Herpes Simplex Virus Protocols

METHODS IN MOLECULAR MEDICINE™

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Herpes Simplex Virus Protocols

Edited by

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Preface

Herpes Simplex Virus Protocols comprises a wide range of experimental protocols that should be especially useful to new workers in herpes virology. Hopefully, it will also provide information for those with experience in the field, as well as those embarking on techniques that are new to them.

Obviously the range of topics covered cannot be comprehensive, but we have tried to provide protocols dealing with those procedures that are most widely used; and we have selected expert authors accordingly. We have also tried to cover the range from the more biological in vivo maneuvers to purely molecular procedures, taking into account the topical interest in the potential use of HSV as a therapeutic tool. In this way there should be sufficient information for most procedures the average herpes virologist is likely to require—at least at this moment in time!

Since the herpesviruses are a large family, we have largely based the protocols on the virus we know best—herpes simplex virus. With this as the prototype, it should be relatively easy to extrapolate and make the necessary modifications required for application to some of the other herpesviruses, especially members of the alpha group, such as PRV and EHV. It would have been an impossible task to include chapters covering the unique aspects of each known herpesvirus.

The point of this series, *Methods in Molecular Medicine*, is to provide a reference source in which a procedure should be able to be followed from A to Z without having to refer to other literature. Much detail is therefore provided, and the pitfalls and shortcuts—which are never mentioned in papers—are addressed as fully as possible. Naturally, many of the methods are now standard and can be found in any molecular biology textbook, but the additional details required for their application to HSV have been provided where necessary.

Like most other scientific disciplines over the last 10 yr, the expansion in technology and knowledge in the herpes field has been exponential. Kits are now available for just about every procedure but, thankfully, we have not yet reached the stage where books of this type are no longer required. It is essential for young virologists to understand what they are doing at the bench, as well as why they are doing it, and this practical experience is what we hope to foster here.

Our grateful thanks go to the numerous people who have contributed to this volume either willingly or under pressure! John Subak-Sharpe introduced me to HSV in 1968. Subsequently, in 1984, Alasdair MacLean came to my lab as a graduate student. We, along with many other herpes virologists world-wide, are indebted to John.

S. Moira Brown
Alasdair R. MacLean

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1

HSV Growth, Preparation, and Assay

June Harland and S. Moira Brown

1. Introduction

Whether herpes simplex virus (HSV) is viewed as a pathogen or as a model eukaryotic system, it is virtually certain that any experimental work will require the virus to be grown and assayed. The following chapter is therefore seen as the fundamental first step before embarking on more intellectually and technically challenging technology. Its importance should not however be underestimated. It never fails to surprize us that people who describe themselves as virologists have little understanding of the basic requirements needed to attain a contamination-free, high-titer, low particle:plaque-forming units (PFU) ratio, genetically pure virus stock

HSV grows well in a wide variety of cell types to yield high-titer stocks. In general, HSV type 1 (HSV-1) grows to a higher titer than type 2 (HSV-2) and is less cell-associated, i.e., more infectious virus is released into the growth medium. Cell lines routinely used to grow HSV include BHK (hamster kidney), RK13 (rabbit kidney), Vero (monkey kidney), and CV1 (monkey kidney).

When HSV infects a single cell, the surrounding cells will also become infected by spread of progeny virus from cell to cell. This focus of infection normally causes cell necrosis, resulting in a hole in the monolayer with rounded cells at the periphery. Alternatively, certain virus strains can pass from cell to cell and cause fusion of the infected cells, resulting in a syncitium. For either type, these foci of infection are called plaques and are a measure of the number of infectious particles within a virus stock. The titer of a virus stock is expressed as the number of PFU per milliliter of virus (PFU/mL).

Spontaneous genomic mutations (point mutations, deletions, insertions) occur relatively frequently within a virus stock and, if nonlethal, they will be maintained. Therefore, to achieve genomic homogeneity, it is essential that a

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virus stock originates from a single virus plaque (single infectious particle) and that subsequent passage numbers are kept to a minimum. To ensure the purity of the isolate from which the stock will be derived, it must be stringently plaque-purified. This is done by serial dilution of the virus until preferably only one plaque is present on a monolayer. This plaque is picked, the virus titrated again, and a single plaque picked. A minimum of three rounds of stringent purification is usually required to yield a pure stock. Once a virus stock has been grown up from this plaque-purified isolate, it should be retained as an elite master stock and used as the only source of virus for generating working virus stocks.

The quality of virus stocks can also be adversely affected if the correct procedures are not followed when growing the virus Defective particles are generated when incomplete virus genomes are packaged If the DNA in the defective particle contains an origin of replication, it can be replicated in the presence of the standard virus, which supplies essential helper virus functions. All virus stocks should be grown from low multiplicity of infection (MOI) inocula. This optimizes amplification and packaging of complete virus genomes as opposed to defectives, during the several cycles of genomic replication required to generate a stock. The proportion of defective particles within a stock is a good indication of the quality of the virus. It is desirable for most experimental procedures to use stock with as low a particle:PFU ratio as possible. Wild-type stocks of HSV-1 with a ratio of 5:1 or less can be achieved, and a stock with a ratio >10:1 should be considered poor. For HSV-2, the average ratio of a good stock is <100·1

2. Materials

2.1. Reagents

1 ETC_{10}· Glasgow modified Eagle's medium with the addition of 10% newborn calf serum, 100 U/mL penicillin, 100 U/mL streptomycin and 10% tryptose phosphate broth (TP)
2 ETMC 10% Glasgow modified Eagle's medium with the addition of 10% newborn calf serum, 100 U/mL penicillin, 100 U/mL streptomycin, 10% TP, and 1% methylcellulose Since the methylcellulose needs to be heated to solubilize, 10X concentrated Eagle's medium is used The requisite amount of low-viscosity carboxymethylcellulose, sodium salt is dissolved in water to give a final concentration in the medium of 1% After autoclaving, the methylcellulose solution is substituted for water when making up the media
3 Phosphate-buffered saline (PBS)/calf serum PBS with the addition of 5% newborn calf serum
4 Brain heart infusion (BHI) agar
5 Blood agar: BHI agar containing 10% horse blood.
6. Giemsa: Giemsa's stain (Gurr)
7 Virkon

2.2. Equipment

1 Trays for Petri dishes
2 Bijoux racks.
3 Cell monolayer scrapers
4 Vortex.
5 Sonibath.
6 Stereo zoom plate microscope.
7 Centrifuge (2000 rpm), e g , Beckman GPR centrifuge
8 Centrifuge (12,000 rpm), e.g , Sorvall RC5C
9 CO_2 incubators
10 Roller bottle incubators
11. Class II hood
12 –70°C Freezer
13. Availability of an electron microscope (for particle counts)

3. Methods

Many tissue-culture lines can be used for the growth of HSV, but for the purpose of this chapter we will concentrate on BHK 21/C13 cells, which are routinely used in Glasgow and which give high yields of infectious virus. BHK 21/C13 cells are grown in ETC_{10} at 37°C in an atmosphere containing 5% CO_2.

For the preparation of large stocks of virus, 10 roller bottles of BHK cells (approx 3×10^8 cells/bottle) are used, which should yield 5–10 mL of stock at approx 10^9–10^{10} PFU/mL. Virus production on this scale requires an incubator capable of accomodating roller bottles If a suitable incubator is not available, it will be necessary to scale down the method appropriately.

Wild-type HSV-1 will grow over a large range of temperatures, between 31 and 39°C with little discernable effect on infectious virus yield. However, it is preferable to grow virus stocks at 31°C, since fewer defective particles are generated than at 37°C If roller bottle space at both 37°C (for growth of cells prior to virus inoculation) and 31°C (for virus growth) is not available, the virus can usually be grown at 37°C with only a marginal impairment in quality.

3.1. Growth of HSV Stocks

Good microbiological practice and sterile techniques need to be used throughout the procedure.

1. Seed each of 10 roller bottles with 3×10^7 BHK cells in 100 mL of ETC_{10} medium and add 5% CO_2 either from a central CO_2 line or from a cylinder In practice, this is done by attaching a sterile Pasteur pipet to the line, inserting the pipet into the bottle, and counting to 5[1]
2. Grow the cells at 37°C for 3 d until they form almost confluent monolayers.
3. Pour off the growth medium, and infect with virus at an MOI of 1 in 300 Assum-

ing 3×10^8 cells/bottle, add 10^6 PFU in 20 mL of fresh ETC_{10}. There is no need to add more CO_2

4. Incubate the infected cells at 31°C Cytopathic effect (CPE) should be apparent after 1–2 d, and the virus will be ready to harvest in 3–5 d when the cells have rounded up and are starting to detach from the plastic
5. The roller bottles should be shaken (unopened) until all the cells are in the medium If this proves difficult, sterile glass beads (approx 2-mm diameter) may be added and swirled around to detach the adherent cells
6 The medium containing the detatched cells should be poured into a sterile 200-mL centrifuge bottle (the glass beads if used will remain in the roller bottle) and spun at 2000 rpm for 10 min to pellet the cells Both the cell pellet and the supernatant should be kept
7 The supernatant should be poured into a sterile 250-mL centrifuge bottle and spun at 12,000 rpm, e g , in a Sorvall GSA rotor for 2 h The resultant pellet will consist of cell-released/supernatant virus (SV) and should be resuspended in 1 mL ETC_{10}/roller bottle
8. To harvest the cell-associated (CA) virus, the cell pellet from step 6, should be resuspended in a small volume (2–5 mL) of ETC_{10} This should be transferred to a suitable container (glass universal bottle) and sonicated thoroughly in a sonibath to disrupt the cells The sonicate should be spun at 2000 rpm for 10 min and the supernatant kept as fraction (1) of the CA virus To re-extract, a further 2–5 mL of fresh ETC_{10} should be added to the pellet, the solution sonicated, and the cell debris spun out again at 2000 rpm for 10 min This CA fraction 2 should be added to fraction 1
9 The CA and SV virus preparations may be kept separate or combined If they are to be kept separate, the virus pellet from step 7 should be resuspended in 5–10 mL of fresh ETC_{10} and sonicated briefly in a sonibath to disrupt the pellet If they are to be combined, then the pellet from step 7 can be resuspended directly by sonication in the CA fraction, since the overall resultant volume will be smaller Usually for HSV-1, SV and CA titers are similar For HSV-2, the CA titer is usually 10 times higher than SV

3.2. Sterility Checks

1. Sterility checks should be carried out on a new virus stock to ensure that it is free from bacterial or fungal contamination before storing at –70°C. This is done by streaking an inoculum of the virus on a blood agar plate using a sterile platinum loop and incubating the plate at 37°C for several days To test for fungal infections, the virus stock can be similarly streaked on a BHI agar plate and the plate incubated at room temperature for up to a week If the stock is contaminated with either bacteria and/or fungi, obvious colonies and/or hyphae will be seen on the plates Usually, a distinct smell will be obvious!
2. It is usual for contaminated stocks to be discarded, but if the virus is "irreplaceable," it can be filter-sterilized to remove bacterial or fungal contamination Unfortunately, this results in a large drop in titer and loss of volume, so it is only

worthwhile if the virus is very important. Clearance of contamination is achieved by passing the virus through a 0.2-μ pore size filter. It may be easier if the stock is first passed through a 0.4-μ filter.

Note: It is important always to wear safety goggles when carrying out this procedure, since there is a risk of the syringe detatching from the filter and spraying virus into the face

3. Mycoplasma contamination of virus stocks is hard to detect, although mycoplasma usually cause blood agar plates to discolor. If the cells used to grow virus test positive for mycoplasma, the virus stock and the cells should be immediately discarded. If the virus is "irreplaceable," it is possible to extract viral DNA, which can be used to transfect clean cells to obtain a mycoplasma-free, virus stock

3.3. Viability

To reduce the number of freeze-thaw cycles, virus stocks should be aliquoted maximally into 1-mL amounts and stored at –70°C.

Note: HSV should never be stored at –20°C, since infectivity will be lost very rapidly. Aliquoted vials should be frozen quickly, and when being thawed, they should be warmed rapidly and kept at 0–4°C until use. The amount of time the virus is at 0–4°C should be kept to a minimum, but it can remain at 4°C for 24 h without a significant drop in titer.

3.4. Titration of Virus Stocks

To quantitate the amount of infectious virus within a stock, it is necessary to titrate the stock on cell monolayers, and count the number of plaques on plates that have been fixed and stained to make the plaques easily visible under a microscope. The titer is expressed as PFU/mL of virus.

1. Seed 60-mm plastic Petri dishes with 3×10^6 BHK cells in 5 mL of ETC_{10}
2. Incubate the plates overnight in a 37°C incubator in an atmosphere with 5% CO_2 The cells should form just subconfluent monolayers.
3. Serial dilutions of virus are made in PBS/calf serum, which is aliquoted in 0.9-mL amounts into the calculated number of bijoux bottles
4. Dilute the virus (1/10) by adding 100 μL of virus to a 0.9-mL aliquot of PBS/calf serum (giving a 10^{-1} dilution). Recap the bottle, and vortex to mix. Using a fresh tip, take 100 μL of the 10^{-1} stock and transfer into another 0.9-mL aliquot of PBS/calf serum giving a 10^{-2} dilution Vortex, and so on Continue with this serial dilution procedure until the appropriate range of dilutions has been achieved. For a large-scale virus preparation, which may yield up to 10^{10} PFU/mL, it is necessary to titrate out to a dilution of 10^{-7} or 10^{-8}

   Note: The tip should be touched against the side of the bottle and not into the liquid, since droplets on the outside of the tip can be carried over, introducing inaccuracies.
5. Pour the growth medium off the 60-mm plates.

6. Plate out 100 µL of the serially diluted virus stock onto the BHK monolayers, taking care not to dislodge the cells from the plates when delivering the inoculum through an Eppendorf tip Starting with the highest dilution and working back to the most concentrated, it is not necessary to change tips, since any carryover will be insignificant. Rock the trays of plates back and forth gently to ensure even coverage of virus
7. Put into a 37°C incubator for 1 h to allow absorption of the virus onto the monolayers
8. Add 5 mL of ETMC 10% to each plate The methylcellulose stops progeny virus from the plaques formed from the inoculum from spreading through the medium and resulting in trailing plaques or secondary satellite plaques
9. Place the titration plates in a CO_2 incubator at the appropriate temperature Wild-type virus can be titrated at 31 or 37°C Temperature sensitive virus is usually titrated at the permissive (e g , 31°C) and nonpermissive (e g , 38.5°C) temperature Incubate plates for 2 d at 37°C or 38 5°C, and 3 d at 31°C
10. The viscosity of the methylcellulose makes it difficult for stain to permeate through to the cell monolayers, and it is therefore preferable to pour off the overlay medium prior to the addition of 2–3 mL of Giemsa's stain The decanted medium will contain virus, and should be autoclaved or treated with an appropriate viricidal agent (e g , Virkon)

    The stain should be left on the plates for 2–24 h at room temperature Staining fixes the cells, and any virus remaining on the plates will be inactivated The stain can be washed off directly under running tap water.
11. Using a plate microscope, count the plaques on the monolayers by inverting the dish, and with a water-soluble pen, mark off each plaque as it is counted. It is best to count the dilutions with 20–200 plaques/plate, since too many or too few plaques give less accurate counts Ideally, duplicates of each dilution should be counted and the average count used In practice, it is usually sufficient to count the number of plaques from two plates with serial dilutions, e.g , 10^{-5} and 10^{-6}. The accuracy of the titration can be measured in this way

    Note: Plaques should always be counted using a microscope Although some may be visible to the naked eye, the size of plaques can vary considerably, and many will be missed if a microscope is not used
12. The titer should be calculated as follows

$$20 \text{ plaques on the } 10^{-7} \text{ plate and } 200 \text{ on the } 10^{-6} \text{ plate} = 2 \times 10^{8} \text{ PFU in the } 100\ \mu\text{L inoculum} \quad (1)$$

    The titer is therefore 2×10^{9} PFU/mL

3.5. Particle Counts

Virus suspensions are mixed with equal volumes of a 1% solution of sodium silicotungstate and a suspension of latex beads of known concentration We use a solution of 1.43×10^{11} particles/mL. A droplet of this suspension is placed on an electron microscope grid and, after 5 min (when the particles have

settled), the excess suspension is removed and the particles are counted. The latex beads are of course used as the reference count

A wild-type stock of HSV-1 should ideally have a particle:PFU ratio of <10:1, and for HSV-2 this figure should be <100.

3.6. Single and Multicycle Growth Experiments

To assay the in vitro growth phenotype of a particular virus stock, it may be necessary to determine its growth kinetics over one or more replication cycles compared with a known standard. This is achieved by infecting multiple plates of cells with virus, under the same conditions, but harvesting at different time-points postinfection. The progeny virus from the different time-points is titrated to monitor progression of the infection.

A single-cycle growth experiment involves infecting every cell in a monolayer and monitoring the growth during one round of replication. To do this, cells are inoculated with an MOI of 5 or 10 PFU/cell to ensure that every cell is infected and the progress of the infection is normally monitored during 24 h.

A multicycle growth experiment amplifies the effect of any small impairment during several rounds of replication. In this case, cells are infected at a low MOI (usually 0.01–0.1 PFU/cell), and the infection is monitored over 72 h.

The method for both is the same with only the virus inoculum and the points of harvest varying.

1. Count a BHK 21/C13 cell suspension and seed 35-mm plates with 2×10^6 cells/dish in 2 mL of ETC_{10} Seed a single plate per time-point for each virus being assayed Especially for large experiments where several viruses are being compared, it is advisable to label the plates at this stage, since it saves time when inoculating with virus
2. Incubate overnight at 37°C
3. Pour off the growth medium
4. Inoculate with virus, e.g , 2×10^6 cells infected at a MOI of 5 PFU/cell means an inoculum of 1×10^7 PFU/plate. Therefore, it is necessary to dilute the virus to 1×10^8 PFU/mL and add 100 µL/plate Make sufficient diluted virus for all of the time-points, so that the inocula going onto a series of plates is fom a single virus solution
5. Incubate at 37°C for 1 h to allow the virus to absorb.
6. Wash the plates with 2 mL of PBS/calf serum to remove any unabsorbed virus
7. Overlay the plates with 2 mL of ETC_{10} (accuracy here is very important) This is 0 h on the time scale
8. Incubate at the appropriate temperature (normally 37°C)
9. Harvest the virus samples at the designated time-points by scraping the cell monolayer into the medium and transferring the suspension to a clearly labeled sterile bottle that is suitable for sonication (black cap vial)

   Time-points for harvesting are arbitrary, but for a high MOI (single-cycle)

experiment, 0-, 2-, 4-, 6-, 12-, and 24-h points are usual, and in some cases, 8- and 16-h points may also be required For a low-MOI (multicycle) experiment, 0-, 4-, 8-, 12-, 24-, 48-, and 72-h samples are usual.

10 Sonicate the samples thoroughly in a sonibath to disrupt the cells, and release the virus into the medium. Store the samples at –70°C until they can be titrated.

11 Titrate the virus as described above, and calculate the titers at each time-point Since virus from 2×10^6 cells is harvested into 2 mL, the final titer per milliliter is equivalent to its titer per 10^6 cells

12 Plot out the titers on a log graph scale with PFU/10^6 cells ($\log_{10}$) on the *y*-axis and time (in hours) on the *x*-axis

References

It will be obvious that we have not included any references Since the step-by-step procedures explained in this chapter are fundamental and have been in operation for many years, we are assuming that it will be unnecessary for the reader to require more detailed information. Most of the basic references are in papers published over 20 years ago!

2

HSV Entry and Spread

Christine A. MacLean

1. Introduction

This chapter deals with assays commonly used to follow herpes simplex virus type 1 (HSV-1) entry into and spread between cells in tissue culture These are complex processes, known to involve several of the 20 or more HSV-encoded membrane proteins (*see* refs. *1* and *2* for recent reviews). HSV entry is mediated by a number of proteins on the surface of the virus particle. Recognition of and binding to target cells are known to involve at least three glycoproteins—gB, gC, and gD. gC mediates the initial interaction with cells, recognizing heparan sulfate proteoglycans on the cell surface. gB also interacts with heparan sulfate proteoglycans, and can substitute for gC in gC negative viruses. This initial, heparin-sensitive attachment to cells is relatively weak, and is followed by a more stable attachment to cells, apparently mediated by gD. Following attachment, the virus particle fuses with the cell membrane to mediate entry. Fusion is known to require gB and gH/gL, and possibly also gD, but their precise functions are uncertain. The roles of other virus-encoded membrane proteins in entry are unclear, but it is possible that different proteins may be required for entry into different cell types.

Following infection, spread of progeny virus in tissue culture occurs via both the release of mature infectious virus particles into the extracellular medium and the direct cell-to-cell spread of virus UL20 plays a role in membrane trafficking events involved in the maturation and egress of virus particles, whereas several virus membrane proteins are probably involved in the membrane fusion event required for cell-to-cell spread, including gB, gD, gE/gI, gH/gL, and gK.

This chapter will describe assays that address virus entry, in terms of the initial attachment of virus to cells (adsorption) and the subsequent fusion between

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the virus and cell membranes (penetration), and virus spread, in terms of both intracellular and extracellular virus yields (virus release) and virus growth under conditions that limit extracellular spread of virus (cell-to-cell spread). Detailed methodology is provided for the assays used in our laboratory, although some attempt will be made to refer to procedures used by others. The assays described here involve the use of tissue-culture cells, the growth of virus stocks, and extensive virus titration. The reader should therefore be familiar with the procedures described in Chapter 1.

2. Materials

1 Cells We standardly use baby hamster kidney 21 clone 13 (BHK C13) cells, although any cell line permissive for HSV infection should be suitable BHK C13 cells are grown in ETC_{10} *(see* step 2*)*, at 37°C in a humidified atmosphere containing 5% (v/v) carbon dioxide Cell monolayers are seeded at 1×10^6 cells/35-mm Petri dish or a well of a six-well tray, or 2×10^6 cells/60-mm Petri dish, 20–24 h before use Cell monolayers are used when about 80–90% confluent, and we assume approx 2×10^6 cells/35-mm dish

2 Media ETC_{10} Eagle's medium supplemented with 10% newborn calf serum, 5% tryptose phosphate broth, 100 U/mL penicillin, and 100 mg/mL streptomycin

EC_5/EC_2 Eagle's medium supplemented with antibiotics, and either 5 or 2% newborn calf serum, respectively

Emet/$5C_2$ Eagle's medium containing one-fifth the normal concentration of methionine and 2% newborn calf serum

MC_5/MC_2 Eagle's medium supplemented with antibiotics, 1 5% carboxymethylcellulose, and either 5 or 2% newborn calf serum, respectively

EHu. Eagle's medium supplemented with antibiotics and 10% pooled human serum

3 PBS 170 m*M* NaCl, 3 4 m*M* KCl, 10 m*M* Na_2HPO_4, 1 8 m*M* KH_2PO_4, supplemented with 6 8 m*M* $CaCl_2$ and 4.9 m*M* $MgCl_2$

4 Citrate buffer. 40 m*M* citric acid, 135 m*M* NaCl, 10 m*M* KCl, pH 3 0

3. Methods

The methods described here are based on the use of HSV-1 strain $17syn^+$ in BHK C13 cells. It is important to remember that growth characteristics and/or kinetics of entry may differ when using different strains of HSV-1 or different cell types

Before undertaking these procedures, consult local regulations for the safe handling of HSV. We generally work with the virus on the bench or in a class II biological safety cabinet, and inactivate all waste either by steeping overnight in a 1% solution of vircon or chloros, or by autoclaving. The most obvious risks from HSV are from splashes to the eye, or contact with areas of broken skin (e.g., cuts, eczema). Because of the large numbers of infected monolayers

that may need to be handled at once in some of the procedures below, it is rather easy to be a little careless.

Unless otherwise stated, manipulations are carried out at room temperature, as rapidly as possible. Seed stocks of virus or cells are generally handled in a biological safety cabinet using sterile technique, but all other procedures are conducted on the bench using good microbiological practice.

3.1. Adsorption of Radiolabeled Virions to Cells

This assay measures the proportion of total virus particles that bind to cells with time. Purified radiolabeled virus particles are allowed to bind to cells for given periods of time, the cells washed extensively to remove unbound virus, and the cells then lysed and the amount of bound radiolabel measured. The radiolabeled virions used in these experiments should be free from contaminating cell membranes or debris. We generally use ^{35}S-methionine-labeled virions, purified by Ficoll gradient centrifugation *(3)*, but other radiolabels (e g., ^{3}H-thymidine) and/or different gradient purification procedures are also suitable (e.g., *see* refs. *4–6*). We generally find that around 15–20% of radiolabeled virions bind to cells within 60–90 min at 37°C.

3 1.1. Step 1: Preparation of Radiolabeled Virions

1 Infect 80–90% confluent cell monolayers in 80-oz roller bottles (assume around 2×10^8 BHK C13 cells/roller bottle) at 0 001 PFU/cell in EC_2 at 31°C (*see* Note 1).
2 Once plaques become visible (12–24 h pi), remove the medium and wash the cells twice with, and subsequently maintain them in, Emet/$5C_2$. Approximately 2–4 h later, add ^{35}S-methionine (Amersham, SA >1000 Ci/mmol) to a final concentration 10–20 mCi/mL. A total volume of 20 mL is usually sufficient, but ensure that the cells do not dry out.
3 Once all the cells appear rounded, but still attached to the roller bottles (3–4 d pi), carefully remove the culture supernatant (avoid detaching the cells), and pellet the cell debris by centrifuging the supernatant in a Fisons coolspin centrifuge (or equivalent) at 2000 rpm for 30 min at 4°C. Care should be taken to avoid cell debris in virus stocks membrane fragments can copurify with virions on Ficoll gradients, and excessive cell debris can trap/sequester virus in large aggregates, resulting in low yields of purified virions
4. Again, carefully remove the supernatant, and then pellet the virus by centrifugation at 12,000 rpm for 2 h at 4°C in a Sorvall GSA rotor. Remove all the supernatant, add 1 mL of Eagle's medium without phenol red, and then very gently scrape the virus pellet into the medium and allow the virus to resuspend overnight at 4°C Virions should be handled very gently at all stages to avoid damage to the virus envelopes.
5. Prepare 35-mL continuous 5–15% Ficoll gradients (Ficoll 400, in Eagle's medium without phenol red) in transparent centrifuge tubes that can be easily pierced by a syringe needle, and cool on ice or at 4°C. We generally use Beckman Ultra-

clear™ centrifuge tubes Gently pipet the virion suspension until homogeneous, layer it onto the Ficoll gradients, and centrifuge for 2 h at 12,000 rpm, in a Sorvall AH629 rotor at 4°C The number of roller bottles of virus loaded per gradient will depend on the yield of virus expected For 17syn$^+$ wild-type, virus from 2–5 roller bottles would normally be loaded onto a single 35-mL Ficoll gradient (approx 1–5 × 10^9 PFU at the end of step 4)

6 Visualize the virion band under a light beam (*see* ref *3* and Note 2) Carefully remove the virion band by side puncture, using a 5-mL syringe and broad (18/19G) gage needle, dilute the virus in Eagle's medium without phenol red, and then recover the virus by centrifugation at 21,000 rpm for 16 h in a Sorvall AH629 rotor at 4°C.

7 The virus pellet should appear as an opaque halo at the base of the centrifuge tube Remove the supernatant carefully, and dry the tube with a tissue to remove excess liquid (avoid disrupting the pellet) Add 500 mL ETC_{10}, gently scrape the virus into the medium, and allow it to resuspend overnight at 4°C

8 Very gently, resuspend the virus preparation until homogeneous, using an Eppendorf pipet, and then determine
   a. The quality of the preparation, by electron microscopy,
   b Particle numbers (particles/mL), by electron microscopy,
   c The virus titer (PFU/mL), and
   d Radioactivity (counts/min/mL), by liquid scintillation counting

9 Virions can be stored at –70°C until use

3.1.2. Step 2: Adsorption of Radiolabeled Virions to Cells

1 Remove the medium from 90–100% confluent monolayers in six-well trays (*see* Note 3), and add ETC_{10} containing 1% BSA for 30–60 min at 37°C This step is included to reduce nonspecific binding of virions, although in practice, we find little difference if this step is omitted

2. Dilute the radiolabeled virions in prewarmed ETC_{10}. The amount of virus added will vary in terms of counts. When comparing different viruses, we aim to use comparable particle numbers, while trying to keep the counts within the range 10–1000 cpm/μL (20–200,000 cpm/well). This is usually 10^1–10^3 particles/cell, and does not reach saturation binding (*see* refs *7* and *8*)

3. Remove the blocking medium Since volume influences adsorption rates, all wells should be drained thoroughly

4 Add 200 μL prewarmed virus/well, in triplicate for each time-point, plating different time-points on separate trays (since shaking will affect the rate of adsorption) Plate all viruses for each time-point together. the first set added should be the last time-point harvested, whereas the last set added should be the first time-point harvested (*see* Note 4) Transfer monolayers to 37°C; this represents 0 time

5 At the relevant time-points, remove the virus supernatant using an Eppendorf pipet, and transfer to a scintillation vial

6 Wash the cells three times with 1 mL PBS, shaking the trays for 5–10 s each time, and transfer each wash to a scintillation vial

7 Harvest the cells (and bound virus) by scraping into 300 µL PBS/1% (v/v) SDS, and transfer this to a scintillation vial.
8 Add 4 mL Ecoscint™-A (National Diagnostics, Atlanta, GA) to each vial, vortex briefly, and count each sample in a liquid scintillation counter for 1 min
9. The percentage of bound virus at each time-point is calculated from:

$$(\text{cpm bound}/\text{total recoverable counts}) \times 100 \quad (1)$$

where cpm bound = cpm in cell harvest and total recoverable counts = (cpm in virus supernatant + cpm in washes1/2/3 + cpm bound).

3.2. Adsorption of Infectious Virus to Cells

In this assay, virus is allowed to attach to cells for given periods of time, the cells washed extensively to remove unbound virus, and the amount of bound virus then measured in terms of subsequent plaque formation. Either crude virus preparations or gradient purified virions can be used as input virus.

1. Remove the medium from 90–100% confluent monolayers in six-well trays, and drain all wells thoroughly
2 Briefly sonicate virus stocks before use (30–60 s), and dilute the virus in prewarmed ETC_{10} to 150–200 PFU/200 µL (*see* Notes 5 and 6).
3. Add 200 µL virus/well, in triplicate for each time-point, plating different time-points on separate trays Plate all viruses for each time-point together the first set added should represent the last time-point handled, whereas the last set added should be the first time-point harvested. Transfer monolayers to 37°C, this represents 0 time.
4 At relevant time-points, remove the virus using an Eppendorf pipet, and discard
5. Wash the cells three times with 2 mL PBS, shaking the trays for 5–10 s each time
6 Drain all wells thoroughly Overlay the monolayers with 2 5 mL MC5 (or MC_2 if the cells are very confluent), and incubate at 37°C until plaques are clearly visible (usually 2 d pi).
7 Stain the cells by adding 1–2 mL Giemsa stain, leaving the cells at room temperature for 2–24 h before washing.
8 Count the plaques under an inverted microscope.
9. The percentage of infectious virus binding to cells at a given time is calculated from

$$(\text{avg. no. of PFU at given time}/\text{avg. no. of PFU at peak or final time-point}) \times 100 \quad (2)$$

3.3. Modifications of the Adsorption Assays

Sections 3.1. and 3.2. describe adsorption of virus at 37°C, under standard conditions. It is obviously possible to modify these procedures in a number of ways: e.g., to wash in the presence of reagents that may interfere with binding (e.g., heparin) or to slow adsorption by incubating at 4°C (*see* refs. *9* and *10*).

To carry out these assays at 4°C, both cells and virus should be precooled before addition of virus to cells, and the experiments carried out in a

4°C cold room. We do not cool the cells on ice, as described by others, since we find that our BHK C13 cells do not survive such treatment well. Time-points are washed at 4°C, before transferring to room temperature for harvesting (Section 3.1.) or addition of prewarmed MC5 (Section 3 2.). A reasonable time course would be 0, 15, 30, 45, 60, 90, 120, 180, and 240 min after virus addition. We find BHK C13 cell monolayers do not survive longer periods at 4°C

3.4. Penetration

Virus penetration is measured as the rate at which attached virus becomes resistant to inactivation by low pH *(11,12)*. Virus is bound to cells at 4°C, a temperature at which very little penetration should occur. Cells are then shifted to 37°C, to allow penetration to begin, and at various times the monolayers are treated with low-pH buffer to inactivate virus that has not penetrated the cell Virus penetration is measured in terms of subsequent plaque formation and expressed as a percentage of the number of plaques formed on control monolayers

To minimize penetration during the 4°C adsorption stage, steps 1–4 below are carried out in a 4°C cold room Warm clothing and gloves are strongly advised!

1. Remove the medium from 90–100% confluent cell monolayers in six-well trays, replace with cold (4°C) medium and incubate at 4°C for 15–30 min
2. Briefly sonicate virus stocks (30–60 s), and dilute in precooled ETC_{10}, to 150–200 PFU/200 µL (*see* Note 7).
3. Remove the medium from the wells, and drain thoroughly Add 200 µL virus/well, and incubate at 4°C for 60 min (*see* Note 8) Note that for each virus, one six-well tray will represent one time-point
4. Remove the virus, and wash the cells twice with cold PBS
5. To start penetration, add 2 mL prewarmed ETC_{10} (37°C), and transfer the cells to a 37°C incubator This represents 0 time Again, add the overlay to all viruses for each time-point together the first set added should represent the last time-point handled, whereas the last set added should be the first time-point harvested (*see* Note 9)
6. At the relevant time-point, remove the medium from the trays, and add 1 mL PBS to three wells (control), and 1 mL citrate buffer, pH 3 0, to the remaining 3 wells of each tray Incubate for 3 min at room temperature with gentle shaking It is important to include a set of PBS control wells for each time-point, since absolute plaque numbers do vary from tray to tray (*see* ref *13*)
7. Remove the buffer and wash the cells twice with PBS, shaking 5–10 s each wash
8. Drain the wells thoroughly, then add 2 5 mL MC5, and incubate at 37°C until plaques are clearly visible
9. Stain and count as described above (Section 3.2)
10. Penetration is measured as the percentage of acid-resistant virus with time

$$\text{Each time-point} = (\text{avg PFU on low pH-treated wells} / \text{avg PFU on PBS-treated wells}) \times 100 \quad (3)$$

3.5. Virus Release

To measure the efficiency of release of virus particles during infection, the percentage of total infectious progeny virus that is present within the extracellular medium is measured with time, following infection at either high or low multiplicity. Infection at high multiplicity (5–20 PFU/cell) follows infection through one infectious cycle. Infection at low multiplicity (0.001 PFU/cell) allows multiple cycles and may amplify small differences in overall virus growth

1. Briefly sonicate virus stocks (30–60 s), and dilute to either 5×10^7 PFU/mL (for infection at 5 PFU/cell, assuming 5×10^6 cells/60-mm Petri dish), or 1×10^4 PFU/mL (for infection at 0.001 PFU/cell) (*see* Notes 10 and 11)
2. Remove the medium from 90–100% confluent cell monolayers in 60-mm Petri dishes. Add 0.5 mL virus/plate, and incubate at 37°C for 1 h.
3. Remove the virus inoculum. Add 1 mL citrate buffer, pH 3.0, per plate, and incubate at room temperature for 2–3 min (*see* Note 12)
4. Remove the buffer, wash the cells twice with PBS, and drain the monolayers thoroughly.
5. Add 2 mL EC_5/plate (EC_2 if the monolayers are very confluent), and incubate the cells at 37°C
6. At the relevant time-points, remove the medium to a glass bijou bottle (or alternative vial suitable for sonication), and measure the volume. Store on ice. This represents the released or supernatant, virus (SV)
7. Wash the monolayer gently twice with PBS—if any cells detach, recover these by centrifugation (e.g., Fisons coolspin, 2000 rpm, 5–10 min). Add 2 mL EC_5 to the monolayer, scrape the cells into the medium (e.g., using a commercial cell scraper, or a plunger from a sterile syringe), and transfer to a glass bijou. Pool any cells recovered from the washes. Store on ice. This represents the cell-associated (CA) virus.
8. Sonicate the SV stocks briefly (30–60 s), and the CA stocks until clarified (around 3×60 s). Store the SV and CA virus stocks at –70°C until they can be titrated.
9. Quantitate the yields of infectious virus by titration. This is basically the method described in Chapter 1 with some minor modifications. Prepare serial 10-fold dilutions of virus in a total volume of 1 mL ETC_{10} in 5-mL bijou bottles, mixing the dilutions by swirling. Because of the large number of titrations being handled, it is relatively easy to contaminate your hands with liquid from the lids when opening and closing the vials—swirling is less likely than vortexing to result in virus contamination on the lids of the vials. Virus should then be plated in duplicate on 60-mm Petri dishes, using 200 µL/plate and adsorbed for 1 h at 37°C. To minimize secondary spread of virus, the inoculum is removed before overlaying with MC_5. Note that MC_5 contains 1.5%, not 1%, carboxymethylcellulose
10. Calculate the yield of virus in the CA and SV samples, since well as the percentage of progeny virus that is released at the different time-points.

$$\text{Virus yield} = \text{titer} \times \text{volume}$$
$$\%\text{SV} = [\text{SV yield/total virus yield (SV + CA yield)}] \times 100 \quad (4)$$

3.6. Cell-to-Cell Spread

Cell-to-cell spread can be assayed by infecting cells at low multiplicity and allowing the virus to grow under conditions that limit the extracellular spread of virus. We standardly use commercial pooled human serum, which contains high levels of neutralizing antibody to HSV in the overlay medium. Neutralizing monoclonal antibodies (MAb) could also be used.

1. Briefly sonicate the virus stocks (30–60 s), and dilute to 1×10^4 PFU/mL (0.001 PFU/cell) (*see* Notes 13 and 14)
2. Remove the medium from 90–100% confluent cell monolayers in 60-mm Petri dishes. Add 0.5 mL virus/plate, and incubate at 37°C for 1 h.
3. Remove the virus inoculum, and add 1 mL citrate buffer, pH 3.0, per plate, and incubate at room temperature for 2–3 min.
4. Remove the buffer, wash the cells twice with PBS, and drain the monolayers thoroughly.
5. Add either 2 mL ETC_{10} or 2 mL Ehu/plate, and incubate the cells at 37°C.
6. At the relevant time-points, remove the medium. If desired, the supernatant from the ETC_{10}-treated plates can be kept, and treated as in Section 3.5. It is also a good idea to titrate one or two of the later EHu supernatants to monitor neutralisation.
7. Wash the monolayers gently twice with PBS—if any cells detach, recover these by centrifugation (e.g., Fisons coolspin, 2000 rpm, 5–10 min). Add 2 mL EC_5 to the monolayer, scrape the cells into the medium, and transfer to a glass bijou. Pool any cells recovered from the washes. Store on ice.
8. Sonicate the SV stocks briefly (30–60 s), and the CA stocks until clarified (around 3–60 s). Store at –70°C.
9. Quantitate the yields of infectious virus by titration (*see* Section 3.5., step 9).

$$\text{Virus yield} = \text{titer} \times \text{volume} \quad (5)$$

Calculate the ratio of CA yields under human serum compared to normal medium, for each virus.

4. Notes

1. If preferred, radiolabeled virions can be prepared following infection at high multiplicity (low multiplicity of infection is simply more economical with virus stocks). In this case, infection is carried out at 37°C. Use 5 PFU/cell, infect in Emet/$5C_2$, add the radiolabel at 4 h pi, and harvest around 24 h pi.
2. Large pellets after Ficoll gradient centrifugation can suggest either the presence of cell debris or overheating of the Ficoll during preparation of the solutions. This can considerably reduce the yield of purified virions. Ficoll solutions can be prepared by leaving overnight in the refrigerator to dissolve, and only minor warming with stirring is then required to get the Ficoll into solution. Solutions are cooled to 4°C before gradients are prepared.
3. Petri dishes (35-mm) can be used instead of six-well trays. We use the latter, since they are easier to handle for the washing stages.

4. The main problem with this assay is in handling the samples quickly enough It is therefore important to be well-organized before starting, e g., have all the trays and scintillation vials labeled, and have all media, buffers, pipets, and so forth, available. For 17syn$^+$, a reasonably detailed time-course would be 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min postvirus addition We find that, with practice, one person can handle 5-min time gaps for two viruses
5 No blocking step is included in this procedure.
6 Virus stocks are titrated shortly before use, adsorbing the virus at 37°C for 1 h under conditions identical to those used in the adsorption studies. Best results are obtained if the peak or final plaque counts are between 100 and 250 For 35-mm wells, counts above 300 are usually inaccurate The actual number of PFU added to the wells can often differ by up to threefold from that expected. This is probably owing at least partly to dilution error To minimize variation, we keep a stock vial of each virus specifically for these experiments and regularly recheck titers. We find errors are less if at least 50 µL of sonicated virus stock are used to prepare serial 10-fold dilutions, and these then used to generate the final dilution required. It is also sensible to test regularly the accuracy of the pipets used to prepare the dilutions!
7 To calculate the amount of virus to use in these experiments, titrate virus stocks at 4°C on 35-mm Petri dishes/six-well trays under conditions identical to those to be used for adsorption in the penetration assay
8. In practice, incubation at 4°C does allow some penetration. Although some laboratories cool cells on ice, we find that BHK C13 cells do not survive well at this temperature. Monolayers become loose after even short periods at 0°C and are often lost If the base level of penetration is too high, virus can be incubated at 4°C for shorter periods (10–15 min) before shifting to 37°C
9 Again, the main problem with this assay is in handling the time-points quickly enough. Be well organized before starting—have all buffers, pipets, and so forth, ready, and all six-well dishes correctly labeled For 17syn$^+$, penetration has usually reached 80–100% by 20–30 min, and so a reasonable time-course would be 0, 5, 10, 15, 20, 30, 45, and 60 min after temperature shift. For more detail, we occasionally use 3-min time-points up to 21 min. Handling these time-points is significantly easier if two people work together.
10 Ideally, viruses should have been recently titrated on the same batch of cells used in the growth experiments. The input dilutions should also be titrated, either immediately after use, or following storage at –70°C.
11. A reasonably detailed time-course would be. 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, and 32 h pi, following infection at high multiplicity; and 0, 4, 8, 12, 24, 36, 48, 60, 72, and 96 h pi, following infection at low multiplicity 17syn$^+$ usually reaches a plateau for total virus yield around 16 h pi, and for SV yields around 24–32 h pi (high MOI), or around 36–48 h pi, and 60–96 h pi, respectively (low MOI) Because of the numbers of samples (and titrations) involved, and because results can depend very much on the health of the cells, we tend to use only a single plate per time-point per virus, repeating the experiment a further 1–2 times

12 The addition of citrate buffer pH 3.0 serves to inactivate any remaining, nonpenetrated virus, ensuring a synchronous infection and giving more accurate values for progeny virus titers at early times. This step is particularly important following a high multiplicity infection
13. Ideally, viruses should have been recently titrated on the same batch of cells used in the growth experiments The input dilutions should also be titrated, either immediately after use or following storage at –70°C
14. Reasonable time-points are 0, 12, 24, 36, 48, 60, 72, and 96 h pi Assuming only two or three viruses were being compared, these time-points would usually be carried out in duplicate for each virus

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3

Preparation of HSV-DNA and Production of Infectious Virus

Alasdair R. MacLean

1. Introduction

This chapter deals with (1) the preparation of herpes simplex virus (HSV) virion DNA of a quality and purity suitable to be used for the generation of infectious virus, and (2) its use in the preparation of infectious virus. An important development in the understanding of virus genetics and gene products has been the ability to carry out reverse genetics. This is dependent on the ability to manipulate the genome in vitro and reconstitute infectious virus. Our understanding of DNA viruses and positive stranded RNA viruses (where DNA and RNA/cDNA, respectively, are generally infectious) is considerably greater than for negative stranded RNA viruses, where until recently, it had been impossible to generate virus from either RNA or cDNA. Within the herpesviridae, knowledge of the function of HSV gene products is one of the more advanced owing to the relatively straightforward techniques required to generate virus from HSV-DNA, and to introduce desired mutations by cloning small parts of the genome, manipulating them, and then reintroducing the mutations by a process of cotransfection and in vivo recombination with intact virus DNA. Other α-herpesviruses, such as EHV-1 and PRV, are equally amenable to such manipulation, and knowledge of their gene products is also well advanced. In contrast, this technology is only now, and with much less success, being applied to other members of the family, such as EBV, HCMV, and VZV, and knowledge of their genetics is much less advanced. The use of cosmids to reconstitute intact virus will aid in the advance of knowledge for these viruses. For examples of uses of recombinant DNA technology, the reader is referred to other chapters (especially those on cloning and mutagenesis). I will concentrate on the techniques currently in use in my laboratory, but will also mention

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other techniques in use elsewhere that may be more appropriate in certain cell types.

2. Purification of HSV-DNA

This chapter deals only with the purification of virion HSV-DNA for use in transfection procedures, although such DNA can also be used for analysis of genome structure by restriction enzyme digestion and Southern blotting. More rapid small-scale procedures for the purification of infected cell DNA for Southern blotting, are described elsewhere in this book. The integrity of HSV-DNA is absolutely crucial for its infectivity. Because of its high molecular weight (150 kbp), HSV-DNA is easily fragmented, and must therefore be handled gently with no vortexing, vigorous shaking, or pipeting. It is important that there is no contaminating nuclear DNA in the preparation, since cellular and noninfectious concatemeric HSV-DNA will inhibit the transfection efficiency by lowering the ratio of infectious to noninfectious DNA.

2.1. Protocol

Our standard cell line for HSV growth is BHK 21/C13 cells *(1)*, but any cell line permissive for HSV growth (such as Vero or CV1 cells) can be used.

1. Ninety percent confluent BHK 21/C13 cells are infected at a multiplicity of infection (MOI) of 0 003 plaque-forming units (PFU)/cell in a minimal volume of ETC_{10} We carry this out in 20-mL roller bottles holding 1×10^8 cells For HSV-1 each roller bottle will typically give a yield of 100 μg DNA and for HSV-2 10–50 μg DNA. We routinely prepare stocks of 5–10 roller bottles, but this can be scaled down to only one roller bottle or less if neccessary.
2. The cells are incubated at 31°C until cytopathic effect (CPE) is complete, usually after 3–4 d.
3. Virus-infected cells are shaken into the medium (glass beads can be used if difficulties are encountered in detaching the cells), and the medium decanted into centrifuge tubes
4. Cells are pelleted by spinning at low speed (2K) in a benchtop coolspin for 15 min at 4°C The supernatant is carefully decanted and stored at 4°C
5. Cytoplasmic virus is extracted from the cells by lysing in RSB containing 0 5% (v/v) NP40, which lyses the plasma, but not the nuclear membrane. The cells are resuspended in 10 mL RSB/NP40 and incubated on ice for 10 min, the nuclei pelleted at 2K in a coolspin for 10 min at 4°C, and the supernatant carefully removed so as not to disturb the pellet This supernatant is added to the cell supernatant from stage 3
6. The nuclei are re-extracted with RSB/NP40, the supernatant again being added to the cell supernatant and the nuclear pellet discarded
7. Virus from the cell supernatant and cytoplasm is pelleted by spinning in a GSA rotor in a Sorvall superspeed centrifuge (or equivalent) for 2 h at 12K at 4°C The supernatant is discarded, and the virus pellet resuspended in 10 mL NTE and trans-

ferred to a glass tube. Virus is completely resuspended by sonication in a water bath.

8. Virus is lysed by the addition of SDS and EDTA to concentrations of 2.5% (w/v) and 10 m*M*, respectively, followed by incubation at 37°C for 5 min. From this stage, the virus DNA is free and hence susceptible to shearing, and all manipulations must be done carefully with gentle shaking and no vortexing.
9. The DNA is phenol-extracted by adding an equal volume of NTE saturated phenol, gently inverting, and allowing to stand for 10 min at room temperature. The aqueous phase containing the DNA is separated from the organic phase by centrifugation in a coolspin at room temperature for 10 min at 2K. The upper aqueous phase is carefully removed from the organic phase, taking care not to disturb the proteinaceous interphase.
10. The aqueous phase is re-extracted with phenol between one and three times, until there is no interphase and the upper layer is clear. If the volume of the aqueous layer drops significantly below 10 mL to minimize physical loss of DNA, the volume should be increased back to 10 mL by the addition of NTE.
11. A chloroform:isoamyl alcohol (24:1; v/v) extraction is carried out in a similar manner to the phenol extraction, except that the incubation and spin are for only 5 min.
12. The DNA is precipitated by the addition of 2 vol of ethanol and gentle inversion. DNA should precipitate immediately as fine strands. No added salt or –20°C incubation is required owing to the high molecular weight of HSV-DNA. The DNA is pelleted by centrifugation in a coolspin at room temperature for 10 min at 2K and washed with two-thirds of the tube volume of 70% (v/v) ethanol.
13. The DNA should be air-dried in an inverted position for 15 min before redissolving in sterile distilled H_2O (dH_2O) containing 50 µg/mL RNase A. It is important not to overdry the DNA, since this may cause difficulty in redissolving. Typically, the DNA from 10 roller bottles is resuspended in 1–2 mL. The DNA is quantitated either by OD at 280 nm with 1 OD unit equaling 40 µg DNA or running on an ethidium-bromide-strand agarose gel against a standard of known concentation.
    RSB Buffer: 10 m*M* Tris-HCl, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$

2.2. Titration of HSV-DNA Infectivity

Each preparation of HSV-DNA will vary in its ability to generate virus. Here its infectivity will be expressed as the number of PFU/µg DNA. The better the quality of the DNA, the higher this figure will be and, in general, the more efficient at generating recombinant virus. Each DNA preparation requires to be titrated (usually in the range of 0.1–2 µg) DNA to establish its PFU/µg DNA, and an optimal figure is chosen. At low levels, there is a linear increase in the number of PFU as the DNA amount is increased, but this plateaus and then begins to fall owing to inhibition at high levels of DNA. A point near the top of the linear response should be chosen as the optimal amount of DNA to be used in transfections.

2.3. Calcium Phosphate Precipitation/DMSO Boost

The standard method for introducing HSV-DNA into cells is the calcium phosphate precipitation/DMSO boost method *(2,3)* All buffers should be stored and reactions carried out in sterile plasticware, since the detergents used to wash glassware may be inhibitory if not properly rinsed.

1 HSV-DNA is added to 400 µL HEBES buffer containing 10 µg/mL carrier calf thymus DNA
2 Calcium phosphate is added to a final concentation of 130 m*M*, and the sample gently mixed and allowed to sit at room temperature for 5 min to allow the calcium phosphate/DNA precipitate to form.
3 The DNA sample is added gently to tissue-culture cells in a 60-mm plate, from which the medium has been removed. For maximum transfection efficiency, the cells should be 50–70% confluent, actively growing, and should have been set up the previous night from freshly typsinized cells, which had not been stored at 4°C
4 After 40 min of incubation at 37°C, the cells are overlaid with 5 mL ETC_{10} and incubation allowed to proceed at 37°C for 4 h prior to boosting with DMSO This is the optimal timing for the DMSO boost, but it will be effective up to 7 h after addition of DNA
5 The next stage is the DMSO boost. The DNA/medium mixture is removed from the cells, which are washed once with 5 mL ETC_{10} One milliliter of 25% (v/v) DMSO in HEBES is added gently to the cells and incubated for exactly 4 min at room temperature. This time must not be exceeded owing to the toxicity of DMSO. The DMSO/HEBES is poured off, and the cells washed twice with, and subsequently overlaid with 5 mL ETC_{10} Speed is extremely important here because of the need to dilute the DMSO as quickly as possible. Initially, it is better not to handle more than 10 plates at a time at the DMSO boost stage and even with experience never more than 20 plates at one time.
6 The plates are incubated at 37°C for 3 d or until CPE is extensive, at which point the infected cells are harvested to recover virus. If individual plaques are required, the ETC_{10} overlaying the transfection should be replaced after 16–24 h by a medium, such as ETMC 10% containing carboxymethylcellulose, to prevent secondary spread of cell released virus (*see* Chapter 1 on virus growth) and incubation continued for a further 48 h. Cells used for transfections in our laboratory are BHK 21/C13, but this procedure will work with most established cell lines.

HEBES Buffer. 130 m*M* NaCl, 4.9 m*M* KCl, 1.6 m*M* Na_2HPO_4, 5.5 m*M* D-glucose, 21 m*M* HEPES

2.4. Lipofection

We do not routinely use lipofection for the generation of infectious virus, since we consistently find it less efficient than calcium phosphate transfection. This contrasts with our results using plasmids for transient expression assays where lipofection has a much higher efficiency than calcium phosphate transfection. However, for cell types refractile to calcium phosphate transfection, it is worth trying.

1. The DNA (1–5 μg) is added to 100 μL Optimem serum-free medium (Life Technologies) and is mixed with.
2. 12-μL Liposomes made up no more than 1 mo ago are added to 100 μL Optimem.
3. The DNA and liposomes are combined, mixed gently, and left to stand at room temperature for 5–15 min.
4. Cells in 35-mm plates (at 10–25% density, since lipofection is significantly more efficient in low-density cells) are washed twice with Optimem
5. DNA/liposomes are mixed with 800 μL Optimem, added to the cells, and incubated for 6 h at 37°C
6. One milliliter of ETC_20 is added, and incubation continued for 24 h at 37°C
7. The medium is removed and replaced with fresh ETC_{10}, and incubation continued at 37°C for 2 d or until CPE is extensive, at which point the infected cells are harvested to recover virus

2.5. Preparation of Liposomes

1. Pipet 1 mL dioleoyl L-α-phosphatidyl ethanolamine (DOPE) (10 mg/mL in chloroform) (Sigma P0510) into a glass universal, add 5 mg dimethyldioctadecyl ammonium bromide solid (DDAB) (Sigma D2779), and dissolve by vortexing
2. Evaporate chloroform using a stream of nitrogen This takes approx 5 min
3. Lyophilize overnight in a freeze dryer
4. Resuspend dried lipids in 10 mL sterile dH_2O either by vortexing or sonicating in sonibath
5. Once suspended, sonicate the lipids using a soniprobe Sonicate at maximum power, using bursts of 30 s, keeping the universal on ice in between, until the suspension clears, indicating liposomes have been formed
6. Store the liposome preparation at 4°C. Prior to use, vortex briefly Discard after 1 mo

2.6. Electroporation

We do not carry out electroporation of HSV-DNA into eukaryotic cells, but for cells that do not transfect/lipofect, such as primary neurons or lymphocytes, this is a possible way to introduce virus DNA into cells.

3. Generation of Recombinant Virus

3.1. Marker Rescue

This procedure is very similar to that for transfection, except that in addition to HSV-DNA, a HSV fragment (usually derived from a plasmid clone) containing the genetic marker to be introduced into the genome is also added to the transfection mix. This fragment should have flanking sequences of ideally >500 bp on either side of the alteration, although recombination at a somewhat reduced frequency will still occur if only 200 bp flanking DNA are present: this is the minimum amount that will allow detectable recombination to occur *(4)* Flanking sequences of >1 kbp will not lead to an increase in recombination frequency. The fragment is usually added at a range of molar ratios. A suggested

range is 1- to 50-fold molar excess. Maximum recombination frequency is usually reached at a 10-fold molar excess As the excess of plasmid and hence the DNA present increases, the overall transfection efficiency will decrease, leading to a subsequent decline in recombination efficiency at high levels of plasmid. To detect recombinants, the transfection mixture should be harvested, plated out at a dilution appropriate to give single plaques, which should be isolated, and a DNA stock grown from these for analysis of genome structure The recombination frequency is variable between experiments, but is generally low, typically being on the order of 0.1–1%. Thus, detection of recombinants by analysis of their DNA profile is time consuming. If a detectable marker, such as the *Escherichia coli* β-galactosidase gene is included in the rescuing fragment, recombinant virus can be detected by blue staining by adding X-gal (150 μg/mL) to the methylcellulose overlay after the plaques have formed.

More recently, other methods of generating recombinant virus where the frequency of recombinants is higher have been developed. One of these, the use of cosmids to generate recombinant virus, is described elsewhere in this book. Two other methods are described below.

3.2. Ligation of a Fragment into a Unique Site

Rixon and McLaughlan *(5)* have described the use of a virus with a unique *Xba*I site in a nonessential site of the genome between genes US9 and 10 Digestion of this DNA to completion almost completely abolishes the ability of the virus to generate virus. To regenerate virus, the two digested halves can be ligated together. If a plasmid with sequences to be introduced, flanked by *Xba*I sites is added to the ligation mix, then recombinant virus is generated at a high frequency (1–10%). This frequency is 10-fold higher than for the classical marker rescue technique. A virus with a unique restriction enzyme site is generated in the desired location by standard marker rescue. This method does not rely on the presence of flanking DNA for the generation of recombinant virus, but is mainly useful for the insertion of extraneous DNA for expression rather than for alteration or deletion in single genes. The transfection procedure is as described above. Usually 0.5–2 μg of HSV-DNA and 1–2 μg fragment are used in the ligation reaction and subsequent transfection.

3.3. Recombination Across Digested HSV

Recently we have developed a method to generate nearly a 100% recombinants in any desired location of the HSV genome. This will be especially useful for the introduction of multiple mutations into one gene. First, a virus with a unique restriction enzyme site is generated in the desired location by standard marker rescue. We have been using a *Pac*I site, since there is no site occuring naturally in the HSV-1 genome For ease of detection of the original

restriction enzyme site positive virus and recombinants, this is usually done with a fragment containing the β-galactosidase gene as a marker, flanked by *Pac*I sites. The HSV-DNA is digested by *Pac*I, and a fragment spanning (at least 500 bp on each side) the digested DNA is added to the mixture. Transfection takes place as described above. For viable virus to be generated, recombination is required to take place across the two ends through the overlapping fragment; thus, almost all progeny will be recombinants. Nondigested parental DNA containing the β-galactosidase gene will produce blue-staining plaques in the presence of X-gal.

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4

HSV Mutagenesis

Robert S. Coffin

1. Introduction

Herpes genomes are large and complex, with many interactions among herpes encoded proteins, herpes DNA and RNA, and the host cell. These interactions begin as the virus enters the cell, and continue as the decision for latency or lytic replication is made. Correctly regulated gene expression then allows herpes genes to be expressed in a temporally regulated manner and to subvert the host cell metabolism in favor of virus production. Finally, infectious progeny virions are assembled and released. Exploring the function of the many herpes-encoded proteins and the mechanisms to control their expression during these processes thus requires a fine dissection of the herpes genome, so as to allow protein-coding regions to be linked with function and control DNA regions to be identified.

Before modern molecular biological techniques, viruses were often studied by the random generation of loss of function mutations giving an altered phenotype, either as they arose spontaneously during virus propagation or in which the natural rate of mutation was increased in some way. In a similar way, temperature-sensitive mutations were identified in which mutations in an essential gene allowed growth at a permissive temperature, but not at a higher non-permissive temperature, allowing the function of a particular gene to be probed. These types of studies can nowadays most usefully be used to select for a virus with a particular phenotype (especially if the locus likely to be responsible for the phenotype is unknown), but cannot allow the finer details of protein or DNA function to be understood. Molecular biology now allows targeted changes to be made in the herpes genome (as long as the virus remains viable or can be grown on a cell line complementing the mutation), which can be used to unravel the details of the virus/host relationship. This chapter will concen-

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trate on the types of targeted mutation, which can be made, what these mutations can tell you, and how the mutant DNA sequences can be generated. The methods by which sequences are introduced into the herpes genome (when required) and recombinant plaques isolated, purified, and the DNA structure checked by Southern blotting, are covered elsewhere in this book.

1.1. Types of Mutation

Mutations can be made that either prevent the expression of a gene (by its deletion or other disruption), alter the protein product of a gene (by deletion or addition of translated sequences), affect the regulation of transcription of a gene (by alteration of promoter regions), or whose primary effect is at the RNA level (sequences affecting RNA processing and stability can be altered). Mutations can also be made that do not affect gene expression, but that otherwise affect the virus replication cycle, such as the alteration of DNA replication origins, or of sequences necessary for packaging herpes DNA into virus particles. The functions of genes and other DNA regions can also be studied by the swapping of DNA regions between closely related viruses, by the replacement of genes with a marker (such as *lac*Z), or by the insertion of an antigenic peptide tag into a protein. Tags can also be inserted into a protein to enable interacting proteins to be copurified and identified. Some mutations are more easily studied when cloned into a plasmid vector, from which a mutant protein can be expressed when transfected into cultured cells, whereas other mutations require the mutant sequences to be inserted into the herpes genome.

Mutations can either be made by the addition or removal of large stretches of sequence from the virus (usually accomplished by the use of naturally occuring restriction sites), or by the insertion, deletion, or alteration of shorter sequences (either by the insertion of "linker" sequences into a natural restriction site, "filling in" and religation of a restriction site, or by the use of site-directed mutagenesis, where small, defined changes can be made). Combinations of these techniques can allow most of the types of alteration outlined above to be made.

2. Materials

1. 50X TAE: 242 g/L Tris base, 57.1 mL/L glacial acetic acid, 50 m*M* EDTA
2. 5X TBE. 54 g/L Tris base, 27.5 g/L boric acid, 10 m*M* EDTA
3. LB. tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, pH 7.0 with 5*M* NaOH, autoclave
4. LB agar. as above with 15 g/L agar
5. X-gal stock: 40 mg/mL X-gal in dimethylformamide.
6. T4 ligase and buffer, restriction enzymes and buffers, T4 DNA polymerase, *Taq* polymerase, and buffer by Promega (Madison, WI).

3. Methods

All the methods described below require the region of interest within the herpes genome to have been previously cloned into a plasmid vector. If the mutated sequence is to be studied in the context of the virus genome, the region to be altered must also be flanked on either side by at least 1 kb of unaltered sequence to allow efficient recombination back into the herpes genome (methods covered elsewhere in this book). Some techniques will require further subcloning of only the sequence to be altered into a second plasmid vector, which can then be returned to the flanking sequences after alteration for recombination into the herpes genome.

3.1. Alterations Making Use of Restriction Sites

3.1.1. Initial Subcloning and Simple Additions or Deletions

Before herpes DNA sequences can be altered, the region of interest often needs to be subcloned from a larger parental plasmid, either to allow useful restriction sites to be made use of, so that the DNA is in a particular plasmid vector (e.g., for the generation of single-stranded [ss] DNA for mutagenesis), or merely so that the plasmid to be manipulated is of a more manageable size General considerations for subcloning include.

1. "Sticky"-end ligations are more efficient than blunt-ended ligations.
2. If possible, use a plasmid vector in which blue/white selection of recombinant colonies is possible
3. Ligation together of DNA fragments whose two ends have each been cut with a different restriction enzyme are more efficient than where only one enzyme is used, since in this case, only the desired recombinant can be produced (especially useful where no blue/white selection is available) The use of two restriction enzymes also allows directional cloning.
4. The larger the DNA fragments to be ligated, the less efficient the subsequent ligation
5. DNA of reasonable purity, e.g., purified by a commercial kit, such as the Wizard system (Promega) or Qiagen (Hilden, Germany) systems, should be used.
6. Full sequence information/restriction maps should be available for all DNA fragments/vectors used

Regions can be deleted from herpes DNA by simple digestion with an individual or pair of restriction enzymes (sometimes after blunt-ending of the DNA) and religation, and sequences added by the same subcloning procedures and with the same considerations as outlined above. The easiest way to inactivate a particular herpes gene is to insert a promoter/marker gene cassette (such as SV40/*LacZ* from pCH101 [Pharmacia, Uppsala, Sweden]) into a unique restriction site within the plasmid-encoded gene, so that after

cotransfection with herpes DNA recombinant virus, plaques appear blue after staining with X-gal.

3.1.2. Subcloning Procedure

3.1.2.1. Digestion/Blunt-Ending of DNA

Approximately 2–5 μg of each DNA (vector and insert) should be digested with the appropriate restriction enzymes in a buffer optimal for activity (*see* manufacturer's instructions) in 100 μL using ≈20 U of each enzyme. If two enzymes are being used, a buffer can usually be found in which both enzymes are reasonably active. Incubate for 1 h at the appropriate temperature (usually 37°C). Do not use "One-Phor-All" or "Multicore" type acetate based buffers if DNA is to be subsequently blunt-ended. If one DNA terminus needs to be blunt-ended (if DNA is being cut with two enzymes), digest DNA with the first restriction enzyme (site to be blunted) for 1 h, add 1 μL 25 m*M* each dNTP and ≈10 U T4 DNA polymerase, and incubate at 37°C for a further 30 min (T4 DNA polymerase works in most restriction enzyme buffers). Heat to 80°C for 20 min (inactivates enzymes), cool to 37°C, add the second restriction enzyme (site not to be blunted), and incubate for a further 1 h. If both ends require blunt-ending add 1 μL 25 m*M* dNTPs and ≈10 U T4 DNA polymerase after restriction enzyme digestion, and incubate at 37°C for 30 min. DNA should then be phenol-extracted and precipitated with ethanol *(1)*. DNA pellets should be washed with 70% ethanol by vortexing and recentrifugation before drying and resuspension in 10 μL of water.

3.1.2.2. Purification of DNA

If the vector DNA has been cut with only a single enzyme, then no further purification is required. If with two enzymes it, like the insert DNA, must be separated from the unwanted DNA fragments in an agarose gel. Cast a 1% LMP (LMP) agarose gel using the smallest wells available in 1X TAE containing 0.4 μg/mL ethidium bromide. Load the entire DNA sample (10 μL), and run until the DNA fragments are well-separated from each other as viewed by UV *trans*-illumination. Excise the desired band, using a scalpel, with the minimum possible excess surrounding agarose and minimal exposure to UV.

3.1.2.3. Ligation of DNA

There is no need to purify DNA from the gel slice. Melted gel can be added directly to the ligation mix Melt the gel slice(s) at 70°C for 5 min, vortex, and add 1 or 2 μL of each melted agarose slice to a 5-μL ligation mix:

Ligation using	One gel-purified fragment	Two gel-purified fragments
Water	<2 µL	2 µL
Gel fragment (insert DNA)	2 µL	1 µL
Gel fragment (vector DNA)		1 µL
Vector DNA	use various amounts to optimize ligation	
(in water from Section 3.1.2.2.)	0.5–2 µL	
5X T4 DNA ligase buffer	1 µL	1 µL
Total	5 µL	5 µL

Remelt agarose by brief incubation at 70°C, and mixing, cool to room temperature, add 1 µL T4 DNA ligase, mix, and incubate at room temperature for 1 h. Add 5 µL of water, heat to 70°C to melt any agarose, mix, and add to competent cells *(see* Section 3.1.2.4.*)*

Controls: (1) cut vector DNA, no ligase: checks efficiency of vector cut—should give no colonies, and (2) cut vector DNA + ligase: checks efficiency of ligation—if cut with one enzyme should give many colonies; if cut with two enzymes, should give many less colonies than when insert DNA is added.

3.1 2.4. Transformation of *E. coli*

Competent cells are prepared by inoculating a single, freshly grown colony of *Escherichia coli* DH5 or similar into 100 mL of LB. This is grown shaking at >200 rpm at 37°C for 2–4 h until swirling, opalescent bacterial growth can just be seen. Cells are harvested by centrifugation at 3000 rpm for 10 min, resuspended in 20 mL ice-cold 100 m*M* CaCl, recentrifuged, and finally resuspended in 4 mL 100 m*M* CaCl. These are stored on ice until used and may be kept for up to 4 d, or frozen in 200-µL aliquots at –70°C indefinitely.

Ligated DNA from Section 3.1.2.3. above is added to 200 µL of competent cells in a 15-mL plastic disposable tube, incubated on ice for 30 min, transferred to a 42°C water bath for exactly 90 s, transferred back to ice for 2 min, and 800 µL LB added. The tube is incubated by shaking at 37°C for 1 h before harvesting the cells by centrifugation as above, resuspension in a small volume of the medium (≈100 µL), plating onto 1% LB agar plates containing the appropriate antibiotic (and X-gal [50 µg/mL] if blue white selection is being used—IPTG is usually unnecessary), and incubated overnight at 37°C. Control: Transform with ≈10 ng uncut vector DNA—checks competence of the cells.

3.1.2.5. Identification of Recombinant Colonies

Recombinant colonies are identified by plasmid minipreparation and restriction digestion. If blue/white selection can be used, then white colonies are picked. If a single fragment is being religated or two fragments ligated in such a way that self-ligation is not possible (i.e., two restriction enzymes used), colonies can be picked at random. However, if the vector is capable of self-religation in the absence of the inserted DNA (no blue/white selection), a colony lift to transfer colonies to a nitrocellulose or nylon membrane must be performed, followed by hybridization with the insert DNA (radiolabel some of the DNA in melted agarose from Section 3.1.2 2., e.g , Ready-To-Go kit from Pharmacia) to identify recombinant clones. The manufacturer's instructions for the membrane used (e.g., Amersham [Amersham, UK], HyBond N, or HyBond N+) should be followed in this case.

Miniprep of plasmid DNA: Single colonies identified as above are inoculated into 5 mL of LB and grown overnight in an orbital shaker (200 rpm) at 37°C. Following incubation, transfer 1 5 mL of the culture to a microfuge tube, centrifuge in a microcentrifuge for 1 min, and aspirate the supernatant. Next sequentially add, and thoroughly mix by vortexing, 100 µL of solution 1 (50 m*M* Tris-HCl, pH 7.5, 10 m*M* EDTA, 100 µg/mL RNase A), 200 µL of solution 2 (0.2*M* NaOH, 1% Triton X-100), and 150 µL of solution 3 (3*M* KOAc pH 4.8). Centrifuge for 3 min, remove the pellet with a bent hypodermic needle, add 500 µL of isopropanol, mix, and recentrifuge for 5 min. The pellet is then washed with 70% ethanol, dried, and resuspended in 50 µL of water containing 20 µg/mL RNase A.

Restriction digestion of miniprep DNA: Digest 3 µL of the miniprep DNA from above with restriction enzymes (total <1 µL) suitable to distinguish recombinant from nonrecombinant colonies in the appropriate buffer in a total of 10 µL for 1 h. Directly run the 10 µL reaction mixture (+ 1 µL loading buffer [50% glycerol, 1X TAE, 0.25% bromophenol blue]) on a 1% agarose gel.

A plasmid maxiprep (100 mL to 1 L culture) should then be performed, either using a commercially available kit (e.g., Qiagen tip-100 or 500) or by a traditional method, such as cesium chloride density gradient centrifugation, before, for example, cotransfection into cells together with herpes DNA to generate a recombinant virus.

3.1.3. Filling in Restriction Sites

Subtle changes to a sequence (e.g., alteration of a promoter element or introduction of a stop codon into an open reading frame) can often be made by "filling in" (5' overhang) or "chewing back" (3' overhang) of a restriction site. In both cases, T4 DNA polymerase (which has both polymerase and exonuclease activity) can be used as in Sections 3 1.2.1.–3.1.2 5., above. The alteration

in restriction pattern observed can then be used both to identify recombinant plasmids and also recombinant viruses should the alterations need to be transferred to the herpes genome. A series of truncated proteins can often be generated by cutting and filling in of different restriction sites as they occur along a sequence. Inspection of the sequences to be altered, and the sequence generated after filling in will determine if the desired result can be achieved in this manner.

3.1.4. Oligonucleotide Insertion

Pairs of complementary oligonucleotides, which when annealed leave protruding "sticky" ends, can easily be inserted into a unique restriction site or (preferably) between two different sites (since here only the desired recombinant can be generated) in a target sequence. These might encode an amino acid sequence or a particular nucleic acid motif. Ideally, the inserted oligonucleotide should contain a restriction site to aid identification of the desired recombinant.

Method:

1. Digest target DNA with appropriate enzyme(s) and gel-purify as in Section 3 1 1
2. Set up two ligation reactions, one containing ≈100 ng of each oligonucleotide, and one without (control), as in Section 3 1 1 Overlay with 50 µL mineral oil Do not add ligase There is no need to phosphorylate the oligonucleotides
3. Heat reaction to 95°C, and cool slowly to room temperature in a large beaker of water
4. Add ligase below the mineral oil, mix, and incubate at room temperature for 1 h before adding the mixture (below the mineral oil) to competent cells
5. Pick colonies, miniprep, and identify recombinants by the introduction of restriction sites encoded by the oligonucleotides or, if necessary, by a colony lift using end-labeled oligonucleotide (*see* Section 3.2.) as a probe.

3.2. Linker Insertion Mutagenesis (Scanning)

Linker scanning mutatagenesis *(2)* allows the fine mapping of regions important for gene function (promoter or protein-coding regions), usually after important regions have been initially defined at a more gross level by deletion analysis. The general goal of a linker scanning procedure is to produce a series of mutants in which overlapping small sequences (≈10 bp) have been individually replaced by a common linker sequence along the length of the sequence under study. Thus, although important amino acid or nucleic acid residues are likely to have been replaced by the procedure, the spacing between promoter elements or protein domains would remain unchanged.

The procedure requires four stages.

i. Cloning of the DNA of interest into a plasmid vector.
ii. Generation of randomly spaced breaks in the sequence of interest.

iii. Deletion of a short sequence on each side of the breaks and insertion of linkers.
iv Identification and characterization of the mutants generated

A number of protocols for stages (ii) and (iii) have been devised, the major stumbling block being the generation of truly random breaks (by chemical means) and the concurrent deletion of sequence together with the insertion of the linker. Simpler procedures can be performed if the breaks in the sequence are produced by partial digestion (to linearize) with frequently cutting restriction enzymes (not allowing "full coverage" of a sequence) and if linker sequences are then inserted without the concurrent deletion of a sequence of equal length (precise spacing between sequences is not maintained). Protocols for this based on the method of De Wind et al. *(3)* are described below.

1 Restriction digestion of target DNA Blunt-cutting restriction enzymes with four-base recognition sequences that cut with reasonable frequency in the DNA of interest should be used. Available enzymes are *Hae*III, *Nla*IV, *Fnu*DII (isoschizomer *Bst*UI), and *Alu*I. Use of the restriction enzymes at low concentration allows the partial digestion of the DNA to give linear fragments, which can be purified on a polyacrylamide gel

Set up five digestions/enzyme, 10 µg of DNA each, with 1, 0.5, 0 25, 0 12, and 0 06 U of enzyme/100 µL reaction, in the manufacturer's recommended buffer Inclusion of ethidium bromide at 50 µg/mL (*Fnu*DII), 5 µg/mL (*Hae*III), or 0.5 µg/mL (*Rsa*I) increases the proportion of linear fragments *(3,4)* Incubate at 37°C for 15 min, and transfer to an 80°C water bath for 20 min to inactivate the enzyme

2 Gel-purification. After partial digestion, phenol-extraction and ethanol precipitation, plasmid DNA that has been cut only once (linearized) is separated from undigested and multiply digested forms by separation on a 1X TBE-5% polyacrylamide gel *(1)* in comparison to plasmid DNA that has been cut with an enzyme that cuts only once. After electrophoresis, the gel is stained in ethidium bromide for half an hour (0.5 µg/mL in 1X TBE), viewed on a UV transilluminator, and the sample with the greatest proportion of linear DNA is quickly excised (in the smallest possible gel fragment) with a scalpel DNA is extracted from the gel by placing the excised fragment into the well of a 1% LMP agarose gel, running the DNA into the gel 1–2 cm, and extracting the DNA from the gel using a commercial kit or by melting and phenol-extraction. Resuspend in water at ≈200 ng/µL (estimated by agarose gel electrophoresis and comparison with DNA of known concentration)

3. Linker insertion· The linker used should be 12 bases in length for optimal ligation efficiency and to keep the protein in frame, and palindromic (i.e , self-complementary) to allow double-stranded insertion into the digested plasmid after annealing has occurred. The linker should also contain a restriction site for an enzyme that does not cut in the plasmid undergoing mutation to allow easy characterization of mutants containing the inserted linker The sequence used should also not contain any translational stop signals (TGA, TAG, TAA) or allow the generation of such stop signals after insertion For this purpose, the inserted

sequence can start with a 5'-C The use of phosphorylated linkers increases the efficiency of insertion

Linker phosphorylation.

Oligonucleotide	500 ng
10X kinase buffer (200 m*M* Tris, pH 7 6, 100 m*M* $MgCl_2$)	2 5 µL
10 m*M* ATP	2.5 µL
T4 DNA polymerase	5 U

Incubate at 37°C for 30 min and use directly in the ligation reaction below

Set up three 5-µL ligation reactions for each eluted linearized DNA band using 10, 30, and 100 ng of linker and 1 µL (50–100 ng) of purified linearized DNA

Purified DNA	1 µL
5X T4 DNA ligase buffer	1 µL
Phosphorylated linker	*x* µL
Water	to 4 µL
T4 DNA ligase	1 µL

After ligation, transform into competent cells, and perform colony lifts onto, e g., HyBond-N (Amersham) following the manufacturer's instructions Transferred colonies are then probed with γ-^{32}P-labeled linker (using T4 polynucleotide kinase· mix 5 µCi [γ-^{32}P]ATP, 5 ng linker, 4 µL 10X kinase buffer [above], water to 40 µL, 10 U T4 polynucleotide kinase, and incubate at 37°C for 1 h) and linker containing colonies are identified by autoradiography Positive colonies are miniprepped, and their structures are analyzed by restriction analysis (using the restriction site introduced with the linker), and finally by DNA sequencing

By this means, many mutants may be identified with near-random insertion of the linker sequence to allow regions of functional significance to be probed, either while still in plasmid form or after transfer to the viral genome

3.3. Site-Directed Mutagenesis

Specific, small changes to a sequence are made by site-directed mutagenesis using specific mutagenic oligonucleotides and target DNAs either cloned into an M13 or phagemid vector, or into any or a more specialized plasmid for the newer PCR or "repair oligonucleotide" methods. These can include introduction of a restriction site, alteration of individual amino acids in a protein sequence, introduction of a stop codon, or alteration of particular promoter elements (to name but a few). However, in each case, the general considerations for all site-directed mutagenesis procedures and the design of the oligonucleotide to be used are the same whichever method is being used:

1. Small mutations (1–3 bases) are more efficiently generated than large mutations (5+ bases).

2. Replacement of nucleotides is much more efficient than deletion or insertion of nucleotides.
3. Highly pure oligonucleotides should be used (HPLC or "cartridge" purified)
4. Longer oligonucleotides generally give a higher efficiency of mutagenesis than do shorter ones. Optimal size is obviously dependent on the number of mismatches. In general, 20–25 mers can be used to replace 1–3 bases in the center of a sequence, and for larger replacements/deletions/insertions (up to ≈10 bases), oligonucleotides with ≈15 unchanged bases on either side of the bases to be changed can be used with reasonable efficiency.
5. Palindromic oligonucleotide sequences should be avoided, as should "unusual" looking sequences. sequences that look truly random are best and should optimally have a G + C content of ≈ 60%.
6. Use of an oligonucleotide that introduces a restriction site (if possible) greatly eases the identification and characterization of potential mutants.

The original and most widely used methods for site-directed mutagenesis devised by Kunkel et al *(5)*, using uracil containing DNA and mismatch repair were relatively complex systems and, although often reliable, were time-consuming. Individual users would usually have to optimize the various steps before successful mutagenesis. However, newer and simpler methods have now been described that are both highly reliable and widely used. These are either based on the use of an oligonucleotide to remove a unique restriction site (or similar alteration to repair an antibiotic resistance gene) or on PCR, and since these provide many advantages over the older methods, these will be described here.

3.3.1. Unique Site Elimination-Based Mutagenesis

Advantages:

Rapid, easy, and efficient
Any plasmid vector can be used.
Kits commercially available
Multiple mutations can be made concurrently.
Multiple rounds of mutagenesis can easily be performed.

A number of commercially available kits are available for unique site elimination-based mutagenesis (e.g., "U.S.E." from Pharmacia; "Chameleon" from Stratagene [La Jolla, CA]), all based on the same original system of Deng and Nickeloff *(6)*, a modification of which will be described here. This method, like the Kunkel method outlined above, relies on a mutagenic oligonucleotide annealing to the plasmid DNA (preferably ssDNA generated by superinfection with bacteriophage, but alkali-denatured dsDNA can be used with lower efficiency), and the use of T4 DNA polymerase/T4 DNA ligase to synthesize and repair the remainder of the complementary DNA strand. However, mutated plasmid is here selected for by the use of a second oligonucleotide, which deletes a

unique restriction site, and thus after digestion with this restriction enzyme and transformation, will only allow growth of bacteria containing plasmid in which the site has been deleted. Experiment has shown that a very high proportion of plasmid in which the restriction site deletion has taken place (up to 95%) will also have incorporated the mutagenic oligonucleotide. Multiple mutagenic oligonucleotides can be used successfully at one time (up to 4) and further rounds of mutagenesis performed by the sequential use of different unique restriction sites in the plasmid vector. A similar method is available from Promega ("Altered Sites") in which, instead of deletion of a unique restriction site, an inactive antibiotic resistance gene is repaired, and thus only plasmid in which repair has occurred will allow growth in the presence of that antibiotic. However, here the DNA to be mutagenized must be cloned into a specialized plasmid vector (purchased from Promega), adding another stage to the mutagenesis process.

Method: For best results, ssDNA should be produced by superinfection of phagemid containing bacteria with helper-phage, although alkali-denatured plasmid DNA can be used with lower efficiency, and both the mutagenic and restriction site elimination oligonucleotide should be phosphorylated (Section 3.2.). Twenty-base oligonucleotides removing any unique site in the vector to be used (without interrupting an open reading frame) can easily be designed, and if these also introduce a new unique site, multiple rounds of mutagenesis can easily be performed Oligonucleotides for use in pUC- and pBR-based vectors are available from Pharmacia and Statagene for this purpose. The repair-deficient *E. coli* strain BMH71-18 *mut*S should also be purchased, e.g., from Promega, which prevents repair of the mutations after the mutagenesis reaction and before final transformation into a standard lab *E coli* strain.

1 Preparation of ssDNA
   a. Pick a single freshly grown colony into 5 mL of selective media, add $\approx 10^{10}$ PFU M13KO7, and grow overnight at 37°C shaking vigorously. (Stock M13KO7 should be bought and prepared according to the manufacturer's instructions, e g , Promega, or by standard methods *[1]*)
   b. Pellet the cells (6000*g*, 4°C, 10 min), and decant the supernatant to a fresh tube
   c Add 900 µL 20% PEG6000, 2 5*M* NaCl, mix, and incubate at room temperature for 30 min to precipitate phage.
   d. Pellet phage at 12,000*g*, 4°C; 20 min, and decant superanatant (white pellet should be visible), aspirate to remove traces of PEG
   e. Resuspend in 500 µL water containing 100 µg/mL RNase A, and transfer to a microfuge tube
   f. Phenol/chloroform-extract at least three times, each time vortexing for at least 30 s
   g. Add 0.5 vol of 7 5*M* ammonium acetate and 3 vol of ethanol, and vortex. Spin in a microfuge for 10 min, discard the supernatant, and wash with 70% ethanol by vortexing and respinning before air-drying the pellet and resuspension in 20 µL of water; 5 µL should give a bright band on an agarose gel.

2 Mutagenesis reaction and restriction digestion
   a. Anneal. Mix 100 ng ssDNA, 2.5 ng restriction site elimination oligo, 1 25 ng mutagenic oligo, 2 μL 10X annealing buffer (200 m*M* Tris-HCl, pH 7 5, 100 m*M* $MgCl_2$, 500 m*M* NaCl), and water to 20 μL
   Heat to 70°C for 5 min, cool slowly to room temperature, and place on ice
   b Synthesis· Add to the annealing reaction 3 μL 10X synthesis buffer (100 m*M* Tris-HCl, pH 7.5, 5 m*M* each dNTP, 10 m*M* ATP, 20 m*M* DTT), 10 U T4 DNA polymerase, 2 U T4 DNA ligase, and water to 10 μL (final annealing/ synthesis reaction vol 30 μL)
   Incubate at 37°C for 90 min Incubate at 80°C for 20 min Add 10 U of the restriction enzyme at the site that is being deleted Incubate at 37°C for 1 h
3. Transformation into *E coli* BMH71-18 *mut*S
   a. Prepare competent BMH71-18 *mut*S cells as in Section 3.1 1.
   b Add the entire synthesis reaction to 200 μL competent cells, incubate on ice for 30 min, heat shock at 42°C for 90 s, and add 5 mL LB before incubation at 37°C for 1 h with vigorous shaking Add ampicillin to a final concentration of 100 μg/mL, and continue incubation overnight
4. Final selection and and transformation into standard host *E coli*
   a Miniprep 1 5 mL of the culture from step 3, and resuspend in 85 μL of water containing 5 μg/mL RNase A Add 10 μL of the optimal 10X buffer for the enzyme site being deleted and 5 μL (50 U) of the appropriate restriction enzyme. Incubate at 37°C for 1 h, add 300 μL of water, and phenol-extract before ethanol precipitation
   b Resuspend the precipitated DNA in 10 μL of water, and transform into 200 μL of competent cells (standard lab strain, e.g , DH5α, JM101) as in Section 3 1 1 Spread $^1/_{10}$ and $^9/_{10}$ onto ampicillin plates, and incubate at 37°C overnight
   Colonies should be checked for the mutation by miniprep and restriction digestion, and finally by sequencing

3.3.2. PCR-Based Methods

Advantages:

DNA in any plasmid vector can be used
Easy and efficient
Kits available

Disadvantage:

Since PCR itself can introduce mutations, the entire cloned DNA should be sequenced after the procedure to check for unwanted mutations.

A number of different PCR-based mutagenesis procedures have been developed, and a method will be described here that has been used many times and with high efficiency in our laboratory to introduce single-point mutations, multiple-point mutations, random mutations (at defined sites) using degener-

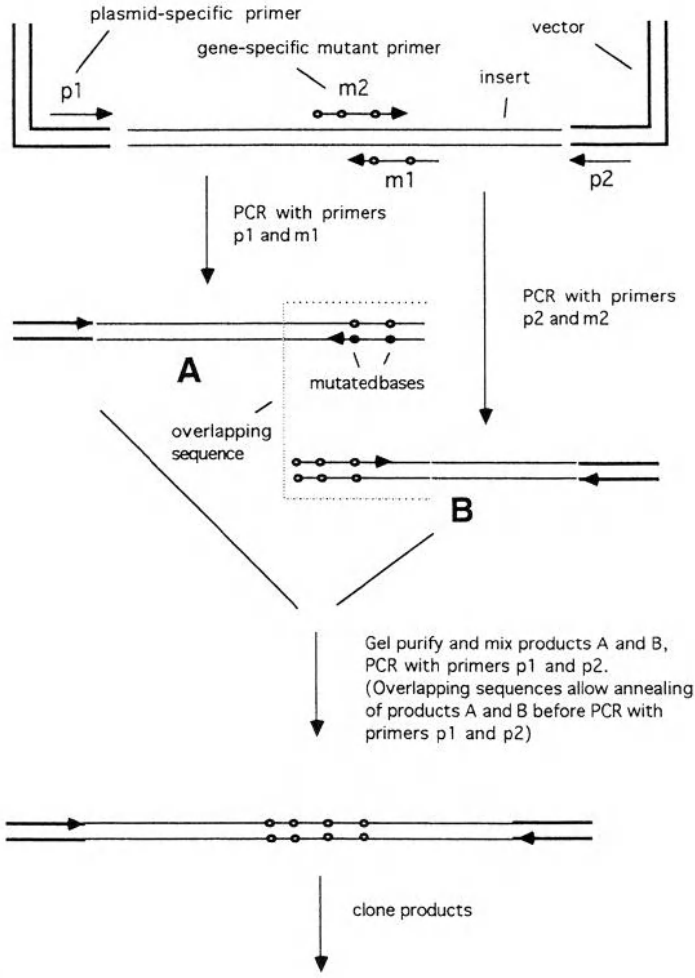

Fig. 1. Outline of the scheme for mutagenesis by overlap extension.

ate primers, insertions, and deletions. The method is based on the site-directed mutagenesis by overlap extension procedure described by Ho et al. *(7),* which requires two plasmid-specific primers, allowing amplification of the entire insert sequence (universal sequencing primers can often be used), and two complementary gene-specific mutant primers.

The method is outlined in Fig. 1, where two initial PCR reactions are performed using in each case a plasmid-specific primer (p1 or p2) and a gene-specific mutant primer (m1 or m2) to generate two mutated PCR products, which overlap at one end. After gel purification, and mixing, this overlap allows

annealing of the two products before a second amplification step with the two plasmid-specific primers alone to generate a full-length mutated sequence that can be cloned into the plasmid of choice.

Gene-specific mutant primers: The design of gene-specific primers is outlined in Fig. 2 and should generally, for simple point mutations, be at least 25 bases long. As with site-directed mutagenesis, greater numbers of mismatches require longer oligonucleotides. Random mutations can be made at defined sites if degenerate oligonucleotides are used (i.e., where all four bases A, T, C, G, or particular mixtures are included at defined positions within the oligonucleotide) Self-complementarity within primers, "unusual" sequences (runs of individual nucleotides or repeated elements) and particularly high or low G + C content should be avoided.

Plasmid-specific primers: The plasmid-specific primers can in many cases be the M13 forward and reverse universal sequencing primers, which have the advantage that the polylinker of the plasmid in which the sequence has been cloned will also be amplified during the second PCR, and can thus be used for digesting and cloning the final PCR product. If these primers are unsuitable, primers containing suitable restriction sites (recognition site starting at least 5 bases from the 5' end to allow efficient digestion) should be designed. However, even without restriction sites, the final PCR product can easily be cloned into commercially available plasmids linearized to include an overhanging 3'-T (e.g., Promega T-vector system). This system takes advantage of the overhanging 3'-A *Taq* polymerase added to all PCR products during amplification.

Method:

1. Set up two PCR reactions containing template DNA and either primers p1 and m1 or p2 and m2 We routinely use *Taq* polymerase and buffer from Promega 10 µL 10X buffer, 7 µL 25 m*M* $MgCl_2$, 1 µL 10 m*M* dNTPs, 300 ng each p1 + m1 or p2 + m2, ≈50 ng template, and water to 100 µL Overlay with 100 µL of mineral oil
2. Denature DNA by heating to 98°C for 5 min in a thermal cycler, remove, add 50 U *Taq* polymerase below the mineral oil, and mix by pipetting.
3 Cycle 20–30 times at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min.
4 Run 10 µL of each reaction on a 1% LMP gel, and excise the appropriate bands in the smallest possible gel volume. If the correct bands cannot be seen, then the annealing temperature (50–65°C) and the $MgCl_2$ concentration (0 5–5 m*M*) should be optimized
5. Set up a second PCR reaction mix containing 300 ng each of primers p1 and p2 and 3 µL of each of the two gel slices (first melt at 70°C, 5 min, and mix), and cycle as before, with optimization if necessary
6. Clone the products· Make up the PCR reaction to 400 µL with water, phenol-extract, and precipitate the DNA Cut with the appropriate restriction enzyme in 100 µL for 1 h at 37°C, phenol-extract and precipitate the DNA, and run the entire reaction on an LMP gel Excise the appropriate band and ligate to suitably prepared vector in the gel as in Section 3 1.1

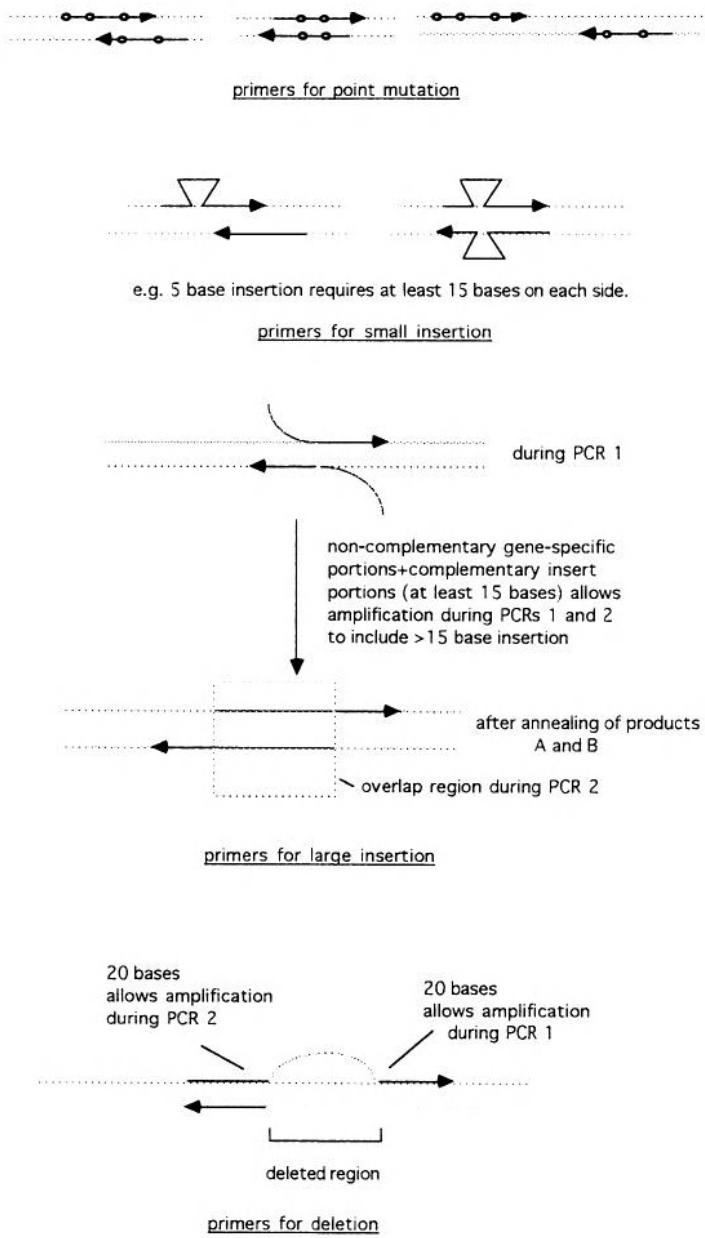

Fig. 2. The design of gene-specific primers for mutagenesis by overlap extension.

Alternatively, PCR products can be cloned directly using, for example, the T-vector system from Promega.

7. Check for the mutation by appropriate restriction digestion, and finally by sequencing.

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5

Saturating Mutagenesis and Characterization of a Herpesvirus Genome Using In Vivo Reconstitution of Virus from Cloned Subgenomic Regions

Niels de Wind, Maddy van Zijl, and Anton Berns

1. Introduction

The study of genome structure and gene function is pivotal in understanding the mechanisms of replication, pathogenesis, and virulence of herpesviruses. In this respect, mutagenesis and sequence analysis of genes encoded by the virus are of great importance. However, the herpesvirus genomes are large, with sizes ranging between 120 and over 200 kbp and encoding between 70 and 200 genes (*see* ref. *1* for a review). This large size hampers handling and systematic mutagenesis of the virus genome using standard modern molecular biology techniques. Most current methods of mutagenesis therefore do not rely on direct modification of the viral genome in vitro but depend on exchange in vivo, by homologous recombination, of a viral gene by a copy of the latter gene that is truncated in vitro by insertion of a marker gene. Mutant virus progeny can be screened or selected for, depending on the marker gene that is used. Commonly used marker genes are thymidine kinase and *lacZ*. This procedure is generally used, reliable, and has yielded a wealth of information on the function of herpers simplex virus type 1 (HSV-1) encoded genes. However, it requires prior mapping and cloning of every gene to be mutagenized and is therefore less feasible if the virus is a novel or less-well-known herpesvirus.

