin, and M. A. Gonda, *J. Virol.* **66,** 6868 (1992).

[16] Retrovirus Reverse Transcription and Integration

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Introduction

Retroviruses contain an RNA genome that is replicated through a double-stranded DNA intermediate. DNA synthesis is initiated from a tRNA primer annealed to the 5′ untranslated region of the viral RNA catalyzed by the virus-encoded reverse transcriptase (RT). During the reverse transcription process the 5' unique (U5) and 3' unique (U3) regions of the viral RNA are duplicated and juxtaposed to form the ends of the linear DNA. Each U3–R–U5 end is referred to as a long terminal repeat (LTR). The double-stranded DNA, flanked by the LTRs, is then integrated into the host chromosome through the action of a second virus-encoded enzyme, integrase (IN). Our understanding of the mechanistic roles of these two enzymes in viral replication has vastly increased with the cloning of the retrovirus genome and its subsequent molecular genetic analysis. In this article we discuss methods both to introduce mutations into the retrovirus genome and to reconstitute *in vitro* the activities of RT and IN in the initiation of reverse transcription and integration of viral DNA, respectively.

Structure of U5 RNA Is Required for Efficient Initiation of Reverse Transcription

The efficiency of Rous sarcoma virus (RSV) RT to initiate DNA synthesis from the $tRNA^{Trp}$ primer is dependent not only on the primer, but also on secondary structures near the primer binding site (PBS) in the viral RNA. Readers are referred to the work of Leis *et al.* (1) for a review of this area. These RNA secondary structures are called the U5–leader and U5–inverted repeat (IR) stems (see Fig. 1). The U5–leader stem is formed by sequences in the middle of U5 and in the leader. Mutations (either deletions or base substitutions) that disrupt this structure impair the initiation of reverse transcription in RSV-infected cells, while mutations that alter the sequence but retain the structure have no effect (2). The U5–IR stem is composed of an IR in U5 adjacent to the PBS. Disruption of this structure results in the same defect to initiation of reverse transcription (3, 4). The U5–IR sequence, at the level of DNA, forms, in part, the IN recognition site, so that mutations that alter its nucleotide sequence cause defects to integration as well as to reverse transcription.

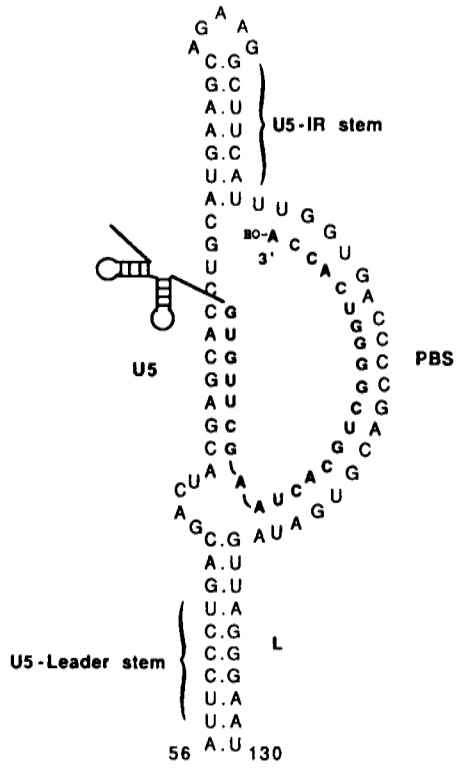

FIG. 1 Predicted RNA secondary structure for the 5′ noncoding region of RSV (Schmidt–Ruppin A) RNA near the primer binding site (PBS). The numbers indicate nucleotides from the 5′ end of the viral RNA. U5, Nucleotide sequences derived from the 5′ end of viral RNA; L, leader sequence. The U5–leader and U5–IR stems are shown. The $tRNA^{Trp}$ primer has been partially unwound to show the interaction with the PBS and U5 RNA.

Construction and Analysis of U5 RNA Mutations

Avian Retrovirus Plasmids

RSV was the first retrovirus to have its genome completely sequenced, and circularly permuted molecular clones of the virus, such as the plasmid pATV8-K are commonly available (5). This plasmid represents a clone of the Prague C strain of RSV, which can be grown on chicken embryo fibroblasts. Another DNA clone of importance is pRCAS–B*neo* (6). This plasmid is a molecular clone of the Schmidt–Ruppin A strain of RSV, with a *pol* gene derived from the Bryan high-titer strain. This mosaic

virus can be propagated to high titers on QT-6 quail embryo fibroblasts, a cell line that is easier to maintain than chicken embryo fibroblasts. Additionally, since this vector expresses the *neomycin phosphotransferase* gene, successfully transfected cells can be selected using the drug G-418. This permits the isolation of cell lines that produce viral particles containing mutations in the RNA genome, independent of the effect of the mutation on viral replication. These mutant particles can be subsequently used to infect cells and the sites of genetic lesions can be defined by quantifying viral replication intermediates.

The plasmid pRCAS–B*neo* contains two complete copies of U5 sequences present in the viral LTRs, so that mutations placed in one copy may accidentally be rescued by recombination with the other during passage in bacteria. To avoid this problem, two derivatives of pRCAS–B*neo* have been constructed by digesting the plasmid with *Eco*RV. This restriction enzyme cleaves the viral DNA at a unique site in the *pol* gene and once in the vector, effectively separating the two copies of U5. Of the two fragments released, the larger contains the 5′ LTR, the PBS, the leader, the *gag* gene, and approximately half of the *pol* sequences (up to nucleotide 3655). This fragment also contains the pBR322 β-lactamase gene (for ampicillin resistance in *Escherichia coli*) and the pBR322 *ori*. This fragment is purified and self-ligated to produce the plasmid pDC101B (see Fig. 2). A second plasmid, pDC102B, is created by digesting pRCAS–B*neo* with *Sac*I (cuts at position 255 in the leader), and partially with *Rsr*II (which cuts at position 42 in U5 and within the *neo* sequence). The large fragment, consisting of part of the leader, *gag, pol, env, neo,* U3, R, and part of U5 (until nucleotide 42), is purified. After overhangs are removed by action of the Klenow fragment of *E. coli* DNA polymerase I, it is blunt-end cloned into *Hinc*II–digested plasmid vector pTZ18. Mutations that define a role for RSV RNA secondary structures in the initiation of reverse transcription have been placed between nucleotides 42 and 100, a region that is present in pDC101B, but missing in pDC102B. To reconstitute an infectious clone, *Hpa*I-digested pDC101B is ligated to *Hpa*I-digested pDC102B (*Hpa*I cuts at position 2743 in the *pol* gene), and the ligated linear DNA is transfected into QT-6 fibroblasts (see Fig. 2).

Site-Directed Mutagenesis of the Viral Genome

M13-based mutagenesis techniques do not work well for introducing mutations into the U5 region of the retrovirus genome. This is due to the presence of the stable RSV U5 RNA secondary structures, which presumably form in single-stranded DNA. However, two alternative methods of mutagenesis have been used successfully. In the first, DNA sequences containing mutations are chemically synthesized from the *Rsr*II site (position 42) in U5 to the *Bst*EII site (position 103) in the PBS (2). These are assembled and ligated *in vitro* and cloned into *Rsr*II/*Bst*EII-digested pDC101B. While this technique is very effective, it is expensive and labor intensive. For this

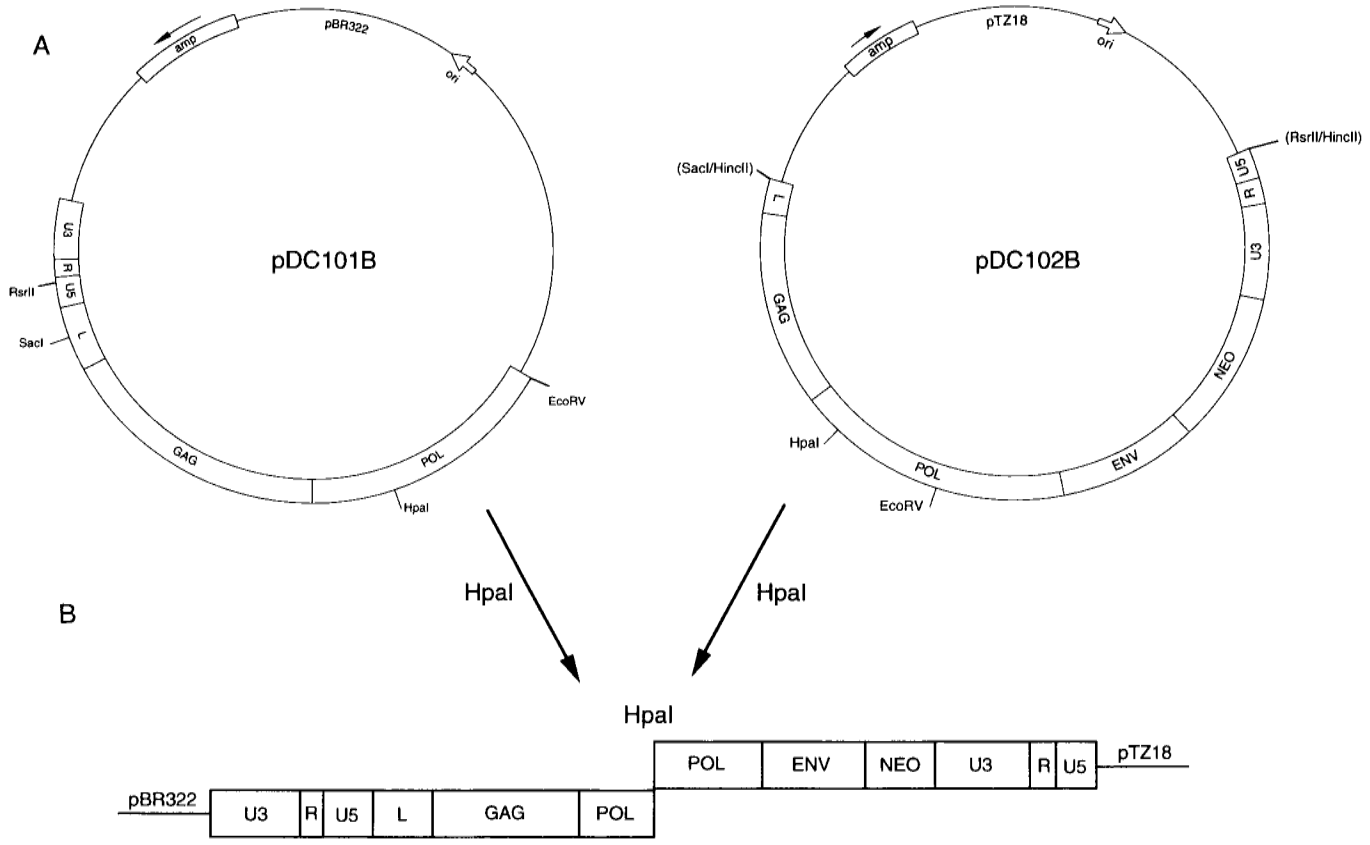

FIG. 2. (A) Structure of the plasmids pDC101B and pDC102B. Wide bars represent viral sequences and thin lines represent prokaryotic vector sequences. U3 and U5 are sequences derived from the 3′ and 5′ ends of untranslated viral RNA, respectively. R is a small direct repeat found at the termini of U5 and U3. L is an untranslated leader RNA sequence between U5 and the *gag* gene. The *gag*, *pol*, *env*, and *neo* genes are as indicated. The sizes of the indicated regions are not to scale. Ori, Origin of the bacterial plasmid pTZ18. (B) pDC101B and pDC102B are digested with *Hpa*I and ligated prior to transfection. The structure of the linear product from which a complete viral genome and *neo* gene can be expressed is shown.

reason an alternative polymerase chain reaction (PCR)-based mutagenesis approach has been developed (7, 8). It is a modification of the procedure of Sarkar and Sommer (9).

PCR Mutagenesis

The PCR mutagenesis technique utilizes four primers, one of which is mutagenic (7). In the first reaction a PCR is performed using the 5′ primer (termed A) and the mutagenic primer (termed B) (see Fig. 3). The product of this reaction (product I) contains the desired mutation. In a separate reaction a PCR is performed using primer C and the 3′ primer D. Primer C is 15–100 nucleotides 5′ to the mutagenic primer, but 3′ to primer A (see Fig. 3). The PCR product from this second reaction (product II) is wild type in sequence, and has a 15- to 100-nucleotide "overlap" with the product of the first PCR reaction. Products I and II are gel-purified and used as templates in a 5:1 molar ratio in a third PCR reaction, using primers A and D. The products of this final reaction are cloned using convenient restriction sites (internal

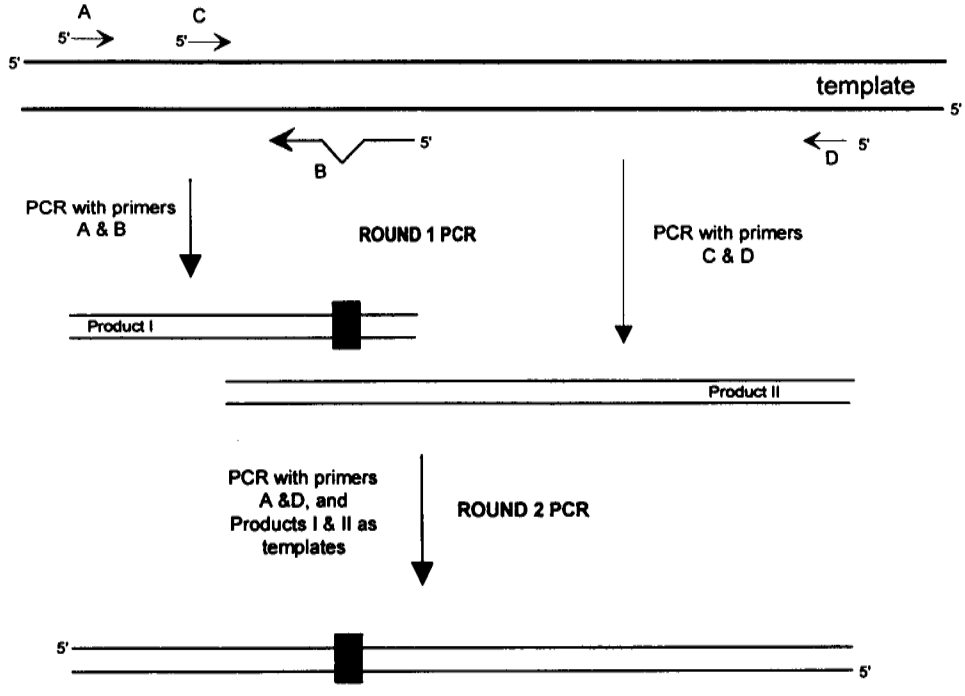

FIG. 3 Schematic representation of PCR mutagenesis. Product I, which contains the desired mutation (stippled box), is amplified using primers A and B. Primer B contains the mutation. Product II is amplified separately using primers C and D. Products I and II are purified and used as templates in a third PCR reaction with primers A and D.

to the final PCR product) into the plasmid pDC101. In all cases mutants are easily identified and the efficiency of the reaction ranges from 60% to 90%.

The following PCR conditions are recommended to reduce aberrant mutations. First, the thermostable Vent DNA polymerase (New England Biolabs, Boston, MA) should be used. Compared to *Taq* DNA polymerase, this enzyme has a much lower rate of misincorporation of deoxynucleotides due to its intrinsic $3' \rightarrow 5'$ proofreading exonuclease activity. Also, it does not add nontemplated 3′ deoxynucleotides (as does *Taq* polymerase) so that the product DNAs are ready for blunt-end cloning. Second, PCR reactions should be limited to 15–18 cycles using 50 pmol of each primer in the 100-μl reactions. Both of these conditions greatly reduce the appearance of "primer-dimers" and other artifactual products. Finally, the Mg^{2+} concentration should be optimized for each set of primers to improve the yield and specificity of amplified products.

Transfection of Viral DNA into QT-6 Quail Fibroblasts

Once mutations have been verified by DNA sequencing, DNAs can be introduced into QT-6 quail fibroblasts. Cells are maintained in Dulbecco's modified Eagle's medium, supplemented with 4% (v/v) fetal calf serum and 20 m*M* L-glutamine. A penicillin–streptomycin solution is also added to a final concentration of 25 U/ml (penicillin) and 25 μg/ml (streptomycin). For transfections the cells are grown to 70% confluence on 100-mm dishes and the DNA is introduced using the calcium phosphate method. Fifteen to 20 μg of cut and ligated DNA is ethanol-precipitated, washed extensively with 70% (v/v) ethanol, dried, and suspended in 225 μl of sterile HEPES-buffered saline [137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* dextrose, and 20 m*M* HEPES (pH 7.1)]. Twenty-five microliters of 1.25 *M* $CaCl_2$ is slowly added to this solution, and the mixture is allowed to sit at room temperature for 20 min to efficiently form the $CaCl_2$–DNA precipitate. The DNA–HEPES–$CaCl_2$ suspension is then slowly deposited onto the surface of the cells and allowed to remain there for 4–5 hr. At this time the cells are shocked with 15% (v/v) glycerol in HEPES-buffered saline for 15–20 sec and the medium is replaced. The cells are fed 1 day after transfection and split on the second day. At the same time, the cells are placed under G-418 selection at a concentration of 100–200 μg/ml. G-418–resistant colonies usually develop within 3 weeks and produce high titers of viral particles that can be collected from the cell supernatants.

RT Assays with Exogenous Primer Templates

To estimate the number of viral particles in the cell supernatants, RT activity is determined using an exogenously added primer/template. Five to 15 μl of cell super-

natant is placed into a solution of 0.07% (v/v) Nonidet P-40 (NP-40; Sigma, St. Louis, MO), 83 m*M* Tris (pH 8.3), 100 m*M* NaCl, 17 m*M* magnesium acetate, 33 m*M* dithioerythritol (DTE), 0.5 optical density unit (at 260 nm) of poly(G) per milliliter, 0.25 optical density unit of oligo$(dC)_{12-18}$ per milliliter, and 0.03 μM labeled dCTP (2.5 μCi, 3000 Ci/mmol). The mixture is incubated at 37° C for 30 min, then 10 μl of each reaction is spotted in duplicate on DE-81 paper in premarked 1.5 × 1.5-cm squares. The paper is washed three times in 2× SSC (SSC: 150 m*M* NaCl plus 15 m*M* sodium citrate) on a rocker at room temperature. Each wash is performed for 15 min. After this the paper is rinsed twice with 95% (v/v) ethanol, dried under a heat lamp, and cut into squares for scintillation spectroscopy.

Assays for Initiation of Reverse Transcription

Detection of Strong Stop DNA

The effect of U5 mutations on initiation of reverse transcription can be directly examined in virus particles collected from the QT-6 cell lines. The first detectable DNA product synthesized from the tRNA primer is strong stop DNA, which is complementary to the template RNA from the PBS to the 5′ end of the viral RNA. It ranges in size from 100 to a few hundred nucleotides, depending on the retrovirus. For RSV, strong stop DNA is 101 deoxynucleotides in length. The synthesis of strong stop DNA can be detected in permeabilized virions as follows: 16 hr after the addition of fresh media, virus-containing supernatants are collected from the producer cells from two 100-mm plates and spun at 4000 *g* for 10 min at 4° C to remove cellular debris. The supernatants from this spin are sedimented through a 5-ml cushion of 20% (w/v) sucrose in 50 m*M* Tris (pH 8.0) plus 1 m*M* EDTA (5TE) for 100 min at 85,000 *g* at 4° C. After removing the media and the sucrose cushion, the tubes are thoroughly drained by inversion and the remaining liquid is carefully aspirated. Virus is suspended in 5TE and quantified using an exogenous primer/template assay. The amount of virus that incorporates 0.1 pmol of deoxynucleotide in 30 min is then incubated in 50-μl reactions in the presence of 15 μg/ml mellitin (Sigma) as described above for the RT assay, but without the exogenous primer/template. [$\alpha - {}^{32}$P]dCTP (3000 Ci/mmol, 10 mCi/ml) is added to a final concentration of 6.5 Ci/mmol. Unlabeled dATP, dGTP, and TTP are present at a final concentration of 1 m*M*. Reactions are stopped by incubation with 10 μg of RNase A for 30 min at room temperature. An equal volume of 5TE containing 1% (w/v) sodium dodecyl sulfate and 3 μg of carrier tRNA is then added, and reaction mixtures are extracted with phenol and precipitated using ammonium acetate (2.5 *M* final concentration) and ethanol. The products of the reaction are analyzed on denaturing 5–8% (w/v) polyacrylamide gels and detected by autoradiography. Borroto-Esoda and Boone (10) have recently refined the

mellitin procedure by the addition of EGTA at a final concentration of 0.5–1.0 m*M*. The authors reported that the EGTA relieves inhibition by CA^{2+}, which is often a low-level contaminant in preparations of mellitin and the nonionic detergent NP-40. The latter has been used as an alternative agent to permeabilize virions at a final concentration of 0.025–0.1%.

Detection of tRNA–dAMP

In RSV, murine leukemia virus (MuLV), and several other retroviruses, the first two nucleotides added to the 3′ end of the primer $tRNA^{Trp}$ are both dAMP. It was therefore possible to examine the addition of these first deoxynucleotides by carrying out the mellitin reactions in the presence of only [$\alpha - {}^{32}$P]dATP. In these reactions, the dATP is present at a final concentration of 0.65 μM and 3000 Ci/mmol. Also, the RNase hydrolysis step is eliminated so that the tRNA–dAMP product can be detected by electrophoresis through denaturing 15% (w/v) polyacrylamide gels.

U5 RNA Interactions with the Primer tRNA

In addition to interactions between the 3′ end of the $tRNA^{Trp}$ primer and the PBS, there is a separate interaction with a 7-nucleotide U5 sequence lying between the RSV U5–IR and U5–leader stems and the TψC loop of the $tRNA^{Trp}$ primer. Base substitutions in the U5 RNA, which disrupt this interaction, result in the same defect to initiation of reverse transcription *in vivo* that is observed with mutations in the U5–IR and U5–leader stems (4, 8). However, to demonstrate that this defect is due to a loss of the interaction between the U5 RNA and the tRNA, an *in vitro* reconstituted system is required to introduce base substitutions into the primer as well as into the viral RNA. The reconstituted system described below uses conditions similar to those defined by Prats *et al.* (11) and Barat *et al.* (12). Viral RNA templates and primer tRNA polymerase. Primers are annealed to the template RNA in the presence of avian myeloblastosis virus (AMV) RT, and the annealed complexes are extended by the addition of deoxynucleotide triphosphates.

Assembly of the Reverse Transcription Initiation Complex in Vitro

Reverse transcription reactions are reconstituted *in vitro* by mixing 50 pmol of RNA primer with 1.2 μg of template RNA in a 10-μl annealing reaction that contains 40 m*M* Tris (pH 8.0), 60 m*M* NaCl, and 60 U of AMV RT (Molecular Genetic Resources, Tampa, FL). A DNA primer (25 pmol) that anneals 5′ to the PBS can also

be added at this step as an internal control to quantify the amount of viral RNA added. The RT and RNAs are incubated at 40° C for 30 min and then brought to a final volume of 20 μl containing 60 m*M* NaCl, 6.7 m*M* $MgCl_2$, 5 m*M* DTE, 25 m*M* Tris (pH 8.0), 0.5 m*M* (each) ATP, dATP, dGTP, and TTP, and 12.5 μM [$\alpha - {}^{32}$P]dCTP (3000 Ci/mmol, 60 μCi). An additional 30 U of AMV RT is added, and the reaction mixture is incubated for 40 min at 37° C. The reaction products are then treated with 10 μg of RNase A for 30 min at room temperature followed by phenol and phenol–chloroform–isoamyl alcohol (25:24:1) extractions. One third of each reaction mixture is analyzed by denaturing polyacrylamide gel electrophoresis. Products are detected by autoradiography.

To produce the synthetic RNA templates, the 5′ noncoding region of the virus is synthesized as a PCR product approximately 400 nucleotides long. Using a modified 5′ primer, a T7 promoter is introduced at the 5′ end during the PCR reaction. The PCR product is gel-purified and then added to a T7 transcription reaction (4, 13). The reaction is stopped by incubation with 10 U of RQ1 DNase (Promega, Mathion, WI) for 1 hr at 37° C, after which the reaction mixture is extracted with phenol, followed by phenol–chloroform–isoamyl alcohol. Sodium acetate (pH 5.2) is added to a final concentration of 0.3 *M*, and the RNA products are precipitated by the addition of 2 vol of 100% (v/v) ethanol. The precipitates are washed with 70% (v/v) ethanol, dried, and suspended in 20 m*M* NaCl, 10 m*M* Tris (pH 7.6), plus 2 m*M* EDTA. Template RNAs are stored in 1.2-μg aliquots at a concentration of 0.3 μg/μl at −20° C. Immediately prior to use, RNAs are heated to 80° C in a sand bath and slowly cooled to room temperature. RNA primers are produced using chemically synthesized DNAs followed by T7 RNA polymerase transcription (14). By changing the sequence and the length of the DNA oligomers, various RNA primers of different sizes can be prepared. The primer RNAs are purified by 15% (w/v) denaturing polyacrylamide gel electrophoresis, eluted with 0.5 *M* ammonium acetate, 5 m*M* EDTA, and 0.1 *M* NaCl, further purified on small C-18 reversed-phase chromatography columns, dried in a Savant Speed Vac, and suspended at a concentration of 50 pmol/μl (50 μM) in RNase-free distilled water.

Integration of Retroviral DNA

An important intermediate in the retroviral life cycle is the provirus. Formation of the provirus marks the end of early steps in the life cycle. Retroviruses utilize a virus-encoded enzyme, IN, to mediate the insertion of the linear viral DNA into the host chromosomal DNA. In the integration reaction 2 nucleotides are lost from the 5′ end of U3 and the 3′ end of U5. In addition, there is a duplication of host sequences at the position of integration. The size of this duplication is dependent on the infecting virus. For RSV the duplication is 6 deoxynucleotides long (15).

Detection of Integrated Viral DNA in Cells

To detect integrated viral DNA, especially at short times after infection, one should use a quantitative PCR approach. There is an insufficient amount of viral DNA to be detected by direct Southern hybridization. The high-molecular-weight Hirt pellet (16) is sheared by passing it through a 21-gauge needle several times. It is then heated to 55° C and layered onto a 10–40% (w/v) sucrose gradient in 0.1 *M* NaCl, 10 m*M* Tris (pH 8.0), and 1 m*M* EDTA. The gradients are spun for 16 hr at 85,000 *g* in a Beckman SW28 rotor at 4° C. Fractions containing DNA over 25 kb in size, which would exclude unintegrated viral DNA, are combined and precipitated with 2 vol of 100% (v/v) ethanol. Five micrograms of this DNA is used in PCR reactions with primers such as those that amplify a 400-nucleotide-long *gag–pol* junction fragment. The cycling conditions are 92° C for 2 min, 55° C for 90 sec, and then 72° C for 1 min. Amplification of 0.01–1 pg of a control DNA is linear under these conditions for up to 25 cycles. To detect the viral DNA, 24-cycle amplified DNA is separated by gel electrophoresis, blotted, and hybridized to a virus-derived ^{32}P-labeled probe (2×10^9 cpm/μg).

In Vitro Integration Assays

Much of our knowledge of the mechanism of action of the viral IN comes from the development of the reconstituted integration systems. The viral recognition sequences for IN are present at the termini of the LTRs. The first reconstituted system used purified AMV IN and double-stranded oligodeoxynucleotide substrates that resembled the terminal 15 nucleotides of unprocessed U3 and U5 LTR termini (17). Using these substrates, it was demonstrated that in the presence of Mg^{2+} or Mn^{2+}, purified IN nicked the duplex substrates 2 nucleotides from the 3′ end on either the U5 plus strand or the U3 minus strand. This specificity is identical to the processing observed *in vivo*. Shortly after this initial report, it was determined that the oligodeoxynucleotide assay could also be used to examine the joining of cut viral DNA with host DNA (18, 19). This was based on the observation that some of the products of the integration reaction were larger than the starting labeled oligodeoxynucleotides. When these larger products were purified and sequenced, they were shown to possess the sequence of the oligodeoxynucleotide until the CA 3′ dinucleotide. After this point the sequence became random. This was expected for authentic integration events, in which the host cleavage site is randomly selected (15).

The duplex oligodeoxynucleotide assay has been used in RSV, MuLV, and human immunodeficiency virus (HIV) systems to examine substrate sequence requirements for cleavage, joining, and the reverse disintegration reactions (17, 3, 20, 21) and to elucidate the chemical basis of the integration reaction (22). The same system has

also been widely used to characterize mutations placed in RSV, MuLV, and HIV INs (23–29) and has provided a simple method to characterize the IN proteins from other retroviruses, such as visna (30). Variants of the duplex oligonucleotide assay have been described, including those in which both strands are synthesized as a single molecule that can fold as a hairpin (31), or in which the products of the cleavage reaction are linked to biotin, thus permitting rapid isolation by affinity chromatography (32).

An Assay for the Integrase Using Oligodeoxynucleotides

Standard 10-μl reactions contain 1 pmol of double-stranded oligodeoxynucleotide. The duplex is 5′ end-labeled with [$\gamma - {}^{32}$P]ATP on the strand nicked by IN prior to annealing the two strands together (specific activity, 10^5 cpm/pmol). The buffer conditions are 25 m*M* Tris (pH 8.3), 10 m*M* 2-mercaptoethanol, and either 2 m*M* $MnCl_2$ or 3 m*M* $MgCl_2$. One pmol of IN is added [in 40% (v/v) glycerol, 0.4 *M* NaCl, 33 m*M* Tris (pH 7.6), 1 m*M* DTE, 0.1 m*M* EDTA, and 0.1% (v/v) Triton X-100, Sigma]. After the addition of IN, the reactions are incubated at 37° C for 90 min, stopped by the addition of 10 μl of loading buffer (95% (v/v) formamide, 10 m*M* EDTA, 0.025% bromphenol blue, and 0.025% (w/v) xylene cyanole), and heated to 95° C for 5 min. Reaction products are separated on 20% (w/v) denaturing polyacrylamide gels and detected by autoradiography.

While the oligonucleotide assays are simple, they do not provide a mechanism to assay the entire process of integration in which left and right LTRs are coordinately inserted into a single site on the target DNA. Brown *et al.* (33) demonstrated that a nucleoprotein complex from RSV-infected cells containing viral DNA, IN, and other viral proteins could correctly integrate the viral DNA into exogenous bacteriophage λ DNA when the latter is used as a target. Several reconstituted assays using λ DNA have now been described (18, 19).

In Vitro Integration Assays Using Linear DNA

Linear substrate DNA (0.5 μg) and 1.5 μg of concatameric λ target DNA are added to 75-μl reaction mixtures that contain 20 m*M* HEPES (pH 7.6), 6 m*M* Tris (pH 7.4), 150 m*M* KCl, 5 m*M* $MgCl_2$, 1 m*M* DTE, 10% (v/v) dimethyl sulfoxide, and 5% (w/v) polyethylene glycol (PEG). The components are chilled on ice (with the exception of PEG), after which 2.5–10 pmol of purified IN is added. Immediately after the addition of IN, PEG is added, and the reactions are incubated at 37° C for 1 hr. Reactions are terminated by the addition of Pronase (Sigma, St. Louis, MO) at 5 mg/ml in 250 μ*M* EDTA and 0.1% (w/v) sodium dodecyl sulfate. After incubation at 37° C for 2 hr, sodium acetate is added to a final concentration of 0.3 *M*, and the reactions

are phenol-extracted, followed by chloroform extraction. The DNA is ethanol-precipitated, washed with 70% (v/v) ethanol, and suspended overnight in 4 μl of 1 m*M* Tris (pH 8.0) plus 0.05 m*M* EDTA. The DNAs are then packaged in an *in vitro* λ packaging extract (Gigapack Gold, Stratagene) and used to infect an appropriate strain of *E. coli.* Since the viral substrate contains ampicillin and tetracycline genes, clones can be rapidly identified.

Fitzgerald and Grandgenett (34) have improved the yield of the reconstituted integration reactions by using a 30-μl reaction mixture containing 100 m*M* NaCl, 20 m*M* Tris (pH 7.5), 10 m*M* dithiothreitol, 5 m*M* $MgCl_2$, 10% (v/v) dimethyl sulfoxide, 5% (w/v) PEG, 1% (v/v) glycerol, and 0.05% (v/v) NP-40. (Note: Omission of the NP-40 severely reduces the efficiency of the reaction.) The DNA, IN, and buffers (minus the PEG) are preincubated on ice for 10 min. The PEG is then added and the reaction mixture is incubated at 37° C for 60 min. Reactions are stopped as described above.

Conclusions

Retroviruses are associated with severe pathogenic effects in a wide range of hosts, including HIV-induced AIDS in humans (35). As such, viral specific enzymes have received much attention as targets for the design of antiviral agents. The techniques described here provide experimental approaches both to mutate and to assay two of the three known enzymes (RT, IN, and protease) involved in retrovirus replication.

Acknowledgments

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References

1. J. Leis, A. Aiyar, and D. Cobrinik, *in* "Reverse Transcriptase" (A. M. Skalka and S. P. Goff, eds.), p. 33. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1993.
2. D. Cobrinik, L. Soskey, and J. Leis, *J. Virol.* **62,** 3622 (1988).
3. D. Cobrinik, A. Aiyar, Z. Ge, H. Huang, and J. Leis, *J. Virol.* **65,** 3622 (1991).
4. A. Aiyar, D. Cobrinik, Z. Ge, H.-J. Kung, and J. Leis, *J. Virol.* **66,** 2464 (1992).
5. R. Katz, C. Omer, J. Weis, S. Mitsialis, A. Faras, and R. Guntaka, *J. Virol.* **42,** 346 (1982).
6. S. H. Hughes, J. J. Greenhouse, C. J. Petropoules, and P. Sutrave, *J. Virol.* **61,** 3004 (1987).
7. A. Aiyar and J. Leis, *BioTechniques* **14,** 366 (1993).

8. A. Aiyar, Z. Ge, and J. Leis, *J. Virol.* **68,** 611 (1994).
9. G. Sarkar and S. S. Sommer, *BioTechniques* **8,** 404 (1990).
10. K. Borroto-Esoda and L. R. Boone, *J. Virol.* **65,** 1952 (1991).
11. A.-C. Prats, L. Sarih, C. Gabus, S. Litvak, G. Keith, and J. L. Darlix, *EMBO J.* **7,** 1777 (1988).
12. C. Barat, V. Lullien, O. Schatz, G. Keith, M. T. Nugeyre, F. Gruninger-Leitch, F. Barre-Sinoussi, S. F. J. LeGrice, and J. L. Darlix, *EMBO J.* **8,** 3279 (1989).
13. R. Sutton and J. Boothroyd, *Cell (Cambridge, Mass.)* **47,** 527 (1986).
14. J. Milligan, D. Groebe, G. Witherell, and O. Uhlenbeck, *Nucleic Acids Res.* **15,** 8783 (1987).
15. A. M. Skalka, *Gene* **135,** 175 (1993).
16. B. Hirt, *J. Mol. Biol.* **26,** 365 (1967).
17. M. Katzman, R. Katz, A. M. Skalka, and J. Leis, *J. Virol.* **63,** 5319 (1989).
18. R. Craigie, A.-T. Fujiwara, and F. Bushman, *Cell (Cambridge, Mass.)* **64,** 829 (1990).
19. R. Katz, G. Merkel, J. Kulkosky, J. Leis, and A. M. Skalka, *Cell (Cambridge, Mass.)* **63,** 87 (1990).
20. S. A. Chow and P. O. Brown, *J. Virol.* **68,** 3896 (1994).
21. G. A. Donzella, C. B. Johnson, and M. J. Roth, *J. Virol.* **67,** 7077 (1993).
22. A. Engleman, Mizuuchi, and R. Craigie, *Cell (Cambridge, Mass.)* **67,** 1211 (1991).
23. F. D. Bushman and B. Wang, *J. Virol.* **68,** 2215 (1994).
24. R. A. Katz, J. P. Mack, G. Merkel, J. Kulkosky, Z. Ge, J. Leis, and A. M. Skalka, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 6741 (1992).
25. C. B. Jonsson and M. J. Roth, *J. Virol.* **67,** 5562 (1993).
26. J. Kulkosky, K. S. Jones, R. A. Katz, J. P. Mack, and A. M. Skalka, *Mol. Cell. Biol.* **12,** 2331 (1992).
27. A. Mazumder, A. Engelman, R. Craigie, M. Fesen, and Y. Pommier, *Nucleic Acids Res.* **22,** 1037 (1994).
28. P. L. Schwartzberg, M. J. Roth, N. Tanese, and S. P. Goff, *Virology* **192,** 673 (1993).
29. K. A. Vincent, V. Ellison, S. A. Chow, and P. O. Brown, *J. Virol.* **67,** 425 (1993).
30. M. Katzman and M. Sudol, *J. Virol.* **68,** 3558 (1994).
31. S. A. Chow, K. A. Vincent, V. Ellison, and P. O. Brown, *Science* **255,** 723 (1992).
32. B. Muller, K. S. Jones, G. W. Merkel, and A. M. Skalka, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 11633 (1993).
33. P. Brown, B. Bowerman, H. Varmus, and J. M. Bishop, *Cell (Cambridge, Mass.)* **49,** 347 (1987).
34. M. L. Fitzgerald and D. P. Grandgenett, *J. Virol.* **68,** 4314 (1994).
35. J. M. Coffin, *in* "Virology" (B. N. Fields, D. M. Knipe, R. M. Chanock, M. R. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman, eds. (2nd ed. Raven, New York, 1990.

[17] *In Vitro* Binding and Transcription Assays Using the Human T-Cell Leukemia Virus Type I Tax Protein

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Introduction

Human T-cell leukemia virus tpe I (HTLV-I) encodes a 40-kDa regulatory protein, Tax, which interacts with the host cell transcriptional machinery to increase expression of viral and cellular RNAs (reviewed in Ref. 1). Because the Tax protein plays a role in both productive viral infection and viral oncogenesis, the mechanism by which Tax interacts with the transcriptional machinery is of considerable interest.

Promoters that are activated by Tax protein contain specific DNA sequences called Tax-responsive elements, or TxREs. Genetic and biochemical analyses have shown that these TxREs coincide with binding sites for host cell transcription factors. The three TxREs in the HTLV-I proviral long terminal repeat (LTR) have been the most extensively studied. These contain a common sequence, TGACG, that is recognized by proteins in the ATF/cAMP response element-binding (CREB) family of transcription factors. TxREs in other promoters have been shown to coincide with recognition sites for NF-κB, serum response factor (SRF), and proteins in the fos/jun family of transcription factors (2–5).

To better understand how Tax activates transcription, we developed a Tax-responsive *in vitro* transcription system (6). In this system, purified recombinant Tax increases transcription of the HTLV-I LTR promoter approximately 10-fold. This Tax-mediated transactivation shows a strong dependence on the *in vivo*-defined TxREs, suggesting that it occurs by the same mechanism *in vitro* as *in vivo*. Interestingly, purified recombinant Tax increases the binding of affinity-purified host cell proteins to the TxREs. We have suggested that this increased binding drives the increase in transcription (6).

Recent work has shown that Tax affects the DNA binding activity of a number of different proteins. Purified Tax enhances the DNA binding of several proteins in the ATF/CREB family (7, 7a). We have developed a quantitative treatment to describe this phenomenon (7b). Tax has also been reported to bind in ternary complexes containing DNA and CREB (8). Tax also enhances the binding of other transcription factors that may be relevant to the effect of Tax *in vivo*, including NF-κB, SRF, and c-jun (9). This ability to influence the DNA binding activity of a number of

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different transcription factors suggests that *in vitro* systems may be useful for studying the effects of Tax on a wide range of promoters.

This article describes methods for establishing *in vitro* systems for studying the biochemical activities of the Tax protein. We describe the purification of recombinant Tax and CREB from *Escherichia coli,* as well as protocols for assaying the effects of Tax on *in vitro* transcription and protein–DNA binding reactions.

Methods

Purification of Tax Protein from E. coli

Our previous studies have used Tax protein purified from insect cells infected with recombinant baculovirus (6). Baculovirus-expressed Tax is posttranslationally modified and is active *in vivo* in infected insect cells (10). Baculovirus expression is relatively inconvenient, however, and in our experience only a fraction of the Tax protein expressed in the baculovirus system can be recovered in the active monomeric form. Therefore, in more recent experiments we have used an *E. coli* expression system. Expression of Tax in *E. coli* has been previously reported (11). Present experiments use a Tax derivative with a histidine tag at the C terminus (12). The presence of the histidine tag does not appear to affect Tax function. Genetic analysis suggests that sequences at the extreme C terminus of Tax are not essential for Tax function, and C-terminal sequences are not conserved between the Tax proteins of HTLV-I and HTLV-II (13–15). Side-by-side comparisons suggest that baculovirus-expressed and *E. coli*-expressed Tax proteins function equivalently in *in vitro* transcription and DNA binding assays (7). The procedure for purification of histidine-tagged Tax protein is adapted from that of Zhao and Giam (12).

Purification of $TaxH_6$ Protein

Materials

The $pTaxH_6$ Tax expression plasmid (12) is in the *E. coli* strain HB101. LB medium contains 5 g of NaCl, 5 g of yeast extract, and 10 g of Bactotryptone (Difco, Detroit, MI) per liter. Buffer A contains 50 m*M* Na_2HPO_4/NaH_2PO_4 (pH 8.0), 500 m*M* NaCl, 10 m*M* 2-mercaptoethanol (2-ME), and 10 μg/ml phenylmethylsulfonyl fluoride (PMSF). Buffer B contains 50 m*M* Na_2HPO_4/NaH_2PO_4 (pH 7.0), 300 m*M* NaCl, 10 m*M* 2-ME, and 10 μg/ml PMSF. HE buffer contains 25 m*M* HEPES (pH 7.9) at 25°C, 150 m*M* KCl, 1 m*M* EDTA, 0.1% (v/v) Nonidet P-40 (NP-40; USB, Cleveland, OH), 10% glycerol, 4 m*M* 2-ME, and 10 μg/ml PMSF. For all buffers PMSF and 2-ME should be added immediately before use. The Ni^+ column is prepared

using 2.5 ml of NTA–agarose (Qiagen, Chatsworth, CA). The resin is washed three times with 2.5 ml of sterile water and once with 2.5 ml of buffer A.

Procedure

All procedures are carried out at 0–4°C unless otherwise indicated. For best results 4–12 liters of the bacterial culture should be used.

1. Inoculate a single colony of *E. coli* strain HB101 containing the $pTaxH_6$ plasmid into LB medium containing 50 mg/liter ampicillin. Incubate at 37°C with shaking to allow the culture to reach saturation.
2. Centrifuge at 6000 *g* for 10 min.
3. Discard the supernatant and resuspend the cell pellet in buffer A containing 0.5% Triton X-100 (Sigma) and 10 mg/ml lysozyme. Use 2.5 ml of buffer per liter of initial culture.
4. Incubate at room temperature for 30 min.
5. Sonicate on ice with 10 bursts of 10 sec each at medium power.
6. Remove any insoluble material by centrifuging for 15 min at 8000 *g*. Retain the supernatant and measure the volume. If desired, the supernatant can be stored frozen at −70°C.
7. Slowly add 3 *M* $(NH_4)_2SO_4$ to a final concentration of 1 *M*. Stir slowly for 1 hr.
8. Centrifuge at 22,500 *g* for 15 min.
9. Discard the supernatant and resuspend the entire pellet in 2 ml of buffer A.
10. Dialyze the sample against excess buffer A for 2 hr. Repeat.
11. Incubate the protein solution with the washed NTA–agarose. Rock the mixture for 6–8 hr.
12. Pour the protein/NTA–agarose mixture into a suitable column. Wash with 20 ml of 10 m*M* imidazole in buffer B. Elute with a gradient of 10–500 m*M* imidazole in 10 column volumes of buffer B. Tax elutes at about 300 m*M* imidazole.
13. Pool the peak fractions and load onto a HiLoad Superdex 200 preparation-grade 16/60 column (Pharmacia, Piscataway, NJ) equilibrated in HE buffer. Run the column in HE buffer. A Peak of Tax protein, identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, elutes at 78 ml (fraction 39) as shown in Fig. 1. This corresponds to a molecular mass of 45 kDa for an average globular protein. Identity of the protein purified by this procedure may be verified by measurement of activity in the electrophoretic mobility-shift assay (EMSA) and the *in vitro* transcription reaction (see below).

Notes

1. It is important that all buffers used prior to the nickel column are made without EDTA or dithiothreitol (DTT) and with 2-ME at concentrations less than 10 m*M*, since these reagents will strip the Ni^+ off the column.

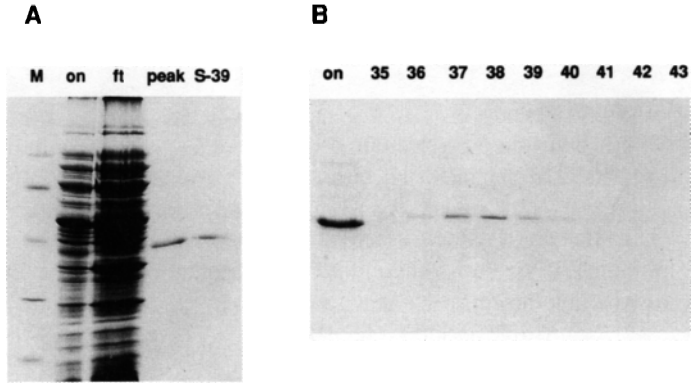

FIG. 1 Purification of bacterially expressed $TaxH_6$. (A) Tax preparation was carried out using 12 liters of bacterial culture. SDS–PAGE analysis of fractions from different stages of purification is shown. The lane marked "M" contains molecular mass markers (Bio-Rad). Molecular masses (top to bottom) are 97.4, 66.2, 45.0, 31.0, and 21.5 kDa. Each of the remaining lanes represents 1/200 of the preparation. The lane marked "on" contains clarified *E. coli* extract loaded onto the NTA–agarose column. The lane marked "ft" contains the flow-through from the NTA–agarose column. The lane marked "peak" contains peak Tax fractions pooled from the Ni^+ column. The lane marked "S-39" contains fraction 39 from the Superdex 200 column. (B) Elution of $TaxH_6$ protein from the Superdex 200 column. The lane marked "on" contains the sample loaded onto the Superdex 200 column [peak from (A)]. The lanes labeled 35–43 contain samples from fractions 35–43.

2. Chromatography of $TaxH_6$ on the Superdex 200 column using HMZ buffer (see below), which contains $MgCl_2$ and $ZnSO_4$ but no EDTA, yields an elution profile over a large volume, without a distinct peak. This is most likely due to heterogeneous aggregation of the $TaxH_6$ caused by an interaction between the H_6 tag and zinc ions in the buffer. Tax protein without the H_6 tag elutes normally in the HMZ buffer (6).

