Preface

Simian virus 40 gained notoriety in the 1960s because it was found to be a contaminant of polio and adenovirus vaccines that had been administered to millions of healthy individuals worldwide. The public health implications of this revelation provided the initial impetus for an in-depth study of SV40 biology. Later work showed that SV40 DNA sequences as well as infectious virus are in fact found in human tumors and may have contributed to oncogenesis. It also turned out that SV40 uses mostly cellular machinery to carry out many steps in viral infection, which makes it a powerful probe for examining many fundamental questions in eukaryotic molecular biology. *SV40 Protocols* consolidates a number of well-tested step-by-step techniques in one volume; experts with hands-on experience in particular methods give detailed accounts of their optimized experimental protocols, so that the beginner, as well as more experienced researchers, may readily overcome problems of ambiguity often present in the literature.

As with other DNA tumor viruses, the response of cultured cells to SV40 infection depends upon the species being infected. Monkey cells support virus production, which leads to their death, whereas rodent cells produce only the early proteins and acquire a transformed phenotype. Thus, *SV40 Protocols* is organized in two sections. The first relates to assays of the lytic cycle of the virus, and the second deals with transformation. The first section starts with a chapter on the basic techniques for growth of SV40 and its mutants in tissue culture, which would be valuable for the beginner. Three chapters follow on techniques for the growth and detection of two SV40-related human viruses, BKV and JCV, which would be of particular interest to those involved in clinical reasearch.

Complicated biochemical processes cannot change radically once solved by evolution. Since SV40 DNA replication is carried out mostly by the cellular machinery, insights gained into its replication are likely to be relevant to eukaryotic DNA replication in general. Similarly, the study of SV40 transcription and its regulation has provided valuable clues to the mechanisms of eukaryotic transcription. Therefore, the next three chapters constitute detailed accounts of protocols for SV40 DNA replication and transcription, both in vivo and in vitro.

The field of gene therapy has evolved from an investigative curiosity to a major focus of medical research. Therefore, the next two chapters of this section are on the use of SV40 virus, or SV40 pseudovirions made in insect cells, as vectors for human gene therapy. The section ends with a chapter on the use of retroviral vectors for the expression of SV40 tumor antigens in cultured cells, an approach that is useful in gene expression in general and prepares the reader for the next section dealing with SV40-mediated neoplastic transformation.

Work on SV40 neoplasia has yielded a plethora of information on cell growth controls and led to the discovery of two families of antioncogenes, p53 and the retinoblastoma susceptibility gene products. This section starts with a detailed account of biological assays of neoplastic transformation that are applicable to any system. The next two chapters describe methods to assess the effects of SV40 large T antigen (SVLT) expression upon adipocytic differentiation and upon establishment of cells in culture. Three chapters follow, each dealing with proteins associated with and affected by SVLT: p53, Rb, and Ras. Chapters on the effects of the second SV40 tumor antigen, small T, cytotoxic T lymphocytes in SV40 infections, and the detection of SV40 sequences in human tissues follow. The section ends with a chapter on the use of SVLT transgenic animals as models for the study of multistage tumor development.

All chapters in *SV40 Protocols* offer thorough technical accounts of the methods treated and include discussions of the problems and pitfalls that may be encountered as well as valuable troubleshooting advice. An Appendix at the end of the volume lists all companies whose products are cited in the text. Since a company may stop manufacturing a given reagent, whereas others may start later, an Internet directory address is also provided. Note that the performance of many reagents may vary from lot to lot and many reagents may come in kits. In these cases, the precise recommendations of the manufacturer for use of a particular reagent must take precedence over the conditions described.

First and foremost I would like to thank all of the contributors: their high-quality submissions and valuable suggestions made this volume possible. Special thanks go to Ximena Montano for her many recommendations of potential contributors. I also thank the series Editor, John Walker, for his valuable advice, especially, at the initial stages.