One such less-well-characterized herpesvirus is the porcine alpha-herpesvirus pseudorabies virus (PRV, synonyms: suid herpesvirus type 1, Aujeszky's disease virus). To explore the contents of the virus genome and to study gene function, we have developed a method to mutagenize the virus in vitro without prior knowledge of the gene structure. Here, we describe how to:

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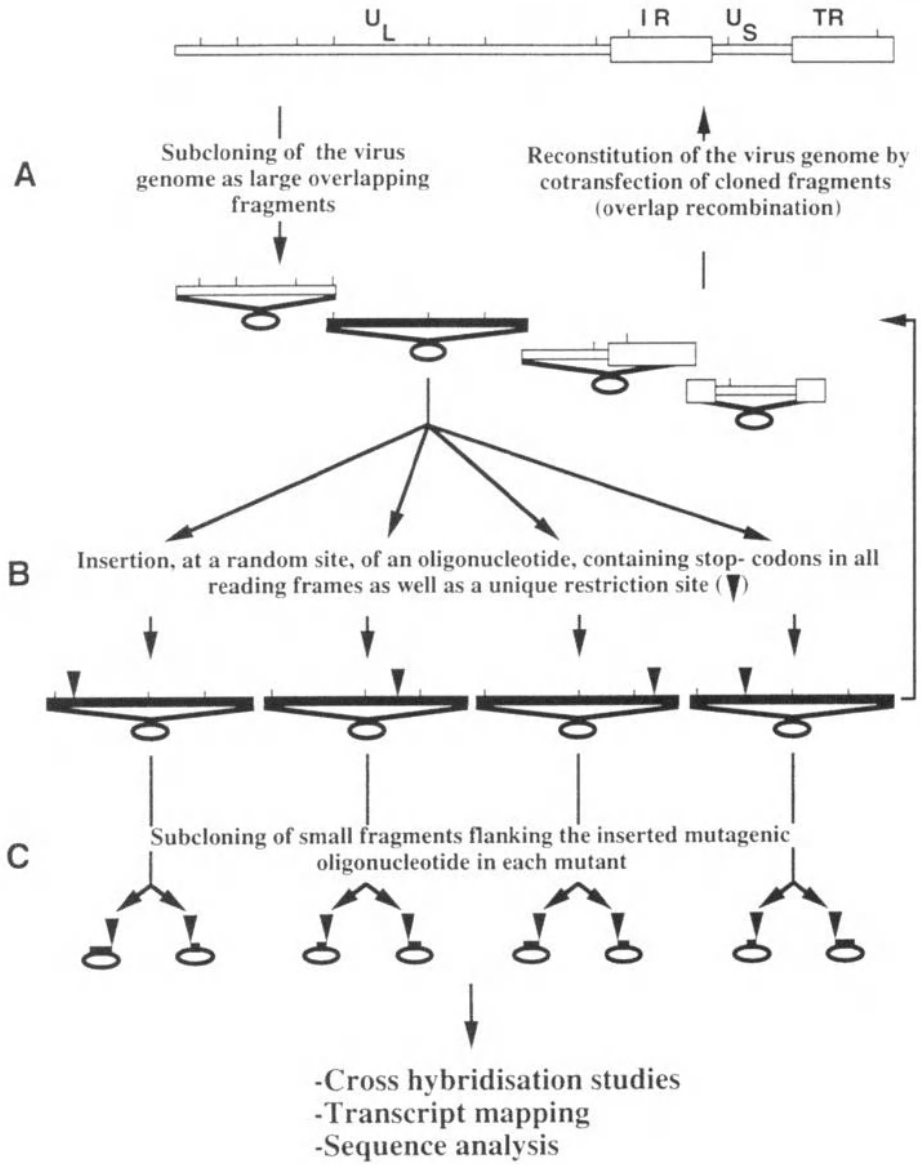

1. Make the genome of a herpesvirus amenable to modification in vitro;
2. Generate a large series of virus mutants, with each mutant carrying an inserion of an oligonucleotide in a single gene, abolishing the expression of that gene; and
3. Use these mutants to obtain gene-specific probes to explore the gene layout of the virus.

The method, as outlined in Fig. 1, relies on cotransfection of four to five cloned overlapping DNA fragments that together constitute the entire virus

genome. In vivo, replicating virus is generated efficiently and faithfully after recombination between the homologous ends of the DNA molecules ("overlap recombination," Fig. 1A). Mutagenesis of these individual clones is performed by insertion of an oligonucleotide that contains stop codons in all reading frames and a unique restriction site, at a random location, in such a large cloned virus fragment (Fig. 1B). This is followed by reconstitution of mutant virus by overlap recombination. To obtain information on the identity of mutagenized genes, small DNA fragments flanking the inserted oligonucleotide in every mutant are subcloned. These subclones are subsequently used for cross-hybridization studies with a prototype herpesvirus, for transcript mapping and for direct sequence analysis (Fig. 1C).

We will describe the background and rationale of the different steps of the procedure outlined in this Section. In Sections 2. and 3., we provide a detailed description of the techniques involved. In addition, in Section 4., we will provide the reader with supplementary information and hints on the techniques described.

1.1. The Generation of Large Overlapping Clones of a Herpesvirus Genome for Overlap Recombination

In 1987 we demonstrated that cotransfection of a PRV genome fragment lacking the Unique Short (US) region, together with a cloned fragment containing a mutagenized US region, resulted in the regeneration of replication

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Fig 1 *(opposite)* Outline of the procedures described in this Chapter Top: the genome of PRV consisting of a Unique Long (U_L) and a Unique Short (U_S) region, the latter being bracketed by the Internal Repeat (IR) and the Terminal Repeat (TR) Small vertical bars indicate the cleavage sites of a (hypothetical) diagnostic enzyme. **(A)** The virus genomic information is cloned as a set of four to five cosmids, each cosmid containing an adjacent but overlapping region of the virus genome. Cotransfection of the insert fragments leads to the regeneration of intact virus genome and progeny virus ("overlap recombination") **(B)** An individual cosmid clone is used for mutagenesis by insertion, at a random site, of an oligonucleotide that contains stopcodons in all reading frames, abrogating translation, and also a restriction site that is normally absent from the cosmid. In this way, a large series of mutant cosmid derivatives is generated, each mutant cosmid derivative bearing the oligonucleotide at a single and unique site Mutant virus strains, each containing the oligonucleotide at a single and unique site, are subsequently generated by overlap recombinations. **(C)** Of every mutant cosmid derivative, small genomic fragments are subcloned, that flank the inserted oligonucleotide at both sides. These "tags" can be used to explore the virus genome, as probes for crosshybridization studies with a prototype herpesvirus, for the mapping of virus-encoded transcripts, and for direct DNA sequencing to identify the gene in which the oligonucleotide is inserted in

competent virus *(2)*. Apparently, ligation in vivo between the ends of both DNA molecules is responsible for this (*see* Note 1). This experiment did show that the virus could efficiently be reconstituted from large subgenomic fragments of which (at least) one was cloned and mutagenized in vitro. Since the wild-type US fragment was absent in the transfection, screening or selection for the introduction of the mutation was avoided. Besides the gain in time, regeneration of virus by cotransfection of large, excluding, genomic DNA fragments enhances both the subtlety and the versatility of mutations to be introduced into the virus genome.

It is well-established that, in cultured cells, extrachromosomal recombination between homologous DNA stretches is a rapid and efficient process, provided that the homology is present at the end of the linearized DNA molecules. In the early 1980s, this property of the cell already was used to reconstitute viable adenovirus after cotransfection of two overlapping (linearized) cloned fragments of the virus genome *(3,4)*. Recombination in vivo also underlies most established methods to mutagenize the HSV-1 genome, as described above (*see* Chapter 4).

These results led us to investigate whether cotransfection of multiple, overlapping, cloned fragments, covering the entire PRV genome, resulted in the efficient reconstitution of viable virus via homologous recombination between the homologous ends of the linearized virus fragments *(2)* To this end, a set of cosmids was constructed, each cosmid containing an adjacent but overlapping region of the virus genome The cloning procedure is depicted in Fig. 2. Briefly, purified PRV genomic DNA was mechanically sheared and subgenomic fragments with sizes between 35 and 45 kbp were isolated and cloned in a cosmid vector, packaged in phage lambda and used to infect *Escherichia coli* The resulting subgenomic clones of the virus were accurately mapped by restriction digest mapping. A set of three clones, overlapping at the ends by 0.5 and 1.5 kbp (together comprising the Unique Long [UL] and a copy of the Internal Repeat [IR] of the virus), was selected. In this specific case, a fourth overlapping fragment, containing the US region, was (for technical reasons) not cloned as a cosmid, but cloned separately as a 28-kbp *Hin*dIII fragment (the overlap here is about 10 kbp). These four overlapping fragments were purified to remove vector sequences and cotransfected into permissive cells (*see* Note 2). This resulted in the highly efficient production of PRV. Even cotransfection of another set of five, instead of four, overlapping frag-

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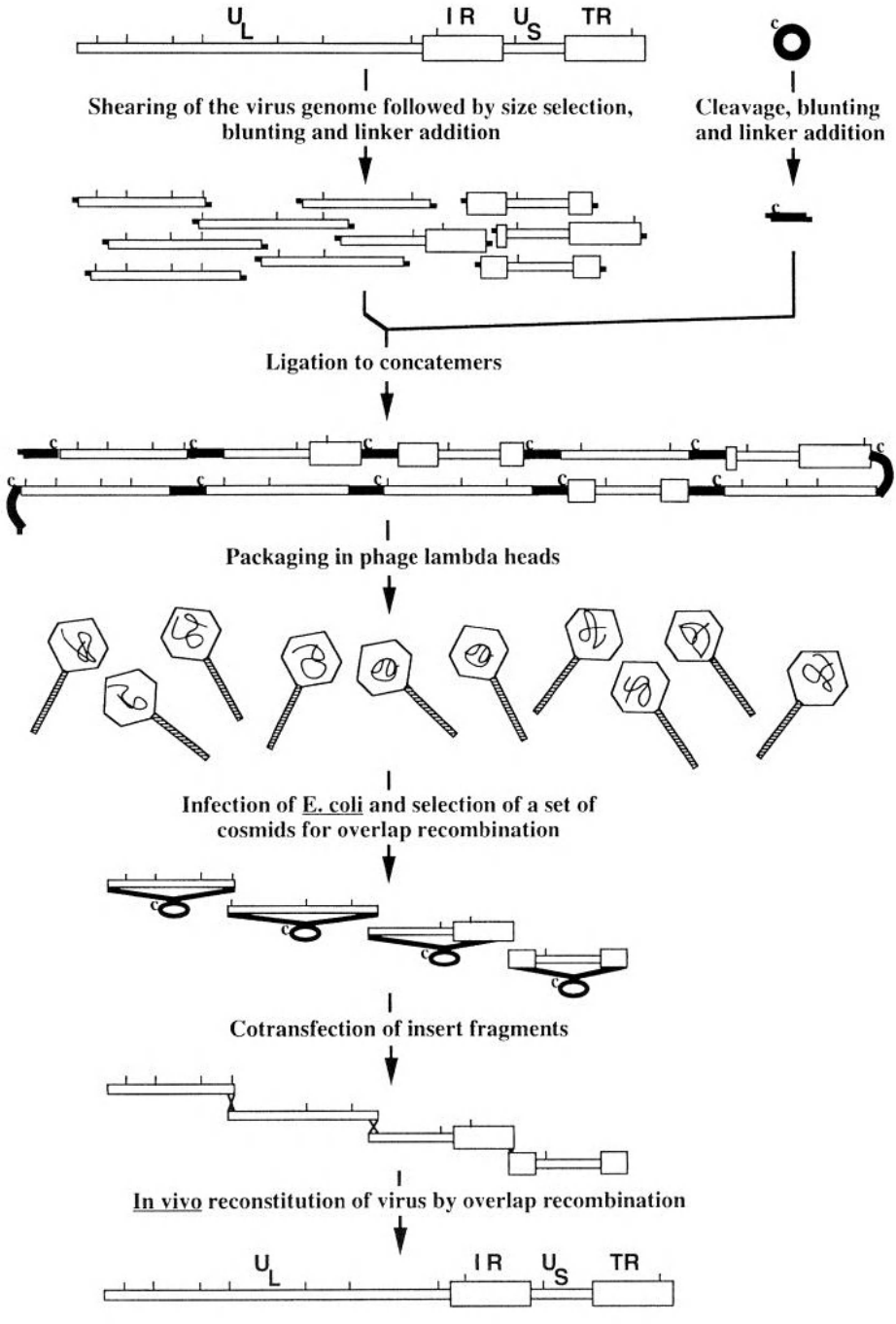
Fig. 2 *(opposite)* Outline for the procedure for cloning the virus genome as a a set of four to five cosmids, each cosmid containing an adjacent but overlapping region of the genome. The small black circle represents the cosmid vector c. *cos* site, required for packaging of the large DNA fragments in phage lambda heads Details of the procedure are explained in the text.


U L
I R
U S
TR
Shearing of the virus genome followed by size selection, blunting and linker addition
Cleavage, blunting and linker addition
Ligation to concatemers
Packaging in phage lambda heads
Infection of E. coli and selection of a set of cosmids for overlap recombination
Cotransfection of insert fragments
In vivo reconstitution of virus by overlap recombination
U L
I R
U S
TR

ments resulted in efficient generation of virus progeny. This confirms that the process of homologous recombination is very efficient (*see* Note 3). Virus thus regenerated is indistinguishable from wild-type PRV, both on the basis of restriction enzyme analysis as well as by phenotypic analysis (virulence and pathogenicity for pigs *[2]*). As described next, these individual virus subclones are easily amenable to large-scale systematic mutagenesis.

1.2. The Generation of Oligonucleotide Insertion Mutants of the Virus Using Large Cloned Subgenomic Regions

Each of the cloned subgenomic regions of the virus can now be subjected to mutagenesis in vitro to efficiently generate large numbers of mutant derivatives of the cosmid clone. This is subsequently followed by overlap recombination, after cotransfection of each mutant cosmid derivative together with the overlapping cloned virus genomic fragments, leading to the regeneration of virus carrying the specific mutation (Fig. 1B).

To systematically inactivate genes located on these large plasmids, we have modified common oligonucleotide insertion mutagenesis techniques (refs. *5* and *6*; Fig. 3). These modifications enable insertion, at a random site within such a large plasmid, a palindromic double-stranded oligonucleotide that contains stop codons in all six reading frames as well as a restriction site that normally is not present in that cosmid (and preferably also not in the rest of the virus genome). Presence of this oligonucleotide in any orientation within a coding region will, as a consequence of the insertion of a stop codon in the reading frame, lead to termination of translation of the messenger RNA, thus eliminating expression of that gene (*see* Note 4). As an example, for the mutagenesis of PRV clones, we used the 20-mer oligonucleotide 5'-*TAG*GC*TAG*GAATTC*TAG*CCTA-3', which contains an *Eco*RI site (underlined, this site is absent from the PRV genome) and amber stop codons in all reading frames (italicized, *see* Note 5). The pivotal step in the procedure of mutagenesis is the linearization of the clone at a random site, leaving a blunt-ended linear molecule that is suitable for the insertion of the double-stranded oligonucleotide This linearization is achieved by very partial digestion using restriction enzymes that have a 4-bp recognition site and that leave a blunt end after cleavage (*see* Note 6) The presence of Ethidium bromide in the digestion mixture, which inhibits multiple cuts of the same DNA molecule (*7*), will result in an increased proportion

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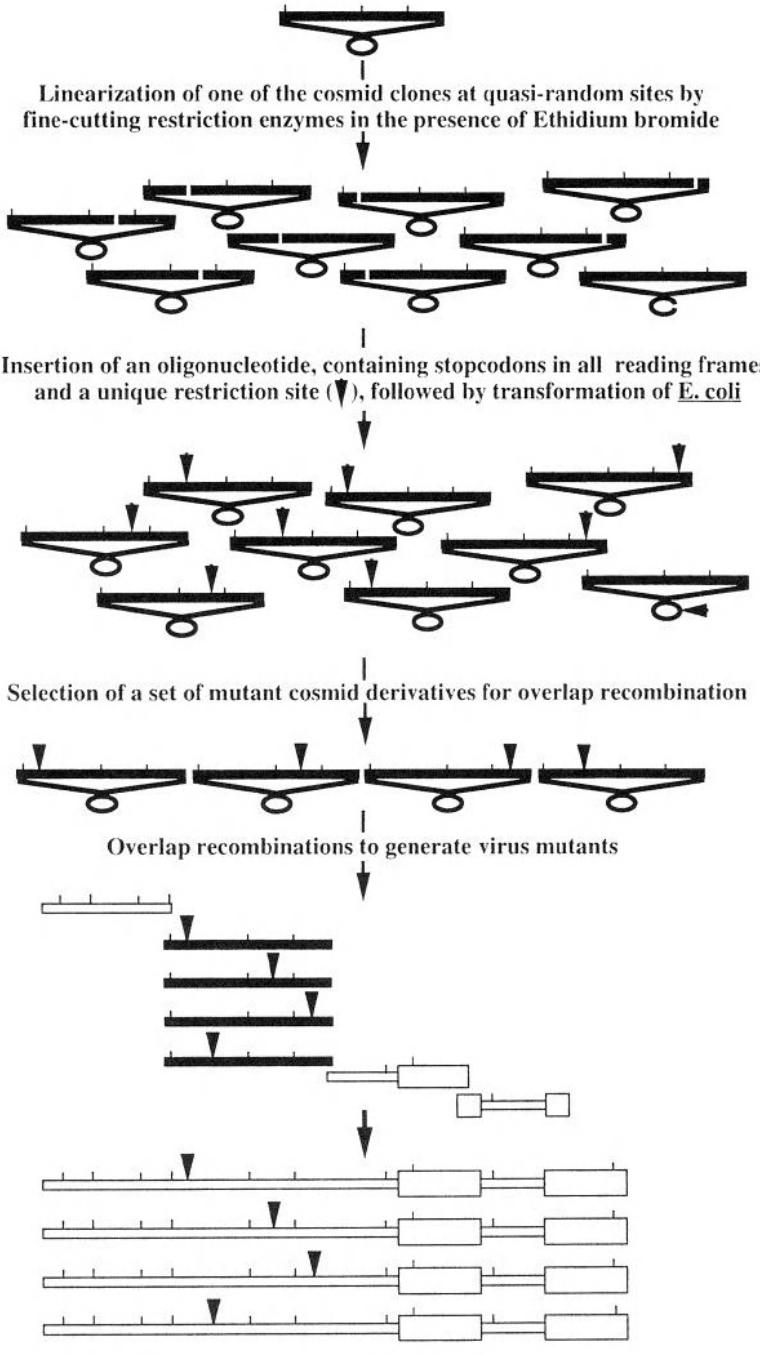
Fig 3. *(opposite)* Outline of the procedure for the insertion of the oligonucleotide at unique and random sites within a cosmid clone. This is followed by overlap recombinations, each including a mutant cosmid derivative and the three overlapping cloned fragments. A viable virus mutant is generated that lacks the expression of a single gene, if the gene into which a specific oligonucleotide is inserted, is not essential for the virus


Linearization of one of the cosmid clones at quasi-random sites by fine-cutting restriction enzymes in the presence of Ethidium bromide
Insertion of an oligonucleotide, containing stopcodons in all reading frames, and a unique restriction site (▼), followed by transformation of E. coli
Selection of a set of mutant cosmid derivatives for overlap recombination
Overlap recombinations to generate virus mutants

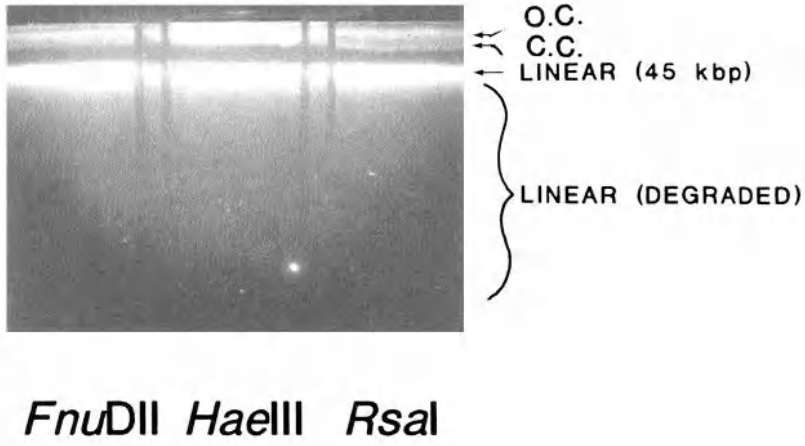


Fig. 4. A 45-kbp cosmid clone that is linearized at random sites by cleavage with blunt cleaving restriction sites with four-bp specificity (*Fnu*DII: CGCG; *Hae*III: GGCC; *Rsa*I: GTAC), in the presence of Ethidium bromide. OC, open circular DNA, CC, covalently closed DNA. Also indicated: (full length) linear DNA and multiply digested (degraded) linear DNA fragments.

(up to about 50%) of linearized plasmids. An agarose gel of such digests is shown in Fig. 4. After purification of the full-length linearized plasmids, the double-stranded oligonucleotide is inserted at the site of linearization. After recirculation of the resulting recombinant plasmids, *E. coli* is transformed and a library is obtained of recombinant plasmids, each carrying the inserted oligonucleotide at a unique and random site, essentially as depicted in Fig. 3. Mutant plasmids thus obtained are characterized for integrity and site of insertion of the oligonucleotide by restriction enzyme analysis (*see also* Note 7). A set of mutant virus strains is subsequently reconstituted by overlap recombination using a selected set of mutant plasmid inserts, together with overlapping cloned virus fragments, as described in Section 1.1.1. (*see* Fig. 3 *see also* Note 8). Viable virus mutants thus obtained completely lack the expression of a single gene (as exemplified in ref. *5*) and are amenable to phenotypic study in tissue culture *(see* refs. *8–14)*. In addition, they can also be used to study the effect of the gene on virulence, pathogenicity, and immunogenicity of the virus in animals *(6,13–16)*. Furthermore, oligonucleotide-bearing cosmid clones can be used as the starting point for further modification of the genome, e.g., for the generation of specified deletions into the virus genome or for the insertion of heterologous genes. We will give an outline of the different steps involved.

1.3. The Generation of Gene-Specific Probes Flanking the Inserted Oligonucleotide in Each Mutant to Explore the Virus Genome

The set of virus clones, each carrying an insertion of the oligonucleotide at a different, random site also permits easy access to gene-specific cloned short DNA fragments. These are subcloned after digestion of the oligonucleotide-bearing large virus clones with the restriction enzyme for which a unique site is present in the inserted oligonucleotide plus a second restriction enzyme having a 4-bp specificity that most likely cleaves nearby.

These subcloned short virus fragments are useful for a number of analyses (*see* Fig. 1C and ref. *6*):

1. As probes in cross hybridization experiments with DNA of a prototype herpesvirus to gain information on the genomic relationship between both viruses,
2. As probes in Northern blotting experiments on RNA isolated from infected cells to acquire information on location, size, and class of transcripts encoded by that specific region of the virus genome; and
3. For direct sequence analysis to identify the gene inactivated in each specific oligonucleotide insertion mutant

Given the scope of this chapter, we will only provide here an outline of the procedures involved in these experiments

The methodology described in this chapter requires careful planning and the labor of preparation and characterization of a cosmid library of the virus genome and of the subsequent mutagenesis procedure. The only prerequisites to the methodology are that viable virus be obtained by transfection of the virus genome (this may be assayed using the protocol given in Section 3 1.16.). In addition, a precise restriction map of the virus for one, or preferably more, diagnostic enzymes must be established in advance. In conclusion, if the herpesvirus under study is less well known, such a set of virus clones for overlap recombination, followed by oligonucleotide insertion mutagenesis, will be a valuable tool to rapidly perform a thorough analysis of gene structure and gene function of the virus.

2. Materials

2.1. Cloning the Virus Genome as a Cosmid Library

1 10X DNase buffer: 500 m*M* Tris-HCl, pH 7.4, 10 m*M* $MnCl_2$, 1 mg/mL bovine serum albumin. A stock solution of 250 µg DNaseI/mL is made by dissolving lyophilized enzyme in 1X DNase buffer. The stock is divided in small aliquots and frozen at –20°C Use each tube only once

2. Phenol: Add 8-hydroxyquinoline to 0.1% of phenol, which is liquified by warming. Extract repeatedly with equal volume of 0 1*M* Tris-HCl, pH 8 0, until the pH

of the aqueous phase is greater than 7 6. Aliquot in 50-mL conical tubes, and store in the dark at –20°C in 5 mL buffer.

3 Phenol chloroform isoamyl alcohol Mix 25 vol phenol with 24 vol chloroform and 1 vol isoamyl alcohol

4 3*M* Sodium acetate pH 5.2 and pH 7 0

5. TE. 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA pH 8.0 Sterilize both solutions by autoclaving.

6 Absolute ethanol

7. Isopropanol

8 70% v/v Ethanol

9 Running buffer (TAE) Usually made as a 50X concentrate· 242 g Tris base, 57 1 mL glacial acetic acid, 100 mL 0 5*M* EDTA pH 8.0/L. Prior to use, this concentrate is diluted 50X and 50 µL of a 10 mg/mL ethidium bromide solution is added per liter. **Warning:** Ethidium bromide is mutagenic. Wear gloves while handling solutions Also, store ethidium bromide solution shielded from light Use a good, preparative, quality agarose

10 5X sample buffer· 50% (w/v) sucrose, 1% (w/v) SDS, 0 1% Bromophenol blue

11 Size markers phage lambda DNA and phage lambda DNA, digested with *Hin*dIII, can best be purchased

12. Glycerol gradients· 50 m*M* Tris-HCl, pH 8 0, 300 m*M* NaCl, 1 m*M* EDTA pH 8 0, 15% v/v glycerol, and 50 m*M* Tris-HCl, pH 8 0, 300 m*M* NaCl, 1 m*M* EDTA pH 8 0, 40% v/v glycerol

13. Acid trituration buffer for preparing transformation-competent *E coli*· 100 m*M* $CaCl_2$, 70 m*M* $MgCl_2$, 40 m*M* sodium acetate pH 5 5 Filter sterilize The solution should be used fresh and be ice cold. **Note:** Preferably use disposable plasticware prerinse glassware thoroughly. Use the purest available water for all solutions. Any *recA*⁻ (recombination deficient) strain of *E coli* is suitable

14 Media for *E coli*. SOB 20 g Bacto tryptone, 5 g yeast extract. 0 58 g NaCl, 0 2 g KCl/L Autoclave, cool to 60°C and add 20 mL of a 1*M* sterile $MgSO_4$ solution/L SOC. SOB to which 20 mL/L of a 1*M* sterile glucose solution is added after autoclaving. LB· 5 g NaCl, 5 g yeast extract, 10 g tryptone, 0 3 mL 10*M* NaOH/L Autoclave. For LB agar: Add 15 g Bacto agar/L prior to autoclaving For ampicillin containing medium or plates. Add 0 001 vol of a filter sterilized aqueous solution of 100 mg/mL Na ampicillin in H_2O Medium must be cooler than 55°C for the addition of ampicillin

15 Plasmid isolation. solution I 25 m*M* Tris-HCl, pH 8 0, 10 m*M* glucose, 10 m*M* EDTA, pH 8 0, 0 02 w/v% NaN_3 **Warning:** NaN_3 is extremely toxic. Can also be prepared as 10X stock. Just before use, add lysozyme to a final concentration of 5 mg/mL. Solution II 0 2*M* NaOH (freshly diluted from a 10*M* stock), 1% SDS. Solution III 3*M* potassium acetate and 115 mL glacial acetic acid/L Store solutions at 4°C. In addition, isopropanol, phenol chloroform isoamyl alcohol, 10 mg/mL RNaseA solution in water, boiled for 5 min to inactivate DNase contamination This solution is aliquoted and frozen at –20°C

16 Transfection: HBS buffer· 140 m*M* NaCl, 5 m*M* KCl, 0 75 m*M* Na_2HPO_4, 6 m*M* dextrose, 25 m*M* HEPES in double distilled (or milliQ) water Shake the solution

well to saturate it with CO_2 (from air) and adjust the final pH to precisely pH 7 05 at room temperature using 0 5*N* NaOH Sterilize using a 0 22-μ filter, aliquot, and store at –20°C Other solutions· 2 5*M* $CaCl_2$, sterilized by filtration, aliquoted in 1-mL quantities, and stored at –20°C and 0.1X TE 1 m*M* Tris-HCl (pH 8.0), 0.1 m*M* EDTA (pH 8.0), filter sterilized, aliquoted, and stored at 4°C.

17. Cell culture a cell line permissive for virus growth (preferably well transfectable), growth medium, growth medium + 15% (w/v) glycerol, growth medium + 1% (w/v) methylcellulose as solidifying agent. Other cell culture requirements are PBS, trypsin, disposables, and so on.
18. DNA isolation from infected cells—lysis buffer 0.1*M* Tris-HCl, pH 8 0, 0.2*M* NaCl, 5 m*M* EDTA pH 8.0. 0.2 w/v% SDS. Add (just before use) proteinase K to 100 μg/mL. Proteinase K is kept as a frozen stock in water at 20 mg/mL at –20°C.
19. Other requirements—enzymes· restriction enzymes, Calf Intestinal Phosphatase (CIP), polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase. Nowadays, enzymes generally come with the appropriate reaction buffers. A neutralized solution containing each of the four dNTPs at a 2-m*M* concentration. A cosmid vector may be obtained from, e.g., Boehringer or Stratagene. A phage lambda packaging kit A Geneclean (Bio 101) or similar kit for elution of DNA fragments from agarose gels. 1-mL syringes, 25-gage needles. Quick Seal 0.5 × 2-in ultracentrifuge tubes Electroelution equipment. A gradient former.

2.2. Oligonucleotide Insertion Mutagenesis of Individual Cosmid Clones

1. 10X Restriction enzyme buffers for digestion in the presence of ethidium bromide· *Fnu*DII: 200 m*M* Tris-HCl pH 7.5, 80 m*M* $MgCl_2$, 500 μg/mL ethidium bromide, *Hae*III: 200 m*M* Tris-HCl pH 7.5, 500 m*M* NaCl, 80 m*M* $MgCl_2$, 50 μg/mL ethidium bromide, *Rsa*I. 200 m*M* Tris-HCl pH 7.5, 500 m*M* NaCl, 80 m*M* $MgCl_2$, 5 μg/mL ethidium bromide Store aliquoted at –20°C.
2. Other requirements. Commercially available ultracompetent cells Any *recA*$^-$ (recombination deficient) strain of *E coli* is suitable. Ultrapure preparative agarose (e g , Agarose NA. Pharmacia). A 10% (w/v) SDS solution.

3. Methods

3.1. Cloning the Virus Genome as a Cosmid Library

The different steps of this procedure are depicted schematically in Fig 2.

Virus can be grown, and virus DNA may be isolated. To obtain random fragments of the virus genome, two methods can be employed: mechanical (*see* Section 3.1.1.) and enzymatic (*see* Section 3.1.2.) shearing. Shearing is followed by further purification of DNA fragments with sizes between approx 33 and 40 kbp (*see* Notes 7 and 9) using centrifugation through a glycerol density gradient (*see* Section 3.1.3.), blunting of the DNA fragments using T4 DNA polymerase (*see* Section 3.1.5.), and addition of synthetic linkers for clon-

ing (*see* Section 3.1.5. and Note 10 for the criteria that the cloning linker should meet; *see also* Note 11). This is followed (if necessary) by ligation to a modified cosmid vector (*see* Sections 3.1.6.–3.1.11. and Note 12), packaging in vitro in phage lambda capsids, and infection of *E coli* (the packaging and infection steps are done using a commercially available packaging kit). Resulting bacterial colonies containing cloned virus DNA fragments are expanded, cosmids are isolated, and size, map location, and integrity of the inserts are analyzed by restriction enzyme digestion and gel electrophoresis. Finally, a set of clones is selected in which the inserts cover all of the genomic information of the virus, and that have overlaps at their ends, as described above and depicted in Fig 2 (*see* Notes 13 and 14). After characterization by restriction enzyme analysis, the selected set of clones is assayed for the regeneration of viable virus by overlap recombination, after transfection of cultured cells. Resulting virus is analyzed by digestion of virus DNA followed by gel electrophoresis.

3.1.1 Mechanical Shearing of the Virus Genome

1 20 µg Purified virus DNA is taken up in 0.5 mL TE and loaded into a 1-mL syringe. Attach a 25-gage needle to the syringe, fix firmly, and squeeze into a 10-mL polypropylene tube Repeat once
2. Transfer the sheared DNA to a microfuge tube and precipitate the DNA by the addition of 0.1 vol 3*M* sodium acetate pH 5 2 and 2 vol ethanol Mix and leave on ice for 15 min; spin down for 5 min in a microfuge at maximum speed Wash the pellet in 1 mL 70% ethanol and let the remaining ethanol evaporate at room temperature (*see* Note 15).
3. Add 100 µL TE to the pellet The DNA is dissolved by heating to 65°C for 5 min, followed by repeated pipeting up and down or by overnight storage at 4°C The DNA may subsequently be kept at 4°C
4 To check the sizes of the sheared fragments, 5 µL of DNA are loaded on a 0 5% agarose minigel Load 0 5 µg unsheared virus DNA as a control As marker fragments, 0.5 µg uncleaved phage lambda DNA and 1 µg of a *Hin*dIII digest of lambda DNA are loaded (*see* Note 16) Run the gel until the Bromophenol blue dye has migrated well off the gel. The DNA smear resulting from the shearing of the virus genome should migrate well below the intact virus genome, starting at around the band of uncleaved lambda DNA and extending below that band.
5 Next, the DNA fragments will be size selected on a glycerol density gradient (Section 3.1 3.)

3.1.2. Enzymatic Shearing of the Virus Genome

1. Mix, in a microfuge tube on ice, 40 µg virus genomic DNA, 100 µL 10X DNase buffer, distilled water to 1 mL. Add 10 ml of the DNaseI stock solution (DNaseI end concentration: 2 5 µg/mL) Rapidly transfer the tube to a 15°C water bath
2 Take 250-µL samples after 6, 9, 12, and 15 min of incubation. Immediately after sampling, add to each sample an equal volume of phenol chloroform.isoamyl-alcohol and vortex gently (*see* Note 17) for 30 s to inactivate the enzyme

3. Spin all samples in a microfuge at maximum speed for 2 min. Remove the upper phases each to a clean tube Add to each of these tubes 0 1 vol 3*M* sodium acetate pH 5 2 and 2 vol ethanol Mix and leave on ice for 15 min, spin down for 5 min in a microfuge at maximum speed. Wash the pellet in 1 mL 70% ethanol and let the remaining ethanol evaporate at room temperature (*see* Note 15).
4 Add 50 µL TE to each pellet The DNA is dissolved by heating to 65°C for 5 min, followed by repeated up-and-down pipeting or by overnight storage at 4°C The DNA can subsequently be kept at 4°C.
5 To check the sizes of the sheared fragments, 5 µL of DNA are loaded on a 0 5% agarose minigel. Load 0 5 µg unsheared virus DNA as a control. As marker fragments, 0.5 µg uncleaved phage lambda DNA and 1 µg of a *Hin*dIII digest of lambda DNA are loaded (*see* Note 16). Run the gel until the Bromophenol blue dye has migrated well off the gel The DNA smear resulting from the shearing of the virus genome, should migrate well below the intact virus genome, starting at around the band of uncleaved lambda DNA and extending below that band
6 Pool the fractions containing sheared DNA of approximately the right size for further fractionation on a glycerol density gradient (Section 3 1 3).

3.1.3. Size Selection of Subgenomic Fragments by Centrifugation Through a Glycerol Gradient

1. Using a gradient former, prepare in an SW40 tube two linear 15–40% glycerol density gradients, each of 11 mL in total
2. Carefully load the sheared DNA preparations, containing approx 20 µg DNA each in a volume of 100–200 µL TE buffer, on top of the gradients
3 Spin the tubes in an SW40 rotor for 5–7 h at 40,000 rpm, 20°C.
4. After centrifuging, hermetically seal the tubes with parafilm and carefully puncture the bottom of the tubes with a 25-gage needle. Take off the parafilm and collect 12-drop (i e , 0 6 mL) fractions in prenumbered microfuge tubes.
5. Load 25-µL aliquots of every second fraction on a 0 5% agarose gel, using as markers uncleaved lambda DNA and lambda DNA, cleaved with *Hin*dIII (*see also* Note 16)
6. Pool fractions containing DNA from the desired size range, dilute with TE to 5 mL (dilute at least twofold), and centrifuge in a SW50 rotor for 5 h at 50,000 rpm, 20°C to precipitate the DNA
7 Carefully discard the supernatant and dissolve the DNA in 20 µL TE.
8. Run 1 µL on a 0.5% agarose minigel together with several loadings of between 0.1 and 1 µg of lambda DNA to estimate its concentration.

3.1.4. Addition of Synthetic Linkers to the Sheared DNA Fragments and to the Cosmid Vector

The ends of the sheared- and size-selected virus DNA fragments are blunted, after which synthetic linkers are to be added at the ends for cloning (*see* Note 10). If this site is not also present in the cosmid, one of the unique cloning sites in the cosmid vector (most cosmid vectors contain, e.g., a unique *Bam*HI site)

must be converted to the new unique cloning site by linker addition, for which we also provide the protocol. For both linker addition to the sheared virus DNA fragments and to the cosmid vector, linkers should be phosphorylated at their 5' ends, as also described in Section 3.1.5.

3.1.5. Blunting the Sheared Virus DNA Fragments and Addition of a Restriction Site for Cloning

This subprotocol includes a number of separate enzymatic steps:

1. Blunting the sheared DNA fragments with T4 DNA polymerase (*see* Note 18),
2. Phosphorylation of the linker,
3. Ligation of an excess of linker (*see* Note 19) containing the cloning site, to both ends of the DNA fragments,
4. Removal of the excess of linkers by cleaving with the restriction enzyme for which the linker has a site followed by purification on an agarose gel.

This cloning procedure may partially be performed simultaneously with the addition of (the same) linker to the cosmid vector (*see* Section 3.1.6.) It is recommended to set aside a sample of 0.1–0.2 µg after each step for analysis on a 0.5–0.8% agarose minigel.

1. Mix on ice in a microfuge tube the following. 5 µg sheared DNA, 2 µL 10X T4 polymerase buffer (*see* Note 20), 1 µL of a 2-m*M* dNTP mix, 1 U T4 polymerase, distilled water to 20 µL Incubate for 15 min in a 12 °C water bath and return the tube to ice.
2. To inactivate the enzyme, add to each tube 80 µL TE and 100 µL phenol:chloroform:isoamyl alcohol. Vortex for 30 s and centrifuge at maximum speed for 2 min in a microfuge Transfer the upper phases to clean microfuge tubes, add to each tube 0 1 vol 3*M* sodium acetate pH 5.2 and 2 vol ethanol. Mix and leave on ice for 15 min. Spin down for 5 min in a microfuge at maximum speed. Wash the pellet in 1 mL 70% ethanol and let the remaining ethanol evaporate at room temperature (*see* Note 15). Dissolve the pellet in 5 µL TE by overnight storage at 4°C or by incubation at 65°C for 15 min
3. Mix in a microfuge tube: 1 µL 10X polynucleotide kinase buffer, 2 µg nonphosphorylated linkers, 1 µL 10 m*M* ATP, 10 U polynucleotide kinase, distilled water to 10 µL Incubate for 1 h at 37°C. Heat the tube at 65°C for 15 min to inactivate the enzyme.
4. Add to the microfuge tube containing the sheared and blunted virus DNA· a 50-fold molar excess of phosphorylated linker (i.e., approx 7.5 ng), 2 µL ligation buffer, 5 (Weiss) Units T4 ligase, distilled water to 20 µL. Incubate overnight at 15°C.
5. Add to each tube 80 µL TE and 100 µL phenol chloroform.isoamyl alcohol, vortex gently for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Subsequently, precipitate the DNA by addition of 0 1 vol 3*M* sodium acetate pH 5 2 and 2 vol ethanol Store on ice for 15 min and centrifuge for 5 min at maximum speed Wash the pellet with 1 mL 70% ethanol and dissolve the DNA in 10 µL TE by heating for 15 min at 65°C or by overnight incubation at 4°C.

6. Add to the DNA: 5 µL of the appropriate 10X digestion buffer, 50 U of the restriction enzyme for which the linker has a site, and distilled water to 50 µL. Mix well using a micropipet and incubate for 4 h at the appropriate temperature for the enzyme.
7. Load the entire digestion mixture on a 0.5% preparative agarose gel and electrophorese until the bromophenol blue marker has migrated 2 cm into the gel.
8. Cut out the DNA smear and elute using an electroelution apparatus.
9. After electroelution, transfer the eluted DNA to a microfuge tube, add 1 vol phenol:chloroform·isoamyl alcohol, vortex for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Repeat if necessary. Subsequently, precipitate the DNA by addition of 0.1 vol Na-acetate pH 5.2 and 2 vol ethanol Mix and store on ice for 15 min and centrifuge for 5 min at maximum speed. Wash the pellet with 1 mL 70% ethanol and dissolve the DNA in 10 µL TE by heating for 15 min at 65°C or by overnight incubation at 4°C.

3.1.6. Generation of a Cosmid Vector Containing a Specified Unique Cloning Site by Linker Addition

Similarly to the addition of the cloning linker to the sheared and blunted virus DNA, the insertion of [the same] restriction site in the cosmid vector consists of a number of separate enzymatic steps.

1 Cleavage of the cosmid vector to linearity with the enzyme at which site a novel cloning site will be generated;
2 Blunting of the linearized cosmid vector;
3. Ligation of an excess of linker (*see* Note 19) containing the cloning site, to the resulting molecule;
4. Removal of the excess of linkers by cleaving with the restriction enzyme for which the linker has a site followed by purification on an agarose gel. Steps 3 and 4 may be performed simultaneously with the addition of a linker to the sheared DNA fragments as described in the previous section;
5. Religation of the cosmid that now contains a single linker molecule; and
6. Transformation of competent *E coli*. Bacterial colonies are expanded and DNA of the linker containing cosmid vector is isolated and analyzed (*see* Note 21).

Set aside a sample of 0.1–0.2 µg after each step for analysis on a 0.5–0.8% agarose minigel. In addition, it is recommended to prepare competent *E coli* cells first (*see* Section 3.1.7.).

1. Mix the following in a microfuge tube: 5 µg covalently closed cosmid vector, 4 µL of the appropriate 10X restriction enzyme digestion buffer, 20 U restriction enzyme for which the site is to be sacrificed for the insertion of a new cloning site, and distilled water to 40 µL. Incubate for 2 h at 37°C.
2. Put the tube containing the digest on ice and add 2 µL 2 m*M* dNTP mix and 5 U T4 polymerase (*see* Note 18). Incubate for 15 min in a 12°C water bath.
3. To inactivate the enzyme, add to the tube 60 µL TE and 100 µL phenol chloroform:isoamyl alcohol. Vortex for 30 s and centrifuge at maximum speed for 2 min

Transfer the upper phase to a clean microfuge tube, add 0 1 vol 3*M* sodium acetate pH 5 2 and 3 vol of ethanol Mix and leave on ice for 15 min Spin down for 10 min in a microfuge at maximum speed Wash the pellet in 1 mL 70% ethanol and let the remaining ethanol evaporate at room temperature Dissolve the pellet in 10 µL TE

4. Mix in a microfuge tube: 2 µg linearized and blunted cosmid vector with a 50-fold molar excess of phosphorylated linker (*see* Section 3.1 5 , step 3, i e , approx 200 ng), 2 µL ligation buffer, 5 (Weiss) units T4 ligase, and distilled water to 20 µL Incubate overnight at 15°C
5. Add to each tube 80 µL TE and 100 µL phenol:chloroform.isoamyl alcohol. Vortex for 30 s and centrifuge at maximum speed for 2 min in a microfuge Transfer the upper phases to clean microfuge tubes, add to each tube 0 1 vol 3*M* sodium acetate pH 5.2 and 3 volumes ethanol Mix and leave on ice for 15 min Spin down for 5 min in a microfuge at maximum speed Wash the pellet in 1 mL 70% ethanol and let the remaining ethanol evaporate at room temperature. Dissolve the pellet in 10 µL TE The DNA may be stored at 4°C or at –20°C
6. Mix in a microfuge tube 1 µg of the DNA, 5 µL of the appropriate 10X digestion buffer, 100 U of the restriction enzyme for which the linker has a site, and distilled water to 50 µL Incubate for 4 h at the appropriate temperature for the enzyme.
7 Load the entire digestion mixture on a 0 8% preparative agarose gel and electrophorese until the dye band has migrated approx 2 cm.
8 Cut out the cosmid vector band and elute using a Geneclean (or similar) kit Take the DNA up in 10 µL TE
9. Add to the DNA. 2 µL ligase buffer, 1 (Weiss) Unit T4 ligase, and distilled water to 20 µL. Incubate for 2 h to overnight at 15°C The ligation mixture can be stored at –20°C (*see* Note 22).
10 Transform competent *E. coli* (*see* Section 3.1.7.) with 2 µL of the ligation mixture, as described below (*see* Section 3.1.8.).
11 Inoculate the next day, using wooden toothpicks, 6–12 tubes containing 2 mL LB plus ampicillin with fresh single transformant colonies. Grow overnight at 37°C in a shaking incubator. Perform minipreps as described in Section 3.1.9
12. Test the quality of the competent cells by transformation of 0 1 ng supercoiled control plasmid (like pBR 322), as described in Section 3.1.8. The efficiency of transformation should be equal to, or higher than, 5×10^6 colonies per µg plasmid. Also include, as a negative control, a mock transformation with no added DNA
13 Reinoculate a colony containing the desired cosmid vector in a flask containing 15 mL LB + ampicillin, grow overnight in a shaking incubator at 37°C, and isolate the plasmid using a 10-fold scaled up version of the protocol described in Section 3.1.9.

3.1.7. Preparation of Competent E. coli *Cells*

Most protocols for making *E. coli* competent for uptake of DNA rely on an incubation of cells in a solution containing $CaCl_2$. The method described here, acid trituration *(17)*, is no exception to this and yielded good results in our hands. We routinely use strain DH5; however, any strain may be suitable provided that the strain is recombination deficient (*recA*$^-$). **Note:** It is important that the glass-

ware used is thoroughly rinsed with sterile ultrapure water prior to autoclaving. In addition, all steps from step 4 on must be carried out at 0–4°C.

1. Pick a fresh colony, inoculate a 200-mL sterile flask containing 25 mL LB, and incubate overnight at 30°C in a shaking incubator
2. Inoculate a 2-L flask containing 500 mL LB with 5 mL cells of the overnight culture
3. Shake the culture at 30°C until the OD_{600} reaches 0 45–0 55
4. Transfer the cells to ice water and leave on ice for 2 h
5. Centrifuge the cells for 15 min at 2500*g*, 4°C
6. Gently resuspend the cells in 20 mL fresh ice-cold trituration buffer and add, subsequently, 480 mL more ice-cold trituration buffer
7. Incubate the cells on ice for 45 min
8. Centrifuge the cells for 20 min at 1800*g*, 4°C.
9. Gently resuspend the cells in 50 mL ice-cold trituration buffer
10. While gently swirling the cells in ice water, slowly add glycerol to a final concentration of 15% v/v
11. Aliquot the cells in 0.2- to 1-mL portions in microfuge tubes and freeze on dry ice The cells can be stored for at least 6 mo at –80°C
12. Test the quality of the competent cells by transformation of 0.1 ng supercoiled control plasmid (like pBR322), as described in Section 3.1.8 The efficiency of transformation should be equal to, or higher than, 5×10^6 colonies per µg plasmid Also include, as a negative control, a mock transformation with no added DNA.

3.1.8. Transformation of E coli

1. Pipet 1–10 µL of a solution containing up to 50 ng DNA on the bottom of a sterile microfuge tube on ice (*see* Note 23)
2. Thaw, on ice, an aliquot of frozen competent cells and gently pipet 200 µL ice-cold cells on the DNA solution
3. Mix by tapping and incubate for 30–60 min on ice
4. Heat the tubes in a 42°C water bath for 90 s.
5. Transfer the tubes to ice water for 2 min.
6. Add 800 µL SOC medium and incubate at 37°C for 30–60 min
7. Plate out aliquots on LB agar plates containing ampicillin (*see* Note 24)
8. Incubate overnight at 37°C.

3.1.9. Small-Scale Isolation of Plasmid DNA ("minipreps")

This procedure is derived from the original alkaline lysis method *(18)*. It can easily be scaled up if more DNA is required. In the latter case, use 15 or 50 mL conical tubes instead of microfuge tubes and centrifuge 5X longer, at 5000*g*.

1. Inoculate numbered tubes with 2 mL LB containing ampicillin with fresh single transformant colonies using wooden toothpicks. Grow overnight at 37°C in a shaking incubator
2. Transfer 1 5 mL of the cultures to microfuge tubes and centrifuge for 1 min at maximum speed

3 Decant the supernatants, tapping off the last drops
4. Resuspend the cell pellets by vortexing in 0 2 mL cold solution 1 Incubate for 5 min on ice
5. Add 0 4 mL solution 2, mix by shaking, and incubate for 5 min on ice.
6. Add 0.3 mL solution 3, mix by shaking, and incubate for 5 min on ice
7 Spin for 5 min at maximum speed
8 Carefully transfer the supernatants to new microfuge tubes containing 2 µL RNase A stock solution Mix and incubate for 15 min at 37°C
9. Add 0 4 mL phenol.chloroform.isoamyl alcohol. Vortex for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to new tubes
10. Add 0 7 mL isopropanol, mix, and centrifuge for 5 min at maximum speed. Discard the supernatants, centrifuge briefly, and remove the last drops using a micropipet
11 Dissolve the pellets in 50 µL TE. The plasmid preps may be stored at 4°C or at –20°C.

3.1.10. Cleavage and Phosphatase Treatment of the Cosmid Vector

Prior to ligation to the virus DNA fragments, the vector, that now contains the appropriate cloning site, is linearized. Subsequently, the 5' phosphate moieties are removed by treatment with calf intestinal phosphatase (CIP). This prevents recirculation of the vector

1. In a microfuge tube, mix the following 2 µg cosmid vector (approx 20 µL of a miniprep), 4 µL of the appropriate 10X restriction enzyme digestion buffer, 10 U restriction enzyme that cleaves the cloning linker, and distilled water to 40 µL Incubate for 2 h at 37°C.
2 Add 80 µL TE and one vol phenol chloroform isoamyl alcohol Vortex for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube Subsequently, precipitate the DNA by addition of 0.1 vol 3*M* sodium acetate pH 5.2 and 3 vol ethanol Mix and store on ice for 15 min, and centrifuge for 10 min at maximum speed. Wash the pellet with 1 mL 70% ethanol and take the DNA up in 48 µL distilled water Take apart a sample of 5 µL (i e , approx 0.2 µg) as a control for the efficiency of the CIP treatment (step 6).
3. To the rest of the DNA, add 5 µL 10X CIP buffer and 1 U CIP Incubate for 30 min at 37°C, add again 1 U CIP, and reincubate for 30 min at 37°C (*see* Note 25).
4. Add 50 µL TE and 1 vol phenol. chloroform. isoamyl alcohol. Vortex for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Repeat the extraction twice, both times using 50 µL chloroform. Subsequently, precipitate the DNA by addition of 0.1 vol 3*M* sodium acetate pH 5.2 and 3 vol ethanol. Mix and store on ice for 15 min and centrifuge for 10 min at maximum speed Wash the pellet with 1 mL 70% ethanol and dissolve the DNA in 10 µL TE. The DNA can be stored at –20°C.
5 To verify cleavage and yield of the vector, electrophorese 1 µL on a 0 8% agarose minigel Also load 0 2 µg uncleaved cosmid vector DNA as a marker
6. To verify the efficiency of the CIP treatment, mix 0 1 µg of the dephosphorylated vector with 1 mL 10X ligase buffer, 1 (Weiss) Unit T4 ligase and distilled water

to 10 µL As a control, perform the same ligation using 0.1 µg of the untreated cleaved DNA, set apart in step 2. Incubate for 4 h to overnight at 15°C and transform *E. coli* with 2 µL of both ligation mixtures, as described in Section 3.1.8. The CIP-treated DNA should yield at least 10-fold less colonies than the untreated DNA

3.1.11 Cloning of Virus DNA Fragments into the Cosmid Vector

The virus DNA fragments are ligated to the linearized dephosphorylated vector fragments under conditions that favor the formation of linear concatemers (*see* Fig. 2). Since the vector does not self-ligate owing to the CIP treatment, an excess of vector is used to ensure the formation of vector-insert hetero concatemers. Subsequently, a monomer is packaged into empty phage lambda heads using a commercially available packaging kit. During the latter process, the concatemers are cleaved at the *cos* sites present in the vector molecules; the virus DNA fragment between two subsequent cleaved *cos* sites is then internalized into the lambda heads, using the packaging signal present in the *cos* sites. After packaging, *E coli* is infected with the phages; this is followed by selection on ampicillin containing LB plates (Fig. 2). Transformant *E. coli* colonies contain the recircularized cosmid containing the inserted virus DNA fragment. We do not provide a protocol here for packaging and infection since the procedure varies somewhat depending on the packaging kit used; a detailed protocol comes with each commercial kit.

1. Mix 1 µg of the prepared sheared virus DNA, 1 µg dephosphorylated cosmid vector DNA, 1 µL 10X ligase buffer, 1 (Weiss) Unit T4 ligase, and distilled water to 10 mL. Incubate for 4 h overnight at 15°C
2. Package 0.5 µg of the concatemers in phage lambda heads and infect (and plate out) *E coli* as described in the manual of the packaging kit.

3.1.12. Analysis of Cosmid Clones

Cosmid clones containing virus DNA must be thoroughly analyzed to fulfil the following criteria: (1) At least one (*see* Note 8) set of clones, containing all of the genomic information of the virus (*see* Note 14) and having overlaps of the optimal size, has to be selected (*see* Note 13); and (2) it should be verified that the selected cloned virus DNA fragments do not contain deletions or rearrangements.

To these purposes, miniprep DNA of the cloned virus DNA fragment is cleaved with mixtures of two restriction enzymes: the enzyme that cleaves the cloning site for the virus DNA (this liberates the insert from the vector), together with a diagnostic enzyme, for which a precise restriction map of the virus genome is available. On an agarose gel, size, overlaps, and integrity of the cloned virus DNA fragments can then be analyzed.

1 Using wooden toothpicks, inoculate, with fresh single transformant colonies, all wells of two sterile marked 96-well microtiter plates containing 100 µL LB containing 15% glycerol as well as ampicillin, per well. Use the first 48–96 toothpicks to also inoculate numbered tubes with 2 mL LB containing ampicillin (*see* Note 26) Cover the well plates and incubate the plates overnight at 37°C in a standing incubator (*see* Note 27). Incubate the tubes overnight at 37°C in a shaking incubator.
2 Freeze the well plates in a –80°C freezer These minicultures are stable and serve as a stock
3. Prepare cosmid miniprep DNA from the 48–96 inoculated 2-mL cultures, as described in Section 3 1 9
4 Mix in a microfuge tube 5 µL miniprep DNA, 2 µL of the appropriate digestion buffer (*see* Note 28), 5 U each of the diagnostic enzyme and of the enzyme that cleaves the cloning linker, distilled water to 20 µL In addition, cleave 4 µg of total purified virus DNA, in a volume of 40 µL, with 20 U of the diagnostic enzyme (this is sufficient for two gel loadings). Incubate the digestions for 2 h at the appropriate temperature
5 Load all digestions on a 0 8% agarose gel, together with a *Hin*dIII digest of phage lambda DNA as a marker (*see* Note 16) and electrophorese until the bromophenol blue dye has migrated at least 10 cm
6 Compare the digests of the various cosmids with that of the cleaved total virus DNA This enables one to determine the location of the cloned fragments on the virus restriction map In addition this enables screening for clones that have overlaps of the desired size. Moreover, large deletions or rearrangements are easily detected. If no complete set for overlap recombination can be constituted using the first 48–96 analyzed clones, inoculate another 48–96 clones from the frozen stocks, and repeat the analysis. Repeat the procedure until sufficient, apparently intact, clones have been found to constitute one or more sets of clones for overlap recombination. Include ample backup clones since minor rearrangements may be found during more detailed analysis
7 Once a set of clones to be tested in overlap recombination is selected, proceed by growing up a 500-mL LB + ampicillin culture of each clone in 2-L flasks Start from the frozen stocks
8. Isolate and purify cosmid DNA by a scaled-up alkaline lysis and Cesium chloride-ethidium bromide density gradient centrifugation, as described in Section 3 1.13

3.1.13. Large-Scale Plasmid Isolation by Alkaline Lysis, Followed by Cesium Chloride-Ethidium Bromide Density Gradient Centrifugation

The first part of this procedure (steps 1–6) is essentially the same as the miniprep procedure described in Section 3.1.9.

1 Centrifuge the cultures for 15 min at 5000*g*.
2 Decant the supernatants, shaking off the last drops
3. Resuspend the cell pellets by vortexing in 8 mL cold solution 1 Transfer the cells to 50-mL conical tubes and incubate for 5 min on ice.
4 Add 16 mL solution 2, mix thoroughly by inversion, and incubate for 5 min on ice

5. Add 12 mL solution 3, mix thoroughly by inversion, and incubate for 20 min on ice.
6. Centrifuge 30 min at 5000*g*, 4°C
7. Carefully transfer the supernatants to new 50-mL conical tubes, taking care not to transfer any of the loose pellet. Precipitate the nucleic acid by adding 0 6 vol isopropanol, mix, and immediately centrifuge 15 min at 5000*g*, room temperature. Discard the supernatant, centrifuge briefly, and remove the last drops using a micropipet
8. Dissolve the pellets in 3.7 mL TE Add 4 3 g CsCl, dissolve, and add 0 6 mL 10 mg/mL ethidium bromide solution (*see* Note 29). Mix and centrifuge for 15 min at 5000*g* at room temperature to remove the precipitated protein
9. Fill with the supernatant 5 mL quick seal (0.5 × 2 in.) centrifuge tubes, equilibrate, seal, and centrifuge for 8–16 h at 65,000 rpm at 15°C in a VTi 80 vertical rotor
10. Pierce the top of the tubes with a 25-gage needle Under long-wave UV light, two bands are visible. The upper one consists of bacterial DNA and relaxed cosmid DNA, the bottom one consists of supercoiled cosmid DNA Pierce the tubes just below the lower band with a 25-gage needle attached to a 1-mL syringe and drain the supercoiled (lower) cosmid band. Transfer the DNAs to 15-mL conical tubes taking care not to shear the DNA by squeezing the syringe too hard
11. Add 3 vol TE and 2–3 (end) vol ethanol. Mix and leave for 15 min on ice Centrifuge the tubes for 15 min at 5000*g* at 4°C Discard the supernatant and remove the last drops using a micropipet after recentrifuging briefly. Dissolve the pellets in 400 µL TE and transfer the solution to microfuge tubes
12. To remove the ethidium bromide, extract the DNA by vortexing gently for 30 s with 1 vol phenol:chloroform:isoamyl alcohol. Centrifuge for 5 min at 5000*g* and transfer the supernatant to a new tube Repeat the extraction until the supernatant does not fluoresce any more when kept in front of a long-wave UV lamp. Precipitate the DNA by adding 0 1 vol 3*M* sodium acetate and 2 vol ethanol After inversion of the tubes, a cloudy DNA precipitate should appear
13. Fish out the DNA precipitate using a drawn-out Pasteur pipet, wash the DNA in 70% ethanol, and transfer to a new microfuge tube. Centrifuge briefly, pipet off the ethanol, let the remaining ethanol evaporate, and dissolve the DNA overnight at 4°C (or 15 min at 65°C) in 200 µL TE. Store the DNA at 4°C.
14. Determine the DNA purity and concentration by measurement of the OD_{260}/OD_{280} ratio (*see* Note 30)
15. Verify the identity and integrity of the cosmids by digesting of 1 µg DNA with appropriate diagnostic restriction enzymes, followed by gel electrophoresis, as described in the previous section (steps 4 and 5).

3.1.14. Regeneration of Virus by Overlap Recombination of Cloned Subgenomic DNA Fragments

Once a set of overlapping cloned subgenomic cloned fragments of the virus, that meets the requested criteria, has been selected and expanded, the fragments will be used to test regeneration of viable virus after cotransfection of permissive cells. For efficient recombination, the cloned inserts have to be excised from the cosmid vector by restriction enzyme digestion, using the

enzyme that cleaves the cloning linker (*see also* Note 2). The digested cosmid clones may subsequently be used for transfection. Regenerated virus is expanded for DNA analysis and, if possible, for experimental infection of model animals. If results of these assays do not show differences with control assays using nonmanipulated virus, the set of cosmid clones is used as a basis for the generation of large series of oligonucleotide insertion mutants, as described in the following part of this chapter (*see* Section 3.2.).

3.1.15. Excision of Cloned Fragments from the Cosmid Vector

1. For every cosmid clone to be used in overlap recombination, mix in a microfuge tube 10 µg CsCl purified cosmid DNA, 5 µL 10X digestion buffer for the restriction enzyme that cleaves the cloning linker, 50 U of the enzyme, and distilled water to 50 µL. Incubate for 2 h at the indicated temperature for the enzyme. Electrophorese 1 µL of each digest on a 0.5–0.8% agarose minigel to verify completeness of the digestions. Load 0.2 µg of each uncleaved cosmid clone as a control.
2. Add 50 µL TE and 1 vol phenol:chloroform:isoamyl alcohol. Vortex gently for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Subsequently, precipitate the DNA by addition of 0.1 vol 3*M* sodium acetate pH 5.2 and 2 vol ethanol. After inversion of the tubes, a cloudy DNA precipitate should appear. Fish out the DNA precipitate using a drawn-out Pasteur pipet, wash the DNA in 70% ethanol and transfer to a new microfuge tube. Centrifuge briefly, pipet off the ethanol, let the remaining ethanol evaporate, and dissolve the DNA overnight at 4°C (or 15 min at 65°C) in 10 µL sterile 0.1X TE. The DNA may be stored at 4°C.

3.1.16. Calcium Phosphate Transfection

The most common method to transfect adherent cells with DNA is by calcium phosphate-DNA coprecipitation *(19)*. These coprecipitates are taken up by the cell by pinocytosis. Intracellularly, the homologous ends of the internalized DNA fragments recombine resulting in the regeneration of an intact virus genome (*see also* Note 14). Ultimately, this will lead to the production of virus particles that infect neighboring cells, thus resulting in plaque formation. This protocol consumes 20 µg DNA (*see* Note 31). It is advised to transfect, as a positive control, total purified virus DNA.

1. One day before transfection, seed a 10-cm dish with permissive cells so that the cell density will be 25–40% at the time of transfection. Add medium to a total of 10 mL to the cells.
2. For every transfection, mix in a sterile microfuge tube, in equimolar amounts, a total of 20 µg of digested cosmid clones. Take up in 950 µL HBS buffer and vortex gently for 10 s.
3. Add 50 mL 2.5*M* $CaCl_2$ and immediately vortex gently for 30 s. Leave the tube for 20 min at room temperature in the dark.

4 Resuspend the precipitate well by pipeting a few times up and down and add the precipitate dropwise to the cells (*see* Note 32). Swirl the medium and incubate the cells 4–5 h at 37°C in the CO_2 incubator (*see* Note 33).
5. Aspire the medium, add 2 mL medium containing 15% glycerol and swirl gently. After 90 s (*see* Note 34), aspire the medium and wash the cells twice with 10 mL sterile PBS. Add 10 mL medium and incubate at 37°C in the CO_2 incubator until a generalized cytopathic effect is visible (*see* Note 35).

3.1.17. Analysis of Reconstituted Virus

The virus obtained by overlap recombination must thoroughly be investigated to ascertain that no mutations, that have escaped detection during the analysis of the individual cosmid clones, have occurred during the process of manipulation, cloning, and transfection of the virus DNA. Several genotypic and phenotypic parameters may be analyzed to accomplish this. First, a thorough comparison should be made between restriction digests of the genomes of both virus that is reconstituted by overlap recombination, and nonreconstituted (i.e , nonmanipulated) virus Although it is helpful to posses a restriction map for a number of diagnostic enzymes, this is not an absolute requirement. Digests may also be performed "at random," i.e., with an enzyme for which cleavage sites are not known; this provides fingerprints of both the nonmanipulated and the manipulated virus genomes. It is clear that both fingerprints should be identical. We are unable to give state-of-the-art protocols for these analyses since protocols somewhat depend on the virus under study, the enzymes used, and so on. We will however provide a protocol of the methodology as was applied for PRV. Next to this type of genotypic analysis, reconstituted virus should be compared with nonmanipulated virus with respect to various phenotypic parameters as, e.g., plaque phenotype, growth in cultured cells and, ultimately, pathogenesis and virulence in experimentally infected animals. Since again there is no exclusive protocol for this, and phenotypic analysis of herpesvirus does not fall within the scope of this chapter, we do not provide protocols for these experiments here. However, one may find applicable protocols in other chapters of this volume.

3.1.18. Genotypic Analysis of the Reconstituted Virus Genome

For PRV, we have been able to study the genome of reconstituted strains by directly isolating total DNA from cells infected for 8 h (i.e., the duration of a complete infection cycle) at high multiplicity. This DNA consists for a major part of virus DNA; agarose gels of restriction enzyme digestions will show a foreground of bands derived from the virus genome and a background smear consisting of digested cellular DNA. A photograph of such a gel is shown in Fig. 5. If the virus

Fig. 5. *(opposite)* Example of a series of oligonucleotide insertion mutants that are generated in a cosmid (named c-448) that carries 41 kbp of the PRV genome *(6)*

Top panel: (A) Physical map of the PRV genome (*see also* Fig. 1A). Indicated are the recognition sites of the diagnostic enzyme *Bam*HI The numbering refers to the respective *Bam*HI fragments (B) The four PRV cloned subgenomic regions used in overlap recombination, (C) PRV subgenomic region cloned in cosmid c-448. Diagnostic enzymes used for mapping the site of insertion were (B) *Bam*HI, Bg, *Bgl*II, K,*Kpn*I, S, *Sca*I. Arrows indicate the position of the insertion of the oligonucleotide in the different mutant derivatives of c-448 that were used in overlap recombination V. the specific mutant yielded a viable virus mutant after overlap recombination. Bottom panel Agarose gel of *Bam*HI + *Eco*RI double digests of most of the oligonucleotide containing mutant PRV strains indicated on the top panel (C) Note that *Eco*RI only cleaves the inserted oligonucleotide. Mutant BΔ420-114 contains a deletion of 0 2 kbp in the thymidine kinase gene, between the oligonucleotide insertion sites of mutants 420 and 114, that is introduced by exchanging *Eco*RI-*Bgl*II fragment of mutant 420 for the corresponding fragment of mutant 114 Left are indicated the *Bam*HI fragments of the virus (*see* top panel [A]) Right. Sizes of the marker fragments (*see also* Note 16). Sizes of the *Bam*HI fragments after cleavage of the inserted oligonucleotide by *Eco*RI, in the various mutant PRV strains.

Mutagenized fragment	Mutant number	New fragments generated by *Eco*RI digestion, kbp
*Bam*HI fragment 2 (18 1 kbp)	B3	7 5,10.6
*Bam*HI fragment 2 (18 1 kbp)	B4	9 1, 9 0
*Bam*HI fragment 2 (18 1 kbp)	B23	10 0, 8 1
*Bam*HI fragment 2 (18.1 kbp)	B1	10.9, 7 2
*Bam*HI fragment 2 (18 1 kbp)	B6	12.0, 6 1
*Bam*HI fragment 2 (18 1 kbp)	B14	14 0, 4.1
*Bam*HI fragment 2 (18 1 kbp)	B31	14 6, 3 5
*Bam*HI fragment 2 (18.1 kbp)	B17	16 4, 1 5
*Bam*HI fragment 2 (18.1 kbp)	B25	17 4, 0 7
*Bam*HI fragment 2 (18.1 kbp)	BΔ420-114	2.8, 0.2
*Bam*HI fragment 2 (18.1 kbp)	B15	0.6, 0.2
*Bam*HI fragment 2 (18.1 kbp)	B35	8.5, 0 9
*Bam*HI fragment 2 (18.1 kbp)	B9	7.8, 1 6
*Bam*HI fragment 2 (18 1 kbp)	B59	7 5, 1.9
*Bam*HI fragment 2 (18.1 kbp)	B267	7 0, 2 4
*Bam*HI fragment 2 (18.1 kbp)	B26	13 7, 2.3
*Bam*HI fragment 2 (18 1 kbp)	B24	11 2, 4.8
*Bam*HI fragment 2 (18.1 kbp)	B5	10 2, 5 8
*Bam*HI fragment 2 (18 1 kbp)	B34	9 5, 6 5
*Bam*HI fragment 2 (18.1 kbp)	B37	8 3, 7 7

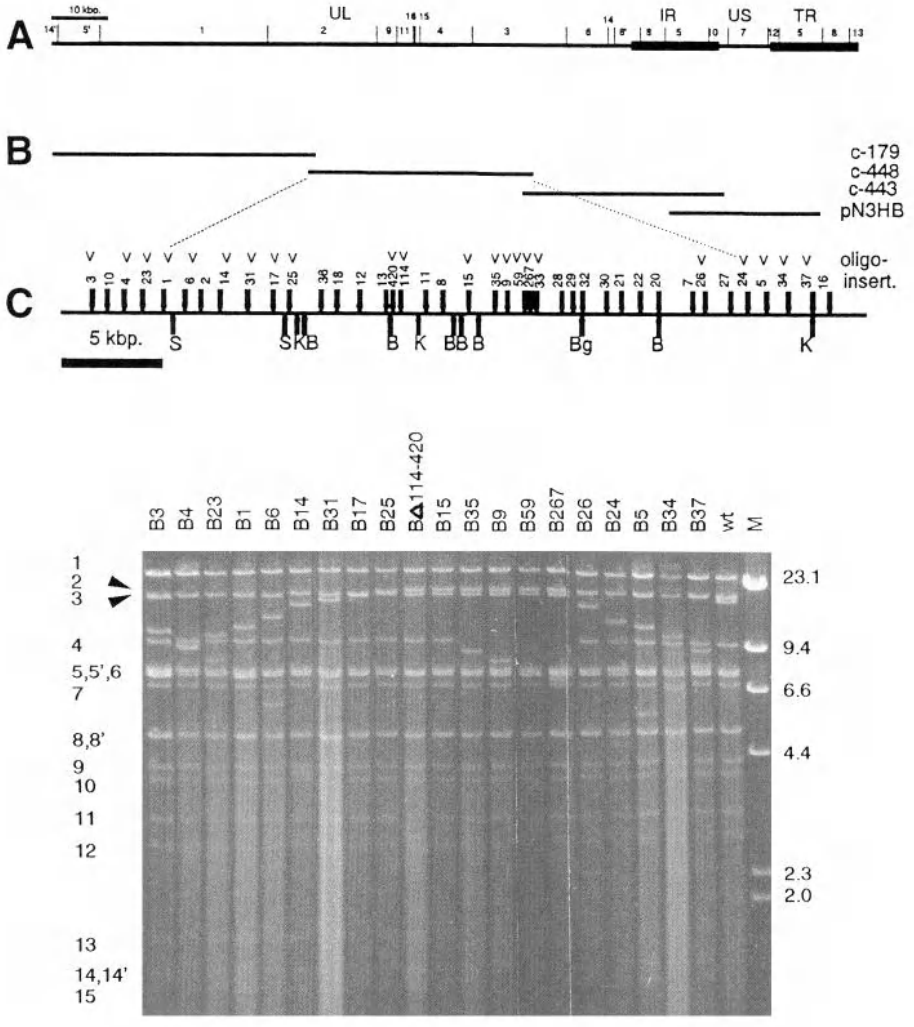


Fig. 5

bands do not light up sufficiently above the background smear, they can be visualized by Southern blotting of the gel, followed by hybridization to a labeled probe consisting of total purified virus DNA, or of cloned restriction fragments of the virus (this method of analysis was done by us in initial experiments *[2]*). We will provide here a protocol for the infection of cells by PRV, of the isolation of DNA from these cells, and of restriction digestion of this DNA.

1. Seed a 10-cm dish with permissive cells 1 d prior to infection, so that cells are subconfluent at the time of infection.

2 The next day, aspire the medium and overlay the cells with 2 mL of a high-titered virus suspension (a multiplicity of infection of at least 10, e.g , a culture supernatant) Note that, in parallel, DNA should be prepared and assayed from both reconstituted as well as nonmanipulated virus Incubate for 1 h at 37°C, wash the cells twice with 10 mL PBS, and add 10 mL medium. Incubate for the length of an infection cycle (which is 8 h for PRV).
3. Wash the cells with 10 mL cold PBS and lyse the cells by the addition of 2 mL lysis buffer
4. Scrape off the lysate and transfer it to a 15-mL conical tube Incubate the tube for 2 h at 55°C or overnight at 37°C
5. Add 1 mL phenol.chloroform:isoamyl alcohol Mix by shaking gently for 5 min, centrifuge for 5 min at 5000*g*, and transfer the supernatant to a clean tube Subsequently, precipitate the DNA by addition of 2 vol ethanol After inversion of the tube, a cloudy DNA precipitate should appear
6 Fish out the DNA precipitate using a drawn-out Pasteur pipet, wash the DNA in 70% ethanol, and transfer to a microfuge tube. Centrifuge briefly, pipet off the ethanol, let the remaining ethanol evaporate and let the DNA dissolve overnight at room temperature (or 15 min at 65°C) in 200 µL TE. Store the DNA at 4°C
7 Using a yellow tip with cut-off end for pipeting the DNA, which should be viscous, run 1 µL on an agarose minigel together with a known quantity of phage lambda DNA to estimate the DNA concentration.
8 Digest DNA of the isolates of both the reconstituted and nonmanipulated viruses with the diagnostic restriction enzyme(s) and preferably also with a number of other restriction enzymes as follows· Mix in a microfuge tube 2–4 µg DNA, 2 µL of the appropriate 10X digestion buffer, 10 U restriction enzyme, and distilled water to 20 µL Incubate for 2–4 h at the appropriate temperature for the enzyme. Analyze the digests by running side to side the different digests of DNAs from reconstituted and nonmanipulated viruses on a large 0 8% agarose gel. If virus bands are not clearly visible, blot the gel and analyze by hybridization with virus probes The digests of both virus preparations should be identical (*see* Note 36)

3.2. Oligonucleotide Insertion Mutagenesis of Individual Cosmid Clones

Once the set of cosmid clones for overlap recombination has been validated, individual clones can be used for modification in vitro. We will describe here the generation of a large series of mutant derivatives of a single cosmid (i.e., one of the cosmids generated for overlap recombination). Each of these mutant cosmid derivatives lacks the expression of (at least a part) of a single gene. This is achieved by the insertion of a mutagenic oligonucleotide, at a random site, within the cosmid clone. Subsequently, virus mutants that harbor the oligonucleotide are generated by overlap recombination using the modified cosmid together with the set of overlapping cosmids. The steps involved in this procedure are described in the Introduction to this chapter and are schematically depicted in Fig. 3.

3.2.1. Linearization of a Cosmid Clone by Restriction Enzymes with 4-bp Specificity in the Presence of Ethidium Bromide

This has proven to be an excellent way to linearize a large DNA clone of the size of a cosmid, at quasi-random sites. The latter is essential since the aim of the procedure is to generate a "saturated" series of virus mutants, i.e., for every gene encoded in the cosmid clone, a mutant virus strain should be obtained (*see also* Notes 4, 6, and 8). Here we provide the protocol that is optimalized for the linearization of PRV cosmids using the enzymes *Fnu*DII, *Hae*III, and *Rsa*I. However, cosmid clones containing virus DNA of lower GC content (the GC content of PRV is 73%) may require slightly different digestion conditions (*see also* Note 37).

1. Prepare, on ice, three microfuge tubes, labeled *Fnu*DII, *Hae*III, and *Rsa*I, respectively, each containing 25 µg DNA of the cosmid clone to be mutagenized (purified by Cesium chloride-ethidium bromide density gradient centrifugation, *see* Section 3.1.13.) Add 12.5 µL of the appropriate ethidium bromide-containing digestion buffer (*see* Section 2.) and add sterile distilled water to 125 µL. Then add, respectively, 2 U *Fnu*DII, 1 U *Hae*III, and 0.5 U *Rsa*I to the tubes. Incubate for 15 min at 37°C.
2. Add 10 µL 10% SDS to inactivate the enzymes. Load the digests on a large 0.5% preparative agarose gel. Include as a control a lane with the uncleaved cosmid. Electrophorese overnight until the bromophenol blue dye has well migrated off the gel.
3. The gel should look, under long-wave UV light, as in Fig. 4. Visible should be (from top to bottom) uncleaved relaxed cosmid, uncleaved supercoiled cosmid, linearized full-length cosmid, and multiply cleaved cosmid (a smear).
4. Cut out the three linear cosmid DNA bands, taking great care to avoid taking along any of the multiply cleaved DNA. Elute the DNA using an electroelution apparatus.
5. After electroelution, transfer the eluted DNAs each to a microfuge tube, add one volume phenol:chloroform:isoamyl alcohol, and vortex gently for 30 s, centrifuge for 2 min at maximum speed and transfer the supernatant to a clean microfuge tube. Repeat if necessary. Subsequently, precipitate the DNA by addition of 0.1 vol 3*M* sodium acetate, pH 5.2, and 2 vol ethanol. After inversion of the tube, a cloudy DNA precipitate should appear.
6. Fish out the DNA precipitate using a drawn-out Pasteur pipet, wash the DNA in 70% ethanol, and transfer to a microfuge tube. Centrifuge briefly, pipet off the ethanol, let the remaining ethanol evaporate, and let the DNA dissolve overnight at 4°C (or 15 min at 65°C) in 20 mL TE. Store the DNA at 4°C.
7. Load 1 µL of each of the three linear cosmid preparations on a 0.5% agarose minigel to estimate its concentration. Use as marker 0.1, 0.2, and 0.4 µg uncleaved lambda DNA.

3.2.2. Ligation of the Mutagenic Oligonucleotide to the Linear Cosmid DNA

To the ends of the randomly linearized cosmid clone, the oligonucleotide is ligated, after which the oligonucleotide containing cosmid is recirculated for high-efficiency transformation. The addition of the oligonucleotide to the

cosmid DNA is essentially performed in the same way as the addition of the cloning linker to the sheared virus DNA (Section 3.1 4.) Finally, the randomly linearized cosmid, which now contains the oligonucleotide added to the ends, is religated at low DNA concentration, to favor the formation of circular cosmid molecules (as opposed to concatemers; *see* Note 38).

1 To phosphorylate the oligonucleotide, mix in a microfuge tube 1 µL 10X kinase buffer, 2 µg nonphosphorylated oligonucleotide, 1 µL 10 m*M* ATP, 10 U polynucleotide kinase, distilled water to 10 µL Incubate for 1 h at 37°C After kinasing, heat the oligonucleotide to 70°C and let it cool down, over a period of 30 min, to room temperature (*see* Note 39)
2 In a microfuge tube, mix 2 µg of each of the three linearized cosmid preparations Add to the microfuge tube 50-fold molar excess of phosphorylated mutagenic oligonucleotide (this will be approx 150 ng), 2 µL 10X ligation buffer, 5 (Weiss) Units T4 ligase, and distilled water to 20 µL Incubate overnight at 15°C
3 Add to each tube 80 µL TE and 100 mL phenol chloroform·isoamyl alcohol, shake gently for 2 min, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Subsequently, precipitate the DNA by addition of 0 1 vol 3*M* sodium acetate pH 5 2 and 2 vol ethanol Store on ice for 15 min and centrifuge for 5 min at maximum speed Wash the pellet with 1 mL 70% ethanol and take the DNA up in 10 µL TE Let the DNA dissolve overnight at 4°C (or 15 min at 65°C)
4. Add to the DNA. 10 µL of the appropriate 10X digestion buffer, 100 U of the restriction enzyme for which the oligonucleotide has a site, and distilled water to 100 µL. Incubate for 4 h at the appropriate temperature for the enzyme.
5 Load the entire digestion mixture on a 0.5% preparative agarose gel and electrophorese until the bromophenol blue marker has migrated 2 cm into the gel
6. Cut out the cosmid DNA band and elute using an electroelution apparatus
7 After electroelution, transfer the eluted DNA to a microfuge tube, add 1 vol phenol·chloroform:isoamyl alcohol, vortex gently for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Repeat if necessary. Subsequently, precipitate the DNA by addition of 0 1 vol 3*M* sodium acetate pH 5 2 and 2 5 vol ethanol Mix and store on ice for 15 min, and centrifuge for 5 min at maximum speed. Wash the pellet with 1 mL 70% ethanol and take up the DNA in 20 µL TE.
8 Load 1 µL of each of the oligonucleotide-containing linear cosmid fragments on a 0 5% agarose minigel to estimate its concentration. Use as marker 0 1, 0.2, and 0.4 µg uncleaved lambda DNA
9 Mix in a microfuge tube 0.1 µg of the cosmid fragments, 40 µL 10X ligase buffer, 2 (Weiss) Units T4 ligase, and distilled water to 400 µL. Incubate for 4 h to overnight at 15°C
10 Precipitate the DNA by adding 0 1 vol sodium acetate pH 5 2 and 2 5 vol ethanol Mix by inversion and leave on ice for 15 min, and centrifuge at maximum speed for 5 min Wash the pellet with 70% ethanol and dry at room temperature until the ethanol has evaporated and dissolve the pellet in 10 µL TE (overnight incubation at 4°C or 15 min incubation at 65°C)

3.2.3. Transformation and Analysis of Mutagenized Cosmids

After addition of the oligonucleotide, *E. coli* is transformed with the recircularized mutant cosmid. Since *E coli* is not transformed very well by large plasmids, we have used a high-efficiency protocol for transformation that is originally developed by Hanahan *(20)*. However, since preparation of these competent cells is somewhat cumbersome and not always reproducible, we recommend purchasing ultracompetent *E coli* cells. These are available from many suppliers and have a competency of at least 5×10^8 colonies/μg transformed test plasmid. A protocol for transformation comes with every batch of cells.

After transformation, a large amount of transformant colonies is stored and, batch by batch, cosmid DNA is prepared of these transformants. To screen against deletions and rearrangements, this DNA is first analyzed by restriction enzyme digestion with the diagnostic enzyme plus the enzyme that cleaves the cloning linker and gel electrophoresis. Subsequently, cosmid clones that appear to be intact are further analyzed to determine the site of insertion of the mutagenic oligonucleotide. This is done by performing minimally two double-digestions, followed by gel electrophoresis: (1) By the diagnostic enzyme and the enzyme that cleaves the mutagenic oligonucleotide; and (2) by the enzyme that cleaves the cloning linker and the enzyme that cleaves the oligonucleotide (*see* Note 40). These digests should enable to map the site of insertion of the oligonucleotide within a few hundred basepairs.

1. Transform ultra competent *E. coli* with 2–5 μL (*see* Note 41) of the recircularized mutagenized cosmid preparation using the protocol provided by the vendor of the cells
2. Using wooden toothpicks inoculate, with fresh single transformant colonies, all wells of (at least) five sterile marked 96-well microtiter plates prefilled with 100 μL LB containing 15% glycerol and ampicillin per well (*see* Note 27). Use the first 48–96 toothpicks to also inoculate numbered tubes with 2 mL LB containing ampicillin. Cover the well plates and incubate the plates overnight at 37°C in a standing incubator Incubate the tubes overnight at 37°C in a shaking incubator.
3. Freeze the well plates in a –80°C freezer These frozen minicultures are stable and serve as a stock
4. Prepare cosmid miniprep DNA from the 48–96 inoculated 2-mL cultures, as described in Section 3.1 9.
5. Mix in a microfuge tube: 5 μL miniprep DNA, 2 μL of the appropriate digestion buffer, 5 U of a diagnostic restriction enzyme, and distilled water to 20 μL. As a control, digest the unmutagenized cosmid clone Incubate the digestions for 2 h at the appropriate temperature.
6. Load all digestions on a 0.8% agarose gel, together with a *Hin*dIII digest of phage lambda DNA as a marker (*see* Note 16) and electrophorese until the bromophenol blue dye has migrated at least 10 cm
7. Thoroughly compare the digests of the mutagenized cosmid clones with the digest

of the nonmutagenized cosmid clone This enables monitoring for deletions or rearrangements Discard aberrant cosmid clones

8 This selected group of clones is further analyzed for the site of insertion of the mutagenic oligonucleotide. For this purpose, repeat steps 5 and 6, now performing two digests per clone, using two enzymes per digest. The first digest contains the diagnostic enzyme and the enzyme that cleaves the oligonucleotide, the second digest contains the enzyme that cleaves the cloning linker and the oligonucleotide (*see* Note 28).

9. Indicate on a physical map of the cosmid, the sites of insertion of the oligonucleotide in the various characterized mutant cosmids.

10 Repeat steps 4–9, inoculating cultures from the frozen microtiter stocks, until a satisfactory density of mutants is achieved (*see* Note 42)

3.2.4. Generation of Virus Mutants by Overlap Recombination Including a Oligonucleotide-Containing Fragment

Once a set of derivatives of a cosmid clone has been generated (each derivative bearing the mutagenic oligonucleotide at a different site), oligonucleotide-bearing virus can simply be generated. This is done by overlap recombination using one of the mutagenized cosmid derivative plus three (if a set of cosmid clones for overlap recombination exists of in total four clones) wild-type cosmid clones (*see* Fig. 3 and Note 43).

For the generation of a saturating set of virus mutants by overlap recombination, we initially select a panel of mutant cosmid derivatives with a distance of, on the average, 1 kbp between two subsequent oligonucleotide integration sites. This distance is a compromise between practical feasibility and the assumption that we will find mutants for almost any gene encoded on the cosmid. The latter assumption is based on the following parameters, derived for the sequenced UL region of the HSV-1 genome *(20)*: (1) The average size of the HSV-1 genes is 1.9 kbp—73% of the open reading frames are larger than 1 kbp; and (2) 89% of the genome is protein-encoding. For a cosmid with an insert size of, e.g., 40 kbp, this will amount to 40 different overlap recombinations. If, at a later stage, one needs additional mutants, one can refer to the set of characterized mutant cosmid derivatives that have not been chosen for initial overlap recombination (*see* Note 42). If, after transfection, no regeneration of viable virus is found after a single overlap recombination, we repeat the overlap recombination up to three times before we assume that the oligonucleotide is likely inserted in a gene that is essential for virus growth (*see* Note 44).

The protocols for preparation of the DNAs for overlap recombination and for transfection itself are almost the same to those described before; we will therefore refer to the appropriate sections in this chapter and elaborate only here on differences we introduced to facilitate the simultaneous performance of a large series of overlap recombinations. The preparation of all mutant

cosmid derivatives for transfection and the repeated transfections make this protocol the most lengthy of this chapter.

To keep the work manageable, we advise that it be divided over multiple portions.

1. Using the frozen stocks in microtiter plates, inoculate flasks containing 50 mL LB + ampicillin with all mutant cosmid clone derivatives selected for overlap recombination
2. Perform a 20-fold scaled-up miniprep protocol (*see* Section 3.1.9.) in 50-mL conical tubes to isolate the cosmids. Dissolve the isolated DNA by incubation for 15 min at 65°C or overnight at 4°C, in 50 µL TE.
3. To estimate the yield and to verify the identity and integrity of the isolated DNAs, perform triple digestions with the diagnostic plus the oligonucleotide-cleaving plus the cloning linker cleaving enzymes, on all preps. Mix in a microfuge tube 1 µL midi prep DNA, 2 µL 10X digestion buffer (*see* Note 28), 5 U of each of the three enzymes, and distilled H_2O to 20 µL. Incubate for 2 h at the appropriate temperature.
4. Load the digests on a 0.8% agarose gel, also loading 1 µg of a *Hin*dIII digest of lambda DNA as a marker (*see also* Note 16). Electrophorese until the bromophenol blue dye has migrated 10 cm. Carefully scrutinize the gel to ascertain the intactness and identity of the isolated cosmids (*see* Note 45).
5. If all mutant cosmids are found to be correct, the cosmids are digested with the restriction enzyme for which the cloning linker has a site, to liberate the mutant virus genomic fragment from the cosmid vector. To this end, mix in a microfuge tube: 5 µg DNA, 5 µL 10X digestion buffer, 25 U restriction enzyme, and distilled water to 50 µL. Incubate for 2 h at the appropriate temperature.
6. Load the digests on 0.5% preparative ultrapure agarose gels. Electrophorese until the bromophenol blue dye has migrated approx 5 cm.
7. Cut out all of the insert bands. Elute each of the DNAs using an electroelution apparatus.
8. After electroelution, transfer the eluted DNAs each to a microfuge tube, add one volume phenol:chloroform:isoamyl alcohol, vortex gently for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Repeat the extraction if necessary. Subsequently, precipitate the DNA by addition of 0.1 vol 3*M* sodium acetate pH 5.2 and 2 vol ethanol. Mix by inversion and store for 15 min on ice. Centrifuge for 5 min at maximum speed and discard the supernatant. Wash the pellets with 1 mL 70% ethanol, dry briefly, and dissolve the DNA in 10 µL sterile 0.1X TE.
9. Electrophorese 1 µL of all gel purified DNAs on a 0.8% agarose gel to estimate integrity and concentration.
10. Prepare 50 µg of each of the other three (nonmutagenized) cosmids to be used for overlap recombination, using a fivefold scaled-up version of the protocol as described in Section 3.1.15.
11. For every overlap recombination to be performed, seed the day prior to trans-

fection two 3.5-cm wells or dishes with permissive cells so that cell density will be 25–40% at the time of transfection

12. Perform overlap recombination using a 10-fold scaled-down version of the protocol described in Section 3.1.16. (*see* Note 46) **Note:** Great care should be taken during these steps to avoid crosscontamination between mutants. To obtain separate plaques for every overlap recombination, add 10 µL of every $CaPO_4$ precipitate to one well, and 90 µL to the other well. After the glycerol shock, overlay the cells with medium containing 1% methylcellulose to obtain separate plaques. As a positive control for the overlap recombination, perform overlap recombination using unmutagenized fragments only (*see* Note 47)
13. Using a micropipet equipped with a yellow tip, pick six separate plaques of every overlap recombination. Resuspend the virus from every plaque in a sterile microfuge tube containing 100 µL tissue culture medium. The tubes may be frozen and stored at –80°C.

3.2.5. Plaque Purification, Expansion, and Restriction Digest Analysis of Virus Mutants

Once a set of viable virus mutants has been obtained, individual mutants are plaque purified and expanded to obtain a pure stock each of the virus mutants. **Note:** Great care should be taken during these steps to avoid crosscontamination between mutants. Especially slow-growing mutants will easily be overgrown, and lost, after infection with a faster growing mutant (or wild-type) virus. We do not provide a protocol for plaque purification and expansion of the mutants here; these protocols may be found in Chapter 4 of this volume. Next, DNA of plaque-purified mutants is isolated and analyzed by restriction enzyme analysis and gel electrophoresis. Virus mutants of which gel patterns of the restriction enzyme digests are in accordance with the expected patterns are amenable to phenotypic analysis. We do not provide protocols for this, instead, we refer to other chapters in this volume.

1. Perform three rounds of plaque purification on three of the plaques that are picked for every overlap recombination, as described in Chapter 4 of this volume.
2. Using high-titered stocks of all plaque-purified virus mutants and also of wild-type virus, infect 10-cm dishes with permissive cells. Isolate DNA of the infected cells as described in Section 3.1.18., steps 1–7.
3. The DNA preparations are digested with a diagnostic enzyme, to verify that no (visible) genomic alterations have occurred, and the same diagnostic enzyme plus the enzyme that cleaves the inserted oligonucleotide, to verify the identity of the mutant. Perform the digestions as follows: Mix in a microfuge tube: 2–4 µg DNA, 2 µL of the appropriate 10X digestion buffer (*see* Note 28), 10 U of each restriction enzyme, and distilled water to 20 µL. Incubate for 2–4 h at the appropriate temperature for the enzymes.
4. Analyze the digests by running side to side the different digests of DNAs from reconstituted and nonmanipulated viruses on a large 0.8% agarose gel. Also, load

1 µg *Hin*dIII digested lambda DNA as a marker. If virus bands are not clearly visible, blot the gel and analyze by hybridization with virus probes The digests with the diagnostic enzyme should be identical for all virus preparations. The double digests should have the diagnostic fragment cleaved into two fragments, with sizes depending of the site of insertion of the oligonucleotide An agarose gel exemplifying this is shown in Fig. 5

5 Of every mutant virus, choose one plaque-pure strain that is found correct by restriction enzyme analysis for further experiments. If no strain is found that meets these criteria, repeat this protocol using the three additionally picked, frozen plaques from the overlap recombination.

3.2.6. Other Uses for Cosmid Clones Bearing a Oligonucleotide Containing a Unique Restriction Site

We will mention here two informative examples showing how the presence of an inserted unique restriction site, combined with overlap recombination, has further expanded the possibilities to modify the virus genome

Although the oligonucleotide abolishes expression of the gene it is inserted in, for the production of a safe vaccine strain of a herpesvirus, one prefers to entirely delete a gene that is found to be a determinant of the virulence of the virus. This deletion can easily be generated using a cosmid bearing an oligonucleotide at the 5' end of the gene of interest and a cosmid bearing an oligonucleotide 3' of the first oligonucleotide insertion in the same gene. A novel cosmid having a deletion between the oligonucleotides in both mutants is obtained by ligating the cosmid insert fragment left of the 5' inserted oligonucleotide to that right of the more 3' inserted oligonucleotide. After overlap recombination, the resulting virus strain, that now contains a specified deletion, can be used as a safe vaccine strain. The strain BΔ420-114, as depicted in Fig. 5, bearing a deletion in the thymidine kinase gene, is an example of an avirulent PRV strain that is generated in this way *(16)*.

The unique restriction site also provides opportunities for the development of the virus as a vector for heterologous genes. In the latter case, the unique site inserted in the cosmid serves as a socket to plug in cassettes containing the gene of interest. In a subsequent overlap recombination the, heterologous gene containing, virus is reconstituted *(22)*.

3.3. The Generation and Use of Probes for the Study of Layout, Expression, and Identity of Virus Encoded Genes

In the previous section (*see* Section 3.2.), we described the generation of a large series derivatives of a cosmid containing 30–40 kbp of the virus genome. The mutagenic oligonucleotide that is present in each of these mutant cosmids contains a restriction site that is unique for the cosmid. The latter enables one to specifically subclone small virus genomic fragments that flank the inserted

oligonucleotide in each mutant. This is done by simply digesting each mutant cosmid with this enzyme and a second enzyme that has a 4-bp specificity (and thus frequently cleaves the virus genome; *see* Note 6) and gives a sticky overhang. The resulting digest contains many short fragments; however, only two of these (at both sides of the inserted oligonucleotide) contain a unique overhang at one end and the overhang of the 4-bp recognizing enzyme at the other end. This enables to subclone these fragments into a vector that is predigested with enzymes that yield the same overhangs as the two enzymes with which the mutant cosmid has been cleaved (*see also* Fig. 1C). The resulting subclones are analyzed for insert size and the orientation with respect to the mutagenic oligonucleotide (facultatively). After this characterization, the subclones may be used for the following purposes:

1. Crosshybridization studies with DNA from a prototype herpesvirus to gain information on gene structure of the virus under study,
2. To map and to determine the class of transcripts in the region of the inserted oligonucleotide; and
3 Direct DNA sequencing to identify (by comparing with the sequence of a prototype herpesvirus) the virus gene that has been mutagenized by insertion of the oligonucleotide

Here, we will provide a detailed description of the subcloning procedure itself, we will only give a global description of the procedures for crosshybridization studies, transcript mapping, and DNA sequence analysis.

3.3.1. Construction and Characterization of Subclones Flanking Each Inserted Oligonucleotide

Every mutant cosmid derivative is to be digested with two restriction enzymes: the enzyme that specifically cleaves the inserted mutagenic oligonucleotide, together with an enzyme that has a 4-bp specificity and thus will cleave in the vicinity of the inserted oligonucleotide For the latter enzyme, *Sau*3A is a good choice; this enzyme recognizes the sequence GATC, leaving a 4-bp 5' overhang that is compatible with the *Bam*HI, *Bgl*II, and *Bcl*I overhangs (*see* Note 48). The vector for subcloning, containing a polylinker (*see* Note 49), is therefore cleaved with, e.g., *Bam*HI plus the enzyme that generates an overhang that is compatible with the overhang, generated by cleavage of the oligonucleotide (*see* Note 50).