Purification of CREB A Protein from E. coli

It appears that Tax interacts with a number of host cell transcription factors. We use the CREB protein in standard assays, since it is apparently an authentic physiologically relevant target of the Tax protein (7, 8, 12, 16). CREB protein binds with high affinity to the TxRE sequences present in the HTLV-I LTR. In addition, it is one of the major proteins selected when total nuclear extract is subjected to DNA-affinity chromatography using the TxRE sequences from the LTR (7). Tax enhances the binding of CREB protein to these TxREs *in vitro* and cooperates with recombinant CREB protein to enhance HTLV-I transcription in a cell-free system (6, 7). CREB protein coimmunoprecipitates with Tax (17). In addition, it may form ternary complexes

with Tax and DNA (8, 9, 12). Human CREB protein exists in two forms. CREB B differs from CREB A by an insertion of 14 amino acids. The procedure below is for purification of CREB A protein from *E. coli.*

Materials

The CREB 327 clone, or CREB A (18), is in the pET 3B expression vector (19). This vector allows for induction of CREB A expression with isopropyl β-D-thiogalactopyranoside (IPTG) when the plasmid is grown in the *E. coli* strain BL21 (DE3) pLysS. HMZ buffer is as follows: 25 m*M* HEPES, 150 m*M* KCl, 12.5 m*M* $MgCl_2$, 10 μM $ZnSO_4$, 4 m*M* 2-ME, 0.1% NP-40, 10 μg/ml PMSF, and 20% glycerol. PMSF and 2-ME should be added immediately before use.

Procedure

All procedures are carried out at 0–4°C unless otherwise indicated. These procedures are based on those described by Hoeffler and co-workers (18).

1. Inoculate a single colony of cells into 5 ml of LB medium with 50 μg/ml ampicillin and 35 μg/ml chloramphenicol. Incubate at 37°C with shaking to allow the culture to reach saturation.
2. Inoculate the overnight culture into 1 liter of LB medium with the same concentrations of antibiotics as in step 1.
3. Grow the cells to an optical density at 600 nm of 0.4–0.6. Add IPTG to a concentration of 0.4 m*M*. Incubate for 2 hr with shaking at 37°C.
4. Harvest the cells by centrifuging at 6000 *g* for 10 min.
5. Discard the supernatant and resuspend the cell pellet in 20 ml of HMZ buffer.
6. Aliquot into microfuge tubes and boil at 96°C for 8 min. Incubate on ice for 5 min, centrifuge in a microcentrifuge at 14,000 *g* for 10 min, and transfer the supernatants to new tubes.
7. Combine the supernatants and load onto a 5-ml heparin–agarose column prepared as previously described (19a). Wash the column with 25 ml of HMZ buffer, followed by 15 ml of HMZ buffer with 0.3 *M* KCl. Elute the CREB protein with 15 ml of HMZ buffer with 0.6 M KCl. Analyze by SDS–PAGE to identify the CREB-containing fractions, and dialyze against 1 liter of HMZ buffer for 2 hr. Repeat the dialysis step twice.
8. Aliquot and store at −70°C.

Notes

For best results the CREB protein should be fully reduced. The presence of dimeric oxidation products can be detected by nonreducing SDS–PAGE. An alternative method (no longer in routine use in our laboratory) is to substitute a HiLoad Superdex 200 preparation-grade 16/60 column equilibrated in HMZ buffer in step 7. The CREB peak elutes near the void volume, possibly indicating multimers and/or an elongated shape.

EMSA

CREB–DNA binding reactions use a 85-bp double-stranded DNA probe containing the sequence TCAGGCGTTGACGACAACCCCTCACCTCA, representing a region of the HTLV-I LTR that contains the third 21-bp TxRE. An oligonucleotide containing this sequence was inserted in the *Bam*HI site of pUC19 to create the pUC–3S plasmid (7). Binding can also be performed using synthetic DNA probes; however, cloned DNA is more homogeneous and often gives more satisfactory results.

Preparation of Probe DNA

1. Digest the pUC–3S plasmid with *Eco*RI and *Hind*III. Typically, 0.5–1.0 mg of plasmid is digested to yield approximately 25 pmol of probe after purification.
2. After the digestion is complete, add 5 U of calf intestinal alkaline phosphatase (Promega, Madison, WI) directly to the restriction digests. Incubate for 15 min at 37°C, then for 15 min at 55°C to inactivate the enzyme.
3. Purify the 85-bp fragment using an 8% (w/v) nondenaturing polyacrylamide gel in a buffer of 44.5 m*M* Tris–base, 44.5 m*M* boric acid, and 1 m*M* EDTA (0.5X TBE buffer).
4. Visualize the DNA by ethidium bromide staining, excise the 85-bp fragment, and elute the DNA into a dialysis bag (20).
5. Extract with phenol–chloroform–isoamyl alcohol [24:24:1 (v/v)]. Add 2.5 vol of a 0.5 *M* solution of NH_4OAc in ethanol to precipitate the DNA.
6. After precipitation radiolabel 2 pmol of the DNA by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP, using the conditions specified by the manufacturer. Inactivate the kinase by incubation at 70°C for 10 min.
7. Purify the radiolabeled DNA fragment using a Sephadex G-25 spin column (5 Prime–3 Prime, Boulder, CO), according to the protocol supplied by the manufacturer.

Binding Reactions

Binding reactions contain final concentrations of 5 μg/ml poly(dI)–poly(dC), 12.5 m*M* HEPES (pH 7.9), 2.5 m*M* $MgCl_2$, 0.4 m*M* EDTA, 75 m*M* KCl, 1.0 μ*M* $ZnSO_4$, 2 m*M* 2-ME, 0.05% (v/v) NP-40, and 6% (v/v) glycerol. This mixture is obtained by combining reaction components as described below. Two sets of 10 binding reactions are set up; one is a titration of CREB A with the Tax protein, and the other is a CREB A titration without Tax.

Reactions are performed as follows.

1. Prepare 3-fold serial dilutions of CREB A in HMZ buffer. Dilutions should cover the range of 1 ng/ml to 25 μg/ml CREB A.
2. Assemble the reactions at room temperature. Set up the reactions by adding, in order, 2.0 μl of 12.5 m*M* $MgCl_2$, 8.0 μl of the Tax protein (160 ng) or HE buffer,

2.0 μl of diluted CREB A, and a mixture of radiolabeled DNA fragment (10 fmol) and poly(dI)–poly(dC) (0.1 μg) diluted in double-distilled water to a volume of 8.0 μl. The final reaction volume will be 20 μl.

3. Incubate at room temperature for 15 min.

4. Load directly on a 5% nondenaturing polyacrylamide gel (acrylamide–*N,N'*-methylene bisacrylamide, 39:1). The electrophoresis buffer contains 0.04 *M* Tris, 0.306 *M* glycine (pH 8.5) and 0.1% NP-40. Run at 15 V/cm until the bromphenol blue in a marker lane reaches the bottom of the gel (Fig. 2).

5. Dry the gels and visualize the complexes by autoradiography or phosphorimage analysis.

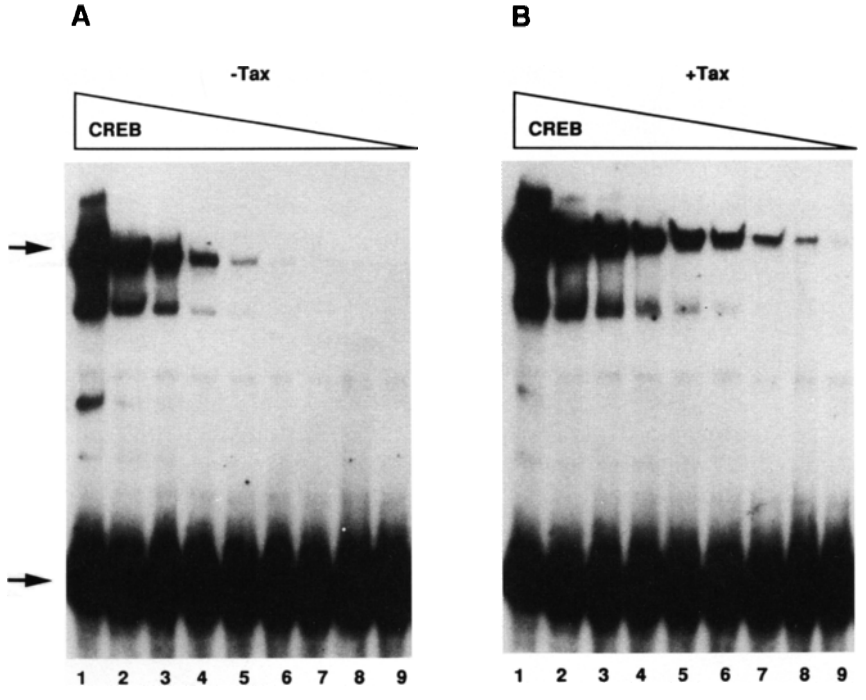

FIG. 2 Electrophoretic mobility-shift assay using sequences from the third 21-bp TxRE of the HTLV-I promoter. (A) Standard binding reactions contained CREB A in the following concentrations (ng/ml): 2500 (lane 1), 833 (lane 2), 278 (lane 3), 92.6 (lane 4), 30.9 (lane 5), 10.3 (lane 6), 3.43 (lane 7), 1.14 (lane 8), and 0.381 (lane 9). Reactions were electrophoresed through a 5% acrylamide–bisacrylamide (39:1) Tris–glycine–NP-40 nondenaturing gel and the products were visualized by autoradiography. The bottom arrow indicates the free DNA probe, and the top arrow indicates the CREB A–DNA complex. CREB A in these experiments was purified by the Superdex method. (B) Autoradiogram of identical reactions, except that 160 ng of Tax is also included.

Notes

1. Note that sample dyes may interfere with CREB protein binding and are not added to the reactions. Dyes may be added to a separate marker lane to monitor the progress of the electrophoresis.
2. Electrophoresis in TBE buffer does not give satisfactory results.

In Vitro Transcription

With any transcription factor, it is important to show that the protein actually affects RNA synthesis, as well as participating in various protein–protein and protein–DNA binding interactions. We find that the Tax protein stimulates *in vitro* transcription of the HTLV-I LTR 4- to 10-fold. Transcription of the LTR can also be stimulated by recombinant CREB. Together, Tax and CREB have an approximately multiplicative effect, suggesting that they cooperate to activate transcription from the LTR promoter (7).

Preparation of Nuclear Extracts

Materials

The T-lymphocyte cell line CEM was used (21). Phosphate-buffered saline (PBS) contains 137 m*M* NaCl, 2.7 m*M* KCl, 8.1 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4 (pH 7.5), and 4.9 m*M* $MgCl_2$. Hypotonic lysis buffer contains 10 m*M* Tris–HCl (pH 7.9 at 25°C), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* DTT, 10 μg/ml PMSF, and 10 μg/ml soybean trypsin inhibitor (SBTI). Nuclear extraction buffer contains 50 m*M* Tris–HCl (pH 7.9 at 25°C), 420 m*M* KCl, 5 m*M* $MgCl_2$, 0.1 m*M* EDTA, 20% (v/v) glycerol, 10% (w/v) sucrose, 2 m*M* DTT, 10 μg/ml PMSF, and 10 μg/ml SBTI. TM 0.1 *M* buffer contains 50 m*M* Tris–HCl (pH 7.9 at 25°C), 100 m*M* KCl, 12.5 m*M* $MgCl_2$, 1 m*M* EDTA, 20% (v/v) glycerol, 1 m*M* DTT, 10 μg/ml PMSF, and 10 μg/ml SBTI. The protease inhibitors and DTT should be added immediately before use.

Procedure

All procedures are carried out at 0–4°C unless otherwise indicated.

1. Grow the CEM cells in spinner cultures at 37°C to a concentration of $1-2 \times 10^6$ cells per milliliter using Iscove's medium supplemented with 5% fetal bovine serum, 5% calf serum, and glutamine. Typical extract preparations use $4-5 \times 10^{10}$ cells, corresponding to 32 liters of culture.
2. Collect the cells by centrifuging in 1-liter bottles at 1350 *g* for 15 min.
3. Resuspend the cell pellets in 0.01–0.05 culture volumes of PBS, consolidate into a single bottle or tube, and centrifuge at 1350 *g* for 15 min. Decant the supernatant. Suspend the cells in PBS to a total volume of no more than 50 ml, transfer to

a conical centrifuge tube, and centrifuge at 700 *g* for 15 min. Decant the supernatant and note the packed cell volume (PCV). A typical PCV is in the range 1.0–2.5 ml per liter of culture.

4. Resuspend the cell pellet in 4 PCV of hypotonic lysis buffer in an Erlenmeyer flask and incubate for 10 min with occasional gentle swirling.

5. Homogenize the suspension with 10 strokes in a Dounce homogenizer with a B pestle. Lysis may be checked by microscopic analysis, and should be at least 90%. If not, the suspension should be homogenized further.

6. Pellet the nuclei by centrifugation at 2300 *g* for 5 min. Decant the supernatant and discard. Resuspend the pellet in 2 PCV of hypotonic lysis buffer. Centrifuge immediately at 2300 *g* for 5 min.

7. Resuspend the nuclear pellet in 4 PCV of extraction buffer. Stir for 30 min. Centrifuge at 26,500 *g* for 30 min. Collect the high-speed supernatant and note the volume (HSSV). If desired, the supernatant can be stored frozen at −70°C.

8. Add solid $(NH_4)_2SO_4$ (ultrapure; Schwarz/Mann) slowly, with gentle stirring, to a final concentration of 0.33 g/ml HSSV. Stir until the $(NH_4)_2SO_4$ is dissolved, then for an additional 60 min. It is desirable to avoid excessive foaming.

9. Collect the precipitated proteins by centrifugation for 20,500 *g* for 10 min. Discard the supernatant.

10. Resuspend the pellet in 0.3 ml of TM 0.1 *M* buffer per milliliter of original PCV. Dialyze against 2 liters of TM 0.1 *M* buffer overnight, then for an additional 4 hr against another 2 liters of TM 0.1 *M* buffer. Clarify the dialyzed extract by centrifugation at 9000 *g* for 10 min and collect the supernatant.

11. Divide the extract into small aliquots suitable for individual experiments. Store the aliquots frozen at −80°C.

12. Determine the protein concentration using the Bradford assay (Bio-Rad, Richmond, CA). Typical values are in the range of 5–10 mg/ml.

Synthesis of RNA

RNA synthesis is assayed by runoff transcription using an isolated promoter DNA fragment.

Materials

The buffer used for transcription reactions contains final concentrations of 10 m*M* Tris–HCl (pH 7.9 at 25°C), 7.5 m*M* HEPES (pH 7.9 at 25°C), 65 m*M* KCl, 3.6 m*M* $MgCl_2$, 0.3 m*M* EDTA, 7.4% (v/v) glycerol, 3.2 m*M* 2-ME, 0.5 μg/ml PMSF, 0.4 μM $ZnSO_4$, and 0.03% NP-40. This corresponds to a mixture of the buffers in which nuclear extract, CREB A protein, and Tax are stored. The stop solution contains 200 m*M* NaCl, 20 m*M* EDTA, 1% SDS, and 100 μg/ml tRNA. TNE buffer contains 10 m*M* Tris–HCl (pH 7.9 at 25°C), 100 m*M* NaCl, and 1 m*M* EDTA. Sample buffer contains 7.5 *M* urea, 10 m*M* Tris–HCl (pH 7.9 at 25°C), 1 m*M* EDTA, 0.25% (w/v) bromphenol blue, and 0.25% (w/v) xylene cyanol.

To prepare the HTLV-I templates, the plasmid pU3RCAT (22) is digested with *Hin*dIII and *Pvu*II. The DNA fragment containing the promoter and the 5′ region of the chloramphenicol acetyltransferase gene is isolated by preparative PAGE. Typically, about 0.5–1 mg of plasmid is used to prepare the template fragment. Recovery is verified by measurement of the ultraviolet absorbance.

Procedure

1. Assemble the reactions at room temperature. Set up the reactions by adding, in order, 8 μl of HE buffer, 2.5 μl of 12.5 m*M* $MgCl_2$, 2 μl of CREB A (0–40 ng) or HMZ buffer, 5 μl of Tax (approximately 100 ng) or HE buffer, and 50 ng of template in 21.5 μl of sterile water. Binding is allowed to proceed at room temperature for 15 min.
2. Add 10 μl of nuclear extract. Incubate the reactions at 30°C for 30 min to allow the formation of preinitiation complexes.
3. Initiate RNA synthesis by the addition of 250 μM (each) ATP, GTP, and UTP, 12.5 μM CTP, and 10 μCi of [α-^{32}P]CTP (3000 Ci/mmol; New England Nuclear, Boston, MA) in a volume of 3.5 μl, which brings the total volume to 50 μl. Incubate for 60 min at 30°C.
4. Terminate the reactions by addition of 150 μl of stop solution and 100 μl of TNE buffer. Extract with 300 μl of phenol–chloroform–isoamyl alcohol [25:24:1 (v/v)] and centrifuge for 5 min at 10,000 *g* at room temperature.
5. Transfer the top phase to a fresh tube and add to it 750 μl of a 0.5 *M* solution of NH_4OAc in ethanol. Mix well by inversion. Chill in a dry-ice ethanol bath for 5 min and centrifuge at 10,000 *g* at 4°Cfor 10 min.
6. Discard the supernatant and wash the pellet gently with 70% ethanol. Centrifuge briefly, discard the supernatant, drain the pellet thoroughly, and dry it *in vacuo*.
7. Resuspend the pellet in 15 μl of sample buffer.
8. Load the samples on a 7.5 *M* urea plus 5% (v/v) polyacrylamide gel. The gel buffer consists of 1× TBE buffer. Run at approximately 30 V/cm until the bromphenol blue reaches the bottom.
9. Dry the gel and visualize the radiolabeled products by autoradiography or phosphorimage analysis (Fig. 3).

Conclusions

We have developed an *in vitro* system to characterize interactions between the HTLV-I viral transactivating protein Tax and the host cell transcriptional machinery. *In vitro* transcription of the HTLV-I LTR is typically stimulated 5- to 10-fold when purified recombinant Tax is added to nuclear extract from uninfected T-lymphocytes. In addition, Tax stimulates the binding of purified CREB protein to DNA, and it may be that this stimulation of binding drives the increase in transcription.

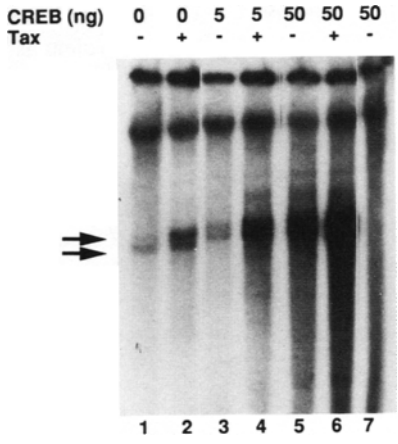

FIG. 3 Stimulation of *in vitro* transcription. All reactions include 50 ng of the HTLV-I promoter and 100 ng of CEM cell nuclear extract. Some reactions also contained Tax (60 ng) and CREB A (amounts incubated, in nanograms). α-Amanitin (2 μg/ml) was added to reaction 7 to inhibit RNA polymerase II transcription. The reactions were analyzed by urea PAGE, and radiolabeled RNA products were visualized by autoradiography. The double arrows indicate the properly initiated transcript and another transcript that initiates approximately 30 bp upstream. CREB A in this experiment was purified by the Superdex method.

It appears that Tax is an unusual transcription factor in many respects. Unlike many RNA polymerase II transcriptional activators, it does not appear to bind directly to specific sequences in the DNA (6, 23). Its action appears to be highly pleiotropic both *in vivo* and *in vitro* (1, 9). Studies with cultured cells and transgenic mice strongly implicate Tax in neoplastic transformation (24). It is to be hoped that studies of the biochemical activities of purified Tax may illuminate a common mechanism for the various biological effects of the protein.

A number of unanswered questions remain about the biochemical properties of Tax. In particular, the mechanism by which Tax brings about the remarkable stimulation of the DNA binding activity of host cell transcription factors is unresolved. Some workers have suggested that Tax may form a stable ternary complex with DNA and transcription factors, thus stabilizing the binding (8, 12). Other workers have failed to detect Tax in the mobility-shifted complexes (7), consistent with the idea that Tax interacts only transiently with the CREB protein–DNA complex.

Another area that needs to be addressed is the apparently broad specificity of the Tax response. It may be that different surfaces of the Tax protein are involved in interactions with different transcription factors. This is supported by findings that different mutants in the Tax protein differ in their relative abilities to activate transcription from the HTLV-I, HTVL-II, and human immunodeficiency virus type 1

(HIV-1) LTR promoters (13–15). The underlying mechanisms responsible for these different specificities may become more clear as Tax protein is studied in more detail.

Acknowledgments

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References

1. J. Sodroski, *Biochem. Biophys. Acta* **1114,** 19 (1992).
2. S. Ruben, H. Poteat, T. Tan, K. Kawakami, R. Roeder, W. Haseltine, and C. Rosen, *Science* **241,** 89 (1988).
3. K. Leung and G. J. Nabel, *Nature (London)* **333,** 776 (1988).
4. D. W. Ballard, D. W. Bohnlein, J. W. Lowenthal, Y. Wano, R. B. Franza, and W. C. Green, *Science* **241,** 1652 (1988).
5. P. Lindholm, S. Marriott, S. Gitline, C. Bohan, and J. Brady, *New Biol.* **2,** 1034 (1991).
6. M.-A. H. Matthews, R.-B. Markowitz, and W. S. Dynan, *Mol. Cell. Biol.* **12,** 1986 (1992).
7. A. A. Franklin, M. F. Kubic, M. N. Uittenbogaard, A. Brauweiler, P. Utaisincharoen, M.-A. H. Matthews, W. S. Dynan, J. P. Hoeffler, and J. K. Nyborg, *J. Biol. Chem.* **268,** 21225 (1993).

7a. S. Wagner and M. R. Green, *Science* **262,** 395 (1993).

7b. M. G. Anderson and W. S. Dynan, *Nucleic Acids Res.* **22,** 3194 (1994).

8. L. J. Zhao and C.-Z. Giam, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7070 (1992).
9. A. P. Armstrong, A. A. Franklin, M. N. Uittenbogaard, H. A. Giebler, and J. K. Nyborg, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7303 (1993).
10. K. T. Jeang, C. Z. Giam, M. Nerenberg, and G. Khoury, *J. Virol.* **61,** 708 (1987).
11. C.-Z. Giam, M. Nerenberg, G. Khoury, and G. Jay, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 7192 (1986).
12. L.-J. Zhao and C.-Z. Giam, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 11445 (1991).
13. W. Wachsman, A. Cann, J. Williams, D. Slamon, L. Souza, N. Shah, and I. S. Y. Chen, *Science* **235,** 674 (1987).
14. A. J. Cann, J. D. Rosenblatt, W. Wachsman, and I. S. Y. Chen, *J. Virol.* **63,** 1474 (1989).
15. M. R. Smith and W. C. Green, *Genes Dev.* **4,** 1875 (1991).
16. J.-I. Fujisawa, M. Toita, and M. Yoshida, *J. Virol.* **63,** 3234 (1989).
17. T. Suzuki, J.-I. Fujisawa, M. Toita, and M. Yoshida, *Proc. Natl. Acad. Sci. U.S.A.* **90,** 610 (1993).

18. J. P. Hoeffler, J. W. Lustbader, and C.-Y. Chen, *Mol. Endocrinol.* **5,** 256 (1991).
19. A. H. Rosenberg, B. N. Lade, D.-S. Chui, S.-W. Lin, J. J. Dunn, and F. W. Studier, *Gene* **56,** 125 (1987).
19a. B. L. Davison, T. Leighton, and J. C. Rabinowitz, *J. Biol. Chem.* **254,** 9220 (1979).
20. T. Maniatis, E. F. Fritsch, and J. Sambrook, *"Molecular Cloning: A Laboratory Manual."* Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
21. D. J. Slamon, M. F. Press, L. M. Souza, D. C. Murdock, M. J. Cline, D. Golde, J. C. Gasson, and I. S. Y. Chen, *Science* **228,** 1427 (1985).
22. J. Brady, K.-T Jeang, J. Duvall, and G. Khoury, *J. Virol.* **61,** 2175 (1987).
23. J.-I. Fujisawa, M. Toita, T. Yoshimura, and M. Yoshida, *J. Virol.* **65,** 4525 (1991).
24. M. Nerenberg, S. Hinrichs, R. K. Renolds, G. Khoury, and G. Jay, *Science* **237,** 1324 (1987).

[18] Polymerase Chain Reaction-Based Techniques for Utilizing Retroviruses as Cell Lineage Markers

Christopher A. Walsh

Introduction

Study of the development of complex systems often begins with an analysis of cell lineage. *Lineage* refers to the patterns of cell division and differentiation that lead from single cells to complex tissues. In order to understand the molecular mechanisms that direct cellular development, precise information about intercellular interactions is indispensable. In principle, the most direct means for tracing patterns of cell lineage is injecting dyes intracellularly into dividing progenitor cells and observing them while they divide. A labeled progenitor should divide to form a labeled clone of cells, with a *clone* defined as the set of daughter cells derived from a single mother cell. Whereas microinjection techniques have provided a great deal of information about lineage in nonmammalian vertebrates (1, 2), microinjection into developing mammalian cells is not technically feasible because of the inaccessibility of the mammalian embryo and the small size of mammalian cells. Consequently, other methods of lineage tracing have been developed for use in mammalian systems.

Replication-defective retroviral vectors are popular tools for cell lineage analysis in mammals. Since retroviruses insert a DNA copy of their genome into the cells they infect, target cells carry and transmit proviruses in mendelian fashion. Retroviral vectors have been designed with histochemical marker genes replacing genes necessary for ongoing retroviral replication; thus, they label the progeny of infected progenitor cells in clonal fashion. Retroviruses have the additional property that proviruses integrate into the host genome at many different sites (see below for further discussion); thus, distinct clones are, in principle, uniquely identified by the DNA sequence at the viral insertion site.

Despite the advantages of retroviruses as lineage markers, they are not without problems. Whereas viruses that encode histochemical marker genes label cells in clonal fashion, they provide no direct means for distinguishing labeled cells that are members of different clones. This is a problem because, ultimately, definitions of clones rely on spatial relationships of labeled cells rather than on a direct demonstration of the clonality of cells. Thus, definitions of clonal relationships require assumptions about cellular behavior; in tissues in which cells are highly or unpredictably mobile, these assumptions are dangerous to make.

The advent of the polymerase chain reaction (PCR) allows direct determination of retrovirally visualized clonal relationships without making assumptions about cell

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behavior. The purpose of this article is to present two techniques that broaden the applicability of retroviral vectors as lineage tracers. In the first method the insertion site of retroviruses can be directly amplified and sequenced using a modification of PCR called inverse PCR. In the second method retroviral vectors are modified to contain short DNA sequences that serve as DNA tags; these tags can them be amplified from single retrovirally labeled cells to allow determination of clonal relationships. Although the two techniques have been developed in the context of retrovirally mediated lineage analysis, both have broader applicability. Several excellent discussions of general techniques for making retroviral vectors are available elsewhere (3), and thus are not covered here in detail.

Inverse PCR for Determination of Retroviral Integration Sites

The inverse PCR technique has been described (4) as a rapid and convenient method for amplifying and cloning unknown DNA sequences adjacent to known sequences. The technique starts with genomic DNA isolated from a large clone of cells carrying a single retroviral integration site. The DNA is digested with restriction enzymes that do not cut the known DNA sequences, but instead generate fragments including both known and unknown DNA sequences. The DNA fragments are ligated to form closed circles, allowing amplification of unknown DNA sequences using primers complementary to known sequences (see Fig. 1). Inverse PCR has been used to identify the ends of yeast artificial chromosome (YAC) clones (5) and to identify the insertion sites of transposable elements (6). For each application, specific restriction enzymes and primer sequences are chosen based on the known target DNA sequence; here, a specific protocol is presented for the identification of retroviral insertion sites of the Moloney murine leukemia virus (7).

Materials

Genomic DNA from a clone of retrovirus-infected cells
Proteinase K (10 mg/ml) in distilled water
Sodium acetate (3 *M*, pH 5.2)
Glycogen (10 mg/ml) in distilled water
Restriction enzymes (*Mbo*I, *Mse*I, and *Taq*I) and appropriate buffers
T4 DNA ligase and appropriate buffer
10× PCR buffer
Primers 19 and 20 (see Fig. 2), as 20 μM solutions in distilled water

Digestion of Retrovirus-Infected Genomic DNA

1. In three reactions digest 20 μg of genomic DNA with 70 U each of *Mbo*I, *Mse*I, and *Taq*I in 200 μl of 1× buffer supplied by the manufacturer. After incubating the DNA for 2–3 hr at 37°C, inactivate the *Mbo*I and *Mse*I by heating to 65°C for

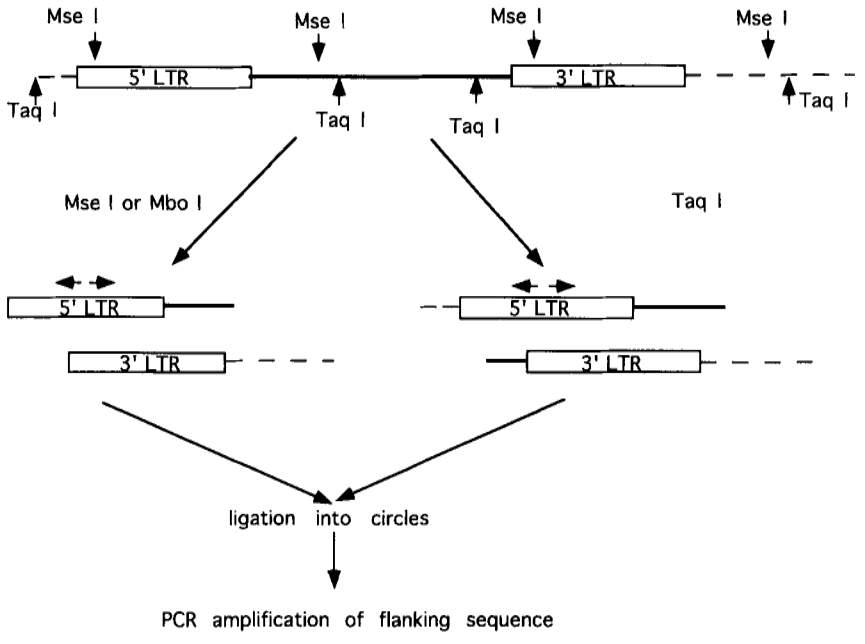


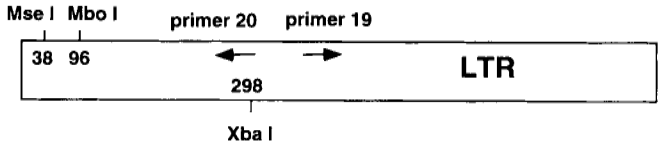
FIG. 1 Summary of the inverse PCR method for amplifying DNA sequences adjacent to retroviral integration sites. Inserted retroviral provirus contains viral regulatory elements (LTRs) as tandem repeats at its 5′ and 3′ ends. The genomic DNA is digested with restriction enzymes that either do not cut within the LTR sequences (e.g., *Taq*I) or cut once within the LTR (*Mse*I or *Mbo*I; only *Mse*I is illustrated). Enzymes such as *Taq*I that do not cleave the LTR generate 5′ and 3′ fragments that include both proviral sequences (solid black line) and flanking host sequence (interrupted line). Enzymes that cut within the LTR (e.g., *Mse*I or *Mbo*I) generate truncated LTR fragments: one fragment includes only proviral flanking sequences (5′ LTR) and one includes only flanking host sequence (3′ LTR). All restriction endonuclease fragments are treated with DNA ligase to form closed circles, then amplified in PCR reactions that use primers complementary to LTR sequences (arrowheads). The PCR products contain proviral DNA, host DNA, or hybrid sequences containing both.

15 min. The *Taq*I reaction is incubated at 65°C for 2–3 hr. Extract the solution once with 200 μl of phenol–chloroform, then once with chloroform.

2. Add 20 μl of 3 *M* sodium acetate solution and mix well. Precipitate the DNA with 450 μl of 100% ethanol.

3. Freeze the solution on dry ice for 20 min, then isolate the DNA by centrifuging the solution in a microfuge at 13,000 rpm for 20 min at room temperature.

4. Wash the DNA pellet once with 70% ethanol and allow to dry. Resuspend the dried DNA in 50 μl of distilled water.



primer 19: GTG CCT TAT TTG AAC TAA CCA ATC AGT TCG

primer 20: AAA CTG CTG AGG GCT GGA CCG CAT CTG G

FIG. 2 Map of a retroviral LTR. The LTR is present as two tandem repeats of identical orientation at the 5′ and 3′ ends of the provirus. Restriction endonucleases *Mse*I and *Mbo*I recognize and cut near the 5′ end of the LTR, forming fragments that contain either viral or host sequences 3′ to the LTR. *Taq*I does not cleave sequences within the LTR. *Xba*I is a rare-cutting restriction endonuclease that cuts once in the LTR and is useful to reopen the ligated closed circles of DNA to facilitate PCR amplification. The positions of PCR primers 19 and 20 are indicated.

5. Use 1–10 μl of the resulting solution to carefully quantitate the DNA concentration, since DNA concentration is critical to the success of the ligation step.

Ligation of DNA

1. Prepare a ligation reaction consisting of 200 ng of the restricted DNA in 200 μl of the DNA ligase buffer recommended or supplied by the manufacturer. (Note: At this low DNA concentration (1 ng/μl), most DNA fragments react intramolecularly and are ligated into closed circles. At higher DNA concentrations, DNA molecules tend to ligate to one another to form larger fragments.)
2. Add 1–10 Weiss units of T4 DNA ligase and react at 14–16° C overnight.
3. Add 1 μl of a 10 mg/ml solution of glycogen and 20 μl of 3 *M* sodium acetate and mix well.
4. Precipitate the DNA by adding 500 μl of 100% ethanol.
5. Freeze the solution on dry ice for 20 min, then isolate the DNA by centrifuging the solution in a microfuge at 13,000 rpm for 20 min. Wash the pellet very carefully with cold 70% ethanol (100 μl), then allow to air-dry.

PCR Amplification

1. Resuspend the DNA in 50 μl of 1 × PCR buffer.
2. Digest 10 μl of the resulting solution with 1 μl ($\approx$10 U) of *Xba*I for 1 hr at 37° C (fig. 2); heat-inactivate the *Xba*I by heating to 65° C for 20 min. Spin the tube in a microfuge for 20 sec to bring down condensation from the sides of the tube, and cover the solution with 50 μl of mineral oil.

3. Denature the DNA by heating it to 95° C for 10 min, then 93° C for 2 min, in an automated thermal cycler. Without cooling the solution, add 90 μl of a 1 × PCR buffer that includes 200 μM (each) dATP, dCTP, dGTP, and dTTP (dNTPs); 2.5 U of *Taq* DNA polymerase per 100 μl of solution; and 1 μM (each) oligonucleotide primers 19 and 20. (Note: This solution can be added above the oil. It will slip beneath the oil and join the aqueous phase.)

4. Continue to cycle the solution through 40 cycles of 94° C for 45 sec, 60° C for 1 min, and 72° C for 1 min. After the final cycle keep the solution at 72° C for 5 min before stopping the reaction.

5. Resolve the PCR products on a 3% NuSieve–1% Seakem agarose (FMC Bioproducts, Rockland, ME) gel, using Tris–borate–EDTA (TBE) (8) buffer as the running buffer.

Expected Results

Typical results are shown in Fig. 3. Each PCR reaction should amplify two bands, one corresponding to each long terminal repeat (LTR). Following *Mbo*I or *Mse*I digestion one band consists exclusively of proviral sequence without any unknown flanking sequence; the size of the proviral fragment is therefore constant from clone to clone (Fig. 3, lanes 3 and 6, lower band; lane 4, upper band; and lane 7, lower

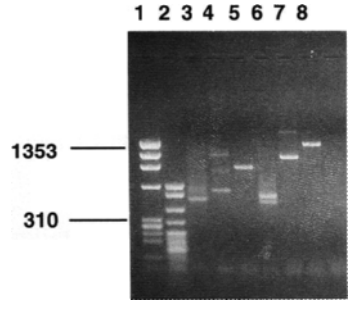
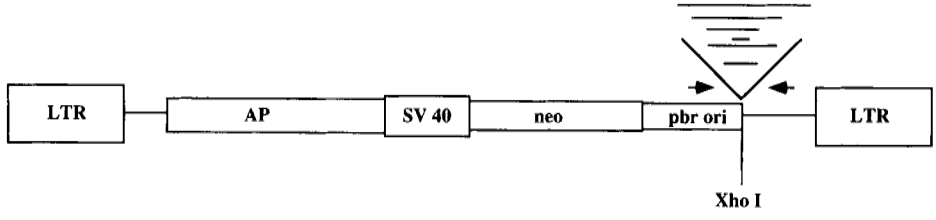


FIG. 3 Results of an inverse PCR experiment. Lanes 1 and 2 contain DNA size standards; lanes 3 and 6 show inverse PCR of two different retrovirus-infected clonal cell lines, using *Mbo*I as the restriction enzyme. Lanes 4 and 7 show inverse PCR with the same PCR conditions and the same two cell lines, using *Mse*I as the restriction enzyme. Lanes 5 and 8 show the results with *Taq*I on the same two cell lines. Since *Mbo*I and *Mse*I both cut once within the LTR, one of the PCR products in each lane is of constant size and DNA sequence in all clones, as it contains only proviral sequences (smaller band in lanes 3 and 6; larger band in lane 4 and smaller band in lane 7). *Taq*I does not cut the LTR, so no amplified bands are of the same size in different clones; however, some *Taq*I fragments are large and do not amplify efficiently in the PCR reaction.

band). For these two enzymes the second band contains a variable length of DNA sequence flanking the retroviral insertion site. For *Taq*I digestions both PCR products contain some flanking sequence, since *Taq*I does not cut within the LTR sequence itself. However, since *Taq*I cuts relatively rarely, some *Taq*I fragments appear to be too large to circularize and amplify efficiently in the PCR, so that in Fig. 3 lanes 5 and 8 show one fragment each instead of two. Thus, each of the three enzymes has distinct advantages and disadvantages. The PCR products can be cloned and sequenced, if necessary, using techniques described elsewhere (8).

DNA-Tagged Retroviral Libraries for Cell Lineage Analysis

For many applications it is preferable to create a library of retroviruses, each of which carries a small variable segment of DNA that can still act as a clonal tag. Direct PCR is far more sensitive than inverse PCR, so that the DNA tags can routinely be amplified from single retrovirally labeled cells (9). The preparation of a library from 100 retroviral constructs has been described previously (9) and has been presented in protocol form (10). However, more recent work has shown that retroviral libraries of greater complexity can be constructed with a simpler protocol and that retroviral libraries can be made from a variety of starting plasmids (Fig. 4). Preliminary data



primer sequences for first PCR reaction:
PBR-5: CGG GTT TCG CCA CCT CTG ACT TGA GCG TCG
BND-4: TGT ACT GCG GCT TGG AGC TGC TGG AAT TGC

primer sequences for second PCR reaction:
PBR-4: GCG GAG CCT ATG GAA AAA CGC CAG C
BND-3: TGA GTG GCC ATT AGA GCA GTA GTC CCT GTT C

FIG. 4 Scheme for generation of a retroviral library. A retroviral plasmid is used (11) that contains two genes, human placental alkaline phosphatase (AP), and the *neomycin transferase* gene that confers resistance to G-418 in culture (neo). A unique cloning site (*Xho*I) downstream of these two genes can be used to insert DNA sequences without affecting gene expression. The library consists of hundreds to thousands of distinct constructs, each containing different DNA sequences inserted into the *Xho*I site. Sequences may be distinguishable by size or by DNA sequence.

suggest that retroviral libraries can be quite complex, so that clones can be essentially uniquely tagged with this method as well as by the inverse PCR method. Thus, an improved protocol is presented that details preparation of a retroviral library from the DAP plasmid (11).

Preparation of Vector

1. Digest 10 μg of the DAP plasmid with 200 U of *Xho*I for 3 hr, followed by heating to 65° C for 20 min to inactivate the enzyme.
2. If necessary, partially fill in the ends of the vector by treating it in the same buffer with 2 U of the Klenow fragment of DNA polymerase, 1 m*M* dGTP, and 1 m*M* dATP for 1 hr to convert the "TCGA" termini formed by the restriction enzyme to "GA" termini.
3. After 1 hr at 37° C, correctly cut vector is separated from uncut vector on a 1% (w/v) agarose gel.
4. Purify the vector from the agarose, using crushed glass.
5. Treat the purified vector with 1 U of calf intestinal phosphatase (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's instructions, then extract the reaction once with phenol–chloroform. After the addition of 0.1 vol of 3 *M* sodium acetate (pH 5.2), precipitate the DNA with a 2.5-fold excess of 100% ethanol.

Preparation of Insert DNA

The inserted DNA that constitutes the tags can, in principle, come from any source. Genomic DNA from *Arabidopsis thaliana* has been used (9), since it is a DNA source with few repeated sequences, so that restriction fragments vary greatly in size. However, the insert DNA could also consist of DNA from any other source, such as synthetic oligonucleotides. Synthetic oligonucleotides of randomly variable DNA sequences have the advantage that libraries of essentially limitless complexity and equal representation are theoretically possible.

1. Digest the insert DNA so it is compatible with the cloning site. In the example case the DNA was digested to completion with an excess of *Mbo*I at 37° C in the buffer recommended by the manufacturer, then heated to 65° C for 20 min.
2. Treat the DNA by the addition of 2 U of the Klenow fragment of DNA polymerase, 1 m*M* dCTP, and 1 m*M* dTTP to convert the "GATC" termini formed by the restriction enzyme to "TC" termini.
3. Separate the DNA by size on low-melting-temperature agarose. DNA fragments less than 450 bp in size can then be cut out of the gel and isolated by phenol extraction of the melted agarose solution (12). Size selection is important because of limitations on the size of fragments that can be amplified efficiently from single cells when using the PCR.

4. The plasmid and the insert are then ligated together. Mix the prepared vector and insert the DNA in several ratios (12). The optimal concentrations were 150 ng of vector and 15 ng of insert in a 15-μl reaction. The reaction also consisted of 1× ligation buffer, 0.5 m*M* ATP, and 2 U of DNA ligase from T4 (Boehringer-Mannheim) and was allowed to continue overnight at 14° C.

5. The ligation mixture is then used to transform competent *Escherichia coli* cells of the DH5α strain. The transformed cells are grown overnight on L(8)uria Broth medium containing kanamycin sulfate at 30 μg/ml, producing about 3400 colonies. However, large-scale transformations could produce 10^6–10^7 colonies.

Preparation of Retroviral Vectors

1. Mix together colonies of retroviral plasmids directly from the plates.

2. Prepare "miniprep" DNA, using an alkaline lysis protocol; then purify the DNA using crushed glass (8).

3. Transfect the purified DNA into the CRE amphotropic packaging line (13), using techniques presented elsewhere (14). Use 10 μg of DNA to transfect 10-cm plates; each 10-cm plate will yield about 3 ml of "transient" viral supernatant with a titer of about 10^4 cfu/ml (i.e., ≈30,000 functional viral particles). To ensure adequate representation of the various tags in the library, harvest more than 10 transient viral particles for each plasmid colony. For example, a library of 3400 colonies should be transfected into at least three 10-cm dishes (≈60,000 transient particles).

4. Each supernatant is now used to infect a 10-cm dish of the ecotropic packaging cell line ψ2 (15), in order to make stable retroviral producer cell lines. Again, more than 10 producer colonies should be made for each member of the library. The ecotropic packaging line produces retroviral vectors that are suitable for infection of rodent cells only.

5. Select stable producer cells by growth in medium containing G-418 for 7–10 days (14), and raise the population of resistant colonies to confluence.

6. Recover the viral supernatants and titer them on NIH 3T3 cells in six-well tissue culture dishes, according to previously described techniques (14).

7. The mixed viral supernatant can be concentrated by centrifugation overnight at 10,000 rpm at 4° C (14).

8. The retroviral library is now ready for use. It should be tested for the presence of helper virus and should be titered on NIH 3T3 cells, according to previously published techniques (14).

9. Inject the library into the brain or other developing tissue of fetal rats or mice. Allow the animals to survive for at least 3–4 days, then kill the animals and perfuse them with fixative. The histochemical processing technique has been presented elsewhere (11, 14, 16). Sections should be stained for alkaline phosphatase using X-phos (Boehringer-Mannheim, Indianapolis, IN) as a substrate (11). Retrovirally labeled cells should be drawn and plotted, after which the clonal analysis can be done using PCR.

Tissue Analysis of Clonal Relationships with PCR

In analyzing the labeled cells, the goal is to create a permanent record of the morphology and relative location of the labeled cells, since the PCR analysis permanently removes them from the tissue. A standard microscope and camera lucida device allows morphological details of cells to be drawn at high magnification, while cell location can be plotted at low magnification. Alternatively, cells can be photographed or plotted on a computerized system. The anatomical analysis is often the most time-consuming aspect of the entire study.

After anatomical analysis PCR amplification of the tags that distinguish the vectors allows clonal analysis. These experiments use two "nested" pairs of oligonucleotides specific for the target sequences (17). First, an initial PCR amplifies the target sequence from the fixed histochemically reacted tissue. A second PCR, using nested oligonucleotides, increases the sensitivity and specificity of the detection and provides sufficient DNA for restriction enzyme digestion or DNA sequence analysis. PCR parameters for each oligonucleotide pair must be optimized, especially the magnesium concentration in the PCR buffer, as discussed elsewhere (18).

Materials

Histological sections, prepared and stained as described in the preceding section, and coverslipped any aqueous-based mounting medium.

Sterile distilled water
1 × PCR buffer
Two nested pairs of oligonucleotides, as 20 μM solutions
Deoxyribonucleotide solution [20 mM (each) dATP, dTTP, dCTP, dGTP]
Proteinase K (10 mg/ml) in sterile distilled water
Tween-20 (Sigma, St. Louis, MO) (10%) in sterile distilled water
Mineral oil (light) (Sigma)
Disposable breakable razor blades and blade holder (Fine Scientific Tools, Foster City, CA)
600-μl microfuge tubes (autoclaved and silanized) or 96-well microtiter dishes and lids (e.g., Falcon; Cat. Nos. 3911 and 3913, Becton Dickinson, Lincoln Park, NJ)
Automated thermal cycler
Dissecting microscope
50-ml centrifuge tubes

[Note: All solutions and containers must be assembled and stored using the most stringent precautions to prevent contamination with DNA that could be amplified in the PCR. Dissections should be performed in a clean laboratory that is spatially remote from the main laboratory where PCR products are analyzed (see below).]

Dissect the Cells and Digest the Tissue

1. Prepare a lysis solution. For 50 samples mix

50 μl (or the optimum amount) of 10× PCR buffer
25 μl of 20 μM (each) outer oligonucleotides PBR-5 and BND-4
10 μl of 10 mg/ml proteinase K (final concentration, 0.2 mg/ml)
25 μl of 10% Tween-20 (final concentration, 0.5%)
375 μl of distilled water

2. Soak off the coverslips in distilled water in a clean, sterile 50-ml centrifuge tube. After the slide has soaked for about 30 min, the coverslip can be carefully pried off with a razor blade. After the coverslip comes off, soak the tissue for about an additional 5 min to remove traces of mounting media.

