CHAPTER 7

Use of Recombinant Vaccinia Virus Vectors for Cell Biology

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I. Introduction

Vaccinia virus has been exploited as a powerful and convenient tool for transient expression of proteins in mammalian cells. This chapter describes the

use of several of the recombinant vaccinia expression systems pioneered by Bernard Moss and colleagues. We will focus on the systems that are most useful for cell biologists, and discuss their advantages and limitations. Vaccinia-mediated expression can be used for assessing cellular localization, post-translational modifications, oligomerization, and transport and turnover rates. The system provides a rapid method for screening mutant proteins for expression and targeting. It is an excellent way of quickly deciding which mutant proteins might be worth further study using stable expression systems. Some uses of vaccinia vectors will not be discussed here, including methods for large-scale production of proteins. For detailed methods for large-scale production of proteins from cDNAs, or other vaccinia methods not discussed here, see Earl and Moss (1991), Moss et al. (1990), and Moss (1991).

A. Biology of Vaccinia Virus

Vaccinia virus is the best-studied member of the Poxviridae, the largest and most complex of the animal viruses. Widespread vaccination with vaccinia virus (probably derived from cowpox virus) resulted in the worldwide eradication of smallpox. The double-stranded linear genome of vaccinia virus is nearly 200 kb, and there are over 250 potential genes (Goebel et al., 1990; Moss, 1991). The virions are enveloped and are approximately 200 × 300 nm in size, with a characteristic brick shape (Fig. 1). Several features of the vaccinia life cycle make it unique as a eukaryotic expression vector. At least 25 kb of DNA can be inserted into the vaccinia genome without detrimental effects on viral replication or assembly. Vaccinia replicates completely within the cytoplasm of the host cell, and thus imports or directs the synthesis of its own polymerases and transcription factors. In addition, the virus has a wide host range, so most cultured mammalian cell lines are susceptible to infection. The virus is easy to grow and purify in large quantities, and is relatively safe to work with.

Considering the size of vaccinia virus, the complexity of replication and assembly is not surprising (Figs. 1 and 2). After binding to the host cell, the viral envelope is believed to fuse directly with the plasma membrane (Doms et al., 1990), releasing the core virion into the cytoplasm. Little is known about the uncoating steps, but transcription of early genes begins within 1 hr of infection. The virion core contains all the enzymes it needs for transcription of the early genes. Transcribed RNAs are capped, methylated, and polyadenylated in the cytosol by viral enzymes. DNA replication begins with 3–4 hr, and occurs in discrete juxtanuclear regions of the cytosol termed "viral factories" near the Golgi complex. After transcription of intermediate and late viral genes (by different viral RNA polymerases), viral assembly begins. This complex process appears to involve two separate membrane "enwrapping" events, each of which results in a double lipid bilayer surrounding the core particle. The first double membrane is derived from the "intermediate compartment" between the endoplasmic reticulum (ER) and Golgi (Sodeik et al., 1993). The viral DNA is
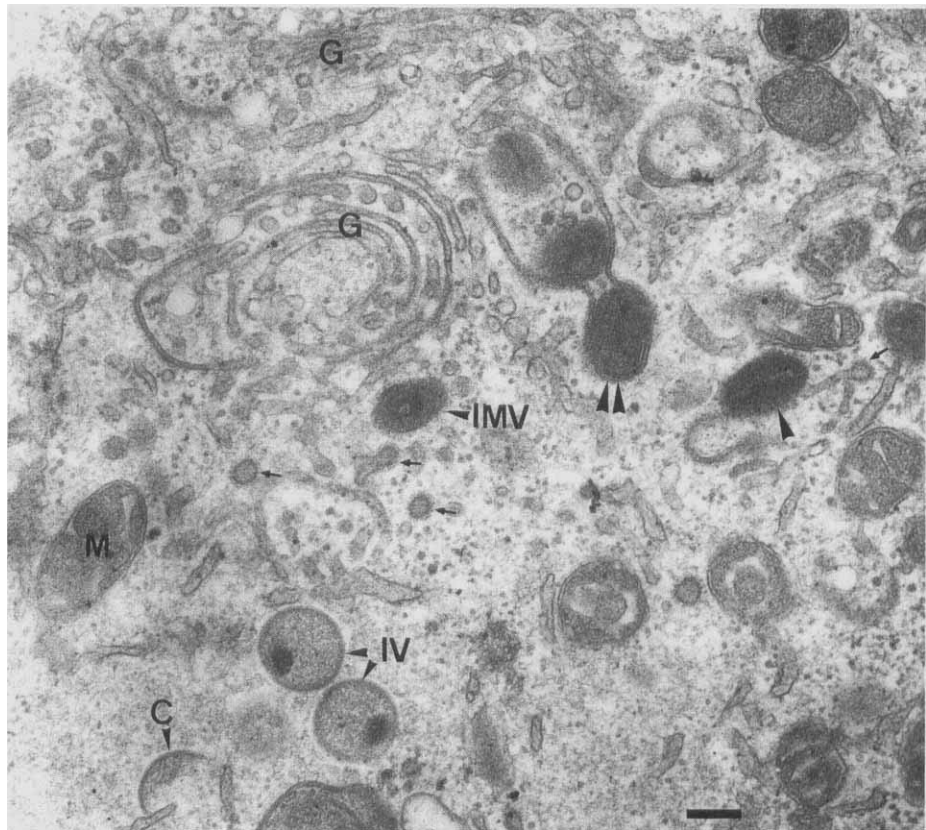
Fig. 1 Ultrastructure of assembling vaccinia virus. Epon section of a HeLa cell infected with vaccinia virus for 8 hr. The viral factories (where viral DNA replication and early assembly events occur) can be seen near the Golgi complex, as can several forms of assembling virions. C, Crescent; IV, immature virus, IMV, intracellular mature virus (first infectious form). The single arrowhead indicates attachment of an IMV to a cisterna of the *trans*-Golgi network (arrows indicate putative clathrin buds and vesicles). The double arrowhead shows wrapping of an IMV by *trans*-Golgi network membrane. All the membranes surrounding the virion are clearly visualized in ultrathin cryosections (Sodeik *et al.*, 1993). Bar, 200 nm. (Micrograph courtesy of Drs. B. Sodeik and G. Griffiths.)