After subcloning, the two different flanking subclones for every oligonucleotide insertion mutant are isolated and their sizes are determined by releasing the inserts from the vector using enzymes that cleave the polylinker of the vector at either side of the insert, followed by agarose gel electrophoresis. In addition, the orientation of the subclones with respect to the oligonucleotide may be determined by hybridization with a blot of a gel containing the respec-

tive mutant cosmid that is digested with the diagnostic enzyme for the virus plus the oligonucleotide cleaving enzyme (the blot may be generated in Section 3.2.4.; *see* Note 45)

1 Mix in a microfuge tube. 10 µg of the appropriate plasmid vector, 10 µL of the appropriate 10X enzyme digestion buffer (*see* Note 28), 50 U of the enzyme that cleaves the oligonucleotide (or an enzyme giving compatible ends, *see* Note 50), 50 U *Bam*HI, and distilled water to 100 µL Incubate for 2 h at the appropriate temperature
2. Load the digest on a 0 8% preparative agarose gel, load as markers (in a small slot) 1 µg uncleaved plasmid, 1 µg lambda DNA digested with *Hin*dIII (*see* Note 16) Electrophorese until the bromophenol blue dye has migrated 5 cm Cut out the linear plasmid band and isolate the DNA using the Geneclean (or similar) kit. Take up the DNA in 100 µL TE
3. Mix in microfuge tubes· 1 µg of each oligonucleotide containing cosmid for which flanking probes are to be obtained, 2 µL of the appropriate 10X digestion buffer (*see* Note 28), 5 U of the of the enzyme that cleaves the oligonucleotide (*see* Note 28), 5 U *Sau*3A, and distilled water to 20 µL As control, digest the unmutagenized cosmid with both enzymes. Incubate for 2 h at the appropriate temperature
4. Add 80 µL TE and one volume phenol·chloroform·isoamyl alcohol. Vortex for 30 s, centrifuge for 2 min at maximum speed, and transfer the supernatant to a clean microfuge tube. Subsequently, precipitate the DNA by addition of 0 1 vol 3*M* sodium acetate pH 5 2 and 3 vol ethanol. Mix and store on ice for 30 min and centrifuge for 15 min at maximum speed. Wash the pellets with 1 mL 70% ethanol and take the DNA up in 200 µL TE
5 For each set of two flanking probes, mix in a microfuge tube: 1 µL digested gel purified vector, 1 µL of a digested mutant cosmid derivative (*see* Note 51), 5 µL 10X ligase buffer, 1 (Weiss) Unit T4 ligase, and distilled water to 50 µL Incubate for 4 h to overnight at 15°C. As a negative control, perform a simultaneous ligation containing the digested gel purified vector plus the digested unmutagenized cosmid.
6. Transform competent *E coli* with 10 µL of each ligation, as described in Section 3 1.8.
7. Count the transformant colonies; the ligations of digests of oligonucleotide-bearing cosmids should clearly give more colonies than the ligation of the digest of the unmutagenized cosmid For every ligation, inoculate six colonies, using wooden toothpicks, in 2 mL LB + ampicillin. Incubate overnight in a shaking incubator at 37°C
8 Perform minipreps on all cultures, as described in Section 3.1.9
9 Mix in microfuge tubes· 2 µL miniprep DNA, 2 µL of the appropriate 10X enzyme digestion buffer (*see* Note 28), 5 U of each of two restriction enzymes that cleave the polylinker at either side of the insert, and distilled water to 20 µL Incubate for 2 h at the appropriate temperature.
10 Electrophorese the digests on a 2% agarose gel, load as markers DNA fragments in the range of 0.1–2 kbp (*see* Note 52) If not two sizes of inserts (representing

fragments flanking both sides of the oligonucleotide) are found, one may characterize more clones (steps 7–10, *see* Note 53)

3.3.2. Determination of the Orientation of the Flanking Probes with Respect to the Inserted Oligonucleotide

If the orientation of the subclones with respect to the oligonucleotide is to be determined, this can be done by hybridization with a blot of a gel containing the corresponding mutant cosmid that is triply digested with the diagnostic enzyme for the virus plus the oligonucleotide cleaving enzyme plus the enzyme that cuts the cloning linker (the blot may be generated in Section 3.2.4., *see* Note 45). Cut out each lane from the blot (containing a digested mutant cosmid) and hybridize the miniblots with the one of the two corresponding, flanking clone containing, radiolabeled plasmids. After exposure of each autoradiogram, two bands will be visible: a band representing the cosmid vector, cross-hybridizing with the radiolabeled plasmid vector, and a second band that represents the side of the oligonucleotide that is represented in the flanking probe.

3.3.3. Crosshybridization Studies with DNA from a Prototype Herpesvirus

The genomes of one or more members of all three subfamilies of herpesviruses have now entirely been sequenced (reviewed in ref. *1*), yielding a wealth of data on gene location and (putative) gene function for each subfamily. Therefore, a feasible way to gain insight into the global layout of the genome of a less-well-known herpesvirus is by investigating crosshybridization with the genome of a prototype herpesvirus from the same subfamily A defined set of well-characterized small probes, derived from large genomic regions of the virus under study is an ideal tool to perform these crosshybridization studies

We successfully investigated crosshybridization between small flanking probes derived from a mutagenized 41-kbp subgenomic region from PRV (derived from cosmid clone c-448, Fig. 5) and cloned fragments form HSV-1 Briefly, a series of cloned *Kpn*I fragments of HSV-1 were digested with *Bam*HI plus *Ssp*I, and with *Bam*HI plus *Eco*RI plus *Ssp*I (*see* Note 54). These specific *Kpn*I fragments of HSV-1 were chosen on the basis of previous cross-hybridization experiments by others, using large PRV fragments as probes, establishing a rough synteny map between both viruses The digested HSV-1 clones were loaded manifold on agarose gels, electrophoresed, and blotted onto nylon membranes. The blots were subsequently cut in small blots, each containing a series of digested HSV-1 clones. Flanking small PRV probes were cut out of their vector, isolated by preparative gel electrophoresis and labeled by random priming (*see* Note 55). After hybridization at low stringency (6X SSC, 25% formamide, 42°C), blots were washed by subsequent multiple

washes at increasing stringency: 6X SSC, 60°C; 6X SSC, 65°C; 3X SSC, 65°C; 0.1X SSC, 65°C. Between each series of washings, blots were exposed to autoradiography film. Bands that crosshybridized after washing at low stringency, but disappeared at the highest stringency washing were marked crosshybridizing. In this way we were able to define four crosshybridizing regions and to establish synteny between a large subgenomic region of both viruses *(6)*.

3.3.4. The Use of Small Probes to Gain Information on Location, Sizes, and Expression of Virus Genes

The availability of a set of well-characterized probes from the virus genome also allows the gaining of information on the transcriptional units of the virus genome. Although the herpesvirus genome contains many transcriptional units, containing several genes with coterminal 3' ends *(21)*, a thorough analysis of the mRNAs encoded in the subgenomic region(s) of the virus that are mutagenized by oligonucleotide insertion yields information on gene location, expression, and on the genes that are inactivated by insertion of the oligonucleotide. This analysis is facilitated by the absence of introns in most genes *(21)*. A chapter of this volume is devoted to the analysis of HSV gene expression (Chapter 23), so we again provide only an outline of the procedure we have followed.

RNA was prepared from cells that were mock infected, or infected for 2 and for 6 h (to be able to discriminate between early, early–late, and late genes, respectively) with a high multiplicity of wild-type virus. These RNAs were loaded in manifold on RNA gels, electrophoresed, and blotted. Small fragments of each blot, containing lanes with the three RNA preps were each hybridized with a flanking probe, under high stringency. Size and class of all transcripts that were detected unambiguously were listed. If a transcript was detected with probes derived from both flanks of a specific oligonucleotide, the oligonucleotide was assumed to be inserted into that transcript. Since most herpesvirus transcripts have coterminal 3', and not 5', ends, we could in many cases obtain indications if the oligonucleotide was inserted in the 5' part of the transcript (and thus likely in the open-reading frame) or in the 3' part of the transcript (and thus possibly in a downstream located open-reading frame).

3.3.5. The Use of Flanking Probes as Sequence Tags

The ultimate and definite way to identify virus genes of interest is by sequence analysis. The cloned small virus DNA fragments, flanking the inserted oligonucleotide in the set of mutant cosmid derivatives, provide an excellent way to rapidly identify genes, and also to determine in what genes and at what site the oligonucleotide is inserted in the various mutants. The inserts of the plasmids may directly be sequenced using double-stranded DNA

sequencing. If the G C content of the virus is high (as in PRV), single-stranded templates may be preferred for sequencing. Single-strand templates can either be achieved by producing single-strand DNA of the plasmid by entire super infection with a helper phage, if it contains a single-strand phage replication origin (like, e.g., the pBluescript series from Stratagene), or otherwise by subcloning in one of the M13mp series of bacteriophages.

4. Notes

1 It should be noted that a small deletion has occurred here at the junction of both fragments during ligation in vivo

2 In the original protocol, insert fragments were, after digestion, purified free from vector sequences before transfection to avoid the risk of incorporation of vector sequences into the reconstituted virus by nonhomologous recombination or ligation *(2)*. This last purification step has proven to be superfluous since we never found incorporation of vector sequences (when the purification step was omitted) after overlap recombination If, however, this purification step turns out to be required when performing overlap recombination with another herpesvirus, the protocol for size selection on glycerol gradients (*see* Section 3 1 3) is suitable for this purpose.

3 The efficiency of virus regeneration when the set of five fragments was used for overlap recombination was at least as high as when four fragments are used *(2)* This may be related to the larger overlaps between the five subgenomic fragments than between the four fragments It is believed that the frequency of homologous recombination strongly depends on the length of the homology (*see also* Note 13)

4 Obviously, this type of mutagenesis is only efficient if most of the genome is protein encoding For HSV-1, this is 89% of the virus genome *(20)*

5. The oligonucleotide should be palindromic to allow double-strandedness. This also prevents polarity, i e , insertion in either orientation within a gene will be functional In addition, it is preferred that the enzyme gives sticky (as opposed to blunt) overhangs after cleavage. The *Eco*RI site (GAATTC) in this specific oligonucleotide is absent from the entire PRV genome. Consequently, this single oligonucleotide would be suitable for mutagenesis of all four cloned subgenomic regions of the virus. If the restriction site that is present in the mutagenic oligonucleotide would also be present (rarely) in the virus genome, a different oligonucleotide should be devised for mutagenization of each region of the virus genome (*see also* Note 10) Although the amber (TAG) stopcodon is the only stopcodon used in this specific oligonucleotide, any of the three stopcodons (TAA, TAG, and TGA) may be used provided that the aforementioned requirements for the oligonucleotide are fulfilled

6. The cleavage site of an enzyme recognizing a 4-base sequence will statistically be present once in every 256 bp (presumed that the virus genome contains 50% G.C bp) The PRV genome, consisting of 73% G C bp, contains considerably more recognition sites for these enzymes, which recognize G C rich quadruplets)

Although linearization of a plasmid by such an enzyme will thus not truly be random, the use of several four-cutter enzymes with different recognition sites to linearize the plasmid will ensure linearization at almost (quasi) random sites

7. It should be noted here that the oligonucleotide insertion mutagenesis as described here has worked well for cosmids up to 46 kbp total length (i e., including the vector). Using a cosmid of 51 kbp, we found deletions in the plasmid after oligonucleotide insertion mutagenesis Thus, approx 46 kbp may be the upper size limit of the cosmid to be mutagenized using this technology.
8. Viable virus mutants are only obtained if the gene carrying the inserted oligonucleotide is not essential for virus replication in cultured cells. It is believed that at least half of the virus genes are dispensable for growth of the virus in tissue culture (*22*) If the gene is essential, however, the mutant virus may be rescued by performing the overlap recombination in cells that provide the essential gene product *in trans* (*see*, e.g., refs. *9* and *10,* and Chapter 6 of this volume) If the oligonucleotide is inserted at the end of the cloned fragment, in the region homologous between two adjacent clones, the site containing the oligonucleotide may be lost in some of the progeny virus generated during each overlap recombination, depending on the site of the crossover. To circumvent this, it may be advantageous to select two complete sets of cosmid clones, each having different overlaps (*see also* Note 13)
9 Although fragments between approx 33 and 45 kbp may be cloned in cosmid vectors (depending on the specific vector), the upper limit in this case is set by experimental limitations (*see* Note 7) to approx 41 kbp
10. Since the insert has to be cleaved out of the cosmid vector for transfection, the restriction site in the linker to use should preferentially be absent from the virus genome. If no such site can be found, different regions of the virus genome may be cloned using different linkers from which the restriction site is absent in that specific cloned fragment (*see* also Note 5) However, a number of 8-bp restriction sites, (e.g , *Asc*I, *Not*I, *Pac*I, *Pme*I, and *Sfi*I [all available from New England Biolabs], *Srf*I [Stratagene], *Sse8387*I [Takaraa], or *Swa*I [Boehringer]) are now available. Since every 8-bp sequence statistically is present only once in every 66 kbp, one will probably be able to find a restriction site that is absent from the entire virus genome. In this case, a single linker suffices for cloning of cosmids containing the entire virus genome It is important to note here that the restriction site in the linker used for cloning the virus genome in cosmids should be another site than the site in the mutagenic oligonucleotide that is later inserted at random sites in the cloned virus genomic fragment. The linker may be purchased commercially However, if no linker containing the desired restriction site is available, it should be newly designed. This linker should be a palindrome, containing the desired restriction site flanked by two bases (preferentially C or G residues) on both sides.
11. The procedure might be shortened by blunting of the virus DNA fragments and addition of the cloning linker (as described in Section 3.1.5., steps 1–6) directly after shearing (Sections 3 1 1 and 3.1.2) and before the glycerol gradient (Sec-

tion 3.1 3.) This also obviates the purification of sheared molecules on a preparative gel after linker addition (Section 3 1.5 , steps 7–9)

12. Although the virus DNA fragments may be also be cloned in low copynumber plasmid vectors like pBR322, cloning into a cosmid vector has the advantage that it is much more efficient, thus facilitating the generation of a set of virus clones that meets the requirements with respect to integrity, insert size, and size of homologous overlaps between the inserts. Any cosmid vector may be used for cloning
13. For efficient recombination, a homologous region of at least 0 5 kbp is required. However, this region should be as short as possible to minimize the risk of loss of an oligonucleotide inserted at the homology at the flank of a virus clone (*see also* Notes 3 and 8).
14. It is sufficient to clone only one complete copy of every inverted repeat present in the virus genome, its counterpart is regenerated during transfection *(2)*
15. The pellet should not dry out completely, as this impairs dissolution.
16. Uncleaved phage lambda DNA gives a single band of 48.5 kbp; a *Hin*dIII digest gives fragments of 23 1, 9 4, 6.6, 4 4, 2 3, 2.0, and 0.6 kbp Heat the lambda DNA for 5 min at 65°C prior to loading to melt out annealing of the terminal overhangs of the phage DNA.
17. Gentle vortexing of DNA fragments smaller than 50 kbp does not shear the fragments significantly further.
18. T4 DNA polymerase is capable of blunting 5' protrusions (using its 5' → 3' polymerase activity) and also 3' protrusions (by its 3' → 5' exonuclease activity) Hence, it is the enzyme of choice to generate blunt ends for linker addition and subsequent cloning of the subgenomic fragments of the virus The Klenow fragment of *E coli* DNA polymerase I might also be used here, however, the latter enzyme has a considerably lower 3' → 5' exonuclease activity than the T4 enzyme.
19. An excess of linker in the ligation will most efficiently drive the reaction toward addition of linkers to all DNA ends.
20. Any restriction enzyme digestion buffer may substitute for the T4 polymerase buffer
21. Theoretically, steps 5 and 6 are not essential since cosmid vectors containing the novel restriction site may directly (i e., after dephosphorylation) be used to ligate to the sheared virus DNA to which the same site is added. However, prior cloning of the restriction site containing cosmid vector (steps 5 and 6) is recommended as this improves the efficiency of cosmid cloning.
22. At this stage, cosmid vectors that have accidently retained the original restriction site may be eliminated by digestion with the corresponding enzyme prior to transformation
23. As a control for transformation, it is advised to also transform 0 1 ng control plasmid (like pBR322) The efficiency of transformation should be equal to, or higher than, 5×10^6 colonies/mg plasmid.
24. Cells may be concentrated by spinning for 2 min at 4000*g*.
25. If the restriction enzyme with which the cosmid vector was cleaved yields blunt or 3' protruding termini, perform the second incubation at 55°C

26 The amount of cultures to be inoculated depends on practical factors like, e.g., the capacity of the microfuges and gel electrophoresis equipment.

27 Frozen storage of fresh transformant *E coli* cells is necessary since large plasmids are often not well maintained by *E. coli*. As a consequence, deletions will occur if transformant colonies are stored too long at 4°C.

28 A digestion buffer and incubation temperature should be chosen in which both enzymes have sufficient activity. Most manufacturers provide lists with tolerated conditions for their enzymes If one of both enzymes requires a low salt buffer and the other enzyme a high salt buffer, one may also cleave first with the first enzyme, and add salt and the second enzyme after the first digestion has completed.

29 Do not expose the DNA to strong light if the DNA solution contains ethidium bromide

30 Pure DNA has an OD_{260}/OD_{280} of 1.8 An OD_{260} of 1 corresponds to a DNA concentration of 50 µg/mL.

31 Depending on the amount of plaques obtained, the transfection may be scaled down to 2 mg DNA In the latter case, 35-mm dishes with cells are used.

32. If separate plaques are to be obtained, different amounts (e.g , 0 1 and 0.9 mL) of the $CaPO_4$ precipitate may be added to different dishes with cells. In addition, medium containing 1% methyl cellulose, as a solidifying agent, should then be used Plaques can be picked using a micropipet equipped with a sterile yellow tip

33 At this time a $CaPO_4$ precipitate will be visible at a ×100 magnification Although a precipitate that is formed at the right pH should be very fine, we often observe some lumps that may be related to the size of the DNA molecules in the precipitate This does not greatly interfere with the efficiency of transfection

34. The length of the glycerol shock may vary for optimal results between 0.5 and 3 min, depending on the cell type

35. The time of incubation is dependent on the length of the viral cycle If this is long and a cytopathic effect is not yet visible when the cells are grown to confluency, the medium may be transferred to a novel dish containing cells at lower density.

36 In PRV we sometimes observe submolar bands that are derived from the repeat regions. These variations are likely caused by expansion or contraction in simple sequence repeats within the PRV IR and TR regions. The major form can usually be plaque purified In naturally occurring isolates of the virus, similar size heterogeneity of the repeats is often observed

37. Enzymes other than *Fnu*DII, *Hae*III, and *Rsa*I may also be used, provided that the enzymes have 4-bp specificities and yield blunt ends. To this purpose, the optimal ethidium bromide concentration and the amount of enzyme to be used should be determined experimentally.

38. It is tempting to perform this ligation at a higher DNA concentration, to subsequently package the resulting cosmid concatemers. However, this would be disastrous as each monomer in the concatemers has the *cos* site at a very different site (owing to the very nature of the random insertion of the mutagenic oligonucleotide). The distance between the *cos* sites would therefore often not be 33–50

kbp, the required distance for packaging. Moreover, the rare cosmids that will be packaged do not carry a contiguous fragment of the virus genome anymore

39 This will inactivate the enzyme and also favor the formation of double-stranded oligonucleotides over self-annealed oligonucleotides

40 If the sites for two diagnostic enzymes have been mapped, the site of insertion of the mutagenic oligonucleotide may also (and possibly more precisely) be determined by two double digestions, each containing a different diagnostic enzyme and the enzyme that cleaves the mutagenic oligonucleotide.

41 The transformation efficiency of ultracompetent cells drops when more than 20–50 ng DNA are added per transformation

42 We continue analyzing mutant cosmid derivatives until we have a density of approximately one oligonucleotide insertion per 250–500 bp

43 To generate double (or triple or quadruple) mutants, one may (in a later stage) also include mutant derivatives of more than one cosmid clone in an overlap recombination

44 The proof that a specific gene is essential (or that an observed phenotype can be attributed to the inserted oligonucleotide) can be given in two ways: (1) by cotransfecting a wild-type cloned DNA fragment overlapping the inserted oligonucleotide *(13)* The oligonucleotide will be replaced, by homologous recombination, with the wild-type fragment This should result in the generation of viable, wild-type virus and (2) by constructing a cell line that provides the wild type gene *in trans* by stable transfection (refs *9* and *10,* and Chapter 6 of this volume). This should now allow the oligonucleotide-containing mutant virus to be viable after overlap recombination

45 It is advised not to discard the resulting gel but to blot it on a nitrocellulose or nylon membrane (*see* elsewhere in of this volume for a protocol on Southern blotting) The resulting blot may later be used to characterize small subclones that flank the inserted oligonucleotides (as described in Fig. 1C and Section 3 3)

46 Of the cloned virus fragments to be cotransfected here, the nonmutagenized fragments are CsCl gradient purified cloned fragments, liberated from the vector (*see* Section 3.1.15), the fourth fragment is the gelpurified mutant insert fragment In total, every transfection consumes 2 mg DNA, each of the four (or five) fragments amounting equimolarly

47. If no viable virus is generated after overlap recombination including a mutant cosmid fragment, repeat the overlap recombination twice to ascertain that the failure of overlap recombination is not owing to failure of the transfection itself Based on our results with PRV *(5,6)* and on mutagenesis studies with HSV-1 (reviewed in ref *23*), about half of the overlap recombinations are expected to yield viable mutant virus

48. Since the PRV genome has a G.C content of 73%, *Sau*3A sites occur less than once in every 256 bp. Consequently, most flanking probes are larger than 200 bp. For another herpesvirus, average insert sizes may be smaller or larger, depending on the G C content of the virus.

49. Any high copynumber vector that contains a polylinker, e.g., one from the pUC,

the pSP, the pGEM, or the pBluescript series, may be used here Vectors containing a single-strand replication origin may be preferred if the inserts are to be sequenced.

50 If no compatible site is found in the polylinker of the vector, the vector may be cleaved with both *Bam*HI and an enzyme that leaves a blunt end (e.g , *Eco*RV) The oligonucleotide-containing cosmid derivatives are in the latter case first digested with the oligonucleotide cleaving enzyme, blunted with the Klenow enzyme, or with T4 DNA polymerase (*see* Section 3.1.5.) and next with *Sau*3A.

51 A light molar excess of vector over insert fragments (note that the insert fragments will, on the average, have a size of a mere 128 bp if the virus genome has a GC content of 50%) and a large volume of the ligation will promote the cloning of a single fragment only.

52 We use an *Msp*I digest of pSP72, yielding fragments of 501, 430, 404, 242, 237, 190, 147, 110, 67, 40, 34 (2X), and 26 bp, in addition to a lambda *Hin*dIII digest.

53. If one of the flanking clones is too small, it may not be detected on the gel. This problem may be circumvented by digesting more DNA, and increasing the agarose percentage of the gel to 3 or 4%. Alternatively, one may be content with only a single flanking probe, or use another four-cutting enzyme than *Sau*3A for the cloning of the flanking probes.

54 *Ssp*I does not cut these HSV-1 fragments but, instead, the vector only Since, for technical reasons, the HSV-1 inserts could not be cut out of the vector, this was done to physically separate the HSV-1 DNA fragments that are present at either side of the vector

55 Most PRV flanking probes have sizes greater than 200 bp Smaller probes are labeled inefficiently by random priming. Therefore, concatemerization of very small probes by ligation is advised to improve labeling efficiency.

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6

Construction and Use of Cell Lines Expressing HSV Genes

Claire Entwisle

1. Introduction

Complementary cell lines have become an accepted tool for the functional analysis of viral genes *(1,2)* and proteins, as well as an essential component in strategies for the construction of mutant viruses. More recent applications include the propagation of replication-defective virus products with potential as viral vaccines *(3,4)* or as vehicles for gene therapy *(5,6)*. The perceived requirements for such systems are low recombination frequencies between complementing cell and recombinant virus, stable expression of the complementing protein within the cell, and efficient complementation. The standard techniques of eukaryotic cell transfection and clonal selection are routinely employed in the generation of complementary cell lines, and are described briefly in this chapter. Perhaps a more novel introduction to this field is the possibility of using transgenic technology. Transgenic animals have the potential to provide both an in vivo model of complementation *(7)* and a comprehensive library of novel complementing cell types, particularly cell types resistant to traditional transfection protocols.

1.1. Principle

The introduction of plasmid DNA into eukaryotic cells can be accomplished by several techniques: calcium chloride precipitation, dextran, electroporation, or liposome fusion *(8)*. Each method has particular advantages and disadvantages, and may be more or less suitable for a particular cell type. Series of trial transfections using a test vector and reporter gene can be set up to determine appropriate transfection conditions *(8)*. Lipofectin™ reagent (Gibco-BRL) is

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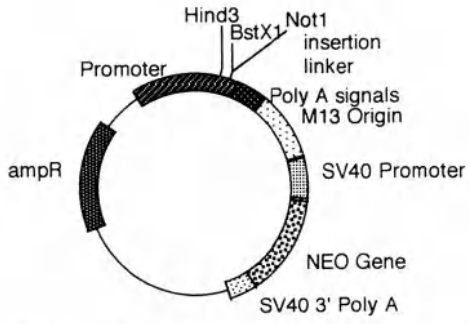

Fig. 1. Expression vector. Unique sites in polylinker *Hin*dIII, *Bst*XI, *Not*I, *Xba*I, and *Apa*I.

generally applicable to most cell types. Methods and recommended uses are supplied by the manufacturer. Calcium chloride precipitation is a simple alternative, and requires no investment in expensive equipment or specialized reagents. A general method is described in this chapter.

Several commercially available plasmids are suitable for mammalian cell expression (Fig. 1). Suppliers are listed Section 2. Expression vectors generally include a selection cassette, consisting of a constitutive promoter driving a selection marker, an amp resistance marker for plasmid maintenance in *Escherichia coli,* and an expression cassette with a promoter sequence, insertion linker, and poly A signal.

When selecting or modifying an available plasmid, it is important to consider the activity and source of the expression promoter, whether to choose a constitutive *(1)* or inducible system, and whether early or late expression within the viral replication cycle is a particular requirement. Viral specific inducible promoters *(4)* are particularly useful for the expression of proteins toxic to cell survival. Even with proteins considered nontoxic, expression from an inducible promoter may increase cell line stability. The homologous promoter and poly A signals should also be considered and may be appropriate for certain experimental protocols. Unfortunately, this approach can sometimes lead to later problems with unacceptable levels of recombination between the cell line and deleted virus.

There are a number of selection systems available. The most widely used dominant selection system is the neoresistance marker, which introduces resistance to the antibiotic Geneticin®. Other useful systems are puromycin, hygromycin, HSV tk, and His D. The particular choice of system will depend on the pre-existing phenotype of the target cell type and the availability of the respective antibiotic/or marker plasmid.

2. Materials

2.1. $CaCl_2$ Transfection of Vero Cells

1. Dulbecco's modified Eagle's medium (DMEM)
2. Fetal calf serum
3. Phosphate-buffered saline, magnesium-, and calcium-free.
4. 1X Trypsin-EDTA, ICN Biomedicals, Inc , cat no 16-891-49.
5. 2X HBS· 280 m*M* NaCl, 50 m*M* HEPES, 1 5 m*M* Na_2HPO_4, in H_2O Adjust to pH 7.1–7 2. Sterilize the stock solution by filtration, and store at –20°C in 20-mL aliquots
6. TE buffer 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, and filter-sterilize
7. Ethanol
8. 3*M* sodium acetate, pH 5 0.
9. Stock solution of G418 (Sigma, St. Loius, MO, Geneticin® product no. G9516) G418 is dissolved in to give a stock solution at 10 mg/mL. Filter sterilize, and store aliquots at –20°C
10. Vero cell growth media (GM). DMEM, 10% FCS, 1% glutamine. Transfectants are selected at a concentration of 600 µg/mL G418 in GM
11. Tissue-culture grade flasks and Petri dishes.
12. Mammalian expression plasmids are available from Invitrogen (R&D Systems, UK), Promega Corporation, and Clontech (Cambridge Bioscience, UK)
13. The pCAT and Luciferase reporter gene systems are available from Promega (Southampton, UK)

2.2. Screening of Cell Lines by PCR

1. Digestion buffer· 5 m*M* EDTA, pH 8.0, 200 m*M* NaCl, 100 m*M* Tris-Hcl, pH 8.0, 0.2% SDS
2. Proteinase K (Sigma cat no P2308) 10 mg/mL in UHP H_2O.
3. 10X PCR buffer. 100 m*M* Tris-HCl, 15 m*M* $MgCl_2$, 500 m*M* KCl, pH 8 3
4. Equilibrated phenol/chloroform· 50/50 mix of Tris-saturated phenol, pH 8.0 (FSA, laboratory supplies), and chloroform
5. *Taq* DNA polymerase (5 U/mL Boehringer).
6. dNTP mix·
   1.25 m*M* of each dNTP diluted into UHP H_2O
   100 m*M* stocks are available from Boehringer
   dATP lithium salt 100 m*M* (cat. no. 1051440)
   dCTP lithium salt 100 m*M* (cat. no. 1051458).
   dGTP lithium salt 100 m*M* (cat. no 1051466).
   dTTP lithium salt 100 m*M* (cat. no. 1051482)
7. Light mineral oil (Sigma cat. no. M5904)
8. PCR primers diluted to 50 pmol/µL. For PCR from genomic templates, 30-mer oligos are recommended For RT-PCR, use 18–22 mer within the coding sequence
9. 1 L TBE buffer (10X) 108 g Tris base, 55 g boric acid, 9.3 g EDTA It should be unnecessary to adjust the pH of this solution However, check that the pH is 8 2–8 5

10. Agarose ultra-pure electrophoresis grade (540–5510UB BRL Life Technologies)
11 DNA electrophoresis mol-wt markers
12 Ethidium bromide made up in water at 10 mg/mL stock.
13 M-MLV Reverse Transcriptase (cat. no. 28025-013 Gibco-BRL)
14 Random Hexamers (Pharmacia Biotech cat no. 27–2166) 100 pmol/μL
15. Diethyl pyrocarbonate-treated (DEPC) H_2O. Add DEPC 0 1% to UHP water, and stand at room temperature for 4 h Then autoclave.
16. RNasin ribonuclease inhibitor (Promega, cat. no N2511)

3. Methods

Many cell types can potentially be used for the production of complementary cell lines. The parental line must be permissive for virus growth and preferably give high titers The cell line must also be susceptible to one or the other of the transfection techniques available. Vero (African Green monkey kidney ECACC no. 84113001), BHK 21 (Syrian hamster kidney ECACC no. 85011433), or MRC5 (Human Fetal Lung Fibroblast ECACC. no. 84101801) cells are appropriate parental lines for many herpes viruses and will transfect with high efficiency. The amount of selective antibiotic or drug required to kill untransfected cells should be determined prior to transfection, for example, G418 should be titrated onto growing cells at concentrations ranging from 200 μg/mL to 2 mg/mL. A suitable selection concentration will give complete destruction of the monolayer after 10–14 d. Cells are media changed every 3 or 4 d.

3.1. $CaCl_2$ Transfection of Vero Cells

1 Aspirate the growth media from a semiconfluent 80-cm^2 flask of vero cells, wash twice with 5 mL of PBS, and add 2 mL of trypsin. Incubate the flask at 37°C for 2–5 min until the cells begin to lift off
2. Neutralize the trypsin by adding 5 mL of growth media, and gently resuspend the cells. Count the cells and adjust the concentration to give 1×10^5 cells/mL.
3. Seed 60-mm Petri dishes (tissue-culture grade) at 5×10^5 cells/plate
4 Incubate at 37°C, 5% CO_2 for 24 h.
5 Aliquot 30 μg of plasmid DNA into an Eppendorf. This is sufficient DNA to transfect three 60-mm plates Precipitate the DNA by adding 2 5 vol of ethanol and $^1/_{10}$ vol 3*M* sodium acetate. Store at –80°C for 5 min (or until frozen solid) Spin Eppendorfs at 12K for 10 min
6. Transfer the Eppendorf to a tissue-culture cabinet, and aspirate the ethanol. Air-dry the DNA pellet Resuspend the DNA carefully in 60 μL of TE buffer. Transfer to a universal containing a further 675 μL of TE and 75 μL of $CaCl_2$
7 Add dropwise 750 μL of 2X HBS A fine precipitate should form Allow to stand for 10 min at room temperature.
8 Aspirate the medium from the cells, and replace with 500 μL of the DNA precipitate Incubate at 37°C 5% CO_2 in a humidified incubator for 4 h.

9 Add 3 mL of growth medium (GM) to each plate, and aspirate off. Add 500 µL of glycerol shock solution, and allow to stand at room temperature for 2 min Add 3 mL of GM, and aspirate Wash once, and then add a further 3 mL GM and return to the incubator.
10. Forty-eight hours later, the cells are changed into selection medium
11. At the concentration suggested, selection in G418 will take about 2 wk. Split or refeed the cells every 3 or 4 d, always maintaining the selection.

Single-cell clones can be isolated by dilution cloning either from a fully selected mass culture or from transfected, but unselected cells 24–48 h posttransfection. Both proceduues will give similar results. There may be advantages with selecting early if the gene is particularly poorly expressed, since a greater diversity of clones with fewer sister colonies will be isolated.

3.1.1. Dilution Cloning of Cell Lines

Plate cells in 96-well plates at concentrations ranging from 0.1–5 cells/well/100 µL. Independent clones will appear after 1–2 wk. Scan the plates under the microscope to select wells that contain single colonies. Clones can be picked and transferred to 24-well plates and expanded. It is advisable to maintain the selection pressure throughout the cloning process to minimize reversion of the clone. As a precaution against losing the transfected cell lines from fungal or bacterial contamination, aliquots should be frozen at frequent intervals and transferred to liquid nitrogen storage containers. A freeze medium of 10% DMSO and 90% serum is suitable for most cell types. When recovering cells from frozen storage, replating in medium containing the selective drug will often decrease cell viability. Selection should be applied 24 h postrecovery.

3.2. Screening of Cell Lines by PCR

Unfortunately not all clones isolated will express the transfected protein. This is sometimes owing to rearrangements and deletions within the plasmid construct following or during integration. A more frequent reason may be inefficient application of the selection. Excess cells can be screened by PCR or Southern blot to check plasmid integrity and to estimate copy number. Protein expression is confirmed by RT-PCR or more frequently Western blot. If a null mutant virus is available, the most precise screening method for "essential gene" expression in a complementing cell line is by plaque assay or complementation assay.

3.2.1. Screening by DNA Analysis

The DNA prepared by this method is suitable for analysis by PCR and Southern or slot-blot probing.

1. Pellet 1–5 × 10^6 cells in a sterile Eppendorf, aspirate the media, and resuspend

the cell pellet in 730 µL of digestion buffer Add 20 µL of a stock solution of proteinase K Incubate at 56°C for 2–4 h

2 When completely digested, add 500 µL of equilibrated phenol/chloroform Vortex briefly (15 s) Spin for 10 min at 12K in an Eppendorf centrifuge. Transfer 500 µL of the aqueous phase (top layer) to a clean Eppendorf Avoid transferring any protein material present at the interface

3. Add 750 µL of ethanol to each sample, spin samples for 10 min at 12K, and carefully aspirate the ethanol without disturbing the DNA pellet Wash the pellet in 70% ethanol, centrifuge, and aspirate Air-dry under vacuum

4. Resuspend the pellet in 50 µL of TE buffer Heat at 65°C for 2 h to inactivate contaminating DNases and aid in resuspension

5 Measure the absorbance at 260 nm (1 OD = 50 µg/mL) Dilute to give a working stock of 100 µg/mL

Prepare a stock PCR mix as follows The volumes are scaled up depending on the number of samples to be screened. For 20 PCR reactions with a final vol of 20 µL/reaction, the mix is made up as follows. Use sterile plasticware throughout.

1 Reaction mix

40 µL of 10X PCR buffer
64 µL of dNTP mix
Primer 1 8 µL
Primer 2 8 µL
76 µL of UHP
4 µL *Taq* polymerase

Mix well and aliquot 10 µL into sterile 0 5-mL PCR tubes Store on ice

2 Add 1 µL (60–400 ng) of genomic template DNA, and make up to 20 µL with UHP H_2O. Overlay with 20 µL of light mineral oil.

3. Following PCR, the mineral oil is aspirated from the reaction and 2 µL of loading buffer is added to the sample

4. Prepare a 1% agarose gel in 1X TBE containing ethidium bromide at 1 µg/mL.

5 Load samples alongside appropriate mol-wt markers and standard samples Suggested PCR protocol

Step 1 Denature	5 min	94°C	1X
Step 2 Denature	1 min	94°C	25X
Annealing	1 min	40–55°C	
Elongation	2 min	72°C	
Step 3	5 min	72°C	1X
Hold		4°C	

Negative control reactions should be prepared in parallel containing genomic DNA from untransfected cells Appropriate plasmid samples are also prepared as positive controls and spiked into control genomic DNA A dilution series rang-

ing from 100 pg to 100 ng of plasmid DNA is a useful range to check the efficiency of the PCR reaction The PCR reaction may be improved by the addition of DMSO to the reaction mix at a final concentration of 7.5%.

3.2.2. Screening by RNA Analysis

Screening for protein expression by Western blot may be appropriate if a suitable antibody is available and if the protein is expressed at detectable levels Screening by RT-PCR can be a quick alternative. Total RNA can be prepared by several methods *(9)*, and many commercial kits are available (Stratagene RNeasy cat. no. 74104). The RNA sample must be free of contaminating DNA.

Prepare a stock PCR mix as follows. The volumes are scaled up depending on the number of samples to be screened. For 10X RT-PCR reactions with a final volume of 100 µL/reaction, the mix is as follows. Quantities can be scaled down appropriately.

1 Reverse transcriptase reaction mix (A).

20 µL PCR buffer
32 µL dNTP mix (1 25 m*M*)
0.25 µL RNasin
10 µL random hexamers
5 µL enzyme
32 75 µL DEPC-treated H_2O

Prepare the above mix in duplicate (B) omitting the RT enzyme

2. Dilute RNA samples into DEPC-treated H_2O to a final vol of 10 µL Use approx 1 µg/reaction. Prepare samples in duplicate Set up negative controls omitting template Add 10 µL of either RT mix A or B to each duplicate
3 Incubate at room temperature for 10 min, and then at 42°C for 1 h.
4. Heat-treat at 90°C for 5 min to inactivate the enzyme
5 Prepare PCR mix: 20X PCR reactions.

20 µL of primer 1 at a conc of 50 pmol/µL
20 µL of primer 2 at a conc. of 50 pmol/µL
200 µL 10X PCR buffer
8 µL *Taq*
1352 µL of DEPC-treated H_2O

6. Prepare appropriate water only controls. Overlay with 100 µL of mineral oil.
7 Suggested PCR protocol·

Step 1	Denature	5 min	90°C	1X
Step 2	Denature	1 min	90°C	1X
	Annealing	1 min	55°C	25X
	Extension	1 min	72°C	1X
Step 3		5 min	72°C	1X
	Hold		4°C	

8 Prepare a 1% agarose gel in TBE 1X buffer, containing ethidium bromide at 1 μg/mL.
9 Load the samples alongside appropriate mol-wt markers.

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7

Analysis of HSV Polypeptides

Lars Haarr and Nina Langeland

1. Introduction

Lytic infection by herpes simplex virus (HSV) efficiently inhibits the synthesis of most cellular proteins while a large number of viral proteins is produced, including a host shut off protein *(1–3)*. The inhibitory effect of the protein obviously needs a certain period of time to be fully efficient, but some cellular proteins involved in virus replication still are produced. Examples are those interacting with the regulatory viral proteins $V_{MW}65$ ($65K_{TIF}$) and IE 110 (V_{MW} 110, ICPO) *(4–8)*. Certain quiescent cellular genes are activated after infection to express their proteins *(9)*.

These basic facts have implications for analysis of HSV proteins. First, one should distinguish between virus-induced and virus-encoded proteins. Second, extracts from infected cells always will contain a mixture of cellular proteins and newly synthesized virus-encoded proteins, although the proportion of the former is markedly reduced at late times of infection. Comparison of uninfected and infected material therefore is crucial for detection of virus-induced proteins.

Analysis of proteins is a large subject that can be divided into a number of different topics. Here we will focus on separation of the proteins in polyacrylamide gels, their identification and localization, and some posttranslational modifications

2. Methods

2.1. Separation of Proteins by Electrophoresis in Polyacrylamide Gels

2.1.1. Separation Capacities of One-Dimensional and Two-Dimensional Gel Electrophoresis

Since the introduction by Laemmli *(10)* 25 yr ago, electrophoresis in a discontinuous polyacrylamide slab gel system has been one of the most widely

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used methods in experimental biology. The solubilized proteins, covered with the negatively charged detergent sodium dodecyl sulfate (SDS), are separated according to their M_rs (SDS-PAGE). Under optimal conditions one can detect a maximum of 50–70 distinct protein bands. The HSV genome has a coding capacity for at least 76 different proteins *(11–14)*. A product from a single gene may exist in several forms because of posttranslational processing. The total number of viral and cellular proteins and their different forms is far beyond the separating capacity of SDS-PAGE, although the separation within a certain range of M_rs can be markedly improved by manipulation with the polyacrylamide concentration

By two-dimensional (2D) gel electrophoresis, as described by O'Farrell *(15,16)* the proteins are separated in a pH gradient according to their charges before running in a SDS-PAGE system. By this technique more than 1000 spots can be separated easily in material from HSV-infected cells *(17)*. Maximal separation is obtained by adjusting the conditions for electrophoresis either in the first or in the second dimension, depending on the molecular charges and weights of the proteins of interest. A variety of ampholyte mixtures are available such that almost any area in the range pH 3.0–11.0 can be expanded in the first dimension. To avoid artifactual charges, a neutral detergent like Nonidet P40 (NP-40) has to be used in the first dimension. The pH gradient gradually is broken down during electrophoresis such that proteins may be lost both from the acidic and the basic end of the gel This is avoided by stopping the electrophoresis before all proteins have reached their isoelectric points, a method referred to as NEPHGE (nonequilibrium pH gel electrophoresis) *(16)*. Recent advances in 2D gel electrophoresis include replacement of the ampholyte system with immobilized pH gradients that do not drift during focusing *(18–21)*

2.1.2. Solubilization of the Proteins and Denaturation

Proteins to be analyzed by SDS-PAGE are efficiently solubilized by boiling in the presence of SDS and 2-β-mercaptoethanol. The original solubilization procedure for 2D gel electrophoresis used NP-40 in combination with high concentrations of urea (9.5*M*) and 2-mercaptoethanol (5%) *(15)*. Some hydrophobic membrane proteins, however, are not dissolved under these conditions *(22)*. To overcome this problem a combination of NP-40 and SDS or 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate (CHAPS) has been used *(23)*. The concentration of the ionic detergent should be low enough not to have any significant influence on the charge of the protein. An example of the effect of SDS is shown in Fig. 1. The spot marked with an arrowhead is almost undetectable in the absence of SDS.

One should keep in mind that the solubility of a protein may change from the state immediately after translation to its final modification and localization. Thus, the spot labeled VP11/12 in Fig. 2, which is supposed to be the

Fig. 1. Effect of SDS on solubilization of proteins for 2D gel electrophoresis. BHK cells were infected with HSV-2 and proteins labeled with [^{35}S]-methionine. One portion of the proteins was solubilized in 9.5*M* urea, 2% NP-40 and 5% mercaptoethanol as described in Section 3.7. Another portion was solubilized in the same mixture except that SDS was added to a final concentration of 1.3%. Undissolved material was removed by centrifugation for 1 h at 156,000*g* in a Beckman SW50.1 rotor. The slab gel contained 5–12.5% polyacrylamide crosslinked with BIS. The arrowhead shows a protein solubilized by SDS only.

tegument protein VP11/12 (U_L 48), is quite substantial in the gel after in vitro translation, more faint in material from infected cells, and almost undetectable at the expected position in material from virions and light particles. This disappearance is related to an increased amount of protein unable to enter the pH gradient gel, as indicated by the arrowhead.

The solubilization procedures mentioned earlier result in denaturation of the proteins. For certain purposes, however, it may be crucial to preserve their native forms. A nondenaturing system for polyacrylamide gel electrophoresis has been used successfully to localize discontinuous epitopes of glycoprotein gD *(24)*. In this system the concentration of SDS was reduced to 0.1% as compared to 2% in ordinary SDS-PAGE.

2.1.3. Artifacts from the Reagents and Influence of the Crosslinkers on the Apparent M_r

Urea used for solubilization and electrophoresis in the pH gradient may be degraded to form cyanate ions that react with the proteins and introduce artifactual charges. One way of avoiding this is to keep the sample on dry ice during all handlings, except when dissolving and loading it on the gel.

Silver staining of the slab gel may result in horizontal streaking that is usually caused by mercaptoethanol. It can be reduced by filtering the reagent. Some researchers have successfully substituted mercaptoethanol with dithiothreitol

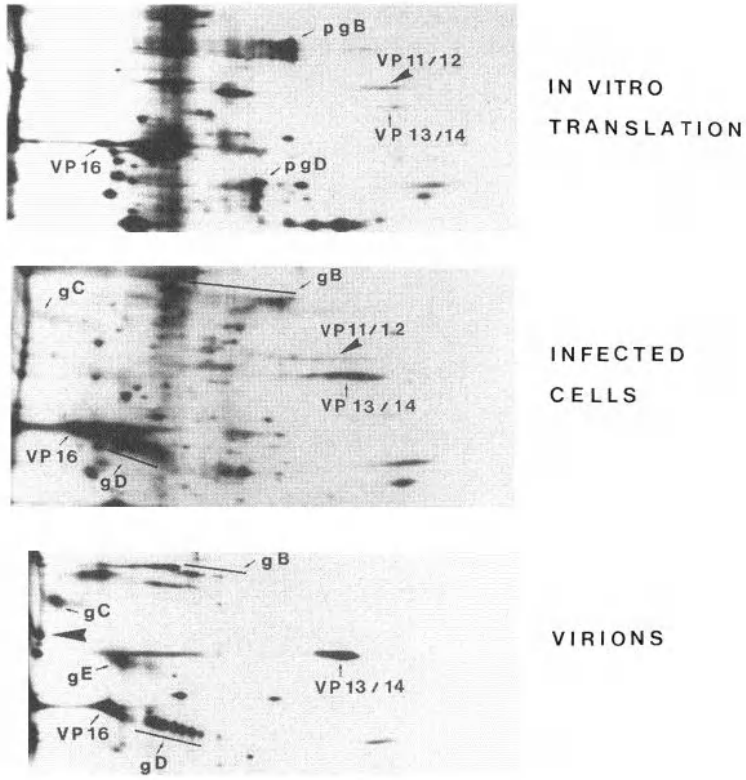

Fig. 2. Variation of the solubility of a protein. Proteins labeled with [^{35}S]-methionine were solubilized in the presence of urea, NP-40, and mercaptoethanol as described in Section 3.7., and then subjected to 2D gel electrophoresis. Gradient gels of 5–12.5% polyacrylamide crosslinked with BIS were used. Upper panel: RNA was isolated from BHK cells infected with HSV-1 and used for in vitro translation as described in Section 3.6. Middle panel: Proteins in extract from BHK cells infected with HSV-1. Lower panel: HSV-1 virions were radioactively labeled and purified in a Nycodenz gradient as described in Section 3.3. Some HSV-1 specific proteins are indicated. The relative amount of VP11/12 migrating into the pH gradient gel is different in the three panels and supposed to reflect different solubility.

(DTT) and observed that 65 m*M* is the optimal concentration *(25)*. Vertical streaking after silver staining may be caused by dust present on the glass plates during casting of the slab gel.

Separation on 2D gels demonstrate clearly that the crosslinkers in the slab gel affect the migration of some proteins. Figure 3 shows analysis of the same

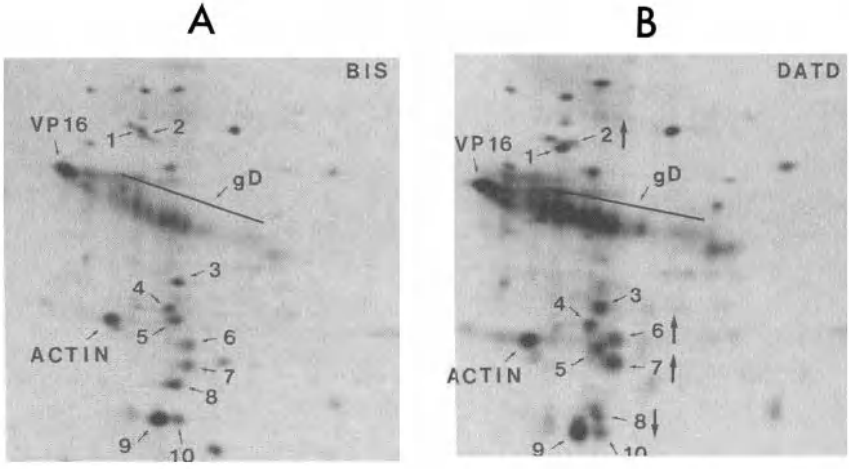

Fig. 3. Effects of the crosslinkers on the relative migration of proteins in polyacrylamide gels. Human fetal lung (HFL) cells were infected with HSV-1, proteins labeled with [^{35}S]-methionine and subjected to 2D gel electrophoresis. Two samples were run identically in the first dimension, then either in a 7.5% polyacrylamide gel crosslinked with BIS **(A)**, or in a 9% polyacrylamide gel crosslinked with DATD **(B)**. Ten different polypeptides were identified in both slab gels and arbitrarily designated 1–10. Those migrating markedly different in the two slab gels are labeled with large arrows, indicating the direction of deviation in B relative to A.

material in identical pH gradients, but on two different slab gels. Proteins of interest are marked 1–10. The relative positions of spots 1, 2, 6, 7, and 8 vary considerably when *N,N'*-diallyltartaramide (DATD) is used as crosslinker instead of *N,N'*-methylene-*bis*-acrylamide (BIS). Such variations are also observed for other proteins in other areas of the slab gel (results not shown). The discrepancies some time ago about the M_rs of certain HSV proteins can thus be explained by the fact that different crosslinkers were used.

2.1.4. Detection of Separated Proteins by Staining, Autoradiography or Fluorography

The sensitivity of silver staining is 50–100 times higher than that of Coomassie blue *(26)*, and comparable to that of autoradiography. Loading of 10–20 μg of total protein per tube gel is usually enough to visualize a large number of spots of varying intensity. Adjustment of the incubation period for optimal staining sometimes is a fine balance between the intensities of the spots and background staining. Radioactive labeling of the proteins has several

advantages. First, the loading on the gel depends on the amount of radioactivity rather than of protein. Second, the same gel can be exposed to films for a variety of periods such that heavily labeled and weakly labeled bands or spots are distinct after short and long exposures, respectively. Third, in our hands the background is clearer than after silver staining.

The low-energy β-emission from [^{3}H] is almost undetectable unless special films with high sensitivity are used. Alternatively, the radioactive signal is converted into scintillation by infusion of the gel with a scintillant. In the original procedure of Bonner and Laskey *(27)* the hydrophobic compound 2-5-diphenyloxazole (PPO) was dissolved in dimethylsulfoxide (DMSO) and precipitated in the gel by addition of water. The dried gels are exposed to film at –80°C. DMSO, however, is toxic and penetrates skin as well as mucous membranes both in the liquid form and as vapor. Less toxic or atoxic scintillants prepared for the same purpose are available commercially (Amplify from Amersham [Arlington Heights, IL] and En^{3}Hance from New England Nuclear [Beverly, MA]). Signals from β-emitting isotopes with energies slightly higher than [^{3}H] ([14C] and [^{35}S]) are increased by a factor of 10 by this technique.

To increase the incorporation of [^{35}S]-methionine into HSV proteins the concentration of methionine in the medium is reduced to 20% *(28)*. Labeling for periods shorter than 2–3 h can be performed in PBS. He et al. *(29)* compared the incorporation of [^{35}S]-methionine and [^{14}C]-amino acids into proteins in mouse fibroblasts during a period of 24 h, and found rather surprisingly that the former method was three to four times better. A few spots, however (3–4% of the total), were labeled with [^{14}C] and not with [^{35}S] Glycoprotein L is one example of a HSV protein that is labeled with [^{35}S]-cysteine rather than with [^{35}S]-methionine *(30)*.

2.1.5. Detection of Protein Processing

Many of the herpes simplex virus proteins are processed further either concomitant with the translation or afterward. Eleven glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM) are described so far *(30–36)*. Most, probably all, of the tegument proteins (at least 11) are phosphorylated (reviewed in ref. *33)*. Kinase activities are induced by HSV-1 in infected cells *(37)*, and the nuclear kinase encoded by gene U_L13 is also present in virions *(37–39)* The structural proteins VP24 and VP21 and the scaffolding protein VP22a, are generated by proteolytic cleavage of precursor proteins from genes U_L26 and $U_L26.5$, respectively *(40–44)* A tegument protein (encoded by gene U_L11) is myristylated *(45,46)*. Another tegument protein, VP22, seems to be modified by poly(ADP-ribosyl)ation *(47)*, and the immediate–early regulatory proteins are subjected to both adenylation and guanylation *(48)*.

Glycosylation is usually detected by the incorporation of [^{14}C] glucosamine or [^{3}H]-mannose added directly to the medium. A number of different com-

pounds inhibit specific steps during glycosylation. Tunicamycin blocks N-linked glycosylation *(49,50)*, whereas monensin impairs Golgi apparatus function, i.e., both O-linked glycosylation and the processing of N-linked moieties *(51)*. Carbohydrate groups already bound to the proteins are cleaved specifically with different enzymes *(52,53)*. High-mannose oligosaccarides are sensitive to endoglycosidase H (endo-β-*N*-acetyl glucosaminidase, endo H) until passage through the Golgi, then resistant. Endoglycosidase F (endoglycosidase F/N-glycosidase, endo F) cleaves N-linked oligosaccarides specifically, both from high mannose and complex glycoproteins. O-linked carbohydrates are sensitive to O-glycanase (endo-α-*N*-acetylgalactosaminidase). Sialic acid, which is charged, is removed by neuraminidase.

Phosphoproteins are labeled with radioactive inorganic phosphate ([^{32}P-P$_i$]). We find that the incorporation is markedly increased by dialysis of the serum and reducing the phosphate concentration in the medium to 10%. Beta-emission from [^{32}P] has a relatively high energy capable of causing damage of different molecules, including nucleic acids. Thus, in the presence of 50–500 μCi/35-mm dish we observed that DNA synthesis was reduced by approx 15% at 6–7 h after infection and addition of isotope, and by 50% at 12 h

Myristylation takes place during translation as covalent binding of myristic acid to a consensus sequence in the N-terminus *(54)*. It is detected by the incorporation of [^{3}H]-myristic acid, and has been described for a large number of different proteins in various viruses *(55–62)*. The only HSV product known so far to belong to this group is encoded by gene U_L11 *(45,46)*. Protein precursors are often labeled in pulses of 10–30 min with the radioactive compound, and the final products detected after a subsequent chase. Some posttranslational modifications, however, may occur even during the shortest possible pulse. Systems for in vitro translation of mRNAs are normally so different from infected cell cultures that posttranslational modification either is deficient or absent. In vitro translated proteins thus may be considered to be more or less identical to the primary translation products. Some large mRNAs may not be translated efficiently in vitro. Removal of posttranslationally added moities like carbohydrate or phosphate groups usually will result in a mobility shift during electrophoresis such that the precursor–product relationship is established. Specific antibodies used in immunoprecipitation or in Western blot may unambiguously demonstrate such relationships.

Cleavage of HSV polypeptides owing to the unspecific action of cellular proteases should be avoided by adding protease inhibitors, for example, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), and by keeping the preparations on ice.

Figures 4 and 5 show examples of processing of HSV-proteins detected by 2D gel electrophoresis in combination with pulse–chase, in vitro translation, and enzymatic treatment.

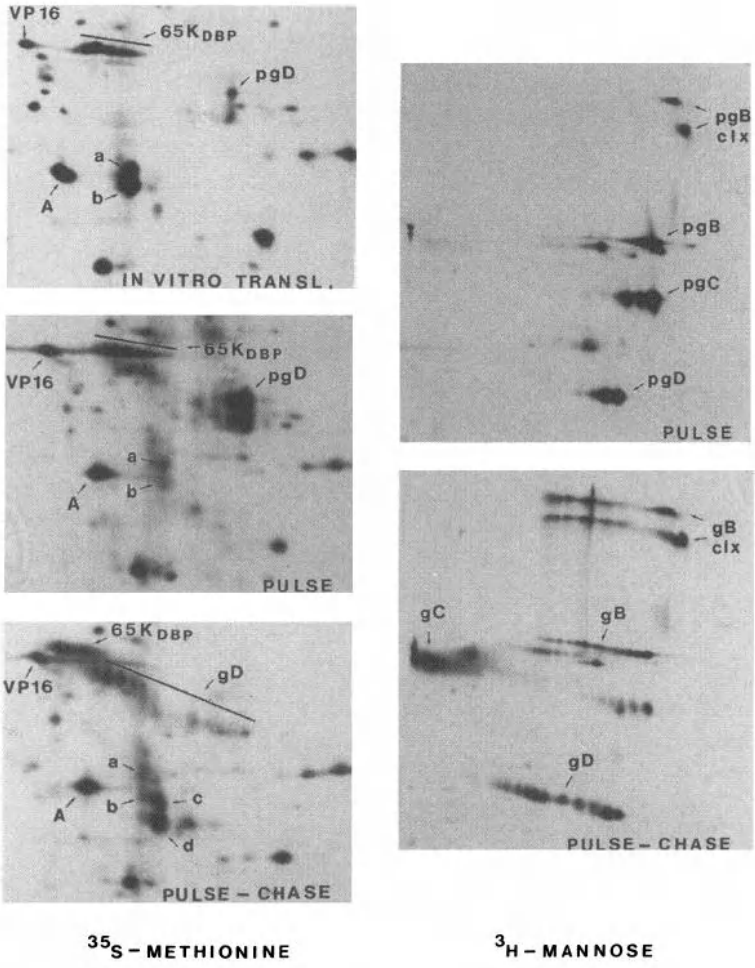

Fig. 4. Posttranslational modifications of proteins detected by 2D gel electrophoresis. BHK cells were infected with HSV-1. The proteins were labeled with [^{35}S]-methionine either by in vitro translation of isolated RNA, by a 30-min pulse at 5 h post infection, or by a 30-min pulse followed by chase for 12 h. The slab gels contained 9% polyacrylamide crosslinked with DATD. In the right panels the glycoproteins were labeled with [^{3}H]-mannose using a 30-min pulse only, or a pulse followed by chase for 6 h. Gradients of 5–12.5% polyacrylamide crosslinked with BIS were used in the slab gels. Processing is indicated for the 65 K DNA-binding protein ($65K_{DBP}$), gB, gC, gD, and for the polypeptides arbitrarily designated *a*, *b*, *c*, and *d*. VP16 and actin (A) are unmodified and used as markers.

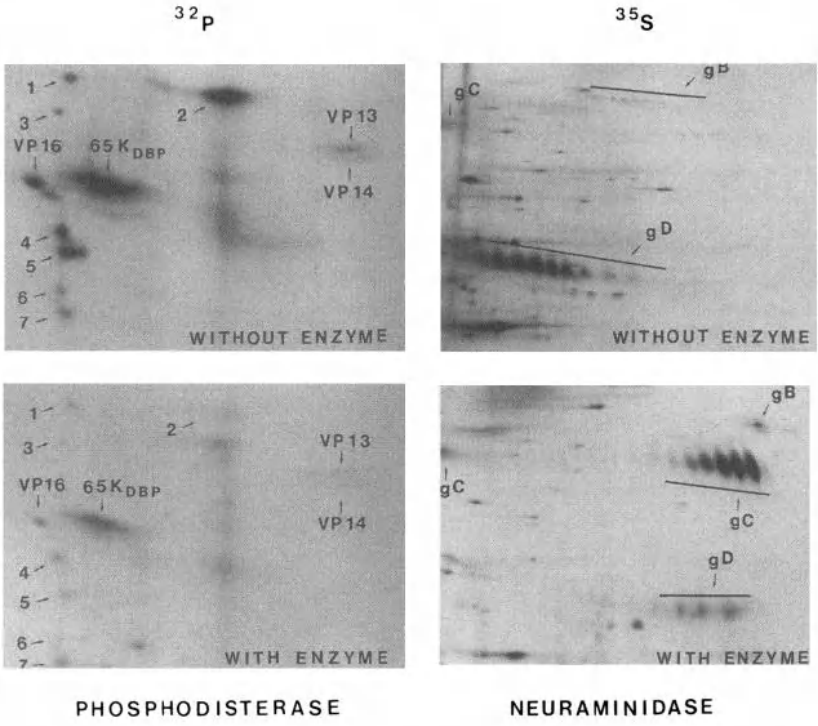

Fig. 5. Enzymatic removal of moities added by posttranslational modification. BHK cells were infected with HSV-1 and the proteins labeled with either [^{32}P]-P_i (left panels or [^{35}S]-methionine (right panels). The phosophoprotein preparation was incubated for 30 min at 37°C with either 15 U/mL of phosphodiesterase (EC 3.1.4.1.) or with water. The slab gels contained 9% polyacrylamide crosslinked with DATD. Other HSV-1-infected cells were labeled with [^{35}S]-methionine and the proteins incubated for 14 h at 37°C with either 0.02 U neuraminidase (EC 3.2.1.18.) in 40 µL total volume, or with water. Two-dimensional gel electrophoresis was performed using a mixture of 20% ampholines pH 3.5–10.0 and 80% ampholines pH 5.0–7.0 such that the region pH 5.0–7.0 was expanded. The slab gels contained 5–12.5% polyacrylamide crosslinked with BIS. Some virus-induced proteins are indicated as well as the polypeptides arbitrarily designated 1–7.

2.2. Detection of Proteins Present in Small Amounts

Herpesvirus proteins present in minute amounts may not be detected unless they are tagged by insertion of an epitope recognized by a powerful monoclonal antibody. This technique was used successfully to identify the product from gene U_L37 *(63)*, and to separate the otherwise indistinguishable proteins

derived from genes U_L26 and $U_L26.5$ *(41)*. In both cases the commercially available antibody was directed against a CMV epitope

A protein accumulating in a certain compartment of the cell, or in a structural component of the virion may barely be detectable in extracts from whole cells but quite evident in a particular cell fraction or in isolated virions.

2.3. Mapping of Proteins to Their Encoding Genes

One of the first successful strategies to map HSV proteins used intertypic recombinants between HSV-1 and HSV-2 and took advantage of the fact that homologous proteins from the two types migrate slightly different in SDS-PAGE gels *(64,65)*. Several [^{35}S]-methionine-labeled proteins, phosphoproteins and glycoproteins were thus assigned to restricted regions of the HSV genome. Temperature sensitive mutants as well as other mutants are important tools for more precise localization. Virus mutants deficient in essential genes, like those encoding gB, gD, gH, and gL can only be propagated in cell lines carrying the appropriate genes

Marker rescue experiments are cotransfections with specific DNA fragments and genomic DNA carrying the defect under investigation. The rescuing fragment contains the gene *(66)*. Analysis with intertypic recombinants and marker rescue are still powerful methods for obtaining an initial and more or less crude mapping, for example of genes encoding viral ligands involved in attachment *(67,68)*.

The gene technology available today allows almost unlimited manipulations with any of the HSV genes, and thus a detailed functional analysis of protein domains. Transfection with plasmids containing the normal or modified HSV gene of interest has been used successfully to study functional regions in gC and gD *(53,69)*, but gene expression from the plasmid is transient. It is generally accepted that the best way of studying a manipulated gene is to have it inserted into the virus and then analyze the effects. The plasmid containing the HSV gene should have HSV-specific flanking sequences for recombination and insertion at the correct position in the virus genome. This technique has been useful for studies of gC *(70)*.

Alternative methods for correlating a gene to its product include hybrid-arrested or hybrid-selected in vitro translation, generation of antibodies to oligopeptides synthesized according to predicted sequences in open reading frames, and finally, sequencing of the amino acids in isolated proteins.

When hybridizing a specific DNA fragment to a mixture of different RNA molecules, those molecules bound to DNA will be inactive in in vitro translation, but reactivated by denaturing the hybrid The disappearance and reappearance of a given protein can be detected then by SDS-PAGE or 2D gel electrophoresis. Three different protein products from the thymidine kinase gene were detected by this technique *(71,72)*. The gene encoding the 65 kDa

DNA-binding protein was identified by in vitro translation of mRNA isolated by hybrid selection using a specific DNA fragment *(73)*.

Antibodies against synthetic peptides are directed against specific proteins or protein regions, and can be used in search for previously unidentified proteins. The products of gene U_L47 were identified by this method *(74)*. Alternatively, the gene encoding a potential protein can be cloned into an expression vector and the protein then isolated for immunization. To facilitate protein purification it may be useful to introduce a tag of histidines *(75)* or make a fusion protein with glutathione-*S*-transferase *(31,76)* such that the expressed protein can bind to columns of Ni^{2+}-nitriloacetic acid agarose or glutathione, respectively.

Sequencing of proteins isolated by 2D gel electrohoresis was originally described by Vandekerchove et al. *(77)* and by Aebersold et al. *(78)*. Proteins are blotted onto membranes before being cut out for further analysis. Membranes of polyvinylidene fluoride (PVDF), or a second generation thereof *(79)* seem to be best. A problem with this type of microsequencing is N-terminal blocking that may require cleavage of the protein by CNBr *(80)* or with a protease such that internal fragments can be separated by HPLC and then sequenced. At least some of the blocking seems to occur during electrophoresis, fixation, or destaining of the gel owing to impurities in the reagents Therefore it is recommended to use pure chemicals during all those steps.

2.4. Localization of Proteins Within Infected Cells and in the Virions

To localize a protein to a certain compartment in the infected cell or in the virion the various components usually will have to be separated. Several methods are available for fractionation of cells. A simple procedure for separation of cytoplasmic and nuclear fractions is given in the following (Section 3.2.). Both mature and immature virus capsids are isolated by gradient centrifugation of material from nuclei of infected cells *(81,82)*. Light particles consisting of tegument and envelope, but devoid of nucleocapsid, are separated from mature virions of HSV-1 by centrifugation in a Ficoll gradient *(83)*. Distinction between localization in the tegument and in the envelope has to be done by chemical stripping of the particle or by specific labeling of surface proteins, or by a combination of these methods. Stripping is usually performed by incubation with 1% NP-40 in the presence of salt followed by centrifugation in an ultracentrifuge to spin down the remaining particles. Complete solubilization of envelope and tegument by this treatment requires a high concentration of sodium chloride (1*M*) Treatment for longer than 15 min at 4°C under these conditions may result in precipitation of already solubilized proteins.

Iodination was used approx 10 yr ago to detect proteins on the surface of the HSV-1 capsid *(84)*. When studying envelope proteins the reagents should not

penetrate the membrane. For this purpose the water insoluble compound 1,3,4,6-tetrachloro-3α-6α-diphenylglucoluril (Iodo-gen) may be useful in combination with iodine *(85)*. It reacts primarily with tyrosine residues. Iodo-gen sticks to the inside of the test tube such that the reaction is stopped by transferring the mixture to another tube. An alternative method is biotinylation with sulfosuccinimidobiotin (sulfo-NHS-biotin), which is a water soluble reagent unable to penetrate membranes. Biotinylated proteins are then detected by binding of streptavidin ([^{125}I]-labeled or linked to an enzyme). In our hands certain proteins(e.g., gC) are more efficiently labeled by the former and others (e.g., gE) by the latter method.

The virion envelope is fragile and may rupture easily as a result of centrifugation, other mechanical treatments and freezing and thawing *(86)* To ensure a minimum of damaged particles we do the iodination on freshly harvested virus, before purification in a gradient. A result of such an experiment is shown in Fig. 6.

3. Methods

In the following section we describe only the procedures with which we have some experience. Unless otherwise stated we use baby hamster kidney (BHK) cells, Eagle's minimum essential medium, newborn calf serum, and 35-mm plastic dishes for cell culture

3.1. Radioisotope Labeling

3.1.1. Infected Cells

3.1 1 1 Labeling with [^{35}S]-Methionine

Almost confluent monolayers of cells in 35-mm dishes are incubated with 10–20 PFU/cell. Virus is removed after 1 h (zero time) and the infected cells washed twice with medium containing one-fifth the normal concentration of methionine and supplemented with 2% serum. The infection then is allowed to proceed for 0.5–1 h to reduce synthesis of cellular proteins before addition of [^{35}S]-methionine to a final concentration of 50–250 μCi/mL, and incubation continued for the appropriate period of time.

Labeling in pulse–chase experiments is performed after washing and replacing the medium with phosphate-buffered saline (PBS). At the end of the pulse (10–30 min), the label is removed and the cells washed three times with medium. Some cells are harvested (pulse) and others incubated further in medium for chase.

3.1.1.2. Labeling with [$^{32}P_i$]

Prior to labeling the cells are incubated 2–3 h in medium containing one-tenth (10 μ*M*) the normal concentration of phosphate and serum dialyzed

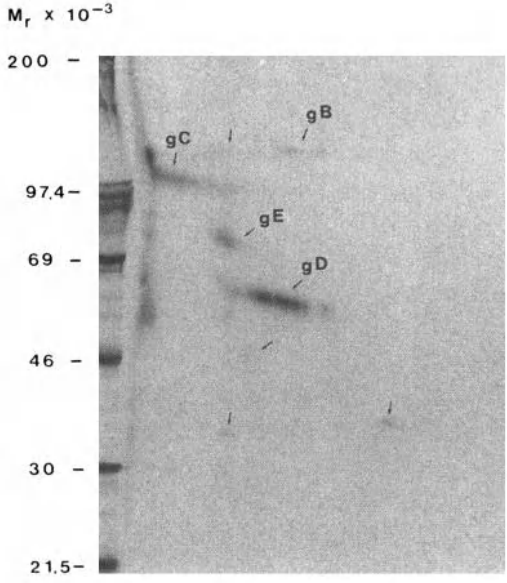

Fig. 6. Two-dimensional gel electrophoresis of [^{125}I]-labeled surface proteins of HSV-1 virions. HSV-1 virions were isolated and iodinated by the Iodo-gen method as described in Section 3.4. before further purification in a Ficoll gradient (*see* Section 3.3.). The slab gel contained 5–12.5% polyacrylamide crosslinked with BIS. M_r markers were run in a separate lane. Labeled proteins are indicated by arrows.

against 0.9% NaCl. Phosphate starving often is started in uninfected cells, and labels added after infection. For 2D gel electrophoresis, 50–150 μCi is used/mL.

3.1.1.3. Labeling with [^{3}H]-Mannose

In the procedure described by Hope et al. *(87)* the medium is supplemented with 2% serum and 100 μCi [^{3}H] mannose/mL is used. The ethanol in which the mannose is supplied is removed before use. Pulse–chase is performed as for [^{35}S]-methionine labeling.

3.1.2. Virions

3.1.2.1. Labeling with [^{35}S]-Methionine

Subconfluent cells in roller bottles are infected with 0.02 PFU/cell in a total volume of 20 mL. One mCi of [^{35}S]-methionine is then added per bottle, and 5-mL portions of normal medium added at intervals such that the final volume does not exceed 40 mL.

3.1.2 2. Labeling with [^{32}P-P$_i$]

Cells in roller bottles are infected as described (*see* Section 3.1.2.1.) and incubated for 5 h in medium with reduced phosphate and 10% dialyzed serum, as described in Section 3.1.1.2. One mCi [^{32}P$_i$] is added and aliquotes of phosphate-reduced medium added at intervals such that the final volume does not exceed 40 mL.

3.2. Preparation of Nuclear and Cytoplasmic Fractions

Infected cells are harvested by scraping with a rubber policeman and centrifugation at 850*g* for 5 min. The pellet is resuspended and incubated for 5–10 min on ice in a solution containing 0 5% NP-40, 0.25% sodium deoxycholate, 10 m*M* Tris-HCl, pH 8.0, and 10 m*M* EDTA. After centrifugation for 5 min at 850–1000*g* the nuclei are in the pellet. The centrifugations were performed in a Kubota 8700 centrifuge using the RS 3000/6 rotor.

3.3. Purification of Virions for Analysis of Structural Proteins

Harvesting starts when the cells are detached. Nuclei are spun down at 850*g* for 10 min, in a Kubota centrifuge, and cellular debris by a subsequent centrifugation in a Sorvall SS 34 rotor for 5 min at 14,500*g*. The supernatant is then centrifuged in the same rotor for 1 h and the pellet resuspended by leaving it overnight in a small volume of medium without mechanical agitation.

Further purification in a Ficoll gradient is described by Szilagy and Cunningham *(83)*. Briefly the resuspended virus pellet from four roller bottles is layered on a preformed gradient of 5–15% Ficoll 400 dissolved in culture medium without phenol red, and centrifugation performed for 2 h at 33,000*g* in a Beckman SW 41 TI rotor. The upper band consists of light particles devoid of nucleocapsids, and the lower band contains the virions. Alternatively, a preformed gradient of 20–40% Nycodenz dissolved in 1 m*M* phosphate buffer (pH 7 4) can be used, and centrifugation performed in a Beckman SW 41 TI rotor for 2 h at 68,000*g*. Light particles and virions are present in the first and second band, respectively, from the top.

In our hands the recovery of infectious virus is approximately the same from these gradients, and superior to that obtained from other gradients.

Virions and light particles are concentrated before electrophoresis by spinning in rotor TLA 120.2 in a Beckman TLX table top ultracentrifuge for 15 min at 157,000*g* or for 3 h at 279,000*g*, respectively. All procedures are carried out at 4°C.

3.4. Iodination of Virion Proteins

The method is adopted from that described by Markwell and Fox *(85)*. Briefly, 2 mg Iodo-gen is dissolved in 2 mL chloroform. Aliquotes of 25–75 µL are transferred to Eppendorf tubes, dried, and then stored at –20°C under nitrogen. [^{125}I] (200 µCi) is then added to each tube and subsequently 30–300 µL virus preparation. Incubation with regular shaking is performed for 10–15 min

at 0–4°C, and the reaction stopped by transferring the solution to another tube without Iodo-gen. To restrict labeling as much as possible to surface molecules the iodination should be performed before loading virus on the gradient. (Some virus membranes may disrupt during gradient centifugation.)

3.5. Biotinylation of Virion Proteins

Virions are purified as described in Section 3.3. and selective labeling of surface proteins obtained as described above (*see* Section 3.4.). When labeling after gradient centrifugation it is recommended to concentrate the virus by adding 1 vol of 100 m*M* Sörensens phosphate buffer pH 8.0, followed by centrifugation in rotor TLA 120.2 in a Beckman TLX table top ultracentrifuge for 15 min at 157,000*g*. The pellet is allowed to resuspend in 100 µL of the same phosphate buffer.

To 100 µL of virus preparation is added an equal volume of sulfo-NHS-biotin 0.4 mg/mL (final concentration 0.2 mg/mL). After incubation for 5 min the reaction is stopped by addition of 7 µL 1*M* citric acid, followed by 100 µL 67 m*M* Sorensens phosphate buffer pH 6.1. All steps are performed at 0–4°C Virus is then spun down in the table top ultracentrifuge as described and subjected to polyacrylamide gel electrophoresis and Western blotting onto a nitrocellulose filter. The filter is incubated overnight in PBS containing 0.05% Tween-20, which is then removed and a new portion containing 0 3–0.4 µCi [^{125}I]-streptavidin/mL added (total volume approx 8 mL). Incubation is carried out for 2 h at 37°C before removing the radioactive solution and extensive washing for 8–12 h using a shaker at room temperature, PBS/Tween as above, and changing the solution minimally six times. The filter finally is rinsed in H_2O, dried, and exposed to film. Sulfo-NHS-biotin is stored dry at 4°C under nitrogen vapor, and the solution is made immediately before use.

3.6. In Vitro Translation and Hybrid Arrest

The protocol developed by C. M. Preston is used *(88)*. Dried DNA is dissolved in a solution of 80 µL formamide and 6 µL 1*M* PIPES (piperazine-*N,N-bis*-2-ethanesulfonic acid) (pH 7.4) and incubated at 90°C for 5 min Then follows sequential addition of 6 µL RNA (approx 6 µg) and 8 µL 5*M* NaCl, and mixing by pipeting up and down, before transferring the tube to 56°C and incubation for 1 h. The reaction mixture is then transferred to ice and the nucleic acids precipitated by addition of 1 mL ethanol and 0.4 mL isopropanol.

The nucleic acids are recovered by centrifugation, the pellet washed with ethanol and allowed to dry thoroughly before dissolving in 7 µL H_2O. An aliquot of 2.5 µL of the solution is stored on ice, and is the hybridized sample. The rest of the solution is heated at 100°C for 60 s to denature the hybrid, transferred quickly to ice and an aliquot of 2.5 µL used for further

reactions. 22.5 µL of an in vitro translation mixture containing 20 µL micrococcal nuclease-treated rabbit reticulocyte lysate and 2.5 µL (2.5 µCi) of [^{35}S]-methionine are then added to each tube. After incubation at 30°C for 60 min the reaction is stopped by adding 25 µL of a solution containing 100 m*M* EDTA (pH 7.5) and 500 µg/mL of RNase A, and incubation continued for 15 min.

When translating mRNA directly, without hybridization and denaturation, approx 5 µg is used per tube.

3.7. 2D Gel Electrophoresis

The following procedure is adapted from that described by O'Farell et al *(15,16)*. Infected cells are lysed by osmotic shock from added water and three cycles of freezing and thawing followed by 1 min of bath sonication at 0°C. Nucleic acids are then degraded by adding to each sample 0 05 vol of 300 m*M* Tris-HCl (pH 7 5) containing 300 m*M* Mg^{2+}, 300 µg/mL DNase 1 and 300 µg/mL RNase A, and incubating at 0°C for 30 min The mixture is then transferred to dry ice and kept there during the subsequent additions

To 187.5 µL sample is added 80 µL 10% NP-40, 16 µL ampholines 3.5–10 (diluted 1:3 in H_2O), 20 µL 2-β-mercaptoethanol and 216 mg urea When dissolved this gives a final volume of approx 400 µL, and the additions can be adjusted proportionally for other sample volumes. The urea is dissolved by gentle shaking at room temperature, the mixture put back on dry ice immediately afterward and stored at –80°C

Solid samples or pellets are dissolved in an appropriate volume of "lysis buffer" containing 9.5*M* urea, 2% (w/v) NP-40, 2% ampholines (1.6% range pH 3.5–10.0 and 0.4% range pH 5.0–7.0) and 5% mercaptoethanol.

Our tubes for the first dimension gels are 10.5 cm long with an inner diameter of 2.3 mm. The solution for the gel is made of 2.75 g urea, 585 µL 30% acrylamide, 900 µL 10% NP-40, 1170 µL H_2O, and 250 µL ampholines pH 3.5–10. Urea is dissolved by gentle shaking at room temperature. At this time 10 µL of 10% ammonium persulfate and 7 µL TEMED are added, and the solution is filled into the vertical tubes that are sealed in the bottom by pushing them against parafilm. Enough space should be left at the top for subsequent loadings. An overlayer of 20 µL H_2O is added and the gels allowed to polymerize for approx 1 h When using more basic ampholines more ammonium persulfate and TEMED are needed for polymerization.

To keep the gel in position during running, a piece of dialysis tubing is fixed with a rubber string at the bottom of the tube, avoiding air bubbles. With the tubes in position in the apparatus for electrophoresis the liquid on top of the polymerized gel is removed and a 10–35 µL sample is loaded per tube. An overlay of 20 µL 8*M* urea (stored at –80°C and thawed immediately before

use) is added. The extra space in the tube and the upper electrophoresis chamber are filled with 10 m*M* phosphoric acid. The lower chamber contains 20 m*M* NaOH. For nonequilibrium pH gel electrophoresis we run for 3 h at 550 V. The gels are then pushed gently and either subjected immediately to electrophoresis in the second dimension, or put into glass tubes that are subsequently filled with "sample buffer" containing 10% (w/v) glycerol, 5% (v/v) mercaptoethanol, 2 3% (w/v) ultrapure SDS, and 62.5 m*M* Tris-HCl, pH 6.8, then stored at –80°C. The tube gel is kept in position on top of the slab gel by pouring a hot (80–100°C) solution of 1% agarose in "sample buffer."

Slab gels used for the second dimension are those described by Laemmli *(10)*, and need no further description. A few points, however, should be noted. A special comb is used to provide a wide slot for the tube gel and a narrow one for application of a molecular weight marker. We use 5% polyacrylamide in the stacking gel. Gradient running gels are allowed to polymerize from the top by using 2.3 times more ammonium persulfate in the solution with lowest concentration of acrylamide *(28)*. Electrophoresis is performed at 35 mA per gel using bromphenol blue as dye.

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8

Protein Purification

Joseph Conner

1. Introduction

The isolation of an individual polypeptide from a heterogeneous mix is an essential process in characterizing a protein of interest. In purified form a protein can be used to generate specific polyclonal and monoclonal antibodies for in vivo studies, in vitro the enzymic properties or interactions with nucleic acids or other proteins can be studied in detail and related to in vivo function and, ultimately, the purified protein can be used in structural determinations that define how polypeptide chains fold and amino acids interact to create a protein with a specific function. Protein purification exploits the properties a polypeptide derives from its unique amino acid composition and separation techniques rely on variations in solubility, size, charge, hydrophobicity and specific affinities to achieve fractionation. A combination of these methods is sufficient to isolate an individual protein from a complex mix. A prerequisite for any purification is the ability to unambiguously distinguish the protein of interest at all stages. This can be achieved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting with specific antisera, or by use of an assay specific for an activity of the protein.

An enormous range of reagents is available to assist in protein fractionation and these include compounds such as ammonium sulfate or polyethylene glycol, which promote protein precipitation, and numerous matrices for size fractionation, ion-exchange, hydrophobicity interaction, and affinity chromatographies. These latter techniques are generally performed in columns in which the matrix forms a stationary phase through which the heterogeneous protein mixture in an appropriate buffer (the liquid phase) is passed. Proteins that interact with the stationary phase and are immobilized on the column can be eluted by alterations, such as ionic strength or pH, in the liquid phase. Numer-

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ous automated chromatography systems, such as FPLC or HPLC, are available to perform column chromatography although similar results can be achieved using a peristaltic pump and gradient mixer Ion-exchange and affinity chromatographies also can be performed in simple batch procedures.

Size fractionation by gel filtration uses a matrix, comprised of gel particles in bead form, with a defined pore size. The passage of a protein through the stationary phase of the column is dependent on its physical size. Small molecules permeate the matrix, taking longer to elute from the column than larger molecules that are excluded from the gel bed and move through the liquid phase out with the matrix. The molecular size separation range of a gel filtration column depends on the pore size of the gel beads and a variety of matrices with different separation ranges is available.

Ion-exchange chromatography is a very versatile technique that utilizes either positively or negatively charged matrices to adsorb proteins in low ionic strength buffers. The net charge of a protein is influenced by the buffer pH and this affects its ability to interact with an ion-exchange matrix. The isoelectric point (p*I*) of a protein is the pH at which it has no net charge. By varying the pH above or below the p*I*, the net negative or positive charge of a protein will be affected and this influences the strength of the interaction with anionic or cationic exchange matrices. Proteins that bind to ion exchange columns are eluted by gradients of salts, usually NaCl or KCl, which compete for charged residues; the stronger the interaction with the matrix, the higher the concentration of salt required for elution

Hydrophobicity interaction chromatography (HIC) uses matrices with hydrophobic groups that interact with surface hydrophobic regions of proteins. High salt concentrations promote and stabilize hydrophobic interactions and proteins are adsorbed onto HIC columns at high ionic strength. Elution from the matrix is achieved by reducing the ionic strength of the buffer.

Affinity chromatography encompasses a wide diversity of matrices that exploit specific interactions that proteins may possess. The affinity may be derived from an antibody, specific or nonspecific nucleic acid sequences, proteins, or peptides with which the protein to be purified is known to interact, substrates or cofactors to which the protein binds or more general matrices with immobilized ligands such as heparin, hydroxylapatite, or Cibacron blue. Proteins are absorbed onto the matrix at low ionic strength and eluted by competition with a relevant ligand or an increase in ionic strength. The advantage of affinity chromatography is the specificity of the protein for the immobilized ligand, although, in some instances, such as antibody affinity chromatography, the strength of the interaction may be so great that conditions required for elution may be detrimental to the protein.

Recent advances in cloning techniques have allowed the expression of proteins with additional amino acid sequences at the N- or C-terminus that confer specific affinities (e.g., glutathione *S*-transferase–glutathione, histidine hexamer-Ni^{2+}) to the protein and assist in purification. Plasmids and other reagents required for producing tagged proteins are available from various companies that provide reagents for molecular biology and this methodology represents a significant advance in protein purification techniques.

It is beyond the scope of a single chapter to consider all aspects of protein purification but, by describing the purification of two proteins from herpes simplex virus (HSV), some insight into protein fractionation techniques will be gained. The proteins involved are the R1 and R2 subunits of HSV ribonucleotide reductase, an essential enzyme for viral DNA synthesis and potential target for antiviral chemotherapy *(1)*. R1 and R2 interact to produce the active form of the enzyme and a peptide, corresponding to the nine amino acids at the C-terminus of the R2 subunit, specifically inhibits enzyme activity by preventing subunit interaction *(2)*. Structural studies on the protein–peptide interaction will be of benefit in designing peptidomimetic compounds as antiviral reagents The proteins were required in large amounts for structural studies and were overexpressed in *Escherichia coli* using the T7 expression system *(3,4)*; the construction of the expression plasmids is described in detail in *(5,6)*. The purification of R2 demonstrates the effectiveness of ion exchange chromatography in generating large amounts of homogeneous preparations whereas R1 purification was complicated by a number of factors that prevented the use of this technique and a method for screening a large number of matrices, to identify those that provide useful purification steps, is described.

2. Materials

1. Equipment: A Pharmacia (Uppsala, Sweden) FPLC system with conductivity monitor should be used for all column chromatography steps. Chromatofocusing and anion exchange chromatography are performed on Pharmacia Mono P and Mono Q columns respectively Five-milliliter Econo-Pac Heparin Affigel and Affigel Blue columns are obtained from Bio-Rad (Hercules, CA). For buffer exchange, a Pharmacia Fast Desalt column was used. Sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting are performed with Protean II mini-gel and Trans-blot kits (Bio-Rad).
2. HEPES buffer: 25 m*M* HEPES, pH 7.6, with 2 m*M* dithiothreitol. Lysozyme is obtained from Sigma
3. $(NH_4)_2SO_4$ was used either as a saturated solution or as a finely ground powder
4. Polybuffer 74 (Pharmacia) and the pH of this and the triethanolamine buffer should be adjusted with a saturated solution of iminodiacetic acid. These buffers are recommended by Pharmacia for use with the Mono P column.

5 *Bis*-Tris should be obtained from Sigma and the pH adjusted with concentrated HCl. Other biological buffers also are obtained from Sigma and used as recommended by Pharmacia in instructions provided with Mono Q and Mono S columns
6. Cibacron blue, reactive red, reactive yellow and ATP agaroses should be obtained from Sigma. Heparin affigel and hydroxylapatite should be obtained from Bio-Rad and phosphocellulose, from Whatman (Maidstone, UK)
7 CNBr-activated Sepharose should be obtained from Pharmacia and protein coupling performed as recommended in the manufacturer's instructions The nonapeptide YAGAVVNDL is synthesized by continuous flow F-moc chemistry *(7,8)* on a Novabiochem peptide synthesizer

3. Methods

1. *E coli* expressing R1 and R2 are lysed by the addition of lysozyme to produce an initial crude extract Cells from 1 L of culture are harvested by centrifugation and resuspended in 20 mL of HEPES buffer and stored frozen at –70°C until required (*see* Note 1) Lysis is performed by the addition of 250 μg/mL lysozyme and incubation on ice for 20 min Cell debris is removed by centrifugation at 18,000*g* for 20 min and the resulting supernatant is the crude extract (*see* Notes 2–4)
2 Using 100-μL aliquots of crude supernatant the optimum percentage of $(NH_4)_2SO_4$ required to precipitate R1 and R2 should be determined. Increasing amounts of saturated solution of the salt are added to give a concentration range of between 20–50% saturation After incubation on ice for 20 min precipitated proteins are obtained by centrifugation at 13,000*g* for 20 min and are resuspended in 100 μL of HEPES buffer Supernatant and precipitated fractions are analyzed by SDS-PAGE and the lowest concentration of $(NH_4)_2SO_4$ that gives maximum precipitation of R1 and R2 determined For both proteins this is 35% and in large-scale purifications finely ground $(NH_4)_2SO_4$ powder gradually is added to the crude extract on ice with constant stirring After incubation on ice for 20 min precipitated proteins are obtained by centrifugation at 12,000*g* for 20 min and resuspended in a minimal volume of buffer (*see* Note 5)
3 The isoelectric point of R2 should be determined using a Mono P chromatofocusing column A pH range of 8.0–4 0 is obtained using a 0 025*M* triethanolamine/iminodiacetic acid buffer, pH 8.0 and Polybuffer 74/immunodiacetic acid, pH 4 0 The R2 fraction (50 μL), partially purified by precipitation with 35% $(NH_4)_2SO_4$ should be diluted 100-fold and applied to the Mono P column, previously equilibrated with the pH 8 0 buffer. The flow-through fraction was collected and bound proteins were eluted by developing a 0–100% gradient of the pH 4 0 buffer over 32 mL (*see* Note 6) The pH of each 1-mL fraction collected was determined using a conventional pH meter and elution of protein is analyzed by SDS-PAGE. The column is washed with 1*M* KCl before reuse. The isoelectric point of R2 should be found to be pH 5.3.
4. Anion-exchange chromatography was performed on a 1-mL Mono Q column at pH 5 8, 0 5 pH units above the p*I* of R2; at this pH R2 will bind weakly to the column and elute at low salt (*see* Note 7) The $(NH_4)_2SO_4$ fraction is desalted

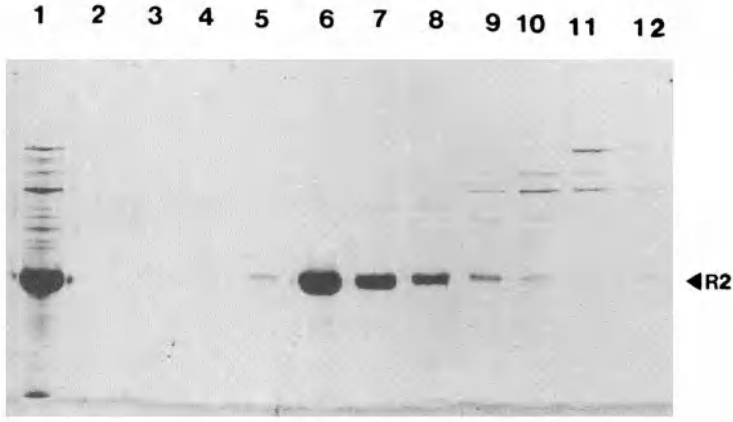

Fig. 1. SDS polyacrylamide gel showing purification of R2 by Mono Q ion exchange chromatography. Lane 1 shows the 35% $(NH_4)_2SO_4$ fraction, lanes 2 and 3, column flow-through material and lanes 4–12, proteins eluted from the column by the 0–1*M* KCl gradient. R2 is indicated by an arrow.

using a Fast Desalt column into a 20 m*M bis*-Tris-HCl buffer pH 5.8 and applied to the column (*see* Note 8). The column is then washed to remove unbound proteins (flow-through fraction) and bound proteins are eluted with a 0–1 KCl gradient developed over 15 mL. Salt concentration is monitored by an in-line conductivity meter. Proteins present in each fraction are analyzed by SDS-PAGE (Fig. 1) and R2 eluted as a distinct peak at 75 m*M* KCl (*see* Note 9). The salt gradient in subsequent runs is adjusted to improve R2 resolution; following binding of proteins to the column, the salt concentration was increased rapidly to 75 m*M* KCl, held at this for 5 column volumes, and then rapidly increased to 1*M* KCl. R2 produced by this method was greater than 95% pure (*see* Note 10). Although a Mono Q column should be used in this purification similar results could be obtained with a variety of other anion-exchange resins.

5. Chromatofocusing and ion exchange analysis of R1 indicated that these methods are of limited use in R1 purification. Using the chromatofocusing protocol described for R2, R1 elution in the pH 8.0–4.0 gradient should not be observed. The behavior of R1 in anion and cation-exchange chromatography was analyzed at a variety of pHs using buffers recommended for use with Mono Q and Mono S columns. An FPLC Fast Desalt column should be used to alter the pH of the 35% $(NH_4)_2SO_4$ fraction to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 and Mono Q and Mono S chromatographies are performed at each pH. Flow-through fractions are collected and bound proteins eluted with a 0–1*M* NaCl gradient developed over 20 mL. The R1 elution profile is analyzed by Western blotting. At neutral pH R1 bound to the Mono Q column, but this is of limited use in purification as the protein does not elute as a distinct peak and was detected in a number of fractions

across the salt gradient R1 is rapidly lost from solution, either by degradation or precipitation (*see* Note 11), at acid or alkali pH

6. The binding of R1 to a variety of affinity matrices should be analyzed using a small-scale batch procedure A variety of matrices, including heparin affigel, phosphocellulose, hydroxylapatite, Cibacron blue, reactive red and reactive yellow agaroses and ATP agarose are tested for R1 binding by incubation with the 35% $(NH_4)_2SO_4$ fraction (*see* Note 12). All manipulations are performed in 1 5-mL Eppendorf tubes. Matrices in powder form are swollen using HEPES buffer for 2 h and washed extensively before use Matrices provided as suspensions are also washed extensively in HEPES buffer One hundred microliters of a 50% slurry of matrix is incubated with 200 µL of $(NH_4)_2SO_4$ fraction for 30 min at 25°C with constant agitation. The supernatant was removed after a brief centrifugation and retained Matrices are washed three times with 1 mL of HEPES buffer and bound proteins were eluted by boiling in SDS-PAGE sample buffer Supernatants and bound proteins are analyzed by SDS-PAGE and Western blotting (Fig. 2) and, by comparison of these fractions, matrices useful in R1 purification can be identified. R1 binds to Cibacron blue and reactive red agaroses and heparin affigel but shows no specific interactions with the other matrices Interaction with a matrix is assumed to be specific when the amount of R1 in the bound fraction is greater than the amount remaining in the supernatant Five milliliter columns of Cibacron blue agarose and heparin affigel are tested for R1 binding and elution conditions assessed using 0–1 and 0–2*M* gradients of NaCl Protein elution is analyzed by SDS-PAGE. A combination of these columns results in R1 preparations of greater than 90% purity Cibacron blue chromatography should be used first as R1 eluted from this matrix as a broad peak between 1–2*M* NaCl R1 containing fractions, identified by SDS-PAGE, are dialyzed overnight against HEPES buffer and applied to the heparin affigel column R1 elutes from this column as a sharp peak at 150 m*M* NaCl (*see* Note 13)
7. An alternative method is used for R1 purification that utilizes the specific interaction of this protein with R2, an interaction that is disrupted by a peptide, YAGAVVNDL, corresponding to the R2 interaction site *(2)* R2 is immobilized, at 1 mg/mL, to CNBr-activated sepharose and the matrix is incubated with the 35% $(NH_4)_2SO_4$ R1 fraction for 20 min at 25°C Five milliliters of affinity matrix is used and purification performed by batch procedures in a 20-mL universal The supernatant is removed after a brief centrifugation at 2500*g* and the matrix washed three times with 10 vol of HEPES buffer, with 2*M* NaCl R1 elution is achieved by incubation of the matrix, for 1 h at 25°C, in a 2-m*M* solution of peptide Peptide is removed by gel filtration on a 100-mL superose 12-FPLC column This method of purification is suitable for producing small amounts (1–2 mg) of purified R1 (*see* Note 14).