Leda Raptis

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

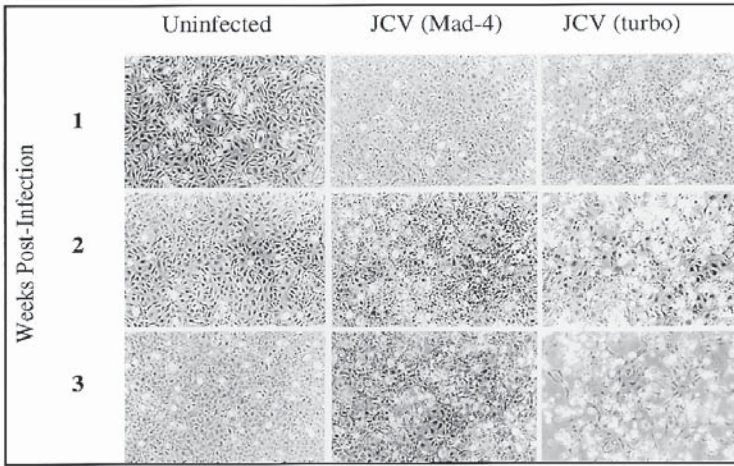

Fig. 1. JC virus-induced cytopathology using an owl monkey derivative of a Mad-4 strain of JCV (M4) and a chimeric JC/SV40 virus (M1SVE) ***(13)***. Infection with commercially available isolates of Mad-1 and Mad-4 does not result in significant amounts of CPE.

(*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-

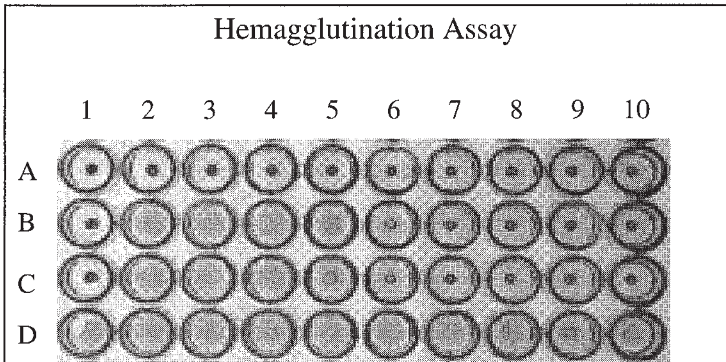

Fig. 2. Hemagglutination assay. Twofold serial dilutions of virus are prepared in duplicate in a U-bottom 96-well plate (rows **B**, **C**, and **D**; columns 1–10). Control wells without virus are also prepared (row A, columns 1–10). Red blood cells (0.5% suspension) are then added to each well and 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

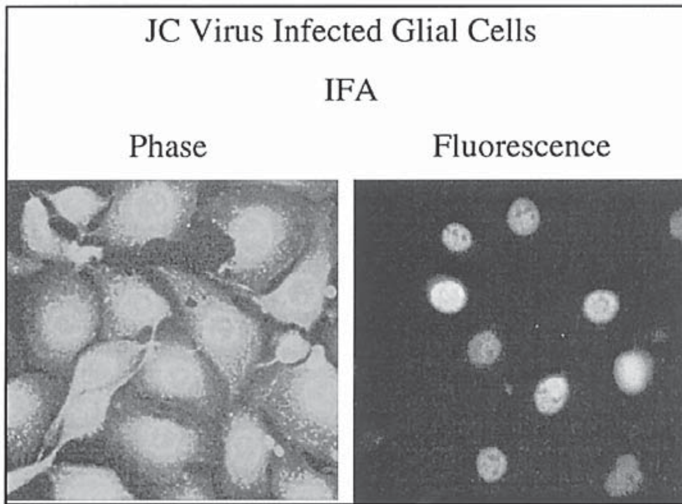

Fig. 3. JCV infected SVG cells stained with an anti-SV40 V antigen monoclonal antibody and a goat antimouse FITC conjugated secondary antibody. There was no detectable staining in uninfected SVG cells. Magnification: ×20.

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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