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SV40 Protocols

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Propagation of Wild-Type and Mutant SV40

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1. Introduction

Simian virus 40 (SV40) is routinely propagated in established kidney cell lines derived from the African green monkey ***(1)***. Generally, one of two cell lines, BSC-1 or CV1, are used for this purpose ***(2,3)***. Both of these cell lines are easy to maintain in culture and can be readily frozen and recovered from frozen storage. Wild-type SV40 grows and plaques equally well in both cell lines.

Because SV40 uses cellular machinery to carry out many steps in viral infection, it has proved to be a powerful probe of molecular mechanisms of transcription, DNA replication, and growth control. SV40 subverts cellular systems toward virus production by acting on key regulatory cellular targets and pathways. The analysis of SV40 mutants that are unable to act upon specific cellular targets has proved extremely useful in identifying key targets and understanding how the virus alters their function ***(4,5)***. In this chapter, we describe methods for propagating and assaying infectious SV40. We also briefly outline the routine assays used in our laboratory to assess the effects of specific mutations on viral productive infection, DNA replication, and transformation.

2. Materials

1. BS-C-1 or CV-1 cells: ATCC (Rockville, MD) cat. no. CCL-26 or CCL-70, respectively.
2. Minimum essential medium (Gibco-BRL, Grand Island, NY) supplemented with 10% or 2% fetal bovine serum (FBS, Hyclone), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco-BRL).
3. 1.8% Bacto-Agar (Difco) in distilled water. Autoclave and cool to 45°C.

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4. 2X Modified Eagle's medium (MEM) (without phenol red) (Gibco-BRL) supplemented with 10% FBS and 200 U/mL penicillin and 200 µg/mL streptomycin (Gibco-BRL).
5. Neutral Red solution, 3.33 g/L (Gibco-BRL).
6. Diethylaminoethyl (DEAE) Dextran (Sigma) solution: 1 mg/mL in 2X MEM.
7. Qiagen plasmid purification columns (Qiagen Inc., Valencia, CA).
8. Transfectamine (Gibco-BRL).

3. Methods

3.1. Cell Culture

1. Culture BSC-1 or CV-1 cells as monolayers in MEM-10% FBS at 37°C in a humidified incubator with 5% carbon dioxide and 95% air.
2. Change medium every 3–4 d and split cells prior to confluence.

3.2. SV40 Virus Stock Production

1. Prepare 80% to 100% confluent T75 flasks of BS-C-1 or CV-1 cells (*see* **Note 1**).
2. Remove medium from dishes and replace with 2 mL of MEM-2% FBS containing the appropriate dilution of SV40 plaque forming units (PFU's) to achieve a multiplicity of infection (MOI) between 0.1 and 0.01 (*see* **Note 2**).
3. Allow infection to proceed for 2 h in an incubator while rocking plates at 15-min intervals to assure complete coverage and even distribution over the monolayer.
4. After this period, MEM-2% FBS should be added to each plate to obtain a final volume of 10 mL of medium per 10-cm plate.
5. After 4 d of incubation, the medium should be replaced with fresh MEM-2% FBS. After this point, cells should be checked daily for signs of cytopathic effects (CPE) by comparing infected monolayers to the uninfected control (*see* **Note 3** and **Fig. 1**).
6. When CPE develops to the point of complete destruction of the monolayer with floating clumps of cells, place flasks at –20°C overnight or until completely frozen and then thaw at room temperature. Repeat for a total of three freeze/thaw cycles.
7. This is the viral stock. It will contain cellular debris, which can be removed by centrifugation at 200 RCF for 5 min if desired.

3.3. Titering SV40 by Plaque Assay

1. Prepare serial dilutions of the SV40 virus stock in MEM-2% FBS by putting 20 mL of stock solution into 2 mL of MEM-2 and vortex vigorously (10^{-2} dilution). Repeat this procedure using the immediately preceding dilution in place of the stock to make 10^{-4} and 10^{-6} dilutions. 10^{-7} and 10^{-8} dilutions should be made by using 200 mL of the preceding dilutions in 2 mL of MEM-2% FBS (*see* **Note 4**).
2. Use 1 mL of each dilution to infect 6-cm dishes of freshly confluent dishes of BS-C-1 or CV-1 after removing the old medium. Be sure to include a dish with 1 mL of MEM-2% FBS without virus as mock control.

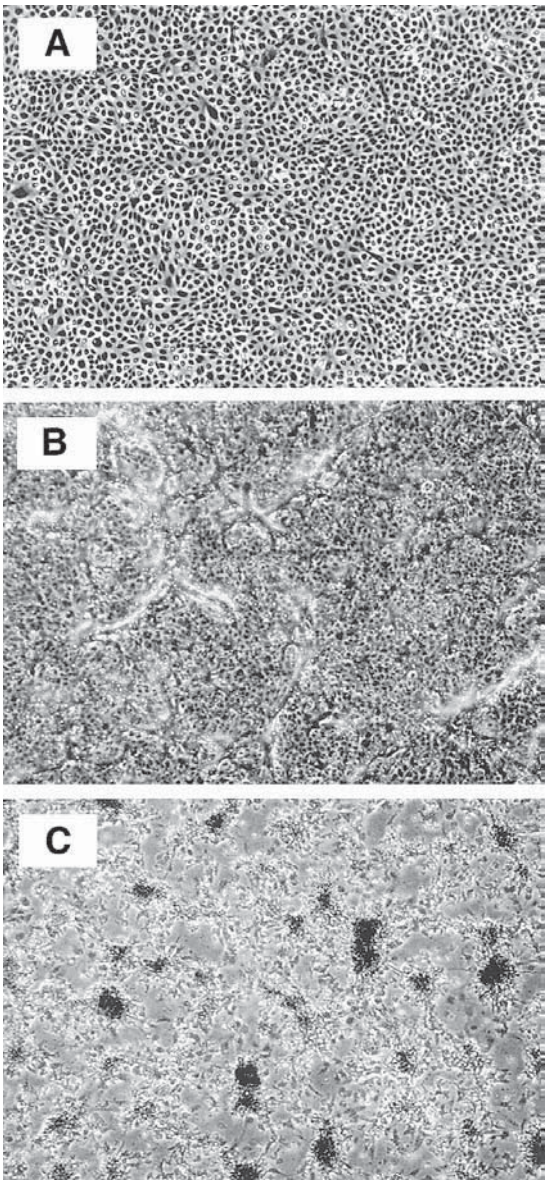

Fig. 1. Cytopathic effect of BS-C-40 cells infected with SV40. (**A**) Confluent monolayer of BS-C-40 cells 17 h after infection. After 7 d, a CPE is clearly discernible by comparing mock (**B**) with SV40 (**C**) infections.

3. As in virus stock production, dishes should be incubated at 37°C for 2 h with rocking at 15-min intervals to assure even coverage of the monolayer.
4. After this 2-h period, remove the 1 mL of MEM-2 and overlay the monolayers with 4 mL of a fresh mixture containing 1 part melted 1.8% Bacto-Agar cooled to 45°C and 1 part 2X modified Eagle's medium (without phenol red) with 10% FBS at 37°C. Allow the overlay to harden at room temperature for approx 15 min before returning dishes to the incubator (*see* **Note 5**).
5. Add an additional 3 mL of this mixture every 3 d with the exception of the sixth day, when neutral red should be added to the overlay mixture at a final concentration of 50 µg/mL, in order to visualize the plaques (*see* **Note 6**).
6. Plaques should be visible and can be counted on the eighth day and beyond. The number of plaques times the inverse of the dilution factor gives the titer of the virus stock in pfu/mL.

3.4. Strategies for Analyzing SV40 Mutants

SV40 mutants continue to be generated for a variety of purposes. We will not review the various methods used for selecting for SV40 mutants in vivo or constructing them in vitro. Rather, we briefly describe the routine assays our laboratory uses to assess the effects of mutations on virus growth and transformation. It is important to realize that the assays described here represent a preliminary analysis of mutant activity. More thorough studies are needed to understand the basis for each mutant phenotype.

Following sequencing to confirm the nature of the mutation, we perform three tests to assess mutant activity. First, a viral plaque assay is performed as aforementioned. In this test, the starting material is viral DNA introduced into cells by transfection, rather than virus particles. In cases where the mutant virus is defective for plaque formation, we attempt to rescue the mutant phenotype by complementation with a characterized defective SV40 mutant. Thus, if the mutation of interest maps to T antigen, it should be rescued by any defective SV40 mutant that expresses a wild-type T antigen. For example, deletion mutant dl 1007, lacking late regions coding for viral coat proteins can complement deletions in early regions, such as mutant 3213, which fails to bind to the tumor suppressor *pRb* ***(4)***. Second, we assess the ability of the mutant to replicate viral DNA. Finally, we perform a focus formation assay to test the transforming ability of the mutant.

3.5. Analysis of Mutants

3.5.1. Transfection and Plaque Assays

1. Transfect freshly confluent 6-cm plates of BS-C-1 cells using 100 mL of 1 µg/mL DEAE Dextran in 2X MEM mixed with equal parts of water containing 10 to 30 ng of purified DNA. Carefully add this mixture to the center of each plate after removal of the old medium.

2. After 15 min, overlay cell monolayers as described in **steps 4–6** of **Subheading 3.3**.
3. Plaques should be scored by number, size, and time of appearance as compared to wild-type SV40.

3.5.2. Complementation Assays

1. Complementation assays are carried out as above (*see* **Subheading 3.5.1.**) except that equal quantities of the two virus mutants to be tested are mixed and added to cells at once.

3.5.3. In Vivo DNA Replication Assay

1. Transfect freshly confluent 6-cm dishes of BS-C-1 cells with 45 ng of wild-type SV40 DNA or 45 ng of each mutant DNA using the DEAE Dextran method aforementioned with an 800 µL total volume.
2. After 15 min, feed dishes with 10 mL of MEM-2% FBS and return to the incubator for 2 or 3 d or until the desired time-point.
3. Trypsinize cells from the plates and extract DNA using Qiagen miniprep columns, according to the manufacturer's instructions.
4. Quantitate extracted DNA and then digest it with *Bcl*I to linearize newly replicated DNA, and with *Dpn*I to remove any residual nonreplicated input DNA (*see* **Note 7**).
5. Separate DNA extracts by gel electrophoresis on a 0.8% agarose gel.
6. Transfer to a nylon membrane and hybridize with a ^{32}P-labeled probe synthesized using random primers on an SV40 genomic or cloned DNA template (***6***, *see* Chapter 12).

3.5.4. Dense Focus Assay for Transformation

1. Transfect subconfluent plates of REF 52 cells with 2 µg of each DNA using Transfectamine according to the manufacturer's instructions (*see* **Note 8**).
2. On the following day, rinse the Transfectamine and DNA from the cells and replace the culture medium.
3. After 24 to 48 h, split cultures 1:3 and feed twice weekly with MEM-10% FBS.
4. After 4 to 6 wk, stain plates with crystal violet and count foci using cells subjected to mock transfections (e.g., vector alone) for comparison (*see* **Fig. 2B**).

For more details on transformation assays, *see* Chapter 11.

4. Notes

1. SV40 virus stock production procedures can easily be scaled up or down to any size or number of dishes.
2. The MOI is the ratio of plaque-forming units (PFUs, i.e., number of infectious particles) to the number of cells being infected. The most important parameter for production of virus stock is keeping the MOI low. This is important to prevent the replication of spontaneously occurring defective mutant viruses in the stock. High MOI infections allow replication of defective viruses because they are complemented in *trans* by wild-type viruses infecting the same cell. Another

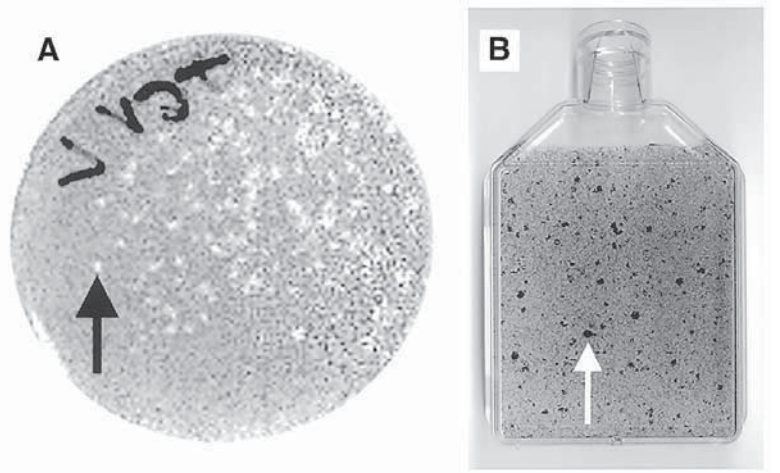

Fig. 2. SV40 viral plaques and dense foci. **(A)** Plaques visible on a 60-mm diameter agar plate after more than 2 wk of growth. The arrow indicates an individual plaque. **(B)** A 75-cm^2 flask stained for foci after 6 wk of culture. The arrow indicates an individual dense focus.

important practice to reduce the occurrence of defective viruses in the stock is to avoid serial passage of the virus when preparing new stocks. Because a relatively small amount of virus is needed to produce a stock, it is best to have one master virus stock which is used exclusively to produce working virus stocks for use in experimental infections.

The next consideration after the MOI is the volume of medium used in the infection. Ideally, the volume should be kept as low as possible while still completely covering the monolayer of cells being infected (approx 0.5 mL for a 3-cm plate, 1 mL for a 6-cm plate, 2 mL for a 10-cm plate). This keeps the virions in close proximity to the monolayer, promoting rapid absorption of the virions to the cell surface and into the cells. Under these conditions most of the virus particles will have entered the cells of the monolayer within the 1.5 h of incubation at 37°C.

3. The cytopathic effect associated with lytic SV40 infections in BS-C-1 cells can be separated into four stages (**Fig. 1**). Stage 1: some rounded, refractile cells appearing on the monolayer. Stage 2: many rounded, refractile cells on the monolayer with some forming groups. Stage 3: clumping of cells and the beginning of deterioration of the monolayer. Stage 4: complete destruction of the monolayer with floating clumps of cells. Some cells, such as CV1 show large vacuoles late after infection.
4. Precise serial dilution is a key step in SV40 stock titering. It is critical that each dilution be thoroughly mixed before it is used to make the subsequent dilutions. It is also important to make the serial dilutions immediately before they are used because the virus in very dilute solutions tends to be less stable.

5. Another critical step in titering plaque assays is the preparation of the overlay mixture. The key is to work quickly. The agar/medium mixture must not be too hot when placed on the cells, so that it will start solidifying very soon after being mixed together. If the mixture begins to harden before it is placed on the cells, the overlay will have a rough texture which will make the plaques harder to see. Also it is important to avoid bubbles in the agar/medium overlay.
6. Although one 3-mL agar/medium overlay with 50 mg/mL of neutral red is sufficient to visualize plaques, the amount of neutral red can be increased in order to improve the contrast between living cells that take up the red color and plaques of dead cells that do not (*see* **Fig. 2A**). It is also important to note that a full 24 h is often required for the neutral red to migrate through the agar/medium overlay and evenly stain the monolayer.
7. There is a single *Bcl*I site in the SV40 genome. Thus, digestion with this enzyme linearizes the circular genome DNA molecule, allowing for a single-band signal during Southern blotting using a genomic SV40 probe. *Dpn*I digests only methylated DNA, The nonreplicated DNA (i.e., input DNA) is of bacterial origin and will therefore be methylated, unlike genomic SV40 DNA replicated in the host cell. *Dpn*I will therefore selectively digest only the DNA added experimentally as original SV40 template ***(6)***.
8. In our hands, transfectamine is at least as sensitive as DEAE-dextran or calcium phosphate and allows the use of smaller amounts of DNA than is possible with these methods.

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2

Propagation and Assay of the JC Virus

Christine K. Liu and Walter J. Atwood

1. Introduction

The human polyomavirus, JCV, is the etiological agent of a fatal central nervous system (CNS) demyelinating disease known as progressive multifocal leukoencephalopathy, or PML. Seroepidemiological studies have indicated that greater than 70% of the human population worldwide is infected with JCV. Like other polyomaviruses, JCV establishes a lifelong latent or persistent infection in its natural host ***(1–5)***. Reactivation of JCV in the setting of an underlying immunosuppressive illness, such as AIDS, leads to virus dissemination to the CNS, infection of oligodendrocytes, and the development of PML ***(1,2,6,7)***. Not surprisingly, the incidence of PML has increased dramatically as a result of the AIDS pandemic. A recent epidemiological study has found that PML increased twentyfold from 0.2 cases per million people in 1979 to 3.3 cases per million people in 1994 ***(8)***. The increase in PML has renewed an interest in studying the biology of this common human polyomavirus.

JCV was initially isolated from the brain of a patient with progressive multifocal leukoencephalopathy whose initials were J.C. ***(9)***. Isolation was achieved by incubation of primary human fetal glial cells (PHFG) with extracts prepared from PML brain taken at necropsy. PHFG cells and a few cell lines derived from these cultures are the only cell types that are fully permissive to the lytic growth of JCV. This chapter will focus on the methods and techniques currently used to propagate and assay this virus in tissue culture.

2. Materials

2.1. Cell Culture

1. Cells: The human glial cell line, SVG is available from the American Type Culture Collection (ATCC) (Rockville, MD), cat. no. CRL-8621 (*see* **Note 1**).

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2. Eagle's Minimal Essential Medium (EMEM, Mediatech, Gaithersburg, MD) supplemented with 10% heat inactivated fetal calf serum (FCS) (Mediatech) and 100 U of penicillin/streptomycin (Gibco-BRL, Gaithersburg, MD). Store the medium at 4°C, and let the solution come to room temperature before adding to the cells. The complete medium is stable for about 60 d, as after this period the serum proteins will begin to degrade and the effectiveness of the antibiotics will diminish (*see* **Note 2**).
3. JCV stock: ATCC, cat. no. VR-1397 (Mad-4 strain) or cat. no. VR-819 (Mad-1 strain). Store at –80°C or in liquid nitrogen (*see* **Note 3**).
4. Trypsin-ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin and 0.53 m*M* EDTA-4Na) (Gibco-BRL).
5. Sterile phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4, pH 7.2; sterilize by autoclaving.
6. 2.5% deoxycholic acid (DOC). Filter sterilize.
7. Receptor destroying enzyme: Crude neuraminidase from *Vibrio cholerae*, 0.2 U/mL (Calbiochem, La Jolla, CA).

2.2. Hemagglutination Assay

1. 96-well U-bottom plate.
2. PBS (*see* **item 5** in **Subheading 2.1.**).
3. Type O human blood collected in a Vacutainer tube containing anticoagulant (*see* **Note 4**).
4. Alsever's solution: 0.1 *M* D-glucose (dextrose), 0.027 *M* sodium citrate, 0.07 *M* sodium chloride. Adjust pH to 6.5 with citric acid and incubate for 30 min at room temperature. Filter sterilize.
5. RBC Fix (ISOLAB, Inc. Akron, OH, cat. no. RS-3200), and RBC Wash (ISOLAB, Inc. cat. no. RS-3100).

2.3. Indirect Immunofluorescence Assay

1. Six-well tissue-culture dishes.
2. Sterile 18-mm glass coverslips.
3. Monoclonal antibody directed against JCV V antigen (Novocastra Laboratories, Newcastle upon Tyne, UK, cat. no. NCL-JC). A rabbit polyclonal anti-SV40 V antigen antiserum (Lee Biomolecular Research, San Diego, CA, cat. no. 6201), or a monoclonal antibody against SV40 T antigen (PAB416-AB2, cat. no. DP02) which crossreacts with JCV T antigen, can also be used (*see* **Note 5**).
4. Goat antimouse secondary antibody conjugated to a fluorogenic dye such as fluorescein isothiocyanate (FITC). If the polyclonal serum was used as a primary antibody, then the secondary must be antirabbit. Both can be obtained from a number of sources.
5. PBS (*see* **item 5** in **Subheading 2.1.**).
6. Copplin jars or other suitable staining dish.
7. Fixative: There are many fixatives that can be used. The two we use routinely are acetone and 70% ethanol/30% 1X PBS (v/v): Mix 100% ethanol with the appro-

priate amount of PBS and mix by gentle shaking. This will result in a slightly cloudy solution. If there is a precipitate in the solution, do not use it. This solution should be made up fresh before each use.
8. Evans blue as a counterstain, if you use FITC as the fluorochrome. Make a solution of 0.01% w/v in PBS.
9. Mounting medium: 90% glycerol/10% PBS, or a commercially available mounting medium such as Permount (Sigma, St. Louis, MO).

3. Methods

3.1. Cell Culture

1. Culture SVG cells as an adherent monolayer in complete medium (EMEM with FCS and antibiotics) at 37°C in a humidified atmosphere of 5% CO_2 (*see* **Notes 1** and **2**).
2. Cells will become confluent in 2–5 d. They should be passaged at a ratio of 1 : 3 once they become confluent. To passage, aspirate medium from the cells, wash once with PBS and incubate in trypsin-EDTA for 10 min at room temperature or until the cells are easily removed from the growth surface by gentle pipetting (*see* **Note 6**). Add an equal volume of medium containing 10% FCS (serum will inhibit trypsin from digesting the cells) and pellet the cells at 400*g* for 5 min. Resuspend the pellet in an appropriate volume of medium and aliquot to new flasks.
3. Cells can be frozen at a density of 2×10^6/mL in 92% FCS and 8% dimethylsulfoxide (DMSO). Store at –80°C or in liquid nitrogen.

3.2. Preparation of Virus Stock

1. SVG cells should be plated so that they reach 50–60% confluence within 1 or 2 d after plating.
2. Remove the medium from the SVG cells to be infected and wash the monolayer twice in EMEM containing 2% FCS.
3. Infect SVG cells for 1 h at 37°C with a low multiplicity of infection (MOI) (0.1–1.0 hemagglutination units (HAU)/cell) of virus diluted in a small volume (enough to just cover the surface of the dish) of EMEM containing 2% FCS. Gently rock the flasks every 15 min to ensure proper distribution of the inoculum.
4. After incubation with virus, add EMEM with 10% FCS and antibiotics. It is not necessary to remove the virus inoculum.
5. Infected cells detach and are found in the medium. Virus remains associated with infected cells and debris. At weekly intervals, remove the medium and any floating cellular debris from the flask and add fresh medium. Spin the old medium at 12,000*g* for 10 min. Resuspend the pellet in 1/10 the volume of the supernatant and assay by hemagglutination. The size of the pellets obtained will not be significant until the cells start to show cytopathic effects (*see* **Note 7** and **Fig. 1**). Store at –80°C.

3.3. Harvest of JCV Stock

1. The cells are ready for harvest when significant hemagglutinating activity is present in the weekly supernatants or when cytopathic effects (CPE) are observed

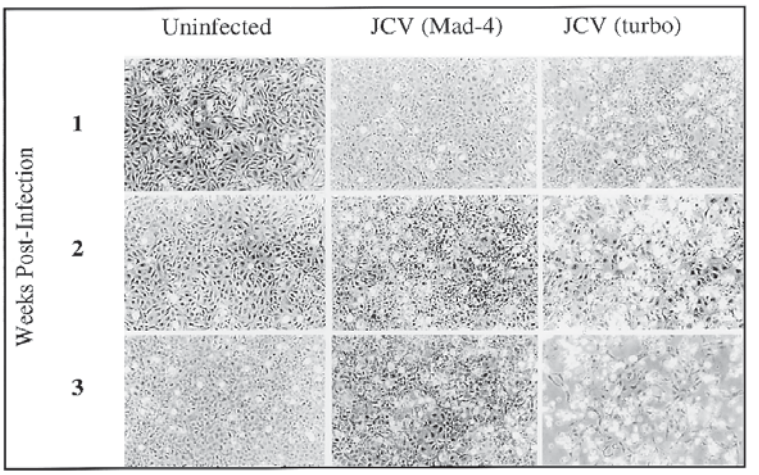

Fig. 1. JC virus-induced cytopathology using an owl monkey derivative of a Mad-4 strain of JCV (M4) and a chimeric JC/SV40 virus (M1SVE) ***(13)***. Infection with commercially available isolates of Mad-1 and Mad-4 does not result in significant amounts of CPE.

(*see* **Fig. 1** and **Note 7**). The amount of CPE and the time between infection and harvest of virus varies considerably between virus isolates.

2. To harvest virus, scrape cells from the dish (trypsin will inactivate the virus) and centrifuge the cells along with the supernatant. Resuspend the pellet in 1/10 the volume of the supernatant and discard remaining supernatant (JCV adheres tightly to cellular membranes and all of the hemagglutinating activity will be associated with the cell pellets). Combine the pellets from the previous 2- or 3-wk harvest that have measurable hemagglutinating activity with this final virus harvest. Freeze-thaw the combined material three times and then incubate for 30 min at 37°C in 0.25% v/v deoxycholic acid to release the virus. Alternatively, cells can be incubated for 30 min in receptor destroying enzyme (0.2 U/mL).
3. Pellet the cellular debris by centrifugation at 12,000*g* for 30 min at 4°C. The supernatant will now contain the virus. Decant or pipet off the supernatant, being careful not to disrupt the pellet of lysed cells and debris. Aliquot the supernatant and store at –80°C. Assay the supernatant by hemagglutination.

3.4. Hemagglutination Assay

All of the polyomaviruses, with the exception of SV40, hemagglutinate red blood cells (RBCs). Human type O red blood cells are the preferred source for hemagglutinating JCV, although other sources of RBCs such as guinea pig have been used (*see* **Note 4**). In a hemagglutination assay, RBCs are added to a dilution series of virus in a U-bottomed microtiter dish. The RBCs sediment to the bottom of the well and form a red "button." In the presence of JCV, how-

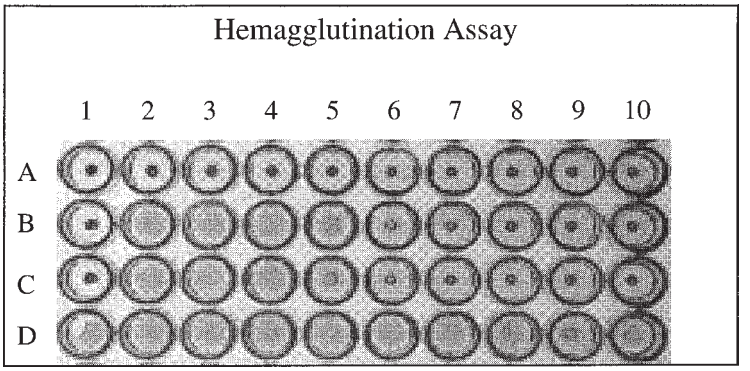

Fig. 2. Hemagglutination assay. Twofold serial dilutions of virus are prepared in duplicate in a U-bottom 96-well plate (rows **B**, **C**, and **D**; columns 1–10). Control wells without virus are also prepared (row A, columns 1–10). Red blood cells (0.5% suspension) are then added to each well and incubated for 2–4 h at 4°C. Note that small "buttons" of RBCs form on the bottom of wells when either no virus is present (row **A**, 1–10) or when very low levels of virus are present (rows **B** and **C**; row **D**, 1–10). The presence of virus hemagglutinates the RBCs and prevents them from forming "buttons" on the bottom of the well. The titer of virus is expressed as the reciprocal of the highest dilution giving hemagglutination. Typically, the titer is expressed as HAU/mL. The titer of virus in rows **B** and **C** is 32 HAU/50 µL. The titer of virus in row D is 256 HAU/50 µL. High concentrations of DOC in the first few wells usually leads to lysis of the RBCs and the wells will be a translucent reddish color (column 1, rows **B**, **C**, and **D**). This represents lysis, not hemagglutination.

ever, the virus will bind to sialic acid residues on the surface of the RBCs, forming a network of RBCs and virus that prevents the formation of the red button; a smooth "carpet" is formed instead (**Fig. 2**).

1. Obtain at least 5 mL of human type O blood. Wash the whole blood three times with Alsever's solution, spinning at 1000*g* for 10 min at room temperature each time. After each wash, carefully remove the whitish/yellow layer of leukocytes that form on top of the RBC pellet. After the washes, resuspend the RBCs in 20 mL of Alsever's for use and storage. Store at 4°C. Under these conditions, RBCs remain usable for about 30 d. If you need to store RBCs longer, they should be treated with a commercially available red blood cell fixative such as RBC Fix.
2. The hemagglutination assay requires a 0.5% suspension of red blood cells diluted in Alsever's solution. This can be prepared by pelleting the RBCs, discarding the supernatant and resuspending the pellet to 0.5% v/v in Alsever's solution.
3. Prepare twofold serial dilutions of your virus stock in PBS. This can be conveniently done by adding 50 µL of PBS to each well of a 96-well U-bottom plate and adding 50 µL of sample to the first well and diluting it 1:2 across the plate. Include a positive control (virus with a known titer) and a negative control (no virus).

4. Add 50 µL of the 0.5% RBC solution to each well. Shake the plate very gently to mix. Incubate the plate for 2 h at 4°C undisturbed (*see* **Fig. 2** and **Note 8**).

3.5. Indirect Immunofluorescence Assay

1. For this assay, the SVG cells are grown on coverslips in six-well dishes. Seed the coverslips so that they are about 60–70% confluent.
2. Make serial dilutions of virus in medium containing 2% FCS. It is recommended to start with a dilution of at least 1:40 as at higher concentrations the DOC will cause some lysis of the SVG cells.
3. Wash the coverslips three times with medium containing 2% FCS and add 200 µL of the virus inoculum.
4. Incubate the coverslips for 1 h at 37°C in a humidified 5% CO_2 incubator for the virus to absorb to the cells. Rock the plate gently back and forth every 15 min to ensure proper distribution of the virus and then add complete medium. It is not necessary to remove the original virus inoculum.
5. At 2–3 d postinfection, wash the coverslips twice with PBS: With a pair of forceps, gently lift the coverslips from the wells and place them in Coplin jars filled with PBS (work quickly so as not to let the coverslips dry).
6. Wash the coverslips once more with PBS and then fill the Coplin jar with either ice-cold acetone or with 70% ethanol to fix the cells. If using acetone, incubate the coverslips in ice-cold acetone for 10 min, remove the acetone, and let the coverslips air-dry. If using 70% ethanol, incubate the coverslips in ethanol for 30 min at room temperature, remove the ethanol, and let the coverslips air-dry. Coverslips can be conveniently stored in parafilm-covered six-well dishes at –20°C indefinitely.
7. To stain the coverslips with antibody, place them cell-side-up into clean, six-well dishes and rehydrate with PBS if they were previously frozen. Alternatively, the coverslips can be stained in the Coplin jars if a sufficient amount of antiserum is available and affordable. Each Coplin jar holds about 10 cm^3 of antibody solution.
8. If staining the entire coverslip with antibody, use between 50 and 100 µL of an appropriate dilution of primary antibody (*see* **Note 5**). Incubate the coverslips in a humidified chamber at 37°C for 30 min. Wash three times with PBS and add between 50 and 100 µL of secondary antibody to each coverslip. Incubate in a humidified chamber at 37°C for an additional 30 min. Wash once in PBS, dip in 0.01% Evans blue, then wash twice in PBS to remove excess counterstain. Mount the coverslips cell side down on glass slides using either 90% glycerol or Permount.
9. View stained cells with an inverted fluorescence microscope. Both T and V antigens give a characteristic nuclear pattern of staining. Nucleoli do not stain and appear dark within a stained nucleus. An example is shown in **Fig. 3**.

4. Notes

1. SVG cells were derived by transformation of primary human fetal glial cells with origin-defective SV40 DNA ***(10)***. As a result, SVG cells constitutively express the SV40 large T antigen. The cells reach crisis at approx passage 50. JCV has

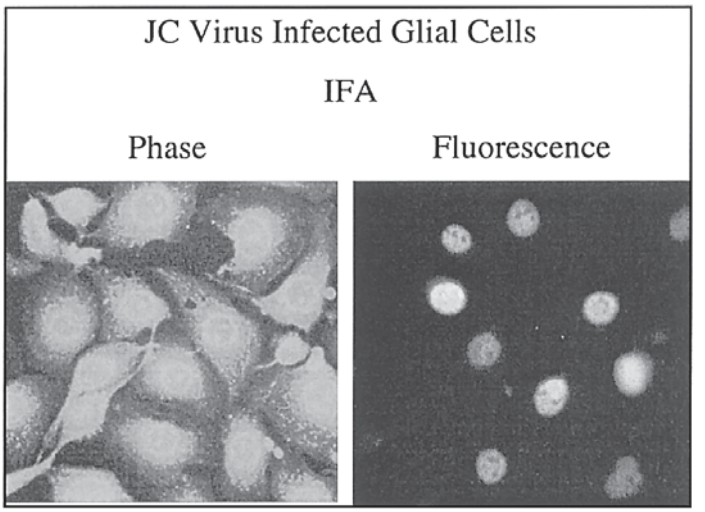

Fig. 3. JCV infected SVG cells stained with an anti-SV40 V antigen monoclonal antibody and a goat antimouse FITC conjugated secondary antibody. There was no detectable staining in uninfected SVG cells. Magnification: ×20.

also been propagated directly in cultures of primary human fetal glial cells and in POJ cells. POJ cells are JC Virus T-antigen transformed primary human fetal glial cells ***(11)***.

2. If the cells become contaminated with fungi or yeast, they can be rescued by passage in the presence of 2.5 µg/mL of Fungizone (Gibco). Fungizone is toxic to the cells and it is not recommended to maintain cells in fungizone for more than 1 or 2 wk.
3. There are several strains of JCV that have been isolated from humans, called Mad-1 through Mad-8. The first strain to be isolated was Mad-1 (isolated in Madison, WI). This is the most common strain used in the laboratory.
4. JCV hemagglutinates erythrocytes from chicken and guinea pig, but does not hemagglutinate erythrocytes from hamster, sheep, African green monkey, or Rhesus monkey ***(12)***. Human type O erythrocytes isolated from JCV seronegative donors are preferred.
5. If infections are done using SVG cells, then a monoclonal anti-SV40 V antigen antibody that crossreacts with JCV V antigen must be used. SVG cells do not express SV40 V antigen. However, SVG cells constitutively express SV40 T antigen and most antibodies directed against JCV T antigen crossreact with SV40 T antigen and vice versa, therefore anti-SV40 T-antigen antibodies cannot be used. On the other hand, if using cells that do not constitutively express SV40 or JCV T antigen, then the mouse monoclonal anti-JCV T antigen or the mouse monoclonal against SV40 T antigen (PAB416-AB2), which crossreacts with JCV T antigen can be used.

6. Add just enough trypsin-EDTA solution to just cover the monolayer (for 25-cm^2 flasks, use 0.5 mL of trypsin-EDTA, for 75-cm^2 flasks, use 1.0 mL, and for 175-cm^2 flasks, use 5.0 mL. Try to work quickly. Trypsin is a protease and will destroy the cells if left to incubate with the cells for a significant amount of time.
7. The amount of CPE varies considerably between virus isolates. The commercially available isolates show very little CPE and it is difficult to recognize. These viruses should be harvested between 4 and 6 wk postinfection when HA activity is readily demonstrable in the cultures.
8. Sometimes the results will change if the plate is incubated for longer than 2 h. It is recommended to read the results immediately.

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3

Propagation and Assay of BK Virus

Wendy A. Knowles

1. Introduction

BK virus (BKV), one of two human polyomaviruses, was first isolated in cell culture from the urine of an immunocompromised renal transplant patient in England in 1971 ***(1)***. Seroprevalence studies have shown that BK infection is widespread in children throughout the world, and the virus thereafter persists latently in the kidney for life. Immunosuppression can lead to reactivation of the virus and prolonged viruria ***(2)***. Although in immunocompetent individuals BK infection is generally asymptomatic, lowering of immunity can occasionally result in life-threatening renal disease ***(3,4)***. An association between active BKV infection and ureteric stenosis in renal transplant recipients, and hemorrhagic cystitis in bone marrow transplant patients has long been established ***(2)***, and recent evidence suggests that BK may contribute to further complications arising in immunocompromised groups, e.g., interstitial pneumonia ***(5)*** and atypical retinitis ***(6)***.

Despite the recent development of molecular techniques for rapid diagnosis of BKV infection and production of recombinant antigens (*see* Chapter 4), viral culture is still necessary, for example, in the detection of neutralizing antibody, the preparation of infected cell substrates as controls for *in situ* hybridization, immunofluorescence or immunohistochemistry, or to obtain viral isolates for basic research.

The interaction of BKV with cells in vitro ranges from fully permissive, in which infectious progeny are produced and cell lysis occurs, to fully transformed in which BK DNA persists in the cells either integrated into the host cell genome or episomally, and BK T antigen is expressed. In general, BKV replicates most efficiently in cells of human origin, cells from nonhuman pri-

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mates are semipermissive, and rodent cells are nonpermissive but are transformed ***(7–10)***. Although many human cell types support lytic BKV infection (embryo kidney, lung, brain and liver, urinary epithelial cells, thyroid, amnion), low-passage human embryo kidney (HEK) cultures are the cells of choice, especially for virus isolation, because of a relatively short incubation time and a distinctive cytopathic effect (CPE) ***(8,9)***. Fibroblast cell strains established from human embryo lung (HEL) are also suitable, can be used through many cell passages, and are more readily available than low-passage HEK cell cultures. A continuous cell line (Vero) derived from African green monkey kidney may also be used for growth of stock virus, although BK production is less efficient in these cells.

Replication of BKV in the cultures can be monitored by several methods ***(1)***. (1) The development of CPE may be followed microscopically, but may be variable and not easily recognized. (2) The production of papovavirus particles may be detected by electron microscopy (EM) if expertise in this technique is available. (3) A hemagglutinin (HA) is produced by BKV during productive infection, and this may be detected and quantified by agglutination of human group O or guinea pig erythrocytes. More rapid techniques for BKV detection in cell culture using immunofluorescence ***(11,12)*** or immunoperoxidase ***(13)*** staining for viral capsid or T antigens have also been reported.

Infectious BKV can be assayed in various ways. Virus titration can be performed in tube cultures ***(14–16)*** or microtiter plate cultures ***(17)***, and the result expressed as tissue-culture infectious doses ($TCID_{50}$). However, the viral titer may be 2 $\log_{10}$ lower when assayed on Vero cells compared to HEL or HEK cultures ***(14)***. BKV will form plaques in certain cell cultures (notably HEK), therefore, if a more accurate quantitative assay is required a plaque assay can be performed and the result expressed as plaque forming units (PFUs). However, the HEK cells must be able to survive under agar for several weeks, and the formation of plaques is dependent upon the passage level of the cells and the incubation conditions ***(18)***. BKV will also plaque in Vero cells, but the plaques are not well defined and the results unreliable ***(18)***. Attempts to plaque BK virus in HEL cells have been unsuccessful ***(18,19)***. Further quantitative methods for the assay of infectious BKV involve the counting of individual infected cells detected by various staining methods such as immunofluorescence ***(19,20)*** or immunoperoxidase ***(13)***. None of these assay methods is easy and all have drawbacks. In this chapter, culture, monitoring, and two methods for assay of BKV are described, both using human embryo fibroblast cells: the microtiter plate assay for virus pools with high titers or when comparing pools with $\log_{10}$ differences in titer, and the fluorescent focus method for lower-titered stocks or when a more accurate result is required ***(19)***.

2. Materials

2.1. Propagation and Harvest of BKV

1. Cultures of low-passage human neonatal kidney cells (70-151; BioWhittaker Ltd.), human embryo lung fibroblasts (HEL, diploid cell strains) e.g., MRC-5 (84101801), W1-38 (90020107) or the continuous cell line, Vero (84113001) (European Collection of Cell Cultures, CAMR; ECACC).
2. a. HEL growth medium (GM): Eagle's minimum essential medium (MEM) with Earle's salts and sodium bicarbonate (Sigma Chemical Co.) supplemented with 2 m*M* L-glutamine, 10% fetal bovine serum (FBS; Gibco BRL) or the FBS substitute, serum supreme (BioWhittaker UK, Ltd.) and 0.025 mg/mL gentamycin. HEL maintenance medium (MM): as above with 2% FBS.
   b. HEK growth medium: Medium 199 with Earle's salts and 1.25 g/L sodium bicarbonate (Sigma Chemical Co.) supplemented with 2 m*M* L-glutamine, 5% FBS, and 0.05 mg/mL gentamycin. HEK MM as above with 2% FBS.
   c. Vero growth medium: Dulbecco's modified Eagle's medium (DMEM) with sodium pyruvate, 4.5 g/L glucose, and 3.7 g/L sodium bicarbonate (Imperial Laboratories) supplemented with 2 m*M* L-glutamine, 5% FBS, and 0.05 mg/mL gentamycin. Vero MM as above with 2% FBS.
3. Sterile phosphate-buffered saline (PBS) pH 7.2 (PBSA; Oxoid Ltd.).
4. Cell scrapers, 23 cm (Nunc).
5. Glass universal containers.
6. Ultrasonic water bath.

2.2. Monitoring of Virus Production

2.2.1. Preparation of Culture Fluid for Electron Microscopy

1. 1.5-mL microfuge tubes (Beckman Instruments Ltd.).
2. Avanti 30 benchtop high-speed centrifuge (Beckman Instruments Ltd.) or equivalent.
3. Fine-tip Pastettes® (Alpha Laboratories Ltd.).
4. Glass-distilled water.
5. Benchkoat (Whatman International Ltd.) or parafilm (American National Can.).
6. PTA stain: 1.5% phosphotungstic acid in distilled water with 0.05% bovine plasma albumin (BPA).
7. 400 mesh copper grids, formvar and carbon coated (Agar Scientific Ltd., Stansted, UK).
8. Fine forceps.
9. Filter paper.
10. Dessicator.

2.2.2. Hemagglutination

1. Nonidet P-40, (NP40; BDH Chemicals Ltd.), diluted to 2.5% in PBS.
2. HA buffer pH 6.34. Prepare three solutions: 0.1 *M* sodium dihydrogen orthophosphate, NaH_2PO_4. $2H_2O$ (A), 0.1 *M* disodium hydrogen orthophosphate,

Na_2HPO_4 (B), 0.3 *M* sodium chloride, 1% dextrose [D(+) glucose], 0.06% gelatin (C). Add sodium azide (final concentration 0.008%) to each solution as a preservative. Mix 6.25 mL of A + 3.75 mL of B + 10 mL of C and add 0.4 mL of 10% BPA. Store at 4°C.
3. V-bottomed 96-well microtiter plates.
4. Human group O erythrocytes (other groups are not suitable). Wash three times in sterile 0.85% saline and make up to a 0.5% suspension in saline. Prepare on day of use and keep at 4°C.
5. Hand lens (×10) or magnifier.

2.3. BKV Assay

2.3.1. Microtiter Plate Method

1. Sterile washing fluid (WF): 1X Earle's balanced salt solution (BSS) with 2.2 g/L sodium bicarbonate and 1 g/L D-glucose (Gibco-BRL).
2. Sterile flat-bottomed 96-well microtiter plate.
3. Human embryo fibroblast cells with growth and maintenance medium (as in **Subheading 2.1., step 2a**).
4. Cell-counting chamber (improved Neubauer, Weber Scientific Int. Ltd.).
5. CO_2 incubator.

2.3.2. Fluorescent Focus Method

1. Coverslips No. 3, 10 × 10 mm (Chance Propper Ltd.)
2. 24-well tissue-culture plate.
3. Human embryo lung fibroblasts with growth and maintenance medium (as in **Subheading 2.1., step 2a**).
4. Cell-counting chamber (as in **Subheading 2.3.1., step 4**).
5. CO_2 incubator.
6. Sterile washing fluid (as in **Subheading 2.3.1., step 1**).
7. PBS.
8. Deionized water.
9. Sharp-ended forceps.
10. Paper tissues.
11. Cold acetone.
12. Watch glasses.
13. Bijoux bottles.
14. Monoclonal antibody NCL-JCBK clone 3.1.1. (Novocastra Laboratories Ltd.). (*see* **Note 1**).
15. Damp box.
16. FITC-conjugated rabbit anti-mouse immunoglobulin (Dako Ltd.).
17. Evans-blue counterstain (Sigma Diagnostics).
18. Microscope slides.

19. PVA/glycerol mountant. Place 3 g of glycerol in a glass universal and add 1.2 g of polyvinyl alcohol (PVA). Stir the mixture well, taking care not to spread the PVA onto the sides of the container. Add 3 mL of deionized water, stir, and leave at room temperature for 4 h. Add 6 mL of Tris-HCl buffer (0.1 *M*, pH 8.5) and leave at 50°C for 10 min for the PVA to dissolve. Clarify by spinning at 1500*g* for 15 min and store at 4°C ***(21)***.
20. Fluorescence microscope.

3. Methods

3.1. Propagation and Harvest of BKV

All procedures must be done using sterile techniques.

The method described is for small plastic flasks (25 cm^2) e.g., Nunclon or Greiner, using 10 mL of medium, but can be scaled up for medium (75 cm^2) or large (175 cm^2) flasks or down for tissue-culture tubes taking 20 mL, 40 mL, and 1 mL of medium, respectively.

1. Prepare a monolayer culture of cells (HEK, HEL, Vero) in a small plastic flask in their corresponding growth medium.
2. When the cells are just confluent, remove the growth medium and add 0.5 mL of seed virus. Rock the flask to distribute the inoculum over the whole of the cell sheet, and incubate the flask at 37°C for 1 h (*see* **Note 2**).
3. Pipet off the inoculum, wash the cell sheet with 5 mL of warm sterile PBS, and refeed the culture with 10 mL of warmed maintenance medium.
4. Incubate the flask at 37°C examining microscopically every few days for CPE. At regular intervals (2–3 d for HEK, 3–4 d for HEL, 7 d for Vero), pipet off the culture fluid and replace with fresh maintenance medium.
5. When a CPE appears, virus production may be monitored by testing the culture fluid using one of the methods below (*see* **Note 3**).
6. Harvest the culture when the CPE is advanced, there are large numbers of papovavirus particles in the culture fluid or the titer of HA in the culture fluid is 1 in 128 or above (*see* **Note 4**).
7. If virus is needed for a stock pool or for hemagglutinin, freeze and thaw the culture three times and scrape the cells down into the fluid.
8. Transfer the suspension to a glass universal container and disrupt the cells by treatment in an ultrasonic water bath until the suspension appears homogeneous (1–3 min) (*see* **Note 5**).
9. Aliquot the suspension and store at –30°C or below.
10. If cell-free virus is required for infectivity studies, remove and clarify the culture fluid by centrifuging at 1500*g* for 15 min to remove cell debris. Aliquot and store the supernatant suspension at –30°C or below.

3.2. Monitoring of Virus Production

3.2.1. Preparation of Culture Fluid for Electron Microscopy (Negative Stain Method)

1. Clarify 1 mL of culture fluid by centrifuging at 1500*g* for 10 min; remove the supernatant but do not discard the pellet.
2. Transfer the supernatant to a microfuge tube and centrifuge in an Avanti 30 centrifuge at 48,000*g* for 45 min at 4°C to pellet the virus particles.
3. Pipet off the supernatant and leave the tube upright for 15 min to drain. Remove any remaining supernatant with a fine-tip Pastette, taking care not to touch the pellet (*see* **Note 6**).
4. Resuspend the pellet in 60 µL of glass-distilled water. If the suspension appears turbid, dilute until clear before the next step.
5. Working in a safety cabinet, place 30 µL of the suspension onto a small piece of BenchKoat or parafilm and add 30 µL of PTA stain to the drop.
6. Place a formvar-coated grid (coated side down) onto the drop and leave for 2 min.
7. Pick up the grid using fine forceps and touch with a piece of filter paper to remove excess fluid.
8. Store the grid in a dessicator until examined in the electron microscope.
9. If no virus particles are seen, go back to the low-speed pellet in **step 1**, resuspend in 1 mL of distilled water to lyse the cells and release any intracellular virus particles, and proceed from **step 2** as above (*see* **Note 7**).

3.2.2. Hemagglutination

1. Place 90 µL of culture fluid in a small vial, add 10 µL of 2.5% NP40 in PBS and leave at 37°C for 30 min (*see* **Note 8**).
2. Make doubling dilutions of the treated fluid in 25-µL volumes with the HA buffer in a V-bottomed 96-well microtiter plate (*see* **Note 9**).
3. Add 25 µL of a cold 0.5% suspension of human O erythrocytes to each well, shake the plate and leave at 4°C for nonagglutinated cells to settle (about 1 h). (*see* **Note 10**).
4. Read hemagglutination using a 10X hand lens or a magnifier: agglutinated cells will be dispersed throughout the suspension, nonagglutinated cells will have formed a button in the center of the well. The titer of the hemagglutinin is taken to be the highest dilution in which >75% of the erythrocytes are agglutinated; this dilution also contains 1 U of hemagglutinin (1 HAU) (*see* **Note 11**).

3.3. BKV Assay (*see* Note 12)

3.3.1. Microtiter Plate Method

1. Make tenfold dilutions (10 µL + 90 µL) of virus suspension (up to 10^{-7}) in sterile washing fluid and transfer 25 µL of each dilution to three wells of a 96-well flat-bottomed microtiter plate.
2. Add to each well 0.15 mL of a HEL cell suspension diluted to contain approximately 3×10^5 cells/mL in growth medium.

3. Incubate the plate overnight at 37°C in a humidified atmosphere of CO_2 to allow the cells to settle and attach and the virus to adsorb.
4. Remove the culture fluid from each well and replace with 0.15 mL of HEL MM.
5. Continue incubation as above for at least 6 wk, recording the CPE and replacing the medium at weekly intervals (*see* **Note 13**).
6. Calculate the titer of the virus suspension, i.e., the virus dilution at which 50% of the inoculated wells would show CPE, using the method of Reed and Muench *(22)*. This involves calculation of the accumulated percentage CPE at each dilution, followed by the proportionate distance between the two values immediately above and below 50%.

3.3.2. Fluorescent Focus Method (see **Note 14***)*

1. Place 1-cm^2 coverslips into the wells of a 24-well tissue-culture plate, and seed wells with HEL cells (approx 1×10^5 cells/mL). Incubate at 37°C overnight in a humidified atmosphere of CO_2 for the cells to attach and form a monolayer.
2. At 24 h when the cultures are just confluent, remove the medium and replace with 200 µL of sterile washing fluid.
3. Make tenfold dilutions (30 µL + 270 µL) of virus suspension up to 10^{-6} in sterile washing fluid and add 0.1 mL of each dilution to duplicate wells (*see* **Note 15**).
4. Rock the plate to distribute the inoculum over the coverslips and incubate the plate for 1 h at 37°C in a humidified atmosphere of CO_2; repeat the rocking after 30 min.
5. Remove the inoculum from the wells, wash the cell sheets with 1 mL of WF and replace with 1 mL of HEL MM. Incubate the plate as above for 7 d with a medium change at 3–4 d.
6. Remove the medium and wash the cell sheets twice with 1 mL of PBS followed by 1 mL of deionized water.
7. Remove the coverslips from the wells with forceps and place on a tissue to dry, being careful to note the identity of each coverslip (*see* **Note 16**).
8. Fix the cells by immersing each coverslip in cold acetone in a watch-glass for 10 min; remove the coverslips to a tissue to dry and then store in bijoux bottles (1 bottle/virus dilution) at –30°C until stained. Place a small piece of crumpled tissue at the bottom of each bijoux bottle first to cushion the coverslips and a second piece of tissue between each coverslip.
9. Stain the coverslips as follows:
   a. Thaw the coverslips and place in the wells of a new 24-well tissue-culture plate, making sure the side with the fixed cells is uppermost (*see* **Note 17**).
   b. Dilute the monoclonal antibody 1 in 100 (or otherwise as recommended by supplier) in PBS and spread 50 µL over each coverslip.
   c. Incubate the plate in a damp atmosphere at 37°C for 1 h.
   d. Wash each coverslip twice for 5 min with PBS and once for 5 min with deionized water on a shaker set at low speed (*see* **Note 18**).
   e. Remove the coverslips from the wells and place on a tissue to dry.
   f. When dry, replace in dry wells.

g. Dilute the anti-mouse conjugate 1 in 40 (or as appropriate for each batch of conjugate) in PBS containing 0.005% Evans blue, and spread 50 µL over each coverslip.
h. Incubate as above for 45 min.
i. Wash and dry the coverslips as in **(d)** and **(e)** above.
j. Mount the coverslips, cell-side down, on glass microscope slides using a drop of PVA/glycerol mountant.
k. Count the number of fluorescing cells on each coverslip under a fluorescence microscope (*see* **Note 19**).

4. Notes

1. This monoclonal will detect polyomaviruses JC and SV40 as well as BK, but the specific crossreactive epitope has not been identified ***(23)***. It was produced using JC virus and it reacts to high titers in immunofluorescence tests with both JC and BK, and in hemagglutination inhibition with JC, but not BK.
2. Defective particles accumulate in tissue culture-grown stocks of BKV ***(8,24,25)*** and may interfere with the production of infectious virus ***(26)***. The proportion of defectives can be reduced ***(25)***, but not eliminated ***(27)*** by subculturing at a low multiplicity of infection (MOI).
3. The CPE produced by BKV varies depending on the type of culture ***(19)*** (**Fig. 1**). In HEK, scattered refractile round cells first appear, followed by foci of distinctive vacuolation in which cell debris is soon evident. Infected cells finally become necrotic and detach from the monolayer, the process being complete within 3–4 wk. The CPE in HEL begins with smaller scattered round cells, accompanied by small indistinct vacuoles, and the fibroblasts become increasingly refractile. Eventually granular debris appears and the whole cell sheet looks "stringy"; this effect is often taken to be nonspecific degeneration in older cultures if the presence of BKV is not suspected. Unlike the HEK cultures, infected HEL cell sheets stay largely intact until detaching from the flask at 4–5 wk. In Vero cell cultures, the CPE consists mainly of cell rounding, scattered small- to medium-sized refractile cells and vacuoles first appearing as in the other cell cultures and subsequently becoming widespread. As infected cells detach from the cell sheet, clumps of small dark granular cells are left. New cells may grow out and infection may persist for between 6 and 8 wk.
4. When primary isolation of BKV is being attempted in HEK cells, it may be necessary to carry out blind passage of the culture before a CPE appears or sufficient virus replication has occurred for virus particles or HA to be detected. At 4 wk, freeze and thaw the cultures, scrape the cells into the medium, disrupt the cells by sonication, and inoculate onto fresh cell monolayers. Incubate and observe the cultures for a further 4 wk.
5. It is sometimes impossible to disperse all cell clumps even by prolonged ultrasonic treatment.
6. The pellet must be well drained to prevent the final preparation being contaminated with salt from the culture fluid, which would form crystals on the grid.

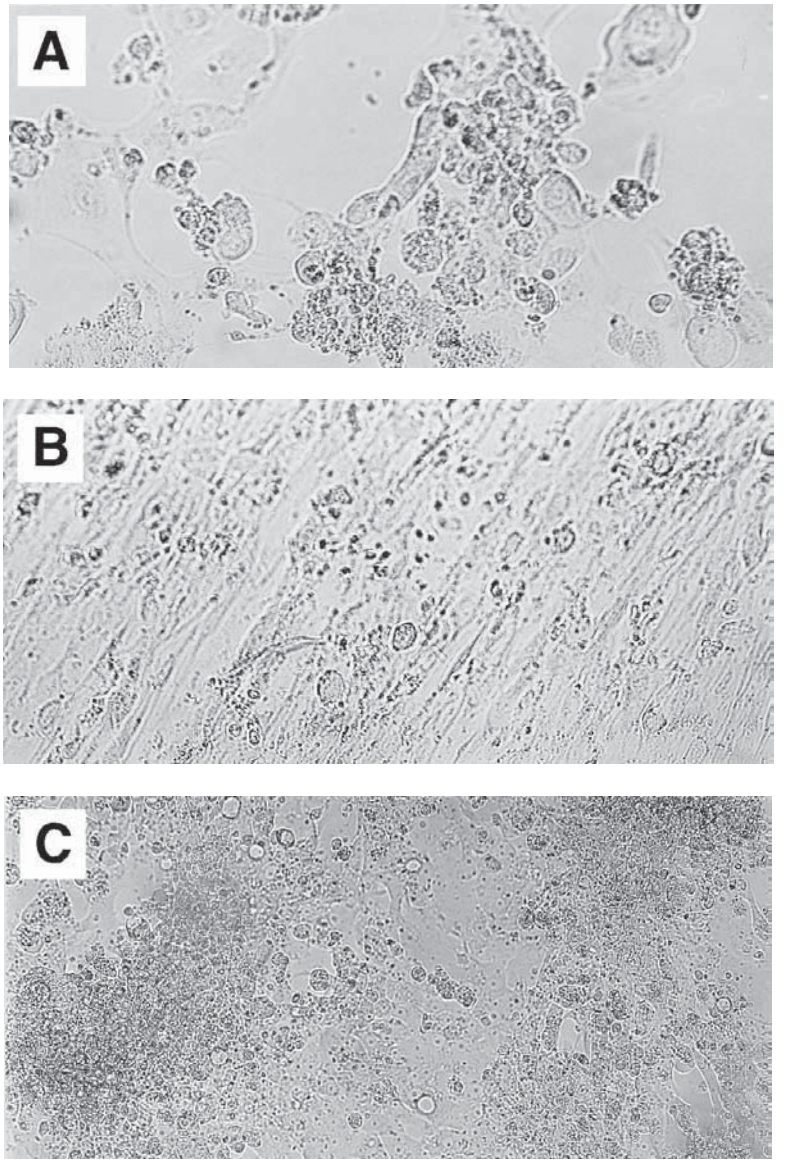

Fig. 1. Cytopathic effect produced by BKV in cell culture. **(A)** HEK. **(B)** HEL. **(C)** Vero.

7. Electron microscopy is a relatively insensitive technique, and to demonstrate virus particles by this method the suspension should contain at least 10^6 particles/mL. Papovavirus particles are icosahedral in shape with 72 capsomeres and non-

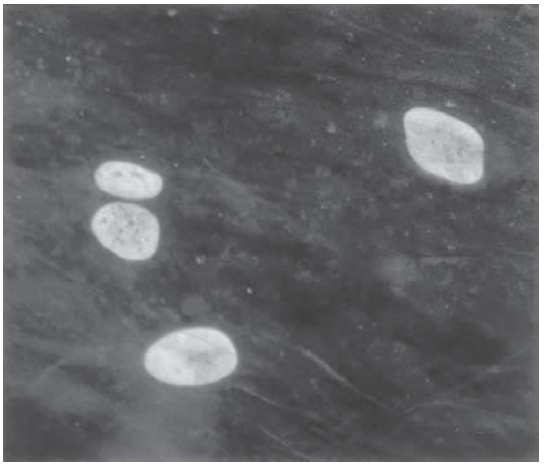

Fig. 2. BK-infected HEL cells stained with monoclonal antibody NCL-JCBK clone 3.1.1 and FITC-conjugated rabbit anti-mouse immunoglobulin.

enveloped; they can only be identified specifically as polyomavirus particles if measurements are done (40–45 nm in diameter).

8. BK readily attaches to cell membranes ***(20)***, and so much of the hemagglutinin remains cell associated ***(7,8,30)***; the HA titer of a suspension derived from disrupted cells is usually about eightfold higher than that in the culture fluid. Treatment of a suspension with the detergent NP40 can increase the HA titer by four- to eightfold. A less-efficient method for freeing virus from cellular material is by treatment with receptor-destroying enzyme (RDE) as up to 90% of virus may be attached to low-density material by RDE-sensitive bonds ***(28)***, but the enzyme must be inactivated before testing for HA. Inhibitors related to $_2$-macroglobulins may also be present in the culture fluid and are eluted from the virus particles by warming ***(30)***.
9. Hemagglutination by BK is sensitive to both rising temperature and pH ***(19,29)***. However, with the acidic buffer used here (pH 6.34) most BK strains (but not all) agglutinate human O erythrocytes to the same titer at 36°C as at 4°C, thus allowing for a rise in ambient temperature during the test without significant elution occurring. The gelatin in the complete buffer improves the settling down of nonagglutinated cells, thus producing more definite and reliable readings ***(19)***.
10. Guinea pig erythrocytes can be used as an alternative to human O erythrocytes, but the reaction is more temperature sensitive with hemagglutination titres being much lower at 36°C even in the complete buffer, and the guinea pig cells tend to settle less well than the human O cells ***(19)***.
11. The NP40 present will lyse the erythrocytes in the first two or three wells, and so the lowest titer of hemagglutination that can reliably be determined is 1 in 16. For pictures of agglutinated red blood cells, *see* Chapter 2.

12. In addition to direct assays for infectious BKV, a measure of the quantity of virus in a pool can be obtained rapidly and simply by titration of the hemagglutinin present, as aforementioned, provided there is a sufficiently high titer of virus; it has been estimated that 1 HAU is equivalent to 3×10^6 virus particles/mL ***(30)***, which includes both complete particles and empty capsids ***(7,8,30)***.
13. If the microtiter method is used for assay of low titer BKV pools, the appearance of CPE may be delayed so long that it is difficult to distinguish a CPE from a degenerating HEL cell sheet especially at the higher dilutions.
14. This method is labor-intensive, but the results are accurate and incubation is for only 1 wk as opposed to several weeks for the other assays. The fluorescent-focus method can also be adapted for 96-well microtiter plates.
15. Clumping of virus particles is a problem for assays that depend on counting infected foci, and can occur after storage. Ultrasonic treatment and warming can help to reduce the problem.
16 Take care not to scrape the cells when handling the coverslips.
17. A good light is needed to see on which side of the coverslip the cells are situated especially with HEL fibroblasts which form a smooth monolayer.
18. Problems can be experienced with the cell sheet detaching from the coverslip.
19. Infected cells are identified by reticulate intranuclear immunofluorescence (*see* **Fig. 2**), although some diffuse cytoplasmic staining may also be seen.

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4

Molecular Methods for Identification and Genotyping of BK Virus

Li Jin

1. Introduction

General properties of human polyomavirus BK (BKV) such as its clinical significance, propagation, and serological assays are described in Chapter 3. In this chapter, two molecular methods for detection of a subtype-specific sequence of BKV are described. Both methods were developed based on polymerase chain reaction (PCR) amplification and subsequent identification of BKV subtypes by either sequencing or restriction enzyme analysis.

A number of BKV strains have now been isolated, mostly from immunocompromised patients. **Figure 1** shows the gene organization in the BKV genome. The BKV noncoding control region (NCR) is about 300–500 basepairs (bp) in length and is highly variable among BKV strains. Partial duplications and/or deletions have been recognized in the NCR sequences of many BKV strains ***(1–5)***. The precise relationship between the variation of the NCR and the capacity for transmission and reactivation remains unknown.

BKV variants have also been described based on a variable region of the major structural protein VP1. Four BKV genotypes with characteristic amino acid sequences at residues 61 to 83 of the *VP1* gene product were identified ***(6)***. These four genotypes correlate well with four serogroups, which show antigenic differences in hemagglutination inhibition (HAI) tests ***(7–10)*** and were shown to be circulating independently in the human population ***(11–13)***. However, the significance of BKV subtypes in relation to particular clinical symptoms or to particular groups of immunocompromised patients has not been clearly established.

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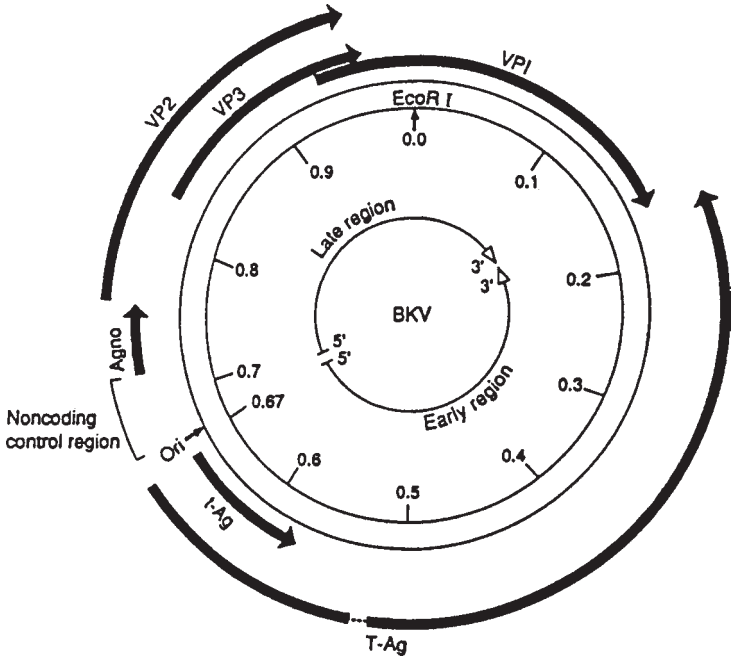

Fig. 1. Diagramatic representation of the genomic organization of BKV ***(26)***. The map coordinates are oriented clockwise from the *Eco*RI cleavage site at 0.0 map units. The inner arcs indicate the early and late regions and the directions in which the early and late mRNAs are transcribed. The origin of replication is indicated at 0.67 map units and the noncoding control region is located outside the genome of BKV. Coding regions are depicted by the thick-black arrows for the early proteins (t-Ag and T-Ag) and for the late proteins (Agno, VP1, VP2, and VP3). **Table 1** shows the nucleotide positions are numbered starting from the origin of DNA replication, which is located within the start codons of the T/t antigens and the agnogene, and proceeds toward the late region ***(25)***.

The value of the serological classification for epidemiological and pathogenicity studies is limited by the need to cultivate the virus and produce antisera. Therefore, PCR assays were developed based on the variable regions of the BKV genome and became important biological tools for direct detection in clinical materials, for use in molecular epidemiological investigation and in studies aimed at understanding BKV pathogenicity and the mechanism of viral variation. Oligonucleotide primers complementary to the 5 -end of the T antigen and the 5 -end of the VP2 regions, which are conserved among the BKV strains, were used to amplify the NCR of BKV by PCR from a variety of specimens ***(5)***. Such PCR products can be subsequently used for characterization of

the NCR of different BKV strains, which may contribute to understanding the basis of the variation in the NCR.

Primers for PCR amplification of the antigenic epitope (aa61-83) in the *VP1* gene product were chosen from the regions where four BKV subtypes have an identical nucleotide sequence ***(7)***. Two genetic subtyping schemes, PCR-sequencing (PCR-S) for DNA when DNA quantities of less than 100 ng are available and a simplified PCR-restriction enzyme analysis (PCR-RE) were developed using the VP1-PCR amplicons (327 nt in length) ***(7,14)***. These methods were reliable for estimating the prevalence of BKV infection in the population by screening urine and other specimens, such as tissues or body fluids, e.g., peripheral blood mononuclear cells (PBMCs) or cerebrospinal fluid (CSF) ***(11,13–15)***. Sequencing the PCR amplicon of the NCR can also identify variants of BKV ***(16)***. Methods are described in this chapter, including PCR assays using different primer pairs for BKV detection, primers for subtype-specific region in protein VP1, and primers for the NCR, and two genotyping methods: PCR-RE and PCR-S (manual and automatic sequencing) using the PCR amplicon of the subtype-specific region.

2. Materials

2.1. Source of Materials From Which BKV DNA May Be Extracted

1. Clinical specimens: urine, PBMC, tissues, CSF.
2. BKV isolates: cell-culture fluid collected from the human embryonic kidney or human embryonic lung cells infected with BKV from different sources.

2.2. Materials for Sample Preparation

1. Phosphate-buffered saline (PBS): 0.137 *M* NaCl, 2.68 m*M* KCl, 7.98 m*M* Na_2HPO_4, 1.47 m*M* KH_2PO_4, pH 7.2.
2. Ficoll-paque (Pharmacia, cat. no. 17-1440-02).
3. Proteinase K (Sigma): 20 mg/mL. Store at –20°C.
4. QIAamp Tissue kit (M-Medical-Genenco, Qiagen, cat. no. 51304).
5. Ethanol.
6. Homogenizer (Griffith's grinder).

2.3. PCR Reagents

1. Oligonucleotide primers (**Table 1**): primers VP1-327-1 and VP1-327-2r used for amplification and sequencing of the type-specific region; primers bioVP1-306-1 and bioVP1-306-2r used for the nested amplification and sequencing of the type-specific region; and primers NCR-1 and NCR-2r used for amplification and sequencing of the entire NCR region.
2. 10X PCR buffer without Mg^{2+}: 200 m*M* Tris-HCl, pH 8.4, 500 m*M* KCl (Gibco-BRL).

Table 1
Oligonucleotide Primers for Identification of BKV

Name	Sequence 5 to 3	Nucleotide position[a]
VP1-327-1	5 -CAAGTGCCAAAACTACTAAT	1630–1649
VP1-327-2r	5 -TGCATGAAGGTTAAGCATGC	1956–1937
BioVP1-306-1	5 -ACTACTAATAAAAGGAGTAG	1641–1660
BioVP1-306-2r	5 -GTTAAGCATGCTAGTTATTC	1947–1928
VP1-5	5 -AGGAGTAGAAGTTCTAGAAG	1656–1675
NCR-1	5 -TCCATGAGCTCCATGGATTCTTC	5110–5132
NCR-2r	5 -CTAGGTCCCCCAAAAGTGCTAGA	657–635

[a]Nucleotide positions are numbered starting from the origin of replication, which is located within the start codons of the T/t antigens and the agnogene, and proceeds toward the late region ***(25)***.

3. 50 m*M* $MgCl_2$ (Gibco-BRL).
4. 100 m*M* deoxynucleotide 5 -triphosphate (dNTP) set consisting of all four deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) at a concentration of 100 m*M* (Gibco-BRL).
5. *Taq* DNA polymerase (Gibco-BRL).
6. Sterile mineral oil (Sigma).
7. Sterile distilled water (Sigma).
8. PCR thermocycler.

2.4. Electrophoresis Reagents

1. Molecular-biology grade agarose for PCR products: NuSieve 3:1 (FMC BioProducts).
2. 1-kb size markers (Gibco-BRL) or any other commercial markers, which give DNA sizes around 300–500 bp.
3. TBE running buffer: for 1 L of 5X: 54 g of Tris-HCl base, 27.5 g boric acid, and 20 mL of 0.5 *M* ethylenediaminetetraacetic acid (EDTA) (pH 8.0).
4. TAE buffer: for 1 L of 50X: 242 g of Tris-HCl base, 57.1 mL of glacial acetic acid, and 100 mL of 0.5 *M* EDTA (pH 8.0).
5. TE buffer (pH 7.4): 10 m*M* Tris-HCl (pH 7.4) and 1 m*M* EDTA (pH 8.0).
6. Gel-loading buffer: 0.25% orange G and 10% Ficoll in TE buffer, filtered. Other commercial solutions are suitable, e.g., gel loading solution, Type I (6X buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose in H_2O, Sigma).
7. 10 mg/mL ethidium bromide in water.

2.5. Restriction Enzymes

1. *Alu*I, *Xmn*I, *Ava*II, and *Rsa*I.
2. 10X stocks of the appropriate restriction enzyme buffers as recommended by the manufacturer.

2.6. DNA Purification Reagents

2.6.1. For Manual Sequencing

1. Primers for producing the single-strand DNA template: VP1-327-1 and bioVP1-306-2r, a biotinylated downstream primer, 9 bp internal to VP1-327-2r (*see* **Table 1**).
2. 10 µg/µL M-280 streptavidin beads (Dynabeads, cat. no. 112.050; Dynal, Norway).
3. 2X binding/washing buffer (B&W): 10 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, and 2 *M* NaCl.
4. Other solutions: 0.1 *M* NaOH; 0.2 *M* HCl; 1 *M* Tris-HCl, and TE buffer (*see* **Subheading 2.4.**).

2.6.2. For Automatic Sequencing

Geneclean kit (cat. no. 800-424-6101; BIO101 Inc.)

1. 6 *M* NaI (provided with the kit).
2. TAE buffer (*see* **Subheading 2.4.**) or,
3. TBE buffer (*see* **Subheading 2.4.**) plus TBE modifier (pH 5.8 ± 0.2, a proprietary mixture of centred salts to aid in melting TBE gel slices and reverses inhibitory effects of borate buffer, provided with the kit).
4. Glassmilk (silica matrix in water), provided with the kit.
5. NEW concentrate, a NaCl/ethanol/water wash, provided with the kit.

2.7. Sequencing Reagents

2.7.1. Manual Sequencing

Sequenase version 2.0 kit (cat. no.71071; US Biochemical)

1. End-labeling reaction mix: DNA template, nested primer specific to the subtype-specific region (VP1-5, **Table 1**). Dithiothreitol (DTT), [^{32}P]dATP (10 µCi/µl), Sequenase. 5X annealing buffer: 200 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 250 m*M* NaCl. 5X labeling mix (dGTP): 7.5 µ*M* dGTP, 7.5µ*M* dCTP, 7.5µ*M* dTTP.
2. Chain termination mixes:
   ddG: 80 µ*M* dGTP, 80 µ*M* dATP, 80 µ*M* dCTP, 80 µ*M* dTTP, 50 m*M* NaCl, 8 µ*M* ddGTP;
   ddA: 80 µ*M* dGTP, 80 µ*M* dATP, 80 µ*M* dCTP, 80 µ*M* dTTP, 50 m*M* NaCl, 8 µ*M* ddATP;
   ddT: 80 µ*M* dGTP, 80 µ*M* dATP, 80 µ*M* dCTP, 80 µ*M* dTTP, 50 m*M* NaCl, 8 µ*M* ddTTP;
   ddC: 80 µ*M* dGTP, 80 µ*M* dATP, 80 µ*M* dCTP, 80 µ*M* dTTP, 50 m*M* NaCl, 8 µ*M* ddCTP.
3. Stop solution: 95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF.
4. Sequencing gel: 8% denaturing polyacrylamide gel with 23% urea in TBE.
5. Gel fixing solution: 10% acetic acid/10% methanol (v/v).
6. Autoradiography film: e.g., Kodak XAR5.

2.7.2. Automatic Sequencing

1. Terminator Premix (*Taq* DyeDeoxy terminator cycle sequencing kit, cat. no. 402079, ABI).
2. Distilled water (dH_2O).
3. Light mineral oil (not necessary if the thermal cycler has a heated lid).
4. 70% ethanol.
5. 3 *M* sodium acetate (pH 4.6).
6. Automatic fluorescent DNA sequencer (ABI 373 or ABI Prism 377, Perkin-Elmer).

3. Methods

3.1. Specimen Preparation for PCR

All specimens are treated in an isolated room to avoid contamination.

3.1.1. Urine

Centrifuge 1 mL of urine at 10,000*g* for 2 min. Wash the sediment in 1 mL of PBS and spin for 2 min. Resuspend the pellet in 100 µL of distilled water and heat at 95°C for 5 min to break up the cells and release the target DNA. Spin for 10 s and collect the supernatant for PCR ***(6)*** or store at –20°C.

3.1.2. CSF and Cell-Culture Supernatant

These need no treatment (***17,18***; *see* **Note 1**).

3.1.3. PBMC

Isolate the PBMC from the heparinised blood sample by gradient centrifugation with Ficoll-Paque as follows:

1. Dilute the blood 1 : 1 with PBS, e.g., add 8 mL PBS to 8 mL blood.
2. Add 8 mL Ficoll layer on top of 16 mL of diluted blood (1/3 + 2/3).
3. Spin at 500*g* for 30 min and stop without a break. Four layers are formed: serum with PBS at top, then the buffy coat with PBMC, and ficoll and red cells at the bottom.
4. Remove serum and transfer buffy coat to a fresh universal bottle. Wash cells twice with PBS at 100*g* for 10 min and store at –20°C.
5. Treat the cells with proteinase K (final concentration, 500 µg/mL) at 48°C for 3 h or overnight and extract DNA with phenol and chloroform. Precipitate DNA with ethanol and suspend the pellet in dH_2O (***19,20***; *see* **Note 2**).

3.1.4. Tissues (e.g., Kidney, Placenta, or Brain)

For fresh or frozen tissues:

1. Mince a small section of tissue with a sterilized homogenizer in 100 µL of dH_2O.
2. Collect the supernatant for DNA extraction and/or for direct PCR amplification (*see* **Note 1**).