4. Notes

1. Lysozyme extraction of soluble proteins from *E coli* is improved if bacteria are frozen overnight Protease inhibitors can be added at this point if the protein of

Fig. 2. Western blot, probed with R1 specific antiserum showing R1 interactions with a variety of affinity matrices. Odd and even numbered lanes show supernatants and bound proteins respectively. Lanes 1 and 2, Cibacron blue agarose, 3 and 4 reactive red agarose, 5 and 6 heparin affigel, 7 and 8 reactive yellow agarose, 9 and 10 ATP agarose, 11 and 12 hydroxylapatite, and 13 and 14 phosphocellulose. R1 is indicated by an arrow.

interest is susceptible to proteolytic degradation. Inhibitors can be tested individually to determine those that prevent degradation or a cocktail of inhibitors, specific for a wide variety of proteases, can be used. Advice on the selection and use of protease inhibitors is available from suppliers, e.g., Boehringer-Mannheim.

2. Other methods of cell lysis exist including sonication and the use of a French pressure cell. Lysozyme extraction, however, requires no additional equipment.
3. In some instances proteins overexpressed in *E. coli* form insoluble aggregates that will be lost during centrifugation. Induction of protein expression in *E. coli* at lower temperatures reduces the formation of these aggregates *(4)*.
4. Lysozyme extraction also releases nucleic acids and the crude supernatant may be viscous. Nucleic acids can be precipitated at this point by streptomycin sulfate (Sigma). To 5 mL of supernatant, 0.3 mL of 5% streptomycin sulfate is added and, after 20 min incubation on ice the precipitated nucleic acids are removed by centrifugation at 18,000*g* for 20 min. Proteins that interact with nucleic acids will be precipitated by this method; protein–nucleic acid interactions usually can be disrupted by the inclusion of 1*M* NaCl, which must be removed, either by dialysis or desalting (*see* Note 5) before continuing with the purification.
5. $(NH_4)_2SO_4$ precipitation is a useful first step in a purification as it greatly reduces the volume of the extract to be used in subsequent steps. Prior to any column chromatography steps it may be necessary to dilute out residual $(NH_4)_2SO_4$ or remove it by dialysis or desalting. Desalting is the method of choice as it can be performed rapidly on a number of commercially available columns, however, the volume applied per run is restricted to 1–3 mL. Dialysis is generally performed overnight at 4°C against a volume of buffer that is at least 10-fold greater than that of the sample. Both dialysis and desalting invariably result in the loss of some protein. Occasionally, $(NH_4)_2SO_4$ precipitation can cause nonspecific

aggregation of proteins and this can be prevented by the inclusion of detergents (e g., 0 1% NP40 or 4 m*M* CHAPS) in the buffers.

6 The volume over which a pH or salt gradient is developed depends on a number of factors, including the size of column and the elution profile of the protein of interest but it is recommended that a gradient is developed over a minimum of five times the column volume. The size of fractions collected during elution from the column also varies according to individual protocols but, in general, smaller fractions improve protein peak resolution.

7 At pH 5 3, R2 did not bind to either Mono Q or Mono S columns and this was initially considered for use in purification However, several *E coli* proteins also shared this property and a pH slightly above the pI was chosen At a pH slightly below the p*I*, R2 would bind weakly to a Mono S column.

8. Manufacturers of ion-exchange and other matrices provide data on the protein binding capacity of the resin Information on recommended running conditions, and buffers, for elution, regeneration, and cleaning are also provided In any separation run less protein than the maximum capacity should be applied to the column to avoid saturation of the matrix

9 Although R2 elutes as a distinct peak, the protein elution trails into later fractions. Inclusion of 5–10% glycerol in the buffers improved elution of R2 by preventing this trailing.

10 Purity can be estimated by densitometry of SDS polyacrylamide gels or accurately determined by amino acid analysis. It is also advisable to confirm the identity of the purified protein by either Western blotting using a number of specific antisera or by N-terminal sequencing

11. Some proteins are known to precipitate at, or close to their isoelectric point and this may have happened with R1. Rapid degradation also was observed.

12. A large number of affinity matrices are available from suppliers such as Sigma, Pharmacia, or Bio-Rad Lectin-affinity chromatography is a useful purification, step if the protein is a glycoprotein

13. Hydrophobicity interaction chromatography was considered for R1 purification, but the protein precipitated at the high salt concentrations was used to promote interactions with the matrix. Concentrations of salts, such as $(NH_4)_2SO_4$, NaCl, or K_2SO_4, in excess of 2*M* stabilize interactions of hydrophobic regions of proteins with hydrophobic groups, such as phenyl-, octyl, or butyl, immobilized on the matrix and bound proteins can be eluted by lowering the salt concentration. The concentrations of $(NH_4)_2SO_4$, NaCl, or K_2SO_4 in R1 preparations were adjusted to between 2 and 4*M* by the careful addition of finely ground salts and the solution centrifuged at 13,000*g* for 10 min Supernatants were analyzed by SDS-PAGE and, in all cases, R1 was precipitated. At salt concentrations below 2*M*, R1 did not interact with the hydrophobic interaction matrices tested HIC is a useful technique to consider following salt elution of proteins from another matrix; the protein of interest may bind directly to an HIC matrix at this concentration or, the salt concentration can be increased directly to achieve binding.

14 Affinity chromatography, using specific protein–protein or protein–nucleic acids interactions, is an excellent method for producing small amounts of highly purified proteins. Strong interactions (such as antibody–antigen) may require extreme conditions, such as high salt, denaturation, or extremes of pH, to elute the bound protein, which may be detrimental to both the immobilized ligand and purified protein The usefulness of these methods is restricted by the availability of the ligand, the conditions required to recover the bound protein, and the ability to regenerate the affinity matrix, such constraints may limit the use of these techniques in large-scale purifications

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9

Expression and Purification of Secreted Forms of HSV Glycoproteins from Baculovirus-Infected Insect Cells

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1. Introduction

Herpes simplex virus (HSV) remains a major human pathogen worldwide *(25)* causing cold sores, eye and genital infections, blindness, encephalitis, and neonatal infections. Most adults have antibodies against the oral form of the virus HSV-1 *(9)*, and a significant number are infected with the genital form, HSV-2. Both serotypes establish lifelong latent infections and reactivate periodically to produce recurrent disease *(25)*. After infection, virus-encoded glycoproteins are expressed on all cellular membranes and are major targets of the host's immune response. The virion envelope contains 10 glycoproteins that are important for infection and pathogenesis of HSV-1 and HSV-2. Because HSV contains so many glycoproteins, sorting out their functions in virus entry remains a difficult task. Our approach has focused on establishing structure–function relationships of the individual glycoproteins with particular emphasis on gC and gD. After many years of studying the properties of these proteins in HSV-infected and plasmid-transfected mammalian cells, we have now begun to overexpress the proteins using a baculovirus expression system.

The first interaction between HSV and a susceptible cell occurs when gC binds to cell surface heparan sulfate proteoglycans *(29)*. The second step of virus entry involves the interaction of gD with a second cellular receptor *(3,17)*, possibly the mannose-6-phosphate receptor (Man6pR) *(2)*. Following this interaction, the virus envelope fuses with the host cell plasma membrane. Four

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viral glycoproteins (gB, gD, gH, and gL) are important for this step, although the precise mechanism is not understood

Immunological, biochemical, and genetic approaches have established working models of gD structure *(6,8,12,14,19,22)*, but a complete understanding requires X-ray crystallographic methods. Therefore, one of our long-term goals is to solve the 3D structure of gD and to learn how alterations in structure relate to function. To obtain the large amounts of gD needed, a baculovirus system is used to express a truncated form of the glycoprotein (gD-1[306t]) that lacks the transmembrane region (TMR) and carboxyl terminus *(27)*. gD-1(306t) is therefore secreted into the culture supernatant but contains all residues associated with its function Having large amounts of a soluble form of purified gD has allowed us to analyze its structure on three different levels. First, we have continued antigenic mapping studies, and now we use biophysical techniques such as mass spectrometry and circular dichroism (CD) to further probe the structure of gD Second, we have utilized scanning transmission electron microscopy (STEM) and 2D electron crystallography to obtain details of gD structure and a topography at the ultrastructural level. Third, we are able to crystallize gD-1(306t) *(34)* and efforts are now underway to obtain crystals suitable for X-ray diffraction.

In addition to the structural studies, we are also analyzing the functional properties of wild-type and variant forms of baculovirus-expressed HSV glycoproteins. For example, experiments using the truncated proteins will enable us to extend five observations about gD function:

1. gD Binds to cell surfaces *(16)*;
2. gD Blocks HSV plaque formation *(16)*,
3 gD Binds to the Man6pR *(2)*;
4. gD Participates in fusion, and
5 gD Mediates superinfection interference *(4)*

Thus far, we have shown that gD-1(306t) binds to cells in a saturable manner and also blocks HSV plaque formation *(31)*.

The production of a soluble form of gC has allowed us to explore the interaction between gC and the cell surface in the absence of detergent. Truncated forms of gC lacking the TMR and carboxyl regions were expressed in baculovirus-infected cells *(31)*. These proteins bind to mammalian cells, bind to immobilized heparin, and block attachment of virus to cells. In addition, each of these proteins bind conformation-dependent monoclonal antibodies (MAbs) and to the human complement component C3b, showing that the proper conformation of gC is maintained when the truncated molecule is expressed in insect cells *(31)*. Thus, our experience with the baculovirus expression system

is that it opens up new areas of investigation by providing a relatively simple means of obtaining large quantities of biologically active and structurally correct forms of HSV glycoproteins.

This chapter therefore is devoted to our colleagues desiring the capacity to obtain large amounts of protein for their studies. The term "large amounts" is relative to the investigator We find that no matter how much protein is made, it is quickly expended and easier ways of getting purified proteins in bulk are constantly being sought. We hope that what we have learned will have an impact on the ability of other labs to produce proteins using a baculovirus infection system. In this spirit, we present protocols based on our experiences, recognizing that other published protocols exist for many of the techniques we describe. Our goal is to bring you as close as possible to our "benchside" with both pleasant and unpleasant experiences from working our way through the procedures for the isolation and purification of baculovirus-expressed HSV glycoproteins

2. Materials and Equipment

The materials and equipment we use as well as where they can be purchased are listed within the body of the text.

3. Methods

Our early attempts to express gD-1(306t) using the wild-type gD signal peptide and standard baculovirus vectors yielded barely detectable amounts of protein either intra- or extracellularly *(27)*. We also studied a construct containing no signal peptide and found that significant amounts of gD-1(306t) were synthesized intracellularly, although as expected, the protein lacked carbohydrate, failed to fold properly, and was not secreted. Therefore the *Autographa california* nuclear polyhedrosis virus (baculovirus) transfer vector pVT-Bac *(32)* was used to insert the gD gene into baculovirus. This vector is derived from pAC373 *(30)* and contains the polyhedrin promoter and sequences that normally flank the polyhedrin gene. The signal peptide sequence is for honeybee melittin, and cloning sites adjacent to this sequence permit in-frame fusion of the coding sequence for a foreign protein with that of the melittin signal peptide. Tessier et al. *(32)* previously showed that use of this particular signal peptide significantly enhanced expression and secretion of the papain precursor (EC 3.4.22.2) from baculovirus-infected *Spodoptera frugiperda* (Sf9) cells. For gD-1(306t) we routinely obtain 20 mg of immunoaffinity-purified protein per liter of infected Sf9 cell growth medium After one additional step of purification, the glycoprotein is free of contaminating proteins and appears to be >99% pure.

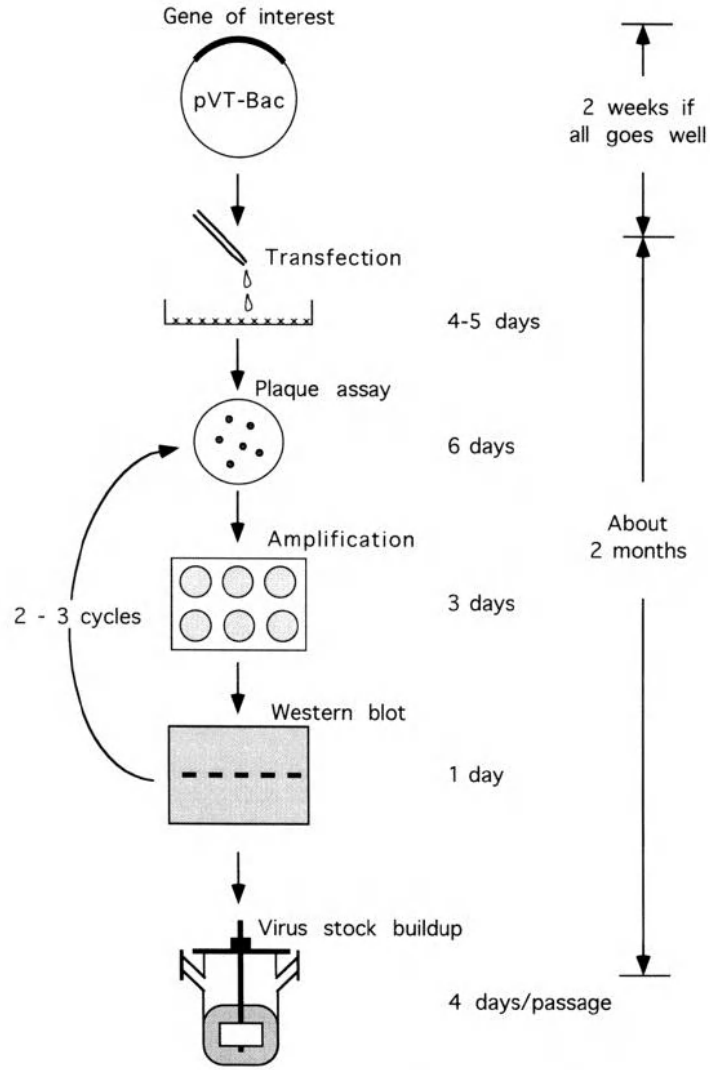

Fig. 1. Construction of a recombinant baculovirus. Plasmids with the glycoprotein gene of interest were used as PCR templates to generate DNA fragments containing the glycoprotein gene. The 5' primer was designed to begin at the first residue of the protein after the signal peptide and to be in frame with the mellitin signal peptide. The 3' primer was designed to truncate the glycoprotein just prior to the hydrophobic TMR. In addition, the 3' primer was designed to add histidine residues to the carboxyl-terminal end of the glycoprotein. Each primer contained an appropriate restriction site *(27,31)*. The vector pVT-Bac and the PCR-amplified glycoprotein gene were each digested with complementing restriction endonucleases. The gene was ligated into pVT-bac using T4 DNA Ligase. The ligated plasmids were used to transform *E. coli*

3.1. Steps Needed to Construct the Baculovirus Recombinant

Figure 1 summarizes the steps involved to obtain the desired recombinant protein, from cloning the gene through purifying the protein. The details are given in Sections 3.2.–3.5. Section 3.6. describes techniques that can be used to analyze the protein, but these by necessity of space are given in less detail. Notes on the protocols are given in Section 4.

3.2. Maintaining Cell Lines

Critical to all of the following steps is a good cell line. Following the next two protocols will ensure that your cell line remains healthy.

3.2 1. Maintaining Monolayers of Sf9 Cells

Note: Monolayers of cells are only used for transfection.

1 Seed 7 × 10^6 Sf9 cells from a spinner into a T75 flask with Grace's medium (Gibco, Gaithersburg, MD) plus 10% fetal bovine serum (FBS, BioWhitaker, Walkersville, MD), 1X antibiotic/antimycotic (Gibco), and 0.1% Pluronic F-68 (Gibco, *see* Notes for choice of cells and medium)
2. When the cells reach confluence, split them (step 3) using a 1 3 or 1·4 dilution Do not split the cells lower than 1:4 because Sf9 cells grow best at a high inoculation density It will take 3–4 d for the cells to reach confluence Incubate Sf9 cells under humid conditions at 27°C with normal air conditions.
3 Splitting Sf9 cells:
   a Remove the old medium from the T75 flask
   b Add a few milliliters of fresh medium and sterile glass beads (3 mm, Thomas Scientific, Swedesboro, NJ) to strip the cells off the wall of the flask, approx 50 beads for a T75 flask.
   c Gently rock the glass beads against cells to dislodge them.
   d Gently pipet up and down to break up the cell clumps. Place the cells into a new flask at the appropriate dilution

3.2.2. Maintaining Suspension Cultures of Sf9 Cells

Log-phase Sf9 cells in suspension have a doubling time of 24 h.

1 We routinely keep stocks of Sf9 cells growing in suspension in a spinner with SF-900 II serum free medium (Gibco) plus 1X antibiotic/antimycotic This

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Fig. 1 (cont'd) XL-1-Blue competent cells. Plasmids from ampicillin-resistant colonies were screened by restriction endonuclease analysis. Positive plasmids were recombined into baculovirus using Baculogold as the source of baculovirus DNA Plaques were picked and amplified Culture supernatants from infected cells were screened for protein production by SDS-PAGE and Western blotting using polyclonal antiserum. Positive baculovirus recombinants were subjected to two additional cycles of plaque purification before amplification of the virus stock. The time required to carry out these steps is indicated on the right.

enables us to have the cells available for scaling up for large infections We do not supplement this medium with FBS

2 All spinners should be placed on a magnetized platform set to spin at the maximum speed. We use a Bell Stir Multi Stir 4 Magnetic Stirrer (Bellco, Vineland, NJ) The surface of this magnetic stirrer does not get hot with prolonged use
3 Splitting cells in suspension. Cells should be split to a density of no less than 5×10^5 cells/mL We split cells twice a week to keep them in the best condition
4 Cells can be grown to as high as 6×10^6 cells/mL for inoculating large volumes of media for large-scale protein production
5 Long-term storage Freeze log phase Sf9 cells from a spinner at 2×10^7 cells/mL in 95% FBS + 5% DMSO. Store in liquid nitrogen

3.3. Cloning, Transfection, and Amplification of Recombinant Baculovirus

3.3.1. Transfection

We will not detail the steps in polymerase chain reaction (PCR) cloning, or the ligation of the PCR product into pVT-Bac (Fig. 1) because these are well described elsewhere *(27,31,32)*. When designing the PCR primers it is important to remember that the coding sequence minus the normal signal peptide must be in frame with the melittin signal peptide in the vector, and that several extra amino acids may be added at the N-terminus of the protein as a result of cloning. The resulting vector transforms well into XL-1 blue cells (Stratagene, La Jolla, CA). DNA yields from maxi preparations are on the order of 1 mg of DNA from a 500-mL culture Both CsCl-purified DNA and column-purified DNA (Qiagen, Chatsworth, CA) work well for transfection. It is important to seed Sf9 cells from suspension into monolayers at least 1 wk ahead of the transfection experiment (Section 3.2 1.)

1 From a monolayer of Sf9 cells, seed $3–4 \times 10^6$ cells into a 60-mm tissue culture plate with Grace's medium supplemented with 10% FBS, 0.1% Pluronic F-68, and 1X antibiotic/antimycotic Let sit for $1^1/_2$ h
2 Prepare the DNA solution in a sterile 1 5-mL microfuge tube: 0.5 μg BaculoGold (Pharmingen, San Diego, CA) linear DNA; 2 μg recombinant plasmid DNA; 1 mL Grace's medium (not supplemented, no FBS), 20 μL Insectin (Invitrogen liposomes, San Diego, CA)
3. Vortex the DNA solution vigorously. Incubate at room temperature for 15 min
4. Remove the old medium from the insect cells and replace it with 2 mL of fresh Grace's medium (not supplemented, no FBS), let sit for 10 min at room temperature.
5. Remove the medium from the plate, but do not let the cells dry (work quickly)
6. Add the DNA solution *dropwise* to the cells
7. Rock the plate slowly at room temperature for 4 h using a side-to-side platform shaker (Rocker Platform, Bellco).
8 Add 1 mL of Grace's medium supplemented with 10% FBS, 0 1% Pluronic F-68, and 1X antibiotic/antimycotic

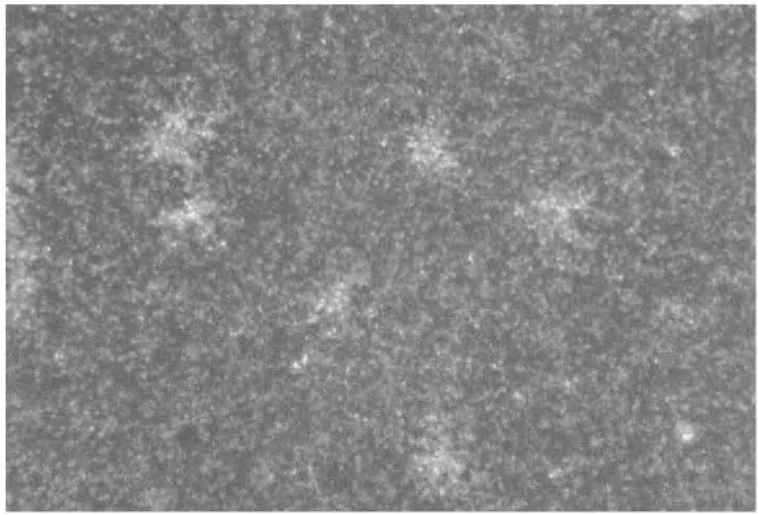

Fig. 2. Plaques of bac-gC1(457t) infected Sf9 cells. Sf9 cells were infected with tenfold serial dilutions of a bac-gC1(457t) baculovirus stock *(31)*. Plaques appeared after 6 d of incubation at 27°C.

9. Incubate the plate at 27°C for 4–5 d.
10. Remove the supernatant from the plate and save it. This is your recombinant virus stock to be used for plaque purification. Store at 4°C in a sterile tube.

3.3.2. Plaque Purification

Plaque purification is a time-consuming but essential step to ensure that 100% of your virus stock is producing the protein of interest. Three rounds of plaque purification will take approx 3 mo to complete (Fig. 1).

1. Split Sf9 cells growing in suspension to 5×10^5 cells/mL one day before the actual experiment. The next day, seed 7×10^6 cells into a 10-cm plate. **Note:** Make sure to pipet up and down to break up any cell clumps. This is necessary to distinguish between a cell clump and a virus plaque (Fig. 2) after 5–6 d of incubation. Allow the plate to sit for at least 30 min at room temperature for the cells to adhere.
2. Make 10-fold serial dilutions from the recombinant virus stock using SF-900 II medium.
3. Remove the medium from the plate.
4. Add 3 mL of diluted viral supernatant (10^{-5}, 10^{-6}, and 10^{-7}) to each plate. For the first round of plaque purification, also use the 10^{-1} dilution to make sure the transfection worked.
5. Let the plates sit for 1 h at 27°C for infection to occur. Rock the plates occasionally.

6 Prepare 2% Seaplaque agarose (FMC) in water Autoclave and keep in a 55°C water bath until ready for use. Bring Grace's 2X medium (20% FBS, 2X antibiotic/antimycotic, 0 2% Pluronic F-68) to 37°C.
7 After the 1 h infection, mix equal volumes of 2X Grace's supplemented medium with the liquefied 2% agarose. The final concentration will be 1% agarose in 1X Grace's supplemented medium.
8 Remove the medium containing the virus inoculum from the plate. Overlay the plate with 10 mL of 1% agarose/1X Grace's supplemented medium by pouring carefully from one side of the plate
9 Let the plate sit undisturbed at room temperature until the agarose is completely hardened (about 30 min).
10 Incubate the plates in a moist 27°C incubator for 5–6 d. After this time the plates can be wrapped in parafilm and stored at 4°C. Leaving the plates too long at 27°C will make counting plaques more difficult Figure 2 shows typical baculovirus plaques observed using a dissecting microscope.
11 Pick a few plaques using a sterile long Pasteur pipet (cotton plugged). Elute the plaque by immersing the pipet tip and releasing the piece of agarose into 500 µL of supplemented Grace's medium (or SF-900 II medium) and incubating overnight at 4°C. Vortex the solution the next day to release virus from agarose Pick 8–10 plaques

3 3.3. Amplification of Virus

Cycling through the preceding steps (Fig. 1) yields a virus stock in a small volume (500 µL). The next step is to make enough virus and protein for screening.

1 Using SF-900 II medium, seed 1 2 × 10^6 Sf9 cells from a spinner into each well of a 6-well plate Incubate the plate for at least 30 min at room temperature to allow the cells to adhere
2 Remove the old medium and add 1 mL of SF-900 II medium with 50 µL of the solution containing the virus from a single plaque (*see* Section 3 3 2 , step 11)
3. Let the infection proceed at room temperature for 1 h Slowly rock the plates on a platform rocker during the infection
4. Add another 1 mL of medium to each well
5 Incubate the cells for 3 d at 27°C and collect the culture supernatant in a sterile tube
6. Run 10–30 µL of the collected culture supernatant on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Check for protein production by staining for protein or Western blotting (Fig 3).
7 Store the rest of the culture supernatant from each well at 4°C in a sterile tube.
8. On the basis of the Western blot (Fig. 3), pick one or two positive wells and process the virus through the plaque assay and amplification cycles again. Repeat this procedure one or two more times (Fig. 1) until 100% of the plaques are positive for protein production by SDS-PAGE staining or Western blot analysis Choose one virus stock to establish the permanent virus recombinant line and freeze the others at –80°C as backups.

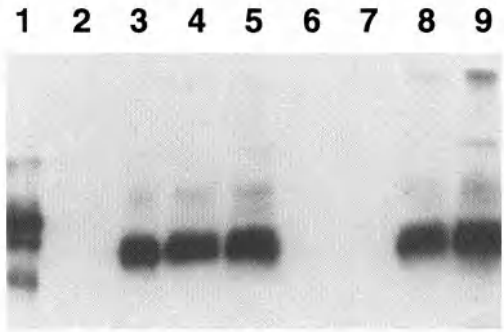

Fig. 3. Western blot analysis of gC-1(457t). Following SDS-PAGE proteins were transferred to nitrocellulose and probed with polyclonal anti-gC antibody R46 *(13)*. Lane 1, cytoplasmic extract prepared from HSV-1 infected BHK cells *(26)*; lanes 2–9, 20 μL of culture supernatants from individual plaques that were picked, placed into medium and used to infect Sf9 cells.

3.3.4. Baculovirus Stock Titration

It is important to titer the virus stocks so that multiplicities of infection (MOI) are known rather than estimated for large scale virus and protein production.

1. Split Sf9 cells growing in suspension to 5×10^5 cells/mL one day before the actual experiment. The next day seed 3×10^6 cells into 60-mm plates. **Note:** Make sure to pipet up and down to break up any cell clumps. This is necessary to distinguish between a cell clump and a virus plaque after 5–6 d of incubation (Fig. 2). Incubate the plate for at least 30 min at room temperature to allow the cells to adhere.
2. Make 10-fold serial dilutions of the recombinant virus stock into SF-900 II medium.
3. Remove the old medium from the plate.
4. Replace the medium with 1 mL of the 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the recombinant virus stock.
5. Let the plates sit for 1 h at 27°C for infection to occur. Rock the plates occasionally.
6. Prepare 2% Seaplaque agarose in water. Autoclave and keep in a 55°C water bath until ready for use. Bring Grace's 2X medium plus 20% FBS to 37°C.
7. Aspirate the virus and overlay the plate with 4 mL of a 1:1 mixture Grace's 2X (plus FBS) and 2% agarose (*see* Section 3.3.2). The final concentration will be 1% agarose in 1X Grace's medium. Leave the plate untouched for 30 min.
8. Incubate the plates for 5–6 d at 27°C in a humidified incubator. After this time the plates can be wrapped in parafilm and stored at 4°C. Leaving the plates too long at 27°C will make counting plaques more difficult. Leaving the plates for less time at 27°C will decrease the accuracy of the titration. Count the plaques using a dissecting microscope (Fig. 2) and calculate the titer. It is easiest to count the plaques from the bottom of the plate.

3.3 5 Growing Baculovirus Stock

As a general rule, virus stocks should be prepared at a low MOI (0.1–0.3 PFU/cell). We have occasionally found that some recombinants do not amplify well unless the moi is at least 1. As with any virus, it is best to make large amounts of low passage stock virus before going to large-scale protein production Our normal procedure is to save the first two passages of recombinant virus stocks for growing more virus stocks in the future. We then use later passages for protein production.

1 Bring at least 100 mL of Sf9 cells in SF-900 II medium to a density of 2×10^6 cells/mL in a spinner
2 Infect cells at an moi of 0.1–1 (depending on the recombinant virus). At the same time, add 5% FBS to the medium This is the only time FBS is added to SF-900 II medium
3 Incubate the cells at 27°C for 96 h in a spinner flask Monitor the cell viability every 24 h by removing 1 mL of the culture, diluting the cells 1/10 with trypan blue (a 0.2% solution in phosphate-buffered saline (PBS) filtered through a 0 22-mm membrane, Gibco), and counting the cells to determine cell density and viability. It is important to wait to harvest the virus until the cell viability drops to 50–60%. This usually occurs by 96 h postinfection (PI)
4 Remove the culture from the spinner and pellet the cells by centrifugation
5 Save the supernatant as the virus stock.
6 Titer the virus stock (*see* Section 3 3 4)
7 Aliquot the virus stock and store at –80°C

3.4. Large-Scale Protein Production

In order to obtain multimilligram quantities of protein it is necessary to scale up protein production to greater than 1 L. We routinely do 3–8 L preparations using either a large Bellco spinner or a Celligen Plus Bioreactor (New Brunswick Scientific, Hatfield, UK). Protocols for both are outlined in the following. Protein production can also be done in a shaker flask. Spinners are easier to work with, however. Proper aeration is very important for maximum protein production. *See* Section 4.7 for a discussion of this topic. Note that all protein production is done in serum-free medium.

Samples are taken every 24 h for each run (described in the following) in order to determine percent of cell viability and so that Western blots can be done to evaluate each run and compare one run to another. Figure 4 shows a typical plot of cell density and percent cell viability vs time for cells infected with bac-gD1(306t) *(27)*. Also shown is a typical time course of protein production by Western blot analysis. Do not be concerned if there is protein in your 0 h time-point. This is protein that is present in the recombinant virus stock used for inoculation.

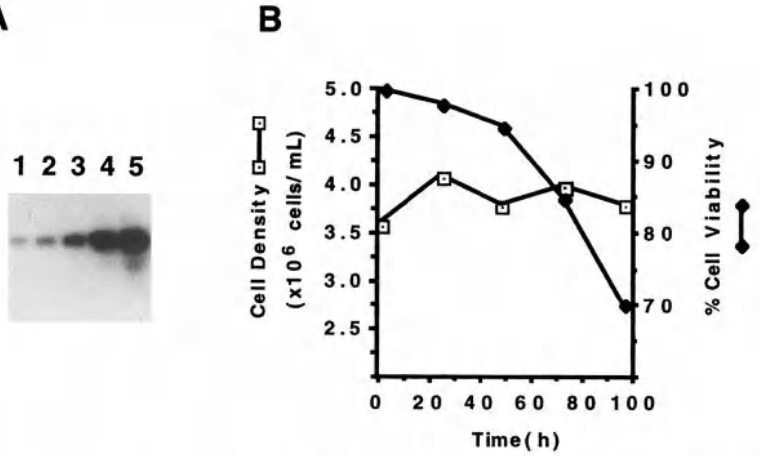

Fig. 4. Evaluation of protein production and cell viability of Sf9 cells infected with bac-gD1(306t). Three L of Sf9 cells were infected with bac-gD1(306t) *(27)* at a moi of 4. Samples were taken daily and the cell density and viability evaluated. **(A)** Western blot analysis of daily samples of gD-1(306t) from the culture supernatant (20 µL) using polyclonal anti-gD antibody R7 *(15)*. Lane 1, 0 h PI; lane 2, 24 h PI; lane 3, 48 h PI; lane 4, 72 h PI; lane 5, 96 h PI. **(B)** Plots of percent cell viability and cell density vs time at 0, 24, 48, 72, and 96 h PI.

3.4.1. Spinner Cultures

1. Bring an assembled and autoclaved Bellco spinner flask (3 or 8 L with double paddle and sparger mechanism) into a sterile hood and add cells and medium to the final desired volume and a cell density of 1×10^6 cells/mL.
2. Place the spinner in a 27°C incubator and attach an overhead motor. Spin at the maximum speed.
3. Hook the sparging apparatus (same apparatus as used in fish tanks) up to a gas tank (40% oxygen/60% nitrogen), and bubble air through the sparger at 5 cc/min.
4. When the cells have reached a density of 4.0×10^6 cells/mL (1–3 d depending on the initial cell density), infect with 4 PFU/cell of recombinant baculovirus. Because recombinant virus stocks are usually 10^8 PFU/mL, 100–200 mL of virus stock will be added for a 3-L culture.
5. Remove an aliquot from the sample line (5–10 mL). Flush the sample line with air from a syringe. Dilute 100 µL of the cell sample 1/10 with 0.2% trypan blue (*see* Section 3.3.5.) and determine the cell density and percent cell viability. The cell viability should be 95–100%. Spin the cells out of 1 mL of the sample. Freeze the supernatant and save it as the 0 h time-point.
6. Every 16–24 h remove a sample as in step 5, count the cells and determine the viability. Spin down 1 mL of the sample and save the supernatant as a time-point.

7 The cell density should remain consistent, although it may increase during the first 24–48 h and then level off
8. Harvest the culture when the cell viability drops to 70% (Fig 4) This occurs between 72 and 96 h PI

3.4.2. Celligen Bioreactor

We have experience using the New Brunswick Celligen Plus Bioreactor with either a 5 L vessel (3.5 L working volume) or a 7-L vessel (5 L working volume) A detailed protocol is supplied by the manufacturer and will not be provided here. We can be contacted for a step-by-step walk-through of how we use our Celligen Plus Bioreactor.

New Brunswick sells two interchangeable systems for stirring the insect cell culture: a marine blade and a cell lift. We have used both successfully but recommend the marine blade because it is less expensive and significantly easier to assemble. Infected cells in the Celligen Plus Bioreactor produce up to three times the amount of extracellular protein compared to similar runs made using a large Bellco spinner. In addition, the length of a run in the Bioreactor is at least 1 d shorter than a comparable run in a spinner. This may be owing to the fact that both pH and O_2 levels are electronically monitored and controlled by the Bioreactor Normally, Sf9 cells at 4×10^6 cells/mL are infected at a moi of 4 Runs are monitored as described for the large spinner cultures (*see* Section 3.4.1) Cell viability generally drops to 70% by 48–52 h PI

Settings for the Celligen Bioreactor should be: O_2 at 50%, pH = 6 2, temperature at 27°C (although the machine should be calibrated at 25°C), agitation set at approximately 140–200 rpm (optimized for the size of the culture), initial cell density is 1×10^6 cells/mL.

3.4.3 Harvesting Large Cultures of Infected Cells

It is easiest to harvest large runs by removing the cells by centrifugation.

1. Large spinner runs· Remove the overhead drive and disconnect the bubbling apparatus Pour the culture into 100- to 150-mL centrifuge bottles and spin (4°C, 1400*g*) for 30 min If the protein is secreted save the supernatant, if not save the cell pellet.
2. Celligen bioreactor: Turn off the agitation and remove the culture via the harvest line according to manufacturer's specifications Pellet the cells as described in step 1
3. Samples can be stored at –20°C until ready to process further

3.4.4. Concentration and Buffer Exchange

The Millipore Easy-Flow Masterflex tangential flow unit is convenient to use and saves time when working with large culture volumes. The unit can be

used with a 0.45-μm cutoff filter to remove cells from the culture supernatant. However, we find it more convenient to remove the cells by centrifugation (*see* Section 3.4.3.). We routinely use tangential flow to reduce culture supernatant volumes from 3, 5, or 7 L runs to 1 L or less. It also is beneficial to use the unit to exchange (dialyze) the sample with PBS. Exchanging the medium and salts is a necessary step for some immunoadsorbent columns, possibly owing to inhibition of MAb binding. In general, the concentration step reduces column-loading times and the buffer exchange increases the efficiency of chromatography. However, this process is not beneficial for all glycoproteins, and for gC-2(426t) we found it to be detrimental. A detailed protocol for setting up the apparatus is provided by the manufacturer.

1. Assemble the unit according to the manufacturer's directions with the appropriate membrane (we use a 10,000 mol-wt-cutoff Pellicon Cassette Filter [Millipore, Bedford, MA])
2. Flush the membrane with 10 L of dH_2O to remove the storing solution (0.1*N* NaOH)
3. Sample preparation. Spin the sample at 4°C and 10,000*g* for 20 min Filter the sample through a 0 45-μm disposable filter (with a prefilter)
4. Cycle the culture sample through the membrane. We recycle the retentate back into the sample and discard permeate. The first time a sample is run through the unit, however, save the permeate to ensure that the sample is not flowing through the membrane Continue until the volume has been decreased to approx 500 mL. It is convenient to use a 1-L graduated cylinder to hold the sample being concentrated so that the exact volume can be monitored.
5 Add PBS to the sample in the 1-L graduated cylinder. We routinely exchange with a total of 5 L of PBS.
6. After all of the PBS has been added, bring the volume to 500 mL or less. Flush the system with an additional 500 mL of PBS to remove any of the sample that remains in the filter. The sample is now ready for chromatography.

3.5. Protein Purification

The next step in the process is to isolate the protein from the medium. This is most easily accomplished by using some form of affinity chromatography. Two types of affinity chromatography successfully used in our lab for large-scale purification are described in the following: immunoaffinity chromatography and heparin chromatography. Histidine-tailed proteins can be purified on a small scale by Ni^{2+}-agarose chromatography (*see* Section 4.10.). Additional purification steps depend on the ultimate use of the protein. We next purify our proteins over a gel filtration column. In addition, we have used anion exchange to further purify our proteins, but have found that this third step of purification is not necessary for the procedures we use. Keep in mind that with every purification step there is a loss of protein. All protein purifications should

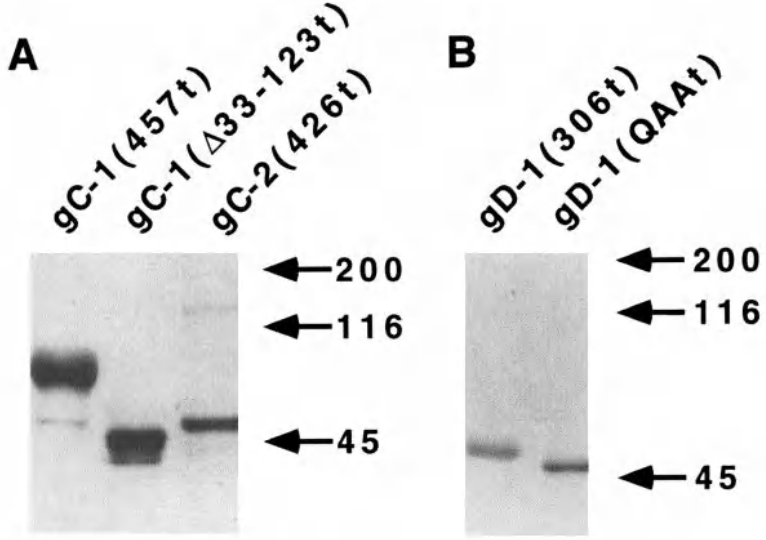

Fig. 5. SDS-PAGE analysis of gD and gC variants expressed using a baculovirus expression system. All proteins are truncated prior to the TMR. Purified glycoproteins (1–5 μg/lane) were electrophoresed on 10% denaturing gels and stained with Coomassie blue. **(A)** gC-1(457t) is a form of HSV-1 gC truncated at amino acid 457 *(31)*. gC-1(Δ33-123t) is a form of HSV-1 gC lacking residues 33-123 in the N-terminus and truncated at amino acid 457 *(31)*. Both proteins were immunoaffinity purified using MAb 1C8 *(13,31)*. gC-2(426t) is a form of HSV-1 gC truncated at amino acid 426 *(31)*. The protein was purified by heparin chromatography *(31)*. **(B)** gD-1(306t) is a form of HSV-1 gD truncated at amino acid 306 *(27)*. gD-1(QAAt) is a form of HSV-1 gD with mutations that remove the signal for the addition of the three N-linked oligosaccharides *(27,28)*. gD-1(QAAt) is also truncated at amino acid 306 *(27)*. Both proteins were immunoaffinity purified using MAb DL6 *(13,27)*.

be monitored at $A_{280\,nm}$. This can be accomplished two ways: using an automated system (Pharmacia [Uppsala, Sweden] Fast Protein Liquid Chromatography [FPLC] or GradiFrac systems) that produces a chromatogram of $A_{280\,nm}$ vs time, or measuring $A_{280\,nm}$ for collected fractions from a nonautomated column run.

3.5.1. Immunoaffinity Column

Immunoaffinity chromatography is the most convenient way to isolate gD and gC from the infected cell culture supernatants (Fig. 5). We use CNBr-Activated Sepharose 4B (Pharmacia, Uppsala, Sweden) to attach the purified IgG (8 mg of IgG/mL of Sepharose) to the column matrix. Details on MAb coupling are according to manufacturer's specifications.

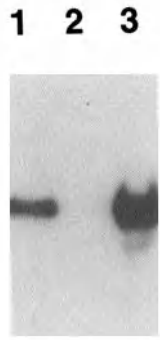

Fig. 6. Western blot analysis of gD-1(306t). The protein was purified by immunoaffinity chromatography on a DL6-sepharose column *(13,27)*. Samples were electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose and probed with polyclonal anti-gD antibody R7. Lane 1, 20 µL of culture supernatant from SF9 cells infected with bac-gD1(306t) for 96 h; lane 2, 20 µL of column flow-through; lane 3, 1.2 µg of immunoaffinity-purified gD-1(306t).

Protein can be eluted from the column with either a high or low pH wash or with a chaotropic agent such as 3*M* KSCN. The choice of eluant depends on the protein and MAb being used. We use 0.1*M* ethanolamine for high pH elution (Fig. 6). Our general running buffer for the column is TS wash (10 m*M* Tris-HCl, pH 7.2, 0.5*M* NaCl).

All procedures are done at 4°C using a jacketed column (XK series, Pharmacia), and all buffers are kept at 4°C in a water bath. If the immunoadsorbent column has been used five times or if the proteins being purified are switched, the column must be cleaned and regenerated according to the manufacturer's specifications. The regeneration protocol is designed to remove proteins and other material that are bound nonspecifically to the column.

Samples should be run across the immunoadsorbent column according to the following protocol:

1. Equilibrate the column at 2 mL/min with at least five bed volumes of TS wash.
2. Filter the sample through a 0.45-µm filter before loading.
3. If you are using an automated system (i.e., Pharmacia GradiFrac or FPLC) write a program with the following steps:
   a. Load the sample onto the column overnight at a flow rate that does not exceed 2.5 mL/min.
   b. Wash the column with five bed volumes of buffer A (TS wash).
   c. Switch to buffer B (0.1*M* ethanolamine) in 2 min and run buffer B for 2 column volumes.

d Collect the eluted protein in an Erlenmeyer flask (on ice) and add 2 5*M* Tris-Cl until the pH is approx 7.0 Fifty millimeters of eluted protein needs approx 10 mL of 2 5*M* Tris-Cl to be neutralized
e. Switch back to buffer A in two minutes and wash the column with 5–10 bed volumes

4. Concentrate the protein to 1–5 mg/mL using the appropriate molecular weight cutoff membrane We use the Amicon 50 mL stirred-cell concentrator with YM10, YM3, or PM10 membranes The choice of membrane depends on protein size However, some proteins unexpectedly go through membranes that should retain them It is wise to check the flow-through the first time a protein is concentrated
5 Dialyze the sample against an appropriate storage buffer
6 Determine the concentration of the protein and store aliquots at –80°C

3.5.2. Heparin Chromatography

This procedure is used for the purification of gC-2(426t) because we have not found an MAb suitable for the purification of this glycoprotein (all of them irreversibly bind the protein), and we found that heparin chromatography is better than Ni^{2+}-agarose chromatography for large-scale purification. The insect medium is not exchanged with PBS, rather it is run directly over the heparin-sepharose column without concentration. By trial and error we found this to be best for this protein. Two consecutive runs on the heparin column are needed to purify gC-2(426t) (Fig. 5A).

The following procedure is written for use with the Pharmacia FPLC or GradiFrac systems. We use a 5-mL heparin column from Pharmacia. The column is run at room temperature. Buffer A is PBS and Buffer B is PBS/1.5*M* NaCl. Gradient composition and times need to be optimized for each individual protein

1 Equilibrate the column with Buffer A then wash the column with Buffer B until the $A_{280\,nm}$ is constant (baseline)
2 Re-equilibrate the column with 5 column volumes of Buffer A
3 Load the filtered sample at 1 0 mL/min. We load 600 mL of supernatant containing 6 mg of gC-2(426t)
4. Wash with 5 column volumes of Buffer A
5. Elute the protein with a gradient from 0–60% Buffer B over 75 min (collect 1-mL fractions). Next run a gradient from 60–100% B over 10 min followed by a 5-min wash with Buffer B in order to ensure that all protein is removed from the column
6 Wash the column with 5 column volumes of Buffer A
7 If this is the final column run, wash the column with 5 column volumes of 20% EtOH Store the column at 4°C for future use
8. Pool the protein-containing fractions, dialyze against PBS, and run the pooled sample over the column again using the same gradient Pool and concentrate the protein-containing fractions and dialyze against an appropriate storage buffer Store the protein at –80°C

3.5.3. Gel Filtration Chromatography

Concentrated samples of immunoaffinity-purified gC and gD are yellowish in color. This appears to be owing to residual components of the culture medium that remain after the initial purification step. Gel filtration chromatography successfully removes the remaining traces of medium from the protein sample. We routinely use a Pharmacia Superose 12 column (useful for proteins in the 1×10^3–3×10^5 mol-wt range).

1. Fully equilibrate the column with PBS not exceeding a flow rate of 1 mL/min This is best done as an overnight wash.
2. For best resolution, the volume of the sample should not exceed 2% of the total bed volume of the column.
3. Filter the sample through a 0.2-μm filter and load the sample onto the column The flowrate should not exceed 1 mL/min for the duration of the run
4. The running buffer for the entire experiment is filtered PBS.
5. Protein size will dictate how long after the void volume the protein will elute To be safe, it is best to start collecting fractions a short time before the void volume of the column has passed through
6. Collect 0.5- to 1.0-mL fractions Analyze the fractions by SDS-PAGE and/or isoelectric focusing (IEF) to determine which ones to pool Pool and concentrate the appropriate fractions, dialyze against an appropriate storage buffer, aliquot, and store the protein at –80°C
7. After all of the protein has eluted, wash the column with 5–10 bed volumes of PBS. Clean and maintain the column according to the manufacturer's specifications
8. Store the column in 20% EtOH

3.6. Characterization of Purified Protein

Below we provide a brief description of the methods we have used to characterize our baculovirus-expressed proteins. Detailed protocols are not given, and certainly more methods are available than the ones we describe.

3.6.1. SDS-PAGE and Western Blotting

SDS-PAGE is useful if it is possible to purify the protein from the cell supernatant or extract, or if enough of the protein is made so that is stands out from the other baculovirus infected cell proteins present in your sample. Silver staining is more sensitive (0.2–0.5 μg of protein/lane; we use the Pharmacia Plustone Protein Silver Staining Kit) than Coomassie staining (1–10 μg of protein/lane; we use the ISS Pro-Blue Staining System, Natick, MA). SDS-PAGE is used to get an approximate molecular weight and to determine the homogeneity of the sample.

A Western blot will indicate which of the stained bands contain the protein (0.05–1 μg of protein/lane). Western blot analysis is better than a simple

immuno-dot blot because the different species present in the sample are separated and can be distinguished from one another. A Western of "native" PAGE *(7)* is useful to probe the antigenic conformation of the protein with MAbs that bind to discontinuous epitopes However, keep in mind that not all MAbs react well in a Western blot of a denaturing or native gel.

3.6.2. Isoelectric Focusing

If the purified protein is going to be used for crystallography, IEF is essential because proteins that are homogeneous on IEF tend to crystallize better We have had success using two IEF systems, the Pharmacia PhastSystem, and the IEF gels that can be purchased from Novex. Both systems give essentially the same results, however the Novex (San Diego, CA) gels are easier to transfer for Western blot analysis. In general, 200–300 ng of protein are needed per lane for a PhastSystem IEF gel and 0.5–1 μg of protein are needed per lane for a Novex IEF gel.

3.6.3. Quantitative ELISA

A quantitative enzyme-linked immunoadsorbent assay (ELISA) more critically assesses the binding of MAbs. The assay is sensitive enough to determine differences in binding among MAbs that are not detectable on Western blots. In addition, some MAbs do not react well in Western blots even of native gels, and we have found that these MAbs react much better in the ELISA. We have also used the ELISA to look at the effect of heating the protein on MAb binding *(23)*.

Briefly, coat 8 μg/mL protein in PBS on 96-well microtiter plates (Corning, Corning, NY) for 2 h at room temperature. Remove the remaining sample and block nonspecific binding by adding 1% bovine serum albumin (BSA), 1% ovalbumin in PBS. Remove the blocking solution and wash the wells with 0.1% Tween-20/PBS. Serially dilute MAb ascites fluids in blocking solution and add for 30 min at room temperature. Remove the unbound MAbs and repeat the wash. Next add protein A-horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) for 30 min (remove and wash as above) followed by the substrate 2,2'-azino-di(3-ethylbenzthiozoline-6-sulfonic acid [ABTS]). Allow the reaction to proceed at room temperature until the optimal color change is achieved, approx 1.5 absorbance units in the darkest well. We assess absorbance with a Dynatech (Dynatech, Chantilly, VA) plate reader using a 405-nm filter blanked against a negative control well *(31)* Alternatively, twofold serial dilutions of protein can be coated onto the plates and probed with a fixed dilution of MAb.

3.6.4. Analytical Gel Filtration

This technique is useful to get an approximate molecular weight of the protein. The molecular weight obtained using gel filtration can be compared to

that obtained using SDS-PAGE. Comparison of the molecular weights by the two techniques will give a good indication if the protein exists as a monomer, dimer, or higher-order structure in solution.

Calibrate the column using a combination of high and low molecular weight standards (purchased from Pharmacia). We use a Superdex 75 column (HR 10/30, Pharmacia) for our analytical gel filtration work. Run a sample of the protein over the column using the same procedure used to run the protein standards (in general use a small volume, 100–200 µL at a protein concentration 0.5–1 mg/mL). The molecular-weight standards come with directions for calculating molecular weight based on the elution volume.

3.6.5. Physical Characterization

We routinely use a number of physical techniques to verify and characterize the sequence, conformation, and oligomeric state of our recombinant proteins. Brief descriptions of each are offered in the following.

3 6.5.1. N-Terminal Sequencing

It is often beneficial to confirm that correct N-terminal processing has taken place in the insect cells by submitting a sample of purified protein for N-terminal sequence analysis. Sequencing takes very little protein (200 pmol or less), and we have found that many of the preparations contain a small percentage of protein (<10%) that has been clipped at the N-terminus.

3.6.5.2. Mass Spectrometry

This technique is useful to get an accurate molecular weight of the protein and to help judge the purity of the sample. The amount of protein needed depends on the type of mass spectrometry being done but should be in the 10-pmol range. We have found matrix-assisted laser desorption ionization mass spectrometry (MALDI) useful in characterizing baculovirus-expressed glycoproteins *(1,18)*. Mass spectrometry must be done in the absence of detergents. Unless dimers and higher order structures are stabilized by covalent bonds, the molecular weight obtained will be of the monomeric form of the protein because dimers and higher order structures are disrupted by the ionization procedure necessary to collect the mass spectrometry data. The molecular weight obtained using this technique will be the most accurate because anomalous effects owing to migration through a column or gel do not have to be considered. Comparing this value to the formula weight will also give a good indication of the extent of posttranslational modification, e.g., glycosylation.

3.6.5.3. Scanning Transmission Electron Microscopy

STEM is another method of looking at the molecular weight of a protein in solution and is also useful to determine the oligomeric state of the protein.

Unlike the more traditional methods of molecular-weight determination that measure averages over a total population (SDS-PAGE, mass spectrometry), STEM quantitates the mass of one molecule at a time and also looks at the molecule's size, shape, and internal mass distribution *(33)* Less than 30 pmol of sample are needed for STEM analysis. A histogram of the data is made to look at the overall mass distribution, and the average molecular weight is determined. The average molecular weight can be compared to that obtained from mass spectrometry to determine the oligomeric state of the protein.

3 6.5.4 Circular Dichroism

CD measures the difference in a protein's absorbance of right and left circularly polarized light *(5)*, and the CD signal in the far UV region is used to probe the secondary structure of proteins. CD experiments can be used to estimate the quantity of secondary structural elements in the proteins being studied and to compare them one to another This technique must be carried out in aqueous solution, preferably in dilute buffers *(10)*. The concentration of sample needed depends on the molecular weight of the protein and the path length of the cuvet. We had success using 0.3 mg/mL solutions (25 m*M* KPi buffer pH 7.2) of gD-1(306t) and gC-1(457t) in cuvets with pathlengths of 1 and 0.02 mm. CD has been useful in comparing a panel of gD variants to determine if any of the amino acid changes have an effect on the secondary structure of the protein

3.6.6. Crystallography

X-ray diffraction of protein crystals is one of the ultimate ways of determining the structure of a protein. In general, viral glycoproteins have not been frequently studied using this method because of the large quantity of protein needed for successful experiments. The baculovirus expression system helps bypass this road block. For crystallization, protein samples need to be highly homogeneous on SDS-PAGE, native PAGE, and IEF gels. The protein also needs to be stable at high concentrations (10 mg/mL) and in low ionic strength buffers

For crystallization we use a two-step purification (immunoaffinity followed by gel filtration chromatography). The protein is then concentrated and exchanged into 25 m*M* HEPES, pH 7 0. It is best to avoid phosphate buffer because phosphate crystals are formed easily. The final concentration of protein should be 10 mg/mL. The best starting point for screening crystallization reagents is to purchase a Crystal Screen Kit (Hampton Research, Riverside, CA). The kits provide a variety of crystallization solutions covering a wide range of pH, precipitants, and salts that have been commonly used in the crystallization of macromolecules. There are a number of crystallography books that are good for the first time crystallographer to read *(11,20,21,24)*.

4. Notes

4.1. Which Cell Line and Which Medium to Use?

Our initial studies to determine the optimal insect cell line and insect medium were confusing. After much deliberation we settled on Sf9 cells grown in serum-free SF-900 II medium. The Sf9 cells were weaned onto this medium by the company and these adjusted cells can be obtained directly from Gibco. Other companies make their own formulation of serum-free medium and have adapted insect cells to their formulation. We suggest you pick one cell line and stay with it. Pluronic F-68 is included in SF-900 II medium as an antishearing agent so there is no need to supplement.

4.2. Cell Line Maintenance

New suspension cultures are started every 3 mo from frozen cells or if there is a noticeable drop of either virus titer or protein production. Cells are thawed and placed directly in suspension culture (27°C, SF-900 II medium, no FBS) and kept at 1×10^6 cells/mL until they start to grow. The cells should be subcultured every 3 to 4 d and should be ready to work with in 1 wk. Thereafter the cells can be maintained at 5×10^5 cells/mL (*see* Section 3.2.2.). We recommend using a 27°C refrigerated incubator for culturing cells and growing virus to avoid the ups and downs of "room temperature."

4.3. Cloning and Purification of Recombinant Virus

We have had excellent results using BaculoGold DNA (Pharmingen) for doing homologous recombination. Our efficiency for positive plaques ranges from 60–100% (Fig. 3). We have not had success using Sf9 cells directly from a spinner flask for transfection. Monolayers should be split at least twice for the best transfection results (*see* Section 3.2.1.).

When doing plaque purification, it is best to choose the plate with the lowest density of plaques. Be sure that each picked plaque is derived from a single recombinant virus, not a possible mixture of clones.

Assessing protein production by Western blotting is better than looking by Coomassie blue staining because there are also baculovirus proteins in the medium. It is also more informative and easier to screen directly for protein expression rather than probing for the gene. The high-salt concentration of the SF-900 II medium influences the migration of proteins on SDS-PAGE. The proteins appear larger (slower mobility) when electrophoresed in the presence of medium

The 2% Seaplaque agarose used for titering the virus can be prepared in large quantities, autoclaved, aliquoted, and stored at room temperature. Microwave aliquots carefully to reheat. Once overheated, the agarose loses its ability to solidify.

4.4. Storage of Virus

Virus stored at 4°C loses one full log of infectivity over a 6-mo period. In addition, the virus is light sensitive and should not be stored in a frequently used cold room. For these reasons, we store all virus in convenient aliquots at –80°C in SF-900 II medium supplemented with 5% FBS. There is no loss of infectivity on thawing, however, we do not refreeze the aliquots. In addition, once you know your cloning has been successful, freeze all of your transfectants and all of your plaque purified clones at –80°C in case you ever need to go back to them.

4.5. High-Titered Virus

It is important to obtain high-titered virus (at least 10^8 PFU/mL) for infection of large volumes of cells In addition, it is critical to titer the virus stock carefully so that the moi can be carefully controlled. Fresh, healthy cells growing in suspension in log phase (95–100% viability) are the key to getting a high titer virus stock. To test for the proper moi, infect 100-mL aliquots of cells at 2×10^6/mL with a range of recombinant virus from 0.1 to 1PFU/cell following the protocol in Section 3.3.5. We have found no evidence for the generation of defective viruses. Each virus stock is then titered and the best moi determined.

4.6. Viewing the Plaques

Viewing plaques is a major drawback of the baculovirus system. The best method for viewing is with a dissecting scope and a stereo light source such as Sage Instruments-Model 281 or a Leitz Wild MZ8 stereoscope. Excellent definition can also be obtained with side lighting using fiberoptics. The picture in Fig. 2 gives an example of what the plaques look like.

4.7. Growing Cells in Suspension Cultures

Adequate aeration is very important for growing Sf9 cells in suspension and can be achieved in any spinner culture of 500 mL or less using a stirring paddle. The problem arises when volumes of greater than 500 mL are needed. Maximum aeration of the culture is absolutely mandatory particularly after infection. If aeration is not maintained at a high level we see no increase in protein production from the zero time point, so although the cells look infected, protein is not being produced

For volumes over 500 mL a double-paddle spinner is necessary (Bellco). The top paddle should be set to break the surface of the culture. We do not see damage to Sf9 cells using these paddles. For larger spinners (3–8 L) we employ an overhead drive (100 rpm) and a sparger to enhance aeration (control valve-MG Industries, Philadelphia, PA). Foaming caused by bubbling the air into the

vessel does no harm. There is no reason to concentrate the cells prior to adding virus to obtain efficient infection and production of protein.

4.8. Protein Production

It is difficult to estimate the yield of protein secreted when working with insect medium. The ionic strength of the medium and the presence of other baculovirus proteins interfere with the protein assay. Accurate protein values are obtained only after the glycoproteins are purified from the medium. We have not found it necessary to use protease inhibitors when isolating proteins from culture supernatants. We routinely obtain 5–30 mg/L of affinity-purified glycoprotein using our baculovirus expression system. In general, we try not to refreeze purified protein so choose aliquot size carefully.

4.9. Immunoaffinity Chromatography

The scale-up from a small to a large immunoadsorbent is relatively straightforward. The total binding capacity of an individual IgG can be determined only by trial and error. For gD-1(306t) we use a 150-mL (8 mg of IgG/mL of sepharose) column of DL6 IgG bound to CNBr-activated Sepharose (Pharmacia, all procedures are according to the manufacturer's instructions). This column is able to bind approx 90 mg of gD. The eluant for this column is 0.1*M* ethanolamine. It is very important to use fresh, colorless ethanolamine. Old ethanolamine stock or old bottles of deteriorated ethanolamine should be discarded. Old ethanolamine will strip antibody from the column. We now buy ethanolamine in 100-mL quantities from Sigma. Our immunoadsorbent columns are remarkably stable and have been used repeatedly for several years with no loss of activity. Between runs the columns are stored in TS wash/ 0.02% azide at 4°C. Do not let the columns dry out.

4.10. Ni^{2+}-Agarose Chromatography

We initially used Ni^{2+}-agarose (Qiagen) to purify small amounts of our proteins from the infected cell culture supernatant to take advantage of the histidine tail that was added to the gC and gD constructs *(27,31)*. When we tried to scale up the purification, we encountered a number of problems. First, we were not able to load multimilligram amounts of protein directly from the culture supernatant onto the column. Initially the protein bound, but adsorption dropped with time. Second, the contents of the medium eventually stripped the Ni^{2+} from the column (changing the color of the matrix from pale blue to white). Third, the pH of the growth medium drops from 6.3–5.9 by 72 h PI (note that the pH is controlled in the Celligen Bioreactor) and inhibits binding. Finally, the Ni^{2+}-agarose beads are easily crushed by extended spins in the microfuge and under the low pressure used for FPLC. Therefore, scale-up to a larger automated column was abandoned.

We tried two different elution schemes for small-scale Ni^{2+}-agarose chromatography. Both are protocols obtained from Qiagen. The first was to elute the protein with a low pH wash and the second was to elute the protein with an imidazole wash (10 m*M* imidazole wash to remove nonspecific binding followed by a 250 m*M* imidazole wash to elute the protein). In general, protein purified with imidazole elution looks cleaner on SDS-PAGE than protein purified by pH elution. Both procedures need to be optimized for the particular protein being worked with.

4.11. Gel Filtration Chromatography

Although we use a UV monitor, we find it convenient to analyze the column fractions using the Pharmacia PhastSystem because small volumes (1 μL) and protein amounts (150–200 ng) can be detected rapidly by silver stain. However, Western blotting or standard SDS-PAGE can also be used.

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10

Crystallization of Macromolecules for Three-Dimensional Structure Determination

Ben Luisi, Marie Anderson, and Graham Hope

1. Introduction

The last decade has seen a remarkable flourishing of the biological structure field. This blossoming has brought an explosion of stereochemical information, and has been made possible by the combined improvement in techniques of X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, and the bulk preparation of biological materials. Most crucial, however, has been the desire of the experimental biologists to follow research problems to the level of stereochemistry, which is the ultimate reductionist limit of molecular biology. This aim has been driven by the anticipation that such knowledge may permit better understanding and even engineering of biological function.

Structural information has indeed proven invaluable in functional studies and has provided a foundation for the interpretation of results from directed mutagenesis. Structural knowledge has often provided clues regarding enzymatic mechanisms even before functional studies have identified catalytic centers *(1)* One engineering aspect of structural studies, still in its infancy, is the field of rational drug design. It seems reasonable that structural information will permit the design of tailored therapeutic compounds, including molecules, which bind defined DNA targets with high specificity and of ribozymes, which, it is hoped, may selectively inactivate defined RNA species *(2,3)*. Three-dimensional structural information coupled with improvements in the evaluation of molecular interaction energies may provide better algorithms for rational design programs.

The methods of structure determination by X-ray crystallography and NMR spectroscopy have grown increasingly more sophisticated, and it is now possible to tackle problems that would have been considered impossible a few years ago. In NMR, signal broadening places a practical upper limit on the size of molecules that

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can be studied, and the molecules must be well-behaved in solution and monodispersed. If these conditions are satisfied, the solution structure may be determined fairly quickly. For crystallography, the practical upper limit is enormous. Even large viruses have been successfully crystallized and studied at high resolution The rate-limiting steps for the success of the method, however, are the capricious acts of growing crystals and suitably doping them with electron-rich atoms.

Here, we describe some practical methods for the preparation and evaluation of macromolecular crystals for 3D structure determination by X-ray crystallography We also discuss preparation of specimens for NMR and electron microscopy. The methods for growing crystals for X-ray diffraction are extremely simple; however, the requirement for specimen purity is exacting. Consequently, we devote a special discussion (Section 4.) of the attention required for specimen preparation and some useful procedures that might sometimes help in obtaining crystals.

2. Materials

2.1. Protein Preparation

It is crucial that the protein samples are of the highest possible purity and readily available in milligram quantities. Section 4. provides comments on the evaluation of protein purity. Preferably, the protein samples are at a concentration of at least 3 mg/mL in distilled water Most proteins will prefer the presence of some salt or buffer, and others might require glycerol (also as a cryoprotectant for frozen storage) or traces of detergent. Each case will be different, and experimentation will be required

2.2. Nucleic Acid Preparation

If preparing synthetic DNA for cocrystallizations, it is most convenient to start with tritylated material. Purification and detritylation can be performed with a NENSORB reverse-phase column (Dupont). The purified material is lyophilized and should be checked for quality by analytical reverse phase HPLC, gel electrophoresis, or mass spectrometry The dried material is then dialyzed against 100 m*M* NaCl and then distilled water to yield the sodium salt Centricon concentrating units (Amicon) are very convenient for the dialysis and concentration of the samples. Joachimiak and Sigler *(4)* make several suggestions for designing oligonucleotides to be used in cocrystallizations with proteins.

2.3. Buffers, Precipitating Agents, Crystallization Wells, and Cover Slips

Table 1 summarizes the precipitating agents commonly used for crystallization experiments. Table 2 summarizes the most commonly used buffers. Crys-

Table 1
Frequently Used Precipitating Agents for Proteins

Salts
- Ammonium citrate
- Ammonium sulfate
- Lithium sulfate
- Sodium chloride
- Sodium bromide
- Sodium/potassium phosphate
- Sodium/potassium citrate

Alcohols
- 2-Methyl-2,4-pentanediol (MPD)
- 2,6-Hexanediol and 1,2-hexanediol
- Isopropanol
- Ethanol
- Propanol

Long-chain polymers
- Polyethylene glycol (PEG), typical mol wt 400–20,000 Dalton
- PEG methyl ether, typical weight range

Special additives (usually used at m*M* concentrations)
- Reducing agents (dithiothreitol, mercaptoethanol, dithionite)
- Bacteriostatic agents (sodium azide)
- Detergents octyl-β-glucoside, CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate)
- Polyvalent cations and anions, such as spermine or spermidine, Mg^{2+}, Ca^{2+}, $[Co(NH_3)_6]^{3+}$, Eu^{3+}

Table 2
Frequently Used Buffers for Crystallization Trials

Buffer	Buffering range, pH
Na acetate	3.6–5 6
Sodium/potassium phosphate[a]	5.0–8.2
Sodium cacodylate	5.2–7 2
Bis-tris: *bis*[2-hydroxyethl]iminotris[hydroxymethyl]-methane	5 8–7.2
PIPES piperazine-*N,N'-bis*[2-ethanesulfonic acid]	6 1–7.5
MOPS 3-[*N*-morpholino]propanesulfonic acid	6 5–7 9
HEPES· *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]	6 8–8.2
Tris: tris[hydroxymethyl]aminomethane	7 0–9 0
CAPS· 3-[cyclohexylamino]-1-propanesulfonic acid	9 7–11 1

[a]Forms inorganic crystals with traces of barium or calcium, so care must be taken when choosing additives.

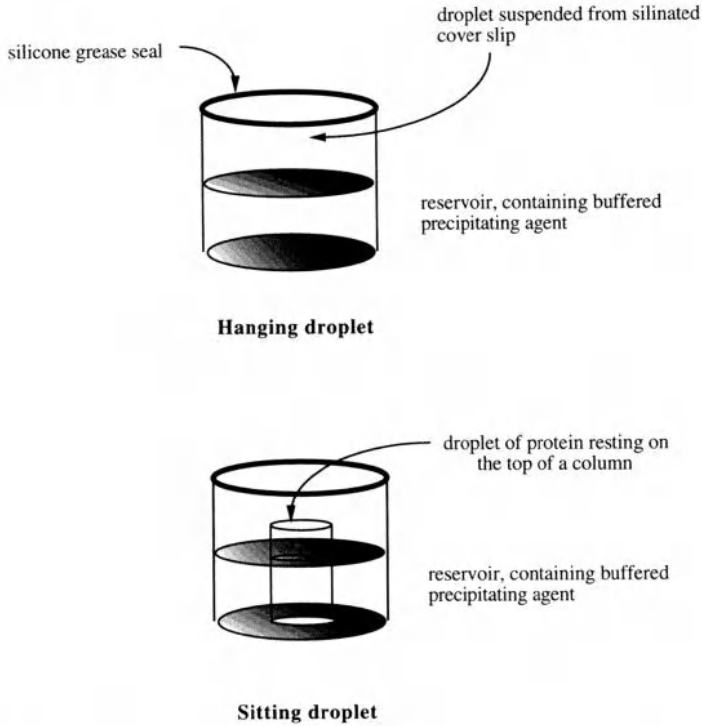

Fig. 1. Schematic diagram of vapor diffusion crystallization setup. A droplet containing the protein solution and ⅓–½ vol of the external reservoir is suspended from a silinated cover slip over a reservoir containing buffered precipitating agent. Water molecules and other volatile species will exchange between the droplet and reservoir and, at equilibrium, their chemical activities in the two compartments will become equal.

tallization kits are commercially available (Hampton Research) and useful for quickly surveying many conditions. Crystallization trials using vapor diffusion crystallization can be undertaken with disposable plastic tissue-culture plates (Limbo model FB16-24-TC) that have 24 wells (2 cm diameter × 2 cm deep) with flat rims. Circular glass cover slips with 2 cm diameter are required for suspending a droplet of protein solution over the reservoir. Application of silicone vacuum grease around the rim of each well enables airtight sealing (*see* Fig. 1). Pretreat the cover slips with silination solution (3% by volume of dimethylsilane in dichloromethane) for one-half hour and then air-dry in a fume hood after washing briefly with dichloromethane. Water will not adhere to properly silinated slides. The cover slips can be easily cleaned before use by immersing in water.

2.4. Quantitation of Protein Concentration for Crystallization

Commercial kits are available that use the Bradford, bicinchoninic acid (BCA), or Lowry assay, and these have different degrees of sensitivity, depending on the sample. As described in Section 3., UV spectrophometry is also a convenient means of quantitation.

2.5. Two-Dimensional Crystallizations for Electron Microscopy

Prepare fresh solutions of a lipid mixture containing 0.05 mg/mL of octadecylamine and 0.45 mg/mL of egg L-α-phosphatidylcholine in 1:1 chloroform/hexane *(5)*. Store anaerobically at –20°C. Two-dimensional crystals can also be prepared on mica, and good-quality mica sheets are required. Use 1% weight uranyl acetate solution for negative staining to evaluate the trials by electron microscopy.

3. Method+s

3.1. Growing Crystals: Introduction

Protein crystals are most likely to grow under conditions where the effective protein concentration exceeds its solubility limit. This condition is known as supersaturation, and it is a nonequilibrium state. Crystallization conditions lurk where the system relaxes to equilibrium, and the protein molecules are excluded into the solid state. The most common strategy in growing crystals is to achieve supersaturation by modifying the solvent with precipitating agents, adjusting the pH, or by altering a physical property, such as the temperature.

Using light-scattering measurements to infer particle sizes, it has been experimentally observed that crystals are most likely to grow when the precipitating agent does not cause the protein molecules to aggregate randomly *(6)* For instance, lysozyme remains monodispersed over a high concentration range of sodium chloride, but in the presence of ammonium sulfate, it aggregates. This observation appears to explain why lysozyme crystals can be readily prepared from solutions of sodium chloride, but not from ammonium sulfate (*see* Section 3.5.). Therefore, in preparing crystals, one is ideally searching for conditions of supersaturation without aggregation, where nucleation and then crystal growth will occur. If, however, the system is pushed to supersaturation under conditions where aggregation results, only noncrystalline precipitate may develop.

To achieve supersaturation, it is necessary to screen a range of conditions, including variations in concentration of precipitating agents, pH, protein concentration, and temperature (given in rough order of importance for the typical case). Summaries of suitable precipitating agents and biological buffers for crystallization trials are presented in Tables 1 and 2. The number of possible

combinations of crystallization conditions is immense; however, the supply of protein is usually limited. One would ideally like to cover as wide a range of conditions as possible in the initial searches, but still be able to correlate trends between trials for subsequent crystallization screens. The best strategy is to strike a balance between these requirements, and this approach is known as factorial analysis *(7)* A number of general crystallization sets have been designed in part on this principle and also on the use of *a posteriori* information from the generally most successful crystallization conditions *(8–11)*. In these general screens, a carefully selected, diverse set of the most successful conditions is chosen as the preliminary crystallization screen

3.2. Effectors of Crystal Growth

3 2.1. Precipitating Agent

The most common procedure to achieve supersaturation is to change the concentration of salts gradually. At very low ionic strength, protein solubility is low. Indeed, some proteins crystallize in distilled water as they are being concentrated. For most proteins, the solubility becomes greater with increasing ionic strength, a phenomenon known as "salting-in." However, the solubilization occurs up to a point when a reverse solubility effect known as "salting-out" is manifested, and both salt ions and protein molecules compete for hydration structures, which are required to maintain their solubility Furthermore, the high ionic strength of the solution may shield unfavorable electrostatic interactions between protein molecules, thereby permitting association.

It is important that a large range of salts be used in attempting to crystallize a macromolecule, since, in addition to salting-out effects, there may also be specific protein–ion interactions that may have secondary consequences. Polymeric alcohols, such as the range of polyethylene glycols, are very popular as effective crystallizations agents. Organic solvents can also be used to induce crystallization, although they are not as popular as salts and polyethylene glycol. Proton concentration can have a tremendous effect on crystal growth. Solubility tends to be minimal at the isoelectric point, but not all proteins crystallize at the corresponding p*I*. Lattice contacts might be made by salt bridges, which can be affected by pH

3.2.2. Temperature

Temperature can have a strong effect on crystal growth owing to its influence on protein solubility. Proteins can have either negative or positive solubility coefficients with temperature. For instance, insulin crystals can be grown by an inverse temperature gradient (for example, *see* ref. *12*). If there is sufficient material available, crystallization trays should be set out at least in dupli-

cate, with one tray being kept at room temperature and the second maintained at 4°C in a cold room Optimally, a bank of controlled temperature incubators should be used at as many different temperatures as possible. Precisely because of the effect of temperature on crystallization, it is a good policy to maintain the crystallization trays at a constant temperature, preferably by using a well-insulated container or a controlled temperature incubator.

3.2.3. Additives

Divalent cations and spermine are proven crystallization agents for nucleic acids *(4,6)*. Detergents are required for some proteins, such as membrane proteins. In some cases, proteins or protein–DNA complexes can be cocrystallized with a heavy atom, such as lanthanide salts *(13)*

3.3. Protein Quantification

Protein concentration can be conveniently quantified using the Bradford, BCA, or Lowry assay, and these have different degrees of accuracy, depending on the sample. Amino acid composition analysis is a reliable method for quantitating protein concentration, providing that accurate amino acid standards are available. UV spectroscopy is yet another convenient method of estimating protein concentration, and the relationship between concentration vs absorption is roughly linear up to about two optical units. The protein concentration is estimated from the absorbance at 280 nm using the calculated extinction coefficient based on the composition of the aromatic amino acids. This estimation is usually made more accurate by using a buffer containing a denaturing agent, such as 6*M* guanidinium hydrochloride. Spectroscopic measurement is also a convenient means of quantifying nucleic acid concentration, again using calculated extinction coefficients based on the base composition. Empirical corrections for hypochromic effect must be made, usually by heating the sample or digesting with nuclease.

3.4. Crystal Preparation by Vapor Diffusion

The most popular method of growing crystals is by vapor diffusion, where the protein solution is mixed with buffered solution of the precipitating agent, and the mixture is permitted to equilibrate against a large reservoir containing buffered precipitating agent at a higher concentration. There are two methods of achieving vapor diffusion: hanging droplets and sitting droplets (Fig. 1). In the hanging droplet method, a microdroplet of protein (as little as 2 μL) is placed on a silinated microscope cover slip, which is then inverted and placed over a well containing 1 mL of buffered precipitating solution. It is important that the cover slip is silinated, since this ensures proper drop formation and prevents droplet spreading. A large number of conditions can be screened by

this method using only a small amount of protein. Disposable plastic tissue-culture plates that have 24 wells (2 cm diameter × 2 cm deep) with flat rims enable airtight sealing by application of silicone vacuum grease around the circumference of each rim. These plates provide a further advantage in that they can be easily examined under a dissecting microscope and allow compact storage.

The design of the sitting droplet is similar to the hanging droplet, except that the drop sits on an elevated table above the precipitating agent (Fig. 1). The sitting droplet approach is advantageous in cases where the protein droplet spreads extensively on coverslips or when detergents are being used in crystallizations, since these cause droplet spreading.

3.5. Crystal Preparation by Dialysis

A macromolecule may be guided gradually toward crystallization by dialysis against a solution of buffered precipitating agent. This method has the advantage that the rate of equilibration can be controlled by adjusting the conditions of the external side of the membrane. If the concentration of precipitant is too high and an amorphous precipitate results rather than crystals, the sample may be redissolved and new conditions established simply by adjusting the external solution.

Various methods have been designed for microdialysis cells or vessels. In most cases, only 10–20 μL of protein solution are injected into a short glass capillary or tube. The vessel is then sealed at one end, and the other is covered with a dialysis membrane. The entire assembly is submerged in a container holding the solution of buffered precipitating agent. Gradual equilibration then occurs through the membrane. One convenient aspect of this procedure is that the protein can be maintained under anaerobic conditions. The droplets can be set out under nitrogen with reducing agent in the degassed buffer.

3.6. Crystal Nucleation by Microseeding

Microseeding is used to induce directed crystal growth to increase crystal size or improve crystal quality. A supersaturated protein solution that contains small crystals from earlier trials may be diluted into a fresh solution that is only slightly supersaturated, so that slow growth of crystals will occur. One problem with seeding with microcrystals is that, if too many crystals are induced into the fresh supersaturated solution, twined or poorly formed crystals may form that are unsuitable for data collection.

3.7. Practical Example: Growing Crystals of Lysozyme

We recommend that the hopeful crystallographer practice the hanging droplet method by crystallizing hen egg lysozyme, which will yield beautiful crystals in 1–2 d. These crystals can be used to practice some of the more difficult

manipulations, such as mounting crystals in capillaries for data collection or flash-freezing in liquid nitrogen.

1. Prepare a stock buffer solution of 0.1*M* Na acetate, pH 4.7.
2. Prepare a stock solution of 50 mg/mL hen egg white lysozyme (use good-quality protein). Spin in an Eppendorf centrifuge at 4°C to remove any insoluble material.
3. Prepare a tray in which the precipitating agent, NaCl, varies in the wells from 2–8% wt/vol in the Na actetate buffer. Apply silicone grease to the rim of the reservoir
4 On a silinated cover slip, mix 5 µL of the lysozyme solution with 5 µL of the precipitating solution from the reservoir. Carefully invert the cover slip and seal against the rim of each reservoir
5. Leave undisturbed in an insulated storage box at room temperature or in a temperature-controlled incubator at 25°C for 1–2 d.

3.8. Evaluation of Crystals

The best means of examining crystallization trials is with a good-quality dissecting microscope. A convenient method of visualizing the smallest microcrystals (i.e., crystals with longest dimensions of 5 µm) is with a set of polarizing filters. Because crystals consist of ordered chiral molecules, light that passes through them will become plane-polarized. (This will occur for all crystals, except those with cubic symmetry.) Consequently, light that is already plane-polarized will change its polarization state on passing through a protein crystal. The field of view will be perceived to change from brightness to darkness as one of the filters is rotated through 90°. Microcrystal that may be too small to visualize directly under the dissecting microscope may be seen as a "glowing" effect under rotating polarizers. Inexpensive polarizers may be constructed from a sheet of Polaroid filter, placing one between the illuminating light source and specimen, and the second between any of the objective lenses.

One of the most disheartening aspects of crystallizations trials is that inorganic salt crystals can often be mistaken for protein crystals. There are a number of simple tests to check if crystals are composed of protein or salt. Protein crystals tend not to have the same mechanical rigidity as salt crystals and will shatter if a small force is applied. A destructive, but quick test is therefore to poke the crystal with a hair or finely drawn glass fiber. Protein crystals will readily shatter with such a force, whereas salt crystals will remain intact or make a discernible crunching noise. Another quick, but destructive test is to stain with filtered Coomassie dye solution. Protein crystals should take up the dye and become much darker than the background.

Although crystals may appear to be of good-quality externally by optical examination, they may not necessarily have satisfactory crystalline order. To test the quality of their internal order, a diffraction pattern must be measured. Most crystals are very sensitive to changes in humidity. The crystals therefore

need to be maintained under the same humidity as that under which they were grown. This can be achieved by mounting the crystal in a capillary with reservoir solution at either end and sealing it to maintain the crystal in an atmosphere with the appropriate humidity. This was the great technique that permitted Bernal and Hodgkins to obtain the first protein diffraction pattern 60 yr ago.

Flash-freezing has become a recent and very popular method to preserve crystals for X-ray data collection *(14,15)* The crystals are first briefly transferred to a cryoprotectant solution (usually a mixture of reservoir solution diluted with antifreezing solute, such as glycerol) and then quick-frozen in a nitrogen stream at 100 K. The low temperature effectively immortalizes the crystals against the damaging effects of X-rays

3.9. Preparation of Derivatives

In order to solve the crystal structure, it is necessary to obtain information about the phase of each reflection that arises from diffraction This can be achieved by doping the crystal with a heavy atom, which will change the intensity distribution of the diffracted X-rays. Details of the optimal conditions for soaking heavy metals into protein crystals are described elsewhere *(12)* Extreme caution is required in handling these deadly compounds, since they bind proteins avidly, including those that comprise the protein crystallographer. Like growing crystals themselves, these soaking methods suffer from a capricious element. Fortunately, procedures have been designed to rationalize the preparation of heavy atom derivatives *(16)* Using site-directed mutagenesis, one can introduce cysteine residues as target sites for modification by mercurial compounds. The best strategy is to choose amino acids that are hydrophilic, since these will most likely be on the surface of the protein and therefore more likely to be exposed for reaction.

3.10. Methods for Growing and Evaluating Two-Dimensional Crystals for Electron Microscopy

Two-dimensional crystallography offers a convenient method for obtaining structural information. Although the resolution of the method is limited, there are a number of advantages for using this approach. Only microgram quantities of protein are required at concentrations as little as 250 μg/mL, and the results can be known within hours rather than weeks, since the crystals can be immediately visualized with the electron microscope.

3.10.1. Crystallization of Protein on Lipid Layers

Crystallization depends on the binding of the macromolecules to a charged water-exposed surface of lipid layers *(17)* Association with this surface orients and concentrates the macromolecule in two dimensions, whereas lateral

diffusion facilitates crystallization. A number of macromolecules have been successfully crystallized using this method, including yeast RNA polymerase II *(5)*. Crystals were formed from polymerase II at a concentration of 50–250 μg/mL. The droplet of protein was coated with a lipid mixture containing 0.05 mg/mL of octadecylamine and 0.45 mg/mL of egg L-α-aphosphatidylcholine in 1:1 chloroform/hexane. The drops were then incubated under nitrogen or argon for 30–60 min; during this time, crystals formed on the droplet surface. The crystals were then transferred to a carbon-coated grid, using a wire loop. This loop was placed over the drop, and a thin layer of protein lipid was carefully lifted and passed onto the electron microscope grid. The grid was allowed to dry and then washed with 2 mL of distilled water. After washing, the sample was stained using uranyl acetate and examined by electron microscopy.

Recently, the lipid surface method has bean made more general through the development of nickel-chelating lipids for histidine-tagged proteins *(18)*.

3.10.2. Crystallization of Protein on Mica Surfaces

Another method of crystallizing proteins in two dimensions is to use freshly cleaved mica. A few microliters of protein solution are spread over the top of the mica and allowed to dry. The protein-coated mica is then covered in a very fine layer of carbon, and using a razor blade, the surface of the protein mica is scored very gently in a crisscross fashion. The sample is then placed in a shallow dish containing distilled water, and the coated specimen floats from the surface of the mica. The electron microscope grid is then placed under the protein sample and lifted from the water. The sample is then dried and stained with uranyl acetate or other suitable negative stain.

4. Notes

1. Preparation of protein—requirement for purity and quantity. Perhaps the most important factors affecting the success of crystallization trials are the purity and abundance of the sample. Because there are no rules dictating the conditions under which a macromolecule will crystallize, many different conditions must be examined before a successful result might be obtained. The more material that is available, the easier the task of surveying a wide range of conditions Even if crystals are readily found in an initial screen, it may be necessary to consume more material in refining conditions to optimize crystal quality and in the search for suitable heavy-atom derivatives Experience has demonstrated repeatedly that specimens that are free of substantial heterogeneity, which include small-molecule contaminants, are most likely to crystallize. Heterogeneity is likely to ruin the uniform interaction of proteins, which is required for generating a crystalline lattice. Protein samples must be free of traces of protease, which can degrade the material into a useless heterogeneous mixture with time. Elsewhere in this book, chapters are dedicated to detailed methods of purification of proteins (e g ,

ammonium sulfate precipitation, ion-exchange, and hydrophobic interaction chromatography), and these should be referred to for advice on designing purification protocols (*see also* ref. *19*) We describe here some procedures for analysis of the purity of the specimen.

The first goal of the hopeful crystallographer is to ensure that the specimen for crystallization is as pure as possible by analysis with denaturing gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, or SDS-PAGE), which is the easiest and most common method of evaluating the sample for contaminating proteins Differences in molecular weight of about 1000 Dalton can be readily detected by SDS-PAGE Coomassie blue staining of SDS-PAGE gels can detect about 0.5 μg of protein, whereas silver-staining increases the level of detection about 10-fold. The response of the silver stain is nonlinear with protein quantity, and it may be difficult to estimate the relative abundance of contaminants

A homogeneous band on SDS-PAGE may, however, not be a sufficiently rigorous test of purity For instance, nonproteinacious substances will not be detected, and highly basic proteins may sometimes carry nucleic acid contaminants. UV spectrophotometry is a useful method to check protein samples for nonproteinacious contaminants. Proteins have characteristic adsorption maxima at 280 nm owing to the presence of aromatic rings in tryptophan, phenyalanine, and tyrosine, whereas nucleic acids absorb maximally at 260 nm Contaminating nucleic acids will affect crystal growth of proteins, but these can be readily removed by hydroxyapatite chromatography.

Microheterogeneity within a protein may also go undetected by SDS-PAGE This heterogeneity may arise from protein modifications, e.g., glycosylation or phosphorylation, which are posttranslational processes, or from effects, such as oxidation and deamination Microheterogeneity may have a strong effect on crystallizations or no effect whatsoever It depends on the case, and every case is different. However, the safest strategy is to ensure that the protein suffers from as little microheterogeneity as possible

A number of sensitive procedures are available to detect microheterogeneity. One method is two-dimensional PAGE, where the first dimension separates according to the isoelectric point of the protein and the second separates by size, as in regular SDS-PAGE. Microheterogeneity owing to charge differences can also be detected conveniently by isoelectric focusing (IEF) gels Any microheterogeneity detected by IEF under nondenaturing conditions may be owing to differences in primary structure or in the type and number of prosthetic groups. For this reason, it is helpful to examine the protein sample by denaturing IEF.

Electrospray mass spectrometry is an extremely sensitive method that can reveal specimen heterogeneity and give a clue regarding its nature *(20)* This method provides accurate mass determination to less than the mass of a single amino acid residue of proteins up to about 100 kDa. Since the protein sequence is usually known, the expected mass can be very accurately calculated. Mass deviations can usually be accounted for by proteolysis or covalent modification A related technique is peptide fingerprinting by mass spectrometry Here, the pro-

tein could be treated with sequencing grade protease to generate a characteristic pattern of fragments in the mass spectrometer. This fingerprint aids in corroborating the identity of the protein and defining possible sites of modification

Capillary electrophoresis has proven to be a useful method for detecting microheterogeneity. A fused silica capillary is filled with electrolyte support buffer and placed between two buffer reservoirs containing high-voltage electrodes Protein is introduced at one end of the capillary and migrates under the influence of the electric field. Because the capillary can be made to very long dimensions, extremely high-resolution separation can be attained, capable of resolving molecules differing by single charges

Deamination of glutamine and asparigine residues may occur at low pH and lead to charge change This can be prevented by avoiding acidic conditions at all stages in protein preparation. Another source of microheterogeneity is oxidation of cysteine and methionine residues. Oxidation can be avoided if the specimen is carefully kept under reducing conditions. One should include gentle reducing agents in all buffers, such as dithiothreitol, dithioerythreitol, or mercaptoethanol Degassing buffers and flushing with nitrogen may also prolong the reducing environment

Microheterogeneity in recombinant proteins can arise from amino acid misincorporation When foreign genes are expressed in *Escherichia coli,* misfolding of the expressed protein and misincorporation of amino acids may occur if the codons used by the foreign genes are relatively rare in the bacteria (reviewed in ref *21*). One possible way to overcome the problem with amino acid misincorporation in recombinant proteins would be to synthesize the gene chemically using codons optimal for expression in *E coli*

Glycosylation inherently produces high levels of microheterogeneity owing to the variation in branching of the carbohydrates. There are also several examples where removal of part or the entire carbohydrate may have assisted crystal growth *(22,23)* It should be pointed out, however, that there are other examples where crystals have been grown from proteins that have a mixture of different carbohydrate moieties. Indeed the presence of carbohydrate may be essential for stability and solubility of some proteins, so complete removal of the carbohydrate may not be helpful in preparing specimens for crystallization in certain cases. Potential problems caused by glycosylation have to be investigated for each case

If the carbohydrate is suspected as being a possible culprit retarding crystal growth, then there are several ways of removing the offending party. For instance, the sialic acid residues of the carbohydrates bear charged carboxylates and are a source of charge heterogeneity. These sugars can be removed using neuraminidase This treatment should result in the protein being less heterogeneous on IEF gels. More drastically, hydrogen fluoride treatment will remove the carbohydrate moiety entirely *(24)* This procedure is extremely dangerous and requires special safety apparatus. It is critical that the protein be lyophilized to complete dryness for this procedure. As a safer alternative, commercially available cocktails of endoglycosidases may also be used to remove carbohydrate. Since the cocktail

may not be very pure, it may be necessary to include protease inhibitors to prevent degradation of the sample Site-directed mutagenesis can also be used to alter known glycosylation sites For instance, Bentley et al. *(23)* mutated asparagines to glutamines in the β-chain of the T-cell antigen receptor to stop amino-linked glycosylation The aglycosylated material yielded crystals, whereas the native glycosylated material failed to do so

2 DNA and RNA purity· Purity of nucleic acids may be readily tested by denaturing gel electrophoresis. Mass spectrometry is also becoming a very useful method for evaluating the nucleic acid specimen. A convenient matrix has been recently developed that is used in matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) *(25)*

3 Useful procedures to increase the chances of growing crystals. If the recombinant proteins are insoluble, one can purify the material under denaturing conditions and refold the specimen *(21)* Some proteins aggregate extensively under low salt conditions. In this case, one can use high salt, a volatile salt, such as ammonium bicarbonate, or ammonium acetate to improve the solubility *(26)* It is also a good strategy to try homologs of the protein from related species This worked very well for structural studies of the RING finger domain from equine herpesvirus *(27)* Here, the corresponding segment of the protein from the viral homologs (human herpes simplex virus) yielded only very poorly behaved proteins. Mutations that change surface residues sometimes provide another useful variation that has successfully resulted in crystallization, which failed for the native protein *(28,29)*

We can suggest that if the protein of interest is poorly behaved, one might trim it down to stable structural cores for analysis by NMR or to be used for crystallization trials. Definition of structural subdomains by proteolysis has been quite successful in a number of cases for structural studies, and it is particularly useful for preparing NMR specimens. Use sequencing-grade trypsin or TPCK-treated chymotrypsin

Cocrystallization of Fab/protein complexes may facilitate crystal growth. If good monoclonal antibodies (MAb) are available that recognize the protein under nondenaturing conditions (and therefore most likely recognize exposed surface epitope), they could be used for generation of Fab fragments. The Fab complexes appear to have been helpful for growing crystals in several cases *(30–33)*

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11

Direct Immunogold Labeling of Herpesvirus Suspensions

Linda M. Stannard

1. Introduction

Gold particles in colloidal suspension are particularly well suited as markers for immune electron microscopy. Their extreme electron opacity ensures that they are detected with accuracy even at particle sizes of less than 3 nm. Gold spheres can be made easily and inexpensively by reduction of gold chloride with mild acid and heat *(1)*, and particles can be prepared in a variety of sizes by varying the nature of the reducing agents *(2)*.

An important feature of the colloidal particles is that they have negative charges on their surfaces, and consequently bind rapidly and spontaneously to proteins at a pH close to, or slightly above, the isoelectric point (p*I*) of the protein. This irreversible electrostatic bond with protein stabilizes the sol and prevents precipitation of the gold particles in the presence of electrolytes. Because proteins (such as lectins, immunoglobulins, enzymes, staphylococcal protein A, and so on) can be stably coupled to colloidal gold without significant loss of their biological properties, gold–protein complexes form excellent probes for the ultrastructural identification of viral antigens or sites of biological activity.

Herpes virions contain multiple structural proteins and much still has to be learned about their physical arrangement in the virion at various stages of morphogenesis, as well as about their biological functions in vivo. The use of immunogold labeling and negative stain electron microscopy makes it possible not only to identify specific proteins within individual virus particles at an ultrastructural level but, moreover, to observe the interactions and affinities of these proteins when they are in a natural conformation. Gold-tagged monoclonal antibodies have been used to identify envelope glycoproteins on herpes simplex virions *(3)* and on human cytomegalovi-

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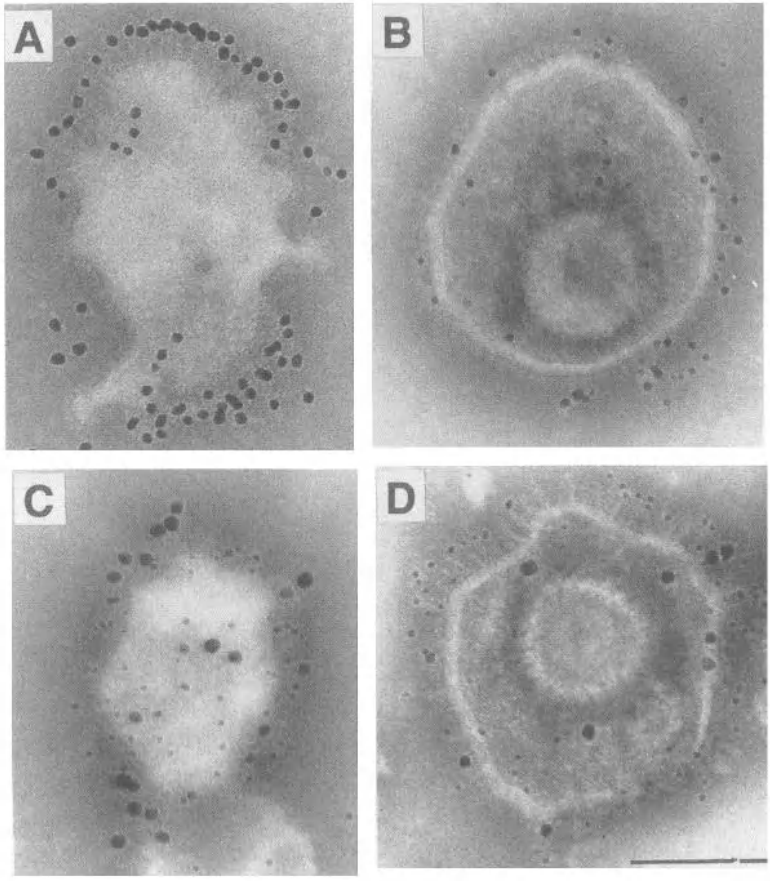

Fig. 1. By direct immunogold labeling of herpes simplex virions with specific probes for gB **(A)**, or gD **(B)** it is possible to detect morphological differences between these two types of glycoprotein spikes in the virion envelope. The gB spikes are long and relatively rigid, whereas gD spikes are shorter and more slender. Virions in **(C)** and **(D)** have been double-labeled with monoclonal antibodies to gB and gD on different sizes of gold particles. In C, anti-gB is coupled to the larger gold particles, and in D the anti-gB is attached to the smaller gold particles. Note how the smaller sized probes allow better visualization of individual glycoprotein spikes. The envelopes of the virus particles in A and C are intact, but in B and D the envelopes have been damaged sufficiently to allow penetration of the negative stain so that the nucleocapsids can be seen. Bar = 100 nm.

rus particles *(4)* (*see* Figs. 1 and 2). In addition, immunogold labeling has demonstrated that both β_2-microglobulin and the Fc portion of human IgG bind to one of the tegument proteins of human cytomegalovirus *(5,6)*.