inserted into a crescent-shaped region of the intermediate compartment membrane which then fuses around it, generating intracellular mature virus (IMV), formerly called intracellular "naked" virus. The second enwrapping membranes are derived from the *trans*-Golgi network (Schmelz *et al.,* 1994), resulting in the intracellular enveloped virus (IEV) form. Both membrane enwrapping events are thought to depend on certain viral membrane-associated proteins that localize specifically to the intermediate compartment or the *trans*-Golgi network. IEV with four membranes (doubly enwrapped) can fuse with the
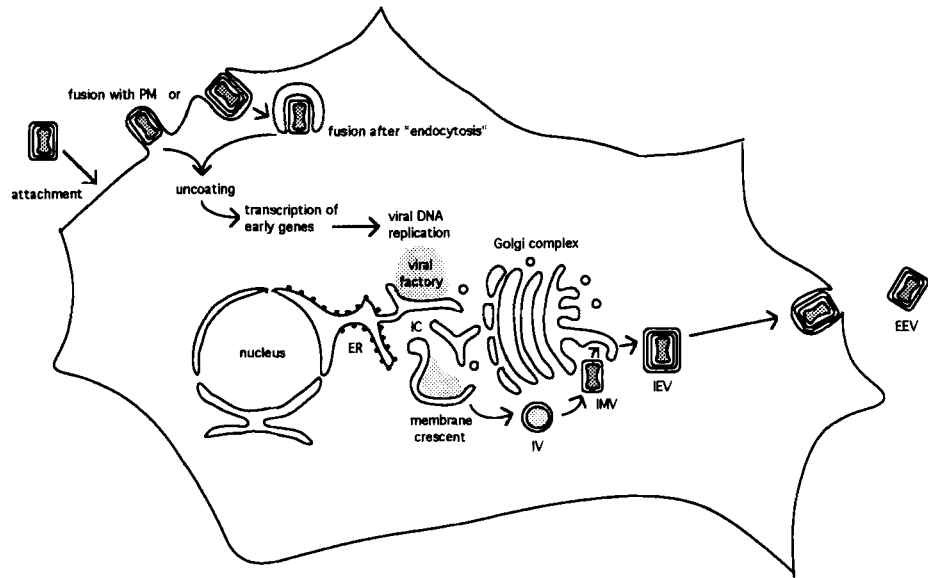
Fig. 2   Infection cycle of vaccinia virus. After virus entry, uncoating, transcription of early genes, and DNA replication, virus assembly occurs (Griffiths and Rottier, 1992). Two membrane enwrapping events result in intracellular mature virus (IMV) and intracellular enveloped virus (IEV). Fusion of IEV with the plasma membrane (an inefficient process for many vaccinia strains) results in release of virions with three membranes (EEV, extracellular enveloped virus). Both IMV and IEV forms are infectious. ER, Endoplasmic reticulum; IC, intermediate compartment; IV, immature virus.

plasma membrane, releasing virions with three membranes into the extracellular space. However, some strains of vaccinia are inefficiently released from cells. Approximately 95% of the commonly used WR strain remains cell associated. Other strains (e.g., IHD-J) produce substantial amounts of extracellular virus. Since both forms of intracellular virus (singly and doubly enwrapped) are infectious, virus is usually purified from cell homogenates rather than from the culture medium.

B. Systems for Protein Expression Using Vaccinia Virus

Expression of foreign genes using vaccinia virus is based on recombinant viruses constructed by insertion of cDNA into the nonessential thymidine kinase (TK) gene. Both direct and indirect methods of expression are possible (Fig. 3). The foreign gene can be inserted into the vaccinia genome by homologous recombination using a plasmid with flanking regions of vaccinia DNA. The recombinant virus is selected, expanded, and used to infect cells, which then express high levels of the foreign protein. In an alternative ("indirect") approach, a recombinant vaccinia virus encoding bacteriophage T7 RNA polymer-
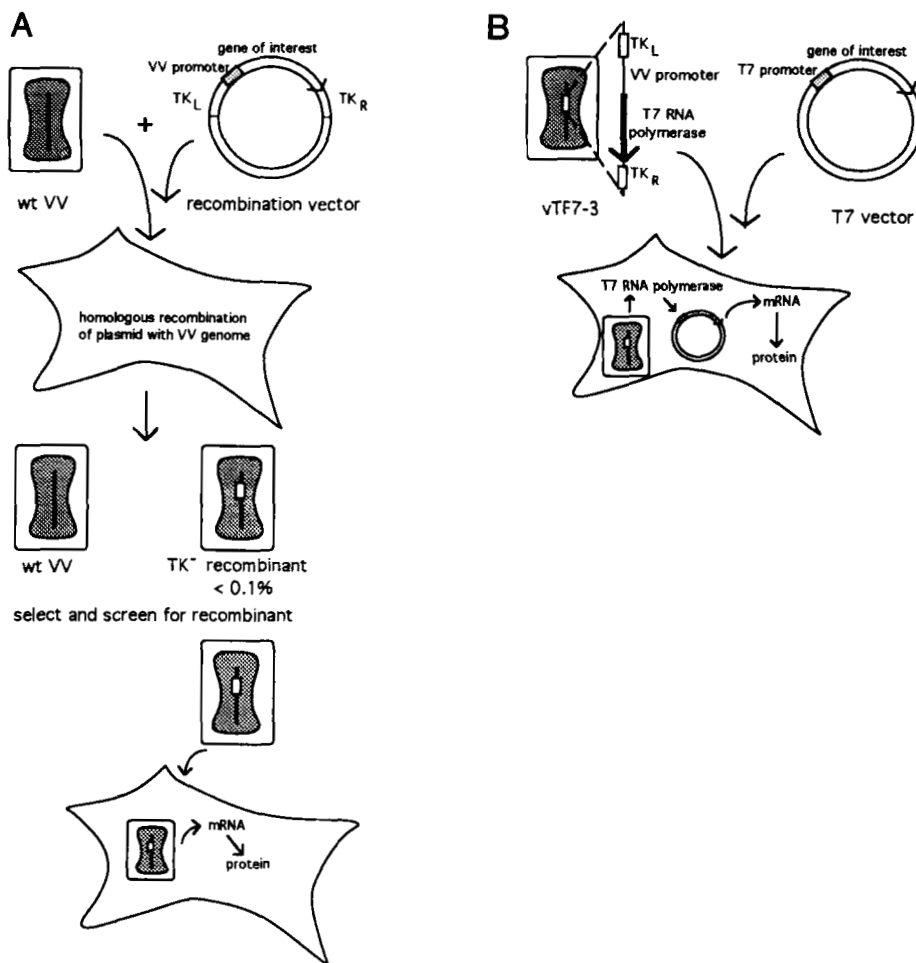
Fig. 3 Comparison of direct expression using a recombinant vaccinia virus and the vaccinia/T7 RNA polymerase hybrid system. (A) Expression using the direct system requires production of a recombinant virus encoding the foreign gene as shown. (B) In the hybrid system, cells are infected with the recombinant virus vTF7-3 encoding T7 RNA polymerase, then transfected with plasmids encoding the foreign gene behind a T7 promoter.

ase (vTF7-3) is used to infect cells (Fuerst *et al.*, 1986). The T7 RNA polymerase is expressed efficiently in the cytoplasm early after infection. Transfection of cells immediately after infection with a vector containing the gene of interest cloned behind a T7 promoter results in rapid and efficient expression of the encoded protein. This system is convenient, since recombinant virus production is unnecessary. Commonly used vectors such as pBluescript (Stratagene, La Jolla, CA) can be used.