3. Pipette 10 μl of the lysis solution into each 0.6-ml microfuge tube (or each well of a 96-well microtiter dish).

4. Break off a fresh fragment (2–5 mm wide at the edge) of the breakable razor blade in the blade holder.

5. Under the dissecting microscope, locate a labeled cell. [Note: Well-stained cells can be seen with a low-magnification (0.8×) objective, but lighter cells may be seen only with a high-magnification (5×) objective.]

6. Using the razor blade, cut a fragment of tissue that includes the nucleus of the labeled cell. [Note: If labeled cells are widely scattered, they can be dissected one at a time, in chunks that contain no more than 1000 unlabeled cells. Chunks are typically less than 500 μm in each dimension, but larger pieces can be used (PCR sensitivity may be less with larger pieces, since they do not dissolve as well). If labeled cells are immediately adjacent, dissect several cells in one chunk.]

7. Transfer the tissue piece, on the razor blade, to the lysis solution. Confirm under the microscope that the labeled cell is in the lysis solution. It is important to keep careful notes of which cell goes where—drawings are helpful here.

8. Cover the lysis solution with 50–100 μl of mineral oil and cap the tube (or cover the microtiter plate).

9. Replace the blade fragment with a fresh one and dissect the next cell (return to step 5). (Note: While dissecting the cells, prepare negative controls. Intersperse samples that contain no tissue, or unlabeled tissue, among the positive samples.)

10. After all of the cells have been dissected and all lysis samples have been covered with mineral oil, cover the tubes (or the plate).

11. Transfer the samples to a thermal cycler. Digest for 2–3 hr at 65°C. [Note: Inspect a few samples after this time to confirm that the tissue is totally dissolved. If not, digest longer (overnight at 37°C is all right). The histochemical precipitate does not dissolve, but does not interfere with the PCR.]

12. Once the tissue is digested, heat the solution to 85°C for 20 min, then 95°C

for 5 min. (Note: This inactivates the proteinase K and denatures the genomic DNA. The samples are now ready for the PCR.)

First PCR Reaction

1. Prepare the PCR solution. For 50 samples use

   100 μl (or the optimum amount) of 10× PCR buffer
   50 μl of 20 μM (each) outermost oligonucleotide primers PBR-5 and BND-4
   10 μl of the mixed-deoxyribonucleotide solution [20 mM (each) dNTP]
   7.5 μl (37.5 U) of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT)
   800 μl of distilled water

(Note: These reactions can be scaled up or down proportionately. *Taq* polymerase from other manufacturers can be used, but should be tested first.)

2. Put the samples in the thermal cycler and start the PCR reaction, which should begin with an initial denaturation at 92–94° C for 3 min.

3. Once the samples have exceeded 85° C, uncover them and add 20 μl of the PCR solution to each sample. (Note: The "hot start" enhances the sensitivity and specificity of the PCR. The added PCR solution does not need to be mixed in. It will sink beneath the oil and join the aqueous phase.)

4. Cover the samples and allow the PCR reaction to proceed. PCR conditions should be optimized, but will comprise 45 repetitions of denaturation (92–94° C for 30–45 sec), annealing (55–70° C for 1–3 min), and extension (72° C for 1 min).

Second PCR Reaction

The samples now contain large amounts of amplified target DNA and should not be opened or even transported into the clean laboratory. Since contamination of the samples is no longer a concern, they may be handled in the main laboratory. The sensitivity of the second PCR reaction is not critical, and *Taq* polymerase from any manufacturer can be used.

1. Prepare the following solution. For 50 samples use

   10 μl of mixed-deoxyribonucleotide solution [20 mM (each) dNTP]
   200 μl (or the optimum amount) of 10× PCR buffer
   50 U of *Taq* DNA polymerase
   1800 μl of sterile distilled water
   20 μl of each of the internal pair (PBR-4 and BND-3) of oligonucleotide primers (final concentration, 0.2 μM)

(Note: The lower concentration of primers and deoxyribonucleotides used in the second reaction does not affect the PCR sensitivity or the product yield.)

2. Pipette 40 μl of this solution into 50 tubes (or microtiter wells).
3. Transfer 2–4 μl of the product of each PCR reaction into each tube (or well). Keep the samples carefully labeled, and take note of any pipetting errors.
4. Run the second PCR for 25–35 cycles. (Note: Conditions for the second PCR will also have to be optimized, but will likely be similar to those used for the first reaction. If the same thermal cycler is used for both the first and second PCR reactions, be sure not to open any of the samples of the second reaction near the machine.)
5. Separate the PCR products on 3% NuSieve–1% Seakem agarose gels. Use 1 × TBE as the running buffer, and include pBR/*Msp*I or ΦX/*Hae*III DNA size standards. (Note: Anticipate that 40–70% of the samples will produce a band. Suspect a problem if yields are consistently below 40%; suspect contamination if many contiguous samples show the same product.)

Analysis of PCR Products

DNA inserts may be distinguished by size or sequence analysis. The goal is to "fingerprint" each tag. The most precise fingerprint of any tag is, of course, a complete DNA sequence, and protocols for the sequence analysis of PCR products are available elsewhere (12). However, restriction mapping of the viral tags offers a convenient and rapid assay and is sufficiently specific for libraries with 100–300 different inserts. One plan uses five restriction enzymes with 4-base recognition sequences (*Cfo*I, *Rsa*I, *Alu*I, *Mse*I, and *Msp*I) that are active in similar buffer conditions (50–100 m*M* NaCl). The mixture cuts small DNA fragments frequently, allowing them to be easily distinguished. Before loading the diagnostic gel, or prior to restriction digestion, sort the PCR products by size. When samples of similar initial size are run side by side on the gel, it allows the most direct comparison of the restriction fragments.

1. Prepare the following solution (for 50 samples):

   250 U of each restriction enzyme
   150 μl of 10 × restriction enzyme buffer, appropriate for the enzymes chosen
   15 μl of 10 mg/ml bovine serum albumin
   Sterile distilled water to a total volume of 500 μl

2. Pipette 10 μl of the solution into individual wells of a microtiter dish (tubes can also be used).
3. Add 20 μl of one of the PCR reaction products to each well.
4. Cover the microtiter plate and incubate at 37° C for at least 3 hr.

5. Terminate the reactions by adding loading buffer, and separate the products of the reaction on a 3% NuSieve–1% Seakem agarose gel in 1 × TBE running buffer.

6. Record which samples contain the same tags, and the overall number of tags seen. Compare this information to the original plots of cell location to get clonal information.

Statistical Analysis

The tentative conclusion from the PCR analysis is that cells containing the same tag are members of the same clone. The confidence of this conclusion rests on (*a*) the number of clones in a given experiment, *n*, and (*b*) the number of tags in the library, *k*. There is a surprisingly large probability that the same tag will appear in two different clones by coincidence, and this probability should be considered in the clonal analysis. The probability of "coincidental double infections" by one tag can be calculated using binomial theory or computer-simulated using a Monte Carlo method (19). Either method requires the assumption that all tags are present in equal ratios, so the ratios of tags in the library must be verified experimentally. If tags are not present in equal concentrations, the library is still usable, but more complicated statistical modeling should be used (19). A computer program (MONTAG) that aids in the calculation of expected frequencies of coincidental double infections given different distributions and numbers of tags has been written by George Church (Department of Genetics, Harvard University Medical School, Cambridge, MA).[1] The best results demand a highly complex library, with very few clones labeled in each experiment (no more than three or four clones). Under these conditions the probability is maximized that observed patterns faithfully reflect clonal patterns..

Reagents and Solutions

10× PCR buffer

100 m*M* Tris buffer, pH 8.3 (purchase as premixed crystals from Sigma)
500 m*M* KCl
0.1% (w/v) gelatin
15–25 m*M* $MgCl_2$

[1] Available through anonymous internet ftp from rascal.med.harvard.edu. This will run on most VMS machines without recompiling. Type *run montag* and answer the queries. If there are problems, contact the program author at church@gnome.med.harvard.edu.

Mixed-deoxyribonucleotide solution

Deoxyribonucleotides may be purchased from Pharmacia (Piscataway, NJ) as separate 200 m*M* solutions of dATP, dCTP, dGTP, and dTTP. Mix them 1:1:1:1 with distilled water to make a working mixture that is 20 m*M* in each solution. Store as 10-μl aliquots at $-70°$C.

Proteinase K

Proteinase K can be purchased from many manufacturers, dissolved in sterile distilled water to make a 10 mg/ml solution, and stored as 20-μl aliquots at $-70°$C.

Oligonucleotide primer solutions

Deprotected oligonucleotides can be passed over an NAP-10 ion-exchange column (Pharmacia) and eluted with sterile distilled water. Adjust the concentration of the effluent to 20 μM by measuring absorbance at 260 nm. We routinely use oligonucleotides without further purification. Oligonucleotides should be stored as 25- to 50-μl aliquots at $-20°$C.

Avoiding Contamination of PCR Reactions

Given well-optimized PCR reactions and sufficient cycles, the target sequence can be successfully analyzed; the biggest long-term threat to the continued success of PCR reactions that involve single DNA molecules is contamination of these reactions. In order to avoid contamination of the PCR reactions, several precautions are necessary. First, a physically separate clean room should be used for the construction of sensitive PCR reactions. Avoid opening clean solutions in the main laboratory, since minute quantities of aerosolized DNA can contaminate reagents and PCR reactions. The clean room should contain dedicated solutions, enzymes, pipettors, etc., and these should never be used in the main laboratory. All PCR reagents (primers, buffer, dNTPs, and proteinase K) should be prepared and divided into aliquots suitable for one experiment. Use one aliquot, then discard whatever remains. For PCR 1 oligonucleotides (PBR-5 and BND-4) prepare 50-μl aliquots; for PCR 2 oligonucleotides use 20- to 30-μl aliquots. Prepare proteinase K in 20-μl aliquots of 10 mg/ml solution in distilled water. Prepare dNTPs as 10-μl aliquots of a mixed solution that is 20 m*M* (each) dATP, dGTP, dTTP, and dCTP. Pipetting steps should be minimized, because pipettors transmit contamination. Prepare sensitive PCR reactions as a "master mix," then use a direct displacement repeating pipettor to dispense the master mix into each reaction.

When preparing PCR reactions, precautions analogous to those of sterile technique should be used. Put on clean gloves when entering the clean room, and change gloves

upon leaving and reentering the room. Any plasticware in the clean room should come directly from new packages, opened only in the clean room. Use direct displacement pipettors only, with ejectable shafts. Use pipette tips once, then eject them directly into the trash without touching them.

Never bring any of the following into the clean room: DNA solutions, pipettors from the main laboratory, or PCR products. Do not use the clean room if the reaction being prepared involves over 100 molecules of DNA as starting material ($\approx$1 ng of DNA). Whenever the clean room is used, all precautions should be followed to avoid the risk of contaminating the room. Plan on setting up sensitive PCR reactions in the morning. Wear clean clothes, since PCR reactions have been contaminated by DNA transmitted by falling hair and lint. Do not set up sensitive PCR reactions on the same day that you are analyzing PCR products of the same reaction. Set up a reaction one day; analyze it on the same day or the next day. Set up another clean reaction in the morning on a new day. Suspect that all solutions are contaminated. Contamination has been shown to affect solutions including buffers, primers, enzymes, gelatin used for subbing slides, coverslipping medium, etc.

Although methods have been reported for the decontamination of solutions contaminated with DNA, decontamination is usually not quantitative. Ultraviolet (UV) light can cross-link double-stranded DNA, but this only works if it shines directly into the solution (not through plastic or glass), and if the DNA sequences are 200 bp or longer. UV light is also only effective if DNA is aqueous. HCl (at least 1 N) is effective at removing DNA from contaminated glassware.

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References

1. S. Fraser, R. Keynes, and A. Lumsden, *Nature (London)* **344,** 431 (1990).
2. S. A. Moody, *J. Neurosci.* **9,** 2919 (1989).
3. C. L. Cepko, *in* "Neuromethods: Molecular Neurobiological Techniques," Vol. 16. Humana, Clifton, New Jersey, 1989.
4. H. Ochman, A. S. Gerber, and D. L. Hartl, *Genetics* **120,** 621 (1988).
5. G. A. Silverman, R. D. Ye, K. M. Pollock, J. E. Sadler, and S. J. Korsmeyer, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 485 (1989).
6. D. J. Earp, B. Lowe, and B. Baker, *Nucleic Acids Res.* **18,** 3271 (1990).
7. E. Y. Snyder, D. L. Deitcher, C. Walsh, S. Arnold-Aldea, E. A. Hartweig, and C. L. Cepko, *Cell (Cambridge, Mass.)* **68,** 33 (1992).

8. T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
9. C. Walsh and C. L. Cepko, *Science* **255,** 373 (1992).
10. C. L. Cepko, E. F. Ryder, C. P. Austin, C. Walsh, and D. M. Fekete, *in* "Methods in Enzymology" (P. S. Wassarman and M. L. DePamphils, eds.),Vol. 225, p. 933. Academic Press, Orlando, 1993.
11. S. Fields Berry, A. Halliday, and C. L. Cepko, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 693 (1992).
12. F. Ausubel, *et al.,* "Current Protocols in Molecular Biology." Wiley, New York, 1989.
13. O. Danos and R. C. Mulligan, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6460 (1988).
14. C. L. Cepko, *in* "Current Protocols in Molecular Biology" (Ausubel *et al.,* eds.), Suppl. 17. Wiley, New York, 1993.
15. R. Mann, R. C. Mulligan, and D. Baltimore, *Cell (Cambridge, Mass.)* **33,** 153 (1983).
16. C. Walsh and C. L. Cepko, *Science* **241,** 1342 (1988).
17. K. B. Mullis and F. A. Faloona, *in* "Methods in Enzymology" (R. Wu, ed.), Vol. 155, p. 335. Academic Press, San Diego, 1989.
18. M. A. Innis, *et al.,* "PCR Protocols." Academic Press, San Diego, 1990.
19. C. Walsh, C. L. Cepko, E. F. Ryder, G. M. Church, and C. Tabin, *Science* **258,** 317 (1992).

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Section IV

RNA Viruses

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[19] New Methods to Study Poliovirus Assembly and Encapsidation of Genomic RNA

Casey D. Morrow, Donna C. Porter, and David C. Ansardi

Introduction

Poliovirus, a member of the Picornaviridae family, is a small nonenveloped icosahedral virus that encapsidates a single genome molecule of the positive sense (1, 2). The capsid shell that encloses the viral RNA genome is composed of 60 copies of each of four structural proteins: VP1 (33 kDa), VP2 (30 kDa), VP3 (26 kDa), and VP4 (7.5 kDa). One or two copies of VP4 and VP2 may be present in the mature virion as a VP4–VP2 polyprotein precursor, termed VP0 (37.5 kDa). Expression of the poliovirus genome occurs by translation of the entire viral genomic RNA of 7500 nucleotides, resulting in the production of a long protein precursor (1, 2) (Fig. 1). Two virus-encoded proteinases, $2A^{pro}$ and $3C^{pro}$ are used to process the precursor polyprotein (3, 4). The 2A proteinase autocatalytically cleaves the polyprotein at a specific tyrosine–glycine amino acid pair, releasing the capsid precursor, P1, from the polyprotein (5). The second viral proteinase, $3C^{pro}$, further processes the P1 precursor, yielding the capsid proteins VP0, VP3, and VP1 (6, 7). Previous studies from this and other laboratories have demonstrated that this processing can occur in *trans* and is mediated by a polyprotein, 3CD, which consists of a fusion of $3C^{pro}$ and the viral RNA polymerase, $3D^{pol}$ (6, 8, 9).

Once the P1 protein is released from the large polyprotein by the autocatalytic activity of $2A^{pro}$, the process of poliovirus assembly commences. Assembly of the capsid is apparently dependent on cleavage of the P1 capsid precursor by 3CD, since capsid particles consisting of uncleaved precursors have not been identified. Early studies have suggested that the assembly pathway proceeds through several well-defined intermediates, which can be separated on sucrose density gradients according to their different sedimentation velocities (10–12) (Fig. 2): a 5 S protomer subunit, consisting of one copy each of VP0, VP3, and VP1; a 14 S pentameric subunit containing five copies each of VP0, VP3, and VP1; a 75 S empty capsid (or procapsid) structure consisting of 60 copies each of VP0, VP3, and VP1; and a provirion intermediate in which the genomic RNA has been encapsidated, but a final proteolytic cleavage event has not occurred. This final cleavage event is the processing of VP0 to VP4 and VP2, two proteins found only in mature RNA-containing virions. The protease responsible for this cleavage has not yet been identified (13).

The availability of an infectious clone for poliovirus has made it possible to use molecular genetics to begin to dissect the assembly and encapsidation process (14,

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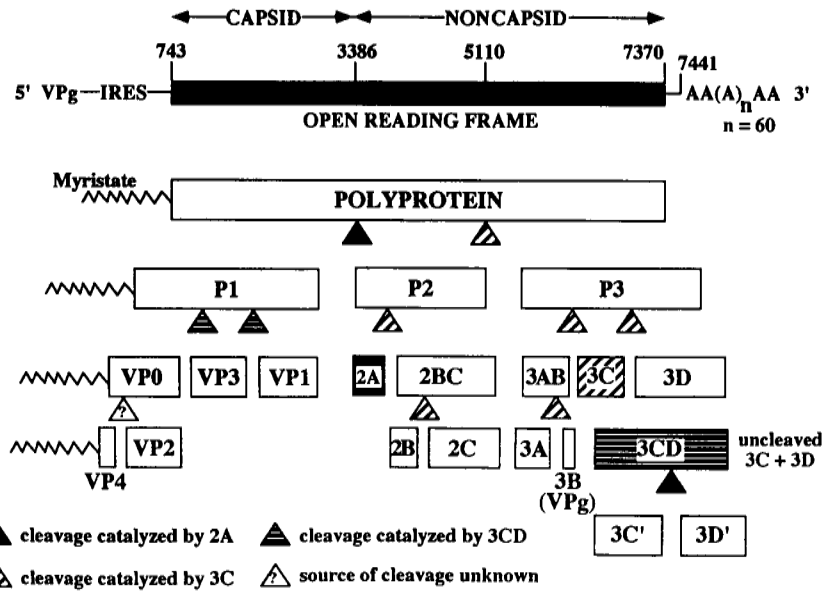

FIG. 1 Genomic organization of poliovirus. The entire poliovirus genome has been cloned and the 7500-base nucleic acid sequence has been determined (1). The viral RNA genome is covalently linked at its 5′ end to a small peptide, designated VP_g. The genome contains a long 5′ nontranslated region [approximately 750 nucleotides containing an internal ribosome entry site (IRES)]. Translation begins at nucleotide 743 and results in the synthesis of a long polyprotein. The N-terminal glycine of the polyprotein is covalently linked to a single molecule of a 14-carbon fatty acid, myristic acid (*n*-tetradecanoic acid). The processing of the polyprotein occurs by two virus-encoded proteinases, 2A and 3C. Following translation of the 2A proteinase, the autocatalytic activity of this enzyme releases the capsid precursor P1 from the polyprotein (7). The capsid precursor is subsequently processed by the 3C proteinase in the form of a polyprotein, 3CD, to generate the viral capsid proteins VP0, VP3, and VP1. Following encapsidation of the viral genomic RNA, VP0 is further processed by an as yet unknown mechanism, generating VP4 and VP2. Further processing of the P2 and P3 region polyproteins is carried out by the viral 3C proteinase. Further details regarding the sequential cleavage of the P2 and P3 proteins and the kinetics of this reaction can be found in the work of M. A. Lawson and B. L. Semler [*Virology* **191,** 309 (1992)]. The processing of the P1 polyprotein by the 3CD viral proteinase has been demonstrated to occur in *trans,* using both *in vitro* and *in vivo* systems (6–8).

15). In early studies numerous mutations were made in the poliovirus infectious clone in the capsid-encoding regions (16–21). In order to assay these mutants, the poliovirus cDNA containing the mutations, or an *in vitro*-transcribed RNA transcript, was transfected into cells and the production of virus was assessed. In some cases no virus was produced, indicating that the mutation was somehow deleterious to viral repli-

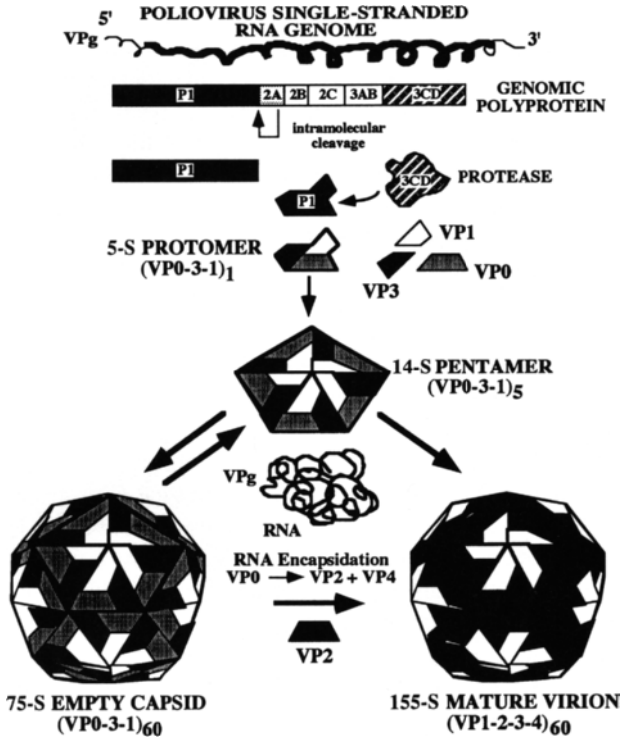

FIG. 2 Assembly of poliovirus. Previous studies have suggested that the assembly of poliovirus proceeds through several intermediates (10–12). The poliovirus single-stranded RNA genome is translated, producing a long polyprotein. Once the ribosomes have proceeded past the 2A proteinase, an intramolecular proteolytic cleavage occurs, resulting in release of the capsid precursor protein, P1. The P1 protein is processed by the 3CD proteinase, a fusion of the $3C^{pro}$ and $3D^{pol}$ proteins of poliovirus. The processed P1 protein assembles into a 5 S protomer (VP0, VP3, or VP1). The 5 S protomers then self-assemble into a 14 S pentamer consisting of five copies each of VP0, VP3, and VP1. At this point, two possible routes to encapsidation have been proposed. One possibility is that the 14 S pentamers assemble into an empty capsid structure, $(VP0-3-1)_{60}$, which then interacts with the viral genomic RNA to form the mature virion. Alternatively, it is possible that twelve 14 S pentamers coalesce around the viral RNA genome to produce the RNA-containing virion. During or subsequent to RNA encapsidation, VP0 is cleaved to VP2 and VP4. Previous studies have demonstrated that coexpression of the viral P1 precursor and the 3CD protease in the absence of viral RNA results in the assembly of 75 S empty capsids.

cation and/or assembly processes; in other cases viruses were recovered from transfection of the mutant viral genomes. Many times, however, the recovered viruses had reverted the original mutation back to the wild type. While these studies were in-

formative, it was not always possible to clearly identify the step in assembly at which the viruses were blocked. In order to further study the mechanism of assembly and morphogenesis of poliovirus, an experimental system was required in which the P1 protein and the viral proteinase 3CD could be manipulated independently.

The experimental approach developed at the University of Alabama at Birmingham laboratory is based on the use of recombinant vaccinia viruses to independently express the poliovirus P1 and 3CD proteins (8, 22–24). We have found that coinfection of cells with recombinant vaccinia viruses expressing the P1 and 3CD proteins independently results in correct processing of the P1 protein and assembly of subviral capsid particles, including 14 S pentamers and 75 S empty capsids (8). This system allows us to investigate aspects of poliovirus assembly in the absence of a viral RNA genome. In addition, we have extended this assembly system further to study the events associated with genomic RNA encapsidation. These studies are accomplished by using the recombinant vaccinia virus expressing the poliovirus P1 capsid protein in conjunction with a defective genome of poliovirus (23) (Fig. 4). The defective genome of poliovirus used in these studies has an in-frame deletion in the P1 capsid gene that results in the synthesis of a deletion-containing P1 protein that cannot serve as a precursor to virions (25). In order for this defective poliovirus genome to be propagated, the capsid precursor protein must be supplied in *trans,* and we have shown that the recombinant vaccinia virus that expresses the P1 precursor can fulfill this requirement (23). This experimental system allows us to mutagenize the P1 protein and to evaluate the mutants for the capacity to be processed, assemble into subviral particles, and encapsidate RNA without concern for reversion due to replication of the viral RNA.

This article describes the growth and characterization of the recombinant vaccinia viruses that express the poliovirus capsid precursor protein and the 3CD proteinase and the use of these recombinant vectors to study P1 cleavage and assembly of subviral particles. Furthermore, the article discusses the use of the recombinant vaccinia virus expressing the P1 capsid precursor in conjunction with the defective poliovirus genome to study the individual steps of poliovirus assembly and encapsidation.

Materials

General laboratory chemicals, restriction enzymes, radioisotopes, tissue culture flasks, and plasticware were obtained from standard commercial suppliers.

Tissue Culture Cells and Viruses

Most of the experiments were performed in HeLa or BSC40 cells. Generally, these cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented

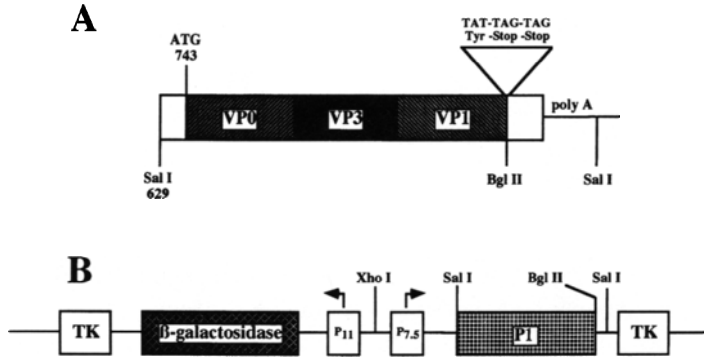

FIG. 3 Plasmids used in the analysis of mutations in the P1 gene. (A) The P1 gene contained within the phagemid pUC119–P1 is shown. The P1 cDNA sequences of poliovirus, consisting of nucleotides 743–3382, were subcloned into a phagemid, pUC119. This construction was made by using restriction sites within the poliovirus cDNA: (1) the poliovirus cDNA clone was digested with *Nde*I, which cuts at nucleotides 3382 and 6427, and two synthetic oligonucleotides were annealed together and ligated into the *Nde*I-digested DNA to create two termination codons, TAG, immediately after the codon for the C-terminal tyrosine residue of P1; (2) the *Bal*I site at nucleotide 629 of the poliovirus cDNA was converted to a *Sal*I restriction site using synthetic DNA linkers; (3) the *Sal*I-flanked P1 insert was subcloned into the unique *Sal*I site in the polylinker of pUC119. Following mutagenesis the P1 insert can be removed from the phagemid by *Sal*I digestion and then subcloned into the vaccinia virus transfer plasmid. (B) The plasmid vector used for construction of the recombinant vaccinia viruses is a derivative of the pSC11 vector in which the unique *Sma*I site was converted to a *Sal*I site. The vector contains the β-galactosidase gene positioned downstream of the vaccinia virus P_{11} promoter. A second vaccinia virus promoter, $P_{7.5}$, is positioned upstream of the *Sal*I restriction site into which the P1 gene is inserted. Sequences derived from the vaccinia virus thymidine kinase gene (TK) flank the β-galactosidase and P1 genes. Once the P1 gene is subcloned into the pSC11–*Sal*I transfer vector, *Xho*I–*Bgl*II double digestions are performed to ensure that the P1 gene is correctly oriented downstream of the promoter. The generation of recombinant vaccinia viruses expressing the mutant P1 gene is accomplished using techniques described in the text.

with 5% (v/v) fetal calf serum and GIBCO's (Grand Island, NY) 1× GMS-G (complete medium). The recombinant vaccinia viruses were derived from the WR strain [American Type Culture Collection (ATCC), Rockville, MD; Cat. No. VR-119]. The derivation of recombinant vaccinia viruses VV-P1, which expresses the poliovirus P1 capsid precursor protein, and VV-P3, which expresses the poliovirus 3CD proteinase, has been described (26).

The cDNA clone of the poliovirus defective interfering genome has already been described (25); the use of this cDNA clone to generate homogeneous stocks of defective poliovirus by using VV-P1 to provide capsid proteins in *trans* has also been described recently (23).

Methods

Overview

The first step in the analysis of the effect of mutations in the P1 capsid gene on assembly and encapsidation is to generate specific mutations in the poliovirus P1 gene, followed by subcloning of the mutated P1 gene into a vaccinia virus transfer vector that facilitates generation of a recombinant vaccinia virus. The vaccinia virus transfer vector is a plasmid that allows homologous recombination of the poliovirus P1 gene into the wild-type vaccinia virus genome to occur via sequences from the vaccinia virus thymidine kinase gene, which flank the P1 gene construct in the plasmid. In addition, the transfer vector contains a *lacZ* (β-galactosidase) gene under the control of a vaccinia virus promoter, also located between the flanking thymidine kinase sequences. Expression of this gene by recombinant vaccinia viruses facilitates their identification because they display a blue plaque phenotype in the presence of X-Gal (5-bromo-4-chloro-3-indoyl-β-*O*-galactopyranoside). The studies described in this article can be performed using the VV-T7/transfection system for transient expression instead of recombinant vaccinia viruses, but we obtain greater consistency between experiments using stable recombinant vaccinia viruses (27). Once the recombinant vaccinia virus expressing the mutant P1 protein is obtained, the capacity of the precursor to be processed and assemble into subviral particles and/or RNA-containing virions can be assayed. The cleavage steps require that 3CD proteinase be provided in *trans,* which can be supplied by either the recombinant vaccinia virus (VV-P3) that expresses the 3CD protein, or by coinfection of cells with stocks of encapsidated defective poliovirus genomes that also express 3CD upon replication. These two alternative infection strategies permit study of the assembly pathway in either the presence or absence of a replicating poliovirus RNA genome.

Plasmids and Molecular Biology Techniques

To develop a method by which mutant P1 genes could be constructed easily, the P1 gene of poliovirus, nucleotides 743–3382 of the poliovirus genome, was subcloned into a phagemid vector, pUC119. This construct has been designated pUC119–P1. To facilitate subcloning of mutant P1 genes out of the phagemid plasmid and into the vaccinia virus transfer vector, we have positioned *Sal*I restriction sites at the 5′ and 3′ ends of the gene (Fig. 3A).

We have used *dut*$^-$ *ung*$^-$ strains of *Escherichia coli* to generate uracil-rich single-stranded template DNA from the phagemid pUC119–P1 in order to facilitate the isolation of mutant P1 genes (28). However, any standard mutagenesis technology can be used in the construction of these mutant P1 genes. Once a suitable mutant has been constructed, the mutant P1 gene is released from the pUC119–P1 plasmid by *Sal*I digestion and is subcloned into the unique *Sal*I restriction site of the vaccinia

virus shuttle plasmid, pSC11–*Sal*I. This plasmid is identical to the pSC11 vector described by Chakrabarti *et al.* (29), except that the *Sma*I site has been converted to a *Sal*I site using synthetic linkers (Fig. 3B). The *Sal*I-flanked P1 construct also contains a unique *Bgl*II site located at the 3′ end of the P1 coding sequences. Upon subcloning of the P1 fragment into pSC11–*Sal*I, the presence of this *Bgl*II site facilitates determination of the orientation of the insert. Double digestion of the pSC11–P1 plasmids with *Xho*I and *Bgl*II is a convenient method for making this determination, as a unique *Xho*I site exists in the pSC11–*Sal*I plasmid, just upstream of the vaccinia virus promoter that directs expression of the inserted P1 sequences.

Generation of Recombinant Vaccinia Viruses

Standard procedures are used to generate the recombinant vaccinia viruses that express poliovirus P1 precursors (8, 29). For these studies BSC40 cells (a derivative of CV1 cells) are used for the initial step of the generation of the recombinant vaccinia viruses. The cells are first infected with wild-type vaccinia virus at a multiplicity of infection of 0.05 plaque-forming units (pfu) per cell. At 2 hr postinfection the cells are transfected with the pSC11–P1 plasmid, using the calcium phosphate method (30). After complete cell lysis (36–48 hr) the cells and the culture medium are collected and subjected to either three cycles of freezing and thawing or mild sonication to release the vaccinia virus. The cell debris is clarified from the extracts by low-speed centrifugation, and the supernatant is used to infect a confluent monolayer of $TK^- 143_B$ cells in the presence of 5-bromo-3′-deoxyuridine (25 μg/ml). After 24 hr complete cell lysis is usually evident, and the culture medium and the cells are collected and subjected to freezing and thawing or mild sonication. The clarified supernatant from this step is then diluted to several different concentrations and used to infect $TK^- 143_B$ monolayers in six-well dishes.

After adsorption of the virus in 0.2–0.4 ml of medium per well, additional medium containing 5-bromo-3′-deoxyuridine is added, and the cells are incubated for 24 hr or until individual vaccinia virus plaques become visible. At this point the liquid medium is removed and the monolayers are covered with a solid overlay consisting of 1 × Basal Medium (GIBCO), 0.9% (v/v) agarose, 10% (v/v) fetal calf serum, and 50 μg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β-*O*-galactopyranoside). Blue plaques, indicative of a recombinant vaccinia virus, are evident within 6–24 hr. Several blue plaques, as well isolated as possible, are picked and used to infect fresh confluent $TK^- 143_B$ monolayers in liquid culture in the presence of 5-bromo-3′-deoxyuridine for approximately 24 hr, followed by overlay with the agarose–X-Gal medium and a repeat of the plaque-picking procedure. The percentage of blue plaques observed generally increases during the second round. After three or four rounds of plaque isolation, several clear well-isolated blue plaques are picked and grown to high titers in $TK^- 143_B$ monolayers in six-well dishes by allowing infections to continue until a complete cytopathic effect appears over the entire monolayer (usually 48 hr). If nec-

essary, a freeze–thaw extract of the infected monolayer can be made and the virus can be passaged again in six-well dishes to further increase the titer. To harvest the recombinant vaccinia viruses, the drug-containing medium should be removed from the monolayer carefully and replaced with approximately 0.5 ml of drug-free complete medium. A freeze–thaw or sonicated extract of the infected cells can then be used to determine whether the individual recombinant vaccinia viruses express the P1 polyprotein, as described in the next section.

Analysis of Mutations

The analysis of the mutant P1 protein is carried out in four steps.

Step 1: Expression of the Mutant P1 Protein by a Recombinant Vaccinia Virus

The first step is to determine whether the mutant P1 protein is expressed by the isolated recombinant vaccinia virus. HeLa cell monolayers in 24- or six-well dishes are infected with the stocks of the recombinant vaccinia viruses containing the mutant P1 gene (VV-P1–mut), prepared as described in the final step. Usually, 0.1–0.2 ml of the extract (~20 pfu per cell) will be sufficient to completely infect the monolayer. At 3.5 hr postinfection the cells are washed and incubated in methionine/cysteine-free medium for 30 min to 1 hr. The cells are then metabolically labeled for 1–2 hr with ~100 μCi of ^{35}S-Translabel (ICN Biochemicals, Irvine, CA) per 10^6 cells. Following the labeling the medium is removed and the cells are lysed in an ice-cold buffer consisting of 25 m*M* Tris–HCl (pH 8.0), 50 m*M* NaCl, 0.2% (v/v) sodium dodecyl sulfate (SDS), 1% (v/v) sodium deoxycholate, and 1% (v/v) Triton X-100 [Sigma, St. Louis, MO]. The labeled P1 proteins are immunoprecipitated using a rabbit antiserum to whole poliovirus (8). The immunoprecipitates are analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), the gels are fluorographed, and the immunoprecipitated proteins are visualized by autoradiography (31). The P1 protein migrates as a 97-kDa protein. Poliovirus proteins that have been metabolically radiolabeled in poliovirus-infected cells and immunoprecipitated with the same antiserum can be used as markers.

Once expression of the P1 protein has been confirmed, selected stocks of the recombinant vaccinia viruses are grown to high titer in 175-cm^2 flasks of BSC40 cells, and the viruses are concentrated from the large-volume extracts by centrifugation [2 hrs at 13,500 rpm (25,000 *g*) in an SW28 rotor (Beckman Inst.) at 15°C] through a 36% (v/v) sucrose cushion. The concentrated virus is then resuspended in a small volume (0.5–1 ml per flask) and titered by conventional plaque assay. Once these titered stocks are obtained, a pulse–chase radiolabeling experiment is performed to determine the intracellular stability of the mutant P1 protein expressed by the VV-P1–mut in comparison to that of the wild-type P1 protein expressed by VV-P1. It is possible that mutations in the P1 gene will destabilize the protein and reduce its half-life in the vaccinia virus-infected cells, thus precluding studies on assembly and en-

capsidation. For this determination parallel cultures of HeLa monolayers are infected with either VV-P1 or VV-P1–mut at a multiplicity of infection of 20 pfu per cell and are labeled for 1 hr as described for the expression analysis. At the end of the labeling, one set of cells is harvested in RIPA buffer, whereas the second set is washed twice and then incubated for an additional 4 hr in medium containing excess methionine and cysteine. The levels of radiolabeled P1 proteins immunoprecipitated from the pulse–chase samples are compared by SDS–PAGE analysis and autoradiography, as described for the expression analysis. The wild-type P1 protein expressed by VV-P1 is generally stable in cells for at least 4 hr.

Step 2: Proteolytic Processing of Mutant P1 Protein

In each of the characterization steps, mutant P1 proteins expressed by a particular VV-P1–mut should be compared with the wild-type protein expressed by VV-P1 in a parallel experiment. The capacity of the mutant P1 protein to be processed by poliovirus 3CD protease is the next step in the analysis. Previous studies from this laboratory have demonstrated that the poliovirus 3CD proteinase expressed by a second recombinant vaccinia virus, VV-P3, will process the P1 protein in *trans* in cells coinfected with VV-P1 (8). These experiments are conducted by coinfecting HeLa cell monolayers with the VV-P1–mut and VV-P3 at a multiplicity of infection of 20 pfu per cell for each virus. At 3.5 hr postinfection, the medium is removed and the infected cells are starved for 30 min to 1 hr in methionine/cysteine-free medium. The cells are then metabolically labeled with ^{35}S-Translabel (approximately 100 μCi per 10^6 cells) for 30 min. At the end of the labeling period, the medium is removed, one set of cells is harvested in RIPA buffer, and the remaining sets are washed twice and then incubated further in complete medium containing excess methionine and cysteine. At 30-min to 1-hr intervals, sets of the infected cells are lysed in RIPA buffer and processed for immunoprecipitation using the antipoliovirus antiserum as described in step 1. If processing of P1 occurs, VP0, VP3, and VP1 cleavage products will be detected in the chase samples upon immunoprecipitation. These cleavage products are easily identified if a marker lane containing radiolabeled proteins immunoprecipitated from a lysate of poliovirus-infected cells is run on the same gel. Poliovirus capsid proteins VP2 and VP4 are not detected in this assay because their cleavage from VP0 occurs either during or subsequent to RNA encapsidation. In several cases we have found that the cleavage products of mutant P1 precursors are unstable, even though the unprocessed precursors displayed a degree of stability similar to that of the wild-type precursor.

Step 3: Assembly of Subviral Particles in the Absence of Genomic RNA

The most convenient method for analyzing the assembly of subviral particles in the absence of viral RNA is to coinfect cells with the VV-P1–mut and VV-P3 and analyze the assembled particles generated in these cells on sucrose density gradients. Sucrose density gradient centrifugation has been the traditional method used for analysis of poliovirus capsid intermediates, as they are easily separated by their char-

acteristic sedimentation velocities. Generally, for these experiments larger numbers of cells are used ($\sim 10^7$ for each set of conditions). HeLa cell monolayers are coinfected with the VV-P1–mut and VV-P3 (20 pfu per cell of each) and are metabolically labeled for 3 hr beginning at 6 hr postinfection. This is usually a sufficient incubation time for subviral particles to become detectable; however, chase periods of 2–12 hr can be added if necessary. At the end of the incubation, the medium is removed and the cell monolayers are lysed in ice-cold RSB buffer [10 m*M* Tris–HCl (pH 7.0), 10 m*M* NaCl, and 1.5 m*M* $MgCl_2$] containing 1% (v/v) Triton X-100. Alternatively, the cells can be scraped into the RSB buffer without detergent and lysed by brief sonication. The cell debris is removed by low-speed centrifugation (1,000 *g* for 10 min at 15°C), and the extract is layered onto a continuous sucrose density gradient prepared in RSB buffer containing 0.01% bovine serum albumin.

The percentages of sucrose in the gradients and the centrifugation conditions to be used are dictated by the type of subviral particle being analyzed. To resolve 75 S empty capsids away from 14 S pentamers and unassembled 5 S monomers, a 15% to 30% (v/v) sucrose density gradient is used and is centrifuged at 40,000 rpm (196,500 g_{av}) in a TH-641 (Sorvall) or similar rotor at 4°C for 4.5 hr. To resolve 14 S pentamers away from unassembled monomers on the gradient, a 5% to 25% (v/v) sucrose gradient empty capsids will be pelleted. Fractions from the gradients are collected from the bottom of the tubes and adjusted to RIPA buffer conditions, and poliovirus capsid-related proteins in the individual fractions are detected upon immunoprecipitation with antipoliovirus antiserum, SDS–PAGE, and fluorography followed by autoradiography. The sedimentation of the capsid particles generated in the recombinant vaccinia virus-infected cells can be compared with those assembled in poliovirus-infected cells to provide a standardization for the gradients. This is accomplished by conducting a parallel gradient fractionation of a lysate from metabolically radiolabeled poliovirus-infected cells. 14 S and 75 S peaks of radiolabeled proteins are identified by precipitating proteins from the poliovirus gradient fractions with trichloroacetic acid and counting the acid-precipitable material in a scintillation counter. Under the conditions described here for the recombinant vaccinia virus coinfections, the wild-type P1 precursor protein expressed by VV-P1 is processed in *trans* by the 3CD protein expressed by VV-P3, and the cleavage products assemble into distinct 14 S pentamer and 75 S empty capsid particles. Since each of the subviral particles sediments at different fractions in the gradient, mutations in the P1 gene that affect the capacity of the processed P1 proteins to assemble result in a lack of poliovirus capsid-related proteins sedimenting into the 14 S and/or 75 S regions of the gradients. In addition, the step at which the block occurs can be determined by analysis of the gradient fractions.

Step 4: Analysis of Genomic RNA Encapsidation by Mutant Capsid Proteins

To analyze whether the cleavage products of a mutant P1 protein can encapsidate genomic RNA, a defective poliovirus genome is used (25). Previous studies have

established that this defective poliovirus genome encodes all of the P2 and P3 region proteins required for replication of the defective RNA, but contains an in-frame deletion in the P1 gene such that it no longer encodes a functional capsid precursor. In order for this defective genome to be encapsidated, the P1 protein must be provided in *trans* from either wild-type poliovirus or VV-P1 (Fig. 4). Capsid mutants expressed by recombinant vaccinia viruses can be assayed for their ability to encapsidate this defective genome in *trans* by transfecting *in vitro*-transcribed defective genomes into VV-P1–mut-infected cells; however, we have found a great deal of

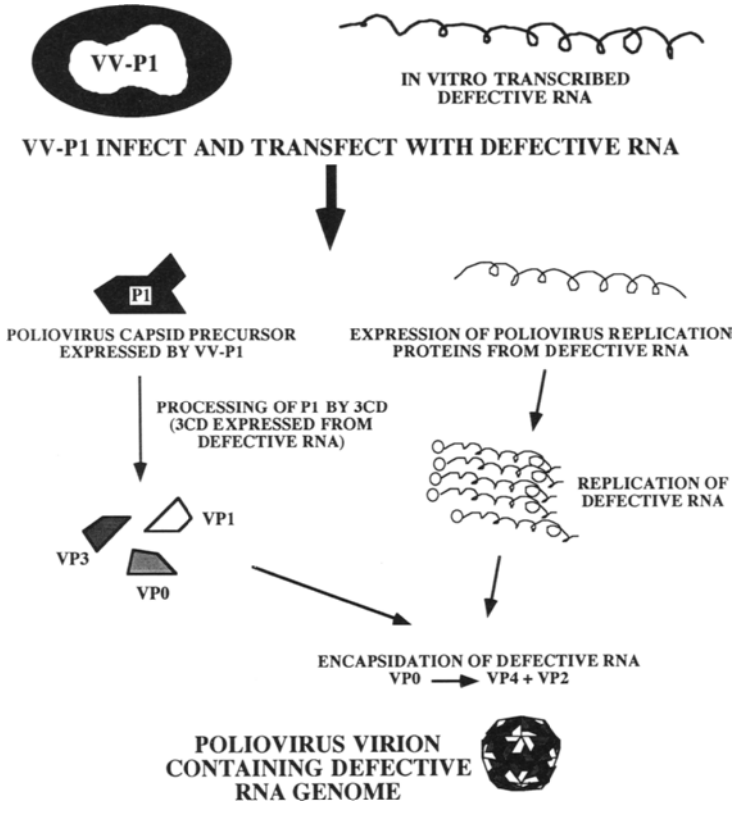

FIG. 4 Encapsidation of poliovirus replicons using VV-P1. Shown is a schematic representation of the encapsidation of the defective poliovirus genome. *In vitro*-transcribed defective poliovirus genomes are transfected into cells previously infected with a recombinant vaccinia virus, VV-P1, which expresses the poliovirus capsid precursor protein. This system is based on the ability of the poliovirus capsid precursor protein, P1, to be processed by poliovirus protease 3CD provided in *trans* upon expression of the defective RNA genome. Once the P1 protein has been cleaved, encapsidation of the defective RNA genome ensues, resulting in the formation of mature poliovirus virions containing the defective genome.

variation between experiments when this method is used. To avoid the limitations of the transfection experiments, we have generated homogeneous stocks of encapsidated defective genomes and have used these stocks to effectively and consistently deliver the defective genome to monolayers of cells.

Stocks of the encapsidated defective poliovirus genomes are maintained by serial passage in cells coinfected with recombinant vaccinia virus VV-P1, which supplies the capsid proteins in *trans* and complements the capsid gene deletion (23). Prior to experimental use, infectious VV-P1 in the preparations is removed by differential centrifugation and treatment with detergents. In order to determine the minimum concentration of a stock of defective polioviruses required to completely infect a monolayer, dilutions of the stock are plated on HeLa cell monolayers (3×10^5 cells total) and are visually inspected for a complete cytopathic effect across the monolayer after overnight incubation (23). A complete cytopathic effect indicates that a particular concentration of the encapsidated defective poliovirus genome is sufficient to infect every cell. Once a suitable stock of the defective viruses has been obtained and the residual infectious VV-P1 in the preparation has been removed, it can be used to effectively deliver the defective poliovirus genome to cells coinfected with the vaccinia virus expressing the mutant P1 protein.