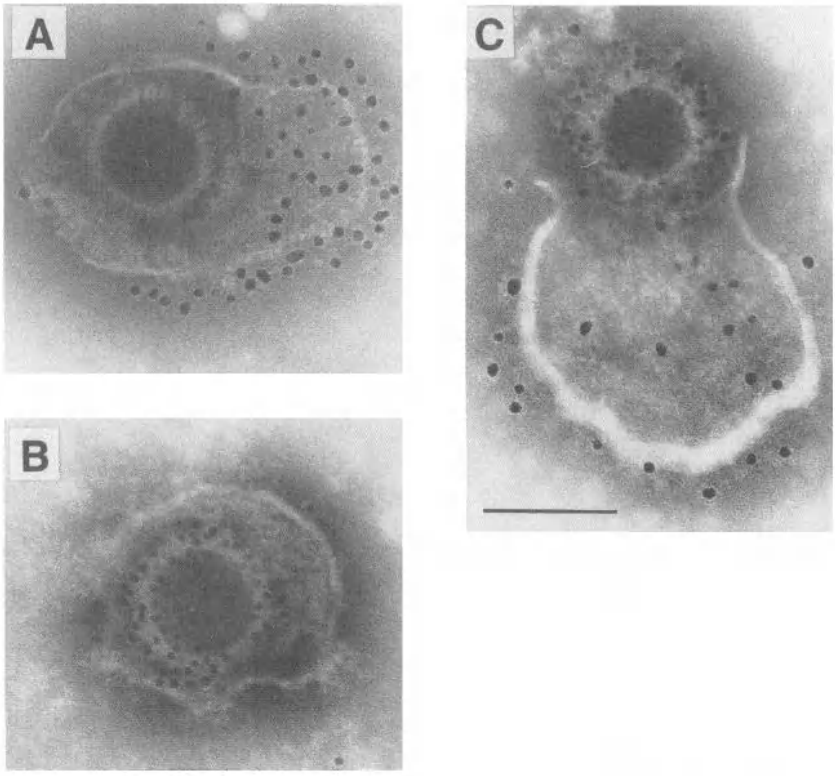

Fig. 2. Immunogold labeling of human cytomegalovirus (HCMV) particles has been used to identify the location of two different viral proteins, one on the envelope and the other in the tegument. **(A)** Gold particles coupled to a monoclonal antibody to the major envelope glycoprotein (gB) were mixed with a suspension of HCMV particles. The probes bind strongly to the virion envelope. **(B)** This virus particle was allowed to dry on the EM grid, then floated on a droplet of colloidal gold coupled to β_2-microglobulin. The virion envelope has collapsed, allowing the β_2-microglobulin to bind to its affinity protein that is situated in the tegument. **(C)** Tegument proteins of virions in suspension can be labeled only if the envelope has ruptured. In this case the virus particle has been double-labeled with anti-gB (larger gold) and β_2-microglobulin (smaller gold). Bar = 100 nm.

Indirect immunogold staining techniques have been used in the study of a variety of viruses and bacteria by making use of colloidal gold coupled to either protein A *(7,8)* or an anti-IgG *(9,10)* to tag unlabeled IgG that have bound to the virion in a primary reaction. However, by linking a specific antibody directly to the gold particles, it is possible to obtain probes that are much more sensitive and specific, and which can be used in a simple one-step labeling protocol. A further

advantage of direct labeling is that it facilitates multiple labeling. By using gold particles of different sizes, each coupled to a different monoclonal antibody (or alternative ligand), the distribution and relationship of more than one antigen can be demonstrated on a single virion (Figs. 1C,D and 2C). This technique is especially rewarding for herpesvirus particles because of their comparatively large size and because of the comprehensive range of monoclonal antibodies that is available.

The optimal parameters for the preparation and application of gold–protein complexes have been extensively studied and reported in comprehensive review articles *(11,12)*. However, this chapter describes a simple and effective protocol to produce sensitive and specific immunoprobes that can be used with minimal effort, and will allow for innovative versatility in the detailed examination of multiple facets of herpesvirus architecture.

2. Materials

2.1. Preparation of Colloidal Gold Particles

All solutions must be prepared with double-distilled or, preferably, deionized water. Filtration through a 0.22-μm pore size Millipore filter is recommended to ensure that traces of organic substances are removed from the water. It is important to ensure that all glassware, magnetic fleas, and so on are scrupulously cleaned and rinsed with distilled water. **Note:** Extraneous ions present during the preparative stage will cause the gold to flocculate.

Colloidal gold particles <15 nm in diameter are made by reduction of chloroauric acid (gold chloride) with a mixture of two reducing agents, sodium citrate, and tannic acid. The size of the particles is determined by the amount of tannic acid used in the reduction mixture; the more tannic acid, the smaller the size of the colloidal particles.

Chloroauric acid is supplied in a crystalline form and is extremely hydrophilic. Therefore, it is advisable to dissolve the entire contents of the vial as soon as it is opened and to store the gold chloride preparation as a 4% aqueous solution. If kept at 4°C in the dark, it should last for many months.

Not all sources of tannic acid are equally good for production of homogeneous gold sols. Successful results can be obtained with tannic acid supplied by Mallinckrodt Inc. USA, as code no. 8835.

To make a 50-mL colloid:

1. Solution A (1% $HAuCl_4$)· Deionized water, 39.875 mL, 4% chloroauric acid, 0.125 mL.
2. Solution B (reduction mixture): 1% trisodium citrate, 2 mL (freshly made); 1% tannic acid, 0–2.5 mL; 0 015*M* K_2CO_3, 0–2.5 mL (equivalent volume to tannic acid used), microfiltered deionized water up to 10 mL.

It is important that the Na-citrate solution for the reducing mixture (Solution B) be made fresh just before use. Choose a volume of 1% tannic acid that will yield gold

particles of the desired size. This volume may vary in the hands of different researchers, but a rough guide of quantities is as follows: 25 µL tannic acid for 10-nm gold particles; 50–100 µL tannic acid for 6–8 nm gold, 1.5 mL tannic acid for 3 nm gold.

2.2. Preparation of Specific Probes

The following solutions are required:

1. 0.2 m*M* borax buffer, pH 9.0. For convenience, make a 10X stock solution that can be stored at 4°C until needed. Dissolve 0.762 g of disodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) in 1 L of distilled water, and adjust the pH to 9.0 with 0.1*N* HCl. This stock solution must be diluted 10-fold before use.
2. 0.2*M* K_2CO_3
3. 0.2*M* H_3PO_4
4. 10% NaCl
5. 30% (100 vol) Hydrogen peroxide.
6. 10% Bovine serum albumin (BSA) in distilled water. Adjust the pH to 9.0 with NaOH, and microfilter using a 0.22-µm filter.
7. TBSA-azide: 0.02*M* Tris-HCl, pH 8.2, in 0.15*M* NaCl, containing 0.5% BSA and 0.02*M* NaN_3

2.3. Labeling of Virus Particles with Gold Probes

2.3.1. Viral Envelope Components

1. Large numbers of virus particles can be recovered from the supernatant culture fluids of infected cell monolayers that exhibit obvious cytopathic effects.
2. Washing diluent: 0.06*M* phosphate buffer, pH 7.2 (PB).
3. Negative stain for electron microscopy: 1% phosphotungstic acid, pH 6.2 (PTA).

2.3.2. Viral Capsids

Triton-X-100 lysis buffer: Prepare a solution of 1% Triton-X-100 in 10 m*M* Tris-HCl, pH 7.5, containing 1 m*M* $CaCl_2$, 0.15*M* NaCl, and 2 m*M* phenylmethylsulfonyl fluoride (PMSF), which is a protease inhibitor. Note that PMSF is not readily soluble in aqueous solutions and therefore it should first be dissolved in a small volume of ethanol such that the final concentration of ethanol in the lysis buffer is 1%.

3. Methods

3.1. Preparation of Colloidal Gold Particles

1. Decide on the size of colloidal gold particles required, and prepare the reducing solution (Solution B) accordingly (*see* Note 1).
2. Preheat both Solution A and Solution B to 60°C by placing them in a 60°C water bath.
3. Place a magnetic flea in Solution A and stir on a magnetic stirrer heated to maintain the temperature of 60°C.

4. Add Solution B to Solution A while rapidly mixing. The color of the gold mixture will change from blue to red, indicating that reduction is complete Continue stirring at 60°C for 20 min, then bring to boiling point and boil gently for approx 2 min (*see* Note 2)

After cooling, a sample may be examined in the electron microscope to monitor the size and homogeneity of the colloidal particles.

Gold sols should be stored in sterile bottles at 4°C in the dark. They can be kept for many months (even years) without deterioration

3.2. Preparation of Specific Probes

1 The protein ligand that is to be coupled to the gold colloid should be as pure as possible (*see* Note 3) You will require 200–500 µL of protein at a concentration of approx 1 mg/mL
2 Dialyze the protein sample against 0 2 m*M* borax buffer to reduce the ionic concentration of the protein solution (*see* Note 4).
3. Measure the pH of the gold colloid by applying one or two drops of the suspension to a strip of good quality pH indicator paper (Particles of colloidal gold are negatively charged and will clog the pores of the electrode of a conventional pH meter) Use 0 2*M* K_2CO_3 to adjust the pH of the gold sol to be slightly more basic than the p*I* of the protein that will be used for the probe For human or rabbit IgG, a pH of approx 9.0 is satisfactory. For monoclonal antibodies, pH 7.5 is preferable. If necessary, the pH of the gold may be lowered using 0.2*M* H_3PO_4
4 Prepare a minititration to determine the protein concentration that is needed to stabilize the gold sol. In glass tubes make doubling dilutions of protein in 10-µL amounts of 0.2 m*M* borax buffer Add 100 µL of gold (at appropriate pH) to each tube, vortex, let stand for 2 min, then add 10 µL of 10% NaCl and vortex again. Salt will cause unstabilized gold to flocculate, which results in a color change from red to blue When colloids have been prepared using substantial amounts of tannic acid, approx 10 µL of 30% hydrogen peroxide also should be added together with the NaCl This will serve to bleach the brownish color caused by polymers of tannic acid, which might otherwise obscure the titration end point The *minimal stabilizing concentration* of protein is indicated by the highest dilution at which the gold remains red. Protein should be used at this concentration (or if desired, increase by about 10%) for the preparation of gold probes (*see* Note 5).
5 To prepare the probe, add 1 vol of protein (appropriately diluted in borax buffer) to 10 vol of colloidal gold at the required pH, vortex, and allow to stand for 2 min Test a small aliquot of the protein–gold mixture to ensure that the gold has been stabilized and remains red after the addition of 10% NaCl Then add 10% BSA solution to give a final concentration of 1% BSA. The BSA acts as an extra stabilizer.
6 Centrifuge the gold–protein complex at 48,000g (20,000 rpm in a Beckman SW50 1 rotor) for 1 h, and resuspend the sedimented gold particles in 5–10 mL TBSA-azide Very small gold particles (3 nm) will require a longer time of centrifugation, approx 2 h, to sediment all the gold. Particles of 10 nm or more will

be sedimented for 45 min at 27,000*g*. Colloidal gold centrifuges to a soft pool rather than a pellet Any underlying hard pellet represents aggregated gold and therefore should be discarded

7. The centrifugation step should be repeated to wash away free unbound protein that might compete with the probe during labeling experiments, and also any remaining tannic acid that might have an adverse effect on the proteins bound to the gold particles (*see* Note 6)
8. After washing, suspend the gold–protein complex in a small volume of TBSA-azide—about one-tenth of the initial volume of gold used in step 4 Store probes in tightly sealed containers at 4°C, and do not expose them to light unduly

3.3. Labeling of Virus Particles for Electron Microscopy

3.3 1. Labeling of Viral Envelope Components

1. Concentrate the virions by centrifugation and resuspend the pellet in approx 250 µL of PB.
2. Add 20–30 µL of the gold probe, mix well (color of the mixture should be very pale pink) and incubate for 2 h at 37°C, or longer at room temperature (*see* Note 7) All immunogold tests should include appropriate controls (*see* Note 8)
3. Increase the volume of the sample to 5 mL with PB, mix by inverting the tube three times, then centrifuge for 20 min at 27,000*g* in a Beckman SW50 1 rotor
4. Decant the supernatant fluid and leave the tube inverted on some absorbent paper in a beaker to allow all the PB to drain from the pellet
5. Use distilled water to suspend the pellet and place a small drop of virus suspension onto a formvar-coated electron microscope grid. Remove excess fluid and immediately add a drop of 1% PTA

   After removal of excess stain, the grid is ready for examination by electron microscopy (EM) (*see* Note 9)

3.3.2. Labeling of Subenvelope Components

To allow gold probes to bind to subenvelope components of intact enveloped virions, the viral envelope must first be ruptured or removed entirely.

3 3.2.1. Viral Capsids

Detergents such as Triton-X-100 will cause dissociation of the membranous viral envelope and allow the release of viral capsids.

1. Concentrate the virions by centrifugation.
2. Resuspend the virus pellet in 750 µL of Triton-X-100 lysis buffer, vortex, leave at 4°C for 1 h, then vortex again.
3. Centrifuge for 5 min in a microfuge.
4. Decant the supernatant lysis buffer, and resuspend the pellet in approx 250 µL of PB

5 Follow steps 2–5 as described for the labeling of envelope components in Section 3.3 1

3.3.2.2 Tegument Proteins

Treatment of virus particles with Triton-X-100 will remove not only the virion envelopes but the tegument proteins as well. To achieve labeling of the tegument proteins (Fig. 2B), the envelope can be ruptured by nonchemical means to allow the gold probes to penetrate·

1 First concentrate the intact virions by centrifugation
2 Suspend the pellet in distilled water and place one drop of virus suspension on a formvar-coated electron microscope grid Remove excess fluid and allow the grid to dry completely (*see* Note 10)
3 Place a drop of dilute gold probe onto a piece of dental wax in a humidified Petri dish. Float the grid face down on the gold and incubate at 37°C for 2 h (*see* Note 11)
4 Rinse the grid gently with distilled water and stain with 1% PTA, then examine by transmission electron microscopy (*see* Note 12)

4. Notes

1 Large-sized probes bind at lower frequency than those prepared with smaller gold particles, probably as a result of steric hindrance It is a useful practice to prepare gold particles of different sizes so that labeling with a specific ligand can be tested using probes of a variety of sizes When performing double-labeling experiments with two probes of different sizes, it is informative to repeat the test and reverse the sizes of the gold that is coupled to each ligand (*see* Fig 1)
2. When preparing gold sols, the beakers or flasks in which Solutions A and B are contained should be of a size that will allow rapid mixing of both solutions Keep these containers covered during the heating process to avoid loss of moisture by evaporation that could cause changes in concentration of the chemicals
3. Monoclonal antibodies (MAbs) have distinct advantages over polyclonal antibodies (PAbs) for use in direct immunogold studies The most obvious advantage is that of specificity, since all the immunoglobulin molecules are identical The relatively low ratio of specific molecules in a PAb mixture can be improved by affinity purification prior to the production of probes A polyclonal preparation contains antibodies with a wide range of isoelectric points, and calculation of the optimal pH for coupling to gold is difficult. A pH of 9 0 generally has been found to be suitable for heterogeneous immunoglobulins *(12)* MAbs have a better defined p*I* value and therefore adsorb more efficiently to the gold particles at a slightly lower pH.
4 As many proteins tend to aggregate if kept in low ionic strength solutions, it is wise to choose conditions that will allow dialysis to occur in the shortest possible time. Large volumes of dialysis buffer and constant stirring will help to speed up the process Protein precipitates that form during dialysis should be removed by

centrifugation of the protein sample before it is coupled to the gold. This precipitated protein will usually redissolve in buffers of higher molarity and can be used without serious loss of potency in other tests

5. Flocculation of small size colloidal particles is sometimes slow Allow 10–30 min after the addition of 10% NaCl before calculating the titration endpoint When preparing probes, the amount of protein that is added should not greatly exceed the minimal stabilizing concentration An excess of free ligand in the final preparation will compete with the ligand attached to the gold and reduce the binding efficiency of the probes.
6 Washing by centrifugation is improved by using swinging bucket-type rotors, such as the Beckman SW50.1 The increased distance of centrifugation over fixed angle rotors allows better separation of large and small particles. Gold probes will pellet more rapidly from suspending fluids containing 0.5% BSA than from 1% BSA. If some of the gold remains in suspension after 45 min at 27,000*g*, remove the supernatant fluid to a new tube and repeat the centrifugation of that fraction. Increasing the time of centrifugation will result in increased amounts of the unbound ligand in the pellet and thus decrease the efficiency of washing
7. Optimal labeling occurs in the presence of excess probe This assures that the maximum number of binding sites are labeled and decreases the chance of crosslinking the virus particles into immune complexes. On the other hand, if too much gold is added to the reaction mixture, it will be difficult to separate the labeled virions from the unbound gold that will hamper the detection of specific binding in the final EM preparation If the volume of the reaction mixture is kept small (less than 300 µL), the total amount of probe that needs to be added can be kept to a minimum. After incubation, labeled virions can be separated from the unbound gold by first diluting the sample 15- to 20-fold and then applying differential centrifugation to pellet the virus particles while leaving most of the free gold in suspension If the amount of background gold is unacceptably high in the first pellet, resuspend the pellet in at least 5 mL of PB, mix by inverting the tube three times, and then repeat the differential centrifugation step.
8. When using whole IgG molecules on probes, the chance of nonspecific Fc-binding is always a problem and should be monitored by including appropriate control experiments with gold probes prepared with an unrelated antibody from the same animal species Preincubation of the virus sample with nonimmune immunoglobulin before exposure to the specific probe may help to eliminate nonspecific Fc-binding. Alternatively, the antibody can be digested with papain and the $F(ab)'_2$ portion isolated for coupling to the gold, although this process is somewhat laborious. Most monoclonal antibodies do not give anomalous Fc-binding, although murine IgG of the subclass IgG1 has been observed to react nonspecifically with cytomegalovirus structural proteins (D. H. Hardie, personal communication) Therefore, it is recommended that tests include control probes comprising MAbs of the same subclass of IgG as those under investigation.
9. Good resolution of fine ultrastructural components of the virion depends to a large extent on successful negative staining The stain should not be too dense

nor too concentrated at the periphery of the virus particles otherwise both structural detail and small gold probes will be obscured Best staining is achieved when a small amount of low-molecular-weight protein is present in the sample This will aid the "spreading" of the stain on the EM grid and create a light grey background that is optimal for visualization of the labeled viral components. When using virus samples concentrated from cell culture fluids, sufficient low molecular weight protein will be present in the pellet When investigating gradient-purified virus samples, however, it may be necessary to apply a coating of 0.01% BSA to the EM grid before adding the stain.

10. When virions in a hypotonic solution are allowed to dry on a grid, the viral envelopes usually rupture and roll back This is a quick and efficient means of exposing subenvelope components which can then be labeled *in situ* (i.e., on the grid) as described Note, however, that this technique does not allow accurate detection of antigens on the surface of the envelope
11. Gold probes should be diluted in TBSA-azide. The BSA content of the diluent will minimize unwanted background staining It is important that the drop of gold–protein complex does not dry out during incubation, as this will result in unacceptable background staining Ensure that the drop of diluted probe is not too small, and that the Petri dish is adequately humidified by placing a thick layer of water-saturated filter paper on the base of the dish
12. Take care not to damage the support film when rinsing the grid Hold the grid carefully with fine-tipped forceps, place a piece of torn filter paper between the tines of the forceps so that it just touches the edge of the grid, and repeatedly apply small drops of water to the opposite edge of the grid Apply the negative stain in the same way

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12

Expression of HSV Proteins in Bacteria

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1. Introduction

Expression of herpes simplex virus (HSV) polypeptides in bacterial expression systems has provided a useful tool for the generation of large quantities of specific viral proteins for use in both biochemical and functional analysis, and as immunogens for antisera production. Proteins can be expressed either in the full-length native form or as fusion proteins with affinity tails.

The pET system *(1,2)* is probably the most widely used for production of native full-length herpes simplex virus proteins, and has been used successfully to generate large quantities of both the large and small subunits of ribonucleotide reductase for functional analysis *(3,4)* In the pET system, the protein-coding sequence of interest is cloned downstream of the T7 promoter and gene 10 leader sequences. Protein expression is generally induced by the addition of IPTG to the growth media. The bacteria are lysed and the expressed protein purified using conventional chromatography techniques.

Several systems for expression of proteins as fusions are available. These are all based on a general theme where the protein of interest is expressed as a fusion with a peptide that can be purified using an affinity matrix. Generally, a specific cleavage site is available to permit release of the expressed protein from its fusion partner. In herpesvirology, one of the most commonly used is the GST system, in which proteins are expressed as fusions with the glutathione-*S*-transferase from Schistosoma japonicum *(5)*. These fusion proteins can be easily purified using glutathione Sepharose 4B, and then eluted under mild conditions using glutathione. The GST portion can then be cleaved from the fusion protein using site-specific proteases.

The Xpress™ Expression and Purification System (Invitrogen, San Diego, CA) produces recombinant proteins that are fused to a short leader peptide,

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which is derived from the bacteriophage T7 gene *10* and contains six histidine residues (the polyhistidine sequence). These histidine residues have a high affinity for divalent cations and bind readily to a nickel-chelating resin. Recombinant proteins can be eluted from the resin using either low pH buffer or by competition with imidazole or histidine. Protein A vectors are also available, these use protein A as the fusion partner and IgG Sepharose 6FF for affinity purification. Recently, PinPoint™ vectors have been introduced by Promega (Madison, WI). In this case, the DNA coding for the protein of interest is cloned downstream of a sequence encoding a peptide that is biotinylated in vivo. The expressed protein binds to monomeric avidin resin and can be eluted under mild denaturing conditions.

Other available vectors are specifically designed for the expression of soluble proteins. In one such system, proteins are expressed as fusions with the *Escherichia coli* thioredoxin protein, which remains soluble even when expressed at levels as high as 40% of the total cellular protein. The fusion protein accumulates at sites in *E coli* called adhesion zones, and is selectively released from the bacterium by either osmotic shock or heat treatment. An enterokinase cleavage signal is present between the thioredoxin gene and the cloned polypeptide to allow release of the protein of interest following purification.

The protocols described in this section provide a general guide to the methods used for assessing levels of expression and protein solubility, and are applicable to almost any of the commercially available protein expression systems. Protocols for purification of GST fusion proteins are also given in this chapter. However, for other specific affinity purification systems, readers should refer to the manufacturer's instructions.

2. Materials

2.1. Expression Plasmids and Bacteria

A wide range of expression plasmids are commercially available, and the application for which the expressed protein is to be used will determine which is most suitable. pET vectors that permit expression of the full-length native protein are really only necessary if the expressed protein is to be used for functional studies, since they have the disadvantage that the recombinant protein must be purified free from contaminating bacterial proteins, which requires a sound knowledge of protein purification techniques. For many other applications, such as antibody production, fusion proteins are more suitable, since they are more easily purified to homogeniety.

Manufacturer's generally supply bacteria with their expression plasmids. For pET vectors, the strain of choice is BL21(DE3), which has a deletion in both the *ompT* and *lon* protease, thus reducing proteolysis of expressed fusion proteins. In this strain, the T7 RNA polymerase is encoded on a lysogenic λ bacteriophage

under *lacUV5* control. Induction of the polymerase with IPTG allows controlled expression of genes placed downstream of the T7 RNA polymerase binding site.

pGEX vectors have no specific host requirements for propagation of the plasmids or for expression of fusion proteins, but it is best to use strains that are protease deficient, e.g., Y1090.

2.2. Bacterial Growth Media

1. LB Agar: In 900 mL dH_2O, dissolve 10 g NaCl, 10 g tryptone, 5 g yeast extract, and 20 g bacteriological agar, pH to 7 0, with 5*N* NaOH, and make volume up to 1 L Aliquot and autoclave
2. LB. In 900 mL dH_2O, dissolve 10 g NaCl, 10 g tryptone, 5 g yeast extract, pH to 7.0, with 5*N* NaOH, and make volume up to 1 L. Aliquot and autoclave.

2.3. Chemicals and Reagents

1. Lysozyme (Sigma, St. Louis, MO) to a final concentration of 500 µg/mL
2. 100 mg/mL Ampicillin (Sigma). filter-sterilized
3. 100 m*M* IPTG (Sigma). filter-sterilized.
4. 20% Triton X-100 in PBS
5. 1X phosphate-buffered saline (PBS). 140 m*M* NaCl, 2 7 m*M* KCl, 10 m*M* Na_2HPO_4, 1 8 m*M* KH_2PO_4 (pH 7.4)
6. 6X SDS loading buffer· 0 35*M* Tris-HCl (pH 6 8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 5% β-mercaptoethanol, 0.012% (w/v) bromophenol blue

2.4. Miscellaneous Equipment

Orbital shaker with variable temperature control, microfuge, benchtop centrifuge (e.g., Fison's coolspin), high-speed centrifuge with SLA-3000 rotor and bottles (or equivalent), 50-mL disposable plastic tubes, apparatus for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reagents for fixing gels, and staining with Coomassie blue.

3. Methods

3.1. Generation of an Expression Vector Containing the DNA Sequences of Interest

A fragment of DNA encoding the chosen polypeptide can be obtained either by purification of a restriction enzyme fragment from an agarose gel or by PCR. Generation of fragments by PCR for subcloning into expression vectors has the advantage that the primers can be designed to incorporate specific restriction enzyme recognition sites, so that the gene of interest can be cloned directionally into available restriction sites in the vector. In the case of fusion proteins especially, the recombinant plasmid should be sequenced across the junction between the fusion partner and the gene of interest to ensure that the protein has been cloned in the correct reading frame. This is particularly import-

ant when the expressed protein is not the expected size as predicted from its amino acid sequence.

The recombinant plasmid is used to transform an appropriate *E coli* strain. A glycerol stock can be prepared by picking a transformed colony into 5 mL 2X YT containing 250 μg/mL ampicillin and growing overnight with shaking at 37°C. An equal volume of glycerol is added before aliquoting into 1 mL amounts and storing at –70°C.

3.2. Small-Scale Production of Fusion Proteins

Small cultures of bacteria containing the fusion protein of interest can be used to determine the optimal factors for expression of soluble fusion proteins in terms of length of induction period and temperature of induction. As a control, bacteria transformed with the parental plasmid vector containing no insert should be used.

Day 1: Scrape a small amount of bacteria from your glycerol stock into 1 mL LB. Plate out 100 μL onto an LB agar plate. Incubate in an inverted position at 37°C overnight

Day 2:

1. Pick a transformed bacterial colony from the agar plate into 5 mL LB (170 m*M* NaCl, 10 g/L Difco bactotryptone, 5 g/L yeast extract) containing 100 μg/mL ampicillin
2. Grow at 37°C for 3–6 h with shaking until an A_{600} of (0.6–0 8) is reached
3. Induce fusion protein expression by the addition of 50 μL of 100 m*M* IPTG
4. Remove 500-μL samples at hourly intervals, and pellet the bacteria in a microfuge by spinning at 6500*g* for 5 min.
5. Resuspend in 250 μL of 1X SDS gel loading buffer and boil.
6. Analyze 15-μL (minigel), or 50-μL (maxigel) aliquots by SDS-PAGE.

Gels can be fixed and stained with Coomassie blue. It should then be possible to visually determine the length of induction time required for optimal protein expression. Similar experiments can be carried out to examine the effect of variations in the concentration of IPTG used for induction on protein expression, since lower or higher concentrations than those given above may be optimal for expression of some proteins. Having assessed the optimal induction times and IPTG concentrations, production can now be scaled up to determine the solubility of the expressed protein.

3.3. Determining the Solubility of the Expressed Protein

To determine the solubility of the expressed protein·

1. Inoculate several 5-mL cultures with a single transformed bacterial colony as described in Section 3.2 , and induce with IPTG for the optimal determined time.
2. Pool the 5-mL cultures, and centrifuge at 2000*g* for 10 min in a Fison's coolspin at 4°C
3. Resuspend the pellet in 5 mL PBS containing 500 μg/mL lysozyme, and incubate at room temperature for 10 min. Fully lyse the bacteria on ice using a probe sonicator

Note: The tip of the soniprobe should never be completely immersed into the tube. Instead, it should be placed just below the surface of the solution. Care should be taken not to allow frothing of the solution as this is indicative of oversonication, which may lead to aggregation or denaturation of the proteins.

4. Spin at 2000*g* for 10 min at 4°C in a Fison's coolspin to pellet the bacterial debris Pour off the supernatant and retain. Resuspend the bacterial pellet in 5 mL PBS Fifty-microliter aliquots of bacterial pellet and supernatant fractions can be added to 10 µL 6X SDS loading buffer and analyzed by SDS-PAGE. If the recombinant protein is soluble, it should be present primarily in the supernatant fraction; if it is insoluble, it will be present in the fraction that contains the bacterial debris.

3.4. Large-Scale Production of Crude Bacterial Lysates.

After carrying out small pilot experiments to establish optimal conditions for expression, a large-scale purification can be performed. Small samples should be retained at each step in the procedure for analysis of the purification method.

1 Inoculate a transformed bacterial colony into 100 mL of LB containing 100 µg/mL ampicillin, and incubate with shaking at 37°C overnight.
2. Dilute this culture in 900 mL LB + ampicillin, and split between two 2 or 3 L flasks. Grow at 37°C for 3 h.
3. Add IPTG to induce fusion protein expression, and continue incubation for the time required to obtain maximal expression.
4 Pellet bacteria by spinning at 5000*g* for 10 min in an SLA-3000 rotor, and resuspend in ~50 mL of PBS containing 500 µg/mL lysozyme Split sample evenly between two 50-mL Oakridge tubes. Incubate at room temperature for 10–15 min.
5 Place the bacteria in a container on ice, and sonicate using five or six 15-s bursts to lyse the cells completely. Following sonication, a detergent solution, e.g., 500 µL Tween-20, could be added to the lysate to aid solubilization of the fusion protein.
6. Centrifuge at 10,000*g* for 15 min in an SS34 rotor to remove bacterial debris.
7 Collect the supernatant, which is the crude bacterial lysate, for further purification.

The next stage in purification will be dependent on the type of expression vector used. For purification of native full-length recombinant proteins, ammonium sulfate purification is commonly used.

3.5. Ammonium Sulfate Fractionation of Expressed Proteins

Ammonium sulfate fractionation can be utilized to purify expressed proteins partially from bacterial extracts. This involves adding increasing amounts of a saturated solution (4*M*) of ammonium sulfate to crude bacterial extracts and determining by SDS-PAGE the optimal concentration for precipitation of the expressed protein.

1. Add increasing amounts of ammonium sulfate to 100-µL aliquots of crude bacterial extracts in Eppendorf tubes, and incubate for 30 min on ice. Table 1 shows the volumes of ammonium sulfate that are required to give final concentrations

Table 1
Precipitation of Crude Extract
Using a Saturated Solution of Ammonium Sulfate

Volume crude extract, µL	Final % ammonium sulfate	Volume 4*M* solution added, µL
100	5	5 3
100	10	11 1
100	15	17.7
100	20	25.0
100	25	33 4
100	30	42.8
100	35	53.9
100	40	66.7
100	45	81.9
100	50	100 0

in the range of 5–50% when added to 100 µL crude extract
2. Spin samples at 13,500*g* for 30 min in a microfuge at 4°C.
3 Redissolve pellets in 300 µL PBS, and bring supernatants up to an equal volume
4 Analyze pellet and supernatant fractions by SDS-PAGE

By comparing pellet and supernatant fractions over a range of ammonium sulfate concentrations, it should be possible to determine the ammonium sulfate concentration at which the majority of the expressed protein is precipitated from solution.

For large-scale ammonium sulfate fractionation, ammonium sulfate powder is slowly added to the extract with constant stirring, at room temperature, followed by incubation on ice for at least 30 min. The protein precipitate is collected by centrifugation at 12K for 30 min in an SS34 rotor at 4°C. The pellet is then resuspended in PBS. Further purification will probably involve techniques, such as FPLC ion-exchange chromatography.

3.6. Purification of GST Fusion Proteins

GST fusion proteins are easily purified from bacterial lysates using glutathione Sepharose 4B that has been equilibrated using PBS. Glutathione Sepharose 4B is supplied from Pharmacia as a 75% slurry. It should be washed twice using 10 mL of PBS, and resuspended in 1 mL PBS for each 1.33 mL of glutathione Sepharose 4B used. This results in a 50% slurry. One milliliter of a 50% slurry is generally sufficient to purify recombinant protein from 1 L of bacteria.

1 Add supernatant from 1 L of bacterial extract to 1 mL of 50% slurry of glutathione Sepharose 4B beads, and mix gently in a rotating wheel at room tem-

perature for 10–15 min. Pellet the beads at 2000*g* for 2 min in a Fison's coolspin, and gently wash three or four times with 50 mL of PBS After the final spin, resuspend the beads in 1 mL PBS and transfer to an Eppendorf tube

2 Microfuge at 13,500*g* for 1 min to pellet the beads, and carefully remove the PBS using a yellow tip

3 Resuspend pellet in 1 mL of 50 m*M* Tris-HCl (pH 8 0)/5 m*M* reduced glutathione Mix on a rotating wheel for 5 min, and pellet beads as before Remove the supernatant which contains the eluted fusion protein, and store. Repeat elution step a further four or five times. Remove small aliquots at each elution step, and analyze using SDS-PAGE to ensure that the protein is totally eluted from the beads It is also advisable to run a small portion of the beads on the gel to check that no protein still remains bound While awaiting this result, the glutathione Sepharose 4B can be stored short term at 4°C in 1 mL of 50 m*M* Tris-HCl (pH 8 0)/5 m*M* reduced glutathione.

3.7. Removal of the GST Affinity Tail

In some situations, it may be desirable to remove the GST portion of the expressed protein. GST is a relatively immunogenic protein, so if the fusion protein that you have produced is to be used for antiserum production, and your protein of interest is known to be of low immunogenicity, it is better to remove the GST portion of the expressed protein if this is feasible

All pGEX vectors contain thrombin or factor Xa protease recognition sites, and cleavage occurs most efficiently when the fusion protein is bound to the glutathione Sepharose 4B:

1. Roughly determine the amount of protein bound to the beads using SDS-PAGE.
2. Wash the beads twice with 20 mL of 1% Triton X-100 in PBS, once with 50 m*M* Tris-HCl, pH 7.5/150 m*M* NaCl and once with either thrombin cleavage buffer (50 m*M* Tris-HCl, pH 7 5, 150 m*M* NaCl, 1 m*M* $CaCl_2$) or factor Xa cleavage buffer (50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 2.5 m*M* $CaCl_2$).
3. Resuspend beads in ~1 mL of the appropriate cleavage buffer containing the appropriate concentration of either thrombin or factor Xa (refer to manufacturer's instructions), and incubate at room temperature for 1 h on a rotating wheel.
4. Pellet beads and collect supernatant by spinning at 13,500*g* for 1 min in a microfuge. Repeat wash steps three or four times and analyze each fraction using SDS-PAGE

3.8. Commonly Encountered Problems

Fusion protein does not elute from glutathione Sepharose 4B, or elution is inefficient: It may be necessary to increase the concentration of reduced glutathione used. Up to 15 m*M* glutathione can be used without any effect on the pH of the buffer. Alternatively, overnight elution may be required.

Expression levels are low. This problem is often encountered with herpes simplex virus proteins, and is probably owing to the difference in codon usage

between the host bacteria and herpes simplex, which has a high G-C content. In this case, it is useful to vary the length of induction and concentration of IPTG used. Delaying the time of induction from 3 h to 5–6 h will give a denser culture of bacteria to begin with and possibly a higher yield of expressed protein. Alternatively, if high concentrations of purified protein are required, it may be necessary to shorten the length of the polypeptide that is being expressed or even use a eukaryotic expression system.

Expressed protein is insoluble: There are several ways in which the solubility of proteins can be improved, the most simple of which is to reduce the induction temperature. We have found on several occasions that proteins that are insoluble when induced at 37°C are more soluble at lower temperatures, e.g., 30 or 28°C. The addition of detergents, e.g., 1% Triton X-100, 1% Tween-20, 10 m*M* DTT, and 0.01% CHAPS, may all also aid solubilization. Alternatively, it may be necessary to express a smaller portion of the polypeptide to exclude regions that are highly charged or hydrophobic.

Bacterial proteins copurify with the expressed protein: This may be owing to oversonication of the fusion protein, leading to aggregation, and can be prevented by reducing the sonication time and frequency. If the copurifying proteins are of a lower molecular weight than the expressed protein, they may be breakdown products. These can be minimized by decreasing the length of the induction period and adding protease inhibitors to the medium used for harvesting the crude bacterial extracts.

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In Vitro Systems to Analyze HSV Transcript Processing

Anne Phelan and J. Barklie Clements

1. Introduction

During lytic virus replication, herpes simplex virus (HSV) exhibits a closely regulated pattern of viral gene expression and of DNA replication, resulting in virion production *(1)*. Broadly, HSV genes can be divided into immediate early, early, and late categories based on the kinetics of their expression. The five immediate early genes are expressed in the absence of prior viral protein synthesis although their expression is stimulated by a viral tegument protein. Two immediate early proteins are essential for virus replication in vitro and act at the transcriptional (IE175) and posttranscriptional (IE63) levels to regulate early and late gene expression. Throughout infection, mRNA is synthesized using cellular RNA polymerase II, which is modified by the action of an immediate early protein *(2)*.

Mature mRNAs are formed in nuclei by extensive posttranscriptional processing of their primary transcripts. Processing events include splicing, for intron-containing RNAs, and formation of the 5'-cap structure and the mature 3'-end. Only five HSV genes contain introns *(3)*, three of which are expressed from immediate early times, whereas all but one (a latency-associated transcript) are polyadenylated. Both polyadenylation and splicing, require a complex coordination of *trans*-acting protein–protein and protein–RNA interactions and, for these posttranscriptional processes, the virus is reliant on host cell functions of transcription. During HSV-1 infection of permissive cells, viral gene expression and polypeptide synthesis occur against a background of declining host macromolecular synthesis and inhibition of host DNA synthesis: the tegument of the infecting virion contains the product of the UL41 gene, the virion host shutoff (VHS) function, which degrades RNA molecules *(4)*.

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HSV-1 infection has wide-ranging effects on posttranscriptional processes. Nuclear extracts from HSV-1-infected cells demonstrate an increase in the efficiency of usage of certain poly (A) sites, predominantly sites that are inherently weak, and the increase appears to be owing to an enhancement in the binding of polyadenylation factors to their substrate RNAs This increase in polyadenylation efficiencies is reliant on the IE63 protein, but its precise mode of action is unclear. HSV infection also causes an inhibition of RNA splicing, both in vitro and in vivo *(5,6)*, which required IE63. This inhibitory effect is thought to be caused by an effect whereby the splicing snRNPs are redistributed from a general diffuse speckled pattern to that of a distinctly punctate pattern within the infected cell nucleus: IE63 is necessary and sufficient for this effect *(7,8)* In this way, the snRNPs are removed from the active sites of transcription and splicing, and a consequent inhibition of splicing would have a severe effect on expression of the extensively spliced host genome while having relatively little effect on processing of viral mRNAs.

The establishment of in vitro systems that faithfully reproduce the events of cleavage, polyadenylation, and splicing in crude cell extracts using RNAs that resemble authentic in vivo pre-mRNAs was a huge step forward on the road to deciphering the mechanism and components required for pre-mRNA processing. These in vitro systems facilitate the analysis of effects of virus gene products on these processes.

2. Materials

2.1. Cells and Media

Mammalian cell nuclear extracts permissive for HSV-1 infection and active for posttranscriptional processes, such as polyadenylation and splicing, are normally generated from HeLa cells.

HeLa cells are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum, 2 m*M* L-glutamine, 1000 µg/mL penicillin, and 100 µg/mL streptomycin. HeLa cells are grown at 37°C in an atmosphere containing 5% (v/v) carbon dioxide. Cell monolayers in plastic dishes (approx 2.5×10^7 cells/140-mm plate) are seeded the day before required. Following infection with HSV1, the cells are harvested as described later.

2.2. Other Reagents

1 Nuclear extraction buffer A· 10 m*M* HEPES, pH 8.0, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, m*M* dithiothreitol (DTT).
2. Nuclear extraction buffer C: 20 m*M* HEPES, pH 8.0, 1.5 m*M* $MgCl_2$, 25% (v/v) glycerol, 420 m*M* NaCl, 0.2 m*M* EDTA, pH 8 0, 1 m*M* DTT, 0.5 m*M* phenyl methyl sulfonyl fluoride (PMSF)
3 Phosphate-buffered saline (PBS)

4 Linearized in vitro transcription DNA template with Sp6, T7, or T3 RNA polymerase promoter
5. In vitro RNA transcription stock reagents. bovine serum albumin (type V) 4 mg/mL, 0.1*M* DTT, 10 m*M* CAP (G[5']ppp[5']GOH), RNasin (30,000 μ/mL), 1 m*M* each of rATP, rGTP, rCTP, and rUTP, 5X Sp6/T7/T3 transcription reaction buffer (Promega), Sp6/T7 RNA polymerase (15,000 μ/mL), DNase (1000 μ/mL)
6. Radio-isotope [α^{32}P] rUTP (800 Ci/mmol)
7. Water saturated phenol chloroform.
8. In vitro polyadenylation stock solutions 25 m*M* cordycepin, 50 m*M* creatine phosphate, 10 m*M* Tris-HCl, pH 7 6, 25% PEG, proteinase K 20 mg/mL, tRNA 20 mg/mL
9 2X proteinase K buffer. 100 m*M* Tris-Hcl, pH 8.0, 20 m*M* EDTA, 20 m*M* NaCl, 0 4% SDS.
10 Sequencing gel loading buffer· 98% deionized formamide, 10 m*M* EDTA, pH 8 0, 0.25 mg/mL XCFF, 0 25 mg/mL bromophenol blue.
11. In vitro RNA splicing stock solutions: 20 m*M* $MgCl_2$, 15 m*M* ATP/50 m*M* creatine phosphate, RNasin (30,000 μ/mL), 100 m*M* DTT
12. Splicing reaction stop solution: 6% SDS, 250 m*M* EDTA, pH 8 0, 250 m*M* Tris-HCl, pH 8 0
13. 6% Sequencing gel mix: 6% *bis*.acrylamide (1:30), 7*M* urea, 1X TBE gel buffer
14. Binding buffer. 60 m*M* KCl, 20 m*M* HEPES, pH 8.0, 1 m*M* $MgCl_2$, 10% glycerol
15 RNase A 10 mg/mL stock.

3. Methods

3.1. Preparation of HeLa Cell Nuclear Extracts Efficient in Pre-mRNA Processing

In order to generate an active nuclear extract which will function in posttranscriptional processing assays, it is essential that extracts contain all the necessary processing components required for both polyadenylation and splicing. These factors include the cellular splicing snRNPs and snRNP-associated proteins, polyadenylation factors CstF and CPSF, poly (A) polymerase, cleavage factors, and so forth. Nuclear extracts active for these processes are routinely prepared from HeLa cells. Extracts from other cell types, such as baby hamster kidney (BHK) cells, have only been shown to have limited activity in the in vitro assays described here.

This procedure is a modified version of the Dignarn method as described by Lee and Green *(9)*.

A minimum of three 140-mm dishes of 70–80% confluent HeLa cells are required to make a useful amount of active extract. Between 5 and 10 plates are routinely used. If HSV-1-infected cell extracts are to be made, the infections (MOI 10 PFU/cell) are allowed to proceed for 8–16 h before the cells are harvested. If any longer, the activity of the extracts will be severely reduced.

1. Harvest the cells by removing the medium and rinsing the monolayer with ice-cold complete PBS. Scrape the cells off the plastic into a final volume of approx 50 mL PBS, and place on ice immediately.

2 Pellet the cells by spinning at ~2000 rpm for 5 min at 4°C
3. Wash the cells gently, but thoroughly with ~30 packed cell volumes (PCV) of ice-cold PBS, using a 10-mL glass pipet.
4 Pellet cells by spinning at 2000 rpm for 5 min at 4°C
5. Resuspend the pellet in 1 PCV of buffer A, transfer to an Eppendorf tube, and allow to swell on ice for 15 min
6. Lyse cells by rapidly pushing through a narrow-gage needle:
   a. Using a 1-mL syringe and a 23- to 26-gage needle, draw up and push out buffer A to remove as much air as possible from the syringe.
   b Draw the extract slowly into the syringe, and then eject with a single rapid stroke. Six rapid strokes normally provide 80% of cell lysis, but this can be checked under a light microscope, and further strokes through the needle can be made if required.
7. Centrifuge the homogenate for 20 s (12,000*g*) in a microfuge to produce a crude nuclear pellet (room temperature).
8 Resuspend this nuclear pellet in two-thirds original PCV (established in step 5) of buffer C.
9 Incubate at 4°C for 30 min with continuous stirring.
10 Pellet nuclear debris by spinning for 5 min (12,000*g*) in a microfuge at 4°C
11. The supernatant is the nuclear extract
12 The extract is then aliquoted and stored at –70°C
13. The extract can be dialyzed into binding buffer if required.

3.2. In Vitro RNA Transcription to Generate Precursor RNA Templates for Processing

In vitro transcription allows the generation of ^{32}P-labeled precursor mRNA from a plasmid borne DNA template using either Sp6, T7, or T3 RNA polymerase The average precursor RNA should be between 400 and 800 bases in length, since longer transcripts (>1.5–2 kbp) are poorly synthesized. For the purposes of posttranscriptional 3' RNA processing assays, precursor RNAs are synthesized that contain a polyadenylation signal (AAUAAA) with surrounding GU- and U-rich sequences. This precursor can then be processed by cleavage immediately downstream of the polyadenylation signal and addition of a poly(A) tail. The poly(A) tail adds stability to the RNA and is important for its subsequent transport to the cytoplasm for translation.

To generate precursor mRNAs for in vitro splicing assays, a template is used that contains a coding gene interrupted by a noncoding intervening sequence or intron. The termini of introns are recognized by highly conserved 5' (AG . .) and 3' (GU . .) splice site recognition sequences. A complex array of splicing proteins interact with these sequences to form the spliceosome, the protein–RNA complex responsible for accurate excision of the introns and rejoining of the exon ends. These spliced mature mRNAs are then ready for translation.

To generate a precursor mRNA, linearized plasmid DNA template is required, since the precursor RNA molecules are synthesized as runoff transcripts:

1. The components required for pre-mRNA synthesis are defrosted and placed on ice. The reaction mix is prepared in an Eppendorf tube at room temperature by adding the reagents as follows:
   - 0.5 µL 4 mg/mL BSA
   - 5.0 µL 5X SP6 RNA polymerase buffer
   - 2.5 µL 0.1*M* DTT
   - 2.5 µL 10X rNTPs (10X mix is 1 m*M* each of rATP, rGTP, rCTP, and rUTP)
   - 1.25 µL 10 m*M* CAP
   - 1.0 µL RNasin (30,000 µ/mL)
   - 4.25 µL dH_2O
2. The reaction mix can be divided into two aliquots of 8.5 mL and used for the synthesis of two precursors.
3. To the 8.5 mL add
   - 40 mCi (2 µL) α^{32}P-UTP (800 Ci/mmol)
   - 1 mg (1 µL) linearized DNA
   - 15 U (1 µL) SP6 RNA polymerase (or T7/T3 polymerase as appropriate)
4. Incubate the mix at 37°C for 1 h
5. Add 2.5 U (2 5 µL) RNase-free DNase Incubate at 37°C for 10 min.
6. Add 185 µL dH_2O to bring the volume to 200 µL.
7. Perform one phenol/chloroform extraction followed by a chloroform extraction
8. Remove the supernatant to an Eppendorf tube with:
   - 2.0 µL tRNA
   - 20 µL 6*M* ammonium acetate
   - 600 µL ethanol
9. Precipitate RNA at –20°C for 4 h or overnight if more convenient.
10. Pellet RNA by spinning for 10 min in a microfuge (12,000*g*).
11. Wash pellet thoroughly with 70% ethanol.
12. Resuspend RNA to give 200–30 cps/µL

3.3. In Vitro 3' RNA Processing Reaction

Normal 3' RNA processing occurs as a two-step reaction. Initially, a precursor RNA is cleaved 10–30 bases downstream of its canonical polyadenylation signal (AAUAAA), and then a poly(A) tail of 200–400 adenosine residues is added by the enzyme poly (A) polymerase *(10)* The process of polyadenylation is achieved primarily by two protein complexes, cleavage stimulation factor (CstF) and cleavage and polyadenylation specificity factor (CPSF), together with other poorly defined cleavage factors *(11,12)* These proteins bind specifically to the highly conserved polyadenylation site sequences, and 3' RNA processing occurs rapidly after transcription. The poly (A) tail adds stability to

the RNA and is thought to have a role in the efficient transport of mature RNA to the cytoplasm for translation *(11)*

During this assay, rather than allowing normal polyadenylation to occur, immediately after the RNA cleavage step, an analog of ATP called cordycepin (3' dATP) is incorporated into the growing adenosine tail. This ATP analog inhibits any further extension of the poly(A) tail. The result is that any processed RNA products can be resolved as a distinct band of a known size on a gel, rather than a smear of RNAs with varying poly (A) tail lengths The precursor constructs commonly used in our group are Sau 5 *(13,14)*, a tandem construct consisting of poly(A) sites from an immediate early gene upstream of a late gene poly(A) site, together with a number of single constructs encoding representative poly(A) sites from all the temporal classes of HSV1 (unpublished data). At the end of a processing reaction, the products are resolved on a 6% sequencing gel. A precursor band is still clearly visible, since the reaction is not 100% efficient The processed RNA product is resolved as a smaller distinct band on the gel. (An example of this assay is shown in Fig 1.)

1 The nuclear extract is defrosted and kept on ice until required.
2 Incubate the nuclear extract at 30°C for 30 min. This depletes any ATP present in the extract
3. Prepare the reaction mix by adding in the following order:
   1 0 µL 25 m*M* 3' dATP (cordycepin)
   2 5 µL 50 m*M* creatine phosphate
   6.5 µL 10 m*M* Tris-Hcl, pH 7 6
   3 0 µL 25% PEG
4 To the 13 µL reaction mix, add 11 µL nuclear extract and 200–300 cps (1 µL) ^{32}P-labeled RNA precursor (*see* Section 3 2).
5 Incubate at 30°C for 2 h
6. To the reaction mix add:
   100 µL 2X proteinase K buffer
   75 µL dH_2O
   2 µL Proteinase K (20 mg/mL)
   2 µL tRNA (20 mg/mL)
7 Incubate at 30°C for 15 min
8 Phenol chloroform extract once.
9. Chloroform extract once.
10 To precipitate RNA, add 600 µL ethanol and 20 µL ammonium acetate Incubate on dry ice for 15 min.
11. Pellet the processed RNA by spinning in a microfuge (12,000*g*) for 10 min
12. Wash pellet with 70% ethanol.
13. Add 5 µL loading buffer, boil for 5 min, and place on ice.
14 Resolve the bands on a 6% urea acrylamide sequencing gel.

Fig. 1. The in vitro polyadenylation assay. Precursor RNA from the tandem HSV-1 poly (A) site construct Sau5, was processed with nuclear extracts from mock-infected (MI) and wild-type HSV-1 (INF)-infected HeLa cells. The cleaved products were resolved on a 6% acrylamide sequencing gel. "Pre" indicates the position of the initial precursor RNA. "IE" represents the cleavage product from the 5'-most immediate early poly (A) site, and "L" represents the cleavage product from the 3'-most late poly (A) site. HSV-1 infection results in increased usage of the late poly (A) site at later times postinfection.

3.4. In Vitro RNA Splicing Assay

The removal of introns to produce mature mRNA molecules ready for translation requires a very complex and accurate sequence of events. This procedure is reliant on a large number of small nuclear ribonuclear protein particles (snRNPs) and associated proteins, which interact to form the spliceosome. These riboproteins recognize and bind to the 5' and 3' RNA splice site sequences, and by protein–protein and protein–RNA interactions, bend the RNA to bring the two ends of the exon together. The RNA is then cleaved and the two exons joined via two distinct transesterification steps, which result in the formation of branched lariat RNAs as intermediates and the mature RNA product. The excised intron remains complexed with the snRNPs until it is degraded (for a review, *see* ref. *15*).

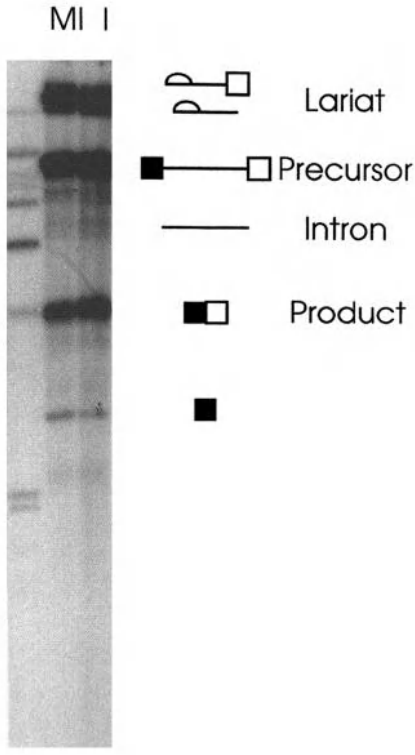

Fig. 2. The in vitro splicing assay. Precursor RNA from the Adenovirus type 2 major late transcript was processed with mock-infected (MI) and HSV-1-infected (I) HeLa cell nuclear extracts. The spliced products were resolved on a 10% sequencing gel. The lariat, precursor, intron, product, and 5'-exon forms of the initial precursor have been indicated on the figure.

This in vitro splicing assay allows the efficient splicing of ^{32}P-labeled precursor RNA molecules, resulting in the generation of spliced product, excised intron, and lariat forms of the spliced intermediate. The template used commonly in our group is that of the Adenovirus type 2 major late transcript as has been described by Moore et al. *(16,17)*. This construct contains a single intron with typical splice site recognition sequences. An example of the splicing assay and the intermediate processed forms can be seen in Fig. 2.

1. The splicing reactions are set up in Eppendorf tubes at room temperature as follows:
   11 µL Nuclear extract
   30 µL 20 m*M* $MgCl_2$
   3 µL 15 m*M* ATP/50 m*M* $CrPO_4$

2.0 µL RNasin (60 U)
1.5 µL 100 m*M* DTT
1.0 µL pre-mRNA (200–300 cps)
8.5 µL ddH_2O

2. Incubate the mix at 30°C for 2 h
3. Add 3 0 µL of splicing stop solution (*see* Section 2 2 , item 12).
4. Add 3.5 mL of proteinase K/tRNA mix:
   16 mg/mL proteinase K
   2 mg/mL tRNA
5. Incubate at 65°C for 45 min.
6. Precipitate RNA on ice with 7 vol of ethanol/ammonium acetate mix for 30 min. Mix consists of 86 mL abs. ethanol + 14 mL 4*M* NH_4OAc, pH 5.0.
7. Pellet RNA by spinning in a microfuge (12,000*g*) for 20 min.
8 Resuspend in 10 µL loading buffer
9. Heat sample to 65°C for 20 min before resolving on a 10% acrylamide sequencing gel

3.5. UV RNA–Protein X Linking

This assay allows closely associated RNA and protein molecules to be covalently crosslinked by UV light *(16,18,19)*. The complexes formed are resistant to heat, detergent, and alkali treatment, and therefore are very stable. RNA crosslinked to protein is protected against the action of RNase A RNase A treatment removes any unprotected RNA leaving the RNA–protein hybrids radiolabeled. The proteins and their interacting RNA can be resolved on a 10–12% protein gel and visualized by autoradiography.

Precursor RNAs labeled with ^{32}P-UTP (containing RNA processing motifs or not) are synthesized as described in Section 3.2. and should be resuspended in binding buffer. The nuclear extracts must be dialyzed against binding buffer to remove the high salt content of buffer C before use in the binding assay. Protein concentrations were determined by Bradford's assay *(20)* and normalized before performing the binding assay, adding 10–15 µg of protein to each reaction.

1 Into a 96-well microtiter plate, add:
   8 µL binding buffer—*see* Section 2.2.
   1 µL precursor RNA (300–400 cps)
   1 µL nuclear extract (10–15 mg protein)
2 Incubate the mix for 15–20 min at room temperature
3. UV X-link the interacting RNA and protein molecules with 250 mJ/cm^2
4. Add RNase A to a final concentration of 1 mg/mL, and incubate at 37°C for 15 min.
5. Add an equal volume of boiling mix, boil for 5 min, and resolve on a 10–12% SDS polyacrylamide protein gel.

4. Notes

1 (a) It is important that the cells are no more than 70–80% confluent if good active extracts are to be prepared. (b) Solutions A and C should be made fresh every time a new extract is prepared (c) The final protein concentration of a nuclear extract should be 15–20 mg/mL.

2 (a) Precursor RNAs should be resuspended in a minimum of 50 µL, 1 µL each of which should count at least 200 cps Poorly labeled RNA will result in poor RNA processing results (b) tRNA must be added to aid precipitation of the precursor RNA, and allow a visible pellet to be formed (c) A "smeary" precursor, which consists of incomplete transcripts can be cleaned up by a gel purification method *(17)*

3 (a) All components of the polyadenylation assay should be stored at –20°C and allowed to defrost on ice. (b) The solutions, such as cordycepin and creatine phosphate, should be stored in small aliquots to avoid repeated freeze-thawing (c) Vigorous vortexing of the samples should be avoided

4 (a) It is important only to heat the samples to 65°C prior to loading on the gel Higher temperatures can destruct the lariat forms of the incompletely spliced precursor. (b) The same principles apply to storage of the components as for the polyadenylation assay.

5. (a) The extracts should be thoroughly dialyzed prior to use in the binding assay, to remove the high salt from buffer C, though it is important that no appreciable amount of protein is lost at this stage. (b) The binding of many factors to RNA is ATP-dependent If ATP has been lost from the extract, it may be necessary to add ATP to the reaction, prior to the incubation at room temperature

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14

Analysis of HSV-1 Transcripts by RNA-PCR

Jordan G. Spivack

1. Introduction

Since the herpes simplex virus type 1 (HSV-1) genome has been sequenced and most HSV-1 RNAs are not spliced *(1)*, detailed information about the structure of many HSV-1 RNAs can be obtained without the considerable time and effort that is required to construct and analyze cDNA libraries. Once the 5' and 3' ends of an RNA have been mapped precisely, the RNA nucleotide sequence can be deduced simply from the genomic DNA sequence. However, there are certain situations, such as the analysis of spliced RNAs or of chimeric RNAs expressed from foreign genes inserted into HSV-1 vectors, where cDNA cloning of HSV-1 transcripts may be informative. There are families of transcripts that arise by alternate splicing in several human herpesviruses: HSV-1, cytomegalovirus (CMV), and Epstein-Barr virus (EBV). For example, HSV-1 encodes several overlapping latency-associated transcripts or LATs *(2–4)*. The splice junctions of the intron within the HSV-1 2.0-kb LAT have been determined by RNA-PCR with primers located on either side of the intron, followed by direct DNA sequence determination of the PCR product *(5)*. The construction of partial cDNAs by PCR saves much time-consuming effort and expense compared with the analysis of cDNA libraries. In addition, by sequencing PCR products directly, the need to analyze several cDNA clones in order to be assured of obtaining the consensus sequence is eliminated.

The protocol presented in this chapter uses total RNA as a template for PCR combined with direct DNA sequence determination of the products. As with all techniques that involve RNA, the preparation of high-quality nuclease-free RNA is of crucial importance These techniques are suitable for isolation of RNA from either tissue culture cells or mammalian tissues. The RNA-PCR and double-stranded DNA sequencing procedures have been modified specifi-

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cally for the high GC content (68%) of HSV-1 DNA. Additional PCR techniques using RNA templates can be found in vol. 15 of this series, *PCR Protocols, Current Methods and Applications (6).*

2. Materials

2.1. RNA Isolation (see Notes 1 and 2)

1. 200 mL guanidinium thiocyanate stock *(7,8)* Add the following in order.
   a 100 g Guanidinium thiocyanate
   b 4 g Sodium-*N*-lauroyl-sarcinate
   c 5 mL 1*M* NaCitrate, pH 7 0
   d 1.4 mL β-mercaptoethanol
   e 0 67 mL antifoam A (Sigma, St Louis, MO).
   f Add H_2O to 190 mL
   g. Stir and warm (35–40°C)
   h Adjust to pH 7.0 with 1*N* NaOH.
   i Add H_2O to 200 mL, filter sterilize, store in a brown glass bottle (light sensitive) at room temperature
2. 100 mL CsCl cushion.
   a. 95 98 g Biochemical grade CsCl
   b 10 mL 1*M* EDTA, pH 7 0.
   c Add DEPC H_2O to 100 mL (*see* Note 2, check pH [should be 7 0])
   d Filter sterilize, store at room temperature.
3. 5*M* NH_4 acetate, store at –20°C in tightly capped microcentrifuge tubes
4. 100% Ethanol, store at –20°C.
5. Phosphate-buffered saline, pH 7.4

2.2. Agarose Gel Electrophoresis of RNA

1. Deionized 6*M* glyoxal (*see* Note 3)
2. DEPC-treated H_2O.
3. DMSO.
4. 0.1*M* NaH_2PO_4, pH 7.0
5. RNA (5 μg/lane, 10 μg max)
6. Loading buffer. 50% glycerol, 0 01*M*, NaH_2PO_4, 0 4% bromophenol blue
7. 10 mg/mL acridine orange in H_2O. Store at 4°C in plastic tube covered with aluminum foil (light-sensitive).

2.3. RNA and DNA-PCR

1. RNA template (1–2 μg) or DNA template (1 ng)
2. DEPC-treated H_2O.
3. 100 μ*M* random hexamer (pdN_6, Pharmacia, Uppsala, Sweden)
4. dNTPs (mixture of 2 m*M* of each).
5. 10X *Taq* buffer (Promega, Madison, WI).
6. Moloney murine leukemia virus (M-MLV) reverse transcriptase (BRL, Richmond, CA)

7. RNasin (Promega).
8. PCR primers (*6,9*; *see* Note 4)
9. *Taq* polymerase (Promega)

2.4. Direct Sequence Determination of PCR Products

2.4.1. Double-Stranded DNA Sequencing Reaction

1. Sequenase Version 2 0 kit (United States Biochemical, Cleveland, OH)
2. 0.1*M* Dithiothreitol (DTT).
3. 10 mg/mL proteinase K.
4. SeaPlaque (FMC, Rockland, ME) or LMP agarose (BRL).
5. 10X TBE, pH 8.0. 1X TBE = 0.089*M* Tris-borate, 0.089*M* EDTA, pH 8.0
6. 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.
7. Phenol.
8. 100% Ethanol.
9. TE· 10 m*M* Tris pH 8.0 and 0 1 m*M* EDTA
10. [^{32}P]dATP (sequencing grade, ≈3000Ci/mmol).

2.4.2. 8% Sequencing Gel

1. 0.6 g *Bis*-acrylamide.
2. 11 4 g Acrylamide.
3. 30.68 g Urea
4. Amberlite MB-3 (Mallinckrodt).
5. Distilled H_2O.
6. 10X TBE, pH 8.0
7. Tetramethylethylenediamine (TEMED)
8. 25% Ammonium persulfate (APS). Make fresh.

2.4.3. Pouring Sequencing Gel

1. Mix the *bis*-acrylamide, acrylamide, and urea.
2. Add water to approx 130 mL total, stir until acrylamide has dissolved, add 1 teaspoon Amberlite, stir gently for 5 min, and filter through Whatman (Maidstone, UK) 1 paper Add 7 5 mL 10X TBE, bring the volume up to 150 mL, and place on ice for 5 min. Just before pouring the gel add 240 µL APS and 60 µL TEMED. Pour the gel immediately and polymerize for 2 h at room temperature before loading sequencing samples The gel can be poured the day before and stored overnight at 4°C.

3. Methods

3.1. RNA Isolation

Analysis of RNA begins with the preparation of clean, undegraded RNA. This extraction procedure, based on the procedure of Chirgwin *(3,7,8)*, should first be followed precisely, before introducing any modifications.

1. Rinse mouse tissues, or tissue culture cells, with phosphate-buffered saline (PBS) Homogenize tissue or pelleted cells (resuspend by vortexing) in guanidinium thiocyanate solution for 20 s with a Polytron (Brinkmann) or Teckmar (Cincinnati, OH) homogenizer at setting 5 The solution to tissue ratio should be 8:1 or greater Use a homogenizer probe that is designed to minimize foaming of the sample
2. Depending upon sample volume, pellet the RNA through a cushion of 5 7*M* CsCl, 0 1*M* EDTA, pH 7 0, by centrifugation at 35 K in a Beckman SW40 1 rotor (11-mL sample/2-mL cushion) or 40 K in a Beckman SW55 rotor (3 5 mL-sample/1 5-mL-cushion) for 20–24 h, at 18°C. After centrifugation, carefully aspirate the supernatant with a Pasteur pipet, invert, and dry the RNA pellet for 2–3 min (*see* Note 5) Resuspend the clear glassy pellet in 100 µL H_2O. The amount of RNA is estimated by measuring the absorbance of a 1·100 dilution of each sample at 260 nm (1 OD U ≈ 40 µg RNA) The RNA yield is ≈80–100 µg from a cell monolayer in a 75 cm^2 flask The purity of the RNA is estimated by the 260:280 nm ratio; for a clean preparation the ratio is between 1 8 and 2 0 Ratios below 1 6 indicate contamination, often with proteins (*see* Note 6) Ethanol precipitate the RNA by adding an equal volume of 5*M* NH_4 acetate (100 µL) and 2.5 vol (500 µL) of 100% ethanol and store at –20°C overnight The RNA is stable at –20°C for at least several months and for as long as 2–3 yr

3.2. Agarose Gel Electrophoresis of RNA

The quality of the RNA can be judged by agarose gel electrophoresis and acridine orange staining. Since approx 80% of mammalian cell RNA is ribosomal, prominent 28S and 18S rRNAs should be visible on acridine orange stained gels, and sometimes 5S RNA can be seen.

1 Vortex the RNA ethanol precipitate Remove the appropriate volume and centrifuge for 30 min at room temperature in a microcentrifuge. Carefully remove the supernatant using a micropipet with a disposable plastic tip.
2 Air-dry the pellet in a well-ventilated hood for 15–20 min When the pellet is dry resuspend in 3 7 µL DEPC treated H_2O Denature RNA (5–10 µg/sample) by incubation with 10 m*M* Na_2HPO_4, pH 7 0 (1 6 µL of 0 1*M* stock), 1*M* deionized glyoxal (2.7 µL of 6*M* stock), and 50% DMSO (8 µL) at 50°C for 1 h. Also denature RNA markers from BRL (either 0 16–1 77 or 0.24–9 5 kb) After cooling to room temperature add 4 µL loading buffer.
3. Electrophorese the RNA through 1 2% agarose in 10 m*M* Na_2HPO_4, pH 7 0, with constant buffer recirculation until the dye marker is 1 cm from the bottom of the gel (*see* Note 7)
4. Stain the RNA by placing the gel in a glass dish, cover with the running buffer from the gel, add 1 µL/mL of the acridine orange stock, rock gently, and incubate in the dark for 20 min *(10)* Transfer the gel to a white metal enamel baking dish, add fresh gel buffer and destain for a minimum of 1 h at room temperature or overnight at 4°C (*see* Note 8). Photograph the gel on a UV light box

3.3. RNA- and DNA-PCR

3.3.1. DNA-PCR

1 Before designing RNA-PCR primers, the ends and splice junctions of the HSV-1 transcript(s) of interest need to be at least partially mapped. PCR primers are chosen upstream and downstream of introns (*see* Note 4).
2. Optimal amplification conditions can be established empirically with 1 ng plasmid DNA template
3 To ensure uniformity between samples, and to reduce the possibility for contamination, make a master mix containing PCR buffer, the dNTP mixture, H_2O, and *Taq* polymerase, and aliquot to each tube.
4 Then, with positive displacement pipets, add the primers, and lastly the DNA template. Always include positive and negative (no DNA added) controls.
5 Perform PCR amplifications with 2 5 U of *Taq* polymerase, in 1X Promega buffer with 1 μ*M* primers, 100 μ*M* dNTPs, in a 25 μL vol overlaid with mineral oil, by 30 cycles of denaturation at 96°C for 1 min, annealing at 55–68°C for 2 min, and extension at 72°C for 3 min (*see* Note 9).
6. Resolve PCR products by agarose gel electrophoresis, stain with ethidium bromide, and visualize on a UV light box

3.3.2. RNA-PCR

1. For RNA PCR, in 18 μL combine 2 μg of total RNA, 1X PCR buffer, 1 m*M* dNTPs and 5 μg $pd(N)_6$, and heat to 80°C for 3 min, then chill on ice.
2. Add 40 U RNasin and 200 U of M-MLV reverse transcriptase, incubate the reaction for 1 h at 37°C, heat to 95°C, and chill on ice (*see* Note 10)
3 To 10 μL of the first-strand reaction add 1 μ*M* PCR primers and 2.5 U *Taq* and increase the vol to 100 μL in 1X PCR buffer
4. Amplify as for the DNA-PCR, and resolve PCR products by agarose gel electrophoresis, as described.

3.4. Direct Sequence Determination of PCR Products

3.4.1. Isolation of PCR Products for DNA Sequence Determination

The best double-stranded sequencing of PCR products is obtained with large amounts of template.

1. Ideally use 1–2 μg DNA per sequencing reaction. This usually requires running several (5–10) identical PCRs.
2 Run the PCR products on a low melting point agarose gel.
3. Use a 123-bp ladder (BRL) for DNA size markers.
4. Find the band of interest, excise the smallest gel slice possible, and transfer to a microcentrifuge tube
5. Add 400 μL 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA and melt gel at 65°C for 10 min.
6. Extract twice with 400 μL phenol, transfer the aqueous phase to a fresh microcentrifuge tube, and precipitate by addition of 40 μL 3*M* Na acetate, pH 5.0, and 1 mL of 100% ethanol.

7 Incubate in a dry ice/ethanol bath for at least 1 h and then microcentrifuge for 30 min at room temperature
8. Drain the sample well and use a disposable 20-µL pipet tip to remove excess ethanol.
9. Reprecipitate from 100 µL TE, pH 8 0, and 100 µL 5*M* NH_4 acetate.
10 Redissolve the sample in 25 µL TE (*see* Note 11).

3.4.2. DNA Sequencing Reaction

The DNA sequencing reaction is based on the USB Sequenase protocol.

3.4.2 1 Annealing Reaction

In a microcentrifuge tube combine: 2 µL 5X Rxn buffer (USB), 1 µL primer (0.5 pmol/µL), and 7 µL DNA (approx 1 µg)

Heat samples in a boiling water bath for 5 min, chill on ice for 3 min, quick spin samples, and incubate at room temperature for 5 min (*see* Note 12).

3.4.2.2. Labeling Reaction

To the annealing reaction, add in order· 1 µL DTT (0.1*M*), 1 µL Mn buffer (USB), 2 µL labeling mix (1X), 1 µL dATP^{32}P, 2 µL Sequenase Version 2.0 (*see* Note 13). Dilute 1:8 with deionized H_2O.

Incubate at room temperature for 5 min, then proceed with normal termination reactions, transferring 3.5-µL portions to each of the four termination reaction tubes (*see* Note 14).

3.4.3. DNA Sequencing Gel

Good resolution is obtained by using wedge spacers with an 8% acrylamide/ 7*M* urea gel. Heat the gel to 50°C and denature the samples at 95°C for 3 min before loading. Then load 1.5 µL of the samples onto the gel (*see* Notes 15 and 16). When gel has run so that the methylene blue is 2–3 cm from the bottom, fix the gel in 10% acetic acid/12% methanol for 1 h at room temperature, and dry the gel at a maximum of 70°C.

4. Notes

1 For RNA work use materials made of sterile disposable plastic, or glass baked overnight at 180°C Wear disposable gloves during all of these procedures to minimize contamination with RNase from hands and fingers.
2. DEPC-treat all solutions (except solutions containing Tris or guanidium thiocyanate) Add 0.1% diethylpyrocarbonate (DEPC), shake vigorously, incubate overnight at room temperature, shake again, and autoclave
3 To deionize a 40% glyoxal solution, add 40–45 mL glyoxal to 10 mL Bio-Rad AG 501-X8 resin in a 50-mL plastic disposable tube. Rock gently for 3–4 min, transfer glyoxal to a fresh tube with new resin During deionization more than

half of the volume is lost within the resin. After repeating this three times, remove 10–15 µL with a micropipet and check pH with a color**pH**ast indicator strip (EM Science/E. Merck, Rahway, NJ). Repeat with fresh resin until the pH is between 6.0–7 0. Store the deionized glyoxal in filled, tightly capped microcentrifuge tubes at –20°C Leave a small amount of room in each tube for expansion during freezing The deionized glyoxal is stable at –20°C for at least several years.

4. Designing PCR primers is an empirical art (*see* ref. *6*; Chapter 2) Often primers that are ideal on paper work poorly in practice. On the other hand, sometimes when compromises are made owing to the need for a primer in a specific location, the primers work well. For HSV-1 PCR primers we have been successful following these guidelines.
   a. Primers are 22 bases long;
   b. Approx 68% GC content (i.e., 15/22 bases) with a mix of all four bases;
   c. Lack of self-homology or complementarity, determined by visual inspection or computer analysis;
   d. Exact alignment in only one position of the HSV-1 genome,
   e Primer pairs are chosen to yield PCR products of different sizes (200–600 bp) so that the primers can be used in combination;
   f Primers span introns so that the PCR products from RNA and DNA templates can be distinguished by size.

   This last point is important because it is difficult to prepare RNA that is completely free of DNA.
5. The supernatant is removed by careful aspiration with baked Pasteur pipets. After removal of the guanidinium layer, a few seconds are allowed for the walls of the tube to drain. A fresh pipet is used to remove the CsCl layer, and after removing the viscous DNA layer, and another pause, another fresh pipet is used These precautions minimize the contamination of the RNA with DNA or proteins Before resuspending the pellet, the tubes are inverted for 2–3 min and the walls gently wiped with a Kimwipe. If this interval is too long, the glassy RNA pellets are very difficult to resuspend
6. If the 260:280 ratio is less than 1.6, sometimes the RNA can be cleaned up by a series of phenol, phenol/chloroform, and chloroform extractions, followed by ethanol precipitation However, these manipulations can lead to RNA degradation. Check by agarose gel electrophoresis and ethidium bromide staining
7. These gels can also be used for Northern blot transfer *(3,5)* For RNA smaller than 1 kb, better resolution can be achieved with 1 5% agarose
8. The acridine orange binds to the white metal enamel dish, which aids the destaining process. To clean the enamel dish use ethanol; acridine orange is soluble in ethanol
9. In our hands, Promega's *Taq* polymerase produces a higher yield of PCR products than *Taq* from other suppliers. For first strand synthesis with M-MLV reverse transcriptase, the yield with the *Taq* buffer from Promega is greater than with the reverse transcriptase buffer from BRL.
10. RNA PCR can be performed with polyA$^+$ RNA However, in my hands, satisfactory results have been obtained with total RNA. Although a PCR primer can be

used in the first step reaction to synthesize cDNA, I have obtained better results with the random hexamer (pdN_6)

11. Make sure that the sample is completely dry before resuspending in TE
12. PCR primers are also used for DNA sequence determination. To achieve high primer and template concentrations, the volume for the annealing reaction always should be 10 µL
13. At each step, it is important to gently mix all the reagents in the tube without introducing air bubbles. The Sequenase should be diluted immediately before use and this diluted mixture should not be left on ice longer than 20 min. When starting the reactions, add Sequenase to one sample, wait 2 min, and then add Sequenase to the next sample. This will allow enough time to terminate each reaction before starting the termination of the next sample
14. Aliquot 3.5 µL of sample into each of the termination reaction tubes containing prewarmed 2.5 µL of appropriate ddNTP. Incubate at 37°C for 3 min, add 4.0 µL stop solution, and immediately place on ice. Just before loading samples on the gel, incubate at 93–95°C for 3 min Chill on ice, quick spin samples, return samples to ice, and load 1 5 µL on sequencing gel.
15. For an 8% urea gel, methylene blue dye (the darker dye) runs at 20 bp and xylene dye (light blue) runs at 40 bases. Run the gel until the methylene blue is approx 1–2 cm from the bottom of the gel At this time, or up to 1 h later, the samples can be reloaded to read farther in the DNA sequence.
16. If loading the samples for a second run, reheat them at 95°C for 3 min

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15

Transient Assays for HSV Origin and Replication Protein Function

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1. Introduction

Investigations of genome replication in DNA viruses involve several facets. These include characterizing the sites at which synthesis is initiated (origins of DNA replication), identifying the viral and host proteins that participate, understanding the enzymatic activities of these proteins, and elucidating the mechanisms of DNA synthesis and maturation. For several viruses cell-free systems capable of carrying out faithful viral origin-dependent DNA synthesis have been described that have provided important insights into these areas. Unfortunately, such an assay is not yet available for HSV and other approaches therefore have been required. One of the most useful and widely employed has involved transient assays for viral origin-dependent DNA synthesis in transfected tissue culture cells. Such assays played important roles in the initial identification of the viral replication origins and the virus-coded proteins essential for DNA synthesis and more recently have helped provide detailed information on the structure and function of these elements. Similar approaches also have been exploited to study genome replication in other herpesviruses

The HSV-1 genome contains three separate origins of replication, a single copy of ori_L close to the centre of the UL segment and two identical copies of ori_S located in the repeat regions IR_S and TR_S that flank the U_S segment (*see* Note 1). A set of seven viral proteins encoded by genes UL5, UL8, UL9, UL29, UL30, UL42, and UL52 are both necessary and sufficient for viral DNA replication. The UL30 and UL42 proteins constitute the catalytic and an accessory subunit of the viral DNA polymerase, the UL29 protein is a nonsequence-specific single-stranded DNA binding protein and the UL9 protein binds to specific sequence elements within the replication origins. The UL5, UL8, and UL52

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proteins form a trimeric complex that exhibits both DNA helicase and DNA primase activities (for excellent reviews on HSV-DNA replication, *see* refs. *1–3*). Recent studies on the enzymatic activities of these proteins has been greatly facilitated by their overexpression using a variety of heterologous expression systems, most notably the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV).

The transient assays for HSV-1 DNA replication are based on earlier observations of the structure of HSV-1 defective genomes that arise on serial high multiplicity passage of virus stocks and were shown to consist of tandem duplications of small segments of the viral genome *(4,5)*. Since the tandemly repeated structure was regenerated when monomeric units produced by restriction endonuclease cleavage were transfected into permissive cells in the presence of wild-type HSV-1 DNA as helper, it was deduced that each unit must contain a functional viral replication origin and that replication probably occurred by a rolling circle mechanism *(6,7)*. It was reasoned, therefore, that if cells were transfected with a circular plasmid into which a functional HSV origin had been cloned and subsequently superinfected with wt HSV-1, the replication proteins produced by the helper virus would be able to recognize and act on the plasmid-borne HSV-1 origin, resulting in amplification of the whole plasmid. Such specific amplification then could be detected by hybridization with a probe containing the vector sequences alone *(8)*. The protocols presented in the following sections describe how this approach can be utilized to assay for the ability of viral sequences to function as replication origins, of wild-type and mutated forms of the replication proteins to participate in DNA synthesis, and of replicated molecules to be packaged into virus particles. However, because these methods rely on transfection of tissue culture cells, it should remembered that, in contrast to the cell free assays available for other viruses, they are unable to provide information on the role of host proteins in these processes.

2. Materials

2.1. Cells and Media

1 Any cell line permissive for HSV infection that can be transfected at reasonably high efficiency should be appropriate for these assays. We have extensively used baby hamster kidney (BHK) 21, clone 13 cells *(9)* grown in Eagle's medium supplemented with 10% calf serum, 10% tryptose phosphate broth, 100 U/mL penicillin, and 100 µg/mL streptomycin (ETC_{10}) at 37°C in an atmosphere containing 5% (v/v) carbon dioxide. Cell monolayers in plastic Petri dishes (approx 2×10^6 cells/35-mm plate) are prepared the day before use, and following infection or transfection they are maintained in Eagle's medium containing 5% newborn calf serum plus antibiotics (EC5). Eagle's medium supplemented with antibiotics is used for the washing of cell monolayers, unless specified.

2. The modified assay in insect cells (*see* Section 3.3.) uses monolayers of *Spodoptera frugiperda* (Sf) cells (strain IPLB-SF-21; ref. *10*). These are maintained at 28°C without carbon dioxide in TC100 medium supplemented with 5% fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin (TC100C). Monolayers of approx 4×10^5 cells in 2 cm^2 plastic Linbro wells are suitable for the replication assays and TC100 without serum or antibiotics can be used in the lipofection of these cells.

2.2. Other Reagents

1. Tris-buffered saline (TBS): 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 5.5 m*M* glucose, 25 m*M* Tris-HCl, pH 7.4.
2. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA
3. 20 mg/mL protease: Sigma grade XIV protease dissolved in TE, predigested for 1 h at 37°C and stored at –20°C.
4. Cell lysis buffer (CLB): 10 m*M* Tris-HCl, pH 7 5, 1 m*M* EDTA, 0 6% (w/v) SDS.
5. 200X RNase mix: 1 mg/mL RNase A, 10,000 U/mL RNase T1 in TE (stored at 4°C)
6. Reticulocyte standard buffer (RSB): 10 m*M* Tris-HCl, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$
7. 10% (v/v) NP40 in H_2O.
8. Hepes-buffered saline (HeBS): 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 5.5 m*M* glucose, 21 m*M* HEPES-NaOH, pH 7.05; autoclaved or filter sterilized and stored at room temperature
9. 2 mg/mL Double-stranded calf thymus DNA (Sigma, St. Louis, MO) in H_2O (stored at –20°C)
10. 2*M* $CaCl_2$: autoclaved or filter sterilized and stored at room temperature
11. 25% (v/v) Dimethyl sulfoxide (DMSO) in HeBS, made up immediately before use.
12. Liposomes· prepared as described *(11,12)* from dimethyl dioctadecylammonium bromide (DDAB) and dioleoyl phosphatidyl ethanolamine (DOPE). A solution containing 4 mg DDAB and 10 mg DOPE in chloroform is evaporated to dryness under vacuum, the lipids resuspended in 10 mL sterile H_2O, and sonicated using a Dawe Soniprobe at approx 100 W until no further decrease in turbidity occurs. Store at 4°C. (Can be used for at least 6 mo)
13. Detailed descriptions of the reagents and methods used for restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer, preparation of [^{32}P]-labeled probes by nick translation or random priming, and hybridization, which are beyond the scope of this chapter, can be found in standard manuals of molecular biological techniques *(13,14)*

3. Methods

3.1. Assay for the Presence of a Functional Origin of HSV-1 DNA Synthesis

In order to determine whether a particular fragment of HSV-1 DNA contains a functional origin sequence, or if a known origin remains functional fol-

lowing mutagenesis, the most convenient procedure is to transfect a bacterial plasmid containing the fragment into mammalian cells and superinfect with wild-type HSV-1 to provide helper functions. The majority of our work has utilized common plasmids such as pAT153 *(15)*, pUC derivatives *(16)* or the pTZ series *(17)* as vectors for the viral origin-containing fragments, and these are propagated in *dam*$^+$ *E. coli* strains such as DH5 or XL1-blue. The DNA is introduced into monolayers of BHK cells in 35-mm Petri dishes by the calcium phosphate technique. Following superinfection, total cellular DNA is prepared and examined for the presence of amplified input plasmid DNA sequences by a combination of restriction enzyme digestion, Southern blot analysis, and hybridization to a labeled probe prepared using the DNA of the initial plasmid vector. The DNA is usually digested with *Dpn*I in the presence of a second enzyme that cuts the input DNA once and consequently can convert the high molecular weight concatemeric replication products to monomeric units that are more readily transferred from the gel and more easily detected since they represent a discrete, uniformly sized species. The use of *Dpn*I allows replicated plasmid sequences to be distinguished from unreplicated input DNA since the latter contains methylated A residues within the *Dpn*I recognition sequence and therefore is susceptible to *Dpn*I digestion Following amplification of these sequences in mammalian cells these sites are no longer methylated and the DNA becomes resistant to cleavage by *Dpn*I. Digestion with the two enzymes therefore should yield a species that comigrates with linearized DNA of the input plasmid and represents DNA that has been replicated subsequent to transfection, and several smaller species that represent the *Dpn*I cleavage products of unreplicated input DNA (an example of this type of analysis is shown in Fig. 1).

1. Prepare the calcium phosphate-DNA coprecipitate for transfection (1 mL of precipitate is sufficient for transfection of two cell monolayers in 35-mm Petri dishes, one of which should be superinfected with the other serving as a mock-infected control) To 1 mL of HeBS add 20 µg calf thymus carrier DNA and 0 5 µg of the test plasmid. Mix gently, then add 70 µL 2*M* $CaCl_2$ and mix rapidly Allow the precipitate to form for approx 5 min at room temperature. This amount of test plasmid is suitable for a plasmid of approx 3.5 kbp. Equimolar amounts of other plasmids should be used (*see* Note 2).
2. Remove growth medium from the monolayers and add 0.4 mL/plate of the fine precipitate.
3. Incubate at 37°C for 45 min then add 2 mL EC5/plate.
4. Continue incubation for a further 3 h 15 min at 37°C
5. Pour off medium and wash the cells once with Eagle's medium Then add 1 mL 25% DMSO in HeBS for 4 min, pour off, wash once more, and add 2 mL EC5
6. Incubate for a further 2 h at 37°C and then mock-infect or superinfect the cells with wt HSV-1 at an MOI of 5 PFU/cell Virus should be adsorbed at 37°C for 45

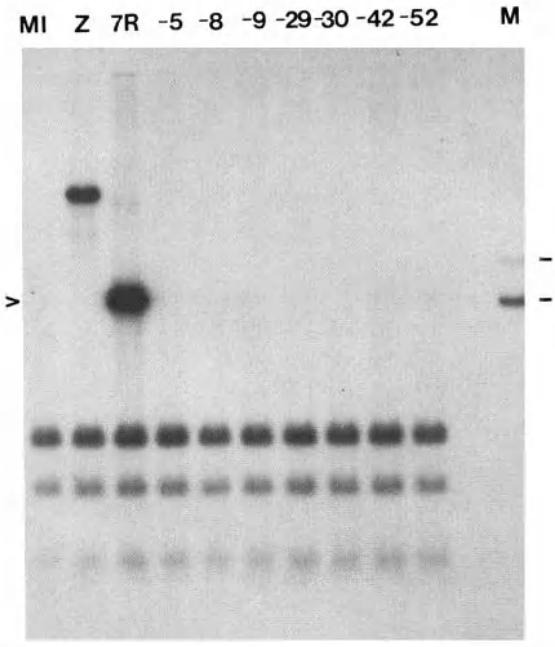

Fig. 1. An example of a transient DNA replication assay in Sf cells (Section 3.3.). Sf cells were transfected with plasmid pST19, which contains a functional copy of HSV-1 ori_S and either mock-infected (MI) or superinfected with the parental baculovirus, AcRP23*lacZ* (Z), a mixture of AcNPV recombinants specifying all seven HSV-1 DNA replication proteins (7R) or similar mixes from which one recombinant in turn had been omitted (–5 indicates the mixture from which the UL5 expressing virus had been omitted, and so on). Total cellular DNA was cleaved with *Eco*RI (which cleaves pST19 once to yield a 2.9 kbp fragment) and *Dpn*I, the fragments separated by agarose gel electrophoresis and transferred to a nylon membrane. Replicated pST19 DNA was detected by hybridization to pTZ19U (the vector used in the construction of pST19) DNA, which had been ^{32}P-labeled in vitro by nick translation. Size markers of 3.8 and 2.8 kbp are shown in lane M. Small fragments resulting from *Dpn*I cleavage of unreplicated input pST19 DNA can be seen in all lanes (except M). A product corresponding in size to linearized pST19 DNA is present in lane 7R (arrowhead) and is indicative of pST19 replication in the presence of all 7 HSV-1 DNA replication proteins. The absence of this band from tracks –5, –8, –9, –29, –30, –42, and –52 confirms that all seven HSV-1 proteins are required for efficient DNA synthesis. The larger band present only in lane Z results from homology between the pTZ19U probe and sequences present in AcRP23*lacZ* but not the other recombinant baculoviruses. Reproduced from ref. *24* with permission.

min and the monolayers then overlayed with 2 mL EC5 prior to continuation of incubation at 37°C.

7 Twenty-four hours after initial transfection, prepare total cellular DNA (*see* Note 3). Remove medium from cells and wash with TBS Add 2 mL CLB containing 0.5 mg/mL protease to the drained cell monolayer and incubate at 37°C for 2–6 h. Transfer the cell lysate to suitable tubes, extract sequentially with phenol, then chloroform and precipitate nucleic acids with ethanol. Recover the nucleic acids by centrifugation and redissolve in 100–200 µL TE containing 1X RNase mix.
8. Digest a sample of the DNA corresponding to that recovered from approx 2×10^5 cells with *DpnI* and an appropriate second enzyme (3 h in a 40-µL final volume in the presence of 3–5 U of each enzyme is adequate usually) and separate the fragments by agarose gel electrophoresis. An appropriate marker is approx 5 ng of the transfected plasmid cut with the second enzyme alone. Blot the gel and hybridize to an appropriate labeled probe (*see* Note 4)
9. After hybridization, wash the filter and detect any signal by autoradiography. Replicated plasmid DNA in the total cellular DNA samples is detected as a band comigrating with the marker DNA

3.2. Assay for HSV-1 DNA Replication Gene Function

The assay for HSV-1 origin function described earlier was modified to provide a convenient method for determining the requirement for specific HSV-1-encoded proteins in viral origin-dependent DNA synthesis *(18,19)*. In this approach, cells were transfected with a known HSV-1 origin-containing plasmid; and viral helper functions, rather than being provided by a superinfecting virus, were specified by a series of cotransfected recombinant plasmids. With this assay Challberg and his colleagues were able to identify the set of seven HSV-1 products that are both necessary and sufficient for viral DNA synthesis. In the initial experiments these genes were all expressed from their own promoters and two additional plasmids encoding the HSV-1 transactivators Vmw110 and Vmw175 were also required to be present to activate their transcription. Subsequently, it was shown that the requirement for Vmw175 and Vmw110 could be circumvented if expression of the seven replication genes was driven by the human cytomegalovirus (HCMV) major IE promoter that allowed constitutive high level expression *(20)*. This assay provides a convenient method to determine whether mutations in any of the seven replication proteins affect their ability to participate in viral DNA synthesis. Cells are cotransfected with an origin plasmid, a plasmid encoding the mutated protein and six other plasmids specifying the *wt* versions of the remaining replication proteins *(21)*.

1. Make up a mix of the DNAs in HeBS. One milliliter should contain 1 µg of each of seven plasmids encoding the essential HSV-1 DNA replication proteins plus 0.5 µg of plasmid containing a functional origin of replication and 12 µg calf thymus carrier DNA (*see* Note 5)
2. Form precipitate and add to cells (0.4 mL/35 mm dish) as described in Section 3 1 (steps 1 and 2)

3. Feed cells with 2 mL EC5 after 45 min and treat with DMSO 4 h posttransfection as described in Section 3.1. (step 5).
4. After DMSO treatment incubate for a further 48 h at 37°C.
5. Prepare total cellular DNA and analyze for replication of the HSV origin-containing plasmid as described in Section 3.1. (steps 7–9).

Weller and colleagues have described a transient replication complementation (TRC) assay that combines several of the features of the described procedures (*see* Sections, 3.1. and 3.2.) and provides an alternative method of determining whether mutations affect the functions of cloned HSV replication genes. In this approach the replication gene to be tested is cotransfected with a plasmid containing a known functional HSV origin, and the cells subsequently superinfected with a mutant helper virus containing a deletion of the gene of interest. Amplification of the origin-containing plasmid indicates that the product of the transfected gene is able to participate in viral DNA synthesis *(22,23)*.

3.3. Assay for the Ability of Baculovirus Expressed HSV-1 DNA Replication Proteins to Participate in Viral DNA Synthesis

The high level expression of HSV-1 DNA replication proteins by recombinant baculoviruses provides an attractive system for the study of the biochemical activities of wild-type and mutated forms of the proteins. However, it was also desirable to demonstrate that the heterologously expressed proteins retained their ability to carry out HSV-1 DNA synthesis. The absence of a cell-free system for origin-dependent HSV-1 replication therefore prompted the development of a suitable transient assay using baculovirus recombinants in Sf insect cells. It was shown that transfection of Sf cells with a plasmid containing a known functional HSV-1 origin and superinfection with a mixture of seven recombinants expressing the HSV replication proteins resulted in amplification of the origin containing plasmid *(24)*. The amplification was dependent on the presence of all seven HSV-1 DNA replication proteins and exhibited the important characteristics of viral DNA synthesis in permissive mammalian cells. This assay therefore provides a convenient method by which a single recombinant baculovirus can be used to evaluate the ability of a mutated replication protein to participate in viral DNA synthesis and to produce amounts of the mutated protein sufficient for biochemical investigations.

1. Set up monolayers of Sf cells in Linbro wells the day before required (final cell number to be approx 4×10^5 cells/2 cm^2 well in 1 mL TC100C medium).
2 The HSV origin containing plasmid is introduced by a liposome-mediated procedure. Separately dilute 0.4 μg plasmid DNA with 0.5 mL TC100 (without serum or antibiotics), and 15 μL liposomes with 0.5 mL TC100. Mix the diluted DNA and liposomes and stand 10 min at room temperature prior to application to the

cells (*see* Note 6) Remove the growth medium from cell monolayers and wash once with TC100

3 Add 0.3 mL DNA–liposome mix per well and incubate for 4 h at 28°C.

4. Prepare a mixture of recombinant baculoviruses in TC100C such that a final volume of 150 µL contains 2×10^6 PFU of each virus Remove DNA/liposome mix from wells and add 150 µL/well of the virus mix (5 PFU/cell of each virus)

5 Incubate for 1 h and add 1 mL TC100C per well

6 Continue incubation at 28°C for a further 48–72 h prior to the preparation of total cellular DNA. This is done essentially as described in Section 3.1 except that the cells are more conveniently harvested by gentle pipeting into the supernatant followed by centrifugation for 15 s in a microfuge. Resuspend the cell pellet in 0.4 mL CLB containing 0 5 mg/mL proteinase and incubate at 37°C for 2–6 h Extract sequentially with phenol then chloroform and precipitate nucleic acids with ethanol Recover the nucleic acids by centrifugation and redissolve in 60 µL TE containing 1X RNase mix

7 Analyze the DNA for the presence of replicated ori-containing plasmid molecules as described in Section 3.1. (steps 8 and 9).

3.4. Assays for Encapsidation of HSV-1 DNA

In the described assays, replication of a plasmid containing an HSV-1 origin in the presence of HSV-DNA replication proteins yields concatemeric molecules composed of tandem head-to-tail repeats of the original plasmid. However, because these products lack specific *cis*-acting sequences required for the site-specific cleavage of the concatemeric DNA and its encapsidation into virus particles, they are not further processed or packaged. It is now known that the essential sequences reside within the viral *a* sequence, an approx 400-bp element that occurs as direct repeats at the genomic termini and in inverted orientation at the junction between the L and S segments. The insertion of the *a* sequence into the origin-containing plasmid allows the concatemeric replication products to be cleaved and encapsidated when transfected cells are superinfected with wild-type helper virus *(25)*. In contrast to nonencapsidated DNA, the packaged molecules are resistant to the action of exogenously added DNase and can be further propagated as defective genomes when virus progeny from the transfected and superinfected cells are used to infect fresh cell monolayers. These properties provide the basis of convenient assays for packaging of HSV DNA that have proved useful for defining the encapsidation signals and should have application in elucidating and characterizing the viral gene products that are required.

3.4.1. Assay for Encapsidated (DNase-resistant) DNA

1 Transfect BHK cells with a plasmid containing a known HSV origin and sequences to be tested for packaging activity, superinfect with *wt* HSV-1 and process as described in Section 3 1 , steps 1–6.