II. Generating a Recombinant Vaccinia Virus

Recombinant vaccinia viruses are generated by subcloning the foreign gene into a plasmid transfer vector so it is flanked by DNA from the vaccinia (TK) gene, which is nonessential for growth of the virus in tissue culture. This plasmid is then transfected into vaccinia-infected cells. Homologous recombination of the plasmid and the vaccinia genome generates a recombinant virus with an inactive TK gene. A lysate from the infected cells is used to infect a thymidine kinase negative (TK$^-$) cell line, usually human osteosarcoma 143B cells (Mackett *et al.*, 1984). Recombinant viruses are enriched by growing the cells in the presence of bromodeoxyuridine (BrdU), since only recombinant viruses (and TK mutants) are able to replicate. If the plasmid transfer vector used for recombination contains the β-galactosidase gene (see subsequent discussion), recombination viruses can be distinguished from TK mutants by color screening using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Chakrabarti *et al.*, 1985).

A. Vectors and Subcloning

Various vectors for recombination into the vaccinia genome are available (Moss *et al.*, 1990; Earl and Moss, 1991; Moss, 1992). The use of an early vaccinia promoter to drive the foreign gene is essential for cell biology applications, since the protein will be expressed before most of the cytopathic effects of the virus infection become evident. The vector we use for production of recombinant virus is pSC11ss (Fig. 4A) or its derivative pSC65. pSC11ss contains the promoter $P_{7.5}$ that is transcribed both at early and late times after infection; pSC65 contains a synthetic early/late promoter. Following the promoter is a short polylinker into which the foreign gene is cloned. Another vaccinia promoter, P_{11} (a late promoter), drives expression of the bacterial β-galactosidase gene and allows color screening of plaques to help identify recombinants. Both expression cassettes are flanked by portions of the vaccinia TK gene.

For subcloning the gene of interest into the recombination vector, several things must be kept in mind. Since splicing of the subcloned DNA will not occur, cDNAs must be used. The 5' and 3' untranslated regions should be kept short. Also, it is important to check for a transcription termination sequence that is recognized by the early RNA polymerase. The sequence TTTTTNT (T_5NT, where N can be any nucleotide) will cause termination of transcripts about 50 bp downstream (Yuen and Moss, 1987). Thus, silent mutations that eliminate any T_5NT sequences should be introduced by site-directed mutagenesis prior to subcloning. Although proteins can still be expressed from recombinant viruses made with pSC11ss or pSC65 when their cDNAs contain T_5NT sequences, expression will only occur during the late phase of virus infection
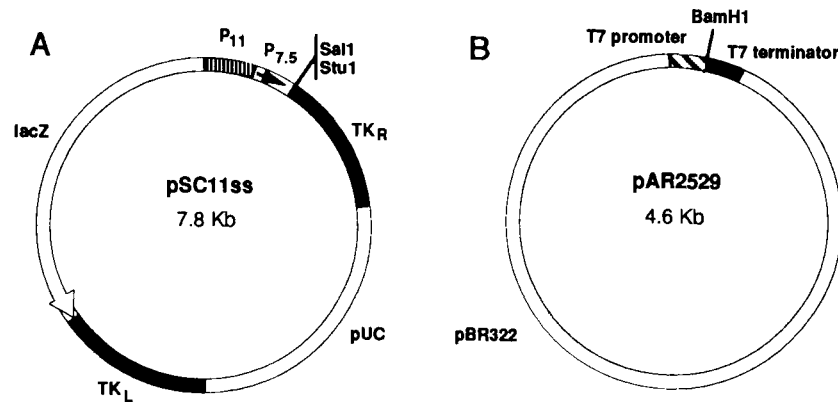
Fig. 4 Examples of vectors used for recombination and for T7 polymerase-mediated expression. (A) pSC11ss is used for producing vaccinia recombinants. The foreign gene (with its own start and stop codons) is subcloned into the *Stu* I or *Sal* I sites behind the vaccinia P$_{7.5}$ promoter. (B) pAR2529 is used for T7 polymerase-mediated expression after infection with vTF7-3 (Section IV,A). Other plasmids such as pBluescript (Stratagene) can also be used; however, the absence of the T7 polymerase terminator sequence and the presence of the G-C rich region in the Bluescript polylinker upstream of the foreign gene can reduce mRNA production and consequently decrease protein expression.

(>6 hr) when the late RNA polymerase (which does not terminate transcripts at T_5NT) is active.

After the subcloning step is completed, purified plasmid DNA should be prepared in CsCl gradients or by the Qiagen method (QIAGEN, Inc., Chatsworth, CA). Nonpurified miniprep DNA does not work when the $Ca_3(PO_4)_2$ method of transfection is used.

B. Recombination

Work with vaccinia virus should be performed under standard Biosafety Level 2 (BL-2) conditions, including the use of class I or II biological safety cabinets. National Institutes of Health (NIH) and Centers for Disease Control (CDC) guidelines recommend that workers be vaccinated every 3 years, although each institution sets its own requirements for vaccination and physical containment. Regulations should be obtained from institutional biosafety offices before initiating projects involving vaccinia virus. We use class II biological safety cabinets, autoclave disposable items that have been in contact with virus, and inactivate used solutions containing infectious virus with bleach. A convenient way of inactivating virus-containing solutions is to aspirate them into the reservoir flask in the hood and, immediately afterward (using the same pasteur pipet), aspirate bleach through the line. Additional precautions for using large quantities of vaccinia virus (for example, when growing and purifying large stocks) are discussed in Section III,A.

1. Production of Recombinant Viruses

1. HeLa or CV-1 cells are usually used for production of recombinant viruses. They are grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). Plate approximately 5×10^5 cells in a 3.5-cm dish for cells to be about 80% confluent the next day.

2a. Rinse the cells once in serum-free DMEM, and add 0.25 ml serum-free medium containing 0.05–0.1 plaque-forming units (pfu)/cell of the WR strain of vaccinia virus (we use 10^5 pfu total).

2b. Some investigators employ a trypsinization step prior to infection, which is required when the virus stock is a cell lysate instead of a purified preparation. Add an equal volume of 0.25 mg/ml trypsin to the amount of lysate that will be required and incubated at 37°C for 30 min, with occasional vortexing. This helps dissociate aggregates and releases infectious virus from cell debris. If trypsinization is used, DMEM containing 2.5% FCS should be used to dilute and adsorb the virus. FCS inhibits the trypsin.

3. After addition of the virus inoculum, cells are returned to the incubator and rocked every 5–10 min for 45 min (or 2 hr if trypsinized virus in serum-containing medium is used).

Toward the end of the infection period, prepare a $Ca_3(PO_4)_2$ precipitate of the DNA for transfection.

4. Add 5 μg DNA to 125 μl 2× HEPES buffer (50 mM HEPES, pH 7.1, 0.28 M NaCl, 1.5 mM sodium phosphate). Add an equal volume of 0.25 M $CaCl_2$ dropwise with continuous vortexing, and allow a precipitate to form by leaving the mixture at ambient temperature for 20–30 min. The precipitate should be very fine, turning the solution slightly opaque.