For fixed tissues:

1. Slice the paraffin-embedded specimens into sections of 2–5 mm with a disposable scalpel.
2. Deparaffinize the sections in 400 µL xylene for 5 min, spin for 5 min, and decant the supernatant.
3. Rinse the sections once with 100% ethanol and once with 95% ethanol by inverting the tube two or three times. Evaporate the remaining ethanol under vacuum and resuspend the sections in 100 µL of TE or dH_2O for proteinase K digestion (*see* **Note 3**).
4. Slice the formalin-fixed tissue into small sections and deformalinize the sections by dipping once in 100% ethanol, once in 70% ethanol, and once in dH_2O. Suspend the sections in 100 µL of TE or dH_2O for DNA extraction and/or for direct PCR amplification (*see* **Note 4**).

*3.1.5. DNA Extraction Using the QIAamp Tissue Kit (**15**)*

This is performed according to the manufacturer's instructions. The kit contains spin columns with different binding buffers for each specific application. A buffer with proteinase K lyses samples of up to 50 mg of tissue or up to 200 µL of body fluid. Nucleic acids bind specifically to the QIAamp silica-gel membrane. Two wash buffers are used to remove impurities, and pure DNA is eluted in water. A comprehensive handbook with instructions is provided with the kit. A number of alternative methods and commercial kits are also available and suitable for BKV DNA extraction (*see* **Note 5**).

3.2. PCR Amplification

Positive (BKV DNA) and negative (pure water) controls are always set up in parallel to exclude false negatives and contamination in all experiments. The following procedures must be performed in separate dedicated rooms. Filter plugged tips are used throughout the PCR amplification.

1. Make a reaction master mix using dedicated PCR reagents in a clean room dedicated to PCR reagent preparation only:

   The reaction mix for one specimen is as follows: 5 µL of 10X *Taq* buffer, 1.5 µL of 50 m*M* $MgCl_2$, 1 µL of each of primer in the pair, VP1-327-1 and VP1-327-2r for the subtype specific region, or NCR-1 and NCR-2r for the entire NCR region) at 50 pmol/µL (*see* **Note 6**), 1 µL of 10 m*M* dNTP mixture, 1.25 U of *Taq* polymerase, 30.5 µL of sterile distilled H_2O (dH_2O). Aliquot the master mixture for 40 µL of each specimen. Add 25 µL (one drop) of sterile mineral oil on top of each micro-tube.
2. Add 10 µL of treated specimen containing the DNA to each reaction mix in a dedicated room and spin for 2 s. Start the thermal cycling with the following conditions: 2 min of denaturation at 94°C, followed by 35 rounds of an amplifi-

cation cycle consisting of 1 min denaturation at 91°C, 1 min annealing at 55°C and 1 min extension at 72°C, and a final extension cycle of 1 min at 55°C and 4 min at 72°C (*see* **Note 6**).

3. Load 5–10 µL of PCR product with 2 µL of orange G/Ficoll solution on a 3% agarose gel with TBE for electrophoresis ***(7)*** in a dedicated room. Load 2 µL of the 1-kb size marker in one of the lanes. Add TBE to submerge the gel after the electrophoresed samples enter into the gel. Continue electrophoresis at approx 120 V for 1 h. Stain with ethidium bromide and photograph.

   Based on results from different strains ***(5)***, PCR products are expected to be 327 bp in length for the subtype-specific region of the VP1 ***(7,8)*** and 600–800 bp for the entire NCR.

3.3. PCR-RE for Subtyping

PCR amplicons for the *VP1* gene (327 bp) can be used to distinguish the four genotypes of BKV using one of two available strategies (*see* **Fig. 2** and **Note 6**).

3.3.1. Strategy 1 (Two Steps)

1. Digest 5–15 µL (depending upon quantity) of PCR product with 1–2 U of *Alu*I in a total of 20 µL of the buffer supplied, at 37°C for 2 h (*see* **Note 7**).
2. Load the reaction mix with 5 µL of orange G/Ficoll solution on a 3% agarose gel made in TBE buffer for electrophoresis. Visualize the DNA fragments after staining with ethidium bromide. Samples that are cut with *Alu* I belong in genotype I or II and the uncut specimens in genotype III or IV.
3. Digest 5–15 µL of PCR product of genotype I or II with 1–2 U of *Xmn*I and of genotype III or IV with 1–2 U of *Ava*II in a total of 20 µL of the supplied buffer at 37°C for 1 h. The cut specimens belong in genotype II (with *Xmn*I) or III (with *Ava*II) and the uncut specimens in genotype I or IV.

3.3.2. Strategy 2 (Three Steps, *see* **Note 8**)

1. Digest PCR products with *Xmn*I to distinguish genotype I (uncut) from other genotypes.
2. Digest unidentified PCR products with *Ava*II to distinguish genotype IV (uncut) from other genotypes (II and III).
3. Digest the remaining PCR products with *Rsa*I to distinguish between genotypes II and III.

Mix all 20 µL of the reaction mixture with 5 µL of the loading buffer (orange G/Ficoll), load onto a 4% agarose gel, and analyze by gel electrophoresis. Include a size marker in one of the lanes. The sizes of fragments cleaved with restriction enzymes *Alu*I, *Xmn*I, *Ava*II, and *Rsa*I, which can reliably determine BKV subtype are shown in **Table 2**. Dual infections can also be detected using this approach ***(8)***.

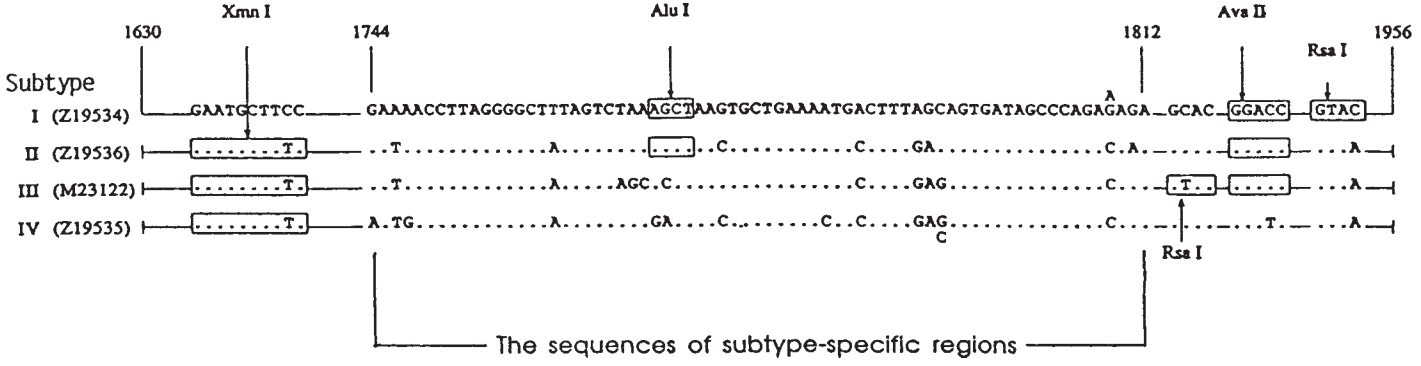

Fig. 2. The sequence variations within VP1 region used as the basis for the genotyping of BKV strains by PCR-RE and PCR-S. The sites of the restriction endonucleases used for BKV subtyping are boxed. Only the complex sequence for the subtype-specific region of subtype I is presented, and only where the different sequences for the other subtypes from this are indicated. A point mutation in the sequence for strain GS (ZI9537, a subtype I strain closely related to strain BKV-PT) and a point mutation occurring in strain DB (a subtype IV strain closely related to strain BKV-IV) are indicated, respectively, by a single letter A above the sequence of subtype I, and a single letter C below the sequence of subtype IV. The nucleotide positions in the sequence are numbered according to the sequence of strain BKV-DUN (JO2038). The accession numbers for the EMBL Data Library are bracketed next to each subtype or strain.

Table 2
The Sizes of PCR-RE Fragments Cleaved with Restriction Enzymes; Implications for BKV Subtyping

	Sizes (nt) of the fragments cleaved with			
BKV subtypes	*Alu*I	*Xmn*I	*Ava*II	*Rsa*I
I	186, 141	327	237, 90	281, 46
II	186, 141	244, 83	237, 90	327
III	327	244, 83	237, 90	203, 124
IV	327	244, 83	327	327

3.4. DNA Purification for Sequencing

3.4.1. Immobilization and Purification of ssDNA from PCR Amplicons for Manual Sequencing

Dynabeads M-280, covalently coupled to streptavidin are used as a solid phase for capturing and purifying one strand of the PCR-generated dsDNA. These beads are superparamagnetic, i.e., they are only magnetic in a magnetic field, so that they are easily resuspended when the magnetic field is removed. The high affinity of the streptavidin/biotin interaction ($K_d = 10^{-5}$), allows the rapid and efficient biomagnetic isolation of biotin-labeled target molecules.

1. Add 2–5 µL of the VP1 PCR (327 bp) amplicon to a fresh (the nested) PCR mix containing 5 pmol of each primer, VP1-327-1 and BioVP1-306-2r. Subject the reaction to the same PCR amplification (25–30 cycles) as mentioned before.
2. Prewash M-280 streptavidin beads (Dynabeads, 20 µL for each PCR product) with an equal amount of 1X B&W buffer. Keeping the microtube on the magnet, which separates the beads from the supernatant, remove the supernatant and resuspend the Dynabeads in 40 µL of 2X B&W.
3. Incubate 40 µL of the nested PCR product with 40 µL (200 µg) of prewashed Dynabeads at room temperature (RT) for 15 min. Remove the supernatant.
4. Denature the DNA on the Dynabeads with 8 µL of 0.1 *M* NaOH at RT for 10 min. Remove the NaOH supernatant, which contains the eluted single-strand DNA.
5. Neutralize the Dynabeads with 4 µL of 0.2 *M* HCl and 1 µL of 1 *M* Tris-HCl.
6. Wash the Dynabeads containing the immobilized ssDNA sequentially with 50 µL of 0.1 *M* NaOH, 40 µL of 1X B&W, and 50 µL of TE buffer. Remove the supernatant while keeping the microtube on the magnet.
7. Add 7 µL of distilled water to the beads as the template of the sequencing reaction.

3.4.2. Purification of dsDNA from PCR Amplicons (see **Note 9***) for Automatic Sequencing Using the Geneclean Kit*

1. Load 40 µL of the PCR amplicon on a 2–3% agarose gel (*see* **Note 10**) for electrophoresis.

2. Excise the gel slice containing the specific DNA fragment (327 bp for the subtype-specific region of the VP1 and approx 600–800 bp for the NCR) visualized under a long wavelength UV light.
3. Add 3X the volume of the slice of *Na*I solution (approx 1 mL) and incubate at 45–55°C for 5 min to dissolve the agarose.
4. Add 5–10 µL of Glassmilk suspension and incubate for 5 min at RT (mix frequently).
5. Pellet Glassmilk/ DNA complex for 5 s and remove the supernatant.
6. Wash pellet three times with NEW Wash.
7. Elute DNA into 20 µL dH_2O and collect the supernatant as the template of the sequencing reaction.

3.5. PCR-S for Subtyping

Sequencing can be performed manually or using an automatic DNA sequencer. Compared with automatic sequencing, manual sequencing is cheaper, available in many laboratories, and subtypes of dual infections can also be detected by inspecting two different nucleotides located at horizontal positions. Disadvantages are that radioactive reagents are involved and the technique is labor intensive. In the Sanger (chain termination) method, either pure single-stranded DNA, or double-stranded DNA that has been denatured with heat or alkali can be used as template, however the best results are obtained using single-stranded DNA templates. Dynabeads M-280 coated with streptavidin were found to be very efficient for the isolation of any biotinylated target and very reliable for manual sequencing. On the other hand, heat-denatured, double-stranded DNA is only suitable for automatic sequencing.

3.5.1. Manual Sequencing with Immobilized ssDNA Templates with Sequenase Version Sequencing Kit

The ssDNA templates are sequenced with oligonucleotide primers specific for BKV by the dideoxy-mediated chain termination method of Sanger et al. ***(21)***.

1. Anneal ssDNA template and primer (molar ratio of template to primer 1:20): 2 µL (approx 0.65 µg/100 pmol) of primer VP1-5 (for subtype-specific region), 6 µL (approx 1 µg/5 pmol) of ssDNA template purified from the PCR amplicon with a biotinylated primer, and 2 µL of 5X annealing buffer (10 µL in total). Incubate the reaction mix at 65°C for 10 min then at RT for 10 min.
2. Labeling reaction: 10 µL of the annealing mix, 2 µL of sequenase (1.6 U/µL), 2 µL of 1X labeling mix (dGTP), 1 µL of 0.1 *M* DTT, and 0.5 µL of [^{32}P]dATP (10 µCi/µL). Mix and keep the reaction (15.5 µL total) at RT for 30–60 s.
3. Termination reaction: add 3.5 µL of labeling mix into 2.5 µL of 37°C prewarmed termination mix (ddA, ddC, ddG, and ddT). Incubate at 37°C for 3 min and then add 4 µL of stop solution to each reaction. Store at –20°C.
4. Heat the reactions at 70–80°C for 2 min before loading onto the gel.

5. Load 2–5 µL of each reaction onto the prerun denaturing gel at a constant 1700 V, 60°C for 30 min.
6. Continue the denaturing gel electrophoresis for up to 3 h for the VP1-PCR generated genotype-specific region of BKV (approx 100 bp) or up to 4–5 h for more than 250 bp.
7. Wash the gel with 10% acetic acid for 15 min, and air-dry the gel for 6–8 h.
8. Expose to X-ray film.

3.5.2. Automatic Sequencing with dsDNA Fragments Generated by PCR-Cycle Sequencing Using Dye-Labeled Terminators

(Dye Terminator sequencing kit, ABI) and ABI 373 automatic DNA sequencer (ABI)

1. Make up sequencing reactions for each sample (*see* **Note 9**): 8 µL of Terminator Premix, 3–9 µL (30–90 ng) of purified dsDNA, 1 µL (3.2 pmol) of primer. Add dH_2O to 20 µL in total.
2. Overlay the reaction mixture with one drop of light mineral oil (not necessary if the thermal cycler has a heated lid).
3. Begin thermal cycling as follows: 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. Repeat for 25 cycles and hold the reactions at 4°C.
4. Precipitate the reaction product (remove the mineral oil, if applicable) by adding 2 µL of 3 *M* sodium acetate, pH 4.6 and 50 µL 95–100% ethanol. Vortex and place on ice for 10 min.
5. Centrifuge in a microcentrifuge at maximum speed for 15–30 min.
6. Remove the ethanol completely.
7. Rinse the pellet by adding 250 µL 70% ethanol. Aspirate all the alcohol solution without disturbing the pellet, which may or may not be visible.
8. Dry the pellet in a vacuum centrifuge or at RT. Store the pellet at –20°C.
9. Resuspend the sample in 3–6 µL of the loading buffer (depending upon the number of wells). Vortex and spin in microfuge.
10. Heat the sample to 90°C for 2 min to denature. Place on ice until ready to load.
11. Run Automatic DNA sequencer according to manufacturer's instructions.

3.5.3. Sequence Data Analysis

Data obtained by manual sequencing are analyzed by reading gel autoradiography. Subtypes of dual infections can also be detected by inspecting two different nucleotides located at horizontal positions (*see* **Fig. 3** and **Note 11**). Data obtained by automatic sequencing can be analyzed using computer assistance. Analysis of nucleotide and deduced amino acid sequences may be performed using a Macintosh PC with the SeqEd version 1.0.3 program and the Clustal routine of Megalign, a multiple-alignment program in the DNASTAR package. Subtypes of dual infections may be detected by limiting dilution of the samples prior to PCR amplification ***(22,23)***.

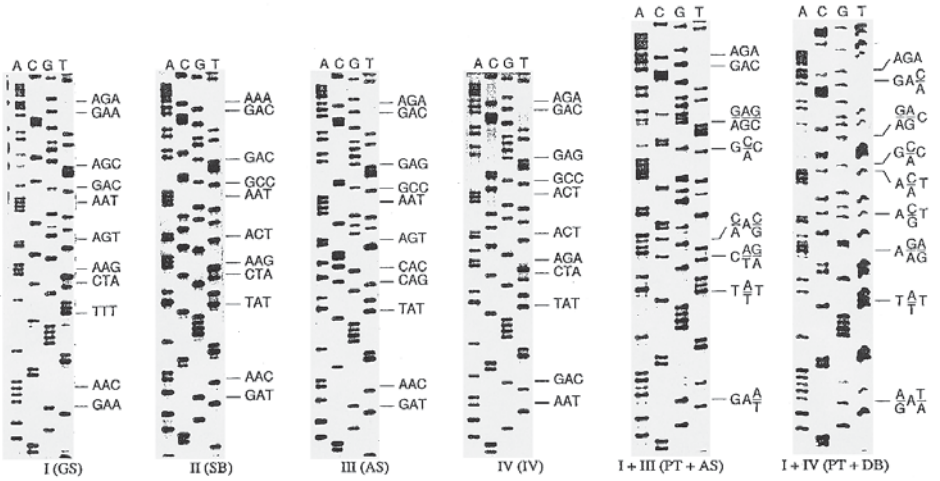

Fig. 3. Sequencing gel illustrating differences in BKV subtype-specific regions. 327-bp DNA fragments generated from urine specimens were subjected to the second PCR amplification with a biotinylated primer and sequenced after capturing the ssDNA using Dynabeads. Electrophoresis was performed on an 8% polyacrylamide gel for 3 h at a constant voltage of 1700. Nucleotide differences leading to amino acid changes between subtypes are highlighted. The sequences detected in urine samples containing two subtypes are indicated by dashes.

4. Notes

1. DNA extraction of BKV from other specimens such as urine, PBMC, and tissue is unnecessary for PCR amplification and could be detrimental because loss of DNA may occur during the process. Once the cells of blood and tissues are broken mechanically or chemically (proteinase-lysis), the supernatant can be directly used for PCR amplification.
2. EDTA-blood sample was found to be more suitable for measles virus detection than PBMC in our lab. Therefore, the EDTA-blood sample is recommended for BKV detection by PCR.
3. PCR amplification from paraffin-embedded tissues is less efficient than amplification from purified DNA and fresh tissues. To compensate for this reduced efficiency, the number of cycles and cycle times may be increased.
4. The integrity of nucleic acid in tissues can be damaged by formalin. Therefore, DNA fragments larger than 250 nt are unlikely to be amplified by PCR. A pair of internal primers of the 327- region can be chosen, which may amplify a smaller region of BKV genome and increase the sensitivity of PCR. This was also successful for other viruses in human tissues in our lab.
5. Other methods such as guanidinium isothiocyanate-silica method ***(24)*** and TRI Reagent (Helena BioScience, UK) can be used for DNA extraction.

6. A nested PCR can be processed using primers bioVP1-306-1 and bioVp1-306-2r, which may increase the sensitivity of the PCR. Alternatively, the first round PCR should be modified by reducing the primer concentrations (VP1-327-1 and VP1-327-2r) from 50 to 20 pmol of each, the annealing temperature from 55°C to 50°C, and the amplification cycles from 35 to 25. Five microliters of the first round of PCR product is transferred to the nested PCR reaction with 50 pmol of each primer, bioVP1-306-1 and bioVp1-306-2r, and the nested PCR is performed with the annealing temperature at 55°C for 35 cycles. The nested PCR produces an amplicon of 306 bp in length, which can be subsequently used for subtyping by either PCR-RE or PCR-S following the procedures for the PCR amplicon amplified using primers VP1-327-1 and VP1-327-2r.
7. It is difficult to achieve complete digestion of the PCR products with *Alu*I (not with other enzymes) and this could not be improved by overdigestion using excess enzyme and extension of digestion time ***(14)***. This may be caused by unknown inhibitors in the PCR and PCR conditions suboptimal for the *Alu*I activity. However, the incomplete digestions were easily distinguished from undigested samples in our previous studies.
8. Subtype I of BKV was found to be the most prevalent strain ***(7–9)***. Therefore, restriction enzyme *Xmn*I can be used first to distinguish subtype I from other subtypes, which will avoid unnecessary work.
9. PCR amplicons produced using any primer pairs aforementioned could be used subsequently for automatic sequencing, and it is necessary to sequence the template with both the sense and antisense primer of the PCR amplification.
10. According to the sizes of DNA the concentration of agarose for gel electrophoresis varies from 2% for the NCR amplicons (600–800 bp), 3% for the VP1 amplicons (327 bp or 306 bp) to 4% for PCR-RE analysis (90–327 bp, **Table 2**).
11. Most dual infections with BKV were found to contain subtype I and one of the other subtypes ***(8)***. Not all of the existing heterozygous nucleotides in the subspecific region were readily detectable on DNA sequencing gels and minor strains presented faint bands (**Fig. 3**).

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5

Methods for Studying Interactions Between Simian Virus 40 T-Antigen and the Viral Origin of Replication

Kodangattil R. Sreekumar, Brett A. Barbaro, Andrea E. Prack, and Peter A. Bullock

1. Introduction

The Simian virus 40 (SV40) DNA replication system has served as a useful model for studies of DNA synthesis in eukaryotes [for reviews, *see* ***(1,2)***]. One major advantage of this system is that, with the exception of one viral protein, termed T-antigen (T-ag), all of the proteins required for DNA replication are supplied by cells that are permissive for SV40 replication ***(3)***. Because the virus depends on host proteins for replication ***(4)***, the SV40 system has been used to identify many of the proteins required for DNA synthesis in eukaryotes [for reviews, *see* ***(5–7)***]. Moreover, because T-ag binds to the SV40 origin of replication in a site-specific manner, this system has provided many insights into the protein–DNA interactions taking place at a eukaryotic origin during initiation of DNA replication [for reviews *see* ***(1,8)***]. Insights gained into the initiation on the SV40 origin are likely to be relevant to initiation events at other origins, because complicated biochemical processes are not believed to change radically once solved by evolution ***(9)***.

Methods used to study particular steps during initiation of SV40 replication, such as origin-specific DNA unwinding and formation of nascent DNA molecules, have been published previously ***(10)***. Herein we describe procedures, including gel mobility shift, footprinting, and nitrocellulose filter binding assays that have proven useful for studies of T-ag's interactions with the core origin. It is hoped that these detailed protocols will facilitate the study of protein/DNA interactions taking place at other origins of replication.

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1.1. Preparation of Oligonucleotides

Methods for studying T-ag's interactions with DNA, such as gel mobility shift, footprinting, and nitrocellulose filter-binding assays, frequently utilize oligonucleotides that have been labeled with ^{32}P. Procedures for preparing oligonucleotides for use in these reactions are described in **Subheading 3.1.**

1.2. Gel Mobility-Shift Assays

Gel mobility-shift assays (also termed electrophoretic mobility-shift assays) were developed by Fried and Crothers ***(11)*** and Garner and Revzin ***(12)*** and subsequently modified for use in the SV40 system ***(13–15)***. The steps required to conduct a band-shift assay with T-ag and linear fragments of duplex DNA, adapted from these references, are presented in **Subheading 3.2.**

1.3. DNA Footprinting of Particular Protein–DNA Complexes Formed in Band-Shift Reactions

Individual protein–DNA complexes formed in gel mobility-shift assays, such as those presented in **Fig. 1**, can be footprinted using the phenanthroline-copper technique ***(16)***. Steps required to perform this technique are described in **Subheading 3.3.**

1.4. Filter-Binding Assays

Filter-binding assays provide an alternative, glutaraldehyde-independent means for quantitating T-ag's interactions with the SV40 origin. In this assay, formation of protein–DNA complexes is measured by using nitrocellulose filters; oligonucleotides are not retained by nitrocellulose filters, but protein–DNA complexes are efficiently bound. The nitrocellulose filter binding-assay used to measure T-ag binding is based on previously published methods ***(17–19)***.

2. Materials

2.1. Preparation of Oligonucleotides

2.1.1. Purification of Oligonucleotides

1. Oligonucleotides: At Tufts University, these molecules are synthesized on an Applied Biosystems 394 DNA synthesizer. Commercial sources of oligonucleotides include Oligos Etc. (Guilford, CT) or Life Technologies Inc. (Rockville, MD).
2. Oligonucleotide elution buffer (*see* **Note 1**): 0.1% sodium dodecyl sulfate (SDS), 0.5 *M* ammonium acetate, and 10 m*M* magnesium acetate.
3. 10X TBE: 890 m*M* Tris-borate and 20 m*M* ethylendiaminetetraacetic acid (EDTA) (pH 8.0).
4. Gel-loading buffer (1X): 90% formamide, 10% 1X TBE.
5. Gel-loading dye (6X): 15% Ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol.

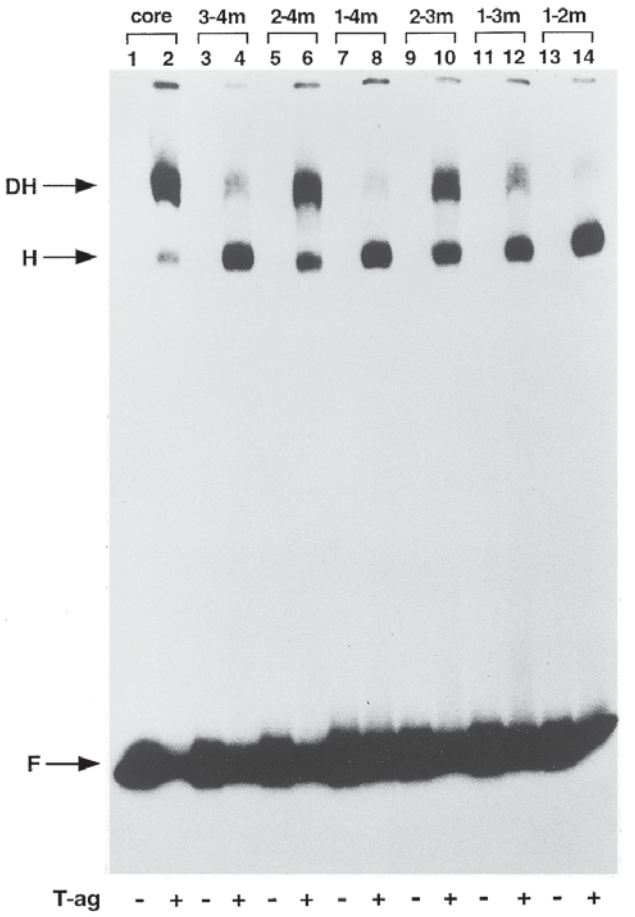

Fig. 1. Autoradiogram showing the products of a gel mobility-shift assay used to assess T-ag oligomerization on wild-type and mutant forms of the SV40 core origin of replication. This set of reactions was conducted in the presence of AMP-PNP, a nonhydrolyzable analog of ATP. A band-shift reaction was conducted with the 64-bp core origin and wild-type T-ag (lane 2). Reaction products formed with T-ag and mutant oligonucleotides (^{32}P labeled at the 5 ends) 3–4m, 2–4m, 1–4m, 2–3m, 1–3m, and 1–2m are presented in lanes 4, 6, 8, 10, 12, and 14, respectively, (*see* **ref. *25*** for details). The products of band-shift reactions conducted in the absence of T-ag and the indicated oligonucleotides are presented in the odd-numbered lanes. The positions of T-ag hexamers (H), T-ag double hexamers (DH), and free DNA (F) are indicated. Reprinted with permission from Joo et al. ***(25)***.

6. Tracking dye: For 1 mL, combine 333 µL gel-loading dye (6X) with 667 µL gel loading buffer.
7. TE: 10 m*M* Tris-HCl and 1 m*M* EDTA (pH 8.0).

8. Phenol-chloroform-isoamyl alcohol (25:24:1, Gibco-BRL, Gaithersburg, MD).
9. Chloroform-isoamyl alcohol (24:1, Gibco-BRL).
10. 10% denaturing polyacrylamide gel (approx 23-cm long, 16-cm wide, and 0.8-mm thick): To prepare the gel, combine 15 mL of 40% acrylamide solution (19:1, acrylamide: *bis*-acrylamide, Intermountain Scientific), 26 g urea, 6 mL 10X TBE, and distilled water (dH_2O) to 59.2 mL. Once the urea has dissolved, add 800 µL 10% ammonium persulfate and filter through Whatman #3 paper. Just before pouring the gel, add 25 µL TEMED.
11. Speedvac (e.g., Savant Instruments Inc., Farmingdale, NY).
12. Vertical gel electrophoresis apparatus.
13. Electrophoresis power supply.
14. Siliconized pipet tips (Marsh Biomedical Inc., Rochester, NY).
15. Low binding microcentrifuge tubes (Marsh Biomedical Inc.).
16. 90°C water bath.
17. Hand-held UV lamp (e.g., UVP Inc., Upland, CA).
18. UV protective glasses.
19. TLC plates with fluorescence indicator (cat. no. 4474-04, Cellulose PEI-F, J. T. Baker Inc., Phillipsburg, NJ).
20. Rocking platform (e.g., Nutator Mixer, available from Fisher Scientific, Pittsburgh, PA).

2.1.2. Hybridization to Form Duplex DNA

1. 5X hybridization buffer: 335 m*M* Tris-HCl (pH 7.6), 65 m*M* $MgCl_2$, 33.5 m*M* dithiothreitol (DTT), 6.5 m*M* spermidine, and 6.5 m*M* EDTA.
2. 90°C water bath.
3. Siliconized pipet tips (Marsh Biomedical Inc.).
4. Low binding microcentrifuge tubes (Marsh Biomedical Inc.).

2.1.3. Labeling Oligonucleotides

1. [^{32}P] ATP: (3000 Ci/mmol, 10 mCi/mL in Tricine (New England Nuclear Life Science Products, Boston, MA).
2. T4 polynucleotide kinase (10 U/µL) (Promega Corp., Madison, WI).
3. 10X polynucleotide kinase buffer (Promega Corp.): 700 m*M* Tris-HCl (pH 7.6), 100 m*M* $MgCl_2$, and 50 m*M* DTT.
4. Oligonucleotide(s) purified as described in **Subheading 3.1.**
5. 0.5 *M* EDTA (pH 8.0).
6. Pyrophosphate stop solution: Mix equal volumes of 100 m*M* tetrasodium pyrophosphate and salmon sperm DNA (1 mg/mL) (Sigma, St. Louis, MO).
7. TE containing 15 m*M* $MgCl_2$.
8. 5 *M* ammonium acetate.
9. Gel loading dye (6X): 15% Ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol.

10. 5% (w/v) trichloroacetic acid (TCA) and 1% (w/v) TCA.
11. Phenol-chloroform-isoamyl alcohol (25 : 24 : 1).
12. Chloroform-isoamyl alcohol (24 : 1).
13. Oligonucleotide elution buffer: 0.1% SDS, 0.5 *M* ammonium acetate, and 10 m*M* magnesium acetate.
14. Filtration unit (e.g., Hoefer single-filter holder for 25-mm filters, Amersham Pharmacia Biotech Inc., Piscataway, NJ).
15. Vertical gel electrophoresis apparatus.
16. Electrophoresis power supply.
17. Siliconized pipet tips (Marsh Biomedical Inc.).
18. Low binding microcentrifuge tubes (Marsh Biomedical Inc.).
19. Glass fiber filters (25 mm, Enzo Diagnostics Inc., Farmingdale, NY).
20. Disposable glass tubes (13 mm × 100 mm) (Fisher Scientific).
21. A 10% nondenaturing acrylamide gel (approx 23-cm long, 16-cm wide, and 0.8-mm thick): To prepare the gel, combine 15 mL 40% acrylamide (acrylamide: *bis*-acrylamide mixed at a ratio of 19 : 1, Intermountain Scientific), 6 mL 10X TBE, and 38.2 mL dH_2O. Add 0.8 mL of 10% ammonium persulfate, filter through Whatman #3 paper, and just before pouring the gel, add 25 µL TEMED.
22. Kodak XAR Film (Eastman Kodak Company, Rochester, NY).
23. Liquid Scintillation Counter (e.g., Beckman Instruments, Fullerton, CA).
24. Heat lamp.
25. Phosphorescent markers (small pieces of paper written on with an autoradiography pen, e.g., Bel-Art Products, Pequannock, NJ).

2.2. Gel Mobility Shift Assays

1. SV40 T-ag: this molecule can be purified using immunoaffinity techniques ***(10,21,22)***. In our laboratory, insect cells (Sf9) infected with a recombinant baculovirus are used for overexpression of SV40 T-ag. SV40 T-ag can also be purchased from CHIMERx (Madison, WI, cat. no. 5800-02).
2. Stock solutions for band-shift reactions: 1 *M* $MgCl_2$, 1 *M* DTT, 0.5 *M* creatine phosphate (pH 7.6), 0.5 mg/mL, creatine phosphate kinase in 50 m*M* imidazole buffer (pH 6.6), 0.5 mg/mL nonspecific competitor DNA (e.g., pBR322 digested with *Hae*III and dissolved in 10 m*M* Tris-HCl, pH 7.4, 0.1 m*M* EDTA), and 10 mg/mL bovine serum albumin (BSA). These solutions are stored at –20°C.
3. 5 end-labeled double-stranded oligonucleotides (25 fmol/µL) (*see* **Subheading 3.1.**).
4. T-ag storage buffer: 20 m*M* Tris-HCl (pH 8.0), 50 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT, 0.2 µg leupeptin/mL, 0.2 µg antipain/mL, 10% (v/v) glycerol, and 0.1 m*M* phenylmethyl sulfonyl fluoride (prepared from a 100 m*M* stock in isopropanol).
5. 0.1 *M* adenosine triphosphate (ATP) (pH 7.5), 0.1 *M* adenosine 5 -diphosphate (ADP) (pH 7.5), and 0.1 *M* AMP-PNP (in 10 m*M* Tris-HCl, pH 7.5) (Roche Molecular Biochemicals, Indianapolis, IN). Store at –80°C.
6. Hexokinase 1 U/µL (Roche Molecular Biochemicals, Indianapolis, IN).

7. 0.1 *M* glucose.
8. 1% glutaraldehyde (freshly prepared): 4 µL 25% glutaraldehyde (Sigma, St. Louis, MO) and 96 µL dH_2O.
9. 6X gel-loading dye: 15% Ficoll, 0.25% bromophenol blue, 0.25% xylene cyanol.
10. A 4–12% gradient polyacrylamide gel (approx 23-cm long, 16-cm wide, and 0.8-mm thick) (*see* **Note 2**).
11. 37°C water bath.
12. Gel electrophoresis apparatus (vertical).
13. Electrophoresis power supply.
14. Gel dryer (e.g., Amersham Pharmacia Biotech Inc.).

2.3. DNA Footprinting of Particular Protein–DNA Complexes Formed in Band-Shift Reactions

1. The "band-shift" reagents listed in **Subheading 2.2.**
2. 50 m*M* Tris-HCl (pH 8.0).
3. 40 m*M* 1,10-phenanthroline (cat. no. 13137-7, Aldrich, Milwaukee, WI) in ethanol (can be stored at 4°C for 6 mo).
4. 9 m*M* $CuSO_4$ (Aldrich) in dH_2O (can be stored at 4°C for 6 mo).
5. 3-mercaptopropionic acid (Aldrich).
6. Solution A: Mix 4 mL 40 m*M* 1,10-phenanthroline, 4 mL of 9 m*M* $CuSO_4$, and 72 mL dH_2O. (Prepare fresh.)
7. Solution B: Mix 400 µL 3-mercaptopropionic acid and 80 mL dH_2O. (Prepare fresh.)
8. Stop solution (28 m*M* 2,9-dimethyl phenanthroline in ethanol): Dissolve 468 mg of 2,9-dimethyl phenanthroline (Aldrich) in 80 mL ethanol. (Prepare fresh.)
9. Elution buffer: Combine 1 mL of 3 *M* sodium acetate (pH 5.2), 200 µL 10% SDS, 200 µL 0.5 *M* magnesium acetate, 10 µL of 10 mg/mL proteinase K, and 8.6 mL dH_2O. (Prepare fresh.)
10. Dimethyl sulfate (Sigma).
11. Dimethyl sulfate buffer (Sigma).
12. Dimethyl sulfate stop solution (Sigma).
13. Salmon sperm DNA (5 mg/mL) (Sigma).
14. 1 *M* piperidine formate (pH 2.0): Add 10 *M* piperidine to a 4% (v/v) solution of formic acid in water until the pH of the solution is 2.0.
15. 3 *M* sodium acetate (pH 5.2) and 0.3 *M* sodium acetate (pH 5.2).
16. 80% formamide resuspension solution: 80% v/v formamide, 10 m*M* NaOH, 0.1% bromophenol blue, 0.1% xylene cyanol, 1 m*M* EDTA. Can be stored at –20°C for 12 mo.
17. A 4–12% gradient polyacrylamide gel (*see* **Note 2**).
18. A 14% denaturing polyacrylamide gel (approx 40-cm long, 33-cm wide, and 0.3-mm thick): To prepare the gel, combine 35 mL of 40% acrylamide (acrylamide:*bis*-acrylamide mixed at a proportion of 19:1), 10 mL of 10X TBE, 46 g urea and dH_2O to 99 mL. After the urea is dissolved, add 1 mL of 10%

ammonium persulfate and filter through Whatman #3 paper. Just before pouring the gel, add 40 µL TEMED.
19. 90°C water bath.
20. Sequencing gel apparatus.
21. Gel dryer (e.g., Amersham Pharmacia Biotech Inc).
22. Kodak Biomax MS Film (Eastman Kodak Company).
23. Teflon PTFE Laboratory matting (e.g., Fisher Scientific).
24. Liquid scintillation counter (e.g., Beckman Instruments).

2.4. Filter-Binding Assays

1. 0.1 *M* Tris-HCl (pH 7.5).
2. 0.5 *M* KOH.
3. The previously described band-shift reagents (*see* **Subheading 2.2.**).
4. Glass microanalysis system (e.g., Millipore Corp., Bedford, MA).
5. Nitrocellulose filters (HAWP 45 µm, Millipore Corp.).
6. Heat lamp.

3. Methods

3.1. Preparation of Oligonucleotides

3.1.1. Washing Crude Oligonucleotides

1. To remove volatile chemicals that may be present following oligonucleotide synthesis, dissolve the oligonucleotide pellet(s) (approx 12 OD units) in 500 µL dH_2O.
2. Vortex vigorously, then dispense 100-µL aliquots into low-binding microcentrifuge tubes and dry using a Speedvac.
3. Redissolve the oligonucleotides in 100 µL dH_2O and dry again. Store oligonucleotides as pellet(s) at –80°C.

3.1.2. Purification of Oligonucleotides by Polyacrylamide Gel Electrophoresis (PAGE)

To separate full-length oligonucleotides from contaminants of smaller length, the washed oligonucleotides are routinely run on denaturing polyacrylamide gels and the molecule(s) of interest purified as follows:

1. To equilibrate the 10% denaturing polyacrylamide gel with the running buffer, prerun the gel in 1X TBE for approx 30 min at 20 W limiting (approx 590 V, approx 33 mA).
2. Dissolve oligonucleotide pellet(s) (approx 2.4 OD U) in 15 µL gel loading buffer (1X). Place in a 90°C water bath for 3 min and load onto the gel; to avoid contamination, samples should be loaded in alternating wells. To ensure uniform electrophoretic migration, load 15 µL of tracking dye into all wells lacking sample. Run the gel in 1X TBE at 20 W limiting for 2 h.

3. Once electrophoresis is completed, disassemble the plates and transfer the gel onto UV-transparent plastic wrap (e.g., Saran Wrap). Fold the plastic wrap around the gel so that it is completely covered. Place a TLC plate underneath the wrapped gel and hold a UV lamp (254 n*M*) at a distance of about 15 cm above it (*see* **Note 3**). Bands of DNA will cast a visible shadow. Using a fresh razor blade for each sample, cut out the band(s) of full-length oligonucleotide(s) and place the gel slice(s) in low-binding microcentrifuge tube(s).
4. Add 350 µL of oligonucleotide elution buffer and incubate at 4°C overnight. Spin the sample(s) in a microcentrifuge and transfer the supernatant(s) into fresh microcentrifuge tube(s) leaving behind the gel slices. To wash the gel slices, add 50 µL of oligonucleotide elution buffer and rock the tube for 10 min. Pool the oligonucleotide elution buffer wash with the initial supernatant.
5. Extract the supernatant-containing oligonucleotide twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol.
6. Determine the volume(s) of the sample(s), add 2.5X volume of 100% ethanol and mix well. Spin at 12,000*g* in a microcentrifuge at 4°C for 30 min. Aspirate off the ethanol. To wash the sample(s), add 800 µL 70% ethanol to the pellet(s) and spin at 12,000*g* in a microcentrifuge at 4°C for 20 min. Remove the ethanol and dry the pellet(s) using a speedvac.
7. Dissolve the pellet(s) in 40 µL TE (*see* **Note 4**).
8. Using a 50-fold dilution of the resuspended sample, calculate the amount of oligonucleotide recovered using the standard ratio of 1 $O.D_{260}$ = 33 µg/mL. Adjust the volume of TE so that the concentration of oligonucleotide in the sample is 10 pmol/µL.
9. Store the purified single-stranded oligonucleotide(s), adjusted to a concentration of 10 pmol/µL, at –20°C until ready to use.

3.1.3. Hybridization to Form Duplex DNA

In many instances, the interactions between T-ag and DNA are analyzed using double-stranded oligonucleotides. In our laboratory, such duplex DNA is formed using techniques described by Kadonaga and Tjian ***(23)***.

1. In a low-binding microcentrifuge tube, combine 2.5 µL of two complementary oligonucleotides (10 pmol/µL; 25 pmol total), 4 µL of 5X hybridization buffer and dH_2O to 20 µL. Vortex until thoroughly mixed and spin briefly.
2. To ensure complete denaturation of the DNA, place the tube(s) in a 90°C water bath for 3 min.
3. Turn off the power and let the sample(s) cool to 25°C in the water bath. (This usually takes several hours.) In general, the annealed oligonucleotide(s) are used immediately in labeling reaction(s), but may be stored at –20°C.

3.1.4. Labeling of Oligonucleotides

Both single-stranded and double-stranded DNA can be used in band shift and nitrocellulose filter-binding assays. Methods used to label and subse-

quently isolate the oligonucleotides used in these assays are described in this section.

3.1.4.1. Labeling Duplex Oligonucleotide(s)

1. On ice, combine 20 µL of hybridized oligonucleotide(s) (*see* **Subheading 3.1.3.**), 7 µL of [^{32}P] ATP, 2 µL dH_2O, and 2 µL T4 polynucleotide kinase (20 U). Mix the reagents and incubate at 37°C for 20–30 min.
2. Stop the reaction(s) by adding 2 µL 0.5 *M* EDTA, mix, and proceed to estimation of ^{32}P-incorporation by TCA precipitation.

3.1.4.2. Determining the Specific Activity of a Given Oligonucleotide

The specific activity of a given ^{32}P-labeled oligonucleotide is defined as the cpm/pmol DNA. One method for establishing the cpm in a particular oligonucleotide preparation is via TCA precipitation.

1. While incubating the labeling reaction, prepare for the TCA precipitation. Add 200 µL of pyrophosphate stop solution into 13 × 100-mm glass tubes and place on ice. Soak the glass fiber filters in dH_2O.
2. Once the labeling reaction has been stopped, withdraw 2 µL of the sample and add it to 8 µL TE (5X dilution).
3. Transfer 2 µL of the 5X diluted sample into the glass tube containing 200 µL pyrophosphate stop mix and fill the tube with chilled 5% (w/v) TCA. Following a 5-min incubation on ice, place the glass fiber filter on the filter holder and pour the contents of the tube over it. Then wash the filter three times with chilled 1% (w/v) TCA solution and once with chilled 95% ethanol. Impale the filter on a pin stuck into an aluminum foil-wrapped polystyrene support. Dry the filter under a heat lamp for 10 min, or air-dry.
4. Count the filter(s) in a scintillation counter without scintillation fluid.
5. To determine the specific activity of the sample (cpm/pmol DNA), divide the cpm on the filter by 0.3 pmol, which is the amount of DNA used for the TCA precipitation.

3.1.4.3. Purification of Labeled Oligonucleotide(s)

Unincorporated [^{32}P] ATP is removed via ethanol precipitation and the labeled oligonucleotides purified by electrophoresis through a nondenaturing polyacrylamide gel.

1. Preelectrophorese the 10% nondenaturing polyacrylamide gel in 1X TBE at 10 W limiting (approx 350 V, approx 28 mA) for approx 30 min before loading the samples.
2. The kinase reaction(s) (31 µL) are processed in the following manner: Add 69 µL of TE containing 15 m*M* $MgCl_2$ and 100 µL 5 *M* ammonium acetate. Add 500 µL 100% ethanol, mix the contents, and pellet the DNA by centrifugation at 12,000*g* in a microcentrifuge at 4°C for 30 min. Remove the ethanol, add 700 µL 70%

ethanol, and centrifuge again at 12,000*g* for 20 min at 4°C. Remove the ethanol and dry the pellet in a Speedvac.
3. Dissolve the DNA pellet in 20 µL TE containing 15 m*M* $MgCl_2$.
4. Add 10 µL of gel loading dye (6X), mix, load the samples onto a nondenaturing 10% polyacrylamide gel, and run the gel at 10W limiting for 2 h.
5. Transfer the gel onto a clean piece of used X-ray film. Tape phosphorescent markers on the sides to facilitate the subsequent alignment of the autoradiogram with the gel and cover with plastic wrap. Expose two films at room temperature (RT) for approx 2 min and develop the autoradiograms.
6. Locate the band(s) of interest on one of the developed autoradiograms and cut them out of the film. Using the phosphorescent markers, align the autoradiogram on the gel and remove the band(s) of interest. Transfer the gel slices into low-binding microcentrifuge tube(s). Add 350 µL of oligonucleotide elution buffer and incubate at 4°C overnight. Spin the sample(s) in a microcentrifuge and transfer the supernatant(s) into fresh microcentrifuge tube(s) leaving behind the gel slices. To wash the gel slices, add 50 µL of oligonucleotide elution buffer and rock the tube for 10 min. Pool the oligonucleotide elution buffer wash with the initial supernatant.
7. Extract the pooled sample twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol. Precipitate the DNA with 1 mL 100% ethanol and spin the tube(s) at 12,000*g* for 30 min in a microcentrifuge at 4°C. Remove the ethanol and add 700 µL 70% ethanol. Spin the tubes at 12,000*g* for 20 min in a microcentrifuge at 4°C. Remove the ethanol and dry the pellet(s) in a Speedvac.
8. Dissolve the pellet in 50 µL dH_2O and aliquot into approx 5 low-binding microcentrifuge tubes. Dry the DNA using a Speedvac. Count the dry pellets in a scintillation counter without scintillation fluid. Calculate the pmols of oligonucleotide in each pellet by dividing the cpm by the specific activity of the oligonucleotide (*see* **Subheading 3.1.4.2.**).
9. Calculate the volume of dH_2O to be added to each pellet to achieve a concentration of 25 fmol/µL; however, add the water to the pellet only when the oligonucleotide is ready to be used in an experiment.

3.1.4.4. Labeling Single-Stranded Oligonucleotides

There are many reasons for preparing single-stranded labeled oligonucleotides; for instance, to study T-ag's nonsequence-specific assembly on single stranded DNA. Moreover, the phenanthroline-copper DNA footprinting technique requires asymmetrically labeled DNA. Therefore, a single-stranded oligonucleotide is labeled and subsequently hybridized with its unlabeled complementary strand to form an asymmetrically labeled duplex DNA molecule. Steps required for labeling single-stranded oligonucleotides with [^{32}P] ATP are in the following section.

1. On ice, set up a 31-µL labeling reaction containing 2.5 µL (25 pmol) of single-stranded oligonucleotide, 3.1 µL of 10X kinase buffer, 7 µL of [^{32}P] ATP, 2 µL (20 U) of T4 polynucleotide kinase, and 16.4 µL dH_2O. Vortex, spin briefly, and incubate at 37°C for 20–30 min.
2. Stop the reaction(s) by adding 2 µL 0.5 *M* EDTA, mix and determine the specific activity of the oligonucleotide by trichloroacetic acid precipitation (*see* **Subheading 3.1.4.2.**).
3. Deproteinize the samples by extracting twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol.
4. Estimate the volume of the solution, add 0.1 volume of 3 *M* sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol; spin at 12,000*g* for 30 min at 4°C to precipitate the DNA. Decant the supernatant and add 700 µL 70% ethanol to wash the pellet(s). Spin at 12,000*g* for 20 min at 4°C, carefully drain the 70% ethanol, and dry the pellet in a Speedvac.

3.1.4.5. Forming Asymmetrically Labeled Double-Stranded Oligonucleotides

1. To the tube containing the labeled single-stranded DNA pellet, add 13.5 µL dH_2O, 2.5 µL (25 pmol) of unlabeled complementary oligonucleotide, and 4 µL of 5X hybridization buffer. Vortex until thoroughly mixed and spin briefly.
2. Incubate in a water bath at 90°C for 3 min. Turn off the power and let the sample cool to 25°C in the water bath.
3. Purify the asymmetrically labeled duplex oligonucleotide by gel electrophoresis (*see* **Subheading 3.1.4.2.**).

3.2. Gel Mobility-Shift Assays

To ensure optimal binding of T-ag to fragments of DNA from the SV40 origin, SV40 replication conditions are used ***(24)***. Glutaraldehyde is used to crosslink the relatively weak protein/DNA complexes prior to gel electrophoresis.

1. Preelectrophorese the 4–12% gradient polyacrylamide gel in 0.5X TBE at 10W limiting (approx 20 mA, approx 500 V) for approx 30 min before loading the samples.
2. For each reaction, combine the following in low-binding microcentrifuge tubes on ice: 0.3 µL of a $MgCl_2$/DTT mix (28 µL 1 *M* $MgCl_2$, 2 µL 1 *M* DTT, and 30 µL dH_2O), 0.8 µL 0.1 *M* ATP, 1.6 µL 0.5 *M* creatine phosphate, 0.96 µL 0.5 mg/mL creatine phosphate kinase, 0.5 µL 10 mg/mL bovine serum albumin (BSA), 1.6 µL 0.5 mg/mL competitor DNA, 1 µL of 25 fmol/µL 5 -end labeled oligonucleotide and dH_2O to 15.1 µL (*see* **Note 5**). Mix by gently tapping the tubes. (To conduct gel mobility shift assays with AMP-PNP or ADP, refer to **Note 6**.)
3. Add 4.9 µL of T-ag to the reaction (freshly diluted with T-ag storage buffer to 100 ng/µL), mix by gentle tapping the tube and incubate for 20 min at 37°C.

4. At the end of the reaction, add 2 µL of 1% glutaraldehyde, mix, and further incubate for 5 min at 37°C.
5. When incubation in the presence of glutaraldehyde is complete, add 5 µL of 6X gel loading dye and load samples on a prerun 4–12% gradient polyacrylamide gel.
6. Run the gel for approx 2 h at 10 W limiting (approx 20 mA, approx 500 V) (assuming a 64 bp DNA fragment—shorter fragments should be run for less time to prevent the free DNA from running off the end of the gel—e.g., 48 bp fragments are usually run for 1 h and 35 min).
7. At the end of the run, transfer the gel onto a sheet of Whatman 3 MM paper, cover with plastic wrap, and dry using a gel dryer.
8. Subject the dried gel to autoradiography and quantitative analysis.

Results of one gel shift experiment, reprinted from Joo et al. ***(25)*** are shown in **Fig. 1.**

3.3. DNA Footprinting of Particular Protein-DNA Complexes Formed in Band-Shift Reactions

3.3.1. In Situ *Footprinting of T-ag/Origin Complexes Formed in Gel Mobility-Shift Assays*

1. Conduct band-shift reactions as described in **Subheading 3.2.** using asymmetrically labeled double-stranded oligonucleotides (*see* **Subheading 3.1.4.5.**). Scale up the reactions fivefold (100-µL reactions) in order to ensure enough product for analysis. Add 25 µL of 6X gel loading dye.
2. Run the products of the gel shift assay on two or three lanes of a 4–12% polyacrylamide gradient gel (described in **Subheading 3.2.**).
3. While the samples are undergoing electrophoresis, prepare solution A, solution B, stop solution, and elution buffer (reagents described in **Subheading 2.3., steps 6–9**) for the footprinting reaction.
4. Once the electrophoresis is completed, gently pry apart the glass plates. Transfer the gel onto a solid support that permits easy drainage (e.g., Teflon PTFE laboratory matting) and place the gel in a glass baking dish (36 cm × 24 cm) containing 800 mL of 50 m*M* Tris-HCl (pH 8.0) for 30 s.
5. Cleavage reaction: Remove the gel from the baking dish by lifting it up with the matting. To the dish, add 80 mL of solution A followed by 80 mL of solution B. Mix the reactants and place the gel back in the dish for 20 min (*see* **Note 7**). To ensure uniform cleavage, gently rock the baking dish.
6. Remove the gel from the baking dish. Add 80 mL of the stop solution to the dish and mix. Place the gel in the dish for 2 min to let the dimethyl phenanthroline quench the cleavage reaction. As in previous steps, the dish should be gently rocked.
7. Remove the gel and place it in a second baking dish containing 800 mL of dH_2O for 2 min while gently rocking the dish.
8. Transfer the gel onto a clean piece of used X-ray film and tape phosphorescent markers onto the sides of the gel to facilitate the subsequent alignment of the

autoradiogram with the gel. Cover the gel and autoradiogram in plastic wrap. Expose two films at room temperature for approx 1 h.
9. Using phosphorescent markers, align the newly exposed autoradiogram with the gel. Cut out the band(s) of interest and transfer the gel fragment(s) into low-binding microcentrifuge tube(s). Add 350 µL of elution buffer and incubate at 37°C overnight. The following morning, spin the tubes in a microcentrifuge and transfer the elution buffer into fresh microcentrifuge tube(s) leaving behind the gel slices. To wash the sample(s), add 50 µL of elution buffer to the gel slices and rock them for 10 min. Collect the supernatant from the tube(s) and pool with the original sample.
10. Extract the sample(s) twice with phenol-chloroform-isoamyl alcohol and twice with chloroform-isoamyl alcohol. Precipitate the DNA with 1 mL 100% ethanol and spin the tubes at 12,000*g* for 30 min in a microcentrifuge at 4°C. Remove the ethanol and add 700 µL 70% ethanol. Spin the tubes in a microcentrifuge at 12,000*g* for 20 min at 4°C. Remove the ethanol and dry the pellets in a Speedvac.
11. Count the dry pellets in a scintillation counter.
12. Dissolve the pellets in 80% formamide resuspension solution (approx 4000 cpm/µL).
13. Boil the samples, and the Maxam and Gilbert sequencing reactions (*see* **Subheading 3.3.2.**) for 3 min. Load equal counts of the footprinting reactions (approx 10,000 cpm/lane) on a prerun 14% denaturing polyacrylamide gel. For the sequencing ladders, load approx 10,000 cpm of G reaction and approx 20,000 cpm of G+A reaction.
14. Run the gel at 60W limiting (approx 37 mA, approx 2100 V) for 2 h.
15. Fix the gel in a solution containing 10% methanol and 10% acetic acid for 15 min. Transfer the gel onto a sheet of Whatman #3MM paper and cover with plastic wrap. Using a gel dryer, dry the gel for 1 h.
16. Subject the dried gel to autoradiography and quantitative analysis.

Results from a representative *in situ* DNA-footprinting experiment are shown in **Fig. 2**.

3.3.2. Maxam and Gilbert Sequencing Reactions

A sequencing ladder formed using the Maxam and Gilbert ***(27)*** technique is used to establish the limits of the footprint formed in a given reaction. Aliquots of the same asymmetrically labeled oligonucleotide used in the *in situ* footprinting assay are used in this procedure. The Maxam and Gilbert sequencing reactions should be completed prior to conducting the *in situ* footprinting assay. For the sake of convenience, the steps required to perform G and G+A reactions are described in this section.

3.3.2.1. Cleavage at G Residues

1. In a low-binding microcentrifuge tube on ice, combine 15 µL of oligonucleotide (approx 25 fmol/µL), 200 µL of DMS buffer, 1 µL of salmon sperm DNA (5 mg/mL), and 0.5 µL dimethyl sulfate.

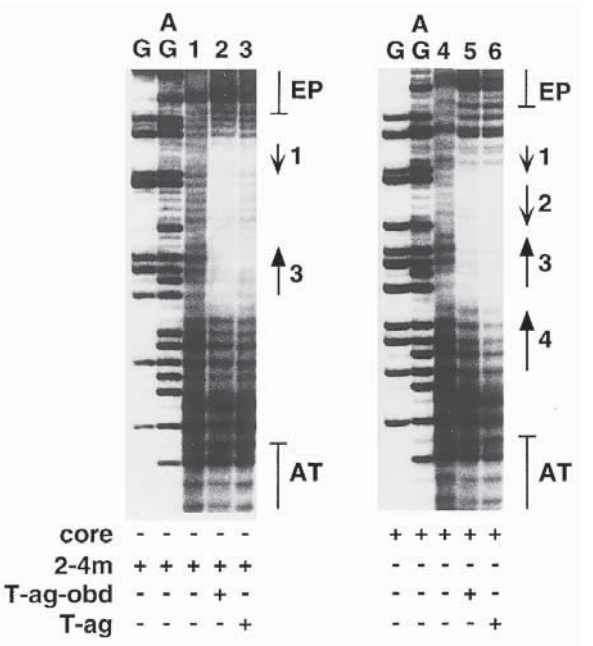

Fig. 2. *In situ* footprinting of T-ag double-hexamer/core complexes with the nuclease activity of 1, 10-phenanthroline-copper ion. Lanes 1 and 4 contain products of control reactions conducted with free DNA isolated from band-shift reactions containing mutant (2–4m) and wild-type (64 bp core) oligonucleotides, respectively (as described in **ref. *25***). Lanes 3 and 6 contain the products of footprinting reactions conducted with the same oligonucleotides, but isolated from T-ag double-hexamer complexes. Lanes 2 and 5 contain products of footprinting reactions conducted with T-ag-obd$_{131–260}$, the DNA binding domain of T-ag (as described in **ref. *26***) and the same pair of oligonucleotides. Size markers were generated by subjecting the indicated oligonucleotides to the G and G+A sequencing reactions described in **Subheading 3.3.2.** Flanking each panel is a map of the relative positions of the early palindrome (EP), pentanucleotides 1 to 4 (arrows) and the AT-rich region in the SV40 core origin. The arrows with smaller heads represent the complementary sequences of a given pentanucleotide. Reprinted with permission from Joo et al. ***(25)***.

2. Mix briefly, spin the tube in a microcentrifuge, and incubate for 5 min at RT.
3. Add 50 µL of DMS stop solution.
4. Add 750 µL 100% ethanol, mix, and spin the tube in a microcentrifuge at 12,000*g* for 20 min at 4°C.
5. Draw off the supernatant and discard into 5 *N* NaOH. Add 225 µL TE to the DNA pellet and vortex. Add 25 µL 3 *M* sodium acetate (pH 5.2) and 750 µL 100% ethanol. Vortex the tube and spin in a microcentrifuge at 12,000*g* for 20 min at 4°C.

6. Draw off the supernatant and add 20 µL dH_2O to the pellet and vortex. Add 900 µL 100% ethanol, vortex, and spin in a microcentrifuge at 12,000*g* for 20 min at 4°C. Remove the supernatant and dry the pellet in a speedvac.
7. To catalyze elimination of the modified residues, resuspend the pellet(s) in 100 µL of 1 *M* piperidine. After vortexing and a brief spin in a microcentrifuge, incubate at 90°C for 30 min.
8. Transfer the entire solution into a fresh low-binding tube and add 100 µL of 0.3 *M* sodium acetate (pH 5.2). Add 800 µL 100% ethanol and spin in a microcentrifuge at 12,000*g* for 20 min at 4°C.
9. Remove the supernatant and add 900 µL 70% ethanol. Spin at 12,000*g* for 15 min at 4°C. Remove the supernatant and dry the pellet using a speedvac.
10. Count the tubes in a scintillation counter.
11. Resuspend the pellet(s) in 80% formamide resuspension solution (approx 4000 cpm/µL).

3.3.2.2. Cleavage at G+A Residues

1. In a low-binding microcentrifuge tube on ice, combine 30 µL dH_2O, 30 µL oligonucleotide (approx 25 fmol/µL), 1 µL salmon sperm DNA (5 mg/mL), and 1.5 µL 1 M piperidine formate (pH 2.0).
2. Mix, spin the tube briefly in a microcentrifuge, and incubate the tube at 37°C for 25 min.
3. Add 162.5 µL TE, 25 µL 3 *M* sodium acetate (pH 5.2) and 700 µL 100% ethanol. Vortex the tube and spin in a microcentrifuge at 12,000*g* for 20 min at 4°C.

The sample is then washed, cleaved with 1 *M* piperidine and prepared for a sequencing gel as described in **Subheading 3.3.2.1., steps 6–11**. Using the 80% formamide resuspension solution, resuspend the G+A reactions at approx 8000 cpm/µL.

3.4. Filter-Binding Assay

3.4.1. Preparation of Nitrocellulose Filters

1. Soak nitrocellulose filters in 0.5 *M* KOH for 20 min at room temperature.
2. Wash the filters extensively (6X) with distilled water.
3. Soak the filters for 45 min in 0.1 *M* Tris-HCl (pH 7.5). Pour off the 0.1 *M* Tris-HCl, then store the filters in the same buffer at 4°C.

3.4.2. Filter-Binding Assay

1. In low-binding microcentrifuge tubes, assemble T-ag "binding reactions" (20 µL) (as in **Subheading 3.2.**). For each reaction combine the following: 0.3 µL of $MgCl_2$/DTT mix (28 µL 1 *M* $MgCl_2$, 2 µL 1 *M* DTT, and 30 µL dH_2O), 0.8 µL of 0.1 *M* ATP, 1.6 µL 0.5 *M* creatine phosphate, 0.96 µL 0.5 mg/mL creatine phosphate kinase, 0.5 µL 10 mg/mL BSA, 1.6 µL 0.5 mg/mL competitor DNA, 1 µL (25 fmol) of 5 -end labeled oligonucleotide, and 7.5 µL dH_2O (*see* **Note 5**).

Table 1
Acrylamide Solutions

	4%	12%	Extra 4%
10X TBE	0.7 mL	0.7 mL	0.7 mL
40% acrylamide	1.4 mL	4.2 mL	1.4 mL
dH_2O	to 14 mL	to 14 mL	to 14 mL

Count the reaction tubes in a scintillation counter to determine the total cpm in the reaction. (To conduct filter binding assays with AMP- PNP or ADP, refer to **Note 6**.)

2. Add 4.9 µL T-ag to the reaction (freshly diluted with T-ag storage buffer to 100 ng/µL), aspirate to mix, and incubate for 20 min at 37°C. Add T-ag to the samples at 1-min intervals, as it takes this amount of time to process each filter.
3. Spin the tubes and remove the entire reaction mix into a siliconized pipet tip. Carefully apply the reaction mix onto a nitrocellulose filter on the glass base of the filtration apparatus. Rinse the reaction tube and the pipet tip with 100 µL 0.1 *M* Tris-HCl (pH 7.5) and spot onto the nitrocellulose filter. Wash the filter with 5 mL of 0.1 *M* Tris-HCl (pH 7.5).
4. Using a heat lamp, dry the filter for 10 min. Count the filter in a scintillation counter.
5. Divide the cpm on the filter by the total cpm in the sample to determine the fraction of DNA retained on the filter in protein/DNA complexes.

4. Notes

1. Preparation of many of these reagents is described in Sambrook et al. ***(20)***.
2. The glass gel plates (approx 23-cm long, 16-cm wide, and 0.8-mm thick) should be thoroughly cleaned with water and ethanol, and (when necessary) treated with Sigmacote (Sigma Chemical Co.). Once assembled, place the gel mold in a vertical position using appropriate supports (e.g., styrofoam boxes). Place a magnetic stirrer on an object such that the stirrer is approx 1–2 ft above the top of the gel. Place a gradient gel maker on the top of the stirrer, and place a small magnetic stirring bar in the chamber proximal to the outlet tubing. Ensure that the valve between the two chambers of the gradient maker is closed. Use a hemostat to prevent the initial flow of the acrylamide solution.

   In 15-ml conical tubes prepare the 4% and 12% acrylamide solutions (acrylamide/*bis*-acrylamide 19:1) as seen in **Table 1**.

   When ready to cast the gel, add 80 µL ammonium persulfate (made fresh) and 8 µL TEMED to the 4% and 12% acrylamide solutions; mix the solutions by inverting the tubes. Pour the 12% solution into the chamber proximal to the outlet tubing and the 4% solution into the second chamber. At the top center of the gel mold, insert the tubing about 1 cm down into the space between the plates. Remove the hemostat, and once the solution has started flowing, open the valve

connecting the two chambers of the gradient maker. If needed, use the "Extra 4%" solution to top up the gel. Insert the 0.8-mm-thick comb, clamp the comb in the middle, and let the gel polymerize overnight.

3. UV shadowing gives best results if carried out in a dark room. Wear UV protective glasses or a face mask while working with UV.
4. In our laboratory, we generally start with approx 2.4 OD U of oligonucleotide and dissolve the final pellet in 40 µL TE. Depending on the amount of oligonucleotide one starts with and the efficiency of recovery, one will have to adjust the volume of TE used to dissolve the oligonucleotide.
5. The final concentrations of the reagents in the reaction are 7 m*M* $MgCl_2$, 0.5 m*M* DTT, 4 m*M* ATP, 40 m*M* creatine phosphate, 24 µg/mL creatine phosphate kinase, 250 µg/mL of BSA, 40 µg/mL competitor DNA, and 1.25 pmol/mL of 5 end-labeled oligonucleotide .
6. To test the effect of different nucleotides or nucleotide analogs on protein/DNA complex formation, ATP can be replaced by ADP, AMP-PNP, and so on. In reactions conducted with ADP, replace the creatine phosphate kinase with an equal volume of 50 m*M* imidazole buffer. Trace amounts of ATP present as a contaminant in other reagents may be converted to ADP by incubating the reaction mix in the presence of 1 U hexokinase and 10 m*M* glucose at 37°C for 5 min. This preincubation should be performed before adding T-ag. Also, the amount of water added must be adjusted to achieve a final reaction volume of 20 µL.
7. Depending on the protein/DNA complex and the concentration of acrylamide in the gel, it may be necessary to conduct pilot studies to determine the optimum time for cleavage.

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6

Cell-Free Transcription of SV40

Richard J. Kraus and Janet E. Mertz

1. Introduction

The development of soluble cell-free systems that accurately transcribe genes has provided a means to investigate the mechanisms of eukaryotic transcription. The results of many studies have shown that specific DNA *cis*-acting elements, together with a complex array of protein factors, are required for transcription to occur. In particular, there has been an explosion of knowledge regarding the general transcription factors required by RNA polymerase II to function efficiently ***(1–4)***. Our laboratory has been investigating the molecular mechanisms by which the genes of SV40 are transcriptionally regulated. To elucidate the biochemical factors involved in the transcriptional regulation of SV40, the development of a cell-free transcription system was required. In this chapter, we describe conditions for growing and harvesting cells that can serve as a source for the cell-free nuclear extract, a method for preparing the extract, conditions for transcribing the SV40 early and late promoters in this cell-free system, and assays for the analysis of the transcription products.

1.1. Growing and Harvesting Cells

HeLa cells, a human cervical cancer cell line, are commonly used as a source for the preparation of transcriptionally active nuclear extracts for several reasons. First, these cells can be grown in suspension, thereby allowing one to easily harvest substantial volumes of cells. Growing cells in suspension also eliminates the need to use trypsin when harvesting cells. Second, *HeLa* cells grow rapidly, doubling every 24 h. Finally, *HeLa* cells cultured in suspension grow well in calf serum, saving one the higher cost of fetal calf serum (FCS). In this chapter, we describe methods for growing and harvesting *HeLa* cells

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from which transcriptionally active extracts can be made. These procedures are also applicable to other cell lines that can be grown in suspension.

1.2. Preparing Nuclear Extracts

The preparation of transcriptionally active nuclear extract described here consists of the selective extraction of transcription components from intact nuclei. Our protocol is adopted from the procedure originally described by Dignam et al. ***(5)***. It is predicated on the fact that salt concentrations of approx 0.3 *M* break the ionic interactions between proteins and nucleic acids. However, the ratio of the salt concentration to the number of cells is critical to obtain transcriptionally active extracts. Salt concentrations that are too low result in only partial extraction of the transcriptional machinery. Excessive salt concentrations extract large quantities of histone proteins that repress transcription.

1.3. Cell-Free Transcription of SV40 DNA

The SV40 genome contains a bidirectional promoter region (reviewed in **refs. *6–8***; *see* **Fig. 1**). The early promoter contains a strong consensus "TATA" box sequence ***(6)***. The late promoter contains multiple basal elements, none of which conform to consensus element sequences ***(8)***. Thus, the development of a cell-free transcription system that met the biochemical requirements of both promoters provided a unique and difficult challenge. The system described here, adopted from the work of Farnham and Schimke ***(9)***, represents the optimal conditions we worked out for the simultaneous cell-free synthesis of RNA from the SV40 early and late promoters (*see* **Fig. 2**).

Fig. 1. *(see facing page)* Schematic of the SV40 genome and primers used to analyze synthesis of the SV40 RNAs. **(A)** Map of the wild-type SV40 genome. The 5243-bp circular genome is represented by the inner circle. The genome is controlled by one bidirectional promoter that can be divided into two transcriptional units, early and late. The thin solid lines represent the structures of the early and late mRNAs. The dashed lines depict the intervening sequences excised from the primary transcripts. The thick lines indicate the regions of the mRNAs encoding the SV40 proteins. **(B)** Representation of the promoter region of the SV40 genome. The arrows indicate the early-early (E-E), the major late (MLP) and minor late transcriptional start sites observed in the cell-free transcription system. The open rectangles below the major late promoter show the locations of the basal proximal elements as identified by Ayer and Dynan ***(8)***. The various other symbols indicate the binding sites for *trans*-acting factors. The SV40 nucleotides are given in the numbering system of Buchman et al. ***(17)***. **(C)** Locations of the primers used in the primer extension analyses and the expected cDNA products of the major RNA species.

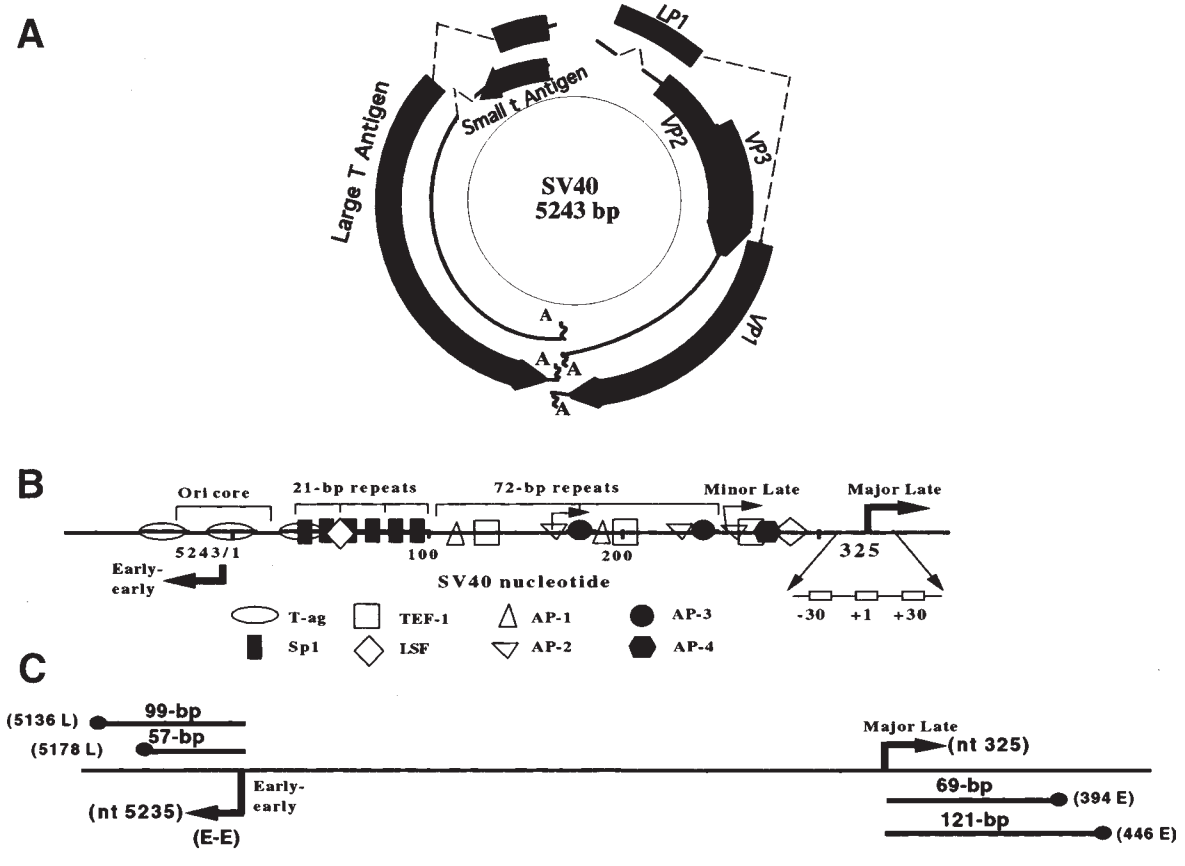
A
LP1
Small t Antigen
Large T Antigen
VP2
VP3
SV40
5243 bp
VP1
A
A
A
A
B
Ori core
21-bp repeats
72-bp repeats
Minor Late
Major Late
5243/1
100
200
325
Early-
early
SV40 nucleotide
-30
+1
+30
T-ag
TEF-1
AP-1
AP-3
Sp1
LSF
AP-2
AP-4
C
(5136 L)
99-bp
(5178 L)
57-bp
Major Late
(nt 325)
Early-
early
(nt 5235)
(E-E)
69-bp
(394 E)
121-bp
(446 E)

Fig. 1.