1. *trans* complementation of a defective poliovirus genome by recombinant vaccinia viruses expressing mutant P1 proteins. An experiment that is routinely performed is to determine whether a mutant P1 precursor is capable of supporting passage of the defective poliovirus genome by *trans*-complementation of the capsid gene defect. For these studies BSC40 or HeLa cell monolayers are infected with 20 pfu of the recombinant vaccinia virus per cell. At 2 hr postinfection the cell monolayers are washed with fresh medium and then superinfected with the stock of encapsidated defective genomes. The cell monolayers are washed again with fresh medium at 2 *and* 4 hr postinfection to remove any unattached or eluted particles. The coinfected cell monolayers are allowed to incubate overnight and are then lysed by four cycles of freezing and thawing. The lysates are clarified by centrifugation at 15°C in a microfuge (13,000 *g*) for 5 min and incubated with RNase A (40 μg/ml at 37°C for 15 min) to digest any unencapsidated RNA still present in the extracts. One half of each of the lysates is then used to infect a fresh monolayer of cells. The infected cells are incubated with ^{35}S-Translabel from 5 to 7 hr postinfection, and the cell lysates are analyzed for the presence of the poliovirus 3CD protein by immunoprecipitation with an antiserum specific for $3D^{pol}$.

The 3CD protein is a P3 region protein that is expressed by the defective poliovirus genome but not by the VV-P1–mut and therefore can serve as a marker for passage of the defective genome. The 3CD protein will only be immunoprecipitated from lysates of cells that had been infected with encapsidated defective genomes (Fig. 5) (23). Therefore, if in the initial coinfection experiment the mutant P1 protein expressed by the VV-P1–mut is incapable of complementing the defective genomic

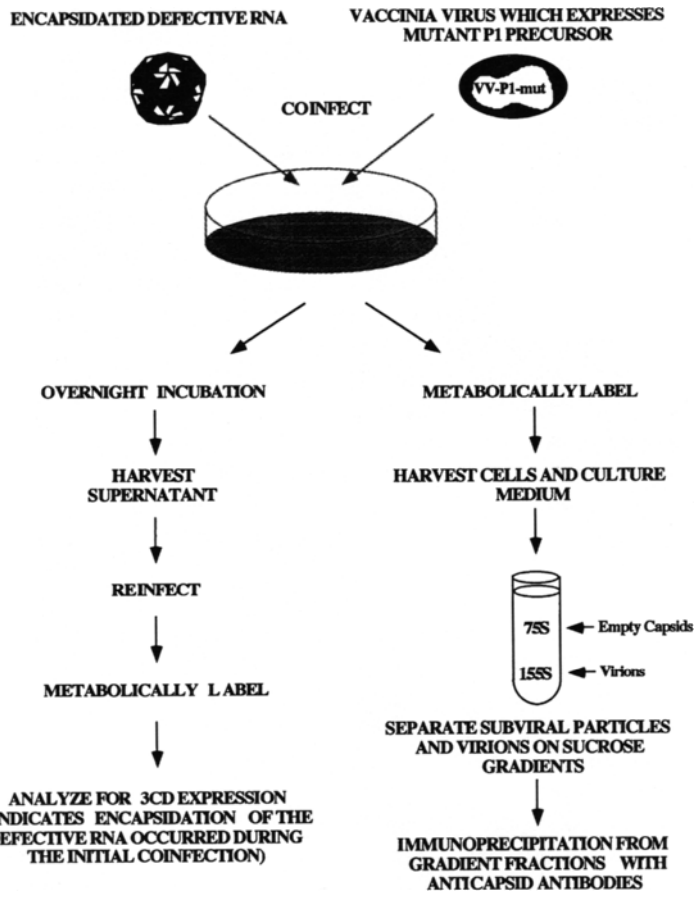

FIG. 5 Analysis of the capacity of mutant P1 genes to encapsidate poliovirus RNA. In order to determine whether the cleavage products of the mutant P1 precursors can encapsidate genomic RNA, we make use of a stock of encapsidated defective RNA genomes. These RNA genomes contained within mature poliovirus capsids are used to coinfect cells with the VV-P1–mut. Two types of experiments may then be performed. In one case the mutant P1 precursor is assessed for its ability to *trans*-complement the defective RNA genome, resulting in the assembly of infectious virion particles that can initiate a new round of infection. This new round of infection is detected by analyzing the infected cells for expression of the 3CD protein, which is encoded by the defective genome. In the second type of experiment, radiolabeled capsid proteins derived from the mutant P1 precursor can be assayed for the ability to assemble subviral particles and/or RNA-containing virions. Assembly of these structures is detected by using sucrose density gradient fractionation to separate the subviral particles and virions by their different sedimentation velocities. In the depicted gradient the 155 S virions sediment approximately two thirds of the way through the gradient, whereas the 75 S empty capsids are easily distinguished from the virions, since they sediment about half of this distance. Different types of gradients and centrifugation conditions are used to analyze the individual types of particles. The details of these experiments are described in the text.

RNA and forming infectious virus particles, expression of 3CD should not be detected above background levels upon passage (23, 24, 32). Background levels of expression can be determined by setting up a coinfection experiment with wild-type vaccinia virus and the encapsidated defective polioviruses. The background levels of 3CD expression that sometimes are obtained are likely the result of residual input defective poliovirus particles remaining associated with the cells. Background levels of 3CD expression or reduced levels of 3CD expression are suggestive of defects in precursor processing, assembly of capsid proteins, encapsidation of genomic RNA, or initiation of a new round of infection.

2. Capacity of mutant P1 cleavage products to assemble into subviral intermediates and mature virions in the presence of genomic RNA (Fig. 5). Assembly of subviral capsid intermediates and RNA-containing virions in the presence of the defective genomic RNA is assayed by using sucrose density gradients to separate the capsid particles. These experiments are performed by infecting parallel BSC40 or HeLa cell monolayers ($\sim 2-3 \times 10^6$ cells per set) with 20 pfu of the designated recombinant vaccinia virus; the cells are superinfected with encapsidated defective poliovirus genomes 2 hr later. After 1.5 hr of infection with the encapsidated stocks of defective poliovirus, the monolayers are washed with methionine/cysteine-free DMEM and incubated with ^{35}S-Translabel for 2 hr. At the end of the labeling, the medium is replaced with complete medium and the cells are further incubated for 2–12 hr. The cells are then lysed into the culture medium by the addition of Triton X-100 to the medium on the monolayers at a final concentration of 1% (v/v). This procedure allows recovery of both cell-associated and released particles.

Following clarification the supernatant is layered onto sucrose density gradients prepared and centrifuged as described previously in step 3 for analysis of 14 S pentamers or 75 S empty capsids. For analysis of the assembly of RNA-containing virions (155 S), a third type of gradient condition is used to separate virion particles from empty capsids, pentamers, and monomers. This separation is done on a 15% to 30% (v/v) sucrose density gradient centrifuged at 27,500 rpm (92,920 g_{av}) in a TH-641 (Sorvall) or similar rotor for 4.5 hr at 4° C. Under these conditions of time and acceleration, the RNA-containing virion particles will sediment approximately two thirds of the way through the gradient, whereas the empty capsids will sediment approximately half this distance. Following centrifugation poliovirus capsid-related proteins are immunoprecipitated from the fractions and analyzed as described in step 3. Fractions containing mature virions will differ from those containing subviral intermediates by the detection of greater amounts of the VP2 protein. Furthermore, the mature virion fractions can be confirmed by comparison with a profile of radiolabeled proteins from poliovirus-infected cells fractionated under the same conditions. Using these gradient analyses, it is possible to determine at which step the assembly or encapsidation process is affected by the mutation in the P1 capsid precursor. Previous studies from our laboratory have identified several different mutations that cause blocks in various stages of the assembly and encapsidation processes (22, 24, 32). Finally, it is also important to note that it is possible to perform the infections at

lower or higher incubation temperatures for analysis of temperature-sensitive defects in the assembly or encapsidation process.

Perspectives and Future Directions

There are several important features about poliovirus that make it an ideal virus in which to study basic features of assembly and RNA encapsidation. Since the three-dimensional structure of poliovirus has been determined (33), it is possible to visualize the locations and interactions of individual amino acid residues on a molecular level and to potentially identify domains important for assembly and RNA encapsidation. These studies are important because little information about capsid–RNA interactions in poliovirus virions was obtained with the solution of the three-dimensional structure, as the genomic RNA molecule was not visible. Furthermore, the infectious cDNA clone of poliovirus allows the use of molecular genetics to create mutants that are essential to test whether individual amino acids or regions of the capsid play important roles in assembly and encapsidation (14, 15). The experimental tools presented in this article make it possible to construct mutations in the poliovirus capsid genome that can be maintained and analyzed for effects on assembly and encapsidation. The systems presented here also allow the analysis of assembly in both the absence and presence of a replicating virion RNA. One of the critical questions to be answered in the coming years regarding poliovirus morphogenesis in general is the mechanism by which the genomic RNA is actually encapsidated. Computer graphic displays of the poliovirus three-dimensional structure have been used to predict certain amino acid residues and regions of the capsid that might be involved in the stabilization of the interaction between the encapsidated genomic RNA and the virion (32). By using this information, mutations have been constructed that have profound effects on the capacity of the capsid proteins to assemble and encapsidate RNA. Future use of this system should help in beginning to unravel the interactions between the capsid and genomic RNA that are required to assemble a poliovirus virion.

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References

1. N. Kitamura, B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer, *Nature (London)* **291,** 547 (1981).

2. F. Koch and G. Koch, "The Molecular Biology of Poliovirus." Springer-Verlag, Vienna, 1985.
3. A. C. Palmenberg, *Annu. Rev. Microbiol.* **44,** 603 (1990).
4. K. S. Harris, C. U. T. Hellen, and E. Wimmer, *Semin. Virol.* **1,** 323 (1990).
5. H. Toyoda, J. H. Nicklin, M. G. Murray, C. W. Anderson, J. J. Dunn, F. W. Studier, and E. Wimmer, *Cell (Cambridge, Mass.)* **45,** 761 (1986).
6. M. F. Ypma-Wong, P. G. Dewalt, V. H. Johnson, J. G. Lamb, and B. L. Semler, *Virology* **166,** 265 (1988).
7. R. Hanecak, B. L. Semler, C. W. Anderson, and E. Wimmer, *Proc. Natl. Acad. Sci. U.S.A.* **79,** 3973 (1982).
8. D. C. Ansardi, D. C. Porter, and C. D. Morrow, *J. Virol.* **65,** 2088 (1991).
9. J. Jore, B. D. Geus, R. J. Jackson, P. H. Pouwels, and B. E. Enger-Valk, *J. Gen. Virol.* **69,** 1627 (1988).
10. M. F. Jacobson and D. Baltimore, *J. Mol. Biol.* **33,** 369 (1968).
11. J. R. Putnak and B. A. Philips, *Microbiol. Rev.* **45,** 287 (1981).
12. A. C. Palmenberg, *J. Virol.* **44,** 900 (1982).
13. W.-M. Lee, S. S. Monroe, and R. R. Rueckert, *J. Virol.* **67,** 2110 (1993).
14. V. Racaniello and D. Baltimore, *Science* **214,** 916 (1981).
15. B. L. Semler, A. J. Dorner, and E. Wimmer, *Nucleic Acids Res.* **12,** 5123 (1984).
16. K. Kirkegaard and B. Nelson, *J. Virol.* **64,** 185 (1990).
17. K. Kirkegaard, *J. Virol.* **64,** 195 (1990).
18. C. Reynolds, D. Birnby, and M. Chow, *J. Virol.* **66,** 1641 (1992).
19. N. Moscufo, J. Simons, and M. Chow, *J. Virol.* **65,** 2372 (1991).
20. D. Marc, G. Masson, M. Girard, and S. v. d. Werf, *J. Virol.* **64,** 4099 (1990).
21. A. V. Paul, A. Schultz, S. E. Pincus, S. Oroszlan, and E. Wimmer, *Proc. Natl. Acad. Sci. U.S.A.* **84,** 7827 (1987).
22. D. C. Ansardi, D. C. Porter, and C. D. Morrow, *J. Virol.* **66,** 4556 (1992).
23. D. C. Ansardi, D. C. Porter, and C. D. Morrow, *J. Virol.* **67,** 3684 (1993).
24. D. C. Ansardi and C. D. Morrow, *J. Virol.* **67,** 7284 (1993).
25. K. Hagino-Yamagishi and A. Nomoto, *J. Virol.* **63,** 5386 (1989).
26. D. C. Porter, D. C. Ansardi, M. R. Lentz, and C. D. Morrow, *Virus Res.* **29,** 241 (1993).
27. T. R. Fuerst, E. G. Niles, F. W. Studier, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8122 (1986).
28. T. A. Kunkel, J. D. Roberts, and R. A. Zakour, *in* "Methods in Enzymology" (R. Wu and L. Grossman, eds.), Vol. 154, p. 367. Academic Press, Orlando, Florida, 1987.
29. S. Chakrabarti, K. Brechling, and B. Moss, *Mol. Cell. Biol.* **5,** 3403 (1985).
30. F. L. Graham and A. J. v. d. Eb, *Virology* **52,** 456 (1973).
31. J. P. Chamberlain, *Anal. Biochem.* **98,** 132 (1979).
32. D. C. Ansardi, M. Luo, and C. D. Morrow, *Virology* **199,** 20 (1994).
33. J. M. Hogle, M. Chow, and D. J. Filman, *Science* **229,** 1358 (1985).

[20] Molecular Studies of Hepatitis Delta Virus RNAs

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Introduction

During the past few years the application of new techniques of molecular biology to the study of hepatitis delta virus (HDV) replication has revealed some of the special and unique aspects by which this virus is reproduced. For example, we now know that the RNA genome of this small subviral agent has a circular conformation (1), that the RNA can undergo self-cleavage (2) and even ligation (3), and that a specific form of posttranscriptional RNA editing is required for the production of an essential viral gene product (4). For a detailed review of HDV, the reader is directed elsewhere (5).

The intent of this article is to describe two of the quantitative techniques used in our laboratory at Fox Chase Cancer Center (Philadelphia, PA) to study the molecular biology of HDV. These techniques are based on the use of the nested polymerase chain reaction (PCR). The first technique is a sensitive quantitative assay for HDV genomic and antigenomic HDV RNAs. The second makes use of the immobilization of PCR products via the avidin–biotin reaction, so as to quantitate RNA editing. The immobilization procedure can also be used for other purposes, such as dideoxy-sequencing of both strands of the PCR products.

Nested PCR for the Quantitation of HDV RNAs

Ten picograms of 1.7-kb HDV RNA represents about 10 million molecules, which is about the lower limit for detection by slot hybridization assays. Despite this limitation such assays are, in fact, a reliable approach for detecting HDV RNA in serum samples. However, if slot hybridization is applied to the detection of HDV genomic RNA—and its complement, the antigenomic RNA—in the presence of cellular RNAs, then background signals become a serious problem. The source of this problem is that the abundant cellular rRNAs (5 million per cell) also hybridize somewhat with HDV RNA probes. This cross-hybridization problem can be partially overcome by carrying out Northern analyses. However, high-resolution electrophoresis is needed, since the small rRNA and the 1.7-kb HDV RNA nearly comigrate. Thus, to both increase the sensitivity of the assays and avoid the problem of cross-hybridization with rRNAs, we have developed and in some cases used a nested PCR that has been quantitated via the use of an internal RNA standard. Many laboratories have reported the application of such assays to other viral and cellular RNAs (6–10).

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However, our approach has been designed to deal with some novel problems typically associated with such assays. Moreover, as explained below, we have also extended our approach to problems other than simple quantitation. In particular, we have used immobilization of the PCR product to achieve a highly sensitive assay for the detection of edited HDV RNA.

Strategy for Quantitative Nested PCR of HDV RNAs

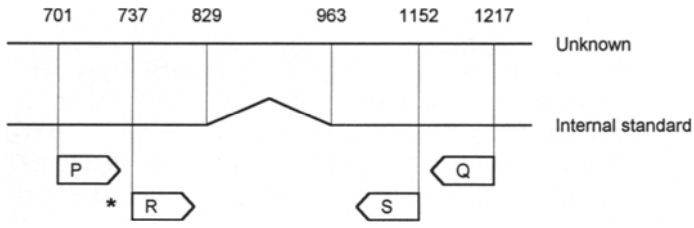
We began by adapting the strategy reported by Gilliland *et al.* (6). Figure 1a shows the region of the HDV RNA chosen for nested PCR. The exact positions and sequences of the four 35-nucleotide primers, P–S, are presented in Fig. 1b. The HDV nucleotide numbering was taken from an earlier report by Kuo *et al.* (11).

To calibrate the nested PCR assay for HDV RNA, we used as an internal standard, an RNA with a 133-nucleotide deletion relative to the wild-type sequence. Thus, with the internal primers, R and S, the length of the amplified DNA product should be 416 bases for unmodified HDV RNAs and 283 bases for the RNA standard with the internal deletion. As described below, to increase the sensitivity of the PCR and to allow direct quantitation, we used an end-labeled primer. In particular, we used T4 kinase to ^{32}P–end-label oligonucleotide R.

Reagents

Aurintricarboxylic acid (ATA; Sigma, St. Louis, MO; Cat. No. A-0885): 10 m*M*
Dithiothreitol (DTT): 200 m*M*
RTase buffer (10×): 100 m*M* Tris–HCl (pH 8.3), 60 m*M* $MgCl_2$, and 10 m*M* DTT
Annealing buffer (5×): 1.5 *M* NaCl, 50 m*M* Tris–HCl (pH 7.5), and 5 m*M* EDTA
PCR buffer (5×): 250 m*M* Tris–HCl (pH 8.5), 5 m*M* $MgCl_2$, 100 m*M* KCl, and 2.5 mg/ml bovine serum albumin (BSA)
Primers: 10 μM (see Fig. 1b for sequences)
TE (10:0.1) buffer: 10 m*M* Tris–HCl (pH 8.0) and 0.1 m*M* EDTA
TE (10:10) buffer: 10 m*M* Tris–HCl (pH 8.0) and 10 m*M* EDTA
TAE buffer (10×): 40 m*M* Tris–HCl (pH 8.0), 40 m*M* sodium acetate, and 10 m*M* EDTA
Kinase buffer (10×): 0.5 *M* Tris–HCl (pH 7.6), 0.1 *M* $MgCl_2$, 50 m*M* DTT, 1 m*M* spermidine, and 1 m*M* EDTA
T7 transcription buffer (5×): 0.2 *M* Tris–HCl (pH 8.0), 40 m*M* $MgCl_2$, 10 m*M* spermidine–$(HCl)_3$, and 125 m*M* NaCl

a



b

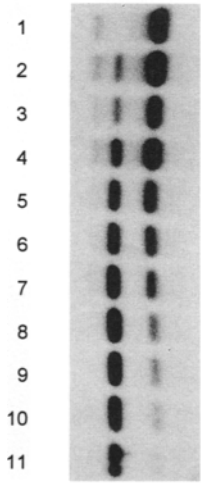
P 701 -AGCCTCCTCGCTGGCGCCGGCTGGGCAACATTCCG-735

Q 1217 -GAGGAACTCAGGAGGTTGACCGAGGAAGACGAGAG-1183

R 737 -GGGGACCGTCCCCTCGGTAATGGCGAATGGGACCC-771

S 1152 -TGGGGGTGTGAACCCCCTCGAAGGTGGATCGAGGG-1118

c



	Ratio [Unknown] / [Internal standard]	
	Predicted	Observed
1	0	< 0.01
2	0.06	0.13
3	0.13	0.27
4	0.25	0.42
5	0.5	0.88
6	1	1.33
7	2	2.46
8	4	3.83
9	8	4.73
10	16	7.10
11	32	13.92

FIG. 1 A quantitative nested RNA–PCR for the detection of HDV RNAs. (a) A representation of the region of the HDV genome that was amplified on both the normal genome, the "unknown," and a genome with a specific deletion, the "internal standard." The notations are from the nucleotide sequence of Kuo *et al.* (11) and indicate the outsides for the four DNA primers P–S, the asterisk indicating that primer R was 5′-labeled. (b) The sequence, again from Kuo *et al.* (11), of the four 35-base primers. (c) An autoradiogram of a denaturing-gel analysis of nested RNA–PCR reactions. For the initial reverse transcription each reaction contained 1 ng of the internal standard and 0–32 ng of the unknown, as indicated by the predicted value of the ratio of the unknown to the internal standard. Quantitation of the two bands on the gel with a Radioanalytic Imaging System allowed calculation of the ratio observed.

SP6 transcription buffer (5×): 0.2 *M* Tris–HCl (pH 7.9), 30 m*M* $MgCl_2$, and 10 m*M* spermidine–$(HCl)_3$
rNTP mix (5×): 2.5 m*M* (each) rATP, rCTP, rGTP, and rUTP
RTase–dNTP mix (10×): 10 m*M* (each) dATP, dCTP, dGTP, and dTTP
PCR–dNTP mix (25×): 20 m*M* $MgCl_2$ plus 5 m*M* (each) dATP, dCTP, dGTP, and dTTP
Self-digested Pronase (Sigma; Cat. No. P-5147): 10 mg/ml in 50 m*M* Tris–HCl (pH 7.4), preincubated for 2 hr at 37° C, then stored as aliquots at −20° C
Dextran (Sigma; Cat. No. D-4133): 10 mg/ml

Protocol

Internal RNA Standard

Specifically deleted forms of HDV RNA were used as internal standards in PCR assays of HDV RNAs. A region of the pGem4Z construct, pGem4Z(D1) (11), in the polylinker region between *Bam*HI and *Hind*III, was first deleted. A deletion was then made in the HDV sequences between *Bst*I and *Sal*I restriction enzyme sites; that is, the DNA was first cleaved with *Bst*I and then blunt-ended with T4 DNA polymerase (12). The DNA was then digested with *Sal*I and filled in using the Klenow fragment of *Escherichia coli* DNA polymerase I (GIBCO–BRL, Gaithersburg, MD). The blunt-ended linear DNA was incubated with T4 ligase (GIBCO–BRL) to obtain the construct designated pGem4Z(D1ΔBS). The resultant deletion removed 133 bases from subsequent HDV transcripts copied from this DNA. To make an antigenomic standard RNA, the DNA was cleaved with *Pvu*II and then copied with phage T7 RNA polymerase.

Plasmid DNA cleaved with *Pvu*II (1 μg)	x μl
T7 transcription buffer (5×)	10 μl
rNTP mix (5×)	10 μl
DTT (200 m*M*)	5 μl
RNasin (40 U/μl)	2 μl
Sterile water	$22 - x$ μl
T7 RNA polymerase	1 μl
	50 μl

Incubate for 1 hr at 37° C, then add 2 μl of RNase-free DNase I (1 U/μl; Promega, Madison, WI) and incubate for an additional 30 min. As described fully in the following section, extract once with phenol and then twice with ether. Layer directly onto a 1% agarose gel containing TAE buffer (1×) supplemented with 0.1% sodium dodecyl sulfate (SDS) and 10 μ*M* ATA. Localize via a parallel standard of labeled RNA, and then collect by electroelution in TAE buffer (0.25×) followed by precipitation

with ethanol in the presence of salt and dextran carrier. Determine the recovery of RNA by optical density measurement.

Similarly, to make a genomic standard, take the plasmid cleaved with *Pvu*II and transcribe it with phage SP6 RNA polymerase.

RNA Preparation

Extract RNA samples using a prior digestion in 0.4 ml of TE (10:10) buffer with SDS (0.1%) and Pronase (1 mg/ml) for 40 min at 37°C. Then extract once with an equal volume of phenol (predistilled and then equilibrated with an equal volume of 50 m*M* Tris–base and 0.1% hydroxyquinoline). Remove the aqueous phase and extract twice with 1 ml of ether. Precipitate the RNA by the addition of 0.1 vol of 3 m sodium acetate, 1 μl of dextran (10 mg/ml), and 2 vol of 95% ethanol.

Premix an aliquot of RNA sample, containing an unknown amount of HDV RNA (genomic or antigenomic; e.g., 0.1–10 ng), with an aliquot containing the internal standard (e.g., 1 ng).

5′ End-Labeling of Primer

For the second PCR primer R was end-labeled with ^{32}P.

Primer solution R	20 μl
Kinase buffer (10×)	5 μl
[γ-^{32}P]ATP (6000 Ci/mmol; DuPont, Boston, MA)	5 μl
Sterile water	18 μl
T4 kinase (GIBCO–BRL)	2 μl
	50 μl

Incubate for 30 min at 37°C and then apply directly to a prepared Sephadex G-25 Mini-Spin column (Worthington, Freehold, NJ; Cat. No. LS04410). Use the flow-through directly as a component of the second PCR. (A similar end-labeling of primer S is used in the RNA editing assay described in the section on RNA editing.

Primer Annealing

Combine the following in a 1.5-ml Eppendorf tube.

RNAs (assay for genomic *or* antigenomic)	x μl
Primer solution (Q *or* P)	1 μl
Annealing buffer (5×)	2 μl
Sterile water	$10 - x$ μl
	10 μl

Denature for 4 min at 95°C, then incubate for 30 min at 55°C, followed by a brief centrifugation.

Reverse Transcription

To the above primer annealing mix, add

RTase buffer (10×)	2 μl
RTase–dNTP mix (10×)	2 μl
RNasin (40 U/μl, Promega)	0.5 μl
Sterile water	5.5 μl
AMV RTase (20 U/μl; Life Sciences, St. Petersburg, FL)	0.5 μl
	20 μl

Incubate for 1 hr at 37° C, then extract as described earlier, followed by precipitation with ethanol in the presence of dextran. Resuspend in 10 μl of sterile water.

First PCR

Distribute aliquots of the following master mixture.

PCR buffer (5×)	2 μl
Primer solution P	1 μl
Primer solution Q	1 μl
PCR–dNTP mix (25x)	0.4 μl
TE (10:0.1) buffer	4.4 μl
Vent polymerase [2 U/μl; New England Biolabs (NEB)]	0.2 μl
	9 μl

For each sample add 1 μl of RTase product to the 9 μl, mix, and then heat-seal in a graduated microdispenser tube (Drummond, Broomall, PA; Cat. No. 105G). The PCR reactions (denaturation for 5 sec at 94° C, annealing for 5 sec at 55° C, and elongation for 15 sec at 72° C) are carried out using an Air Thermo-Cycler (Idaho Technologies, Idaho Falls, ID; Cat. No. 1605). The design of this device is such that 35 cycles are completed in less than 30 min.

Second PCR

Distribute aliquots of the following master mixture.

PCR buffer (5×)	2 μl
Primer solution R, end labeled	1 μl
Primer solution S	1 μl
PCR–dNTP mix (25x)	0.4 μl
TE (10:0.1) buffer	4.4 μl
Vent polymerase (2 U/μl; NEB)	0.2 μl
	9 μl

Add 1 μl of the product of the first PCR. Heat-seal and amplify as described for the first PCR.

Analysis of PCR Products

Take the labeled PCR products, extract, and denature with glyoxal prior to analysis on a gel of 2% agarose (2) to resolve the 416- and 283-base DNA products. Such denaturing conditions are sometimes necessary to remove mixed hybrids between full-length and deleted PCR products (6). This heteroduplex formation can arise because of suboptimal PCR conditions during the amplification of the unknown and related internal standard; specifically, if complimentary strands of the PCR products hybridize with each other rather than with the provided PCR primers, they will not undergo DNA-directed DNA synthesis. Such suboptimal conditions can arise for many different reasons, such as depletion of a primer or inactivation of the polymerase.

If the PCR conditions are optimal, such heteroduplexes are, by definition, not a problem and the products can be examined on a native rather than a denaturing gel.

After electrophoresis the gel is dried on DE-81 paper (Whatman, Clifton, NJ) and subjected to autoradiography and/or direct quantitation with a Radioanalytic Imaging System (AMBIS, San Diego, CA).

Comments and Applications

Strand Specificity

Depending on the choice of primer used in the RTase reaction, this assay can quantitate either genomic or antigenomic HDV RNA, using the corresponding strand of the internal standard. HDV RNAs are known to be about 70% self-complementary. Thus, one might expect that in an assay for one strand, say, antigenomic RNA, there might be false-positive signals if an excess of the genomic RNA were present. However, reconstruction experiments indicated that in the presence of even a 10,000-fold excess of genomic RNA relative to internal antigenomic RNA standard, there was no detectable false-positive signal.

The assay for antigenomic RNA spans the site at nucleotide 900/901 where self-cleavage can occur (2). The assay detects molecules that have never self-cleaved, and of course, those that had both self-cleaved and ligated.

Calibration

Figure 1c shows a reconstruction experiment in which we assayed for antigenomic RNA. A range of amounts of the "unknown" were quantitated in the presence of a fixed amount of internal standard. A comparison was made of the predicted ratios of the unknown to the internal standard, based on direct quantitation of the gel analysis

of the products. As can be seen in Fig. 1c, the two values were in closer agreement around the regions of equivalence. We would estimate that the assay is quantitative for unknown samples at least within a factor of 10-fold more to 10-fold less, relative to the internal standard. In other words, the dynamic range for a single assay is at least 100-fold. Of course, by carrying out the assay with different amounts of the internal standard, one can quantitate samples over a much larger total range.

The lower limits for the assay have been determined. In 8 hr of autoradiography, we can readily detect the radioactive signal produced from 100 molecules of internal standard (10^{-7} ng). Also, in a 10-day exposure we can detect 10–30 molecules.

Applications

This assay has been used to quantitate HDV RNAs (*a*) in serum samples in which the amount was less than that detectable by the slot analysis, (*b*) in tissues of animals with low-level HDV infections, and (*c*) in HDV RNA-directed RNA synthesis by nuclear extracts (13) and by purified transcription factors.

RNA Editing Assay Utilizing an Immobilized PCR Product

During the replication of HDV RNA inside an infected cell, an unusual posttranscriptional RNA editing occurs. A specific nucleotide change at position 1012 converts the amber termination codon UAG, for the small form of the delta antigen, to the tryptophan codon UGG, which leads to the translation of the longer form of the delta antigen (4, 13, 14). As cited in the introduction, this increase from 195 to 214 amino acids changes the properties of the delta antigen in ways that are critical for the life cycle of HDV. At this time the mechanism of this editing reaction remains to be clarified. Simple and sensitive assays are needed to quantitate the change and ultimately to determine its molecular basis.

If the RNA molecules undergo editing, RNA-directed RNA synthesis, and then translation, one can, of course, use a Western blot analysis to detect the altered form of the delta antigen (15). The sensitivity of this assay is tied to the resolving power and the detection limits of the Western blot procedure. However, in some studies of RNA editing, there is no associated translation. For example, with nonreplicating RNAs or with RNAs edited in cell-free extracts, only the full-length HDV is changed but no mRNA is made (16). Thus, it is necessary to develop sensitive, quantitative, and convenient assays for the editing. Nucleotide sequencing is not a sensitive strategy for detecting a variant sequence in the presence of a large excess of unaltered sequence; others have claimed to detect as little as 10% of a single variant sequence in the presence of 90% unaltered sequence (17). Of course, prior cDNA cloning and sequencing or screening could be used, but the sensitivity would be limited by the number of clones screened; for example, one would need to screen at least 100 clones to detect 1% editing (4). Workers in the HDV field, like those working with other

RNA-editing reactions, have developed procedures that have various combinations of sensitivity, quantitation, and convenience. As described here, we have improved on an RNA–PCR procedure, previously detailed both by us (16) and by Casey *et al.* (14), that assays for editing via the appearance of a novel restriction enzyme site. The current method will readily detect as little as 0.1% of edited RNA. The novel site detected is that for *Nco*I; editing of U to C at position 1012 on the genomic RNA sequence (11) converts the cDNA sequence from CTATGG to CCATGG, which now corresponds to a site for *Nco*I.

In terms of the quantitative nested RNA–PCR discussed earlier, it was pointed out that under suboptimal PCR conditions heteroduplex double-stranded DNAs can accumulate. In the present editing assay such heteroduplexes pose a potentially serious problem; such a heteroduplex, by definition, would not contain the indicator restriction enzyme site. However, as described here, we have devised an assay strategy that solves this problem. In brief, by using 5′-biotin on one of the primers in the PCR, it is possible to specifically immobilize one strand of the product and then, using a 5′-labeled primer and RTase, convert it to double-stranded DNA, which, by definition, is totally free of heteroduplex.

Reagents

Biotin was added to the 5′-end of oligonucleotide R, as the last step of chemical synthesis in an Applied Biosystems DNA synthesizer (Foster City, CA). The reagent used was Biotin-On from Clonetech (Palo Alto, CA).

Superparamagnetic beads, designated as Dynabeads M-280 Streptavidin, were obtained from Dynal (Great Neck, NY)

Binding and washing (B&W) buffer (2×) was prepared as recommended by Dynal (18): 10 m*M* Tris–HCl (pH 7.5), 1 m*M* EDTA, and 2 *M* NaCl.

*Sal*I buffer (10×): 100 m*M* Tris–HCl (pH 7.9), 100 m*M* $MgCl_2$, 1.5 *M* NaCl, 10 m*M* DTT, and 1 mg/ml BSA

*Nco*I buffer (10×): 200 m*M* Tris–acetate (pH 7.9), 100 m*M* magnesium acetate, 500 m*M* potassium acetate, and 10 m*M* DTT

TE (10:1) buffer: 10 m*M* Tris–HCl (pH 7.5) plus 1 m*M* EDTA

PBS buffer: 0.14 *M* NaCl, 2.7 m*M* KCl, 44 m*M* KH_2PO_4, and 10 m*M* Na_2HPO_4 (pH 7.4)

Protocol

Nested RNA–PCR

The sample containing HDV RNA is first subjected to nested RNA–PCR as indicated in Fig. 2a. The procedure is essentially as described earlier in the section on nested

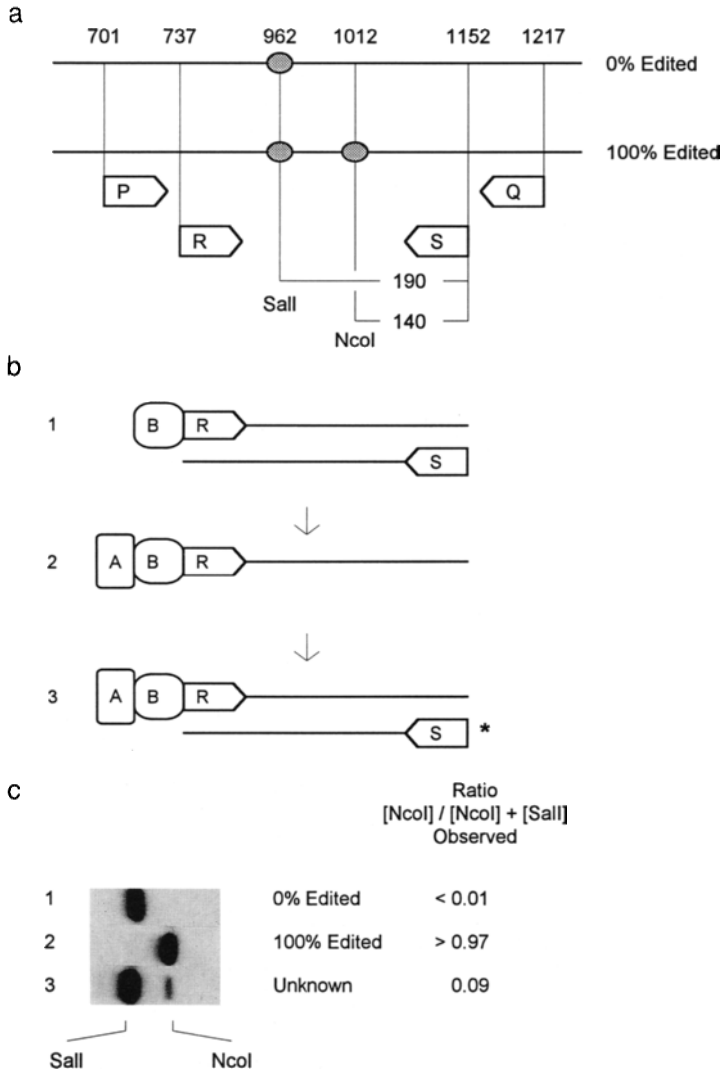


FIG. 2 A nested RNA–PCR approach to quantitate RNA editing on the genome of HDV RNA. (a) The four primers used in relation to the site of editing at position 1012, and the two restriction enzyme sites used, *Sal*I and *Nco*I, as indicated by the shaded circles. The sequence of the four primers are as in Fig. 1a. (b) A summary of the three steps subsequent to PCR. Step 1 begins with the double-stranded PCR product as made with primers R and S. Primer R has a 5′-biotin (B). In step 2 this PCR product is immobilized on the avidin-coated paramagnetic beads (A). Then, by treatment with alkali, one of the DNA strands is specifically removed. In step 3 primer S, with a 5′ ^{32}P label, is hybridized to the immobilized DNA and then extended in a DNA synthesis reaction using RTase. After this the immobilized product is digested with *Sal*I and *Nco*I to release end-labeled double-stranded DNA fragments, ready for separation by gel electrophoresis. (c) The results of such electrophoresis for three specific examples of the editing.

PCR, except that the internal standard is not used. Also, in the second PCR, primer S is not labeled, and primer R is biotin labeled.

Biotin Affinity

1. For each PCR sample transfer 5 μl of Dynalbeads into a 1.5-ml Eppendorf tube.
2. Wash with 10 μl of PBS containing 0.1% BSA. Use the Dynal magnet (MPC-E) to attract beads to the side wall, then carefully remove the supernatant.
3. Resuspend in 10 μl of B&W (2x) buffer, then add 10 μl of PCR product.
4. Incubate for 1 hr (at room temperature), with constant rocking to keep the beads in suspension.
5. Use the magnet to attract the beads, remove the supernatant, and wash with 40 μl of B&W buffer.

Alkali Denaturation

1. Wash the beads with 40 μl of sterile water.
2. Resuspend in 20 μl of 0.1 N NaOH and incubate for 10 min.
3. Use the magnet to attract the beads, and remove the supernatant.
4. Wash with 40 μl of 0.1 N NaOH, then with 40 μl of B&W (1×) buffer, and finally with 40 μl of TE (10:1) buffer.

Primer Annealing to Immobilized DNA

1. Remove the supernatant.
2. Resuspend in 20 μl of annealing buffer (1×) and incubate for 10 min.
3. Remove the annealing buffer and add the following.

Annealing buffer (5×)	2 μl
^{32}P–end-labeled primer S	1 μl
Sterile water	7 μl
	10 μl

4. Incubate for 1 hr at 50° C.
5. Remove the supernatant and wash twice with 40 μl of B&W (1×) buffer.

Elongation with RTase

1. Remove the supernatant.
2. Resuspend in 20 μl of RTase buffer (1×) and incubate for 10 min.
3. Remove the RTase buffer and add the following.

RTase buffer (10×)	2 μl
RTase–dNTP mix (10×)	2 μl
Murine leukemia virus RTase (200 U/μl)	0.5 μl

Sterile water	15.5 μl
	20 μl

4. Incubate for 1 hr at 37° C.
5. Remove the supernatant and wash the beads with 40 μl of B&W (1×) buffer.

First Digestion of Immobilized DNA

1. Remove the supernatant.
2. Resuspend in 20 μl of *Sal*I buffer (1×) and incubate for 10 min.
3. Remove the *Sal*I buffer and add the following.

*Sal*I buffer (10×)	2 μl
*Sal*I (10 U/μl)	0.5 μl
Sterile water	17.5 μl
	20 μl

4. Incubate for 2 hr at 37° C.
5. Attract the beads and collect the supernatant.

Second Digestion of Immobilized DNA

Repeat as above, but use *Nco*I buffers and enzyme.

Analysis of Products

Directly analyze the combined products released from the beads by electrophoresis using a gel of 12% polyacrylamide in TBE buffer (0.1 *M* Tris–HCl, 90 m*M* boric acid, and 2 m*M* EDTA. (This gel procedure gives optimum resolution of the two DNA cleavage products). Dry the gel on DE-81 paper. [3MM paper (Whatman) is not appropriate, in that small DNA species pass through the paper during the vacuum-drying process.) Directly quantitate the dried gel using a Radioanalytic Imaging System or Phospho-Imager (Fuji). Typically, an adequate sensitivity of 0.1% is readily achieved in a 5-hr scan. Greater sensitivity could be obtained using a longer scan time or by a combination of autoradiographic exposures. Figure 2c gives some examples.

Comments and Applications

Technical Comments

1. It has been noted that when a supernatant is removed from the beads, it can be contaminated by relatively small amounts of the total beads. If this is a problem, it can be readily solved by submitting the supernatant to an additional magnetic separation.

2. If the extent of completeness of a digest with restriction enzyme is in doubt, an unlabeled internal standard can be added. Then, after the electrophoresis, the digestion can be monitored by visualization of the ethidium bromide-stained gel.

3. We have noted two potential problems of steric hindrance associated with DNA bound to the beads. First, activity of the restriction enzyme *Xba*I, which cuts double-stranded product at position 781 (only 44 bp from the biotin site, and hence relatively close to the bead), is partially inhibited. Second, elongation by RTase of primer S hybridized to single-stranded DNA bound to the bead is likewise impeded in the region of the *Xba*I site close to the 5′-end of the template, and hence close to the bead. These two problems can be solved by different strategies, one of which is simply to change from *Xba*I to a restriction enzyme such as *Sal*I, which cuts at a greater distance from the bead, actually 225 bases away.

4. It should be possible to digest simultaneously with two restriction enzymes in a mutually compatible buffer, and thereby reduce the time and manipulations involved as well as the possible errors in quantitation. However, there are unique advantages to consecutive digests. If the enzymes really do have different optimal buffers, as they do in this method, then consecutive digestions are very easy when using immobilized substrates.

Applications

We have described here a strategy of using immobilized RNA–PCR products in order to assay RNA editing. The immobilization step makes it very convenient to change from one reaction to another. The beads are simply prewashed in the next buffer to be used (e.g., restriction enzyme buffer prior to a restriction enzyme digest). The immobilization allows not only such changing of reaction buffers but also the removal of unused components, such as radioactive primer.

Of course, the concept of immobilized products can also be applied for other purposes. We and others have found that bidirectional dideoxy-sequencing is readily achieved (18, 19). Alkali is used to specifically elute one DNA strand, while the other strand is maintained on the beads. These two strands can now be separately sequenced via the dideoxy-sequencing procedure. Another convenient application has been in the preparation of inserts as part of oligonucleotide-directed PCR for mutagenesis and cloning. The PCR product, with one primer biotinylated, is prepared, bound to beads, cut once at a distal site with a unique restriction enzyme, and washed, then the insert is released by cutting at a proximal site by a second unique restriction enzyme. The insert released in this way is ready for use in forced cloning.

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References

1. K.-S. Wang, Q.-L. Choo, A. J. Weiner, J.-H. Ou, C. Najarian, R. M. Thayer, G. T. Mullenbach, K. J. Denniston, J. L. Gerin, and M. Houghton, *Nature (London)* **323,** 508 (1986).
2. L. Sharmeen, M. Y.-P. Kuo, G. Dinter-Gottlieb, and J. Taylor, *J. Virol.* **62,** 2674 (1988).
3. L. Sharmeen, M. Y. Kuo, and J. Taylor, *J. Virol.* **63,** 1428 (1989).
4. G. Luo, M. Chao, S.-Y. Hsieh, C. Sureau, K. Nishikura, and J. Taylor, *J. Virol.* **62,** 1855 (1990).
5. D. W. Lazinski and J. M. Taylor, *Adv. Virus Res.* **43,** 187 (1994).
6. G. Gilliland, S. Perrin, and H. F. Burn, *in* "PCR Protocols" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 60. Academic Press, San Diego, 1990.
7. G. Gilliland, S. Perrin, K. Blanchard, and H. F. Bunn, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2725 (1990).
8. T. Jalava, P. Lehtovaara, A. Kallio, M. Ranki, and H. Söderlund, *BioTechniques* **15,** 134 (1993).
9. M. Becker-André and K. Hahlbrock, *Nucleic Acids Res.* **17,** 9437 (1989).
10. P. D. Siebert and J. W. Larrick, *Nature (London)* **359,** 557 (1992).
11. Y.-P. Kuo, L. Goldberg, L. Coates, W. Mason, J. Gerin, and J. Taylor, *J. Virol.* **62,** 1855 (1988).
12. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
13. T.-B. Fu and J. Taylor, *J. Virol.* **67,** 6965 (1993).
14. J. L. Casey, K. F. Bergmann, T. L. Brown, and J. L. Gerin, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7149 (1992).
15. M. Chao, S.-Y. Hsieh, and J. Taylor, *J. Virol.* **65,** 4057 (1991).
16. H. Zheng, T.-B. Fu, D. Lazinski, and J. Taylor, *J. Virol.* **66,** 4693 (1992).
17. T. Leitner, E. Halapi, G. Scarlatti, P. Rossi, J. Albert, E.-M. Fenyö, and M. Uhlén, *BioTechniques* **15,** 120 (1993).
18. "Dynal Technical Handbook: Dynabeads® Magnetic Separation System." Dynal AS, Norway, 1993.
19. H. J. Netter, T.-T. Wu, M. Bockol, A. Cywinski, W.-S. Ryu, B. C. Tennant, and J. M. Taylor, *J. Virol.* **69,** 1687 (1995).

[21] Systems to Express Recombinant RNA Molecules by the Influenza A Virus Polymerase *in Vivo*

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Introduction

Viruses are obligate intracellular parasites that depend on cellular functions for their reproductive cycle. Two of the critical processes for viral reproduction are replication and transcription of the viral genome. The strategies developed by RNA viruses that require an RNA-dependent RNA polymerase activity to replicate and express their genomes are particularly interesting since such an activity has not been detected in the rest of the living organisms and therefore it has to be encoded for by the viral genome. In this article we focus on recently described methodologies to study the strategy developed by influenza A virus to express and replicate its genome. We briefly review what is known about the replication and transcription processes of the influenza virus genome and describe detailed procedures to (*a*) express synthetic RNA molecules in influenza virus-infected cells and (*b*) express functional polymerase *in vivo* from cloned cDNAs.

Influenza Viruses

Influenza A viruses are members of the Orthomyxoviridae family and contain a genome consisting of eight single-stranded RNA segments of negative polarity. The RNA segments are associated with four virus-specific proteins, the nucleoprotein (NP) and the three P proteins (PB1, PB2, and PA), to form ribonucleoprotein (RNP) complexes and contain conserved regions of 12 and 13 nucleotides at the 3 and 5′ termini, respectively (reviewed in Refs. 1 and 2).

On viral entry, the viral genome (vRNPs) is released into the cytoplasm and transported to the cell nucleus, where RNA replication and transcription take place (3). The incoming RNPs are copied by the viral transcriptase to yield monocistronic mRNAs (+ strand) (primary transcription) that contain a type 1 cap structure followed by 10–15 nucleotides derived from cellular pre-mRNA at their 5′ ends and a poly(A) tail at their 3′ ends. These poly(A) tails appear to be acquired by reiterative copying of a stretch of five to seven uridine residues starting 15–17 nucleotides from the 5′ end of vRNA. Newly synthesized proteins from these mRNAs direct the replication of the viral genome. For replication to occur, the genomic vRNPs (− strand)

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are transcribed into full-length complementary cRNPs (+ strand), which, in turn, serve as templates for the synthesis of new genomic vRNPs. These vRNPs are then transcribed into mRNA molecules (secondary transcription) and ultimately will be packaged into progeny virus (reviewed in Ref. 4).