5. After the adsorption period, remove the virus inoculum and add 2.25 ml DMEM/5% FCS to the dish. Allow the pH to equilibrate in the CO_2 incubator for 15 min.

6. Add the precipitated DNA solution dropwise while gently swirling the dish, and return the cells to the incubator.

7. Replace DNA-containing medium with fresh growth medium the following morning.

8. Prepare a cell lysate 2 days after infection by scraping the cells into their medium and centrifuging for 5 min at 650 g at 4°C.

9. Resuspend the cell pellet in 0.25 ml complete medium and lyse the cells by freezing (in dry ice/ethanol) and thawing (at 37°C) three times.

10. The lysate is then stored at $-80°C$ until selection and screening is performed.

C. Selection and Screening of Recombinants

1. Prepare a BrdU stock solution (5 mg/ml) in water, sterilize by filtering, and store at $-20°C$.

2. Grow human TK$^-$ 143B cells in DMEM containing 10% FCS and 25 μg/ml BrdU. Plate 143B cells in a 6-well tissue culture dish at 5 × 10^5 cells/well in 2 ml complete medium and grow to near confluence (usually overnight).

3. Thaw an aliquot (100 μl) of transfected cell lysate, add an equal volume of 0.25 mg/ml trypsin, and incubate the mixture at 37°C for 30 min.

4. Make 10-fold serial dilutions (to 10^{-4}) of trypsinized lysate in DMEM with 2.5% FCS.

5. Aspirate the medium from the cells and infect cells in the wells in duplicate with 1 ml 10^{-2}–10^{-4} dilutions for 2 hr with intermittent rocking.

Toward the end of the infection period, the agarose overlay is prepared. Although most of the virus remains cell-associated, an overlay helps insure that a plaque is derived from a single virion.

6. Melt a previously autoclaved solution of 2% low melting point agarose (No. 5517; GIBCO/BRL, Grand Island, NY) in water, aliquot enough for 1 ml per well into a tube, and equilibrate to 50–55°C. [Some lots of Difco (Detroit, MI) agar work well, but some are toxic to cells and/or plaque formation.]

7. Warm 2-fold concentrated DMEM containing 10% FCS and 50 μg/ml BrdU (1 ml/well) to 37°C.

8. At the end of the infection period, aspirate the inoculum from the cells, mix the agarose and 2× medium, and overlay each well with 2 ml. After the overlay has solidified at room temperature, return the dishes to the CO$_2$ incubator.

9. After 2 days, the infected cells will be rounded and dead and can be seen as clear areas (plaques) in the cell lawn. To see these plaques more clearly and to identify recombinants, prepare a second overlay by mixing an equal volume of melted agarose with 2× DMEM containing 0.1 mg/ml neutral red (from a 10 mg/ml stock) and 0.6 mg/ml X-gal (from a 60 mg/ml stock in dimethyl sulfoxide or dimethyl formamide).

10. Overlay each well with 2 ml, allow the agarose to solidify, and incubate the dishes overnight.

11. The following morning, pick any blue plaques by inserting a pasteur pipet or a yellow pipet tip through the agarose until it contacts the cell layer; scrape the cell monolayer gently and discharge the agarose plug into a sterile microfuge tube containing 0.4 ml DMEM/5% FCS.

12. These plaque isolates should be vortexed vigorously, then frozen and thawed three times, and stored at −80°C until further purification. Typically 6 plaques are picked and taken through two additional rounds of plaque purification, as described subsequently, to ascertain that the recombinant virus is clonally derived and devoid of nonrecombinant virus.

For each round of plaque purification, a 6-well dish of 143B cells is plated as just described for each isolated plaque. The frozen and thawed cell lysate is diluted serially from 10^{-1} to 10^{-3} and duplicate wells of the confluent mono-

layers are infected with 1 ml of each dilution of virus for 2 hr. After infection, the wells are overlaid first with BrdU–agarose for 2 days, and then with a second overlay containing X-gal, as described.

The plaque-purified recombinants can be tested by several different methods (Northern or Southern blotting, immunofluorescence, dot blotting, or immuno-precipitation) to insure that they contain the gene of interest inserted into the vaccinia TK gene. We commonly use immunofluorescence after 2–3 plaque purifications. A portion of an isolated plaque is used to infect cells on a cover slip, and cells are fixed and stained 1–2 days later (see protocol in Section IV,C,3). Cells surrounding the plaques should express the foreign protein.

For other methods of screening, plaques should be expanded by successively infecting larger and larger numbers of cells. This is usually done by infecting cells in one well of a 12-well dish with half of an isolated plaque; after 2 days a cell lysate is made (as described) and is used to infect cells on a 35-mm dish. Before growing a large-scale preparation of the recombinant, we also test that the expressed protein is the correct size by immunoprecipitation from radiola-beled infected cells, followed by electrophoresis in SDS polyacrylamide gels (see Section IV,C,1).

D. Potential Problems and Practical Considerations

After subcloning into the recombination vector, approximately 3 wk will be required for generating and purifying the vaccinia recombinant. If all goes well, and if cells for successive plaque purifications are plated to be ready the same day plaques are picked, the time required may be somewhat shorter.

The recombination frequency should be about 1 in 1000, which is relatively high. However, occasionally only a few blue and many clear plaques are ob-tained. One problem might be that the vaccinia virus used to infect the cells for the recombination contains a large population of TK$^-$ virus. The solution is to plaque purify the wild-type vaccinia, and test several plaques for their ability to grow in 143B cells in the presence of BrdU. Select a plaque that *fails* to grow (i.e., TK$^+$) for future use.

Another problem might be that expression of the foreign protein is incompati-ble with vaccinia replication or assembly. We have had this problem with several proteins that accumulate in the intermediate compartment and *cis*-Golgi network. Either the recombinant is never obtained, or DNA encoding the foreign gene is lost, giving the virus a growth advantage over virus that still contains the foreign gene. In this case, one alternative is to use a double infection system (Moss *et al.*, 1990; Earl and Moss, 1991). The cDNA encoding the foreign gene is cloned into a recombination vector with the T7 RNA promoter instead of the $P_{7.5}$ promoter (pTM-1), and a recombinant virus is produced as described already. Since the protein is not expressed, it will not interfere with recombinant virus production. Expression is mediated by co-infecting the cells

with another vaccinia virus encoding T7 RNA polymerase (vTF7-3). Alternatively, the infection/transfection system can be used (Section IV,B).

III. Large-Scale Growth and Purification of Vaccinia Virus Stocks

A. Growth of Vaccinia Virus

1. Grow HeLa cells in DMEM with 5% FCS and expand into four 15-cm dishes. Grow the cells until they are approximately 80% confluent.