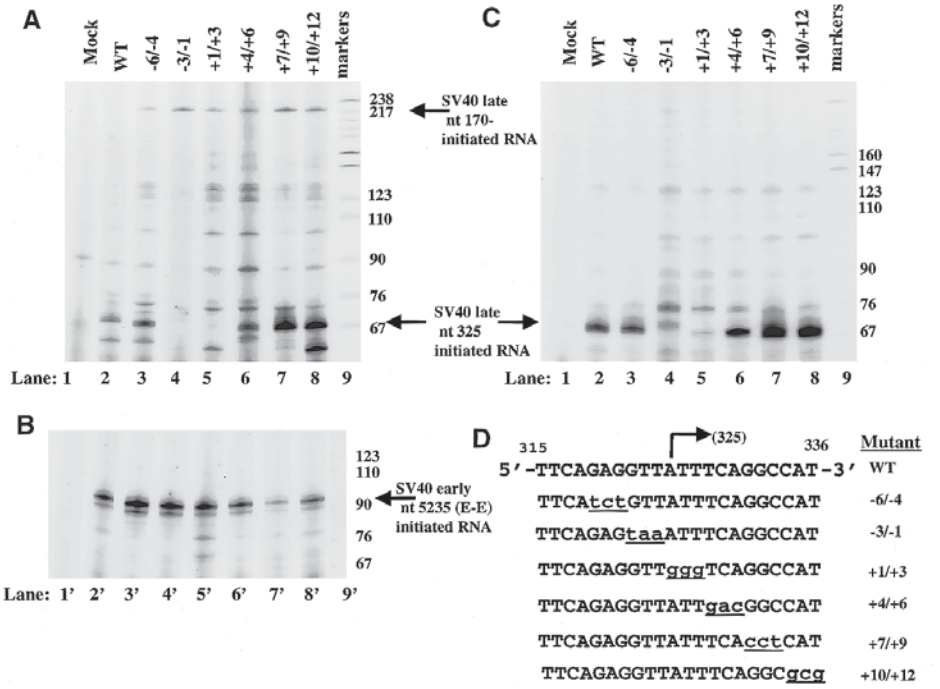

Fig. 2. *HeLa* cell nuclear extract accurately and efficiently transcribes the early and late genes of SV40. **(A)** Autoradiogram of a primer extension analysis showing the quantities and locations of the 5 -ends of the late RNAs synthesized from the indicated SV40 wild-type (WT) and mutant genomes in the cell-free transcription system described here. The SV40 wild-type and mutant templates transcribed here are in the pSV1773 background. pSV1773 contains a 7-bp deletion at the *Acc*1 restriction endonuclease site of pSVS. This mutation disrupts the synthesis of the capsid protein VP1, thereby disabling the encapsidation of SV40 DNA and the formation of infectious virions. pSVS contains wild-type strain SVS combined at its unique *Eco*RI site with the *Pvu*II-to-*Eco*RI fragment of pBR322 in which the *Pvu*II site has been replaced with an *Eco*RI recognition site ***(18)***. The mutants differ from pSV1773 by the major late alterations depicted in panel D. The construction of the late promoter mutants is described in detail in **ref.** ***12***. **(B)** Autoradiogram of a primer extension analysis of the 5 ends of the early RNAs synthesized concurrently in the reactions shown in panel A. **(C)** Autoradiogram of a primer extension analysis of the 5 -ends of the SV40 late RNAs accumulated in CV-1PD cells by 42 h after transfection with the indicated SV40 late promoter wild-type and mutant genomes. The wild-type and mutant templates are identical to the ones studied in panel A except the pBR322 cloning vector sequences had been excised from the viral ones prior to transfection (*see* Chapter 7). **(D)** Sequences of the initiator region of the cluster point mutants analyzed here (*see* **ref.** ***12*** for details). The lower case, underlined letters indicate the alterations from the wild-type sequence.

1.4. Primer Extension Analysis of SV40 Early and Late RNAs Synthesized by Cell-Free Transcription

Primer extension is one of several techniques used to map the 5 -ends of RNA, as well as to quantitate levels of RNA. The test RNA is initially hybridized with an excess of single-stranded DNA primer that is complementary to RNA sequences located usually within 100 nucleotides of the 5 -terminus of the RNA. This primer is either radiolabeled or fluorescently labeled at its' 5 end. Reverse transcriptase, in the presence of excess deoxynucleotide 5 -triphosphate (dNTPs), is then used to extend the primer by synthesizing the strand complementary to template RNA. The remaining single-stranded RNA is hydrolyzed and the resulting complementary DNA strand (cDNA) product is quantified and sized by electrophoresis through a denaturing polyacrylamide gel. The length of the cDNA reflects the distance between the 5 -end of the primer and the 5 -terminus of the RNA. The yield of the cDNA approximates the amount of the RNA. Thus, primer extension provides an excellent analytical technique to quantitate RNA that is correctly synthesized by RNA polymerase II in a cell-free transcription system ***(10)***.

2. Materials

2.1. Growing and Harvesting Cells

2.1.1. Equipment

1. Spinner flasks: 250 mL, 1 L, and 3 L (Bellco Glassware Co., Vineland, NJ). In the absence of spinner flasks, one can substitute Erlenmeyer flasks and magnetic stirrers.
2. Bright-line hemocytometer (Cambridge Instruments, Buffalo, NY).

2.1.2. Cell Lines

1. The cells used here were *HeLa* S3 cells obtained from Dr. Peggy Farnham (University of Wisconsin-Madison). Prior to use, the cells were stored frozen in liquid nitrogen at a cell density of approx 10^7 cells/mL.

2.1.3. Reagents and Solutions

1. Calf serum (Hyclone Laboratories, Logan, UT).
2. Joklik's minimal essential medium, MEM (Gibco-BRL Life Technologies, Grand Island, NY).
3. Penicillin /streptomycin, 10,000 U/mL each (Gibco-BRL Life Technologies).
4. Storage solution A: 5 m*M* KH_2PO_4, 25 m*M* KOH, 30 m*M* NaCl, 0.5 m*M* $MgCl_2$, 20 m*M* L-lactic acid (Sigma cat. no. L-1750, Sigma Chemical Co., St. Louis, MO), 5 m*M* glucose, 0.2 m*M* sorbital. Filter sterilize and store at 4°C.

5. Storage solution B: same ingredients as storage solution A, but make up initially at 1.25X, filter-sterilize, then add dimethylsulfoxide (DMSO) (Fisher Scientific, Fair Lawn, NJ) to 20% and store at 4°C.

2.2. Preparing Nuclear Extract

2.2.1. Equipment

1. 15-mL Dounce homogenizer tube, with A and B pestles (Kontes Scientific Glassware/Instruments, Vineland, NJ).
2. Dialysis tubing (6–8-kDa cutoff, The Spectrum Companies, Gardena, CA). Boil for several minutes in 30% ethanol prior to use.
3. 15- and 30-mL Corex heavy-walled centrifuge tubes (Kontes Scientific Glassware/Instruments).

2.2.2. Reagents and Solutions

1. Glycerin (ACS grade, Fisher Scientific, certified ACS).
2. Buffer A: 20 m*M* HEPES (pH 7.9), 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 1 m*M* DTT (dithiothreitol).
3. Buffer C (high salt buffer): 40 m*M* HEPES (pH 7.9), 25% glycerol, 0.42 *M* NaCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* ethylenediaminetetraacetic acid (EDTA), 0.5 m*M* PMSF (phenylmethylsulfonyl fluoride, ultrapure grade, Boehringer Mannheim Biochemicals, Indianapolis, IN), 1.0 m*M* DTT. PMSF is freshly prepared as a 0.1-*M* solution in isopropanol and added immediately before use. DTT can be stored frozen, but is also added immediately before use.
4. Dialysis buffer D: 40 m*M* HEPES (pH 7.9), 20% glycerol, 0.1 *M* KCl, 6 m*M* MgC_{l2}, 0.2 m*M* EDTA, 0.5 m*M* PMSF, 1.0 m*M* DTT.

2.3. Cell-Free Transcription of SV40 DNA

2.3.1. Reagents and Solutions

1. 25X rNTPs: 5 m*M* ATP, 15 m*M* CTP, 15 m*M* UTP, 15 m*M* GTP (rNTPs are available from Amersham Pharmacia Biotech, Piscataway, NJ).
2. Phenol/chloroform/isoamyl alcohol (25:24:1, pH 7.9).
3. *HeLa* cell nuclear extract (approx 10–15 mg protein/mL).
4. Supercoiled plasmid SV40 DNA.
5. Stock transcription buffer: 20 m*M* HEPES (pH 7.9), 7.3 m*M* $MgCl_2$, 10% glycerol, 50 m*M* KCl, 0.1 m*M* EDTA, 1 m*M* DTT, 143 m*M* potassium glutamate (potassium salt of glutamic acid, available from Sigma Chemical Co.).
6. Stop buffer A (SV40 early stop buffer): 0.1 *M* sodium acetate (pH 5.2), 0.4% sodium dodecyl sulfate (SDS), 1.0 m*M* EDTA, 0.9 mg/mL yeast tRNA (tRNA available from Sigma Chemical Co.).
7. Stop buffer B (SV40 late stop buffer): 0.1 *M* sodium acetate (pH 5.2), 0.4% SDS, 1.0 m*M* EDTA, 0.4 mg/mL Poly (A) [Poly (A) is available from Amersham Pharmacia Biotech].

2.4. Primer Extension Analysis of SV40 Early and Late RNAs Synthesized by Cell-Free Transcription

2.4.1. Preparation of Radiolabeled Primers for Detection of SV40 Early and Late RNAs

2.4.1.1. Reagents and Solutions

1. [^{32}P]ATP (5000–6000 Ci/mmol, 10 µCi/µL, Amersham Pharmacia Biotech).
2. T4 polynucleotide kinase (10U/µL, New England Biolabs, Beverly, MA).
3. SV40 late primer (called 394 E) 5 -TTCAGTCCATGACCTACGAACCTTA-3 (gel purified, *see* **Note 1**).
4. SV40 early primer (called 5136 L) 5 -AATCCTCTCTGTTTAAAACTTTATC-3 (gel purified, *see* **Note 1**).
5. 10X kinase buffer: 700 m*M* Tris-HCl (pH 7.6), 100 m*M* $MgCl_2$, 10 m*M* EDTA, 10 m*M* spermidine (Sigma Chemical Co.). Alternatively, use the buffer supplied by the manufacturer of the T4 polynucleotide kinase.

2.4.2. Primer Extension Reaction

2.4.2.1. Reagents and Solutions

1. AMV reverse transcriptase, 25 U/µL (Boehringer Mannheim Biochemicals).
2. RNasin (Promega Corp., Madison, WI).
3. Actinomycin D (Calbiochem, La Jolla, CA).
4. dNTPs (Amersham Pharmacia Biotech).
5. 5X annealing buffer: 1.5 *M* NaCl, 50 m*M* Tris-HCl (pH 7.5), 5 m*M* EDTA.
6. 1.25X AMV reverse transcriptase buffer (RT Buffer): 12.5 m*M* Tris-HCl (pH 8.0), 10 m*M* $MgCl_2$, 12.5 m*M* DTT, 1.25 m*M* each dNTP, 62.5 µg/mL Actinomycin D.
7. Termination buffer: 2.5 *M* ammonium acetate (pH 5.2), 3 m*M* EDTA, 100 µg/mL tRNA.
8. Formamide loading buffer: 80% formamide, 1 m*M* EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol (Sigma Chemical Co.).

3. Methods

3.1. Growing HeLa Cells in Suspension

1. Quickly thaw 1 vial (10^7) cells at 37°C.
2. Using a laminar flow hood (*see* **Note 2**), transfer the cells to a sterile 250-mL spinner flask containing 250 mL Joklik's MEM, 5% calf serum, and 100 U/mL penicillin /streptomycin (*see* **Note 3**).
3. Place the flask on top of a magnetic stirring plate within a 37°C incubator and gently stir (*see* **Note 4**).
4. When there are approx 4–6 × 10^5 cells/mL, transfer the contents of the flask to a 1-L sterile spinner flask containing 750 mL of Joklik's MEM, 5% calf serum, and 100 U/mL penicillin/streptomycin. Gently stir at 37°C for 48 h. Cell density should again reach 4–6 × 10^5 cells/mL.

5. Transfer the contents to a 3-L spinner flask containing 2 L of the growth medium aforementioned. Again, grow the cells with gentle stirring for 48 h at 37°C to densities of 4–6 × 10^5 cells/mL, at which time they are ready to harvest.

3.2. Harvesting Cells

1. To harvest the cells, distribute 2 L of the cell suspension equally among four 500-mL centrifuge tubes.
2. To the remaining 1 L of cell suspension, add 2 L Joklik's MEM, 5% calf serum, 100 U penicillin/streptomycin (*see* **Note 5**), and continue incubation as aforementioned.
3. Pellet the cells by centrifugation at 4°C, 800*g* for 10 min. Decant the medium.
4. Wash the cells by the addition of 10 mL cold PBS per tube, gently swirl to resuspend the cells, then pellet the cells by centrifugation.
5. Resuspend the cells in 10 mL of cold PBS per centrifuge tube and combine the cells in one 50-mL centrifuge tube.
6. Pellet the cells again by centrifugation at 4°C, 800*g* for 10 min.
7. Gently wash the pellet with 10 mL of cold PBS without resuspending the cells.
8. To the pelleted cells, add 25 mL of storage buffer A, resuspend the cells by gently swirling and place on ice for 10 min.
9. Add 25 mL of cold storage buffer B. Carefully and completely resuspend the cells by inverting the tube several times.
10. Place directly in a –70°C freezer and store the cells frozen at –70°C (*see* **Notes 6** and **7**).

3.3. Preparing Nuclear Extract

1. Quickly thaw the frozen cells at 37°C, carefully mixing to accelerate thawing. We typically start with 1–2 × 10^{10} *HeLa* cells.
2. Centrifuge the cells at 800*g* for 10 min and pipet off the supernatant; carefully estimate the packed cell volume (pcv). For example, at 1–2 × 10^9 cells/tube, the pcv should be 5 mL (*see* **Note 8**).
3. Add a 4X volume of the pcv (i.e., for 5 mL pcv add 20 mL) cold buffer A, resuspend the cells, and allow them to swell at room temperature for 10 min.
4. Pellet the cells at 800*g* for 10 min at 4°C.
5. Add 2X volume of the pcv (as determined in **step 2** above) buffer A. Resuspend the cells, transfer to a cold 15-mL Dounce homogenizer and lyse the cells with 15 strokes of the Dounce pestle A (loose-fitting).
6. Transfer lysed contents to 30-mL Corex centrifuge tubes and pellet at 900*g* for 10 min. Remove most, but not all, of the supernatant.
7. Respin the nuclei at 12,000*g* for 30 min at 4°C. Remove the remaining supernatant.
8. Resuspend the nuclei in 2.5 mL buffer C/1 × 10^9 cells.
9. Transfer the suspension to a cold 15-mL Dounce homogenizer tube and break up the nuclei with 15 strokes using a Dounce B pestle (tight-fitting).
10. Pour the contents into a beaker and stir for 30 min at 4°C.
11. Pellet the debris by centrifuging at 12,000*g* for 30 min at 4°C in 15-mL Corex tubes.

12. Transfer the supernatant to a dialysis bag and dialyze against a minimum of 50 volumes of buffer D at 4°C overnight (at least 5 h).
13. Clear the dialysate of particulate matter by centrifugation at 6500*g* for 10 min at 4°C.
14. Determine protein concentrations (*see* **Note 9**).
15. Aliquot the dialysate as desired, quick freeze the aliquots on dry ice, and store at –70°C.

3.4. Cell-Free Transcription of SV40 DNA

1. Perform cell-free transcription reactions in 1.5-mL microfuge tubes containing the following: 28 µL stock transcription buffer, 4 µL DNA (500 ng of wild-type SV40), 16 µL *HeLa* cell nuclear extract (10–15 mg/mL), and 2.0 µL 25X rNTPs (*see* **Notes 10–13**).
2. After incubation at 30°C for 30 min, transfer half of the mixture to another tube (tube 2).
3. Terminate the reaction in tube 2 by adding 75 µL of SV40 early stop buffer.
4. Stop the reaction in the original tube by adding 75 µL of SV40 late stop buffer.

3.4.1. Isolation of RNA

1. Add 100 µL of phenol:chloroform:isoamyl alcohol to each of the two reaction mixtures. Thoroughly extract by mixing with a vortex mixer.
2. Separate the phases by centrifugation with a microcentrifuge at high speed for 5 min.
3. Transfer the aqueous phase, which contains the nucleic acids, to a new 1.5-mL tube.
4. Back extract the original phenol phase by adding a further 100 µL of the appropriate stop buffer, mixing thoroughly, and centrifuging as aforementioned.
5. Pool the aqueous phase with the aqueous phase from the first extraction.
6. To the pooled aqueous phases, add 600 µL absolute ethanol.
7. Precipitate the nucleic acids by incubating on dry ice for 15–30 min. Pellet the nucleic acids by centrifugation with a microcentrifuge at 4°C, high speed, for 20 min.
8. Decant the ethanol. Gently wash the pellet with 400 µL of 70% ethanol that has been stored at –20°C. Remove the residual ethanol by drying the pellet in a speed-vacuum centrifuge. The precipitated RNA can be stored indefinitely at –20°C.

3.5. Primer Extension Analysis of SV40 Early and Late RNAs Synthesized by Cell-Free Transcription

3.5.1. Preparation of Radiolabeled Primers for Detection of SV40 Early and Late RNAs

1. In a final volume of 30 µL, add 100 µCi of [^{32}P] ATP and 10 units of T4 polynucleotide kinase to 100 ng of the desired oligonucleotide. Incubate at 37°C for 1 h.

2. To aid the precipitation of the SV40 Early Primer, add 25 µg carrier tRNA to the reaction mixture (*see* **Note 14**). To the SV40 Late Primer add 25 µg Poly (A) carrier (*see* **Note 14**).
3. To precipitate the radiolabeled primers, add 100 µmol of ammonium acetate, 125 µL of absolute ethanol and incubate on dry ice for 15–30 min.
4. Pellet by centrifugation in a microcentrifuge at high speed for 20 min at 4°C. Decant the ethanol.
5. Gently wash the radiolabeled pellet with 40 µL cold (–20°C) 70% ethanol. Remove the residual ethanol by drying in a speed vacuum-centrifuge.
6. Dissolve the pellet in 50 µL H_2O. In a typical labeling reaction, approximately half of the radioactivity is present, with nearly complete recovery of the oligonucleotides. The specific activity of the radiolabeled primers is typically 0.5 µCi/ng or 5 µCi/pmol. The primers can be stored for up to 1 wk at –20°C, after which time fresh primer must be prepared.

3.5.2. Primer Extension Reaction

1. Dissolve the dried pellet of RNA obtained from the cell-free transcription reaction in 5 µL H_2O. Add 2 µL annealing buffer and 3 µL, 5 ^{32}P-labeled primer (total volume, 10 µL).
2. Heat at 80°C for 1 min to denature the RNA.
3. Immediately incubate at 62°C for 15 min or longer to enable annealing of the primer to the RNA. Briefly centrifuge in a microcentrifuge to bring down any condensed water and to ensure concentrations of ingredients are correct.
4. Add 40 µL RT buffer, 1 µL RNasin (20–40 U), and 12.5 U of AMV reverse transcriptase (RT).
5. Gently mix. Incubate at 48°C for 90 min to ensure that the reverse transcriptase transcribes all the way to the 5 -end of the RNA (*see* **Note 15**).
6. Stop the reaction by adding 6 µL of 1 *N* NaOH and 1 µL of 0.25 *M* EDTA.
7. Incubate at 48°C for 45 min to hydrolyze the remaining single-stranded portion of the RNA.
8. Add 6 µL of HCl to neutralize the reaction mixture.
9. Precipitate the newly synthesized cDNA by the addition of 30 µL termination buffer and 300 µL absolute ethanol. Thoroughly mix and place on dry ice for 15–30 min.
10. Pellet the radiolabeled cDNA by centrifugation with a microcentrifuge at high speed for 20 min at 4°C. Decant the ethanol phase.
11. Wash the pellet with 50 µL cold 70% ethanol and dry with a speed-vacuum centrifuge.
12. Immediately before loading onto a gel, dissolve the cDNA pellet in 7 µL formamide loading buffer. Incubate the mixture for 2–4 min in a 90–100°C sand bath to ensure complete denaturation of the double-stranded cDNA.

3.5.3. Analysis of Primer Extension Products by PAGE

1. The cDNA products synthesized by the primer extension reaction are analyzed for size and quantity by electrophoresis in a 7 *M* urea, 10% polyacrylamide (19 : 1

acrylamide: *bis*-acrylamide) gel ***(11)***. Run the gel at room temperature with 1X TBE as the running buffer.
2. Preheat the gel by running at 400–450 V for approx 15 min before loading the samples.
3. Electrophorese the samples approx 2 h at 400–500 V alongside appropriate radiolabeled size markers. The cDNA products generated here are 69 and 101 bp for the SV40 late and early products, respectively. We routinely run radiolabeled *Msp*I-digested pBR322 as a marker (size range from 67–606 bp). Because the xylene cynanol dye migrates as a 55-bp oligonucleotide would ***(11)***, one can electrophorese the samples until this dye is near the bottom of the gel.
4. Expose the dried gel to X-ray film overnight at –70°C. Alternatively, analyze the gel by using a PhosphorImager (Molecular Dynamics, Palo Alto, CA).

4. Notes

1. Accurate and clean single-strand synthesis by reverse transcriptase is highly dependent on the use of pure primers. We purify the primers as follows. Dissolve the dry pellet of primer in formamide loading buffer and electrophorese in a preparative 7 *M* urea, 15% polyacrylamide gel for 3 h at 400 V. Using a long-wavelength UV light, shadow the band and cut it out. Crush the gel by forcing it through a 1-mL disposable syringe and soak it in extraction buffer (0.5 *M* ammonium acetate, 0.01 *M* $MgSO_4$, 0.1 m*M* EDTA, and 0.1% SDS) overnight at 37°C. Separate the gel debris by centrifugation at 500*g* for 5 min and collect the supernatant. If necessary, reduce the volume, either by sequential extraction with isobutanol or use of a speed-vacuum centrifuge. When the volume is approx 1 mL, desalt by loading onto a PD 10 column (PD 10 is a prepacked G-25 Sephadex gel-filtration column, Amersham Pharmacia Biotech) that has been equilibrated with 10 mL of H_2O. Elute with 12 mL of H_2O, collecting 1-mL fractions. Assay fractions 2–6 for the primer by reading the absorbance at 260 nm. If necessary, dry down the fractions containing the primer using a speed-vacuum centrifuge and resuspend in desired volume of H_2O.
2. Because cells are being serially passaged, it is crucial to always practice strict sterile technique while culturing the cells. Thoroughly wash the bottles containing media, serum, and antibiotics, and so on, as well as the ports of the spinner flasks with 70% ethanol. Pour in medium, serum, antibiotics, and cells through the opening on one side of the spinner flask. Pour out the contents of the flask through the other port.
3. The starting culture should be seeded at approx $3–4 \times 10^4$ cells per mL. To stimulate growth, spike the 250-mL starting culture with 2% FCS (Gibco-BRL Life Technologies). Twenty-four hours later, assess the cell density with a hemocytomoter. If lower than expected, reseed the culture with more cells.
4. The rate of stirring is critical for optimal cell growth. This often has to be determined empirically. Cells stirred too slowly will be insufficiently oxygenated and grow poorly. However, stirring too quickly will damage cell membranes. Under

the proper conditions, *HeLa* cells should double approx every 24 h. Check the cell density with a hemocytometer.

5. Determine the cell densities before every harvest, checking to see that growth rates are as expected.

   To obtain transcriptionally active nuclear extracts, it is important that cells be harvested in exponential growth phase, i.e., 2–4 × 10^5 cells/mL. If densities are too low, delay harvesting. If too high, dilute to the appropriate density of 0.5–1 × 10^5 and harvest 2 d later.
6. Cells stored frozen for up to 1 yr at –70°C retain suitability for nuclear extract preparation.
7. After culturing for 10–12 wk, the cells begin to show changes in doubling time, become multinucleated, and often clump together. These cells are unsuitable for preparing transcriptionally active extracts. If more cells are desired, we start the process again with a new vial of frozen cells to avoid possibly working with cells whose properties may have changed during prolonged serial passaging.
8. After the cells are thawed and pelleted, it is important to accurately estimate the pcv in order to determine the cell density. If a significant percentage of cells break open during the thawing process, overestimation of pcv is likely to occur. To correct for this, use the rule of thumb: 1 mL pcv = 1 L of cells at 2–4 × 10^5 cells/mL = 2–4 × 10^8 cells per pcv.
9. The protein concentration may be determined by a calorimetric assay (we typically use the Bradford Reagent, Bio-Rad Corp., Hercules CA) or spectrophotometrically by determining absorbances at 280 and 260 nm and correcting for nucleic acid content as described in **ref. *11***. Typical protein concentrations are in the range of 10–15 mg/mL. These extracts contain approx 6% nucleic acids. The amount of nucleic acid extracted, as well as the levels and types of proteins extracted, is also important. A crude determination of the quality of the nuclear extract can be made by monitoring the A_{280}/A_{260} ratio. A ratio typically observed for high-quality extracts is 0.8, which corresponds to a nucleic acid content of approx 6%. Excessive nucleic acid interferes with transcriptional efficiency by nonspecifically binding promoter-specific factors.
10. The quality of the template DNA is critical. We usually use DNA that is purified by $CsCl_2$-ethidium bromide density gradient centrifugation. We have occasionally failed to detect RNA when using column-purified DNA templates. This latter method includes an RNase treatment step. Likely, minor contamination with RNase affects the quality of cell-free synthesized RNA.
11. Many standard cell-free transcription assays use runoff transcription from a linear template. During the course of experiments in which we analyzed RNA synthesized in vitro, we found that transcription of the SV40 late promoter is significantly more efficient from supercoiled plasmid DNA than from linear DNA. Because a supercoiled template more closely mimics transcription of the natural state of the SV40 genome, we recommend using supercoiled DNA.
12. The transcription reaction conditions described in **Subheading 3.3.** were optimized for the simultaneous transcription of the SV40 early and late genes. They

are as follows: 25 m*M* HEPES (pH 7.9), 60 m*M* KCl, 6 m*M* $MgCl_2$, 12% glycerol, 80 m*M* glutamate (K salt), 0.1 m*M* EDTA, 1 m*M* DTT, 0.2 m*M* ATP, 0.6 m*M* CTP, 0.6 m*M* UTP, 0.6 m*M* GTP, 500 ng (0.1 pmol or 2.0 n*M*) supercoiled plasmid wild-type SV40 DNA (pSV1773), and 150–250 µg *HeLa* cell protein. We have found that concentrations of salt greater than 0.1 *M* KCl or glycerol greater than 15% markedly inhibit transcription of the *SV40* genes. We have found 30°C to be the optimal temperature for cell-free transcription of the *SV40* genes. Synthesis of both early and late SV40 RNA is significantly reduced at 37°C as previously observed with the murine DHFR promoter ***(9)***.

13. Another important parameter in achieving efficient transcription in vitro is the DNA template concentration. For any given template DNA, we have found that a threshold concentration exists below which no RNA can be detected. For example, when assaying the plasmid described in **Fig. 3**, *pm*322C × LS26 in the XS13 background ***(13)***, we found the threshold concentration to be 0.1 n*M*. In addition, we failed to detect RNA when protein concentrations were below 2.5 µg/µL of reaction mix.

    The addition of nonspecific carrier DNA to the reaction mixture often lowers the minimum template requirement. Presumably, the carrier DNA binds nonspecific DNA-binding factors present in the nuclear extract that interfere with the binding of promoter-specific factors. The presence of vector DNA sequences within the template can also function as nonspecific carrier in the cell-free transcription system. Thus, it is possible to avoid the addition of carrier DNA and empirically determine the optimal amount of template DNA-to-protein ratio needed to achieve efficient transcription. High concentrations of template or carrier DNA can also inhibit transcription, presumably by binding promoter-specific factors.

    We have found that the SV40 late promoter contains binding sites for members of the steroid/thyroid/retinoid receptor superfamily ***(13–16)***. Two of these sites map to sequences directly surrounding and approx 55 bp downstream of the major late initiation site. Some of these receptors are abundant in *HeLa* cell nuclear extracts and function as sequence-specific repressors of the SV40 major late promoter (*see* **Fig. 3A–C**). Cell-free transcription of the SV40 late promoter is dependent upon the ratio of these repressor-binding sites to the cellular repressors. At low-template DNA concentrations, cellular repressors saturate the sequence-specific binding sites and inhibit transcription. Therefore, template DNA concentrations must be sufficiently high to titrate out repressors, yet remain low enough as not to bind promoter-specific factors (*see* **Fig. 3A**, lanes 2 and 3).

14. The method described here utilizes two different stop buffer systems containing two different carriers. This arose because the 394E primer hybridized to nonspecific RNA present in the tRNA carrier, whereas the 5135L primer hybridized to nonspecific nucleic acids present in Poly(A). More recently, we have found that the use of a different set of primers can overcome these interferences. This allows the use of only the Poly (A) stop buffer system and primer extension analysis of both SV40 early and late RNA concurrently in a single tube (**Fig. 4**).

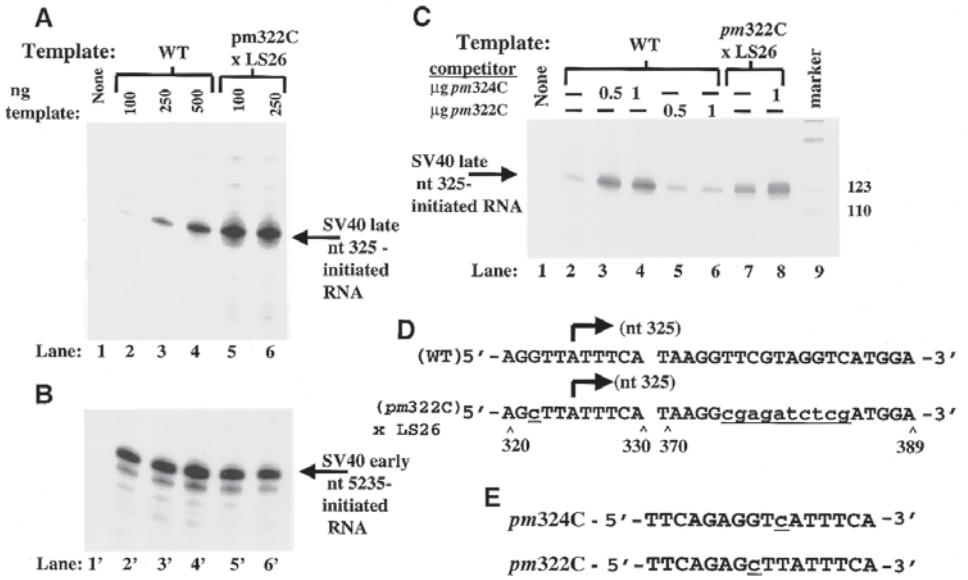

Fig. 3. Binding of sequence-specific factors present in *HeLa* cell nuclear extract represses transcription from the SV40 late promoter. **(A)** Primer extension analysis of the SV40 late RNA synthesized in the cell-free system with wild-type and the double repressor-binding site mutant *pm*322C × LS26 (in the XS13 background, i.e., deletion of the 72-bp repeats from the promoter region of wild-type SV40, pSVS; *see* **ref. *18***) at various template concentrations (for details regarding construction of these plasmids, *see* **ref. *13***). The SV40 RNAs synthesized in the experiments shown in panels A and C were analyzed by primer extension using a primer corresponding to SV40 nucleotides 446-422 (called 446E), 5 - GCAGCAAAAGCTCTAAAACAAACAC-3 . **(B)** Primer extension of the SV40 early RNA synthesized concurrently in the same reactions analyzed in panel A. **(C)** The addition in *trans* of a double-stranded synthetic oligonucleotide relieves repression of the SV40 late promoter. **(D)** Representation of the bases near and downstream of the initiation site of the SV40 major late promoter altered in the double-mutant *pm*322C XLS26 studied here. **(E)** Sequences of the competitor oligonucleotides used in the experiment shown in panel C.

15. Template RNAs rich in secondary structure, such as GC-rich regions, can cause reverse transcriptase to pause or stop, resulting in cDNAs that are not full length. We try to minimize this artifact by performing the reaction at 48°C.
16. To assess the accuracy and efficiency of the cell-free system, we compared the primer extension products of the SV40 RNAs synthesized in transfected CV-1PD cells versus the cell-free system described here. Overall, synthesis of SV40 late RNA in the cell-free system was remarkably similar to that observed in the transfected cells (compare **Fig. 2A** and **C**). First, the 5 -end of the major late RNA

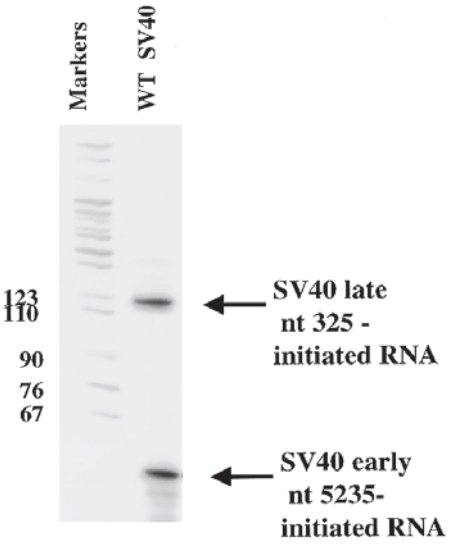

Fig. 4. SV40 early and late transcripts can be analyzed by primer extension simultaneously using the 446E and 5178L set of primers. The 5178L primer corresponds to SV40 nucleotides 5178-5201, 5 -TTGCAAAAGCCTAGGCCTCCAAAA-3 . The wild-type SV40 genome used in this experiment was in the XS13 background ***(13,18)***.

maps to the same site, nucleotide (nt) 325, of the SV40 genome. Second, for each comparable template, the percentage of the total RNA mapping to nt 325 is similar in both systems. Although the SV40 major late promoter contains a weak "TATA" box, the degree of heterogeneity and the locations of most of the 5 -ends of the RNAs seen in the cell-free system mimic those seen in the SV40-transfected cells. The one exception is the appearance of late RNAs initiated at SV40 nt 170 in the cell-free system, observed by others as well ***(8)***. Third, the 5 -ends of the SV40 early RNAs synthesized in the cell-free system also map to the same site as observed in transfected cells (**Fig. 2B**), with little 5 -end heterogeneity. Therefore, we conclude that the cell-free transcription system described here mediates the accurate and efficient transcription of both the SV40 early and late genes simultaneously.

Acknowledgments

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7

Quantitation and Structural Analysis of SV40 RNAs

Richard J. Kraus and Janet E. Mertz

1. Introduction

The 5243-bp genome of SV40 contains two transcriptional units (*see* **Fig. 1**); the early one, which is first expressed early in the lytic cycle of infection, and the late unit, which is expressed at a significant level only after the onset of viral DNA replication ***(1)***. The early genes encode the viral regulatory proteins, small, and large T-antigens. These proteins play roles in replication of the viral genome and transcriptional transactivation of the late genes (*see* **refs. *2–4*** and references therein). The late genes encode the capsid proteins that encapsulate the viral DNA into new virions. The *cis*-acting transcriptional elements that regulate the synthesis of both the early and late viral RNAs (*see* **refs. *5,6*** and references therein), the mechanisms that regulate the temporal expression of the SV40 genome (*see* **ref. *4*** and references therein) and the patterns by which the viral transcripts are spliced (*see* **refs. *7,8*** and references therein) are well understood. In this chapter, we describe methods for quantifying and mapping the 5 ends of the viral RNAs synthesized in SV40-transfected cells. Minor variations of these methods can be used to map the 3 ends as well ***(9)*** and the splice sites ***(8)*** of the SV40 RNAs synthesized, not only in SV40-transfected cells, but also in SV40-infected and transformed cells.

1.1. Preparation of SV40 DNA for Transfection

Today, few researchers work with SV40 DNA obtained from SV40-infected cells. In the Mertz laboratory, we perform most experiments using derivatives of pSVS ***(10)***. This plasmid consists of the larger *EcoR*I-to-*Pvu*II fragment of pBR322, which contains the ampicillin resistance gene and the bacterial origin of replication, cloned into the *EcoR*I site of the wild-type SV40 strain 776 genome. We have made a derivative of pSVS, called pSV1773, which contains

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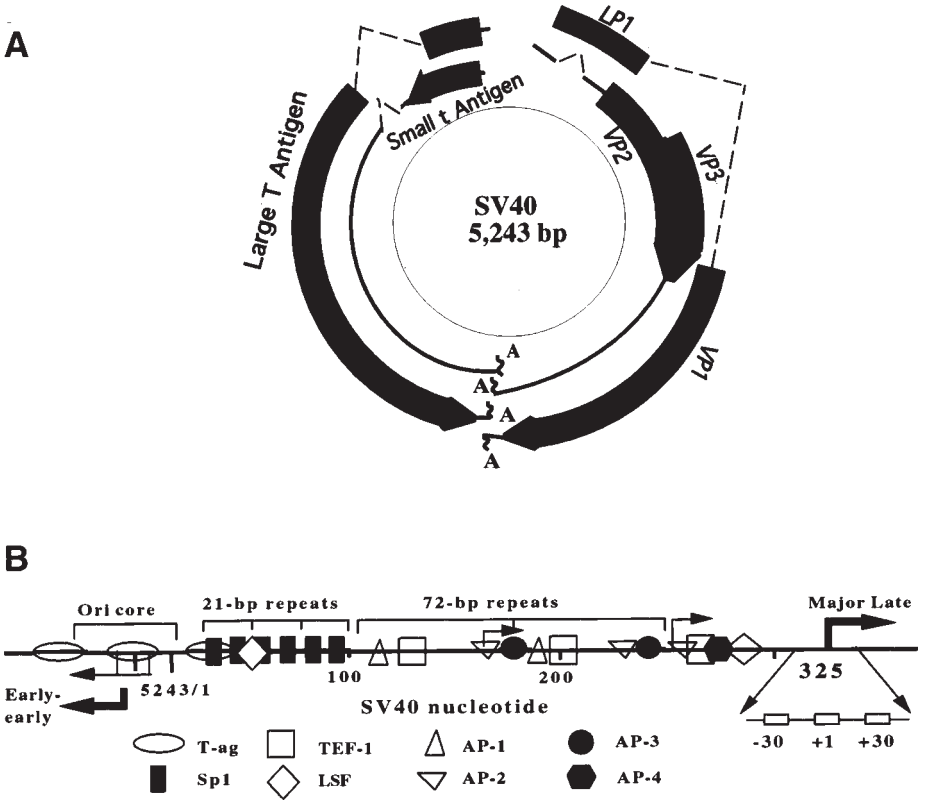

Fig. 1. Schematic of the SV40 genome. **(A)** Map of wild-type SV40 DNA. **(B)** Schematic of the origin-promoter region of SV40. This bidirectional promoter controls both early and late transcription. The thick and thin arrows indicate the positions at which transcription initiates and the direction transcription proceeds. The objects indicate the *cis*-acting sequences and the transcription factors that bind in trans. The nucleotide numbering system is that of Buchman et al. ***(21)***.

a 7-bp deletion within the VP1 codon region. Because of this frameshift mutation,VP1 is not synthesized with this SV40 variant. Thus, viral DNA is not packaged into virions, infectious particles are not formed, and second cycles of infection do not occur.

To recreate intact SV40 genomes, we excise the pBR322 DNA sequences by cleavage with *EcoR*I and ligate the SV40 DNA back into circular molecules prior to introduction into cells by the diethylaminoethyl (DEAE)-dextran method. Using pSV1773 and derivatives of it, one can study viral expression in a single cycle of growth. Using the original pSVS, one can produce infectious virus.

1.2. Introduction of SV40 DNA into Cells

Techniques permitting the introduction of cloned eukaroytic DNA into cultured mammalian cells have contributed greatly toward understanding how genes are expressed. The development of efficient delivery systems is still ongoing. The use of DEAE-dextran, an anion-exchange resin, was first described by Vaheri and Pagano ***(11)*** as a way to introduce poliovirus RNA into cells. Shortly thereafter, this method was successfully used to transfect SV40 and polyoma DNA into cells ***(12,13)***. This method is still widely used and is particularly effective for the transfection of viral genomes that contain a eukaryotic origin of replication. The exact mechanism by which DEAE-dextran delivers DNA into cells is still unknown. It is thought that the polymer, which binds nucleic acids through ionic interactions, promotes endocytosis of the DNA. In addition, the incorporation of chloroquine into the method increases the efficiency of transfection several fold ***(14)***. In this subsection, we will describe the DEAE-dextran/chloroquine method used in our laboratory to introduce SV40 DNA into simian cells.

1.3. Isolation of Whole-Cell RNA

Several rapid techniques are available to isolate RNA from mammalian cells grown in culture ***(15,16)***. These methods depend on the differential partitioning of RNA in various organic solvents or the precipitation of RNA based on molecular weight. The method described here represents a fairly rapid technique for the isolation of whole-cell nucleic acids. It employs the initial hydrolysis of cellular proteins, followed by extensive extraction of the protease digestion mixture with phenol : chloroform : isoamyl alcohol and, finally, the precipitation of the nucleic acids. Regardless of the method used to isolate RNA from eukaroytic cells, care must be taken to minimize the activity of RNases that can be liberated during cell lysis or introduced from outside sources ***(17)***.

1.4. Analysis of SV40 RNA

Several methods exist that permit the quantitation and structural analysis of RNAs extracted from cells. Our goal was to develop a method whereby the structures and relative amounts of both the early and late RNAs of SV40 could be analyzed simultaneously. In Chapter 6 of this volume, we describe the use of the primer extension technique to achieve this goal. Here, we describe an S1 nuclease mapping technique that also meets these requirements and enables one to examine the entire primary structures of the RNA, not just their 5 ends.

Analysis of RNA by S1 nuclease mapping is based on the principle that single-stranded fragments of genomic DNA can hybridize with RNA that had been transcribed from this template ***(18,19)***. The region of the DNA that

remains single stranded after hybridization is enzymatically hydrolyzed with S1 nuclease. However, duplex DNA and DNA:RNA hybrids are resistant to digestion. As a result, the sizes of the resistant duplexes correspond to the structures of the RNAs. The amounts of radioactivity associated with the duplexes correlate with the abundance of the respective RNAs.

We describe here the use of a DNA probe that spans the entire promoter region of the SV40 genome. Radiolabeling the 5 ends of both strands of this probe permits the simultaneous measurement of both the early and late SV40 RNAs.

2. Materials

2.1. Preparation of SV40 DNA for Transfection

2.1.1. Excision of SV40 Genome from Bacterial Cloning Vector Sequences

2.1.1.1. Reagents and Solutions

1. *Eco*RI restriction endonuclease (20 U/µL, New England Biolabs, Beverly, MA).
2. 10X *Eco*RI buffer (NEB #3, New England Biolabs).
3. 5 µg pSV1773 or other SV40-containing plasmid DNA grown in *Escherichia coli* and CsCl-gradient purified.

2.1.2. Intramolecular Ligation of Viral DNA

2.1.2.1. Reagents and Solutions

1. T4 DNA ligase (1U/µL, New England Biolabs).
2. 10X ligation buffer: 0.5 *M* Tris-HCl (pH 7.8), 0.1 *M* $MgCl_2$, 0.1 *M* dithiothreitol (DTT), 10 m*M* spermidine (Sigma Chemical Co. St. Louis, MO), 10 m*M* adenosine triphosphate (ATP) (Amersham Pharmacia Biotech, Piscataway, NJ), 1 mg/mL bovine serum albumin (BSA) (high quality, New England Biolabs). Alternatively, the 10X ligation buffer provided by the manufacturer can be used.
3. TE: 10 m*M* Tris-HCl (pH 7.5), 1 m*M* ethylenediaminetetraacetic acid (EDTA). Sterilize by autoclaving.

2.1.2.2. Equipment

1. Centricon 30 microconcentrators (Amicon Inc., Beverly, MA).

2.2. Introduction of SV40 DNA into Cells

2.2.1. Culturing Simian Cells

2.2.1.1. Cell Lines

1. African green monkey kidney cell line, CV-1PD.
2. SVLT-expressing, African green monkey kidney cell line, COS-M6 (*see* **ref. *20***).

2.2.1.2. Reagents and Solutions

1. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL Life Technologies, Grand Island, NY).
2. Fetal calf serum (FCS) (Gibco-BRL Life Technologies).
3. Penicillin/streptomycin 10,000 U/mL(Gibco-BRL Life Technologies).

2.2.1.3. Equipment

1. Humidified incubator at 37°C and 5% CO_2.
2. 100-mm tissue-culture dishes.

2.2.2. Transfection by the DEAE-Dextran Method

2.2.2.1. Reagents and Solutions

1. Transfection mixture: 20 m*M* HEPES (pH 7.25), 0.5 mg DEAE-dextran (Amersham Pharmacia Biotech) in DMEM (*see* **Note 1**).
2. Washing medium: 20 m*M* HEPES (pH 7.25) in DMEM.
3. DMEM-chloroquine medium: 100 µ*M* chloroquine (chloroquine diphosphate salt, Sigma Chemical Co.), 2% FCS (Gibco-BRL Life Technologies), in DMEM (*see* **Note 2**).
4. Posttransfection medium: 2% FCS, 100 U penicillin/streptomycin per mL in DMEM.

2.3. Isolation of Whole-Cell RNA

2.3.1. Reagents and Solutions

1. Proteinase K (15.5 U/µL Boehringer Mannheim, Indianapolis, IN).
2. Proteinase K buffer: 0.2 *M* Tris-HCl (pH 7.5), 25 m*M* EDTA, 0.3 *M* NaCl, 2% sodium dodecyl sulfate (SDS).
3. phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v : v : v pH 7.9).
4. chloroform : isoamyl alcohol (24 : 1).
5. TD buffer: 0.14 *M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4 , 2.5 m*M* Tris-base. (8 g NaCl, 0.38 g KCl, 0.1 g Na_2HPO_4, 3.0 g Tris-base.) Adjust pH to 7.5 and sterilize by autoclaving.

2.4. Analysis of SV40 RNA

2.4.1. Preparation of S1 Nuclease Mapping Probe by PCR

2.4.1.1. Reagents and Solutions

1. *Taq* DNA polymerase 5 U/µL (Promega Corp., Madison WI).
2. dNTPs (Boehringer Mannheim).
3. 10X polymerase chain reaction (PCR) buffer: 500 m*M* KCl, 100 m*M* Tris-HCl (pH 9.0 at 25°C), 1% Triton X-100 (Promega Corp.).
4. 25 m*M* $MgCl_2$ (Promega Corp.).

5. SV40 late primer (called 446E), 5 -GCAGCAAAAGCTCTAAAACAAACAC-3 .
6. SV40 early primer (called 4924L), 5 -TGCATCCCAGAAGCCTCCAAAGTC-3 .
7. SV40 DNA (25 ng/µL).

2.4.2 Radiolabeling of the S1 Nuclease Mapping Probe

2.4.2.1. Reagents and Solutions

1. [^{32}P] ATP (5000–6000 Ci/mmol, 10 µCi/µL, Amersham Pharmacia Biotech).
2. T4 polynucleotide kinase (10 U/µL, New England Biolabs, Beverly, MA).
3. 10X kinase buffer: 700 m*M* Tris-HCl (pH 7.6), 100 m*M* $MgCl_2$, 10 m*M* EDTA, 10 m*M* DTT. Alternatively, use the buffer supplied by the manufacturer.
4. G-50 Quick Spin Column (Boehringer Mannheim).

2.4.3. S1 Nuclease Mapping

2.4.3.1. Reagents and Solutions

1. S1 nuclease, 400 U/µL (Boehringer Mannheim).
2. Hybridization buffer: 40 m*M* PIPES (pH 6.4) (Boehringer Mannheim), 1 m*M* EDTA, 0.4 *M* NaCl, 80% formamide (Ultrapure grade, Fischer Scientific, Fair Lawn, NJ).
3. Reaction buffer: 0.28 *M* NaCl, 0.05 *M* sodium acetate (pH 4.5), 4.5 m*M* $ZnSO_4$, 20 µg/mL salmon sperm DNA (Sigma Chemical Co.).
4. Termination buffer: 2.5 *M* ammonium acetate, 3 m*M* EDTA, 100 µg/mL yeast tRNA (Ribonucleic Acid Type III. cat. no. R7125, Sigma Chemical Co.).
5. Formamide loading buffer: 80% formamide, 1 m*M* EDTA, 0.1% (w/v) bromophenol blue (Sigma Chemical Co.), 0.1% (w/v) xylene cyanol (Sigma Chemical Co.).

3. Methods

3.1. Preparation of SV40 DNA for Transfection

3.1.1. Excision of SV40 Genome from Bacterial Cloning Vector Sequences

1. Digest 5 µg of the SV40 plasmid DNA with 20 U of the restriction endonuclease *Eco*RI in *Eco*RI buffer for 1 h at 37°C. Heat inactivate *Eco*RI by incubation of the reaction mixture for 15 min at 65°C (*see* **Note 3** and **Fig. 2A**).

3.1.2. Intramolecular Ligation of Viral DNA

1. In a final reaction volume of 1.5 mL, incubate 5 µg of the *Eco*RI-digested DNA with 2 U of T4 DNA ligase overnight at 16°C (*see* **Note 4**).
2. Load the entire ligation reaction mixture into the microconcentrator (Centricon 30) and centrifuge at 2800*g* for 20 min. To prevent crosscontamination during the centrifugation, seal the tops of the microconcentrators with parafilm and poke a tiny hole in the top prior to centrifugation. The retained volume should be approx 100 µL. If the volume is larger than this, centrifuge for several additional minutes.

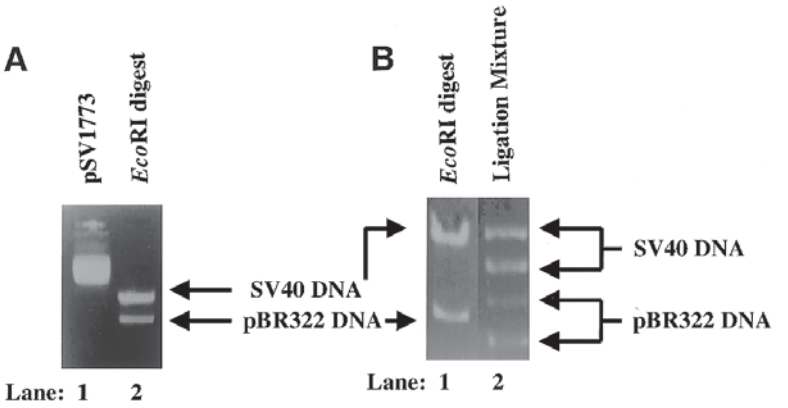

Fig. 2. Formation of SV40 monomer circular DNA from plasmids grown in *E. coli*. **(A)** Excision of the pBR322 vector sequences from the SV40-containing plasmid, pSV1773. The samples were electrophoresed in a 1% agarose gel before (lane 1) and after (lane 2) digestion with *EcoR*I. **(B)** Formation of circular monomers following the intramolecular ligation of the *EcoR*I-digested SV40-containing plasmid DNA. The samples were electrophoresed in a 1% agarose gel before (lane 1) and after (lane 2) treatment with ligase.

3. Desalt the sample by adding 1 mL of sterile TE to the microconcentrator and centrifuging at 2800*g* for 30 min. The resulting volume should again be approx 100 µL.
4. Remove the plastic tube that contains the flowthrough. To collect the retentate, place the retentate cup over the microconcentrator, invert, and centrifuge at 3 K for 5 min.
5. Check the extent of the ligation by electrophoresing 5 µL of the retentate (approx 250 ng) alongside an aliquot of the unligated sample in a 1% agarose gel (**Fig. 2B**). The relaxed circular viral DNA is now ready for transfection.

3.2. Introduction of SV40 DNA into Cells

3.2.1. Culturing Simian Cells

1. Grow CV-1 and COS cells in 100-mm tissue-culture dishes in a humidified incubator at 5% CO_2 and 37°C in DMEM containing 5% and 10% FCS, respectively, and 100 U penicillin/streptomycin per mL.
2. Two days before transfection, replate the cells from fully confluent dishes at a 1:5 splitting ratio. At the time of transfection, the cell monolayer should be approx 70–80% confluent.

3.2.2. Transfection by the DEAE-Dextran Method

1. Working in a laminar flow hood, pipet 1 mL of the DEAE-transfection mixture into a sterile 1.5-mL tube containing 1–2 µg of circular SV40 DNA (*see* **Note 5**).

2. Withdraw the dishes from the incubator, place in a laminar flow hood (*see* **Note 6**), and remove the medium from the cells by aspiration.
3. Wash the cells twice with 5 mL of washing medium.
4. Gently apply 1 mL of the DNA/DEAE-transfection mixture to each dish of cells.
5. Rock the dishes to ensure that the mixture is evenly and completely covering the monolayer. Place the dishes back into the incubator.
6. At 10–15-min intervals, withdraw the dishes and gently rock in all directions to redistribute the DNA/DEAE-transfection mixture over the cells.
7. After incubation for 35–45 min (*see* **Note 7**), transfer the dishes from the incubator to the hood and remove the DNA/DEAE-transfection mixture by aspiration. Record this as time zero. Removal of the DNA/DEAE-transfection mixture requires the use of a different Pasteur pipet for each dish to prevent cross contamination among dishes.
8. Wash the cells twice with 5 mL of washing medium, again using a different Pasteur pipet for each plate during the first wash.
9. Working in a darkened hood and tissue-culture room, apply 10 mL of DMEM-chloroquine medium to each dish. Incubate in the CO_2 incubator for 3–4 h (*see* **Note 8**).
10. Replace the DMEM-chloroquine medium with posttransfection medium.

3.3. Isolation of Whole-Cell RNA

1. At the time of harvest, remove the medium from the cells and wash them twice with 5 mL of cold (4°C) TD (*see* **Note 9**).
2. Add 0.5 mL proteinase K buffer/dish and 500–600 U of proteinase K.
3. Scrape the cells into a 1.5-mL microfuge tube and incubate at 37°C for 1 h to hydrolyze the proteins. If desired, the procedure can be stopped at this point and the incubation mixture stored at –20°C.
4. Add 0.5-mL phenol:chloroform:isoamyl alcohol and mix by vortexing for 15–20 min to extract the digested proteins and shear the cellular DNA.
5. Separate the phases by centrifugation at high speed for 10 min using a desktop microcentrifuge.
6. Transfer the aqueous phase to a new microfuge tube and repeat the extraction on this phase.
7. Again, transfer the aqueous phase to a new microfuge tube, add 0.5 mL chloroform:isoamyl alcohol, mix by vortexing for 1 min, and separate the phases by microcentrifugation.
8. Transfer the aqueous phase to a 15-mL conical centrifuge tube.
9. Precipitate the nucleic acids by adding 50 µL 3 *M* sodium acetate and 1.5 mL absolute alcohol. Thoroughly mix and incubate on dry ice for 15–30 min.
10. Pellet the nucleic acids by centrifugation for 15 min at 3200*g* in a benchtop centrifuge.
11. Wash the pellet with cold (–70°C) 70% alcohol.
12. Remove the residual alcohol by drying for several minutes in a dessicator.
13. Resuspend the nucleic acids in100 µL TE and store at –20°C (*see* **Note 10** and **11**).

14. Calculate the yield of nucleic acids by determining the absorbance at 260 nm. Yields of approx 1 µg/µL are routinely observed.

3.4. Analysis of SV40 RNA

3.4.1. Preparation of S1 Mapping Probe by PCR

1. In a 200 µL PCR tube, mix the following reagents: 100 ng SV40 DNA, 20 µL 10X PCR buffer, 300 nmol $MgCl_2$, 40 nmol of each dNTP, 200 pmol of each primer, and 2 U *Taq* polymerase. Adjust to a final volume of 200 µL with H_2O.
2. Incubate in the PCR machine (Perkin Elmer Model 9600, Norwalk, CT) programmed to cycle 30 times at a denaturation temperature of 94°C for 30 s, a hybridization temperature of 52°C for 30 s, and a polymerization temperature of 72°C for 2 min. Following the 30 cycles, incubate at 72°C for 10 min and store at 4°C.
3. Electrophorese the PCR mixture in a 1% preparative agarose gel (**Fig. 3B**), excise the region of the gel that contains the 765-bp probe band and electroelute the DNA from the gel slice (*see* **Note 12**).
4. Precipitate the probe DNA by adding 1/10th volume of 3 *M* sodium acetate (pH 5.2), three times the volume of absolute alcohol and incubating overnight at –20°C.
5. Collect the probe DNA by centrifugation at high speed with a desktop microcentrifuge at 4°C.
6. Wash the pellet with –20°C, 70% ethanol and remove residual ethanol by high-speed vacuum centrifugation.
7. Dissolve the probe DNA in 100 µL H_2O. A yield of approx 10 µg of synthetic oligonucleotide can be expected.

3.4.2. Radiolabeling of the S1 Nuclease Mapping Probe

1. Incubate 200 ng (0.4 pmol) of purified probe DNA in a final reaction volume of 30 µL with 150 µCi of [^{32}P] ATP, 50 U of T4 polynucleotide kinase for 1 h at 37°C.
2. Extract with 50 µL of phenol : chloroform : isoamyl alcohol to separate the T4 polynucleotide kinase from the labeled DNA probe.
3. Separate the unincorporated nucleotide from the labeled probe DNA using a G-50 Quick Spin Column. Incorporation of ^{32}P is quite low in this reaction. Typical specific activity yields are 10–20,000 cpm/ng probe or 50–100,000 cpm/pmol of probe.

3.4.3. S1 Nuclease Mapping

1. For each RNA sample to be analyzed, prepare in a 1.5-mL microfuge tube a mixture that contains 42 µmol ammonium acetate, 25 µg tRNA, and 20,000–80,000 cpm of the probe DNA (approx 1 pmol) brought up to a final reaction volume of 120 µL with TE.
2. Add the RNA obtained from the cells, typically 5% of a dish (*see* **Note 13**) and 350 µL of absolute alcohol. Mix thoroughly.

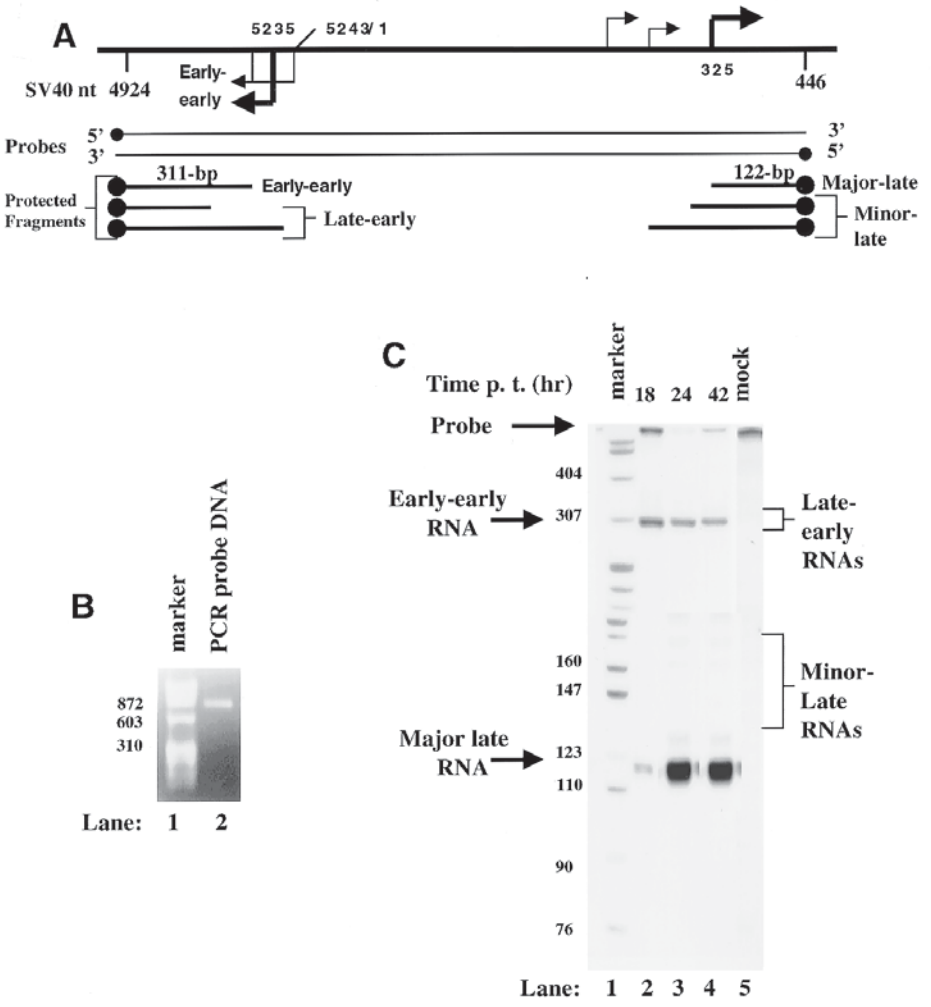

Fig. 3. S1 nuclease mapping analysis of the early and late SV40 RNAs synthesized in CV-1PD cells. **(A)** Schematic diagram of the SV40 promoter region and the structure of the 5 -labeled probe used in the S1 nuclease mapping experiment shown in panel C. Also shown are the fragments protected by hybridization of the probe with the major and minor species of early and late SV40 RNAs. **(B)** Synthesis and purification of the PCR-generated DNA probe. Shown here is a preparative 1% agarose gel in which the products of the PCR were electrophoresed. **(C)** Audioradiogram of the products of quantitative S1 nuclease mapping of the early and late SV40 RNAs accumulated by the indicated times after transfection. CV-1PD cells were transfected with 2.0 µg of SV1773, and whole-cell RNA was harvested at the indicated times.

3. Precipitate the RNA together with the probe DNA by incubation on dry ice for 10 min.
4. Collect the precipitate by centrifugation at high speed for 20 min in a microcentrifuge at 4°C.
5. Wash the pellet with cold (–20°C) ethanol. Thoroughly dry the pellet using a speed-vacuum centrifuge (*see* **Note 14**).
6. Resuspend each sample in 10 µL of hybridization buffer by vigorous vortexing.
7. Incubate at 85°C for 5 min to denature the nucleic acids, then immediately transfer to a 48°C circulating water bath for 8 to 30 h.
8. Add 100 µL of reaction buffer containing 120 U of S1 nuclease to the annealing reaction mixture. Mix thoroughly by pipetting up and down.
9. Incubate at 25°C for 1.5 h (*see* **Note 15**).
10. Stop the reaction by the addition of 30 µL cold termination buffer.
11. Precipitate the DNA products by the addition of 300 µL of absolute alcohol containing 1 µmol of $MgCl_2$ and incubate on dry ice for 10 min.
12. Pellet the precipitate by centrifugation at high speed and 4°C, for 20 min using a benchtop microcentrifuge.
13. Wash the pellet with cold 70% ethanol. Dry by high-speed vacuum microcentrifugation. Samples are now ready for polyarcylamide gel electrophoresis (PAGE) analysis and can be safely stored at –20°C if necessary (*see* **Note 16**).

3.4.4. Analysis of Primer Extension Products by PAGE

1. Analyze the S1 nuclease reaction products for size and quantity in a 7 *M* urea, 6% polyacrylamide (19 : 1 acrylamide: *bis*-acrylamide) gel. Run the gel with 1X TBE as the running buffer.
2. Preheat the gel by running at 400–450 V for approx 15 min before loading the samples. Electrophorese the samples approx 2 h at 400–500 V at room temperature (*see* **Note 16**). The DNA products generated with the probe described here are 121 and 311 bp for the SV40 late and early RNAs, respectively (**Fig. 3A**). Therefore, we routinely run radiolabeled *Msp*I-digested pBR322 as a marker (size range from 67 to 606 bp). Alternatively, because the xylene cyanol dye migrates as a 110-bp oligonucleotide would, one can electrophorese the samples until this dye is near the bottom of the gel.
3. Expose the dried gel to X-ray film overnight at –70°C (*see* **Note 17**). Alternatively, analyze the gel with a PhosphorImager (Molecular Dynamics, Palo Alto, CA).

4. Notes

1. The efficiency of transfection by the DEAE-dextran method is highly dependent on the molecular weight of the DEAE-dextran polymer. Introduction of DNA into cells with DEAE-dextran polymers having molecular weights of less than 2×10^6 is inefficient for reasons that remain unknown. In addition, the efficiency of transfection is highly dependent upon the pH of the transcription mixture. We have found that a pH of 7.25 is optimal for efficient transfection of CV-1 cells. Ionic binding of the DNA by DEAE is pH-dependent and may be one of several reasons this pH is optimal.

2. Chloroquine is light-sensitive and must be handled appropriately. That is, following its addition to the medium, the bottle must be wrapped in aluminum foil or stored in the dark.
3. **Figure 2** shows typical results obtained when preparing SV40 DNA for use in transfections. Digestion of the plasmid with *EcoR*I yields the 5236 bp and 2299 bp SV40 and pBR322 fragments, respectively, indicating excision of the pBR322 sequences from the plasmid. Failure to heat-inactivate *EcoR*I will interfere with the subsequent ligation reaction and, thus, the formation of the circular monomer DNA.
4. We carry out the ligation reaction under conditions that favor intramolecular ligations of the *EcoR*I ends, that is, at low DNA concentrations. Thus, it is unnecessary to purify the SV40 DNA away from the pBR322 DNA prior to ligation. Formation of faster migrating DNAs upon treatment with ligase confirms successful intramolecular ligation of the DNAs into monomer circles (**Fig. 2B**). With at least half of the SV40 DNA converted to monomer circles, the ligation mixture is ready for transfection into cells.
5. Another important parameter of transfection efficiency is the amount of DNA that is applied to the cells. At low DNA concentrations, i.e., less than 100 ng DNA/100-mm dish, the percentage of cells transfected is proportional to the amount of viral DNA applied to the cells. However, the maximal percentage of cells capable of being transfected by the method described here is approx 10%. This maximum is reached with 1–3 µg DNA/100-mm dish. Above that amount, the efficiency of transfection begins to fall.
6. We typically work with no more than 16 dishes in the hood at one time. Prolonged times out of the humidified CO_2 chamber is detrimental to the cells.
7. Cells can not tolerate lengthy exposures, i.e., greater than 45 min, to DEAE-dextran because it is toxic to the cells.
8. Treat the transfected cells with chloroquine for 3–4 h. Times less than 3 h are insufficient for inhibiting lysosomal hydrolases that degrade DNA. At exposure times longer than 4–5 h, chloroquine is toxic to the cells. Treatment of the cells with chloroquine improves the transfection efficiency several fold ***(14)***.
9. Harvest the cells at the desired time point, usually no later than 48 h.
10. Because we do not see interferences from DNA by the S1 nuclease analysis method we employ, we do not treat the isolated RNA with DNase. Thus, incubation of the sample with RNase-contaminated DNase is avoided. If a DNase treatment is desired, we incorporate the following method. To 90 µL of the RNA sample, add in this order, 20 µL 10X DNase buffer [0.1 *M* HEPES (pH 7.0), 50 m*M* $CaCl_2$, 0.1 *M* $MgCl_2$, 10 m*M* DTT], 30 U RNasin (Promega Corp.), 10 U DNase I (RNase-free, Amersham Pharmacia Biotech). Incubate for 20 min at 37°C. Add another 5–10 U of DNase I and repeat the incubation. Stop the digestion by the addition of 2.5 µmol EDTA, followed by the addition of 1 mL TES Buffer [0.1 *M* Tris-HCl (pH 9.0), 1 m*M* EDTA, 0.5% SDS]. Heat at 70°C for 10 min and extract with 1 mL of phenol:chloroform:isoamyl alcohol. Back extract the phenol:chloroform:isoamyl alcohol phase with 1 mL of TES buffer. Com-

bine with the first aqueous phase. Precipitate the RNA by adding 1 mL of 8 *M* ammonium acetate, 7.5 mL of absolute alcohol, and incubating on dry ice for 20–30 min. Collect the precipitate by centrifugation at 3200*g* using a benchtop centrifuge. Wash the pellet with cold 70% ethanol. Remove residual ethanol by vacuum-drying. Resuspend in 100 µL TE. Store at –20°C.

11. We have described in this chapter a method for the isolation of whole-cell RNA. We employ the following method to isolate separately cytoplasmic and nuclear RNA (*see* **ref. *8*** for details). At the desired time after tranfection, wash the cells twice with 5 mL cold TD buffer and scrape them off the dish into a 15-mL conical tube. Pellet the cells at low speed (2000 rpm) in a table-top centrifuge. Resuspend the cells in 250 µL ice-cold TD. Add an equal volume of TD containing 1% Nonidet P-40 (NP40) (Sigma Chemical Co.) and 10 m*M* vanadyl ribonucleoside complex (*see* **ref. *17*** and references therein). Gently mix. Incubate on ice for 5 min, then pellet the nuclei by centrifugation at 900*s* for 5 min. Carefully and completely remove the supernatant, i.e., the cytoplasmic fraction. Wash the pelleted nuclei with TD buffer containing 0.5% NP40. Resuspend the nuclei in 450 µL TD and treat both the nuclear and cytoplasmic fractions with 250 µg proteinase K (Boehringer Mannheim) in the presence of 1% SDS for 30 min at 37°C. Extract both fractions twice with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform: isoamyl alcohol (24:1). Precipitate the nucleic acids with a 3X volume of absolute ethanol and incubation on dry ice for 15–20 min. Recover the precipitate by centrifugation at 3200*g* for 15 min. Resuspend in 50 µL of RNase-free TE. Save an aliquot of each fraction for DNA analysis, then proceed with the DNase I treatment as aforementioned in **Note 10**.
12. A number of methods are available to recover DNA from agarose gels and can be used to collect the DNA probe synthesized here. The method we use is as follows. Run the preparative agarose gel to separate the desired PCR product from the primers and incompletely synthesized products. Locate the band of interest using a long-wavelength UV lamp. Excise the band with a sharp blade. Place the gel slice in a dialysis bag filled with 0.5X TBE (50 m*M* Tris-borate, 50 m*M* boric acid, 1 m*M* EDTA). Allow the gel slice to sink to the bottom of the dialysis bag. Seal the dialysis bag just above the top of the gel slice so as to minimize the volume. Immerse the bag in a shallow layer of 1X TBE in an electrophoresis tank. Elute the band out of the gel into the surrounding buffer by passing an electric current through the dialysis bag (100–150 V for 10–15 min). Reverse the polarity for 20–30 s to release any DNA that is bound to the inner wall of the dialysis bag. Open the dialysis bag and remove the buffer that surrounds the gel slice, usually 250–300 µL. Wash the gel slice with 50 µL TE and combine with the original buffer. Add 30 µL 3 *M* sodium acetate (pH 5.3) and 900 µL absolute ethanol. Mix and precipitate overnight at –20°C. Collect the precipitate by microcentrifugation for 20 min using a high-speed microcentrifuge. Wash the pellet with 100 µL cold (–70°C) 70% ethanol. Remove any residual ethanol using a speed-vacuum microcentrifuge. Dissolve the dried pellet in 100 µL H_2O and calculate the DNA concentration by determining the absorbance at 260 nm.

13. We have successfully mapped SV40 RNA from as little as 0.5% of a 100-mm dish when looking at late times posttransfection. When working with samples harvested at early time-points, we have assayed RNA from as much as 25% of a 100-mm dish without interference from cellular RNAs.
14. It is critical that the salt concentrations that promote the annealing of the DNA probe with RNA be exact. Therefore, it is important that no residual water be present at this step.
15. We have empirically determined the optimal reaction conditions for S1 nuclease digestion of the DNA:RNA hybrid assayed here. At insufficient concentrations of enzyme, there is incomplete hydrolysis of single-stranded DNA. Concentrations that are too high result in partial digestion of the DNA:RNA duplex.
16. Immediately before PAGE analysis, resuspend the pellet in 7 µL of formamide loading buffer, taking care to ensure that the pellet is thoroughly dissolved. Heat in a 95°C sand bath for 3–4 min prior to loading onto the gel to denature the radiolabeled probe DNA from the RNA that has been protecting it from degradation with S1 nuclease.
17. Analysis of the SV40 early and late RNAs accumulated in CV-1PD cells at various times after transfection is shown in **Fig. 3C**. By 18 h after transfection, both early and late RNA can be detected by this method. Late SV40 RNA accumulates with time, whereas early RNA levels remain fairly constant, changing from early-early to late-early 5 ends. At late times after transcription, relatively large amounts of late RNA have accumulated, whereas early RNA synthesis is partially repressed.

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8

Generation of Recombinant SV40 Vectors for Gene Transfer

David S. Strayer, Maria Lamothe, Danlan Wei, Joseph Milano, and Rumi Kondo

1. Introduction

1.1. Gene Therapy and Gene Delivery Vectors

The field of gene therapy has evolved from an investigative curiosity to a major focus of medical research. To date, however, its clinical successes have been few. There are many reasons for this lack of success in clinical studies, but among the most important of these is the inadequacy of gene-delivery vectors, both viral and nonviral. In part because of this problem, we began work on a system of viral gene-delivery vehicles based on SV40.

Virus-mediated gene transfer has focused principally on Moloney murine leukemia virus and adenovirus, and more recently adenoassociated virus and lentiviruses as delivery vehicles ***(1–6)***. Although each vector has its strengths and weaknesses, these viral delivery systems do not address the range of potential therapeutic needs. Each vector has advantages and disadvantages, but the deficiencies of these vectors have been problematic in that they restrict the progress of gene therapy. These shortcomings include limited target cell range, low efficiency necessitating ex vivo selection, low yields in production, short-lived expression, immunogenicity that limits expression or how often the vector can be given, contamination by replication-competent or helper virus, and many others. This chapter describes our experimental approaches to applying another virus, Simian virus-40 (SV40) to gene transfer.

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1.2. SV40 Biology

SV40 is a papovavirus with a genome of 5.2 kb. Its genes encode three structural proteins (VP1, VP2, VP3), large T (for transforming) antigen (T-ag), and small t antigen. T-ag can immortalize cultured cells of many animal species and transform cultured rodent cells. In animals however, SV40 infection is not clearly associated with tumor development in any animal except suckling hamsters. In its natural host, monkeys, it generally causes a mild, transient infection. Other animals, including humans, show few, if any, immediate clinical effects when given SV40 *(7)*.

Several reports indicate that MHC class I, or a closely associated molecule, may act as a receptor for SV40 *(8,9)*. After binding, the virus is endocytosed. It is then transported directly and very rapidly through the nuclear membrane to the nucleus, where it is released and uncoated *(10)*. The lack of a cytoplasmic phase during early infection means that viral antigens are not processed.

Immunity to wild-type (wt) SV40-infected cells is principally against T-ag. Although it is primarily a nuclear protein, T-ag inserts into the cell membrane and is the major target for antibody- and cell-mediated immune responses *(11)*. Our constructs lack T-ag, and thus impart no immunogenicity to virus-infected cells. Capsid antigens may elicit immune responses not, as indicated above, during the early phase of infection, but rather when they are produced by the cell during virus assembly, which follows viral DNA replication. Viral DNA replication and capsid gene expression both require T-ag, therefore they do not occur with the T-ag-deleted SV40 derivatives that we have generated.

1.3. rSV40 vectors

These features of wt SV40 biology allow recombinant SV40 (rSV40) vectors to circumvent the immune system and provide long-term expression of transduced genes. Without activating the immune system, rSV40-infected cells may carry the viral genome integrated into host DNA, as well as in episomal form. In addition, multiple inoculations with rSV40 vectors are possible, with no evidence that gene delivery diminishes with repeated transduction. SV40-based vectors have been used to deliver several genes, including reporter genes, as well as genes of therapeutic interest, to experimental animals (mice and rats) in vivo, to human and other cells in culture, and following reimplantation in vivo of cells transduced ex vivo *(12,13)*. Following transduction, stable transgene expression and activity have been documented for over 1 yr in vivo. In various experimental settings, both in vitro and in vivo, we have transduced high levels of active enzymes (e.g., bilirubin UDP-glucuronyltransferase), secreted proteins (e.g., 1-antitrypsin), viral envelope components (e.g., HIV-1 gp120), intracellular antibodies (e.g., single-chain Fv fragments), active

ribozyme molecules (e.g., against mutant cellular transcripts), and others. Thus, SV40 has considerable potential as a gene-delivery vehicle for a variety of applications. The purpose of this chapter is to describe the general approaches and selected specific methodologies that can be used to apply SV40-derived vectors to studies of gene transfer in vitro and in vivo.