2. Twenty-four hours pi, remove medium from monolayers and scrape the cells into 1 mL RSB.
3. Transfer to a 5-mL plastic tube and add 50 µL 10% NP40 to lyse the cells followed by 5 µL of a 10-mg/mL stock solution of DNase I (*see* Note 7).
4 Incubate for 2 h at 37°C
5 Add 1 mL 2X CLB containing 1 mg/mL proteinase and incubate for a further 2–6 h at 37°C
6 Extract the DNA sequentially with phenol and chloroform and analyze for the presence of replicated plasmid sequences as described in Section 3.1. steps 7–9 The presence of a *DpnI* resistant fragment in DNase I treated samples that comigrates with the marker DNA is indicative that the test plasmid has been both replicated and encapsidated into virus particles (*see* Note 8)

3.4.2. Assay for Propagation of Plasmid Molecules as Defective Genomes

1 Carry out transfection and superinfection of BHK cell monolayers as described in Section 3.4.1., step 1.
2 Twenty-four hours pi, scrape the cells into the growth medium and sonicate extensively to prepare a virus stock from the transfected cells
3 To determine whether amplified plasmid sequences have been encapsidated as defective genomes, this stock is further passaged at high MOI. Use 0.5 mL of the stock to infect a fresh monolayer of BHK cells in a 35-mm Petri dish. Allow virus to adsorb for 1 h at 37°C, remove inoculum, and add 2 mL EC5 to the cells
4. Incubate at 37°C for 24 h and prepare and analyze total cell DNA for the presence of replicated plasmid sequences as described in Section 3.1. Sequences corresponding to the test plasmid will be detected only if it is both replicated and packaged into virus particles in the original transfected cells (*see* Note 9)

4. Notes

1. HSV-1 ori_S and ori_L share considerable sequence homology and appear to be essentially equivalent in transient replication assays. Although both origins contain palindromic sequence elements, the palindrome in ori_L is significantly longer and is susceptible to deletions when cloned in most *E coli* strains. In contrast, the ori_S sequence is stable on cloning and plasmids containing it therefore are more frequently employed in the replication assays *(1–3)*.
2 The efficiency of the calcium phosphate transfection procedure is influenced by several factors, including the concentrations of plasmid and carrier DNAs and the pH of HeBS, and these should be optimized. A convenient method for evaluating and comparing transfection efficiencies is to use a plasmid that expresses the *E coli lacZ* gene from a strong constitutively expressed promoter (e.g., the HCMV major IE promoter; *see* Section 3.2.) and to stain and count cells expressing the β-galactosidase product *(26)*. The inclusion of such a plasmid can also provide a valuable internal control to confirm that the transfection efficiency does not vary significantly between different plates within an experiment. Alternative

methods for introducing plasmid DNAs into cells (e.g., lipofection, electroporation, and various modifications to the calcium phosphate procedure) are probably suitable for transient replication assays, with the optimal procedure being influenced by the cell type used.

3. In some experiments it may be desirable to isolate DNA from the nuclei of transfected cells. A simple method of preparing nuclei is to lyse the cells in RSB containing 0.5% NP40 and to recover the nuclei by centrifugation at 600*g* for 2 min. The nuclei are resuspended and DNA isolated following digestion with protease in CLB as described for total cellular DNA *(27)*.
4. Although we use probes labeled in vitro with ^{32}P by either nick translation or random priming, nonradioactive probes should, in principle, be equally suitable.
5. In our hands the introduction of multiple plasmid species into cells seems to be much more effective using the calcium phosphate precipitation method than lipofection, but the reason for this is not clear.
6. Sf cells respond much better to lipofection than to calcium phosphate transfection that has a cytotoxic effect and causes significant disruption of cell monolayers. A variety of reagents for liposome-mediated transfection are available commercially, and the lipid composition of homemade preparations can be varied to optimize transfection efficiency.
7. Initially sonication rather than exposure to NP40 was used to break open the cells prior to DNase I treatment *(25)*, but in practice the latter method *(28)* proved to be simpler and more reproducible.
8. DNA fragments resistant to *Dpn*I cleavage, but smaller than the input plasmid, also will be detectable at lower abundance. These represent the terminal fragments of packaged molecules generated by specific cleavage of concatemers at the *a* sequence during the encapsidation process *(25,29)*.
9. Although the use of virus superinfection to provide helper functions for replication and encapsidation represents a convenient and reproducible method to analyze DNA encapsidation, this is not the best method to generate stocks containing the maximum proportion of defective genomes. If this is the objective it is better to provide the helper functions by cotransfection with intact infectious HSV-1 DNA *(6,7)*.

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16

HSV Amplicons in Gene Therapy

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1. Introduction

Herpes simplex virus (HSV) amplicons are defective virus vectors capable of introducing amplified foreign genes into variable types of eukaryotic cells, such as fibroblasts, macrophages, glia, and neurons in different organisms including rodents, monkeys, and human (refs. *1–3*; reviewed in ref. *4*). The defective viruses follow their nondefective counterparts in the ability to infect mitotic, as well as postmitotic cells. This makes them potentially useful vectors for use in nondividing cells, such as in nerve cells. Available retrovirus vectors employed to date for gene therapy require cell division and therefore cannot be used to target neurons.

HSV resides latently in the host, from which it is reactivated, producing recurrent infections *(5–7)*. HSV latency takes place in ganglia, with the virus traveling through nerve cells moving from the sites of replication to the sites of latency. Being a neurotropic vector, HSV is used to localize nerve cell connections synaptically between peripheral sites of entry and the central nervous system and interbrain conections (reviewd in refs. *8* and *9*). During latency, the virus enters a benign stable state that does not alter electrophysiological properties of the cells *(10,11)*. Recently, Farkas and colleagues have examined the electrophysiological properties of cholinergic and dopaminergic neurons after infection with HSV-1-derived vectors for a few days and have found no alterations *(12)*. These neurotropic properties of the virus have made it an attractive vector for experimantation in nerve cells in vivo. Actually, constructed amplicon-type vectors were shown to be expressed in neuronal cells in vitro *(13,14)*, as well as in nondividing neurons in the adult animal in vivo *(15–18)*

Since the amplicons are defective virus vectors, they depend on the helper virus that supplies *in trans* DNA replication functions needed for the replication of the amplicon DNA sequences, as well as *trans*-acting virion polypep-

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tides and functions required for the packaging of the replicated amplicon DNA *(19)*. In addition, the helper virus must contain specialized functions, essential for gene expression, within the target cells and tissues. Cotransfection of cells with the seed amplicon along with helper virus DNA, or alternatively, transfection of cells with amplicon DNA and then superinfection of the cells with the helper virus result in virus stocks that typically consist of two components: (1) Defective genomes containing multiple identical head-to-tail repeats of the HSV seed amplicon, most likely arising by rolling circle replication. The concatomeric genomes are cleaved to unit-length genomes at the cleavage/packaging signals (pac-1-pac-2 signals), which they contain. The full size of packaged defective virus particles is close to 150 kbp, which is the size of the HSV-1 genome *(20–22)* Therefore, defective genomes and standard genomes are not separable by their DNA sizes in the virus stocks. (2) Helper viruses: The resultant virus stocks can be routinely propagated at high multiplicity of infection (MOI) for several passages to amplify defective-to-helper virus ratios. The state of the input amplicons in resultant defective virus stocks is analyzed, e.g., by restriction enzyme analyzes of viral DNAs, by using marker genes to quantify the ratios of helper and defective viruses in Southern blots, or employing β-galactosidase as an expressed marker gene *(23,24)*.

Virus propagation at high MOI results in typical fluctuations in infectious virus yield, reflecting the fact that the cells are dually infected with the helper/standard virus and the defective genomes. After efficient replication of defective genomes, above the threshold level, the stocks with very high ratios of defective genomes were found to interfere with the replication of the standard virus, eventually reducing the production of the helper virus to minimal infectious virus/cell, eliminating altogether the standard replicating viruses in the majority of the cells. At that point in the cycle, in the absence of helper virus, the defective viruses cannot be replicated, the ratio of helper-to-defective virus was found to be reversed, interference was reduced, the series recovered, and repeatedly so.

The amplicon-derived genomes replicate efficiently and have advantages over the helper virus DNA. The 152 kbp of HSV-1 DNA contain three origins of replication. By comparison, the vectors contain one origin of replication in each repeat unit sizes smaller than 15 kbp (reviewed in refs. *7* and *19*).

Inasmuch as the amplicon depends on the helper virus for its preparation and large-scale preparation of the amplicon stocks, they are potentially hazardous owing to the lytic properties of HSV. This will be dealt with later in the text, where amplicon and helper combination will be dealt with specifically.

Once generated, virus stocks containing mixtures of helper virus and ample constructed amplicons can be potentially used for experimental studies aimed at future development of gene therapy.The stocks containing defined ratios of helper virus and amplicon can be repeatedly reproduced.

The defective viruses cannot propagate and spread in the recipient cells in the absence of their helper virus counterparts, as documented in vitro using wild-type *(19,25),* as well as mutant helper viruses *(13,26,27).*

1.1. Amplicon Plasmid Properties

The amplicon contains three kinds of genetic elements:

1. Sequences that allow its propagation in bacteria, including the *Escherichia coli* origin of replication and the ampicillin resistance gene.
2. Sequences from HSV that support propagation of the amplicon in virus stocks These include an HSV origin of replication *(ori)*—*ori*S or *ori*L *(25,28),* and the two HSV packaging signals, pac-1 and pac-2, located within the "a" sequence of the HSV-1 genome. The pac-1 and pac-2 are consensus sequences of all herpes-viruses, which define distance and structure of the corresponding genomic termini (Deiss and Frenkel, 1986; Deiss et al, 1986).
3. A transcription unit that includes a promoter element (e.g., HSV-1 immediate early [IE] 4/5 promoter *[2]* and CMV promoter *[15]*) This is then followed by an expressed foreign gene and the SV40 early region polyadenylation site

Consideration of *cis*-acting functions required for amplicon DNA propagation, includes the HSV origin (*ori)* of DNA replication that can be variable. HSV contains two types of origins of replication: the *ori*S- two copies of that are located within the *c* reiterated sequence of the S component, and the *ori*L—one copy of which is mapped in the middle of the L component. By comparison, both S and L origins contain *ori* binding proteins—two copies of that are present in both the S *ori* and the L *ori*. The *ori*L is slightly longer because of A + T-rich perfect palindrome sequences, which make it unstable in DNA fragments cloned in *E. coli*. Both types of origins are sufficient for propagation of standard HSV inasmuch as both copies of *ori*S have recently been deleted without impairing the ability of the virus to multiply *(29).* In terms of *ori*S and *ori*L, they were both shown to provide proper origins for amplicon replication *(1,19,30).*

The promoter element, HSV IE promoters in the amplicon-based vectors are not subjected to the promoter inactivation that occurs with many types of virus vectors, including defective HSV vectors *(31).* HSV IE promoters can support expression in human, monkey, and mouse in vitro, which is very important, because it is needed for preparations of stocks to be used in different animals in vivo and in gene therapy *(27,32).* The cytomegalovirus (CMV) IE promoter can also directly express foreign genes, but downregulation of the expressed gene after 2 wk was reported in in vivo experiments *(15,17).* Promoters from housekeeping genes may be more effective at causing sustained changes in central nervous system function. Cell-type-specific promoters may allow restricted expression of a gene in the vector to a chosen cell type.

The gene in the amplicon can be subjected to experimental manipulation. The *E coli lacZ* gene could be expressed and serve as a marker gene employing *in situ* β-galactosidase activity assay. This gene can be replaced with virtually any gene. In addition, by fusing the gene to subcellular targeting sequences, the expressed protein can be localized to a particular part of the cell One can construct a plasmid that contains the *lacZ* gene as a marker gene together with a cellular gene under different promoters.

There is a limit to repeat unit sizes. We have found that constructed amplicons with repeat units below 15 kbp were stably propagated, whereas amplicons with larger seed repeats underwent systematically random deletions, generating deleted repeats smaller than 15 kbp, which thereafter retained their stability *(32)*. The reasons for this limitation are as yet unclear, but they may have to do with rolling circle replication.

1.2. Helper Virus Properties

In terms of the helper virus, the original work with the amplicon has been done with nondefective helper virus, as well as a temperature-sensitive *(ts)* mutant defective with respect to ICP4 *(1,26)*. The wild-type HSV-1 in the virus stock invariably causes cell death. However, intracerebral injection *(33)* and infection of mouse neuroblastoma cells with HSV-1 *ts* mutants allow persistence of the virus without cell death *(13)*. The *ts* mutants are affected in the ICP4 gene (also termed IE3,Vmw174), the IE gene responsible for turning on β-gene expression, and hence a critical turning point in the cascade regulation of HSV gene expression in the infected cells *(34)*. At the restrictive temperature of 37 or 39°C, the *ts* mutations block the lytic cycle and thereby prevent cell damage. Virus is grown at the permissive temprature of 31–34°C.

Deletion mutants are better than *ts* mutants as the helper, resulting in lower frequencies of reversion to wild-type *(16,27)*. An ICP4 gene deletion mutant, D30EBA *(35)*, which requires for its propagation an ICP4-containing cell line, has a reversion frequency of 5×10^{-5} compared to the reported reversion frequency of *ts*K- 2×10^{-3}. By using HSV-1 deletion mutant viruses (grown on complementing cells), it is possible to obtain virus stocks that are unable to replicate on normal noncomplementing cells. Additional helper viruses are currently beeing developed *(36)*.

1.3. Amplicon Propagation: Preparation and Analyses of Transfection-Derived Virus Stocks

DNA transfections are done using rabbit skin (RS) cells, whereas the virus is grown in Vero (African Green Monkey kidney) or Hep-2 (Epidermoid carcinoma) cells. Mixtures of plasmid DNA and HSV-1 helper virus DNA are used to transfect RS cells in 25-cm^2 flasks by the calcium-phosphate coprecipitation

technique *(37,38)*. Transfection efficiencies should be first determined by estimating the proportion of blue cells/total cells after transfecting with plasmid containg β-galactosidase driven by an adequate promoter. Alternatively, amplicon DNA can be transfected followed by superinfection with the helper virus. The derived virus stocks are harvested by three cycles of freeze (–80°C) thawing (37°C). The virus stocks derived from the cotransfection or transfection-superinfection (passage 0) are serially passaged, taking a fixed ratio for each passage (e.g., 1:4 dilution) in Vero or Hep-2 cells, to generate subsequent virus stock passages. Propagation continues while determining the ratio of defective:helper virus in each passage. Required passage numbers depend on the amplicon and helper virus employed. The choice of passage to be used as a constructed amplicon stock depends on the amount of foreign gene expression aimed for. Concentrations of the helper virus are determined by titration plaque assay. Defective virus can be assayed in culture via histochemical detection of bacterial β-galactosidase—each blue cell is considered to represent one infectious defective virion.

2. Materials

2.1. DNA Transfection of RS Cells

1 RS cells (Epidermis, cottontail rabbit): cells are available from the American Type Culture Collection as CCL-68.
2 Hep-2 cells (Epidermoid carcinoma)· cells are available from the American Type Culture Collection as CCL–23.
3. Vero cells (African Green Monkey kidney): cells are available from the American Type Culture Collection as CCL-81.
4 DMEM: Dulbecco's modified minimum essential medium.
5. Maintenance medium: DMEM supplemented with 10% inactivated fetal calf serum (IFCS-30 min at 56°C).
6. 199-V medium—M199 (Hank's) medium containing 1% inactivated calf serum (ICS—30 min at 56°C) and 100 U/mL penicillin/100 mg/mL streptomycin.
7 Sterile microtubes
8 2X HeBS· Dissolve 8 2 g of NaCl, 5.95 g HEPES, and 0.105 g Na_2HPO_4 in double-distilled (dd) H_2O to a final volume of 500 mL. Bring to pH 7.05 with 5*N* NaOH. Sterilize by filtering through 22-μm filter, and store at –20°C.
9. 2*M* $CaCl_2$· sterilize through 22-μm filter, and store at –20°C.
10 Sterile ddH_2O.
11 Sterile glycerol.
12 Amplicon and helper virus DNAs/helper virus stock

2.2. X-Gal-Based Histochemical Reaction

1. Stock solutions of 1*M* Na_2HPO_4, 1*M* NaH_2PO_4, and 1*M* $MgCl_2$· Prepare in ddH_2O and store at room temperature.

2 Stock solutions of 50 m*M* potassium ferricyanide ($K_3Fe[CN]_6$) and 50 m*M* potassium ferrocyanide ($K_4Fe[CN]_6$)· Prepare in ddH_2O and store in foil-wrapped glassware (in the dark) at 4°C, where they are stable for at least 3 mo
3. X-gal stock: Dissolve in *N,N*-dimethyl formamide at 20 mg/mL, and store in a glass container (not polycarbonate or polystyrene) in the dark at –20°C.
4 4% Paraformaldehyde (wear a mask and gloves when handling paraformaldehyde). In a fume hood, dissolve 8 g of powder in 150 mL 0 1*M* sodium phosphate, pH 7.3 (80 m*M* Na_2HPO_4, 20 m*M* NaH_2PO_4) while stirring and heating to 60°C. Add 10*N* NaOH at a rate of 1 drop/min until the solution clears Bring the volume to 200 mL with 0 1*M* sodium phosphate, pH 7 3 Store at 4°C for up to 1 mo
5 25% Gluteraldehyde.
6 Working fixative (2% paraformaldehyde, 0.2% gluteraldehyde in 0.1*M* sodium phosphate): combine 50 mL of 4% paraformaldehyde with 49.2 mL of 0.1*M* sodium phosphate, pH 7 3, and 0.8 mL of 25% gluteraldehyde This can be stored at 4°C for up to 1 wk.
7. X-gal stain: 100 m*M* sodium phosphate, pH 7.3 (80 m*M* Na_2HPO_4, 20 m*M* NaH_2PO_4), 1 3 m*M* $MgCl_2$, 3 m*M* $K_3Fe[CN]_6$, 3 m*M* $K_4Fe[CN]_6$, and 1 mg/mL X-gal Filter through a 0 45-μ disposable filtration unit prior to use
8 Phosphate-buffered saline (PBS)· 15 m*M* sodium phosphate, pH 7 3, 150 m*M* NaCl

3. Methods

3.1. DNA Transfection of RS Cells

1 Prepare RS cell cultures in 25-cm^2 flasks
2 Decant the medium and overlay with fresh maintenance medium.
3. Preparation of DNA/CaCl reaction: For each transfection prepare two sterile microtubes In the first tube, put 250 μL of 2X HeBS; in the second tube, which is kept cold in ice, put 30 mL of 2*M* $CaCl_2$, DNA, and ddH_2O to a final volume of 250 μL Add the DNA mixture to the 2X HeBS, mix gently (do not vortex), and allow to precipitate at room temperature for 20 min
4. Add transfection mixture onto the RS cells monolayer, apply to the middle of the monolayer and ensure that it is properly distributed
5. Incubate at 37°C for 4–6 h.
6 Remove the medium, and add 2 mL DMEM containing 10% glycerol Incubate for 1 min, and quickly remove the medium. Rinse twice with DMEM The glycerol-containing medium is toxic to the cells and should not be left in contact with the cells for longer times
7. Overlay with maintenance medium. Incubate for 4–5 d, or until the viral infection is spread in the entire culture

3.2. X-Gal-Based Histochemical Reaction—Detection of Defective Viruses with lacZ Bacterial Gene as a Marker

1. Remove media
2. Rinse the cell monolayer twice with PBS.
3. Overlay the culture with the fixative. Incubate at 4°C for 5 min.

4. Remove the fixative, and rinse twice with PBS at room temperature.
5. Remove PBS and overlay the fixed cells with the X-gal stain. Incubate at 37°C until blue color develops (30 min to overnight).

4. Notes

1. The amplicon plasmid can contain either the *ori*S and *ori*L, which are both functional. The *ori*L sequences are less stable in DNA fragments cloned in *E coli*
2. Amplicon propagation could begin with cotransfection of the cells with amplicon and helper virus DNA or, alternatively, it could involve transfection of the cells with amplicon DNA followed by superinfection with the helper virus. For transfection-superinfection, begin by transfecting the cells with amplicon DNA, and follow by infection with the helper virus at a MOI of 1 PFU/cell. Incubate in 199-V medium.
3 The amplicon virus stocks containing defined ratios of helper virus and amplicon can be repeatedly reproduced.
4. It is most important to change pipet or tips for each dilution to prevent carryover of virus, which will result in anomalous high titers. Make a short vortex before using the virus and after each dilution Pipet up and down because the viruses adhere to the plastic.
5. The titers of the defective virus stocks can be assayed in culture via histochemical detection of bacterial β-galactosidase Alternatively, serially passaged viral DNA can be ^{32}P-labeled, and restriction enzyme analyzes can be done by eletrophoresis in agarose gel *(1)*

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17

Analyses of HSV Proteins for Posttranslational Modifications and Enzyme Functions

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1. Introduction

In order to identify the nature of posttranslational modifications and enzyme functions of herpes simplex virus 1 and 2 (HSV-1 and HSV-2) proteins, it is necessary to apply both biochemical and genetic analyses. The experimental methods described in this chapter have been applied to cells cultured in vitro and infected with HSV-1 or to isolated nuclei of infected cells, to nuclear or cytoplasmic fractions, and, in some instances, to purified extracts of infected eukaryotic cells or of prokaryotic cells expressing a viral gene.

2. Materials

1. Chemicals and reagents: *N,N'*-diallyltartardiamide (DATD), endogylcosidase H, galactosyltransferase, glutathione-agarose, tetrahydrofuran, phosphocreatine, creatine kinase, NaF, Polygram CEL 300 PEI/UV plastic sheets, and DEAE-cellulose were from Sigma Chemical Company (St Louis, MO) Neuraminidase are available from Boehringer Mannheim (Indianapolis, IN) Lectins and the Vectastain kit are available from Vector Laboratories (Santa Cruz, CA) Nitrocellulose membranes (type BA 83) and DEAE filters are available from Schleicher and Scheull (Keene, NH). Pure poly(ADP-ribose) glycohydrolase was a gift from Myron Jacobson (University of Kentucky). pGEM plasmids, RNA polymerase, and rabbit reticulocyte lysate is available from Promega (Madison, WI). Cap analog is available from New England Biolabs (Beverly, MA). Sephadex G-200 and poly(dT-dA) is available from Pharmacia (Uppsala, Sweden). Glass fiber filters (type G/C F) are available from Whatman (Maidstone, UK).
2. Radioactive materials. [^{32}P]orthophosphate, [α^{32}P]ATP, [α^{32}P]GTP, [8-^{3}H]GTP,

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[2-^{3}H]ATP, and [^{3}H]CDP are available from Amersham (Arlington Heights, IL) [^{35}S]methionine, [β^{32}P]NAD, [^{3}H]thymidine, [^{3}H]UDP-galactose, [^{3}H]TTP, [^{3}H]uracil, [^{3}H]glucosamine, and [α^{32}P]ATP are available from New England Nuclear (Boston, MA)

3 Animal cells: Human HEp-2, human HeLa, mouse Ltk-, and BHKtk- cells are obtained from the American Type Culture Collection (Bethesda, MD) All cell lines are maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% newborn calf serum HSV-1(F) is a limited-passage clinical isolate of HSV-1 that is temperature-sensitive and is used as the prototype HSV-1 strain *(1)*

4 Immunochemicals: Rabbit preimmune serum and polyclonal antisera against specific viral polypeptides are produced by Josman Laboratories (Napa, CA) Rabbit polyclonal antibody to poly(ADP-ribose) is a gift from Mark Smulson (Georgetown University) Monoclonal antibody H725 to ICP35 is a gift from Lenore Pereira (UCSF) *(2)* Monoclonal antibody MAB30 to the HSV-2 large subunit of ribonucleotide reductase should be requested from Laure Aurelian (Johns Hopkins) Goat antirabbit immunoglobulin immunobeads are from Bio-Rad (Duarte, CA) Protein A-sepharose CL4B is from Sigma

3. Methods

3.1. Denaturing (Sodium Dodecyl Sulfate [SDS]) DATD Polyacrylamide Gels

Denaturing gel electrophoresis is one of the most convenient assays for the detection of HSV protein modifications and enzyme functions. The assay is based on the observation that most modifications change the electrophoretic mobility of viral proteins in denaturing gels. In our laboratory, we exclusively use polyacrylamide gels that are crosslinked with *N,N'*-diallyltartardiamide (DATD). DATD has many advantages over *bis*-acrylamide in resolving glycosylated viral proteins *(3)*. For example, these gels enable the resolution and detection of the three posttranslationally modified forms of the ICP4 protein *(4)*.

1. Use any standard vertical electrophoresis apparatus
2. Pour and set the following 9 3% separating gel using 0 5-mm spacers: 11 2 mL water, 10 mL 0 14% ammonium persulfate (w:v), 5-mL 3*M* Tris-HCl (solution A), pH 8 5, 13 mL DATD:acrylamide (0 735% 28%, w.w.v) (solution C), 200 µL 20% SDS, 2 µL TEMED (*see* Note 1)
3. Pour and set the following stacking gel: 6 45 mL water, 12 mL 0 14% ammonium persulfate, 3 mL solution B (1*M* Tris-HCl, pH 7 0, 20% SDS), 2.55 mL solution C, 8 µL TEMED
4. To any solution of soluble protein (*see* Note 2), add 1/3 vol disruption buffer (2 mL 55% sucrose, 4 mL 20% SDS, 2 mL 1*M* Tris-HCl, pH 7 0, 2 mL 2-mercaptoethanol, 25 mg bromophenol blue), boil for at least 60 s, load and run gel (at 0 5 mA/cm for 16 h using 0.5 mm spacers) in the following running buffer: 6 g Tris-base, 28 8 glycine, 2 g SDS, and water up to 2 L (*see* Note 3).

5 The most efficient electrical transfer of viral polypeptides from a 0 5-mm gel to nitrocellulose requires 120 V for 3 h at 4°C in running buffer containing 0.025% SDS
6 To do fluorography, the membranes are sprayed with En^{3}Hance (NEN), dried, and placed directly on X-ray film Exposures may take as long as 4 mo

3.2. Phosphorylation

Phosphate cycles on and off several viral proteins during the course of productive infection. Moreover, not all phosphoproteins are made at the same time and, as a consequence, the identity of the labeled proteins may vary depending on the time at which they are pulse labeled *(5)*. The technique described in the following was developed to label α proteins; it is included here because these are the most difficult proteins to label (*see* Note 4). Labeling proteins made later in infection is easily accomplished by delaying the time of addition of the radioactive phosphate.

1. With few exceptions, HEp-2 cells are the cells of choice in our laboratory for analyzing viral polypeptides (*see* Note 5).
2. Monolayer cultures containing 4×10^6 cells are maintained in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) minus phosphate for 6 h.
3 Cells are then exposed to 10–20 PFU of HSV-1(F) per cell for 1 h
4 After absorption, the inoculum is replaced with Dulbecco's modified Eagle's minimal essential medium minus phosphate containing 10 μCi [$^{32}P_i$] (*see* Note 6) and maintained for 6 h at 37°C.
5. The cells are harvested, washed (*see* Note 6) with phosphate-buffered saline (PBS; 140 m*M* NaCl, 3 m*M* KCl, 10 m*M* Na_2HPO_4, 1.5 m*M* K_2HPO_4 [pH 7.4]), and either analyzed immediately (*see* Section 3.1.) or fractionated (*see* Section 3 6)
6. Since the viral phosphoproteins are abundant and highly labeled, exposure of the electrophoretically separated proteins to film for autoradiography is generally relatively short

3.3. Poly(ADP-ribosyl)ation

Poly(ADP-ribosyl)ation is a posttranslational modification that takes place in the nucleus. Modified proteins are thought to participate in DNA repair and synthesis, as well as other cellular processes including differentiation, transformation, and signal transduction. In vivo analysis of this modification has relied on the use of specific antibodies and purified poly(ADP-ribose) glycohydrolase, which specifically degrades the poly(ADP-ribose) chains *(6)*. Although interest in this modification is growing rapidly, these key reagents remain commercially unavailable and must be obtained from researchers in the field.

1 Infected cell nuclear extracts are prepared as described in Section 3 6
2. Nuclear extracts are denatured by boiling for 60 s in 0.1% SDS.

3 After denaturation, SDS is removed by three serial acetone precipitations and acidifications with 10% acetic acid To do this, 4–5 vol of cold (–20°C) acetone is added, the mixtures are stored at –70°C for 30 min, prior to pelleting of the proteins in a high-speed centrifuge After lyophilization, proteins are resuspended in 1 vol of acid and the procedure is repeated
4 After the final lyophilization, the polypeptides are digested for 30 min at 37°C with (0 1 U)-poly(ADP-ribose) glycohydrolase in 50 m*M* KPO4, pH 7 5, 50 m*M* KCl, 0 1 mg/mL bovine serum albumin, and 10 m*M* β-mercaptoethanol *(6)*
5. The digestions are stopped by the addition of SDS to 0.1% and the proteins are electrophoretically separated in a denaturing polyacrylamide gel (*see* Section 3.1.), electrically transferred to nitrocellulose and reacted with antibody to poly(ADP-ribose) (*see* Note 7)

3.4. Glycosylation

The definitive proof that a viral gene product is glycosylated is to label the protein with tritiated glucosamine *(7)*. To do this, simply add 50 μCi of [^{3}H]glucosamine to the culture medium during infection and then harvest the cells and analyze the proteins by fluorography, as described below (*see* Section 3.4.1.). An alternative and more detailed approach to show glycosylation is described in Section 3.4.1. This technique extends preexisting sugar chains on proteins with labeled galactose using purified galacosyltransferase (G.C-F., personal communication); this enzyme will transfer galactose from UDP-galactose to *N*-acetylglucosamine. Herpesviral glycoproteins contain both N-linked and O-linked oligosaccharides. Proteins containing N-linked sugars are predicted to contain the sequence Asn-X-Thr/Ser. The technique for identifying N-linked sugars is described in Section 3.4.2. and it involves cleaving the sugar chains attached to proteins in cellular extracts with endoglycosidase H, which degrades high mannose chains *(7)*, and then testing for an altered electrophoretic mobility of the protein of interest. The determination of O-linked sugars utilizes the fact that *N*-acetylgalactosamine(GalNAc) is added to the proteins very early in the processing *(8)*. To do this, simply transfer infected cell protein to nitrocellulose as described in Sections 3.1. and 3.3. and then probe the blot with a lectin specific for GalNAc. We recommend the lectin from *Helix pomatia* and the Vectastain system from Vector Labs as the developing reagents. Finally, both N-linked and O-linked glycoproteins contain sialic acid that may be removed by neuraminidase *(8)* This is done exactly as described in Section 3.4.2., but substituting neuraminadase for endoglycosidase H.

3.4.1. Galactosyltransferase Activity

1. Prior to use, galactosyl transferase must be autogalactosylylated. In 500 μL, add 10 U of enzyme in 50 m*M* HEPES, pH 7.3, 5 m*M* $MnCl_2$, 1 m*M* β-mercaptoethanol, 1% aprotonin (v.v), and 0.4 m*M* UDP-galactose, and incubate at 37°C for 30 min.

2. Add 326 mg of ammonium sulfate, incubate on ice 20 min, and precipitate the enzyme by centrifugation (microfuge 30 min at 4°C). The enzyme is resuspended in 250 µL of 25 m*M* HEPES, pH 7 3, 5 m*M* $MnCl_2$, 50% glycerol, and stored at –20°C in 50-µL aliquots.
3. Infected cell extracts (5 µL), prepared as described in Section 3 4.1., but without the pulse-labeling, are added to reactions (50 µL) containing 5 m*M* HEPES, pH 7.3, 7 5 m*M* NaCl, 2 5 m*M* $MgCl_2$, 0.1% NP40, 2.5 m*M* ATP, 5 m*M* galactose, 1 µCi [^{3}H]UDP-galactose, 100 mU of autogalactosylylated transferase, and reacted at 37°C for 30 min.
4. The reactions are terminated by boiling in 0 1% SDS prior to denaturing gel electrophoresis (*see* Section 3.1.) and fluorography (*see* Note 8). To specifically identify viral proteins, it is recommended that they be immunoprecipitated prior to galactosylylation (*see* Note 9)

3.4.2. Endoglycosidase H Cleavage

1. Approximately 4 × 10^6 infected cells are pulse-labeled with [^{35}S]methionine as follows At 5 h postinfection, the media is replaced with DMEM containing 50 µCi [^{35}S]methionine, 1% newborn calf serum, and 1/10 the normal amount methionine and the cells are incubated for 2 h, prior to washing the cells three times with PBS and replacing the media with DMEM containing 1% newborn calf serum.
2. At 16 h postinfection, the medium is removed, washed three times in PBS, and the cells are lysed by the addition of 300 µL PBS containing 1% NP40, 1% deoxycholate, 10 µ*M* tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK), and 10 µ*M* α-tosyl-L-lysine chloromethyl ketone (TLCK) and briefly sonicated. This technique is standard for obtaining infected whole-cell extracts; for unlabeled cells, simply omit the methionine label and use DMEM throughout
3. Cellular extract (10 µL) is reacted with endoglycosidase H (0.1 mU) in 50 µL of 100 m*M* sodium citrate (pH 5.5) and 100 m*M* β-mercaptoethanol at 37°C for 30 min.
4. The reactions are terminated by boiling in 0.1% SDS prior to denaturing gel electrophoresis (*see* Section 3 1). To specifically identify viral proteins, it is recommended that they be immunoprecipitated prior to electrophoresis (*see* Note 9).

3.5. Sulfation

Some viral glycoproteins are sulfated *(9)*. To analyze the sulfation of specific viral gene products, add carrier-free H_2[^{35}S]O_4 (50 µCi/mL) directly to the infected cell culture media at four hours postinfection. Cellular extracts are then made at 8 h postinfection and the labeled polypeptides are visualized in denaturing gels (*see* Section 3.1.) following fluorography, as described in Section 3.4.

3.6. Myristylization

At least two HSV-1 polypeptides have been shown to be myristylated, the product of the U_L11 gene *(10)* and the large subunit of HSV-2 ribonucleotide reductase *(11)*. Myristylated proteins contain a consensus modification sequence, Gly-X-X-X-Ser/Thr, at their amino terminus. Identification of

myristylated viral gene products requires metabolic labeling of cells infected with HSV. In general, either [^{3}H]myristic acid or [^{3}H]palmitic acid (125–250 μCi/mL) is simply added to the media of infected cells at 3 h postinfection and the infected cells are labeled for as long as 20 h, prior to harvesting and analyzing the viral polypeptides in denaturing gels (*see* Section 3.1.) and by fluorography (*see* Section 3.4.)

3.7. Isolation of Purified Infected Cell Nuclei

Since infected cell nuclei are extremely fragile, care most be taken during their isolation. The following procedure uses HeLa cells (*see* Note 5) and provides a simple, highly reproducible source of infected cell nuclei, as well as nuclear extract proteins. However, this technique is not sufficient for the determination of the intracellular partitioning of viral polypeptides; more rigorous techniques should be used for these cases.

1. Infected HeLa cells are harvested at 16–20 h post infection as follows The cells are scraped into the medium, pelleted at 1000 rpm for 3 min in a Beckman tabletop centrifuge, washed once with 1 mL of-phosphate-buffered saline, resuspended in 100 μL of 50 m*M* Tris-HCl, pH 7 5, 5 m*M* $MgCl_2$
2. The plasma membrane is solubilized by the addition of 4 μL of 10% Nonidet P-40 and storage at 25°C for 5 min.
3. The nuclei are separated from the cytoplasm by centrifuging for 1 s in a Brinkman microcentrifuge, washed in 100 μL 0 1% Nonidet P-40, 50 m*M* Tris-HCl (pH 7 5), 5 m*M* $MgCl_2$, and centrifuged again for 1 s. The supernatant fluid from the first pelleting may be saved and used as a crude cytoplasmic extract
4. Nuclear proteins are extracted following the addition of 100 μL of 20 m*M* Tris-HCl, pH 8.0, 420 m*M* NaCl, 1.5 m*M* $MgCl_2$, 0 2 m*M* EDTA, 0.5 m*M* DTT, 0 5 m*M* PMSF, 25% glycerol, and incubation at 4°C for 30 min, prior to pelleting of the nuclear debris and saving the supernatant fluid. The nuclear extract should be stored in 20-μL aliquots at –70°C if not assayed immediately.

3.8. (ADP-ribosyl)ation in Nuclei

Section 3.3. describes a technique for detection of protein poly(ADP-ribosyl)ated in infected cells. The addition of (ADP-ribose) to viral polypeptides in isolated nuclei is catalyzed by the cellular (ADP-ribose) synthetase using NAD as substrate. The modification that occurs in nuclei may differ from that which occurs in intact cells *(6)* and may result from either the elongation of pre-existing chains or addition at novel sites on the proteins.

1. Infected cell nuclei are isolated as described in Section 3.7.
2. Nuclei are resuspended in 25 m*M* Tris-HCl, pH 8 0, 3 m*M* $MgCl_2$, 20 m*M* KCl, 0 2 m*M* PMSF, 4 m*M* 2-mercaptoethanol containing 5 μ*M* (100 μCi) [^{32}P]NAD
3. The nuclei are incubated at 25°C for 30 min, pelleted, and the label is removed and discarded

4 The nuclei are then washed three times with 200 µL of reaction buffer minus NAD.
5. The nuclear proteins are then extracted as described in Section 3 7. and analyzed by using denaturing gels (*see* Section 3.1), followed by autoradiography

3.9. Nucleotidylylation in Nuclei

At least eight HSV proteins are nucleotidylylated *(12)*. This conclusion is based on the fact that viral proteins are labeled in isolated nuclei by either [α^{32}P]ATP, [α^{32}P]GTP, or [2-^{3}H]ATP, where the tritium atom is in the purine base.

1 Infected cell nuclei are isolated as described in Section 3.7
2. Nuclei are resuspended in 50 µL of 50 m*M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 30 m*M* of [α^{32}P]ATP or [α^{32}P]GTP (3000 Ci/mmol) plus 150 µ*M* ATP or GTP, respectively. The specific activity of [2-^{3}H]ATP is approx 25 Ci/mmol.
3 The nuclei are incubated at 15°C for 30 min and nuclear proteins are then extracted as described in Section 3.7
4. Visualization of the proteins is done conveniently by denaturing gel electrophoresis, followed by either autoradiography or fluorography (*see* Note 9).
5. As a result of the extremely long fluorographic times required to observe proteins labeled with tritium (>4 mo), we have developed a simple nitrocellulose filter binding assay (*see* Section 3.12) in order to measure the nucleotidylylation of proteins

3.10. Phosphorylation in Nuclei

Phosphorylation of viral nuclear proteins can be done exactly as described for nucleotidylylation in Section 3.9. simply by substituting [γ^{32}P]ATP for the labeled nucleotide. To differentiate between phosphorylation and nucleotidylylation, the unlabeled ATP or GTP is substituted for nonhydrolyzable nucleotide analogs, such as GTPγS or GDPβS (guanosine 5'-*O*-[2-thiodiphosphate]) *(13)*.

3.11. Induction and Purification of HSV Protein–GST Fusion Proteins

In general, herpesvirus proteins are not tolerated well when expressed in bacteria (*see* Note 10), presumably owing to either the high G-C content of the viral DNA or the high proline-rich content of the proteins. The following protocol reproducibly generates highly purified, intact fusion protein to yields as high as 8 mg/mL of bacterial culture.

1. Fresh single colonies of *E coli* BL21 cells containing glutathione-*S*-transferase (GST) fusion protein expressing plasmids are picked and incubated in 3 mL of Luria broth (L-broth) for no more than 6 h.
2. This 3-mL culture is used to inoculate a 50-mL overnight culture that is incubated no longer than 12 h.
3. This 50-mL overnight culture is used to inoculate 500 mL of L-broth, which is

grown to an OD_{600} of ~0.4–0.6, at which point 60 µL of fresh 20% IPTG is added and the cells are incubated for an additional 1 h All bacterial growth is at 37°C under ampicillin selection (100 µg/mL)

4. All of the following techniques are done at 4°C (wet ice). Pelleted cells are resuspended in 5 mL PBS, briefly sonicated, and 500 µL of Triton X-100 is added, prior to pelleting the cellular debris at 8000*g* in a Sorvall SS34 rotor
5. The supernatant fluid is mixed with 2.5 mL of a 50% slurry (v:v in PBSA) of glutathione-agarose (Sigma) and rotated for 30 min
6. The agarose beads are pelleted and then rinsed:
   a Two times in 10 mL of PBS;
   b. Two times in 10 mL of 0 02% Tween-20 in PBS (v·v), and
   c. Two times in 10 mL of 50 m*M* Tris-HCl, pH 8.3.
7. Fusion protein bound to the glutathione beads is purified following two sequential 1.5 mL elutions with 5 m*M* glutathione-50 m*M* Tris-HCl, pH 8.3.
8 Fusion protein are either used immediately or dialyzed into 50 m*M* Tris-HCl, pH 8.3, aliquoted, stored at –70°C, and thawed once prior to use.
9. The viral fusion polypeptides generated in this fashion are frequently suitable substrates for protein modification reactions. To test the usefulness of fusion proteins in nucleotidylylation reactions, the protocols described earlier for reactions in nuclei was done with nuclear extracts (*see* Section 3 7) by simply mixing the extract with an equal volume of fusion protein (~10 µg) in a 2X reaction cocktail (this brings the NaCl to 0.2*M*). The modified fusion proteins are then analyzed by either denaturing gels (*see* Section 3 1.) or by nitrocellulose filter binding (*see* Section 3.12)

3.12. Nitrocellulose Filter Binding of Posttranslationally Modified Proteins

The methods for specifically modifying viral proteins described above ultimately yield soluble radiolabeled polypeptides. The extent of protein labeling can be easily quantified in a nitrocellulose filter binding assay. This technique is sensitive and the method of choice for the analysis of tritiated polypeptides (*see* Note 11).

1 Nitrocellulose membranes (Schleicher & Scheull, BA83) are first wet in phosphate-buffered saline.
2. Mixtures containing radiolabeled polypeptides are then passed through the nitrocellulose under vacuum using a "dot-blot" manifold (Gibco-BRL, Gaithersburg, MD).
3. The filters are washed by sequentially passing 0 5 mL of phosphate-buffered saline through the membranes three times
4. The membranes are then dried for 30 min in an oven (130°C)
5. The radioactivity retained is measured by liquid scintillation using a Beckman scintillation counter To do this, the membranes are first dissolved in 1 mL tetrahydrofuran (Sigma) prior to adding 5 mL BCS-NA scintillant (Amersham)
6 The radioactivity of of ^{32}P-labeled proteins bound to membranes may also be quantified using a β-counter (Betagen)

3.13. DNA Polymerase

The DNA polymerase of HSV-1, the product of U_L30 gene, forms a complex with the product of the U_L42 gene. The U_L42 protein increases the processivity of the complex *(13)*. The polymerase activity is measured by using purified enzyme by measuring the formation of acid-precipitable counts starting with radiolabeled nucleotides *(15)*.

1. Infected cells are harvested 18 h postinfection and are lysed in 20 m*M* Tris-HCl, pH 7.5, 0.5 m*M* DTT by sonication.
2. The salt concentrations are adjusted to 1 7*M* KCl, 5 m*M* EDTA, 500 µg/mL bovine serum albumin (BSA) and the mixture is incubated on ice for 30 min, prior to pelleting the cellular debris and dialysis in 50 m*M* Tris-HCl, pH 7 5, 0.5 m*M* DTT, 0 2% NP40, and 20% glycerol.
3. The extract is passed over a DEAE-cellulose column and the polypeptides retained are eluted using a 0–0 3*M* KCl linear gradient. (Additional steps of purification may be performed in the same manner using sequential phosphocellulose and DNA-cellulose chromatography.)
4. Fractions are assayed for acid-precipitable radioactivity as follows Fifty microliters of sample is added to mixtures (200 µL) containing 6 6 m*M* Tris-HCl, pH 7.5, 3 m*M* $MgCl_2$, 2 m*M* β-mercaptoethanol, 100 µg of denatured salmon sperm DNA, 5 µM [^{3}H]TTP (50 Ci/mmol), and 0.3 m*M* each of dATP, dCTP, and dGTP and incubated at 37°C for 10 min.
5. The mixture is immediately spotted onto glass-fiber filters (Whatman GC/F) and washed three times in ice-cold trichloroacetic acid (10%), and then two times in ice-cold ethanol (95%), prior to drying
6. The incorporation of radioactive precursors into acid precipitable oligodeoxynucleotides is measured by liquid scintillation counting (*see* Section 3.12.).

3.14. Thymidine Kinase

Thymidine kinase (tk), the product of the HSV-1 U_L23 gene, has been used as a highly sensitive reporter gene product *(16,17)* in studies of viral gene regulation. In general, chimeric genes containing the coding sequences of the tk gene are either transfected into cells, which are then infected with a tk– virus, or the chimeric gene itself resides within the genome of the virus that is used to infect cells. In both cases, cells deficient in the cellular enzyme homolog are used (murine Ltk–, hamster BHKtk–, or human 143tk– cells). Thymidine kinase activity is measured in infected cell extracts and quantitated as cpm of [^{3}H]thymidine converted to thymidylate per µg of total cellular protein. Thymidylate formation is assayed by its retention on DEAE filter membranes.

1. The medium from a 25 cm^2 dish of infected cells (~4 × 10^6) is aspirated off and the cells are washed two times with 5 mL phosphate-buffered saline
2. Cellular proteins are extracted by adding 400 µL of 10 m*M* Tris-HCl, pH 7.5, 10

mM KCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol, 50 μM thymidine, 0 5% NP40 and incubating at 25°C for 5 min

3 KCl is added to 0 15M by the addition of 10 μL of 3M KCl, and the mixture (containing cellular debris) is shaken off of the plate into a microcentrifuge tube, which is then spun in a Brinkman microfuge for 2 min

4. Fifty microliters of the supernatant is then mixed with 10 μL of [^{3}H]thymidine (71 Ci/mmol), 5 μL cold thymidine (1 mg/mL) and 25 μL of 0 76M Tris-HCl, pH 7 5, 7 6 mM ATP, 7.6 mM $MgCl_2$, 12 mM phosphocreatine, 40 mM DTT, and 40 mM NaF, and 10 μL of creatine kinase (33 U/mL)

5 The mixture is reacted at 37°C for 30 min and is terminated by boiling in 0 1% SDS for 1 min

6 Duplicates of 50 μL of each reaction are spotted onto DEAE filter paper, which is then dried using a heat lamp

7 The filters are sequentially washed using the following solutions 10 mM ammonium formate for 15 min, 10 mM ammonium formate for 15 min, distilled water for 10 min, and ethanol (95%) for 5 min (*see* Note 12)

8 The filters are finally dried and the tritiated thymidylate bound is measured by liquid scintillation using a toluene-based fluor (e g., POPOP/PPO)

3.15. Protease

The HSV protease is encoded by the U_L26 gene. Its substrate is the protease precursor molecule itself that is cleaved twice, and ICP35, the more abundant product of the $U_L26.5$ gene cleaved once. U_L26 and $U_L26.5$ are 3' coterminal *(18,19)*. ICP35 forms the scaffolding of the capsid; during packaging of DNA, ICP35 is removed from the capsid. Two assays for the protease activity have evolved in the past 5 yr. The first assay employs the intact, transfected or infected cell. The second assay is based on in vitro cleavage of the substrates (the protease precursor and ICP35).

3.15.1. Protease Cleavage Following Transfection

The U_L26 proteolytic activity is assayed conveniently by transfecting a copy of the U_L26 gene (which inherently contains $U_L26.5$) that is driven by the viral α4 promoter, infecting the transfected cells with HSV-1(F) at 39.5°C, and then analyzing the ICP35 polypeptides by immunoblotting. Because HSV-1(F) contains a ts lesion in the α4 gene, it does not express its own U_L26 protease or its own ICP35 protein at 39.5°C.

1 Approximately 4 × 10^6 BHKtk- cells in a 25-cm^2 screw-capped culture flask are transfected *(15)* with 10 μg of a plasmid containing the U_L26 gene driven by the HSV-1 α4 promoter (*see* Note 13) and incubated at 37°C for 20 h

2. The cells are then cooled to 10°C for at least 30 min, prior to exposing them to 10 PFU of HSV-1(F) for 2 h at 10°C

3. Following absorption, the viral inoculum is quickly removed and replaced with

cold (4°C) medium The flask immediately is immersed in a circulated water bath preequilibrated at 39.5°C and incubated for 20 h (*see* Note 14)

4. The infected cells are washed twice in phosphate-buffered saline and harvested by scraping them off the dish After pelleting, the infected cell proteins are extracted by resuspension in 50 m*M* Tris-HCl, pH 7 0, 8 5% sucrose, 5% β-mercaptoethanol, 2% SDS, sonication on ice, and boiling for 1 min
5. The polypeptides are separated in denaturing gels (*see* Section 3 1), electrophoretically transferred to nitrocellulose, and the probed for ICP35 using monoclonal antibody H725 (*see* Note 7).
6. In order to control for the synthesis of the $U_L26.5$ gene product without proteolytic cleavage, it is necessary to repeat this procedure using plasmid that contains only $U_L26.5$ driven by α4 but not the amino portion of U_L26 *(18)*

3.15.2. Protease Cleavage in Cell-Free Extracts

The protease used in this assay is derived from the 635-amino acid open reading frame of U_L26 and it is either transcribed and translated in vitro *(20)* or expressed in bacteria as a fusion protein *(21)*. The induction and purification of bacterial fusion proteins was described in Section 3.11. The technique described below is for the in vitro expression of the protease from a recombinant plasmid. The cleavage measured is autoproteolysis and it is assayed by denaturing gel electrophoresis. Cleavage of the ICP35 substrate is performed easily by adding exogenous substrate to the reaction. The substrate may be either pure ICP35 protein, an ICP35 fusion protein, or a synthetic peptide that contains the core cleavage site Leu-Val-Asn-Ala/Ser *(22)*.

1 Linear DNA containing the U_L26 gene in the polylinker of a pGEM plasmid (Promega) is transcribed in the presence of the cap analog GppG (New England Biolabs) with RNA polymerase from either bacteriophage T7 or SP6 (Promega).
2. The U_L26 RNA is added to a mixture (50 µL) containing nuclease-treated rabbit reticulocyte lysate (Promega) and 10 µCi [^{35}S]methionine and reacted for 10 min at 4°C
3. Translation is inhibited by the addition of cycloheximide to 100 µg/mL.
4. The mixtures are then incubated for up to 6 h in order to allow the proteolysis to go to completion. At this time, the reactions are terminated by the addition of SDS to 0.1%.
5. The cleavage products are visualized following denaturing gel electrophoresis and autoradiography (*see* Section 3 1.).
6. Since the U_L26 protease is a serine protease, in vitro synthesis and incubation in the presence of 10–25 m*M* phenylmethylsulfonyl floride (PMSF) enables the formation of full length, noncleaved precursor protein.

3.16. Protein Kinase U_S3

The U_S3 gene product was predicted to encode a protein kinase based on its sequence *(23)*; this prediction later was confirmed experimentally *(24,25)*. The

assay for the U_S3 kinase is essentially identical to that previously described for the viral kinase of pseudorabies virus, inasmuch as both enzymes phosphorylate basic peptide substrates in vitro *(26,27)* In order to differentiate U_S3 activity from that of cellular kinases it is necessary to partially purify the kinase from infected cells by anion exchange chromatography *(27)*.

1. Infected cells are lysed by Dounce homogenization in 10 m*M* Tris-HCl, pH 7.5, 10 m*M* KCl, 1 5 m*M* magnesium acetate, cellular debris is pelleted, the supernatant is removed, and dialyzed in 20 m*M* Tris-HCl, pH 7 5, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol, 10% glycerol
2. The crude fraction is chromatographed on a DEAE-cellulose column and the bound polypeptides are eluted using a linear gradient from 0–0.4*M* KCl and tested for activity
3 Partially purified U_S3 is added to a mixture (100 μL) containing 20 m*M* Tris-HCl, pH 7.4, 50 m*M* KCl, 10 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, 0.1 m*M* [γ^{32}P]ATP (0.5 μCi), 0 8 mg protamine sulfate/mL, and incubated at 30°C for 30 min
4 The reactions are terminated by spotting them onto Whatman 3MM paper disks and washing them sequentially for 15 min each in the following: two times in 20% TCA, four times in 10% TCA; one time in ethanol (95%)
5 The disks are then dried and the amount of radiolabeled protamine remaining is measured by liquid scintillation (*see* Section 3 12)

3.17. U_L13 Kinase

Evidence that the product of the U_L13 gene is associated with protein kinase activity has been published *(28,29)*. Thus, in cells infected with U_L13– mutants, ICP22, ICP47, and VP22 are not processed and the accumulation ICP0 and the U_S11, U_L26, and $U_L26.5$ gene products is decreased *(27–31)*.

The gene product is a component of the virion *(32,33)*. However, since the protein itself has not been purified, evidence has not been presented that it has enzymatic activity.

3.18. Alkaline Exonuclease

The HSV-1 alkaline exonuclease is encoded by the U_L12 gene. The enzyme plays a role in viral growth and DNA synthesis The DNase activity can be easily assayed using agarose gel electrophoresis and measuring the conversion of form I DNA to form III as a function of the concentration of DNase *(34)*

1 Infected cell nuclear extracts are prepared as described in Section 3 7
2. Covalently closed circular DNA (0.5 μg) is mixed with the infected cell nuclear extract (5 μL) in reactions (25 μL) containing 50 m*M* Tris-HCl, pH 8.0, 4 m*M* β-mercaptoethanol, 10 m*M* $MgCl_2$ and incubated at 37°C for 30 min (*see* Note 15)
3. The reaction is stopped by the addition of 5 μL of 5% SDS, 0 15*M* EDTA, 0.1% bromophenol blue, and 30% glycerol.
4 The DNA is then resolved in an agarose gel using 40 m*M* Tris-acetate, 2 m*M*

EDTA buffer (pH 8.3), followed by ethidium bromide (0.5 μg/mL) staining and visualization by fluorescence enhancement.

3.19. Helicase/Primase

The HSV helicase-primase consists of three polypeptides that are the products of the U_L5, U_L8, and U_L52 genes *(35)*. Also, the HSV origin binding protein, encoded by the U_L9 gene, was shown to contain both a helicase activity *(36)* and a triphosphatase activity *(37)*. Although detailed biochemical studies of the helicase-primase were done using purified protein *(38)*, a convenient technique for measuring the helix-destabilizing activity is to assay its ability to decrease the melting temperature of DNA *(39)*.

1. Infected cell nuclear extracts are prepared as described in Section 3.7. and dialyzed against the extraction buffer to remove small molecules
2. The extract is passed over a Sephadex G-200 gel filtration column that pre-equilibrated in extraction buffer and fractionated
3. The helix-destabilizing activity is determined by measuring the affect of the fractions on the melting temperature (T_m) of poly(dT-dA), measured at 254 nm, using a temperature-controlled microcuvet and spectrophotometer (e g., Varian, Palo Alto, CA).
4. Twenty-five microliters of each fraction is mixed with 25 μg of poly(dT-dA) in 200 μL of 10 m*M* potassium phosphate, pH 7 8, 10% glycerol, and 50 μL of 100 m*M* $MgCl_2$. UV absorption measurements at 254 nm are taken each minute for 30 min at temperatures between 0 and 75°C
5. The reference sample contains buffer without DNA and control measurements are done using the same conditions but without any added protein.

3.20. Uracil–DNA Glycosylase

Uracil–DNA glycosylase is encoded by the U_L2 gene of HSV-1. Its function is to remove uracil residues from DNA generated by the insertion of dUTP or the deamination of dCTP *(40,41)*. The activity is assayed by measuring the release of tritiated uracil from DNA by infected cell protein.

1. Infected cells are harvested at 12 h post infection in 0 2*M* potassium phosphate, pH 8.0, 1 m*M* EDTA, 2 m*M* $MgCl_2$, 2 m*M* DTT, 1% Triton X-100, 1 m*M* PMSF, and 20% glycerol, briefly sonicated, and dialyzed in 50 m*M* Tris-HCl, pH 7.5, 1 m*M* DTT, 1 m*M* $MgCl_2$, and 20% glycerol.
2. Infected cell extract (10 μL) is added to mixtures (200 μL) containing 50 m*M* Tris-HCl, pH 7 5, 2 m*M* DTT, 100 μg/mL BSA, and 4 μg/mL of [^{3}H]DNA (*see* Note 15), and reacted at 37°C for varying times.
3. Reactions are terminated by rapid chilling to 4°C, followed by the addition of 25 μL of sheared calf thymus DNA (1 mg/mL) and 25 μL of 4*M* perchloric acid.
4. After 10 min at 4°C, the samples are microcentrifuged, the supernatant is removed, and the radioactivity present is measured by liquid scintillation (*see* Section 3.12.).

3.21. dUTPase

The herpesvirus dUTPase is encoded by the U_L50 gene and is not essential for virus replication in tissue culture cells. dUTPase was first described by Wohlrab and Francke as a deoxyribopyrimidine triphosphatase activity that was specific for cells infected with HSV-1 *(42)*. In this study, triphosphatase activity is assayed by measuring the production of radiolabeled deoxynucleoside monophosphates from the corresponding triphosphates.

1. Infected cell nuclei are prepared as described in Section 3 6 and are resuspended in 20 m*M* HEPES, pH 7 8, 1 m*M* DTT, 1 m*M* $MgCl_2$, 80 m*M* potassium acetate
2. Approximately 3×10^6 nuclei are added to reactions (50 µL) containing 1 m*M* DTT, 3 m*M* EDTA, 2 m*M* ATP, and 50 µ*M* [^{3}H]dUTP (2 Ci/mmol)
3. Reactions are initiated by the addition of $MgCl_2$ to 80 m*M* and incubated at 4°C, prior to termination with 20 µL of 0 1*M* EDTA and 125 µL cold methanol (4°C).
4. Four-microliter aliquots of the reaction mixture are mixed with unlabeled nucleotide markers (e g , 0 1 m*M* each of dUTP, dUDP, and dUMP) and spotted on polyethylimine(PEI)-cellulose thin-layer chromatography plates that were prewashed with 100% methanol The plates may be dried following the methanol wash, as well as prior to development without consequence
5. The TLC plates are developed with 1*M* HCOOH-0 5*M* LiCl at room temperature, dried, and examined by illumination with UV light
6. Spots containing nucleoside mono-, di-, and triphosphates are excised and the radioactivity contained in them is determined using liquid scintillation (*see* Section 3.12)

3.22. Ribonucleotide Reductase

The HSV-1 ribonucleotide reductase consists of two subunits, encoded by the U_L39 and U_L40 genes, which are tightly combined in an $\alpha_2\beta_2$ complex; both subunits are required for activity *(43)*. The enzymatic assay requires a high multiplicity of infection in order to get consistent results *(44,45)*.

1 Cells should be infected at an MOI of at least 20 and the infection should proceed for at least 7 h, prior to making cellular extracts (*see* Section 3.4.1.).
2 The infected cell extract is passed through a small cation exchange column (e g., AGI-X8, Bio-Rad), previously equilibrated 50 m*M* Tris-HCl, pH 8.0, 1 m*M* DTT, to remove nucleotides
3. Approximately 0.5 mg of infected cell protein is added to mixtures (100 µL) containing 5 m*M* Tris-HCl, pH 7.0, 50 m*M* $FeCl_3$, 4 m*M* NaF, 6.5 m*M* magnesium acetate, 3 m*M* ATP, 5 m*M* dithioerythritol, 5 m*M* CDP plus 1×10^5 cpm of [^{3}H]CDP and incubated at 37°C for 60 min
4 The reaction is stopped by the addition of 50 µL of 4*M* perchloric acid and boiling for 10 min. During this step the nucleotides are converted to monophosphates.
5. The mixture is then neutralized by adding 15–20 µL of 10*M* KOH prior to pelleting insoluble material by centrifugation

6 Thirty microliters of the supernatant are applied to the center of a PEI-cellulose plates prespotted with 2 5 m*M* cold CMP and cCMP markers.
7. The plate is washed by ascending irrigation once with distilled water, dried, and the buffer front at the top is removed and discarded.
8 The plate is then turned 180° and developed in the opposite direction in a solution of 20.40:100:0.5 (v:v) 5*M* ammonium acetate, pH 9.8:saturated sodium tetraborate:95% ethanol:0.25*M* EDTA.
9 After drying, the CMP and dCMP spots are identified by UV light illumination, excised, and the radioactivity present in each is determined by liquid scintillation (*see* Section 3.12.).

3.23. HSV-2 Ribonucleotide Reductase Protein Kinase

A protein kinase activity is associated with the large subunit of ribonucleotide reductase of HSV-2, but not that of HSV-1 *(10)*. Although the kinase autophosphorylates itself, the exact substrate of the kinase is not known. Therefore, to study this kinase it is necessary to separate it from the other viral kinases. This is done readily by immunoprecipitation (*see* Note 8) using an antibody specific for the large subunit.

1 Unlabeled infected cell extracts (25 µL) (*see* Section 3 4.1) are mixed with monoclonal antibody MAB30 and 20 µL of protein A-sepharose CL4B and incubated for 30 min at 4°C.
2. The beads are washed three times with 500 µL of 0.15*M* NaCl, 20 m*M* Tris-HCl, pH 7.4, resuspended in 50 µL of 20 m*M* Tris-HCl, pH 7 4, 5 m*M* $MgCl_2$, 0.1 µ*M* [γ^{32}P]ATP (10 µCi), and incubated at 30°C for 10 min
3. The reaction is terminated by boiling in 0.1% SDS prior to denaturing gel electrophoresis (*see* Section 3.1).

4. Notes

1. It is a standard and necessary practice to degas the separating gel solution prior to adding SDS and TEMED for optimum resolution of electrophoretically separated proteins in denaturing DATD gels. Note that 9.3% gels are aesthetically the most pleasing (!), but at times it is necessary to increase the percentage of polyacrylamide to as much as 17% to detect and resolve small mol-wt proteins.
2. The major cause of poor quality viral protein gels is overloading. Always determine protein concentration of the sample prior to loading Never load more than 75 µg viral protein on a 0.1-mm gel.
3 Protein gel solutions A, B, and C should be made as stocks; store A and B at room temperature and C in the dark at 4°C. Use a fresh ammonium persulfate solution and running buffer
4 When labeling viral proteins in vivo with phosphate, especially the α proteins, the trick is to keep the extent of labeling of host proteins to a minimum. The easiest way to do this is to be sure that you get an excellent infection of your cells; thus, be sure all cells are infected and the cell monolayer is at 70–80%

confluency at the time of labeling. Another way to get the same result is to starve the cells for phosphate prior to labeling, this helps when labeling early in infection. Late in infection it may have little or no effect and it may even be detrimental

5. In all cases, cells are grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 5% newborn calf serum. HEp-2 and BHK cells are the most commonly used cells for studies of viral proteins in this laboratory HeLa cells are useful for studies on proteins in nuclear extracts but these cells are partially restrictive to HSV replication Vero cells frequently express large amounts of a specific protein, but they also contain rather active proteases that have a predilection for numerous denatured viral proteins. Although we recommend against using cells other than HEp-2s and Helas, if you must, be certain that you solubilize the cells in SDS and boil them immediately. We recommend the use of HeLa S3 cells for the isolation of infected cell nuclei. Although infected cell nuclei from all cell types are extremely fragile, nuclei from S3 cells are the most stable that we have found.
6. It is extremely difficult to identify specific viral proteins if one labels infected cells with phosphate for a long period of time The preferred interval is less than 1 h at any time and two h late in infection. A major cause of uninterpretable results is the use of way too much [$^{32}P_i$] This also leads to radioactive waste problems Even amounts as low as 10 μCi/4 × 10^6 cells requires at least three monlayer washes with phosphate-buffered saline Caution should taken during this procedure to reduce the amount of radioactive "splatter" during these manipulations
7. Most standard immunoblot protocols (Western blots) can be used for the analysis of viral polypeptides. However, we have found that the lowest background levels are obtained following blocking for at least 1 h with 5% nondairy lowfat milk (e g , Carnation by Borden) in phosphate buffered saline (w v)
8. Two fluorographic techniques are used. The first involves impregnating denaturing gels with fluors (either 20% sodium salicylate or 20% 2,5-diphenyloxazole [PPO] in DMSO) prior to drying The second requires that the polypeptides are electrically transferred to nitrocellulose and dried prior the spraying them with En^{3}Hance (NEN). In both cases, fluorography is done by placing either the dried gel or membrane directly against X-ray film (Kodak X-OMAT) Depending on the specific activity of the labeled proteins, the film may require exposure times of 4 d to as long as 4 mo in order to get convincing signals.
9 In order to differentiate specific viral polypeptides from other viral or cellular proteins, it may be necessary to immunoprecipitate the proteins of interest prior to analyzing them. In our hands, efficient precipitations require preclearing the infected cell extracts prior to adding the antibody. In the case where a rabbit polyclonal antiserum is used, add preimmune serum, protein A-sepharose, and goat antirabbit immunoglobulin immunobeads directly to the extract, mix the slurry, pellet the beads, and finally, use the supernatant for the immune precipitation reactions.
10 Bacteria seldom tolerate long viral polypeptides. Depending on the piece of viral DNA used to make the fusion protein, varying extents of proteolysis will occur during the growth of the bacteria. The major factor affecting this is the length of the final fusion protein· thus, the length of viral peptide added should be kept as

short as possible. Although incubation of the bacterial cultures at a lower temperature (28–30°C) reduces nonspecific proteolysis sometimes at the expense of longer incubation times, the best way to reduce proteolysis is to delay the induction by IPTG as long as possible and to keep the induction period short; as little as 30 min of induction is sufficient for a late log phase culture

11 Although the nitrocellulose filter binding assay is able to pick up proteins labeled to low specific activities, one drawback is that the protein of interest must be pure Thus, it is ideal for use with viral fusion proteins that can be highly purified and can be compared easily to the parental (GST) protein. However, intact viral proteins must be purified partially either by immunological or chromatographic techniques prior to doing the filter binding assay.

12 Since the actual number of TK assays that are done at one time is significantly large, the process of washing the filters can be quite laborious. A convenient trick is to attach the numerous filters to a 1-mL glass pipet using paper clips and then dangle the filters into a plastic tray that contains the various wash solutions. In this manner, as many as 80 filters can be handled easily at one time.

13 Although most transfection techniques appear to give satisfactory results, we prefer the procedure described by Graham and van der Eb *(46)*. Although the purity of the transfected DNA is of obvious importance, it is not necessary to purify it by CsCl gradients, as long as the contaminating RNA is removed by polyethylene glycol precipitation In order for this transfection/infection technique to work, it is necessary that the U_L26 gene be driven by an α promoter; we recommend the promoter of α4.

14 At >10°C, HSV-1 binds but does not penetrate cells For consistent results, it is very important that one works fast when removing the inoculum and placing the flasks in the water bath so as not to allow the expression of post α genes The temperature of the water during must be very precisely regulated

15. Any covalently closed circular DNA (e.g., plasmid DNA) may be used, but care must be taken during the purification to insure that it is maintained as form I; CsCl gradients are recommended. Since the amount of viral DNase will be high, the nuclear extracts should be diluted in reaction buffer and several different dilutions should be tested. To prepare DNA specifically labeled in uracils, 1 mCi of [^{3}H]uridine is simply added to a 200-mL bacterial culture in log phase, which is then used to prepare the plasmid DNA

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18

HSV–Cellular Protein Interactions

David S. Latchman

1. Introduction

The herpes simplex virus (HSV) lytic cycle is dependent on a precise temporal pattern of viral gene expression with the initial expression of the immediate-early (IE) genes, followed by the early genes, and finally late gene expression *(1)*. Although such a temporal cascade of viral gene expression involves the action of virally encoded regulatory proteins, such factors act, at least in part, by interacting with cellular transcription factors that are present in the uninfected cell. Thus, although the HSV virion protein Vmw65 is essential for transactivation of the viral IE genes in lytic infection by binding to the TAATGARAT sequences in the promoters *(2)*, it can only achieve this by forming a complex with the cellular transcription factor Oct-1 *(3,4)* and other cellular factors *(5)*. Similarly, the IE promoters contain binding sites for other cellular transcription factors such as Sp1 *(6)* and this is also observed in the promoters for viral genes of other kinetic classes, such as the early gene encoding thymidine kinase *(7)*.

In addition to their role in the viral lytic cycle, cellular factors binding to viral promoters also are likely to play a critical role in producing asymptomatic latent infections of neuronal cells with HSV. Thus, viral IE gene expression is undetectable during latent infections *(8,9)*, and these infections can be established by viral mutants unable to express one or more of the viral IE genes *(10,11)*. Hence, latent infection is likely to involve a failure of viral IE gene expression leading to an abortion of the lytic cycle at an early stage. Although it was originally thought that the absence of IE gene expression could arise from the failure of Vmw65 to reach ganglionic neurons *(12)* this is now known not to be the case, since

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latency can be established readily in transgenic mice expressing Vmw65 in every cell *(13)*. Hence the failure of IE gene expression in neuronal cells must arise from an absence of a positively acting cellular transcription factor required for IE gene expression or from the specific expression of a negatively acting cellular factor that inhibits IE gene expression.

Hence an understanding of the processes whereby HSV promoters are regulated by cellular transcription factors is essential for our understanding of lytic and latent infections. In this chapter, I describe techniques that allow the identification of the regulatory elements in viral promoters that produce a particular pattern of expression. Subsequently, I describe the manner in which the cellular transcription factors binding to such sites can be identified. By using these methods, we have been able to identify a neuronally expressed cellular transcription factor, Oct-2, which is responsible for the inhibition of HSV IE gene expression in these cells *(14)*.

2. Assays

2.1. Promoter Assays

1. HEPES-buffered saline (HBS) 10X stock, 8.18% NaCl (w/v), 5 94% HEPES (w/v), 0.2% Na_2HPO_4 (w/v). Prior to transfection make a 2X HBS solution and adjust to pH 7 12 with 1*M* NaOH Filter sterilize. The pH is absolutely critical
2. Phosphate-buffered saline (PBS)· 8 g NaCl, 2 g KCl, 1.5 g Na_2HPO_4, 2 g KH_2PO_4/L
3 Dye reagent· 100 mg Coomassie brilliant blue G, 30 mg SDS, 50 mL 95% (v/v) ethanol, 100 mL 85% (v/v) phosphoric acid/L.

2.2. DNA-Binding Assay

1. Buffer A· 10 m*M* HEPES, pH 7.9, 1.5 m*M* $MgCl_2$ 10 m*M* KCl, 0.5 m*M* dithiothreitol (DTT)
2. Buffer C. 20 m*M* HEPES, pH 7.9, 25% glycerol, 1.5 m*M* $MgCl_2$, 0.25 m*M* ethylenediamine tetra-acetic acid (EDTA)
3. STE. 10 m*M* Tris-HCl, pH 7 6, 1 m*M* EDTA, 100 m*M* NaCl.
4 TBE 10 m*M* Tris-HCl, 10 m*M* boric acid, 2 m*M* EDTA, pH 8 3.
5 Buffer F· 50 m*M* NaCl, 20 m*M* HEPES pH 7.9, 5 m*M* $MgCl_2$, 0 1 m*M* EDTA, 20% glycerol, 1 m*M* $CaCl_2$, 1 m*M* DTT.
6. Sample loading buffer: 950 µL formamide, 25 µL 1% bromophenol blue, 25 µL 1% xylene cyanol.
7. Renaturation buffer· 10 m*M* HEPES pH 7 9, 1 m*M* DTT, 100 m*M* KCl, 0.1% NP40
8 Blocking buffer: 10 m*M* HEPES pH 7 9, 1 m*M* DTT, 5% nonfat dried milk
9 Hybridization buffer: 10 m*M* HEPES pH 7 9, 50 m*M* NaCl, 0.1 m*M* EDTA, 1 m*M* DTT, 0 25% nonfat dried milk
10 Washing buffer 10 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl.

3. Methods

3.1. Promoter Assays (see Section 4.1.)

3.1.1. Transfection

In order to test the features of an HSV promoter that result in it having cell-type specific activity or that allow it to be activated by a particular inducer or in response to viral infection, it must be linked to a marker gene encoding a readily assayable product, such as chloramphenicol acetyl transferase *(15)* or β-galactosidase *(16)*. A number of plasmid vectors containing the coding regions of these genes are now available and contain multiple cloning sites upstream of the coding region to facilitate insertion of a heterologous promoter *(17)*. Once this has been done, the hybrid construct is introduced by transfection into different cell types or into the same cell type treated in different ways, for example, with or without superinfection with HSV and any effect of the regulatory sequences on production of the assayable product is assessed.

In order to test promoter activity, it is necessary to introduce the construct containing it into cultured cells. A number of techniques exist for doing this, including treatment with calcium phosphate *(15)*. DEAE dextran *(18)*, and electroporation *(19)*. We have found the calcium phosphate procedure to be effective for many cell types and it is therefore presented here.

1. On the day before transfection (d 1), replate the cells to be used at a density of $10^4/cm^2$
2. On d 2, replace the culture medium with 5 mL of fresh medium containing 10% fetal calf serum. DNA is added to the cells 2 h later.
3. To prepare the calcium phosphate-DNA precipitate for a 90-mm dish containing 5 mL of medium, set up the following solutions. In tube A, place a solution containing 5–20 ng of DNA together with 31 mL 2*M* $CaCl_2$ and bring the final volume to 0.25 mL with water. To tube B, add 0.25 mL of 2X HBS.
4. To make the precipitate, the contents of tube A must be added to the HBS in tube B. The order of addition is crucial. Add the DNA solution dropwise to the HBS. The precipitate will form immediately.
5. Pipet the precipitate onto the cells by slightly tilting the dish and adding the precipitate to the medium. Put the cells back into the incubator immediately to ensure that the pH does not change.
6. Incubate the cells for 4–12 h. The longer incubation is sometimes required for promoters that are expressed weakly
7. Wash the cells in serum-free medium and then feed them with complete medium.
8. Harvest the cells on d 4. A test of transient expression can be carried out at this stage

3.1.2. Assay of Promoter Activity

Once the transfection protocol has been carried out, the cells can be harvested and promoter activity determined by assaying the activity of the enzyme

encoded by the test gene. The activity of the enzyme following transfection of different cell types or in differently treated cells provides a measure of the relative promoter activity under these conditions. In experiments of this sort, however, it is necessary to control for differences in the efficiency of DNA uptake between different cell types or under different conditions. This can be achieved by transfecting with constructs containing another promoter whose activity is unchanged in the different cell types. The constructs containing this promoter are transfected in parallel with those containing the regulated promoter and the activity in the different samples compared. However, it is preferable to transfect each cell sample with both the regulated and control promoter constructs. Hence, the activity of each promoter can be assessed in the same sample, controlling for variations in transfection efficiency between different plates of cells. To do this, the control and regulated promoters must drive the expression of different assayable proteins. We therefore give protocols for assaying the activity of chloramphenicol acetyl transferase and β-galactosidase in the same extract. All assays are carried out on samples that have been equalized for their content of total protein as described. The choice of which enzyme should be expressed from the control promoter and which from the regulated one is entirely arbitrary and will depend on the availability of control promoter constructs, vectors, and so on.

3 1.2.1. Chloramphenicol Acetyl Transferase Assay

This assay relies on allowing the enzyme to acetylate [^{14}C]-chloramphenicol and assaying the level of acetylated chloramphenicol by thin-layer chromatography (TLC).

1. Following transfection, wash the cells with PBS, harvest and transfer them to a 1 5-mL microcentrifuge tube
2. Add 100 µL of 0 25*M* Tris-HCl, pH 7.5 to the cell pellet
3. Disrupt the cells by freezing and thawing. To freeze–thaw, immerse the tubes in liquid nitrogen for 2 min, and then transfer them to a 37°C water bath Repeat the cycle three times.
4. Spin down the cell debris and save the supernatant to test for enzyme activity Samples may be saved at this point by storage at –20°C.
5. Depending on the cell type and promoter to be assayed, the amount of extract assayed may vary.
   The reaction mixture contains:
   a. 70 µL 0.25*M* Tris-HCl, pH 7.5.
   b 35 µL Water
   c, 20 µL Cell extract
   d 1 µL [^{14}C]-chloramphenicol (40–50 Ci/mmol) (Amersham, Arlington Heights, IL)
   e. 20 µL 4 m*M* acetyl CoA.

6. Incubate the reaction mixture for 30 min at 37°C The incubation time can be increased up to 60 min, provided enough active acetyl-CoA is added to keep the assay linear.
7. Extract the chloramphenicol with 1 mL ethyl acetate, by vortexing for 30 s.
8 Spin for 2 min in a microcentrifuge tube and save the top organic layer which will contain all forms of chloramphenicol.
9. Dry down the ethyl acetate under vacuum. This will take approx 2 h
10. Resuspend the chloramphenicol samples in 15 µL ethyl acetate and spot them onto silica gel TLC plates.
11. These plates are subjected to ascending chromatography with a 95:5 mixture of chloroform:methanol.
12 After air-drying, expose the chromatography plate to X-ray film After exposure, the regions corresponding to acetylated and nonacetylated chloramphenicol can be cut out and counted.
13. The percentage of total chloramphenicol converted to the monoacetate form gives an estimate of the transcriptional activity.

3.1.2.2. Beta-Galactosidase Assay *(16)*

1. Following transfections, treat the cells as described in Section 3 1 2 1 , steps 1–4 of the chloramphenicol acetyl transferase assay
2. Take 40 µL of cell extract, add 2 µL 0.1*M* DTT, 6 µL glycerol and 500 µL of 60 m*M* Na_2HPO_4, 40 m*M* NaH_2PO_4, $2H_2O$, 1 m*M* $MgSO_4$ $7H_2O$, 10 m*M* KCl, 40 m*M* β-mercaptoethanol. Vortex and incubate for 5 min.
3 Add 100 µL (2 mg/mL) ONPG (*O*-nitrophenyl-β-D-galactopyranoside), and incubate the reaction at 37°C until a visible yellow color is achieved. (This can take from 5 min to 24 h.)
4 Stop the reaction with 250 µL 1*M* Na_2CO_3.
5. Measure the colorometric change in a spectrophotometer at 420 nm.

3.1.2.3 Bradford Assay for Protein Concentration *(20)*

1. Following transfection, the cells are harvested and then lysed as described The lysed cells are then spun to remove the cell debris and an estimate of protein concentration can be performed on the supernatant to allow the assay of chloramphenicol acetyl transferase or β-galactosidase to be carried out on equal amounts of total protein.
2. Add 1 mL of dye reagent to 5 µL of each sample.
3. Measure the absorbance of the sample at 595 nm after 15 min.
4. If the absolute concentration of protein is needed, then a standard curve can be constructed using BSA as standard (draw A_{595} vs [BSA] mg/mL)

Once a region of the promoter that can confer a pattern of regulation on another gene has been identified, the promoter can be truncated until the effect is lost allowing identification of the precise region of the promoter that confers this effect (*see*, for example, ref. *21*). Subsequently, this region can be cloned into a

vector in which a heterologous promoter drives the CAT gene in order to determine if it can confer a specific pattern of regulation on a heterologous promoter *(22)*

3.2. Identification of Cellular Transcription Factors

The ability of a particular region of the gene promoter to produce a specific pattern of gene expression normally is dependent on its ability to bind one or more specific transcription factors that are present only in a specific cell type, that are activated in response to a particular stimulus, or that can interact with a virally encoded regulatory protein (for review, *see* ref. *23*). Hence, once a region of the promoter that produces a particular pattern of regulation has been identified, it is necessary to identify the transcription factors that bind to it so they can be characterized and their activity in different cell types and under different conditions investigated. To do this, whole-cell or nuclear extracts containing these factors are prepared and their binding to the specific DNA sequences investigated by several different techniques.

3 2 1 Preparation of Cellular Extracts

Extracts can be prepared either from whole cells or from isolated nuclei. All procedures are carried out at 4°C and the extracts stored at –70°C after preparation.

3 2.1.1. Preparation of Nuclear Extracts *(24)*

1 Harvest 5×10^7 to 10^8 cells and wash them with PBS.
2 Resuspend the cells in 5 vol of hypotonic buffer A and protease inhibitors, 0 5 m*M* PMSF 1 µg/mL pepstatin A, 1 µg/mL aprotinin, and 10 m*M* β-glycerophosphate, and stand them on ice for 10 min
3 Spin the cells at 1000*g* for 10 min and resuspend in 3 vol of buffer A
4 Add NP40 to 0.05% and homogenize the cells with 20 strokes in a tight-fitting homogenizer
5 Check the samples for release of nuclei by phase–contrast microscopy.
6. Spin at 1000*g* for 10 min to pellet the nuclei
7 Resuspend the nuclei in 1 mL buffer C with protease inhibitors as in step 2
8 Measure the volume of solution and add NaCl to 400 m*M* Incubate the solution on ice for 30 min
9 Spin at 16,000*g* for 20 min at 4°C in a refrigerated microfuge
10. Aliquot the supernatant and snap–freeze in liquid nitrogen. Store aliquots at –70°C

3.2.1.2. Preparation of Whole-Cell Extracts *(25)*

1 Resuspend the cells in buffer C.
2 Homogenize the cells with 20 strokes in a tight-fitting Dounce homogenizer
3 Add NaCl to a final concentration of 400 m*M* and incubate the solution on ice for 30 min.
4 Spin at 16,000*g* for 20 min at 4°C in a refrigerated microfuge.
5. Snap–freeze supernatant aliquots and store them at –70°C

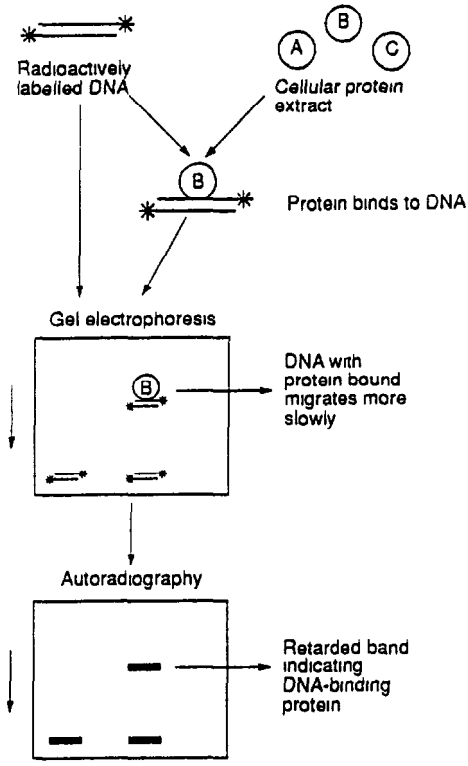

Fig. 1. DNA mobility shift assay in which the binding of a protein (B) to a radioactively labeled DNA sequence is detected by its ability to form a slow moving complex with the DNA

3.2.2. DNA Mobility Shift Assay

Once extracts have been prepared from the cells of interest, they can be used in a number of ways to study the proteins binding to a particular sequence. The simplest of these is the gel retardation or DNA mobility shift assay *(26,27)* that relies on the principle that a DNA fragment to which a protein has bound will move more slowly in gel electrophoresis than the same fragment without bound protein. Hence, the proteins binding to a particular DNA fragment can be investigated by radioactively labeling the fragment, incubating it with cell extract and electrophoresing on a nondenaturing gel. The retarded DNA–protein complexes can then be visualized by autoradiography (Fig. 1). Moreover, by carrying out the assay using proteins prepared from different cell types or from the same cell type under different conditions, the nature of the factors binding to a specific piece of DNA in different situations can be determined.

Once the proteins binding to this sequence have been identified in this way, it is possible to investigate the precise sequence specificity of this binding. This is achieved by including a large excess of an unlabeled oligonucleotide of specific sequence in the binding reaction. If the protein binding to the labeled oligonucleotide can also bind to this unlabeled oligonucleotide it will do so and the retarded band will disappear. The sequence specificity of a novel binding protein that can be determined in this way, may provide clues about its relationship to previously characterized transcription factors with identical or related DNA-binding specificities. Further information about the relationship of a novel factor to known factors can also be obtained by including antibody to a previously characterized factor in the binding reaction. If this antibody reacts with the protein of interest, it will either prevent its binding to DNA, so abolishing the complex, or produce a so-called supershift of the complex by binding to the DNA-bound protein and decreasing the mobility of the complex. Similarly, the interaction of a viral protein with the cellular protein can be followed either by comparing the patterns obtained from infected or uninfected cells or by adding a small amount of the purified viral protein and investigating whether a super-shifted complex containing the antibody is formed (*see*, for example, ref. *4*).

3.2.2.1. Labeling of Oligonucleotide Probes for DNA Mobility Shift Assay

1. Anneal the separately synthesized strands of the oligonucleotide by heating equimolar amounts of each to 80°C for 2 min, and then cooling slowly to room temperature
2. Incubate 2 pmol of annealed oligonucleotide with 20 μCi gamma [^{32}P]ATP in 50 m*M* Tris-HCl, pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* DTT, 0.1-m*M* EDTA and 4 U of T4 kinase at 37°C for 30 min.
3. Separate the labeled oligonucleotide from the free probe on a Sephadex G25 column and recover the void volume in 200 μL of STE One microliter of probe should be sufficient for each band shift incubation.

3.2.2.2. DNA Mobility Shift Assay

1. Set up a 20-μL binding reaction containing 4% Ficoll, 20 m*M* HEPES pH 7.9, 1 m*M* $MgCl_2$, 0.5 m*M* DTT, 50 m*M* KCl, 2 μg poly dIdC (Pharmacia, Uppsala, Sweden), 10 fmol double-stranded end-labeled oligonucleotide or DNA fragment probe and approx 2 μg of whole-cell or nuclear protein extract.
2. Incubate on ice for 40 min.
3. Load on to 4% polyacrylamide:*bis*-acrylamide (29:1) gel in 0.25X TBE and run in 0.25X TBE at 150 V for approx $2^1/_2$ h
4. Dry the gel under vacuum onto 3MM paper (Whatmann, Mainstone, UK) and autoradiograph.

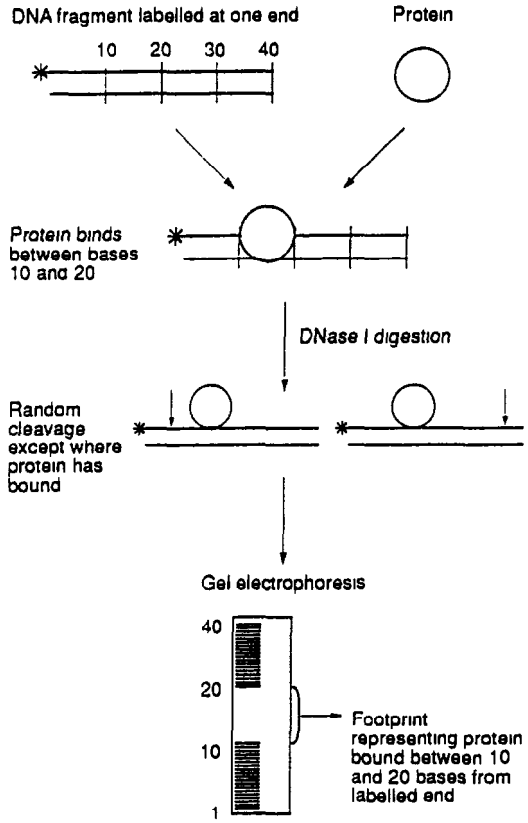

Fig. 2. DNase I footprinting assay in which the region bound by a protein is identified by its resistance to digestion by DNase I.

3.2.3. DNase I Footprinting Assay

Having identified a protein binding to a specific DNA sequence, the area of contact between the DNA and protein can be localized by using the same extracts to carry out a DNase I footprinting assay *(28,29)*. To do this, a double-stranded DNA fragment labeled at only one end is incubated with the protein extract and then digested with a small amount of DNase I. Each molecule will be cut only once or a very few times by the enzyme, giving rise to a ladder of bands when the sample is run on a denaturing gel. Regions where protein has bound to the DNA, however, will be protected from digestion and hence will appear as a blank area or footprint on the gel (Fig. 2).

1 Set up 100-µL binding reactions as for band-shift assay Incubate on ice for 40 min.

2 Dilute a 2-μg/μL stock of DNase I 1·100 immediately before use in buffer Add 1 μL to each sample and incubate at room temperature for a carefully timed 15–30 s
3 Stop the reaction by adding 100 μL 50 m*M* Tris-HCl, pH 8 0, 2% SDS, 10 m*M* EDTA, 10 μg glycogen, 0.4 mg/mL proteinase K Incubate at 37°C for 30 min, and then at 70°C for 2 min
4 Extract the reaction with phenol chloroform (1 1) and then with chloroform And 15 μL 5*M* LiCl and 600 μL ethanol and leave the samples overnight at –20°C to precipitate the DNA
5 Spin down the DNA and resuspend it in 5–10 μL of sample loading buffer. Load 20–50 counts per second in each well and run on a 6% denaturing polyacrylamide:*bis*-acrylamide (19:1) gel containing 1X TBE and 42% urea (w/v)
6. Dry the gel under vacuum, and autoradiograph

3.2.4. Methylation Interference Assays

The interaction between a DNA-binding protein and its specific DNA-binding site can be more precisely studied using the methylation interference assay in which the effect on the binding of the protein of methylating specific G residues in its binding site is assessed *(30)*. This method allows the precise assessment of the interaction of the DNA binding protein with individual nucleotides within its binding site. To do this, the DNA is partially methylated so that on average only one G residue per DNA molecule is methylated, and used in a standard DNA mobility shift assay. Following electrophoresis, the DNA that has bound protein and that which does not are both excised from the gel, and their level of methylation at specific G residues compared by cleaving methylated Gs with piperidine *(31)*. A lack of methylated G residues at a particular site in the protein-bound DNA indicates that methylation at this G blocks protein binding, and that it therefore plays a critical role in protein binding (Fig. 3).

Methylation interference can therefore be used as a supplement to DNase I footprinting by identifying the precise protein: DNA interactions within the footprinted region.

1. To prepare partially methylated probe, add end-labeled DNA to 200 μL 50 m*M* sodium cacodylate pH 8 0, 1 m*M* EDTA
2. Chill on ice and add 1 μL dimethyl sulfate (DMS) Incubate at 20°C for 3 min
3. Add 2 5 μL 3*M* sodium acetate pH 7 0 and 600 μL ethanol and incubate at –20°C overnight to precipitate the DNA.
4 Following centrifugation, discard the DMS-containing supernatant into a 5*M* NaOH solution. Wash the pellet with 70% ethanol, dry it, and resuspend it in 10 μL of water.
5 Carry out a 120–200 μL DNA mobility shift reaction using 50–100 fmol of partially methylated probe (approx 4×10^5 cpm).
6. Incubate the samples for 1 h on ice and load them onto three to five wells of a

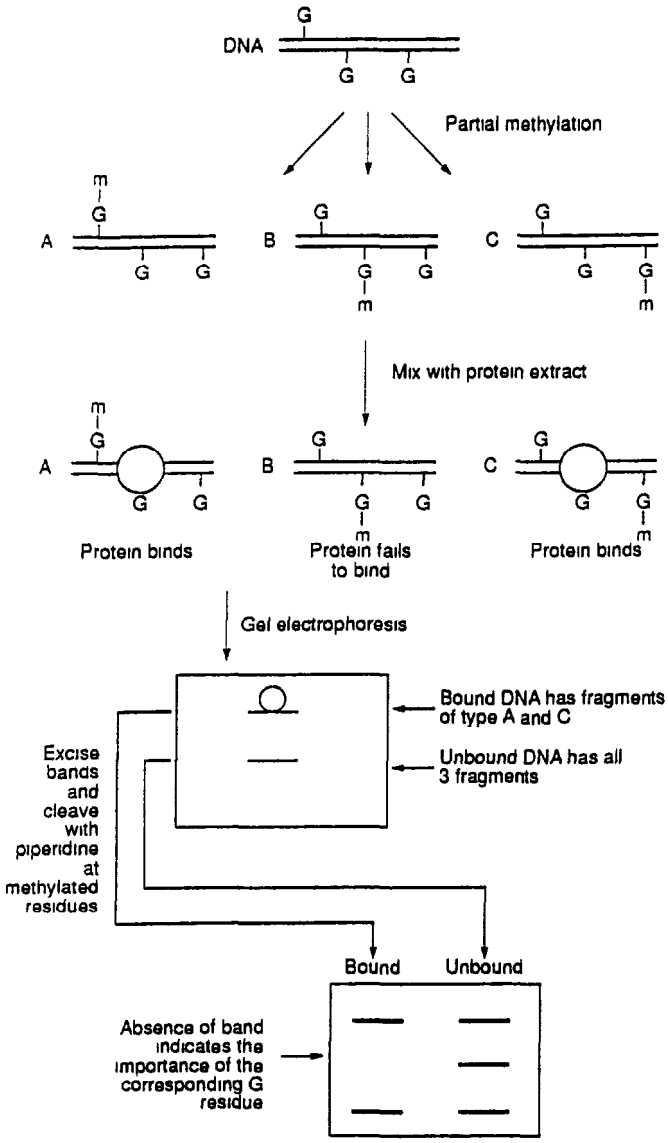

Fig. 3. Methylation interference assay in which the guanine residues that are critical for binding of a protein to the DNA can be identified by the fact that their methylation prevents protein binding and hence the formation of a retarded complex in a DNA mobility shift assay

polyacrylamide gel prepared as for the DNA mobility shift assay

7 After running, cover the gel with clingfilm and expose it to X-ray film for approx 4 h

8 Excise the retarded and unretarded bands and extract the DNA with 1*M* LiCl Add 10 μg glycogen, phenol extract, and then ethanol precipitate the DNA
9. Redissolve the DNA in 10 μL 1*M* piperidine (freshly diluted) and heat it to 90°C for 30 s
10 Cool the sample on ice, pulse-spin it in a microfuge, and freeze-dry the sample
11 Redissolve the DNA in 50 μL of water and freeze-dry again
12 Resuspend the DNA in 5 μL sample loading buffer, heat at 90°C for 3 min and freeze it until ready to load onto a denaturing polyacrylamide gel.

3.2.5. Southwestern Blotting

The techniques of DNA mobility shift, DNase I footprinting, and methylation interference discussed in previous sections can provide considerable information on the nature of the interaction between a particular DNA sequence and transcription factors. They do not however, provide information on the protein itself and its characteristics. Ultimately, such information can be obtained by cloning the gene encoding the transcription factor using one of a number of different methods that are beyond the scope of this chapter (for a discussion, *see* refs. *32* and *33*) Prior to this, however, it is possible to determine the size of the protein by using the technique of Southwestern blotting *(34)*. In this method, protein extracts are electrophoresed on a standard SDS-polyacrylamide gel and the separated proteins transferred to nitrocellulose membrane and probed with a radioactively labeled oligonucleotide containing the DNA-binding site of interest. A protein capable of binding to this specific site will do so, producing a radioactive band, and its size can be determined by comparison to marker proteins of known size.

1 Electrophorese approx 50 μg of protein extract on a standard SDS-polyacrylamide gel and transfer onto a nitrocellulose membrane at 20 mA overnight in 25 m*M* Tris-HCl, 200 m*M* glycine, 20% methanol.
2 Denature the protein bound to the filter in 6*M* guanidine-hydrochloride for 30 min
3. Renature the protein overnight in renaturation buffer
4 Incubate the filter in blocking buffer for 60 min at room temperature with gentle agitation.
5 Incubate the blot for at least 2 h (or overnight) in hybridization buffer with about 3.5×10^7 cpm/mL of [^{32}P]-labeled concatamerized oligonucleotide probe.
6. Wash the blot in washing buffer, wrap it in clingfilm, and autoradiograph.

4. Notes

4.1. Promoter Assays

1 The amount of DNA added to each transfection should be the same This can be achieved by adding appropriate amounts of salmon sperm DNA
2 Great care is needed in making up the HBS buffer since the pH is very critical for these experiments.