Influenza virus transcriptase activity has been detected in disrupted virions (5) and in extracts prepared from infected cells (6, 7). RNP complexes exhibit mRNA synthesis activity and are infectious when transfected into susceptible cells (8, 9). However, naked genomic RNA is not infectious, since the host cell lacks the RNA-dependent RNA polymerase activity required to copy the viral genome into messenger sense (+ strand) RNA molecules.

Biochemical and genetic studies (reviewed in Ref. 10) have demonstrated that the three P proteins form a complex that exhibits the viral RNA-dependent RNA polymerase activity. It has been shown that the PB2 protein binds the cap 1 structure of host cellular mRNAs used as primer for influenza transcription and that PB1 polypeptide is involved in the elongation of the growing mRNA chain. The PA protein appears to be associated and to move together with the other two P proteins during mRNA synthesis (11). However, no role in transcription has yet been assigned to this polypeptide. Analysis of temperature-sensitive (*ts*) mutants indicates that both PB1 and PA proteins are involved in vRNA synthesis (reviewed in Ref. 10) but the specific functions of the P proteins for the synthesis of cRNA and vRNA molecules remain to be determined. The P proteins have been shown to form highly stable complexes (12) that can be isolated in association with genomic RNAs after removing the NP from the RNP complexes (13). The functions of NP during virus-specific RNA synthesis are unclear. It is known that NP interacts with cRNA and vRNA molecules and plays a major structural role in maintaining the RNP structure. In addition, the NP protein is required for RNA replication since *ts* mutants in the *NP* gene show defects in RNA synthesis (reviewed in Ref. 10), antibodies to the NP protein inhibit the transcriptase activity associated with disrupted virions (14), and nucleocapsids in which most of the NP has been removed are unable to synthesize template-sized RNA transcripts (13). Furthermore, it has been shown that the NP is required for full-length synthesis of cRNA and vRNA molecules (4).

Until very recently, a detailed characterization of the *cis*-acting elements and *trans*-acting factors involved in the replication and transcription processes of the viral genome has been hampered by the inability to reconstitute functional RNP complexes from synthetic RNAs and the lack of systems in which to test the functionality of cDNA copies encoding the viral polymerase functions. In 1989 Parvin and coworkers (15) described a system to reconstitute functional RNP complexes using RNAs obtained from cloned genes and RNA-free protein preparations (which contained NP and the three P proteins) isolated from purified influenza virions. In this way it was possible to express and package synthetic genes encoding nonviral gene products (16) and to exchange influenza virus segments for genes made *in vitro* from recombinant plasmids (reviewed in Ref. 17). On the other hand, Seong and Brownlee

(18) developed an alternative protocol to obtain active viral polymerase using purified RNP complexes that had been treated with micrococcal nuclease to remove the genomic RNA. Studies on *in vitro*-reconstituted RNPs have demonstrated that the sequences conserved at the 3′ ends of cRNA and vRNA molecules are the minimum promoters for virus-specific RNA synthesis (15, 18–21).

In Vitro Reconstitution of Active Viral RNPs

In this section we describe the detailed experimental procedures to obtain active nucleoprotein polymerase (NP-Ps) preparations from influenza virus-infected cell extracts instead of from purified virus, as described by others. These NP-Ps preparations are able to reconstitute active RNP complexes *in vitro* and to rescue an artificial virionlike RNA into infectious virus when complemented in *trans* with influenza helper virus (22). The protocol includes several steps, which are diagrammed in Fig. 1 and described below in detail.

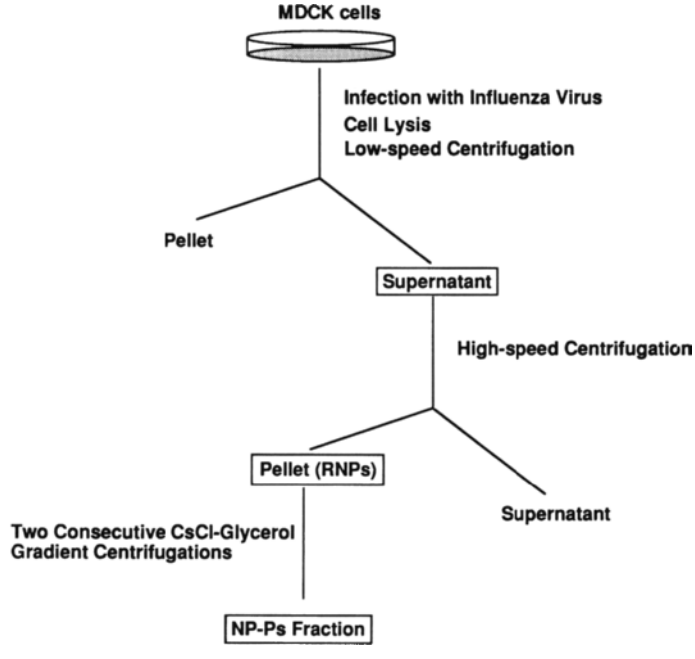

FIG. 1 Diagram of the procedure to isolate active nucleoprotein polymerase preparations. The main steps in the isolation of active nucleoprotein polymerase preparations are shown, starting from the infection of MDCK cells. See text for further details.

Preparation of Active NP-Ps Fractions

Influenza Virus Infection

Madin–Darby canine kidney (MDCK) cells are grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS). The cells are seeded in seven petri dishes (150-mm diameter) and incubated until they reach confluence. At this point the medium is removed and the cells are washed with DMEM (without FCS) and inoculated with an aliquot (2 ml per dish) of influenza virus A/PR8/34 [1.5×10^7 plaque-forming units (pfu)/ml in DMEM; multiplicity of infection (MOI), 1]. Dishes are kept for 1 hr at room temperature, with rocking every 15–20 min to allow virus adsorption. The virus inoculum is then aspirated, 20 ml of DMEM is added to each dish, and the cell cultures are incubated in a CO_2 incubator at 37° C for 18–20 hr.

Preparation of the Cell Extract

Special precautions should be taken in this and the rest of the protocols described in this article to avoid the action of nucleases. In particular, gloves should be always worn, every material should be sterilized, and every reagent should be prepared under RNase-free conditions, using diethyl pyrocarbonate (DEPC)-treated distilled water. The procedure to prepare cell lysates is a modification of that described by Dignam *et al.* (23). The cells are washed three times with ice-cold phosphate-buffered saline (PBS) solution, scraped off the dishes with a rubber policeman, and harvested by low-speed centrifugation at 3000 rpm in a bench centrifuge for 5 min at 4° C. The supernatant is aspirated and 15 ml of hypotonic buffer [10 m*M* HEPES (pH 7.5), 1.5 m*M* $MgCl_2$, and 10 m*M* KCl] is added. The cells are centrifuged again, resuspended in 10 ml of the same buffer, and transferred to a glass Dounce homogenizer. The cells are lysed by applying 20 strokes with a pestle and then examined under the microscope to check for cell lysis. The homogenate is then transferred to a 15-ml Corex tube and centrifuged at 10,000 rpm in an SS-34 rotor for 10 min at 4° C to remove nuclei and cell debris.

Sedimentation of the RNP Complexes

The supernatant resulting from the previous centrifugation step is transferred to two 5-ml tubes and centrifuged at 45,000 rpm in an SW50.1 rotor (Beckman, Palo Alto, CA) for 4 hr at 4° C. The supernatant is aspirated with a Pasteur pipette, leaving 100–200 μl of liquid at the bottom, which is removed by inverting the tube. The pellet, which contains the RNP complexes, is first resuspended in the liquid remaining, using a Pasteur pipette with a blunted tip. Then, 300 μl of RNP resuspension buffer [40 m*M* HEPES (pH 7.5), 200 m*M* KCl, 8.7% (v/v) glycerol, and 1 m*M* dithiothreitol (DTT)] is added and resuspension is continued. This latter step is repeated three or four times until a final volume of 1.2 ml is reached. Resuspension can be assisted by passing the suspension through a 25-gauge needle, using a syringe.

Dissociation of RNP Complexes

The RNP suspension is loaded onto a CsCl–glycerol gradient that contains four layers [buffered with 20 m*M* HEPES (pH 7.5)]: 1 ml of 3 *M* CsCl–43.5% (v/v) glycerol, 1 ml of 2 *M* CsCl–34.8% (v/v) glycerol, 1 ml of 1 *M* CsCl–26.1% (v/v) glycerol, and 1 ml of 0.5 *M* CsCl–17.5% (v/v) glycerol. The sample is centrifuged at 35,000 rpm in an SW50.1 rotor for 24 hr at 4° C (the centrifuge is set to slow acceleration and deceleration rates to avoid disturbing the gradient). Fractions of 500 μl are collected from the top, and a sample of 10 μl of each fraction is analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomasie blue staining. The upper fractions of the gradient contain most of the cellular and viral proteins, whereas the middle to bottom fractions should contain the NP and P proteins. The fraction(s) with the highest content of NP (around fraction 7) is then diluted with 2 vol of RNP dilution buffer [40 m*M* HEPES (pH 7.5), 200 m*M* KCl, 1 m*M* DTT, and 8.7% (v/v) glycerol] and centrifuged again through a second CsCl–glycerol gradient as described above. Fractions collected from the gradient are analyzed by SDS–PAGE, and those containing most of the NP protein are pooled and dialyzed at 4° C against buffer I [50 m*M* Tris (pH 7.5), 100 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, and 10% (v/v) glycerol] for 2 hr and against buffer II [50 m*M* Tris (pH 7.5), 100 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT, and 50% (v/v) glycerol] for an additional 2 hr. The preparation is distributed in small (10- to 30-μl) aliquots and kept frozen at −80° C. Frozen aliquots are stable for several months. Typically, 200–500 μl of dialyzed NP-Ps fraction is recovered, containing 0.2–1 μg/μl of NP protein.

Preparation of the Influenza Virus-like RNA Template

To reconstitute biologically active functional RNP complexes, we have used RNA templates derived from plasmid pPB2CAT9 (courtesy of Mark Krystal, Brystol-Myers-Squibb). This plasmid, which mirrors pIVACAT1 (16), contains, in 5′-to-3′ order, the T7 RNA polymerase promoter, a negative polarity copy of the 5′ noncoding region of the influenza virus PB2 segment, the chloramphenicol acetyltransferase (*CAT*) gene and the corresponding 3′ noncoding region, and a linker including a restriction site for enzyme *Hga*I. To prepare the RNA template, the plasmid is digested with *Hga*I and treated with *Escherichia coli* DNA polymerase I (Klenow fragment) to fill in the protruding ends. The digested plasmid is then transcribed *in vitro* with T7 RNA polymerase to yield − sense RNA molecules containing the 5′ and 3′ untranslated sequences of the viral segment encoding the PB2 protein flanking the bacterial gene encoding CAT enzyme. This RNA template will only be expressed to yield CAT enzyme if it is transfected into cells expressing an RNA-dependent RNA polymerase activity.

More than 100 μg of the CAT–RNA template can be obtained from 2 μg of *Hga*I-digested plasmid using the MEGAscript transcription kit (AMBION, Austin, TX). Following transcription the reaction mixture is treated with RNase-free DNase (Pro-

mega, Madison, WI) to eliminate the template DNA and then filtered through a Sephadex G-50 (Pharmacia, Piscataway, NJ) column. We routinely include [^{3}H]GTP in the transcription reaction to quantitate the amount of RNA synthesized.

In Vivo Determination of the Biological Activity of RNP Complexes

In Vitro Reconstitution

It is convenient to carry out a preliminary experiment to test the efficiency of the NP-Ps preparation to reconstitute functional RNP complexes from synthetic RNAs. To this aim, cells (MDBK or COS-1) are grown in 35-mm petri dishes and infected with influenza virus at an MOI of 1 as described above. During viral adsorption several Eppendorf tubes are prepared containing 500 ng of pb2CAT RNA, 5 U of human placental RNase inhibitor (HPRI; Boehringer-Mannheim, Indianapolis, IN), different amounts of the NP-Ps fraction (1–10 μl), 10 μl of buffer III [200 m*M* Tris (pH 7.9), 30 m*M* $MgCl_2$, 50 m*M* DTT, and 10 m*M* spermidine], and sterile water up to a final volume of 50 μl. The mixtures are incubated for 15 min at 37° C and added to polystyrene tubes that contain 47 μl of DMEM and 3 μl of Lipofectin reagent (1 μg/μl; GIBCO–BRL, Gaithersburg, MD). This mixture is incubated for an additional 15 min at room temperature and added to the infected cell cultures containing 1 ml of DMEM (lacking FCS and antibiotics).

Alternatively, RNP complexes can be reconstituted by including the influenza virus polymerase proteins during the T7 transcription reaction. For this protocol a 50-μl mixture containing 500 ng of *Hga*I-digested plasmid, an aliquot of the active polymerase fraction, 10 μl of buffer III, 500 μ*M* NTPs, and 5 U of HPRI is prepared. After a 30-min incubation at 37° C, 1 U of RNase-free DNase is added and the incubation is continued for another 5 min. The mixture is then added to a tube containing Lipofectin and transfected as described in the previous paragraph. Finally, the infected/transfected cultures are incubated for 16–20 hr at 37° C in a CO_2 incubator.

Determination of CAT Activity

Expression of the *CAT* gene can be monitored by analyzing the transfected cell extracts for *CAT* activity as described in the next two subsections.

Preparation of Cell Extracts

Cell cultures are washed twice with 1 ml of TNE solution [100 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris–HCl (pH 8.0)], scraped off the dish with a rubber policeman and sedimented by centrifugation at 12,000 rpm in an Eppendorf tube for 1 min at 4° C. The supernatant is aspirated and the cells are resuspended in 100 μl of a 0.25 *M* Tris (pH 7.5) solution. The cells are lysed by three consecutive freezing (−80° C)–thaw-

ing (37° C) cycles, and the resulting homogenates are then centrifuged at 12,000 rpm for 5 min at 4° C. The supernatant is saved and stored at −20° C.

CAT Assay

An aliquot of the cell extract is incubated in a 150-μl mixture containing 0.25 *M* Tris (pH 7.5), 0.5 m*M* acetyl-CoA (Boehringer-Mannheim), and 0.025 μCi of [^{14}C]chloramphenicol (New England Nuclear, Boston, MA; Cat. No. NEC 408A). Following incubation at 37° C, 1 ml of ethyl acetate is added and the mixture is vortexed and centrifuged at 12,000 rpm for 1 min at room temperature. The top layer (ethyl acetate) is transferred to a new Eppendorf tube and dried in a Speedvac (SAVONT, Hicksville, NY) evaporator. The sample is resuspended in 20 μl of ethyl acetate and spotted (5 μl at a time) on a thin-layer chromatography (TLC) aluminum sheet (Merck, Darmstadt; Cat. No. 5554). The TLC is developed in a chromatographic tank (equilibrated for 2 hr before adding the thin layer) containing 200 ml of 19:1 chloroform–methanol. When the solvent is close to the middle of the TLC sheet, the sheet is removed, air-dried, and exposed for autoradiography. To determine the amount of radioactivity in the acetylated species, the film and the TLC sheet are aligned, and the corresponding areas of the sheet are cut and added to a scintillation vial containing scintillation fluid, and the radioactivity is counted. The amount of extract to be assayed can vary with different polymerase preparations. Routinely, a 10-fold dilution of the cellular extract incubated for 2 hr as indicated above gives a 5% conversion of chloramphenicol precursor to acetylated forms.

Reconstitution of Polymerase Activity from Cloned Genes Expressed in Mammalian Cells

The use of a helper virus infection to provide the polymerase and other accessory proteins for RNA synthesis precluded the study of *trans*-acting factors involved in the transcription and replication processes. The description of a completely artificial system in which defined vaccinia virus recombinants capable of expressing the viral core proteins substituted for the helper virus infection (24) constituted an important breakthrough in this direction. Thereafter, a number of alternative *in vivo*-reconstituted systems have been described (25–27). Using any of these systems, it has been concluded that the three polymerase subunits and the nucleoprotein make up the minimal requirements for viral RNA transcription and replication, although the involvement of some other virus factor in the processes has not been rigorously excluded. Two main differences became apparent when these reconstituted systems were compared with those that use influenza virus helper virus as a source for the required *trans* factors: on the one hand, naked virion-sense RNAs can be used to drive the systems, that is, can be packaged *in vivo* (25–27), and on the other hand, virion-sense RNAs with additional nucleotides at their 3′ end are recognized by the

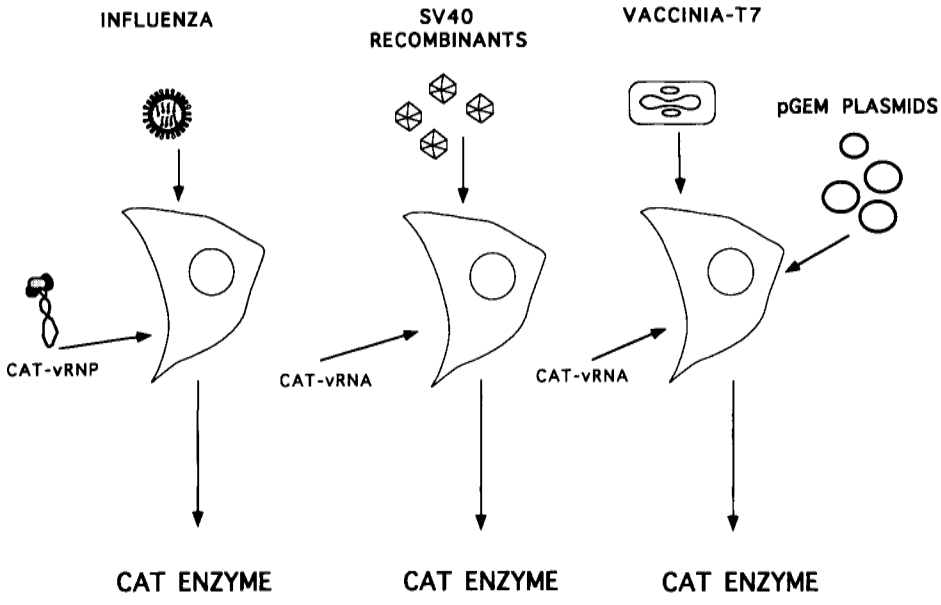

FIG. 2 Comparison of different *in vivo*-reconstituted transcription/replication systems. The scheme shows the three approaches described in the text to reconstitute influenza virus transcription/replication in living cells. Three ways to express the influenza virus *trans* factors are indicated: influenza virus helper (left), SV40 recombinant viruses (center), and vaccinia–T7 plus pGEM recombinant plasmids (right). The latter two recognize naked RNA transfected into the cells as template, but the former requires transfection of an RNP.

polymerase, albeit at reduced efficiency (27). Two of the reported systems rely on the expression of the required virus proteins by infection or transfection of recombinant viruses or plasmids (26, 27). These systems are therefore more amenable to the study of the virus *trans* factors than the other reported system, which is based on cell lines that express the full set of viral genes required (25). In this section the procedures used for the reconstitution of influenza virus transcription/replication systems based on simian virus 40 (SV40) recombinant viruses or on vaccinia–T7 virus (Fig. 2) are reviewed and updated.

SV40 Recombinant Virus System

Generation of the SV40 Recombinant Viruses

The double-stranded cDNAs of influenza virus were synthesized using purified virion RNAs as templates and oligodeoxynucleotides corresponding to the conserved terminal sequences. After selection of the complete cDNA segments by chromatog-

raphy on a Sepharose 4B-CL column, they were cloned into the filled-in *Bam*HI site of pUC18 plasmid (28). After sequencing of the appropriate inserts by the dideoxynucleotide method, they were subcloned into the pBSL intermediate vector to obtain the pSE plasmid series. Plasmid pBSL contains the SV40 genome late region, from the *Hin*dIII site (position 5171) to the *Bcl*I site (position 2770), cloned between the *Hin*dIII and *Bam*HI sites of Bluescript plasmid (Stratagene, La Jolla, CA). In this way, by removing the bacterial sequences of the pSE plasmid series by digestion with *Xba*I restriction nuclease, DNA molecules were obtained that contained pseudo-SV40 DNA in which the influenza virus genes substituted for the SV40 early region (29). On circularization by ligation at 2 μg/ml, the DNAs were transfected into COS-1 cell cultures. Routinely, 100 ng of ligation mixture is mixed with Lipofectin (GIBCO) at a 1:2 ratio and added to subconfluent COS-1 cell cultures covered with DMEM without FCS. After an overnight incubation the cultures are washed and fresh DMEM medium containing 5% (v/v) FCS is added. Usually, a cytopathic effect is observed by 5–7 days of incubation. The cells are collected and opened by freezing and thawing and the cellular debris is eliminated by low-speed centrifugation at 10,000 rpm for 10 min. The supernatant constituted the P0 stock and is used to prepare high-titer virus stocks by infection of fresh COS-1 cell cultures. Usually, P1 viruses are kept as reserve stocks and P2 and P3 are used as working stocks. Their titers are estimated by infection of COS-1 cells with the appropriate dilutions and immunofluorescence determination of the proportion of infected cells, using an anti-VP1 monoclonal antibody.

Reconstitution of the in Vivo Transcription/Replication System

Cultures of COS-1 cells are inoculated with a mixture of SV40 recombinant viruses expressing the three subunits of the polymerase (PB1, PB2, and PA) and the NP at an MOI that ensured that each cell is infected with each of them. After a 1-hr adsorption time the cells are washed with DMEM, and fresh DMEM containing 5% (v/v) FCS was added. After an incubation of 60 hr at 37°C, the cultures are transfected with a virionlike RNA containing the *CAT* gene flanked by the terminal sequences of an influenza virus RNA segment, usually pb2CAT or nsCAT RNA (16), prepared as described above. Sixteen to 20 hr thereafter, cell extracts are prepared to determine the *CAT* activity accumulated, following the procedures indicated in the previous section.

Vaccinia–T7 Virus System

As an alternative way to express the four influenza virus core proteins, a system based on the vaccinia virus recombinant expressing the T7 RNA polymerase (vTF7-3) (30) was used.

Construction of T7-Driven Recombinant Plasmids

To generate plasmids pGEM–PB1, pGEM–PB2, pGEM–PA, and pGEM–NP, the cDNAs encoding the influenza virus PB1, PB2, PA, and NP polypeptides (28) were transferred from pUC18 plasmids to the polylinker of pGEM–3 vector under the control of the T7 RNA polymerase promoter. These plasmids directed the expression of the cloned genes by transfection of COS-1 cells infected with vaccinia vTF7-3 recombinant virus (27).

Reconstitution of the in Vivo Transcription/Replication System

To analyze the functionality of the influenza virus-expressed proteins, COS-1 cell cultures are infected with vTF7-3 virus at an MOI of 2–5. After an adsorption period of 1 hr, the virus inoculum is removed and the cells are washed with DMEM and transfected with a mixture of 1 μg each of plasmids pGEM–PB1, pGEM–PB2, pGEM–PA, and pGEM–NP, using Lipofectin at a 1:2 ratio as described above. Five hours later the cultures are transfected with virionlike *CAT* RNA (0.5 μg). The transfected cultures are incubated an additional 16–20 hr and cell extracts are prepared and tested for *CAT* activity as described above.

Optimization of the Vaccinia–T7 Virus System

The standard vaccinia–T7 virus system described above provided a transcription/replication level, as determined by the *CAT* activity, comparable to that obtained with the SV40 recombinant virus system. However, since it was not expected that equal amounts of each of the influenza virus core proteins were required for optimal activity and plasmid transfection is more amenable to standardization than virus infection, a series of experiments were carried out to optimize the vaccinia–T7 system. COS-1 cell cultures were transfected with variable amounts of the plasmid pGEM–PB1 and constant amounts of the other required plasmids, and 6 hr later a second transfection was carried out in which the template RNA, pb2CAT vRNA, was added. The *CAT* activity of the cultures was determined 16 hr later; these results are presented in Fig. 3. Similar experiments were carried out in which variable amounts of each of the other plasmids were used. The results of this are also shown in Fig. 3. It is clear that the plasmid pGEM–PA was previously transfected in amounts larger than required and that the plasmid pGEM–NP was required in larger amounts than those used before. Further experiments of the same type were performed until an optimal proportion of transfected plasmids was obtained (Table I). Under these optimized conditions about 10 times more *CAT* activity was achieved than that previously obtained; that is, a 1000-fold dilution of the cellular extract gave a 5% conversion of chloramphenicol precursor to acetylated forms in a 2-hr incubation.

A fundamental difference exists between the SV40 recombinant virus and the

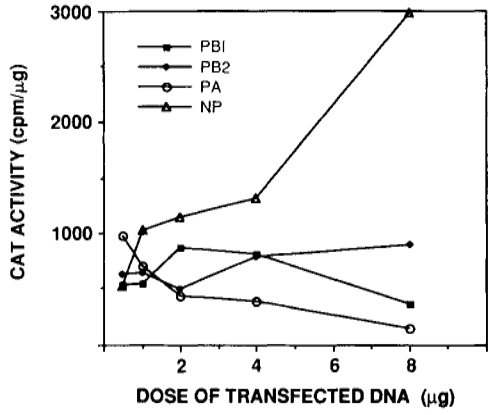

FIG. 3 Dependence of *CAT* activity on the dose of recombinant plasmids transfected. Cultures of COS-1 cells were infected with vaccinia–T7 and transfected with pGEM recombinant plasmids as indicated in the text. Extracts were prepared and assayed for *CAT* activity. Each curve indicates the activity obtained using a fixed dose (1 μg) of every plasmid except one.

vaccinia–T7 reconstitution systems. Whereas influenza virus can carry out a productive infection cycle in an SV40-infected COS-1 cell culture (S. de la Luna, unpublished observations, 1989), complementation of viral genes can only be detected under limited experimental conditions in a vaccinia virus-infected culture (31). This fact restricts the possibilities of use of the vaccinia–T7 system in those experiments aimed at the analysis of complementation of genes expressed by a superinfecting influenza virus. To avoid these problems, a modification of the transfection protocol described above was introduced. The COS-1 cell cultures were treated with cytosine arabinoside (40 μg/ml) before infection with vaccinia–T7 virus. Under these experimental conditions the cultures can be productively infected with influenza virus (32). After the transfection of plasmids pGEM–PB1, pGEM–PB2, pGEM–PA, and pGEM–NP, the levels of *CAT* activity obtained were reduced by only 40–50%.

TABLE I Optimal Doses of Recombinant Plasmids in the Reconstituted System

Plasmid	Doses (μg)[a]	Plasmid	Doses (μg)[a]
pGPB1	1	pGPA	0.1
pGPB2	1	pGNP	4

[a] Amount to be transfected per 35-mm dish.

Functional Analysis of Mutant Polymerase Subunits and NP Genes

The systems described above, based on the expression of the influenza virus *trans* factors by SV40- or vaccinia–T7-based vectors, allowed an easy way to determine the activity of mutant versions of the genes involved. Thus, mutations in the cloned genes were prepared (either deletion or point mutations) and the mutant genes were included in the reconstituted systems, along with wild-type (*wt*) versions of the rest of the genes involved. In all cases the expression and accumulation of the mutant proteins were ascertained by Western blot analysis of total extracts of infected or transfected cells. Examples of the *CAT* activities obtained with mutants of the *PA*, *PB2*, and *NP* genes are shown in Table II. Some of the mutant genes lead to a *CAT* activity comparable to that obtained from the corresponding *wt* gene (see mutants PB2/D4 or NP175K) or even higher than that of the *wt* gene (mutant PA157), whereas others resulted in activity levels that were not detectable (mutants PAΔ670, PB2/P, or NP169D). Obviously, the *CAT* activity is the result of a complex process including several different steps, and other assays should be used to further characterize the mutant genes.

Conclusions and Perspectives

The *in vitro* reconstitution of active RNPs from synthesized vRNA and preparations of viral nucleoprotein and polymerase subunits (15, 18, 22) has opened the possibility

TABLE II Activity of Mutants of the Polymerase Subunits and the Nucleoprotein

Mutant	Mutation[a]	CAT Activity[b]
wt	—	100
PA157	Thr157Ala	300–400
PAΔ670	Δ 670–716	ND
PB2/P	Δ 1–66	ND
PB2/D4	In 298Ile	55–80
NP169D	Gly169Asp	ND
NP175K	Arg175Lys	50–100

[a] Amino acid substitutions are indicated by the sequence position preceded by the *wt* residue and followed by the mutated residue. Deletions (Δ) are denoted by the numbers that limit the deletion. Insertions (In) are shown by the number of the sequence position and the identity of the inserted residue.

[b] The activity was standardized to that of the corresponding *wt* gene. ND, Not detectable.

of analyzing the *cis* elements involved in influenza virus transcription and replication. These new experimental alternatives have been completed by the *in vivo* reconstitution of systems capable of transcribing and replicating viral RNA, in which the required *trans* factors have been expressed from cDNA clones (24–27). As shown with a few examples here for the *PB2*, *PA*, and *NP* genes and recently reported for the *PB1* gene (33), systems of this type are helping to elucidate the functions of these *trans* factors. Advances in this direction will expand the prospect of viral gene rescue in the absence of helper virus.

Acknowledgments

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References

1. P. Palese and D. W. Kingsbury, "Genetics of Influenza Viruses." Springer-Verlag, Vienna, 1983.
2. R. M. Krug, "The Influenza Viruses." Plenum, New York, 1989.
3. C. Herz, E. Stavnezer, R. M. Krug, and T. Gurney, *Cell (Cambridge, Mass.)* **26,** 391 (1981).
4. R. M. Krug, F. V. Alonso-Kaplen, I. Julkunen, and M. G. Katze, *in* "The Influenza Viruses" (R. M. Krug, ed.), p. 89. Plenum, New York, 1989.
5. D. H. L. Bishop, J. F. Obijeski, and R. W. Simpson, *J. Virol.* **8,** 74 (1971).
6. A. R. Beaton and R. M. Krug, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 4682 (1984).
7. L. del Rio, C. Martinez, E. Domingo, and J. Ortín, *EMBO J.* **4,** 243 (1985).
8. O. M. Rochovansky and G. K. Hirst, *Virology* **73,** 339 (1976).
9. J. A. Lopez-Turiso and J. Ortín, *Microbiologia (Madrid)* **4,** 167 (1988).
10. B. W. J. Mahy, *in* "Genetics of Influenza Viruses" (P. Palese and D. W. Kingsbury, eds.), p. 192. Springer-Verlag, Vienna, 1983.
11. J. Braam, I. Ulmanen, and R. M. Krug, *Cell (Cambridge, Mass.)* **34,** 609 (1983).
12. B. M. Detjen, C. St. Angelo, M. G. Katze, and R. M. Krug, *J. Virol.* **61,** 16 (1987).
13. A. Honda, K. Ueda, K. Nagata, and A. Ishihama, *J. Biochem. (Tokyo)* **104,** 1021 (1988).
14. K. L. van Wyke, W. J. Bean, and R. G. Webster, *J. Virol.* **39,** 313 (1981).
15. J. D. Parvin, P. Palese, A. Honda, A. Ishihama, and M. Krystal, *J. Virol.* **63,** 5142 (1989).
16. W. Luytjes, M. Krystal, M. Enami, J. D. Parvin, and P. Palese, *Cell (Cambridge, Mass.)* **59,** 1107 (1989).
17. A. García-Sastre and P. Palese, *Annu. Rev. Microbiol.* **47,** 765 (1993).

18. B. L. Seong and G. G. Brownlee, *Virology* **186,** 247 (1992).
19. K. Yamanaka, N. Ogasawara, H. Yoshikawa, A. Ishihama, and K. Nagata, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 5369 (1991).
20. X. Li and P. Palese, *J. Virol.* **66,** 4331 (1992).
21. M. E. Piccone, S. A. Fernandez, and P. Palese, *Virus Res.* **28,** 99 (1993).
22. J. Martín, C. Albó, J. A. Melero, J. Ortín, and A. Portela, *J. Gen. Virol.* **73,** 1855 (1992).
23. J. D. Dignam, R. M. Levovitz, and R. G. Roeder, *Nucleic Acids Res.* **11,** 1475 (1983).
24. T. S. Huang, P. Palese, and M. Krystal, *J. Virol.* **64,** 5669 (1990).
25. N. Kimura, M. Mishida, K. Nagata, A. Ishihama, K. Oda, and S. Nakada, *J. Gen. Virol.* **73,** 1321 (1992).
26. S. de la Luna, J. Martín, A. Portela, and J. Ortín, *J. Gen. Virol.* **74,** 535 (1993).
27. I. Mena, S. de la Luna, C. Albo, J. Martín, A. Nieto, J. Ortín, and A. Portela, *J. Gen. Virol.* **75,** 2109 (1994).
28. S. de la Luna, C. Martinez, and J. Ortín, *Virus Res.* **13,** 143 (1989).
29. A. Nieto, S. de la Luna, J. Bárcena, A. Portela, J. Valcárcel, J. A. Melero, and J. Ortín, *Virus Res.* **24,** 65 (1992).
30. T. R. Fuerst, P. L. Earl, and B. Moss, *Mol. Cell. Biol.* **7,** 2538 (1987).
31. D. A. Simpson and R. A. Lamb, *J. Virol.* **66,** 790 (1992).
32. T. Huang, J. Pavlovic, P. Staeheli, and M. Krystal, *J. Virol.* **66,** 4154 (1992).
33. S. K. Biswas and D. P. Nayak, *J. Virol.* **68,** 1819 (1994).

[22] Analysis of Membrane Association *in Vivo* and Targeting of Viral Proteins in Polarized Epithelial Cells

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Introduction

Many viruses (both RNA and DNA viruses), such as influenza, mumps, measles, human immunodeficiency virus (HIV), herpes simplex viruses, and others, possess an envelope that they acquire at the final stage of maturation from the infected cell. At both the beginning and end of the infectious cycle, these viruses interact with cellular membrane. In the beginning of the infectious cycle, viruses interact with the cell surface receptor and subsequently, during the process of uncoating and disassembly, viral envelope fuses with the cellular membrane either at the cell surface or in the internal organelles (endosome) after internalization by endocytosis, and thus some of the viral proteins remain associated with the membrane while others are released into the cytoplasm either as free proteins or as part of the viral nucleocapsid. At the end of the infectious cycle, enveloped viruses are formed and released by budding with the viral nucleocapsid enclosed in a viral envelope. During the assembly process viral membrane proteins form quasicrystalline structures with the plasma membrane to which the viral nucleocapsids bind, thereby initiating the process of virus budding. Without envelopment during budding, infectious virus particles are not released and virus replication becomes abortive. Therefore, membrane–protein interactions are crucial during both the entry and exit of enveloped viruses.

Basically, there are two types of membrane proteins. (*a*) The first consists of proteins that are cotranslationally translocated into the endoplasmic reticulum (ER) and then vectorially transported sequentially through the exocytic pathways from the ER, pre-Golgi, *cis*-Golgi, medial Golgi, *trans*-Golgi, and *trans*-Golgi network (TGN) to the plasma membrane. Alternatively, during the exocytic transport translocated proteins can stop en route at any of these organelles, depending on the specific retention signal for a specific organelle (1, 2). These integral membrane proteins possess one or more hydrophobic transmembrane domains embedded in the lipid bilayer and a cytoplasmic tail that functions as a stop transfer signal. These transmembrane proteins become glycosylated (either N-glycosylated or O-glycosylated) as they travel through the exocytic pathway if the protein possesses the appropriate N- or O-linked glycosylation sites. Furthermore, these proteins, if transported to the cell surface, can be targeted specifically to either the apical or basolateral plasma membrane in polarized epithelial cells (3). (*b*) Proteins of the second type associate posttranslationally

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with membranes either via a direct interaction with the lipid bilayer or by an indirect interaction with the cytoplasmic tail/transmembrane domain of another transmembrane protein, or by a combination of both.

Membrane association and targeting of viral proteins during the assembly process are important steps in the biology of enveloped viruses. (*a*) When the specific interaction between membrane components is inhibited, budding of mature virus particles does not take place and therefore virus infection becomes abortive. (*b*) Elucidation of the steps in the assembly process would explain why some viruses bud selectively from the ER, the Golgi apparatus, or the plasma membrane. (*c*) An understanding of the steps involved in the membrane association of viral proteins may facilitate the development of antiviral agents that may interfere with one or more steps involved in virus assembly and budding. Similarly, targeting of viral proteins to specific domains of the plasma membrane plays an important role in viral pathogenesis. Viruses such as influenza, Sendai, and parainfluenza viruses bud from the apical membrane, whereas viruses such as vesicular stomatitis virus (VSV) or retroviruses bud from the basolateral membrane in polarized epithelial cells. A Sendai virus mutant that buds from both the apical and basolateral membranes became more virulent in mice than the parent virus, which buds from the apical plasma membrane (4). In this article we discuss morphological and biochemical methods we have used for studying the posttranslational membrane association of viral proteins and for targeting viral proteins to either the apical or basolateral plasma membrane.

Membrane Association of Sendai Virus Matrix Protein

Sendai virus is a member of the Paramyxoviridae family, which encompasses major groups of human and animal pathogens (5). These viruses are spherical pleomorphic particles possessing a lipid envelope with outer spikes and internal nucleocapsids. The spikes consist of two virally encoded glycoproteins (F and HN) that span the lipid bilayer. Underneath the lipid bilayer resides a shell of matrix (M) protein that encloses the helical nucleocapsid. The viral nucleocapsid consists of a single negative-stranded RNA and nucleoprotein (NP) as well as two other proteins [phosphoprotein (P protein) and L protein] that, together, constitute the viral RNA-dependent RNA polymerase complex (5).

During the assembly and budding process the Sendai virus M protein must interact with the viral envelope and the transmembrane/cytoplasmic tail of one or both spike proteins (F and HN) on one side and the viral nucleocapsid on the other side in order for virion formation to occur (6). M protein becomes membrane associated during the assembly process. However, since M protein does not possess a signal sequence for translocation, it is not cotranslationally translocated into the ER but becomes membrane associated posttranslationally. Therefore, it becomes important to deter-

mine where and how M protein becomes membrane associated and the requirements of F and HN glycoproteins for the membrane association of M protein.

Morphological Analysis to Show That M Protein Becomes Membrane Associated in the Presence of Viral Glycoproteins

Rationale

Essentially, two independent approaches can be used to analyze the interaction of cytoplasmic and membrane components by light microscopy. In each case the assay is based on the following assumption: if the relative subcellular distribution of an integral membrane protein can be shown to affect the localization of a cytoplasmic protein or complex, then the two components must interact and be intimately associated *in vivo*. This type of interaction may be demonstrated in two ways. First, by the use of an antibody-induced capping system, viral glycoproteins present within the plasma membrane can be clustered into distinctive patches (7). Under these conditions M protein becomes concentrated in areas directly below the clustered viral glycoproteins, thereby demonstrating a physical association between M protein and the cytoplasmic domains of the viral spike proteins. Second, when cells are incubated with the drug monensin, secretory transport is blocked at some point between the medial and *trans*-Golgi apparatus, resulting in the accumulation of viral glycoproteins within phenotypically distinct perinuclear membranes (8). Consequently, for some [Semliki forest virus (8), Sendai virus (9, 10), and retroviruses and Moloney murine leukemia virus (11)] but not all viruses [VSV (12) and influenza virus (D. P. Nayak, unpublished observations)], it is possible to demonstrate M protein–glycoprotein interaction by showing that the M protein accumulates on perinuclear Golgi membranes in the presence of monensin when coexpressed with viral glycoproteins. For this reason, recombinant vaccinia viruses were constructed that encoded Sendai virus F, HN, or M protein, thereby facilitating the selective expression of different combinations of membrane and cytoplasmic proteins.

Protocol

The method for antibody-induced capping of viral glycoproteins within the plane of the plasma membrane is not discussed here, since it has been described in detail previously (7).

Construction of Recombinant Vaccinia Viruses

Recombinant vaccinia viruses were constructed according to the method of Chakrabarti *et al.* (13). Sendai virus Z strain cDNAs encompassing the open reading frames of the F, HN, or M proteins were inserted into the multiple cloning site of the vaccinia

virus expression vector pSC11, which contains the 7.5 promoter sequence upstream of the multiple cloning site and the thymidine kinase gene. All vaccinia viruses were propagated and plaque-titered in HeLa cells.

Subcellular Localization of Competent Proteins in the Presence of Monensin

Accumulation of viral glycoproteins within the medial compartment of the Golgi apparatus was performed essentially according to the method of Griffiths *et al.* (8). Baby hamster kidney (BHK) cells were infected with recombinant vaccinia viruses encoding Sendai virus F, HN, or M proteins as indicated in figure legends (Fig. 1). In all cases viral infection was performed at 37° C for a period of 1 hr. Following infection, the cells were washed in cold phosphate-buffered saline (PBS) and incubated in prewarmed (37° C) minimum essential medium (MEM) containing 2.5% (v/v) fetal bovine serum, streptomycin, and penicillin. The addition of the prewarmed medium was used as the zero time point in all experiments. Infected cells were then incubated at 37° C for 5 hr before monensin was added to a final concentration of 20 μM. The cells were then incubated in the presence of monensin at 37° C for a further 3 hr before being processed for immunofluorescence (IF) as described below. The cells were washed thoroughly in PBS and fixed in a 1 : 1 mixture of methanol and acetone at −20° C for 10 min. Following fixation the cells were blocked in PBS containing 0.2% (w/v) gelatin in PBS (blocking buffer) for 1 hr at 37° C. As a general rule, primary antibodies were diluted to 1/100 for monoclonal antisera or 1/1500 for polyclonal antisera (using blocking buffer) and incubated with the cells for 1 hr at 37° C. The cells were then washed three times for 15 min each in blocking buffer before the addition of secondary fluorescein-conjugated antibodies at a dilution of 1/100. After staining, the cells were further washed in PBS plus 0.05% (v/v) Tween-20 (Bio-Rad Lab, Hercules, CA) and then in PBS alone before being mounted in a gelvatol–glycerol solution containing 2.5% (w/v) 1,4-diazabicyclo-[2.2.2] octane (DABCO, Sigma, St. Louis, MD).

Results

The results show that a significant fraction of Sendai viral M protein became colocalized with viral glycoproteins (F and HN) in the perinuclear Golgi membranes when coexpressed in the presence of monensin (Fig. 1A and B), demonstrating an interaction of M protein with viral glycoproteins (9, 10). Furthermore, this interaction was specific and required the coexpression of M protein with F and HN proteins. When M protein was expressed alone, it did not exhibit Golgi staining in the presence of monensin (Fig. 1C).

Comments

As stated above, this technique cannot be used for all viruses. Presumably, in some viruses maturation of the viral glycoproteins during the later stages of exocytic transport as well as other viral components are essential prerequisites to M protein bind-

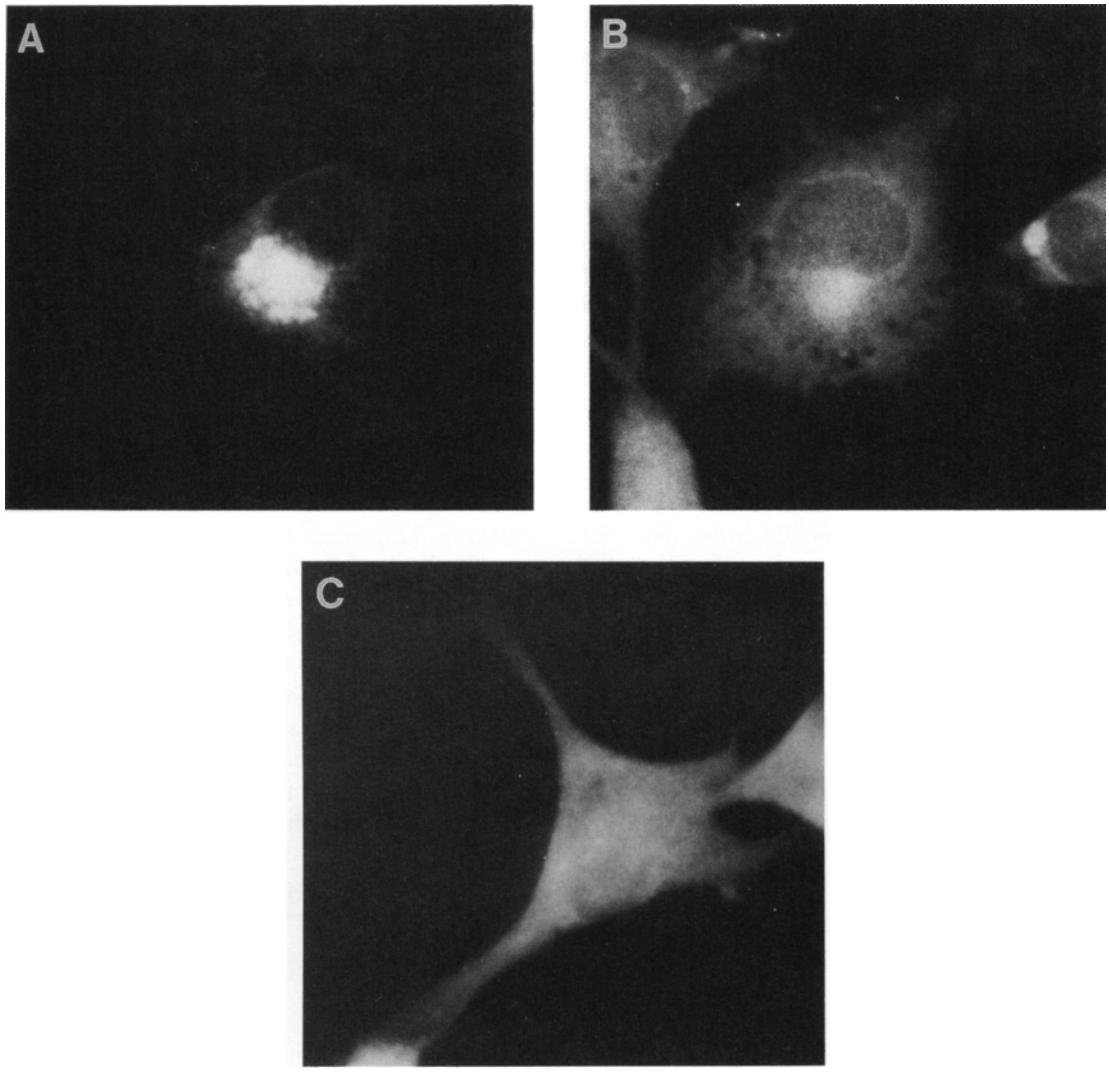

FIG. 1 M protein binds to intracellular Golgi complex when coexpressed with viral glycoproteins (F and HN) in the presence of monesin. BHK cells were infected with either (A and B) a combination of three different recombinant vaccinia viruses encoding Sendai virus F, HN, and M proteins or (C) a single recombinant vaccinia virus encoding the Sendai virus M protein. Monensin (20 μM) was added to all cells 5 hr postinfection, and the cells were incubated for an additional 3 hr before being processed for immunofluorescence. Cells shown in (A) were stained with a bispecific rabbit polyclonal antiserum against F and HN proteins. Cells shown in (B) and (C) were stained using a monoclonal antiserum against the Sendai virus M protein. [From Sanderson *et al.* (10) with permission of the American Society for Microbiology.]

ing. In these cases interaction of M proteins with viral glycoproteins should be analyzed by specific cell surface capping of the viral glycoproteins. In addition, when attempting to visualize the interaction of M proteins with internal cellular membranes, it is advisable to work at an early time point within the infectious cycle, as the proportion of M protein that binds to internal membranes is small in comparison to that of the total cytoplasmic pool. Consequently, as the concentration of the total amount of M protein increases, the intensity of the diffuse cytoplasmic staining tends to mask the distribution pattern of that M protein which is bound to internal membranes. In all cases results obtained by IF should be confirmed by cell fractionation and membrane flotation as described below.