1.4. Approaches

1.4.1. General Principles

The principles behind the use of rSV40 vectors for gene transfer in vivo have been reported by us ***(12,13)*** and their application to gene transfer in vitro have been published by ourselves and others, although approaches used by individual investigators differ ***(14–18)***. There are four basic steps involved:

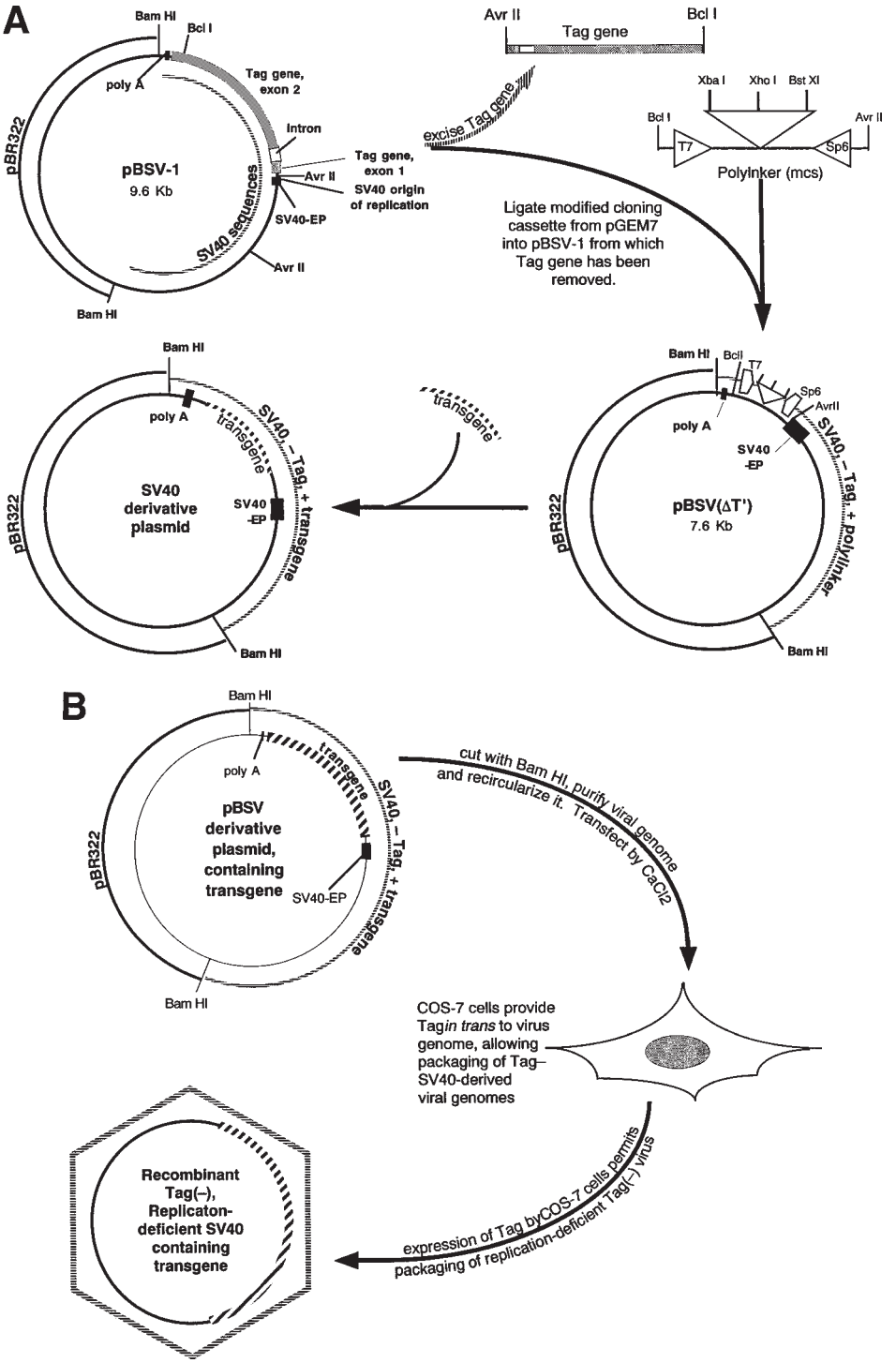
1. Manipulation of cloned SV40 genome for the intended purpose.
2. Excision of modified SV40 genome from carrier plasmid, and transfection into a packaging cell line to produce the desired rSV40.
3. Production, purification, and titering of rSV40.
4. Use of this virus for the desired gene transfer application.

1.4.2. Manipulation of Cloned Viral Genomes

Our approach to the construction of SV40-derivative viruses is illustrated in **Fig. 1** and described below. An advantage of SV40 is the ease with which the virus genome can be manipulated. Our approach is illustrated here, but comparable manipulations can be done in other ways to produce SV40 derivatives that differ in one or more aspects from those we have made to date.

The SV40 genome had been as a *Bam*HI fragment cloned into pBR322 to make pBSV-1 (gift of Dr. J. S. Butel). We replaced the 2.4-kb *T-ag* gene with a polylinker derived from pGEM7® (Promega). This plasmid, pBSV(T), was used effectively to produce replication-deficient virus that could express luciferase as a reporter gene, as described in our initial published gene-transfer studies ***(12,13)***. Many subsequent modifications have been made for various purposes. Additional carrier plasmids have been exploited, with the rSV40 genome cloned differently into each. We have added other promoters (e.g., cytomegalovirus intermediate early promoter or met tRNA promoter), internal ribosomal entry sites (IRES), introns, expanded polylinkers, and so on. In some constructs the orientation or location of the expression cassette has been changed. We have also made constructs in which the majority of the late genome was removed, in order to accommodate larger transgenes. In these cases, all viral capsid proteins are provided by the COS-7 cells.

In all of our constructs, we have avoided altering the viral origin of replication, early and late promoters, enhancer, and packaging signals. These over-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Bam HI
Bcl I
poly A
Tag gene, exon 2
Intron
Tag gene, exon 1
Avr II
SV40 origin of replication
SV40-EP
SV40 sequences
Avr II
Bam HI
pBR322
pBSV-1
9.6 Kb
Avr II
Tag gene
Bcl I
excise Tag gene
Xba I
Xho I
Bst XI
Bcl I
Avr II
T7
Sp6
Polylinker (mcs)
Ligate modified cloning cassette from pGEM7 into pBSV-1 from which Tag gene has been removed.
Bam HI
BclI
T7
Sp6
AvrII
poly A
SV40 -EP
SV40 – Tag + polylinker
pBR322
pBSV(ΔT')
7.6 Kb
Bam HI
transgene
Bam HI
poly A
transgene
SV40 – Tag + transgene
pBR322
SV40 derivative plasmid
SV40 -EP
Bam HI
B
Bam HI
poly A
transgene
SV40 – Tag + transgene
pBR322
pBSV derivative plasmid, containing transgene
SV40-EP
Bam HI
cut with Bam HI, purify viral genome and recircularize it. Transfect by CaCl2
COS-7 cells provide Tag in trans to virus genome, allowing packaging of Tag– SV40-derived viral genomes
expression of Tag by COS-7 cells permits packaging of replication-deficient Tag(–) virus
Recombinant Tag(–), Replicaton-deficient SV40 containing transgene

Fig. 1.

lapping regions are found in DNA sequences beginning at the 3 end of the SV40 early promoter (SV40-EP) and extending 5 approx 400 bp ***(19,20)***. We have also left intact the early and late polyadenylation signals, immediately 3 to the *T-ag* gene.

1.4.3. rSV40 Virus Production

Producing rSV40 viruses that lack T-ag requires a cell line to supply in *trans* the necessary virus products that are lacking in the modified genomes aforementioned. T-ag is necessary for SV40 replication and packaging ***(19)***. Because this gene is absent in all constructs, the packaging cells must supply T-ag protein. COS-7 cells contain integrated SV40 DNA lacking an effective origin of replication. Thus COS-7 cells express T-ag, but cannot themselves produce virus ***(21)***. Their ability to supply T-ag in *trans* was exploited to package our recombinant viruses.

Viral DNA is excised from the carrier plasmid, gel purified, recircularized, and transfected into COS-7 cells. After 1–2 wk, an initial stock of virus is obtained. This initial seed stock is amplified to produce working stocks by infecting COS-7 cells. No further transfection is needed to make working virus preparations. This virus may be used either as a crude cell lysate or band-purified (*see* **Subheading 2.3.**). The SV40-derivative virus stock produced in this fashion is replication-defective ***(12,13)***. No helper virus is involved in this system.

It should be emphasized that the restriction enzyme that is used to excise the rSV40 genome from carrier plasmid must not cut elsewhere in the rSV40

Fig. 1. *(see opposite page)* Construction of SV40-derivative vectors. SV40 genome, cloned as a *Bam*HI fragment into the unique *Bam*HI site of pBR322 (to produce pBSV-1) was reengineered to produce a genome capable of incorporating foreign genes, and incapable of replicating itself. To excise the *T-ag* gene, pBSV-1 was digested with *Bcl*I and partially digested with *Avr*II. This procedure left the SV40 early promoter, just upstream to the *T-ag* gene, and the poly A addition site, just downstream from the *T-ag* gene, intact. Into this opened plasmid, we inserted a modified polylinker from pGEM7: modified to eliminate the *Bam*HI site and to incorporate *Bcl*I and *Avr*II restriction sites flanking the T7 and Sp6 bacteriophage promoters, respectively. Cloning this polylinker into the linearized T-ag-deficient viral genome in pBR322 provided a potential target for gene transfer, pBSV(T). Potential transgenes cloned into this plasmid in orientation (5 3) Sp6 T7 will be expressed under the control of the SV40 early promoter (SV40-EP).

To produce virus from this construct, the viral genome is excised from the carrier plasmid using (in this case) *Bam*HI, gel purified, and religated to itself. This recircularized viral genome is transfected into COS-7 cells, which supply the requisite T-ag in *trans*, and allow packaging of a viral genome that is otherwise replication-defective because it is lacking a *T-ag* gene.

genome, particularly the transgene. For this reason, we reengineered cloning targets in several ways using restriction enzymes such as *Pme*I and *Not*I to excise rSV40 from carrier plasmid. Both enzymes have 8-base recognition sequences therefore they cut DNA infrequently.

1.4.4. Titering Virus

Classically, SV40 has been titered in monkey cells under agar overlays ***(22)***. We have used this method, but it has limitations. In our hands, it does not always provide reproducible titers. More importantly, it is cumbersome and requires weeks for clear results. Only a cell line that supplies T-ag can be used to plaque replication-defective virus. As a virus' infectivity may vary from one target cell to another, titers derived from only the packaging cell line may not reflect the virus' infectivity for cells from different tissues and animals. For all of the above reasons, we developed a rapid and reliable technique for titering replication-defective SV40 based on *in situ* polymerase chain reaction (*in situ* PCR). This technique can be applied to any DNA virus, and adapted to testing infectivity in any target cell type ***(23)***. In general, crude cell lysates prepared as detailed below from SV40-infected cells yield 10^9 infectious units (IU)/mL. Virus may be purified by banding on a sucrose cushion. We have found that in addition to concentrating virus, virus recovery is substantially improved by this technique. We have achieved yields of up to 10^{12} IU/mL in purified rSV40 stocks.

1.4.5. Using rSV40 Viruses for Gene Transfer

We and our collaborators have used SV40-derived viruses for gene transfer both in vivo in mice and rats, and in cultures of human, monkey, and many other mammalian cell types. Choice of route and dosage in vivo vary, depending on the individual application. SV40 infects almost every cell type we have examined, whether the cells are resting or dividing. To infect cells grown in culture, we have used SV40 at multiplicities of infection (MOI) from 1 to 5000 infectious virus particles per cell. Even at the highest MOI, we have detected no more than minimal cytotoxicity with these SV40 derivatives, as measured by levels of "housekeeping" transcripts or by sustained cell viability.

We have found that, in general, effectiveness of gene delivery with SV40 increases with the number of exposures, both in vivo and in vitro. Increasing the MOI for a single exposure does not improve delivery as effectively as does increasing the number of deliveries. Thus, transduction efficiencies of 95% have been achieved with rSV40 vectors in several different organs and cell types both in vitro and in vivo, by using three administrations rather than one without altering the total amount of virus administered.

2. Materials

Unless otherwise indicated, concentrated stock solutions should be diluted to the final concentrations cited in **Subheading 3.** with double-distilled (dd) H_2O. In brackets are the storage conditions.

2.1. Cell Lines

1. COS-7 cells (ATCC, Rockville, MD).
2. CV1 cells (ATCC).

2.2. Tissue-Culture Media and Supplies

1. Double-distilled water (ddH_2O).
2. Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) [4°C].
3. Fetal (bovine) calf serum (FCS, Hyclone) [–20°C].
4. Penicillin/streptomycin stock (100X); glutamine; 10X trypsin (Gibco-BRL) [–20°C].
5. Tissue-culture plasticware (60-mm dishes; 75 cm^2 or 150 cm^2 flasks, Costar).
6. 1X PBS: NaCl (Fisher) 8 g, KCl (Mallinckrodt) 0.2 g, Na_2HPO_4 (Mallinckrodt) 1.44 g, KH_2PO_4 (Sigma) 0.24 g, ddH_2O to 1 L, adjust pH to 7.4.
7. CO_2 incubator (maintained at 5–6% CO_2, NAPCO).
8. Tissue-culture scraper (Fisher Scientific).

2.3. Band-Purification of Virus

1. 75% sucrose: 75 g sucrose per 100 mL, in 0.2 *M* Tris-HCl, pH 7.4 [RT].
2. 20% sucrose: 20 g sucrose per 100 mL, in 0.02 *M* Tris-HCl, pH 7.4 [4°C].
   Solutions are warmed carefully to dissolve the sucrose. This step should be done carefully, avoiding overheating, because sucrose will caramelize at high temperatures. Filter sterilize using a 0.45-µm filter and store at 4°C.
3. Double detergent: 10% Triton X-100 (Aldrich), 5% deoxycholate (Aldrich) [RT]. Filter sterilize.
4. Ultracentrifuge (Beckman).
5. SW28.1 Rotor and matching tubes (Beckman) [4°C].
6. 70% ethanol.
7. 0.45-µm solution filters (Costar).

2.4. Virus Titration by In Situ *PCR*

1. Teflon chamber slides (Erie Scientific, Erie, PA, cat. no. SLS-0311).
2. Proteinase K (Boehringer-Mannheim) 6 µg/mL [–20°C].
3. 3-aminopropyltriethoxy-silane (Sigma) [RT].
4. 4% paraformaldehyde (Fisher) [RT].
5. 10X PCR buffer with gelatin (Perkin-Elmer-Cetus, GeneAmp® cat. no. N808-006) [20°C].

6. dNTPs (0.25 m*M*, Perkin-Elmer-Cetus) [–20°C].
7. Primer pairs (5 primer: ACTGTGACTGGTGTGAGCGCTG; 3 primer: TGGACCCAATGTCTGGGGTC), both made to order [–20°C].
8. *Taq* polymerase (Perkin-Elmer-Cetus) [–20°C].
9. ddH_2O.
   To make 100 µL PCR cocktail, mix:
   a. 10 µL 10X buffer with gelatin (Perkin-Elmer-Cetus).
   b. 2.5 µL of each dNTP (final concentration, 250 µ*M* each).
   c. Primer pair 0.5 µ*M* each (final concentration).
   d. 2 µL *Taq* polymerase, final concentration, 0.1 U/µL ddH_2O to 100 µL.
10. 50% formamide (Fisher Scientific) [4°C].
11. 1 mg/mL salmon sperm DNA (Calbiochem) [–20°C].
12. 0.1% SDS (sodium dodecyl sulfate) (Sigma) [RT].
13. 16 n*M* 5 biotinylated probe made to order: (CCAGGAATGGCTGTAGATT TGTA-TAGGCCAGATGATTACGA) [–20°C].
    Hybridization solution (final concentrations): 50% formamide, 2X SSC, 10X Denhardt's solution, 1 mg/mL salmon sperm DNA, 0.1% SDS, 16 n*M* 5 biotinylated probe (added immediately before use).
14. Streptavidin-alkaline phosphatase (stock solution from Vector Laboratories) in PBS [4°C], to be diluted 1:300 in PBS immediately prior to use.
15. 20X SSC: 175.3 g NaCl (Fisher), 88.2 g Na Citrate (Fisher), ddH_2O to 1 L [RT].
16. 30% H_2O_2 (Fisher) [4°C].
17. BCIP/NBT Alkaline Phosphatase Substrate (kit IV, Vector Laboratories). Make up immediately before use, according to package insert.
18. 100 m*M* Tris-HCl, pH 9.5.
19. 50X Denhardt's solution: polyvinylpyrrolidone (Sigma) 5 g, bovine serum albumin (BSA) (Sigma) 5 g, Ficoll 400 (Sigma) 5 g, ddH2O to 500 mL [–20°C].
20. Thermal cycler for *in situ* PCR (MJ Research, Boston, MA).

3. Methods

3.1. Production of Virus Stocks

3.1.1. Preparation of Initial Sample of Recombinant SV40 (see **Note 1***)*

All steps should be performed under sterile conditions.

1. Transfect COS-7 cells, subconfluent in a 60-mm tissue-culture dish, using any desired protocol [e.g., *see* $CaCl_2$ transfection protocols, Chapters 10 and 13 in this volume and **ref.** ***24***] with 10 µg gel-purified and recircularized virus genome, free of carrier plasmid. Cytopathic effect is observed within 1–2 d and is extensive by 7 d.
2. Harvest the virus by removing the medium. Because SV40 remains associated with the cell, most virus will be in the monolayer. We do not discard the supernatant, however, because it contains some virus. It should be stored at –80°C. Freeze-thaw the cell monolayer three times. Add 1 mL DMEM-2% FCS to the 60-mm dish, and scrape off the monolayer using a rubber policeman.

3. Place the crude lysate in a conical tube and sonicate it using a bath sonicator for 2 min. The crude lysate may be stored at –80°C. Most of the virus remains associated with cell cytoskeletal debris. Thus, the debris must not be removed. This initial sample is then used to generate a working virus stock.

3.1.2. Amplification of the Initial rSV40 Sample

1. Plate COS-7 cells in a 150-cm^2 tissue-culture flask and allow them to achieve approx 80% confluence in DMEM-10% FCS.
2. Infect by removing the medium and adding 2 mL DMEM-2% FCS + 1 mL viral stock made as above. Place the flask on an orbital shaker for 2 h at room temperature. Add 17 ml DMEM-2% FCS and place at 37°C in an atmosphere of 5–7% CO_2 for about 2 wk. Foci of infected cells should be noticeable within 1–2 d and most cells will be infected as judged by cytopathic effect within 10–14 d, when the virus is ready to harvest.
3. Harvest the virus as above, by removing the medium and freezing and thawing the monolayer three times. Add 5 mL DMEM-2% FCS to each flask and scrape the cells off with a rubber policeman. Place the crude lysate in a 15-mL conical tube, sonicate it in a bath sonicator for 2 min, and store at –80°C.
4. Do not discard cellular debris. In cell lysates SV40 is mostly associated with cytoskeletal aggregates and remnants. The virus may be used as crude lysate or after banding.

*3.2. Band Purification of SV40 (*adapted from ref. *14)*

1. Sterilize the buckets of a Beckman SW28.1 rotor or equivalent by autoclaving. Tubes should be sterilized by soaking in 70% ethanol for at least 10 min.
2. From four 150-cm^2 flasks, approx 20 mL of crude lysate should be obtained. Split this into two 10-mL aliquots and add 1 mL of the double-detergent solution. This step dissociates the virus from cell cytoskeletal debris. Incubate on ice for 15 min. Centrifuge at 16,000*g* for 20 min at 4°C to remove cellular debris. Save the supernatant and bring aliquots to 13 mL.
3. To concentrate virus from the cell lysate on a discontinuous sucrose density gradient, add 1.5 mL 75% sucrose to a sterile SW28 tube. Then, carefully layer 2.5 mL 20% sucrose over it. Layer the 13-mL double-detergent supernatant over the sucrose solutions. Place the tubes into sterile buckets and spin at 16,000*g* for 3.5 h. The virus band is not usually visible at the interphase.
4. Pierce the bottom of the centrifuge tube with an 18-gage needle. Drip the gradient into sterile microfuge tubes (0.5 mL per fraction). Collect only the first eight fractions. The virus is usually in fractions 4, 5, or 6. Pool these and dialyze sterilely (we use sterile dialysis cassettes for this purpose) against three changes of 1000 mL PBS. You may detect the virus by ultraviolet spectrophotometery: the A_{260}/A_{280} ratio is usually 1.3–1.5.
5. Virus may be stored in suspension at –80°C in aliquots. It maintains good activity when stored in this way, but does lose activity with repeated freezing and thawing. Thus, the latter is to be avoided by judicious aliquoting where possible.

6. Virus preparations may be lyophilized after banding. Lyophilization itself causes approx 20% loss of infectivity. In this form however, viral infectivity is relatively stable, even to harsh storage conditions: rSV40 preparations lyophilized and maintained at room temperature for 1 mo retain approx 20% of their infectivity.

3.3. Titering Replication-Defective SV40 Viruses

Titering replication-defective SV40 viruses is done by *in situ* PCR (*see* **Note 2**). This technique measures the ability of virus to infect (i.e., to release its DNA into) target cells ***(15)***. The procedure must be standardized for each different target cell type, as each cell type is different and, in particular, may require different times of proteinase K digestion. The approach described as follows is for TC7 cells.

1. Plate TC7 monkey cells (kind gift of Dr. J. S. Butel) in six-well cluster dishes so they will grow to about 80% confluence overnight (approx 1.5×10^5 cells/well). It is critical to determine the number of cells/well. If TC7 cells are unavailable, CV1 cells (ATCC) can also be used. These cells do not carry any part of the SV40 genome, and therefore will not support replication of T-ag-defective, SV40 derivative viruses. Thus, only the ability of the recombinant virus to enter cells is measured.
2. Make serial 10-fold dilutions of virus stock in DMEM-2% FCS. Be sure to include a negative control (no virus) and 100 µL of undiluted viral stock. Remove medium from cultures. Plate 100 µL of each dilution + 400 µL DMEM-2% FCS. Be sure to completely cover the cell monolayer.
3. Incubate for 2 h at RT with rocking.
4. Bring each well to 3 mL with DMEM-2% FCS, and incubate overnight at 37°C.
5. Prepare Teflon slides by precoating them with 3-aminopropyl-triethoxy-silane (AES).
6. Remove cells from plate with 250 mL 10X trypsin (Gibco-BRL) and wash with 500 mL PBS.
7. Place 750 µL of infected cell suspension in 1.5-mL microfuge tubes, spin for 2 min in a microfuge, and remove supernatant. Resuspend cell pellet in 20 µL of serum-free medium.
8. Spread cell suspension onto the glass surface of AES-treated slides and allow to dry.
9. Heat-fix for 1 min at 105°C, then soak in 4% paraformaldehyde for 3 h.
10. Wash 3X in PBS for 10 min/wash, then twice in 1X PBS for 5 min. Store at 4°C until ready for *in situ* PCR.
11. Place slides in PBS containing 6 µg/mL proteinase K for exactly 15 min at RT. This step is highly variable, and depends on the particular cell type used. The cells must be proteinase K-treated sufficiently to allow penetration of the PCR and *in situ* hybridization reagents, but not so extensively that no cellular material remains on the slide. In our experience, each and every cell line must be studied beforehand to optimize this step prior to attempting titering.

12. Stop the reaction by heating the slides to 92°C for 1 min.
13. Wash in 1X PBS followed by ddH_2O and allow to air-dry.

3.4. In Situ *PCR*

For this purpose, we chose a primer pair that would provide a PCR product derived from among the late virus genes, away from the transgene. Clearly, the choice of primer pair for this purpose is empirical. An oligonucleotide probe that has been biotinylated at the 5 end can also be used. This can be commercially prepared and obviously will also vary depending on the virus and the sequence of the region amplified by PCR.

1. Apply 13 µL PCR mixture to the slide and place a cover slip over the reaction mixture, taking care to avoid air bubbles.
2. Seal the cover slip with one light coat of clear nail polish and allow it to dry. Then apply a second, heavier, coat of nail polish and allow it to dry.
3. Place in the thermal cycler and set the following cycle parameters: 94°C for 60 s, followed by 50°C for 1.5 min, then 72°C for 1 min for a total of 35 cycles, followed by a soaking step at 4°C.
4. Remove the sample from the thermal cycler and place it in 95% EtOH for 10 min to remove the cover slip.
5. Be sure to scrape all nail polish from the slide.
6. Heat to 95°C for 1 min to fix the amplified DNA.

3.5. In Situ *Hybridization*

3.5.1. Preparation of Slides for In Situ *PCR*

1. Soak slides in 2X SSC for 15 min.
2. Place 13 µL hybridization mix on the slide and cover slip.
3. Heat to 95°C for 5 min, then place in a humidified chamber at 37°C overnight.
4. Wash twice in 2X SSC for 5 min, then in 1X PBS for 5 min each time.
5. Prepare a solution of streptavidin-alkaline phosphatase to 1:300 final dilution in PBS.
6. Place 13 µL of this solution on the slide, add a cover slip, and keep at 37°C for 1 h.
7. Wash in 1X PBS for 2 min.

3.5.2. Development

1. Prepare the reaction solution: 0.33 mL 1.5 *M* Tris-HCl pH 9.4, 4.66 mL ddH_2O and 2 drops of each BCIP/NBT reagent from the kit.
2. Flood the slides with this solution and incubate at RT in a light-shielded box. Blue color may take between 3 and 20 h to develop in the positive control slides (COS-7 cells). Negative control slides (TC7 or VERO cells alone) should remain colorless.
3. Check the color. When the positive control is clearly blue, mount in 50% glycerol 3.5% gelatin, and seal the slide with clear nail polish.

4. Using a microscope, identify a slide with 1–50% positive (i.e., virus-infected) cells. Determine the fraction of total cells that are positive for virus infection. Using the dilution factor and the number of cells infected (both of which are known), the infectious titer of the virus stock can be determined using the following formula: Titer (IU/mL) = [fraction of positive cells] × [number of cells/well] ÷ [dilution factor]. Thus, if 70% of the cells are positive at a dilution of $1:10^5$, the titer will be $0.7 \times 1.5 \times 10^5 \div (1/1000)$, or 1.05×10^{10} infectious U/mL in the original stock.

3.6. Transduction of Cultured Cells with SV40-Derived Vectors

Precise methods to be used for transducing cultured cells using SV40 vectors will vary depending on the effect desired, the number and type of cells, and so on. We have found that the approach described below is uniformly effective for our purposes, and routinely achieves very high levels of virus infection and transgene expression (95%).

1. Plate cells in a 60-mm tissue-culture dish to about 80% confluence. Cells may be trypsinized and allowed to adhere in 3 to 5 mL DMEM-10% FCS just before infection.
2. Remove medium and add 0.4 mL DMEM-2% FCS + 0.1 mL virus stock at the appropriate dilution to achieve an MOI of approx 10.
3. Place culture dish on an orbital shaker for 2 h at RT. *Do not remove virus-containing medium from the culture mixture*.
4. Add 2.5 mL DMEM-2% FCS and place at 37°C overnight.
5. Repeat procedure on each of the following days, at MOI approx 3.
6. Culture until the desired assay time.

We prefer to assay for transgene expression by immunocytochemistry, Northern analysis, Western blotting, and other techniques. The assay method chosen will depend on the nature of the transgene and its desired effect (*see* **Note 3**).

4. Notes

1. The development and use of SV40-based viruses as gene transduction vectors is in its infancy. Because of relative safety, high levels of infectivity, efficient transduction, longevity of expression in vivo, and ease of use, such vectors are attractive vehicles for gene transfer. Much work remains to be done, and several important parameters need to be optimized. The greatest limitation of the system at the moment is the size of the DNA insert that can be cloned into the virus without loss of efficiency in packaging. We are currently investigating this question. Other pressing issues involve maintaining long-term high levels of expression both in vitro and in vivo. Studies to date have used unselected cells, but

selection ex vivo may provide additional assurance of high levels of sustained transgene expression.

2. *In situ* PCR is not an easy method of titering to use. It requires use of a specific machine that is not commonly available. It should also be stressed that the seal made on the slides using nail polish is critical: if it is leaky, the slide will dry out and the reaction will be useless. This may be minimized, but not totally eliminated, using a damp cloth or filter paper in the *in situ* PCR chamber, to maintain a moist atmosphere throughout.
3. No technique of enumerating replication-defective virus provides a universally applicable measure of the amount of virus in a preparation. Unlike many other approaches to measuring the amount of virus in a preparation, the technique described provides a clear index of infectivity, as opposed to the much more ambiguous measurement of virus particles by uv absorbance readings. The ratio of infectious:total (infectious + defective) virus particles varies from preparation to preparation and virus to virus. Generally between 1% and 5% of virus particles are infectious. *In situ* PCR gives a specific type of information: the number of virus particles that deliver their DNA to cells. It does not measure expression of the gene of interest, although there are variations (*in situ* RT-PCR) that do so. Some investigators may prefer other approaches to obtain virus titers. If antibody or other techniques (e.g., immunocytochemistry) are available for detecting cells that express a transgene, such approaches may be preferable for many investigators.

   In an atmosphere in which serious deficiencies in all of the commonly used gene transfer vectors are widely acknowledged, SV40-based vectors may be a useful addition to the gene therapy armamentarium.

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The use of SV40-derived viral vectors for gene transfer to animals and humans, or to cells to be administered to animals and humans, is the subject of pending United States and international patent applications.

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9

Production of SV40 Proteins in Insect Cells and In Vitro Packaging of Virions and Pseudovirions

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1. Introduction

The use of insect cells and lytic baculovirus for expression of biologically active mammalian proteins has been the method of choice of many investigators. Although prokaryotic expression systems provide higher yields and are technically simpler to use, obtaining biologically active eukaryotic proteins in these systems can be problematic. Posttranslational modifications such as glycosylation and phosphorylation do not occur in prokaryotic systems owing to the lack of enzymatic machinery. On the other hand, insect cells provide an appropriate environment for posttranslational modifications and lead to proper folding and correct assembly of recombinant proteins ***(1)***.

Autographa californica nuclear polyhedrosis virus (AcNPV) infects *Spodoptera frugiperda* (Sf9) cells. During AcNPV infection, two forms of viral progeny are produced: extracellular virus particles (ECV) and occluded virus particles (OV) ***(2)***. The latter are embedded in polyhedra, which are proteinaceous viral occlusions created by polyhedrin protein. This protein is nonessential for infectious ECV production and the gene of interest can be inserted in the place of the polyhedrin coding region. In so doing, the gene is regulated by the strong polyhedrin promoter providing high level of expression ***(2–4)***.

The baculovirus expression system has been successfully used to express early ***(5–7)*** and late ***(8)*** SV40 gene products. To study mRNA splicing in the baculovirus-host cell system, Jeang et al. ***(5)*** constructed a recombinant baculovirus in which the entire SV40 early transcriptional unit has been cloned under control of the polyhedrin promoter. Their experiments showed that small t-antigen splice donor and acceptor sites are recognized by the insect splicing

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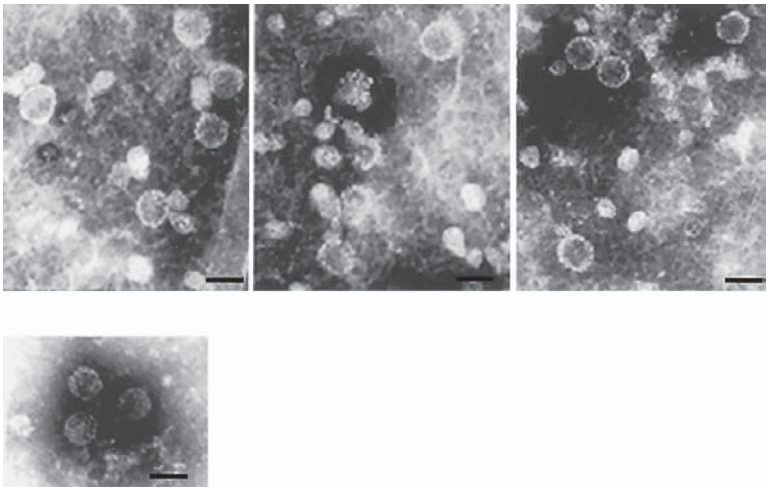

Fig. 1. Self-assembly of SV40 capsid proteins in insect cells. Samples were adsorbed onto Formvar-carbon-coated copper grids and stained with 1% phosphotungstate, pH 7.2. The samples were viewed in a Philips CM-12 electron microscope, using a voltage of 100 kV, and photographed at a magnification of ×60,000. The bar represents 50 nm. Top: Three fields of nuclear extracts of SF9 cells infected with three recombinant baculovirus, expressing VP1, VP2, and VP3. Bottom: wt SV40, shown for comparison.

machinery. Other groups expressed the T-antigens (T-ag) separately using the baculovirus system. The experiments showed that insect cells are capable of producing large T-ag and modifying it by phosphorylation, glycosylation, and oligomerization ***(6)***. Functional assays demonstrated that the *ori*-specific DNA binding, adenosine triphosphatase (ATPase) and helicase activities of insect cell-derived T-ag are comparable to T-ag synthesized in mammalian cells. In addition, it was found that the recombinant small t-antigen is capable of associating with two of the cellular proteins (56k and 32k) that are known to bind to it during wild-type SV40 infection ***(6,7)***.

Late viral proteins have been produced in insect cells for studies on the mechanism of protein–protein interactions that participate in the packaging process and the assembly of SV40 virions ***(8)***. Recombinant baculoviruses carrying each of the three genes for the capsid proteins were constructed and the corresponding proteins were produced in Sf9 cells. The results demonstrated that SV40 capsid proteins, expressed either separately or together, are localized in the nucleus of the infected cells, indicating that their nuclear localization signal is functional in insect cells. In addition, electron microscopy analysis (**Fig. 1**) demonstrated an abundance of virion-like particles (VLPs) and heterogeneous aggregates of variable size, mostly 20–45 nm. Under the same stain-

ing conditions, wild-type SV40 virions are 45 nm. VLPs were also observed when the major capsid protein VP1 was produced alone ***(8)***. Additional studies demonstrated that these VLPs can be successfully used for packaging SV40 DNA or heterologous plasmid DNA in vitro leading to the production of functional SV40 virions and pseudovirions ***(9)***.

A fourth late protein is the agnoprotein or LP1 encoded by the leader region of the 16S late mRNA. In vivo studies showed that agnoprotein mediates efficient localization of VP1 to the nuclear region ***(10,11)***, and facilitates release of mature virus from infected cells ***(11)***. To investigate the role of agnoprotein in the packaging process, the recombinant protein was expressed in Sf9 cells. Preliminary results showed that the recombinant agnoprotein enhanced packaging efficiency in vitro (Sandalon and Oppenheim, unpublished data).

The aforementioned studies suggest that the baculovirus system is useful for the production of authentic SV40 proteins for detailed biochemical and biophysical studies. Furthermore, this expression system can be used for genetic dissection of structure–function relationships. This chapter describes techniques to express SV40 proteins using recombinant baculoviruses for use in gene therapy.

2. Materials

1. Sf9 cells: ATCC no. CRL-1711 (*see* **Note 1**).
2. Grace's insect medium supplemented with 3.3 g/L Lactalbumine, 3.3 g/L yeastolate, 10% fetal calf serum (FCS), 50 U/mL penicillin, 50 µg/mL streptomycin and 2.5 µg/mL amphotericin B (Biological Industries, Israel) (*see* **Note 2**).
3. Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+} (Biological Industries).
4. Tris-Tween-buffered saline (TTBS) buffer: 50 m*M* Tris-HCl pH 7.5, 200 m*M* NaCl, 0.1% Tween-20.
5. Low-melting-point agar (SeaPlaque).
6. Neutral Red (Sigma).
7. BaculoGold kit (PharMingen, CA) (*see* **Note 3**).
8. Tris-glycine buffer: 20 m*M* Tris-base, 200 m*M* glycine, 20% methanol.
9. Buffers for harvesting the capsid proteins: Buffer A: 10 m*M* HEPES pH 7.9, 10 m*M* KCl, 0.1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1 m*M* EGTA, 1 m*M* dithiothreitol (DTT), 0.5 m*M* phenyl methyl sulfonyl fluoride (PMSF) (Sigma), 0.5 m*M* Leupeptin (Sigma). Buffer C: 20 m*M* HEPES pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1 mM DTT, 1 m*M* PMSF, and 1 m*M* Leupeptin.

3. Methods

3.1. Cell Culture

Sf9 cells are grown as monolayers at 27°C in supplemented Grace's insect medium (*see* **Note 4**).

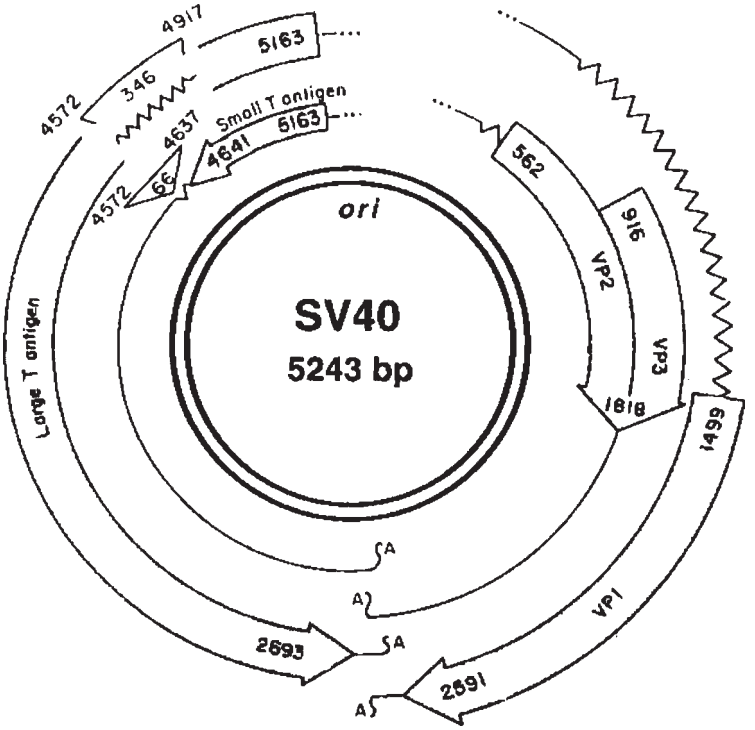

Fig. 2. SV40 genome map [adapted from *(18)*]. The clockwise arrows present the late genes transcripts, the counterclockwise show the early genes transcripts.

3.2. Construction and Purification of Recombinant Baculoviruses

The first step toward the construction of a recombinant baculovirus is to clone the gene of interest in an appropriate plasmid transfer vector. In the second step, recombinant baculovirus is produced by homologous recombination in vivo. Expression the gene of interest is driven by the strong promoter for the viral occlusion protein polyhedrin *(2,3)*.

3.2.1. Construction of Recombinant Plasmids

SV40 DNA fragments of the late region (**Fig. 2**) were cloned into the plasmid vectors pVL1393 and pVL1392 (PharMingen, San Diego, CA) (**Fig. 3**), derived from AcMNPV *(3)* (*see* **Note 3**). The genes for the capsid proteins were cloned into pVL1393 as follows: *VP1* was cloned by introducing a *Stu*I-*Bcl*I DNA fragment (SV40 coordinates 1463–2770) into the plasmid cleaved by restriction endonucleases *Sma*I and *Bgl*II. The *VP2* gene was cloned by ligating a *Hin*cII-*Eco*RI fragment (519–1782) between the *Sma*I and *Eco*RI

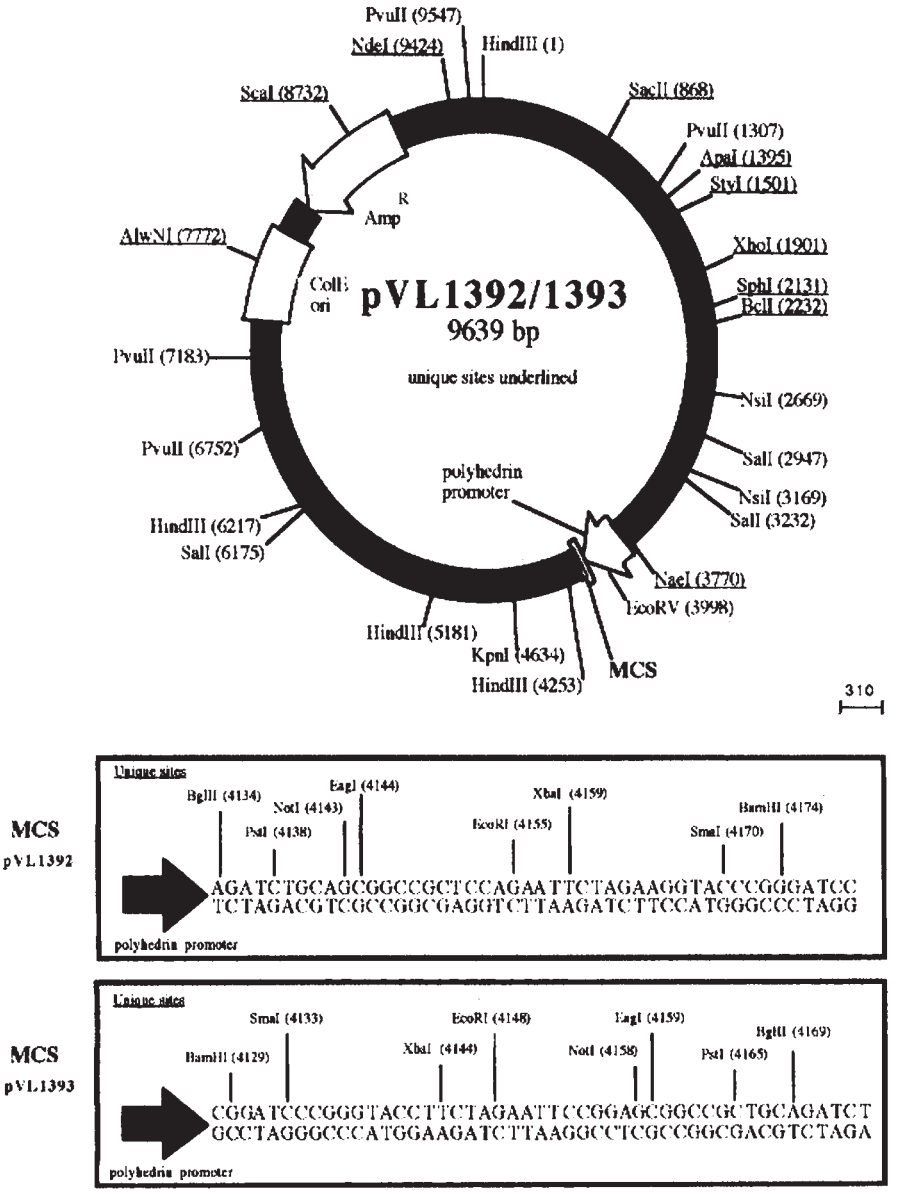

Fig. 3. Genomic map of pVL1392 and pVL1393 baculovirus transfer vectors. (adapted from the PharMingen research products catalog). This vectors contains the complete polyhedrin gene locus of AcNPV virus cloned into the pUC8 vector, but lacks part of the polyhedrin gene coding region. A multiple cloning site (MCS) region has been inserted 37 nucleotides downstream of the ATG polyhedrin start codon, which has been changed into an ATT. The difference between pVL1392 and pVL1393 is: the MCS regions are in opposite orientation to one another. The insert of choice must provide its own ATG start signal at the 5 end of the gene.

sites. The *VP3* gene was cloned by using a *Sau*3AI-*Eco*RI fragment (873–1782) and the *Bam*HI and *Eco*RI sites of pVL1393. The agnogene, a *Pvu*II-*Mbo*I fragment (270–873), was cloned between the *Sma*I and *Bam*HI sites of plasmid pVL1392.

3.2.2. Transfection of Insect Cells and Preparation of Recombinant Baculoviruses

Recombinant baculoviruses carrying each of the three respective genes were produced using the BaculoGold kit (PharMingen) (*see* **Note 3**). The kit provides a tool for efficient selection of the recombinant baculovirus. The method relies on a modified baculovirus carrying a lethal deletion. This virus cannot infect insect cells and does not survive in tissue culture. Only recombinants that emerge following cotransfection with the complementing plasmid construct are viable.

1. Split cells 1:3 into a 60-mm tissue-culture plate a night before the transfection. Cells should be about 60–70% confluent.
2. Remove the culture media from the plate and add 1 mL of BaculoGold transfection buffer A. Make sure that all areas of the plate are covered with transfection buffer.
3. Mix 0.5 µg of linearized BaculoGold virus DNA and 2 µg of your recombinant plasmid DNA, in a sterile 1.5-mL tube and incubate for 5 min in room temperature.
4. Add 1 mL BaculoGold transfection buffer B to the DNA mix and do vortex.
5. Add 1 mL of the BaculoGold transfection buffer B/DNA solution dropwise to the cells. Gently rock the plate back and forth to mix the newly added solution with transfection buffer A that is on the plate. During this procedure a fine precipitate should form, making the solution slightly milky.
6. Incubate the plate at 27°C for 4 h.
7. After 4 h, wash the cells 3× with 1X PBS and add fresh medium. Incubate at 27°C for 4 more days.
8. After 4 d, collect the supernatant containing the recombinant ECV and infect fresh cultures for amplification.

3.2.3. Amplification of Recombinant Baculovirus and Preparation of Viral Stock

Recombinant viruses have to be amplified to obtain high titer stock solution. To this end, freshly seeded cells are infected at a multiplicity of infection (MOI) greater than 1.

1. Infect cells growing in a 25-cm^2 flask at approx 80% confluency (approx 2×10^6 cells) with 500 µL of the ECV-containing supernatant from the transfected cells.
2. For a second round of infection, use 100 µL supernatant taken from the first round of infection.

3. Two rounds of infection usually yield a virus titer of 1–5 × 10^8 particles/mL. Infective virus particles can be stored for up to 6 mo at 4°C. After that period, a decreases in the titer is expected. For best storage, stock virus should be kept in aliquots at –20°C.

3.3. Plaque Assay

Plaque assay is performed using subconfluent Sf9 cells in 25-cm^2 flasks. The culture is split 1:2 the day before the infection.

1. Prepare serial 10-fold dilutions (10^{-3}–10^{-7}) of the recombinant virus.
2. For infection, dilute 100 µL from each sample with 400 µL medium and place on the cells.
3. Incubate the cells at 27°C for 1 h and rock every 20 min to distribute the virus evenly and to keep the cells moist.
4. In the meantime, mix 1.5% low melting agarose, preheated to 37°C, with an equal volume of 2X Grace's medium.
5. Overlay 3 mL from the mix gently on the cells. Incubate the cultures for 4–6 d in a humid environment at 27°C.
6. To visualize the plaques, overlay additional 3 mL of the agar/Grace medium containing 1% neutral red.

3.4. Infection of Sf9 Cells for Recombinant Virus Production

Sf9 cells are infected at a multiplicity of 10 PFU/cell. The virus is allowed to adsorb to the cells for 1 h and then medium is added. The infected cells are grown at 27°C. Protein analyses showed increasing levels of each of the SV40 recombinant proteins from 3 to 6 d postinfection.

3.5. Harvest of SV40 Recombinant Capsid Proteins (*see* Note 5)

As the capsid proteins are localized to the nucleus, they are harvested from isolated nuclei (*see* **Note 6**).

1. 5 d postinfection Sf9 cells are pelletted by centrifugation and the pellet is washed three times with 1X PBS.
2. Resuspend the cell pellet in 400 µL of cold hypotonic buffer A by gentle pipetting, and allow to swell on ice for 15 min.
3. Add 25 µL of 10% Nonident P-40 (NP40) (Fluka) and mix vigorously by vortex for 10 s.
4. Spin the homogenate for 30 s using max speed in the cold. At this stage, the supernatant, containing the cytoplasm, can be transferred to a fresh tube and kept at –70°C for further analyses.
5. Resuspend the nuclear pellet in 50 µL ice-cold buffer C and vigorously shake at 4°C for 15 min on a shaking platform to extract the soluble proteins.
6. Spin the nuclear extract for 5 min. Divide the nuclear extract into aliquots and keep frozen at –70°C.

3.6. Recombinant Protein Analysis

The proteins are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The capsid proteins are separated on 12% SDS-PAGE and then electrotransferred onto a nitrocellulose membrane (Shleicher & Schuell) using Tris-glycine buffer.

1. To eliminate nonspecific binding, block the membrane by treatment with 5% BSA in TTBS buffer overnight at 4°C.
2. Wash the immunoblot with TTBS/0.1% bovine serum albumin (BSA) and incubated with the appropriate antibody (*see* **Note 7**) for 2 h at room temperature.
3. Incubate with a second enzyme-conjugated anti-rabbit IgG antibody (Promega) for an additional hour at room temperature.
4. Each incubation is followed by washing the membrane twice with TTBS/0.1% BSA.
5. The signal is developed using alkaline phosphatase assay (Promega).

3.7. In Vitro Packaging of SV40 Virions and Pseudovirions

Nuclear extracts of Sf9 cells containing either VP1 alone, or all three capsid proteins were used as a source of capsids for in vitro packaging reaction.

1. Mix (by vortex) 2 µL of nuclear extracts (2–4 µg of total nuclear proteins—*see* **Note 8**) with 1 µg of DNA (*see* **Note 9**) in a total volume of 4 µL and place at 37°C for 6 h (*see* **Note 10**).
2. Add to the reaction mixture $CaCl_2$ and $MgCl_2$ to final concentrations of 100 µ*M* and 8 m*M*, respectively, and to a total volume of 6 µL, and incubate on ice for an additional 1 h.
3. To eliminate DNA that is not stably packaged DNaseI digestion is performed. Add 0.5 U of DNaseI to the reaction and incubate for 10 min on ice. The reaction is stopped by the addition of EDTA to a final concentration of 5 m*M*.
4. To test the efficiency of pseudoviral formation and their titer, reaction products are assayed for infectious units on CMT4 monolyers (*see* **Note 11**) using a standard SV40 infection protocol.

4. Notes

1. Since our study was completed ***(8)***, other cell lines that have higher production level have become available. Sf9 and Sf21 cells were derived from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. These two lines are used extensively for the isolation and propagation of recombinant proteins. Sf9 cells are suitable for the formation of monolayer ***(2)***, whereas Sf21 cells can grow to high density in suspension cultures. The cell line high Five (Hi5) was developed from embryonic ovarian cells of the cabbage looper, Trichoplusia ni ***(12)***. This cell line readily grows in suspension and has increased protein production, as much as five- to 10-fold higher than Sf9 cells ***(13)***.
2. Grace's medium does not contain a pH indicator. The normal pH for Sf9 cells in this medium is 6.2 and unlike mammalian cell cultures, the pH rises gradually

as the cells grow. This medium is also available from Gibco, Sigma, and other manufacturers.

3. There is a variety of commercially available complete insect-baculovirus expression systems. Some of the major companies in this area are Clontech, Invitrogen, Life Technologies, Novagen, and Stratagene.
4. Sf9 cells should be subcultured 2–3 times a week. The optimal growing temperature is 27°C. They grow reasonably well at temperatures from 25–28°C. CO_2 is not required and they can be maintained in a closed flask. Since Sf9 cells are not anchorage dependent, it is possible to grow them in suspension cultures. Suspension cultures are maintained at the same conditions as the monolayer cultures.
5. The protocol was adapted from Schreiber et al. ***(14)***.
6. T-antigens are also localized in the nucleus, therefore, they can presumably be harvested by the same method.
7. Polyclonal antibodies against each of the late SV40 proteins were prepared. VP1, VP2, and VP3 and agnoprotein were expressed as fusion proteins to glutathion-S-transferase (GST) in *Escherichia coli*. The four GST-fusion proteins were used to raise polyclonal antibodies in rabbits ***(15,16)***. As expected, antibodies against GST-VP2 and GST-VP3 reacted both with VP2 and VP3, as VP3 is translated from the same coding sequence and at the same reading frame as VP2 (the VP3 coding sequence is nested within the *VP2* gene).
8. Based on coomassie blue staining of SDS-PAGE, we estimated the amount of SV40 capsid proteins at 200–400 ng/reaction.
9. We have used SV40 DNA and plasmid DNA that carry the SV40 origin of replication and the *CAT* gene as a reporter (pSO3*cat*). We have also successfully packaged larger plasmids, 7–7.5 kb ***(9)***.
10. Preliminary experiments showed that shorter incubation periods (1–4 h) gave lower yields of infectious particles.
11. CMT4 cells were derived from CV-1, a permissive African green monkey kidney cell line. CMT4 cells harbor the gene for SV40 T-ag expressed from the inducible metallothionein promoter ***(17)***.

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10

Using Retroviral Vectors to Express SV40 Tumor Antigens

Van Cherington and Cynthia Higgins

1. Introduction

Retroviral vectors for polyomavirus tumor antigens, including SV40 large and small tumor antigens (SVT), have been extensively utilized to study the function of these proteins in a wide range of cell types. Properties of retroviruses that make them particularly efficient vectors include the capacity for dual gene expression in a single vector under the control of a single promotor or separate promotors, and efficient, stable integration of viral genes into the target cell genome ***(1)***. In addition, most cell types have receptors for retroviral infection and vectors may be engineered to further enhance their broad host range ***(2)***. A common application well suited to retroviral vectors is the generation of immortalized cell lines from different tissues following expression of SVT. Conditional, temperature-sensitive mutants of SVT have been used extensively for this purpose. Studies utilizing retroviral vectors to express SVT in a variety of cell types are represented by the following selected references ***(3–20)***.

Retroviral vectors do have limitations. For applications where high-level transient expression is the goal, plasmid or DNA virus-based vectors are better suited because these vectors enter cells at high copy number and express gene products without integrating into the recipient cell genome. In addition, Moloney Murine Leukemia Virus-based vectors, or avian retroviral vectors only integrate their genome into proliferating cells making nonproliferating or slow-growing cell populations poor targets for these vectors. Lentivirus-based vectors are being developed that overcome this problem. However, because the vast majority of retroviral vectors published for SVT are Moloney-based, procedures for use of these vectors are the focus of this chapter.

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Retrovirus particles enter the cytoplasm of target cells after binding to cell surface receptors. Following retrovirus internalization, the single-stranded RNA genome and reverse transcriptase, the product of the viral *pol* gene, are released from the virus particle. Viral RNA is copied by reverse transcriptase into dsDNA with identical sequences, termed long-terminal repeats (LTRs), on each end. LTRs consist of unique sequences derived from both ends of the virion RNA (U3 and U5) that are linked in tandem. The dsDNA is integrated into the cellular DNA where it is referred to as the provirus. The integrated proviral DNA is transcribed by the cellular machinery utilizing the promotor and transcription termination sequences contained within the 5 LTR and the 3 LTR, respectively.

The wild-type retroviral provirus is transcribed and processed into two differentially spliced mRNAs. One mRNA is a full genomic, unspliced transcript containing the packaging signal ( ) and encoding the *gag-pol* genes. The other mRNA lacks the signal and the *gag-pol* reading frames and encodes the *env* gene only. For more detailed discussions of the retrovirus life cycle, see **refs. *1*** and ***2***.

Replication-incompetent retroviral vectors have their *gag-pol* and *env* coding sequences removed, leaving the LTRs and signal. Many retroviral vectors make use of the dual gene capacity by inserting cDNA's into the *gag-pol* position and into the *env* position. Often one of these cDNAs encodes a selectable marker for antibiotic resistance. The retroviral RNA splice donor and splice acceptor are present in so-called splicing vectors as well, but such sites are inactivated in other retroviral vector types. In these vectors, the internal genes are expressed from a separate promotor (e.g., CMV in LNCX, **Fig. 1**).

Two reagents are central to the generation of retroviral vectors ***(2)***. These are the retroviral vector plasmids derived and modified from proviral DNA and the packaging cells; the cell line used to package RNA transcribed from the vector plasmid into replication-incompetent, infectious virus. **Figure 1** outlines

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Fig. 1. *(see facing page)* Examples of retrovirus vector plasmids. This is not a comprehensive list of retroviral vectors and the maps are not drawn to scale. They are examples of published vectors, some of which are available commercially.

MFG: Splicing vector—No selection. Potential for higher titers and larger insert. Gene expressed from the *env* site ***(31)***. This vector must be obtained by special arrangement.

LNCX: Nonsplicing vector with internal promoter—*Neo*-resistance expressed from LTR. Inserted gene expressed from strong cytomegalovirus immediate early promoter and enhancer. May express toxic levels of SVT ***(32)***. Available from Clontech, Palo Alto, CA.

LXSN: Nonsplicing vector with internal promotor—Inserted gene expressed from LTR; *Neo*-resistance expressed from SV40 early region promotor and enhancer ***(32)***. Available from Clontech.

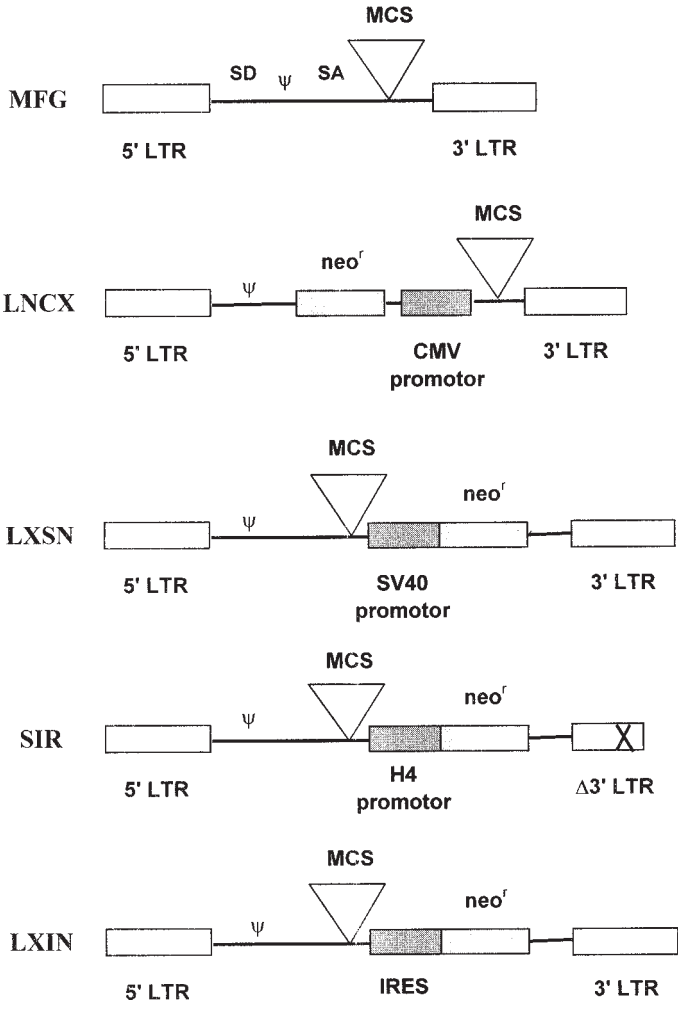

Fig. 1.

Fig. 1. *(continued)* SIR: Self-inactivating vector—Inserted gene must be cloned into vector with a promotor. Inactivated 3 LTR is copied during integration so that integrated provirus has two defective LTRs. *Neo*-resistance expressed from an internal histone H4 promotor and enhancer ***(32)***. Available from Clontech.

LXIN: Bicistronic vector—Vector contains an internal ribosome entry site (IRES) between the MCS and the *neo*-resistance gene. Thus, both the inserted gene and the antibiotic resistance gene are expressed from a single unspliced transcript. As a result, antibiotic resistance correlates with expression of the gene inserted at the MCS ***(32)***. Available from Clontech.

LTR = long-terminal repeat; = packaging signal sequence; MCS = multiple cloning site; SD or SA = splice donor or splice acceptor.

representative vector plasmid types. All vectors retain provirus LTR's at each end of the genome and the LTRs contain all functions required for vector transcription initiation and termination. The 3 -LTR is modified in selfinactivating vectors.

One vector that has been used often for SVT expression, pZIP*neo*SVX ***(2,4,6,8,9,19,20,26)***, is not specifically described in **Fig. 1**. It is a splicing vector (similar to MFG, **Fig. 1**) with a cloning site for SVT in the *gag-pol* location between the splice donor and splice acceptor sites and with the *neo*^{r} gene in place of the multiple cloning site (MCS) in the *env* location after the splice acceptor.

Viruses are produced in culture by transfecting vector plasmid DNA into a packaging cell line. Packaging cell lines express retroviral *gag-pol* and *env* proteins from expression vectors lacking viral transcription (LTR) and packaging ( ) sequences. Therefore, after transfection of the vector plasmid, vector-derived RNA containing LTR's and signal are packaged using the structural proteins expressed by the cell in *trans*. The *env* protein expressed by the packaging cell line, coding for envelope proteins, determines the host range of the virus that is produced. Thus, an individual vector plasmid can be packaged as a narrow host-range (ecotropic) or broader mammalian host-range (amphotropic) virus depending upon the packaging cell line used. It is also possible to form pseudotypes with the Vesicular stomatitis virus glycoprotein (VSV-G) at the virus surface that can infect nonmammalian and mammalian species and can be concentrated by ultracentrifugation.

Retroviral vectors are biohazards. This is particularly true for broad host-range vectors, including amphotropic and VSV-G pseudotyped vectors, which can infect humans. National Institutes of Health (NIH) guidelines require that retroviral production be performed under Biosafety Level 2 precautions. These precautions should include, but are not limited to, the use of lab coats and gloves, limited-access work areas, and laminar flow hoods with microfilters. Studies utilizing infectious viral vectors must be registered with the biosafety committee at the institution where the work is carried out. These committees must be consulted regarding local regulations, beyond those recommended by the NIH. Details of the NIH guidelines are available over the Internet (http://www.niehs.nih.gov/odhsb/biosafe/bmbl/bmbl-1.htm). When following NIH and local guidelines, it is best to work with investigators experienced in the use of amphotropic retroviruses when first working with these vectors.

2. Materials

2.1. SVT Expression

1. Representative retroviral vector plasmids and their sources are outlined in **Fig. 1**. SV40 DNA (ATCC 45019) is available from the American Type Culture Collection (ATCC, Manassas, VA).

2. Representative packaging cell lines and their sources are outlined in **Table 1**.
3. Tissue-culture media: Dulbecco's modified Eagle's medium (DMEM, high glucose)+10% serum. Antibiotic/antimycotic supplementation with penicillin, streptomycin, and amphotericin B is optional. Calf serum is used for NIH 3T3 cells and derived cell lines. Newborn calf serum is used for GP+E86 and GP+envAM12 packaging lines. Although some protocols state that fetal calf serum (FCS) is required for all lines derived from human 293 lines, some investigators use calf serum because it is less expensive and the cells grow well. All cultures are kept in a humidified, 37°C, 5% CO_2 incubator.
4. Selective media: For HXM selection, Hypoxanthine (15 µg/mL; prepare stock in water at 10 mg/mL and store at –20°C), xanthine (250 µg/mL; prepare stock in 0.1*N* NaOH at 10 mg/mL and store at –20°C), and mycophenolic acid (25 µg/mL; prepare stock in 0.1*N* NaOH at 10 mg/mL and store at –20°C) are added to the culture medium. Hygromycin (200 µg/mL; prepare stock at 50 mg/mL in PBS or HEPES buffered saline, pH 7.2, and store at –20°C) may also be added for those lines that are resistant to it, such as GP+envAM12. All may be purchased from Sigma (Sigma, St. Louis, MO) and all stock solutions aforementioned are stable for at least 1 yr and should be filter-sterilized. G418 sulfate (Geneticin, Life Technologies, Inc.) is added to lines that are resistant to it, to a final concentration of approx 400 µg/mL. Note that the G418 concentrations recommended refer to the active compound and G418 preparations are not 100% active. Check the label to determine the active amount of G418 per mg dry weight sold. This is often approx 400–500 µg/mg. Prepare stock solutions of G418 in PBS or HEPES-saline pH 5.0, so that they are 40 mg active G418 per mL. Filter-sterilize and store at –20°C. Stable for more than 2 yr.
5. Glass cloning cylinders are available from Bellco Biotechnology (Vineland, NJ).
6. Polybrene (Sigma) is prepared as a 0.8 mg/mL (100X) stock in water and filter-sterilized. This solution is stable for at least 1 yr at 4°C.
7. Calcium phosphate precipitation transfection reagent kits are available from Life Technologies, Inc. or from Sigma. pHCMV-VSV-G, a plasmid expressing the VSV-G protein, must be obtained through a collaboration ***(21)***. Hank's balanced salt solution (HBSS) may be purchased from Life Technologies, Inc. After transfection, packaging lines may be selected for hypoxanthine-xanthine-mycophenolic acid resistance as in **step 4** above.
8. NIH-3T3 mouse fibroblasts are available from ATCC.
9. Dulbecco's phosphate-buffered saline (PBS, Life Technologies, Inc.).
10. 37% formaldehyde solution and methylene blue may be purchased from Sigma. Methylene Blue should be used as a 0.05% solution in deionized water.
11. Covered microplate carriers for spin infections are recommended to contain aerosols of retroviral vectors because they represent a biohazard. They should be purchased from the centrifuge manufacturer, e.g., Beckman or Sorvall.
12. Mitomycin C may be purchased from Sigma in vials for direct reconstitution in the vial. It is added to medium from a 1 mg/mL stock prepared in sterile water or sterile PBS to make 10 µg/mL in the medium.

Table 1
Examples of Packaging Cell Lines

Cell line name	Host range of virus generated	Features	Reference	Source
GP+E-86 ATCC CRL 9642	Ecotropic	Derived from NIH3T3; grows in HXM medium	***(33)***	*ATCC or Genetix Pharmaceuticals
GP+envAM12 ATCC CRL 9641	Amphotropic	Derived from NIH3T3; grows in HXM medium + hygromycin b	***(34)***	*ATCC or Genetix Pharmaceuticals
PT67	Amphotropic	Derived from NIH3T3; virus can use AM12 and Gibbon ape leukemia virus (GALV) receptor.	***(35)***	Clontech
Anjou65 ATCC CRL 11269	Expresses gag-pol, but no env product	Derived from 293; for production of retrovirus pseudotyped with VSV-G	***(35,36)***	**Rockefeller University and ATCC
Bosc23 ATCC CRL 11270	Ecotropic	Derived from 293; for transient production of high titer retrovirus; this line may produce a low level (approx 100 CFU/mL) of hygromycin resistant recombinants. Grows in HXM medium + hygromycin b	***(36)***	**Rockefeller University and ATCC
Bing ATCC CRL 11554	Amphotropic	Derived from 293; for transient production of high titer retrovirus; this line may produce a low level (approx 100 CFU/mL) of hygromycin resistant recombinants. Grows in HXM medium + hygromycin b	***(36)***	**Rockefeller University and ATCC

This is not a comprehensive list of packaging cell lines.

* These may be obtained directly from American Type Culture Collection (ATCC) (Manassas, VA) or by contacting Genetix Pharmaceuticals Inc. (Cambridge, MA; www.genetixpharm.com).

**These cell lines may be obtained from the ATCC with prior written permission from the Office of the General Council, Attn: Teresa L. Solomon, Esq., The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399. Phone: 212-327-7598. FAX: 212-327-7688. Submit by mail or FAX a letter describing the experiment utilizing the lines in detail. Include the name and the ATCC number of the lines requested.

13. Access to an appropriate radiation source must be arranged with your institution or with a local collaborator. Dosing is calibrated for each source.

2.2. SVT Detection

2.2.1 Immunocytochemistry

1. Cell-culture chamber slides or 35-mm dishes.
2. Normal goat serum for blocking.
3. Anti-SVLT mouse monoclonal antibody pAB108 ***(22)***. This antibody is an IgG2a subclass that binds to an epitope in the N-terminus of SVLT that is identical in both SVLT and the alternate splice early region protein product of SV40, SV40 small t (SVst). pAB108 can recognize SVLT, SVst, and N-terminal fragments of SVLT, therefore, we have used this antibody extensively in our work. pAB108 is available commercially from PharMingen (San Diego, CA, cat. no. 14121A). The hybridoma producing pAB108 is available from the ATCC (cat. no. TIB-230). Many other anti-SVLT antibodies have been described that can be used for immunoblotting and immunocytochemistry of SVT (*see* Chapter 17).
4. Goat antimouse, alkaline phosphatase conjugated antibody.
5. Alkaline phosphatase detection kit using 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chloride, *p*-toluidine salt (NBT/BCIP) (Life Technologies, cat. no. 18280016).

2.2.2. Immunoblotting

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment and reagents.
2. 0.45-µm-pore size, polyvinylidene fluoride (PVDF) membrane (Schleicher and Schuell, Keene, NH, cat. no. 78377). We have found that this membrane works better than nitrocellulose for immunoblotting detection of SVst and N-terminal fragments of SVLT.
3. Semidry blotting apparatus or tank transfer unit.
4. Goat antimouse immunoglobulin G conjugated to horseradish peroxidase (Roche Molecular Biochemicals, Indianapolis, IN, cat. no. 60530), enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, cat. no. RPN2109), film, and film developing equipment. This has worked best in our hands, but other secondary antibodies and detection reagents exist such as alkaline-phosphatase conjugates and colorimetric detection reagents.
5. Protein Assay reagent from Bio-Rad.
   All wash and extraction buffer components are available from Sigma.

3. Methods

The SVT vector plasmid or virus producer line you need may already exist. The path of least resistance to obtaining and using retroviral vectors for SVT is to obtain, through collaboration, a vector plasmid or a packaging cell line producing a retrovirus suitable for the desired target cell type. A search of the literature will reveal investigators who have generated and used SVT retroviral

vectors on a variety of cell types (examples of some references are cited in **Subheading 1.**). Unless your goal is to create a novel vector plasmid or viral pseudotype or to express a new SVT mutant, you can save time and effort by obtaining your vector plasmid or producer lines through a collaboration.

3.1. Selecting a Vector Plasmid and Inserting SVT

If a new vector plasmid encoding SVT must be constructed, plasmids containing SV40 early region DNA and vector plasmids may be obtained collaboratively or purchased from the ATCC or other commercial sources. Examples of vector designs are presented in **Fig. 1** (*see* **Note 1**). The portion of the SV40 genome encoding SVT (*see* **Note 2**) should be cloned into the appropriate site in the vector.

Because SV40 virus, through differential splicing, encodes two proteins involved in transformation (SV40 large T and SV40 small T antigens, *see* Chapter 7), it may be important to obtain or create plasmid constructs engineered to express only large T if large T effects, independent of small T, are of interest (*see* **Note 2**). This is particularly important in large T structure-function analyses. Some examples of expression plasmids expressing large T independently of small T have been published ***(23–26)***.

Always check a retroviral vector plasmid for gross rearrangements after propagation in a bacterial host. Digest the plasmid with a restriction endonuclease that generates a limited number of plasmid fragments of distinct sizes resolvable by electrophoresis. This will provide a quick check for gross recombination events or deletions that may occur as a result of the homologous LTRs contained within each vector.

3.2. Selecting a Packaging Cell Line

Deciding which packaging cell line to use depends upon the species from which the target cells are derived (*see* **Notes 3** and **4**).

Although the practice is not universal, many investigators subject packaging lines to selection in the first passage after thawing a cryopreserved vial to ensure that no subpopulation lacking *gag-pol*, *env*, or vector genes proliferates resulting in diminished titers or virus packaging capacity. This generally involves thawing the cells into nonselective medium, changing the medium after cells have attached, e.g., the next day, to selective medium containing HXM or HXM plus hygromycin b or G418 depending upon the resistance markers present (*see* **Note 3**), and growing to confluence. Cells are then split 1 : 3 into selective medium containing half-strength selection (half of the prior dose of HXM, hygromycin b, or G418) and grown to confluence. However, no selective agents should be present in media used to prepare virus stocks because these will be toxic to target cells.

3.3. Transient Virus Production in Packaging Cell Lines Following Transfection

If stable packaging cell lines producing SVT are not already available, the quickest and easiest way to generate retrovirus from vector plasmid and packaging cells is to transfect vector plasmid into packaging cells and collect virus released transiently into the medium. Packaging lines derived from human 293 cells produce higher titers following transfection than murine NIH3T3-derived packaging cells *(2,21,27,28)*. Transient expression through transfection results in virus available for use within 2 d of initiating the transfection. This process is outlined as follows:

1. Seed packaging cells at 10–20% confluence in a 100-mm dish. This is referred to as a 1 : 5 to 1 : 10 split.
2. Transfect cells the next day with 10 µg vector plasmid using the calcium phosphate precipitation technique (*see* **Note 5**). Change the medium on the day following addition of the calcium phosphate precipitate.
3. Remove the medium 18–24 h later and filter through a 0.45-µm syringe filter, to eliminate cells from the producer line present in the supernatant. Use this "transient" virus supernatant immediately (*see* **Note 6**) to obtain a titer (*see* **Subheading 3.8.**) or to infect target cells, or freeze at –80°C. It is particularly important to harvest supernatants from 293-derived producer cells within 24 h of medium change to avoid the accumulation of a cytostatic factor released by these cells [*(2,29)*; *see* **Note 7**].

3.4. Isolating a High Titer Virus Producer Line Following Direct Transfection

Identifying stable lines producing the highest titers takes many weeks from start to finish (*see* **Notes 6** and **8**). One approach involves direct selection and screening of virus-producing clones following plasmid transfection (*see* **Note 9**). This process is outlined as follows:

1. Transfect the packaging line as aforementioned. If the vector plasmid does not contain an antibiotic resistance gene and transfected cells are to be selected for stable transfectants, 1 µg of a plasmid encoding antibiotic resistance, e.g., pSV*neo* or pRSV*neo*, should be included with the 10 µg of vector plasmid.
2. Split the transfected culture 1 : 10 to 1 : 20, 2–3 d after the transfection into selective medium containing 400 µg/mL G418.
3. Replace the selective medium twice a week (every 3–4 d).
4. Once colonies appear (7–14 d) and are large enough to be macroscopically visible from the underside of the plate, they should be transferred to 24-well plates (one colony per well) for expansion in selective medium (*see* **Note 11**). Pick 24 colonies and pool the remaining colonies from the dish into a fresh 100-mm dish in selective medium. Occasionally, pooled colonies will produce a sufficiently high titer.

5. When the cultures within the wells are 50–90% confluent (they may reach this stage at different times), replace selective medium with medium lacking G418 and collect 18–24 h later to determine virus titer (*see* **Subheading 3.8.**).
6. Refeed producer cell clones or cryopreserve (*see* **Note 12**).

3.5. Isolating a High Titer Virus-Producing Line Following Cross Infection

The second approach to obtaining a stable line producing a high retrovirus titer involves the use of virus produced following transfection to cross infect a susceptible packaging cell line. This second line is then selected and screened for high titer virus production. In this way, the second line will have several integrated genomes, hence, it will produce a much higher titer. This process is outlined as follows:

1. Generate a "transient" virus supernatant as aforementioned. Use a packaging line that generates virus capable of infecting the target packaging line to be used. Normally, retroviruses cannot reinfect cells that are expressing the *env* protein contained within that virus because the receptor on the cell is blocked by the env proteins produced (*see* **Note 13**), therefore, this secondary packaging line must have a different envelope protein.
2. On the day before the "transient" virus supernatant is to be collected, i.e., the day after the transfection, seed the secondary packaging line at a 1 : 10 to 1 : 20 split in 100-mm dishes.
3. Filter the "transient" virus supernatant (*see* **Note 6**) and add polybrene (8 µg/mL final concentration). Add 1 mL to a 100-mm dish containing 3 mL fresh producer cell medium also supplemented with polybrene at 8 µg/mL.
4. Incubate 1–3 h, then add an additional 6 mL of medium lacking polybrene. Incubate an additional 2–3 d.
5. Split each 100 mm dish 1 : 10 into 2 100-mm dishes containing selective medium. Feed twice a week until colonies appear (7–14 d).
6. Once colonies are large enough to be macroscopically visible from the underside of the plate, they should be transferred to 24-well plates (one colony per well) for expansion in selective medium (*see* **Note 11**). Pick 24 colonies and pool the remaining colonies from the dish into a fresh 100-mm dish in selective medium. Often even the pooled colonies will produce a sufficiently high titer when generated using cross infection as described here.
7. When the cultures within the wells are 50–90% confluent (they may reach this stage at different times), replace selective medium with medium lacking G418 and collect 18–24 h later to determine virus titer (*see* **Subheading 3.8.**).
8. Refeed producer cell clones or cryopreserve (*see* **Note 12**).