3. If the precipitate looks dense and opaque, rather than translucent, the HBS has not been prepared at the correct pH

4.1.1. Chloramphenicol Acetyl Transferase Assay

4. Acetyl-CoA is very unstable and should be made up fresh or kept at –20°C for not more than 10 d
5. The TLC tank should be lined with filter paper around the inside to assist equilibrium. The solvent should be made up fresh every day, since chloroform is very volatile

4.1.2. β-Galactosidase Assay

6 It is important to remember that mammalian cells contain a eukaryotic isozyme for β-galactosidase. Therefore a blank containing a nontransfected cellular lysate should be included in the experiment

4.2. Identification of Cellular Transcription Factors

4.2.1. Preparation of Whole-Cell Extracts

7. To prepare extracts from tissues, grind the tissue in liquid nitrogen to a fine powder before resuspending in buffer C
8. For tissue extracts it may be necessary to use a tissue macerator instead of a Dounce homogenizer to get efficient disruption of cells.

4.2.2. DNA Mobility Shift Assay

9 For fragment probes, the phosphates at the end of the purified fragment must be removed with calf intestinal phosphatase prior to kinase labeling
10. For preparing concatamerized oligonucleotide probes for use in Southwestern blotting kinase, treat the annealed oligonucleotide in 50 m*M* Tris-HCl, pH 7.6, 10 m*M* $MgCl_2$, 5 m*M* DTT, and then add DNA ligase and ATP to 5 m*M* and allow to ligate overnight at room temperature. The kinased, ligated concatamers are then separated from free nucleotides on a Sephadex G50 column by collecting the first peak.
11. Prior to use, the protein concentration of different extracts is equalized based on assays of their protein content
12. For competitor assay, competitor oligonucleotides are added to the mixture at onefold, 10-fold, and 100-fold molar excess before addition of the extract.
13. For antibody assay, 1 μL of each of a series of dilutions of the antiserum under test is added to the binding reaction before addition of the extract Similar dilutions of preimmune serum are added to parallel reactions as a control.
14. The gel is pre-electrophoresed before addition of the samples until the current drops from 30–10 mA (approx 2 h).
15. The gel is run until bromophenol blue in a separate marker track has run approx two-thirds of the way down the gel.

4.2.3. DNase I Footprinting Assay

16. In initial experiments it will be necessary to titrate the amount of extract used, the amount of poly dIdC and the magnesium concentration in order to obtain the appropriate level of digestion with DNase I.

17 In contrast to the DNA mobility shift assay where the probe can be labeled at both ends, the probe must be labeled at one end only. The probe is therefore prepared in the same way as before and the labeled fragment is then digested with a second restriction enzyme and gel purified to isolate a fragment with only one labeled end Use approx 5 fmol of probe per reaction labeled to 50–100 counts per second.
18. The gel is prerun for approx 30 min prior to loading and then run at 1600 V/30 mA

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Models of Recurrent Infection with HSV in the Skin and Eye of the Mouse

Terry J. Hill and Carolyn Shimeld

1. Introduction

Animal models remain essential for studies of many aspects of the biology of herpes simplex virus (HSV). Such studies include basic experiments on pathogenesis (including characterization of viral mutants), tests of antiviral drugs, and methods of immunization. With reference to models of recurrent infection, high levels of recurrence and clinical disease have been achieved with guinea pigs (particularly with genital infection) and rabbits (particularly with ocular infection; reviewed in ref. *1*). However, in contrast to these animals, with the laboratory mouse there are many inbred and congenic lines; a major advantage for immunological studies. To this can now be added the growing technology of transgenic and "knockout" animals. For these reasons we have expended considerable effort in developing various mouse models of infection, particularly with HSV type 1 (HSV-1).

This chapter focuses primarily on murine models of experimentally induced recurrent infection in the skin or eye. The basic pattern of these models is similar and consist of two stages: first, the production of latently infected animals by means of a primary infection, usually at a peripheral site; and second, in animals that have recovered as fully as possible from the primary infection (usually after a period of at least 4 wk following inoculation), the experimental induction of reactivation involving either a systemic or local stimulus, leading to virus shedding with or without clinical disease in the skin or eye. In the first stage, animals usually are infected at a particular site in the skin or eye with doses and strains of virus that produce as many latently infected survivors as possible and under circumstances that leave as little residual damage as possible at the inoculation site. It is particularly important to maintain the integrity

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of the peripheral nerves, as these transport reactivated virus from the ganglion to the periphery and may be involved in the transmission of signals to the neuronal nucleus during reactivation *(2)*. Moreover, recognition of a recurrent lesion is not possible on the background of a badly damaged tissue. Corneal nerve damage with a resultant loss of corneal sensitivity that accompanies chronic severe stromal keratitis is the most common outcome of primary infection of the eye in the mouse *(3)*. A secondary bacterial infection may contribute to this severity, but even when the infection is prevented by antibiotic treatment the keratitis remains severe *(3)*. We were the first group to report recurrent corneal disease and shedding of virus in the tears of mice, but such signs were only seen in the small proportion of mice that survived primary infection with undamaged eyes *(4)*. To increase the incidence of mice that have the potential to develop recurrent corneal disease now we routinely use passive immunization prior to corneal inoculation of virus *(5,6)*. Such treatment protects from stromal keratitis and death while allowing a high incidence of latent infection to be established in the ophthalmic part of the trigeminal ganglion.

In addition to these models of true recurrent infection, the zosteriform spread model is also described since it has been argued that, even though this involves only the primary infection, it provides a useful analog of recurrent disease *(7,8)*. In this zosteriform model, virus is inoculated in the skin of the neck where primary infection occurs. From this site virus spreads, probably by retrograde axonal flow to the cervical sensory ganglia (mainly the second to fourth) and the central nervous system (CNS). Further spread, probably at various points within the nervous system, allows virus to reach axons that do not supply the inoculation site itself but that supply skin within the same dermatome as this site. Via these axons, the virus then spreads, probably via orthograde flow, at approx d 3 after the primary inoculation, to these more "distant" skin sites; in this case including the ear pinna on the inoculated side. This in turn produces clinical herpetic lesions on the pinna (erythema as early as d 4 or 5 and lesions on d 5 or 6). Hence, like true recurrent lesions following reactivation of latent infection (described later), zosteriform lesions on the pinna are relatively short-lived, virus is delivered to the skin via nerves and since the zosteriform lesions do not develop until d 5 or 6, like recurrent lesions they develop in the presence of some degree of immunity. The phenomenon of zosteriform spread is not restricted to skin; virus can be delivered to the eye via nerves following inoculation of the snout *(9)*. Zosteriform disease also has the advantage of developing within a well defined time with respect to the primary inoculation and after inoculation in the neck can occur in 100% of animals. The analogy between zosteriform and recurrent disease, however, is not perfect since the dose of virus delivered to the skin in zosteriform spread almost certainly is far greater than that in a true recurrence. Moreover, the nature of the immunity present in

the two situations will be different since with zosteriform disease the immune responses to the virus will be in the early stages of development, whereas in recurrent disease the animal may have had immunity for many months.

In both the ear and the zosteriform model, the pinna offers a number of advantages for the observation of herpetic disease. These include the following: the provision of a flat, relatively hairless area that is easy to inoculate and, in albino animals, on which lesions are easily visible (in albinos, even the mildest inflammatory response is visible as erythema). Other indicators of disease are provided by measurement of ear thickness, isolation of virus or histological observations from small punch biopsies, development of ear paralysis resulting from demyelination in the seventh cranial nerve root *(10,11)*. Moreover, the flat relatively hairless pinna allows easy application of drugs.

It is commonly stated that mice do not show spontaneous reactivation of latent infection, as evidenced by the development of spontaneous recurrent disease or the presence of virus at the periphery in the absence of clinical disease. In fact, spontaneous shedding of virus in the tear film does occur in mice *(4,12–14)*, but at a much lower incidence (3–0.8%) than in the rabbit In addition, virus has been isolated from clinically normal mouse skin *(15)* and, more rarely, spontaneous recurrent skin lesions are observed *(16)*. It has been argued that this apparently low incidence of spontaneous reactivation in the mouse is a disadvantage of this animal for experimental studies with HSV. However, the absence of frequent spontaneous disease gives the advantage of a clean "background" for experimentally induced reactivation. Moreover, as already mentioned, the immunological advantages of the mouse, provide strong arguments for the continued use of this animal.

Three models will be described: for brevity these will be referred to as the ear model, eye model (both involving latent and recurrent infection), and the zosteriform model (the analog of cutaneous recurrent infection but involving primary disease only).

2. Materials

1. Strain and dose of virus: in the eye model a dose of 10^4 PFU of HSV-1 strain McKrae; in the ear model doses of 10^5–10^6 PFU of HSV-1 strain SC16 have been used routinely (*see* Note 1).
2. Mice: 8- and 4-wk-old female NIH mice (inbred strain, H2 q) for the eye and ear models, respectively (*see* Note 2) Four- or eight-week animals are suitable for the zosteriform model This strain is available from Harlan UK Ltd (Bicester, Oxfordshire). All animals should be Specific Pathogen Free (*see* Note 3)
3. Anesthetics:
   a. 60 mg/mL Sodium pentobarbitone (Sagatal, Rhone Merieux, Harlow, Essex, UK);
   b. 10 mg/2 mL Midazolam hydrochloride (Hypnovel, Roche Products, Welwyn Garden City, UK);

c. 0.315 mg/mL Fentanyl citrate and 10 mg/mL fluanisone (Hypnorm, Janssen Pharmaceutical, Oxford, UK),
d. 100 mg/mL Ketamine (Vetalar, Parke-Davis Veterinary, Pontypool, Gwent, UK); and
e. 20 mg/mL Xylazine (Rompun, Bayer plc, Bury St. Edmunds, Suffolk, UK)

4. Antibiotic cream Chloramphenicol eye ointment BP 1% w/w (Daniels Pharmaceutical, Derby, UK).
5. Corneal stains
   a. 2% w/v Fluorescein sodium BP (Fluorescein minims eye drops); and
   b. 1% w/v Rose Bengal (Rose Bengal minims eye drops), both from Smith and Nephew Pharmaceuticals, Essex, UK)
6. For retinal examination.
   a. Mydriatic: 1% cyclopentolate hydrochloride BP with 0.01% benzalkonium chloride (Mydrilate 1%, Boerhinger Ingleheim, Berkshire, UK); and
   b. 2% Sodium carboxymethyl cellulose with 0.01% benzalkonium chloride eye drops
7. Shredded paper bedding supplied by W. M. Lillico & Son (Betchworth, Surrey, UK).
8. Hanovia UV lamp (Slough, Buckinghamshire, UK) or similar that emits a peak of 4.02 mj/cm^{2}s at 320 nm

3. Methods

3.1. Setting Up Infected Mice

3.1.1. Anesthesia

1. 0.2 mL of neat Sagatal injected intraperitoneally is used for terminal anesthesia (*see* Note 4)
2. For surgical anesthesia two methods are used (*see* Note 5). Either dilute hypnorm 1:8 in sterile water or dilute hypnovel 1:8 in sterile water. Inject as two separate injections (0.2 mL of each for a 25–30 g mouse) subcutaneously in the scruff of the neck or add 1 mL Vetalar and 0.5 mL Rompun to 8.5 mL sterile water and use the mixture at 0.25–0.3 mL for a similar size animal, given by intraperitoneal injection.
3. For sedation inject 0.2 mL of the described dilution of hypnorm subcutaneously in the scruff of the neck

3.1.2. Labeling Mice

Mice should be individually labeled by tattooing the tail with Indian ink. For this, use a 1-mL syringe containing ink and fitted with a 26-gage needle. Marks are made on the tail by injecting a very small amount in a line under the skin. A mark at the tip of the tail represents a single unit (up to 4 can be tattooed) and a mark at the base of the tail 5 U. Thus an animal with four marks at the tip is number 4, one with one mark at the tip and one mark at the base is number 6 and so on. If done carefully these marks last for the lifetime of the animal.

3.1.3. Methods and Sites of Inoculation

In all cases animals must be given surgical anesthesia before inoculation.

3.1.3.1. The Eye Model

1. The eyes of all mice used for ocular experiments should be examined using a slit-lamp microscope *(see the following)* before use; any with ocular abnormalities should be discarded (*see* Note 6).
2. To produce mice suitable for the induction of recurrent infection, 24 h before inoculation of virus on the cornea mice are given an ip injection of 0.5 mL of rabbit serum containing antibodies to HSV-1 (*see* Note 7). The serum is diluted to give a dose of 800 ED50 (*see* Note 8).
3. While viewing through a dissecting microscope, the mouse is held on its side, the index finger and thumb are placed either side of the eye and by application of gentle downward pressure the globe is proptosed and partially immobilized
4. The required dose of virus, in a volume of 5 µL is placed onto the eye and the cornea is lightly scarified in a grid pattern of five horizontal and five vertical lines using the sharp edge of the bevel of a 26-gage needle. The amount of damage done to the cornea by scarification is critical, since the corneal epithelium is very heavily innervated by sensory nerves and slight deviations in the extent of damage will directly affect the amount of virus entering nerve endings and thus the severity of disease The inoculator must establish the "correct" amount of damage for his system and then aim to repeat this amount consistently (*see* Note 9).
5. With the drop of inoculum remaining on the cornea, the animal is then left on its side to recover from anesthesia
6 For all ocular studies it is recommended that saw dust bedding is avoided since the small particles can enter the eye easily and produce corneal damage. To avoid this, mice inoculated on the cornea are routinely housed on shredded paper.

3.1.3.2. The Ear Model

Two methods of inoculation can be used. In the first, as originally described *(16)*, virus is inoculated under the epidermis of the upper surface of the pinna (*see* Note 10). Because of the thinness of the pinna, this is technically demanding and some practice is necessary. Use of an illuminated large low power magnifying glass may be helpful.

1. With the mouse anesthetized and the dorsal surface of the pinna uppermost, the pinna is grasped gently between the index finger and thumb.
2. The inoculum, usually 10 µL, is delivered from an accurately graduated glass syringe through a short bevel, 26-gage 3/8-in (0 45 × 10 mm) needle. The needle should enter in the middle area of the dorsal surface of the pinna The most commonly encountered problem is penetration of the needle through the pinna Therefore, it is recommended that a rubber guard is worn on the tip of the index finger. A suitable guard can be made from a cutoff small rubber teat.

An alternative site of inoculation for the ear model is to make use of the phenomenon of zosteriform spread by inoculating the skin of the neck. It has been shown that this site of inoculation also leads to latent infection of ganglionic neurons that supply the pinna. Hence, recurrent disease can be induced on the pinna, even though this was not the original site of inoculation *(7)*.

i Before inoculation of the neck it is necessary to remove the fur from the site Do not use chemical preparation or soaps in this process, since these agents may cause inactivation of the virus in the inoculum With the mouse anesthetized, the fur to be removed is liberally wet with clean, warm water. With the mouse lying under slight tension in one hand the fur is carefully shaved from the ventral surface of the neck and the skin and surrounding fur is then dried, a size 10 round-end scalpel is best for the shaving

ii With the mouse lying on its back, a 10-µL drop of virus suspension is placed onto the dry, shaved skin on the ventral surface of the neck, just caudal to the angle of the jaw and ventral to the bottom edge of the pinna

iii The drop is spread out slightly on the skin with the shaft of a 26-gage needle and with the sharp edge of the bevel, 10 parallel scarifications of the neck skin are made in the craniocaudal direction, through the inoculum fluid During the scarification it helps to keep the skin under slight tension in a craniocaudal direction. Care should be taken to avoid scarifying over the midline otherwise herpetic lesions will occur on both ears As in the eye model, the amount of scarification is critical and more control can be gained by observing the procedure under a low power dissecting microscope

iv The mice are left to recover on their back, with the inoculum left in place.

In setting up the zosteriform model itself, the procedure for inoculation of the neck with virus is the same as described.

3.2. Reactivation of Latent Infection

Mice usually are left for at least 4 wk after the primary infection before reactivation is attempted. For reactivation in the eye model mice must have been passively immunized before inoculation of virus. In all cases, animals must be given surgical anesthesia for the reactivation procedure.

3.2.1. In the Eye Model by UV Irradiation

1 Turn on a UV lamp and leave to warm up according to the manufacturer's instructions

2 Collect eyewashings, from the originally inoculated eye, for the isolation of virus *(see the following)* to check for spontaneous shedding of virus.

3 Put the mice under the UV lamp, using the thumb and index finger exert a slight downward pressure on the skin around the appropriate eye to proptose the eye slightly (*see* Note 11) and hold the mice in position 22 cm from the UV bulb for 90 s (*see* Note 12) The operator should wear protective glasses that screen out UV and protect hands with a high factor sun screen and gloves.

4 Apply antibiotic cream to the eye immediately after UV irradiation and daily for the next 7 d

3.2.2. In the Ear Model by Mild Skin Trauma

For this, cellophane tape is pressed gently onto the upper surface of the pinna of the originally inoculated ear (or the pinna on the same side as the original neck inoculation). This is repeated with a fresh area of tape a further five times (*see* Note 13).

3.3. Methods Applicable to the Observation of Recurrent Infection, Zosteriform Disease, and the Detection of Latent Infection

3.3.1. Clinical Disease

3.3.1.1. In the Eye

1. Surgically anesthetize the mice.
2. Arrange the slit lamp microscope so that you can rest your elbows on the bench and your hands on the chin rest (to reduce shake) and the hands are more or less in focus. Hold and support the mouse in a cupped left hand, use the thumbs to open its eyelids and slightly proptose the eye Focus on the eye by moving the mouse (rather than moving the microscope, which is the normal procedure when examining human patients) (*see* Note 14)

3.3.1.2 On the Pinna of the Ear

In the ear and zosteriform models in albino mice, erythema provides an easily observed (*see* Note 15), early, and sensitive indicator of disease in the pinna *(16)*. With respect to recurrent disease obviously it is important to distinguish between erythema owing to the trauma of the reactivating stimulus itself, e.g., cellophane tape stripping, and erythema due to recurrent disease. With cellophane tape stripping it is possible to define criteria to make this distinction *(17)*. For example, if erythema persists after stripping it is considered to be significant only if it lasts longer than 3 d, since by this time 98% of stripped uninfected control mice are normal. If erythema develops after a period of normality it should remain for at least two consecutive days. By these criteria, the incidence of recurrent disease following stripping is found to be between 7 and 32% in different groups of animals.

Observation of lesions such as vesicles, pustules, and small scabs, however, requires the mouse to be anesthetized or at least sedated to allow careful observation of the skin of the pinna under a dissecting microscope. In the ear model, vesicles are first visible on d 3 after cellophane tape stripping. Vesicles last only a few hours and rapidly develop into pustules which then scab, ulcerate, and heal *(18)*. The angle of illumination is critical in observing lesions such as early vesicles (*see* Note 16).

In the zosteriform model, again the earliest sign of clinical disease in the pinna is erythema. However, presumably because of the larger amounts of virus

reaching the pinna, it is often difficult to see discrete lesions, such as isolated vesicles, under the dissecting microscope. In this case, confluent areas of lesion in the epidermis rapidly reach a scab stage.

3.3.2. Other Indicators of Recurrent Infection in the Skin or Eye

3.3 2 1 Isolation of Virus from Sites of Recurrent Infection

In the ear model, swab skin lesions or take small punch biopsies of skin.

In the eye model, the tear film can be sampled by placing 20 µL of tissue culture medium onto the eye (surface tension will hold this in place). Using a pipet tip, irrigate and aspirate the fluid 10 times, being careful to avoid touching the cornea. This procedure is done on surgically anesthetized mice and therefore can be repeated on subsequent occasions. More rigorous searches for virus require the mouse to be killed so that ear or eye homogenates or scrapings can be taken. Material collected from such samples is inoculated onto tissue culture cells sensitive to HSV-1, e.g., Vero cells, and these are observed for cpe

3.3.2.2. Detection of Antigens

As further evidence of recurrent infection in the skin or cornea, viral antigens can be sought. Obviously this is applicable to sections of the pinna or eye but, without the tedious procedure of serial sectioning, it is easy to miss small foci of infection. To overcome this problem, we have developed methods for examining preparations of whole tissue sheets from the cornea or the pinna *(19–21)*

3.4. Detection of Virus in Ganglia

Clearly, a number of different techniques such as virus isolation in culture, immunocytochemistry, polymerase chain reaction *(22)* can be used to detect the virus in ganglia at different times after the primary and/or reactivated infection. Since all these require removal of the ganglion from the animal, we describe here the necessary dissection procedures and also techniques we have found useful to detect latent infection by explant culture All instruments that come into contact with the ganglia should be sterile. Immediately before dissection mice are terminally anesthetized.

3.4.1. Removal of the Trigeminal Ganglia (Eye Model)

1 Cut the heart through an incision in the rib cage to produce as much exsanguination as possible
2. Pin the mouse in a prone position onto a cork board
3 Remove the skin from the cranium, remove the top of the cranium, and carefully lift away the brain to expose the trigeminal ganglia lying at the base of the cranial cavity. Pin the head securely to the cork board.
4 Remove the ganglia by careful dissection with small bow sprung (Noyes) scis-

sors and pointed stainless steel watchmakers forceps (no. 5) while viewing the procedure through a dissecting microscope. A no 11 scalpel can be used to assist in the dissection. Cut the ophthalmic/maxillary and mandibular nerves as close as possible to their points of entry into their foramina in the base of the skull. A final cut is made on the peripheral side of the dorsal root entry zone. The nerve changes from an opaque white on the peripheral side to a more translucent white in the CNS side (*see* Note 17).

3.4.2. Superior Cervical Ganglia (Eye Model)

1. *See* step 1 in Section 3 4 1.
2. Pin the mouse in a supine position on a cork board. Make a midline incision from the lower chin to the thorax and remove the thymus gland
3. Move the trachea to the right for dissection of the left ganglion or to left for the other ganglion and pin the trachea in place.
4. The vagus nerve is a good landmark, locate this and then find the carotid vessels which run parallel to it. The ganglion lies at the bifurcation of the carotid artery and can be exposed by blunt dissection (*see* Note 18).

3.4.3. Removal of the Cervical Ganglia (Ear Model)

1. Make a complete circular incision in the skin just below the rib cage and pull the skin back over the head.
2. Cut the heart through an incision in the rib cage to produce as much exsanguination as possible
3. Pin mouse in a prone position to a cork board. Remove the top of the cranium, the brain and the dorsal half of the vertebral column in the cervical region. Remove the exposed spinal cord (*see* Note 19)
4. The relevant cervical ganglia (*see* Note 20) are removed from their sockets in the vertebrae using the instruments described earlier. A fine seeker may also be helpful for easing the ganglia out of their "sockets"

3.4.4. Explant Cultures for the Demonstration of Latent Infection

Two methods are used widely to detect latent infection in ganglia removed from experimentally infected animals. In one, the ganglia or fragments of ganglia are cocultured in small flasks with monolayers of a cell line such as Vero, which is sensitive to HSV. These cultures can be maintained for several weeks, with changing of the medium, and the monolayers examined for evidence of cpe. In the second method, the ganglion tissue is maintained for 5 or 6 d in 0.5-mL tissue culture growth medium at 35°C. The tissue is then ground in a tissue grinder and 50-µL samples are placed in triplicate onto monolayers of Vero cells. After 2 d, the monolayers are fixed and examined for virus cpe.

In our experience there is little difference in sensitivity between these methods, and, since the method involving the 5- or 6-d incubation gives more rapid results, it is generally preferred (*see* Note 21).

3.5. Blood Samples

When the eye or ear models are used for some purposes, the taking of blood samples may be required at different times following the primary infection or following induction of recurrent infection. In the ear model, either of the two commonly used methods, from the tail vein or the retroorbital sinus, may be used (*see* Note 22). However, in the eye model, sampling should be restricted to the tail vein; 400–600 µL of blood will provide 50–150 µL of serum.

4. Notes

1 HSV-1 strain SC16 was first isolated in 1974 from a facial lesion *(16)* This was originally chosen as a strain of medium virulence when compared with other isolates in mice Since in the rabbit ocular model, HSV-1 strain McKrae (which was first isolated from an eye lesion) *(23)* consistently has given a high incidence of both spontaneous and induced recurrent infection, this strain was chosen for the mouse eye model

Clearly, strains such as KOS should be avoided, since this strain produced no recurrent eye infection in rabbits *(24)* and no recurrent disease in the mouse ear model *(7)*. There have been very few comparisons of different strains with respect to their ability to produce recurrent infection *(25)*.

With the zosteriform model, even relatively avirulent strains, such as KOS will give a high incidence of zosteriform spread from the inoculation site to the pinna *(7)*. Hence, in contrast to the model of true recurrent infection, in this model such strains may have advantages since few if any animals are lost from lethal CNS disease

It is difficult to make general statements about the dose of virus since many other factors such as the strain, age, and sex of mouse will affect the outcome at any particular dose

2 Greater resistance to death following primary infection with HSV in mice, at least after cutaneous or intraperitoneal infection, appears to be linked to pigmentation However, we have found the reverse to be true when the cornea was the inoculation site. For example, a dose of 10^3 PFU of HSV-1 strain McKrae killed 17/30 (57%) 8-wk-old CB57 BL/J female mice compared to only 1/19 (5%) NIH mice of a similar sex and age. Hence, in studies of recurrent infection it would seem sensible to use a non pigmented strain. Moreover, lesions of the cornea, iris, and retina and features such as erythema in the skin, are more easily visible in nonpigmented animals.

In a comparison of five different albino strains in the ear model, BALB/c, gave a low incidence of recurrent disease *(26)* There was little difference between the other strains but the inbred strain NIH gave a consistently good incidence of recurrent disease and therefore was used in our subsequent work and by others who have used the eye model *(27,28)*

Since, on the whole, males are more susceptible to infection with a wide variety of infectious agents, it might be argued that using males might help to maximize recurrent disease However, the propensity of males to fight can, by leading

to skin damage, seriously interfere with detection of recurrent skin lesions or in severe cases necessitate killing the mice for humane reasons. Moreover, following primary infection without the cover of immune serum, particularly in the cornea, the greater susceptibility of males can lead to unacceptable losses from severe CNS disease For example, with 8-wk-old NIH mice inoculated with 10^4 pfu of HSV-1 strain McKrae the mortality rates were 37% for males compared to 15% for females Hence, for routine purposes, to avoid these problems females are used for ear, eye, and zosteriform models (although the eye model was originally established in male mice) However, for particular purposes, e g , experiments on local immune responses following ocular infection, males may have an advantage since local production of IgA is higher in the male lacrimal gland *(29)*

Finally, it has been known for many years that the resistance of mice to infection with HSV increases significantly with age In the first development of the ear model, 4-wk-old animals were first used as a means of ensuring a high susceptibility to both primary and therefore, it was hoped, recurrent infection. In the eye model, 4-wk-old mice even when infected under cover of immune serum have an unacceptably high mortality rate (44%) that has reduced to 5% by 8 wk old. For this reason and because they are immunologically mature, older mice are used in the ocular model.

3 On various occasions, bitter experience, in particular with subclinical infections with mouse hepatitis virus and/or infection with the common pin worm, has taught us the absolute necessity for using SPF animals in these models Animals with such infections show a significant increase in resistance to primary infection, a reduction in the incidence of latent infection in sensory ganglia, and a concomitant reduction in the incidence of recurrent infection following a suitable reactivating stimulus

4 Surgical anesthesia with sodium pentobarbitone gives a narrow safety margin and is not to be recommended.

5 The doses for surgical anesthesia are: Midazolam hydrochloride 0 024 mg/10 g and Fentanyl citrate 0.0015 mg/10 g and fluanisone 0.048 mg/10 g or a combination of 0.1 mg/10 g xylazine and 1 mg/10 g ketamine. The first combination has two disadvantages, the two ingredients cannot be mixed and therefore have to be delivered as two separate injections and the most consistent result is obtained following subcutaneous delivery (which is technically more demanding than intraperitoneal delivery). However, the recovery rate with this combination is extremely good. It should be noted that sterile water should be used to dilute these drugs since some of the components will precipitate in saline These doses may need adjustment for different strains and ages of mice. It is important to keep anesthetized animals warm during the recovery period

6. We have seen the following abnormalities in uninfected mice: corneal vascularization of varying degrees, cataract, supraorbital abscesses, and band-shaped stromal opacities The incidence of such abnormalities varies with the strain of mouse.

7. If a primary eye infection is required without passive immunization of the mice it is necessary to minimize the risk of a secondary bacterial infection by treating the eyes daily from d 1 to 10 with antibiotic eye cream

8 The dose of anti-HSV-1 rabbit serum is higher than was used originally since in experiments where a range of doses were tested the higher dose was found to give statistically significantly more mice that shed virus on reactivation than animals given the original dose. Normally we use rabbit serum for passive immunization; however, we and others *(27,28)* also have used human serum and have found no difference between the two. There is considerable variation between mice in the levels of anti-HSV-1 neutralizing antibodies acquired following passive immunization Attempts to decrease these variations by delivering the antiviral serum (appropriately diluted) via the tail vein were successful. However, this procedure was not adopted, since it failed to increase the incidence of mice that reactivated and was far more time-consuming than intraperitoneal immunization.

9. In our experience, application of virus to the eye in the absence of corneal trauma produces blepharitis but no corneal disease and a low incidence of latency even when young mice are inoculated with high doses of virus *(30)*.

Only gentle pressure is required to damage the corneal epithelium and slightly greater pressure will extend the inoculation site to include the corneal stroma Inoculating the corneal stroma alone is best done using an injection into this tissue Excessive pressure during scarification will result in puncture of the cornea and collapse of the anterior chamber, and if this occurs the mouse should be killed

In the United Kingdom, for humane reasons, inoculation of virus is restricted to one eye, we recommend this restriction should be adopted elsewhere.

10 Rather than subcutaneous inoculation, virus can be inoculated into the pinna by scarification of the upper surface of the pinna through a small volume (10 µL) of virus suspension. However, the thinness of the pinna makes this difficult to do uniformly without cutting through the pinna and/or causing bleeding.

11. If the mice are just placed under the lamp they will tend to close their eyes and most of the cornea will not be irradiated. If only irradiation of the cornea is required this can be achieved by proptosing the eye through a hole in a piece of cardboard of sufficient size to expose the cornea but shield the lids

12. UV is a known stimulant to recurrent disease in humans The optimum dose of UV is a matter for discussion, in our studies we have tested a range of doses and have found that treatment of the eye for 90 s with a lamp emitting a peak of 4 02 mJ/cm^2 results in the maximum incidence of viral shedding In contrast, others also using our model suggest that a lower dose increases viral shedding, however, they provide no statistical evidence to support this claim *(27)* In our eye model, the incidence of reactivation, as judged by shedding of virus in the tear film, varies from 25 to 60%; the addition of systemic steroids to the UV irradiation *(4,5)* does not appear to increase this incidence However, these drugs do appear to increase the severity of recurrent ocular disease and to increase the duration of shedding of virus

13 In the ear model, successful induction of recurrent disease on the pinna has only been achieved by local treatments to this site These have included UV light *(31)*, local injections of prostaglandins *(31)*, application of irritants such as retinoic acid and xylene *(32)*, stripping the epidermis with cellophane tape *(17)* Systemic

treatments, in particular immunosuppression, produced insignificant results *(33)*. It is noteworthy that some stimuli like DMSO, may produce ganglionic reactivation but little or no recurrent disease *(32)*. Of these various local stimuli, the most widely applied has been physical trauma induced by cellophane tape stripping of the skin A technique originally used to study the effects of mild physical trauma on factors such as epidermal cell mitosis *(34)*

14. Although eyes can be examined under a dissecting microscope only gross disease will be discernible; for detailed examination a slit-lamp microscope is essential. Unanesthetized animals can be viewed but for a more rigorous examination anesthesia, or at least sedation, is essential

    Two types of slit-lamp illumination are useful. Direct illumination with a narrow beam directed obliquely into the eye that provides an optical section of the tissues under examination. With this method the shape and thickness of the cornea can be assessed and any irregularities in the corneal epithelium, such as small lesions, are easily detected. Retro illumination with a wide slit beam directed straight into the eye is a useful method for slit-lamp examination of albino mice; the eye, lids and surrounding skin can be scrutinized. Generally the highest power will be required to examine mouse eyes

    Before examining experimental eyes the operator should be completely familiar with normal eyes. Particular attention should be paid to the shallowness of the anterior chamber and the position of the iris vessels that can be confused easily with corneal vessels by the inexperienced.

    The following tissues can be examined for signs of damage or disease. cornea, limbal vessels, anterior chamber, pupil, iris, lens (not fully seen with an undilated pupil), lids, and surrounding skin The conjunctiva is only partially accessible to examination since it is not possible to evert the mouse eyelids.

    Fluorescein stain can be used to identify the bed of ulcers but requires observation with blue light to see fluorescence. Rose Bengal can be used to stain the margins of ulcers. One to two microliters of stain should be placed on the cornea, the eyelids opened and closed several times to spread the stain evenly and the animal examined immediately. If necessary, any excess stain can be removed by rapidly washing the eye with sterile saline. The normal adult NIH mouse cornea, unlike the human cornea, shows fine punctate staining with Rose Bengal; and, in addition, this stain should be used with caution since it has antiviral properties *(35,36)*.

    Using retro illumination, the retina and vitreous can be examined. In an anesthetized mouse, place one drop of cyclopentolate hydrochloride on the eye; after approx 10 min the pupil will be dilated. A drop of carboxymethyl cellulose is placed on the eye and a glass cover slip is pressed gently onto the cornea to neutralize the refraction of the cornea. By moving the mouse, almost the entire retina can be brought into focus. Examination of the mouse retina by direct ophthalmoscopy does not appear to be feasible with a human ophthalmoscope. Use of phenylephrine hydrochloride as a mydriatic rapidly causes the lens to become opaque; usually this clears by the next day.

    In the eye model, it is important to be able to distinguish damage to the cornea

caused by UV irradiation and that produced by viral disease, especially the early stages of disease, which may occur concurrently with damage from UV Control animals (uninfected) should be included in each experiment UV damage in such animals is predominantly ulceration of the corneal epithelium that conforms to a regular pattern, the cornea may be slightly swollen and have a diffuse light infiltrate There will also be a mild iris hyperemia and eyelids may be swollen slightly In such eyes, healing is complete by d 3–4 after UV treatment In contrast, in eyes with virus induced disease, epithelial lesions are irregular in shape, and accompanied by dense focal infiltration In addition, there is usually a severe iris hyperemia Recurrent lid lesions start as vesicles that rapidly progress to ulcers and then scabs

It should be noted that the light from slit-lamp microscopes is powerful and a lengthy exposure of the eye may produce drying and break up of the tear film that may be misinterpreted as a sign of disease, especially if vital stains are used Occasional opening and closing of the lids will restore the integrity of the tear film

15 Assessment of erythema should be done only on unanesthetized animals since anesthetics may alter the picture by causing changes in the peripheral blood vessels.

16. Good illumination is essential for dissections and so on, under the microscope; fibre optic lamps that easily can be adjusted to different positions are the most useful

17. For certain experiments, such as tracing viral spread, it may be necessary to divide the ganglion into its three parts· ophthalmic, maxillary, and mandibular; this is best done *in situ (12)* For immunohistochemical studies, perfusion with fixatives under terminal anesthesia gives the best tissue preservation Fixation before dissection helps the somatotopic organization of the tissue to be maintained and this is of particular value if you wish precisely to locate evidence of infection

18 The carotid vein usually is full of blood, whereas the artery often is empty and more difficult to find. Superior cervical ganglia vary in size in different mouse strains They are bean-shaped and translucent and they may be confused with lymph nodes that are of a similar size and shape but usually are opaque and yellowish. Standard histological techniques can be used to check that the correct tissue has been removed

19 There is no danger of accidentally removing cervical ganglia during the preparatory dissection since they are held quite firmly in place in small concavities in the vertebrae

20 The major sites of latency in the ear model are the second, third, and fourth cervical ganglia with the highest incidence in the second

21. When sensitivity in detecting latency is important in particular studies, the addition of hypomethylating reagents to the explant medium should be considered since these significantly increase the incidence of reactivation Concentrations of these drugs found to be effective in the medium are 8 m*M* azacytidine for mouse ganglia *(37)*, 50 m*M* for guinea pig ganglia *(38)*, 8 m*M* L-ethionine for mouse ganglia *(37)*, 5 m*M bis*-acetamide for guinea pig ganglia *(39)*.

More recently, in an adaptation of the coculture method, attempts were made to provide better oxygenation of the explanted ganglionic tissue in the hope that this would aid its survival and thereby increase the chance of reactivation For this, ganglia or ganglion fragments are placed in glass tubes, 1 × 10 cm, on the

inside face of which, at one end, a monolayer of Vero cells has been grown. The tubes contain 1 mL of tissue culture maintenance medium and are rotated at 10 rev/h on a roller drum at 35°C. The monolayers are examined daily for 3 wk for evidence of cpe caused by HSV. Medium in the tubes is changed every 2–3 d and precautions are taken to avoid crosscontamination between tubes. This roller tube method gave a significantly higher incidence of latent infection compared with the static, 5-d culture/homogenization procedure *(40)*.

In the ear model, depending on the studies involved, the three cervical ganglia can be incubated separately or together

22. Before sampling from the tail vein, put the mice in a cage and warm them with one or two angle poise lamps until the tail veins are dilated A close watch must be kept; hyperthermia, which is preceded by sweating, may result in death. Useful blood letting instruments can be made by breaking off the plastic mount from 23-gage × 1-in. needles For bleeding, place the mouse in a restrainer, positioned so that the tail hangs downward and is freely accessible Insert a needle into a tail vein and catch the blood in a sterile Eppendorf tube or A4 cup. Remove the needle and press on the vein with a pellet of cotton wool. Bleeding pigmented mice from the tail vein is difficult Mice of a more nervous disposition than NIH's may have to be anesthetized before this procedure and should not be warmed. Blood flow will therefore be considerably slower than in warmed mice

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Pathogenesis and Molecular Biology of HSV Latency and Ocular Reactivation in the Rabbit

James M. Hill, Renjie Wen, and William P. Halford

1. Introduction

Research on herpesvirus infections has commanded the attention of a diverse group of scientists for over a century. Until the advent of the human immunodeficiency virus, the herpes simplex viruses (HSV) were the most intensively studied of all viruses. During the early part of the nineteenth century, long before the infectious agent responsible for cold sores (fever blisters) was identified, studies suggested that damage to the trigeminal nerve could induce peripheral herpetic vesicles *(1)*. Gruter demonstrated that a particle of material from a herpetic blister inoculated into a rabbit eye could cause herpes and that, in this way, the disease could be transmitted in series from one rabbit to another *(2)*. Following Gruter's basic discovery, research conducted in many parts of the world showed a variety of clinical forms of herpes to be similarly transmissible by inoculation *(3)*. At roughly the same time, Goodpasture proposed that "the virus remains in the ganglia in a latent state after the local lesion has healed" and discussed in detail the pathology of herpetic infection in humans and animals *(4,5)*.

Forty years later, Stevens and colleagues *(6–8)* were the first to isolate the virus from the peripheral sensory ganglia of latently infected rabbits and mice. Over the past 20 yr, there has been substantial progress in our understanding of the pathogenesis and natural history of ocular herpetic infection. In recent years, intensive efforts have been focused on unveiling the molecular mechanisms and the virus–host interactions associated with latent HSV infection, reactivation, and recurrence.

The herpesviruses, which are enveloped, double-stranded DNA viruses, are grouped into three classes:

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1 The α-herpesviruses are neurotropic, tend to have a broad cell specificity, and can grow to high titers with a rapid productive cycle in cell cultures These include herpes simplex virus types 1 and 2 (HSV-1 and -2) and varicella zoster virus.
2 β-herpesviruses have restricted host ranges and a lengthy replication cycle with low yields of infectious virus. These include human cytomegalovirus and human herpesvirus type 6.
3 γ-herpesviruses are lymphotrophic, have a highly restricted range of cells in which they can replicate, and have a highly regulated latent state of infection *(9)*. This group includes Epstein-Barr virus and the newly characterized human herpesvirus type 7

HSV infections are common in humans *(10)*. More than 90% of the population has antibodies to HSV, which generally infects mucocutaneous surfaces, especially epithelial cells, as in the lip (herpes labialis) or genitals. Ocular HSV infection is a leading cause of blinding keratitis in industrialized countries. Both HSV-1 and -2 can infect the eye and produce recurrent ocular infection, similarly to the recurrences of oral and genital HSV infection. Whereas most genital infections are caused by HSV-2, approx 85% of ocular isolates are HSV-1 *(11,12)*. HSV-2 ocular infections often are associated with infants born to mothers with active herpetic genital lesions.

Primary ocular infection is an acute keratoconjunctivitis with or without involvement of the periorbital skin. After primary infection, HSV travels along a neuronal pathway to regional ganglia where it remains dormant (latent). Under an ill-defined set of circumstances, the virus reactivates and travels to the site of primary infection to cause recurrent disease. Recurrences in the eye can produce corneal disease and stromal scarring as the result of an incompletely defined immunological response to the reactivated virus *(13–15)*.

HSV establishes latency in ganglionic neurons following primary infection at a peripheral site. Viral latency, as operationally defined, is a state in which cell-free infectious virus particles cannot be demonstrated in tissues at the time of sacrifice, but infectious virus can be isolated from the same tissues after prolonged in vitro cultivation. Under certain circumstances the latent virus can be reactivated, resumes replication, and causes recurrent disease in vivo.

The rabbit has proven to be an excellent model of herpetic eye disease *(16)*. Inoculation of the cornea results in herpetic keratitis that may be quantified both clinically and virologically. Rabbit eye models have been useful in examining the role of viral genes in latency and reactivation. For example, recent studies using HSV-1 latency-associated transcript (LAT) mutants have shown that LAT is not required for establishment of latent infection in rabbit trigeminal ganglia, but is important for efficient reactivation of latent HSV-1 in vivo *(17,18)*. The goal of this work is to describe a reliable rabbit model of ocular HSV infection, latency, and reactivation.

2. Materials

1. Growth of virus and cells: HSV is propagated in primary rabbit kidney (PRK) cell monolayers and titered by plaque assay on African green monkey cell (CV-1) monolayers. The virus is frozen in small alilquots at –70°C so that the same batch can be used for multiple experiments.
2. Rabbits. New Zealand white rabbits (1.5–2 5 kg) can be used for the experiments.
3. ^{3}H-dTTP,^{3}H-dCTP,^{3}H-dATP (Amersham, Arlington Heights, IL)
4. Thymidine-5'-Triphosphate (α-^{32}P) (ICN Biomedicals, Irvine, CA).
5. TRIzol Reagent (Life Technologies, Gaithersburg, MD)
6. Kodak NTB-2 Emulsion (Eastman, New Haven, CT)
7. Kodak fixer and Kodak developer D-19 (Eastman)
8. 2% Epinephrine (Epifren, Allergan, Irvine, CA).
9. 0 5% Timoptic (timoloi maleate; 0 5%, Merck, Sharp and Dohme, West Point, PA)
10. Mineral oil—light white (Sigma, St. Louis, MO)
11. DNA digestion buffer: 100 m*M* NaCl, 10 m*M* Tris-HCl, pH 8.0, 25 m*M* EDTA, 0 5% SDS, and 0.2 mg/mL Proteinase-K.
12. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 9 0 (at 25°C), 1% glycerol, 25 m*M* $MgCl_2$ (Promega, Madison, WI)
13. PCR assay buffer: 2.5 U *Taq* polymerase, 10 µL of 10X PCR buffer, 50 µ*M* dNTPs, 50 ng of each HSV-1 primer, and 100 ng samples of ganglionic DNA.
14. RNase-free water To prepare RNase-free water, distilled water is added to RNase-free glass bottles and diethylpyrocarbonate (DEPC) is added to a concentration of 0 01%, after which the bottles are allowed to stand overnight and then autoclaved
15. 10X RT buffer: 100 m*M* Tris-HCl, pH 8 8, at 25°C, 500 m*M* KCl, and 1% Triton X-100 (Promega).
16. RT reaction buffer. 5 m*M* $MgCl_2$, 0 5 µg oligo (dT) primer, 1 m*M* each dNTP, 2 5 U ribonuclease inhibitor (RNasin), 15 U AMV reverse transcriptase, in 20 µL 1X RT buffer (Promega).
17. 2X Denhardt's solution: 0.22% Ficoll, 0 02% polyvinyl pyrrolidone, 0.02% BSA (fraction V)
18. 2X Hybridization buffer "mix": 4X SSC, 0.2*M* sodium phosphate buffer (pH 6.5), and 2X Denhardt's solution.
19. Terminal deoxynucleotidyl transferase (Promega)
20. *Taq* DNA polymerase (Promega)
21. T_7 promoter and T_7 polymerase (Stratagene, La Jolla, CA).
22. RQ1 RNase-free DNase (Promega).
23. Rabbit DNA primers: Actin 1: 5'AAGATCTGGCACCACACCTT-3', and Actin 2: 5'CGAACATGATCTGGGTCATC-3' The actin 1 and actin 2 primer pair amplify a 125-bp product from the rabbit α-actin gene Probe Actin 1 5: 5'CTCGGTGAGCAGAGTGGGGTG-3' HSV DNA primers and probes for latency-associated transcript (LAT), thymidine kinase (TK), and ribonucleotide reductase (RR) genes are shown in Table 1.

Table 1
HSV Gene, Primer Pairs, Probe, Sequence Location, Size After PCR, and References

HSV-1 gene	Primer pairs (5 → 3')[a]	Sequence location[b]	Size of PCR product, bp	Ref.
LAT				
SK-1.	5'-GACAGCAAAAATCCCCTGAG-3'	120702→	195	*26*
SK-2·	5'-ACGAGGGAAAACAATAAGGG-3'	←120896		
Probe	5'-CACACCAGCGGGTCTTTTGTGTTGG-3'	←120762		
TK				
TK-1.	5'-CTGCAGATACCGCTCCGTATT-3'	47053→	273	*27*
TK-2	5'-CATCTTCGACCGCCATCCCAT-3'	←47326		
Probe.	5'-GTCAAGCTGCCCATAAGGTAT-3'	←47255		
RR				
RR-1:	5'-ATGCCAGACCTGTTTTTCAA-3'	88517→	243	*15*
RR-2·	5'-GTCTTTGAACATGACGAAGG-3'	←88759		
Probe	5'-GGACACCAGCATGTCGCTCGCCGACTTTCA-3'	88594→		

[a]The topmost primer in each pair is the upstream (mRNA sense) primer, the bottom is the downstream (mRNA antisense) primer Primers are chosen by use of a computer algorithm

[b]Location is based upon the complete sequence of the 17 Syn^+ strain of HSV-1 Only the location in the long internal repeat is indicated for LAT gene Arrows designate orientation of oligonucleotide 3' end

24. 96-Well dot blot apparatus (Life Technologies)
25. Phosphorimager (Molecular Dynamics, Sunnyvale, CA)
26 Programmable PTC-100 thermal cycler (MJ Research, Watertown, MA)
27 Storage phosphor screen (Molecular Dynamics).

3. Methods

3.1. Rabbit Ocular Inoculation

All rabbit studies should conform to the ARVO Resolution on the Use of Animals in Research.

3.1.1. Topical Inoculation

1 Scarified or unscarified eyes of New Zealand white rabbits (1 5–2 5 kg) are infected with a suspension of HSV at 0 5 × 10^6 plaque forming units (PFU) in 25 μL
2 Before scarification, rabbits are anesthetized with intramuscular ketamine (20 mg/kg) and xylazine (4 mg/kg), and topical 1% proparacaine is applied to the eye.

3 Scarification is done with a sterile needle, a double cross-hatch on the epithelium is made with care to avoid wounding the stroma
4. After virus is applied to the eye, the eye is held open by pulling up on the eyelids; then the eye is closed and gently rubbed

3.1.2. Intrastromal Injection

1. Rabbits are anesthetized as described earlier.
2. With observation by means of an operating microscope, the central corneal is inoculated intrastromally with a 32-gage needle to form five focal blebs (dice pattern, total volume 50 μL containing 4×10^5 PFU of virus)
3. The cornea is then scarified superficially with eight needle scratches, forming a square around the central intrastromal injection
4. Finally, the lids are closed and the eye is massaged

3.1.3. Superior Cervical Ganglionic Injection

1. Rabbits are anesthetized, the neck area is shaved, and the skin is washed with 70% alcohol.
2 A midline neck incision is made, followed by blunt dissection to separate the neck muscles
3 The cervical sympathetic trunk is identified adjacent to the common carotid artery and vagus nerve By following the sympathetic trunk to the bifurcation of the carotid artery, the superior cervical ganglion is identified as a fusiform structure
4. HSV-1 (10^5 PFU/mL) is injected (25 μL) into the superior cervical ganglion with a 30-gage needle
5. Subcutaneous tissue and skin are closed separately with 4-0 silk sutures.
6 An antibiotic powder (ampicillin) is placed in the wound area before the skin is closed *(19)*. Aseptic technique is used for all procedures.

3.2. Identification of Viral Infection

3.2.1. Slit Lamp Biomicroscopic Examination

1. Primary corneal infection is verified by slit lamp examination with fluorescein staining on postinoculation d 4–8.
2 Corneal epithelial disease is graded on a 0–4 scale following fluorescein staining (0, no stain; 1, 25% of the corneas surface stained, 2, 50% staining, 3, 75% staining; 4, 100% staining)
3. The epithelial lesions generally are characterized as deep epithelial punctate, dendritic, or geographic.
4 The severity of corneal stromal disease can be graded on a 0–4 scale (0, clear cornea with iris details distinctly visible, 1, detectable edema with iris details clearly visible, 2, gross edema with stromal swelling, iris details still visible, 3, gross edema with stromal swelling, pupillary border no longer distinctly visible, some cellular infiltration possibly present; 4, an opaque cornea, anterior chamber structures not visible) *(20–22)*.

3.2.2. Assay of Infectious Virus (Qualitative and Quantitative)

3.2.2.1. Swabs

1. The eye is opened by holding upper and lower eyelids as close to the hairless border as possible
2. A sterile Dacron-tipped swab is used to collect the tear film. The swab is gently rotated in the upper cul-de-sac and then into the lower fornix, where the swab is allowed to rest in the nasal fornix to absorb the tear film
3. Each swab is placed into a screw-cap test tube containing 1 0 mL Eagle's minimum essential medium (EMEM) with 2% fetal bovine serum (FBS)
4. The tubes are shaken in a water bath (37°C) for 90 min and the swabs are transferred to tubes containing confluent PRK cells.
5. The remaining solution in the original tube is frozen (–70°C) for later use in viral titration Following a 18–24 h incubation at 37°C in a CO_2 incubator, the swabs are removed from the PRK cell-containing tubes and EMEM with 2% FBS is added
6. The tubes are monitored daily for 14 d for cytopathic effect consistent with HSV-1 infection.
7. The frozen aliquots from all the samples that are positive for cytopathic effect are titrated on CV-1 cells *(23,24)*

3.2.2 2 Eyewash

1. The eye is opened by holding the upper and lower eyelids as close to the hairless border as possible
2. Wash medium (0.1 mL) containing EMEM with 2% FBS is instilled into the eye. The eyelids are then closed and lightly massaged for 30 s
3. The eyelids are opened and the wash medium is collected
4. Next, 0 3 mL of wash medium is used to rinse the cornea and upper and lower cul-de-sacs for viral collection.
5. The eyes are massaged after each collection
6. The sequence of wash, collection, and massage is repeated until a total of 1.5 mL per eye is collected
7. An aliquot of the collected wash medium is inoculated onto indicator cells *(24,25)* (Table 2)

3.3. Identification of Latency

3.3.1. Removal of Neural Tissue

3.3.1 1 Rabbit Trigeminal Ganglia

Aseptic technique is used to perform these procedures.

1. Rabbits are sacrificed by intravenous injection of sodium pentobarbital, the hair and skin are removed from the head, and a bone cutter is used to break away the skull and expose the brain
2. The brain is removed and the trigeminal ganglia are exposed

Table 2
Frequency and Titer of Ocular HSV-1 Shedding After Adrenergic Induction

Days after induction	EP/TE[a] (%) swab only[b]	Titer[b] $\log_{10}$ PFU	EP/TE (%) swab only[c]	Titer, $\log_{10}$ PFU[c]
1	0/10 (0)	None	0/12 (0)	None
2	7/10 (70)	1.2	8/12 (67)	1 4
3	9/10 (90)	1.7	10/12 (83)	3.0
4	8/10 (80)	2.6	9/12 (75)	3 3
5	7/10 (70)	2.2	11/12 (92)	3.7
6	4/10 (40)	2.0	10/12 (83)	3.4
7	1/10 (10)	2.5	8/12 (67)	2 7
8	0/10 (0)	None	Not done	Not done

[a]EP/TE = eyes positive/total eyes
[b]Data are from ref. *25*.
[c]Data are from ref *24* (avg Figs 1 and 2)

3 The large centrally located ophthalmic nerve is severed from the optic chiasma
4. The third (oculomotor), fourth, and sixth cranial nerve pairs are laterally located and cut
5. The trigeminal nerve is cut and the trigeminal ganglia are exposed and removed, and then either frozen immediately in liquid nitrogen for nucleic acid extraction, or put in 4 0% paraformaldehyde for *in situ* hybridization (ISH), or cut into several pieces and cocultured in the medium containing PRK cell monolayers

3 3.1.2. Rabbit Superior Cervical Ganglia

1. The ventral neck area is shaved and the skin is washed with 70% alcohol
2. Make a midline neck incision and blunt dissection to separate the neck muscles.
3 Following the sympathetic trunk to the bifurcation of the carotid artery, the superior cervical ganglion is identified and removed as noted.
4. The superior cervical ganglion is processed in the same way as the trigeminal ganglion.

3.3.2. Coculture of Trigeminal Ganglia and Superior Cervical Ganglia

1. The trigeminal ganglia and superior cervical ganglia are removed, washed in cold (4°C) EMEM, and their outer sheaths are removed.
2. The ganglia are cut into several pieces and cocultured on PRK cell monolayers.
3. The cultures are monitored for cytopathic effect for up to 28 d

3.3.3. Determination of HSV DNA in Trigeminal Ganglia and Superior Cervical Ganglia

3.3.3.1. DNA Extraction

1. The frozen trigeminal ganglion or superior cervical ganglion is rapidly thawed,

ground with a pestle, and homogenized in a specific volume (600 µL for trigeminal ganglion, 300 µL for superior cervical ganglion) of digestion buffer
2 After an 18-h incubation at 50°C, the DNA is phenol extracted and the DNA concentration is adjusted to 10 ng/µL

3 3.3.2 PCR Amplification of HSV-1 and Rabbit α-Actin DNA

3.3.3.2 1 Standard PCR

1 Three pairs of oligonucleotide primers are chosen from regions of HSV-1 The primer pairs correspond to regions located in the latency-associated transcript (LAT), thymidine kinase (TK), and ribonucleotide reductase (RR) genes (Table 2)
2 100 µL of PCR assay buffer is overlaid with mineral oil and heated for 5 min at 97°C, *Taq* polymerase (2 5 U) are then added under the mineral oil layer
3 PCR is performed for 40 cycles of 75 s at 94°C, 75 s at 56°C, and 30 s at 72°C
4. After the PCR, 20 µL of each reaction is electrophoresed in a 2 0% Tris-borate-EDTA agarose gel.
5 The PCR products can be visualized on the gel, or can be transferred to a nylon membrane for dot or Southern blot analysis.

3 3 3 2 2. Quantitative PCR. This procedure involves the simultaneous amplification of a target sequence and a control sequence from the same DNA sample. The target is the sequence whose concentration is to be measured. The control is a sequence whose concentration is constant in all DNA samples assayed. The yield of control PCR product provides a measure of the amplification efficiency and consistency of each reaction.

The HSV-1 genes, RR, TK, and LAT (Table 2), are amplified to determine the concentration of viral genomes in DNA samples. One genome equivalent of HSV DNA was calculated to be 0.166 fg, assuming a mass of 10^8 Dalton for HSV DNA. The rabbit a-actin gene is used as the internal control sequence. The primers used to amplify portions of these sequences are Actin 1 and Actin 2. The pair of primers amplifies a 110-bp product from the rabbit α-actin gene.

A standard PCR procedure is modified to permit quantification of HSV DNA.

1. Uninfected rabbit ganglionic DNA (100 ng) spiked, respectively, with known amounts (5, 10, 20, 40, 80 copy numbers) of HSV DNA (as standards to determine gene copy numbers) are mixed with 50 pmol each of actin primers and HSV DNA primers
2. For precision, the assay must be repeated using very dilute HSV DNA samples (i.e., 0.1, 0.5, 1, 2, 5 copy numbers). This establishes the sensitivity and precision of the reaction. These PCR samples, referred to as HSV DNA standards, are the standard curve for each quantitative PCR assay
3 Then, infected rabbit ganglionic DNA (100 ng) is mixed with 50 pmol each of actin primers and HSV DNA primers
4. All these PCR samples are assembled as described
5. The samples are heated at 97°C for 5 min, after which 2.5 U of *Taq* polymerase are added.

6 The quantitative PCR assay is performed for 30 cycles of 1 min 15 s at 94°C, 1 min 15 s at 56°C, and 10 s at 72°C *(28,29)* These optimal conditions have been determined by standard procedures *(29)*

3 3 3.2 3. Analysis of PCR Products The Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, CA) and a 96-well dot blot apparatus are used for dot blotting. HSV-specific and actin-specific [^{32}P]-end-labeled oligonucleotide probes are used for hybridization. The radioactive membranes are scanned with a Phosphorlmager. Images are analyzed with ImageQuant software (Molecular Dynamics) to quantitate the [^{32}P]-HSV and [^{32}P]-α-actin probe hybridization to their respective PCR products.

3.3.3 2.4. Calculation of HSV-1 Genome Copy Number

1 The ratio of [^{32}P]-HSV gene probe hybridization to [^{32}P]-α-actin probe hybridization in the HSV DNA standards are calculated.
2 A straight line is generated by plotting the log of these ratios vs the log of the HSV-1 copy number obtained from the standards.
3. The HSV-1 genome copy number per 100 ng of genomic DNA in the trigeminal ganglion or superior cervical ganglion DNA samples is determined by fitting their measured ratios to the standard curve.

3.3.4. Determination of HSV RNA in Trigeminal Ganglia and Superior Cervical Ganglia

3 3.4.1. RNA Extraction

1 TRIzol reagent is used for RNA extraction as described in the manufacturer's protocol The samples are homogenized in 1 mL of TRIzol reagent/50–100 mg of tissue and incubated for 5 min at room temperature
2. Chloroform (0.2 mL/1 mL of TRIzol reagent) is added.
3. Then, the samples are shaken for 15 s, incubated at room temperature for 2–3 min, and centrifuged at 12,000*g* for 15 min at 4°C.
4. The mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains in the aqueous phase
5. The aqueous is transferred to 2-mL tubes and isopropanol (0 5 mL/1 mL of TRIzol reagent) is added.
6. The samples are incubated at room temperature for 10 min and centrifuged at 12,000*g* for 10 min at 4°C. The RNA precipitate forms a gel-like pellet on the bottom of the tube.
7. The supernate is removed and the RNA pellet is washed once using 1 mL of 75% ethanol per 1 mL of TRIzol reagent.
8. The samples are mixed and centrifuged at 7500*g* for 5 min at 4°C.
9. The RNA pellet is air-dried for 5–10 min. Do not let the pellet dry completely as the solubility will greatly decrease.
10 The RNA is dissolved in 100 μL RNase-free water or 0.5% SDS solution by mixing, and then incubated for 10 min at 55–60°C The resulting solution of RNA has an A 260·280 ratio between 1.8 and 2.1.

11. The solution can be stored at –70°C by adding 5*M* NaCl solution (8 µL) and 100% ethanol (500 µL)

3 3 4.2. Reverse Transcription and PCR Analysis

1 Immediately prior to the reverse transcription reaction, the RNA standards are diluted to 10^8–10^2 molecules/µL in 5 0 µL ribonuclease(RNase)-free water
2. For each sample, approx 1 µg RNA in 5 0 µL RNase-free water is needed
3 The RNA is denatured at 90°C and cooled to 67°C
4 The samples and standards are reverse-transcribed in 20 µL RT reaction buffer
5 The reaction mixtures are incubated at 23°C for 10 min followed by 40 min at 42°C
6 The reaction mix is extracted by the phenol-chloroform method and quantified spectrophotometrically at 260 nm.

3 3.4.3. Quantitative Determination of HSV Latency Associated Transcripts (LAT)

The concentration of the cDNA, which contains the LAT and actin products, is adjusted to 10 ng/µL for quantitative PCR analysis. The same procedure as is described above for quantitation of copy number of the HSV genome can be used to calculate the number of LAT per cellular genome. For another approach to quantitation of LAT, *see* ref. *30* for a detailed description of competitive quantitative RT-PCR.

3.4. Identification of Latency Associated Transcripts (LAT)

3.4.1. Northern Analysis

Northern blot analysis is used widely to determine the molecular size, distribution, and concentration of RNA. Relative quantities of RNA often are calculated from optical density by densitometric scanning of Northern blot autoradiograms. The method estimates the amount of a specific RNA by comparing the intensity of RNA hybridization in a test sample to that of an internal standard consisting of a homologous copy RNA (cRNA)

1. After coelectrophoresis and transfer to nylon membrane, the internal standard cRNA and targeted RNA are detected by a radiolabeled nucleic acid probe.
2 In each case, variable amounts of internal cRNA control can be added to create a standard curve *(31)*
3. The internal standard RNA can be synthesized from the specific DNA template using T_7 RNA polymerase by the standard in vitro transcription protocol (Promega)
4 The transcription mix is treated with RQ1 RNase-free DNase to remove the DNA template and is quantitated spectrophotometrically.

3.4.2 In Situ *Hybridization*

The production of two types of ^{3}H probes is described: one by PCR *(29)* and the other by random hexamer priming *(32–34)*. No other *in situ* probes are

described. Other investigators have used [^{35}S]-labeled nucleotides *(35–37)* or biotin-labeled nucleotides *(38,39)* for ISH.

3.4.2.1. LAT-Specific Probes

The PCR-created [^{3}H]-LAT probe *(29)* is prepared as follows.

1. The dNTPs (dGTP, [^{3}H]-dCTP, [^{3}H]-dTTP, [^{3}H]-dATP) are combined and dried.
2. LAT-probe primer (SK1, SK1.5) and LAT-probe DNA template (SK1, 2, 195 bp post-PCR product) are added to the dried dNTPs.
3. PCR is performed as described except that 45 cycles are used.
4. The PCR products are transferred to a Millipore Ultrafree-MC unit
5. TE buffer is added and the solution is centrifuged at 7000*g* for 20 min
6. The [^{3}H]-DNA probe in the upper cup of the microcentrator is removed and tested for purity in a 2.0% agarose gel. The 61-bp probe lies between bases 120702 and 120762 in the genome *(40)*
7. Stevens *(41)* and Hill et al *(17)* used a single-stranded [^{3}H]-RNA probe prepared by random hexamer priming.
8. The LAT probe is prepared from a truncated *Sal-Bcl* fragment spanning 0 790–0.798 μ on the HSV genome This fragment is cloned into pGEM and transcribed by a T_7 promoter using a T_7 polymerase
9. The same [^{3}H]-dNTPs are used as noted. The probe is degraded to 50–200 nucleotide fragments by incubation in 200 m*M* NaOH prior to hybridization

3.4.2.2. Tissue Processing

1. The tissues (trigeminal ganglia and superior cervical ganglia) are immersed in 4% paraformaldehyde and fixed at 4°C for 18–24 h.
2. Immediately after the fixation period, the tissue is dehydrated and embedded in paraffin according to the following schedule: Ethanol at concentrations of 70, 80, 95, 100, 100, 100, each for 30 min at room temperature; chloroform for 45 min at room temperature, followed by a change of chloroform for another 45 min at room temperature; paraffin for 30 min at 60°C, followed by a change of paraffin for another 30 min at room temperature.

3.4.2.3. Paraffin Embedding

1. A metal mold is heated briefly over an open flame and filled with hot paraffin.
2. The ganglion is taken from the paraffin bath and oriented as desired.
3. The metal mold is placed on ice to solidify the bottom of the paraffin.
4. The mold is filled with hot paraffin, and the embedding ring (Fisher, Pittsburgh, PA) is placed on top.
5. The plastic ring is filled completely to the top with more hot paraffin and refilled two to three more times as the paraffin solidifies.
6. When the paraffin is solid, the specimen is kept overnight at 4°C. The resulting tissue blocks are stored at room temperature.

3 4.2.4. Paraffin Sectioning

1 The paraffin block is trimmed into a rectangular shape, mounted on the microtome, and cut into a ribbon of 7-μm sections
2. The sections are separated in a water bath (45°C) and floated individually, shiny side down
3 When the sections have smoothed out, they are placed on Fisher "plus" slides and oriented
4. The slides are dried vertically and stored in a slide box containing desiccant

3.4.2 5. *In Situ* Hybridization

1 Xylene or toluene can be used to deparaffinize the sections
2. Then the sections are washed with 100% ethanol for 5 min
3. The slides are incubated in a proteinase-K solution for 3 min at 37°C and washed three times in 1X PBS for 5 min each time
4 The sections are refixed in 100% acetone for 2 min and air-dried
5 Circles are drawn around each section with a PAP pen (KIYOTA International, Elk Grove, IL)

The probe is prepared as follows:

1. The amount of probe needed is calculated:

$$\text{Vol (probe)} = 86{,}400 \text{ dpm} \times n/\text{dpm}/\mu\text{L of probe}$$

where n = the number of sections to be processed

2 This amount is added to a sterile Eppendorf tube with an equal amount of 4X hybridization buffer "mix " The 4X solution and a solution of 20% dextran sulfate in formamide *(42)* are heated to 65°C
3. The total volume of diluted probe needed is calculated

$$\text{Total vol of diluted probe} = n \times 25\ \mu\text{L}$$

4. The volume of 2X hybridization buffer "mix" needed is calculated·

$$\text{Vol} = 1/2\ (\text{total vol of diluted probe}) - 2\ (\text{vol of probe})$$

and this volume is added to the probe in the Eppendorf tube.

5 The volume of 20% dextran sulfate needed is calculated

$$\text{Vol} = 1/2\ (\text{total vol of diluted probe})$$

6 Aliquots of the diluted probe (25 μL/section) are added to each slide
7 The section is covered with a siliconized cover slip.
8 Air bubbles over sections are eliminated
9 The slides are heated to 100°C for 5 min to denature the DNA, after which the slides are placed in moist chambers, incubated overnight at 42°C, and washed in 0.2X SSC three times for 15 min each.
10 The slides are completely dried before autoradiography

3.4.2.6. Autoradiography, Developing, and Staining

1. The radiolabeled slides are dipped in emulsion in a dark room with only sodium light
2. The slides require 10 d exposure, to ensure dryness, a desiccant is placed in the slide box.
3. The slides are developed by washing in Kodak developer D-19 (17°C) for 4 min, followed by washing in dH20 (18°C) for 1 min and immersion in Kodak fixer (19°C) for 5 min.
4. The slides are washed again in dH_2O (20°C) for 5 min, and then dried and stained with Gill's H&E *(43)*.

3.5. In Vivo Reactivation of Latent HSV Infection

3.5.1. Iontophoresis of Adrenergic Agents

1. Rabbits are anesthetized as described.
2. A sterile eye-cup is centered on the eye within the limits of the corneoscleral limbus and filled with epinephrine (0 01%).
3. The epinephrine is prepared from commercial 2% epinephrine and must be made up immediately before use
4. The epinephrine solution is diluted with sterile, distilled, deionized water
5. The solution should be protected from heat and light Any iontophoresis unit can be used *(17,44)*
6. The anode (+) makes contact with the epinephrine solution in the eye-cup, and the cathode (–) is attached to the ear over a saline-moistened cloth pad. Iontophoresis is at 0 8 mAmp for 8 min once daily and is done on three consecutive days
7. Successful drug delivery is verified when pupillary dilation (mydriasis) occur within 30–60 min after iontophoresis.

Other solutions that can be used in iontophoretic reactivation include 6-hydroxydopamine and timolol. 6-Hydroxydopamine (1%) must be prepared immediately before administration *(24,45,46)*.

1. The powdered drug is dissolved in sterile, deionized, distilled water and the pH is adjusted to 6 1–6.8 using sodium hydroxide.
2. Timolol (0.01%) is prepared by dilution of 0 5% Timoptic with sterile, deionized, distilled water *(47)*.
3. Eye swabs and eye washes are taken to monitor virus shedding for 7 consecutive days after initiation of iontophoresis.

3.5.2. Injection of Cyclophosphamide and Dexamethasone

1. Rabbits are given an iv injection of cyclophosphamide (75 mg/kg) followed 24 h later by an iv injection of dexamethasone (4 mg/kg) *(48)*
2. Swabs and slit lamp examinations are performed to monitor the virus shedding and corneal lesions for 8 consecutive days beginning on the day after injection of cyclophosphamide *(49)*.

3.5 3. Surgical Damage to Corneal Nerves

1. The rabbits are anesthetized and an anterior superficial keratectomy performed by partial trephination into the superficial corneal stroma with a 7 0-mm trephine
2. The edge of the trephined area is grasped with forceps and peeled from the central surface of the cornea.
3. The transection of the corneal nerves is performed with visualization under the operating microscope Eight to ten corneal nerves can be identified as they enter the cornea near the limbus where they are cut with the tip of a no 11 Bard-Parker blade *(50)*

3 5 4. Cryogenic Injury

The rabbits are anesthetized and a cryogenic injury is created by holding a circular 8- to 9-mm diameter piece of dry ice against the surface of the cornea for 30 s This can form an ice ball over the entire cornea that thaws within 1 min after the ice is withdrawn *(51)*.

3 5.5. Intrastromal Injection of Water

1. The rabbits are anesthetized and 100 HI sterile, deionized water is injected intrastromally *(52)*
2. The rabbit eyeball is proptosed with a wooden cotton applicator and stabilized in that position by an assistant An operating microscope can be used to facilitate all surgical manipulations
3. A 30-gage short bevel needle attached to a 0.25-mL tuberculin syringe containing sterile water is inserted into the central corneal sitroma and advanced until the bevel is beyond the entry wound
4. The water is injected slowly; a grayish white circular bleb appears, which slowly increases in size
5. The needle is withdrawn and the proptosed globe is returned to the orbit *(52)*

4. Notes

1. Molecular mechanisms of latency: Establishment and maintenance· HSV can establish a lifelong latent infection in host neuronal tissues. Latent virus can be reactivated and produce recurrent disease *(17,18,47,49,53)* Two avenues are available for the virus to reach a neuronal cell body to establish latent infection from the periphery by axonal transport; and from an adjacent cell in which HSV is replicating Since HSV is a lytic virus, initiation of the viral replication cycle eventually will shut off cellular protein synthesis and leads to cell death. This results in either the establishment of a latent infection in an adjacent cell or infection of an adjacent cell, followed again by viral replication and cell death.

   The establishment of the latent phase of infection probably is controlled and executed by the neuron. Evidence for this idea came from studies showing that HSV DNA of biologically retrievable virus with deletions in the immediate-early transcriptional regulatory gene ICP4 (which regulates replication) persists in the

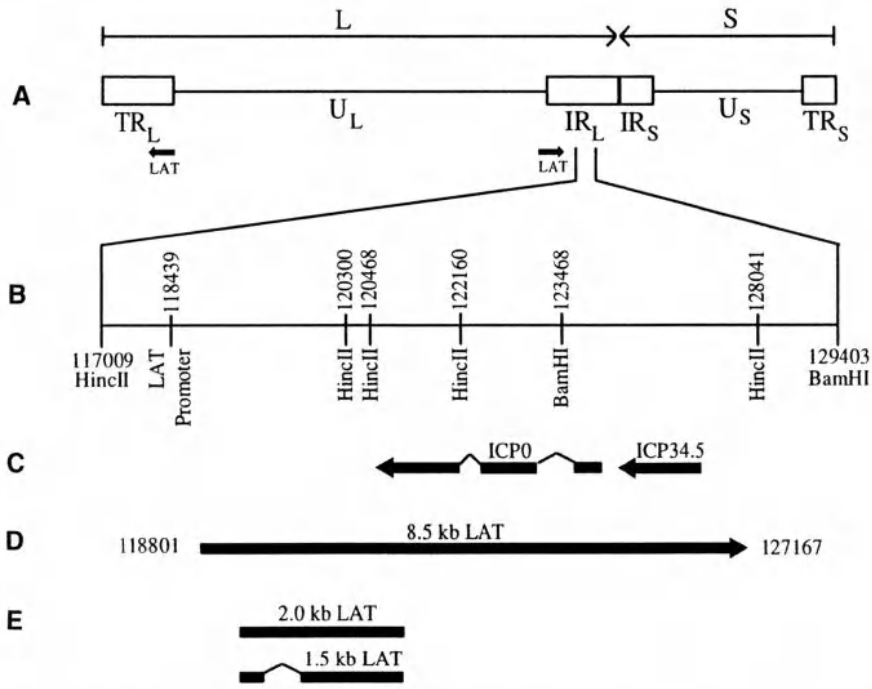

Fig. 1. HSV-1 transcription in LAT region. **(A)** Graphic representation of HSV-1 genome which codes 75 proteins. L, long; S, short; TR, terminal repeat; IR, internal repeat. **(B)** LAT genome is in 60th TR_L and IR_L region. Only the segment in the IR_L is shown. **(C)** Two HSV proteins (ICPO and ICP 34.5). **(D)** Transcription of 8.5-kb LAT during acute infection. **(E)** The region 2.0-kb LAT and region 1.5-kb LAT.

ganglia, demonstrating that strains with very limited replication are capable of establishing latent infection *(54)*. Also, latent infection was found in ganglionic neurons that showed no prior synthesis of viral proteins *(55)*. Finally, neurons with different developmental surface markers were found to differ in their capacity to harbor latent viral DNA, providing evidence for the presence of neuronal cell surface phenotypes that favor the establishment of latent infection *(56)*.

Although the molecular mechanisms by which HSV establishes and maintains latency are a focus of intense study, much remains unknown. LAT is the only HSV-RNA that has been detected during neuronal latency. HSV-1 LAT consists of a family of overlapping transcripts encoded by diploid genes that map to the long repeat region of the viral genome (Fig. 1B). Two colinear LAT, 2.0 and 1.5 kb, have been detected by Northern blot analysis and/or ISH of RNA from latently infected ganglia (Fig. 1E) *(41,57,58)*. The LAT overlap the 3'-terminus of ICPO mRNA (Fig. 1C) and are transcribed from the complementary DNA strand (Fig. 1D) *(40,41,57,58)*. The function of LAT in neuronal latency, however, has not been identified.

The role that LAT might play in HSV-1 replication and in the pathogenesis of latent infection in rabbits has been examined with mutant virus constructs *(17,18,59–61)* These constructs, which are deficient in the production of LAT, are capable of establishing and maintaining latent infections in vivo Additionally, all of the mutants tested so far are replication-competent in cell culture and in the rabbit eye. Thus, LAT is not required for HSV-1 replication or for the establishment or maintenance of latent infection *(17,60,62)*

LAT expressed during HSV-1 latency is encoded by an unusual gene Theoretically, either this gene could act via a gene product or its function could be the outcome of the action of a functional RNA. The 3' end of the LAT region overlaps with that of ICPO Therefore, LAT was suggested to act as an antisense agent *(58)*, but no supporting evidence has been published. One LAT promoter is located more than 650 nucleotides upstream of the 5' end of the most abundant LAT (2.0 kb). Analysis of LAT promoter viral mutants indicates that transcription is initiated close to and/or possibly interacts with components such as the TATA box sp 1, LAT promoter binding factor, cAMP response element, long-term expression region, and CAAT box The transcription and splicing of the 8.5 kb LAT are very complex and have not been elucidated fully *(63–65)* Also, the relationship between the neuronal expression of this LAT promoter and the repression of other possible HSV-1 promoters during latent infection is not understood *(63–65)*. To date, no HSV-specific LAT translational product has been identified in vivo *(63–65)*, and whether any HSV-1 LAT protein is expressed during latency remains to be determined.

2 Molecular mechanisms of reactivation· Activity in neural tissue and shedding at peripheral site: The molecular mechanisms of reactivation, including the precise event(s) that serve as triggers, remain unknown. A key unanswered question regarding reactivation is how replication begins in the absence of Vmw65, a viral protein that is assumed to stimulate the early stage of the HSV-1 replication cycle Perhaps a crucial step in the ability of HSV-1 to reactivate is the presence of some viral or cellular factors that compensate for the initial lack of Vmw65, permitting transactivation of immediate-early gene expression *(63,65)*. Nerve growth factor (NGF) could have a role in rendering a cell nonpermissive for viral replication. Deprivation of NGF in the rabbit ocular model resulted in reactivation of latent HSV-1 *(66)*

HSV-DNA encodes 75 proteins. However, only 37 are required for growth in culture *(64)* No virally encoded functions are required for establishment of the latent state, but a specific gene(s) could be essential for efficient reactivation of latent virus *(64)*. The 8 5-kb LAT, or more likely some derivative of it, probably functions to facilitate adrenergically induced (but not spontaneous) reactivation in the rabbit eye model *(17,18)*

Although LAT is not required for establishment of latent infection in rabbit trigeminal ganglia, this transcript appears to be important for efficient reactivation of latent HSV-1 in vivo *(17,18)* Recent studies have shown that the rates of ocular reactivation in vivo are significantly lower for constructs lacking the LAT

Table 3
Strain Specificity of HSV-1 Ocular Reactivation

HSV-1 strain	Spontaneous reactivation during Pi d 20–39, PE/TE (%)[a]	Adrenergic induction after Pl d 42, PE/TE (%)
Rodanus	8/8 (100)	1/6 (17)
McKrae	93/120 (78)	110/120 (90)
17 Syn+	9/12 (75)	8/12 (67)
RE	2/4 (50)	0/4 (0)
E-43	7/14 (50)	8/14 (57)
KOS	3/6 (50)	0/6 (0)
F	4/8 (50)	0/8 (0)
SC-16	3/14 (21)	3/12 (25)
Macintyre	2/20 (10)	0/20 (0)
CGA-3	0/10 (0)	0/20 (0)

[a]PE/TE = HSV-1 shedding positive eyes/total eyes *(68)*

region, compared with the rates for strains with the LAT region intact *(17,18,59–61)* Perng et al *(59)* reported that rabbits latently infected with an HSV-1 McKrae strain mutant (dLAT2903) having a LAT deletion showed a 33% decrease in the spontaneous reactivation rate, compared with the parental strain. These authors proposed the following possibility. In the rabbit ocular model of HSV-1 latency, two-thirds of spontaneous reactivations are LAT-dependent and could, therefore, represent in vivo induced reactivation for which the inducing factors have not yet been identified. The other one-third of spontaneous reactivations are independent of LAT and represent a subgroup of spontaneous reactivation.

3. Spontaneous shedding· With HSV-1 latent in the trigeminal ganglia, shedding of virus on the ocular surface is the most commonly used sign of viral reactivation in experimental models, including the rabbit. However, reactivation rates and rates of spontaneous and induced shedding vary among HSV-1 strains (Table 3) In general, the McKrae and 17 Syn$^+$ strains have the highest rates of both spontaneous *(67,68)* and induced *(68)* shedding, making them the most useful in rabbit model studies of latency and reactivation. The Rodanus strain, which has a high rate of spontaneous reactivation, cannot be induced, which limits its value in the laboratory The Macintyre and CGA-3 strains show neither spontaneous nor induced shedding, suggesting that for these strains, the mechanisms that allow the establishment and maintenance of neuronal latency are separate from the processes that trigger reactivation and ocular shedding in vivo *(68)*.

Shedding frequencies for individual HSV-1 strains, as determined by the ocular swab procedure, vary with time after inoculation. For the McKrae strain, spontaneous shedding was found in 80% of 20 eyes during postinoculation d 40–80, but only 35% over d 81–180, and 25% over d 181–220 *(69)*. In another study,

shedding was observed in 78% of 120 eyes over d 20–39 pi, however, approximately three-fourths of these episodes occurred in the first half of this period and only one-fourth in the second half *(23)*.

The frequencies of shedding after adrenergic induction also vary significantly with the strain, but not necessarily in correlation with spontaneous shedding (Table 3). In a study of rabbits latently infected with 10 strains of HSV-1 *(68)*, four groups of viruses could be distinguished in terms of reactivation frequencies.

a. Very little or no spontaneous reactivation and no induced reactivation (Macintyre, CGA-3),
b Little spontaneous or induced reactivation (SC16),
c Spontaneous reactivation, but no induced reactivation (Rodanus, RE, F, and KOS), and
d Spontaneous and induced reactivation (McKrae, 17 Syn^+, and E-43).

Despite the differences in spontaneous and induced reactivation frequencies, all of the wild-type HSV-1 strains produce LAT However, as noted, studies with LAT deletion mutant have demonstrated reduced spontaneous reactivation rates, suggesting that LAT gene also has an effect on the frequency of spontaneous shedding *(59)*. Taken together, these results suggest that one or more components outside of the LAT gene are involved in HSV spontaneous and induced reactivation *(59,68)*

4. Induction of HSV-1 specific corneal epithelial lesions Both adrenergic iontophoresis *(46)* and immunosuppression *(49)* can induce specific herpetic epithelial lesions In the first report of adrenergic induction of corneal lesions, Hill et al *(46)* described a positive association of lesions and positive eye swabs after ocular iontophoresis of 6-hydroxydopamine followed by topical application of Propine (dipivefrin hydrochloride, a prodrug of epinephrine) in HSV-1 latently infected rabbits. Deep punctate, dendritic, and geographic corneal lesions were observed Of the 36 eyes with positive swabs, 24 (67%) also had corneal lesions. Of the 13 eyes with dendritic lesions, 10 (77%) were associated with positive HSV-1 eye swabs. Conversely, of 121 eyes with negative swabs, 105 (87%) were also negative for HSV-1 corneal lesions In the first report of immunosuppression-induced ocular herpetic lesions *(49)*, deep punctate, dendritic, and geographic corneal epithelial lesions were observed after intravenous administration of cyclophosphamide and dexamethasone *(49)* Also, a positive correlation was noted between the induced lesions and recovery of HSV-1 from ocular swabs
5. HSV-1 corneal latency One of the most controversial aspects of HSV-1 latency is whether the herpesvirus is capable of existing in the latent state in the cornea, and if so, can corneal latency lead to virus reactivation, shedding, and disease The definition of HSV-1 latency proposed by Gordon et al. *(70)* states that the virus must be capable of existing in a host cell for an extended period of time in a virion-free state and, upon appropriate stimulation, must reactivate to produce infectious progeny virions. To verify the existence of corneal latency, four necessary and sufficient experimental conditions must be fulfilled:
   a. A functional HSV-1 genome capable of reactivation to produce infectious HSV-1 progeny must be detected,

b. No intact virion must be visible by electron microscopy;
c. No HSV-1 transcription can occur, with the exception of LAT; and
d. No HSV-1 protein expression can occur.

Three alternatives that must be ruled out are.

a. Residual defective HSV-1 DNA from a previous corneal infection;
b. Viral persistence in the cornea with a very slow turnover of a very small number of infectious virions; and
c. Spontaneous shedding of virus from neuronal cells via anterograde axoplasmic flow with coincidental detection in the cornea (with or without replication).

One problem, however, is that these strictures are based on the assumption that corneal latency mimics ganglionic latency; if this is not the case, some or all of these criteria may not apply.

ISH studies *(71)* have found that HSV-specific probes hybridized to DNA extracted from corneal epithelial, stromal, and endothelial cells obtained from HSV-1 latently infected rabbits More recently, PCR demonstrated LAT-RNA in 9% of corneas and in 100% of trigeminal ganglia from latently infected rabbits during postinoculation d 41–147 *(72)*; HSV-DNA was detected in 60% of the corneas In one study *(73)*; HSV was recovered from 8–11% of long-term cell cultures from rabbit corneas with no signs of active clinical disease at postinoculation d 118 *(73)*; in another study *(74)*, HSV-specific DNA (TK gene region) was recovered in 55% of corneal cell cultures from HSV-infected rabbits at postinoculation d 118, even though no virus was detected in the cell cultures over a 44-d culture period. Taken together, these findings indicate that at least two populations of HSV-containing cells exist in the cornea after herpetic keratitis One population (approx 10%) contains virus that can be reactivated using conventional culture methods and a second population (approx 50%) contains viral DNA, but in a form that does not reactivate with routine cell culture techniques.

Although the results of many studies provide data that are consistent with the idea of HSV-1 corneal latency in rabbits *(15,72–74)*, a review of the literature indicates that no single study has investigated the issue of corneal latency using all of the three essential approaches: cocultivation, electron microscopy, and biochemical analyses *(70)*. Thus, definitive answers remain elusive.

In the rabbit model, a variety of evidence points to the largely neuronal origin of reactivated HSV-1. Twenty-five years ago, Brown and Kaufman *(75)* reported the recovery of HSV from the ocular secretions of eight of 22 enucleated rabbits latently infected with HSV-1. This study, indicating that virus liberation occurs in the absence of the globe, further confirms that the globe, i.e., the cornea itself, cannot be the sole source of HSV-1 reactivation.

Transplantation studies *(76)* have also indicated that, at least for induced reactivation in the rabbit model, the source of the virus is not the cornea. Uninfected rabbits that received donor corneal tissue from latently infected rabbits did not shed virus after either adrenergic iontophoresis within the limits of the donor cornea or systemic immunosuppression with cyclophosphamide and dexamethasone. However, latently infected rabbits receiving corneas from uninfected

donors did shed virus with systemic immunosuppression, but not with adrenergic iontophoresis only within the limits of the donor cornea This suggests that neuronal latency in the host is necessary for reactivation in the rabbit model, and that intact corneal nerves are necessary for adrenergic iontophoretic induction of ocular shedding of latent HSV-1

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21

HSV Latency In Vitro

In Situ *Hybridization Methods*

Christine L. Wilcox and R. L. Smith

1. Introduction

We have developed an in vitro model of herpes simplex virus (HSV) latency in primary neurons that mimics many aspects of HSV latency in animal models and the human disease *(1–3)*. Using this model, we demonstrated that HSV-1 and HSV-2 establish latent infections in vitro in the same neuronal cell types that are shown to harbor latent HSV in humans *(3)*. Latent HSV infections can be produced in neuronal cultures from ganglia of rodents and primates with similar results *(3)*. In all cases examined, the neurotrophin, nerve growth factor (NGF), is required to maintain the latent infections. Depletion of NGF results in the reactivation of latent virus *(1–3)*. Depending upon the conditions and the use of a high multiplicity of infection, latent HSV-1 infections are established in the majority of primary sensory or sympathetic neurons in tissue culture *(2,4)*. To achieve high efficiency of establishment of latency with little or no evidence of lytic infection, an antiviral agent (e.g., acyclovir) is added to the neuronal cultures during the first week after inoculation with virus. However, latency can be established in the absence of antiviral treatment provided that the multiplicity of infection (MOI) is very low *(1,2)*. At least one of the actions of the antiviral treatment is to prevent amplification of the input virus in the nonneuronal cells that are present in the culture at the outset of the infection. These nonneuronal cells are destroyed in the presence of acyclovir and virus *(4)*. Latency is maintained in neurons in culture for as long as 10 wk in the presence of NGF. Viral transcripts and antigens associated with the productive infection are not detected during the latent infection *(2,3,5)*. Viral transcription is restricted to the latency-associated transcripts (LAT) during the latent infec-

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tion and is present in the nuclei of 80–90% of the neurons by 3 wk postinfection *(4,5)* Upon removal of NGF from the culture medium, for as brief as 1 h, reactivation of latent virus is induced *(3)*, and viral antigens associated with the productive infection and infectious virus are detected between 48–72 h after NGF deprivation.

The in vitro model is well suited for molecular studies of the regulation of latency and cellular signaling since the model can produce a population of neurons with a very high frequency of latent virus, and the establishment and reactivation of latency are controlled, synchronous events. The in vitro model of latency in neurons also allows manipulations using pharmacological methods *(6)* and has the potential for examination of signaling pathways in individual neurons using microinjection methods.

The in vitro neuronal latency system that is currently used in our respective laboratories and that has been best characterized is prepared in sensory neurons from dorsal root ganglia (DRG) obtained from embryonic d 15 rats DRG neurons are used because they are a natural site of HSV latency in humans *(7)* and they are relatively easy to isolate and maintain in tissue culture. Recently we described in detail the methods for the preparation of the neuronal cultures for the establishment of latent infections and the induction of reactivation *(8)*

To further understand the regulation HSV latency, examination of the pattern of viral transcription during the establishment of latency in the in vitro neuronal model has proved information that could not be readily obtained in animal models *(4)*. In this chapter, we have focused on a specialized method for the analyses of HSV latency in vitro that we feel is not well documented by other sources: *in situ* hybridization (ISH). For neurons in culture, radiolabeled probes and emulsion methods are not well-suited. The techniques that we have found work well for ISH in neurons in culture are somewhat unique. In some cases relatively subtle changes in techniques result in significant changes in the success of the methods. The techniques presented here have evolved and have been simplified over the years of working with the in vitro neuronal system.

1.1. Unique Problems with ISH Methods in Neurons In Vitro

ISH data have been invaluable for correct interpretation of transcription detected by other methods in the neuronal cultures. A significant population of quiescent nonneuronal cells (fibroblasts and Schwann cells) remain in the neuronal cultures even after extensive exposure to antimitotic agents and may be a significant source of viral transcription during the establishment of latency *(4)*. Furthermore, only ISH data can provide information about the population of cells expressing a transcript. However, the characteristics of the neuronal culture system imposes several unique problems. The neurons in vitro require a substrate for attachment. We have found collagen to work the best. The neurons grow on and extend their neuronal processes through the 3D matrix pro-

duced by collagen-coating of cover slips. Upon establishment of DRG neurons in vitro, the cell bodies of the neurons are relatively large compared to many cell lines. Neuronal cultures, even with appropriate dissociation of the ganglia, will form clusters composed of a few to dozens of neurons. For these reasons, the neurons produce a three-dimensional character that differs significantly from cell lines in culture. Therefore the detection of transcripts using ISH methods with radiolabeled probes and emulsion provide poor spatial localization. Consequently, we have switched to using only nonradioactive detection of transcripts with digoxigenin-labeled riboprobes.

A second problem is the direct result of the extensive cytoskeletal structure present within the neurons. A more aggressive permeabilization protocol of neurons in culture is required for penetration of macromolecules (such as riboprobes), yet such protocols must not disrupt the attachment of the neurons to the cover slips. In our laboratories, ISH techniques have been developed that have proven to be both reliable and reproducible. These methods assume knowledge of the neuronal culture system, which has been described elsewhere *(8)*, and a general familiarity with the establishment and maintenance of RNAse-free conditions (*see* ref. *9*) for general methods for working with RNA).

2. Materials

1. NTP labeling mix 10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 6 5 m*M* UTP, 3 5 m*M* dig-UTP. Boehringer-Mannheim (Mannheim, Germany) #1277 073.
2. 2 m*M* $CaCl_2$
3. 50X Denhardts
4. DEPC-dH_2O.
5. DEPC-treated phosphate-buffered saline (PBS).
6. 100 m*M* dithiothretol (DTT)
7. 20% Dextran sulfate in formamide.
8. 5% Dimethyldichlorosilane in chloroform
9. DNA-Dependent RNA polymerase.
10. 0.2*M* EDTA.
11. Geneclean II Kit (BIO 101, La Jolla, CA)
12. 1.5 m*M* levamisole.
13. 4*M* LiCl
14. 1 *M* $MgCl_2$
15. 5*M* NaCl.
16. 60 m*M* Na_2CO_3/40 m*M* $NaHCO_3$, pH 10.2
17. 75 mg NBT/mL of 70% v/v DMF
18. 4% Phosphate-buffered paraformaldehyde (prepared fresh)
19. 10 mg/mL Proteinase K (Boehringer-Mannheim).
20. RNasin.
21. 50 µg/mL RNAse A (Qiagen)
22. 2 U/mL T1 RNase

23 20% Sodium dodecyl sulfate (SDS)
24 0 2*M* Sodium acetate, 1% (v/v) acetic acid, pH 6.0
25 20X SSC.
26. 10X Transcription buffer
27. 1*M* Tris-HCl, 9.5.
28. 20 m*M* Tris-HCl, pH 7 5
29. 10 mg/mL yeast tRNA.
30. 50 mg X-phosphate/mL in DMF.
31 RNase buffer. 20 m*M* Tris-HCl, pH 8 0, 0.5*M* NaCl, 1 m*M* EDTA
32. Buffer 1: 100 m*M* Tris-HCl, pH 7 5, 150 m*M* NaCl.
33 Buffer 2 Prepare only the volume that is needed, as precipitation occurs with storage 100 m*M* Tris-HCl, pH 9 5, 100 m*M* NaCl
34. 4 mL Buffer 2 + 18 µL of NBT stock (75 mg NBT/mL of 70% v/v DMF) +14 µL of X-phosphate stock (50 mg X-phosphate/mL in DMF) + 1 5 m*M* levamisole.

The molecular biology reagents have been used interchangeably from Promega (Madison, WI) and Boehringer-Mannheim, with the exception of the nucleotide mixture, which has only been purchased from Boehringer-Mannheim. Other chemicals are purchased from the supplier indicated or Sigma (St. Louis, MO).

3. Methods

3.1. Fixation and Storage of Cultures Prior to Hybridization

1 Grow neuronal cultures on plastic, collagen-coated cover slips in 24-well cluster dishes
2 To begin fixation, rinse cultures with DEPC-treated PBS
3. Fix with 4% phosphate-buffered paraformaldehyde for 12 h at 4°C
4. Rinse with DEPC-treated PBS, and dehydrate through grades of ethanol (70, 95, 100%) and air-dry.
5. At this point fixed cultures are removed from the cluster dishes with a sterile disposable hypodermic needle that has been bent at the tip to facilitate removal of cover slips from the culture dish
6. Store the cover slips at –20°C in the presence of a desiccant Strong hybridization signal has been obtained from cultures stored for 6 mo under these conditions

3.2. Silanization of Glass Cover Slips to Be Placed Over Neuronal Cultures During Hybridization

Glass 15-mm round cover slips are used to cover the samples during the hybridization to prevent drying. To prevent nonspecific sticking of probe, glass cover slips are silanized at least 1 d in advance.

1. In a fumehood, individual glass cover slips are dipped in 5% dimethyldichlorosilane in chloroform and placed in a 100-mm glass Petri dish and air-dried.

2. The cover slips are then washed, in mass, by repeatedly filling the Petri dishes with distilled deionized H_2O, replacing the cover, gently swirling, and decanting
3 The cover slips are then baked in an oven at 250°C for at least 4 h to destroy RNAse activity These can be stored at room temperature for at least 6 mo

3.3. Preparation of Template DNA

To obtain template DNA, we have used a plasmid preparation method based on the differential precipitation of DNA *(9)*. We have avoided methods that require addition of exogenous RNAse to facilitate the purification. Considerable RNA contamination remains in the preparation without presenting a problem.

1 The plasmid is linearized with the appropriate restriction enzyme and template is purified by electrophoresis in an agarose gel followed by purification of DNA using the Geneclean II Kit (BIO 101).
2 The recovery of DNA is then estimated by running on a gel against a set of DNA standards. All efforts are made to use RNAse-free reagents in preparing the template. In our hands, provided reagents are handled to avoid introduction of RNAse, no further purification steps are required Templates subjected to phenol extraction have produced lower yields in transcription reactions in our hands.

3.4. Transcription of Riboprobes

(The methods described below were derived from those provided by Boehringer-Mannheim with the Genius Non-Radioactive Detection Kit).