2a. If the virus inoculum is a purified preparation, rinse the dishes with phosphate-buffered saline (PBS) and infect with vaccinia virus (or a vaccinia recombinant) at 0.05–0.1 pfu/cell in 2 ml serum-free DMEM per dish (a confluent dish contains approximately 2×10^7 cells). Return the infected cells to the incubator and rock every 5–10 min for 30 min.

2b. If the virus inoculum is a crude cell lysate, the volume required to give 0.05–0.1 pfu/cell should be trypsinized as described in Section II,C and diluted in medium with 2.5% serum for infection. Rock the infected cells every 15 min for 2 hr.

3. At the end of the infection period, add 20 ml DMEM/5% FCS to each dish.

4. Purify the virus 2–3 days after infection, at which time most or all of the cells should appear rounded, but should remain attached to the dish.

B. Purification of Vaccinia Virus

Although a frozen and thawed preparation of infected cells can be used for expression work, purified virus stocks are recommended. Sonication and trypsinization of cell lysates is the only way to disrupt virus aggregates generated by freezing in medium, and must be performed before infecting cells. Purified virus is stored at high pH in a buffer lacking salt, which minimizes aggregation. Thus trypsinization and sonication are unnecessary when infecting with purified preparations.

1. Virus purification should be performed in a laminar flow hood and gloves should be worn. Pipets and any glassware used should be soaked in bleach or autoclaved after use to destroy infectious virus.

2. Scrape the infected HeLa cells into their medium using a rubber policeman; then combine and transfer to 50-ml disposable sterile tubes and centrifuge for 5 min at 200 g (1000 rpm in a tabletop centrifuge) at 4°C.

3. Resuspend the pellets in a total of 8 ml of 10 mM Tris HCl, pH 9.0, and homogenize (in two batches) on ice in a 7-ml dounce with 40–60 strokes using the tight pestle.

4. Centrifuge the homogenate in two 15-ml disposable tubes for 5 min at 200 g at 4°C to remove nuclei. Collect the supernatant and save on ice while the pellets are washed.

5. Resuspend the pellets in a small volume of 10 mM Tris-HCl, pH 9.0, dilute to 10 ml with the same solution, and recentrifuge as in step 4.

6. Transfer the supernatants along with those from the previous spin into 50-ml tubes and centrifuge at 650 g (2000 rpm) for 10 min at 4°C to remove any remaining debris.

7. Sonicate the supernatants for 2–5 min in a water bath sonicator; then distribute into two SW28 tubes and underlay with an equal volume of 36% (w/v) sucrose in 10 mM Tris-HCl, pH 9.0, to fill the tubes.

8. Centrifuge the tubes at 13500 rpm (33000 g) in a SW28 rotor for 80 min at 4°C.

9. After centrifugation, remove the supernatant, treat with bleach, and discard. Resuspend the pellets in 2 ml of 1 mM Tris-HCl, pH 9.0, per tube (they should be easy to resuspend but can be sonicated if necessary). Combine and dispense into small (50–100 μl) aliquots.

10. Purified virus is stored at −80°C. Usually, virus purified with this protocol yields 2–8 × 10^9 pfu/ml. After an aliquot is thawed for use, any remainder can be refrozen at −80°C and used once or twice more without a substantial loss of titer.

C. Titering a Vaccinia Virus Preparation

Titering the virus is performed by infecting cells (typically HeLa, CV-1, or BSC-1) with various dilutions of virus and determining the number of infectious particles per milliliter by counting the number of plaques that form. We usually use HeLa cells, but plaques formed on BSC-1 monolayers are easier to distinguish and quantify. The protocol for titering the virus is basically the same as that described for selection of vaccinia recombinants (Section II,C). However, the purified virus does not require treatment with trypsin prior to plaquing. Also, an agarose overlay is not necessary, since most of the virus remains cell-associated and thus does not spread readily through the culture medium. An agarose overlay containing neutral red stain and/or X-gal added to the cells just prior to counting the plaques can aid in visualizing them.

1. Grow cells in a 6-well dish until they are nearly confluent (usually 4–5 × 10^5 cells/well plated the day before).

2. To titer the virus, thaw an aliquot of purified virus and dilute serially into serum-free DMEM to 10^{-8}. Include a negative control (no virus) and a positive control (purified vaccinia virus of known titer diluted to a concentration designed to give 50–100 plaques).

3. After rinsing the cells in PBS, add 100 μl serum-free DMEM to each well; then add 100 μl diluted virus (use dilutions ranging from 10^{-5} to 10^{-8}).

4. Place the dish in the incubator and rock every 5–10 min.

5. After 30 min, replace the inoculum with 2 ml complete medium.

6. After 3 days, aspirate the medium and stain the cells with 0.1% crystal violet in 20% ethanol for 5 min. Aspirate the stain and allow the well to dry before counting the plaques. Alternatively, the wells can be overlaid with 3 ml agarose containing neutral red and/or X-gal. The overlay procedure is described in Section II,C. Count the plaques in each well (where possible) and determine the titer (pfu/ml) based on the dilution of virus used to infect the cells.

IV. Vaccinia Virus–Mediated Protein Expression

A. Vectors for Use with the T7 RNA Polymerase System

As mentioned in the introduction, the T7 RNA polymerase method of vaccinia-mediated expression is quite convenient since only one recombinant virus is needed (vTF7-3). In addition, the gene to be expressed can be cloned into commonly used vectors such as pBluescript. However, specially designed vectors containing a slightly extended version of the T7 promoter and the T7 terminator sequence give better expression (Fig. 4B). For very high levels of expression, vectors have been constructed that contain an untranslated leader region from encephalomyocarditis virus as well as the T7 promoter and terminator. The message produced by T7 polymerase is inefficiently capped by the vaccinia capping enzyme, and the leader region from encephalomyocarditis virus allows cap-independent translation (Elroy-Stein *et al.*, 1989). However the 5- to 10-fold increase in protein expression obtained with this system may not be practical for cell biological studies (see Section V,B).

B. Infection and Infection/Transfection

The protocols for the direct and the indirect methods of expression are essentially the same, with the exception of an additional transfection step following the infection for the T7 polymerase-mediated system. We discuss both protocols for protein expression together in this section, with additional steps for the T7 system described in the appropriate places.

A wide variety of cell lines can be used for expression of the protein of interest. Chinese hamster ovary (CHO) cells are an exception, since they are nonpermissive for vaccinia infection. In addition, polarized Madin–Darby canine kidney (MDCK) and Caco-2 cells are poorly infected.

1. Protein Expression

1. To examine protein expression using metabolic labeling, immunoblotting, or immunofluorescence, cells are plated the day before infection to reach

40–70% confluence (approximately 2×10^5 cells/35-mm dish). Larger dishes may be used for some applications; however, expression is usually high enough that this is not required.

2. Cells are rinsed once in PBS, and then infected with vTF7-3 or another recombinant virus. The virus (10–20 pfu/cell or 10^7 pfu/35-mm dish) is added in 0.3 ml serum-free DMEM per dish.