3.6. Vesicular Stomatitis Virus G Protein (VSV-G) Pseudotyped Retrovirus

VSV-G pseudotyped viruses have a very broad host range (*see* **Note 4**) and may be concentrated by centrifugation, unlike conventional retroviruses, which fuse and lose titer when pelleted. Such viruses may be generated by cotransfecting (using calcium phosphate) equal amounts of a VSV-G expression vector (3–5 µg), e.g., pHCMV-VSV-G, expressing the envelope protein of VSV (21), and the retroviral vector plasmid (3–5 µg) into packaging cells lacking *env* gene expression, e.g., line Anjou65. Such producer lines may also be prepared after stable cross infection with retroviral vector. Because of their lack of envelope protein, these cultures will not produce infectious virus, until transfected with VSV-G expression plasmid. VSV-G expression must be transient because constitutive expression is cytotoxic. Collect virus supernatants 48–72 h after transfection and concentrate immediately or freeze ***(2)***.

1. To concentrate VSV-G pseudotyped virus supernatant, centrifuge for 90 min at 50,000*g* at 4°C.
2. Resuspend the pellet overnight in 1/200 the original volume in 0.1% HBSS at 4°C.
3. Use immediately for infection, freeze at –80°C, or concentrate further through another round of centrifugation (*see* **Note 6**).

3.7. Collection of Virus Supernatants

Packaging cell lines in general should be maintained as dense cultures (split 1:5 to 1:10 once or twice a week). They may be subjected to rounds of selection as described in **Subheading 2.** following recovery from cryopreservation. Virus supernatants should be collected from packaging cell lines when cultures are at 70–90% confluence. Fresh medium should be added to packaging cells and collected 18–24 h later (*see* **Note 6**). Filter through a 0.45-µm filter to remove floating cells and debris.

3.8. Titering Retroviral Stocks on NIH-3T3 Cells

Virus titers (using NIH3T3 as target cells) produced transiently following transfection of NIH-3T3–derived packaging cells will be relatively low (10^3–10^4 colony-forming units, or CFU/mL). Cloned producer lines may produce 100-fold higher titers. Transient titers from 293-derived packaging cells are usually three orders of magnitude higher (10^6–10^7 CFU/mL) than NIH-3T3–derived transient virus titers. VSV-G pseudotyped virus, following con-

centration, should have titers of about 10^9 CFU/mL. Titers are lower for vectors with large cDNA inserts (>4 kb).

1. Change the medium on virus-producing cultures when cells are 50–90% confluent with medium lacking selective agents, and collect 18–24 h later (*see* **Note 6**). The same day, seed 3 60-mm dishes of NIH-3T3 cells at 1:10–1:20 split for each viral stock to be titered.
2. The following day, remove the virus supernatant to be titered and filter through a 0.45-µm syringe filter. Prepare medium for NIH-3T3 containing 8 µg/mL polybrene. Prepare serial dilutions of virus supernatant in this medium so that there are three tubes containing 100 µL, 1 µL, and 0.01 µL virus supernatant per 2 mL. Place each 2 mL dilution on a 60-mm dish of NIH-3T3 cells after removing the growth medium.
3. After 1–3 h, add 4 mL NIH-3T3 medium (without selective agents) to dilute the polybrene. Incubate 2–3 d or approx three population doublings.
4. Split each 60-mm dish 1:10 into 1 100-mm dish (a quarter of a 60-mm into a 100-mm dish) containing selective medium (400 µg/mL G418 for NIH-3T3, *see* **Note 10**). Feed twice each week until colonies can be counted (7–10 d). Alternatively, 60-mm dishes may be used to select G418 resistant colonies, but colony counts are easier using 100-mm dishes.
5. Colonies can be counted directly on the plate without staining, although smaller colonies may be missed unless plates are stained with 0.05% methylene blue following fixation with 3.7% formaldehyde in PBS.
6. Titer is calculated as follows:

$$\text{G418 resistant CFU/mL} = \frac{\text{\# colonies per 100-mm dish}}{\begin{array}{c}\text{virus stock volume used (mL)} \times 8\ (2^{3(\text{population doublings})}) \\ \times\ (\text{fraction of infected cells plated})\end{array}}$$

3.9. Prior to Transduction of Target Cells

Before infection of target cells with the vector the following properties of the target cells must be determined:

1. Although we have seen no specific examples of cells that are sensitive to polybrene, lack of polybrene toxicity should be confirmed by incubating growing cells with 8 µg/mL polybrene for 1–3 h and then for up to 2–3 d in three–fourfold diluted polybrene. If toxicity is indicated by cytolysis or by diminished proliferation relative to untreated cultures, then try removing polybrene supplemented virus supernatant completely after 1–3 h (virus adsorption is mostly complete by then) and replacing with standard medium. If that fails, try lowering the polybrene to 4 µg/mL, then 2 µg/mL.
2. Sensitivity to G418 must be determined in those cases where neomycin resistance will be utilized as a selectable marker. A good starting dose is 400 µg/mL and should be used for NIH-3T3 cells and for most producer cell lines, but some

target cell cultures will be refractory to this concentration, e.g., some nonadherent cell types, whereas others are hypersentive. Set up a dose curve when first selecting virus-infected cells to determine the optimal G418 level to use. Split the cells following infection and feed the next day with medium containing 25, 50, 100, 200, 400, 800, and 1600 µg/mL G418. In general, if cell growth slows during the first 3–4 d of selection and then major cell death occurs after the second feeding with selective medium, then the G418 concentration is about right. The optimal G418 dose will yield the best resolved and the largest number of colonies for a given titer of virus.

3. Do the target cells tolerate packaging cell medium (see the transduction protocols for the level and duration of exposure they must tolerate)? If they do not proliferate well under these conditions, virus supernatant must be collected in target cell medium. This should not be a problem because packaging cells will grow and produce virus in most media.

3.10. Transduction of Adherent Cells by Incubation with Virus Supernatant

1. Plate target cells at approx 10% confluence.
2. Add polybrene to filtered virus supernatant (8 µg/mL final concentration). Add 1 mL to 100-mm dish containing 3 mL fresh target cell medium also supplemented with polybrene at 8 µg/mL.
3. Incubate 1–3 h and add an additional 6 mL of medium lacking polybrene. Replace medium with fresh medium lacking polybrene for polybrene sensitive cells (*see* **Subheading 3.9., step 1**).
4. **Steps 2** and **3** may be repeated to increase the time of exposure to virus and potentially increase the proportion of infected cells.
5. After changing the medium, incubate for an additional number of days sufficient for 2–3 population doublings. This will vary depending upon the growth rate of the target cells. SVT expression may be determined directly at this stage by immunoblotting or by immunodetection *in situ* (*see* **Subheading 3.15.**).
6. If transduced cells are to be selected using antibiotic resistance, split each 100-mm dish 1 : 10 into 100-mm dishes containing selective medium. Feed twice each week until colonies can be seen.
7. Positive selection for cells transformed by SVT is also possible using focus formation or growth in semisolid media (*see* **Note 14**).

3.11. Transduction of Adherent Cells by Spin Infection to Increase the Virus Concentration at the Monolayer Surface

1. Target cells must be seeded at 10^5 cells per well into six-well trays 12–18 h prior to infection. Covered microplate carriers for a centrifuge will be needed.
2. Combine retroviral supernatant and target cell medium to a total of 4 mL infection cocktail. Add polybrene to a final concentration of 8 µg/mL. Be sure the retroviral supernatant is less than or equal to one-half of the total volume (*see* **Note 7**).

3. Replace the medium in the wells to be infected with the 4 mL infection cocktail. The large volume will prevent dehydration of the cells during centrifugation.
4. Centrifuge the trays for 1.5–2 h at 1000*g* at room temperature.
5. Return trays to incubator. Replace the medium the next day and incubate for an additional number of days sufficient for two to three population doublings. This will vary depending upon the growth rate of the target cells.
6. SVT expression may be determined directly at this stage by Western blotting or by immunodetection *in situ* (*see* **Subheading 3.15.**).
7. If transduced cells are to be selected using antibiotic resistance, split each well 1:10 into a 100-mm dish (one-half well per 100-mm dish) containing selective medium. Feed twice each week until colonies can be seen.
8. Positive selection for cells transformed by SVT is also possible using focus formation or growth in semisolid media (*see* **Note 14**).

3.12. Transduction of Nonadherent Cells by Suspension in Virus Supernatant

1. Combine retroviral supernatant and fresh target cell medium to a total of 4 mL infection cocktail. Add polybrene to a final concentration of 8 μg/mL. Be sure the retroviral supernatant is less than or equal to one-half of the total volume (*see* **Note 7**).
2. Pellet 4×10^5–4×10^6 target cells at 500*g* for 5 min.
3. Suspend in 4 mL infection cocktail, add to 60-mm dish, and incubate for 24 h.
4. **Steps 1–3** may be repeated to increase the time of exposure to virus and potentially increase the proportion of infected cells.
5. Pellet target cells, resuspend in fresh target cell medium and incubate an additional 24–48 h.
6. Assay for SVT expression and phenotype or add antibiotic for selection of infected cells.

3.13. Transduction of Nonadherent Cells by Spin Infection

1. For each well to be used, combine retroviral supernatant and target cell medium to a total of 3 mL infection cocktail. Add polybrene to a final concentration of 8 μg/mL. Be sure the retroviral supernatant is less than or equal to one-half of the total volume (*see* **Note 7**).
2. Add 1–2 × 10^6 target cells to each well of a six-well tray and centrifuge tray for 5 min at 500*g* (the cell number should be sufficient to completely cover the bottom of the well, forming a monolayer following centrifugation).
3. Replace target cell medium with infection cocktail prepared in **step 1**.
4. Centrifuge for 1.5–2 h at 1000*g* at room temperature.
5. Immediately after centrifugation, or within 6 h, transfer cells in the infection cocktail to a 100-mm dish and dilute to 10 mL with target cell growth medium. This prevents the medium in the dense cultures in the wells from getting too acidic. Incubate 2 d.

6. Assay for SVT expression and phenotype or add antibiotic for selection of infected target cells.

3.14. Transduction of Nonadherent Cells by Cocultivating with Virus-Producing Cells

Coculture of target cells and virus-producing cells effectively increases the time of target cell exposure to retroviral vector and, like spin infection, increases the concentration of retrovirus at the target cell surface. Prior to coculture, virus-producing cells must be rendered permanently nonproliferative by lethal irradiation. This is accomplished by exposing virus producing cells to irradiation (*see* **Note 15**), 24 h following transfection if transient virus production is used, or at least 24 h following plating of a producer line if stable producers are used. About 1500 rad should be used for 293-derived packaging lines and about 2800 rad should be used for NIH-3T3–derived virus producers. Alternatively, virus-producing cells can be exposed to 10 µg/mL mitomycin C for 3 h, followed by several rinses to ensure removal of mitomycin C. Irradiation and mitomycin C treatments result in viable producer cells that cannot proliferate, but that do continue to release virus for several days.

1. Combine retroviral supernatant and target cell medium to a total of 3 mL infection cocktail. Add polybrene to a final concentration of 8 µg/mL. Be sure the retroviral supernatant is less than or equal to one-half of the total volume (*see* **Note 7**).
2. Pellet 4×10^5–4×10^6 target cells at 500*g* for 5 min.
3. Suspend in 3 mL infection cocktail, add to 60-mm dish containing lethally irradiated or mitomycin C treated producer cells, and incubate for 24 h.
4. Remove medium and any floating target cells. Leave small amount of residual medium in plate so producers and residual target cells do not dry out.
5. Pellet target cells and suspend in additional infection cocktail as described in **refs. *1–3***. Add back to 60-mm dish containing lethally irradiated producer cells, taking care not to dislodge producer cells, and incubate for another 24 h.
6. Remove medium and as many target cells as possible from the dish, taking care not to suspend producer cells, then pellet, and suspend in target cell medium. Incubate for 24 h in the absence of producer cells.
7. Assay for SVT expression and phenotype or add antibiotic for selection of infected target cells.

3.15. Detection of SVT

The proportion of cells infected with an SVT vector may be determined using immunofluorescent or immunocytochemical detection of SVT. This is particularly necessary when using vectors lacking a selectable antibiotic resistance marker. Increasing the time of exposure to fresh virus supernatant may

increase the proportion of T-antigen expressing cells. Confirmation of SVT expression and appropriate size of the product may be confirmed by immunoblotting.

3.15.1. Immunocytochemistry

1. Plate cells at subconfluency into 35-mm-diameter wells or into wells of a chamber microscope slide.
2. Grow overnight, fix for 10 min in 4% paraformaldehyde in PBS, and then permeabilized in 90% ethanol for 10 min.
3. Rinse cells three times in PBS and block 20 min in 10% normal goat serum (NGS) in PBS.
4. Alternatively, TS (50 m*M* Tris-HCl, pH 7.6, 150 m*M* sodium chloride) can be used instead of PBS and permeabilize with 100% methanol chilled to –20°C which is a variation of the fixation method used in ***(30)***.
5. Apply primary antibody pAB108 ***(22)***, 1:5 in 10% NGS in PBS for 1 h at room temperature.
6. Rinse three times in PBS, and apply a second antibody (goat antimouse alkaline phosphatase conjugate; 1:250 in 10% NGS in PBS) for 1 h at room temperature.
7. Rinse three times with PBS, and detect using 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chloride, *p*-toluidine salt, (NBT/BCIP) as instructed by the manufacturer (Life Technologies).

3.15.2. SDS-PAGE and Immunoblotting

1. Rinse target cells in wash buffer [137 m*M* NaCl, 20 m*M* Tris-HCl (pH 8.0), 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$].
3. Extract in wash buffer plus 1% Nonidet P-40 (NP40), 10% glycerol, 2 m*M* phenylmethylsulfonylfluoride, 20 µg of aprotinin per mL, and 1 µg of leupeptin per mL, by rocking at 4°C for 20 min.
4. Clarify extracts by centrifugation (full speed in a microcentrifuge, approx 12,000*g*, 10 min, 4°C) to remove insoluble material. Place supernatant in a fresh tube.
5. Determine the total soluble protein concentration of extracts for immunoblotting analysis using Bio-Rad protein assay reagent according to the manufacturer's instructions. Use bovine serum albumin as the standard.
6. Store extracts at –70°C until used.
7. Resolve proteins by SDS-PAGE using prestained protein standards (Life Technologies) as molecular weight markers, and transfer onto Immobilon-P (0.45-µm pore size, polyvinylidene fluoride membrane; Millipore), using a tank electrotransfer unit (25 m*M* Tris-HCl, pH 8.0, 200 m*M* glycine, 20% methanol transfer buffer) or by semidry blotting (12.5 m*M* Tris-HCl, pH 8.0, 100 m*M* glycine, 10% methanol transfer buffer).
8. Block nonspecific binding on the membrane by soaking in 1% gelatin (enzyme immunoassay grade; Bio-Rad) in Tris-buffered saline (10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl) for 1 h at room temperature.

9. Probe overnight with mouse monoclonal anti-SVLT pAB108 [hybridoma conditioned medium, diluted 1 : 100 in Tris-buffered saline plus 0.05% Tween-20, ***(22)***].
10. Rinse 3×, 5 min in TBST.
11. Apply the secondary antibody, goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Boehringer Mannheim) or alkaline phosphatase (Life Technologies, diluted in TBST) for 1 h at room temperature and detect by using enhanced chemiluminescence (Dupont) or NBT/BCIP (Life Technologies), respectively, according to the manufacturer's instructions.

4. Notes

1. All vector plasmids are propagated in *Escherichia coli*, usually under ampicillin selection. The LXSN or the LXIN vectors (**Fig. 1**) represent the types of vectors that will most likely be of use in studies requiring expression of SVT. Both of these vectors would express SVT off of the 5 LTR and both express neomycin resistance in eukaryotic cells for the independent selection of infected target cells. A splicing vector lacking a eukaryotic antibiotic resistance gene, e.g., the MFG vector, could also be used in those situations where independent antibiotic selection is not necessary. The smaller vector size and the lack of an internal promoter can result in higher titers. Disadvantages of vectors lacking antibiotic selection include the inability to ensure 100% infection of target cells and the difficulty of creating stable packaging cell lines. The maximum transcript size, which can be packaged efficiently as a retrovirus, is approx 10,000 bases. Selfinactivating vectors are only necessary when a gene with its own promoter is used and promoter interference from the LTRs must be eliminated. The strong CMV promoter in LXCN may produce toxic levels of SVT.
2. When constructing your own vector expressing SVT, here are a few points to consider. All coding sequences must contain translational start and stop codons. Avoid 5 SV40 derived untranslated sequences. This is also true in theory for 3 SV40-derived untranslated sequences and for SV40 splice donor and acceptor sequences. However, many published SVT vectors have functioned well with 3 untranslated sequences present. For example, introducing transcriptional stop and polyadenylation signals, or splice donor and acceptor sequences from SV40 into the vector should, in theory, prevent or decrease generation of genomic vector transcript formation. In fact, many functional vectors retain these sequences for cloning convenience, if for no other reason, and they have generated adequate virus titers and expressed adequate levels of SVT. When first testing a new construct in packaging cells, prepare virus using the unmodified vector in parallel. If the new construct gives 10–100-fold lower the titer than the vector containing no inserted sequences, then modifications should be considered.
3. Packaging cell lines express the *gag-pol* and *env* genes that encode the structural proteins for assembly of infectious virus. The safest packaging cell lines, i.e., least likely to produce replication competent virus through recombination with vector plasmid, encode these genes off of separate plasmid expression vectors stably incorpo-

rated into the genome of the cell line. Some examples are summarized in **Table 1**. Note that some packaging lines will grow under HXM selection and some are also hygromycin b resistant (**Table 1**). The varying drug selection of these lines depends upon how the cell lines were engineered to express the *gag-pol* and *env* genes.

4. Packaging cells express the envelope (*env*) protein that is ultimately incorporated into the infectious virus. It is the *env* protein that determines the host range of the virus. The ecotropic viral *env* glycoprotein, such as that expressed in GP+E86 and Bosc23 cells, interacts only with receptors on mouse and rat cells. The amphotropic *env* glycoprotein, expressed in GP+envAM12 and Bing cells for example, confers a broader mammalian host range, including rodent, rat, chicken, dog, cat, mink, and human. Packaging cells that express the 10A1 envelope protein, such as PT67, produce virus that can utilize two receptors on human cells, the amphoteric and the gibbon ape leukemia virus (GALV) receptor. Some cell types that are refractory to virus containing only the AM12 envelope may be more efficiently infected with virus containing the 10A1 envelope ***(2)***. Viruses with the VSV-G protein in their surface (VSV-G pseudotyped) have a very broad host range, infecting nonmammalian, as well as mammalian cells ***(21)***. They also can be concentrated by centrifugation whereas viruses expressing retroviral *env* protein have a limited capacity to be concentrated.
5. The optimal transfection protocol will vary between packaging lines. Critical variables include the time the cells are in contact with the calcium phosphate-DNA precipitate prior to changing the medium (usually 4 h to overnight) and whether or not to "shock" the cells with glycerol or DMSO after removing the calcium phosphate-DNA precipitate. Additional variables include chloroquine treatment of 293 derived packaging cells during transfection and/or sodium butyrate treatment following transfection ***(2)***. Most of these treatments can reportedly increase titers up to two–three-fold, but this experience is not universal. Tips for specific cell lines are often available from the manufacturers of transfection reagents.
6. Virus supernatants have the highest titer immediately upon collection. Retrovirus degrades rapidly (half-life is approx 4 h) following release from producer cells into tissue culture medium. Therefore, filter virus supernatants and prepare dilutions as close to the time of use as possible. They may be frozen at –80°C for later use with only modest loss of titer. Use polypropylene tubes for freezing (less likely to break) and do not freeze/thaw.
7. It is particularly important that less than half of the total infection cocktail volume be virus supernatant if 293-derived packaging cells are used. These cells reportedly ***(2,29)*** secrete cytostatic substances into the medium that must be diluted out.
8. The advantage of generating a stable virus producing line is the ability to screen for high virus titer production and the ability to generate large volumes of virus supernatant without having to scale up plasmid transfection. Scaled-up plasmid transfections are much less reliable than a good virus producing line.
9. Plasmid transfection reportedly results in higher frequency of vector rearrangement than secondary virus infection (*see* below), but is the only available option

if the investigator wishes to avoid working at any step with amphotropic virus. It is also available for generating amphotropic producers, but because of the rearrangement issue secondary infection is recommended.

10. The G418 concentrations recommended refer to active concentration and G418 peparations are not 100% active. Check the label to determine the active amount of G418 per mg dry weight. This is often 400–500 µg/mg. Prepare G418 solutions at 40 mg active G418 per mL.
11. We have used 8-mm glass cloning cylinders for this purpose. These are prepared for use by autoclaving in a glass Petri dish with an open-end embedded in a thin film of silicone grease. After removing the medium from the dish, the rings are then placed over individual colonies using sterile forceps with the greased end forming a seal around the colony. The cells are then trypsinized and transferred to wells in a 24-well tissue culture trays. Each well contains 1 mL selective medium.
12. Freeze vials of prospective virus producer clones at $1–5 \times 10^6$ cells per vial in 1 mL medium containing 10% serum and 10% DMSO. Vials should be frozen (–80°C) overnight prior to long-term storage in liquid nitrogen. High-titer clones should be expanded and >20 vials frozen at low passage. A producer cell line can be maintained for up to 6–8 wk at which point a fresh vial should be thawed.
13. Using the cell lines in **Table 1** as an example, virus produced by GP+E86 can only infect GP+envAM12 because the cells are murine and do not express the E86 envelope protein. Virus produced by GP+envAM12 cannot infect Bing, which expresses AM12, but can infect GP+E86 and any of the other 293-derived cells.
14. Infected cells expressing SVT may be selected directly for anchorage independent growth in semisolid media or for monolayer overgrowth by focus formation (*see* Chapter 11). Although SV40 transformed cells do display these phenotypes, the degree of foci formation or growth in agar depends upon the level of SVT expression. For this reason, only a subset of vector-infected cells may be detected using this approach.
15. It is advisable to confirm the best radiation dose with the radiation source available. After irradiation of a test culture of producer cells, confirm that the cells are nonproliferative by plating at low density and assaying for colony growth. Use a nonirradiated culture as a control. In addition, confirm virus production by irradiated cells using NIH 3T3 as the target cells.

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11

Neoplastic Transformation Assays

Leda Raptis and Adina Vultur

1. Introduction

Although a tumor cell can be distinguished from its normal counterpart by a wide range of phenotypic alterations, only a few of these properties provide useful indicators of malignant transformation. These include changes in cellular morphology, decreased dependence on serum growth factors, loss of density-dependent growth inhibition, reduction of gap-junctional intercellular communication (GJIC), the ability to proliferate in the absence of anchorage to a solid support and the ability to form tumors when injected into the appropriate animal host. These components of the SV40-transformed phenotype, shared with phenotypes of cells transformed by many other oncogenes, constitute the basis for the majority of currently used assays. In certain systems, such as polyoma virus-transformed rat F111 cells, a hierarchy of transformation properties was observed, with morphological changes, focus formation, loss of GJIC, anchorage-independent growth, and tumorigenicity requiring progressively higher levels of oncogene expression ***(1,2)***, although this is not the case in all systems ***(3,4)***.

A variety of cell types have been used to test the transforming potential of the Simian virus 40 large (SVLT) and small (SVst) tumor antigens and their mutants. The most commonly used ones are mouse NIH3T3 and Rat-1 or Rat F111 fibroblasts ***(1,3,5)***. Because these established lines are considered to be preneoplastic, rather than fully normal, they provide very sensitive recipients for transformation assays. For the expression of these genes, a variety of techniques may be used, such as calcium phosphate precipitation, retroviral infection, lipofection, or electroporation. The basic transfection techniques and expression of SVLT proteins through the use of retroviral vectors are described

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in Chapter 10. Once stable populations have been established, either pools of drug-selected cells or individual clones expressing the transfected gene can be used and their phenotype in relation to transformation examined using the biological assays described below.

1.2. In Vitro and In Vivo Assays for Cellular Transformation

1. Morphological Changes—Focus Formation: SVLT expression into cells such as the NIH3T3 or Rat-1 fibroblasts results in morphological changes and loss of density-dependent growth regulation. These alterations result in the appearance of clusters of morphologically transformed cells, that is cells that are rounded or spindle-shaped, appear highly refractile and grow in a disorganized pattern when viewed under phase-contrast microscopy. Such cells are readily detectable against a background of a confluent monolayer of normal cells (**Fig. 1A**, *see* **Note 1**).
2. Accelerated Growth Rate: This can be measured by assaying the increase in cell numbers during the exponential growth of subconfluent cell cultures. This assay can be combined with the saturation-density assay for evaluating the loss of density-dependent growth inhibition and with the serum growth factor dependence assays described below.
3. Elevated Saturation Densities: The normal, established rodent lines used for transformation assays are subject to density-dependent growth inhibition so that they cease to proliferate when confluent. Transformed cells, on the other hand, continue to proliferate even after they reach confluence, thereby achieving higher cell densities at saturation.
4. Reduced Serum Growth Factor Requirements: Compared to normal cells, the proliferation of transformed cells is generally less dependent on serum growth factors. Mouse NIH3T3, C3H10T½, or Rat F111 fibroblasts, for example, are usually maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% calf serum. For these cells, growth in low levels of serum (0.5–2% calf serum) results in a significantly reduced growth rate, whereas after SVLT expression they are able to proliferate under these restrictive conditions.
5. Reduced GJIC: One of the targets of a variety of signals stemming from growth factors or oncogenes may be the gap junctions, that is membrane channels that serve as conduits for the passage of small molecules between the interiors of cells ***(6)***. The investigation of junctional permeability, which is invariably reduced after oncogene expression, is usually conducted through introduction of the fluorescent dye, Lucifer yellow, followed by observation of its migration into neighboring cells. This can be most easily conducted by *in situ* electroporation on a partly conductive slide ***(2)***; cells are grown on a glass slide, half of which is coated with electrically conductive, optically transparent, indium-tin oxide. An electric pulse is applied in the presence of Lucifer yellow, causing its penetration into the cells growing on the conductive part of the slide, and the migration of the dye to the nonelectroporated cells growing on the nonconductive area is microscopically observed under fluorescence illumination (**Fig. 2**). Interruption of

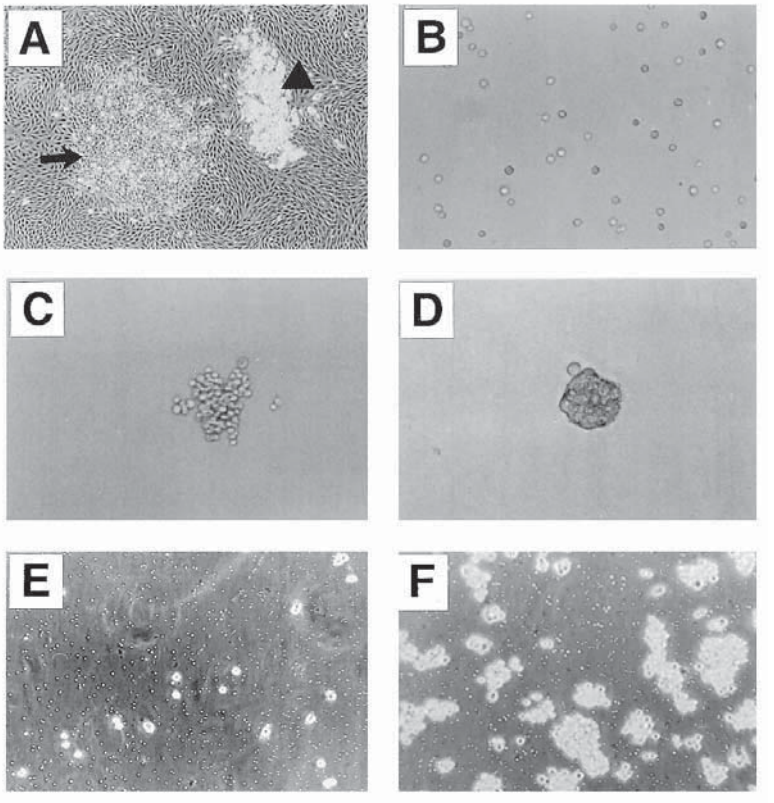

Fig. 1. **(A)** Focus formation on rat F111 cells. Rat F111 cells transformed by SVLT (arrow) or the polyoma virus middle tumor antigen (arrowhead) were plated together with an excess of the parental line. 14 d later, photographs were taken under phase contrast illumination using a ×10 objective. Note the irregularly shaped, refractile appearance and growth to many layers, compared to the background F111 cells. **(B–D)** Growth in soft agar. Rat F111 cells **(B)** or their SVLT-transformed derivatives **(C)** were analyzed for their ability to grow while suspended in agar-containing medium. In **(D)**, SVLT-transformed, mouse NIH3T3 fibroblasts were treated in a similar manner. Photographs were taken 10 d later under brightfield illumination using a ×10 objective. Note the difference in morphology between the SVLT-transformed F111 and NIH3T3 colonies (**C** and **D**, respectively). **(E** and **F)** Growth on polyHEMA-coated Petri dishes. Three-centimeter plates were coated with a 1:60 dilution of a 3% polyHEMA solution. Rat F111 cells, before **(E)** or after **(F)** transformation by SVLT were seeded on the coated plates in DMEM containing 10% calf serum. Cells were allowed to grow and photographs taken 6 d later under phase contrast illumination and a ×10 objective.

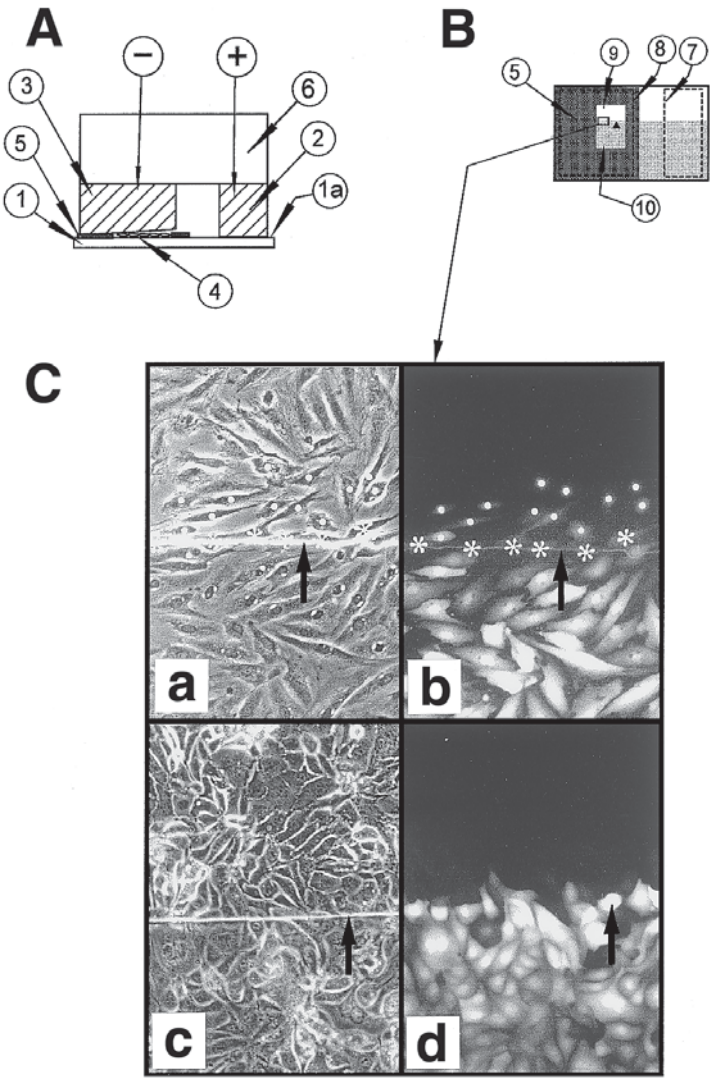

Fig. 2. Determination of gap-junctional, intercellular communication. **(A)** Side view. A coating of indium-tin oxide [1A] on the upper surface of the glass slide [1] makes a conductive path between the positive contact bar [2] and the base of the negative electrode [3], through the cells [4] and the electroporation buffer containing 2 mg/mL Lucifer yellow. An insulating teflon frame [5] defines an area of cell growth and supports the negative electrode [3]. An insulating carrier [6] holds the electrodes together. "+" and "–", denote connections to the positive and negative poles of the pulse source, respectively. **(B)** Top view: The outline of the positive [7] and negative [8] electrode and their relative position on the conductive glass slide in relation to the window where

GJIC has been shown to require lower levels of oncogene expression than anchorage-independent proliferation in a number of systems ***(2,7)***.

6. Anchorage-Independent Growth: One of the best in vitro indicators of malignant growth potential is the ability of cells to grow in the absence of anchorage. Most normal cells require adherence to a solid substratum to proliferate, whereas SVLT-transformed ones can grow while suspended in liquid or semisolid media, such as agar ***(8)***, or methyl cellulose (methocel) ***(9)***. Growth in agar is the most common assay for anchorage independence, although recovery of the cells from the agar for biochemical analysis is difficult. Growth in methocel, on the other hand, permits efficient recovery of the cells because, contrary to agar, this material is water-soluble. Alternatively, cells can be grown on a poly(2-hydroxyethyl methacrylate) (polyHEMA)-coated surface ***(10)*** in which case the degree of cell adhesion can be controled while the cells are amenable to manipulations such as treatment with antisense oligonucleotides ***(11)*** or microinjection, as well as protein extraction.
7. Tumorigenicity: Although the aforementioned assays provide some of the best in vitro approaches currently available to identify growth properties deregulated as a consequence of expression of a particular oncogene, no one parameter, or com-

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Fig. 2. *(continued)* the cells are grown [9, 10] are indicated. The lightly shaded area represents the conductive coating. The etched [9] and conductive [10] parts of the slide are shown relative to the position of the electrodes and the insulating-tape window where the cells are grown. Note that cells growing on the conductive part of the slide [10] will be loaded with the dye by electroporation, while cells growing on the nonconductive surface [9] would not receive any pulse, therefore they would not be permeated ***(16)***. Arrowhead on the conductive side points to the transition line between conductive and nonconductive areas. **(C)** GJIC Quantitation. Rat F111 fibroblasts (a and b), or their SVLT-expressing counterparts (c and d) were grown on partly conductive slides and electroporated (0.1 μF, 30 V, six pulses) in the presence of 5 mg/mL Lucifer yellow. After washing, cells from the same field were photographed using a ×20 objective under phase contrast (**a** and **c**) or fluorescence (**b** and **d**) illumination. The bottom part of both slides is conductive. Electroporated cells growing at the border with the nonconductive zone (**b**, white stars), and fluorescing cells growing on the nonconductive side of the slide, into which the dye had transfered through gap junctions (**b**, white circles) were identified by superimposing the fluorescence and phase contrast pictures. In order to quantitate intercellular communication, the number of cells into which the dye has transfered, per electroporated border cell can be calculated by dividing the total number of fluorescing cells on the nonconductive side (circles) by the number of cells growing at the border with the conductive coating (stars). Arrows on the conductive side point to the transition line between conductive and nonconductive areas. Note the extensive transfer of Lucifer yellow through gap junctions in **b** (rat F111 cells), which is almost absent once the cells are transformed by SVLT **(d)**.

bination of parameters, is an infallible predictor of malignant growth potential. Definitive assessment of the malignant nature of a cell is provided by the use of experimental animal models for tumor formation.

2. Materials

2.1. Determination of Growth Rate, Saturation Density, and Serum Dependence

1. DMEM, supplemented with 10% or 1% calf serum and antibiotics.
2. Trypsin (Life Technologies Inc., Grand Island, NY, cat. no. 15400-054).
3. Humidified, 37°C, 10% CO_2 incubator.
4. Laminar-flow hood to maintain sterility.
5. Hemocytometer or Coulter counter for counting cells.

2.2. Determination of GJIC—Electroporation on a Partly Conductive Slide

1. *In situ* electroporation apparatus (Ask Science Products, Kingston, Ont., Canada).
2. Lucifer yellow (Sigma, cat. no. L-0259). Make a solution of 5 mg/mL in calcium-free DMEM.

2.3. Determination of Anchorage-Independent Growth

2.3.1. Growth of Cells in Soft Agar

1. Agar solution: 1.6% and 0.7% (w/v) agar in distilled water (50 mL each). "Bacto-agar" (Difco, Detroit, MI, cat. no. 0140-01-0), "Noble-agar" (Difco, cat. no. 0142-01-8), or agarose (electrophoresis grade, ICN, Aurora, OH, cat. no. 820723) can be used (*see* **Note 2**). Autoclave, cap tightly to prevent evaporation, and store at 4°C.
2. DMEM, 5X concentrated. 1X DMEM used in cell culture requires 3.7 g/L $NaHCO_3$ to adjust the pH to approx 7.2. However, at a concentration of 5X, DMEM must be prepared without $NaHCO_3$, because at this pH, several of its ingredients may precipitate.
3. 25X $NaHCO_3$ solution: Dissolve 92.5 g/L of glass distilled water. Autoclave and store at room temperature.
4. Antibiotic and antimycotic solutions: gentamycin sulphate (Sigma, St. Louis, MO, cat. no. G3632), Nystatin (LTI, cat. no. 15340-052).
5. Autoclaved distilled water suitable for tissue culture.
6. Calf serum (ICN, Costa Mesa, CA).
7. 1M HEPES. Weigh 23.8 g HEPES (Sigma) and dissolve in 100 mL water appropriate for tissue culture. Autoclave and store at room temperature.
8. Cells growing in 3 or 6 cm plates (*see* **Note 3**). As controls, normal cells as well as cells expressing a highly transforming oncogene, such as *Src* or the polyoma virus middle tumor antigen, must also be included.
9. Trypsin solution (LTI).
10. 1X DMEM pH 7.2, containing serum (*see* **Note 4**).

2.3.2. Growth of Cells in Methylcellulose

1. Agar: 1.6% (w/v) in distilled water. Autoclave, cap tightly to prevent evaporation, and store at 4°C.
2. 5X concentrated DMEM, 25X $NaHCO_3$ solution, antibiotics, 1 M HEPES, trypsin, serum, and cells, as in **Subheading 2.3.1, steps 2–10.**
3. Methyl cellulose: Dissolve 1.2 g of methyl cellulose (methocel, Sigma, cat. no. M-0512) in 50 mL of glass distilled water, suitable for tissue culture. Shake vigorously at 37°C overnight, then autoclave. Autoclaving makes the preparation look "curdly," but upon subsequent cooling to 4°C, it becomes homogeneously viscous.

2.3.3. Growth of Cells on PolyHEMA-Coated Surfaces

1. polyHEMA: It can be purchased from Aldrich (cat. no. 19,206-6). Prepare a 3% solution in 95% ethanol by rocking overnight at 37°C. Centrifuge at 10,000*g* to remove any undissolved particles.
2. Tissue-culture media and solutions as above.

2.4. Tumorigenicity Assays

1. 1X DMEM, serum-free.
2. 1X DMEM with 10% calf serum.
3. Trypsin solution.
4. 6–8 wk old, athymic nude (*nu*/*nu*) mice.

3. Methods

3.1. Determination of Growth Rate, Saturation Density, and Serum Dependence

1. Trypsinize cells from subconfluent cultures.
2. Seed at 1×10^5 cells per 6 cm dish in growth medium supplemented with the desired amount of serum (either 10% or 1% calf serum). Prepare triplicate petris for each measurement.
3. Incubate the cells at 37°C in a humidified, CO_2 incubator.
4. Feed the cells with fresh growth medium every 2 or 3 d during the assay period.
5. At 2-d intervals for 14 d, determine the number of cells per dish. Harvest the cells by trypsinization and resuspend in phosphate-buffered saline (PBS) for determination of cell numbers using a Coulter counter or a hemocytometer.
6. Growth rates are determined by calculating the doubling times from the initial slope of the logarithmic growth curve before saturation occurs.
7. Saturation densities are the cell densities achieved during the final, plateau phase of growth.
8. Serum dependence is determined by comparing the growth rate of the untransformed vs transfomed cells using growth medium supplemented with different amounts of serum.

3.2. Determination of GJIC

Cells are grown on glass slides that are partly coated with conductive and transparent indium-tin oxide. A stainless-steel electrode is placed on top of the cells and an electrical pulse of the appropriate strength applied, as described in **Fig. 2**. After washing the unincorporated dye, the migration of the dye to the nonelectroporated cells growing on the nonconductive area is microscopically observed under fluorescence illumination. The apparatus, which can be used for the introduction of a wide variety of nonpermeant molecules, is available from AskScience Products Inc.

1. Plate the cells on partly conductive slides as shown in **Fig. 2**.
2. Aspirate the medium. Wash the cells with calcium-free DMEM (*see* **Note 5**).
3. Add the Lucifer yellow solution.
4. Apply a pulse of the appropriate strength, according to the manufacturer's instructions.
5. Incubate the cells for 5 min.
6. Wash the unincorporated dye with PBS or calcium-free growth medium. The inclusion of dialysed serum at this point helps pore closure.
7. Microscopically examine under fluorescence and phase contrast illumination (**Fig. 2**).

3.3. Determination of Anchorage-Independent Growth

3.3.1. Growth of Cells in Soft Agar

1. Melt the agar in a microwave oven and place in a 50°C water bath (*see* **Note 6**).
2. Prepare the 2X DMEM solution: 40 mL 5X DMEM, 60 mL sterile water, 6 mL serum (for a final concentration of 3%, *see* **Note 4**), 0.1 mL gentamycin solution, 0.1 mL mycostatin solution. Adjust the pH to 7.2 by adding 4 mL of the 25X $NaHCO_3$ solution dropwise while swirling the bottle and a few drops of the HEPES solution until a color indicating a pH of 7.2 is reached. Place the bottle in a 50°C water bath.
3. Prepare the bottom agar solution: Mix 50 mL of 2X DMEM solution with 50 mL 1.6% agar. Place in a 50°C water bath.
4. Pipet 5 mL of bottom agar solution into each 6 cm plate or 2 mL into each 3 cm plate. Leave the Petris undisturbed at room temperature for approx 30 min for the agar to solidify.
5. Prepare the top agar solution: Mix 50 mL of 2X DMEM solution with 50 mL 0.7% agar in a 100 mL bottle. Depending upon the stringency desired, 50–100 µL of 1X DMEM may be added per mL of top agar, to reduce the agar concentration. Place the bottle in a 50°C water bath (*see* **Note 6**).
6. Remove the growth medium and wash the cells once with PBS.
7. Add 3–4 drops of trypsin and place the cells in the incubator for a few seconds as required.

8. Prepare the cell suspension in top agar: Aspirate some top agar solution in a sterile, 9-in. Pasteur pipet and pipet it onto the cells to dislodge them from the Petri dish. Depending upon cell confluence, it may be desirable to prepare serial dilutions of the cells in the top agar, to achieve a concentration of approximately 1000 to 10,000 cells/mL (*see* **Notes 3** and **7**).
9. Alternatively, cells can be suspended in a small volume of 1X DMEM and added to the top agar. In this case, it is important to ensure that the same amount of 1X DMEM is added to all tubes, so that the agar concentration is the same for all lines (*see* **Note 6**).
10. Add the cells suspended in top agar to the Petri containing the solidified bottom agar (*see* **Note 8**).
11. Leave the Petris undisturbed for 30 min at room temperature for the agar to solidify, then place them in a humidified, 37°C, CO_2 incubator.
12. To prevent drying of the agar medium, and to feed the cells, carefully add 1–2 mL of 1X DMEM containing serum, on top of the agar. Alternatively, medium containing methocel (*see* below) may be added and changed as needed. In this case, disturbance of the top agar, spillage of medium or contamination are minimized. However, depending on the number of cells plated, the extent of their growth, and the length of the experiment, this step may not be necessary.
13. Observe the cells every day with an inverted, phase contrast microscope using a ×4 or ×10 objective. SVLT-transformed cells divide while in soft agar suspension and form colonies that are macroscopically visible after 10–15 d (*see* **Notes 4** and **6**). Because for a number of normal lines anchorage is required only at a specific point of their division cycle ***(12)***, a proportion of cells may divide once after being placed in agar, depending upon the exact point in their cycle when they are placed in agar from a nonsynchronized monolayer culture, even though they are normal (**Fig. 1B**).
14. For record keeping, photograph the cells using a ×4 or ×10 objective and phase-contrast or brightfield illumination (*see* **Note 9**). The morphology of colonies of Rat F111 cells transformed by polyoma virus or SV40 is distinctly different from their NIH3T3 counterparts; each transformed F111 cell appears to retain its shape in the colony, whereas the NIH3T3 cells appear to "fuse" together, so that it is difficult to determine the number of cells forming the colony (*see* **Fig. 1C**, **D**).
15. Individual colonies can be isolated by gently aspirating them with a Pasteur pipet and adding them to a 3-cm Petri containing 2 mL of growth medium. Pipet the clone up and down to release the cells from the agar, and place the Petri in the incubator. The cells will attach and grow out from the clone in a few days.

3.3.2. Growth of Cells in Methylcellulose

1. Prepare the 2X DMEM solution: 40 mL 5X DMEM, 60 mL sterile water, 6 mL serum, 0.1 mL gentamycin solution, 0.1 mL mycostatin solution. Adjust the pH to 7.2 by adding 4 mL of the 25X $NaHCO_3$ solution dropwise while swirling the

bottle and a few drops of 1 *M* HEPES until a color indicating a pH of 7.2 is reached. Place the bottle in a 50°C water bath.
2. Prepare the bottom agar solution: Mix 50 mL of 2X DMEM solution with 50 mL 1.6% agar. Place in a 50°C water bath (*see* **Note 10**).
3. Pipet 2 mL of bottom agar solution into each 3-cm plate. Leave the Petris undisturbed at room temperature for approx 30 min for the agar to solidify.
4. Combine 40 mL of Methocel with 10 mL 5X DMEM, the desired amounts of serum (1–5 mL, *see* **Note 4**) and antibiotics. Add 2 mL, 25X $NaHCO_3$ solution slowly while shaking and adjust the pH to 7.2 with 1 *M* HEPES.
5. Trypsinize the cells and resuspend in a small volume of 1X DMEM. Add approx 1000–10,000 cells in 100 µL DMEM to 1 mL methocel-containing medium. Mix thoroughly and add to the Petri containing the bottom agar.
6. Alternatively, the methylcellulose-cell suspension can be placed in loosely capped, 5 mL polystyrene tubes, in which case there is no need for an agar underlay.
7. Place the Petri dishes or tubes in a humidified, 37°C, CO_2 incubator.
8. Colonies will appear in a few days and can be easily picked individually with a Pasteur pipet and placed in liquid medium. To recover the cells for biochemical analyses, the methocel can be diluted with PBS or growth medium and the cells pelleted out by centrifugation.
9. To achieve a quantitation of anchorage-independent growth, [^{3}H]-thymidine (1–10 µCi/mL) can be added to the methocel-containing medium for the desired number of days, followed by recovery of the cells as above and TCA precipitation of their DNA ***(1)***.

3.3.3. Growth of Cells on PolyHEMA-Coated Surfaces

1. Prepare a solution of 3% polyHEMA in 95% ethanol.
2. Depending on the stringency desired, prepare dilutions of 1:20 to 1:200 in 95% ethanol. Add 150 µL of the desired dilution per cm^2 of cell-growth surface. Let it dry undisturbed for 48 h (*see* **Notes 11** and **12**).
3. Trypsinize the cells and add the cell suspension to the polyHEMA-coated plate. Because of the fact that the potential points of adhesion of the cells to the substratum are reduced, it takes a number of hours for a substantial proportion of the cells to attach.
4. Grow the cells for 5–10 d. Individual colonies can be picked with cloning rings.

3.4. Tumorigenicity Assays

1. Using sterile technique, harvest mycoplasma-free cells ***(13)*** (*see* **Note 13**) by trypsinization or EDTA release from log-phase cultures, wash once, and suspend in serum-free DMEM.
2. Inoculate a 0.1 to 0.2 mL suspension of cells containing 1×10^6 cells subcutaneously into the dorsal flanks of nude mice left and right of the midline. The needle should be at least 21-gage to avoid damage to the inoculated cells, but not more than 18 gage to avoid excessive trauma. At least three, but preferably four or more, mice should be inoculated with each cell line, and both positive and

negative control cell lines should be included in each experiment. One mouse can serve as the host for two different cell lines if care is taken to keep the injections physically separate. The mice should be monitored for tumor formation twice a week (*see* **Note 14**). Tumors typically appear at 6–10 wk.

3. Tumor sizes are commonly reported as tumor diameter, measured with calipers; tumor volume, usually calculated by multiplying tumor diameters measured in different dimensions; or tumor weight, measured by weighing the excised tumor after sacrifice of the tumor-bearing host. All tumor-bearing mice should be sacrificed by the time the tumor reaches a maximum diameter of 1 cm, and preferably well before that time. Depending on the size of the inoculum, tumors may appear as early as 1 mo after injection. If desired, following sacrifice of the tumor-bearing animal, the tumors may be excised using sterile technique, minced finely, resuspended in growth medium, and plated for in vitro passage and characterization. The medium should be changed as soon as the explants (or single cells) have attached. Cells will begin to migrate from the explants within a few days and they can be cultured like the parental line.

4. Notes

1. Morphology-based or focus-formation assays can be straightforward, but several important conditions must first be met. Because cells such as NIH3T3 are preneoplastic, they have a strong tendency to be spontaneously transformed; therefore, it is critical to use a subclone that does not contain a subpopulation of spontaneously transformed variants. To minimize spontaneous transformation, cells should be subcultured at regular intervals to maintain cells in subconfluent cultures in logarithmic growth phases ***(14)***. However, lines such as Rat-1 or Rat F111 have a low frequency of spontaneous transformation. In general, cells transformed by SVLT do not look as refractile as cells transformed by the middle tumor antigen of polyoma virus (**Fig. 1A**).
2. The type of agar used can influence colony growth. In general, the presence of sulfated polysaccharides in "Bacto-agar" restricts cell growth and results in a more stringent assay than "Noble-agar," agarose, or methyl cellulose.
3. Dispersion of the cells is very important. Cells in a clump may "anchor" onto each other and grow even if they are not transformed. In general, NIH3T3 cells and their derivatives tend to adhere to each other when very confluent, so that it is difficult to separate them. Therefore, for all anchorage-independence assays it is important to use subconfluent cultures and pipet the cells well to achieve a single-cell suspension.
4. Higher percentages of serum will permit the formation of larger colonies.
5. For GJIC determination, it is important to wash the dye using a calcium-free solution (growth medium or PBS). If calcium-containing growth medium is used instead, the values obtained may be significantly reduced, presumably because of the calcium influx, which was shown to close gap junctions.
6. The precise agar content of the top agar suspension is very important. Many of the ingredients of the DMEM are not stable at high temperatures, and many cell

lines cannot tolerate temperatures above 45°C. Therefore, although it is important to ensure that the agar is uniformly melted, because localized lower concentrations of agar can permit the growth of even normal cells, it is important to let the agar cool down before addition to the 2X DMEM. It is also important to ensure there is no loss of water during microwaving, because in this case even transformed cells may not grow because of high agar concentration. Uniformity of the agar is also important because lumps of agar interfere with photography.

7. When the growth of cells expressing different mutants is compared, it is important to examine areas with similar numbers of cells. After several days, cells that did not grow may lyse so that they are not taken into account when the percentage of cells that grew in the absence of anchorage is determined.
8. If the cell suspension in top agar is added in a small volume (e.g., less than 0.5 mL for a 6 cm Petri), then the cells are at approximately the same depth so that it is easier to focus with the microscope and photograph them.
9. In general, it is easier to photograph cells growing in agar under brightfield, rather than phase contrast illumination. It is more difficult to focus under phase contrast on cells that are not at precisely the same level, besides the agar may create a dark, uneven background. Photographing cells growing in methocel is more difficult because the cells tend to drift in the viscous liquid.
10. If the cell suspension in methocel is added directly to tissue-culture grade Petris without an agar underlay, the cells may slowly settle to the bottom of the plastic Petri and grow as a monolayer. The same may happen in the case of agar assays if the bottom agar has not solidified at the time when the cell suspension in top agar is added.
11. It has been proposed that cell shape is critical for the growth of normal cells ***(10)***. Cell shape can be precisely controlled by varying substratum adhesiveness by applying increasing concentrations of polyHEMA to a tissue-culture dish. When an alcoholic solution of this material is added to a plastic tissue-culture dish, a thin, hard, sterile film of optically clear polymer remains tightly bonded to the plastic surface after the alcohol evaporates. Serial dilutions of the polymer result in decreasing thicknesses of the polymer film. The mechanism by which polyHEMA reduces the ahdesion of cells to polystyrene is not completely understood. It might act by reducing the net negative electrostatic charge of the polystyrene as a hydrophilic hydrogel of neutral charge, or, depending on its thickness, it might permit few spicules of plastic to protrude through the coating and offer multiple contact points to which cells can adhere, so that increasing thickness of polyHEMA decreases the number of available contacts with the plastic ***(10)***.
12. The edge of the Petri dish (2–3 mm) may receive a thicker coating because of the meniscus formed. In addition, some brands of tissue-culture Petri dishes are not uniformly flat, in which case small “grooves” in concentric circles at approx 5 mm from the edge may be present, where the coating would be thicker, which reduces cell attachment and growth. It was also noted that in certain instances, slight vibration (e.g., from a fan in a laminar-flow hood) can cause ripples and uneven coating in concentric circles, with the same effect.

13. It has been reported that mycoplasma infection can reduce the growth of transformed cells under anchorage-independent conditions, but increase the tumorigenic potential of cells injected into nude mice ***(13)***. Therefore, all cell lines to be tested should first be monitored for mycoplasma contamination.
14. In the case of SV40, injection of virus or transformed cells into immunocompetent hamsters causes tumors at the site of injection which might even metastasize. Cells from other species however, fail to become highly tumorigenic and can only cause tumors in athymic nude mice. It has been proposed that SVLT-induced immortalization primes the cells for secondary mutations that cause multiple phenotypic changes, including loss of cytolytic susceptibility and increased tumorigenicity ***(15)***.

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12

Methods to Assess Inhibition of Adipocyte Differentiation by SV40 Large T Antigen

Cynthia Higgins and Van Cherington

1. Introduction

Murine preadipocyte cell lines represent a convenient and informative model system for determining whether the mechanisms underlying oncogene-induced neoplastic transformation and the suppression of terminal differentiation are related. Preadipocytes transformed by a variety of oncogenic proteins, such as the Simian virus 40 (SV40) large-tumor antigen (SVLT) or activated *ras* oncogene ***(1–6)*** and others, fail to differentiate into adipocytes. However, the transformation and differentiation-inhibition functions are separable. Genetic analysis of SVLT has shown that at least two functional domains block differentiation independently. These include the N-terminal domain that binds to the cellular protein encoded by the retinoblastoma gene (*Rb*) (*see* **Note 1**) and a C-terminal function. However, only one of these two, the *Rb*-binding domain, is involved in neoplastic transformation ***(1,3)***. That is, the C-terminal function blocks differentiation independently of *Rb*-binding and independently of transformation ***(3)***. In addition, it was also shown that at low doses activated *ras* prevents adipocytic differentiation without inducing a fully transformed phenotype ***(5,6)***. Thus, structure-function analysis or dose analysis in the adipocyte system has helped distinguish oncogene functions not detectable using standard transformation assays alone.

The methods described in this chapter are intended for the investigation of the relationship between cellular differentiation and neoplastic transformation. We have used SVLT as a model, but this approach can be applied to the study of other oncogenes or gene products that suppress differentiation. These studies rely on creating preadipocyte cell lines that constitutively express wild-type

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or mutant SVLT following transfection with SVLT expression vectors and selection for stable lines.

The early region of the SV40 virus encodes two proteins involved in transformation; SVLT and SV40 small tumor antigen, svst. When deciding which SV40 sequences to clone and express in preadipocytes, it is important to consider the effect svst may have on differentiation. It may be necessary to obtain SV40 early region plasmids engineered to express only SVLT, if SVLT effects, independent of svst, are of interest. This is particularly important in SVLT structure-function analyses. Some examples of expression plasmids expressing SVLT alone have been published ***(3,7–9)***. These SVLT-expressing preadipocyte lines are then assessed for their ability to differentiate using differentiation-dependent gene expression, lipogenic enzyme activity, triglyceride production, and morphological and histochemical staining criteria for adipogenesis. The assay of phenotypes associated with neoplastic transformation is also an important part of this analysis and is described in Chapter 11.

The availability of many preadipocyte cell lines and cell strains has allowed the study of the process of adipocyte differentiation and the physiology of adipose cells in vitro [reviewed in ***(10–19)***, *see* **Note 2**]. The work presented here focuses on results obtained with the preadipocyte cell line 3T3-L1 ***(20)***; a clonal line developed from mouse embryo cultures (*see* **Note 2**). When 3T3-L1 cells are induced to differentiate, they undergo several rounds of cell division, stop proliferating, alter gene expression by increasing the level of enzymes necessary for triglyceride synthesis, and change in morphology becoming rounded and full of microscopically visible lipid droplets (*see* **Fig. 1**). Many transcription factors have been identified that are regulated early during differentiation [*see* ***(10–19)***] including the CAAT/enhancer binding proteins (C/EBPs) (21–25) and the peroxisome proliferator-activated receptors (PPARs) ***(23,26–32)***. Although growth arrest is a prerequisite for initiating differentiation, much work has shown that there exists a growth-arrest state, where the cells are unable to differentiate ***(1,3,33–37)***. Analysis of SVLT functions that block differentiation without preventing growth arrest will help to biochemically define this differentiation-incompetent, growth arrested state.

This chapter describes methods to assess the effect of SVLT upon adipocytic differentiation. Techniques to assess transformation are described in Chapter 11. Methods to express SV40 tumor antigens in cultured cells using retroviral vectors and methods to detect SV40 tumor antigens are described in Chapter 10.

CAUTION: Many of the materials used in the following protocols are hazardous (e.g., radioactive isotopes and mutagenic chemicals) and proper laboratory safety procedures must be followed in handling them. Consult your institution's policies for proper storage and disposal of hazardous materials. Some specific precautions are included in the text.

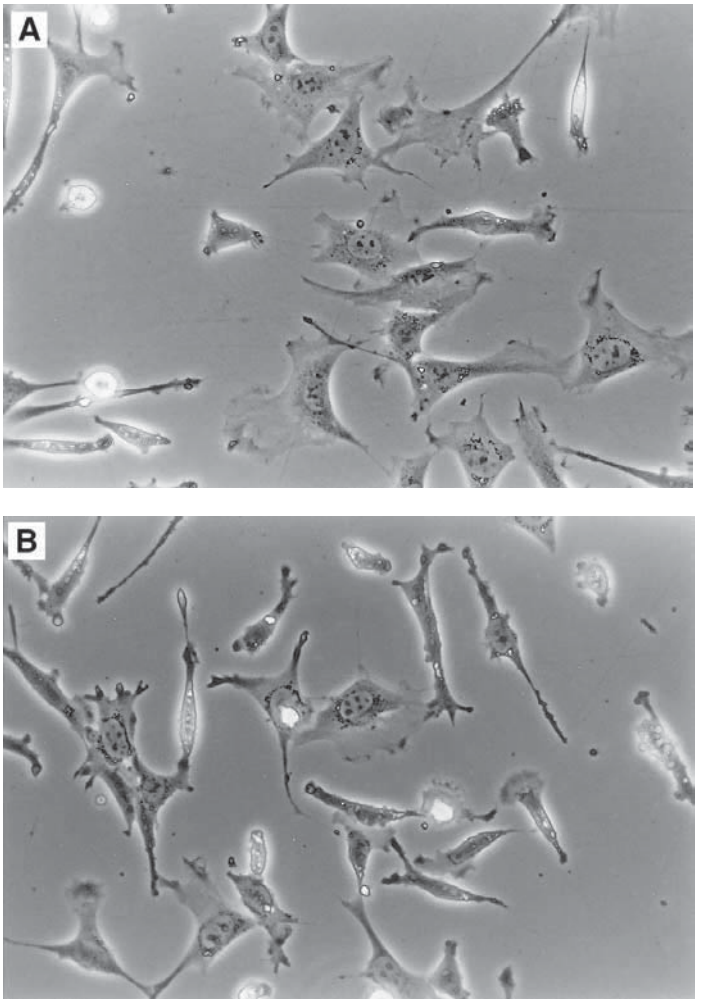

Fig. 1. Photomicrographs of 3T3-L1 cells (original magnification 200X). **A, C, E**, 3T3-L1 neomycin-resistant control cells. **B, D, F**, 3T3-L1 cells expressing SVLT. a and b, cells growing in DMEM, fetal calf serum and insulin. **C** and **D**, cells confluent, 1 d after treatment with DEX/IBMX (induced to differentiate). **E** and **F**, 7 d after DEX/IBMX addition. Control cells expressing only neomycin resistance are fully differentiated and contain numerous intracellular lipid droplets (c, arrow), although cells expressing SVLT are not differentiated **(F)**. The lipid droplets can be identified by their round shape and refractile appearance. They stain bright red with Oil Red O.

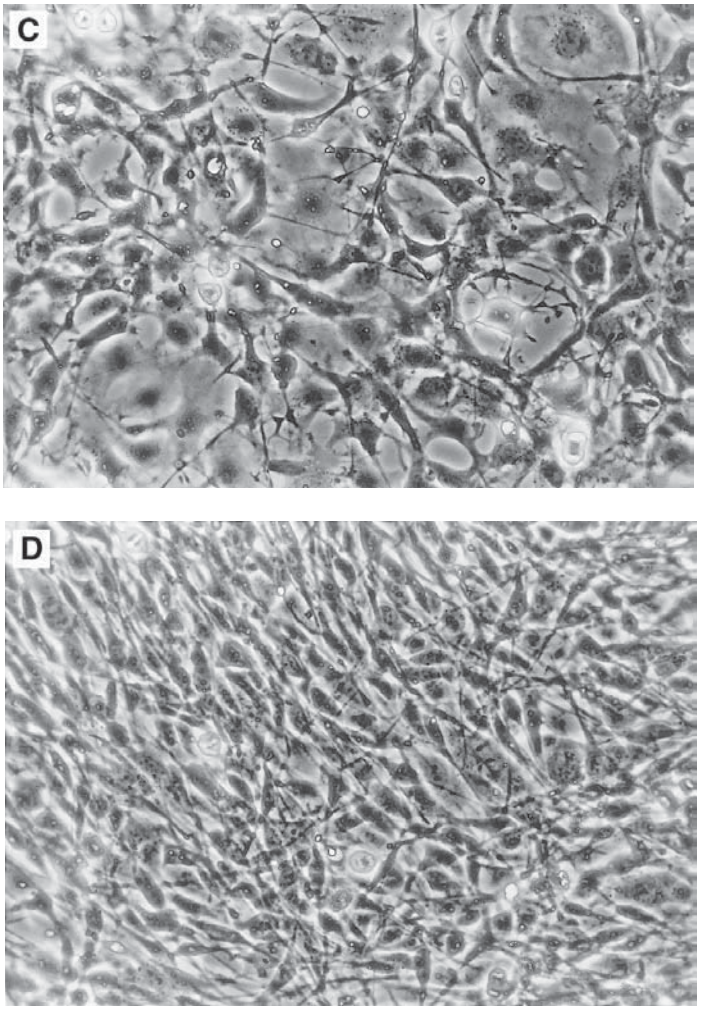

Fig. 1. *(continued)*

2. Materials

2.1. Cell Culture and Differentiation Induction

1. Preadipocyte cell line 3T3-L1 ***(20)*** (American Type Culture Collection [ATCC], Manassas, VA, cat. no. CL-173, *see* **Note 2**).
2. 37°C, humidified, 10% CO_2 cell-culture incubator.
3. Predipocyte culture medium; Dulbecco's modified Eagle's medium (DMEM), high glucose (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% bovine calf serum (BCS), 100 U/mL penicillin, 100 µg/mL streptomycin,

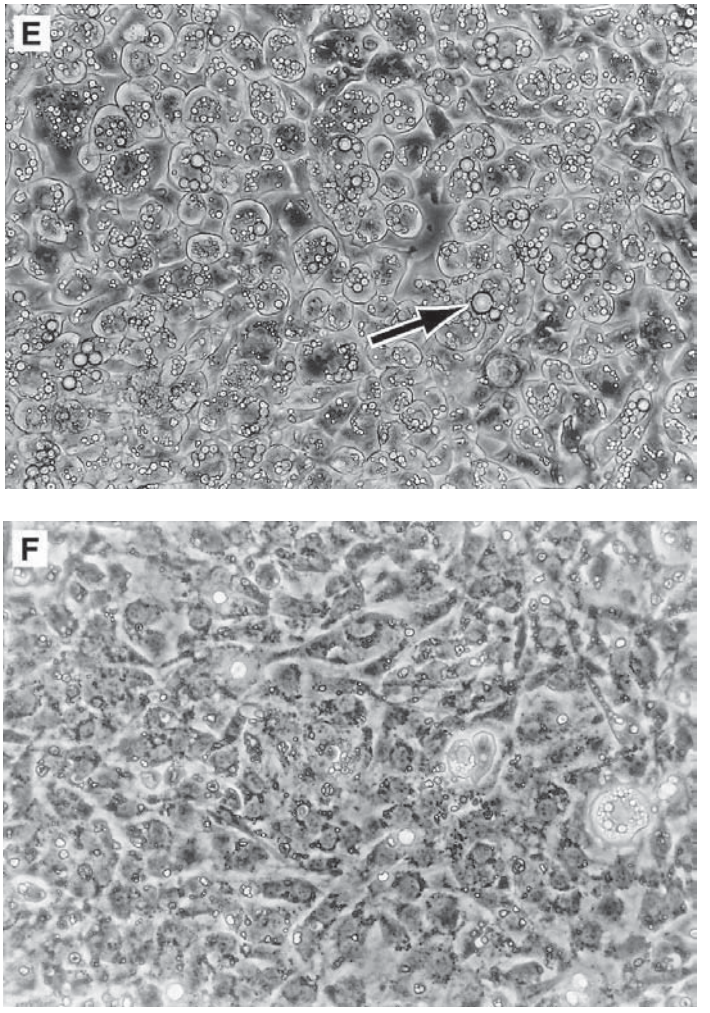

Fig. 1. *(continued)*

and 2.0 m*M* glutamine. 100X solutions of penicillin/streptomycin and glutamine may be purchased from Life Technologies, Inc.

4. Differentiation culture medium: same as preadipocyte culture medium, except that calf serum is replaced with 10% bovine fetal calf serum (*see* **Note 3**) and insulin (5.0 µg/mL) is added (Sigma, cat. no. I1882; 100 mg, lyophilized, sterile). Insulin may be prepared as a 5 mg/mL stock using sterile water and aliquoted into sterile microfuge tubes at 0.25 mL per vial. Thaw a vial to prepare the medium and discard the unused portion. Do not refreeze.

5. Differentiation-induction medium: Differentiation culture medium supplemented with 250 n*M* dexamethasone (DEX) and 500 µ*M* isobutylmethylxanthine (IBMX). Prepare dexamethasone (MW = 392.45; Sigma, cat. no. D2915) as a 10 m*M* stock in water (100 mg/25.5 mL). Dilute 1:40 with water to make a 250 µ*M* (1000X) stock and filter into a sterile 15-mL polypropylene tube. Store at 4°C. Alternatively, a 10-m*M* stock in ethanol can be prepared, which is sterile because of the ethanol which would kill contaminating microorganisms. Therefore, there is no need to filter. The solution is stable for more than a year at 4°.

   Prepare IBMX (MW = 222.2; Sigma, cat. no. I7018) as a 75-m*M* stock in ethanol (16.6 mg/mL) in a sterile, 15-mL polypropylene tube. Store at –20°C. The solution is sterile. Add 6.7 µL per mL medium for a final concentration of 500 µ*M*.
6. 100-mm and 60-mm diameter tissue-culture dishes. Six-well tissue-culture trays with 35-mm diameter wells.

2.2. Assessing Adipocyte Differentiation

2.2.1. Detection of Differentiation-Dependent Gene Expression by Northern Blotting

RNA is easily degraded by RNases, therefore, separate solutions and equipment should be maintained for RNA work; agarose, formaldehyde, gel electrophoresis boxes, and pipetors should all be separate from those used for DNA work and work with RNase-treated or contaminated material (cell extracts, and so on). Wear gloves, because hands can be a source of RNases. Glassware can be baked (150°C, 4 h) and pipet tips can be purchased that are nuclease-free and presterilized. Otherwise, pipet tips can be autoclaved. Pipet tips containing a filter barrier are useful for both RNA work and for radioactive work so that pipetors do not become contaminated.

1. Equipment for agarose gel electrophoresis and Northern blotting.
2. Total RNA isolation solution: Trizol (Life Technologies, Inc., cat. no. 15596026).
3. cDNA probes for differentiation-dependent mRNA detection (*see* **Note 4**).
4. 50X Tris-acetate buffer: 242 g Tris base, 57.1 mL glacial acetic acid bring to 1 L with water. Use at 1X concentration for gel and running buffer for probe fragment purification.
5. For labeling of the probe: Random-priming cDNA radiolabeling kit (Life Technologies) and [^{32}P]dCTP (DuPont New England Nuclear, Boston, MA, or ICN, Costa Mesa, CA) (*see* **Note 4**).
6. DEPC (diethylpyrocarbonate)-treated deionized sterile water: Add 0.2 mL DEPC (Sigma, cat. no. D5758) to 100 mL of deionized water, shake vigorously, autoclave. (**CAUTION:** DEPC is a carcinogen. Handle it in a fume hood. Autoclaving removes remaining DEPC.)
7. Nitrocellulose membrane (Schleicher and Schuell, Keene, NH, cat. no. 21640).
8. 50X Denhardt's solution: 5 g Ficoll 400, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (BSA), deionized water to 500 mL. Store at –20°C in 25-mL

aliquots. Commercially prepared Denhardt's is also available from a variety of vendors.
9. 20X SET buffer: 100 m*M* Tris, pH 8.0, 10 m*M* EDTA, pH 8.0, 1 *M* NaCl.
10. Formamide (RNase-free, deionized.) (**CAUTION:** Handle in a fume hood.)
11. 5% sodium pyrophosphate. Store in aliquots at –20°C.
12. 1 *M* sodium phosphate monobasic, store in aliquots at –20°C.
13. 10% w/v sodium dodecyl sulfate (SDS) in water, store at room temperature.
14. Herring or salmon sperm DNA (10 mg/mL in water). Shear by passing through a syringe and boil for 3 min. Store aliquots in microfuge tubes at –20°C.
15. 10X MOPS buffer: 0.2 *M* MOPS [3-(*N*-morpholino)-propanesulfonic acid] (Sigma, cat. no. E5134), 10 m*M* Na_2 ethylenediaminetetraacetic acid (EDTA), 50 m*M* sodium acetate. Adjust pH to 7.0 with HCl. Filter sterilize and store protected from light at 4°C.
16. 37% formaldehyde. (**CAUTION:** Handle in a fume hood.)
17. Agarose, gel electrophoresis grade.
18. Low-melting-point agarose (SeaPlaque, FMC BioProducts, Rockland, ME, cat. no. 50101). FMC BioProducts also sells SeaPlaque GTG (cat. no. 50111), for use with DNA fragments greater than 1 kb. They indicate that its greater purity results in less interference with DNA modifying enzyme reactions. This is not applicable for the aP2 or GPD probes used in this work because these are only 400 and 300 bp, respectively.
19. 10X ethidium bromide: 1 mg/mL in water. Store at 4°C protected from light. (**CAUTION:** Mutagenic, wear gloves when handling.)
20. 0.1% w/v bromophenol blue in 50% glycerol in water.
21. 50 m*M* NaOH. Store at room temperature.
22. 20X SSC: 3 *M* sodium chloride, 0.3 *M* sodium citrate dihydrate, adjust to pH 7.0 with 1 *M* HCl.
23. Ultraviolet (UV) transilluminator, short and long wavelength. (**CAUTION:** Wear eye protection around UV light sources.)
24. UV crosslinker such as "Stratalinker" made by Stratagene, La Jolla, CA.
25. Hybridization oven with tubes for holding filters and hybridization solutions such as ovens manufactured by Fotodyne, Hartland, WI.
26. Vacuum blotting apparatus (e.g., from Bio-Rad, Hercules, CA, cat. no. 165-5000).
27. X-ray film, film cassettes with intensifying screens, and developing equipment for autoradiography.
28. Marker which emits a signal to imprint on X-ray film, such as Glo-Bug fluid (Dot Scientific, Burton, MI), to help superimpose the blot with the autoradiogram.
29. 3 *M* sodium acetate, prepared in DEPC-treated water.

2.2.2. Glycerol Phosphate Dehydrogenase (GPD) Assay

1. Spectrophotometer capable of measuring the change in absorbance over time. The capacity to perform multiple kinetic assays simultaneously is very helpful.
2. Disposable polystyrene semimicro- or quartz cuvets for use with 340 nm light.
3. Branson Sonifier model 250 or equivalent instrument, with microtip.

4. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8 m*M* Na_2HPO_4, pH 7.4.
5. Cell extraction buffer: 25 m*M* Tris, pH 7.4, 1.0 m*M* EDTA.
6. NADH: Sigma, cat. no. 340-102, disodium, 2 mg/vial.
7. 5X GPD assay buffer: 250 m*M* triethanolamine, 5 m*M* EDTA, 5 m*M* -mercaptoethanol. Adjust pH to 7.5 with HCl, filter, and store at room temperature.
8. Dihydroxyacetone phosphate (DHAP, lithium salt, Sigma, cat. no. 37440) solution: Prepare a 10. 8 m*M* stock solution in water (MW = 181.92) and freeze in small aliquots. For a working solution, thaw an aliquot and dilute 1:10 to 1.08 m*M* and use for GPD assay (it is diluted 1:5 in the final assay mixture to 216 μ*M*).
9. Bio-Rad protein assay reagent (Bio-Rad, cat. no. 500-0002).

2.2.3. Oil Red O Staining for Triglycerides

1. Oil Red O Stock (Sigma, cat. no. O-0625): Weigh 0.5 g/100 mL isopropanol. Stir for several hours or overnight. Immediately before use, dilute three parts dye stock with two parts water. Filter through Whatman #1 filter paper to remove precipitate. Use a filtering flask and funnel dedicated to Oil Red O because it is difficult or impossible to wash it off. Alternatively, Oil Red O can be prepared in propylene glycol (Sigma, cat. no. P1009).
2. PBS (*see* **Subheading 2.2.2., item 4**).
3. 4% formaldehyde in PBS.

3. Methods

3.1. Cell Culture and Differentiation Induction

1. Maintain stock cultures of 3T3-L1 cells as preadipocytes by culturing at subconfluence in preadipocyte culture medium.
2. Change the cell culture medium every 3–4 d and split the cells (maximum 1:50) just before, or upon reaching confluence. Use the cells for experiments at a low-passage number, i.e., thaw a fresh cryopreserved vial of low-passage preadipocyte stock every 6–8 wk.
3. Induce adipocyte differentiation by plating cells in differentiation culture medium. Plate 10^5 cells in 2 mL of this medium in a 35-mm diameter plate.
4. Dishes become confluent within 3–5 d. When confluent, treat overnight with fresh differentiation induction medium containing 250 n*M* dexamethasone and 500 μ*M* isobutylmethylxanthine (DEX-IBMX medium). On the following day, replace with differentiation culture medium (lacking DEX-IBMX).
5. Replace with fresh differentiation culture medium every 3–4 d.
6. Differentiation-competent cultures exhibit 75–100% lipid-containing cells (Oil Red O staining) and express maximal glycerophosphate dehydrogenase (GPD) activity and adipocyte P2 (aP2) mRNA within 7 d after DEX-IBMX treatment. Over these 7 d, mRNA induction precedes GPD enzyme activity and this, in turn, precedes lipid accumulation.

7. Differentiation can be assessed using the assay of GPD enzyme activity (*see* **Subheading 3.2.2.**), the increase in lipid accumulation (Oil Red O staining) (*see* **Subheading 3.2.3.**) and the appearance of, and increase in, expression levels of differentiation dependent mRNA, such as aP2 (*see* **Subheading 3.2.1.**). The measure of GPD enzyme activity gives a quantitative evaluation of differentiation. Lipid accumulation, visualized with Oil Red O, allows a quantitative evaluation of the proportion of the cells in the culture that contain lipid. Northern blotting gives a qualitative assessment of changes in gene expression and can detect the earliest events in the differentiation-dependent cascade.