Biochemical Analysis of Membrane Association of Sendai Virus M Protein

Rationale

Sendai virus M protein is synthesized as a cytoplasmic protein that binds posttranslationally to cellular membranes. Consequently, the process of M protein membrane binding can be monitored by physically separating membrane and cytoplasmic fractions and analyzing the relative distribution of membrane-associated M protein when expressed in either the absence or presence of Sendai virus glycoproteins. Separation of membrane and cytoplasmic fractions is best achieved by a method of membrane flotation. In essence, flotation is a simple but stringent assay for determining the relative degree of membrane association of a soluble cytoplasmic protein or complex, but the assay is not designed to separate different populations of cellular organelles. This procedure essentially involves dispersing a crude postnuclear extract of infected cells into a buffered sucrose solution, the density of which is greater than that of cellular membranes. When the gradient is subsequently centrifuged, the more buoyant membranes are forced to float, while soluble cytoplasmic proteins or complexes that are denser than the sucrose solution remain at the bottom of the gradient.

Protocol

Subcellular Fractionation

Preparative fractionation of cultured BHK cells was performed as follows. The cells were washed twice in ice-cold PBS before being removed from the dishes by scraping. The cells were then harvested by centrifugation for 30 sec in a microfuge at 12,000 g at 4° C, resuspended in hypotonic lysis buffer [10 m*M* Tris–HCl (pH 7.5), 10 m*M* KCl, and 5 m*M* $MgCl_2$), and incubated on ice for 10 min before disruption of the cells by repeated passage (usually 15 times) through a 26-gauge hypodermic needle. Breakage of the cells was monitored under a light microscope; unbroken cells and nuclei were removed by centrifugation at 1000 *g* for 5 min at 4° C, and the resulting postnuclear supernatant was subjected to flotation as described below.

Flotation Analyses

Flotation analyses were performed according to the method of Sanderson *et al.* (9), with the following modifications. Crude postnuclear supernatants were prepared as described above. Aliquots (0.5 ml) were then dispersed into 2 ml of 72% (w/w) sucrose in low-salt buffer (LSB) containing 50 m*M* Tris–HCl (pH 7.5), 25 m*M* KCl, and 5 m*M* $MgCl_2$ and overlaid with 2.5 ml of 55% (w/w) sucrose LSB and approximately 0.6 ml of 10% (w/w) sucrose LSB. The gradients were then centrifuged using a Beckman SW 55 Ti rotor (Beckman, Palo Alto, CA) at 4° C for approximately 8 hr at 38,000 rpm. Following centrifugation 0.8-ml fractions were collected from the top of the gradient using a Hacki-Buchler Auto Densiflow II gradient remover (Hacki-Buchler, Lenexa, Kansas). Any material that pelleted during centrifugation was resuspended within the bottom 0.8-ml fraction of each gradient. Prior to immunoprecipitation all fractions were diluted with 3 ml of LSB before the addition of 1 ml of 5×-concentrated radio immune precipitation assay (RIPA) buffer (22). Samples were shaken at 4° C for 2 hr before the addition of antisera. Each fraction was immunoprecipitated with a cocktail of 1 μl of polyclonal rabbit anti-Sendai virus antibodies and 0.2 μl of monoclonal antibodies against the Sendai virus M protein. In the context of these experiments, this assay was not meant to resolve different populations of cellular membranes (all of which, empirically, should float) but rather to simply determine the relative proportion of M protein that binds to membranes in the presence or absence of selected Sendai virus glycoproteins.

Results

The results (Fig. 2) show that Sendai virus M protein was initially synthesized as non–membrane-bound cytoplasmic protein that subsequently became bound to cellular membranes in the presence of both Sendai virus glycoproteins. Integral membrane proteins (F and HN), on the other hand, were always present in the membrane fraction (fraction 1). Although not shown here, M protein does not require both F and HN for membrane binding and can bind to cellular membrane in the presence of either HN or F glycoprotein (10). The majority of Sendai virus M, when expressed alone, did not become membrane bound and remained within the bottom fractions of the gradient even after chase (10).

Targeting Viral Proteins to the Apical and Basolateral Plasma Membranes in Polarized Epithelial Cells

Epithelial cells in differentiated organs of animals are polarized and possess two distinct structural and functional domains of plasma membranes: one facing the external or luminal side (apical) and the other facing internally, which is in contact with the basal layer (basolateral). Tight junctions joining the lateral domains of adjacent cells

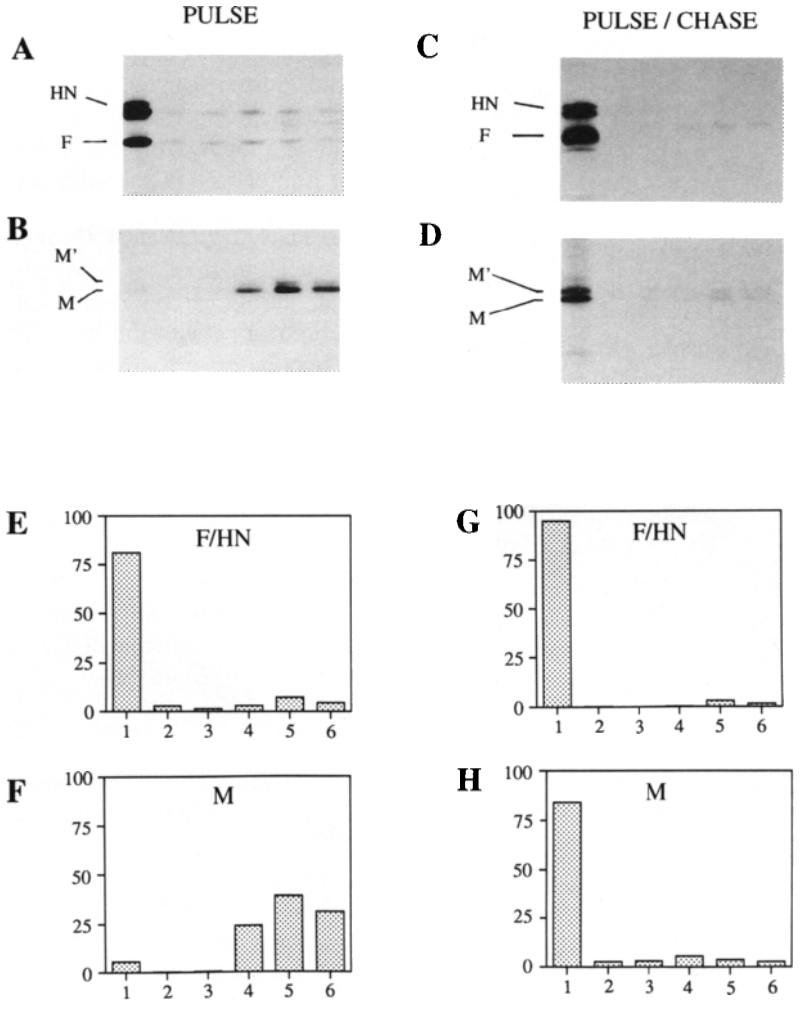

FIG. 2 M protein binds to cellular membrane in the presence of both viral glycoproteins (F and HN). BHK cells were infected with a combination of recombinant vaccinia viruses encoding Sendai virus F, HN, and M proteins. The cells were pulse-labeled for 20 min at 7 hr postinfection and either (A and B) processed immediately or (C and D) chased for 2.5 hr longer before being processed for membrane flotation. The quantification of autoradiographs (A–D) was performed by laser densitometric scanning using an LKB laser scanner (Pharmacia Biotech, Uppsala, Sweden); and the results are shown in panels E–H, respectively. Values shown in graphs represent the percentage of total detected protein. [From Sanderson *et al.* (10) with permission of the American Society for Microbiology.]

block free lateral diffusion and comingling of lipids and proteins between apical and basolateral domains of the plasma membrane, and thereby ensure that each membrane domain (i.e., apical and basolateral) retains distinct lipid and protein compositions. Each domain of the plasma membrane appears to possess distinct functions. For example, secreted proteins are usually released into the lumen via the apical domain, whereas hormones and metabolites to be absorbed by the blood capillaries exit via the basolateral domain. Similarly, some of the plasma membrane proteins, such as aminopeptidase, are targeted to the apical surface, whereas others, such as Na^+,K^+-ATPase are concentrated on the basolateral surface of polarized epithelial cells.

Secreted and transmembrane proteins reach the outside medium or the plasma membrane, respectively, via the same exocytic pathway. Proteins destined for the apical or basolateral plasma membrane can comingle and may travel through the same exocytic pathway up to the TGN (14, 15). However, from the TGN, apical and basolateral proteins exit via distinct vesicles targeted to either the apical or basolateral surface. How proteins are separated at this stage into distinct vesicles and how these proteins are targeted to specific domains of the plasma membrane are highly relevant to both cell biology and viral biology (1, 3, 16). Obviously, targeting viral glycoproteins to a specific plasma membrane will dictate the site of budding of viruses and may play an important role in viral pathogenesis (4, 17). Influenza virus HA, NA, and VSV G proteins have been extensively used to study polarized transport. Influenza virus HA and NA proteins are directly targeted to the apical surface, whereas VSV G protein is targeted directly to the basolateral surface of polarized epithelial cells (16, 18, 19).

A number of cell lines, such as Madin–Darby canine kidney (MDCK), CaCo-2, and Vero cells, as well as primary cultured cells such as African green monkey kidney (AGMK) cells exhibit polarity in tissue culture. Our interest has been to define the structural features that direct a protein to the apical or basolateral surface. We have been particularly interested in defining the structural domains involved in the polarized sorting in influenza virus glycoproteins (HA and NA), paramyxovirus glycoproteins (F and HN), and VSV glycoprotein G. For these studies viral proteins are expressed either individually or in combination in cells that maintain polarity in culture. Subsequently, modified proteins such as chimeric proteins obtained by exchanging specific domains between the heterologous proteins, or mutated proteins obtained by point mutation(s) or deletion(s) within a specific domain(s) of the protein, are expressed in polarized cells and the distribution of these proteins on the apical and basolateral plasma membranes is determined. We have used two approaches for studying the polarized expression of viral proteins in MDCK cells. (*a*) Polarized MDCK cells are infected with recombinant vaccinia viruses expressing the desired protein(s) (20). (*b*) Alternatively, permanent MDCK cell lines are generated that express the desired protein(s) from the viral cDNA under an inducible promoter (21). Polarized expression is assessed morphologically by surface IF as well as by internal staining using confocal microscopy, or biochemically by surface labeling and im-

munoprecipitation as well as by monitoring secretion of the protein into the medium from the apical or basolateral surface. Each approach presents some advantages and disadvantages. The transient vaccinia virus expression system is efficient and less labor intensive, since many cDNA constructs can be created and analyzed rather quickly. But since vaccinia virus itself is highly lytic, the window for polarized analysis is limited to between 3 and 7 hr postinfection. Furthermore, the level of expression in MDCK cells by the vaccinia 7.5 (early–late) promoter is rather low.

On the other hand the selection of stable MDCK cell lines expressing the desired protein is time consuming. Furthermore, multiple cell lines must be examined and the DNA (or RNA) from the expressing cells must be sequenced to check that the expressed protein has not been altered. Currently, both of these methods are used to demonstrate the distribution of proteins on the apical and basolateral surfaces of polarized epithelial cells. However, we prefer, at present, the stable cell line approach to study polarized distribution (21).

Morphological Analysis of Cell Surface Proteins by Indirect IF

Morphological distribution of proteins on the cell surface can be demonstrated either by immunoelectron microscopy or by IF. Analysis by indirect IF is rather quick and simple. However, this technique is not quantitative and requires a sufficient concentration of protein on the plasma membrane to be detected by IF. Therefore, the quality of the antibodies (both primary and secondary) is also critical for both the specificity and sensitivity of the assay.

Protocol

MDCK cells are grown for 3–4 days to ensure that they are completely confluent and have become polarized, with tight junctions separating apical and basolateral surfaces. To ensure polarity in these cultured cells, coverslips from 3-, 4-, 5-, and 6-day-old cultures should be examined with an appropriate control for apical and basolateral proteins. For basolateral and apical proteins appropriate viruses (e.g., VSV or retroviruses for basolateral expression and influenza virus or paramyxovirus for apical expression) should be used as controls. For inducible proteins cells grown on the coverslips should be induced for 6–10 hr with the appropriate inducing agents before use for the IF assay.

To examine the apical or basolateral surface expression of the protein, monolayers of MDCK cells of both intact and EGTA-treated cells are analyzed by indirect IF. Using intact monolayers, proteins that are localized on the apical surface can be detected by IF, whereas cells expressing basolateral proteins will be negative. To determine whether the glycoprotein is expressed basolaterally, monolayers are treated with EGTA, a chelating agent that disrupts tight junctions and exposes basolateral surfaces to antibodies (20).

For surface staining, cell monolayers are washed with PBS–1% (w/v) bovine se-

rum albumin (BA) (intact) or with EGTA (30 m*M*) for 30 min at 37°C. Both intact and EGTA-treated cells are washed with PBS–BA and fixed with 3.7% (w/w) formaldehyde for 15 min and rinsed with PBS–BA. Specific antibodies are added to the coverslips containing the fixed cells and incubated for 30 min at room temperature. The antibody-treated cells are then washed with PBS–BA and layered with an appropriate dilution of fluorescein isothiocyanate-conjugated species-specific anti-IgG for 30 min at room temperature. Antibody-treated cells are washed repeatedly with PBS–BA and layered with gelvatol–glycerol solution and examined under a Nikon optiphotomicroscope (Nippon Kogaka, K., Tokyo, Japan) by epifluorescence.

Results

To examine for protein expression, cells are fixed with acetone–methanol and used for internal staining, and all cells expressing either the apical or basolateral proteins will be positive by this procedure. However, when cells expressing apical proteins such as influenza virus HA or NA are fixed with formaldehyde and stained for cell surface IF, these cells exhibit positive staining at the cell surface before EGTA treatment, indicating that the influenza virus HA or NA is present on the apical surface. However, control MDCK cells as well as cells expressing basolateral proteins such as VSV G protein (without EGTA treatment) are negative, indicating that VSV G is not present on the apical surface (20). However, when cells expressing VSV G protein are treated with EGTA, these cells become positive by surface staining. EGTA releases the tight junction and thus facilitates the lateral diffusion and comingling of basolateral protein throughout the plasma membrane. This would indicate that VSV G protein is expressed on the basolateral surface and after EGTA treatment becomes accessible for surface staining.

Comments

A number of problems may be encountered in this protocol. (i) Protein concentration on the cell surface may not be sufficient for detection by IF. This method detects the steady-state concentration of protein present on the cell surface, and the steady-state concentration of a plasma membrane protein may be affected by a number of factors: (*a*) the efficiency of transcription and translation, which may vary depending on the strength of the promoters, inducing agents, mRNA stability, etc.; (*b*) protein turnover, particularly at the cell surface, including endocytosis; and (*c*) protein structure, including folding, oligomerization, and kinetics of transport. These factors become particularly important for chimeric or mutated proteins, which may behave differently. (ii) Specificity and sensitivity of the antibodies. (iii) Confluence of the cell monolayer, as this may affect the generation of the tight junction and polarity.

Quantification of Surface Protein by Biotinylation

Morphological analysis of surface proteins by IF can demonstrate whether the protein is present on the apical or basolateral surface of polarized epithelial cells. However,

such a procedure, as mentioned above, is only qualitative, not quantitative. It also cannot distinguish whether the protein is present on both apical and basolateral surfaces versus only the apical surface. Furthermore, the above procedure cannot determine whether the protein was targeted directly to the apical or basolateral surface or was first transported to one surface and then to the other surface by transcytosis (1). To avoid these problems, a more direct method for the quantification of surface protein is required. Usually, two methods are used: (*a*) *Antigen trapping,* in which the labeled cell surface proteins are tagged with specific antibodies, and excessive free antibodies are washed away. Cells are then lysed and these antigen–antibody complexes are recovered by protein A– or protein G–sepharose (Pharmacia Biotech, Uppsala, Sweden) or another antibody recovery system. The labeled proteins are dissociated and then analyzed and quantified by polyacrylamide gel electrophoresis (PAGE). However, a number of problems, such as in the specificity and stability of antibodies, dissociation of the antigen–antibody complex during cell lysis, and recovery of the antigen–antibody complex, often affect such analysis. (b) *Surface biotinylation.* We have routinely used biotinylation of surface proteins and recovery of the biotin–protein complex by immunoprecipitation with specific antibodies (21).

Protocol

Step 1

MDCK cells are grown in Costar Transwell (Cambridge, MA) units. The cells are grown for 3–4 days until the filter is completely covered with a monolayer of cells with tight junction. Polarity of the cells is determined by the development of increased electrical resistance between the upper and lower chambers. Alternatively, labeled protein (e.g., inulin) is added on the top chambers to determine whether the labeled protein leaks into the lower chamber. In fully polarized cells there should be no transfer of externally added protein from one chamber to the other. Experiments are performed only after the conditions for cell polarity have been standardized.

Step 2

Polarized cells, grown on Costar Transwell units, are metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine in MEM lacking cysteine and methionine. The labeling time (or the pulse and chase) is determined by the time the desired protein takes to reach the cell surface after synthesis. The monolayers are then washed three times with ice-cold PBS$^+$, and the apical or basolateral surfaces of parallel filters are biotinylated twice for 20 min with 1 mg/ml sulfo-*N*-hydroxy-succinimidyl (NHS)–biotin (Pierce Chemical Co., Rockford, IL) in PBS$^+$. The reaction is quenched by washing with PBS$^+$ containing 50 m*M* lysine. The filters are then cut off the holder and the cells are lysed in RIPA buffer containing 1% Triton X-100 for 30 min under constant agitation at 4° C. The postnuclear supernatants are precleared with Pansorbin (Calbiochem, La Jolla, CA) and immunoprecipitated using polyclonal or monoclonal antibodies for 2 hr, and protein A (Pharmacia–LKB) is added for an additional

2 hr. The protein A beads containing the immunoprecipitates are washed three times with RIPA buffer [50 m*M* Tris–HCl (pH 7.6), 150 m*M* NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), and 1 m*M* phenylmethylsulfonyl fluoride]. The immunoprecipitate is eluted by boiling twice in 100 μl of elution buffer [1% SDS plus 0.2 *M* Tris–HCl (pH 8.5)] for 2 min and then washing the protein A beads with 300 μl of RIPA buffer. The eluates and the wash are pooled, then incubated with 50 μl of a slurry of streptavidin–agarose beads for 30 min at 4° C. The beads are washed three times with RIPA buffer and boiled in sample buffer, and the supernatant is analyzed by SDS–PAGE and quantified.

Results

Figure 3 shows the steady-state distribution of an apical (influenza virus NA) and basolateral [transferrin receptor (TR)] protein on the apical and basolateral surfaces, respectively. It can be seen that the proteins are targeted asymmetrically to either the apical or basolateral surface. Over 90% of NA is present on the apical surface, whereas the reverse is true for TR. Nonspecific distribution of these proteins on both

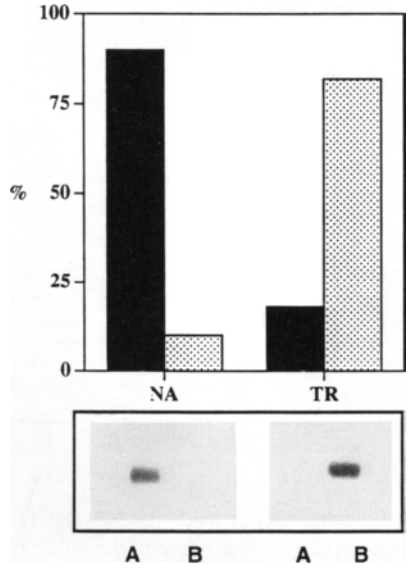

FIG. 3 Polarized surface distribution of wild-type NA and TR. Monolayers of polarized MDCK cells expressing NA and TR were grown on Transwell units and labeled for 4 hr with 400 μCi each of [^{35}S]cysteine and [^{35}S]methionine. The cell surface proteins were isolated by domain-selective biotinylation, immunoprecipitated with specific antibodies, selected with streptavidin–agarose, and analyzed by SDS–PAGE and fluorography. Fluorograms were quantified and the results are expressed as a percentage of the apical (lanes A) and basolateral (lanes B) surface expression of each protein. [From Kundu and Nayak (21) with permission of the American Society for Microbiology.]

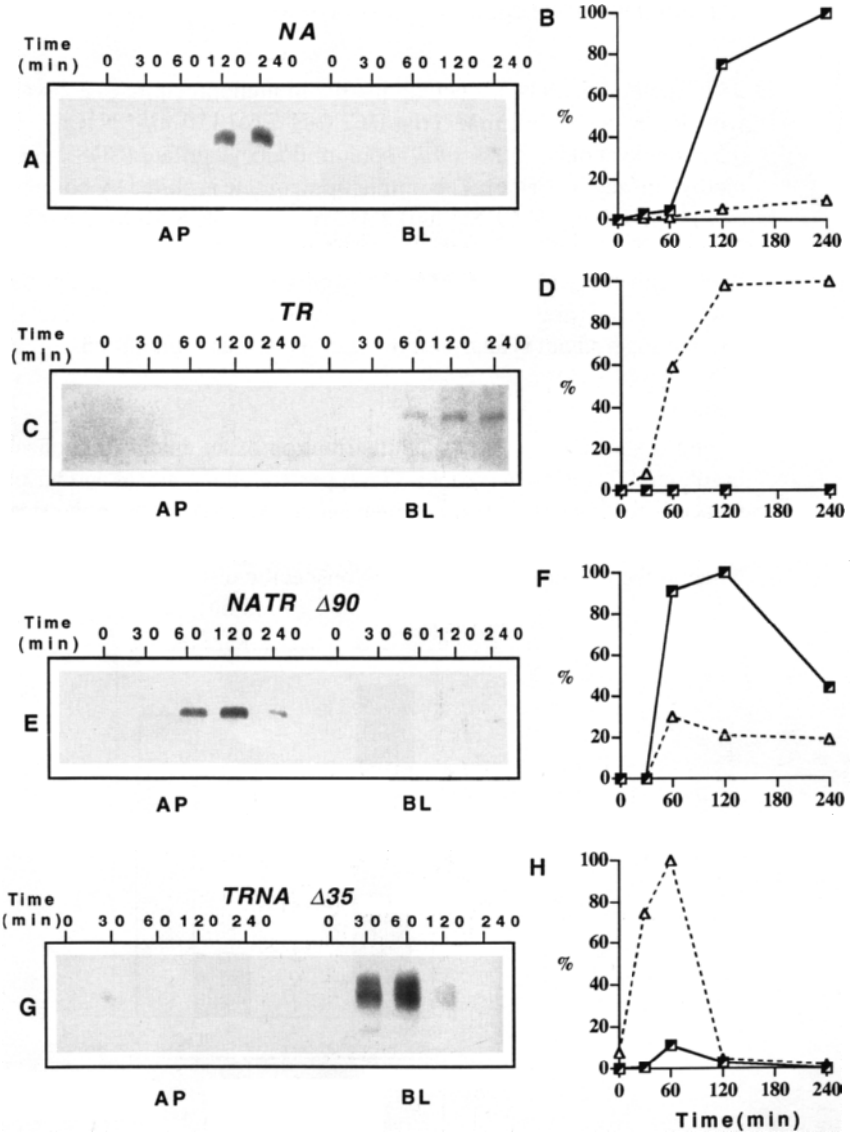

FIG. 4 Pulse–chase analyses of wild-type NA and TR and chimeric NATR Δ90 and TRNA Δ35. Filter-grown MDCK cells expressing the wild-type (NA and TR) and chimeric (NATR Δ90 and TRNA Δ35) proteins were metabolically labeled for 20 min with 500 μCi each of [^{35}S]cysteine and [^{35}S]methionine and chased for the times indicated. The apical (AP, □) or basolateral surface (BL, △) proteins were biotinylated and isolated. Samples were analyzed by SDS–PAGE and processed for fluorography. Fluorograms were quantified and the results were expressed as a percentage of the amount at the time of maximal expression at the cell surface. (A and B) NA; (C and D) TR; (E and F) NATR Δ90; (G and H) TRNA Δ35. [From Kundu and Nayak (21) with permission of the American Society for Microbiology.]

surfaces would indicate that either the polarity of the cells was not reached (i.e., proteins on both surfaces were comingling in the absence of tight junctions) or free biotin was not completely quenched by lysine. Alternatively, cell surface may be damaged, causing biotinylation of internal proteins.

Pulse–Chase Analysis of Polarized Transport

The above results demonstrate the steady-state distribution of proteins on the cell surface but do not distinguish between two possibilities: (*a*) whether the protein can be targeted directly to a specific surface and remain there or (*b*) the protein undergoes transcytosis after first arriving at a specific cell surface before reaching the other surface. Kinetics of polarized transport would distinguish between these two possibilities. For this, cells are pulse-labeled for a short period (e.g., 20 min) and the free label is removed and chased with medium containing an excess of unlabeled amino acids. At different times during the chase, cell surface proteins are biotinylated and examined as above. Figure 4 shows the typical kinetics of targeting proteins directly to the apical or basolateral surface for NA or TR, respectively. If, on the other hand, transcytosis is directing the protein from one surface to the other, the kinetics data would show the transport of the protein first to one surface, and only with increasing chase time will more protein be transported to the other surface.

Chimeric and mutant proteins can be used to define the domain of a specific protein involved in targeting that protein to either the apical or basolateral surface. For example, the results in Fig. 4 show that the cytoplasmic tail of TR contains the signal for basolateral transport. Similarly, it appears that a signal(s) for apical transport is present in the transmembrane/cytoplasmic tail of NA (21).

Costar chambers can also be used for determining the apical or basolateral secretion of proteins. Transmembrane proteins can be made secretory by removing the transmembrane domain. Cells expressing these secretory proteins can be pulse-labeled and chased as above. In this case the medium in the upper (apical) or lower (basal) chamber can be examined directly by immunoprecipitation. For example, NA, when made secretory, secretes predominantly in the upper chamber, indicating that the ectodomain of NA also contains an apical targeting signal (21). Taken together, these results show that the cytoplasmic tail of TR was sufficient for basolateral transport, whereas influenza virus NA possesses two sorting signals, one in the cytoplasmic/transmembrane domain and the other within the ectodomain of NA, both of which are independently able to transport to the apical plasma membrane (21).

Acknowledgment

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References

1. K. Mostov, G. Apodaca, B. Aroeti, and C. Okamota, *J. Cell Biol.* **116,** 577 (1992).
2. K. Simons and A. Wandinger-Ness, *Cell (Cambridge, Mass.)* **62,** 207 (1990).
3. D. P. Nayak and M. A. Jabbar, *Annu. Rev. Microbiol.* **43,** 465 (1989).
4. M. Tashiro, M. Yamakawa, K. Tobita, J. T. Seto, H.-D. Klenk, and R. Rott, *J. Virol.* **64,** 4672 (1990).
5. D. W. Kingsbury (ed.), "The Paramyxoviruses." Plenum, New York, 1991.
6. M. E. Peeples, *in* "The Paramyxoviruses" (D. W. Kingsbury, ed.), p. 427. Plenum, New York, 1991.
7. D. I. Tyrell and A. Ehrnst, *J. Cell Biol.* **81,** 396 (1979).
8. G. Griffiths, P. Quinn, and G. Warren, *J. Cell Biol.* **96,** 835 (1983).
9. C. M. Sanderson, N. L. McQueen, and D. P. Nayak, *J. Virol.* **67,** 651 (1993).
10. C. M. Sanderson, H.-H. Wu, and D. P. Nayak, *J. Virol.* **68,** 69 (1994).
11. M. Hansen, L. Jelinek, S. Whiting, and E. Barklis, *J. Virol.* **64,** 5306 (1990).
12. J. E. Bergman and P. J. Fusco, *J. Cell Biol.* **107,** 1707 (1988).
13. S. Chakrabarti, K. Brechling, and B. Moss, *Mol. Cell. Biol.* **5,** 3403 (1985).
14. K. S. Matlin and K. Simons, *J. Cell Biol.* **99,** 2131 (1984).
15. M. J. Rindler, I. E. Ivanor, H. Pleskin, E. Rodriguez-Boulan, and D. D. Sabatini, *J. Cell Biol.* **98,** 1304 (1984).
16. M. G. Roth, R. W. Compans, L. Giusti, A. R. Davis, D. P. Nayak, M. J. Gething, and J. Sambrook, *Cell (Cambridge, Mass.)* **33,** 435 (1983).
17. G. Griffiths and P. Rottier, *Semin. Cell Biol.* **3,** 367 (1992).
18. L. V. Jones, R. W. Compans, A. R. Davis, T. J. Bos, and D. P. Nayak, *Mol. Cell. Biol.* **5,** 2181 (1985).
19. E. B. Stephens, R. W. Compans, A. Earl, and B. Moss, *EMBO J.* **5,** 237 (1986).
20. N. L. McQueen, D. P. Nayak, E. B. Stephens, and R. W. Compans, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 9318 (1986).
21. A. Kundu and D. P. Nayak, *J. Virol.* **68,** 1812 (1994).
22. C. N. Gujuluva, A. Kundu, K. G. Murti, and D. P. Nayak, *Virology* **204,** 491–505 (1994).

[23] Cloning of Viral Double-Stranded RNA Genomes by Single Primer Amplification

Paul R. Lambden and Ian N. Clarke

Introduction

Several cloning strategies have been devised for viruses that possess segmented double-stranded (ds)RNA genomes (1–3). However, these procedures have relied on the availability of large amounts of dsRNA extracted from purified virus preparations. Of necessity, these procedures depend on a renewable source of viral RNA, either by propagation in cell culture or in a suitable experimental host. There are many situations in which a renewable resource of virus is not available. One such circumstance occurred in our laboratory during the characterization of a virus obtained from a fatal case of gastroenteritis in Bristol, England: the causative agent was later identified as a group C rotavirus (4).

Several groups of rotaviruses are currently recognized based on the antigenic differences in the major inner shell protein and on the characteristic banding pattern of their 11 dsRNA genome segments. Rotaviruses from only groups A, B, and C are associated with human disease. Group A rotaviruses are the major cause of acute gastroenteritis in children under 5 years of age. The group A viruses can be propagated in cell culture, and a number of sequences for all genome segments are available for direct reverse transcription and polymerase chain reaction (PCR) amplification. Group B rotaviruses have been responsible for major outbreaks of adult gastroenteritis in China; group C rotaviruses cause sporadic outbreaks in both adults and children throughout the world. No cell culture systems are available for the "atypical" group B and C human rotaviruses; hence, the only source material for the molecular characterization of these viruses is human fecal specimens. Thus, single-primer amplification of viral dsRNA (5) was developed for the express purpose of cloning atypical rotaviruses of unknown sequence directly from very small quantities of human fecal specimens. The human stool sample is an extremely hostile environment from which to extract undegraded RNA, but the successful cloning of all 11 genome segments of group C rotavirus RNA suggests that this method may be generally applicable to other dsRNA viruses from a variety of biological sources.

The success of the procedure is critically dependent on the preparation of highly purified dsRNA, and it is strongly recommended that enzymes and reagents are obtained from the suppliers stated in this protocol. The procedure can be divided into four sections: (*a*) purification and analysis of dsRNA from fecal samples; (*b*) radiolabeling and purification of primer 1; (*c*) ligation of primer 1 to dsRNA; and (*d*) cDNA synthesis and amplification by PCR.

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Purification and Analysis of dsRNA

Highly purified rotaviral dsRNA can be prepared directly from fecal samples using the guanidinium–phenol technique followed by binding to silica particles in the presence of chaotropic agents. The guanidine thiocyanate–phenol reagent is conveniently supplied as RNAzol reagent by Cinna/Biotecx Laboratories. However, after the addition of chloroform and removal of the aqueous phase containing the RNA, the manufacturers recommend precipitation with isopropanol to recover the RNA. We have modified the procedure to make use of the observation by Boom *et al.* (6) that rotaviral dsRNA will bind to silica particles in the presence of high concentrations of sodium iodide. This procedure permits extensive washing of the RNA in chaotropic reagents to remove potential inhibitors of the RNA ligase reaction. It is critical to extract the dsRNA exactly as described, because RNA extracted by the more usual technique of phenol extraction and ethanol precipitation does not accept primer 1 in the RNA ligation reaction. It is also advisable to check the quality and quantity of dsRNA by polyacrylamide gel electrophoresis (PAGE) before proceeding with the ligation reaction.

Extraction of dsRNA

This method works best on clinical specimens that are preferably less than 0.5 ml in volume, so that all the manipulations can be performed in a 1.5-ml microfuge tube. It is also preferable that the samples contain at least 10^9 rotavirus particles to allow visualization of the extracted dsRNA by silver staining (7). In addition, pretreatment of the fecal sample with RNase A or RNase T1 to remove contaminating rRNA or tRNA prior to guanidinium–phenol extraction gives a much cleaner genomic RNA profile as judged by sodium dodecyl sulfate (SDS)–PAGE and silver staining.

Reagents and Solutions

RNAzol B
Geneclean (Bio 101)
RNase T1 [GIBCO—Bethesda Research Laboratories (BRL), Gaithersburg, MD]
New Wash (NaCl–ethanol–water; provided in the Geneclean kit)

Method

1. Add 1 μl (10,000 U) of RNase T1 to a liquid fecal suspension (~50%). Bring the sample volume up to 0.5 ml with sterile water.
2. Incubate at 55° C for 15 min.
3. Add 0.5 ml of RNAzol B and mix thoroughly.

4. Let stand at room temperature for 5 min.
5. Add 0.1 vol of chloroform and vortex.
6. Let stand at 4° C for 5 min.
7. Centrifuge for 15 min at 13,000 *g* at room temperature.
8. Transfer the aqueous phase to a fresh tube (<300 μl per tube).
9. Add 3 vol of 6 *M* sodium iodide (Geneclean kit) and mix thoroughly.
10. Add 5 μl of Glasssmilk (Geneclean kit) and vortex for 5 min.
11. Quick-spin by centrifugation at 13,000 *g* for 5 sec at room temperature.
12. Resuspend the pellet by vortexing in 300 μl of New Wash buffer held at −20° C.
13. Quick-spin.
14. Repeat steps 12 and 13 twice.
15. Wash with 80% (v/v) ethanol, quick-spin, and remove as much ethanol as possible using a fine tip (suitable tips are provided in the kit).
16. Place the tubes in a vacuum desiccator for 2 min to evaporate any residual ethanol from the Glassmilk pellet.
17. Resuspend the pellet in 10 μl of sterile water and incubate at 55° C for 2 min to elute the dsRNA from the Glassmilk.
18. Centrifuge at 13,000 *g* for 2 min to remove the silica matrix.
19. Recover the supernatant containing the dsRNA.
20. Analyze 1–5 μl on a polyacrulamide gel.

Gel Analysis of dsRNA

Polyacrylamide Gels

dsRNA can be separated on Laemmli discontinuous SDS–PAGE gels designed for the separation of proteins (8). Prepare a 20 cm × 15 cm × 1.5-mm gel and electrophorese at a constant 120 V for 16 hr. It is essential to use high-quality reagents and filtered solutions throughout the procedure to avoid excessive background discoloration during silver staining.

Silver Staining

For the best results and minimum background staining it is essential to use glassware washed in nitric acid: do *not* use plastic containers for staining. It is also advisable to use ultrahigh-quality deionized water for all solutions.

Reagents and Solutions

Solution I: 0.0034 *M* potassium dichromate in 0.0032 N nitric acid, prepared as a 10× stock

Solution II: 0.012 *M* silver nitrate, prepared as a 10× stock and stored away from light

Solution III: 0.28 *M* sodium carbonate containing 0.5 ml of 37–41% (w/v) formaldehyde per liter

Method

1. Fix the SDS–PAGE gel in 50% methanol–12% acetic acid for 30 min.
2. Wash the gel three times for 10 min each in 10% ethanol–5% (v/v) acetic acid.
3. Soak for 5 min in solution I (100 ml).
4. Wash four times for 30 sec each in water (100 ml).
5. Soak in solution II for 30 min.
6. Wash twice for 10 sec each in 200 ml of solution III (quick rinse). At this stage a milky precipitate appears and the gel has a tendency to stick to the glass dish. Therefore, keep the gel agitated during the quick rinses.
7. Place in 200 ml of solution III; illuminate on a light box until the bands are visible.
8. Add fresh solution III after 5 min.
9. Stop development by replacing solution III with 1% (v/v) acetic acid.

Agarose Gels

Occasionally, it is not possible to resolve individual genome segments on SDS–PAGE gels. This can cause serious difficulties when attempting to perform genome segment coding assignments. We have found that genome segments that do not resolve well on polyacrylamide gels can frequently be separated by standard agarose gels run in Tris–acetate buffer (9). Moreover, if sufficient sample is available, it is possible to extract individual genome segments from the agarose gel using the Geneclean procedure. This problem arose during attempts to make a genome coding assignment for full-length clones of the outer capsid spike protein (VP4) (10). The results in Fig. 1A show that it is difficult to resolve genome segments 3 and 4 on polyacrylamide gels to obtain an unequivocal coding assignment by Northern blot analysis, whereas a reasonable separation can be obtained on a standard agarose gel (Fig. 1B).

Northern Blotting

To prove that cDNA clones originated from the original viral cDNA, it is essential to perform a Northern blot analysis. As the availability of dsRNA is limited, we outline here a useful procedure to prepare nylon strips of gel-separated genome profiles. The strips can be used several times in standard hybridization experiments.

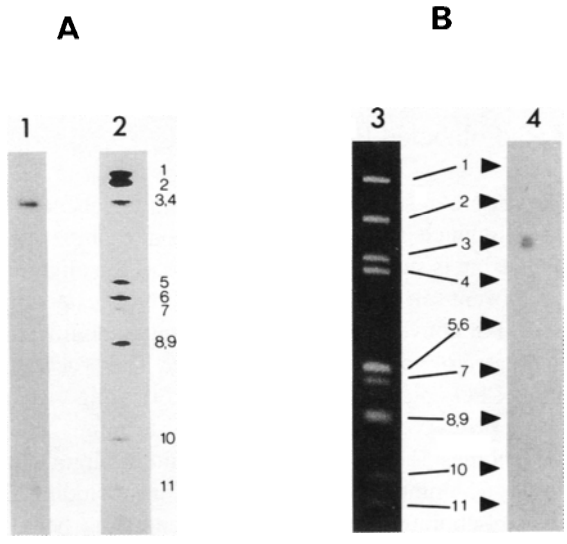

FIG. 1 Gene coding assignment for human group C rotavirus outer spike protein VP4. (A) Northern blot analysis of rotavirus dsRNA genome segments separated by SDS–PAGE. Lane 1, Nylon strip probed with the insert from a recombinant M13 clone carrying the full-length insert of the *VP4* gene; lane 2, nylon strip showing each of the 11 genome segments following probing with radiolabeled total rotaviral cDNA. (B) Agarose gel separation of rotavirus dsRNA genome segments. Lane 3, Ethidium bromide-stained genome profile; lane 4, dot blot of gel-purified genome segments probed with the recombinant VP4 M13 clone, showing unequivocal assignment of genome segment 3 to protein VP4. (Modified from Ref. 5 with permission of the American Society for Microbiology.)

Reagents and Solutions

Hybond–N^+ nylon membranes (Amersham, UK)
Whatman (Clifton, NJ) 3MM paper
Transfer buffer [10 m*M* Tris plus 10 m*M* boric acid (pH 7.3)]

Method

1. Following electrophoresis of dsRNA on an SDS–PAGE gel, the separated genome segments are transferred to nylon membranes by electroblotting. Electrophoretic transfer of the RNA is performed in transfer buffer at 1.2 mA/cm^2 for 2 hr using a "semidry" blotter (Sartoblot II, Sartorius, Gottingen, Germany).
2. Transfer of RNA to the nylon membranes is confirmed by silver staining the remaining gel.
3. Fix the RNA to the membrane by baking at 80°C for 1 hr and then cut into strips approximately 5 mm wide.
4. Immediately prior to use in hybridization experiments, the membrane strips are

boiled in water for 2 min to denature the RNA duplexes. Hybridization is then performed according to standard procedures (9).

Preparation of Radiolabeled Primer 1

The procedure of single-primer amplification depends on the successful ligation of a unique oligonucleotide to the 3′ terminus of each strand of all genome segments. The primer design is such that the 3′ terminus of the oligonucleotide is chemically modified to prevent self-ligation and subsequent concatenation during the T4 RNA ligase reaction. For convenience we chose a simple modification that can be performed on an automated oligonucleotide synthesizer. The procedure involves using a 3′ amino-modifier CPG column on which to build the oligonucleotide. This has the effect of placing a primary amine residue at its 3′ terminus, thereby blocking the possibility of phosphodiester bond formation with other oligonucleotides. Several manufacturers supply 3′ amino-modifier CPG columns, including Glen Research (Sterling, VA), Peninsula Laboratories (Belmont, CA), and Cruachem (Glasgow, Scotland). The actual sequence of the primers is largely a matter of personal preference, but it is worth incorporating one or two restriction sites to facilitate further subcloning.

Kinase Reaction

Oligonucleotide primer 1 is kinased in two stages. The first stage incorporates a radioactive trace using [γ-^{32}P]ATP, and the second stage uses unlabeled ATP to ensure complete phosphorylation of the 5′ terminus of the oligonucleotide.

Set up a 20μl reaction containing the following: 10 μl of primer 1 (20 ng/μl), 2 μl of 10× buffer [0.7 *M* Tris–HCl (pH 7.5), 0.1 *M* $MgCl_2$, and 50 m*M* dithiothreitol], 5 μl (50 μCi) of [γ-^{32}P]ATP, 2 μl of water, and 1 μl (10 U) of T4 polynucleotide kinase. Incubate the reaction mix at 37° C for 1 hr and then add a further 1μl of 10× buffer, 5 μl of water, 3 μl of ATP (20 m*M*), and 1 μl (10 U) of T4 polynucleotide kinase. Incubate for 1 hr longer at 37° C. Labeled oligonucleotides are purified from unincorporated [γ-^{32}P]ATP by adsorption on a NENSORB 20 cartridge (see below).

Purification of Radiolabeled Oligonucleotides

Reagents and Solutions

NENSORB 20 cartridges (DuPont–New England Nuclear, Boston, MA)
Reagent A: 0.1 *M* Tris–HCl, 10 m*M* triethylamine, and 1 m*M* disodium EDTA (pH 7.7)
Reagent B: 50% (v/v) methanol

Method

1. Clamp the NENSORB cartridge and wash through with 2 ml of 100% methanol, using a Pasteur pipette to wash loose packing onto the column bed. This step is essential to prewet the column. Slowly push air, using gentle pressure, through the cartridge using a disposable 10-ml syringe.
2. Prime the cartridge with 3 ml of reagent A, using a disposable syringe as described above.
3. Add 200–400 μl of reagent A to the radioactive primer 1 reaction and mix. Using a micropipette, apply the sample to the top of the cartridge. Push the sample through the column using a 5-ml syringe to apply gentle pressure. Collect the eluate and reapply to the column.
4. Wash the cartridge with 3 ml of reagent A followed by 3 ml of water.
5. Elute the sample with two 500-μl aliquots of reagent B.
6. Dry the sample in a SpeedVac (Framlingdale, NY) vacuum concentrator using gentle heat.
7. Dissolve primer 1 in 5 μl of water.

Ligation of Primer 1 to dsRNA

The most difficult step in the whole procedure is the reaction catalyzed by T_4 RNA ligase. This commercially available enzyme is able to covalently join single-stranded RNA (or DNA) molecules, provided that they contain 5′-phosphate and 3′-hydroxyl termini (9). T_4 RNA ligase is routinely used for 3′ end-labeling of RNA with [^{32}P]mononucleoside 3′,5′-biphosphate and has proven especially useful for defining the 3′ termini of dsRNA genome segments (11). A profile of genome segments radiolabeled at their 3′ termini using T_4 RNA ligase and cytidine 3′,5′-[5′-^{32}P]bisphosphate is shown in Fig. 2.

In the single-primer amplification procedure we used T_4 RNA ligase to join primer 1 (single-stranded DNA) to the 3′ termini of purified dsRNA. This is the most exacting step in the procedure, as dsRNA is not the optimal substrate for T_4 RNA ligase. To amplify the whole virus genome (11 dsRNA segments), it is crucial to monitor the successful ligation of primer 1 to each of the genome segments; this can only be achieved by gel separation of the linkered dsRNA.

For valuable virus samples such as fecal specimens, the amounts of available dsRNA are usually very limited. Therefore, it is often not possible to quantify accurately the RNA without compromising the whole sample. We have therefore chosen, as the arbitrary practical minimum amount of dsRNA for an individual ligation reaction, the amount of dsRNA that can be visualized in a single gel track by silver staining. The kinased primer 1 labeled with [γ-^{32}P]ATP as described in the previous section is used in a single tube ligation reaction with all 11 dsRNA genome segments. A schematic diagram summarizing the overall strategy is shown in Fig. 3.

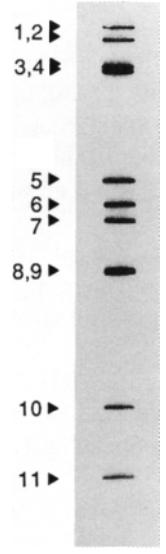

FIG. 2 Autoradiograph of human group C rotavirus genome segments labeled at the 3′ termini with T_4 RNA ligase and cytidine 3′,5′-[5′-^{32}P]bisphosphate (pCp; Amersham, UK). Numbers refer to the dsRNA genome segments. (Reproduced from Ref. 5 with permission of the American Society for Microbiology.)

Reagents and Solutions

Bovine serum albumin (BSA; Promega, Madison, WI): nuclease free and qualified for molecular biology use .

Dimethyl sulfoxide (DMSO): high-purity spectroscopic grade, kept frozen in small aliquots

T_4 RNA ligase (GIBCO–BRL): The quantity of this enzyme added to a reaction is usually quoted in micrograms rather than units. Although other manufacturers, such as New England Biolabs (Beverly, MA), also produce this enzyme, the unit definition and reaction conditions are different from those of the GIBCO–BRL product; therefore, alternative sources of T_4 RNA ligase must be used with caution in the system described below.

Method

Set up the following 30-μl reaction on ice.