3. After addition of the virus, dishes are returned to the incubator and rocked every 5–10 min for 30 min. If the protein of interest is expressed directly by the recombinant virus, the inoculum is replaced after infection with 2 ml regular growth medium containing serum and Step 4 is omitted.

Note: If expression is mediated via T7 polymerase (vTF7-3), the infection period can be used to prepare the DNA for transfection. Transfection can be performed using either cationic lipid or calcium phosphate as a carrier.

4a. For lipid-mediated transfection, 2–5 µg plasmid DNA (CsCl-purified or Qiagen-purified works best, but miniprep DNA can also be used) is added to 0.75 ml serum-free DMEM per 35-mm dish of cells. After mixing, 10 µl LipofectACE (No. 18301; GIBCO/BRL) is added to the side of the tube and immediately vortexed for 5 sec. [Lipofectin (No. 18292; GIBCO/BRL) also works well, but is more expensive.] The mixture is incubated at ambient temperature for up to 30 min. At the end of the viral infection period, the inoculum is replaced with the DNA–TransfectACE mixture.

4b. For calcium phosphate-mediated transfection, a precipitate containing 5 µg purified plasmid DNA (encoding the foreign gene behind the T7 promoter) is prepared and added to cells exactly as described in Section II,B. We no longer use the $Ca_3(PO_4)_2$ method of transfection because it is much less reproducible than the cationic lipid method. After transfection, dishes are returned to the incubator. The incubation time varies depending on the type of analysis that will be performed (see the next section).

C. Analysis of Protein Expression

1. Metabolic Labeling

Metabolic labeling can be initiated as early as 3 hr postinfection. Cells should be starved for 15 min in medium lacking the amino acid that will be used to radiolabel cells if a short pulse label will be used. Because of the high expression level, pulse periods can be short (5 min in 100 µCi/ml [^{35}S]methionine typically gives an overnight exposure). After the appropriate pulse–chase period, cells are lysed in nonionic detergent. We use a detergent solution containing 50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA, 0.4% deoxycholate, 1% NP-40, and 0.04 TIU aprotinin/ml. Other typical lysis buffers contain 1% Triton X-100 in Tris-buffered saline with protease inhibitors. After spinning out nuclei and debris (1–15 min in a microfuge), the protein of interest is immunoprecipitated and

electrophoresed as desired. Solubilization and immunoprecipitation conditions must be determined empirically for each antibody–antigen combination.

a. Cell lysis and immunoprecipitation

1. We typically lyse cells from a 35-mm dish in 0.5 ml detergent solution (described earlier). All polyclonal antibodies and many monoclonal antibodies seem to work well in this mixture.

2. After 10 min on ice, lysates are transferred to a microfuge tube and nuclei and debris are removed by spinning for 1 min.

3. The supernatants are transferred to a fresh tube, and 10% SDS is added to a final concentration of 0.2%. The SDS helps reduce background binding. For immunoprecipitation with monoclonal antibodies, we generally omit the SDS.

4. Antibody is added to the tubes, and they are rotated (2 hr to overnight) at 4°C.

5. We use fixed *Staphylococcus aureus* cells (No. 507861; Calbiochem, San Diego, CA) to collect the antigen–antibody complexes (20 min at 4°C); protein A–Sepharose can also be used.

6. The immunoprecipitates are washed 3 times in RIPA buffer (0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, 1% NP-40, 1% deoxycholate, 0.1% SDS).

7. The samples are usually eluted directly in SDS sample buffer containing reducing agent and are electrophoresed in SDS–polyacrylamide gels. Alternatively, the samples can be eluted and treated with endo- or exoglycosidases to analyze carbohydrate modifications. Our protocol for digestion with endoglycosidase H is given in the legend to Fig. 7.

2. Immunoblotting

1. If the expressed protein is to be detected by immunoblotting, it is advisable to wait until 6–8 hr postinfection before lysing the cells to allow chemical amounts of protein to accumulate. Cells are infected (and transfected if necessary) as described already.

2. After the appropriate incubation time, the medium is removed (and trichloroacetic acid precipitated if the protein is secreted) and the dishes are rinsed once in cold PBS.

3. After removing the last trace of PBS, cells are lysed on the dish by adding 50 μl Laemmli sample buffer containing a reducing agent and swirling with a pipet tip to draw the lysate together (the DNA from the lysed nuclei makes a viscous solution).

4. The samples are transferred to microfuge tubes, heated at 100°C for 3 min (or longer if required to fully shear DNA), and loaded onto SDS–polyacrylamide gels.

5. After electrophoresis, the proteins are transferred to nitrocellulose (or other suitable membrane) and incubated with primary antibodies.

6. Proteins are detected using colorimetric or chemiluminescence methods after incubation with an appropriately conjugated second antibody. An example of immunoblotting used to follow the time course of protein expression is shown in Fig. 5.

3. Immunofluorescence

Significant levels of expressed proteins can usually be detected by immunofluorescence staining between 4 and 8 hr after infection, depending on the cell type used and the subcellular localization of the protein. Proteins that become concentrated in subcellular compartments (e.g., nuclear, Golgi, or lysosomal proteins) may be detectable at shorter times after infection than proteins with diffuse localizations (e.g., plasma membrane or cytoplasmic proteins). At long times after infection (>7–8 hr), localization by immunofluorescence becomes
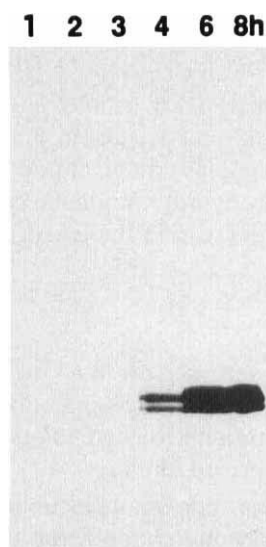

Fig. 5 *Time course of VSV G protein expression after vTF7-3-mediated expression.* BHK cells were infected with vTF7-3, then transfected as described with 5 µg per dish of pAR/G, which encodes the G protein of vesicular stomatitis virus (VSV). At each time point, individual dishes were rinsed once with PBS, then solubilized in 50 µl Laemmli sample buffer containing 5% β-mercaptoethanol. Samples were heated to 100°C for 3 min prior to electrophoresis in a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated overnight with anti-VSV polyclonal antibody after blocking in Tris-buffered saline containing 0.05% Tween-20 and 5% nonfat dry milk. Primary antibody was detected with horseradish-conjugated goat anti-rabbit IgG and enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

increasingly difficult as the cells become rounded. The change in cell shape makes it difficult to focus on certain intracellular compartments. If long infection times must be used, confocal microscopy is recommended.

1. When proteins are to be detected by immunofluorescence, cells are plated on glass cover slips in 35-mm dishes the day before infection. For many cell types, plain cover slips can be used (after sterilization by autoclaving). However, some cells attach better if the glass is pretreated by acid washing, or by coating with poly-L-lysine (1 mg/ml) or extracellular matrix components such as fibronectin (1 μg/ml).