3.2. Assessing Differentiation

3.2.1. Northern Blotting for Adipocyte-Specific RNA

The appearance of differentiation-dependent mRNA can be determined by Northern blotting. Use Northern blotting and randomly primed radiolabeled cDNA probes to detect changes in the expression of adipocyte-specific mRNA (*see* **Note 4**).

1. Seed cells for differentiation in 60- or 100-mm plates to ensure that enough RNA will be obtained. As controls, include cells that are growing and undifferentiated and cells that are confluent but have not been induced to differentiate.
2. Seven days after DEX/IBMX treatment, extract total cellular RNA by the acid guanidinium thiocyanate-phenol-chloroform extraction method ***(39)*** using one of the commercially available kits that are based on this method, such as Trizol and following the detailed protocol provided by the manufacturer. Extraction by other methods that rely on RNase inhibitors such as RNasin (Promega) has been successful with preadipocytes but not with differentiated adipocytes in our hands, presumably because of a high concentration of RNase activity in mature adipocytes.
3. The final pellet of RNA should be dissolved in DEPC-treated, autoclaved deionized water and the concentration of RNA determined spectrophotometrically by reading the OD_{260} of 1 µL of the sample in 1 mL of water. [Absorbance at 260 nm] X 40 = # µg of RNA per µL of original dilution.
4. Aliquot the RNA into convenient amounts for future use in Northern blotting, such as 5–20 µg per microfuge tube and use right away or precipitate with ethanol (add 10% the volume of sample of 3 *M* sodium acetate plus at least 2 vol of 100% ethanol), and store at –20°C.
5. Prepare RNA samples for separation on an agarose/formaldehyde denaturing horizontal gel. Microcentrifuge samples for 10 min at 16,000*g*, look for pellet, carefully pour off the supernatant, drain on a Kimwipe for 5 min, and air-dry. Allowing the RNA pellet to become too dry, e.g., by using Speedvac centrifugation under a vacuum, may make it difficult to dissolve the RNA pellet.
6. Add 4 µL DEPC-autoclaved water to the RNA pellet, 1.75 µL 10X MOPS buffer, 2.85 µL formaldehyde, 8.75 µL formamide.

7. Heat to 55°C for 15 min, add 2 µL of 0.1% bromophenol blue gel sample loading solution, and 2 µL of 10X ethidium bromide. Then the sample is ready to load.
8. Prepare a 1.5% agarose/formaldehyde denaturing gel; boil (microwave) 3.00 g RNase-free agarose in 145 mL water, cool to 60°C.
9. In a fume hood, add 20 mL 10X MOPS buffer and 32.4 mL, 37% formaldehyde, swirl quickly, pour into gel mold, allow to cool, and let set in a fume hood (about 30 min).
10. Load samples after placing gel in tank under 1X MOPS running buffer. Run gel overnight at 20 V in 1X MOPS buffer (can be run in 2 h at 100 V).
11. When the blue tracking dye is visible two-thirds the way down the gel, remove the gel carefully to avoid breaking it and view on a UV transilluminator light box. If the gel is cracked, you will not be able to use vacuum transfer. The 28S and 18S ribosomal RNA should be clearly visible as two sharp, well-separated bands migrating with apparent molecular sizes of approx 5000 bases and approx 2000 bases, respectively. Measure the distance of the leading edge of the 28s and 18s bands from the wells on the gel and use as markers for the final blot. The relative intensity of the bands gives an indication of equal loading between lanes on the gel and the sharpness of the bands indicates intact, nondegraded RNA. Missing bands or smearing may indicate degraded RNA and the gel should not be used for Northern blotting.
12. Immerse in 50 m*M* NaOH for 20 min to nick RNA thus facilitating transfer, rinse in deionized water, then equilibrate in 20X SSC for at least 30 min.
13. Transfer the RNA onto nitrocellulose using a vacuum blotter, according to the manufacturer's instructions. Cut a nitrocellulose sheet slightly larger than the gel, wet by floating on deionized water, and then soak in 20X SSC. Always handle nitrocellulose with gloved hands. Assemble the components of the vacuum blotter according to the manufacturer's instructions; first 3-mm Whatman filter paper cut to the size of the blotter and wetted in 20X SSC, then the wetted nitrocellulose, then the mask, and finally the gel, having it overlap the mask so that a seal will form when the vacuum is applied. Apply vacuum and cover with 20X SSC. One hour should be sufficient to transfer the RNA. Do not exceed the maximum recommended vacuum or the gel will compress too quickly and prevent efficient RNA transfer.
14. When transfer is complete lift the gel slightly to break the vacuum, shut off the vacuum, mark on the nitrocellulose the corners of the gel, location of the wells, and the side of the blot closest to the gel with lead pencil.
15. Rinse the nitrocellulose in 6X SSC and air-dry on Whatman 3-mm filter paper for 5 min. The nitrocellulose can be viewed on a UV transilluminator to see the transferred RNA.
16. UV crosslink both sides of the nitrocellulose using the crosslinker.
17. Prehybridize the blot. Prepare the prehybridization solution on ice:
    a. 4 mL 50X Denhardt's solution.
    b. 5 mL 20X SET.
    c. 10 mL formamide.

d. 400 µL 5% sodium pyrophosphate.
e. 2 mL 1 *M* sodium phosphate monobasic.
f. 200 µL 10% SDS.
g. 150 µL denatured herring or salmon sperm DNA (10 mg/mL). Add last and mix well.

18. Place the blot in prehybridization buffer in the tube, with the side of the blot that was toward the gel facing the inside of the tube (squeeze out bubbles between the filter and the side of the tube by rolling a disposable plastic pipet across the filter), in the hybridization oven at 45°C for approx 4 h while you are labeling the cDNA probe(s).
19. Label mouse actin and aP2 cDNA probes with 50 µCi of [^{32}P] dCTP, 3000 Ci/mmol, 10 µCi/µL in separate microfuge tubes using random-priming (*see* **Note 4**).
20. Denature the probe DNA by boiling for 3 min. Quick-spin down. Use safety lock caps so that the tubes do not pop open when placed in the boiling water bath.
21. Add both probes directly to the prehybridization buffer and hybridize overnight at 45°C.
22. Remove filter from hybridization solution and rinse three or four times in 3X SSC heated to 55°C. Check blot for radioactive signal (concentrated counts) using a Geiger counter. You should hear counts in the area where you expect the bands to be and other areas of the filter should be background. Continue washes until this is observed. If necessary, more stringent washes can be used but be careful not to lose the specific signal. (More stringent conditions: high temperature, low salt concentration; 55°C, 0.2X SSC, 0.1% SDS. Less-stringent conditions: low temperature, high salt concentration; room temperature, 2X SSC, 0.1%SDS.)
23. Mark the lower left-hand corner and the lanes with the Glo-bug marker that will emit a signal to imprint on the film. Wrap the filter in Saran Wrap. Put the filter on film in a cassette in the following order: intensifying screen—film—filter, at –70°C. Expose the film overnight and develop. Alternatively, you can scan and quantitate in a phosphorimager.
24. To strip the blot before reprobing with a different cDNA: prepare the following solution:
    a. 50% formamide.
    b. 10 m*M* Tris-HCl pH 7.4.
    c. 1 m*M* EDTA.
    d. 0.1% SDS.

    Heat this stripping solution to 68°C. Incubate the filter with 20 mL of this solution for 20–30 min at 68°C. Repeat with another 20 mL. Expose overnight to X-ray film to ensure that the blot has been stripped.

3.2.2. Detection of GPD Activity

A modification of the GPD assay described by Wise and Green ***(38)*** is performed to detect the differentiation-dependent increase in GPD enzyme activ-

ity. Extracts of cells grown in duplicate 35-mm tissue-culture wells (plated at 10^5 cells per well) are sufficient for this assay. Only assay as many samples as you can quickly mix and measure at the same time. The number of assays that can be performed at one time depends upon the capacity of your spectrophotometer to perform multiple kinetic assays simultaneously. Have solutions ready along with cuvets and use parafilm to cover the cuvets while inverting to mix.

1. At 7 d postinduction (post-DEX/IBMX treatment), rinse cell monolayers with PBS and scrape directly into cell extraction buffer on ice.
2. Disrupt cells by sonication on ice (25 W for 10 s at 4°C, using a microtip on a Branson model 250 Sonifier). Do not allow sample to foam or overheat.
3. Pellet insoluble material by centrifugation at full speed in a microcentrifuge (approx 12,000*g*) for 10 minutes at 4°C and place supernatant in a fresh microfuge tube. GPD extracts can be stored at –80°C or assayed immediately. Avoid repeated freezing and thawing.
4. Determine total soluble protein concentration using the Bio-Rad protein assay kit.
5. Assay GPD activity in extracts containing 5 to 100 µg of total protein. Use 10 µL of extract for the assay if cells are well differentiated or 100 µL of extract if cells are poorly or not differentiated. Bring the volume of cell extract up to 100 µL final volume with extraction buffer.
6. Thaw an aliquot of DHAP and dilute 1:10 in water.
7. Add 5.0 mL of water to a 2.0 mg vial of NADH. Keep cell extracts on ice while performing the assay, while the other reagents, and the assay itself, remain at room temperature.
8. To each cuvet, add 100 µL 5X GPD buffer, 200 µL NADH, 100 µL cell extract (or extraction buffer for blank). Add 100 µL DHAP last to start the reaction. Mix immediately by holding parafilm over cuvets and inverting several times. Read the change in O.D. at 340 nm over 2 min.
9. A unit of GPD is defined as 1 nanomole NADH oxidized per minute and results are normalized to protein concentration by calculating units per mg of protein in the cell extract in the assay. The rate of oxidation of NADH (decrease in absorbance at 340 nm) must be linear over the course of the assay to accurately measure enzyme activity. If it is not linear over the entire 2-min assay time, then less extract should be used for the assay. Units are calculated as follows:

   Nanomoles NADH oxidized per minute =

$$\frac{\text{(Absorbance change per min at 340 nm)}}{\text{(6.22 m}M^{-1}\,\text{cm}^{-1}\text{)} \times \text{(1 cm light path)}} \times \text{(0.0005 L assay vol)} \times \frac{10^6}{\text{(conv. mmoles to nmoles)}}$$

   where 6.22 is the m*M* extinction coefficient of NADH. Activity is never reported as 0, but as less than the limit of detection, which depends on the limit of resolution of the spectrophotometer being used and the amount of protein added to the reaction.

3.2.3. Staining with Oil Red O to Visualize Lipid Accumulation

1. Plate cells in duplicate as for GPD assay.
2. Induce differentiation and at 7 d post-DEX/IBMX treatment stain with Oil Red O.
3. Rinse monolayers with PBS.
4. Fix for 15 min with 4% formaldehyde in PBS.
5. Remove formaldehyde and stain with diluted/filtered Oil Red O for 10–15 min at room temperature.
6. Rinse with deionized water.
7. Keep water on cells for microscopy. If fat droplets or cells dry out, they will not maintain their morphology. Lipid droplets stain bright red.
8. Determining the morphology and size of the lipid droplets, as well as the proportion of cells in the culture-containing lipid, can provide a quantitative measure of differentiation. At least 300 cells overall should be counted for an accurate determination. A variety of random fields should be counted because differentiated cells are usually not evenly distributed over the monolayer.

4. Notes

1. This is consistent with a functional *Rb* being important for adipocyte differentiation ***(40,41)*** as shown in genetically altered mice. In fact, Rb contributes to regulated adipocyte differentiation both through growth inhibition and through direct interaction with transcription factors involved in the differentiation-dependent gene expression.
2. Our studies of the SVLT differentiation block ***(1,3)*** have used two preadipocyte lines, 3T3-L1 and 3T3-F442A, which were cloned from the same mouse embryo derived 3T3 line ***(17,20,42,43)***. The 3T3-L1 line has three advantages in these studies over 3T3-F442A:
   a. 3T3-L1 transfects more efficiently than 3T3-F442A and thus is preferable when retroviral vectors for SVLT are not available and plasmid transfections must be used to create lines expressing SVLT.
   b. 3T3-L1 cells are available from ATCC whereas 3T3-F442A are not, and
   c. 3T3-F442A cells have a greater tendency to peel off from the culture dish when transformed and confluent, making comparisons of monolayer differentiation difficult.

   When a cell line is initially obtained, a sufficient enough number of cells should be grown to prepare a large number of low passage cryopreserved stock vials at 1–5 × 10^6 cells per vial in growth medium + 10% DMSO. A fresh vial should be thawed every 6–8 wk.

   Other preadipose cell lines exist that may be applicable to these studies. 3T3-T are preadipocytes derived from Balbc/3T3 cells ***(44)***, and TA1 preadipocytes were derived from pluripotential mesenchymal C3H10T1/2 fibroblasts after treatment with 5-aza-cytidine ***(45)***. Other clonal cell lines have been derived from mature adipose tissue by selecting the fibroblast-like, nonlipid filled cells that differentiate under induction culture conditions. An example of such a cell line is

the ob17, which was derived from the stroma of epididymal fat pads from adult genetically obese mice ***(46)***. Primary preadipose cells can be obtained from adult mammalian adipose tissue, e.g., from rat epididymal fat or from human omental adipose tissue, and from bone marrow stromal cells (*see* reviews cited in **Subheading 1.**).

3. Fetal calf serum and calf serum can be purchased from a variety of vendors. It is important to test several (>5) individual lots of fetal calf serum for the ability to promote adipocyte differentiation, before ordering. When testing new serum, compare test lots to a control lot known to support differentiation. Purchase a test lot that promotes differentiation at a similar rate and promotes differentiation in the same percentage of cells. The cells should also have a similar morphology. Testing calf serum lots before purchasing is also wise to ensure that similar growth properties of cells are observed, based upon saturation density, growth rate and morphology, as compared to the previous lot in use.
4. A variety of cDNAs have been cloned that encode genes that are regulated during differentiation and that could be used as probes for Northern blots. These include GPD, Adipsin and aP2 ***(47)***, and the transcription factors C/EBP ***(21)*** and PPAR ***(26–28)*** (*see* also reviews cited in **Subheading 1.**). Messenger RNA for the transcription factors C/EBP and PPAR increase early in 3T3-L1 adipocyte differention (during days 0–3 postinduction). Messenger RNA for proteins such as aP2, GPD, and adipsin that are involved in adipocyte function begin to increase later (during d 1 to 3 postinduction) ***(16)***. A housekeeping gene should be included as a differentiation-independent control. We have often use actin, although this mRNA fluctuates with growth state and is less abundant in adipocytes than in preadipocytes. Others use 2-microglobulin as a control and it reportedly does not change with differentiation ***(5,6)***. We have used Northern blots probed with cDNA radioactively labeled by random-priming ***(48)***, using a commercially available kit (Life Technologies). Mouse cDNA probes can be obtained commercially from the ATCC or through collaboration. We often used actin as a control and the fatty-acid binding protein gene, *aP2*, together because we could hybridize Northern blots with both at the same time (they are well separated on a gel). Also, *aP2* is a convenient end point differentiation marker (*see* **Fig. 2**).

   To prepare probes, the cloned cDNA is amplified in *Escherichia coli*, plasmid isolated as a large preparation and digested with a suitable restriction enzyme to give fragments for labeling (100–1000 bp). The fragments are then resolved on low-melting-point agarose, such as SeaPlaque using 1X Tris-acetate running buffer, visualized with long-wavelength UV, and cut from the gel using a sterile scalpel blade or new straight-edge razor blade. Dispose of blade after use. Reuse could result in cross contamination of DNA fragments. The gel slices are then stored in microfuge tubes at 4°C. When needed, the slice is melted in a 70°C water bath and an aliquot containing 25 ng is removed and used for labeling (the concentration of DNA is determined from the known amount of DNA loaded on the gel), the size of the fragment cut out, and the volume of the gel slice (weigh the tube before and after adding the slice and assume a density of 1 gm/mL). We

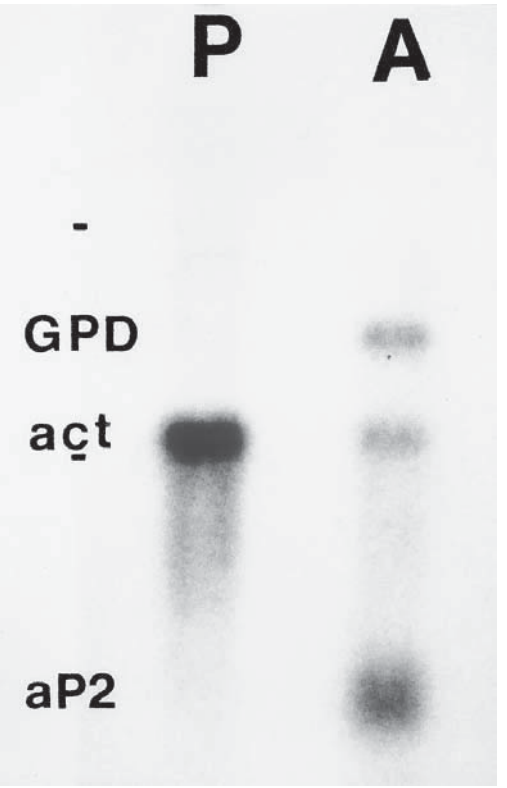

Fig. 2. Autoradiogram of Northern blot of a 1.5% agarose gel probed with [^{32}P]dCTP labeled cDNAs for mouse actin, mouse GPD, and mouse adipocyte P2 mRNA. Probes were labelled by random priming. Fragments of cDNAs for mouse GPD, actin, and *aP2* were excised from PBR322 using *Pst*1 restriction endonuclease. Mouse GPD, actin, and *aP2* mRNAs are 3.5kb, 2.15 kb, and 0.65 kb, respectively. Dashes mark the position of 28S and 18S ribosomal RNAs, respectively. Each lane was loaded with 10 µg of total RNA. Lane P: RNA prepared from preadipose 3T3-F442A. Lane A: RNA prepared from adipose-differentiated 3T3-F442A (7 d after reaching confluence). The plasmids containing adipocyte-specific cDNAs that we used were originally described by Spiegelman ***(47)***, although in this reference the *aP2* product is referred to as "13k." The adipocyte-specific and the actin probes were obtained from Dr. Bruce Spiegelman (Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA, 02115).

have found that fragments isolated this way can be labeled well by random priming, even without prior ethanol precipitation.

The following is based on instructions provided along with the Life Technologies random priming radiolabeling kit. Follow the instructions of the manufac-

turer of the particular product you use. Melt the gel slice containing the cDNA probe at 70°C, remove volume containing 25 ng DNA and bring to 23 µL with water. The low-melting-point agarose and Tris-acetate buffer in the gel slice do not interfere with the random priming reaction when diluted into the reaction mix. Boil the DNA for 5 min to denature then cool on ice. Add 6 µL of dNTP mix (dATP, dGTP, and dTTP nucleotide mix), 15 µL of Random Primers Buffer Mixture, and 5 µL (50 µCi) of [^{32}P]dCTP, 3000 Ci/mmol, 10 µCi/µL. Mix briefly. Add 1 µL of Klenow fragment of DNA polymerase I, mix gently, and briefly centrifuge to place all liquid at the bottom of the tube. Incubate at room temperature for 3 to 4 h. Boil for 5 min to stop the reaction and denature the probe. Spin down to bring all liquid to the bottom of the tube and add to hybridization solution.

Other techniques, such as nonradioactive labeling of cDNA for Northern blotting ***(49)*** and RT-PCR ***(50)***, may be used to detect changes in the expression of differentiation-dependent mRNA.

5. One method to assay for transformation is to plate cells as a suspension in semisolid cell culture medium (e.g., soft agar) to determine if cells can grow in the absence of attachment to the culture dish (Chapter 11). We have observed that when preadipocytes expressing SVLT, unable to differentiate under monolayer culture conditions, are grown in suspension in differentiation inducing medium, all or a high proportion of the cells are able to differentiate. This demonstrates that SVLT-blocked cells are still preadipocytic under certain conditions, although the basis for this reversal of the SVLT effect is not known.

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13

SV40-Mediated Immortalization

Mary Judith Tevethia and Harvey L. Ozer

1. Introduction

Normal cells in culture divide a defined number of times before reaching replicative senescence ***(1)***. The number of divisions that cells can undergo varies with the species of animal and tissue from which the cells are derived. Cells that have exceeded their typical number of divisions are said to have an extended life-span. Cells that have permanently bypassed the natural senescence point are said to have been immortalized. Immortalization can be brought about by the accumulation of mutations in cellular genes (spontaneous immortalization) or by the introduction of specific types of oncogenes, including T antigen (T-ag).

Among rodent cell types, primary mouse C57BL/6 cells are especially useful for studying the immortalizing activity of T-ag. These cells undergo only approx four or five divisions before reaching senescence ***(2)***. Thus, when placed in culture vessels at a low cell density, the cells cannot divide a sufficient number of times to form a monolayer. If the concentration of serum in the culture medium is maintained at 10%, the only altered growth properties selected are extension of life-span and immortalization ***(3)***.

Expression of wild-type SV40 T-ag appears to be sufficient to immortalize rodent cells ***(4)***; and T-ag has been used extensively to generate cell lines from a wide variety of primary cell types. In addition to its capacity to permit cells to bypass natural senescence, T-ag can contribute activities that allow cells to escape oncogene-induced senescence. Recently, the activated *ras* oncogene was shown to induce premature senescence in primary cells ***(5)***. Cells expressing T-ag escape this senescence pathway ***(6)***. Current evidence suggests that the T-ag activities involved in escape from natural senescence and from *ras*-induced

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senescence are separable ***(7)***. Methods for assaying T-antigen-mediated escape from natural senescence (*see* **Subheading 3.1.**) and from *ras*-induced senescence (*see* **Subheading 3.2.**) in rodent cells are described below.

SV40 T-ag has also been a major effector in the immortalization of human fibroblasts. Several aspects demonstrate that additional factors are involved in these cells ***(8)***. For example, human normal diploid fibroblasts rarely, if ever, undergo spontaneous immortalization in culture. Introduction of T-ag results in the temporary bypass of replicative senescence; however, these human cells are not immortal despite persistent expression of T- (and t-) antigen. This has led to the development by the laboratory of one of us ***(9)*** and others ***(10)*** of a two-stage model of SV40-mediated immortalization of human cells. In the first stage, T-ag results in the extension of life-span beyond natural senescence. However, these SV40-transformed cells subsequently die (termed "crisis"). In the second stage, cells either survive or bypass crisis and are immortal. These stages have often been termed M1 and M2, respectively. T-ag is required throughout because both human ***(9)*** and mouse cells ***(11)*** expressing an SV40 genome encoding a temperature-sensitive T-ag are temperature-dependent for persistent growth, when SV40 is introduced into normal cells. This finding is to be contrasted to the situation in which the temperature-dependence of the transformed phenotype, but not cell proliferation, is seen when SV40 is introduced into already immortal mouse cells, e.g., 3T3 ***(12)***. Whereas rodent cells expressing T-ag immortalize at high frequency (i.e., 100%) ***(2)***, T-ag positive human fibroblasts become immortal (i.e., successful transit through M2) at low frequency ***(13–15)***.

Studies have identified two classes of cellular genes that are altered as a necessary condition of immortalization of human fibroblasts. First, a growth suppressor, termed SEN6 is inactivated ***(16)***. Second, telomere shortening characteristic of both normal and nonimmortal SV40 transformed human fibroblasts is stopped and telomere length becomes stabilized ***(17)***. Recent studies have shown that introduction of the catalytic subunit of human telomerase (hTERT) will markedly extend the lifespan and increase the frequency of immortalization of both types of cells ***(18–20)***. Indeed immortal cells have typically reactivated endogenous telomerase ***(17)***. The role of both cellular genes in rodent cells is unclear since mouse cells have considerably longer telomeres than human cells ***(21)*** and Syrian hamster fibroblasts express telomerase in vitro before and after immortalization, but rarely immortalize spontaneously ***(22)***. Methods for recovery of T-ag-mediated immortalization of human cells are described in **Subheading 3.3.**

We propose that both rodent and human experimental systems offer the opportunity for comparisons that should not only provide for the generation of

useful cell lines in a wide range of experimental systems, but also point out areas for further insights into the differences and similarities in the mechanisms involved.

2. Materials

2.1. Solutions

1. 70% ethanol.
2. Tris-buffered saline pH 7.4 (TBS): 20 m*M* Tris-HCl, pH 7.4, 150 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 0.9 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, and 0.1% dextrose. Filter-sterilize and store at 4°C.
3. Versene: 1 m*M* ethylenediaminetetraacetic acid (EDTA)Na_2, 0.15 *M* NaCl, 8 m*M* Na_2HPO_4, 1 m*M* NaH_2PO_4, 3 m*M* KCl.
4. 250 mL trypsinization (fluted) flask.
5. Trypsin-versene: 0.25% trypsin in versene.
6. DMEM 10 × 1: Dulbecco's modified Eagle's medium supplemented with 100 µg streptomycin/mL, 100 µg kanamycin/mL, 100 U penicillin/mL, 0.03% glutamine, 0.075% $NaHCO_3$, and 10% fetal bovine serum (FBS).
7. DMEM: F10 (DF) 10 × 1: Dulbecco's modified Eagle's medium and Ham's nutrient F10 medium at a 1 : 1 mixture supplemented with 100 µg streptomycin/mL, 100 U penicillin/mL, 0.03% glutamine, 0.24% $NaHC0_3$, and 10% FBS. This is used in a 7.5% CO_2 atmosphere.
8. Trypan blue solution: 0.4% in 0.85% saline.
9. Freezing medium for rodent cells: 50% FBS, 40% DMEM, 10% dimethyl sulfoxide (DMSO).
10. Freezing medium for human cells: 10% FBS, 80% DF, 10% DMSO.
11. 2.5 *M* $CaCl_2$.
12. Crystal violet staining solution: 0.2% crystal violet, 0.08% ammonium oxalate, 2% ethanol.
13. 10X HEBS stock solution: 1.37 *M* NaCl, 42 m*M* KCl, 7 m*M* Na_2HPO_4, 1% dextrose, and 0.21 *M* N-hydroxyethylpiperazine-N -2-ethane sulfonic acid (HEPES).
14. Cell culture plasticware: 50-mL conical-bottom centrifuge tubes; 25 cm^2 flasks; 75-cm^2 flasks; 150-cm^2 flasks; 12-well (4.5-cm^2) plates; cryovials.

2.2. Cells and Cell Lines

1. Preparation of primary cultures of rodent cells are described in subsequent sections: primary C57BL/6 mouse (B6MEF) (3.1) (B6MEF) or primary rat (3.2) embryo fibroblasts.
2. Human fibroblasts of limited life-span can be obtained from the National Institute of Aging Collection in the Cell Culture Repository at the Coriell Institute, Camden, NJ or the American Type Culture Collection (ATCC), Rockville, MD. Preparation of primary cultures of human fibroblasts or other cell types from normal and patient sources is dependent on their respective specific requirements.

3. Methods

3.1. Escape from Natural Senescence in Mouse Fibroblasts

3.1.1. Preparation of C57Bl/6 Mouse Embryo Fibroblasts (*see* **Note 1**)

1. Obtain six timed-pregnant C57Bl/6 mice from an animal supplier at approx 12–14 d of gestation.
2. On the following day (*see* **Note 2**), harvest the embryos as follows. Following sacrifice by cervical dislocation, saturate the abdominal area with 70% ethanol (*see* **Note 3**). Using sterile, surgical scissors, make a longitudinal incision in the skin to expose the abdominal body wall. Make a similar incision in the body wall. Using sterile forceps, pull up through the incision one horn of the uterus. Cut the base of the uterus just above the cervix, freeing the second uterine horn. Transfer the uterus containing the fetuses to a 50-mL conical tube containing 25 mL TBS, and place the tube in an ice bath.
3. Once all uteri are collected, transfer the tubes to a laminar flow hood. Place each uterus in a sterile 100-cm Petri dish. Using sterile fine-tipped forceps, make an incision in the uterine wall that extends the entire distance from one end to the other. In this manner, the pups, contained in the amniotic sacs, and the placentas will be exposed. Carefully make a small cut in the amniotic sac. Generally, a single small cut will expose the fetus. Then cut between the fetus and the placenta to release the fetus. One pregnant female typically contains 6 to 9 fetuses.
4. Place the fetuses in a clean 100-mm dish. Using a sterile scalpel, remove the heads, limbs, and tails. Place the torsos in a clean dish; using forceps and scapels, mince the torsos into 1–2 mm pieces of tissue. Add approx 10 mL TBS to a dish containing the minced tissue. Use a wide bore pipet to transfer the minced tissue and liquid to a sterile 50-mL plastic screw-capped conical tube maintained on ice. Allow the tissue pieces to settle for at least 10 min (*see* **Note 4**).
5. Remove most of the TBS in each 50-mL tube following settling. Resuspend the minced tissues in 40 mL versene, prewarmed to 37°C. Using a wide-bore pipet, divide the suspension equally between two sterile 250-mL trypsinization (fluted) flasks, each containing a sterile magnetic stirring bar, 40 mL trypsin (2.5 µg/mL), and 25 mL versene, prewarmed to 37°C. Agitate the suspension on magnetic stirring devices at slow to moderate speeds for 15 min. Avoid the formation of a vortex and frothing. Turn off the magnetic stirrers and allow the tissues to settle to the bottom of the flasks. Remove about 40 mL of the fluid above the settled tissues from each flask and transfer it to sterile 50-mL conical screw-capped tubes held on ice and containing 10 mL of DMEM 10 × 1 medium. The FBS and cold temperature effectively halt further enzymatic digestion. Add 40 mL trypsin and 25 mL versene, prewarmed to 37°C, to each flask and repeat **step 5** three more times.
6. Centrifuge the 50-mL tubes containing the single-cell suspensions at 4°C for 10 min at a speed of 800 rpm (approx 200*g*) using a HL-4 rotor of a Sorvall GLC-2 centrifuge, or equivalent. Pool cells derived from the second, third, and fourth trypsinizations (*see* **Note 5**) in a total volume of 40 mL TBS. Dilute a representa-

tive sample of cells in trypan blue solution and count the number of viable cells in a hemocytometer (*see* **Note 6**). Again pellet the cells by centrifugation; and resuspend them in DMEM 10 × 1.

7. Seed the cells into T-150-cm^2 flasks containing 35 mL DMEM 10 × 1 at a density of 1.5–2 × 10^7 cells per flask. Incubate the flasks at 37°C with caps tightened until confluent monolayers form (*see* **Note 7**).
8. After monolayers form, remove the medium, rinse each monolayer with 10 mL TBS, remove the rinse, and add 5 mL of trypsin-versene. Incubate the flasks at 37°C for 5 to 10 min or until the cells are released from the plastic surface when the flask is struck sharply against the palm of the hand. Add an equal volume of DMEM 10 × 1. Combine the cell suspensions from five flasks and transfer to a 50-mL conical centrifuge tube. Repeat with the remaining flasks. Pellet the cells by centrifuging at 800 rpm in a HL-4 rotor (approx 200*g*) at 4°C for 10 min. Add 10 mL freezing medium to each tube and resuspend the pellets. Combine the cells from all tubes and distribute the pooled cells into sterile freezing cryovials using 2 mL per vial. Freeze the cells according to standard procedures (*see* **Note 8**). Maintain the vials in liquid nitrogen. These cells are considered passage "1."

3.1.2. Immortalization Assay

1. Recover a vial of frozen B6MEF at passage 1 from liquid nitrogen. Immediately place the vial in a 37°C water bath, gently shaking the vial, until the cell suspension is thawed. Immediately remove the cell suspension from the vial and place it in a 75-cm^2 tissue-culture flask containing 20 mL DMEM 10 × 1. Incubate the flask with the cap tightened at 37°C for 2–3 d, until a confluent monolayer has formed.
2. On the day before the assay is to be initiated, passage the cells 1:2 and incubate them overnight at 37°C (*see* **Notes 9** and **10**).
3. Remove the cells from the flask(s) by using trypsin-versene as aforementioned. Count the cells by the trypan blue exclusion method. For each DNA (or virus) to be tested, seed each of two 75-cm^2 culture flasks (*see* **Note 11**) containing 20 mL DMEM 10 × 1 medium with 2 × 10^5cells (*see* **Note 12**). Incubate the flasks overnight at 37°C.
4. For each DNA to be transfected into the cells, prepare a calcium phosphate-DNA precipitate. To prepare 2 mL of calcium phosphate-DNA precipitate, add the following to a sterile polystyrene tube in order: 880 µL sterile water (*see* **Note 13**), 20 µg carrier DNA (*see* **Note 14**), and 2 µg plasmid or viral DNA. Mix. Add 100 µL 2.5 *M* $CaCl_2$. Mix.

   To a second tube, add 1 mL 2 × HEBS (*see* **Note 15**) pH 7.10 ± 0.05 (*see* **Note 16**). Slowly add the DNA-$CaCl_2$ mixture to the tube containing 2 × HEBS with constant slow mixing. Note: slow mixing can be accomplished by passing a stream of air bubbles through the solution. Specifically, attach a sterile 1-mL plastic pipet to a hose. Introduce this pipet into the tube containing 1 mL of 2X HEBS. Attach the other end of the "bubbler" hose to the air jet. Adjust the airflow so that bubbles rise from the tip of the pipet at a rate of approx 1 per second.

Then, using a 1-mL sterile plastic pipet, or a pipetman fitted with a sterile tip, add the $CaCl_2$-DNA solution dropwise to the tube (*see* **Note 17**).

Allow the precipitates to stand at room temperature for 45 min. Resuspend the precipitate by gentle shaking. Add 1.0 mL of the precipitate directly into the medium in each of two flasks of cells. Incubate the flasks at 37°C for 16–24 h (*see* **Note 18**).

5. Remove the medium containing the precipitate and replace it with 20 mL DMEM 10 × 1. Close the flask tightly and incubate at 37°C. Twice a week, replace the medium in each flask with DMEM 10 × 1 (*see* **Note 19**). Colonies should form within 3–4 wk. At this point, the colonies can be stained and counted; alternatively, they can be expanded into cell lines.
6. To immortalize C57 BL/6 mouse embryo fibroblasts (B6MEF) by infection with SV40 virus, remove the medium from flasks of cells prepared as described in **step 3**. Immediately add 2 mL TBS containing 1×10^7 plaque-forming units (PFU) of virus. Incubate the flasks at 37°C for 3 h, redistributing the fluid in the flask occasionally. Then add 20 mL DMEM 10 × 1. Incubate the flasks at 37°C with the caps tightened. Twice a week, replace the medium in each flask with DMEM 10 × 1 (*see* **Note 32**).
7. To stain the colonies, remove the medium from the flask. Rinse the flask with approx 10 mL TBS two times. Add sufficient crystal violet staining solution to cover the bottom of the flask and stain for 5–10 min at room temperature. Pour off the stain and rinse the flasks with tap water until the water poured from the flask is clear. Allow the flasks to dry. Count the colonies.
8. To expand individual colonies into cell lines, remove individual colonies from the surface of the flask by using cloning pipets (*see* **Notes 20–22**). Transfer the colony to a well of a 12-well plate (*see* **Note 23**) containing DMEM 10 × 3 (*see* **Note 11**). Incubate at 37°C in a CO_2 atmosphere until the cells in the well have formed a confluent monolayer. Remove the cells from the well by treating with trypsin and transfer them to a 25-cm^2 flask (*see* **Note 24**). Passage the cells as needed.

3.2. Escape from ras-Induced Senescence in Rat Fibroblasts

3.2.1. Preparation of Rat Embryo Fibroblasts (REF) (see **Note 25**)

1. Obtain six timed-pregnant rats at approx 14–16 d of gestation.
2. Prepare, freeze, and store REF as aforementioned for B6MEF (*see* **Note 26**).

3.2.2. Ras-Cooperation Assay

1. Proceed as described for the immortalization assay, except seed 5×10^5 cells per flask. Dense colonies (*see* **Note 27**) will appear on the monolayer (*see* **Note 28**) of REF after 10–14 d (*see* **Note 29**).
2. Stain the cultures or expand colonies into individual cell lines as aforementioned (*see* **Notes 29–31**).

3.3. Immortalization of Human Diploid Fibroblasts

1. Subculture fibroblasts or other adherent cells in DF 10 × 1 or other more specialized suitable serum-containing medium (*see* **Notes 33** and **34**).
2. Plate 5 × 10^5 cells per 100-mm dish at 37°C, 24 h prior to transfection of DNA.
3. Transfect SV40 DNA as described in **step 4** of **Subheading 3.1.2.** (*see* also accompanying **Notes 13–18**; **Notes 35** and **36**).
4. Remove the medium containing the precipitate and replace it with 10 mL DF 10 × 1. Replace the medium twice a week.
5. Cultivate cells to reach confluence, changing the medium twice per week. Continue to incubate cultures in the same manner until foci appear (*see* **Notes 37** and **38**).
6. Resuspend cells with trypsin-versene once foci are clearly visible. Subculture at high cell density (1:5 ratios or 1–3 × 10^6 cells/100-mm dish). It is advised to culture all cells in order not to lose dl independent transformants (*see* **Notes 39** and **40**).
7. Cultivate cultures to confluence while observing for the reappearance of foci. Freeze replicate cultures under conditions for viable cell storage (*see* **Note 41**).
8. Subculture cells repeatedly at high cell density until the normal cells in the culture become senescent (*see* **Note 42**).
9. The cultures of the transformed cells should eventually exhibit a gradual decrease in the rate of growth. For example, there is an increased interval between subculture to reach confluence, although cells are still proliferating. This indicates the approach of the entry into crisis. Once crisis has been entered, subculture at 1:3 rather than 1:5 ratios, without discarding any cells. In the absence of cell proliferation, maintain cultures without subcultivation (*see* **Note 43**).
10. Passage cells that survive crisis at high cell densities to ensure that immortal cells are actively growing (*see* **Note 44**).
11. Freeze immortal cells under conditions for viable cell storage (*see* **Notes 45** and **46**).

4. Notes

1. Other primary rodent cell cultures can also be used for immortalization assays. However, it is important to be sure that the cells do not divide enough times to form a cell monolayer. In the event that a monolayer forms, the assay tests simultaneously for immortalization and ability to form colonies on top of a cell monolayer. Different activities of T-ag are involved in conferring these two altered cell properties. Cells expressing wild-type T-ag will acquire these properties simultaneously; however, some mutant forms of T-ag will immortalize, but will not permit formation of colonies on a cell monolayer. If primary rodent cells other than C57BL/6 mouse embryo fibroblasts are used to investigate immortalizing activities in T-ag, it may be necessary to include in the immortalization assay a dominant selectable marker such as a plasmid containing a neomycin (*G418*) resistance gene to eliminate cells that have not incorporated T-ag-coding segments. If the cell population contains cells with sufficient life-span to form a

colony, then individual colonies must be removed from the culture vessel and passaged as described below to ensure that the colonies are composed of immortalized cells.

2. Embryos should not be harvested after 16 d to term (21 d).
3. Alternatively, the entire mouse can be immersed briefly in 70% ethanol.
4. In general, the fetuses from each uterus are processed to this point individually. Thus by the time all have been harvested, the tissue pieces have settled.
5. The first trypsinization mostly contains cellular debris and is, therefore, discarded.
6. A typical 13–15-d-old fetus yields about 1×10^7 cells. Viability as assayed by trypan blue exclusion typically exceeds 95%.
7. Typically monolayers form by 3 d after seeding.
8. One procedure is to place the vials containing cells suspended in freezing-medium in Styrofoam boxes (Sarstedt) containing holes that exactly accommodate cryovials and hold at –70°C for 24–48 h. Then transfer the vials to liquid nitrogen. Vials of frozen cells can be maintained for short periods of time at –70°C, although the plating efficiency of the cells will decrease steadily with time at that temperature.
9. Primary cell cultures consist of many cell types. During passage, the composition of the cultures changes. Cells with the shortest life-span senesce and cells with longer natural life-span are enriched for. Freezing a large batch of cells at P1 and following a strict protocol of incubation time and cell passage after recovery of the cells from liquid nitrogen increases the likelihood that the population is relatively constant in composition from experiment to experiment.
10. These cells will be released from the surface of the flask after brief (1–2 min at 37°C) treatment with trypsin. Longer times of trypsin treatment may result in reduced plating efficiency of the cells.
11. The immortalization assay can be performed in 100-mm dishes. However, because the time required for colonies to develop is fairly long (3–5 wk) the risk of contamination is higher in open dishes than in closed flasks. If the assay is performed in dishes, then DMEM 10 × 3 (Dulbecco's modified Eagle's medium supplemented as for DMEM 10 × 1 except with 0.225% $NaHCO_3$) should be used.
12. Individual batches of MEF will differ in plating efficiency. Therefore, the first time a batch of cells is used, the number of cells to be seeded should be standardized. If the plating efficiency is high, 2×10^5 cells per flask may result in the formation of a cell monolayer. If the plating efficiency is low, then the number of immortalized colonies of cells will be low. In general, flasks containing 2×10^5 cells should yield approx 100 colonies when transfected with 1 µg SV40 DNA or plasmid containing the T-ag coding region, and a cell monolayer should not form. Note that small t-ag is not needed for immortalizing rodent (or human) cells and its presence does not enhance the frequency of immortalization of rodent cells.
13. This amount of water is based on the assumption that the total volume of carrier DNA and plasmid DNA does not exceed 20 µL. If the total volume exceeds that amount, adjust the amount of water added to compensate so that the total volume of the water-DNA solution is 900 µL.

14. Carrier DNA is prepared as follows. Place 300 mg of calf thymus DNA in a sterile flask or beaker containing a sterile magnetic stir-bar. Add 100 mL sterile water. Place the flask on a stirring device and stir the contents at room temperature until the DNA is dissolved. If not dissolved by the end of the day, place the flask at 4°C until the following morning, and then continue stirring. Add several drops of chloroform to the flask and stir the contents briefly. Allow the DNA solution to "sit" at 4°C for approx 24 h. Then remove any remaining chloroform with a sterile pipet. Store the carrier DNA in screw capped tubes at 4°C.

    When using calf thymus or salmon sperm DNA as carrier, the precipitate is very fine. The liquid in the tube will appear slightly cloudy. The precipitate can be visualized easily by observing the liquid under a microscope. High-molecular-weight cellular DNA extracted from cells can also be used as carrier and produces a heavy flocculate precipitate. The immortalization efficiency, however, does not substantially differ with the source of the carrier DNA or the nature of the precipitate.
15. To prepare 2X HEBS buffer dilute a 10X stock solution 1 to 5 with sterile distilled water. Adjust the pH to 7.10 ± 0.05 with NaOH. Sterilize the solution by passing it through a sterile 0.2-micron filter. The 10X HEBS can be stored frozen at –20°C indefinitely. The 2X HEBS pH 7.10 ± 0.05 can be stored at 4°C for several months.
16. The pH of the 2X HEBS is critical. Graham and van der Eb ***(23)*** showed that deviation from the pH 7.10 ± 0.05 sharply diminishes the frequency of transformation.
17. Instead of using a gentle stream of bubbles to slowly mix the DNA-$CaCl_2$ solution with the 2X HEBS, the tube containing the 2X HEBS can be gently shaken while the DNA solution is added slowly. Draw the solution up into a 1-mL sterile plastic pipet or a disposable tip fitted to a pipetman set at 1000 µL. Add the DNA mixture dropwise if using a 1-mL pipet or add three to five drops of the mixture if using a pipetman pipettor to the tube containing 2X HEBS. Shake the HEBS-containing tube gently after each addition.
18. The time between adding the precipitate and removing the medium on the following day is fairly flexible. Sixteen to 24 h exposure to the precipitate is appropriate. Leaving the precipitate in the cultures for longer than 24 h results in decreased cell viability.

    In our hands, the technique of calcium-phosphate transfection is simple, inexpensive, and sufficiently effective for this purpose. Other expression techniques (e.g., retroviral vectors, lipofection) could also be used but are generally not necessary. DEAE dextran is toxic to mouse cells and does not permit efficient integration.
19. The concentration of FBS should be maintained at 10% in order to avoid simultaneous selection of cells that are immortalized and have lowered requirement for growth factors.
20. Cloning pipets are prepared as follows. Place a rubber bulb on the end of a Pasteur pipet. Hold the other end of the pipet with forceps. Place the pipet in a flame

such that the flame heats a portion of the narrow end of the pipet approx 1 inch from the end. When the glass is softened, bend the heated portion to give a 45° angle and at the same time use the forceps to pull the end of the pipet downward to narrow the diameter. Break off the end of the pipet that was held with the forceps at a point that leaves no more that approx one-half inch between the bend and the narrowed end of the pipet. Fire polish the narrow end of the pipet by heating in a flame while depressing the rubber bulb to prevent the end from sealing. To remove a colony, insert the pipet with rubber bulb attached into the flask immediately over the colony. With the pipet still above the medium, depress the rubber bulb and lower the pipet to the colony. Gently scrape the colony from the surface while releasing the rubber bulb to draw the colony into the pipet.

21. If the immortalization assay was performed in 100-mm dishes, then a sterile tip fitted to a 100-µL pipetman can be used to scrape the colony from the dish.
22. A mass culture representing a pool of all colonies in a flask can be generated by removing the cells by treating with Trypsin-versene and adding the single-cell suspension to a T75 flask containing DMEM 10 × 1.
23. Surface area of 4.5 cm^2 per well.
24. After reaching this stage, the culture is said to be immortalized once it has been passaged ten times at a 1:10 split ***(2)***. Colonies that develop from cells expressing wild-type T-ag are uniformly immortalized. However, either an N-terminal or a C-terminal fragment of T-ag can extend the life-span of B6MEF cells and allow the formation of colonies, although neither fragment will fully immortalize the cells ***(2)***.
25. B6MEF are not suited for *ras*-cooperation assays. T-ag apparently cannot overcome the combined effects of the natural short life-span and *ras*-induced senescence.
26. A typical 14–16-d-old fetus yields about 2×10^7 cells. Viability typically exceeded 95%.
27. Colonies expressing T-ag and *ras* are very dense and increase in size rapidly. The colonies have a tendency to lift off of the cell monolayer. The remaining cells will grow to fill in the "hole" left when the colony dissociated from the monolayer. The cells in these centers usually are spatially separated from one another, smaller than surrounding cells, and stain more darkly with the crystal violet stain. Thus, there appear to be holes in the monolayer that contain a single layer of cells with transformed morphology as well as dense foci. Both types represent colonies of *ras*-transformed cells.
28. REF divide a sufficient number of times in culture to form a cell monolayer when seeded at the cell density described. At lower cell density, the cells will divide to form individual colonies. An activated *ras* oncogene will induce immortalized cells, but not primary cells to form dense foci. In T-ag-*ras*-cooperation assays, because all of the activities needed to form dense foci are provided by the *ras* oncogene, only immortalization-related activities of T-ag are monitored.
29. It is often necessary to increase the buffering capacity of the cell-culture medium during the assay. If the assay is performed in closed vessels, such as 75-cm^2 flasks with the caps tightened, DMEM 10 × 1 is usually sufficient for the initial week of the assay. If the medium appears to become acidic during the 2–3 d

following addition of fresh medium, medium with twice the normal amount of sodium bicarbonate should be used. Medium containing three times the normal amount of sodium bicarbonate also can be used, if necessary. If the assay is performed in open dishes incubated in a CO_2 atmosphere, then DMEM 10 × 2 containing 25 m*M* HEPES can be used.

30. Essentially, all colonies expressing wild-type T-ag and *ras* can be expanded into immortal cell lines.
31. Mass cultures can also be generated. The first few passages of the culture will contain a mixture of normal REF and transformed REF. However, the transformed cells will rapidly outgrow the normal cells. The replicative capacity of the normal cells diminishes with each passage, and their growth rate is substantially less than that of the transformed cells.
32. SV40 virus must be handled according to BL-2 procedures. Because the medium will contain virus that has not adsorbed to cells, handle and disinfect the medium removed from the flasks according to BL-2 procedures.
33. Human fibroblasts can be used at different population doublings for the purpose of immortalization. Normal fetal fibroblasts characteristically undergo 60–65 population doubling prior to senescence whereas fibroblasts from adult sources undergo fewer population doublings. Hence, the prior subculture history is of predictive value in respect to the life-span of the nonimmortal cell population.
34. Human fibroblast cell lines obtained from reference sources cited earlier (*see* **Subheading 2.2.2.**) can be obtained at particular population doublings. It is, however, possible to determine life-span of cells of interest by subculture at 1 : 4 split ratio in DF 10X1 growth medium or more specialized medium in one's own laboratory. The number of population doublings achieved at each subculture can be calculated using a formula such as $NC/NS = 2X$ (where C = number of cells harvested at confluence; S = initial number of cells seeded; X = population doublings). Replicative senescence is reached when there is a less than a twofold doubling of cell number over a 2-wk period at the terminal subculture, the labeling index and/or rate of DNA synthesis is less than 5% of that found for early passage cells, or the percentage of cells positive for senescence-associated -galactosidase is greater than 90% ***(24)***.
35. Methods for introduction of SV40 sequences into rodent fibroblasts by the calcium phosphate—DNA precipitation procedure are also applicable to human diploid fibroblasts although the incubation is more typically for 4–16 h. A variety of other methods for DNA transfection have been described in the literature and are also applicable. It should be pointed out, however, that the frequency of expression of transfected sequences using a transient assay is far greater (> 1000-fold) than the frequency of stable transfectants in human fibroblasts. When SV40 sequences encoding T-ag are introduced, the frequency of stable transfectants is higher than in the absence of such sequences, even when a separate plasmid encoding a drug resistance marker is being assessed ***(25)***.
36. Human fibroblasts are permissive for replication of plasmids containing an SV40 origin of DNA in the presence of T-ag and semipermissive for virus production

(26). Consequently both integrated and nonintegrated sequences are persistently present when wild-type SV40 virus or DNA sequences are introduced. Many commonly available constructs contain an SV40 origin embedded in the SV40 promoter utilized for selection of transfectants for drug resistance. Although these plasmids cannot replicate (amplify) on their own, they can when introduced into a cell expressing T-ag, as by cotransfection. To avoid these complications, we have generated a construct pRNS-1 *(25)* which contains an SV40 genome with a 6 basepair deletion in the origin, precluding its replication. It also has a gene encoding resistance to the neomycin analog G418 driven by the LTR of Rous Sarcoma Virus. Cell lines stably transfected with pRNS-1 persistently maintain only one to a few integrated copies.

37. Human fibroblasts expressing wild-type T- and small t-antigens form dense "transformed" foci. It is, therefore, possible to calculate the transformation frequency as evidence of a successful transfection by crystal violet staining of these postconfluent cultures (*see* **Subheading 3.1.2., step 7**). Because immortalization is a distinct two step process in human cells as contrasted to rodent cells, cells in such foci are not immortal; similarly, the immortal phenotype is not dependent on expression of all parameters of the transformed phenotype.
38. When constructs encoding T-ag, but not small t-ag are used, transformed foci are not evident *(27)*. Consequently, in those cases or when the investigator desires to avoid secondary changes related to the growth properties of transformed cells, it is preferable to obtain stable transfectants as discrete colonies containing SV40 sequences by selection for a drug-resistance marker as described in **Note 1**, keeping in mind the consideration in **Note 36**.
39. Individual foci may be isolated using cloning pipets, as described in **Subheading 3.1.7.** and accompanying **Notes**.
40. The frequency of immortalization of even SV40 transformed human fibroblasts is quite low and varies among transformants *(13–15)*. Hence, the likelihood that any particular transformant will yield an immortal subline is not assured. By using pooled transformants, one increases the likelihood of recovery of at least one immortal subline. Because this procedure involves subculture of pooled cell populations, it usually results in appearance of immortalized sublines that are not independent. We have found, however, that distinct transformants can be recognized by comparison of the Southern blot analysis of the integrated SV40 sequences when replication-defective constructs are used.
41. Frozen storage of cells that have been held postconfluence may show reduced viability. It is advisable therefore to subculture cells at a 1:2 ratio and harvest the cells for freezing on the next day using the method described in **Note 8**.
42. In the event that immortal cell lines are not isolated following crisis (**step 10**), cells at this earlier passage should be thawed and **steps 8–11** repeated with larger cell numbers.
43. SV40 transformation results in extension of life-span for an additional 20–30 population doublings beyond that of normal fibroblasts. Hence, the population of cells should all be positive for T-ag at this point.

44. Entry into crisis is evident by the appearance of detached and floating cells under subconfluent conditions. There may not be a net increase in viable cell numbers because of a balance of cell proliferation of attached cells and their detachment. Detached cells are predominantly nonviable by trypan blue exclusion tests. Crisis may extend over a several-week period.
45. The mechanism of crisis is still unclear, but cultures in crisis undergo progressive and massive cell death, with up to 50% of the cells in apoptosis late in crisis. Nonetheless, crisis is a stochastic process; as is replicative senescence. Consequently, it is not unusual to see areas of residual cell clusters and proliferation even 2–3 wk after the onset of crisis. Passage of these populations of cells show them not to be immortal. A minority may be immortal. Microscopic examination of the cell clusters may be informative because the nonimmortal clusters of long-lived cells typically show a mixture of viable and nonviable cells. Fungizone (25 µg/mL) is generally added to the medium to minimize contamination, since individual culture dishes are incubated for the several weeks of crisis.
46. As in SV40-immortalized rodent cells, it is useful to prepare frozen stocks soon after isolation for reference purposes. By convention, a subline is considered immortal when it has undergone more than 100 population doublings. Detailed characterization of the immortal cells may not be warranted. However, they should be 100% positive for T-ag by immunohistochemical methods, and most, although not all, immortalized derivatives show reexpression of telomerase enzymatic activity in cell extracts ***(17)***.

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14

Biology of *p53* and SV40 Large T Association

Ximena Montano

1. Introduction

p53 was discovered because of its capacity to bind to SV40 large T antigen (SVLT) in transformed cell lines ***(1,2)***. It is a phosphorylated, 393 amino acid protein, with five blocks of sequences that are highly conserved throughout evolution. Early research showed that *p53* could act as a dominant oncogene; however, comparisons at the DNA level of cloned *p53* from different tumor cell lines showed that these results represented the action of the mutated rather than the wild-type gene, and that the product of the wild-type gene had a tumor suppressor activity instead ***(3,4)***. *p53* is the most frequently mutated gene in a variety of malignancies, as well as in transformed cell lines ***(5)***; inactivation of *p53* is generally associated with loss of the wild-type allele. In other cases, such as the Li-Fraumeni Syndrome, where one allele is in the form of a dominant-negative mutant, both the mutant as well as the wild-type allele can be detected ***(6)***.

Wild-type *p53* has an average half-life of 30 min and is present in small amounts in normal tissues, whereas mutated *p53* has a prolonged half-life and is found at high levels in transformed cell lines and tumors ***(3,4)***. Wild-type *p53* protein possesses features of a transcription factor, located at the N-terminus ***(7)*** and can activate target genes by binding to specific DNA sequences located in their promoters ***(8)***. On the other hand, *p53* can also repress transcription from promoters that do not contain *p53* binding sequences ***(9,10)***. Furthermore *p53* can inhibit helicase activity and DNA replication ***(11,12)***.

Overexpression of wild-type *p53* induces growth arrest at the G1 and G2/*M* phases of the cell cycle ***(13,14)***, as well as differentiation and apoptosis ***(15,16)***. There is evidence suggesting that *p53* may play a key role in the prevention of genetic instability induced by DNA damaging agents ***(17)***.

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Analysis of growth arrested and mitogen-stimulated cells has shown that the localization of *p53* varies during the cell cycle. After mitogenic stimulation, cells contain *p53* in the cytoplasm during G1 and in the nucleus during *S* phase; on progressing through the *S* phase, the *p53* locates mostly to the cytoplasm ***(18–20)***. Amino acid analysis has shown that *p53* contains both nuclear localization, as well as nuclear exclusion signals ***(21)***.

Aside from SVLT, both mutated and wild-type *p53* have the capacity to bind to several proteins such as *Hsp*70, *TBP*, *CDC2*, *trk A*, and *WT1* ***(4,15)***. More careful analysis has been performed on its ability to bind to MDM-2 and it showed that there is a tight regulatory feedback between the two proteins ***(4)***.

This chapter describes techniques to analyze the *p53*/large T association, which can also be used to analyze the association of *p53* with other proteins. The most common methods used to identify *p53*/large T complexes, or the association of *p53* with other proteins are: immunoprecipitation followed by Western blotting, immunostaining, two-site enzyme-linked immunosorbent assay (ELISA), solid-phase ELISA, and in vitro association assays.

2. Materials

2.1. Cell Culture

1. SV40 transformed cell lines such as SVA31-E7 or SV3T3 are maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS).
2. Cell lines derived from human tumors, e.g., melanoma cell lines such as HMB-2 or MJM, are maintained in DMEM supplemented with 10% heat-inactivated FCS.
3. Nontransformed cell lines such as NIH3T3 cells are maintained in DMEM supplemented with 10% heat-inactivated calf serum.
4. In all cases, cells are grown to 80% confluency, then either passaged or used for further analysis. All cell lines are maintained at 37°C, in a humidified incubator with 10% CO_2.

2.2. Antibodies

2.2.1. Antibodies to p53 and SV40 Large T Antigen

There is currently a large collection of monoclonal, as well as polyclonal antibodies against *p53* that recognize a variety of epitopes. Some are specific for wild type (wt) *p53*, whereas others recognize both wt and mutant *p53*. Most of them are available commercially from a variety of sources (e.g., Sigma). The most commonly used primary antibodies are:

1. PAb DO-1, a mouse monoclonal antibody that recognizes only wt human *p53* at a denaturation-resistant epitope located between amino acids 20 and 25 ***(22)***.

2. PAb 1801, a mouse monoclonal antibody to human and *p53* that recognizes a denaturation-resistant epitope located between amino acids 32 and 79 of wt *p53* ***(23)***.
3. PAb 240, a mouse monoclonal antibody that recognizes mutant *p53* across species at an epitope located between amino acids 212 and 217 ***(24)***.
4. PAb 421 recognizes human and murine, mutant and wt *p53* at a denaturation-resistant epitope located between amino acids 373 and 381 ***(25)***.
5. The most efficient polyclonal is CM-1. This is a rabbit polyclonal antiserum raised against recombinant human *p53* ***(26)***.
6. In the case of SVLT, the most commonly used and efficient antibody is PAb 419 ***(25)*** that recognizes an epitope within the first 82 amino acids of large T, a sequence that includes st (*see* Chapter 17).
7. There are several commercially available rabbit antilarge T polyclonal antisera and all recognize SVLT with similar efficiencies, although there are significant lot-to-lot variations.

2.2.2. Secondary Antibodies

1. Western blotting and immunocytochemistry: horseradish peroxidase (HRP)-conjugated, antimouse or antirabbit antibodies (cat. no. PO447 and PO399, respectively, Dakopatts; Denmark). Biotinylated antibodies and streptavidin/HRP conjugate (cat. no. P0397, Dakopatts; Denmark).
2. For immunofluorescence, FITC, rhodamine, or Texas Red–conjugated antimouse or antirabbit antibodies, available from a number of suppliers (e.g., Amersham or Organotechnica, West Chester, PA, or Vector Laboratories, Burlingame, VT).

2.3. Solutions

2.3.1. Immunoprecipitation and Western Blotting

1. NET-N: 20 m*M* Tris-HCl pH 8.0, 100 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), and 0.5% Nonidet P-40 (NP40).
2. EBC buffer: 50 m*M* Tris-HCl pH 8.0, 120 m*M* NaCl, 0.5% NP40.
3. Tris-buffered saline (TBS): 137 m*M* NaCl, and 20 m*M* Tris-HCl pH 8.0.
4. TBS-T: 137 m*M* NaCl, 20 m*M* Tris-HCl pH 8.0, and 0.2% Tween-20.
5. Lysis buffer: 20 m*M* Tris-HCl pH 8.0, 137 m*M* NaCl, 10% glycerol, 1 m*M* phenylmethylsulfonyl fluoride, 0.15 U/mL aprotinin, 20 µ*M* leupeptin, and 1% NP40.
6. 3 3 Diaminobenzidine (DAB) tablet set (Sigma, cat. no. D-4168), used according to the manufacturer's recommendations, or DAB-free base powder (Sigma, cat. no. D-8001) prepared as 1 mg/mL in PBS with 11 µL/mL 30% H_2O_2. For increased sensitivity, enhanced chemiluminescence (ECL) can be employed, using reagents available from a variety of sources (e.g., Tropix, Amersham).
7. Protein G fast-flow Sepharose beads purchased from Pharmacia (cat. no. 17-0618-01). Resuspend in lysis buffer containing 1 mg/mL bovine serum albumin (BSA), or in NET-N buffer containing 1 mg/mL BSA.
8. Transfer buffer: 3.05 g Tris-base, 14.42 g glycine in 1 L.

2.3.2. Immunostaining, ELISA, and In Vitro Associations

1. 50:50, acetone:methanol.
2. DAB solutions, as in **step 6**.
3. 10 m*M* phosphate buffer pH 7.5.
4. Protein assay kit purchased from Bio-Rad and used as indicated by the manufacturer (cat. no. 500-0006).
5. Protein G fast flow, prepared as in **step 7**.
6. BSA for coating plates (Sigma, cat. no. A-7030).
7. 3,3,5 -5 Tetramethylbenzidine (TMB): From Sigma (cat. no. T-2885), prepared as 10 mg/mL in DMSO (TMB buffer).
8. Substrate buffer: 0.1 *M* citrate/phosphate pH 6.0 (0.1 *M* Na_2HPO_4 and 0.1 *M* citric acid).
9. Vitrogen-100 collagen purchased from Biomaterials, Palo Alto, CA (cat. no. PCO701).
10. 1 *M* H_2SO_4.

2.4. Equipment

1. Gel electrophoresis apparatus for SDS polyacrylamide gels, available from a number of suppliers (e.g., Bio-Rad, Gibco-BRL).
2. Gel transfer apparatus (e.g., from Bio-Rad, Novex, Hoeffer).
3. 10-cm and 3.5-cm plastic tissue-culture dishes.
4. Glass cover slips and slides (Fisher).
5. 96-well plates, available from a number of suppliers (e.g., Falcon, Nunc). They are used for cell growth for immunocytochemistry, as well as for ELISA assays.
6. Nitrocellulose (Schleicher and Schuell Protan BA 83, cat. no. 401-391).
7. Humidified staining chambers (Fisher).

3. Methods

3.1. Immunoprecipitation and Western Blotting

Two methods of immunoprecipitation and Western blotting for the detection of SVLT/*p53* binding are described. In both cases, immunoprecipitation can be with either anti-*p53* or anti-SVLT antibodies and the blots probed for SVLT or *p53*, respectively. Method 1 is optimal for the immunoprecipitation of *p53*-associated proteins; however, if this approach gives too much background, then Method 2 (*see* **Subheading 3.1.2.**) should be employed.

The position of the 53-kDa band relative to the heavy chain of IgG depends upon the IgG used. To avoid this background that may completely obscure the *p53* band, it is better to immunoprecipitate SVLT with an antibody from one species (e.g., mouse), then probe the blot with an antibody from a different species (e.g., rabbit). The secondary antirabbit in this case will not recognize the mouse anti-SVLT IgG present in the blot.

3.1.1. Method 1

1. Grow approx 5×10^6 cells in 10-cm plates. Rinse cells three times with TBS.
2. Lyse in 200 µL of ice-cold lysis buffer and incubate for 10 min on ice (*see* **Note 1**).
3. Spin at 4°C for 5 min at 10,000*g*.
4. Determine protein concentration in the supernatants using the Bio-Rad protein assay kit according to the manufacturer's instructions.
5. Incubate with monoclonal antibodies against *p53* or large T for 2–3 h at 4°C, using the concentration suggested by the manufacturer of the particular lot.
6. Collect the precipitates by binding onto 20 µL of protein G Sepharose beads resuspended in lysis buffer containing 1 mg/mL BSA, for 1 h at 4°C. Protein G is preferable to protein A because it reacts with a wider range of antibodies.
7. Wash three times with ice-cold lysis buffer.
8. Boil the beads for 2 min in sample buffer.
9. Run the samples on sodium dodecyl sulfate (SDS)-polyacrylamide gels (*see* **Notes 2** and **3**).
10. Transfer the resolved proteins onto nitrocellulose (*see* **Note 4**).
11. Block the membrane for 1 h in TBS containing 2% BSA.
12. Incubate with CM-1 rabbit anti-*p53* or rabbit anti-large T polyclonal antibodies [diluted 1 : 1000 in TBS containing 0.2% Tween (TBST)] at 4°C overnight.
13. Wash three times with TBST for 5 min at room temperature.
14. Detect bound antibody by incubating with peroxidase-conjugated, antirabbit antibodies (diluted 1 : 20,000 in TBST) for 1 h at room temperature.
15. Wash three times with TBST and visualize by chemiluminescence (ECL) according to the manufacturer's instructions.
16. If desired, blots can be stripped of antibody by incubating with 2% SDS, 100 m*M* 2-mercaptoethanol, 62.5 m*M* Tris-HCl pH 6.8 for 1 h at 70°C. Rinse three times for 15 min with TBS, then probe with a different antibody.

3.1.2. Method 2

1. Wash cells three times with PBS.
2. Wash twice again with ice-cold PBS to reduce protease activity.
3. Drain thoroughly and lyse in 200 µL EBC buffer containing 50 m*M* phenyl methyl sulphonyl fluoride (PMSF) at 4°C.
4. Clarify the extracts by centrifuging for 5 min, 10,000*g* at 4°C. Collect the supernatant.
5. Add anti-*p53*, PAb DO-1 hybridoma supernatant and incubate by mixing at 4°C for 1–2 h.
6. Add 20 µL protein G Sepharose beads, resuspended in NET-N buffer containing 1 mg/mL BSA, and incubate for a further 30 min.
7. Pellet the beads with a brief spin in a microcentrifuge.
8. Wash the resulting immunoprecipitates twice with NET-N containing 0.5 *M* LiCl.
9. Wash once with NET-N.

10. Boil the final pellet in SDS sample buffer.
11. Run the samples on SDS-polyacrylamide gels.
12. Transfer the resolved proteins onto nitrocellulose as in Method 1 (*see* **Subheading 3.1.1.**).
13. Block the blot for 1 h in PBS containing 0.1% Tween-20 (there is no need for BSA addition).
14. Wash twice with PBS.
15. Incubate with rabbit polyclonal anti-*p53* CM-1, or rabbit polyclonal antilarge T antiserum at 4°C overnight, according to the manufacturer's instructions.
16. Wash the blot three times for 5 min at room temperature.
17. Detect antibody binding by incubating with pig antirabbit, peroxidase-conjugated immunoglobulins (dilution 1:5000 in PBS, or according to the manufacturer's instructions), for 1 h at room temperature.
18. Wash three times with PBS and visualize by adding 3 3 DAB/hydrogen peroxide until color develops.
19. Stop the reaction by rinsing with distilled water.
20. Alternatively, the bands can be visualized by ECL as in **Subheading 3.1.1.**

3.2. Immunostaining

1. Grow the cells on collagen-treated cover slips.
2. Fix with ice cold, 50:50 acetone:methanol.
3. Once the fixative has evaporated, place the slides in phosphate-buffered saline (PBS) for 5 min.
4. Incubate for 2 h at room temperature (or overnight at 4°C) with the test monoclonal antibody hybridoma supernatant or polyclonal antibody diluted in DMEM or PBS containing 10% FCS (*see* **Note 5**).
5. Rinse cover slips three times in PBS for 2 min.

The sensitivity of detection of the primary antibody required, depends upon the level of *p53* present in the cells. wt *p53* is found at low levels, hence, an additional amplification step is necessary. On the other hand, the levels of mutant *p53* are higher, so that good results can be obtained with an HRP-conjugated secondary (*see* **Note 6**).

3.2.1. Detection of Endogenous *wt* p53—*Indirect HRP Staining*

1. Depending on the primary antibody used, incubate the cover slips with biotinylated, affinity purified, antimouse or antirabbit immunoglobulins (Dakopatts) for 45 min at room temperature.
2. Wash with PBS as before and incubate with a streptavidin/HRP conjugate, prepared according to the manufacturer's instructions (Dakopatts), for 45 min at room temperature.
3. Wash three times in PBS.
4. Visualise the immune complexes formed using DAB/hydrogen peroxide. Allow the reaction to develop for 1–3 min.

5. Stop the reaction using tap water.
6. Mount the cover slips in Univert (BDH, cat. no. 36118 2E) and leave to dry overnight. Examine under a fluorescence microscope.

3.2.2. Detection of Mutant p53*—Direct HRP Staining*

1. Incubate the cover slips with HRP-conjugated, antimouse, or antirabbit immunoglobulins for 45 min at room temperature, depending on the primary antibody.
2. Wash three times in PBS.
3. Visualize *p53* using DAB/hydrogen peroxide as before.

3.3. Immunofluorescence

Immunostaining works well for the detection of a single antigen (SVLT or *p53*). However, to detect *p53* and large T simultaneously, the immunofluoresce technique is prefered. The same procedure of plating, fixing the cells, and incubating with the primary antibody aforementioned can be used. The two primary antibodies must be from different species, e.g., the first from rabbit and detected by a rhodamine-coupled, antirabbit secondary, the second one from mouse, detected by an FITC-coupled antimouse secondary.

1. Incubate cells with rabbit CM-1 anti-*p53* antibody and rinse three times with PBS.
2. Incubate further with antirabbit Texas Red or rhodamine-conjugated immunoglobulins (Amersham) (diluted 1:30 in PBS containing 10% FCS) for 2 h at room temperature in a humidified chamber in the dark to avoid quenching.
3. Rinse cover slips three times in PBS.
4. Incubate with the mouse anti-SVLT, PAb 419 hybridoma supernatant.
5. Rinse three times and incubate with antimouse, FITC-conjugated immunoglobulins (diluted 1 : 50 in PBS containing 10% FCS) for 2 h at room temperature in a humidified chamber, in the dark to avoid quenching.
6. Rinse and mount in Univert as before.

3.4. Two-Site ELISA

Two methods for two-site ELISA are described. In both cases, an anti-*p53* antibody, which is bound to an ELISA well, will attract *p53* from a lysate added. After washing, the *p53* bound to the plate is detected with a second anti-*p53* antibody. Method 1 has a high sensitivity, whereas Method 2 can be used when samples have easily detectable *p53* such as mutated or overexpressed wt *p53*.

3.4.1. Method 1

1. 96-well plates are incubated overnight at 4°C with 50 µL/well of, e.g., PAb DO-1 ascites fluid or purified monoclonal antibody diluted to 30 µg/mL in 100 m*M* Na_2CO_3 and 100 m*M* $NaHCO_3$ pH 9.8 (*see* **Note 7**).
2. Rinse plates once in PBS.
3. Block with PBS containing 3% BSA for 3 h at room temperature.

4. Rinse plates with PBS and further incubate for 3 h at 4°C with serially diluted cell extracts (>100 µg/mL protein) prepared as described in the immunoprecipitation section of this chapter.
5. Wash plates twice with PBS and twice with PBS containing 0.1% NP40.
6. Incubate for 2 h at 4°C with rabbit polyclonal serum CM-1 diluted in PBS containing 10% FCS.
7. Wash plates as before and incubate for 2 h at 4°C with HRP-conjugated, pig antirabbit immunoglobulins (Dakopatts) diluted 1:1000 in PBS containing 5% FCS (*see* **Note 8**).
8. Wash plates as before.
9. Visualize using 50 mL of 3,3,5 -5 tetramethylbenzidine substrate for 20–30 s (mix 6 mL of substrate buffer with 60 mL of TMB buffer, then add 15 mL of 30% H_2O_2).
10. Stop the reaction with 1 *M* H_2SO_4 and read the results at 450 nm in a Titertek plate reader ***(22)***.

3.4.2. Method 2

1. Pipet 50 µL of purified anti-*p53* monoclonal antibody (30 µg/mL in 10 m*M* phosphate buffer pH 7.5) into each well of a microtiter dish and allow to adsorb overnight at 4°C in a humidified chamber.
2. Rinse the plate with PBS and block in PBS containing 3% BSA for 3 h at room temperature.
3. Rinse plate in PBS and either use immediately or store at –20°C.
4. Add a 50-µL sample of clarified cell lysate (*see* **Subheading 3.1.**) to each well of the antibody coated plate.
5. Incubate overnight at 4°C.
6. Rinse three times with PBS.
7. Pipet 50 µL of rabbit CM-1 polyclonal serum diluted in PBS containing 10% FCS into the wells.
8. Incubate for 3 h at room temperature.
9. Rinse the plate with PBS.
10. Visualise the presence of *p53* with 3,3,5 -5 tetramethylbenzidine as before.

3.5. In Vitro Assay of p53/SV40 T Association

This protocol is based on the two-site ELISA method. An anti-*p53* antibody is bound to an ELISA plate and a mixture of lysates containing *p53* and SVLT are added. After washing, bound SVLT is detected with a specific antibody. Alternatively, an anti-SVLT antibody can be bound to a plate, lysates added, and *p53* detected with a specific antibody.

1. Mix 50 µL of clarified cell lysate from T3T3 cells (which produce detectable amounts of mutated *p53*) and 50 µL of an SVLT-expressing cell line for 3 h at 4°C.
2. Transfer mix to an anti-*p53*, CM-1 antibody-containing plate, or a plate containing anti-SVLT, PAb 419 antibody, prepared as in **Subheading 3.4.1.**

3. Incubate overnight and rinse twice with PBS.
4. Add 50 µL of anti-SVLT, PAb 419 antibody (10 mg/mL supernatant or purified antibody diluted in PBS containing 10% FCS) for the anti-*p53* plates or anti-*p53*, CM-1 antibody (diluted as 30 mg/mL in PBS containing 10% FCS) for the anti-SVLT, PAb 419 plates, to each well.
5. Incubate for 3 h at room temperature and rinse twice with PBS.
6. Incubate the plates with HRP-conjugated, antimouse, or antirabbit immunoglobulins, depending on the second antibody added.
7. Detect the complex with 3,3,5 -5 tetramethylbenzidine.
8. Serially dilute the T3T3 or any other cell line in lysis or EBC buffer for titration purposes.

4. Notes

1. Cell extracts can be quick-frozen in dry ice/ethanol and stored at –70°C. These have to be quick-thawed by incubating at 37°C. However, there is always a risk in losing some of the associated proteins.
2. To be able to detect *p53* and large T, 7.5% or 10% SDS polyacrylamide gels are optimal. These gels can also detect the presence of polymorphic *p53*.
3. Gels are run at 170 V at room temperature. There is no need to run gels overnight as this tends to produce diffused bands.
4. Western transfer is carried out at 25 V overnight at 4°C. However, good results can be obtained when transferred at 500 mA for 4 h at 4°C.
5. The most common antibodies against murine *p53* are PAb 242, PAb 246, and PAb 248. These antibodies do not crossreact with human *p53* ***(27,28)***.
6. Although very good results are obtained with HRP-conjugated secondary antibodies, alkaline phosphatase conjugated antibodies can also be used. There are several substrates for alkaline phosphatase either for ECL or for colorimetric analysis, such as the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chloride, p-toluidine salt (NBT/BCIP) (Life Technologies), which gives a blue color.
7. Primary antibody dilutions have to be determined based on the instructions provided by the manufacturer or obtained from colleagues. For ELISAs (to either coat 96-well plates or to use the antibody as a primary antibody), as a rule, 30 µg/mL diluted in PBS containing either 10% FCS (if the antibody stock was in PBS) or 3% (if the antibody preparation contains some protein, such as gelatin).
8. Secondary antibody dilutions for ELISAs: As a rule, commercially available conjugated antibodies are diluted 1 : 1000 in PBS containing 10% FCS (if the antibody was in PBS) or 3% FCS (if the antibody preparation contains some protein, such as gelatin) for good results. If background develops fast, it is better to use a dilution of 1 : 2000 or 1 : 3000.