0.5 *M* HEPES (Na^+, pH 7.5)	3 μl
0.18 *M* $MgCl_2$	3 μl
30 m*M* dithiothreitol	3 μl

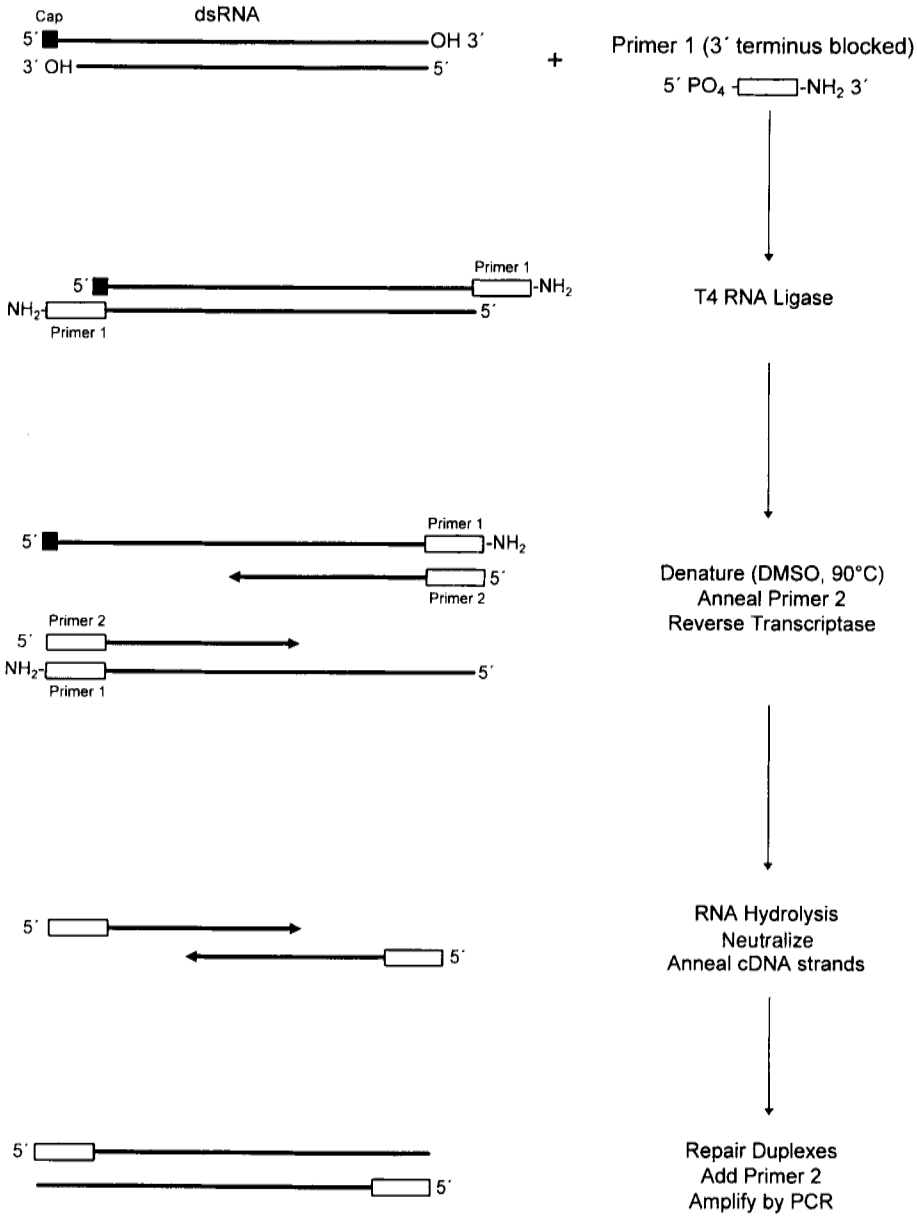

FIG. 3 Schematic showing the principle of single-primer amplification of viral dsRNA genome segments. (Adapted from Ref. 5 with permission of the American Society for Microbiology.)

10 m*M* ATP	3 μl
100 μg/ml BSA	3 μl
10% (v/v) DMSO	3 μl
Primer 1	5 μl
Purified dsRNA	6 μl
T_4 RNA ligase (GIBCO–BRL)	1 μl ($\sim$1.5 μg of enzyme)

Incubate the reaction overnight (for 16 hr) at 4° C. On the following day purify the linkered RNA away from excess primer 1, using spin-column chromatography. Suitable columns are obtainable from Promega and can be packed with the desired column matrix. We routinely use columns packed with Sephacryl S-400 (Pharmacia, Uppsala, Sweden), although recovery of the smallest rotaviral genome segment (600 bp) can be low when using this matrix. If high yields of low-molecular-mass genome segments are required, it may be preferable to use a matrix such as Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Analysis of Reaction Products

It is advisable to check for successful tailing of the rotaviral genome segments before proceeding to cDNA synthesis and PCR amplification. This can be done by analysis of 5 μl of the spun-column eluate on a 1% agarose gel. Although the separation of genome segments on agarose is not as clear as on SDS–PAGE, the procedure is quicker and the resolution is adequate enough to monitor the ligation of radiolabeled primer 1 to the 3′ termini of the dsRNA. After electrophoresis the gel is fixed in 10% (v/v) trichloroacetic acid for 30 min, followed by a 10-min soak in 95% (v/v) ethanol. The gel can be dried rapidly by attaching to an old sheet of unexposed X-ray film. The gel is covered with a sheet of 3MM filter paper and a stack of paper towels and then pressed with a heavy weight. The gel adheres tightly to the X-ray film and can be dried completely with a hot-air drier. The dried gel is then subjected to autoradiography at -70° C overnight. An example of group C rotavirus dsRNA ligated to primer 1 is shown in Fig. 4.

cDNA Synthesis and PCR Amplification

In the following steps the tailed dsRNA is used to prime cDNA synthesis using a primer complementary to primer 1. DMSO is incorporated into the cDNA synthesis reaction to improve reverse transcription of the dsRNA templates. In addition, the mutant form of Moloney murine leukemia virus (MMLV) reverse transcriptase (RTase) lacking RNase H activity (Superscript II RTase, GIBCO–BRL) is used for its increased thermal stability and ability to generate longer transcripts. Following

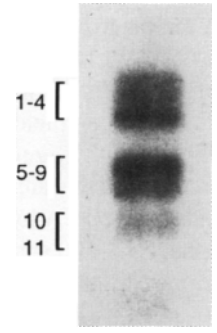

FIG. 4 Agarose gel analysis of the products of the T_4 RNA ligase reaction. The 11 dsRNA genome segments were tailed with radiolabeled primer 1, purified by spin-column chromatography, and analyzed on a standard 1% agarose gel and visualized by autoradiography. The dsRNA genome segment numbers are marked by brackets. (Modified from Ref. 5 with permission of the American Society for Microbiology.)

cDNA synthesis the RNA templates are destroyed by alkali treatment and cDNA strands are annealed overnight. Incomplete cDNA duplexes are then repaired with *Taq* polymerase before thermal cycling.

cDNA Synthesis

The dsRNA is first denatured as follows. Add 2.5 μl of primer 2 (90 ng of total oligonucleotide) to 15 μl of dsRNA ligated to primer 1. Next, add 3.5 μl of DMSO to the RNA primer mix to give a final volume of 21 μl and heat at 90°C for 5 min. Cool rapidly on ice, spin briefly in a microfuge and then add the cDNA synthesis reagents to give a final reaction volume of 50 μl.

10 μl of 5× buffer (supplied with GIBCO–BRL MMLV RTase)	
0.1 *M* dithiothreitol	5 μl
10 m*M* dNTP	2.5 μl
BSA (10 mg/ml)	0.5 μl
RNasin (40 U; Promega)	1 μl
[α-^{32}P]dCTP (50 μCi)	5 μl
Water	2.5 μl
Superscript II RTase (500 U)	2.5 μl

Incubate the reaction mix at 42–45°C for 60 min and then stop the reaction by the addition of 1 μl of 0.5 *M* EDTA.

The genomic RNA templates are then hydrolyzed by the addition of 5 μl of 1 *M* NaOH and heating to 65° C for 60 min. Cool to room temperature and neutralize by the addition of 5 μl of 1 *M* HCl followed by 5 μl of 1 *M* Tris–HCl (pH 7.5). Before amplification by PCR, any incomplete dsDNA duplexes are repaired by annealing cDNA strands and filling in with *Taq* polymerase.

After neutralization the reverse transcription reaction is heated to 65° C overnight to anneal cDNA strands. The cDNA hybrids are then purified by spin-column chromatography using Sephacryl S-400 and collection into water. Strand completion can be conveniently performed by preincubation of the thermal cycling reaction in the absence of primer 2.

PCR Amplification

Set up the PCR reaction mixes, omitting primer 2.

Annealed cDNA in water	34.5 μl
10× buffer (Promega) with $MgCl_2$	5 μl
2 m*M* dNTP	5 μl
Taq polymerase (Promega)	0.5 μl

Incubate at 72° C in the thermal cycler for 5 min to complete duplex DNA synthesis. Remove the tubes, cool on ice, and add 5 μl of primer 2 (50 ng/μl).

Program the thermal cycler with appropriate parameters for the desired fragment size. The annealing temperature will be dependent on the composition of the oligonucleotide primer. A 35-cycle program is usually sufficient to generate enough material to give strong bands on ethidium bromide-stained agarose gels. The amplified genome segments can then be cloned into a vector of choice, making use of the restriction sites in the oligonucleotide primer. However, if the primer restriction sites are present in any of the genome segments, it is probably safer to use blunt-end cloning or a proprietary PCR cloning vector.

Conclusion

We have developed a novel sequence-independent strategy for the amplification of full-length cDNA copies of dsRNA virus genomes. The method relies on the use of T_4 RNA ligase to add a single amino-modified oligonucleotide (primer 1) to both 3′ termini of each dsRNA segment. A complementary primer (primer 2) is then used to prime cDNA synthesis from both genomic RNA strands and, following annealing and repair of the cDNA, the same primer 2 is used in the PCR to amplify the desired genome segments.

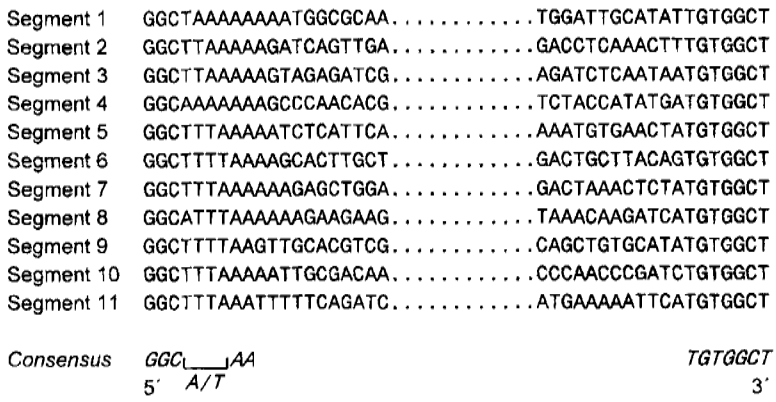

FIG. 5 cDNA sequences of the 5′ and 3′ termini for each of the 11 genome segments of a human group C rotavirus obtained by the single-primer method.

We have selected recombinants representing all 11 genome segments of human group C rotavirus, and the 22 terminal sequences are presented in Fig. 5. The complete cDNA sequences for genome segments 3, 5, 8, and 10 from this virus have been determined (5, 10, 12, 13). The technique has been further validated by the amplification of orbivirus and avian reovirus genomes (G. Viljoen, personal communication, 1994) and other workers have used single-primer amplification to clone and sequence dsRNA from a bovine group C rotavirus (14).

Single-primer amplification is a powerful technique that allows the amplification of the complete dsRNA virus genome in a single experiment. The method also defines the termini of each genome segment, as primer 1 is ligated to the 3′ termini of each RNA strand prior to cDNA synthesis and thus obviates the necessity to perform primer extension reactions on what are frequently rare and valuable samples.

Acknowledgment

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References

1. G. W. Both, A. R. Bellamy, J. E. Street, and L. J. Siegman, *Nucleic Acids Res.* **10,** 7075 (1982).

2. M. Imai, M. A. Richardson, N. Ikegami, A. J. Shatkin, and Y. Furuichi, *Proc. Natl. Acad. Sci. U.S.A.* **80,** 373 (1983).
3. M. A. McCrae and J. G. McCorquodale, *J. Virol.* **44,** 1076 (1982).
4. E. O. Caul, C. R. Ashley, J. M. Darville, and J. C. Bridger, *J. Med. Virol.* **30,** 201 (1990).
5. P. R. Lambden, S. J. Cooke, E. O. Caul, and I. N. Clarke, *J. Virol.* **66,** 1817 (1992).
6. R. Boom, C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-Van-Dillen, and J. Van der Noordaa, *J. Clin. Microbiol.* **28,** 495 (1990).
7. L. Xu, D. Harbour, and M. A. McCrae, *J. Virol. Methods* **27,** 29 (1990).
8. U. K. Laemmli, *Nature (London)* **227,** 680 (1970).
9. J. Sambrook, E. F. Fritsch, and T. Maniatis, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
10. P. A. Fielding, P. R. Lambden, E. O. Caul, and I. N. Clarke, *Virology* **204,** 442 (1994).
11. I. N. Clarke and M. A. McCrae, *J. Gen. Virol.* **64,** 1877 (1983).
12. S. J. Cooke, I. N. Clarke, R. B. Freitas, Y. B. Gabbay, and P. R. Lambden, *Virology* **190,** 531 (1992).
13. A. S. Grice, P. R. Lambden, E. O. Caul, and I. N. Clarke, *J. Med. Virol.* **44,** 166 (1994).
14. B. Jiang, H. Tsunemitsu, J. R. Gentsch, L. J. Saif, and R. I. Glass, *Nucleic Acids Res.* **21,** 2250 (1993).

[24] Using the RNA-Capture Assay to Assess the RNA-Binding Activity of Viral Proteins

John T. Patton and Jian Hua

Introduction

Characterization of RNA-binding proteins can provide key insights into the mechanisms of viral replication, as well as basic information on protein and RNA structure and function. Several methods have been used successfully to describe the interactions of protein and nucleic acids; these include gel mobility-shift electrophoresis (1), nucleic acid-conjugated column chromatography (2), filter binding assay (3), nucleic acid–protein photo–cross-linking (4), nuclease protection (i.e., footprinting) (5), electron microscopy (6), and, recently, crystallization of nucleic acid–protein complexes (7). Because each of these methods has its own unique combination of advantages and disadvantages, the selection of the appropriate technique for the detection and analysis of nucleic acid-binding proteins is often influenced by a number of factors, with those of most importance related to the character and size of the nucleic acid probe and to the purity, availability, structure, and activity of the nucleic acid-binding protein. Here we describe an alternative technique, the RNA-capture assay, which we have successfully used to detect and analyze the specificity of two rotavirus RNA-binding proteins. The RNA-capture assay employs immunoadsorbed protein as an affinity matrix to analyze RNA-binding activity. As described below, the RNA-capture assay depends primarily on the availability of a monospecific antibody that binds with reasonable affinity to the protein whose RNA-binding activity is to be assessed. Additionally, the assay requires that the selected antibody not perturb the RNA-binding activity of the protein. Some of the major advantages of the RNA-capture assay are as follows: (*a*) RNA probes of unlimited size can be used to examine RNA-binding activity; (*b*) the RNA-binding protein need not be purified a priori or subjected to harsh, possibly denaturing, conditions; and (*c*) the RNA-binding protein may be a component of large multimeric protein complexes.

Rotaviruses, members of the family Reoviridae, contain 11 segments of generally monocistronic double-stranded (ds)RNA that code for as many as six RNA-binding proteins (8). The rotavirus genome is replicated asymmetrically with viral mRNA serving as the template for the synthesis of minus-strand RNA (9). To understand the mechanism of rotavirus replication, we have performed studies designed to identify and characterize the role of the viral RNA-binding proteins in genome packaging and assortment and in the synthesis of dsRNA. One particular rotavirus protein, NS53, was found previously to bind zinc (10) and to contain an animo-terminal cysteine-rich region that was proposed to form one or two zinc fingers (11). Because such zinc

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finger motifs often impart nucleic acid-binding activity to proteins (12), the RNA-capture assay was used to determine whether NS53 exhibited affinity for RNA and was subsequently used to examine the specificity of the RNA-binding activity of the protein. Importantly, while RNA-binding activity could not be demonstrated for NS53 by the Northwestern blot assay (13) and while only nonspecific RNA-binding activity could be detected for NS53 by gel mobility-shift electrophoresis (10), the RNA-capture assay revealed that the protein specifically recognizes the 5′ termini of rotavirus mRNAs (14). The varied results obtained for NS53 by the three different assay systems point to the importance of trying multiple approaches in detecting and assessing the RNA-binding activity of suspected RNA-binding proteins.

We have also used the RNA-capture assay to demonstrate that multimers formed by NS35, a basic protein encoded by the rotaviruses, possess nonspecific RNA-binding activity (15). This result is consistent with earlier studies that showed by gel mobility-shift electrophoresis, nucleic acid-conjugated column chromatography, and RNA–protein cross-linking that the protein binds RNA nonspecifically (16). Similarly to NS53, NS35 in the Northwestern blot assay did not exhibit affinity for RNA (13), possibly because the denaturants used in the assay [sodium dodecyl sulfate (SDS)] inactivated the RNA binding domain of the protein. The establishment of the RNA-capture system as a tool to examine the RNA-binding properties of rotavirus proteins was based on a previous study by Koster *et al.* (17), which used immunoadsorbed protein as an affinity matrix to analyze the RNA-binding activity of the XFG 5-1, a zinc finger protein of *Xenopus*. Although we have not tested this, it seems likely that immunoadsorbed proteins might also be useful in the characterization of DNA-binding proteins.

Protocol for the RNA-Capture Assay

The protocol for the RNA-capture assay is illustrated in Fig. 1. Overall, the RNA-capture assay can be divided into two parts: (*a*) immobilization of the RNA-binding protein onto protein A–Sepharose and (*b*) characterization of the RNA-binding activity of the immunoadsorbed protein. The steps in the RNA-capture assay as it was used to characterize the RNA-binding activity of the rotavirus nonstructural proteins, NS53 and NS35, are presented below.

Immobilization of the RNA-Binding Protein

1. To overproduce NS53 and NS35, nearly confluent monolayers of CV-1 cells were infected with approximately 20 plaque-forming units (pfu) per milliliter of vTF7-3, a recombinant vaccinia virus that constitutively expresses bacteriophage T7 RNA polymerase (18). After allowing the virus to adsorb for 1 hr, the inoculum was removed and the cells were mock-transfected or transfected with the plasmid pSP72g5

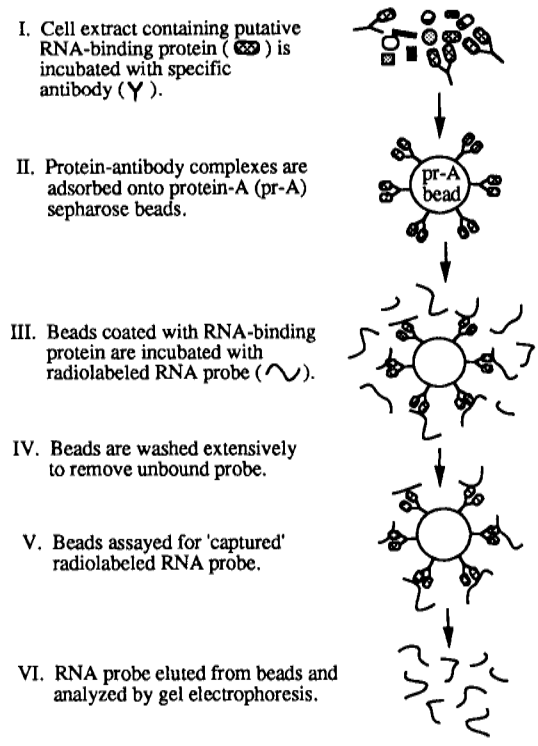

FIG. 1 Overview of the RNA-capture assay.

or pSP72g8(R) by the calcium phosphate method (19, 20). pSP72g5 and pSP72g8(R) are T7 transcription vectors that code for NS53 and NS35 of simian rotavirus SA11, respectively (14, 15). Beginning at 5 hr post infection, the cells were maintained in serum-free minimum essential medium (MEM). In some cases, to label the recombinant protein, the cells were maintained instead in 80–90% methionine- and cysteine-free MEM containing 10 μCi of ^{35}S-labeled amino acids per milliliters (1200 Ci/mmol New England Nuclear, Boston, MA).

2. At 15 hr postinfection the cells were scraped into the medium and pelleted by centrifugation at 1000g for 10 min at 4° C. The cell pellets were resuspended in cold lysis buffer [50 mM HEPES (pH 7.9), 150 mM NaCl, 0.5 mM Na_2EDTA, 5 mM 2-mercaptoethanol, 100 μM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 1% v/v Triton X-100 (Sigma, St. Louis, MO)] at a concentration of approximately 5×10^6 cells per milliliter. After incubation for 5 min on ice with intermittent mixing, the cytoskeletal component of the lysates were disrupted by extensive Dounce homogenization (50–75 strokes). The homogenization was particularly important for the efficient recovery of NS53, as the protein appears to accumulate on the cytoskeleton.

The lysates were centrifuged at 1000 *g* for 10 min at 4° C to remove nuclei and large debris and the soluble fractions were recovered.

3. Depending on the number of RNA-binding assays necessary, 250 to 300-μl aliquots of the soluble fractions were placed into the appropriate number of 1.5-ml microfuge tubes. The volume in each tube was brought to 1 ml with buffer A [50 m*M* HEPES (pH 7.9), 150 m*M* NaCl, 0.5 m*M* Na_2EDTA, 5 m*M* 2-mercaptoethanol, 100 μM $ZnCl_2$, 5% (v/v) glycerol, and 0.1% v/v Triton X-100]. A monospecific antibody directed against the RNA-binding protein was added to the samples to a final dilution of 1:500 to 1:1000. In the case of NS53, a hyperimmune rabbit antiserum was used that specifically recognizes the C-terminal 19 amino acids of NS53 (14). For NS35 a hyperimmune antiserum was used that was prepared by immunizing a guinea pig with recombinant baculovirus-expressed NS35. The NS35-specific antiserum was generously provided by Dr. D. Bass (Stanford University, Stanford, CA). After overnight incubation at 4° C with mixing, 30 μl of a 50% slurry of protein A–Sepharose beads were added to the samples and incubation was continued for an additional
1–2 hr. (Where appropriate for the efficient recovery of the antibody–RNA-binding protein complex, protein G–Sepharose beads may be used in lieu of the protein A beads.) The beads were then recovered by centrifugation for 30 sec in a microfuge and washed twice with 1 ml of buffer A and once with 1 ml of RNA-binding buffer [30 m*M* Tris–HCl (pH 8.0), 200 m*M* NaCl, 0.5 m*M* Na_2EDTA, 10 m*M* 2-mercaptoethanol, and 200 μM $ZnCl_2$]. The RNA-binding buffers were made with diethylpyrocarbonate-treated water.

4. To assess the quality and quantity of the immunoadsorbed RNA-binding protein, some washed beads were resuspended in SDS–sample buffer and subjected to electrophoresis on SDS polyacrylamide gels (21). To analyze RNA-binding protein that was radiolabeled, the gel was processed for fluorography (22). Alternatively, in cases in which the eluted protein was not radiolabeled, the gel was analyzed by Western blot assay (23).

Assay for RNA-Binding Activity

1. After washing with RNA-binding buffer, the NS53- and NS35-coated beads in each microfuge tube were resuspended in 600 μl of RNA-binding buffer containing 2–5 $\times$ 10^5 cpm of ^{32}P-labeled RNA probe (Table I). The RNA-binding reactions were incubated for 40 min at 23–37° C on a rotating wheel. The length of the probes used in binding assays ranged from 34 to 3300 nucleotides and these were synthesized *in vitro* by transcriptionally active rotavirus particles (24) or by T7 transcription of rotavirus cDNAs (25). Prior to use, the probes were deproteinized by phenol–chloroform extraction and, in the case of T7 transcripts, were also gel-purified by electrophoresis on 6% polyacrylamide gels containing 8 *M* urea (described below).

To test the specificity of the RNA-binding activity of the immunoadsorbed pro-

TABLE I Generalized Conditions for RNA-Capture Assay[a]

Conditions	Binding	Washing
^{32}P-labeled RNA probe	2–5 × 10^5 cpm (0.5–5 ng)	
RNA-binding protein linked to protein A–Sepharose	30 μl of 50% slurry	
Tris–HCl (pH 8)	30 m*M*	30 m*M*
Na_2EDTA (pH 8)	0.5 m*M*	0.5 m*M*
2-Mercaptoethanol	10 m*M*	10 m*M*
NaCl	200 m*M*	150–350 m*M*
$ZnCl_2$	0.2 m*M*	0.2 m*M*
Reaction volume	600 μl	3 × with 750 μl
Temperature	23–37°C	23°C
Time	30–60 min	

[a] Elutions are performed in binding buffer containing 0.8 *M* NaCl.

teins, cold RNA probe or nonspecific competitor RNA was also included in the binding reactions. The competitor RNAs were normally added in molar excesses of 5- to 50-fold. Brome mosaic virus mRNA and yeast tRNA were routinely used as the nonspecific competitor RNAs.

2. After incubation the protein A–Sepharose beads were pelleted from the binding reactions by centrifugation for 20 sec at 4° C in a microfuge. Following aspiration of the supernatants, the protein A beads were washed three times with 750 μl of the RNA-binding buffer.

3. To measure the amount of ^{32}P-labeled RNA probe that bound to the immunoadsorbed RNA-binding protein, the protein A beads were resuspended in 150 μl of RNA-binding buffer containing 0.8 *M* NaCl. The high concentration of salt in this buffer disrupts RNA–protein complexes containing either NS53 or NS35. A 10-μl aliquot of the slurry was then added to scintillation cocktail and assayed for radioactivity using a scintillation counter.

4. To electrophoretically analyze the bound RNA probe, following suspension of the beads in RNA-binding buffer containing 0.8 *M* NaCl and incubation for 5 min at room temperature, the beads were pelleted by centrifugation and the supernatant was transferred to a new tube. Thirty microliters of the supernatant was then mixed with 15 μl of RNA sample buffer (8 *M* urea, 3 m*M* Na_2EDTA, 15% (v/v) glycerol, 0.18% w/v bromphenol blue, and 0.18% w/v xylene cyanole) and electrophoresed on a 6% polyacrylamide gel containing 8 *M* urea (26). Each gel (15 × 15 × 1.5 cm) was prepared by dissolving 20 g of urea in 6.3 ml of 38% polyacrylamide solution (37 g of acrylamide and 1 g of bisacrylamide), 4 ml of 10× TBE [0.5 *M* Tris–borate (pH 8.3) plus 10 m*M* Na_2EDTA], and 14 ml of water. Immediately before casting the gel, 37.5 mg of ammonium persulfate and 10 μl of TEMED (N, N, N′, N′-tetramethylethylenediamine) were added. Gels were run at 250 V until the bromphenol blue dye

had run off (approximately 2 hr). ^{32}P-labeled RNA in the gels was detected by autoradiography.

Factors in Using the RNA-Capture Assay to Study RNA-Binding Proteins

The reaction conditions we established for the RNA-capture assay as being optimal for the study of NS53 and NS35 are summarized in Table I. However, when using the assay for other RNA-binding proteins, the reaction components of the capture assay should be modified to maximize the binding of the RNA probe to the immunoadsorbed protein, while at the same time allowing the protein to retain any associated sequence specificity. Possible modifications of the binding reaction to be considered include the addition of metal ions (magnesium or manganese), protease and RNase inhibitors, or nucleotides (ATP or GTP), the adjustment of the final pH of the binding reaction, and the replacement of sodium salts with potassium salts. In some instances the addition of glycerol or nonreactive proteins such as bovine serum albumin may be found to enhance the binding activity of the immunoadsorbed protein. Other factors to be considered when using the RNA-capture assay are addressed below.

Because each binding reaction in the RNA-capture assay is performed in an individual tube, by performing several binding reactions in parallel, it is possible to rapidly establish the optimal binding conditions for the immunoadsorbed protein. Once the conditions are determined, they may be applied to the analysis of the RNA-binding activity of the protein by other assay systems. For example, the buffer determined to give optimal binding in the RNA-capture system may serve as a guide for developing a suitable running buffer to be used when analyzing RNA-binding proteins by gel mobility-shift electrophoresis.

Salt Concentration

In the RNA-capture assay two specific molecular interactions are sensitive to disruption by exposure to high concentrations of salt: (*a*) the binding of the RNA-binding protein to the antibody and (*b*) the binding of the RNA probe to the immunoadsorbed RNA-binding protein. Since the strength of the affinity of an antibody for a protein and that of a protein for an RNA can vary considerably, the concentration of salt used to prepare the protein affinity matrix and to wash the RNA captured on the matrix must be experimentally determined in order to achieve the maximal level of RNA-binding activity, while at the same time minimizing the nonspecific (background) association of probe with the matrix. In the preparation of the affinity matrix, it is particularly important to use the maximum possible concentration of salt in the wash buffer, as this will reduce the level of proteins that nonspecifically contaminate the matrix. Without their removal the contaminates may significantly increase the level

of nonspecific RNA-binding activity associated with the matrix. In the case of NS53, the optimal salt concentration was determined by analyzing the effects of varying the concentration of NaCl in the RNA-binding buffer from 50 to 400 m*M* on the level of RNA probe that bound to NS53-coated and mock-coated protein A beads (data not shown). At 50 m*M* NaCl the level of probe that bound to the mock-coated bead was higher than that observed at 200 and 400 m*M* NaCl, suggesting a high level of nonspecific RNA binding at low salt concentrations. At 400 m*M* NaCl the RNA-binding activity of NS53 was lost. Because at 200 m*M* NaCl the NS53-coated beads retained strong RNA-binding activity that was specific for viral mRNA, this concentration of salt was used subsequently in the RNA-capture assay.

Zinc and Reducing Agents

From our studies on NS53, it is apparent that both zinc and a reducing agent such as 2-mercaptoethanol should be included in all reaction mixtures when using the RNA-capture assay to characterize zinc finger proteins. While zinc would be required for the coordination of zinc fingers in the protein, the reducing agent would appear to act to enhance RNA-binding activity by inhibiting the formation of spurious disulfide bonds between cysteine residues present within the zinc fingers. Likewise, we have found that, to achieve maximal RNA-binding activity, reducing agents should also be included in all buffers used to lyse cells in which the zinc finger proteins are to be recovered.

Ratio of Probe to Immunoadsorbed RNA-Binding Protein

Clearly, the sensitivity of the RNA-capture assay will be greatest when the amount of the RNA-binding protein immobilized on the affinity matrix is greatest. To achieve this requires that sufficient quantities of both the RNA-binding protein and the antibody by which it is recognized are used in the preparation of the matrix. In cases in which only a limited quantity of an RNA-binding protein is available for analysis, it may be worthwhile to determine experimentally the minimum level of protein that can be incubated with a given quantity of antibody and yet generate an affinity matrix with a suitable level of RNA-binding activity.

In our experiments we have used both vaccinia virus- and baculovirus-based expression systems to prepare the quantities of recombinant NS53 and NS35 necessary to examine their RNA-binding activities by the RNA-capture assay. We have also found that lysates prepared from rotavirus-infected cells can serve as a suitable source of NS35 for the capture assay. While it is true that infected-cell lysates can be used as a source of the protein under study, the limitation of this approach is that it introduces the possibility that a second viral protein, due to its specific affinity for

the immunoadsorbed protein, might contaminate the affinity matrix. The outcome of this contamination may be that the RNA-binding properties of coated beads will be altered in such a way that, while authentically representing the activity of the immunoadsorbed protein complex, they no longer possess the inherent binding activity associated with the protein recognized by the antibody. However, given that most viral RNA-binding proteins appear to be components of multiprotein complexes in the infected cell, the characterization of such complexes by the RNA-capture assay may provide important insight into their function and activity during virus replication, which would not be available using individually expressed recombinant proteins.

The ratio of RNA probe to immunoadsorbed protein used in the RNA-capture assay is of critical importance, especially in experiments designed to examine the specificity of RNA-binding activity by competitive analysis in which it is required that the RNA probe must be in molar excess over the immunoadsorbed RNA-binding protein. An example of the experimental procedure that we used to determine the appropriate ratio of RNA probe to NS53 in the RNA-capture assay is illustrated in Fig. 2A. In brief, a constant amount of the ^{32}P-labeled rotavirus-specific RNA probe, s5–5′ (14), was assayed for the ability to bind to 5–30 ng of immunoadsorbed NS53. The sequence of this probe is identical to the first 278 nucleotides of the gene 5 mRNA of simian rotavirus SA11. The experiment revealed that the amount of probe that bound to the affinity matrix increased only until the level of 25 ng of immunoadsorbed NS53 was reached, indicating that up to this level of the protein, probe was in excess. From this analysis a ratio of 20–25 ng of NS53 per nanogram of s5–5′ probe was routinely used in the RNA-capture assay, as under these conditions not only was probe in excess, but also the absolute level of RNA bound was near maximum.

Incubation Length

The length of incubation required to achieve maximum binding of the RNA probe to the immobilized RNA-binding protein will be affected by the concentrations of the binding protein and the probe, the temperature of incubation, and the volume of the reaction mixture. Because the presence of contaminating RNase activity in binding reactions may lead to significant degradation of the RNA probe, excessive lengths of incubation should be avoided. The kinetics of the binding of the probe, ^{32}P-labeled s5–5′, to immunoadsorbed NS53 at 37°C as a function of time is presented in Fig. 2B. The analysis showed that the formation of NS53–probe complexes in the RNA-capture assay reached maximal levels by 90 min of incubation, but that most complexes (80% or more) were formed during the first 30 min of incubation. The complexes present at 90 min were stable for at least another 90 min of incubation.

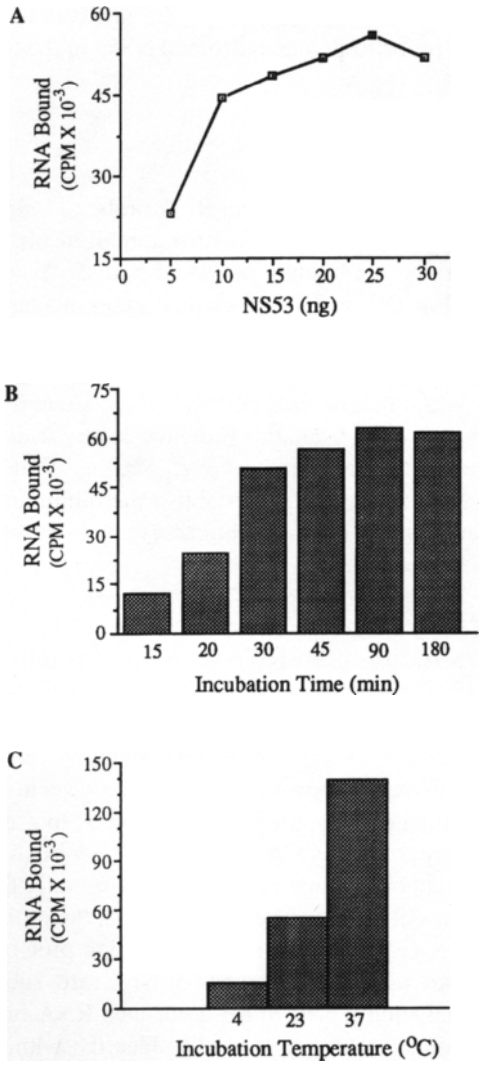

FIG. 2 Parameters affecting the RNA-capture assay. Binding reactions contained 2×10^5 cpm (1–2 ng) of ^{32}P-labeled s5–5′ and, unless otherwise indicated, 20 ng of immunoadsorbed NS53, and were incubated for 40 min at 37° C. Afterwards the beads were recovered from the reactions by centrifugation, washed and assayed for the level of bound probe. (A) Binding of radiolabeled probe as a function of increasing amounts of immunoadsorbed NS53. (B) Binding of radiolabeled probe to immunoadsorbed NS53 as a function of length of incubation. (C) Binding of radiolabeled probe to immunoadsorbed NS53 as a function of incubation temperature.

From these data we routinely used an incubation time of 30–60 min to assay the binding of probe to immunoadsorbed NS53 in the RNA-capture system.

Temperature

The efficiency of binding of an RNA probe to immunoadsorbed RNA-binding protein can be influenced by the temperature of incubation. An example of the effect of temperature on the binding of ^{32}P-labeled s5–5′ to immunoadsorbed NS53 is presented in Fig. 2C, which shows that, upon incubation at 37° C, the level of RNA-binding by NS53 is 2- to 3-fold greater than that observed at 23° C and 6- to 7-fold greater than that observed at 4° C. From such experiments we usually perform RNA-binding assays that involve NS53 at 37° C. However, given that the temperature optimum between RNA-binding protein can vary widely, and for many such proteins is as efficient at low temperatures (e.g., 4° C) as at high temperatures (e.g., 37° C), it is important to experimentally establish the minimum temperature of incubation that can be used in the RNA-binding assays and yet retain maximum levels of binding activity.

Analyzing the Specificity of an RNA-Binding Protein

Identifying a Suitable RNA Probe

In designing experiments to test whether a protein has associated RNA-binding activity, deciding on an appropriate RNA probe to use in an assay system can be difficult. This is particularly true when using RNA-binding procedures (e.g., gel mobility-shift electrophoresis) that generally allow for only the use of relatively short probes, in which case it is not necessarily clear that the probe will indeed contain the appropriate target sequence for the RNA-binding protein. One of the advantages to the RNA-capture assay is that probes of large size, such as full-length mRNAs, can be used in analyzing a protein for associated RNA-binding activity. Additionally, the RNA-capture system can be used to identify, within a mixed population of RNAs, that which contains the recognition signal for the binding protein. In this case, by simply eluting the RNA that binds to the immunoadsorbed protein and then analyzing the RNA by electrophoresis, reverse transcription–polymerase chain reaction (27), cDNA sequencing (28), Northern blot assay (29), or some other technique, it is possible to identify the RNA containing the recognition signal. Such a technique was used initially to determine whether NS53 possessed RNA-binding activity and to ascertain which of the rotavirus mRNAs was recognized by NS53. Specifically, the 11 different species of rotavirus mRNAs were incubated as a mixture with immunoadsorbed NS53 using the RNA-capture assay (14). Subsequent analysis of the

bound RNA revealed that all 11 species contained the recognition signal for NS53. Upon identification of the RNA recognized by an RNA-binding protein, if not already done, it is often useful to prepare a cDNA to the RNA and then, by inserting it in a T7 or SP6 transcription vector, use the cDNA to produce large amounts of the RNA for subsequent binding experiments (25).

Identifying the Recognition Signal of an RNA-Binding Protein

Once a protein is shown to have affinity for RNA, answers to the following two questions are usually pursued: (*a*) Is the binding activity specific or nonspecific?, and (*b*) What is the nature of the recognition signal (target) in the RNA that is recognized by the protein? To address specificity using the RNA-capture system, ^{32}P-labeled probe and varying levels of cold homologous and heterologous RNA are incubated with the immunoadsorbed RNA-binding protein. By measuring the effects of the competitor RNAs on the binding of the radiolabeled probe, it is possible to determine whether the RNA-binding activity of a protein is specific or nonspecific. Proteins such as NS53 that exhibit specific RNA-binding activity will anneal radiolabeled probe in a manner that can be successfully competed away with cold probe but not with unrelated RNA (Fig. 3). In contrast for proteins such as NS35 that exhibit nonspecific RNA-binding activity, radiolabeled probe is competed away by both cold probe and unrelated RNA (Fig. 4).

Two approaches can be used in conjunction with the RNA-capture system to identify the location within an RNA of the target sequence recognized by an RNA-binding protein. The first is to generate a set of radiolabeled probes, which, with respect to the original RNA probe, contain either 5′- or 3′-terminal or internal deletions. The radiolabeled deletion probes are most easily generated by run-off transcription from T7 or SP6 transcription vectors that contain portions of cDNAs of the original probe and are gel-purified prior to use to ensure that they are homogeneous in nature. The purified deletion probes are then assayed in the RNA-capture system for the ability to bind the immunoadsorbed RNA-binding protein. By comparison of the probes that bind with those that do not, it is possible to map the location of the recognition signal within the RNA. Because some proteins that specifically recognize targets within RNA may also possess associated nonspecific binding activity, it is important that the deletion probes be challenged with cold homologous and heterologous RNA to ensure that they are recognized in a manner analogous to that of the original full-length probe. As shown in Fig. 3, this approach revealed that the recognition signal for NS53 resides in the probe s5–5′ and therefore must exist within the first 278 nucleotides of the rotavirus gene 5 mRNA. In contrast, NS35 does not specifically recognize the probe s5–5′ (Fig. 4). Alternatively, the location of the recognition signal in the original probe can be mapped by analyzing the ability of cold deletion probes to reduce, through competition assay, the binding of a radiolabeled

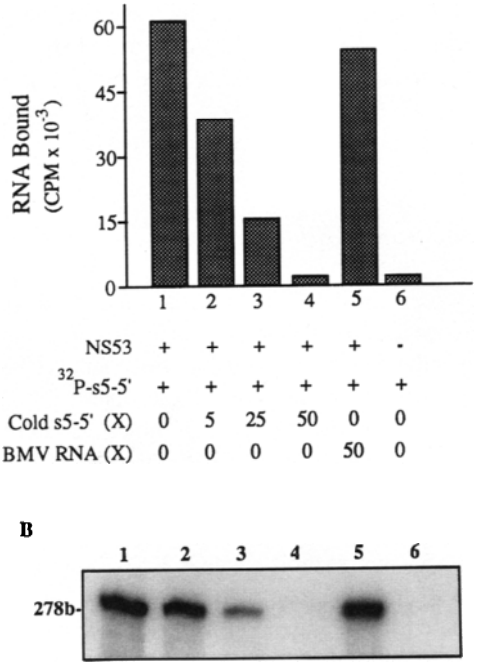

FIG. 3 Analysis of the RNA-binding specificity of NS53. Each binding reaction contained approximately 25 ng of immunoadsorbed NS53 and 6 × 10^5 cpm (1–2 ng) of gel-purified ^{32}P-labeled s5–5′ and was incubated at 37°C for 45 min. As competitor RNA, a molar excess of unlabeled s5–5′ or brome mosaic virus mRNA was added to some reactions. After incubation the beads containing the "captured" RNA were washed three times with RNA-binding buffer and then resuspended in the same buffer, except that it contained 0.8 *M* NaCl. (A) The total level of bound RNA probe was measured by assaying 10-μl aliquots of the resuspended beads for radioactivity. (B) The beads were removed by centrifugation and 30 μl of the supernatant was analyzed for the presence of ^{32}P-labeled s5–5′ by electrophoresis on a 6% polyacrylamide gel containing 8 *M* urea.

full-length probe to the immunoadsorbed RNA-binding protein. One technical advantage of this approach to map the target signal is that it requires the synthesis of only a single radiolabeled probe.

Concluding Remarks

The RNA-capture assay uses immunoadsorbed protein as an affinity matrix to study the activity and specificity of RNA-binding proteins. The nature of the antisera that

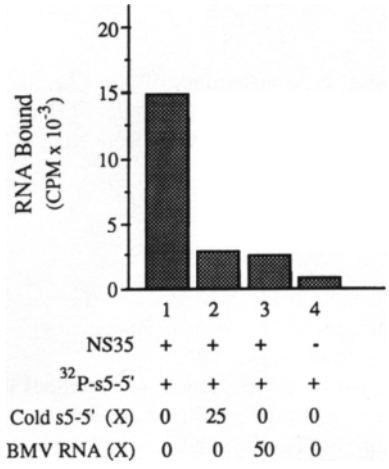

FIG. 4 Detection of nonspecific RNA-binding activity associated with NS35. Binding reactions included 2×10^5 cpm (1–2 ng) of ^{32}P-labeled s5–5′ and in some cases cold s5–5′ RNA or brome mosaic virus mRNA, and were incubated for 40 min at 23° C. The beads were recovered by centrifugation, washed, and assayed for bound radioactivity.

can be used to prepare the affinity column without blocking the RNA-binding activity of the bound protein is diverse, including both peptide-specific antisera and, perhaps surprisingly, polyclonal antisera. By the RNA-capture assay, we were able to show that one rotavirus protein, NS53, possesses specific affinity for viral mRNAs. Given that such an activity was not detected for NS53 by gel mobility-shift electrophoresis or Northwestern blot assay, it is apparent that at least for some proteins, the RNA-capture assay is superior over other assay systems for analyzing RNA-binding activity. It is not clear what advantage the RNA-capture assay imparts to NS53 that allows it to exhibit sequence specificity, but it may be that the mild conditions used to prepare the affinity matrix and the indirect (i.e., antibody-linked) method by which the protein is immobilized to the matrix precludes the denaturation or stearic hindrance of the RNA-binding domain of the protein. Alternatively, the mild conditions may prevent the disruption of NS53 multimers (e.g., dimers) that may be essential for the specific binding activity of the protein. Another strength of the RNA-capture assay concerns the nature of the RNA probes that can be used in the assay system. Not only can they be of unlimited size, but they also can consist of a mixed population of RNAs. The fact that the system can be easily used to isolate those RNAs that possess the recognition signal for an RNA-binding protein from a mixed population suggests that the RNA-capture assay can be a useful tool for characterizing the structure and function of proteins and RNA.

Acknowledgments

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References

1. M. M. Konarska and P. A. Sharp, *Cell (Cambridge, Mass.)* **49,** 763 (1987).
2. J. T. Kadonaga, *in* "Methods in Enzymology" (R. T. Sauer, ed.), Vol. 208, p. 10. Academic Press, San Diego, 1991.
3. L. Hennighausen and H. Lubon, *in* "Methods in Enzymology" (S. L. Berger and A. R. Kimmel, eds.), Vol. 152, p. 721. Academic Press, Orlando, Florida, 1987.
4. K. C. Smith, *in* "Photochemistry and Photobiology of Nucleic Acids" (S. Y. Wang, ed.), p. 187. Academic Press, New York, 1976.
5. A. Hochschild, *in* "Methods in Enzymology" (R. T. Sauer, ed.), Vol. 208, p. 343. Academic Press, San Diego, 1991.
6. M. Dodson and H. Echols, *in* "Methods in Enzymology" (R. T. Sauer, ed.), Vol. 208, p. 168. Academic Press, San Diego, 1991.
7. A. K. Aggarwal, *Methods (San Diego)* **1,** 83 (1990).
8. M. K. Estes and J. Cohen, *Microbiol. Rev.* **53,** 410 (1989).
9. J. T. Patton, *Curr. Top. Microbiol. Immunol.* **185,** 107 (1994).
10. P. Brottier, P. Nandi, M. Bremont, and J. Cohen, *J. Gen. Virol.* **73,** 1931 (1992).
11. J. Hua, E. A. Mansell, and J. T. Patton, *Virology* **196,** 372 1993).
12. J. M. Berg, *Annu. Rev. Biophys. Chem.* **19,** 405 (1990).
13. J. F. Boyle and K. V. Holmes, *J. Virol.* **58,** 561 (1985).
14. J. Hua, X. Chen, and J. T. Patton, *J. Virol.* **68,** 3990 (1994).
15. M. D. Kattoura, X. Chen, and J. T. Patton, *Virology* **202,** 803 (1994).
16. M. D. Kattoura, L. L. Clapp, and J. T. Patton, *Virology* **191,** 698 (1992).
17. M. U. Koster, U. Kuhn, T. Bouwmeester, W. Nietfled, T. El-Baradi, W. Knochel, and R. Pieler, *EMBO J.* **10,** 3087 (1991).
18. T. R. Fuerst, E. G. Niles, F. W. Studier, and B. Moss, *Proc. Natl. Acad. Sci. U.S.A.* **83,** 8122 (1986).
19. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, "Current Protocols in Molecular Biology." Wiley, New York, 1987.
20. J. Hua and J. T. Patton, *Virology* **198,** 567 (1994).
21. U. K. Laemmli, *Nature (London)* **227,** 680 (1970).
22. W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46,** 83 (1974).
23. H. H. Towbin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350 (1979).
24. B. B. Mason, D. Y. Graham, and M. K. Estes, *J. Virol.* **33,** 1111 (1980).
25. E. A. Melton, P. A. Krieg, M. R. Regagliati, T. Manisatis, K. Zinn, and M. R. Green, *Nucleic Acids Res.* **12,** 7035 (1984).
26. T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.