2. Vaccinia infection and subsequent DNA transfections are performed as described in Section IV,B.

3. After the desired incubation period, cells are fixed in formaldehyde or methanol–acetone. Methanol–acetone fixation may be required if components of the cytoskeleton are being analyzed, and usually consists of 5–10 min in methanol at $-20°C$, followed by 30 sec to 1 min in acetone at $-20°C$. No further permeabilization is required.

Note: We find that formaldehyde fixation works well for all the antibodies we use (see subsequent discussion). As for other methods of analysis, optimal antibody dilutions and other parameters should be determined for each protein. As a guideline, polyclonal sera are usually diluted 1 : 200–1 : 1000, monoclonal antibodies from tissue culture supernatant are used straight or diluted up to 1:200, and affinity-purified antibodies or purified IgGs are used at 2–10 μg/ml. Commercially prepared secondary antibodies conjugated to fluorescein, rhodamine, or Texas red are available from many suppliers. We have had excellent success with affinity-purified preparations from Jackson Immunoresearch (West Grove, PA).

4. Our protocol includes fixation in 3% paraformaldehyde in PBS for 20–30 min at room temperature.

5. The fixative is quenched using PBS–gly (PBS with 10 mM glycine).

6. If an intracellular epitope is being labeled, the cells are permeabilized for 4 min with 0.5% Triton X-100 in PBS–gly.

7. After rinsing once with PBS–gly, we typically add a 5 min incubation in blocking solution (0.25% ovalbumin in PBS–gly.)

8. After removing aggregates by centrifuging antibodies in a microfuge for about 30 sec, both primary and secondary antibodies are diluted into the blocking solution. Throughout the staining procedure, it is important that the cover slips never dry out.

9. The cover slips are lifted with forceps, a corner is touched to a Kimwipe® to drain as much liquid as possible, and the slip is placed cell-side down onto a drop (50 μl) of primary antibody on a sheet of parafilm and incubated 20 min at room temperature.

10. The cover slips are then returned (cell-side up) to the dishes, washed for 10 min in several changes of PBS–gly, and incubated in 50 μl fluorochrome-conjugated secondary antibody, as described, for 20 min in the dark.

11. After washing in PBS–gly for 20–30 min, the backs of the cover slips are gently wiped and they are inverted onto a small drop of glycerol on a microscope slide. We use glycerol containing 100 mM n-propyl gallate to reduce photobleaching; glycerol containing phenylaminediamine or a commercial "mounting medium" can also be used.

12. The excess mounting medium is aspirated (and the cover slip may be sealed to the slide with nail polish) before viewing in a microscope equipped with epifluorescence and the appropriate barrier filters. An example of indirect immunofluorescence detection of proteins expressed using the T7 polymerase–vaccinia hybrid system is shown in Fig. 6.
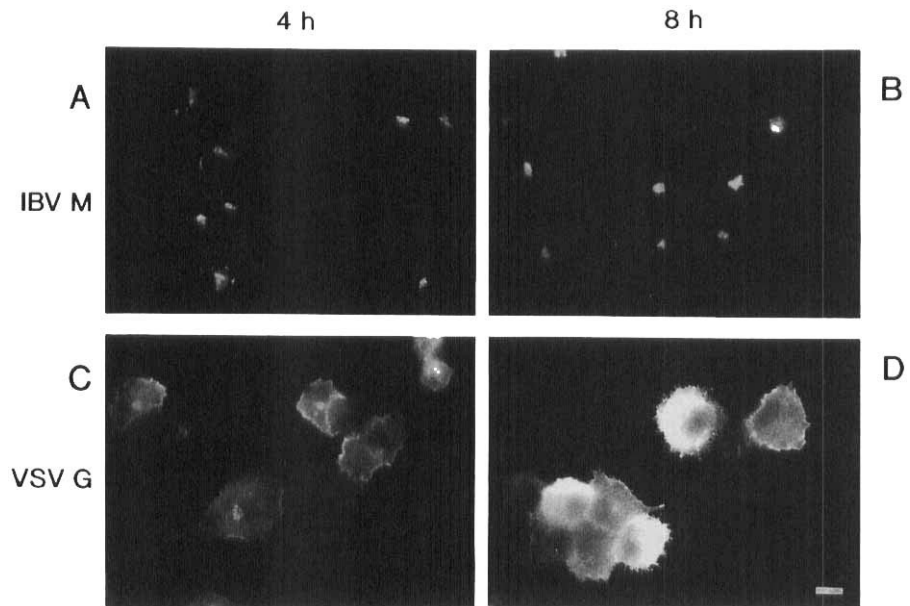

Fig. 6 Indirect immunofluorescence staining of vaccinia-infected BHK cells. BHK cells were infected with vTF7-3 followed by LipofectACE-mediated transfection with pAR2529 encoding either the Golgi-resident M protein of avian infectious bronchitis virus (A,B) or the cell surface VSV G protein (C,D). At 4 (A,C) or 8 (B,D) hr postinfection, cells were rinsed with PBS, fixed, and labeled using appropriate antibodies followed by Texas red-conjugated secondary antibody. Note that cell rounding at 8 hr postinfection makes it difficult to focus on the Golgi complex (compare A and B), whereas it is easier to detect VSV G at the plasma membrane at the later time point. Bar, 10 μm.

═══════ V. Discussion

This chapter has discussed the preparation and use of recombinant vaccinia viruses to express proteins in mammalian cells. This method offers many advantages over other expression systems for the expression of some proteins (see below). However, the limitations of this method should be taken into account before choosing this technique.

A. Advantages of Vaccinia Virus–Mediated Protein Expression

1. Vaccinia virus-mediated expression is rapid and efficient: experiments can be performed in 1 day. Furthermore, most of the cells in a dish can be infected, resulting in a much higher efficiency of expression than other in transient expression systems.

2. Vaccinia has a wide host range, so most mammalian tissue culture cell lines are susceptible to infection. One exception is the CHO cell line, which is not susceptible to infection with the virus. However, cowpox virus does infect CHO cells, and a gene that allows this infection has recently been characterized (Spehner et al., 1988). It is possible that construction of a vaccinia virus recombinant containing this cowpox gene will allow productive infection of CHO cells.

3. Because of the high efficiency of expression, coexpression of two or more proteins is simple using this technique. The expression level of each protein can be varied with the amount of virus or DNA used, depending on the method of expression (e.g., Zagouras et al., 1991).

4. Because this expression system operates cytoplasmically, it can be used to express genes from RNA viruses that contain cryptic splice sites that are used when the cDNA is inserted into a vector that must be transcribed in the nucleus (Machamer and Rose, 1987).