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15

Binding of SV40 Large T Antigen to the Retinoblastoma Susceptibility Gene Product and Related Proteins

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1. Introduction

SV40 large T antigen (T-ag) is an oncoprotein that induces transformation of cells through the binding and inactivation of the *p53* tumor suppressor protein and the retinoblastoma family of proteins. This chapter focuses on the interaction of T-ag with the product of the retinoblastoma susceptibility gene (pRB), and two related proteins, p107 and p130 ***(1–3)***.

Binding of T-ag to the pRB-family proteins requires a special motif, the *LxCxE* motif, where *x* can be any amino acid. The *LxCxE* motif is conserved between the viral oncoproteins SV40 large T-ag, adenovirus E1A, and papillomavirus E7. Mutation of this motif renders these oncoproteins unable to bind to RB-family proteins and unable to fully transform cells ***(4–6)***. In SV40 T-ag (708 amino acids), the *LxCxE* motif encompasses residues 103–107.

T-ag binds specifically to the un(der)phosphorylated form of pRB (the fastest migrating species on a polyacrylamide gel). This observation was used to correctly infer that this is the active form of pRB. This form is present in G0 and early G1 of the cell cycle. In mid-G1, pRB becomes phosphorylated and inactivated by the cdk4 and cdk 6 kinases in a complex with cyclin D. The phosphorylation state of pRB has been used as one indicator of the cell cycle phase of a population of cells.

T-ag must bind and inactivate not just pRB itself, but also the RB-family proteins p107 and p130 to elicit full transformation ***(2,3,7)***. Proteins p107 and p130 are related to pRB, and are highly homologous to each other (52% identity and 61% similarity between them). Like pRB, p107 and p130 undergo cdk4/6-cyclin

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D-dependent phosphorylation and inactivation in mid-G1 ***(8–11)***. These phosphorylations can be easily visualized as slower migrating species of protein in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels ***(8,11,12)***. However, in cells expressing T-ag, only the un(der)phosphorylated forms of p107 and p130 can be detected. It is clear that binding to T-ag alters the phosphorylation state of these proteins; it is possible that binding to T-ag prevents the phosphorylation event. T-ag also significantly shortens the half-life of p130, and may affect p107 as well ***(7,12)***.

Although the pRB-family proteins are phosphorylated at the same time in the cell cycle, their expression patterns are very different. Human cells generally express significantly higher levels of pRB than mouse cells. pRB expression remains relatively constant throughout the cell cycle of cycling cells, but protein levels are decreased in serum-starved fibroblasts and induced as quiescent cells re-enter the cell cycle. p107, on the other hand, is virtually undetectable in quiescent cells, but becomes strongly induced in G1 and S phase. p107 levels remain high in cycling cells. p130 exhibits almost the reverse expression pattern: levels are highest in quiescent cells, then decline as cells enter the cell cycle. Cycling cells express quite low, but detectable, levels of p130 ***(13)***.

The function of pRB-family proteins that T-ag inactivates is most likely their ability to bind and repress members of the family of *E2F* transcription factors ***(14)***. Inactivation of the pRB-family proteins by T-ag leads to transcription of *E2F*-dependent genes required for cell-cycle progression and inappropriate entry into S phase.

Our work on pRB-family proteins has focused on the inactivation of pRB-related proteins in T-ag-expressing cells. We describe in this chapter the procedures necessary to analyze the interaction of T-ag with pRB, p107, and p130, and to assess the phosphorylation status of these proteins.

2. Materials

1. T-ag expressing cells: We have used mouse embryo fibroblasts (MEFs) immortalized by T-ag, COS-1 cells, and cell lines in which T-ag is expressed transiently (*see* **Note 1**).
2. EBC lysis buffer: 50 m*M* Tris-HCl, pH 8.0, 120 m*M* NaCl, 0.5% Nonidet P-40 (NP40), 10 µg of aprotinin per mL, 10 µg of leupeptin per mL, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), 4 m*M* sodium fluoride, 0.1 m*M* sodium Vanadate.
3. NET-N wash buffer: 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.5% NP40).
4. Antibodies: anti-T-ag, anti-pRB, anti-p130, and anti-p107 (*see* **Note 2**).
5. Materials for immunoprecipitation, SDS-PAGE, electrotransfer, and Western blotting. Detailed information on these techniques cannot be given in this chapter, but we refer to ***(15)*** for more information.

3. Methods

3.1. Cell Culture and Extract Preparation

1. Grow cells until they are at the appropriate density (*see* **Note 3**).
2. Wash cells 2X with cold PBS, add EBC directly to the tissue culture dish (0.5 to 1 mL per 100-mm plate). Alternatively, for a more concentrated lysate, trypsinize cells, wash with PBS, then add cold EBC.
3. Incubate for 10 min at 4°C.
4. Scrape the cells off the plate with a rubber policeman and collect in a microcentrifuge tube. Spin at highest speed (e.g., 10,000*g*) for 10 min at 4°C. Transfer the supernatant into a new microcentrifuge tube. Discard the pellet.
5. Normalize samples according to protein concentration (Bradford assay or equivalent).
6. If immunoprecipitation is to be performed before the analysis by Western blot, incubate the extracts with the relevant antibody (*see* **Note 2**) for at least 1 h. Then add protein A Sepharose, continue the incubation for another hour, and wash the complexes four times with NET-N wash. If the samples are to be treated with phosphatase, do so at this step, subsequent to washing the beads (*see* **Note 5**).
7. Boil the sample in SDS-containing sample buffer.

3.2. Electrophoresis and Detection

1. Run the samples in an SDS-PAGE gel with 6% acrylamide (*see* **Note 6**).
2. Transfer the proteins to a PVDF or nitrocellulose membrane and Western blot using appropriate antibodies (*see* **Note 2**).

4. Notes

1. We have extensively used MEFs immortalized by T-ag for our functional studies. Because T-ag provides a growth advantage to these cells, its expression is likely to be maintained at high levels. COS-1 and COS-7 cells are also a widely used system when studying the biology of T-ag. They are easy to use, express high levels of T-ag, and they are derived from African green monkey, the natural host of SV40. Nevertheless, we have found one peculiarity in this system. Specifically, p107 migrates as three distinct phosphatase-resistant bands in cells derived from African green monkey, and no mobility shift is observed in monkey p107 as cells progress through late G1 phase (our unpublished data).

   Another possible system for studying T-ag–pRB-family protein interactions is cotransfection in a cell line ***(7,12,14)***. Although this is an excellent system, the following should be considered: Whereas most cell types can phosphorylate overexpressed pRB-family proteins, this is not universally true: SaOS2 cells are the notable exception ***(16)***. Also, if human or monkey cells are to be used, be aware that T-ag will cause replication of plasmids containing an SV40 origin of replication (for a review, *see* **ref. *17***). This will lead to significantly increased expression of a cotransfected protein. T-ag also significantly enhances transcription on a wide range of simple promoters (RSV, CMV, and so on) ***(18,19)***, hence,

significantly increased expression of a cotransfected protein is usually observed even with plasmids that do not have an SV40 origin of replication. Finally, T-ag will repress transcription from an SV40 promoter *(20)*, hence, this may not be an optimal promoter to drive expression of a protein in the presence of T-ag.

2. There are several excellent antibodies that can recognize T-ag both in Western blot and immunoprecipitation. Examples include the monoclonal antibodies Pab419 (Calbiochem) and PAb101 (Santa Cruz Biotechnology). Both have worked very well in our experiments. PAb419 recognizes an epitope in the N-terminal region of T-ag, and also recognizes SV40 small t antigen because the large and small T antigens share the first exon of large T-ag. PAb101 recognizes an epitope in the C-terminal half of the protein, and does not react with small t antigen. For p107 and p130, the rabbit polyclonal antibodies C-18 and C-20, respectively, from Santa Cruz Biotechnology work well; however the C-20 anti-p130 serum crossreacts with p107 under certain conditions. In the case of pRB, the monoclonal antibody G3-245 from Pharmingen is excellent, and it recognizes pRB from a variety of species, including mouse and human. Unfortunately we have observed some batch-to-batch variability in the quality of this antibody. The monoclonal antibody XZ77 (Upstate Biotechnology) is also very good, but it is specific for human pRB, and does not react with pRB from mouse or rat.
3. The appropriate cell density depends on the nature of the experiment. The phosphorylation of pRB-family proteins is affected by density. In confluent cultures (mainly G0/G1 cells), pRB-related proteins are mostly in the unphosphorylated, active form. In the case of pRB, this is the only form bound by T-ag. Hence, an interaction between T-ag and pRB is most easily detected in confluent or G0/G1 arrested cultures. In rapidly growing cells, most of the pRB protein is phosphorylated, hence, it is difficult to detect an interaction between pRB and T-ag under these conditions. p130 is most abundant in confluent or G0/G1 arrested cells, hence, a T-ag–p130 interaction is also most easily observed under these conditions. However, because T-ag causes a destabilization of p130 *(7)*, this protein is never very abundant in T-ag-expressing cells. An interaction between T-ag and p107, in contrast, is most easily detected in growing cells, because these express the highest level of the p107 protein.
5. Phosphatase treatment of proteins is used to demonstrate that any differential mobility of the proteins in SDS-PAGE is caused by phosphorylation. We find that lambda phosphatase (NEB) works very well for this. Before treatment, wash the beads twice with 50 µL 1X phosphatase buffer (as supplied). Incubate the beads with 500 U of lambda phosphatase in 50 µL buffer for 30 min at 30°C. Then wash the beads once with NET-N and proceed to **step 7** in the protocol.
6. For detection of pRB-family proteins, the percentage of acrylamide is not very important. If the goal is to resolve phosphorylation states of these proteins, on the other hand, it is important to use low percentage gels. Five to 8% acrylamide will work, but high percentage gels (10% or above) and gradient gels tend to resolve the phosphorylation states of pRB-family proteins poorly. We recommend using 5 or 6% gels. Also, for optimal resolution, we use big (15 cm) gels, run at 10 V/cm

for 4 h. The gel should be run at least long enough to run the dye front off the bottom edge of the gel (longer is better). We also find that running the gel slowly (overnight) actually results in markedly *poorer* resolution of phosphorylated species.

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16

In situ Electroporation for the Measurement of c-Ras Activation by SVLT

Heather L. Brownell and Leda Raptis

1. Introduction

Simian virus 40 large-tumor antigen (SVLT) is a largely nuclear oncogene product ***(1,2)***. Evidence from a number of laboratories has indicated that the interaction between cellular nuclear antioncogenes, such as the retinoblastoma-susceptibility gene product family (Rb) and p53, mediates neoplastic transformation by SVLT, as outlined in Chapters 14 and 15. However, cytoplasmic deletion mutants, lacking the nuclear localization signal, can still transform established cell lines with efficiencies similar to that of wt SVLT. The demonstration that SVLT requires the function of the membrane-bound, cellular Ras protooncogene product to elicit neoplastic transformation of mouse or rat fibroblasts ***(3)***, provided an additional link between the nuclear cell cycle machinery and the membrane-signaling apparatus. Later work further revealed that Rb inactivation per se leads to Ras activation ***(4)***. This communication describes a novel technique to measure c-Ras activation by SVLT, as well as by other Ras stimulators.

Ras are GTP-binding proteins that possess intrinsic GTPase activity and exist in two distinct, guanine nucleotide-bound conformations, the active Ras.GTP state, and the inactive Ras.GDP form ***(5,6)***. A large number of stimuli activate Ras by increasing the proportion of GTP-bound Ras, and this activation has been shown to play a key role in signal transduction ***(7)***. A variety of methods of Ras activity assessment have been described ***(4,8,9)***. The most commonly used method of direct measurement consists of the addition of ^{32}P-orthophosphate to the growth medium followed by Ras immunoprecipitation and guanine nucleotide elution, and is expressed as the percentage of Ras bound to GTP ***(10–12)***. This is relatively inefficient due to the fact that the

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isotope is incorporated into all phosphate-containing cellular components. In addition, most cells contain much higher levels of ATP than guanine nucleotides. ATP is difficult to separate from GTP or GDP by thin-layer chromatography (TLC), and can be a significant source of background. To circumvent these problems, [^{32}P]GTP itself has been introduced into intact cells and its breakdown into [^{32}P]GDP after Ras binding monitored as above. Since, contrary to free bases or nucleosides, most nucleotides do not cross the cell membrane, cell membrane permeabilization is necessary for [^{32}P]GDP introduction. In this communication a technique is described where the delivery of [^{32}P]GTP into adherent cells is performed through *in situ* electroporation. Cells are grown on a glass surface coated with electrically conductive, optically transparent indium–tin oxide and an electrical pulse is delivered. Unlike other techniques of cell membrane permeabilization, such as streptolysin O (SLO) treatment, *in situ* electroporation does not substantially affect cellular physiology, presumably because the pores reseal rapidly so that the cell interior is restored to its original state. Ras activity measurement through electroporation of [^{32}P]GTP can be performed using approximately 50 times lower amounts of radioactivity, whereas due to the fact that the ^{32}P is in the form of [^{32}P]GTP exclusively, this technique offers higher specificity compared to labeling through the addition of [^{32}P]-orthophosphate to the culture medium.

2. Materials

2.1. Solutions

1. Electroporation solution: HEPES-saline (10 m*M* HEPES, pH 7.0, 140 m*M* NaCl. Autoclave and store at room temperature). Electroporation can also be conducted in phosphate-free growth medium (e.g., phosphate-free Dulbecco's Modified Eagle's Medium, Sigma, St. Louis, MO), with similar results.
2. Lucifer yellow CH (dilithium salt, cat. no. LO259, Sigma) solution, 5 mg/mL in HEPES-saline: Stable at 4°C for at least 3 mo. A precipitate may form upon longer storage, which may show up as bright dots under fluorescence, but otherwise does not appear to affect the results.
3. [^{32}P]GTP: It must be of the highest purity. A number of lots were found to contain varying amounts of [^{32}P]GDP, therefore it is wise to test the preparation by TLC before use, as described below. Prepare a solution of 200–1000 µCi/mL in HEPES-saline.
4. Ras extraction buffer: 50 m*M* HEPES pH 7.4, 150 m*M* NaCl, 5 m*M* $MgCl_2$, 1% Triton, 0.5% sodium deoxycholate, 0.05% sodium dodecyl sulfate (SDS), 1 mM EGTA. This solution can be made in advance and the following protease or phosphatase inhibitors added from frozen stocks on the day of the experiment: 1 m*M* phenylmethyl sulfonylfluoride (PMSF) (Sigma, cat. no. P7626, 100 m*M* stock in isopropanol), 10 µg/mL aprotinin (Sigma cat.# A-6279, 10 mg/mL stock in 10 m*M* HEPES pH 8.0), 10 µg/mL leupeptin (stock of 10 mg/mL in H_2O),

10 m*M* benzamidine (Sigma, cat. no. B-6506, stock of 1 *M* in H_2O) and 1 m*M* sodium orthovanadate (from a stock of 100 m*M* in H_2O).
5. Guanine nucleotide elution buffer: 2 m*M* Ethylenediaminetetraacetic acid (EDTA), 2 m*M* dithiothreitol (DTT) (Sigma cat. no. D5545), 0.2% SDS, 0.5 m*M* GTP, 0.5 m*M* GDP.
6. TLC running buffer: 66% Isopropanol, 1% concentrated ammonia. Make fresh on the day of the experiment. The amount necessary depends on the size of the chromatography tank.
7. pan-Ras Ab2 antibody: It was purchased from Oncogene Science (Cambridge, MA, cat. no. OP22).
8. Protein A-Sepharose beads (Pharmacia, cat. no. 17-0780-03): Swell with distilled water for at least 2 h before use. The beads are stable for a month at 4°C in 0.001% sodium azide.

2.2. Equipment

1. System for electroporation *in situ* (Epizap model EZ-16, Ask Science Products Inc., Kingston, Ontario, Canada, phone: 613-545-3794). It consists of a capacitor-discharge pulse generator, a number of glass slides coated with electrically conductive and transparent indium–tin oxide and a set of electrodes, negative electrode above the cells and a positive contact bar (*see* **Fig. 1**).
2. Inverted, phase contrast and fluorescence microscope, equipped with a filter block for Lucifer yellow (excitation: 435, emission: 530).
3. Chromatography tank, available from Sigma.
4. TLC plates: Polyethyleneimine (PEI) cellulose or silica gel plates with 254 nm fluorescent indicator (Sigma, cat. no. T-6765 and Merck, Poole, Dorset, UK, cat. no. MO5735-01, respectively).

3. Methods

The manufacturer's instructions should be followed. Briefly, cells are grown on conductive and transparent glass slides with a cell growth area of 32 × 10mm, which are placed in a Petri dish to maintain sterility (*see* **Fig. 1**). The cell growth area is defined by a "window" formed with an electrically insulating frame made of Teflon. The pulse is transmitted through a stainless steel negative electrode placed on top of the cells (–), resting on the Teflon frame. Another stainless steel block is used as a positive contact bar (+). A complete circuit is formed by placing the electrode set on top of the slide and connecting the electrodes to the pulse source supplied, as shown in **Fig. 1**.

The appropriate institutional regulations concerning radioisotope handling must be followed.

3.1. Cell Preparation

1. Plate the cells: Two slides with cell growth areas of 32 × 12 mm each are sufficient in most cases. However, for some lines with low Ras levels, cells from three

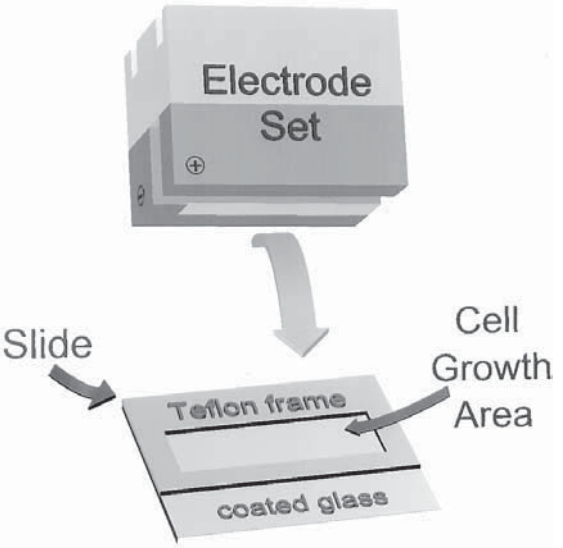

Fig. 1. Electroporation electrode assembly. Cells are grown on conductive, indium-tin oxide–coated glass slides within a "window" cut into a Teflon frame, as indicated. The [^{32}P]GTP solution is placed on the cells and introduced by means of an electrical pulse delivered through the electrode set placed directly on the frame. The slide and electrode fit into a 6-cm Petri dish which is locked in place on a stand ***(14)*** (not shown).

to five slides may be required to obtain an adequate signal. Since the [^{32}P]GTP solution can be aspirated and used again (*see* **Subheading 3.2.5.**), using several smaller slides instead of one larger one requires lower amounts of material.
Place the sterile slides provided by the manufacturer in 6 cm Petri dishes. Pipet the cell suspension in the window (*see* **Note 1**). Add a sufficient amount of medium to cover the slide, and place the Petri dishes in a tissue-culture incubator.
2. Starve the cells from serum for 24 h.
3. Starve the cells from phosphates: Change the medium to DMEM lacking phosphates (Sigma) for 2–5 h, depending on the particular cell line.

3.2. Pulse Application

1. Remove the growth medium and gently wash the cells with HEPES-saline, or serum- and phosphate-free growth medium.
2. Add the [^{32}P]GTP solution: Carefully *wipe* the Teflon frame with a folded Kleenex tissue to create a dry area on which a meniscus can form (*see* **Note 2**). The volume of the solution under the electrode is approx 120 µL, which will contain approx 25–240 µCi [^{32}P]GTP in HEPES-saline or phosphate-free growth medium.
3. Carefully place the electrode on top of the cells and clamp it in place on the stand provided. Make sure there is a sufficient amount of electroporation buffer under

the positive contact bar to ensure electrical contact. Make sure that there are no air bubbles between the negative electrode and the cells.
4. Apply six pulses of the appropriate strength (50–60 V, 5–10 µF, depending upon the cells, *see* **Note 3**).
5. Remove the electrode set: Because usually only a small fraction of the material penetrates into the cells, the [^{32}P]GTP solution can be carefully aspirated and reused up to three times.
6. Add prewarmed phosphate-free medium and incubate the cells for 2 h at 37°C.
7. Remove the unincorporated material: Wash the cells twice with the same phosphate-free medium lacking serum.

3.3. Ras Assay

1. Extract proteins: Wash the cells once with HEPES-saline. Add 1 mL of extraction buffer to the window area of the slide. Scrape the cells into a 15-mL tube using a rubber policeman and rock the tubes on ice for 20 min. Centrifuge for 30 min at 1,000 rpm in a Beckman J-6 centrifuge or equivalent (1000*g*) to clarify the extract. Preclear the lysates by adding 100 µL packed Staph A-Sepharose beads, incubating on ice for 1 h, and centrifuging at 1000*g* for 1 min to pellet the beads.
2. Immunoprecipitate Ras: Incubate the precleared supernatant overnight with 2 µL pan-Ras Ab2 antibody bound to Protein A-Sepharose beads while rocking on ice.
3. Wash the immunoprecipitate four times with 1 mL of extraction buffer lacking the inhibitors. Use a Hamilton syringe to completely remove all traces of wash solution.
4. Elute GTP and GDP off the beads by adding 5–10 µL elution buffer to the beads and incubating at 68°C for 20 min.
5. Spot the eluate onto the TLC plate (*see* **Note 4**). Spot 1 µL each of the stock GTP and GDP solutions to serve as cold standards, easily visible under ultraviolet light. Develop the plate using a solution of 1% ammonia-66% isopropanol for about 3–4 h.
6. Dry the TLC plate and expose to Kodak X-OMAT AR film. Excise the spots for liquid scintillation counting or submit to phosphorimager analysis.

4. Notes

1. Uniform spreading of the cells is very important, as the optimal voltage depends in part upon the degree of cell contact with the conductive surface ***(13,14)***. If necessary, the electrode can be sterilized with 80% ethanol before the pulse, and the procedure carried out in a laminar flow hood, using sterile solutions.
2. Care must be taken so that cells do not dry during wiping of the frame. It was found that serum-starved cells are especially susceptible. The morphology of cells that have been killed by drying is very similar to cells that have been killed by the pulse (*see* **Fig. 2C** and **D**, arrow).
3. Determination of the optimal voltage and capacitance: Electrical field strength has been shown to be a critical parameter for cell permeation, as well as viability

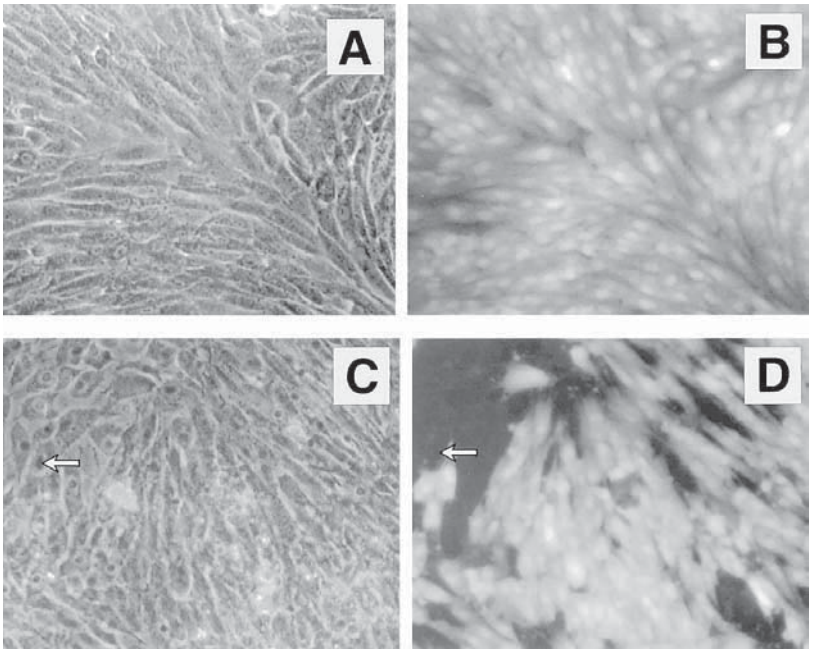

Fig. 2. Determination of the optimal voltage. Rat F111 fibroblasts growing on conductive slides (*see* **Fig. 1**) were electroporated in the presence of 5 mg/mL Lucifer yellow using six pulses of 60 V (**A** and **B**), or 90 V (**C** and **D**) delivered from a 5-μF capacitor. After washing the unincorporated dye, cells were photographed under phase contrast (panels **A** and **C**) or fluorescence (panels **B** and **D**) illumination. Arrows in **(C)** and **(D)** point to a cell that has been killed by the pulse. Note the dark, pycnotic, and prominent nucleus under phase contrast **(C)** and the flat, nonrefractile appearance. Such cells do not retain any electroporated material as shown by the absence of fluorescence **(D)**. It is especially striking that cells at the corner of the electroporated area, often receive a larger amount of current and are killed by the pulse.

(15). It is generally easier to select a discrete capacitance value and then control the voltage precisely. The optimal voltage depends on the strain and metabolic state of the cells, as well as the degree of cell contact with the conductive surface. Densely growing, transformed cells or cells in a clump require higher voltages for optimum permeation than sparse, subconfluent cells, possibly due to the lower amounts of current passing through them, compared to extended cells ***(13,16)***. In addition, cells growing and electroporated on collagen, poly-L-lysine or CelTak™-coated slides require substantially higher voltages than cells growing directly on the slide.

The margins of voltage tolerance depend on the size and electrical charge of the molecules to be introduced. For the introduction of small molecules like Lucifer yellow or [^{32}P]GTP, a wider range of field strengths permits effective

permeation with minimal damage to the cells than does the introduction of antibodies or DNA ***(10,13)***. For all compounds, the application of multiple pulses at a lower voltage can achieve a better permeation and is better tolerated by the cells than a single pulse of higher voltage. This is especially important for electroporation of serum-starved cells where the margins of voltage tolerance are substantially narrower, compared with their counterparts growing in 10% calf serum ***(10)***. The Lucifer yellow offers a convenient marker for cell permeation and it was found not to affect the results. Cell damage is microscopically manifested by the appearance of dark nuclei under phase contrast illumination. For most lines, this is most prominent at 5–10 min after the pulse. Such cells do not retain Lucifer yellow and fluoresce very weakly, if at all (*see* **Fig. 2D**). Current flow along the corners of the window is slightly greater than the rest of the conductive area. For this reason, as the voltage is progressively increasing, damaged cells will appear on this area first. This slight irregularity has to be taken into account when determining the optimal voltage.

The results of a typical experiment are shown in **Fig. 3**. The application of six exponentially decaying pulses of an initial strength of 60 V from a 5-μF capacitor to SVLT-transformed, rat F111 cells [line FSV1a ***(3)***] growing on a conductive growth area of 32 × 10 mm, resulted in essentially 100% of the cells containing the introduced dye, Lucifer yellow. Under the same conditions, incorporation of ^{32}P onto Ras.GTP and Ras.GDP was strong as revealed by the spots obtained by TLC.

Measurement of steady-state Ras activity is possible using this method because, contrary to other methods of cell permeabilization, such as streptolysin-O [SLO (11)], the cells are not detectably affected by the procedure, so that they can be incubated for long periods of time after electroporation before protein extraction. In addition, electroporation does not appear to induce a rapid breakdown of intracellular GTP in any of the lines tested, even under conditions where a substantial fraction of the cells are killed by the pulse ***(17)***. As a result, the determination of the Ras-bound, GTP/GTP+GDP ratio is made easier by the fact that, although the optimal voltage must be empirically determined as in all electroporation experiments, excessively high voltages, in spite of the fact that they may kill a substantial proportion of the cells, do not alter the ratios obtained ***(10)***, presumably because such cells rapidly lyse without affecting the results.

The technique can be applied to a large variety of adherent cell types ***(14,17)***. Cells that do not adhere can be grown and electroporated on the same conductive slides coated with CelTak, poly-L-ysine, or collagen. In addition, a large variety of molecules, such as peptides ***(18–21)***, nucleotides ***(10,22)***, oligonucleotides ***(23)***, proteins ***(13,24)***, or drugs ***(25)*** can be introduced, alone or in combination, at the same or different times ***(14)***.

4. In our hands, silica gel plates offered superior resolution to PEI-cellulose.

Acknowledgments

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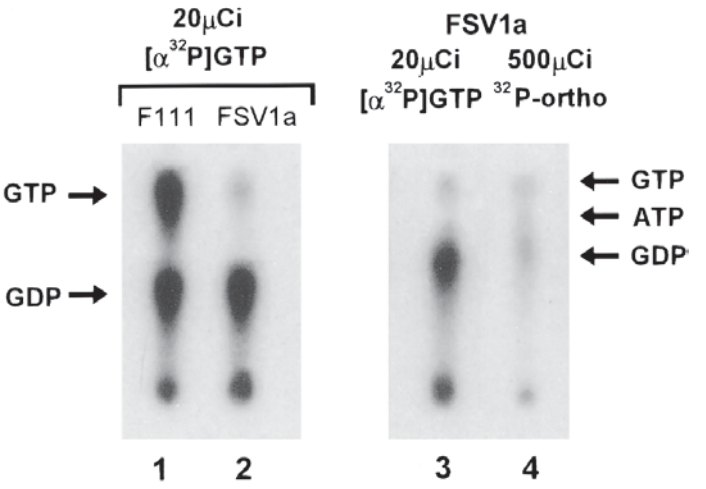

Fig. 3. Assessment of Ras activity through electroporation of [^{32}P]GTP in normal rat F111 cells or their SVLT-transformed counterparts. Rat F111 cells (**lane 1**) or their SVLT-transformed counterparts (line FSV1a, **lanes 2–4**) were grown on conductive glass and starved from serum and phosphates. A solution containing 20 μCi [^{32}P]GTP (in 10 m*M* HEPES, 140 m*M* NaCl) was added to the cells and six capacitor-discharge pulses of 5 μF, 60 V applied (**lanes 1–3**). Cells were subsequently placed in a humidified 37°C, CO_2 incubator for 3 h. Ras was extracted and precipitated with the pan-Ras Ab2 monoclonal antibody, the bound GTP and GDP eluted and separated by TLC. The plate was exposed for 15 h to Kodak XAR-5 film with an intensifying screen. As a control (**lane 4**), cells growing in a 3-cm Petri dish were starved the same way, metabolically labeled with 500 μCi ^{32}P-orthophosphate in 0.5 mL phosphate-free DMEM, and processed as before.

thin-layer chromatography and Dr. Thomas Roberts for advice on the use of the Molecular Dynamics Phosphorimager. The financial assistance of the Natural Sciences and Engineering Research Council of Canada and the Cancer Research Society Inc. to LR is gratefully acknowledged. HLB was the recipient of a studentship from the Medical Research Council of Canada and Queen's University graduate school and Microbix Biosystems Inc. travel awards.

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17

Role of SV40 Small t in Cell Lysis, Transformation, and Signaling

Ximena Montano and Kathy Rundell

1. Introduction

Simian virus 40 small t antigen (st) is encoded on a differentially spliced transcript of the SV40 early region. It shares its aminoterminal 82 amino acids with the large T antigen and contains a unique cysteine-rich carboxyterminal half. Small t mutant viruses were obtained originally as deletion mutations within the large T intron ***(1,2)***. These are fully viable, although they grow somewhat more slowly and to slightly lower yields, as compared to wild-type (wt) viruses.

Large T is able to immortalize certain cell types, induce DNA replication, as well as cell division. Large T-expressing fibroblasts and epithelial cells can overgrow a monolayer of untransformed cells, grow in semisolid media, and can be tumorigenic when injected into nude mice (***3***, and Chapter 11). Small t alone does not have the capacity to transform established cell lines or immortalized primary cells. However, it can enhance large T transforming ability when large T is present in limited concentrations ***(4)***. There is evidence showing that the transforming ability of large T depends upon a sequence between amino acids 1-82 (which is a region common to large and small t); st can restore the transforming ability of a truncated large T protein lacking the first 82 amino acids ***(5)***.

The initial analysis of st cell localization was ambiguous. Monoclonal or polyclonal antibodies preferentially recognized the common amino acid sequences between large and small t and made this task difficult ***(6)***. The production of PAb 280 facilitated this process. PAb 280 is a monoclonal antibody that uniquely recognizes st. It can detect this protein in the nucleus and cytoplasm of transformed or infected mammalian cells ***(7)***. Interestingly, PAb 419

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can only recognize nuclear st, suggesting the possibility that st might have two different molecular configurations with distinct biological functions; however, this result needs to be analyzed further ***(8)***.

Small t antigen binds to two cellular proteins, now known to be the catalytic (C) and regulatory (A) subunits of protein phosphatase 2A (PP2A) ***(9–11)***. The st:PP2A interaction, which is required for host-cell induction ***(12)*** and transformation ***(13)***, maps to the unique C-terminal half of st, and several mutations alter this activity. Three single-point mutations (C97S, P101A, and C103S) are useful in studying the st:PP2A interaction, but the C103S mutation is the best choice for most other studies because it is reasonably stable yet defective in this activity ***(14)***. Like large T, small t has a dnaJ motif in its N-terminal sequences ***(15)*** and has been shown to function as a dnaJ protein, stimulating adenosine triphosphatase (ATPase) activity of dnaK proteins in biochemical studies ***(16)***. In addition, st has a transactivation activity ***(13,17)***, which maps to the dnaJ region, in the N-terminal half of the protein.

In productively infected monkey cells, st is present at a level of about 0.1% of the total cellular protein. Far less st is found in transformed cells or in cells infected with related papovaviruses, such as polyomavirus. Small t has been expressed in baculovirus ***(18–21)***, retrovirus ***(4)***, and adenovirus systems ***(13,22,23)***. Large-scale preparation of st has been accomplished through expression in bacteria under control of IPTG-inducible promoters ***(24,25)***. All such systems express full-length 17-kDa st and an internally initiated 14-kDa product that initiates at met52 of the full-length protein. The 14-kDa product occurs because of ribosome binding sites found in the natural st sequence and it has not been possible to introduce silent mutations that would prevent expression of the 14-kDa protein. The vector that expresses the largest amount of st is pTR865 ***(26)***, where st expression is under control of a trp-lac, IPTG-inducible promotor. Good levels of expression have also been obtained with the ptrc99A vector and others ***(27)***.

Nearly all the st expressed in bacteria following induction is insoluble, and can be separated from the bulk of the bacterial proteins. Small t can be solubilized from bacterial cell wall fractions using urea or guanidinium chloride. Dialysis or dilution of st results in preparations that are extensively aggregated even when large amounts of reducing agents are present during handling. It is now known that st is a zinc-binding protein and that the presence of zinc is essential for refolding of the protein into the monomeric form. Refolding to produce monomers has worked well only when proteins are solubilized in urea (not guanidinium chloride) and they are recovered by slow dilution, rather than by dialysis. About 30–50% of the small t can be refolded into monomers and these can be rapidly separated from remaining aggregates by passing the preparations over bulk DEAE-cellulose columns, collecting proteins that do not bind

this resin. Early studies found that st from SV40 infected cells was partly monomeric, and could be purified by gel filtration, ion-exchange, and thiol Sepharose chromatography ***(28,29)***, procedures that are also useful for work with solubilized, bacterially expressed st. DEAE-cellulose chromatography has been particularly useful. At neutral pH, the monomeric fraction of st does not bind DEAE-cellulose, although st associated with PP2A or aggregated forms of st do bind this resin. Aggregated forms of st also bind DEAE-cellulose. Monomeric protein purified from bacterial sources is fully capable of interacting with the cellular phosphatase, PP2A ***(14)***, and of performing dnaJ protein activities ***(16)***.

2. Materials

2.1. Tissue Culture and Infections

1. SV40 transformed cell lines such as SVA31-E7, SV3T3, or CV-1 infected with wt or mutant viruses are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS).
2. For transient infections, Balb/c 3T3 clone 31 cells are maintained in DMEM supplemented with 10% heat-inactivated calf serum (CS).
3. All cell lines are grown at 37°C and kept in a humidified incubator with 10% CO_2. Cells are grown to 80% confluency and then are either passaged or used for further analysis.
4. For plaque assays of st mutant viruses, 2% "Bacto-agar" (Difco, Detroit, MI, cat. no. 0140-01-0) is used, as described for wt virus in Chapter 1.

2.2. Antibodies

2.2.1. Primary Antibodies

The most commonly used antibodies are as follows:

1. PAb 419, which recognizes an epitope within the first 82 amino acids of large T and small t ***(6)***, [available from Oncogene Research Products, Cambridge, MA, cat. no. DP01].
2. PAb 280, which recognizes an epitope within amino acids 83–114 and therefore uniquely recognizes st ***(7)***, [Oncogene Research Products, cat. no. DP014].
3. PAb 430, which recognizes the first 82 amino acids of large T and small t ***(6)***.
4. There are commercially available (e.g., from Oncogene Research Products), rabbit anti-large T polyclonal antisera.

2.2.2. Secondary Antibodies

1. Western blotting and immunocytochemistry: Horseradish peroxidase (HRP)-conjugated, antimouse or antirabbit antibodies (cat. nos. PO447 and PO399, respectively, Dakopatts, Denmark). Biotinylated antibodies and Streptavidin/HRP-conjugates (Dakopatts, cat. no. P0397).

2. For immunofluorescence, FITC, rhodamine or Texas Red-conjugated antimouse or antirabbit antibodies, available from a number of suppliers (e.g., Amersham, UK or Organotechnica, West Chester, PA, or Vector Laboratories, Burlingame, VT).

2.3. Solutions

2.3.1. Small t Immunoprecipitation and Western Blotting

1. NET-N: 20 m*M* Tris HCl pH 8.0, 100 m*M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA) and 0.5% Nonidet P-40 (NP40).
2. EBC buffer: 50 m*M* Tris-HCl pH 8.0, 120 m*M* NaCl, 0.5% NP40, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.15 U/mL aprotinin, 20 μ*M* leupeptin, and 1% NP40.
3. Protein assay kit purchased from Bio-Rad and used according to the manufacturer's instructions (cat. no. 500-0006).
4. Protein G fast-flow Sepharose beads (Pharmacia, cat. no. 17-0618-01), resuspended in NET-N buffer containing 1 mg/mL BSA.

2.3.2. Coimmunoprecipitation of st and Cellular PP2A Subunits

1. Extraction buffer: 20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 0.2% Triton X-100 (or 0.2% NP40), 1 m*M* 2-mercaptoethanol (2-ME).
2. Immunoprecipitate washing buffer: 100 m*M* Tris-HCl, pH 8.6, 500 m*M* LiCl; diluted 1:5 for the final wash.
3. Protein AG-coupled agarose beads, which bind most types of antibodies (Santa Cruz Biotechnology, Inc., cat. no. sc-2003).

2.3.3. Immunocytochemistry, ELISA, and In Vitro Association

1. 50% acetone:50% methanol.
2. 3 3 diaminobenzidine (DAB) tablet set from Sigma (cat. no. D-4168), used as indicated by the manufacturer, or DAB-free base powder from Sigma (cat. no. D-8001), prepared as 1 mg/mL in phosphate-buffered saline (PBS) with 11 μL/mL 30% H_2O_2.
3. 10 m*M* phosphate buffer pH 7.5.
4. Bovine serum albumin (BSA) for coating plates (Sigma, cat. no. A-7030).
5. 3,3,5 -5 tetramethylbenzidine (TMB): from Sigma (cat. no. T-2885), prepared as 10 mg/mL in DMSO (TMB buffer).
6. Substrate buffer: 0.1 *M* citrate/phosphate pH 6.0 (0.1 *M* Na_2HPO_4 and 0.1 *M* citric acid).
7. 1 *M* H_2SO_4.
8. Vitrogen-100 collagen purchased from Biomaterials, Palo Alto, CA (cat. no. PCO701).

2.3.4. Large-Scale Purification of Small t from Bacteria

1. Column equilibration buffer: (20 m*M* Tris-HCl, pH 8.0, 80 m*M* NaCl, 0.02% NP40, 0.5 m*M* 2-ME).

2. Isopropyl- -D-thiogalactopyranoside (IPTG) (Sigma, cat. no. I-5502).
3. Deoxyribonuclease I (Sigma, cat. no. DN-25); stock 1 mg/mL in 20 m*M* Tris-HCl, pH 8.0, stored at –70°C.
4. Zinc sulfate (Sigma, cat. no. Z-0501); stock 100 m*M* in water, stored at room temperature.
5. Dithiothreitol (DTT) (Sigma, cat. no. D-5545); stock 1 *M* in water, stored at –70°C.
6. G200 Sephadex, rehydrated and stored in column equilibration buffer.
7. Hydroxylapatite (HA-Ultrogel, bead size 60–180 microns, from IBF Biotechnics, Villeneuve-la-Garenne, France, cat. no. 247751).

2.4. Equipment

1. Gel-running apparatus for SDS polyacrylamide gels, available from a number of suppliers (e.g., Bio-Rad, Gibco-BRL).
2. Gel-transfer apparatus (e.g., from Bio-Rad, Novex, Hoeffer).
3. 10-cm and 3.5-cm plastic tissue-culture dishes.
4. Glass cover slips and slides (Fisher), coated with collagen.
5. 96-well cell-culture plates, available from a number of suppliers (e.g., Falcon, Nunc). They can be used both for cell growth for immunocytochemistry and for ELISAs.
6. Nitrocellulose (Schleicher and Schuell Protan BA 83, cat. no. 401-391).
7. Humidified staining chambers (Fisher).

3. Methods

3.1. Growth and Assay of Small t Mutant Viruses

1. Infect confluent monolayers of monkey kidney cells with virus stocks. After infection, add agar-containing medium as described for wt SV40 in Chapter 1.
2. Add 0.5 mL medium containing 5% FCS on top of the agar every 3–4 d to maintain humidity and provide nutrients to the cells (*see* **Note 1**).
3. Macroscopically visible plaques appear at 12–14 d.

3.2. Small t Immunoprecipitation and Western Blotting

3.2.1. Small t Detection by Western Blotting

1. Grow approx 5×10^6 cells in 10-cm plates and infect with virus. Wash infected cells three times with PBS.
2. Wash twice with ice-cold PBS, to reduce protease activity.
3. Drain thoroughly and lyse in 200 µL of EBC buffer at 4°C.
4. Spin cell extracts at 4°C for 5 min at 10,000*g*.
5. Discard the pellet and add anti-T/t monoclonal antibody (2–3 µg) to the lysate (*see* **Note 2**).
6. Incubate by mixing at 4°C for 1–2 h.
7. Add protein G Sepharose and incubate for a further 30 min.
8. Pellet by briefly spinning in a microcentrifuge.

9. Wash twice with ice-cold NET-N containing 0.5 *M* LiCl.
10. Wash once with ice-cold NET-N.
11. Boil pellet in sodium dodecyl sulfate (SDS) sample buffer.
12. Run samples on SDS polyacrylamide gels. Transfer the resolved proteins onto nitrocellulose using standard techniques (*see* **Note 3**).
13. Block the membrane for 1 h in PBS containing 0.1% Tween-20. There is no need for addition of any protein, such as BSA or milk.
14. Wash twice with PBS.
15. Incubate with PAb 419, PAb 280 (2–3 µg/mL), or rabbit polyclonal antilarge T (diluted 1 : 1000 in PBS containing 5% FCS) at 4°C overnight (*see* **Note 4**).
16. Wash the blots in PBS containing 1% NP40 three times for 5 min at room temperature.
17. Detect antibody binding by incubating with rabbit anti-mouse or pig anti-rabbit, peroxidase-conjugated immunoglobulins (dilution 1 : 5000 in PBS containing 5% FCS) for 1 h at room temperature, or according to the manufacturer's instructions for the particular lot.
18. Wash three times with PBS containing 1% NP40.
19. Visualize the st band by adding 3 3 DAB/hydrogen peroxide (prepared as described in **Subheading 2.3.3.**) until color develops, then stop by rinsing with distilled water.
20. Bound antibody can also be visualized by chemiluminescence (ECL, Amersham; follow manufacturer's instructions). In this case, incubate with a secondary antibody diluted 1 : 20,000 in PBS containing 5% FCS.
21. If desired, blots can be stripped of antibody by incubating with 2% SDS, 100 m*M* 2-ME, 62.5 m*M* Tris-HCl pH 6.8 for 1 h at 70°C. Rinse three times for 15 min with PBS containing 1% NP40. Then the blot can be probed with a different antibody.

3.2.2. Coimmunoprecipitation of st with Cellular Protein Phosphatase Subunits

1. Radioactively label cells to be extracted with 100–200 µCi ^{35}S-methionine either for 1–2 h in methionine-free medium or overnight in medium containing 6–8% the normal methionine concentration.
2. Extract cells expressing st with buffers containing 0.2–0.5% nonionic detergent such as Triton X-100 or NP40. Do not use SDS in extraction buffers (*see* **Note 5**).
3. If labeled extracts are derived from uninfected cells, add a source of st (purified st or a second extract of SVLT-expressing cells), then incubate the mixture at 30°C for 10 min to promote complex formation (*see* **Note 6**).
4. Add rabbit anti-T-polyclonal serum or monoclonal antibody PAb430 ***(6)***, then incubate on ice for 45 min (*see* **Note 7**).
5. Collect immune complexes on protein AG-coupled agarose beads.

3.3. Immunocytochemistry

Cells are fixed on cover slips and permeabilized. The primary antibody is added, which will react with the st antigen. In a second step, this antibody is

directly visualized using an HRP-coupled secondary. Alternatively, to amplify the signal further and increase the sensitivity of detection, the secondary antibody can be conjugated to biotin and visualized using HRP-coupled streptavidin.

3.3.1. Cell Fixation and Permeabilization

3.3.1.1. Acetone-Methanol

This treatment simultaneously fixes and permeabilizes the cells.

1. Plate SV40-infected, transfected, or transformed cells onto cover slips or 96-well plates.
2. Rinse twice with PBS and fix on the plate for 3 min with 5 mL of 50:50 acetone:methanol.
3. Rinse again with PBS. Avoid drying.

3.3.1.2. Formaldehyde Fixation

1. Rinse cells twice with prewarmed DMEM, pH 7.3 (to obtain this pH, leave the DMEM in tissue-culture flasks in a 10%-CO_2 incubator overnight).
2. Fix with 3% formaldehyde in 85% PBS for 20 min on ice.
3. Rinse twice with 85% PBS.
4. Incubate with 50 m*M* NH_4Cl in 85% PBS for 15 min to quench the reaction.
5. Rinse twice with 85% PBS.
6. Permeabilize the cells: This can be done either by
   a. Freeze-thawing (drain the PBS from the plates, place the plate on a dry-ice/ethanol bath until frozen, then thaw at room temperature and add 85% PBS immediately), or
   b. Triton treatment (add 0.1% Triton X-100 in PBS, incubate for 4 min at room temperature, then wash twice with 85% PBS).

3.3.1.3. Glutaraldehyde Fixation

1. Rinse cells twice with DMEM pH 7.3 (as with the formaldehyde method).
2. Add 4% glutaraldehyde in PBS and keep on ice for 20 min.
3. Rinse twice with 85% PBS.
4. Add 50 m*M* NH_4Cl in 85% PBS and keep the plate on ice for 10 min.
5. Rinse twice with 85% PBS between steps.
6. Repeat **step 4** and rinse again.
7. Permeabilize the cells by freeze-thawing or Triton treatment as before (this step is optional).

3.3.2. Direct HRP Staining

1. Rinse cells once with PBS.
2. Incubate with hybridoma supernatant or purified monoclonal antibody (2 µg/100 µL diluted in DMEM with 5% FCS), or rabbit polyclonal antibodies overnight at 4°C.

3. Rinse cells three times with PBS.
4. Incubate with 100 µL of HRP-conjugated rabbit antimouse or swine antirabbit antibodies (diluted 1:500 in PBS containing 5% FCS) for 4 h at 4°C.
5. Visualize st by adding 3 3 DAB/hydrogen peroxide, prepared as described in **Subheading 2.3.3.** Incubate for 1–20 min, or until brown color develops.
6. Discard DAB mixture and rinse thoroughly with tap water to stop the reaction.
7. Mount the cover slips in Univert (BDH, cat. no. 36118 2E) and let dry overnight. Observe microscopically under brightfield illumination.
8. If 96-well plates were used, add 50% glycerol and observe with an inverted microscope. Because of the thickness of the plastic, depending upon the microscope, it may be difficult to use as high a magnification as when the cells are stained on cover slips, in which case an upright microscope can be used.

3.3.3. Indirect HRP Staining for Detection of Very Low st Levels

1. Incubate with biotinylated, affinity-purified immunoglobulins (diluted 1:1000 in PBS containing 10% FCS for 45 min) at room temperature.
2. Wash with PBS as before and incubate with streptavidin/HRP (diluted 1:5000 in PBS containing 10% FCS) for 45 min at room temperature.
3. Wash three times with PBS.
4. Visualise the immune complexes using DAB/hydrogen peroxide as above.
5. Allow the reaction to continue for 1–30 min, then stop it with tap water.
6. Observe microscopically under brightfield illumination.

3.4. Immunofluorescence

Immunostaining can efficiently detect a single antigen (e.g., large or small t, or nuclear st detected with pAB419 vs total st). However, it is usually difficult to discern two different colors (e.g., from HRP or alkaline-phosphatase substrates) when they are superimposed on the same cell structure. Therefore, to detect both antigens simultaneously, the immunofluoresce technique is prefered. The same procedure of plating, fixing the cells, and incubating with the primary antibody aforementioned can be used. The two primary antibodies must be from different species, e.g., the first from rabbit and detected with a rhodamine-coupled, antirabbit secondary, the second one from mouse, detected with an FITC-coupled, antimouse secondary.

1. The same procedure of cell fixation and incubation with the first (e.g., rabbit) primary antibody aforementioned can be used. Rinse cells three times with PBS.
2. Incubate with antirabbit Texas Red- or rhodamine-conjugated immunoglobulins (diluted 1:30 in PBS containing 10% FCS) for 2 h at room temperature in a humidified chamber, in the dark to avoid quenching.
3. Rinse three times with PBS.
4. Incubate with the second, mouse monoclonal antibody diluted to 10 µg/mL in PBS containing 10% FCS.

5. Rinse three times with PBS.
6. Incubate with anti-mouse, FITC-conjugated immunoglobulins (diluted 1:50 in PBS containing 10% FCS) for 2 h at room temperature, in a humidified chamber, in the dark to avoid quenching.
7. Rinse three times in PBS.
8. Mount in Univert as before and observe microscopically under fluorescence illumination using the appropriate spectra.

3.5. ELISA

1. Add 25 µL of purified st (approx 10 µg/mL as control, from a bacterial cell extract), or SV40-infected or transformed-cell extracts (prepared as described for immunoprecipitations in **Subheading 3.1.**) to each well of a 96-well plastic microtiter plate. Allow the plate to dry overnight at 37°C (*see* **Note 8**).
2. Wash plates once with PBS and incubate with 50 µL of PBS containing 10% BSA for 24 h at 4°C.
3. Discard blocking mix and rinse plates thoroughly with PBS.
4. Add 25 µL of PAB 280 hybridoma supernatant or purified antibody (5 µg/mL) and incubate for 2 h at room temperature (*see* **Note 9**).
5. Wash plates five times with PBS.
6. Add 25 µL of HRP-coupled rabbit antimouse immunoglobulin diluted 1:100 in PBS containing 5% FCS and incubate for 2 h at 4°C
7. Wash plates three times with PBS.
8. Visualize using 50 mL of 3,3,5 -5 tetramethylbenzidine substrate for 20–30 s (mix 6 mL of substrate buffer with 60 mL of TMB buffer, then add 15 mL of 30% H_2O_2).

3.6. Large-Scale Purification of Small t from Bacteria

1. Inoculate 5 mL luria broth containing 50 µg/mL ampicillin with bacteria containing the pTR865 plasmid and incubate overnight with shaking at 37°C.
2. Transfer the overnight culture into 250 mL LB+amp and incubate with shaking for 4 h; induce the TRP-LAC promotor by adding 5 m*M* IPTG to the medium and continue the incubation for an additional 4 h.
3. Collect bacteria by centrifugation. Pellets can be frozen at this stage for future use (*see* **Note 9**).
4. Suspend bacteria in 20 m*M* Tris-HCl, pH 8.0, containing 20% sucrose, 50 m*M* EDTA, and 10 mg/mL lysozyme and incubate on ice for 30–60 min.
5. Collect spheroplasts by centrifugation, then lyse them in 20 m*M* Tris-HCl, pH 8.0, containing 80 m*M* NaCl, 50 m*M* $MgCl_2$, 0.1% Triton X-100, and 1 µg/mL DNase I. Gently mix at room temperature using a teflon rod until viscosity is reduced. Residual EDTA may interfere with DNase activity, in which case additional $MgCl_2$ may be required.
6. Collect small t-containing inclusions, cell walls, and other cellular debris by centrifugation. This material can be stored frozen for future use (*see* **Notes 10** and **11**).

7. Suspend the inclusions in 5 mL of 10 *M* urea in 20 m*M* Tris-HCl, pH 8.0, 80 m*M* NaCl that contains 2 m*M* DTT, and 0.25 m*M* zinc sulfate and incubate at 37° for 30 min. The total volume of solubilization buffer depends on the expected amount of st in the inclusions. There must be an excess of zinc ions and each molecule of st binds two Zn^{2+} ions. Zinc sulfate cannot be prepared at higher concentrations, and this limits the concentration of st that can be achieved at this step.
8. Centrifuge in several microfuge tubes and collect the soluble protein fraction. A second centrifugation may be necessary to remove insoluble material.
9. Using preparation buffer (20 m*M* Tris-HCl, pH 8.0, 80 m*M* NaCl, 0.5 m*M* DTT, 0.03% Triton X-100 or NP40), dilute the soluble proteins 20-fold slowly over the period of 1 h. After the concentration of urea is reduced to less than 5 *M*, proteins can be moved to a cold room or onto ice. The use of magnetic stirring tends to increase aggregation, and best results are obtained with gentle manual swirling. As an alternative to this dilution, the initial urea-soluble protein fraction may be pumped onto a 2.5 × 40-cm column of G200 Sephadex poured and run in peparation buffer. The large column volume is necessary for good separation of the relatively large sample volume.
10. Pass the diluted urea fraction over a 2 × 10-cm column of DEAE-cellulose suspended in preparation buffer. Aggregated proteins bind this resin, and flowthrough (unbound protein) is almost entirely monomeric st. If fractions from the monomeric region of a G200 Sephadex column are used, they should be passed over a small DEAE column (2 × 4 cm). This removes some bacterial proteins that are of similar size to monomeric st. The 80 m*M* NaCl present in preparation buffer is essential in the DEAE step, and st binds DEAE if salt concentrations are reduced below 10 m*M*.
11. Protein fractions can be concentrated using Amicon filtration, or or hydroxylapatite (HA Ultrogel). Small t can be recovered using 100 m*M* potassium phosphate, pH 7.6, 80 m*M* NaCl, 2 m*M* DTT, and 0.02% Triton X-100. The use of nonionic detergents in elution buffers from hydroxylapatite (or thiol-sepharose) is critical for good recovery of st.

4. Notes

1. Procedures used to grow and maintain stocks of these viruses are identical to those used for wt virus, although plaques appear more slowly and are smaller than those formed by wt virus. Virus yields are increased slightly by adding fresh medium containing 5% FBS twice a week until cytopathic effect is microscopically obvious. In plaque assays performed at the standard temperature (37°C), st mutants give 1–2-mm diameter plaques after 12–14 d, compared to 3–5-mm plaques formed by wt virus in 10–11 d.
2. Both PAb 419 and PAb 280 recognize native and denatured protein. Hence, they can be used for both immunoprecipitation and Western blotting experiments.
3. To detect st by SDS polyacrylamide gel electrophoresis, it is advisable to use 15% acrylamide gels. If both small t and large T need to be detected, 12.5% gels work best.

4. As a rule, large T is more easily detectable than small t. Detection of both large T or st can be achieved with nonpurified PAb 419 or polyclonal antilarge T antibody. Purified PAb 280 (which uniquely recognizes st) should be used to observe the presence of st.
5. The addition of 0.1% SDS to buffers containing deoxycholate and/or NP40 completely disrupts the interaction between t and the AC subunits of PP2A. However, once t:AC complexes have been isolated by immunoprecipitation, gentle incubation with RIPA buffer releases only the C subunit, leaving st and the A subunit together with the antibody ***(18)***. This suggests that the primary st interaction is with the A subunit.
6. It is possible to form t:AC complexes in cell-free extracts, although gentle incubation (30°, 10–15 min) is often required ***(11,30)***. During this incubation, st will displace B subunits that have lower affinity interactions with AC. Some B subunits have higher affinity than st and these are not displaced by st in these in vitro experiments. In vitro complex formation allows analysis of in vitro translated proteins, an approach that has been used extensively to map binding sites on st and on the A subunit ***(14,31)***. Purified st can also be added to extracts or in vitro translated proteins to initiate complex formation.
7. For studies of associated cellular proteins, it is important to note that many frequently used monoclonal antibodies ***(4,30)*** disrupt the complex, resulting in the precipitation of only small t-ag. One monoclonal antibody, Pab430, maintains the integrity of the complex allowing cellular A (63 kDa) and C (37 kDa) subunits to be coprecipitated with the 17-kDa small t antigen ***(14)***.

   For all studies, although very good results are obtained with HRP-conjugated secondary antibodies, alkaline phosphatase conjugated antibodies can also be used. There are several substrates for alkaline phosphatase either for ECL or for colorimetric analysis, such as the 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chloride, p-toluidine salt (NBT/BCIP) (Life Technologies), which gives a blue color.
8. As a rule, st detected in mammalian cell extracts is soluble, but to avoid st aggregates, it is advisable not to use very concentrated extracts (200 µL/well, containing less than 200 µg total cell extract).
9. If partially purified components are available, it is also possible to distinguish t:AC from cellular forms of PP2A (AC dimers and BAC trimers that contain varying B subunits) using native gel electrophoresis. Differences between these forms of PP2A are subtle ***(32)*** and this may explain why there are no published reports that native gel analyses have been used successfully with crude extracts.
10. Rapid purification from baculovirus-infected cells has been accomplished using immunoaffinity columns ***(21)***, modeled on those used to isolate the viral large T-ag. Elution of biologically active st was achieved using 100 m*M* triethylamine, pH 11.5. The monoclonal antibody Pab419 ***(4)*** has been used most extensively for column preparation. This antibody binds aminoterminal sequences of small t and large T-ag, but can be used to purify st when no large T is present as is the case in heterologous expression systems. Pab419 also has the advantage that it does not recognize the truncated 14-kDa form of st found in bacterial expression systems.

11. Preparations of monomeric st with concentrations of 0.2–1 mg/mL have been achieved using pTR865 as a source of st. It is reasonable to expect to obtain 2–3 mg total st per liter of bacterial culture, up to 40% being recoverable as monomeric protein. Such preparations are fully active in interacting with cellular PP2A subunits. Bacteria express a 14-kDa internally initiated form of st, which has the same affinity for PP2A as the full-length 17-kDa protein. If it is necessary to separate the 14-kDa truncated st from the full-length protein however, several approaches can be employed:
    a. the 17-kDa, but not the 14-kDa product binds Pab419, so that antibody affinity chromatography can be used to separate these proteins;
    b. both the 14-kDa and 17-kDa proteins bind DEAE cellulose when NaCl concentrations are less than 10 m*M*. Full-length st elutes ahead of the 14-kDa protein when a 0–200 m*M* linear gradient of NaCl is used;
    c. both proteins bind hydroxylapatite (HA-Ultrogel), but the 14-kDa protein elutes before the 17-kDa full-length protein when a 0–80 m*M* potassium phosphate, pH 7.6, gradient is applied.

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18

Cytotoxic T Lymphocytes in SV40 Infections

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1. Introduction

Simian virus 40 (SV40) infects monkeys and persists in the latent form in the kidneys of this natural host. However, in nonpermissive hosts, such as rodents, the virus induces neoplasia and the outcome is controlled by the immune response of the host ***(1,2)***. Virus-neutralizing antibodies are responsible for limiting the amount of circulating virus in the natural host, leading to the establishment of latency. In contrast, the nonpermissive host undergoing tumorigenesis develops antibodies specific for the large tumor antigen, or T antigen (T-ag) encoded by SV40. In addition, the T-ag in transformed or tumor cells also serves as the target for cytotoxic T lymphocytes (CTL) that are capable of controlling tumor development ***(2)***. CTL responses directed against SV40 virion proteins have not been described.

Peptides corresponding to CTL recognition epitopes, ranging in length from 8–10 amino acids, associate with major histocompatibility complex (MHC) class I heavy chains and $_2$-microglobulin in the endoplasmic reticulum. Formation of this trimolecular complex is followed by transport to the cell surface where it is recognized by the T-cell receptor (TCR) of epitope-specific CTL. The association of a peptide with a particular MHC class I molecule depends on the presence of anchor residues within the peptide that promotes stable binding to the MHC molecule ***(3,4)***. MHC class I binding peptides are derived by proteolysis of intracellular proteins, such as T-ag, in the cytoplasm followed by transport into the endoplasmic reticulum ***(5,6)***. CTL typically recognize their target peptide bound to class I MHC molecules derived from a single allele (e.g., H-2K^b, but not H-2D^b) and thus are designated as restricted by that MHC molecule (e.g., H-2K^b-restricted) ***(7)***.

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Table 1
H-2^b-Restricted CTL Recognition Epitopes from SV40 T-ag and Corresponding CTL Clones

T-ag epitope	T-ag sequence	Amino acid sequence	Epitope-specific CTL clones	Class I MHC restriction
I	206–215	SAINNYAQKL	Y-1 and K-11	H-2D^b
II/III	223–231	CKGVNKEYL	Y-2, Y-3 and K-19	H-2D^b
IV	404–411	VVYDFLKC	Y-4	H-2K^b
V	489–497	QGINNLDNL	Y-5 and H-1	H-2D^b

In this chapter, we describe the methodology used in our laboratory for the demonstration of a CTL response in C57BL/6 (H-2^b) mice to SV40 T-ag. We have identified four distinct CTL-recognition epitopes in T-ag by establishing CD8$^+$ CTL clones specific for each epitope ***(8–13)***. The location of the four T-ag-specific CTL recognition epitopes and the corresponding CTL clones that recognize them are listed in **Table 1**. Three of the CTL epitopes (I, II/III, and V) are H-2D^b restricted, whereas one epitope (IV) is H-2K^b restricted. These CTL clones can distinguish between T-ag from SV40 and the related human papovaviruses, JC and BK ***(14)***. The identification of multiple T-ag CTL-recognition epitopes allows the response against individual epitopes to be monitored and compared following immunization with T-ag.

In addition, we describe the use of limiting dilution analysis to estimate the frequency of CTL that respond to a given T-ag epitope following immunization of mice against T-ag ***(11,13,15)***. In the limiting dilution analysis, titrated amounts of lymphocytes from immune mice are stimulated in vitro and tested for lysis of target cells in order to determine the dilution at which a single cell gives rise to a clonal population of CTL specific for a particular epitope ***(16,17)***. Finally, we describe the use of limiting dilution analysis to isolate T-ag epitope-specific CTL clones. These procedures should be readily adaptable using a variety of immunization strategies and various strains of mice.

2. Materials

2.1. T-ag Immunization of Mice

1. C57BL/6 mice (Jackson Laboratories).
2. Purified SV40 (*see* Chapter 1) or SV40 T-ag-transformed cell line derived from C57BL/6 mice (*see* Chapter 13).
3. Dulbecco's modified Eagle medium (DMEM) supplemented with 100 U of penicillin (Gibco-BRL) per mL, 100 µg of streptomycin (Gibco-BRL) per mL, 100 µg kanamycin (Gibco-BRL) per mL, 2 m*M* L-glutamine (Gibco-BRL),

10 m*M* HEPES buffer (Sigma), 0.075% (wt/vol) $NaHCO_3$, and 5–10% heat-inactivated fetal bovine serum (FBS) (Hyclone, characterized).
4. 0.25% trypsin (w/v) (Gibco-BRL) in versene. Versene consists of 1.1 m*M* ethylenediaminetetraacetic acid (EDTA) (Sigma), 137 m*M* NaCl (Gibco), 7.7 m*M* Na_2HPO_4 (Sigma), 1.4 m*M* NaH_2PO_4 (Fisher Scientific), and 2.7 m*M* KCl (Fisher Scientific), pH 7.4 and sterilized by autoclaving.
5. Hank's balanced salt solution (HBSS) (Gibco-BRL) without phenol red, filter sterilized.
6. Phosphate-buffered saline (PBS) (Gibco-BRL), filter sterilized.
7. 0.04 % trypan blue solution (Gibco-BRL) in PBS, filter sterilized.
8. PBS containing 0.1% bovine serum albumin (BSA) (ICN Biomedical, fraction V, pH 7.0).
9. Hemocytometer (Hausser Scientific).

2.2.1. In Vitro Restimulation of Primed Spleen Cells

1. Cellector™ tissue sieve with 80 mesh wire strainer (Bellco Glass, Inc.), steam sterilized.
2. 5-mL syringe plunger, sterile.
3. RPMI-1640 medium (Gibco-BRL) supplemented with 100 U of penicillin per mL, 100 µg of streptomycin per mL, 2 m*M* L-glutamine, 5×10^{-5} *M* -mercaptoethanol (Sigma), 25 µg of pyruvic acid (Sigma) per mL, 5 m*M* HEPES (Gibco-BRL), and 10% FBS.
4. HBSS without phenol red (*see* **Subheading 2.1., item 5**).
5. Tris-buffered ammonium chloride: mix 90 mL of 0.16 *M* NH_4Cl with 10 mL of 0.17 *M* Tris-HCl, pH 7.65. Adjust to pH 7.2 with HCl. Filter sterilize and store at 4°C.
6. 100-mm plastic Petri dishes (Falcon).
7. 12-well tissue-culture plates (Costar).

2.2.2. Analysis of CTL Reactivity by ^{51}Cr-Release Assay

1. Appropriate target cells (*see* **Note 1**).
2. T-25 tissue-culture flasks (Falcon).
3. Mouse recombinant gamma-interferon (Pharmingen).
4. RPMI-1640 complete medium (*see* **Subheading 2.2.1., item 3**).
5. Sodium ^{51}chromate in saline (NEN, Cat. no. NEZ-030S).
6. Synthetic peptides corresponding to T-ag CTL epitopes and an unrelated CTL epitope.
7. Versene (*see* **Subheading 2.1., item 4**).
8. 96-well V-bottom plates (Costar).
9. 5% sodium dodecyl sulfate (SDS) (Sigma) in water.

2.3. Limiting Dilution Analysis

1. C57BL/6 mice immunized with syngeneic T-ag transformed cells.
2. Naive C57BL/6 mice.

3. Iscove's modified Dulbecco's medium (IMDM) supplemented with 100 U of penicillin per mL, 100 µg of streptomycin per mL, 2 m*M* L-glutamine, 5×10^{-5} *M* -mercaptoethanol, 25 µg of pyruvic acid per mL, and 10% FBS.
4. RPMI-1640 complete medium (*see* **Subheading 2.2.1., item 3**).
5. PBS (*see* **Subheading 2.1., item 6**).
6. 96-well round-bottom microtiter plates (Costar).
7. Syngeneic T-ag-transformed stimulator cell line.
8. Recombinant human interleukin 2 (IL-2) (Amgen).
9. Supernatant from concanavalin A stimulated rat spleen cells (rat T-stim, Collaborative Biomedical, Bedford, MA).
10. 2 m*M* methyl- -D-mannopyrannoside (Sigma) in IMDM.
11. RMA cells or other appropriate target cell.
12. Sodium ^{51}chromate in saline (NEN, Cat. no. NEZ-030S).
13. Synthetic peptides corresponding to T-ag CTL epitopes and an unrelated CTL epitope.
14. 96-well V-bottom plates.
15. 5% SDS.
16. Program for calculating minimal ^{2}.

2.4 Isolation of T-ag Epitope-Specific CTL Clones

1. Bulk CTL culture specific for SV40 T-ag.
2. Naive C57BL/6 mice.
3. Syngeneic T-ag transformed stimulator cells.
4. RPMI-1640 complete medium (*see* **Subheading 2.2.1., item 3**).
5. Recombinant human IL-2 (Amgen).
6. 96-well flat-bottom plates, sterile (Costar).
7. 24-well tissue-culture plates (Costar).

3. Methods

3.1. T-ag Immunization of Mice

3.1.1. Immunization of Mice with SV40

1. Thaw an aliquot of purified SV40 and prepare a dilution of 7.5×10^8 PFU per mL in PBS containing 0.1% BSA.
2. Inject 0.2 mL (1.5×10^8 PFU) subcutaneously into the nape of the neck or intraperitoneally for groups of three mice (*see* **Note 2**).

3.1.2. Immunization of Mice with T-ag Transformed Cells

1. Grow syngeneic T-ag transformed cells in complete DMEM medium at 37°C until confluent (*see* **Notes 3** and **4**).
2. To harvest T-ag transformed cells, remove media and add 0.25% trypsin in versene until cells detach from the surface of the flask (1–3 min). Dilute with

3 vol complete DMEM and pellet cells by centrifugation at 250*g*. Resuspend cell pellets in HBSS and combine.
3. Remove a small aliquot of cells and dilute with an appropriate volume of 0.04 % trypan blue in PBS. Count the viable cells, which do not take up trypan blue, on a hemocytometer and determine the total number of cells.
4. Pellet cells and resuspend in HBSS at a concentration of 1×10^8 cells per mL.
5. Inject 0.3 mL of cells (3×10^7) intraperitoneally into groups of three mice.

3.2. T-Antigen-Specific Bulk CTL Responses

3.2.1. In Vitro Restimulation of Primed Spleen Cells

1. An appropriate syngeneic T-ag-transformed stimulator cell line should be seeded 2–3 d prior to harvest of spleens from immunized mice (*see* **Note 3**).
2. Harvest spleens from immunized animals at 9 d postimmunization into 5 mL of ice-cold complete RPMI-1640 medium.
3. Pour spleens onto wire mesh apparatus seated in a 100-mm plastic Petri dish and tease cells from spleen capsule using downward pressure and a grinding motion with a plunger from a 5-mL syringe (*see* **Note 5**).
4. Rinse screen with 10 mL complete RPMI-1640 medium and form a single-cell suspension by repeated pipeting through a 5-mL plastic pipet (*see* **Note 5**).
5. Transfer cells to a conical tube and centrifuge at 250*g* for 7 min at 4°C.
6. Aspirate the supernatant and lyse red blood cells by adding 2 mL of 37°C Tris-buffered ammonium chloride per spleen to the cell pellet. Resuspend the cells by pipeting and allow the suspension to stand for 3–5 min with occasional agitation. Dilute with 2 volumes of complete RPMI-1640 medium and centrifuge at 250*g* for 7 min at 4°C.
7. Resuspend cells in 10.5-mL complete RPMI-1640 medium and place on ice for 5 min to allow cell debris to settle.
8. Transfer approx 10 mL of cells to a new tube, excluding the debris.
9. Take a small aliquot of cells, dilute with trypan blue solution 1 : 10 per spleen, and count viable cells on a hemocytometer to determine the total number of cells.
10. Resuspend cells at 1×10^7 per mL in complete RPMI-1640 medium and place on ice.
11. Harvest T-ag-transformed stimulator cells by brief trypsinization and combine a small aliquot with an appropriate amount of trypan blue to determine the total number of viable cells.
12. Centrifuge T-ag-transformed cells at 250*g* for 7 min at 4°C and resuspend in complete RPMI-1640 medium at 1×10^6 cells per mL.
13. Inactivate stimulators with 10,000 Cgy of gamma irradiation using a gamma-source cell irradiator. Alternatively, cells can be inactivated by treatment with mitomycin C (*see* **Note 6**).
14. Plate cells in 12-well tissue-culture plates by combining 1×10^7 spleen cells and 5×10^5 irradiated T-ag-transformed stimulator cells in 4 mL of complete RPMI-1640 medium per well. Generally, 3–6 wells are plated for each group.
15. Incubate cultures in a humidified incubator at 37° C, 5% CO_2 for 5–6 d.

3.2.2. Analysis of Bulk CTL Reactivity by ^{51}Cr-Release Assay

1. Assays for CTL lysis are performed on day 6 after in vitro restimulation.
2. Seed T-ag-transformed cell lines to be used as target cells in T-25 tissue-culture flasks 3 d prior to the assay (*see* **Note 1**). Seed RMA cells 1–2 d prior to the assay in order to yield a density of 0.5–1 × 10^6 cells per mL on the day of the assay.
3. Add -interferon (IFN) at 40 U per mL to T-ag-transformed cells 48 h prior to the assay to induce upregulation of cell surface class I MHC molecules (*see* **Note 7**). RMA cells do not need to be treated with -IFN.
4. On the day of the assay, reduce the media on target cell monolayers to 2 mL and add 200 µCi of sodium ^{51}chromate (^{51}Cr) to each flask. Incubate for 3–5 h at 37°C. Alternatively, the cells can be labeled overnight. Use proper protection and adhere to the guidelines of the Nuclear Regulatory Commission when using radioactivity.
5. Label RMA cells with 100 µCi ^{51}Cr per 1 × 10^6 cells in an equivalent volume of complete RPMI-1640 medium for 1 h at 37°C, 5% CO_2 in a loose-capped test tube.
6. Wash labeled RMA cells once with PBS, resuspend at 5 × 10^5 per mL in complete RPMI-1640 medium and add 2-mL aliquots of cells to 15-mL conical tubes.
7. Add synthetic peptides corresponding to T-ag and control CTL epitopes to RMA cell aliquots at a final concentration of 1 µ*M* (*see* **Note 1**) and rock the resulting suspensions for 2 h at 37°C.
8. Harvest effector CTL from 5–6 d splenocyte cultures (*see* **Subheading 3.2.1.**) by aspiration of the overlying medium from each well and addition of 1 mL versene, prewarmed to 37°C, to each well for 1 min (*see* **Note 8**). Pipet versene up and down onto the surface of the well to suspend cells and transfer to conical tubes containing complete RPMI-1640 medium.
9. Determine the total number of viable cells harvested by performing cell counts in trypan blue solution.
10. Resuspend CTL effectors in complete RPMI-1640 medium at a concentration of 6 × 10^6 per mL (*see* **Note 9**).
11. In order to test varying concentrations of effector CTL, prepare threefold dilutions of effector CTL in complete RPMI-1640 medium for a total of four dilutions. The volume of dilutions prepared will be determined by the number of different targets which are to be tested (*see* **Note 9**).
12. Add 100 µL of each CTL dilution in triplicate to 96-well V-bottom plates for each target to be tested.
13. Place plated effector CTL at 37°C, 5% CO_2 until target cells have been harvested.
14. Wash target cell monolayers three times with 3 mL of PBS. Discard all washes into radioactive waste. Harvest cells by trypsinization and dilute with complete RPMI medium. Perform cell counts in trypan blue using a designated radioactive hemocytometer and determine the total number of cells.
15. Wash trypsinized and peptide-pulsed target cells three times and resuspend in complete RPMI-1640 medium at 1 × 10^5 cells per mL (*see* **Note 10**). Discard all

supernatants into the radioactive waste. Add 100 µL of cells (1×10^4) to wells containing the prediluted effector CTL (*see* **Note 9**). Set up 3–6 wells that contain only target cells, plus 100 µL of medium, and 3–6 wells that contain only target cells, plus 100 µL of detergent (5% SDS) to establish the spontaneous and maximum release of ^{51}Cr for each target cell, respectively.