27. E. S. Kawasaki, *in* "PCR Protocols: A Guide to Methods and Applications" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds.), p. 21. Academic Press, San Diego, 1990.
28. F. Sanger, S. Nicklen, and A. R. Coulsen, *Proc. Natl. Acad. Sci. U.S.A.* **74,** 5463 (1977).
29. L. G. Davis, M. D. Dibner, and J. F. Battey, "Basic Methods in Molecular Biology." Elsevier, New York, 1986.

[25] Protein RNA-Binding Activity Measured by Northwestern Blot Analysis: The Interferon-Inducible RNA-Dependent Protein Kinase PKR

Stephen J. McCormack and Charles E. Samuel

Introduction

The Northwestern RNA blot assay is an important method for the identification and initial characterization of RNA-binding proteins. Several viral RNA-binding proteins have been characterized using the Northwestern assay, including proteins encoded by bovine rotavirus (1), human hepatitis delta virus (HDV) (2, 3), mouse coronavirus (4, 5), human immunodeficiency virus (HIV) (6), and human reovirus (7, 8). Cellular RNA-binding proteins likewise have been characterized using the Northwestern assay, including two proteins that bind HIV TAR RNA: a cellular RNA-binding protein of unknown function (9), and the interferon-inducible RNA-dependent protein kinase PKR (also known as P1/eIF-2α, p68, DAI, or dsI) (10, 11). For these viral and cellular RNA-binding proteins (1–11), like the well-established situation for DNA-binding proteins (12), multiple structural motifs exist that constitute functionally different binding domains (13).

The Northwestern RNA blot assay provides an efficient approach for the identification of regions of a protein responsible for its RNA-binding activity. This assay has been utilized in our laboratory at the University of California, Santa Barbara, to localize the RNA-binding subdomains of two proteins: the reovirus σ3 protein and the cellular protein kinase PKR. Truncation of cDNA clones encoding σ3 and PKR and subsequent expression with efficient inducible bacterial systems permitted the production of deletion mutant σ3 and PKR proteins that could be analyzed rapidly for RNA-binding activity (8, 10).

The σ3 protein is the major capsid protein of human reovirus, a segmented double-stranded (ds) RNA virus (14). The 41-kDa σ3 protein binds dsRNA, but not ssRNA or dsRNA (15). However, there is no evidence that the dsRNA binding activity of σ3 is selective for either sequence- or structure-specific dsRNAs (16). Northwestern blot analysis of *Staphylococcus aureus* V8 protease-derived fragments of σ3 localized the dsRNA-binding activity to the C-terminal 16-kDa peptide fragment (7, 8). Northwestern analysis of truncated σ3 proteins expressed from the s4 cDNA in *Escherichia coli* using a modified pET3 vector led to the identification of an 85-amino-acid domain responsible for RNA-binding activity (8).

PKR is an interferon-inducible RNA-dependent protein kinase of central impor-

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tance in the antiviral actions of interferon (17). Activation of PKR requires the presence of a suitable activator RNA and involves the autophosphorylation of PKR. The RNA-binding activity of PKR appears to be selective for structure-specific ssRNAs such as adenovirus VA_I RNA, HIV TAR RNA, and reovirus s1 mRNA (18, 19). Northwestern analysis of substitution and deletion mutant PKR proteins expressed in *E. coli* as TrpE fusion proteins clearly defined the N-terminal 98 amino acids of PKR as both necessary and sufficient for RNA-binding activity (10, 11). Furthermore, a repeated subdomain core of 20 amino acid residues, predicted by computer algorithms to be an amphipathic α-helix, was identified within the N-terminal RNA-binding region of PKR. This core sequence, designated motif R, was present at amino acid positions 55–75 and 145–166 of PKR (10). The RNA-binding R motif is conserved in several viral and cellular proteins from both prokaryotic and eukaryotic sources, proteins now known to be dsRNA-binding proteins (10, 20).

We describe in this article the procedure for analysis of RNA-binding activity by the Northwestern RNA blot assay. The procedure is illustrated with the human RNA-dependent protein kinase PKR.

Strategy

The strategy for measurement of RNA-binding activity by Northwestern analysis involves immobilization of target proteins on a filter membrane. Proteins fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) are electroblotted onto a nitrocellulose filter membrane by standard techniques. The fiber-bound proteins are then analyzed for RNA-binding activity using a radioactive RNA probe; this RNA–protein blot analysis constitutes the Northwestern assay. Subsequently, a Western immunoblot analysis is carried out using the same filter membrane as was used for the Northwestern analysis in order to verify that comparable amounts of test proteins were present. The Western analysis is especially important in the cases of proteins that do not register as RNA-binding proteins in the Northwestern assay.

Materials and Methods

Reagents

The chemicals used in the following procedures are of reagent grade. Solutions are prepared with deionized glass-distilled water. The pH of buffers is measured at 25° C.

Tris–HCl (1 *M*, pH 7.9)
Tris–HCl (1 *M*, pH 7.4)
HEPES (0.5 *M*, pH 7.5)

NaCl (5 *M*)
KCl (3 *M*)
NaOAc (3 *M*)
$MgCl_2$ (1 *M*)
EDTA (0.5 *M*)
2-Mercaptoethanol (14.4 *M*)
Dithiothreitol (DTT; 0.1 *M;* Sigma, St. Louis, MO)
Phenylmethylsulfonyl fluoride (PMSF; 0.2 *M* in ethanol)
Spermidine (0.1 *M;* Sigma)
Nonidet P-40 [NP-40; 10% (w/v); Sigma]
Triton X-100 [10% (w/v); Sigma]
Ficoll 400 [20% (w/v); Sigma]
Polyvinylpyrrolidone [20% (w/v); Sigma]
ATP, GTP, UTP, and CTP (each 0.1 *M;* Sigma)
[α-^{32}P]UTP (650 Ci/mmol; ICN, Costa Mesa, CA)
[^{125}I]Protein A (ICN)
Pansorbin *S. aureus* cells (Calbiochem, La Jolla, CA)
Restriction endonucleases (United States Biochemical, Cleveland, OH)
T4 DNA ligase (Bethesda Research Laboratories, Gaithersburg, MD)
RNase-free DNase (RQ1, Promega, Madison, WI)
RNasin (Promega)
SP6 RNA polymerase (New England Biolabs, Beverly, MA)
Sequenase version 2.0 (United States Biochemical)
T7 RNA polymerase (United States Biochemical)
Thermus aquaticus DNA polymerase I (Boehringer-Mannheim, Indianapolis, IN)
Yeast total RNA (5 Prime–3 Prime, Boulder, CO)
Bovine serum albumin (Sigma)
Nitrocellulose filter membranes (0.45 μm; Schleicher & Schuell, Keene, NH)
Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY)
Fetal bovine serum (FBS; HyClone, Logan, UT)

Vector Constructions

The transcription vector construction pSP6S1(416–576) was previously described (21). The vector construction pT7TAR was provided courtesy of N. Hernandez (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The P1 KIN cDNA clone of human PKR (22) was used for the construction of the pSV(PKR) (11, 23) and pATH–PKR expression plasmids (10, 11).

Expression of TrpE–PKR Fusion Proteins

Freshly transformed *E. coli* C600 cells were used for the expression of the TrpE–PKR fusion proteins essentially as previously described (22, 24). Colonies were grown at 37° C in 2 ml of minimal medium (24) supplemented with 200 μg/ml am-

picillin and 20 μg/ml tryptophan. When the cells were at an optical density of 0.5 measured at 600 nm, a 0.5-ml aliquot of the culture was transferred to a 1.5-ml microfuge tube and the cells were collected by centrifugation at 4000 *g* for 5 min at 4° C. The supernatant solution was discarded and the cells were suspended in 3 ml of minimal medium supplemented with 200 μg/ml ampicillin, but lacking tryptophan. The cells were starved for 3 hr at 37° C in a New Brunswick (New Brunswick, Sci. Co., Edison, NJ) incubator shaker (250 rpm). Indoleacrylic acid (IAA; 5 mg/ml in ethanol) was then added to yield a final concentration of 100 μg/ml, and incubation was continued at 37° C for an additional 3 hr. The IAA-induced cells were collected by centrifugation as above, washed in 1 ml of phosphate-buffered saline (136 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, and 2 m*M* KH_2PO_4), and then used to prepare cell-free extracts. Both whole-cell lysates and the insoluble fraction were analyzed for the presence of expressed PKR protein product.

Isolation of TrpE–PKR Fusion Proteins

To analyze whole-cell lysates for protein expression, 100 μl of either induced or uninduced cells was pelleted and resuspended in 200 μl of 1 × Laemmli buffer (25). The cell suspension was sonicated (three times for 5 sec each) and then boiled for 10 min. Insoluble material was removed by centrifugation for 5 min at 4° C with a microfuge and the supernatant solution was analyzed on an SDS–polyacrylamide (10%, w/v) gel. Proteins were visualized by staining the gel [0.06% (w/v) Coomassie brilliant blue R-250, 10% (v/v) acetic acid, and 25% (v/v) isopropanol] or by Western analysis.

To isolate the insoluble proteins, the whole-cell pellets obtained above were resuspended at 4° C in 20 m*M* Tris–Cl (pH 7.4), 150 m*M* NaCl, 5 m*M* EDTA, 0.5% (w/v) NP-40, 5 m*M* PMSF, and 5 m*M* 2-mercaptoethanol, sonicated (three times for 5 sec each), and microfuged at 14,000 *g* for 10 min at 4° C. The supernatant solution was removed by aspiration, and the insoluble pellet was then resuspended by sonication in 500 μl of the extraction buffer [20 m*M* Tris–Cl (pH 7.4), 150 m*M* NaCl, 5 m*M* EDTA, 0.5% NP-40, 5 m*M* PMSF, and 5 m*M* 2-mercaptoethanol]. This procedure was repeated four times and the final insoluble pellet was then suspended in 500 μl of 1 × Laemmli buffer.

Cell Maintenance and Transfection

Monkey COS-1 cells were grown as monolayer cultures in DMEM supplemented with 5% (v/v) FBS. Transfection was by the diethylaminoethyl (DEAE)–dextran/chloroquine phosphate method (26, 27).

Preparation of the PKR Protein Kinase from Transfected COS Cells

Cell-free extracts were prepared from monkey COS cells at 48 hr after transfection with plasmid constructions prepared using the simian virus 40 (SV40)-based pJC119 vector. Transfected monolayer cell cultures (60-mm dishes) were rinsed twice with 1 ml of PBS, and the cells were lysed at 4° C by the addition of 600 μl of modified

NP-40 lysis buffer [20 m*M* HEPES (pH 7.5), 50 m*M* KCl, 5 m*M* EDTA, 5 m*M* 2-mercaptoethanol, and 1 m*M* PMSF]. PKR proteins were immunoprecipitated using rabbit antiserum raised against recombinant PKR; immune complexes were collected using formalin-fixed *S. aureus* cells. Dilutions of the immunoprecipitated PKR proteins were then fractionated on an SDS–polyacrylamide (10%, w/v) gel and subsequently processed for the Northwestern and Western analyses.

Preparation of RNA Probes

^{32}P-Labeled reovirus s1(416–576) mRNA, adenovirus VA_I RNA, and HIV TAR RNA were prepared as *in vitro* transcripts with bacteriophage polymerases (28) as previously described (21), except that the standard transcription mixture was modified to contain 40 m*M* Tris–Cl buffer (pH 7.9), 6 m*M* $MgCl_2$, 2 m*M* spermidine, 10 m*M* NaCl, 0.5 m*M* ATP, 0.5 m*M* CTP, 0.5 m*M* GTP, 0.2 m*M* UTP, [α-^{32}P]UTP (500 μCi/ml), 10 m*M* DTT, 0.25 mg/ml bovine serum albumin, 250 U/ml RNasin, and 400 U/ml either T7 RNA polymerase or SP6 RNA polymerase as indicated. The vector pSP6S1(416–576) and SP6 RNA polymerase were used to prepare the reovirus s1(416–576) RNA; the vector pT7VA_I and T7 RNA polymerase were used to prepare adenovirus VA_I(1–160) RNA; and the vector pT7TAR and T7 RNA polymerase were used to prepare the HIV TAR(1–82) RNA. Following incubation of the transcription reaction mixture at 37° C (T7 polymerase) or 40° C (SP6 polymerase) for 90 min, RNase-free DNase (5 U) was added and the incubation was continued for an additional 15 min at 37° C, after which the reaction mixtures were extracted with phenol–chloroform (1 : 1) and then chloroform. RNA was chromatographed on a Sephadex (Pharmacia Fine Chemicals, Sweden) scolumn (G150-40) to further remove unincorporated nucleotides. The amount of RNA synthesized was quantitated by trichloroacetic acid precipitation on Whatman (Clifton, NJ) GF/C filter disks; the radioactivity was measured in Aquasol II (DuPont, Boston, MA) with a Beckman LS 1801 liquid scintillation system. The integrity of the RNA was ascertained by electrophoresis under denaturing conditions, using formaldehyde–agarose gels and autoradiography (21, 29).

Northwestern Gel Blot Analysis

Northwestern gel blot analysis for the RNA-binding activity of wild-type and mutant PKR proteins was carried out essentially as described by Boyle and Holmes (1). Briefly, PKR-containing protein samples were fractionated by discontinuous SDS–polyacrylamide slab (1.5 mm thick) gel electrophoresis (25). Proteins were electroblotted to nitrocellulose filter membranes at 4° C (>10 hr at 500 mA) in buffer containing 25 m*M* Tris, 192 m*M* glycine, and 20% (v/v) methanol. Following transfer the filter membranes were washed with water and enclosed in Seal-a-meal bags containing RNA binding buffer [RBB: 50 m*M* HEPES (pH 7.5), 50 m*M* KCl, 0.05% (v/v) Triton X-100, 0.04% (v/v) Ficoll, 0.04% (v/v) polyvinylpyrrolidone, 0.08% (w/v) bovine serum albumin, and 2.5 m*M* EDTA] and 20 μg/ml either human U-cell or *E. coli* total RNA. The filter membranes were pretreated in RBB containing an

RNA blocker at room temperature for 3 hr, with gentle agitation using New Brunswick gyratory shaker G-2. The filters were then incubated at room temperature in RBB with ^{32}P-labeled reovirus s1(416–576) mRNA, ^{32}P-labeled adenovirus RNA, or ^{32}P-labeled HIV TAR RNA as indicated ($>1 \times 10^5$ cpm/ml). Following incubation with the probe, the nitrocellulose membrane filters were removed from the Seal-a-meal bags, washed four times with RBB for 30 sec at room temperature, and then air-dried. Autoradiography was carried out with a screen at $-80°$C using Fuji RX film (Fuju Photo Film Co., Japan).

For the competition experiments filters were pretreated with RBB lacking U-cell RNA; the competitor RNAs were then added at the same time as the probe. Following incubation with the probe–competitor RNA mixture, the nitrocellulose membrane filters were washed (four times for 30 sec each) in RBB at room temperature and then air-dried. Autoradiography was carried out with a screen at $-80°$C.

Western Immunoblot Analysis

Following Northwestern analysis using ^{32}P-labeled TAR RNA or ^{32}P-labeled s1(416–576) RNA, the nitrocellulose filters were routinely washed with water for 30 min at room temperature to remove the RNA probe, which was verified by autoradiography. The filter membrane was then subjected to Western analysis using anti-PKR antibody.

Western immunoblots were performed by the method of Towbin *et al.* (30) essentially as previously described (31). Filter membranes were blocked in PBS containing 0.05% (v/v) Tween 20 (Sigma Chem. Co.) and 5% (w/v) nonfat dry milk at room temperature for 1 hr. Binding of antibody was performed overnight at 4° C with antiserum diluted 1/750 in PBS containing 0.05% (v/v) Tween 20. The filters were washed three times for 20 min at room temperature with PBS containing 0.05% (v/v) Tween 20, then probed with [^{125}I]protein A diluted 1/5000 in PBS containing 0.05% (v/v) Tween 20. The filters were then washed (three times for 20 min each) at room temperature with PBS containing 0.05% (v/v) Tween 20 and air-dried. Autoradiography was then carried out at $-80°$C with a screen. Western signals detected with [^{125}I]protein A and antibody against PKR correlated with the intensity of staining by Coomassie brilliant blue R-250 of the various TrpE–P1 fusion proteins.

Preparation of PKR for Gel Mobility-Shift Analysis

Recombinant PKR protein for RNA gel mobility-shift analysis was prepared from transfected COS cells. COS cells grown as monolayer cultures in 150-mm dishes (Nuclon) with DMEM containing 5% (v/v) FBS were transfected by the DEAE–dextran/chloroquine phosphate method using 40 μg of expression vector DNA in 4 ml of serum-free DMEM. Three culture dishes (150 mm) were routinely transfected with each pSV(PKR) expression vector construction; harvest was at 48 hr posttransfection. Monolayer cultures were washed three times with 5 ml of ice-cold PBS before cell lysis with 3 ml of kinase lysis buffer [containing 20 m*M* HEPES (pH 7.5), 50 m*M* KCl, 5 m*M* EDTA, 0.65% (v/v) NP-40, 5 m*M* 2-mercaptoethanol, and 5 m*M*

PMSF]. All subsequent steps were carried out at 4° C. Ribosomes were separated from S10 extracts and crude 0.8 *M* ribosome salt-wash fractions were prepared essentially as previously described (32), except that the A.12 and A.8 buffers were modified to contain 20 m*M* HEPES (pH 7.5), 120 m*M* or 800 m*M* KCl, 5 m*M* EDTA, 0.1% (w/v) Triton X-100, 5 m*M* 2-mercaptoethanol, and 1 m*M* PMSF. Eluate fractions from the DEAE–cellulose step containing PKR were pooled, frozen in liquid nitrogen, and stored at −80° C until further analysis.

Gel Mobility-Shift Analysis

Gel mobility-shift analysis (32a) was performed essentially as described by Judware and Petryshyn (33) and Gatignol *et al.* (9). The final reaction volume (10 μl) contained approximately 1 ng of the recombinant PKR protein and varying concentrations of ^{32}P-labeled probe RNA (specific activity, $>1 \times 10^7$ cpm/pmol) in 15 m*M* Tris–HCl (pH 7.8), 70 m*M* NaCl, 10 m*M* KCl, 1 m*M* EDTA, 6% (v/v) glycerol, 0.01% (v/v) Triton X-100, 25 ng of ovalbumin, 2 U of RNasin, and 10 ng of yeast total RNA. Reaction mixtures were incubated for 20 min at room temperature before loading onto a native acrylamide (10%) gel (80:1 acrylamide–bisacrylamide) in 0.5× Tris–borate–EDTA buffer. The gel was prerun at 4° C for over 1 hr at 200 V; 40 μl of 2% (v/v) glycerol solution was added to the wells prior to loading the samples. A dye mixture containing 0.05% (w/v) xylene cyanol, 0.05% (w/v) bromphenol blue, and 10% (v/v) glycerol was loaded on the outside lanes of the gel. Electrophoresis was conducted at 280 V for 5 hr or until the xylene cyanol dye was approximately 3 cm from the bottom of the gel. The gels were then dried and subjected to autoradiography.

Discussion

The Northwestern assay is an effective approach both for identifying RNA-binding proteins and for characterizing the subdomain regions within them necessary for RNA-binding activity. The procedures described here for analysis of protein RNA-binding activity measured by the Northwestern blot assay have been used in our laboratory to identify the RNA-binding domain within the interferon-inducible RNA-dependent protein kinase PKR from human cells (10, 11).

Identification of an RNA-Binding Domain within the N-Terminal Region of PKR

The availability of cDNA clones of the PKR kinase provided an opportunity to elucidate the region of PKR responsible for its RNA-binding activity. As an approach to determining the residues of PKR necessary and sufficient for RNA-binding activity, deletion mutants of the P1 KIN cDNA encoding PKR were expressed in *E. coli* as TrpE–P1 fusion proteins (10, 22). The strategy was to determine whether a contiguous subdomain region expressed from the PKR cDNA could convert the bacterial TrpE protein (24) into an RNA-binding protein (10). A similar strategy involving

TrpE fusion proteins was also used to characterize the binding of hepatitis delta antigen to HDV RNA (2).

TrpE–P1 fusion proteins of PKR were examined at varying protein concentrations, as measured by Western immunoblot analysis, for their ability to bind ^{32}P-labeled reovirus s1(415–576) RNA. Reovirus s1(416–576) RNA is known to activate the autophosphorylation of PKR (21), and thus was presumed to bind PKR. As shown by Fig. 1, ^{32}P-labeled reovirus s1 RNA selectively bound to TrpE–P1 fusion

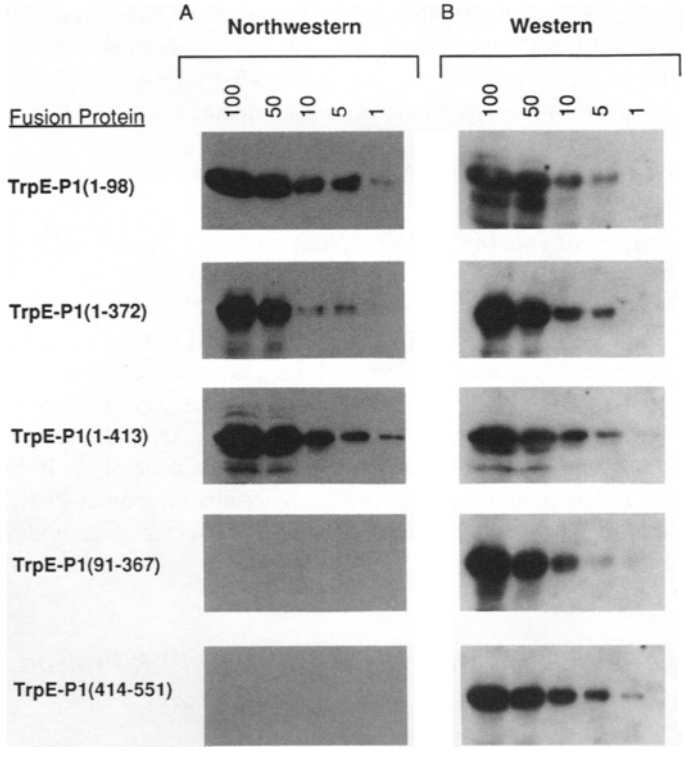

FIG. 1 Effect of protein concentration on the RNA-binding activity of PKR deletion mutant proteins expressed in *E. coli* as TrpE–P1 fusion proteins. (A) Northwestern RNA blot assay using ^{32}P-labeled reovirus s1(416–576) RNA as the probe. (B) Western immunoblot assay carried out with rabbit immune serum generated against recombinant human PKR. Different regions of the P1 KIN cDNA open reading frame, which encodes the human PKR protein kinase (22), were expressed as fusion proteins with TrpE (10). The nitrocellulose fiber membrane containing the TrpE–P1 fusion proteins analyzed in the Northwestern RNA binding assay shown under (A) was stripped and subsequently analyzed in the Western immunoblot assay shown under (B). The amount of fusion protein analyzed (expressed in picomoles) is indicated above the gel lanes. [From S. J. McCormack, D. C. Thomis, and C. E. Samuel, *Virology* **188,** 47 (1992). Reprinted with permission.]

proteins that included the N-terminal region of PKR. The TrpE–P1 fusion proteins that possessed PKR amino acid residues 1–98, 1–372, or 1–413, when immobilized on nitrocellulose, all bound the ^{32}P-labeled reovirus s1 RNA probe. By contrast, TrpE–P1 fusion proteins that lacked the N-terminal 98 amino acid residues but included either the internal or C-terminal regions of PKR or amino acids 91–367 or 414–551, respectively, did not bind the reovirus s1 activator RNA (Fig. 1). In the case of HDV, the middle one third of delta antigen expressed as TrpE fusion was necessary and sufficient for binding of HDV RNA in the Northwestern RNA blot assay (2).

The specificity of RNA-binding detected by Northwestern RNA blot assay can be assessed by the use of competitor RNAs. For example, in the case of PKR, the binding of the ^{32}P-labeled reovirus s1(416–576) RNA to TrpE–P1(1–98) was competed by poly(rI)–poly(rC) but was not competed by total RNA isolated from human U cells (10).

Both Activator and Inhibitor RNAs Bind to the N-Terminal Region of PKR

As shown by Fig. 2, both adenovirus VA_I RNA, an antagonist of PKR kinase activation (34, 35) and HIV TAR RNA, described as both an agonist (36, 37) and an antagonist (38) of the PKR kinase, bound to the same region of PKR, as did the reovirus s1(416–576) RNA, an activator of the kinase (21). Adenovirus VA_I RNA, HIV TAR RNA, and reovirus s1 RNA all bound to the TrpE–P1 fusion proteins that included the N-terminal 98 residues of PKR, whereas none of the RNAs bound to the TrpE–P1 fusion proteins that lacked the N-terminal region of PKR. Finally, the TrpE portion of the fusion protein expressed from vector alone without PKR insert did not bind RNA.

RNA-Binding Activity of Mutant and Wild-Type PKR Proteins Measured by Northwestern Blot and Gel-Mobility Shift Assays

The RNA-binding activity of PKR measured by Northwestern analysis maps to the N-terminal half of PKR within a region that includes the repeated subdomain R motif, the core of which is about 20 amino acids (10). The R motif first identified in human PKR is conserved in many other RNA-binding proteins, is often repeated, and represents the prototype for a new RNA-binding motif with the consensus sequence G–X–G–S/T–K–X–X–A/S–K–X–X–A–A–X–X–A–X–X–X–L (10, 17, 20).

Among the most highly conserved residues of the core R motif is a lysine residue, present at amino acid position 64 within the N-terminal proximal copy of R in PKR (10). In order to assess the importance of the conserved core motif R in the RNA-binding activity of human PKR, lysine-64 was converted to glutamic acid by site-

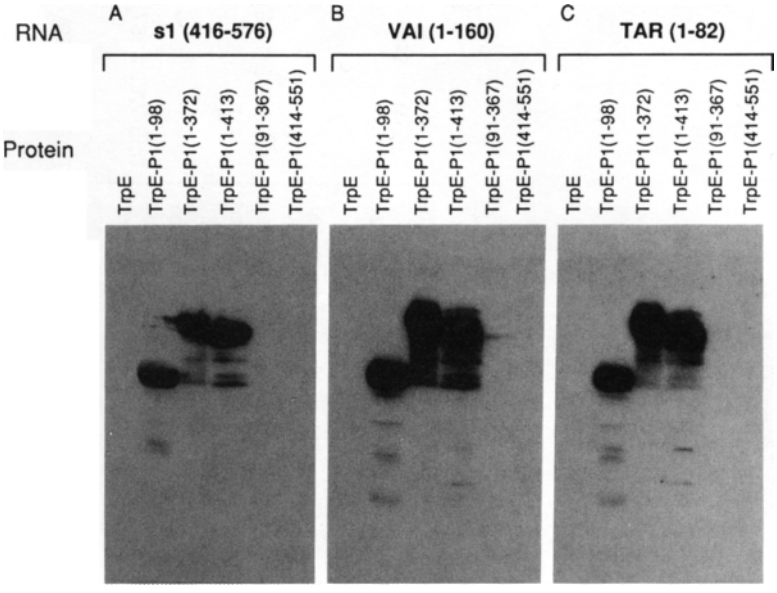

FIG. 2 PKR RNA-binding activity measured with reovirus, adenovirus, and HIV RNA probes. PKR deletion mutant proteins expressed from P1 KIN cDNA as TrpE–P1 fusion proteins were analyzed for RNA-binding activity. (A) Northwestern RNA blot assay using as the probe ^{32}P-labeled reovirus s1(416–576) RNA, an activator of the PKR kinase (21). (B) Northwestern RNA blot assay using as the probe ^{32}P-labeled adenovirus VA_1(1–160) RNA, an inhibitor of PKR kinase activation (19). (C) Northwestern RNA blot assay using as the probe ^{32}P-labeled HIV TAR(1–82) RNA, described as both an activator and an inhibitor of the PKR kinase (18). [From S. J. McCormack, D. C. Thomis, and C. E. Samuel, *Virology* **188,** 47 (1992). Reprinted with permission.]

directed mutagenesis (11). Serial dilutions of TrpE fused with the N-terminal 98 amino acids of PKR, either wild-type (Wt) PKR or the K64E substitution mutant, were first analyzed by the Northwestern assay. Although the TrpE–Wt(1–98) fusion protein efficiently bound RNA (Fig. 3, lanes 1–5), the K64E substitution mutant protein TrpE–K64E(1–98) did not bind RNA, even at concentrations at least 20-fold higher than those of the TrpE–Wt(1–98) protein (Fig. 3, lanes 6–10). The TrpE–Wt(91–367) fusion protein lacking the N-proximal copy of R, but retaining the more C-terminal variant repeat of the R motif, likewise was unable to bind RNA as measured by the Northwestern assay (Fig. 3). The TrpE–Wt(1–372) fusion protein that included both copies of the core motif R efficiently bound RNA (Fig. 3, lane 16). These results suggest that the N-proximal copy of the R motif is both necessary and sufficient to confer RNA-binding activity to TrpE as measured by the Northwestern assay. This result was confirmed by gel-mobility shift analysis of the full-length 551-amino-acid PKR protein (Fig. 4).

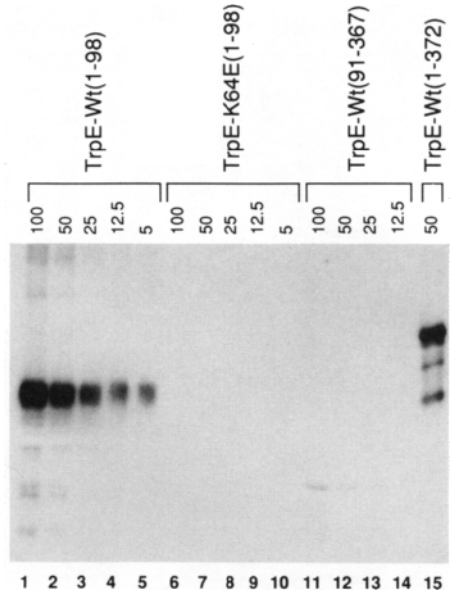

FIG. 3 RNA-binding activity of wild-type (Wt) and mutant PKR proteins expressed in *E. coli* as TrpE–P1 fusion proteins. The Northwestern RNA blot assay was performed with ^{32}P-labeled HIV TAR RNA as the probe. Recombinant PKR proteins expressed from P1 KIN cDNA as TrpE fusion proteins were analyzed as a function of protein concentration; the amount of each protein (in picomoles) is indicated above the gel lanes. [From S. J. McCormack, L. G. Ortega, J. P. Doohan, and C. E. Samuel, *Virology* **198,** 92 (1994). Reprinted with permission.]

The full-length catalytic subdomain II K296R(1–551) mutant, which lacks kinase catalytic activity (23, 39), bound ^{32}P-labeled adenovirus VA_1 RNA in the gel mobility-shift assay (Fig. 4, lane 2), whereas the ability of the K64E/K296R(1–551) double mutant to bind RNA was greatly diminished (Fig. 4, lane 4). Thus, the K64E substitution was sufficient to severely impair RNA-binding activity of the PKR protein, measured both by the Northwestern assay using truncated PKR TrpE fusion protein immobilized on nitrocellulose (Fig. 3) and by the gel mobility-shift assay with full-length PKR protein in solution (Fig. 4).

RNA-Binding Activity of Wild-Type and Mutant PKR Proteins Expressed in Mammalian Cells

The Northwestern blot assay may also be utilized to analyze the RNA-binding activity of proteins present in the crude extracts prepared from mammalian cells. This

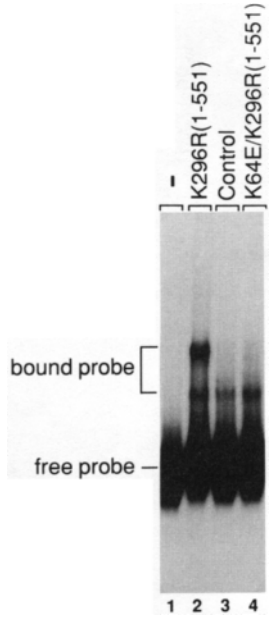

FIG. 4 Gel mobility-shift analysis of recombinant PKR proteins expressed in transfected monkey COS cells. PKR proteins K296R(1–551) and K64E/K296R(1–551), expressed from human P1 KIN cDNAs in monkey COS cells using the pJC119 vector, were analyzed for their ability to bind ^{32}P-labeled VA_1 RNA in a gel mobility-shift assay. The PKR proteins were isolated from the 0.8 *M* KCl ribosomal salt-wash fraction by DEAE–cellulose chromatography. Lane 1, ^{32}P-labeled adenovirus VA_1 RNA probe (6×10^{-9} *M*) alone; lane 2, PKR K296R(1–551) catalytic activity-deficient mutant, with an arginine substituted for the catalytic subdomain II lysine at amino acid position 296 (23); lane 3, control protein sample prepared from COS cells transfected with pJC119 vector containing no PKR cDNA insert; and lane 4, PKR K64E/K296R(1–551) double mutant, with a glutamic acid substituted for the conserved lysine at amino acid position 64 (11) as well as the K296R mutation (23). Comparable amounts (5×10^{-10} *M*) of PKR proteins were present in lanes 1 and 3, as determined by Western immunoblot analysis and comparison to a HIS–K296R(1–551) standard of known concentration.

is exemplified by the results shown in Fig. 5. PKR proteins isolated by quantitative immunoprecipitation from extracts of vector-transfected COS cells were fractionated by SDS–PAGE, transferred to nitrocellulose, and subjected to sequential Northwestern and Western blot analyses. The same nitrocellulose filter blot was first used to measure the RNA-binding activity of PKR proteins by Northwestern RNA blot analysis with ^{32}P-labeled RNA probe; then, after removal of the RNA probe, the amount of PKR protein present on the filter membrane was quantitated by Western analysis with [^{125}I]protein A and polyclonal antibody probe against PKR.

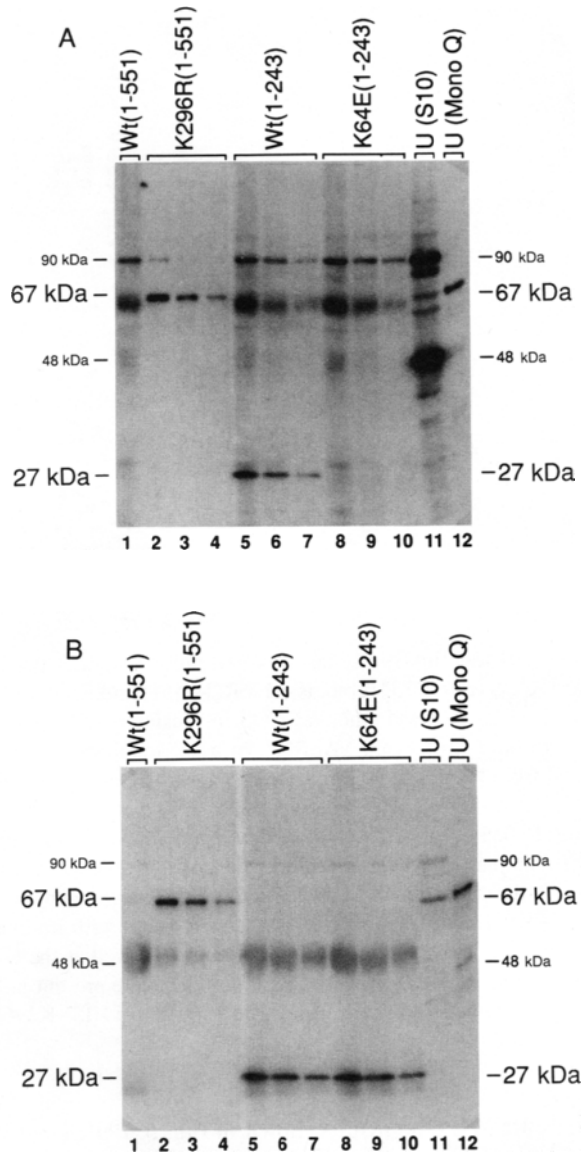

FIG. 5 Effect of protein concentration on the RNA-binding activity of wild-type and mutant PKR proteins expressed in transfected monkey COS cells. The SV40-based vector pJC119 was used to express wild-type (Wt) and mutant forms of the P1 KIN cDNA in monkey COS cells. At 48 hr after transfection, cell-free extracts were prepared and the PKR proteins were immunoprecipitated with anti-PKR antibody. The immunoprecipitates were then analyzed by (A) Northwestern RNA blot assay for RNA-binding activity and (B) Western immunoblot assay for amounts of PKR protein. *Dilution* refers to the fold-dilution prior to SDS–PAGE of

As shown in Fig. 5A, the 67-kDa catalytic subdomain II K296R(1–551) mutant PKR protein (lanes 2–4) and the 27-kDa C-truncated Wt(1–243) PKR protein (lanes 5–7) both retained RNA-binding activity in the Northwestern assay. By contrast, the 27-kDa C-truncated K64E(1–243) substitution mutant did not bind RNA (lanes 8–10). The Northwestern RNA-binding signal (Fig. 5A) was dependent on the concentration of the K296R(1–551) and Wt(1–243) PKR proteins and correlated with their Western signals (Fig. 5B). The K64E(1–243) mutant protein did not display detectable RNA-binding activity at concentrations comparable to those of Wt(1–243) that efficiently bound RNA. Because full-length Wt(1–551) is expressed inefficiently due to translational autoregulation (23, 40), the Western and Northwestern signals are difficult to discern over background (Fig. 5, lane 1). As internal standards, crude S10 extract prepared from interferon-treated human U cells (lane 11) and PKR isolated from the ribosomal salt wash from interferon-treated U cells (lane 12) were included. Both samples contained a 67-kDa RNA-binding protein. The relatively abundant immunologically cross-reactive 90-kDa RNA-binding protein present in crude extracts (lane 11) from human cells (23, 41) was not detected with the partially purified PKR preparation (Fig. 5, lane 12). Other investigators, using the Northwestern assay to analyze homogenates of human brain tissue, also detected a 90-kDa RNA-binding protein (42).

Concluding Comments

The Northwestern RNA–protein blot assay represents one approach to identifying RNA-binding proteins. The Northwestern assay also represents an efficient method for rapidly screening mutant proteins, with the objective of defining the subdomain responsible for RNA-binding activity. This is especially true when a cDNA encoding the protein of interest is available. The RNA-binding analysis of proteins can be facilitated by the use of inducible bacterial systems that permit the overexpression of nested-set deletion mutants of the target protein (2, 8, 10). The Northwestern assay may also be used to examine the *in vitro* RNA-binding specificity of proteins (2, 3,

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the immunoprecipitate derived from 100 μl of lysate. Lane 1, Wt(1–551) at 1/1 (v/v) dilution; lanes 2–4, K296R(1–551) mutant at 1/10, 1/20, and 1/40 (v/v) dilutions, respectively; lanes 5–7, truncated Wt(1–243) at 1/1, 1/2, and 1/4 (v/v) dilutions, respectively; lanes 8–10, truncated K64E(1–243) at 1/1, 1/2, and 1/4 (v/v) dilutions, respectively; lane 11, S10 cell-free extract prepared from interferon-treated human U cells; and lane 12, PKR kinase purified by Mono Q FPLC chromatography (Waters-Millipore, Bedford, MA) from the ribosomal salt-wash fraction of interferon-treated U cells. The positions of the 67-kDa full-length PKR protein and the truncated 27-kDa PKR protein, as well as the positions of the endogenous 90-kDa and 48-kDa RNA binding proteins, are indicated at the sides of the autoradiograms. [From S. J. McCormack, L. G. Ortega, J. P. Doohan, and C. E. Samuel, *Virology* **198,** 92 (1994). Reprinted with permission.]

10). However, because RNA-binding activity as measured by the Northwestern assay is sensitive to pH, false-positive responses may occur at pH below 6.5 (8). Also, because the candidate target proteins first are resolved by SDS–PAGE, the Northwestern assay may not be suitable for proteins whose RNA-binding activity depends on a dimeric structure, particularly a heterodimeric structure in which the two subunits possess different mobilities in SDS–PAGE gels. Therefore, it is important to attempt to confirm RNA-binding results obtained by the Northwestern RNA blot assay with results obtained by a second independent approach, for example, the gel mobility-shift assay (32a).

Acknowledgments

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References

1. J. F. Boyle and K. V. Holmes, *J. Virol.* **58,** 561 (1986).
2. J.-H. Lin, M.-F. Chang, S. C. Baker, S. Govindarajan, and M. M. C. Lai, *J. Virol.* **64,** 4051 (1990).
3. M. Chao, S.-Y. Hsieh, and J. Taylor, *J. Virol.* **65,** 4057 (1991).
4. S. G. Robbins, M. F. Frana, J. J. McGowan, J. F. Boyle, and K. V. Holmes, *Virology* **150,** 402 (1986).
5. S. A. Stohlman, R. S. Baric, G. N. Nelson, L. H. Soe, L. M. Welter, and R. J. Deans, *J. Virol.* **62,** 4288 (1988).
6. C. Aepinus, R. Voll, M. Broker, and B. Fleckenstein, *FEBS Lett.* **263,** 217 (1990).
7. L. A. Schiff, M. L. Nibert, M. S. Co, E. G. Brown, and B. N. Fields, *Mol. Cell. Biol.* **8,** 273 (1988).
8. J. E. Miller and C. E. Samuel, *J. Virol.* **66,** 5347 (1992).
9. A. Gatignol, C. Buckler, and K.-T. Jeang, *Mol. Cell. Biol.* **13,** 2193 (1993).
10. S. J. McCormack, D. C. Thomis, and C. E. Samuel, *Virology* **188,** 47 (1992).
11. S. J. McCormack, L. G. Ortega, J. P. Doohan, and C. E. Samuel, *Virology* **198,** 92 (1994).
12. J. W. Mattaj, *Cell (Cambridge, Mass.)* **73,** 837 (1993).
13. P. J. Mitchell and R. Tijan, *Science* **245,** 371 (1989).
14. W. K. Joklik, "The Reoviridae." Plenum, New York, 1984.
15. H. Huismans and W. K. Joklik, *Virology* **70,** 411 (1976).
16. B. N. Fields, "Fundamental Virology." Raven, New York, 1990.
17. C. E. Samuel, *J. Biol. Chem.* **268,** 7603 (1993).
18. C. E. Samuel, *Virology* **183,** 1 (1991).
19. M. B. Mathews and T. Shenk, *J. Virol.* **65,** 5657 (1991).
20. D. St. Johnston, N. H. Brown, J. G. Gall, and M. Jantsch, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10979 (1992).

21. J. R. Bischoff and C. E. Samuel, *Virology* **172,** 106 (1989).
22. D. C. Thomis, J. P. Doohan, and C. E. Samuel, *Virology* **188,** 33 (1992).
23. D. C. Thomis and C. E. Samuel, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 10837 (1992).
24. C. L. Dieckmann and A. Tzagoloff, *J. Biol. Chem.* **260,** 1513 (1985).
25. K. H. Levin and C. E. Samuel, *Virology* **106,** 1 (1980).
26. H. Luthman and G. Magnusson, *Nucleic Acids Res.* **11,** 1295 (1983).
27. S. M. Munemitsu and C. E. Samuel, *Virology* **163,** 643 (1988).
28. D. A. Melton, P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green, *Nucleic Acids Res.* **12,** 7035 (1984).
29. J. P. Doohan and C. E. Samuel, *Virology* **186,** 409 (1992).
30. H. Towbin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350 (1979).
31. N. Ulker, X. T. Zhang, and C. E. Samuel, *J. Biol. Chem.* **262,** 16798 (1987).
32. C. E. Samuel, G. S. Knutson, M. J. Berry, J. A. Atwater, and S. R. Laskey, *in* "Methods in Enzymology" (S. Pestka, ed.), Vol. 119, p. 499. Academic Press, Orlando, Florida, 1986.
32a. S. J. McCormack and C. E. Samuel, *Virology* **206,** 511 (1995).
33. R. Judware, J. Li, and R. Petryshyn, *J. Interferon Res.* **13,** 153 (1993).
34. J. Kitajewski, R. J. Schneider, B. Safer, S. M. Munemitsu, C. E. Samuel, B. Thimmappaya, and T. Shenk, *Cell (Cambridge, Mass.)* **45,** 195 (1986).
35. R. O'Malley, T. Mariano, J. Siekierka, and M. B. Mathews, *Cell (Cambridge, Mass.)* **44,** 391 (1986).
36. D. N. SenGupta and D. N. Silverman, *Nucleic Acids Res.* **17,** 969 (1989).
37. I. Edery, R. Petryshyn, and N. Sonenberg, *Cell (Cambridge, Mass.)* **56,** 303 (1989).
38. S. Gunnery, A. P. Rice, H. D. Robertson, and M. B. Mathews, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 8687 (1990).
39. G. N. Barber, J. Tomita, A. G. Hovanessian, E. Meurs, and M. G. Katze, *Biochemistry* **30,** 10356 (1991).
40. G. N. Barber, M. Wambach, M.-L. Wong, T. E. Dever, A. G. Hinnebusch, and M. G. Katze, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 4621 (1993).
41. A. P. Rice, M. Kostura, and M. B. Mathews, *J. Biol. Chem.* **264,** 20632 (1989).
42. X. Chen, J. Sadlock, and E. A. Schon, *Biochem. Biophys. Res. Commun.* **191,** 18 (1993).

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Methods in Molecular Genetics, Volume 5, Article 5

Analysis of T-Cell Receptor and Immunoglobulin Transcripts by Nonpalindromic Adaptor Polymerase Chain Reaction
by
Chris D. Platsoucas, Pei-Feng Chen, and Emilia L. Oleszak

On page 83, the 5′ mixed end primers described for the amplification of the μ-chain transcripts by classical two-sided PCR were incorrect. The correct 5′ mixed end primers used for the successful amplification of the μ-chain cDNAs by classical two-sided PCR were ATGGACTGGACCTGGAGG(A/G)TC(C/T)TCT(G/T)C, and were originally reported by Larrick *et al., Biochemical, Biophysical. Research. Communication.* **160,** 1250 (1989) (Reference 59), as were the 5′ mixed end primers incorrectly described. These results are described in detail in a paper by Chen *et al.* published in *Human Antibodies and Hybridomas* **5,** 131–142 (1994).

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