5. Infection with recombinant vaccinia viruses results in high expression levels in >90% of cells on a dish soon after infection. This method is therefore ideal for subcellular localization of expressed proteins by immunoelectron microscopy (Machamer et al., 1990; Krijnse Locker et al., 1992). Early times postinfection (<6 hr) should be analyzed to avoid complications from viral cytopathic effects. Localization of proteins expressed via the vaccinia–T7 polymerase hybrid system is more difficult since the cellular morphology can be altered by the DNA carriers used for transfection, especially cationic lipid.

6. Vaccinia virus-mediated expression can be useful for production of biologically active proteins. Because proteins are expressed in mammalian cell lines, glycosylation and other post-translational modifications that may be necessary for activity are preserved.

7. An advantage specific to the T7 polymerase hybrid expression system is that it can be used to screen rapidly for expression of newly cloned cDNAs

and of mutant proteins generated by site-directed mutagenesis. The same vector can be used for mutagenesis, sequencing, *in vitro* transcription/translation, and vaccinia virus-mediated expression. In addition, a high percentage of cells is transfected because the DNA only needs to reach the cytoplasm and not the nucleus to be expressed.

B. Disadvantages and Limitations

1. A serious disadvantage of the vaccinia virus system is that the expression level may be so high that it overwhelms cellular translation and translocation machinery. This can result in decreased efficiency of membrane translocation, post-translational modifications, and transport through the secretory pathway. At very high expression levels, newly synthesized proteins that enter the secretory pathway may accumulate in the ER. We have observed reduced rates of transport of the vesicular stomatitis virus (VSV) G protein to the cell surface, compared with VSV-infected cells, when the protein is expressed with the vaccinia–T7 hybrid system (Fig. 7). Whereas VSV G becomes resistant to endoglycosidase H (as it moves through the Golgi complex) with a half-time of <25 min in VSV-infected HeLa cells, the half-time is considerably slower (~40 min) in vTF7-3-infected and -transfected cells. The difference in transport kinetics may be less dramatic in other cell types.

2. The cytopathic effects of vaccinia infection can be a problem for certain types of analysis. For example, an early effect of the infection is rounding of cells (probably due to changes induced in the cytoskeleton), which can make conventional immunofluorescence difficult to interpret (Fig. 6). In addition, host cell protein synthesis is inhibited in infected cells. Vaccinia encodes homologs of cellular proteins including superoxide dismutase, epidermal growth factor, and profilin (Goebel *et al.,* 1990), expression of which could affect cell morphology and behavior in unpredictable ways. Although viral DNA replication and early assembly steps can be blocked (by treating infected cells with hydroxyurea or rifampicin, respectively), early cytopathic effects such as cell rounding are unfortunately not prevented.

3. Recombinant vaccinia viruses can be difficult and time-consuming to produce. Because they inhibit transcription from early promoters, any T_5NT sequences in the gene of interest must be mutagenized prior to subcloning into the recombination vector. Even then, some recombinant viruses are impossible to produce, perhaps because expression of the foreign protein is incompatible with vaccinia replication or assembly.

4. A final consideration prior to using the vaccinia virus-mediated protein expression system is the safety of laboratory workers. Institutional guidelines for use of vaccinia virus recombinants must be followed. Some institutions require a vaccination against smallpox by all vaccinia users once every 3 years (as recommended by the NIH and CDC; however, see Grist, 1989; Wenzel and

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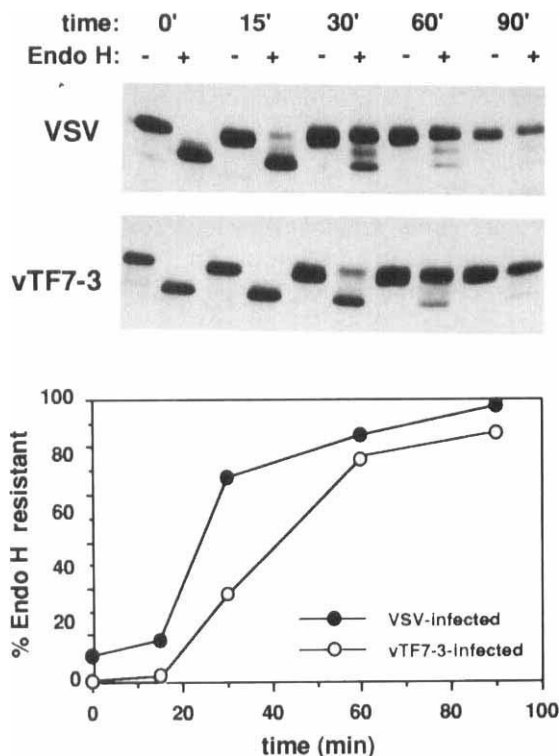Endo H: - +   - +   - +   - +   - +

VSV

vTF7-3

Fig. 7 Comparison of intracellular transit kinetics of VSV G protein in VSV-infected versus vTF7-3-infected/transfected cells. HeLa cells grown in 35-mm dishes were infected with vTF7-3 followed by LipofectACE-mediated transfection with 2 μg pAR/G per dish, as described in Section IV,B, or with VSV (20 pfu/cell). At 3.75 hr postinfection, cells were rinsed once in PBS, starved for 15 min in methionine-free DMEM, and pulse-labeled for 5 min with 50 μC ^{35}S *in vivo*-labeling mix (Amersham) in 0.5 ml methionine-free DMEM per dish. Cells were chased in growth medium. At the indicated chase times, individual dishes were lysed in detergent solution and immunoprecipitated using a polyclonal anti-VSV antibody, as described in Section IV,C,1. Samples were eluted in 20 μl 50 mM Tris pH 6.8, 1% SDS for 3 min at 100°C. Eluents were divided in half, and either 10 μl 0.15 M citrate, pH 5.5, or the same amount of buffer containing 0.3 mU endoglycosidase H (endo H) was added. After overnight incubation at 37°C, concentrated Laemmli sample buffer with β-mercaptoethanol was added; the samples were heated to 100°C for 3 min and electrophoresed on Laemmli SDS–polyacrylamide gels. The percentage of VSV G protein that was resistant to endo H at each time point was quantitated by densitometric scanning of fluorographed gels.

Nettleman, 1989). BL-2 restrictions also apply. If available, a virus-only laminar flow hood is recommended to avoid accidental infection of cell lines during routine tissue culture, although we have not had problems with accidental infection when the ultraviolet light is left on for an adequate time following work with infected cells.

C. Availability of Vaccinia Virus Stocks and Plasmids

A small aliquot of vaccinia virus (both wild-type and the recombinant vTF7-3) and vectors for producing recombinant viruses and for T7 polymerase-mediated expression can be obtained by writing to Dr. Bernard Moss (Laboratory of Viral Diseases, NIAID, NIH, 9000 Rockville Pike, Bldg 4-Rm 229, Bethesda, MD 20892). New users must demonstrate that their facilities meet biosafety requirements, and that they have followed the institutional guidelines regarding vaccination. In addition, a material transfer agreement from the NIH must be completed and signed by your institution prior to receipt of these stocks.

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