16. Centrifuge plates for 1 min at 50*g* and incubate for 4–5 h at 37°C, 5% CO_2.
17. Centrifuge plates for 7 min at 250*g* and harvest 100 µL of supernatant from each well into an appropriate collection vial using a multichannel pipet (*see* **Note 11**).
18. Count radioactivity in a gamma-counter and determine the percent specific lysis using the following formula:

$$\% \text{ specific lysis} = \{(\text{experimental} - \text{spontaneous})/(\text{maximum} - \text{spontaneous})\} \times 100$$

where "experimental" is the counts per minute (cpm) released from target cells incubated with CTL effectors, "spontaneous" is the cpm released from target cells incubated with media alone, and "maximum" is the cpm released from target cells added to wells containing 100 µL of detergent.

19. Plot data as effector to target cell ratio vs percent specific lysis (*see* **Fig. 1**).

3.3. Determination of T-ag Epitope-Specific CTL Precursor Frequency by Limiting Dilution Analysis

1. Harvest spleens from 3–4 C57BL/6 mice at 9 d postimmunization and 2–3 naive C57BL/6 mice into separate tubes containing 5 mL complete IMDM (*see* **Note 12**).
2. Process spleen groups through wire mesh as outlined in **Subheading 3.2.1., steps 3–9**.
3. Inactivate naive C57BL/6 spleen cells with 2000 Cgy of gamma-irradiation and place on ice until needed (*see* **Note 12**).
4. Resuspend spleen cells from immunized mice at 2.56×10^6 per mL in complete IMDM and prepare twofold dilutions (*see* **Note 13**).
5. Add 100 µL of spleen-cell suspension per well to 24–60 inside wells of a 96-well, round-bottom plate for each dilution to yield a range of 256,000–250 cells per well. Add 200 µL of PBS to the outside wells of each 96-well plate in order to prevent evaporation.
6. Prepare syngeneic T-ag transformed stimulator cells as in **Subheading 3.2.1., steps 11–13** (*see* **Note 3**).
7. Prepare stimulation media containing 2×10^4 irradiated T-ag-transformed cells per mL, 1×10^6 irradiated naive C57BL/6 spleen cells per mL, 2.5 U rIL-2 per mL, 11% rat T-stim, and 220 m*M* methyl- -D-mannopyrannoside in complete IMDM. Add 100 µL per well to all wells containing immune spleen cell dilutions, plus an additional 24 wells without any immune spleen cells to serve as controls (*see* **Note 14**).
8. Incubate 7 d in a humidified incubator at 37°C, 5% CO_2.
9. Grow RMA cells to $0.5–1 \times 10^6$ per mL (*see* **Note 15**). Label an appropriate number of cells with 100 µCi ^{51}Cr per 1×10^6 cells in an equivalent volume of complete RPMI-1640 medium for 2 h at 37°C, 5% CO_2.

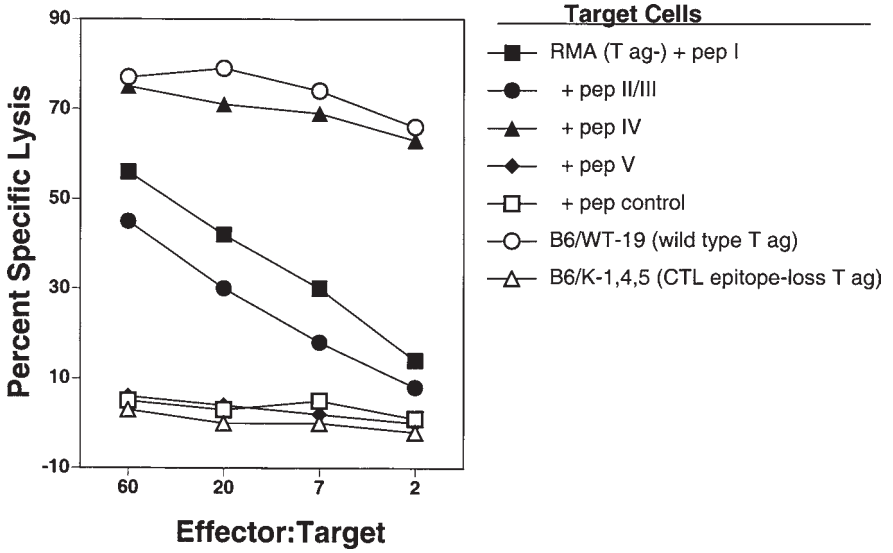

Fig. 1. Lysis of T-ag-transformed and peptide-pulsed target cells by SV40 T-ag-specific CTL. Spleen cells from C57BL/6 mice immunized intraperitoneally with 3×10^7 T-ag-transformed B6/WT-19 cells were cultured in vitro with irradiated B6/WT-19 cells for 6 d. The presence of T-ag epitope-specific CTL was analyzed using T-ag-negative RMA cells that had been pulsed with synthetic peptides corresponding to T-ag epitopes I, II/III, IV, V, and an unrelated control peptide. CTL specific for epitopes I, II/III, and IV were detected. These CTL also lyse the T-ag-transformed cell line B6/WT-19, but not B6/K-1,4,5 cells, which lack the T-ag epitopes.

10. Wash RMA cells one time with PBS, resuspend in 4 mL complete RPMI-1640 medium, and split between two 15-mL conical tubes (1×10^6 cells).
11. Add 1 μ*M* of synthetic peptide corresponding to the appropriate T-ag epitope or an unrelated CTL epitope and rock the resulting suspensions for 2 h at 37°C.
12. Add 30–40 μL of complete IMDM to each effector well to be harvested (*see* **Note 16**).
13. Resuspend effector cells by repeated pipeting with a multichannel pipet and transfer 100 μL to duplicate sets of 96-well V-bottom plates. Place at 37°C, 5% CO_2 until the target cells are prepared.
14. Wash RMA cells three times to remove excess peptide.
15. Resuspend peptide-pulsed RMA cells in complete IMDM at 2×10^4 per mL and add 100 μL per well to one set of plates containing the diluted effector cells.
16. Prepare six wells containing only peptide-pulsed RMA target cells plus medium and six wells containing target cells plus detergent to serve as spontaneous and maximum release controls, repectively, for each target cell (*see* **Subheading 3.2.2., step 18**).
17. Incubate 5 h at 37°C, 5% CO_2.

18. Centrifuge plates at 250*g* and collect 100 µL of supernatant.
19. Determine the cpm released for each sample using a -counter.
20. Determine the number of wells per effector dilution containing at least one CTL precursor as those with ^{51}Cr-release values that exceed the controls (no stimulators) by three standard deviations (*see* **Note 17**).
22. Estimate the frequency of epitope-specific CTL using the minimal ^{2} method (*see* **Notes 18** and **19**).

3.4. Isolation of T-ag Epitope-Specific CTL Clones

1. Prepare syngeneic T-ag-transformed stimulator cells as described in **Subheading 3.2.1.** (*see* **Note 3**).
2. Harvest spleens from naive C57BL/6 mice and prepare irradiated feeder spleen cells as described in **Subheading 3.3., steps 1–3**.
3. Harvest 1–2 wells of bulk T-ag-specific CTL from **Subheading 3.2.1.** 7 d after stimulation.
4. Determine the number of viable cells by trypan blue exclusion and resuspend in complete RPMI-1640 medium at 900 cells per mL.
5. Prepare threefold dilutions of effector cells in complete RPMI-1640 medium to yield approximate cell concentrations of 300, 100, 33, 11, and 3 cells per mL (*see* **Note 20**).
6. Add 100 µL of each dilution to 24 inside wells of a 96-well flat-bottom plate. Add 200 µL of PBS to the outside wells of the plate to prevent evaporation.
7 Prepare stimulation media containing 1–2 × 10^5 irradiated T-ag-transformed cells per mL, 1–2 × 10^6 irradiated C57BL/6 feeder cells per mL and 10 U per mL recombinant human IL-2 in complete RPMI-1640 medium. Add 100 µL to wells containing the prediluted effector cells and an additional set of wells containing only 100 µL of complete RPMI-1640 medium for control wells.
8. Allow CTL to expand for 4–5 d and then screen under the microscope for positive cell growth. Look for dense cell clusters indicating cell expansion. Determine the number of wells per dilution that are positive for cell expansion. Dilutions at which less than 67% of the wells are positive are considered to contain clonal growth.
9. On day 7–10 after stimulation, pick clones from individual wells into one well of a 24-well plate containing 2 × 10^5 irradiated T-ag-transformed cells and 5 U per mL recombinant human IL-2 in 2 mL of complete RPMI-1640 medium.
10. Continue to expand individual clones every 4–7 d until enough cells can be collected to test T-ag epitope-specific recognition of target cells in a ^{51}Cr-release assay as outlined in **Subheading 3.2.2.**

4. Notes

1. Target cells should include T-ag-expressing and T-ag-negative cell lines of the same haplotype (e.g., H-2^b) in order to show T-ag-specific recognition. Other important control target cells include T-ag-transformed cells of a different haplo-

type (e.g., H-2^d) to demonstrate class I MHC-restricted CTL killing. If T-ag epitopes are known, synthetic peptides representing these epitopes may be pulsed onto syngeneic T-ag-negative cells to detect T-ag epitope-specific CTL. We have shown that RMA cells work well in the H-2^b system ***(18)***. Be particularly careful to prevent crosscontamination of peptide stock solutions as this may result in false positives for CTL lysis. We suggest the use of barrier pipet tips when working with synthetic peptides.

2. We have also immunized mice in the hind footpad with 0.5–2 × 10^7 PFU SV40 in order to isolate SV40 T-ag-specific CTL from the draining popliteal lymph nodes ***(19)***.
3. T-ag-transformed cells should be syngeneic with the strain of mice to be immunized (e.g., H-2^b cells for immunization of C57BL/6 mice, H-2^d cells for immunization of Balb/c mice) in order to avoid the induction of alloreactive CTL. Cells that grow in monolayers can be grown in T-150 flasks or roller bottles if larger amounts of cells are required. We have found that cells seeded 2–3 d prior to use are more efficient for immunization and for stimulation in vitro than cells that are seeded for shorter lengths of time. This may allow the accumulation of T-ag within the cells.
4. Alternative immunogens may be used to elicit CTL responses to SV40 T-ag including purified T-ag and recombinant vaccinia viruses expressing full-length T-ag or portions of T-ag. Dose and route schedules for each of these immunogens include 1–10 µg purified T-ag subcutaneously or intraperitoneally ***(20)***, and 1 × 10^7 PFU recombinant vaccinia virus intravenously ***(18)***. Spleens from vaccinia virus infected mice should be harvested 3 wk following immunization to avoid nonspecific lysis caused by vaccinia virus specific immune responses.
5. To achieve maximal yields from spleens, the screen should be placed in 5 mL of medium to facilitate passage of cells through the screen. We use the same medium into which the spleens were harvested. Continue to grind the spleen until the capsule turns white, indicating that the cells have been removed, and discard the capsule. Rub the pipet plunger across the underside of the screen to remove any clumps that have accumulated. Thoroughly rinse the screen, focusing on any clumps, and pipet the cell suspension until the majority of clumps are dispersed.
6. For inactivation of stimulator cells with the DNA synthesis inhibitor mitomycin C, trypsinize monolayers of T-ag-transformed cells and resuspend at 3 × 10^6 cells per mL in complete DMEM containing 0.1 mg per mL mitomycin C. Incubate cells at 37°C for 1 h. Wash the cells three times to remove excess mitomycin C prior to use as stimulators. Mitomycin C-inactivated cells may be stored at –80°C in 90% FBS/10% dimethlysulfoxide (DMSO) for 2–3 mo.
7. Although pretreatment of target cells with -interferon is not necessary to detect lysis by T-ag-specific CTL, this treatment has been shown to enhance the sensitivity of target cells to CTL mediated lysis. -interferon is known to increase the expression of class I MHC molecules, as well as other proteins involved in the processing and presentation of antigens from full-length proteins ***(21)***. Because many Tag transformed cell lines express low levels of surface class I

MHC molecules, -IFN pretreatment may optimize the sensitivity of the target cells.

8. The addition of versene to spleen cell cultures enhances detachment of lymphocytes from the surface of the well, requiring less vigorous pipetting for retrieval. The time in versene should be kept to a minimum in order to preserve cell viability. Cells are generally placed on ice until all effector populations have been harvested.
9. The concentration of effector CTL is determined by the desired effector to target cell ratio. If 1×10^4 target cells are plated in 100 µL per well, the addition of 6×10^5 effector CTL in 100 µL will yield an effector to target ratio of 60:1. If too few effector CTL are obtained to achieve the intended effector:target cell ratio, the number of target cells plated per well may be reduced (e.g., by half), thereby reducing the number of effector CTL required to achieve the desired effector to target cell ratio.
10. When washing target cells, avoid pipetting cells to resuspend. The cells should be resuspended by flicking the bottom of the tube to achieve a vortex action prior to addition of wash buffer or media in order to avoid clumping. Unnecessary pipeting may lead to high spontaneous release of radioactivity.
11. Collection vials may vary because of the format of the -counter used. The use of microvials in a 96-well format aids in efficient transfer of supernatants.
12. Because a varied number of immune spleen cells are aliquoted into microtiter wells, a constant number of naive spleen cells that have been inactivated by -irradiation are added to each well to serve as a feeder layer and to provide a source of professional antigen-presenting cells.
13. We have found that the range of 256,000 to 250 spleen cells plated per well allows the estimation of CTL precursors specific for T-ag H-2^b epitopes. The range of spleen cells plated per well may need to be adjusted depending on the strain of mice used, immunization protocol, and efficiency of target-cell recognition (*see* **Note 15**). Although we typically have used twofold dilutions, higher dilutions may be used if a larger range of spleen cells are plated.
14. As a source of IL-2, we include both recombinant human IL-2 and supernatant from concanavalin A stimulated rat spleen cells. Because these two sources can differentially lead to the expansion of distinct CTL clones from a bulk population of spleen cells ***(22)***, we use a combination of sources in order to induce an inclusive response. Methyl- -D-mannopyrannoside is added for a final concentration of 100 mM to inactivate any concanavalin A still present in the supernatant, which could nonspecifically stimulate T lymphocytes. The stimulation medium is prepared at a 2X concentration because 100 µL is added to 100 µL of effector cells. Control wells containing only stimulation medium should receive an additional 100 µL of complete IMDM to maintain an equivalent concentration of media components.
15. The choice of target cell used in limiting dilution analysis (LDA) should be based on the sensitivity achieved vs. the level of nonspecific lysis that can occur because of activation of some spleen cells by addition of IL-2 alone. Thus, it is important to determine if the target cell can be sensitized to CTL lysis by incuba-

tion with a synthetic peptide corresponding to the appropriate T-ag epitope. In addition, if T-ag-transformed cells are used as target cells an appropriate T-ag-negative cell line should be used as a negative control.

16. Because some evaporation of media will occur during the 7-d incubation period, we add a small amount of medium to each well prior to harvesting the cells to ensure that enough volume is present to result in two equivalent aliquots. The plating of effector cells in only the inside wells of the 96-well round-bottom plates as described in **step 4** minimizes the amount of evaporation that occurs during the incubation period.
17. For example, if the control wells containing only stimulator cells yielded mean cpm of 3100 ± 300 cpm, then all test wells within a given effector dilution with cpm higher than 4000 would be considered as positive. Alternatively, a particular percent specific lysis, such as 10%, may be used to determine which wells yield positive vs negative responses.
18. The most accurate analysis of LDA is currently the minimal ^{2} method ***(16)***. Computer software for calculation of frequencies using the ^{2} method is now commercially available ***(17)***. The data are typically reported as the reciprocal frequency of CTL and including the 95% confidence limits and the ^{2} values for both the relevant and unrelated targets. Lefkovits et al. ***(17)*** provide a detailed description of the methods used for data analysis, as well as the theory behind LDA.
19. Recent technologic advances have led to the development of additional methods for the determination of frequencies of epitope-specific $CD8^+$ T lymphocytes. The ELISPOT ***(23)*** and flow-cytometric measurement of intracellular cytokines ***(24)*** allow the direct measurement of cytokine production from individual cells in response to a stimulus. These methods are advantageous in that they do not require cells to proliferate and divide in vitro in order to be detected, in contrast to the LDA. The effector CTL must be able to secrete a specific cytokine in order to be detected, however. A more recent approach is the use of fluorescence conjugated class I MHC/peptide complex tetramers, which can stain CTL expressing a TCR specific for a given epitope ***(25)***. In this way, the stained CTL within a population of cells can be visualized and quantitated by flow cytometry. The combination of class I MHC tetramer or intracellular cytokine staining with the use of antibodies specific for cell surface markers allows the additional determination of activation status for individual epitope-specific CTL.
20. The number of effector CTL plated per well may need to be adjusted depending on the cloning efficiency of the particular cells.

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Detection of SV40 DNA Sequences in Human Tissue

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1. Introduction

Several recent investigations using polymerase chain reaction (PCR) detection methods have identified SV40-like DNA sequences in human neoplasms, particularly choroid plexus tumors ***(1)***, ependymomas ***(1)***, mesotheliomas ***(2)***, and osteosarcomas ***(3,4)***. Difficulties have arisen because of the use of paraffin-fixed tissue, loss of possible viral episomes during DNA isolation, low copy numbers of viral DNA, PCR contamination, and confusion with BKV and JCV sequences. Nonetheless, the authentication of amplified products by DNA sequencing, the finding of a single 72-bp viral-enhancer repeat element, variability of large tumor antigen (T-ag) carboxy terminal DNA sequences in many specimens, and the isolation of SV40 virus from a choroid plexus tumor all support the validity of the basic observation that SV40 is present in humans, and may therefore contribute to oncogenesis in special situations.

The protocols described here have been optimized for PCR detection of several regions of the SV40 genome in DNA extracted from human tissue. **Subheading 4.** highlights areas for particular attention that have been concerns among investigators with respect to detection efficiency and fidelity.

2. Materials

1. Oligonucleotide primers and probes: Reference nucleotide positions (nt) are given and identify the relevant nt of BKV strain Dunlop, JCV strain MAD-1, SV40 strain 776, or of the human A-gamma hemoglobin gene sequence (GenBank # M32724). The following oligonucleotide (5 –3) sequences were used as primers: for BKV and JCV: PYVfor, TAGGTGCCAACCTATGGA ACAGA (nt 4569-4547 from BKV, nt 4429-4407 from JCV); PYVrev, GGAAAGTCTTTAGGGTCTTCTACC (nt 4388-4411 from BKV, nt 4251-4274

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from JCV); BK probe, GAGAATCTGCTGTTGCTTCTT (nt 4443-4463); JC probe GTTGGGATCCTGTGTTTTCAT (nt 4303-4323). For SV40, 574-bp product spanning intron: SVfor2, CTTTGGAGGCTTCTGGGATGCAACT (nt 4945-4921 from SV40), SVrev, GCATGACTCAAAAAACTTAGCAATTCTG (nt 4372-4399); for SV40, 105-bp product SVfor3, TGAGGCTACTGCTGACTC TCAACA (nt 4476-4453 from SV40) SVrev, GCATGACTCAAAAAACTTA GCAATTCTG (nt 4372-4399); SV probe, ATGTTGAGAGTCAGCAGTAGCC (nt 4452-4473); SV40 enhancer/origin R1, AATGTGTGTCAGTTAGGGTGTG (nt 266-245 from SV40), R2, TCCAAAAAAGCCTCCTCACTACTT (nt 5195-5218), R3, GCGTGACAGCCGGCGCAGCACCA (nt 358-336), R4, GTC CATTAGCTGCAAAGATTCCTC (nt 5119-5142); SV40 VP1 294 bp product LA1, GGGTGTTGGGCCCTTGTGCAAAGC (nt 2251-2274 from SV40), LA2, CATGTCTGGATCCCCAGGAAGCTC (nt 2545-2522), LA3, CAGCAGTGG AAGGGACTTCCCAG (nt 2336-2358); SV40 carboxy-terminus T1, GACC TGTGGCTGAGTTTGCTCA (nt 3070-3049 from SV40), T2, GCTTTATT TGTAACCATTATAAG (nt 2630-2652); T3, ACCACAACTAGAATGCA GTGAAAAAA (nt 2581-2598), T4, GAAGACAGCCAGGAAAATGCTG ATAA (nt 2909-2884), T probe AACCTCTACAAATGTGGTATGGCT (nt 2741-2764); globin controls AG1, CTCAGACGTTCCAGAAGCGAGTGT (nt 1252-1229), AG2, AAACGGCTGACAAAAGAAGTCCT (nt 876-897), AG3, AAACTAGCTAAAGGGAAG (nt 923-940).

The positions and directions of primer pairs relative to the viral genome are shown in **Fig. 1**. GenBank accession numbers of polyomavirus reference strains: JCV-MAD-1, J02226; SV40-776, J02400; BKV-Dunlop; J02038.

2. TE buffer: 10 m*M* Tris-HCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA), pH 8.0.
3. 50X TAE buffer: 242 g Tris-base, 57.1 mL glacial acetic acid, 100 mL 0.5 *M* EDTA pH 8.0, to final volume of 1 L with water.
4. 6X Loading buffer: 0.3% (w/v) bromphenol blue and/or xylene cyanol, 30% glycerol, 6 m*M* EDTA, pH 8.0.
5. 20X SSC: 175.3 g NaCl, 88.2 g sodium citrate in 800 mL of water, adjust pH to 7.0 with NaOH, and bring to 1 L with water.
6. 20X SSPE: 175.3 g NaCl, 27.6 g NaH_2PO_4-H_2O, 7.4 g EDTA in 800 mL water, adjust pH to 7.4 with NaOH, and bring to 1 L with water.
7. 50X Denhardt's solution: 5 g ficoll (Type 400, Pharmacia), 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (BSA) (Fraction V, Sigma), to 500 mL with water.
8. Hybridization buffer A: 2X SSC, 1% sodium dodecyl sulfate (SDS), 20 m*M* sodium biphosphate, and 50 µg/mL salmon sperm DNA.
9. Hybridization buffer B: 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS, 25 µg/mL denatured salmon sperm DNA.

3. Methods

3.1. Isolation of DNA

1. Perform all manipulations within a dedicated facility in which SV40 plasmids and virus have been excluded, ideally in a safety hood whose working surfaces

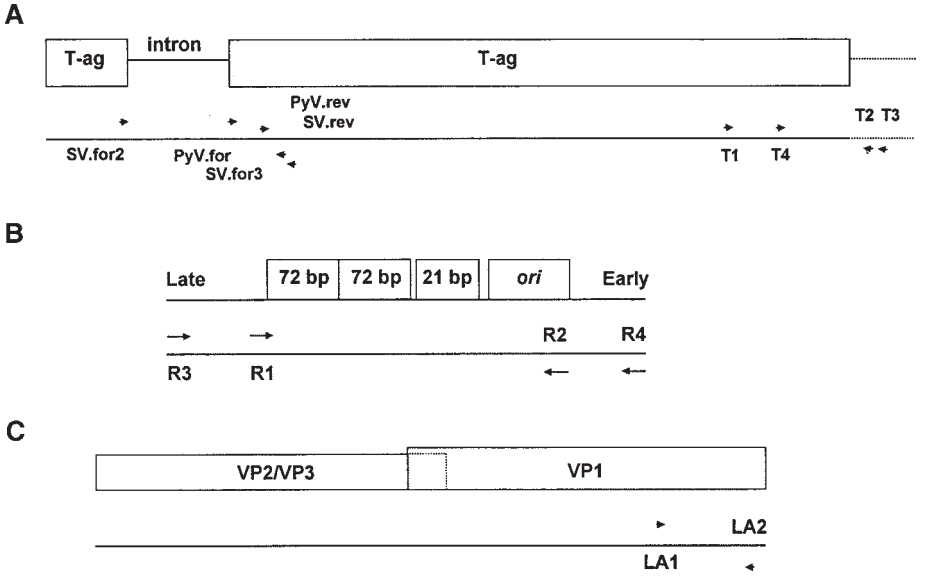

Fig. 1. Schematic representation of PCR and DNA sequencing primer binding sites in SV40-776. The arrows denote the relative positions and directions of the primers (5 –3) during amplification of the T-ag gene **(A)**, regulatory region **(B)**, and the VP1 gene **(C)**. T-ag, large tumor antigen gene; 72 bp, 72 bp enhancer element; 21 bp, 21 bp repeat region (GC-rich region); *ori*; origin of DNA replication; VP2/VP3, viral coat protein 2 and 3 genes; VP1, viral coat protein 1 gene.

have been exposed to sterilizing UV rays prior to DNA extraction. Mince frozen tumor samples and digest overnight at 55°C with proteinase K ***(5)***. Extract the digested sample once with equal volume of phenol (pH 8.0) and collect the DNA-containing aqueous layer. Repeat extraction procedure once more using phenol (pH 8.0) : chloroform : isoamyl alcohol (25 : 24 : 1), and transfer the DNA-containing aqueous layer into a suitable centrifugation tube. Adjust to 2 *M* ammonium acetate, precipitate the DNA with 2 vol of ethanol or 0.8 vol of 2-propanol, wash once with 70% ethanol, dry the pellet, then slowly rehydrate DNA pellet in TE buffer for 24–48 h at 4°C. Triturate the solubilized DNA by gentle resuspension using a large bore pipet (*see* **Notes 1** and **2**).

2. Analyze DNA from frozen tumor samples by gel electrophoresis and ethidium bromide staining for the presence of mitochondrial DNA, which indicates that low-MW ds supercoiled DNA (such as episomal polyomavirus DNA) has been successfully purified. Test by PCR with primers AG1 and AG2 as described below, and continue if the expected 379-bp AG1- and AG2-primed PCR product is observed by gel electrophoresis (*see* **Note 4**).
3. Extract DNA from paraffin-embedded samples as described previously ***(1)*** or by using dedicated commercial kits. Dewax by transferring 1–5 paraffin sections

(5–10 μm) to an autoclaved 1.5-mL microcentrifuge tube and pulverizing with a sterile barrier tip pipet. Deparaffinize by immersion in 1 mL xylene, 37°C for 15 min. Centrifuge for 2 min to pellet the tissue, and remove xylene mixed with solubilized paraffin with a sterile barrier tip pipet. (Samples older than several years will require additional xylene extractions). Add 1 mL 100% ethanol, and incubate at 20°C for 5 min. Centrifuge for 2 min, remove ethanol, and air-dry. Either digest with proteinase K or boil and use directly (*see* **Note 5**).

4. Analyze DNA from paraffin samples by gel electrophoresis and ethidium bromide staining to ensure DNA recovery. DNA from paraffin-embedded tissues is usually linear ds DNA less than 1 kb in size. Intact mitochondrial DNA and higher MW DNA are rarely recovered from these types of specimens. PCR with primers AG1 and AG2 as described below, and continue if the expected 379-bp AG1- and AG2-primed PCR product are observed by gel electrophoresis.

3.2. PCR Conditions

1. Observe general precautions of Kwok and Higuchi ***(6)***. Manipulate samples and prepare PCRs in a facility in which polyomaviruses or plasmids containing polyomavirus inserts have been excluded.
2. Use presterilized filter pipet tips (barrier tips), and perform all manipulations in a laminar flow hood or in a UV light-equipped modular PCR workstation. Each PCR mixture contains 250–600 ng of test DNA, 50 pmol of each primer, all four deoxynucleotide triphosphates (dNTPs) (Promega, Madison, WI) at a final concentration of 200 m*M*, 5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin-Elmer Roche), 1.5 m*M* magnesium, and PCR buffer (Perkin-Elmer Roche) in a final reaction volume of 50 μL (*see* **Note 6**).
3. Thermocycle profiles:
   a. For bottom-heating PCR machines that require 200 μL capacity PCR tubes and an oil or wax overlay of the samples (such as the Perkin-Elmer Cetus DNA thermal cycler), initial denaturation is at 94°C for 3 min, followed by cycling 30 to 45 times for 1 min at 94°C, 1 min at the proper annealing temperature (*see* **Table 1**), and 1 min at 72°C, and terminated with one final extension step at 72°C for 15 min.
   b. For PCR machines in which the heat source is positioned above the samples (and for which an oil overlay is omitted, such as a Perkin-Elmer Cetus Gene Amp PCR System 2400), samples are initially denatured for 2 min, cycled 30–45 times using 15-s annealing, denaturation, and extension steps, and the terminal extension step performed for 7 min. Use high stringency annealing temperatures (**Table 1**) for high-quality DNA extracted from fresh or frozen tissue samples. For these types of samples, 30 to 45 denaturation, annealing, and extension cycles are used. DNA extracted from paraffin-embedded samples is usually highly fragmented, and low-stringency annealing temperatures (**Table 1**) are used. For additional detection sensitivity, add a fresh aliquot of enzyme (5 U) after the first terminal extension step, and repeat the

Table 1
PCR, DNA Sequencing, and Probe Oligonucleotides Used for Detection of Papovavirus DNA

Oligonucleotides		Reference positions[a]	Temperature[b]	Size (bp), PCR product [SVSph21-N	SV21-N	SV2X21-N][c]
SV40 regulatory region oligonucleotides						
R1:	5 -AATGTGTGTCAGTTAGGGTGTG-3	nt 266–245	63(52)	245	317	386
R2:	5 -TCCAAAAAAGCCTCCTCACTACTT-3	nt 5195–5218				
R3:	5 -GCGTGACAGCCGGCGCAGCACCA-3	nt 358–336	63(52)	413	485	554
R4:	5 -GTCCATTAGCTGCAAAGATTCCTC-3	nt 5119–5142				
SV40 VP1 carboxy proximal oligonucleotides						
LA1:	5 -GGGTGTTGGGCCCTTGTGCAAAGC-3	nt 2251–2274	63(52)		294[d]	
LA2:	5 -CATGTCTGGATCCCCAGGAAGCTC-3	nt 2545–2522				
LA3:	5 -CAGCAGTGGAAGGGACTTCCCAG-3	nt 2336–2358	(N/A)		(N/A; VP1 probe)[d]	
SV40 T-ag amino proximal oligonucleotides						
SV.for3:	5 -TGAGGCTACTGCTGACTCTCAACA-3	nt 4476–4453	63(52)		105[d]	
SV.rev:	5 -GCATGACTCAAAAAACTTAGCAATTCTG-3	nt 4372–4399				
SV40 T-ag carboxy terminal oligonucleotides						
T1:	5 -GACCTGTGGCTGAGTTTGCTCA-3	nt 3070–3049	60(52)		441[e]	
T2:	5 -GCTTTATTTGTAACCATTATAAG-3	nt 2630–2652				
Human A -hemoglobin gene						
AG1:	5 -CTCAGACGTTCCAGAAGCGAGTGT-3	nt 1252–1229[f]	60		379	
AG2:	5 -AAACGGCTGACAAAAGAAGTCCT-3	nt 876–897				

[a]Reference nucleotide (nt) positions in SV40 reference strain 776 (SV40-776).
[b]PCR annealing temperature (°C) under stringent and nonstringent conditions (number in parentheses).
[c]Artificial SV40 control templates ***(12)***.
[d]pSV21-N and other full-length SV40 genomes that have been currently tested.
[e]pSV21-N and other plasmids derived from SV40-776.
[f]GenBank human A- hemoglobin gene sequence, accession #M32724.

entire thermocycle for a total of 60–92 denaturation, annealing, and extension cycles (*see* **Note 7**).

4. Plasmids pSVSph21-N, pSV21-N, and pSV2X21-N ***(7)***, each containing artificial SV40 genomes, are used as positive control plasmids (*see* **Note 10**).

3.3. Restriction Enzyme Analysis of PCR Products

1. Digest with *Sal*1 or *Xho*1 using 4 µL of PCR-amplified DNA, 1 µL of 10X commercial buffer, 4 µL of H_2O, and 1 µL of either *Sal*1 (10 U/µL) or *Xho*1 (20 U/µL), with incubation at 37°C for at least 30 min. Distinguish the SV40 regulatory region from the corresponding sequence amplified from BKV or JCV (with primers RA1, -2, -3, and -4), with *Sfi*1 using 4 µL of PCR-amplified DNA at 50°C for at least 30 min [BKV and JCV lack an *Sfi*1 site at the regulatory region, although resistant SV40 regulatory regions have been recently detected ***(8)***].

3.4. Agarose Gel Electrophoresis of PCR Products

1. Electrophorese 5 µL of a PCR product at 60 mA on a 2% SeaKem LE agarose (FMC BioProducts, Rockland, ME) gel in 1X TAE buffer, and stain with ethidium bromide. Load samples amplified with primers SV.for3 and SV.rev with buffer containing xylene-cyanol dye (because the PCR product formed with these primers comigrates with bromophenol blue). Load all other samples with loading buffer containing bromophenol blue dye.

3.5. DNA Sequence Analysis

1. Purify the PCR product after gel electrophoresis. Use asymmetric PCR to generate a single-stranded template by performing 30 cycles of PCR with a reduced concentration of one primer, and sequence using Sequenase or Promega kits. Alternatively, sequence the ds PCR product from the gel purified DNA using the Sequenase PCR Product Sequencing kit (USB Corp, OH) or ThermoSequenase Radiolabled Termination Cycle Sequencing Kit (USB Corp., *see* **Note 13**).

3.6. Southern Blot Analysis

1. Separate PCR products by agarose gel electrophoresis and transfer to a nylon membrane (Duralon UV, Biocrest). Crosslink DNA molecules to the membrane under UV light (UV Stratalinker). Hybridize transfer membrane in buffer A at 42°C with ^{32}P-labeled probes.
2. Alternatively, separate the PCR products by agarose gel electrophoresis and vacublot transfer to a nylon membrane (Hybond N+ membrane, Amersham Life Sciences) using alkaline transfer following the manufacturer's specifications. Hybridize in buffer B at 42°C with ^{32}P-labeled probes.
3. Radiolabeled probes for Southern blots are made by end-labeling oligonucleotides with [^{32}P]ATP using standard conditions ***(9)***.

4. Notes

1. Oligonucleotides are available from many sources. It is advisable to obtain "desalted" oligonucleotides for PCR, and to store them at –20°C in TE buffer.
2. For the preparation of TE buffer or TAE for gel-purification of DNA, extra precautions are mandatory. Distilled, deionized or double-deionized PCR quality water, prepared at a separate facility, should be used. We use double-deionized Milli-Q water (Millipore Corp.). Mixing vessels and stir bars should be thoroughly cleaned with a DNA contaminant removal solution (detergent) such as DNA-OFF (CPG Inc.) prior to use. New containers or tubes should be used for storing TE buffer. The sterilized buffer should be aliquoted into small volumes, and each aliquot used only once. For routine (analytical) purposes, large batches of buffer are made using molecular biology grade distilled, deionized water. Smaller batches are prepared using PCR-quality water and the same precautions for preparing TE buffer.
3. A primary assessment of DNA quality begins with an agarose-gel electrophoretic analysis. Reliance on spectrophotometric readings alone to calculate DNA concentrations is inadequate, as the degree of DNA fragmentation/degradation is not taken into account and impurities within the DNA preparation can lead to aberrant spectrophotometric values. Ideal samples contain intact mitochondrial DNA.
4. Commercial kits for purifying DNA from tissue samples isolate largely intact high-molecular-weight DNA with decreased or absent mitochondrial DNA. Pilot studies should be performed to test the suitability of a particular kit, paying particular attention to the efficiency of recovery of mitochondrial DNA and episomal polyomavirus genomes.
5. DNA extraction methods for paraffin samples typically utilize xylene ***(10)*** or hexane ***(11)*** to dewax the samples, followed by digestion with proteinase K. However, the time used for dewaxing varies widely (<0.5 to <24 h), and may affect the amount and quality of recovered DNA. DNA recovery is also affected by other factors, such as the method used for tissue preservation ***(12)***. Many commercial kits are currently available that offer convenience and simplicity of extraction. Currently, we utilize the EX-WAX™ DNA Extraction kit for paraffin-embedded tissue (Intergen), which, in our experience, produces PCR-quality DNA.
6. Aliquot premixed cocktails (4°C) containing buffer, dNTPs, water, and enzyme into reaction tubes. Tubes containing reaction mixes should be kept cold (maintained in a shallow ice bath at 4°C) to minimize nonspecific amplification by *Taq* polymerase prior to cycling. Add DNA samples (4°C) to the tubes (typically), then an oil overlay if required (*see* **Note 3**). Add positive control DNAs to the appropriate tubes in a separate room, and perform thermocycling in yet another room. Instead of keeping samples cold prior to PCR, "Hot Start PCR" can be used. We have used AmpliWAX PCR Gem 100 (Perkin-Elmer) wax beads, following the manufacturer's recommendations for combining the components of the PCR.
7. Appropriate thermocycle parameters must be chosen for the type of PCR machine to be used. Different PCR protocols are required for "archival DNA" (DNA puri-

fied from preserved tissue embedded in paraffin blocks) and for DNA purified from frozen or fresh tumors. The major difference is that DNA from fresh or frozen tissues can often be purified in a largely intact form, whereas archival DNA is usually fragmented/degraded (with a mean length that can measure <500 bp) ***(4,12–14)*** and PCR with these templates are significantly less efficient ***(4,12–14)***. Annealing temperatures for particular primer pairs must be adjusted according to the quality of the purified test DNA. The problem of sample inferiority for PCR when utilizing DNA extracted from paraffin-embedded samples is partially alleviated by using lower-stringency annealing temperatures. In contrast, higher stringency annealing temperatures are appropriate for properly extracted DNA from fresh or frozen tumors, as nonspecific (artefactual) amplification of heterologous DNA sequence can occur. Low stringency and high stringency annealing temperatures for some of our primers are given in **Table 1**. Archival samples may require re-amplification. Crosscontamination caused by splattering or aerosol formation when adding enzyme for the second amplification series, especially when small tubes (e.g., 100 µL capacity PCR tubes) must be avoided by careful uncapping of tubes at this step.

8. PCR may be more sensitive with prior linearization of DNA or topoisomerase treatment ***(15–17)***.
9. To confirm the presence of SV40, primers are directed at several sites of the SV40 genome (**Fig. 1**). The regulatory region serves as a unique genetic marker for discriminating among BKV, JCV, and SV40, and may distinguish between commonly encountered SV40 laboratory strains and natural strains of SV40 ***(7)***. To differentiate among SV40 strains, an analysis of the DNA sequence that encodes the carboxy terminus of T-ag is imperative ***(4,13,18,19)***. Because primers TA1 and TA2 amplify a DNA sequence that includes the T-ag-VP1 intragenic sequence and because of T-ag variations ***(4,13,18–21)***, different TA1- and TA2-primed PCR products may differ slightly in mobility on a 2% agarose gel.
10. Contamination of test samples by positive control DNA templates is a major problem, leading to false positives. Artificial SV40 templates are, therefore, used in control reactions. Plasmids pSV2X21-N, pSV21-N, and pSVSph21-N contain multiple nucleotide changes within the regulatory region of SV40-776, and these changes distinguish between laboratory contamination and true positives ***(7)***. PCR products arising from these artificial templates are cleaved by restriction enzymes *Sal*1 or *Xho*1 (lacking in natural SV40 strains), providing a rapid method for assessment of authenticity. Additionally, the SV40 regulatory regions of these plasmids can be used to conjecture the type of SV40 regulatory region in a specimen; the regulatory region of pSVSph21-N is similar in size to that of typical archetypal SV40 regulatory regions, the regulatory region of pSV21-N is similar in size to that of the nonarchetypal regulatory region of SV40 reference strain 776, whereas the nonarchetypal regulatory region of pSV2X21-N is 72 bp larger than that of SV40 reference strain 776 ***(7)*** (*see* **Table 1**). All three plasmids are identical in nt sequence except at the viral regulatory region, and all are *Eco*R1

clones. Thus, any of the three plasmids can also be used as a PCR positive control template for regions of SV40 that are not interrupted by the EcoR1 site. Because all three are derivatives of SV40-776, sequence analysis of DNA amplified using primers TA1 and TA2 is often informative, as we have detected sequence variation within this region ***(4,13,18–21)***, and so sequence identity with SV40-776 might indicate contamination with positive control DNA. Also, most commercially available plasmids that might be a source of SV40 DNA contamination of a laboratory are based on SV40-776.

11. RA1 and RA2 work well as PCR primers at an annealing temperature of 52°C (little or no unrelated sequences were amplified after 30 denaturation, annealing, and extension PCR cycles using 0.5 µg of DNA), whereas many human-chromosome-derived sequences were amplified from any human tissue using primers RA3 and RA4 at that temperature (confirmed by DNA sequencing of the products; data not shown). Thus, when "low-stringency amplifications" were performed with primers RA3 and RA4, PCR products in the size range of 100–500 bp were sequenced.
12. Nonpolyomavirus encoded sequences can be amplified with primers TA1 and TA2 at an annealing temperature of 52°C when more than 600 ng of intact human DNA is present. These PCR bands range from 100–500 bp. At temperatures higher than 60°C, artifactual T-ag bands appear with SV40 templates. These consist of truncated T-ag sequences (approx 330 bp), as well as a PCR product that migrates more slowly than expected (approx 600 bp) and may be a DNA heteroduplex. Thus, confirmation by Southern hybridization and/or DNA sequencing is mandatory.
13. DNA sequencing is essential for the confirmation of PCR results. PCR-amplified products may not be unrelated to the intended product. For example, we observed that both JCV and BKV regulatory region sequences could be amplified by our SV40 regulatory region primers under certain conditions ***(7)***, although these primers were predicted to be SV40-specific. A similarly unexpected situation occurred with primers designed to amplify BKV and JCV T-ag sequences; instead, an SV40 sequence was amplified ***(1)***.
14. For DNA-sequence analysis, adequate results are obtained using direct sequencing of the PCR product from high-stringency PCR runs when only one PCR product is formed. From low-stringency runs, if multiple products are observed, individual bands are extracted from an agarose gel, reamplified, and sequenced. Extreme measures, such as washing of electrophoresis chambers, combs, and casting stands with DNA-OFF, followed by UV treatment prior to usage with individual samples, are performed to diminish the chances of contamination by DNA from other samples. Purification of PCR DNA for sequencing should use "ultrapure" TAE buffer. Occasionally, cloning of the low-stringency PCR products into a commercially available direct-PCR cloning vector is performed prior to sequencing. Multiple clones of the same type are sequenced and compared, as individual clones may contain artefactual changes as a consequence of base misincorporation by *Taq* polymerase.

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Guide to Techniques in Creating Transgenic Mouse Models Using SV40 T Antigen

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1. Introduction

The experimental introduction of foreign DNA into the germ line of a mouse is a powerful tool used to identify genetic elements required for appropriate tissue expression, to determine the developmental specificity of gene expression and to determine the consequences of inappropriate expression of cellular oncogenes. Moreover, by fusing a tissue-specific regulatory region of a particular gene to transforming genes, it is possible to direct expression of the transforming gene to specific cell types, thereby producing lines of mice that develop tumors in specific organs. Transgenic mice may then serve as useful models to study the multistage development of certain diseases such as cancer and may also be used to test various types of therapies for treatment of these diseases.

One such transforming gene is the SV40 large T antigen (T-ag). This viral oncogene causes transformation by blocking the action of two growth suppressors, the retinoblastoma susceptibility gene product (*Rb*) and *p53*, leading to the disregulation of cell cycle control ***(1,2)***. Transgenic mice carrying tissue-specific expression of SV40 T-ag have been produced for a number of regulatory regions from different genes, many of which are listed in **Table 1**. Included in this table are the regulatory regions used and the resultant phenotypes.

This chapter describes some of the methods used to construct a transgenic animal. It is organized into three sections. The first describes the molecular methods used in the construction of the vector DNA. The second section describes the preparation of DNA for microinjection into the pronucleus of

(Text continues on page 276)

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Table 1
Creation of Transgenic Mouse Models for Diseases Using SV40 T-ag as an Oncoprotein

Regulatory region	Tumor formation or tumor type	Cell type	Other proliferative effects	Other characteristics	Ref.
Metallothionein	Brain: choroid plexus	Epithelial	Thymus hypertrophy, Kidney: stroma cells		*(3)*
Rat insulin II	Pancreatic	Cells			*(4)*
Metallothionein-human growth hormone fusion gene without the 72bp enhancer of SV40	Hepatocellular carcinomas	Hepatocellular	Demyelinating peripheral neuropathies Islet Adenomas		*(5)*
Elastase I	Pancreatic	Acinar cells			*(6)*
Murine alpha A-crystalin	Ocular lens	Epithelial and fiber			*(7)*
Bovine vasopressin	Pancreas	Cells			*(8)*
	Anterior pituitary	Somatotroph cells			
Mouse Mammary Tumor Virus (MMTV) long terminal repeat	Lung	Epithelial			*(9)*
	Kidneys	Renal			
	Prostate and/or Seminal Vessicles	Epithelial			
	Testes	Leyding			
	Lymphoma	Pre-B			
Mouse protamine I	Heart: rhabdomyo-sarcomas Bone: Osteosarcoma	Myocytes			*(10)*
Rat preproglucagon	Pancreatic glucagonomas			CNS neuroblasts, neurons, (no proliferation, no apparent consequences, either phenotypic or tumorigenic	*(11)*
Human phenylethanolamine N-methyltransferase (PNMT)	Adrenal medulla	Adrenal medullary cells			*(12)*
	Eye: retina	Inner nuclear cell layer at the retina: macrine neurons			

Atrial natriuretic factor (a hormone intimately involved in the regulation of blood pressure)			Right atrium hyperplasia	***(13)***
Mouse renin	Intestinal		Peripheral neuropathies Choroid plexus Kidney Atypical hyperplasia of vascular smooth muscle	***(14)***
Human -1-antitrypsin	Liver	Hepatocytes	Stomach, pancreas, kidney	***(15)***
Mouse major urinary protein	Liver	Parenchymal	Skin sebaceous gland, preputial gland, and occasional Kidney and mammary connective tissue	***(16)***
Rat gonadotrophin-releasing hormone (GnRH)	Neuronal: hypothalamus	Hypothalomic: neurosecretory neurons		***(17)***
Moloney murine Sarcoma Virus (enhancer)	Pineal organ: neuro-ectodermal brain tumors Endocrine: pancreas, fibrosarcoma Kidneys: adenocarcinoma		Lens: cataract	***(18)***
Human glycoprotein hormone -subunit	Anterior pituitary	Retinoblastoma cells (photoreceptor cells, cuboidal cells)		***(19)***

(continued)

Table 1 *(continued)*

Regulatory region	Tumor formation or tumor type	Cell type	Other proliferative effects	Other characteristics	Ref.
Luteinizing hormone -subunit	Retinoblastoma retina ocular tumors				***(20)***
Human Immunodefificiency virus Type 1 (HIV) long terminal repeat			Lymphoid tissue: cells, thymic stromal cells Skin		***(21)***
gp91-phox	Histiocytic lymphoma	Monocyte/ macrophage			***(22)***
Albumin	Hepatocellular carcinomas	Hepatocellular			***(23)***
Bovine thyroglobulin	Thyroid	Epithelial			***(24)***
Rat -cardiac myosin heavy chain		Myocardium: hyperplasia of cardiomyocytes			***(25)***
Human surfactant protein C (SP-C)	Pulmonary	Epithelial			***(26)***
Human cystic fibrosis transn-membrane conductance regulator (CFTR)	Brain: ependymoma	Ependymal (glial origin)			***(27)***
Mouse opsin				Retina: rod photoreceptor degeneration	***(28)***
Rat glucagon	Neuroendocrine carcinoma of the large intestine				***(29)***
Homeo domain protein GHF-1	Brain: pituitary	Somato/ lactotrophic progenitor			***(30)***
Proopiomelanocortin (POMC)	Brain: pituitary	Melanotroph			***(31)***
Myelin basic protein	Schwannomas Facial bone	Spindle-Schwann Mesenchymal			***(32)***

Whey acidic milk (WAP)	Mammary gland	Epithelial			***(33)***
Mouse F-crystallin	Ocular lens	Fiber			***(34)***
Rat prostatic steroid binding protein C3(1) gene	Prostate Mammary gland	Epithelial Epithelial	Chondrodysplasia Sal. gland, lung, thyroid, harderian		***(35)***
Thyrotropin (thyroid-stimulating hormone,TSH) -subunit gene	Brain: anterior pituitary	Pituitary			***(36)***
Mouse growth hormone-releasing hormone (+ 0.9 kb flanking region + 20 nt from first axon)	Adrenal medulla	Neuroepithelial			***(37)***
Mouse epidermal growth factor gene	Prostate Stomach		Submaxillary gland: hyperplasia		***(38)***
Murine glial fibrillary acidic protein (GFAP)	Glioma: astrocytomas	Astrocytes			***(39)***
Human COL2A1				Skeletal abnormalities: chondrocytes	***(40)***
Mouse inhibin subunit	Testicular Ovarian	Leyding Granulosa	Adrenal glands, pituitary, brain		***(41)***
Rat probasin	Prostate	Epithelial			***(42)***
Rabbit uteroglobin	Lung	Clara			***(43)***
SV40	Thymic Brain: choroid plexus	Epithelial Epithelial	Dysplastic renal tubule		***(44)***
Mouse c-kit	Neuroendocrine: Pituitary Thyroid	Neuroendocrine -MSH C cells or their precursors			***(45)***
Tyrosine-related protein 1	Pigment epithelium of retina	Epithelial			***(46)***
Mouse hypothalamicgrowth hormone-releasing hormone			Hypothalamus Testes	Lens: cataract	***(47)***
Mouse cryptdin-2	Prostate	Neuroepithelial			***(48)***

fertilized eggs. The resulting transgenic animals generally carry one or more copies of the foreign DNA integrated into one of their chromosomes. Most often, the injected DNA integrates into the mouse genome in multiple head-to-tail copies. The third section describes how to screen for the inserted construct, how to quantitate copy numbers, and the methods used to detect the transgenic mice among live-born pups.

2. Materials

2.1. Construction of Plasmids

1. All restriction enzymes and ligase were purchased from New England Biolabs and used in accordance with the manufacturer's instructions.
2. Horizontal gel electrophoresis unit (Gibco-BRL, cat. no. 21069-018).
3. Agarose (Gibco-BRL cat. no. 15510-019).
4. Tris-borate-EDTA (TBE) buffer: 0.09 *M* Tris-base, 0.09 *M* boric acid, 0.002 *M* ethylenediaminetetraacetic acid (EDTA), pH 8.3. Tris-acetate-EDTA (TAE) buffer: 0.04 *M* Tris-base, 0.04 *M* glacial acetic acid, 0.001 *M* EDTA pH 8.3.
5. Agarose gel-loading buffer: 0.25% each of bromophenol blue and xylene cyanol; 40% sucrose.
6. Ethidium bromide: 10 mg/mL ethidium bromide (Sigma) in water).

2.2. Preparation of DNA for Microinjection

1. TE: 10 m*M* Tris-HCl, pH 7.4; 1 m*M* EDTA.
2. 40% sucrose solution: 40% sucrose (weight/volume); 50 m*M* Tris-HCl, pH 8.0, 1 *M* NaCl; 10 m*M* EDTA.
3. 10% sucrose solution: 10% sucrose (weight/volume); 50 m*M* Tris-HCl, pH 8.0, 1 *M* NaCl; 10 m*M* EDTA.
4. Dual-chamber gradient maker (Hoeffer, cat. no. SG15).
5. Ultracentrifuge (Beckman), SW60 rotor.
6. Centricon microconcentrator (Amicon, cat. no. 4240), or dialysis tubing (Fisher Scientific, cat. no. 08-670A).
7. Agarose gel electrophoresis apparatus.
8. 0.2-micron syringe filter (Fisher, cat. no. 09-730-218).
9. Cesium chloride (Fisher, cat. no. BP210-500).
10. Injection buffer: 10 m*M* Tris-HCl, pH 7.5, 0.1 m*M* EDTA. Filter twice through a 0.2-μ filter to remove particulates.
11. Elutip (Schleicher and Schuell, cat. no. 27370).

2.3. Preparation of DNA from Tail Clippings

1. STE solution: 0.1 *M* NaCl, 0.05 *M* Tris-HCl, pH 7.5, 1 m*M* EDTA pH 7.5, 1% sodium dodecyl sulfate (SDS).
2. 10 mg/mL proteinase K.
3. 3 *M* sodium acetate pH 6.0.

4. 100% ethanol.
5. Phenol saturated in 0.1 *M* Tris-HCl, pH 8.0.
6. Chloroform.
7. TE buffer.
8. TAE buffer.
9. For the hybridizations:
   a. Random primer extension kit (Stratagene, cat. no. 300385).
   b. DuralonUV hybridization membrane (Stratagene, cat. no. 420101).
   c. QuickHyb hybridization solution (Stratagene, cat. no. 201220).
   d. Hybridization oven or shaking water bath.
   e. Dot/slot blot apparatus (Gibco-BRL, cat. no. 11055-019).

3. Methods

We discuss here some considerations for construct design. For optimum expression, the gene of interest must have an enhancer/promoter unit placed 5 to the coding sequence and a 3 polyadenylation signal. The promoter must be known to function appropriately in vivo. The construct must also be designed so the entire expression unit can be released from the vector by convenient restriction enzymes. All vector sequences must be removed prior to injection.

Here we describe the design of a vector containing the regulatory region of the rat prostatic steroid binding protein *C3*(1) gene ***(49)***, driving the SV40 T-ag, which was used to create an animal model for prostate and mammary adenocarcinoma ***(35)***. This is shown diagrammatically in **Fig. 1**.

3.1. Construction of Vector DNA

1. A 5.7-kb *Sac*I fragment was isolated from the rat *C3*(1) 5 -flanking region including the first exon.
2. This fragment was blunt-ended using Klenow ***(50)*** and ligated into a blunt-ended *Xba*I site of Bluescipt SK(+) (Stratagene).
3. A 161-bp *Nco*I/*Not*I fragment was created using polymerase chain reaction (PCR) in which a unique *Not*I site was introduced in the first exon of *C3*(1) gene prior to ATG translational start site in the *C3*(1) gene.
4. The PCR fragment was substituted for the 890-bp *Nco*I-*Sac*I of the *C3*(1) gene in the 5.7-kb subclone.
5. To create the *C3*(1)/T-ag fusion construct, a 2.7-kb *Sfi*I/*Bam*HI fragment containing the coding region for the SV40 large and small T antigens, as well as the polyadenylation signal was inserted by blunt-end ligation into the *Not*I site of the subclone aforementioned. As shown in **Fig. 1**, this cloning strategy places the SV40 early region and *C3*(1) genes into the plasmid in opposite orientations (3 –>5) in order to place an active transcription unit to the promoter of the T-ag genes. The final *C3*(1)/T-ag fusion product was 7.25 kb and was excised by digestion with *Bam*HI and *Sac*I.

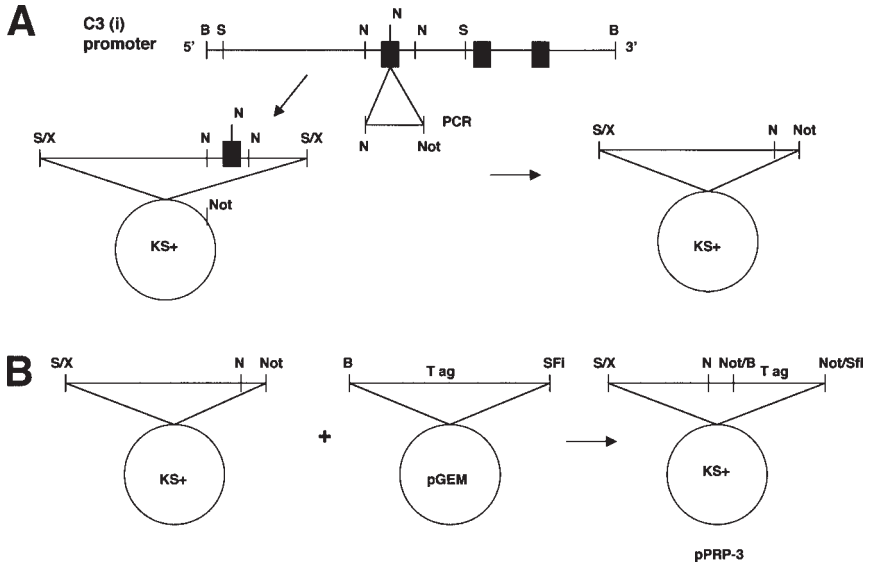

Fig. 1. Diagrammatic representation of the cloning strategy to obtain the *C3*(1) regulatory region driving SV40 T-ag expression. **(A)** Initial cloning of the *C3*(1) regulatory region plus the region from which the PCR product was generated. **(B)** Insertion of the T-ag downstream of the *C3*(1) cassette. The abbreviations are as follows: B (*Bam*HI), S (*Sac*I), X (*Xba*I), N (*Nco*I), KS+ (Bluescript KS cloning vector, Stratagene), pGEM (Fisher/Promega).

3.2. Preparation of DNA for Microinjection

One of the most critical steps in making transgenic mice is preparing the DNA for microinjection (*see* **Note 1**). Plasmid DNA containing the construction is prepared by standard techniques; preferably, plasmid DNA is purified by CsCl–EtBr equilibrium density gradients ***(50)***. The vector DNA is removed from the insert DNA by complete digestion and separated by sucrose gradient centrifugation (*see* **Note 2**). Alternatively, if the insert and vector sequences are relatively close in size, agarose gel electrophoresis (SeaKem, FMC, low-melting-point agarose), followed by Elutip purification can be used (*see* **Note 3**).

3.2.1 Sucrose Gradient Centrifugation

This method can sufficiently separate fragments of approx 6.0–3.0 kb.

1. Digest 50 µg plasmid to completion.
2. Electrophorese a small amount on an agarose gel to check for complete digestion.
3. Precipitate DNA with 0.1 times the volume of 3 *M* sodium acetate pH5.2 and 2.5 times the volume of ethanol.

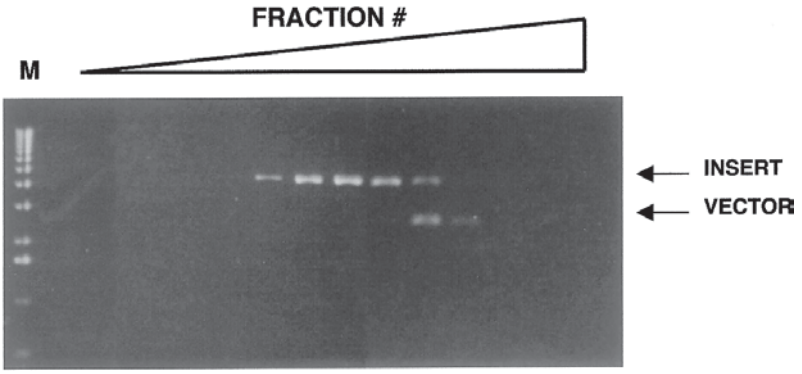

Fig. 2. Agarose gel electrophoresis showing the separation of vector sequence from insert DNA. Fractions containing only insert DNA was pooled and used for microinjection.

4. Air-dry pellet for a few minutes.
5. Resuspend the pellet in 50 µL of TE to 1 µg/mL.
6. The DNA solution is layered onto the top of the sucrose gradient, the amount of which depends on the volume of the gradient; 30 µg for 5-mL gradients or 50 µg for a 10-mL gradients
7. Make sucrose gradient. A linear 10–40% sucrose gradient, in 1 *M* NaCl, 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, is prepared using a dual-chamber gradient mixer. To pour a linear gradient, equal amounts of the two sucrose solutions are placed in the gradient mixer with the low percentage solution in the chamber closer to the front, which contains a stirring bar. While the solution is mixed, and after all air bubbles are removed, stopcocks are opened and the gradient is poured in the centrifuge tube.
8. Centrifuge in a Beckman SW60 rotor at 35,000 rpm at 15°C for 16 h.
9. Collect fractions (300 µL, approx 4 drops/fraction) into Eppendorf tubes in a rack, letting gradient drip by gravity, from the bottom of the tube.
10. Use 2 µL of every fifth fraction to determine which fractions contain DNA, by agarose electrophoresis. Then take 2 µL of every DNA fraction, particularly at region of overlap of bands, to determine which fractions contain only insert DNA (**Fig. 2**).
11. Pool fractions containing only insert DNA and remove gradient medium by five centrifugation rinses with injection buffer in a Centricon 100 microconcentrator. Alternatively, common dialysis procedures can be used to remove the residual sucrose.
12. To determine the final concentration of DNA, compare the staining intensity of 1–3 µL of DNA on an agarose gel to different amounts of a standard of known concentration.
13. Rinse Eppendorf tubes with the filtered injection buffer to remove any contaminants in the tube.

14. Dilute the purified insert with injection buffer to 2.5 µg/mL (concentration for routine use) and store 100-mL aliquots in rinsed Eppendorf tubes at –20°C. The injection buffer should be filtered twice through a 0.2-µ filter to remove particulates. Before storing aliquots of DNA for microinjection, rinse Eppendorf tubes with the filtered injection buffer to remove any contaminants in the tube.

3.3. Microinjection

The working concentration of transgene construct DNA for microinjection is usually 2 µg/mL per pronuclear microinjection of the fertilized egg. Generally a specific transgenic mouse facility is needed for the embryo manipulation: Collection of fertilized eggs, microinjection, and transplantation of the manipulated fertilized eggs into the reproductive tract of recipient pseudopregnant mother ***(51)***. The integration frequency of DNA purified by the above procedure should result in at least 25% live births, regardless of the construct used, provided that it does not induce embryoinc lethality (*see* **Notes 4** and **5**).

3.4. Genetic Analysis of Transgenes

3.4.1. Selection of Positive Animals

Once the pups are born, careful records of the date of birth, the number born, and number weaned are important. Be certain to include the animal care personnel with this record keeping and observation of the mice. Keep a separate record including amount of DNA injected, date injected, percentage of surviving embryos, number of embryos transferred per animal and per uterine horn, number of females pregnant, number of pups born, and the number of positive mice generated. Thus, any trend showing fewer than expected pregnancies or poor survival from DNA injection can be detected early before an extensive amount of time is wasted (*see* **Note 6**).

Pups can be weaned at 3 wk. Separate male and female pups, use toe clips or ear tags for identification, and cut off a 1.0- to 1.5-cm piece of tail for DNA isolation. The animal whose genomic DNA contains the integrated transgene is referred to as the founder of a new transgenic line (*see* **Note 7**). Founder litters are given a number based on the chronological order of birth. A record is noted on the data sheet for the litter of the DNA mix injected, date of birth, date weaned, and sex of each animal relative to the toe clip or ear tag.

3.4.2. Preparation of DNA from Tail Clippings

1. Anesthetize the mouse.
2. Remove 1–1.5 cm of tail with a sterile razor blade.
3. Place the tail piece in a 1.5-mL Eppendorf centrifuge tube and add 0.5 mL of STE solution and 35 µL of a 10-mg/mL solution of proteinase K and mix well.

4. Incubate at 55°C overnight. If the tail is not totally submerged, the tube should be on a rocking platform.
5. After incubation, extract the solution with an equal volume (0.5 mL) of phenol (previously equilibrated with Tris-HCl, pH 8.0). Shake vigorously for 1–2 min.
6. Centrifuge at high speed for 3 min to separate phases.
7. Transfer the aqueous (top) phase to a clean tube, avoiding the interface.
8. Add 0.5 mL phenol : chloroform (1:1), to the aqueous phase and mix vigorously for 1–2 min.
9. Centrifuge for 3 min and transfer aqueous phase as before.
10. Precipitate the DNA by the addition of 50 µL of 3 *M* sodium acetate (pH 6.0) and two volumes of 100% ethanol at room temperature. Invert to mix thoroughly. The DNA should immediately form a visible precipitate. If a pH below 6.0 is used, the EDTA will also precipitate.
11. Spool the DNA from each tube using a pipet tip and transfer into a new tube. Decant or aspirate the ethanol. Add 1 mL of 70% ethanol and gently vortex.
12. Centrifuge for 1 min, remove the ethanol, and dry the DNA under vacuum for 4–5 min.
13. Redissolve in 100 µL TE. This may require overnight incubation, or heating to 65°C for 5–10 min. Depending on yield, 10–20 µL should be sufficient for a Southern blot, and 1–2 µL for PCR.

Once resuspended in Tris-EDTA buffer, the DNA is ready for analysis. Simple dot-blot hybridizations or PCR analysis will identify the presence of the injected DNA. Additionally, Southern blot hybridization analysis will yield information regarding integration site, pattern of repeats, and copy number. Distinguishing heterozygous from wild-type is simply a matter of detecting the construct by an appropriate probe or by the appropriate primers using PCR. Determing the copy number of the transgene is best done by Southern blotting and comparing band intensities from the positive founder mice with the band intensity from different amounts of a standard of known concentration control bands.

For detection of copy number a random-primer generated probe directed against the transgene, with a specific activity of 0.8–2 × 10^8 cpm/mg DNA single copy genes (approx 10 µg genomic DNA) can be detected in Southern blot analysis of genomic DNA. For example, **Fig. 3** shows a Southern blot analysis of genomic DNA from 15 different animals digested with an enzyme that cuts within the *C3*(1) promoter construct. The bands in lanes 3 and 10 reveal the animals that are positive for the transgene. The copy number can be determined by addition of transgene DNA to genomic DNA in different concentrations and then comparing hybridization intesity. Each plasmid control (e.g., pSVB-1) at 100, 50, or 10 pg corresponds to 10, 5, or 1 copy of the transgene, respectively. Founder animal number 3 contains approx 100 copies

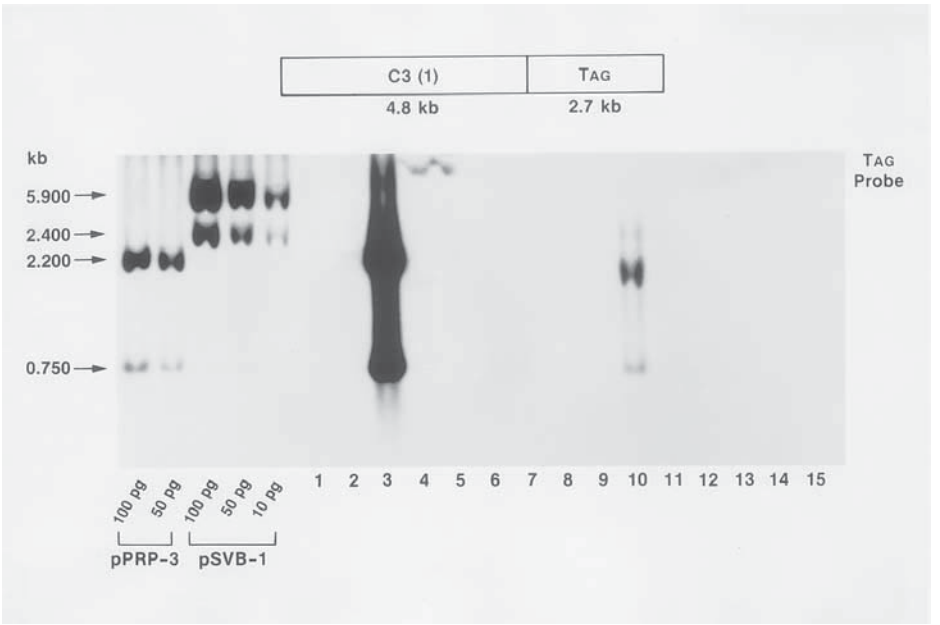

Fig. 3. Twenty micrograms of tail DNA was digested with *Bst*XI, electrophoresed through 0.8% agarose, transferred to nitrocellulose, and probed with T-ag. Control lanes: PRP-3 plasmid (final *C3*(1) T-ag construct) and SVB-1 (original T-ag plasmid). Lanes 1–15 are the offspring from the initial microinjection. Lanes 3 and 10 are representative positive founder mice.

of the transgene because the intensity of the signal is approx 100 copies of the transgene and because the intensity of the signal is approx 10-fold higher than that detected in the 100 pg pSVB-1 control (10 copies).

3.5. Analysis of Transgenics

Table 2 shows a typical analysis for 12 founder animals. Included in the analysis is the founder ID, followed by copy number of the transgene, as determined by Sourthern blot analysis and the sex of the animal (*see* **Note 8**). The resultant phenotypes were determined by macro- and microscopic analysis by pathologists. Potential interesting phenotypes can be determined by commonalities among the founder animals. Some phenotypes, of course, depend on the sex of the animal. To make a direct correlation between transgene and the resultant phenotype, the transgene must be expressed in the affected tissue. Further analyses would include Northern or *in situ* hybridization to determine the expression of the gene or common immunological techniques used to detect protein expression. To establish lines hemizygous for the transgene, each

Table 2
Phenotypic Abnormalities of *C3*(1)/T-ag Founder Mice *(35)*

FOUNDER MICE				PHENOTYPE											
Female	Male	Copy no.	Age at death	Prostate hyperplasia	Prostate adenoma	Prostate carcinoma	Breast adenocarcinoma	Chondrodysplasia	Sal. gland proliferative lesions	Nasal turbinate proliferative lesions	Harderian hyperplasia	Thyroid proliferative lesions	Osteosarcoma	Lung carcinoma
A*		2	1.5 yr											
E		10	9 wk					— N.D. —						
C		6	20 wk				+							
L		4	16 wk				+	+	+	+	+			+
F		50	12 wk				+	+	+	+	+	+[a]		
J		10	4.5 wk					+	+	+	+	+[b]		
	G	40	7 wk					+	+	+	+	+[c]		
	K	5	10 wk	+				+	+	+			+	
	I	4	6 wk	+				+	+	+	+	+[b]	+	+
	D	40	5.5 wk					—N.D.—						
	B*	1	—					—N.D.—						
	M	>100	1 d					—N.D.—						

[a] = Carcinoma, [b] = hyperplasia, [c] = adenoma.
N.D.: Not determined.
*No transmission.

founder animal carrying *C3*(1)/T-ag is subsequently bred to control FVB/N mice. In each case a heterozygous (T-ag±) founder mouse is bred to a normal FVB/N mouse. The offspring are tested for the genetic transmision of T-ag DNA as aforementioned. Progeny that are T-ag DNA positive are then bred to normal FVB/N mice and the process is repeated.

4. Notes

1. Poorly prepared DNA can be toxic to the fertilized egg and contaminants can clog the injection needles, which typically have diameters of 0.5 µ.
2. Vector DNA must be removed prior to microinjection because vector DNA can be toxic to the fertilized egg. Additionally, the presence of vector DNA sequences may inhibit expression of the integrated transgene.
3. Separation of the insert from plasmid sequences through agarose gel electrophoresis may lower integration frequency, regardless of subsequent purification.

4. If fewer than expected positive animals are found and few or none express the gene, either the DNA was of poor quality (*see* **Subheading 3.2.**), the pronucleus was not injected with the DNA, or the gene was toxic.
5. Toxicity can be manifest at a particular time in gestation or be more generalized. At this point, isolation of DNA and RNA from fetuses generated from injected embryos at various times in gestation will allow a comparison of the number of positive fetuses vs the number of positive live-born pups.
6. The efficiency of positive animals also depends on the choice of mouse strains. FVB/N mice is the established strain for creation transgenic mice.
7. If lethality is suspected, check the integration frequency in the embryos.
8. The existence of multiple integration sites, each potentially allowing different levels of expression, may contribute to experimental variation. It is thus important to verify the presence of multiple integrants in any progeny.

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APPENDIX

Alphabetical List of Companies

Aldrich Chemical Co.
Techware Division
940 W. St. Paul Ave.
Milwaukee, WI 53233
Phone: 414-273-3850
Fax: 414-273-4979
Toll Free: 800-558-9160
Website: www.sial.com

American Type Culture Collection (ATCC)
10801 University Boulevard
Manassas, VA 20110
Phone: 703-365-2700
Fax: 703-365-2750
Website: www.atcc.org

Amersham Pharmacia Biotech Inc.
800 Centennial Ave.
Piscataway, NJ 08855-1327
Phone: 732-457-8000
Fax: 800-FAX-3593
Toll Free: 800-526-3593
Website: www.apbiotech.com

Amicon
72 Cherry Hill Drive
Beverly, MA 01915

Beckman Coulter Inc.
4300 N. Harbor Blvd.
Fullerton, CA 92834-3100
Fax: 800-643-4366
Toll Free: 800-742-2345
Website: www.beckmancoulter.com

Becton Dickinson/DIFCO
BD Biosciences
7 Loveton Circle
Sparks, MD 21152
Phone: 410-316-4000
Website: www.difco.com

BD PharMingen
10975 Torreyana Road
San Diego, CA 92121
Phone: 800-848-MABS (6227)

Bel-Art Products
6 Industrial Rd.
P.O. Box 152
Pequannock, NJ 07440-1992
Phone: 973-694-0500
Fax: 973-694-7199
Toll Free: 800-423-5278
Website: www.bel-art.com

Bio101 Inc.
1070 Joshua Way
Vista, CA 92083
Website: www.bio101.com

Biomaterials
Palo Alto, CA, 94303

Calbiochem Corp.
P.O. Box 12087
San Diego, CA 92112

CHIMERx
5520 W. Burleigh St.
Milwaukee, WI 53210-1347
Phone: 414-871-7199
Fax: 414-871-1273
Website: www.chimerx.com

Dynal
10 Thursby Rd.
Croft Bussiness Park, Bromborough,
Wirral, Merseyside L62 3PW, UK
Website: www.dynal.no

Eastman Kodak Co.
Scientific Imaging Systems Division
343 State St.
Rochester, NY 14652
Phone: 716-722-5813
Fax: 203-624-3143
Toll Free: 800-225-5352
Website: www.kodak.com/go/scientific

Enzo Diagnostics Inc.
60 Executive Blvd.
Farmingdale, NY 11735
Phone: 516-694-7070
Fax: 516-694-7501
Toll Free: 800-221-7705

Fisher Scientific Co.
2000 Park Lane
Pittsburgh, PA 15275
Phone: 412-490-8300
Fax: 800-926-1166
Toll Free: 800-766-7000
Website: www.fishersci.com

FMC BioProducts
191 Thomaston St.
Rockland, ME 04841
Phone: 207-594-3400
Fax: 207-594-3426

FMC Bioproducts/Flowgen Instruments Ltd.
Lynn Lane
Shenstone, Lichfield
Staffordshire, WS14 0EE, UK
Website: www.philipharris.co.uk/flowgen

Gibco-BRL
Life Technologies, Ltd.
9800 Medical Center Drive

P.O. Box 6482
Rockville, MD 20849- 6482
Phone: 800-338-5772
Website: www.lifetechnologies.com

Gibco-BRL
Life Technologies Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Website: www.lifetech.com

Helena BioScience
Colima Avenue
Sunderland Enterprise Park
Sunderland, Tyne & Wear, SR5 3XB, UK
Website: www.helena.biosciences.com

Hoeffer
654 Minnesota Street
Box 77387
San Francisco, CA 94107-0387
Phone: 415-282-2307

Hyclone Laboratories, Inc.
1725 South HyClone Road
Logan, UT 84321
Phone: 800-HyClone (492-5663) or 435-753-4584; Messages accepted after hours.
Fax: 800-533-9450 or 435-792-0297; available 24 hours a day.
Office Hours: Monday through Friday 7:00 a.m. to 6:00 p.m., Mountain daylight/standard time.
Website: http://www.hyclone.com/order/index.html

IBF Biotechnics
35 avenue Jean-Jaures,
92390 Villeneuve-la-Garenne, France

Intermountain Scientific Company (ISC)/ BioExpress
420 N. Kays Dr.
Kaysville, UT 84037
Phone: 801-547-5047
Fax: 801-547-5051
Toll Free: 800-999-2901
Website: www.bioexpress.com

ISOLAB Inc.
Drawer 4350
Akron, OH 44321

J.T. Baker
Mallinckrodt-Baker Inc. Division
222 Red School Lane
Phillipsburg, NJ 08865
Phone: 908-859-2151
Fax: 908-859-9318
Toll Free: 800-582-2537

Marsh Biomedical Products Inc.
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Toll Free: 800-445-2812
Website: www.biomar.com

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Toll Free: 800-MILLIPORE
Website: www.millipore.com

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Website: www.nenlifesci.com

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Novocastra Laboratories, Ltd.
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Oligos Etc. Inc.
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Sigma Chemical
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