1. Transcribe by adding to a microfuge tube: 1 µg DNA template; 0 5 µL 100 m*M* DTT; 2 µL NTP labeling mix (10 m*M* ATP, 10 m*M* CTP, 10 m*M* GTP, 6 5 m*M* UTP, 3.5 m*M* dig-UTP), 2 µL 10 X transcription buffer; 2 U of DNA-dependent RNA polymerase, 2 µL RNasin Adjust final vol to 20 µL with DEPC-dH_2O
2. Centrifuge briefly.
3. Incubate at 37°C for 2 h.
4. Remove template by adding 2 U of RNase-free DNase I, incubate 15 min, 37°C
5. Add 1 µL of 0.2*M* EDTA and precipitate the riboprobe with 2 5 µL 4*M* LiCl, and 75 µL 100% ethanol, overnight at –20°C.
6. Centrifuge, wash pellet with cold 80% ethanol, dry, and resuspend in 100 µL DEPC-H_2O + 1 µL RNasin + 2 µL 100 m*M* DTT Incubate at 37°C for 15 min to dissolve the riboprobe
7. Generally, 10–20 µg of riboprobe will be produced per each reaction, depending upon template. Methods for confirming the yield and analyzing the size of the probe are well described *(9)*.

3.5. Fragmentation of the Riboprobe by Alkaline Hydrolysis

1. Hydrolyze digoxigenin-labeled RNA by adding an equal volume of DEPC-H_2O and 2 vol of 60 m*M* Na_2CO_3, 40 m*M* $NaHCO_3$, pH 10.2 Incubate at 60°C for 10 min.
2. Stop hydrolysis by adding an equal volume of 0 2*M* sodium acetate/1% (v/v) acetic acid, pH 6.0

3 Aliquot 1 μg of riboprobe per microfuge tube, add 5 μL of 10 mg/mL tRNA per tube, and 3 vol of ethanol Store indefinitely at –70°C Each tube contains a sufficient amount of riboprobe to hybridize 10–15 cultures on 13-mm cover slips

3.6. Pretreatment of Samples

1 The cultures on cover slips are hydrated in 20 m*M* Tris-HCl, pH 7 5 at 37°C, 2 m*M* $CaCl_2$ with 5 mg/mL proteinase K for 30 min at 37°C This can conveniently be performed by placing up to eight cover slips, culture side up, in a 60-mm sterile plastic Petri dish. Some care is required to be certain that trapped air bubbles have been removed to prevent the cover slips from floating
2 Thereafter, the samples are washed once in DEPC-treated PBS, dehydrated through graded ethanol and allowed to air-dry

3.7. Addition of the Riboprobe-Hybridization Mix to the Samples

1 For one aliquot of an ethanol precipitated riboprobe, spin, rinse pellet with 70% ethanol, and resuspend in 20 μL DEPC-dH_2O
2 Heat to 75°C for 3 min, cool to room temperature, and then add in order.

Stock	Volume
RNasin	2 μL
100 m*M* DTT	2 μL
20% Dextran sulfate in formamide	100 μL
20X SSC	40 μL
50X Denhardt's	4 μL
10 mg/mL yeast tRNA	5 μL
20% SDS	2 μL
DEPC-dH_2O	23 μL
02*M* EDTA	2 μL
Final volume	200 μL

3. Vortex vigorously to mix completely and then centrifuge to remove trapped bubbles

3.8. Hybridization

1. To the samples on the cover slips that have been pretreated with proteinase K and air dried, place culture surface up in a Petri dish, and add 20 μL of the probe-hybridization mixture to each cover slip Great care must be taken to avoid introduction of air bubbles
2. The hybridization mixture is then covered with a silanated glass cover slip We have not found it necessary to seal the edges of the cover slip if a humidified environment is provided for the hybridization
3 The Petri dish containing the cover slips must be leveled carefully If excessive problems occur with the silanated cover slips sliding off of the samples, either the volume of hybridization mix can be reduced or an edge of the cover slip can be tacked in place with a bead of rubber cement delivered via a syringe

4. Place Petri dish containing samples covered with a lid and place in a sealable container (i e., Tupperware) containing water-saturated paper towels Carefully place the sealed container in an oven at 52°C overnight. Temperatures for hybridization and washes are for HSV-specific probes, however, we have found them to be equally successful for probes for many cellular genes

3.9. Posthybridization Washes

1 Remove cover slips and wash samples at 50°C in 5 mL/Petri dish of 2X SSC + 50% formamide for 45 min.
2. Rinse 2X in RNase buffer and then incubate 30 min at 37°C with 50 μg/mL RNase A + 2 U/mL T1 RNase.
3 Wash at 50°C in 2X SSC + 50% formamide for 40 min
4 Wash with buffer 1 for 10 min
5 Block nonspecific antibody binding with buffer 1 + 0.3% Triton-X100 and 2% normal sheep serum for 30 min.
6 Incubate with buffer 1 + 0.3% Triton, 2% normal sheep serum and 1·5000 antidigoxigenin antibody for 4 h at room temperature
7 Wash with 2X with buffer 1 for 10 min each
8. Wash with buffer 2 for 10 min
9. Incubation of the cover slips in a vertical position during the colorimetric development reduces deposition of precipitate that forms. This results in reduced background staining and greatly improves the final appearance of the cultures. Cover slip holders are commercially available from Lipshaw or Thomas. Incubation with buffer 2 + NBT + X-phosphate Incubation in the dark for either 2-4 h at room temperature or 6–12 h at 4°C produce similar color development reactions.
10. Stop in 20 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA
11 Several approaches can be taken to mounting the slides A simple method is to place the cover slip on a standard microscope slide with a small drop of Aquamount below the slide. A second drop is placed on the slide and a glass cover slip is then mounted over the sample Slides prepared by this method are best photographed immediately Some fading and loss of cell morphology appears over time.

4. Notes

1 Currently, 13-mm plastic tissue culture cover slips (Sarstedt) are used for grow the neuronal cultures. This has been an area for problems, with plastic cover slips from some suppliers proving to be toxic to neurons. Unfortunately, although the cover slips from Sarstedt have not been toxic, some of the cover slips curl during processing for ISH. Use of acid-washed, glass cover slips is likely to be feasible, however, we have not yet used them for ISH. Retention of cultures and nonspecific sticking of probe to glass may require some modifications.
2 During the development of *in situ* methods, it became clear that the signal-obtained ISH protocol was greatly altered by the degree of fixation. In initial experiments brief fixation protocols were used, however, the results were highly

variable. Once the importance of permeabilization was appreciated, it became clear that variations could be eliminated with relatively long fixation times.

3 Two approaches for the generation of appropriate sized probes can be used. Either small (less than 300 bp) probes can be used, or longer probes can be reduced in length by limited alkaline hydrolysis, as described

4 As noted in the introduction, permeabilization of neurons is of critical importance for success of ISHs. With use and familiarity, adjustments of the concentration of the proteinase may be required with different lots of proteinase K. To facilitate this adjustment process, the proteinase K is made up as a 10 mg/mL stock and immediately frozen in many small aliquots These aliquots are only thawed once and used immediately In our hand, the best ISHs have been obtained when some degradation of the neuronal processes is detectable. Extensive loss of neurons from the cover slips is a sign of excessive proteinase treatment In addition, inclusion of SDS has been necessary for neurons in culture

5 We have included a figure to illustrate some of the patterns of transcript distribution that have been encountered by our laboratories in using the described system (Fig 1) In **(B)** the commonly observed nuclear LAT signal is demonstrated during the latent infection with a probe specific for the major LAT (For review, *see* ref. *10*) In **(C)** is shown another relatively common pattern of staining observed during the latent infection with a probe homologous to the leader region of the minor LAT. This consists of discrete punctate staining in the nuclei. We have obtained similar results using probes for regions 3' of the major LAT Arthur et al have reported similar results in DRG neurons in the mouse model *(11)* The significance of the punctate staining remains unknown, but may indicate RNA processing This punctate nuclear *in situ* signal can easily be overlooked because the staining is very limited and it is usually in more than one focal plane Finally, shown in **(E)** is a mostly cytoplasmic pattern of staining of neurons during reactivation with a probe specific for the IE 1 transcript The almost exclusively cytoplasmic pattern of staining is more difficult to see in this micrograph because the 3D character of the cells produces a slightly diffuse signal. Staining of the nucleolus has been observed. Probably this is related to nonspecific staining and suggests that inadequate RNase treatment was used

6 The methods described here can be directly applied to sections of ganglia. However, several modifications can be made that improve the histology. First, proteinase K pretreatment can be performed under much milder conditions, such as with 1 mg proteinase K/mL Second, SDS is not required in the hybridization mixture. With these modifications LAT has been detected readily in paraformaldyhde or Bouin's fixed tissue If one of the recently introduced noncross linking fixatives (e g., Amresco HC fixative) are used, proteinase K pretreatment should absolutely not be done and the use of SDS in the hybridization mixture can cause a loss in signal

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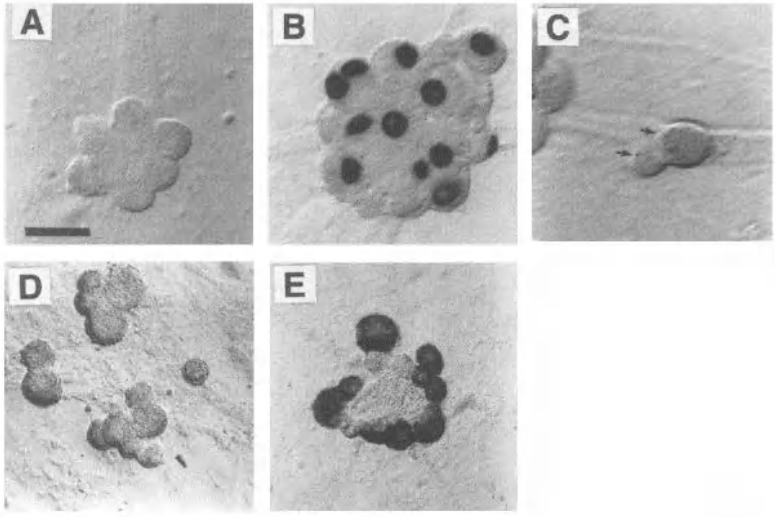

Fig. 1. Patterns of ISH signals in the in vitro neuronal HSV latency model. DRG neurons were hybridized for specific transcripts using the methods described in this review. Shown are **(A)** mock-infected neurons and **(B)** neurons during the latent infection hybridized with a LAT-specific riboprobe (for a review of the LAT region transcripts, *see* ref. *10*). Shown in **(C)** are the punctate nuclear hybridization signals (arrows) observed with a riboprobe to the 5' leader sequence of the minor LAT. Hybridization with an IE 1-specific riboprobe is shown in **(D)** neuronal cultures during the latent infection and **(E)** neuronal cultures during reactivation (12 h after NGF deprivation). The scale bar in (A) is 50 mm and applies to all panels.

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Assessing Cell-Mediated Immune Responses to HSV in Murine Systems

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1. Introduction

Protective immunity against a majority of viral infections is mediated by a combination of both humoral and cell-mediated immune responses. However, in the case of herpesvirus infections, where viral spread is largely cell-to-cell, cell-mediated immune mechanisms (which facilitate the clearance of virally infected cells) are particularly important *(1–4)*. Moreover, cell-mediated immunity (CMI) has also been implicated in the establishment and/or reactivation of latent herpes simplex virus (HSV) infection *(5,6)*. Thus, a major focus of herpesvirus immunology continues to be the identification of those herpesvirus antigens that serve as targets for CMI and the means by which protective responses can be optimally induced. Clearly this information is critical for the rational development of effective vaccine strategies.

In the first sections of this chapter, we detail various in vitro assays commonly used to measure HSV-specific CMI. In later sections, we describe methods that allow for the identification of the viral proteins and the minimal epitopes within these proteins that serve as targets for CMI. Lastly, we discuss a murine challenge model that allows herpesvirus antigens to be tested in vivo for their ability to confer protection from lethal doses of HSV. It should be noted that in this chapter HSV refers specifically to HSV-1.

1.1. Cytotoxic T-Cell (CTL) Assay

A major player in the cell-mediated immune response against herpesvirus infections is the cytotoxic T-cell (CTL). In general, virus-specific CTL are $CD8^+$ and recognize small peptides derived from viral proteins that are presented by class I major histocompatibility complex (MHC) molecules on the surface of

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virally infected cells *(7)*. Thus, the primary function of CTL is to first recognize and then lyse cells in an antigen-specific manner. Moreover, cell lysis is not the only way in which CTLs mediate their antiviral effects. CTLs also appear to stimulate intracellular enzymes that can degrade viral genomes, and CTLs can induce the secretion of cytokines with apparent interferon activity *(8)*. To study CTLs in vitro, CTLs are mixed with ^{51}Cr-labeled target cells which express the appropriate viral antigens. Lysis of the target cells releases ^{51}Cr and serves to indicate the activity of CTL against these target antigens. To induce herpesvirus-specific CTL in vivo, murine systems exist in which mice can be immunized with human strains of HSV. Alternatively a variety of viral and nonviral systems in which individual HSV antigens are expressed (e.g., recombinant vaccinia viruses, recombinant adenoviruses, recombinant retroviruses, and plasmid DNA) can also be used to immunize mice in order to generate CTL *(9–18)*. However, for the purposes of this chapter, the primary focus will be the use of HSV and recombinant vaccinia viruses expressing HSV antigens as tools to study viral-specific cell-mediated immune responses.

1.2. Limiting Dilution Analysis to Determine CTL Precursor Frequency

Although the standard CTL assay as described in the previous section does detect the presence of antigen-specific CTLs, it does not allow for their quantitation. For this reason, it is not possible to directly compare on a per-cell basis the CTL responses generated from different effector populations. In contrast, limiting dilution analysis (LDA) allows one to quantitate the CTL present in a given effector cell population. LDA is a retrospective process based on the measurement of the number of activated CTL clones generated from a given population of cells. Since each of these clones is originally derived from an individual precursor clone, one can retrospectively determine the frequency of precursors that led to the generation of the active clones. Thus, LDA lets you determine how many CTLs in a given population have participated in response to a particular antigen, and allows the number of such precursors to be measured at the clonal level.

In particular, LDA has proven to be invaluable for assessing immune responses to various immunization protocols. For example, LDA can be used to make decisions regarding the most effective vector delivery systems (e.g., recombinant vaccinia virus vs recombinant adenoviruses), as well as doses of immunogen, frequency of administration, and routes of immunization. It should also be noted that LDA can be used to determine precursor frequencies for B cells and for helper T cells as well *(21)*. However, in this section we have confined our discussion to LDA of CTL directed against HSV *(22,23)*.

1.3. T-Helper Cell-Proliferation Assay

Helper T cells (Th) are an important group of cells that not only provide the help needed for B cells to mount antigen-specific humoral immune responses, but they also provide help for the differentiation and maturation of CTL precursors into effector CTL. Th cells are generally of the $CD4^+$ phenotype and are MHC class II restricted. Two distinct subsets of Th cells, Th_1 and Th_2, have been well documented and characterized in both the human and murine systems *(25–29)*. This separation into either Th_1 or Th_2 subsets is based on their distinct cytokine secretion patterns. In most cases, Th1 cells secrete interferon-γ (IFN-γ), tumor necrosis factor (TNF-β), interleukin-2 (IL-2), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF), whereas Th_2 cells express IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, and GM-CSF. Their ability to express different cytokine profiles enables Th_1 and Th_2 cells to mediate different effector functions. For example, Th_1 cells appear most effective in mediating antiviral and antitumor immune responses, whereas Th_2 cells predominate in the immune clearance of extra cellular pathogens (e.g., parasites and bacteria).

Since Th_1 responses appear critical for the induction of effective antiviral immune responses, one important goal of herpesvirus immunology is to identify herpesvirus antigens capable of inducing Th_1 cells. By the same token, it may be equally important to identify viral antigens that induce Th_2 cells, since the inclusion of such antigens could impact negatively on the ability of an immunogen to induce protective immune responses.

When antigen-specific Th cells are stimulated with antigen in the presence of the appropriate antigen-presenting cells, cellular proliferation and cytokine secretion is induced. Thus T-helper responses can be determined by measuring antigen-specific cellular proliferation and/or cytokine production. Specifically, cellular proliferation can be quantitated by measuring the amount of tritiated thymidine incorporated into dividing cells, whereas cytokine levels can be measured by using bioassays or enzyme-based immunoassays (ELISA, ELISPOT). Typically, detection of IL-2, IFN-γ and TNF-β is regarded as indicative of a Th_1-type response, whereas detection of IL-4 and IL-6 indicates a Th_2-type response. What follows are the details needed to assay for HSV-specific lymphoproliferation. These methods have been adapted from a protocol originally described by Horohov et al. *(30)*.

1.4. Mapping CTL Epitopes

A variety of methods can be used to identify the minimal antigenic regions (epitopes) within a viral protein that serve as targets for CMI. In this section we will focus on mapping CTL epitopes, but the same approaches are applicable to mapping Th epitopes as well.

Once a viral protein has been identified as a CTL target, several options can be pursued. One option is to scan the amino acid sequence of the protein for the presence of allele-specific peptide-binding motifs *(31,32)*. These motifs, which allow one to identify putative CTL epitopes, reflect the actual sequence of naturally processed peptides eluted from MHC class-I molecules. For example, peptides eluted from class-I molecules of the H-2K^d haplotype are invariably 9–10 amino acids in length, with a tyrosine always at amino acid position 2 and one of five favored, predominantly hydrophobic amino acids at position 9 Thus, an appropriate protein sequence can be most easily scanned by identifying the locations of all tyrosine residues and fixing this as position 2 of a putative epitope 9 amino acids in length. Peptides representing these regions can then be synthesized and tested for their ability to sensitize appropriate targets for CTL lysis.

An alternative strategy is to synthesize a series of overlapping peptides that span the entire length of the protein of interest. One recommended strategy for CTL epitope screening is to synthesize overlaping peptides that are 15 amino acids in length (15 mer) with 10-mer overlaps This ensures that all putative CTL epitopes of $\leq$10 amino acids will be represented. Although this approach is extremely rigorous, it can be costly depending on the size of the protein For example, to screen a protein of 512 amino acids in length using 15 mers with a 10-mer overlap, 86 peptides need to be synthesized! However, once positive peptides are identified, the minimal CTL epitope can be quickly defined by sequentially deleting amino acids from both the amino and carboxyl ends of the peptide.

The identification of CTL epitopes in herpesvirus proteins continues to be an important goal for several reasons. From a research perspective, peptides representing CTL epitopes remain a powerful tool. In particular, peptides can be used to restimulate CTL cloned lines in vitro, thus eliminating the need to continually set up bulk effectors from the spleens or lymph nodes of immunized mice From a therapeutic standpoint, CTL peptides can be used alone or in conjunction with Th and neutralizing antibody epitopes to test the efficacy of subunit vaccine strategies for the induction of protective immune responses.

1.5. Murine Challenge Model

Several animal models exist for assessing the protective immune responses induced by herpesvirus antigens *(36–38)*. In particular, the mouse has proven useful since many different immunization protocols can be tested, allowing one to evaluate a wide variety of immune responses *(38,39)*. In this section we will detail a murine challenge model that is easy to perform and can be adapted for use with other viruses besides HSV. However, we encourage the reader to explore the use of other models as well. For example, the zosteriform model *(39)* may be used to mimic recurrent infection and thus allows one to test the ability of immunization to protect against peripheral neurologic spread of HSV

2. Materials

2.1. Cytotoxic T-cell (CTL) Assay

1. Inbred mouse strains (ideally 5–9 wk old) to immunize with HSV Three commonly used strains of mice with different MHC class I haplotypes are C3H/HeN ($H\text{-}2^k$), C57BL/6 ($H\text{-}2^b$), and BALB/c ($H\text{-}2^d$).
2. Needles (18, 26, and 30 gage) and syringes (1 and 3 mL) for mouse immunizations
3. Sterile dissection instruments to remove spleens and lymph nodes from immunized animals· scissors, forceps, and scalpels Sterile wire-mesh screens to prepare single-cell suspensions of spleens and lymph node cells.
4. MHC class I-compatible permanent cell lines to serve as targets in the CTL assay LT^-A^- cells ($H\text{-}2^k$), C57SV40 ($H\text{-}2^b$), and EMT6 ($H\text{-}2^d$) *(9)*
5. Complete culture medium for growing permanenT-cell lines. DMEM supplemented with (final concentrations shown): 10% heat-inactivated fetal calf serum (FCS), 2 m*M* L-glutamine,100 IU/mL penicillin,100 µg/mL streptomycin
6. CTL medium for lymphocyte cultures. RPMI-1640 medium containing 10% heat-inactivated FCS, 10 m*M* L-glutamine, 100 IU/penicillin, 100 µg/mL streptomycin, and 5×10^{-5}*M* 2-mercaptoethanol
7. Tissue-culture grade trypsin/EDTA solution (0.5 g trypsin and 0 2 g EDTA/L) used to detach adherent cell lines for routine passage
8. EDTA (0.02%) solution to detach cell lines used as targets in CTL assay.
9. Hemocytometer and trypan blue solution to determine live cell counts.
10. Multichannel *(12)* pipettor (50–200 µL) and an adjustable pipettor (50–200 µL).
11. Sterile plasticware. 15- and 50-mL polypropylene tubes, various sizes of vented tissue culture flasks, 96-well round-bottom tissue-culture plates, 6-well plates, tips for pipettors (200 and 1000 µL capacity), 5- and 10-mL pipets, and Petri dishes (6 and/or 10 cm)
12. Ultraviolet (UV) light souce (e.g., germicidal lamp)
13. $(Na)_2{}^{51}CrO_4$
14. γ-Counter.
15. Table-top centrifuge with carriers that hold tubes as well as microtiter plates.
16. 37°C Incubator with humidified atmosphere of 5% CO_2/95% air.
17. 37°C Water bath

2.2. Limiting Dilution Analysis to Determine CTL Precursor Frequency

1. Mice immunized with HSV or with other vector systems as decribed previously.
2. Spleens from naive, unimmunized, syngeneic mice to serve as feeder cells during in vitro culture
3. Same materials as required for CTL assays.
4. CTL medium as described previously is also used for LDA, but it is additionally supplemented with the following (final concentrations) 5% concanavalin A-derived T-cell growth factor (Collaborative Research, Bedford, MA), 50 m*M* α-methyl mannoside, human recombinant interleukin-2 (5 U/well of 96-well plate,

Collaborative Research), 1 m*M* oxaloacetic acid, 0 2 U/mL bovine insulin, and 5 × 10^{-5}*M* of 2-mercaptoethanol

5 Computer program(s) for data analysis based on formulas provided by Taswell *(24)*.

2.3. T-Helper Cell-Proliferation Assay

1 Mice immunized with HSV or recombinant vaccinia viruses expressing HSV proteins as described previously
2 Spleens from naive, unimunized mice to serve as stimulator cells. These mice must be the same strain as the immunized mice (i e , same haplotype)
3. ^{3}H-Thymidine
4 Concanavalin A.
5 RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 25 m*M* HEPES buffer, 5 × 10^{-5}*M* 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin
6 96-well U-bottom plates
7. Cell harvester and glass fiber filter strips.
8 Liquid scintillation counter, scintillation fluid, and scintillation vials

2.4. Mapping CTL Epitopes

1 Mice immunized with HSV or other vectors systems from which HSV antigens are expressed
2 Appropriate target cells (MHC class I compatible) to which peptides are added (target sensitization) Positive controls set up in parallel should include target cells infected with HSV or with recombinant vaccinia viruses expressing HSV antigens This ensures that the effector cells are capable of cytolytic activity. It is also important to include negative controls in which irrelevant peptides are added to target cells This rules out the possibilty of nonspecific peptide-mediated cytolysis Finally, wells should be set up during the CTL assay containing only target cells and peptide (no effectors added) If lysis occurs in these wells this indicates that the peptide itself is toxic to the target cells.
3. Synthetic peptides of defined lengths (e.g., 15 mers with 10-mer overlap), spanning the length of the protein or individual peptides representing motif-predicted epitopes. Peptides should be stored at –20°C.
4 All reagents necessary to perform CTL assay

2.5. Murine Chalenge Model

1 Inbred mouse strains Mice should be 5–6 wk old at the beginning of the experiment. Each group of mice needs to be genetically identical, the same age, and the same sex; littermates are ideal.
2. Immunogen to be tested (e.g., recombinant vaccinia virus expressing a herpesvirus protein, or other vector system as described earlier)
3. Virulent strain of HSV-1 (McKrae strain).

3. Materials

3.1. Cytotoxic T-cell Assay

3.1.1. Immunizing Mice with HSV

1. Inject mice intraperitoneally (ip) with approx 5×10^6 plaque-forming units (PFU) of HSV-1-KOS. This is a commonly used lab-adapted strain well-suited for immunization. If possible, immunize groups of 10 or more mice at one time in order to be able to perform several CTL assays using mice from the same immunized "stock."
2. Virus for immunization should be diluted in sterile PBS and drawn into a 3-mL syringe, using an 18-gage needle. A 26-gage needle is then used to administer 0.5 mL of virus ip.
3. Mice are boosted ip 2 wk later with the same amount of virus and then rested for 1–2 wk before collecting their spleens for testing in the CTL assay
4. Footpad injection represents an alternative route of immunization. Specifically, a 30-gage needle is used to inject both rear footpads of the mouse with 5×10^6 PFU of virus per footpad. Five days postimmunization the draining popliteal lymph nodes are collected for testing in the CTL assay

3.1.2. Immunizing Mice with Recombinant Vaccinia Viruses, Recombinant Adenoviruses, Recombinant Retroviruses, and Plasmid DNA

Although the immunization of animals with whole HSV remains an excellent way to induce CMI, viral and nonviral vectors expressing herpesvirus antigens can also be successfully used to induce HSV-specific CMI. In particular, this latter approach allows one to identify individual HSV proteins capable of inducing CMI. Moreover, once an immunogenic viral protein is identified, a variety of techniques, several of which will be detailed in later sections of this chapter, can be used to map the exact location of the minimal epitope within the viral protein.

What follows here are immunization recommendations for several viral and nonviral vector systems.

1. For immunization with recombinant vaccinia viruses, mice can be injected IP or in the footpads exactly as described above for HSV, with the exception that the viral doses given should be increased to $1–10 \times 10^7$ PFU IP and 1×10^7 PFU/footpad
2. Similar doses to those used for vaccinia immunization are also recommended for recombinant adenoviruses, except that 5×10^7 PFU is injected per footpad.
3. Although immunization with retrovectors expressing HSV antigens has not, to our knowledge, been reported, retrovectors expressing other antigens have been used to induce CMI. For example, a retrovector expressing the human immunodeficiency virus (HIV) env and rev proteins has been shown to successfully induce CMI in mice *(17)* Intramuscular injection of the vector into two sites ($1–5 \times 10^5$ colony forming units [CFU] per site) is followed by a booster injection 1 wk later. One week following the booster, spleens can be harvested for in vitro testing.

4 Regarding the injection of plasmid DNA encoding HSV antigens, intramuscular injection in at least two sites using 1–100 μg of DNA/site is recommended; boosting is also advisable. However, it should be noted that the amount of DNA needed for either the primary or booster injection appears highly dependent on the particular expressed antigen Thus, the optimal doses need to be empirically determined It is also possible to use a "gene gun" to immunize animals with DNA *(19)* In this procedure, gold beads coated with DNA are propelled from a helium-driven gun and delivered directly into cells (usually into cells of the skin and into cells beneath the skin's surface) In this system, the optimal amounts of DNA to deliver must also be determined for each antigen In general, smaller amounts of DNA (e g , nanograms) can be delivered using the gene gun as compared to other methods of delivery

3.1.3. Collecting Spleens or Lymph Nodes from Immunized Mice

1. Sacrifice each mouse by cervical dislocation, saturate with 70% ethanol, and lay the animal with its left side facing up Cut the epidermis and carefully tear the skin away from the underlaying spleen
2 Lift the mesentary, which covers the spleen, then cut and peel it back before removing the spleen with sterile forceps Finally, trim away any remaining fat and connective tissue Avoid letting the spleen touch nonsterile areas Place the spleen into a 50-mL tube containing 10 mL of CTL media.
3 Draining popliteal lymph nodes are removed 5 d following footpad immunization Mice are placed on their abdomens, and a scalpel is used to shave the hair from the back of both rear legs A vertical incision is then made down the length of the back of each leg, and the popliteal lymph nodes found at the rear of the knee joint are removed using sterile forceps and placed into an appropriate tube containg CTL media

3.1.4. In Vitro Bulk Culture of CTL Effector Cells (Splenocytes or Lymph Node Cells)

1 Pour spleen(s) or lymph nodes from the 50-mL tube into a sterile 6- or 10-cm Petri dish.
2. Tease the spleen(s) or lymph nodes into single-cell suspensions by using the end of the plunger from a 3-mL syringe to gently press the cells through a sterile wire screen. This procedure allows the lymphocytes to pass through the screen while trapping tissue remnants in the screen
3 Use a pipet to rinse the screen several times with media This ensures that very few lymphocytes are left behind in the screen. After disposing of the screen,the lymph node suspension is spun at 1600 rpm for 5 min and resuspended at 2×10^7 cells/mL. Each well of a 6-well plate is then seeded with 1 mL of the suspension, and CTL media is added to bring the volume of each well to a 5-mL total. Plates are incubated for 3 d at 37°C in 5% CO_2
4 Unlike draining lymph node cells, which can be removed from the animal and directly cultured in vitro, splenocytes must be restimulated in vitro with HSV

antigen before bulk CTL cultures can be set up. This is because draining lymph nodes presumably still contain viral antigen trapped within the node itself In contrast, splenocytes from immunized mice are comprised of memory T cells, which need to be re-exposed to antigen in order to become activated UV-irradiation of HSV reduces the viral titer to a low enough level such that splenocytes are exposed to viral antigens without being lysed as the result of a productive viral infection UV-inactivation of HSV is achieved by exposing 2×10^8 PFU of virus to a germicidial lamp for 2 min at a distance of 3 cm and generally reduces viral titer to approx 100 PFU or less. Thus, splenocytes are restimulated in vitro with UV-irradiated HSV-1 KOS at a multiplicity of infection (MOI) of 2 for 1 h at 37°C For example, if 1×10^8 splenocytes are to be restimulated at a MOI of 2 with HSV-1 KOS, whose titer is 1×10^9 PFU/mL, then the neccessary calcuation is as follows. (1×10^8 splenocytes)(MOI of 2) = 2×10^8 PFU of virus needed, thus, 2×10^8 PFU needed/1×10^9 PFU (actual viral titer before UV-irradiation) = 0.2 mL of UV-irradiated virus needed to restimulate 1×10^8 splenocytes at an MOI of 2. Cultures are set up in 6-well plates at 1×10^7 cells/well in a total volume of 5 mL/well for 5 d at 37°C in 5% CO_2.

3.1.5. Radiolabeling Target Cells for CTL Assay

1 Cells to be used as targets should be in log-phase growth. Thus, it is usually best to split cells the day before such that they will be 70–80% confluent on the day of the assay
2. On the morning of the assay, wash cells with phosphate-buffered saline (PBS) and add enough of the 0 02% EDTA solution to cover the cells Incubate flasks at 37°C until cells easily detatch when pipetted with a 10 mL pipet
3 Harvest cells by gently pipeting up and down in the flask The cells must be treated gently and should not at any point in time become clumped.
4. Cells are washed once by centrifugation using CTL media and then resuspended at 1×10^6 cells/mL Two milliliters of cells (2×10^6 cells total) are placed in a 15-mL tube and labeled with 200 µCi of ^{51}Cr. At the same time that the cells are being labeled they are also infected with either HSV (MOI of 5) or a recombinant vaccinia virus expressing an HSV antigen (MOI of 10). The total time for labeling and viral infection is approx 3–4 h Although labeling with ^{51}Cr only requires 1 h, viral antigen expression by HSV and the recombinant vaccinia virus takes approx 3–4 h.
5. Both the ^{51}Cr labeling and the viral infection occurs optimally when a 37°C water bath is used, and the tubes are gently mixed every 10 min during the first hour of incubation
6. Following the incubation period, cells are washed three to four times by centrifugation with CTL media.
7. The washed target cells are then resuspended in CTL media at 1×10^5 cells/mL and placed on ice until the splenocyte or lymph node cultures have been harvested.

3.1.6. Harvesting CTL Effector Cells (Cultured Splenocytes or Lymph Node Cells)

1. Harvest cultured cells from the wells of the plate by gently pipetting up and down with a 10-mL pipet

2. Pool the contents of all wells together, wash once with CTL media, and resuspend pellet at 1×10^7 cells/mL. Keep cells on ice until they are added to the 96-well microtiter plate.

3.1.7. Setting up 96-well Microtiter Plate for the CTL Assay

1. To rows B,C, D, and F of a 96-well microtiter plate add 100 µL of CTL media Add 200 µL of splenocytes or lymph node cells (effector cell population) at 1×10^7 cells/mL to row A Using a 12-channel pipettor, remove 100 µL from row A and sequentially add, mix, and remove 100 µL of cells to the next row continuing this process through row D
2. Next add 100 µL of labeled target cells (1×10^5 cells/mL) to triplicate wells in rows A–D For example, if one has 4 different labeled targets, then target no 1 would be added to wells 1–3 of rows A–D, target no 2 would be added to wells 4–6 of rows A–D, target no 3 would be added to wells 7–9 of rows A–D, and target no. 4 would be added to wells 10–12 of rows A–D.
3. Similarly, 100 µL of each target are added to triplicate wells of row F This row serves to indicate the amount of spontaneous release of ^{51}Cr, since the wells in this row only contain targets and media (no effector cells are added). In contrast, 100 µL of target cells are added to triplicate wells in row G along with 100 µL of 1*N* HCL This row serves to indicate total 51Cr release of target cells, since 1*N* HCL induces maximal lysis of the cells
4. Following the addition of all the target cells to the appropriate effector cells, all wells should contain a total volume of 200 µL. Moreover, row A should contain an effector to target (E T) ratio of 100·1, row B, a ratio of 50 1, row C, a ratio of 25:1; and row D, a ratio of 12.5.1

3.1.8. Harvesting Samples and Data Calculation

1. Remove assay plates from the incubator after a 4-h incubation period.
2. Using the multichannel pipettor, carefully remove and transfer 100 µL of supernatant from each well into the corresponding γ-counter tubes. It is critical that the cell pellet is not disturbed when performing this procedure and that fresh tips are used for each triplicate set of samples.
3. Place tubes in gamma counter for target cell lysis determination and include empty tubes for machine background evaluation. Count tubes for 1 min at settings appropriate for ^{51}Cr γ emission and tabulate ^{51}Cr release as counts/minute (CPM)
4. The percent-specific target-cell lysis is determined by the following formula [(experimental release – spontaneous release)/(maximum release – spontaneous release)] × 100

3.2. Limiting Dilution Analysis to Determine CTL Precursor Frequency

1. Remove lymph nodes (for acute effectors) or spleens (for memory effectors) from immunized mice, press through sterile stainless steel mesh, and prepare single-cell suspensions. Wash once, count the cells, and resuspend in fresh medium.

2. Make serial dilutions of the effector cells titrating from 4×10^5 cells/mL down to 100 cells/mL. Ideally 11 dilution tubes should be set up (4×10^5/mL, 2×10^5/mL, $1\ 5 \times 10^5$/mL, 1×10^5/mL, 8×10^4/mL, 6×10^4/mL, 4×10^4/mL, 2×10^4/mL, 1×10^4/mL, 5×10^3/mL and 1×10^3/mL), plus a 12th "mock" tube to which no cells are added.
3. Prepare 96-well U-bottom plates (one for each dilution) by filling the outer wells with PBS (200 µL/well) and then add 100 µL of each dilution to the 60 remaining wells in the center of each plate Leave plates (should be 12 in total) in the 37°C incubator while preparing feeder spleens
4. For feeder cells, harvest spleens from naive syngeneic mice, make single-cell suspension, lyse RBC with Tris-NH_4Cl, wash three times, and resuspend in fresh medium Irradiate cells (2400 Rads using X-ray machine), wash, and resuspend at 5×10^6 cells/mL.
5. Add 100 µL/well feeder cells to all the dilution plates of effector cells prepared earlier, and incubate the plates for 7–9 d at 37°C with 5% CO_2 Monitor for outgrowth of pleomorphic-shaped CTL from these wells Usually these cells are apparent by d 5 or 6 of culture.
6 When LDA plates are ready to be tested (between d 7 and 9), set up target cells for CTL assay Briefly, add 3×10^6 cells of each target cell in a total of 2 mL medium, add 400 µCi ^{51}Cr, infect with HSV or another virus and incubate as detailed previously Wash targets five times, resuspend at 1×10^4 cells/mL. Remove 100 µL of each target and count in gamma counter. Ideally 2000–3000 cpm is ideal, but there should be a minimum of 1000 cpm/100 µL However, should the cpm be too low, increase the target cell concentration to 3×10^4/mL in order to increase the total cpm/100 µL-aliquot.
7. Prepare effectors while targets are incubating at 37°C Flick the effector plates quickly, add 200 µL PBS/well, and centrifuge briefly to pellet cells Flick PBS and add 200 µL fresh medium/well and make a 4-way split of each well (50 µL/well) into 96-well, V-bottom plates. Incubate plates at 37°C until targets are prepared and ready. Note that a 4-way split allows one to test effectors against four targets Two- or three-way splits can also be done when fewer targets are needed. However, splits greater than 4 are not recommended because the cell numbers become prohibitive.
8. Add 100 µL of appropriate target to each well (resulting in a total of 150 µL/well), spin plates gently, and incubate for 4–5 h at 37°C as in the case of a standard ^{51}Cr release assay.
9 Harvest supernatant fluids from the wells and count as usual in a γ-counter. Calculate CTL precursor frequency according to the method described by Taswell *(4)*. This provides provides the frequency of precursors within a confidence limit of 95%

3.3. T-Helper Cell-Proliferation Assay

1. Prepare antigen presenting cells by infecting naive, syngeneic spleen cells in vitro with UV-inactivated HSV at an MOI of 5 for 3–4 h at 37°C, and then irradiate these infected cells with 3000–4500 rads. Wash one or two times in medium and resuspend cells at 5×10^6 cells/mL These cells are often referred to as stimulator cells (antigen presenting cells) in the proliferation assay

2 Harvest spleens or lymph nodes from HSV or recombinant vaccinia virus-injected mice. Collect effector cells as described previously and resuspend at 1×10^7 cells/mL Effector cells are usually referred to as responder cells in the proliferation assay
3 Titrate responder cells and stimulator cells by plating the cells at different concentrations in 96-well U-bottom plates to achieve a final volume of 200 µL/well Responder cells are usually titrated down the plate using serial twofold dilutions Stimulator cells ($2 \times 10^5 - 1 \times 10^4$ cells/well) are then added to the responder cells and, ideally, all wells are set up in triplicate and the plates are incubated for 72 h It is important to include appropriate positive and negative controls in this assay As a positive control the mitogen concanavalin A (Con A) is used at a final concentration of 2–4 µg/mL and naive, uninfected spleen cells serve as negative controls. Con A control wells need to be harvested within 3 d, since this is the time of maximal mitogen-induced proliferation Therefore, it is advisable to set up the Con A controls in their own separate plate Lastly, don't forget to include control wells that contain responder cells alone or stimulator cells alone as part of the assay
4. At 72 h poststimulation, add 1 µCi/well ^{3}H-thymidine to all wells and incubate for an additional 8–18 h **Note:** For Con A controls, ^{3}H-thymidine should be added after 54–64 h of incubation, since harvesting occurs at 72 h
5. Harvest cells onto glass fiber filters using a cell harvester, transfer filters to scintillation vials, add 5 mL of appropriate scintillation fluid per vial, and count for ^{3}H-thymidine incorporation using a liquid scintillation counter
6 Stimulation index (SI) is calculated as follows.

SI = CPM in HSV-stimulated wells/CPM in nonstimulated wells

Cytokine assays can be performed most easily by setting up duplicate plates of responder cells and stimulator cells exactly as you would for a proliferation assay After 12–48 h of incubation (depending on the particular cytokine of interest), supernatant fluids can be harvested and frozen at –20°C until ready to be assayed for the presence of cytokines Although bioassays for some of the cytokines exist, ELISA kits or paired antibodies are available commercially for the detection of a wide variety of cytokines

3.4. Mapping CTL Epitopes

1. Ideally lyophilized peptides can be reconstituted and diluted appropriately in the same media used for the CTL assay. However, this is sometimes not possible since some peptides are insoluble in these types of solutions In these cases a stock solution of peptide can be made in dimethylsulfoxide (DMSO) and then diluted with CTL media
2. Regardless of the solvent used, it is advisable to prepare a peptide stock solution (e g , 2 m*M* works well) from which dilutions can be made To prepare a 2-m*M* stock solution of peptide, one needs to calculate the molecular weight of the pep-

tide. This is done by adding up the molecular weights of each of the individual amino acids from which the peptide is comprised. Next, divide the actual amount (in mg) of peptide synthesized by the molecular weight of the peptide to determine the number of moles of peptide present For example, if the molecular weight of a peptide with an amino acid sequence of GGHQAAMQL is 1204, and the actual amount of peptide synthesized is 2 mg, then 2 mg/1204 equals 1 66 μM of peptide present in a total weight of 2 mg. Thus, to prepare a 2-mM stock solution of this peptide, reconstitute the 2 mg of peptide in 830 µL solvent.

3. To sensitize target cells with peptide, 8–10 µL of peptide (2 mM stock) is added to 1–3 × 10^6 target cells in a total of 1–2 mL of media also containing ^{51}Cr (200 µci) After 1 h incubation at 37°C, the target cells are washed three to four times in CTL medium, and used to set up a standard CTL assay as described previously. It is important to wash away excess peptide from the target cells before adding them to the effector cells This prevents excess peptide present in the medium from binding to the effector cells themselves and resulting in effector cell.effector cell lysis *(33)*
4 The same steps detailed above can be used to identify T-helper epitopes. However, instead of CTL assays, proliferation and/or cytokine assays are used to detect antigen-specific proliferation and cytokine production

3.5. Murine Challenge Model

1 Before starting the actual experiments, pilot studies need to be performed to determine the minimal dose of McKrae virus needed to kill the different strains of mice This dose will vary between strains. For example, C3H/HeN mice are the most susceptible to McKrae, followed by Balb/c, and then C57/BL6 mice, which are the most resistant of these three strains
2 Once the dose of McKrae virus has been established for each strain, the following groups of mice (10/group) should be injected as follows: group 1 represents the experimental group to be injected with the test immunogen. For example, we have tested the ability of a recombinant vaccinia virus expressing an herpes antigen in this model by injecting both rear footpads with 1 × 10^7 PFU of recombinant vaccinia virus Of course, other sites and other vector delivery systems can be tested as well Group 2 is a specificity control group. For example, if group 1 is injected with the recombinant vaccinia expressing an herpes antigen, then group 2 should be injected with a recombinant vaccinia virus expressing an irrelevant (non-HSV) antigen Group 3 animals represent the negative control group and should be injected with PBS, whereas, in group 4 (positive controls), the animals are injected with HSV-1 KOS strain.
3 Immunized mice are rested for approx 2 wk before challenge with the McKrae virus Depending on the immunogen being tested, animals may be boosted once or twice before challenge. Mice are challenged with an appropriate dose of McKrae virus administered ip and then observed for 2 wk for signs of morbidity and mortality Typically, all mice in group 4 survive, whereas all mice in groups 2 and 3 die by approx d 10–12. The number of mice surviving in group 1 indicates the level of protection afforded by the immunogen.

4. Notes

1. It is not neccesary to remove red blood cells (RBC) when setting up splenocyte cultures. However,the absence of RBCs will make the cultures appear cleaner and easier to visualize under the microscope during their 5-d incubation period. Remove RBC as follows· pellet single-cell splenocyte suspension as described in methods, but do not resuspend the pellet directly in CTL media Instead, resuspend cell pellet in a few milliliters of Tris-buffered ammonium chloride (Tris-NH_4Cl) solution and incubate for 2 min at 37°C Then add 10 mL of media containing FCS to neutralize the activity of the ammonium chloride, centrifuge, and resuspend cell pellet in CTL media for restimulation with UV-irradiated virus. Note that lymph nodes do not contain RBC thus precluding the need for Tris-NH_4Cl treatment Tris-buffered ammonium chloride (Tris-NH_4Cl) solution is prepared by first making stock solutions of 0 16*M* NH_4Cl (stock A) and 0 17*M* Tris, pH 7 65 (stock B). These stocks are then mixed together to make the working solution (90 mL stock A and 10 mL stock B) The final pH of the solution is adjusted to 7.2 with HCl *(20)*
2. Novice CTLers may find that their ^{51}Cr-labeled targets demonstate high levels of spontaneous lysis. This high background level is often the result of washing the labeled targets too vigorously Care must be taken to avoid this type of nonspecific lysis of target cells. Furthermore, during washing, the target cells should be gently but thoroughly resuspended in order to prevent cell clumping, a condition that will also adversely affect CTL-specific lysis
3 The success of LDA is particularly dependent on the quality of the supplements added to the culture medium. For example, it is critical that several lots of fetal calf serum be tested in order to select the lot that correlates with the best assay results. It is also important to try different combinations of Con A-derived T-cell growth factor and recombinant IL-2. It also appears that the presence of 2-mercaptoethanol in the medium is critical for the proper outgrowth of CTL.
4. Proliferation assays performed on splenic cultures often demonstrate high background counts (i e , substantial incorporation of ^3H-thymidine by negative control cultures). One solution is to set up proliferation assays using lymph-node cultures, since, in many cases, these cultures do not exhibit high background counts However, if problems with high backgrounds persist, then the amount of FCS in the culture medium should be reduced to levels below 5%.
5 Although the general guidelines given above should work for most peptides, a certain amount of trial and error is often necessary in order to find the optimal peptide concentration needed to sensitize targets for CTL lysis It is also recommended that peptide assays be performed in medium containing reduced levels of FCS (2–5% final). This reduces the potential for protease-mediated peptide breakdown.
6. When one is faced with a large number of peptides to screen, one can combine as many as four peptides together before adding this pool to target cells. Peptides from positive pools can then be tested individually
7. In general, it is advisable for screening purposes to synthesize peptides containing unblocked amino and carboxyl ends. This holds true for both CTL and Th

epitope mapping. However, for Th epitope mapping, peptides with blocked ends may be more effective especially when peptides less than 15 amino acids are used *(34,35)*.

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23

Assays for HSV Gene Expression During Establishment and Maintenance of Latent Infection

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1. Introduction

Assays in use for the analysis of herpes simplex virus (HSV) gene expression during the establishment and maintenance phases of infection in the nervous system include:

1. The use of reporter genes, for example, the *lacZ* gene from *Escherichia coli*, which is inserted by homologous recombination into the viral genome, and which may be driven either by viral promoters or by an exogenous promoter, such as the major immediate early (IE) promoter of cytomegalovirus. In our hands, the detection of *lacZ* activity in neuronal tissue infected with recombinant HSV constructs has proven to be a simple and effective means of monitoring viral activity in the peripheral nervous system.
2. Analysis of virally encoded RNA transcripts, either by *in situ* hybridization (ISH) using radioactive or nonradioactive indicator molecules, or by Northern analysis (this technique is described in Chapters 13 and 24)
3. Immunohistochemistry to demonstrate the presence of viral proteins, which technique can also be used in combination with ISH (dual labeling)

2. Methods

2.1. Use of the Reporter Gene lacZ

This bacterial gene encodes the enzyme β-galactosidase, a substrate for which is 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, NovaBiochem, Nottingham, UK), which is cleaved by the enzyme to yield an insoluble blue product. This assay for β-galactosidase in infected animal tissue is an adaptation of a standard method *(1)* for the detection in cell monolayers of

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plaques produced by recombinant virus expressing *lacZ*. The kinetics of expression of *lacZ* in neurons infected with recombinant HSV has been examined during acute and latent phases of infection *(2,3)*

2.1.1. Method

1. Dissect tissue (e g , sensory ganglia) from infected animal and place in phosphate-buffered saline (PBS) at 4°C until ready to fix
2. Fix tissue in 2% paraformaldehyde, 0.5% glutaraldehyde in PBS at 4°C for 1 h in the case of mouse ganglia (larger pieces of tissue may require longer times in fixative), and then wash out fixative with PBS
3. Permeabilize tissue by incubation at 4°C for 30 min in 2 m*M* $MgCl_2$, 0.01% sodium deoxycholate, and 0 02% Nonidet P-40 (Fisons, Loughborough, UK)
4. Remove this mixture, and replace with the same solution containing 5 m*M* potassium ferricyanide-ferrocyanide plus X-gal (1 mg/mL) Incubate at 37°C for 2–6 h, checking periodically using dissecting microscope for the appearance of blue-stained neurons
5. Wash out X-gal solution with several changes of PBS, and clear by incubation for 2–12 h in 20% glycerol at 4°C Stained tissue can be visualized under a microscope by placing between two slides in a wet mount If desired, tissue can be embedded in paraffin, and sections cut on a microtome In our experience, the blue color produced in the procedure above is not washed out during the embedding process

2.2. Detection In Situ of Viral Nucleic Acids

ISH is a powerful technique that, via hybridization of indicator molecules, permits visualization of the cellular and possibly subcellular location of target sequences. An increasing number of indicator molecules, radioactive and nonradioactive, are available. Compared to radioactive indicator molecules, nonradioactive alternatives, such as digoxigenin, which show the presence of a probe by a colored precipitate or by fluorescence, offer improved resolution at the subcellular level *(4)* and long storage life, while avoiding difficulties associated with radioactivity. However, enumeration of target molecules from such signals remains problematic. Autoradiographic detection of radioactivity provides a final readout in the form of silver grains, which can be counted, permitting an approximation of the number of target molecules to be made, based on specific activity of the label and assumptions regarding efficiencies of hybridization and autoradiography. On this basis, we have estimated that mouse neurons latently infected with HSV may each contain on the order of 10^4 copies of LAT *(5)*. For excellent discussions of the quantification of autoradiography, *see* Rogers *(6)* and Gowans et al. *(7)*

As radioactive and nonradioactive systems, each have advantages. Therefore, both are described in this chapter.

2.3. Fixation of Tissue

In our hands, the fixative of choice for tissue samples is periodate-lysine-paraformaldehyde (PLP; ref. *8*) owing to its suitability for preservation of nucleic acids and antigens *(9–11)*. However, if the preservation of nucleic acids is the sole consideration, then other fixatives, such as 4% paraformaldehyde or 0.5% gluteraldehyde, each in PBS, may give satisfactory performance (*see* Note 1). After impregnation with paraffin, tissue sections should be collected onto slides treated with a suitable coating to ensure adhesion of sections during subsequent procedures. Glutaraldehyde-activated 3-aminopropyltriethoxy-silane-coated slides *(12)* are adequate for this purpose.

2.4. Choice of Probe

Probes synthesized from recombinant DNA are recommended because they are less likely to contain contaminating sequences than even highly purified naturally derived nucleic acids *(13)* RNA probes have a number of advantages: first, being single stranded, there is less tendency to self-anneal, which effectively increases probe concentration, thereby maximizing sensitivity *(13)*, second, opposite sense transcripts can be used as controls for nonspecific binding. These benefits are considered sufficient to justify the effort necessary to subclone DNA sequences into suitable plasmids, such as pBluescript®, which contain bacteriophage T3 and T7 promoters.

2.5. Choice of Indicator Molecule

2.5.1. Radioactive Isotopes.

The ideal radioactive label would provide high resolution coupled with high sensitivity at short exposure times. Five isotopes are in common use in ISH: ^{3}H, ^{125}I, ^{35}S, ^{33}P, and ^{32}P. In practice, none of them are ideal. A detailed discussion of the decay characteristics of each isotope is beyond the scope of this chapter (*see* ref. *6* or *14*). However, the approximate path lengths in nuclear emulsion of emissions from the above isotopes are 1, 2, 10–20, 15–20, and >20 μ, respectively *(6)*. ^{125}I therefore provides much higher resolution in ISH than does ^{32}P, ^{33}P, or ^{35}S, while retaining sufficiently high energy to permit exposure times similar to those used with ^{32}P. Exposure times with ^{3}H are on the order of weeks or months, which may be considered unacceptably long. Based on the preceding considerations, ^{125}I-CTP is recommended for use in RNA probes. Despite the 60-d half-life of ^{125}I, in our hands, it is found that the signal-to-noise ratio is compromised if label is used later than 1 mo after manufacture. A disadvantage of the use of ^{125}I-CTP is that in our hands, there appear to be large batch-to-batch variations in the signal-to-noise ratio obtained.

2.5.2. Digoxigenin: A Nonradioactive Indicator Molecule

In our hands, the nonradioactive indicator molecule digoxigenin (available as digoxigenin-UTP for transcription reactions; Boehringer Mannheim, Mannheim, Germany) yields excellent results in ISH applications, providing excellent resolution at the subcellular level and giving sensitivity comparable to that achieved with ^{125}I. Preparations of this probe do not appear to suffer loss of sensitivity after >2 yr of storage at –20°C, nor have we experienced any batch-to-batch variations in the behavior of digoxigenin-UTP. Digoxigenin is therefore the indicator molecule of choice, unless there is an absolute requirement that the ISH signal be quantified.

2.6. Preparation of RNA Probes

2.6.1. Radioactive Probes

RNA probes are prepared according to the method supplied by the makers of the Riboprobe® RNA labeling kit (Promega, Madison, WI), and efficiency of incorporation is measured by differential precipitation of RNA by trichloroacetic acid *(15)* In our experience, optimal performance of an RNA probe is associated with >75% incorporation of available radiolabel into RNA transcripts. Probes of 0.5–1 5 kb in length are used without hydrolysis The recommended specific activity of ^{125}I-labeled RNA probes is 5×10^8 dpm/μg, and probes should be used at a concentration of 40 pg/μL *(7)*

2 6.2. Nonradioactive Probes

Incorporation of digoxigenin into RNA probes is carried out by following the method given by the makers (Boehringer Mannheim), which resembles that used for incorporation of radioactively labeled nucleotides into RNA, instead using digoxigenin-UTP. Optionally, a small amount (0.5 μL) of ^{32}P-dCTP may be included in the reaction mix, so that the efficiency of incorporation may be measured by the trichloroacetic acid differential precipitation method *(15)*.

2.7. Preparation of Tissue Sections for ISH

This method is an adaptation of that of Gowans et al *(7)*

1. Dewax paraffin sections in xylene, and rehydrate gradually through graded ethanol/water mixtures over a period of 1 h (*see* Note 4)
2. Fix in 0 1% glutaraldehyde in PBS for 30 min at 4°C, and wash in PBS (2 × 5 min)
3. Digest tissue sections with proteinase K in 20 m*M* Tris-HCl, pH 7.4, 2 m*M* $CaCl_2$ for 15 min at 37°C. The optimum concentration of proteinase K should be determined empirically and may vary from batch to batch In our hands, a concentration of 100 μg/mL gives strong autoradiographic signal with good preservation of tissue morphology

4. Wash sections in phosphate-buffered saline (PBS), refix in 0.1% glutaraldehyde for 15 min, and wash twice more in PBS
5. Acetylate sections by immersing in a freshly made solution of 0.25% acetic anhydride in 0.1*M* triethanolamine (pH 8.0) for 10 min at room temperature with gentle agitation (*see* Note 5)
6. Wash sections twice in PBS, dehydrate gradually, and dry prior to application of hybridization mix

2.8. Hybridization

ISH follows the same general principles as filter hybridization, so that the optimum temperature for hybridization is T_m–25°C (T_m, the temperature at which 50% of double-stranded nucleic acid hybrids are in liquid dissociated into single-stranded molecules, is calculated as in Note 6), and the most stringent wash should be at T_m–10°C. By careful adherence to this guideline, we have found that a posthybridization ribonuclease digestion step is not required. The addition of 50% formamide to the reaction mixture has the effect of lowering the reaction temperature to a level compatible with preservation of histological detail.

The final hybridization mix contains 40 pg/µL RNA probe in 50% deionized formamide, 1X SSC, 100 m*M* Tris-HCl, pH 7.6, 10 m*M* Na_2HPO_4, 10 m*M* NaH_2PO_4, 0.02% Ficoll, 0.12% polyvinyl pyrollidone, 500 µg/mL sheared denatured salmon sperm DNA, 500 µg/mL yeast tRNA, 1.25 mg/mL nuclease-free bovine serum albumin (BSA), 20 m*M* dithiothreitol (DTT), and 1 U/µL ribonuclease inhibitor.

2.8.1. Method

1. Heat mix to 80°C for 5 min, and store on ice prior to addition to slides
2. Apply hybridization mix (3 µL) to sections, and cover with a 13-mm sterile siliconized cover slip, taking care not to trap air bubbles. For larger sections increase the volume of probe mix in proportion to cover slip area
3. Seal cover slips with rubber cement (e.g., repair adhesive 4051, PMD, Bridlington, Yorkshire, UK), and incubate slides for 8–15 h at T_m–25°C. For probes complementary to the LAT region of HSV (i.e., having a G + C content of approx 75%), this temperature is 75°C.

2.9. Washing Procedure

1. Using fine-tipped forceps, remove rubber cement and cover slips, and immediately place slides in a large volume of 2X SSC at room temperature. If the probe solution dries onto the section, this will be associated with a significant increase in background. Wash in 2X SSC for 1 h, and then twice in 0.1X SSC for 1 h at room temperature.
2. High-stringency wash. With gentle agitation, wash slides for 20 min at T_m–10°C, which for probes complementary to the LAT region of HSV is 75°C in 30% deionized formamide, 0.1X SSC.

3. Wash in 0.1X SSC for 30 min at room temperature to remove traces of formamide from sections.
4. Dehydrate slides through graded ethanol solutions, and air-dry prior to dipping in nuclear emulsion

When using radioactive iodine as an indicator molecule, potassium iodide (100 m*M*) is included in all washes to reduce background.

2.10. Autoradiography and Staining for Radioactive Labels

For a comprehensive discussion of the properties and uses of nuclear emulsions, *see* Rogers *(6)*. We have found Amersham LM-1 emulsion suitable for the detection of ^{125}I-labeled RNA probes. To minimize background grains, care is required in the handling of nuclear emulsion, in particular, sudden changes in conditions, such as pH or temperature, should be avoided.

A suitable quantity of Amersham LM-1 emulsion is mixed with an equal volume of water in a darkroom equipped with safelight recommended by Amersham and melted at 42–44°C for 30–40 min. The diluted emulsion is carefully mixed, and slides are dipped vertically, set immediately on an ice-cold flat metal plate for 30 min, dried for 1 h to overnight at room temperature, and stored in a light–tight box at 4°C for 2–5 d. Exposed slides are developed in Kodak D-19 developer for 4 min at 23°C, with gentle agitation for 5 s at 30-s intervals, rinsed in 1% acetic acid stop bath for 30 s, and placed in Ilford Hypam rapid fixer (diluted 1:4) for 8 min. Optimal development time should be determined empirically. Slides are then washed in tap water for 30 min, stained with rapid hematoxylin *(7)* for 30–45 s, and "blued" in 0.1X SSC for at least 30 min. Sections are dehydrated in graded ethanol solutions, stained with eosin for 1 min, washed in ethanol (3 × 1 min), washed in xylene (2 × 10 min), and "coverslipped" using DePex (BDH, Poole, UK) mountant.

2.11. Color Development: Digoxigenin ISH

This method is based on that described by the manufacturer (Boehringer Mannheim).

1. Using coplin jars, wash slides for 5 min in buffer 1 (100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl).
2. Incubate for 30 min in blocking reagent (Boehringer cat no. 1096-176, 1% w/v in buffer 1 This can be dissolved by heating with stirring on a hot plate for 30 min)
3. Wipe slides to remove bulk of blocking reagent, and incubate sections with antidigoxigenin alkaline phosphatase conjugate, 1/1000 in blocking solution, for 30 min at 37°C in a humid box, about 50 µL/section
4. Wash 2 × 15 min in buffer 1, rinse briefly in buffer 3 (100 m*M* Tris-HCl, pH 9 5, 100 m*M* NaCl, 50 m*M* $MgCl_2$).
5. In fresh coplin jars, place slides in substrate solution (5-bromo-4-chloro-3-indolyl

phosphate/Nitro blue tetrazolium, made up according to the manufacturer's instructions) for 1–5 h in a dark box.

6. Wash several times in running water and counterstain briefly (10–15 s) in hematoxylin with further washes in water for several minutes to ensure color differentiation of counterstain.
7. Visualization of slides under wet mounting is sufficient to enable photography.

2.12. Immunohistochemical Detection of Viral Antigens

The peroxidase–antiperoxidase method *(16–18)* using 3,3'-diaminobenzene (DAB; Life Technologies, Paisley, UK) as a substrate, when optimized, gives strong signal over HSV-infected cells with very low levels of background. DAB also has the benefit of forming a precipitate that is insoluble in the reagents used in any subsequent ISH procedure. The primary antibodies could be polyspecific, for example, rabbit antiserum to HSV-infected cells (Dakopatts, Glostrup, Denmark), or monospecific, such as monoclonal antibody (MAb) 58-S (American Type Culture Collection HB8183) to ICP4. Binding of primary antisera is detected by using as secondary antibody swine antirabbit or goat antimouse immunoglobulin as appropriate, followed by rabbit or mouse peroxidase–antiperoxidase conjugate, respectively (all from Dakopatts, Glostrup, Denmark). We have used this method to detect a number of HSV-encoded proteins, including ICP8 and Vmw65, in addition to those detected as above *(5)*.

1. Dewax slides in xylene, and hydrate through decreasing concentrations of ethanol, ending in Tris buffer (Tris-HCl, pH 7.5, 50 m*M*)
2. Immerse sections in Tris buffer containing 0 01% H_2O_2 for 10 min at room temperature, to block endogenous peroxidase activity.
3. Overlay sections with blocking solution (Tris buffer containing 10% normal serum of the species providing the secondary antibody), and leave at 37°C in a humid box for 30 min (*see* Note 2).
4. Wash for 2 × 5 min in Tris buffer.
5. Incubate sections first with primary antibody, followed by secondary antibody, and then peroxidase–antiperoxidase conjugate, with washes as in step 4 between each incubation. Antibodies and conjugate should be diluted in blocking solution, with the optimum dilutions determined empirically (*see* Note 3). Allow all reactions to proceed for 30 min at 37°C in a humid box.
6. Wash sections as above, and then incubate slides in a solution of DAB (0 5 mg/mL, containing 0.1% H_2O_2) for 2–5 min in the dark, with the actual time determined empirically. If sections are not to be subsequently subjected to ISH, they may be lightly counterstained with hematoxylin

2.13. Dual-Labeling

Simultaneous detection of nucleic acids and proteins on the same tissue section (dual-labeling; refs. *7* and *19–21*) enables the presence of viral mRNA to

be correlated with the presence of virally encoded proteins. Using dual-labeling for viral antigens and for LAT during acute infection with HSV in mouse spinal ganglia, we have demonstrated that neurons with the characteristics of latency (i.e., expressing LAT in the absence of antigens) are present from the earliest detectable stages of acute infection in the nervous system *(22)*

In our experience, the dual-labeling method of Gowans et al. *(7)* (in which immunohistochemistry precedes radioactive ISH) has proven satisfactory. An alternative approach in which in nonradioactive ISH is carried out first has been described *(23)* We have not attempted to quantify formally the loss of sensitivity of ISH owing to preceding immunohistochemistry. However, in our hands, there does not seem to be a marked reduction in signal strength. It should be borne in mind that any dual-labeling procedure may lead to some loss of sensitivity in each of the component assays.

As a precaution against degradation of RNA in sections, all immunohistochemical reagents used should be sterilized.

2.13.1. Method

1 Fix sections, and carry out immunohistochemistry as above, having filter-sterilized all reagents using 0 2-μm filters (Sartorius, Gottingen, Germany). Reagents should contain 1 U/μL ribonuclease inhibitor and 1 m*M* DTT
2. After immunohistochemistry, wash sections in 0.1% Triton X-100 in PBS for 10 min at room temperature to minimize nonspecific binding of probe and to increase hybridization efficiency on immunohistochemically stained cells *(7)*
3 Sections are then subjected to ISH using ^{125}I-labeled RNA probe as above.
4. Counterstain: Because nuclear emulsion is rapidly dissolved in low pH, a brief immersion of 10–30 s in fast hematoxylin *(7)* is sufficient Eosin staining is not used due to the presence of DAB precipitate

3. Notes

1 As a general rule, the optimal preservation of antigens is attained by fixing for the minimum time compatible with preservation of morphology, which we find is 60 min in fresh PLP in the case of mouse spinal ganglia Many laboratories make use of central tissue embedding services. However, if this procedure is adopted, it is worthwhile checking whether a formaldehyde fixation step is included, since this will have the effect of greatly increasing the time spent in fixative
2. In the case of severe background problems, it may be necessary to increase the concentration of serum in the blocking solution, conversely, if background is found to be very low, then satisfactory blocking may be achieved in 10% calf serum
3. As an example, we have obtained good results with Dako polyclonal antisera to HSV, diluted 1 in 50, followed by swine-antirabbit antisera at 1 in 25 dilution followed with rabbit peroxidase–antiperoxidase at 1 in 100 dilution
4. Increased background can result from using impure reagents at this point: it is recommended that analytical-grade (AR) reagents be used throughout

5. If excessive background is encountered, it may be useful to repeat the acetylation step Acetic anhydride has a very short half-life and must be made up freshly.
6. Calculation of T_m: the temperature (T_m) at which 50% of double-stranded RNA hybrids will dissociate in liquid into single-stranded molecules is defined by the equation:

$$T_m \text{ (RNA/RNA °C)} = 79.8 + 18.5 \log_{10}[Na^+] - (0.35 \times \%\text{formamide}) + 58.4 (G + C) + 11\ 8 (G + C)^2$$

where G + C is (%G + C)/100 *(24,25).*

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Analysis of HSV-DNA and RNA Using the Polymerase Chain Reaction

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1. Introduction

The polymerase chain reaction (PCR) technique is a sensitive method for detection of nucleic acids that can be used to detect herpes simplex virus (HSV)-DNA and RNA in tissue samples with greater sensitivity than hybridization with specific probes *(1,2)*. In its most basic form, PCR involves multiple cycles of denaturation of DNA, annealing with specific primers and replication of specific DNA using a thermostable DNA polymerase like *Taq* polymerase, resulting in amplification of a specific DNA sequence. Reverse transcriptase polymerase chain reaction (RT-PCR) employs a preliminary reverse transcription step of RNA, using either a specific 3' or an oligo (dT) primer, to produce complementary DNA (cDNA), followed by PCR using primers specific for the transcript of interest. In its standard application, PCR offers qualitative information regarding the presence or absence of target sequences, and has been used to analyze latently infected ganglia and brain for HSV-DNA *(6,8–11)* and RNA *(6–10,12)*. As described in the following, with the inclusion of mutated templates as internal standards, PCR can be used to determine a quantitative estimate of the number of HSV genomes and transcripts in tissue extracts. Histologically, *in situ* hybridization (ISH) can be used to detect HSV-DNA and RNA in specific cells in the nervous system *(3–5)*, although it has not been successfully applied to detect HSV genomes during latency *(6,7)*. PCR methods can be applied to tissue sections (*in situ* PCR), making it possible to identify individual cells harboring HSV genomes, even during latency *(9,13)*.

1.1. Quantitative DNA-PCR

Several methods have been used to adapt PCR to quantify specific DNA or RNA sequences *(14,15)*. One of the simplest methods to quantitate latent HSV-

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DNA is to amplify known amounts of standard and latently infected ganglionic DNA in parallel reactions *(7,10)*. This method is subject to error, because significant tube-to-tube variations can occur even when the same dilution of template is amplified *(15)*. In a second method, a cellular DNA template of known concentration is amplified along with the target DNA in the same tube *(16–18)*. However, it is known that the kinetics of amplification may vary for different substrates of differing length and/or sequence *(15,16)*.

These limitations are overcome in competitive quantitative PCR *(8,9,14)*, in which a competitor template is constructed that is identical in size and sequence to the target DNA, except for a single-base mutation, resulting in either the addition or loss of a unique restriction site. The competitor DNA is quantified, serially diluted, and coamplified with a fixed amount of the target DNA in a series of tubes, using the same set of primers. After coôâplification, the two PCR products can be distinguished from each other after appropriate restriction enzyme digestion. The amount of DNA product is proportional to the initial amount of both DNA templates; the rate of the PCR in both cases is the same because the conditions of amplification are identical for the target and competitor DNA. The relative amount of PCR product is determined for target as well as competitor by ethidium bromide staining or radioactive incorporation during the assay. The tube in which the target and competitor DNAs are equivalent alâôws determination of the amount of the target DNA.

We describe a method to quantitate the number of HSV-1 genomes in latently infected nervous tissue by competitive quantitative PCR *(8,9)*. A great degree of care has to be exercised, since amplification can be affected by a number of factors, including the concentration of the different components of the reaction (Mg, dNTPs, *Taq* polymerase, template DNA, primers, and so on), and the temperatures for annealing and extension. We use a two-step PCR method to eliminate heterodimer formation *(14)*. At the completion of the initial PCR, an aliquot is diluted into a fresh reaction mix, followed by only two additional cycles, using a radioactive tracer to monitor amplification. We used the AMBIS radioanalytic imaging system for radioimaging and direct quantitation of the PCR products, since it is more sensitive than densitometric scanning of autoradiographs with a dynamic range of 5 orders of magnitude, compared to less than 3 for autoradiography.

1.2. Quantitative RNA-PCR

Conventional methods such as Northern hybridization or ribonuclease protection assays are limited when attempting to quantitate low levels of viral RNA transcripts in nervous tissue during HSV latency. RNA can be analyzed quantitatively by competitive quantitative RT-PCR, analogous to competitive quantitative PCR for DNA. The same principles considered for quantitative DNA-PCR hold for RNA quantitation One strategy *(15)* involves coamplifi-

cation of known amounts of a DNA template along with the target cDNA, using the same set of primers. The amount of RNA can be underestimated by this method, since it assumes that the efficiency of reverse transcription is 100%, whereas it can actually range from 5–90% *(19)*. Another method *(20)* involves reverse transcription of the target RNA along with a synthetic RNA standard, followed by coamplification of the cDNAs. The standard has flanking sequences complementary to the same primers as the target mRNA, but possesses a different internal sequence so that PCR products of different sizes are obtained. This technique might not be quantitative because the kinetics of reverse transcription reactions involving RNA of different sizes and sequences often varies *(21)*

During HSV latency, the only viral transcript detected is the latency-associated transcript or LAT *(5,22)*. We describe a method to quantitate the amount of LAT-RNA in latently infected nervous tissue using competitive quantitative RT-PCR *(8,9)*. First, we construct a mutant competitor RNA molecule identical to the LAT message to be amplified, except for the loss of a unique *Bsa*HI site. We have bypassed the need for cloning into a T3 polymerase transcription vector by directly attaching a T3 polymerase promoter site to our mutant LAT-DNA *(8,9)*. The mutant LAT-RNA is used to compete with target RNA from latently infected tissues by competitive RT-PCR. In this method, both the standard and target LAT molecules are subjected to the same kinetics of reverse transcription and DNA amplification in the same tube. After coamplification and digestion with *Bsa*HI, reaction products are detected and quantified as described earlier for the DNA amplification.

1.3. In Situ *PCR*

In contrast to solution techniques that measure the total number of molecules extracted from tissue, *in situ* techniques determine which cells in the tissue contain the target molecules. However, *in situ* hybridization is limited in sensitivity when low levels of nucleic acid are involved. *In situ* PCR *(9,13,23)* can be used to localize DNA in appropriately prepared tissue samples. We describe an *in situ* technique to detect HSV-1 DNA in selected sections from latently infected rat trigeminal ganglia *(9)*. Specific oligonucleotide primers to the HSV-1 glycoprotein B *(gB)* gene and digoxigenin-labeled nucleotides are used to produce amplified digoxigenin-labeled DNAs *in situ*, which are localized using an alkaline phosphatase conjugated antidigoxigenin antibody detected with the BCIP/NBT reagent system. We have identified neurons in infected trigeminal ganglia containing HSV-1 genomes using this method *(9)*.

2. Materials

2.1. Buffers and Enzymes

1. DNA extraction buffer: 50 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, 0.5% Tween-20, 400 mg/mL proteinase K

2 TRI total RNA isolation reagent (Molecular Research Center, Cincinnati, OH)
3. 10X PCR buffer I: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8 4 at 20°C, 15 m*M* $MgCl_2$, 1 mg/mL gelatin
4. PCR assay buffer. 10 µL of 10X PCR buffer, 4 mL dNTPs (10 m*M* each), 2 µL of specific primer pairs, 2 µL *Taq* polymerase, sterile distilled water to volume Top the PCR mixture with 50 µL autoclaved mineral oil
5 RT-PCR assay buffer. 10 µL 10X PCR buffer-II (Perkin-Elmer, Norwalk, CT), 2 µL of each dNTPs (10 m*M* each), 5 m*M* $MgCl_2$, 4 mL of LAT primer pairs, 8 µL RNA template extract, 2 µL *Taq* polymerase, 1000 U MMLV reverse transcriptase, distilled water to 100 µL and topped off with 50 µL autoclaved mineral oil
6 100-ng/µL Primers
7 *Taq* polymerase· Any commercial variety available can be used, but optimal concentration to be used must be first determined by titrating with appropriate substrate
8 Mineral oil: Light white from Sigma (St. Louis, MO)
9. Reverse transcriptase Either MMLV or AMV
10. DNase I, RNase free.
11 NuSieve GTG agarose (FMC BioProducts, Rockland, ME)
12 T3 RNA polymerase
13 *In situ* PCR reaction mix. 1X PCR buffer II, 1 m*M* $MgCl_2$, 1X digoxigenin DNA labeling mix, 100 ng of primer mix, 10% glycerol, and 1 µL of *Taq* polymerase

2.2. Primers

The primer pairs used were:

1 For *gB*. 5' primer ATT-CTC-CTC-CGA-CGC-CAT-ATC-CAC-CTT; 3' primer AGA-AAG-CCC-CCA-TTG-GCC-AGG-TAG-T
2. For LAT: 5' primer GAC-AGC-AAA-AAT-CCC-GTC-AG; 3' primer ACG-AGG-GAA-AAC-AAT-AAG-GG *(6)*, and
3 For glyceraldehyde phosphate dehydrogenase (GAPDH): 5' primer ATT-GGG-GGT-AGG-AAC-ACG-GAA, 3' primer ACC-CCT-TCA-TTG-ACC-TCA-ACT-A

3. Methods

Any one of several methods can be used to extract DNA and RNA from tissue. One simple method for preparing DNA and RNA extracts that we have used to quantify DNA and RNA from infected rat hippocampus by competitive quantitative PCR *(8)*, is described below The samples are taken from tissue mounted on glass slides

3.1. Extraction Procedure for DNA

1. Chill 10-µm sections on glass slides at –20°C.
2. Scrape the region of interest from the brain sections at –20°C with a prechilled sterile razor blade and transfer the tissue to sterile Eppendorf tubes containing 1.0 mL of absolute ethanol at room temperature Vortex for 1 min and pellet in a microfuge at 10,000*g* for 2 min
3 Resuspend the pellet in absolute ethanol and repeat this step twice.

4. Dry the pellet, resuspend in 100 µL of DNA extraction buffer, incubate at 37°C for 1–12 h, boil for 15 min, and take 10 µL for PCR

3.2. Extraction Procedure for RNA

1. Transfer tissue scraped from the slide to a sterile Eppendorf tube containing 0 8 mL TRI and homogenize using a Polytron homogenizer.
2. Store homogenized samples for 5 min at room temperature
3. After addition of 0.2 mL of chloroform, vortex samples for 15 s, and store for 2–3 min at room temperature.
4. Centrifuge samples in a microfuge for 15 min. RNA is found in the upper phase
5. Precipitate RNA from the upper aqueous phase with 0 5 mL of isopropanol for 10 min at room temperature. Microcentrifuge samples for 10 min at room temperature
6. Wash the RNA pellet with 70% ethanol and allow to air-dry.
7. Dissolve extracted RNA in 50 µL distilled water.

3.3. Amplification of DNA

Amplify 10-µL samples of extract in a final volume of 100-µL in PCR assay buffer for 30 cycles, at 95°C for 15 s, 54°C for 15 s, and 71°C for 1.5 min. Amplified *gB* DNA is 191 bp long, using the *gB* gene primers described above.

3.4. Construction of Competitor gB DNA

1. A mutant 191 bp competitor *gB* PCR fragment can be constructed, with a *Hpa*II restriction site 29 nucleotides from the 3' end, by using the wild-type *gB* PCR product as template for amplification with the wild-type *gB* 5' primer and a new 3' primer (5' AGA-AAG-CGC-CCA-TTG-GCC-AGG-TAG-TAC-TCC-GGC-TG3') in which nucleotide 29 is changed from G to C, introducing a *Hpa*II site internal to the original 3' primer. The amplification conditions are as described earlier
2. Gel purify the mutant *gB* product and quantify by optical density

3.5. Competitive Quantitative DNA-PCR

The amount of viral DNA in the extracts is first approximated by titration against a tenfold dilution series of the mutant *gB* fragment, followed by a more precise quantitation by titrating the extracts against a 2-fold dilution series spanning the first determination.

1. Add 10 µL of extract to samples of a tenfold dilution series of the mutant competitor *gB* fragment ranging from 100 pg to 1 fg.
2. Amplify the mixtures for 30 cycles using the original primer pair as described
3. Dilute reaction products 200-fold and amplify again for two cycles with radioactive tracer in a 20-µL final volume of PCR Assay Buffer, to eliminate heterodimer formation *(13)*.
4. Add 20 U of *Hpa*II and incubate the mixture at 37°C overnight.
5. Electrophorese the entire volume on a 4% NuSieve GTG agarose gel in 1X TBE

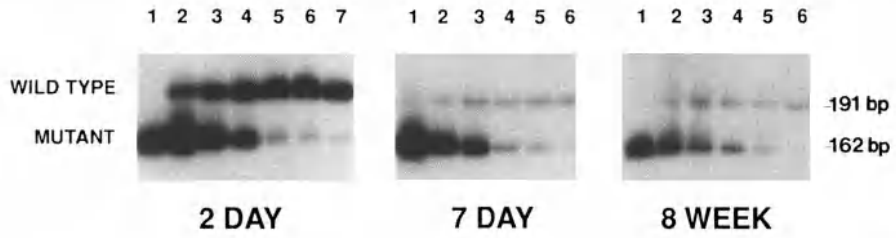

Fig. 1. Competitive Quantitative DNA-PCR of *gB*. DNA from 2 d, 7 d, and 8 wk hippocampal extracts were coamplified with a twofold dilution series of competitor mutant *gB* DNA using *gB* primers as described in the text. The radioactive amplified PCR products were digested with *Hpa*II and electrophoresed on 4% NuSieve agarose gels. Gels were dried and exposed to X-ray film. A representative autoradiograph is shown here. (2 d) Lane 1, 100 fg of mutant *gB* DNA coamplified with an uninfected hippocampal extract; lanes 2–7, twofold dilution series of competitor mutant *gB* DNA ranging from 100 fg (lane 2) to 3.1 fg (lane 7), coamplified with infected trigeminal extracts. (7 d and 8 wk) Lane 1, 100 fg of mutant *gB* DNA coamplified with an uninfected hippocampal extract; lanes 2–6, twofold dilution series of competitor mutant *gB* DNA ranging from 100 fg (lane 2) to 6.25 fg (lane 6), coamplified with infected trigeminal extracts.

buffer. Dry the gel and expose to X-ray film (Hyperfilm-MP, Amersham, Arlington Heights, IL) at –70°C.

6. Visual comparisons of the wild-type and mutant bands allow for a rough approximation of the amount of viral DNA in the extract.
7. Add 10 µL of extract to an appropriate twofold dilution series of mutant competitor *gB* fragments, usually from 100 to 3.1 fg.
8. Repeat steps 2–5. A representative autoradiograph is shown in Fig. 1.
9. Count the amount of radioactivity in each band using a system like the AMBIS Radioanalytic Imaging System.
10. Prepare standard linear regression curves by plotting net counts per minute for each sample in the dilution series against the amount of input mutant *gB* DNA in the series. A software program like the Graph PAD INPLOT software program (Graph PAD Software, San Diego, CA) may be used for this. Using the regression coefficient obtained from the standard curve and the net cpm from the wild-type bands, the point of equivalence is determined and the amount of *gB* DNA in the original extracts calculated.

3.6. Amplification of RNA

RNA templates are amplified using a combined RT-DNA amplification method *(9,24)* in a single tube, using a single buffer system.

1. Treat extracts with DNase I (1000 U) for 60 min at 37°C to destroy the DNA template.
2. Incubate extracts in a final volume of 100 mL of RT-PCR assay buffer, at 37°C for 30 min to permit reverse transcription.

Fig 2. Schematic of the use of a 12-bp bridge oligonucleotide to stabilize the T3 RNA polymerase promoter and the 5' LAT primer for ligation

3. Amplify using LAT primers at 95°C for 1 min and 60°C for 1 min for 30 cycles, followed by a single extension at 72°C for 10 min. This results in a 195-bp product with a *Bsa*HI restriction enzyme site located 21 nucleotides from the 3' end

3.7. Construction of Competitor LAT-RNA

A mutant LAT-PCR product can be constructed in which the cytosine at residue 22 from the 3' end is converted to thymidine, resulting in the loss of the *Bsa*HI site. An oligonucleotide coding for the T3 RNA polymerase promoter (5' GCT-CGA-AAT-TAA-CCC-TCA-CTA-AA 3') is ligated to the 5' end of the LAT 5' primer (5' GAC-AGC-AAA-CCC-GTC-AG 3') using a 12-bp bridge oligonucleotide (5' TGA-TTT-CTG-TCG 3') complementary to six bases of the T3 promoter oligonucleotide and six bases of the LAT 5' primer as shown in Fig. 2.

1. Treat 5 µg of LAT 5' primer with 10 U of T4 polynucleotide kinase Extract the reaction products with phenol-chloroform, precipitate in ethyl alcohol, dry, and resuspend in 10 µL autoclaved distilled water
2. Ligation of the T3 promoter oligonucleotide and the kinased LAT 5' oligonucleotide in the presence of the bridging oligonucleotide (5 µg each) is accomplished using 30 U of T4 DNA ligase in a final volume of 30 µL for 12 h at 14°C.
3. The T3 promoter-5' LAT oligonucleotide ligation product (5 µL of the ligation reaction mixture) is used in combination with the 3' LAT mutant primer (5' ACG-AGG-GAA-AAC-AAT-AAG-GGA-TGC-C-3') to amplify the wild-type LAT fragment creating a T3 promoter-mutant LAT DNA fragment that has lost the *Bsa*HI site 22 nucleotides from the 3' end of the PCR product.
4. Electrophorese the PCR products on a 1% low-melt agarose in 1X TBE buffer, excise the corresponding band and confirm the presence of the mutation by *Bsa*HI digestion. The wild-type 3' LAT primer anneals downstream to the point of mutation, permitting the amplification of the mutant LAT using the original LAT primer pair.
5. Confirm the nature of the T3-mutant LAT construct by PCR amplification using the T3 oligonucleotide as the 5' primer and the 3' LAT primer and the original primer pair. Both primer sets should amplify a mutant LAT fragment that has lost the *Bsa*HI site.
6. Subject 5 µL of the T3-mutant LAT DNA template to in vitro transcription using a T3 RNA polymerase kit (Promega, Madison, WI) in a final volume of 200 µL at 37°C min according to the directions of the manufacturer After 60 min of incubation, add 20 more units of RNA polymerase and continue the reaction for an additional 60 min

Fig. 3. Competitive Quantitative RT-PCR of LAT-RNA. RNA from 2 d, 7 d, and 8 wk infected hippocampal extracts were reverse transcribed and coamplified with a twofold dilution series of competitor mutant LAT-RNA using LAT primers as described in the text. Radioactive RT-PCR products were digested with *Bsa*HI and electrophoresed on 4% NuSieve agarose gels. Gels were dried and exposed to X-ray film. A representative autoradiograph is shown. (2 and 7 d) Lane 1, RT-PCR products from infected hippocampal extract after RNase treatment; lanes 2–6, RT-PCR products from a twofold dilution series of competitor LAT-RNA, ranging from 50 fg (lane 2) to 3.1 fg (lane 6), added to infected hippocampal extracts; lane 7, RT-PCR products from infected hippocampal extract; lane 8, RT-PCR products from infected hippocampal extract without addition of reverse transcriptase. (8 wk) Lanes 1–5, RT-PCR products from a twofold dilution series of mutant LAT-RNA, ranging from 50 fg (lane 2) to 3.1 fg (lane 6), added to infected hippocampal extracts; lane 6, RT-PCR products from infected hippocampal extract; lane 7, RT-PCR products from infected hippocampal extract after RNase treatment; lane 8, RT-PCR products from infected hippocampal extract without addition of reverse transcriptase.

7. Digest the DNA template with 10 U of RNase-free DNase, for 60 min at 37°C. Extract the reaction products with phenol-chloroform, precipitate with ethyl alcohol, dry, and resuspend in 100 µL of autoclaved distilled water. Quantitate RNA by measuring optical density.

3.8. Competitive Quantitative RT-PCR

1. To obtain a first approximation of the amount of LAT-RNA in the brain extracts, 5 µL of extract is mixed with a 10-fold dilution series of mutant LAT-RNA ranging from 1 µg to 1 fg and subjected to RT-PCR as described.
2. Amplify the samples for 30 cycles, dilute 200-fold, and following reamplification for an additional two cycles, electrophorese the entire reaction mixtures in 4% NuSieve GTG agarose gels, dry the gels, and expose to X-ray film, from which visual estimates of RNA quantities can be made.
3. To arrive at more accurate determinations of the amount of LAT-RNA in the extracts, coamplify 5-µL samples with an appropriate twofold dilution series of competitor mutant LAT-RNA ranging from 50 to 3.1 fg, using the same procedures as described above.
4. Process the RT-PCR products, quantify radioactivity using the AMBIS system and analyze counts by linear regression as described for the viral DNA determinations. A representative autoradiograph is shown in Fig. 3.

3.9. In Situ *PCR*

1. Latently infected animals are perfused with 4% paraformaldehyde, the ganglia embedded in paraffin, and cut into 6-μ sections on a microtome
2. Sections of infected trigeminal ganglia on glass slides are deparaffinized in xylene (3 × 2 min)
3. Sections are successively rehydrated with graded ethanols in the following order: 100% ethanol (2 × 2 min), 70% ethanol (2 min), and 50% ethanol (2 min)
4. Wash sections in PBS (pH 7.5) for 5 min, twice.
5. Sections are then treated with 1% HCl in PBS for 5 min and then washed in PBS (3 × 5 min).
6. The sections are rinsed in PCR Buffer II (3 × 5 min)
7. 25 μL of *in situ* PCR reaction mixture is layered onto the sections, which are each covered with a glass cover slip and sealed using nail polish, taking care to ensure that the polish does not seep into the reaction mix.
8. PCR amplification is carried out in a BioOven II Thermal Cycler (BioTherm, Fairfax, VA) in two stages, first for 3 cycles at 92°C for 1 min, 54°C for 30 s, and 72°C for 30 s, followed by 25 cycles at 92°C for 15 s, 54°C for 15 s, and 72°C for 15 s.
9. After amplification, the cover slips are removed and the sections washed successively with 1X SSC (2 × 5 min), 50% formamide in 1X SSC (3 × 15 min, 56°C), and 1X SSC (2 × 15 min)
10. Following a rinse in Tris-buffered saline (pH 7 5) and 5% normal goat serum, the digoxigenin-labeled amplified DNA is treated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (1:250 Boehringer Mannheim, Mannheim, Germany), and visualized with BCIP/NBT (Vector, Burlingame, CA)
11. Color development is monitored visually, and stopped typically after approx 30 min, by washing with 0.1*M* Tris-HCl, pH 7 5, 1 m*M* EDTA. Typical positive neurons are shown in Fig. 4A

3.10. Controls for the In Situ *PCR*

A number of controls are required to determine that the *in situ* PCR results are specific for the HSV-1 *gB* gene sequence.

1. *In situ* PCR is carried out using *gB* primers and uninfected ganglia to show absence of any labeled nuclei (Fig 4B)
2. Using a primer pair for the HIV *Tat* gene not present in the tissue, no *in situ* signals should be detected in infected ganglia (Fig. 4C)
3. DNase treatment of sections from infected ganglia prior to PCR should completely eliminate the PCR signals (Fig. 4D)
4. Finally, DNA extracted from sections of infected ganglia after PCR is completed should hybridize to *gB* specific probes in Southern blot analyses. Single radioactive bands of the expected length should be observed (Fig 5)

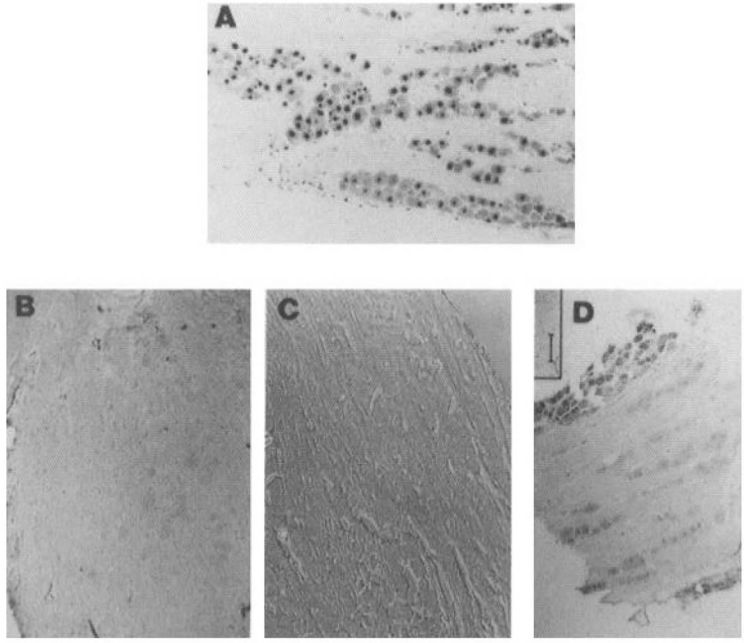

Fig. 4. *In Situ* PCR of HSV-1 DNA in Trigeminal Ganglia. **(A)** *In situ* PCR of a 6-μ infected trigeminal ganglionic sections with oligonucleotide primers for HSV-1 *gB* DNA sequences and digoxigenin-labeled nucleotides at 8 wk postinnoculation. **(B)** Control, showing uninfected ganglion amplified *in situ* with HSV-1 *gB* primers. **(C)** Control, showing 8-wk postinoculation ganglion amplified *in situ* with primers for HIV *tat*. **(D)** Control, showing 8-wk postinoculation ganglion, amplified *in situ* with primers for HSV-1 *gB* gene after overnight DNase-1 treatment. Bar = 100 μ.

4. Notes

1. PCR: A number of factors can contribute to nonspecific amplification. These can include:
   a. Contamination of PCR reagents: Use the best quality of reagents possible, and use them exclusively for PCR. We routinely aliquot out all our solutions and freeze them away at –20°C. We also use aerosol-resistant tips for pipeting to minimize crosscontamination. Designate one area in the lab exclusively for PCR work. When possible, DNA and RNA samples are prepared in separate rooms. In addition, it is absolutely essential to run suitable negative controls (i.e., without target sequence) each time an experimental PCR is performed.
   b. Titrate the *Taq* polymerase with the specific target. Too much enzyme can result in nonspecific background.
   c. Titrate primers from 50 ng to 1 μg.

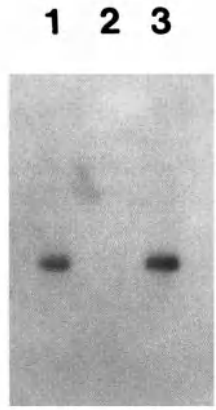

Fig. 5. The specificity of the *in situ* PCR was confirmed by Southern hybridization. DNA was extracted from a single 6-μ section after *gB in situ* PCR, and hybridized with a [γ^{32}P]-labeled *gB* oligonucleotide probe that recognized a region of the LAT product between the pair of primers used for amplification. Lane 1, DNA from an 8-wk infected ganglionic section after amplification; lane 2, DNA from an uninfected ganglionic section after amplification; lane 3, control 191-bp *gB* fragment.

   d. Find the optimal amount of target DNA.
   e. Use the minimum number of cycles possible. We use not more than 30.
2. Competitive quantitative PCR:
   a. Ensure that the restriction enzyme digestion is complete. Be sure to run controls.
   b. Dilute the product of the first coamplification at least 200:1 to avoid heteroduplex formation.
3. Competitive quantitative RT-PCR:
   a. The most important point is to ensure that the DNase treatment is complete. Be sure to run controls in which reverse transcriptase is absent.
4. *In situ* PCR:
   a. In our experience, perfused paraffin sections work the best.
   b. Vary the number of cycles to find the appropriate number that gives the best signal-to-noise ratio.
   c. Observe the same precautions as noted above for PCR.
5. Normalization of DNA and RNA extracts:

   Ten microliters of each extract can be amplified using primer pairs for the cellular glyceraldehyde phosphate dehydrogenase (*GAPDH)* gene in the presence of [α^{32}P]dCTP as tracer, to ensure that the extracts contain equivalent amounts of DNA, using the same conditions described for *gB* amplification. If necessary, the amounts of DNA can be adjusted.

   In order to normalize the amount of starting RNA, 10-μL aliquots of each RNA extract can be amplified by RT-PCR using primer pairs for the GAPDH transcript, using the same conditions described for LAT RT-PCR. The reaction

products can be electrophoresed on 1% agarose gels, gels dried, and amount of radioactivity per reaction product band quantitated as before If necessary, the amounts of RNA can be adjusted

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25

HSV Vectors for Gene Therapy

David C. Bloom

1. Introduction

A number of aspects of the natural biology of herpes simplex virus (HSV) make it an attractive candidate for a vector to express foreign genes within the nervous system. Some of the advantages of an HSV vector are:

1. Establishment of a life-long latent infection within peripheral and central nervous system neurons (for a review, *see* ref *1*);
2. Latent HSV genomes exist as multiple episomal copies/neuron and integration is not known to occur *(2)*, and
3. Nonreplicating HSV recombinants can establish a latent infection efficiently *(3)*

This last point is perhaps the most important in that it permits the construction of safe, attenuated vectors for humans. In addition, there are other biological properties of HSV that enhance its suitability as a vector from a practical standpoint. The virus is easy to manipulate in vitro, so that recombinants containing foreign genes can be constructed rapidly and its genome can accept large inserts of DNA, making feasible the construction of vectors that express multiple therapeutic genes. This potential has understandably generated a great deal of interest in exploiting HSV as a vector *(4)* and, to date, a number of recombinants have been generated expressing reporters such as β-galactosidase *(5–8)*, as well as biologically relevant peptides such as glucouronidase *(9)*, tyrosine hydroxylase *(10)*, and nerve growth factor (NGF) *(11,12)* These vectors include both recombinant HSV as well as a derivative termed "amplicons." Here we will be discussing only the former.

Early viral constructs expressed their respective markers transiently at high levels. However, the expression declined rapidly with time. Although for some therapeutic applications transient expression of a peptide within target neurons may be sufficient, for most uses, long-term expression from the latent infection

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is desirable. Stable expression of genes from the context of the latent viral genome has proven the most difficult problem to solve in the development of HSV vectors. Since latent infections of HSV are characterized by the absence of viral transcription, with the exception of the latency-associated transcript (LAT) *(13)*, the LAT promoter would be the ideal candidate for the expression of foreign genes during latency. However, recombinant viruses containing the genes for nerve growth factor (NGF) and β-galactosidase driven by the LAT promoter, express β-galactosidase and NGF RNA at high levels initially, but not during the latent infection *(12)*. A weaker promoter downstream of the LAT promoter (LAP2) has also been proved to be insufficient for long-term expression. A number of cellular promoters, including those for neuronal housekeeping genes, have been evaluated for their ability to express genes long term in the nervous system within the context of the HSV genome. These constructs have expressed reporter genes at high levels during the acute infection (for a period of 2–10 d postinfection), but the levels of expression drop off dramatically following the latent infection *(14)* The Moloney Murine Leukemia Virus (MoMuLV) LTR has been demonstrated however to afford long-term expression of β-galactosidase *(7,15)* when in the context of the LAT promoter *(16)* This combination of the LAT core promoter and the MoMuLV LTR allows extended expression of transgenes at high levels within the sensory neurons of the peripheral nervous system *(16)*, but only minimal levels of sustained expression within the CNS *(15)*. Work is still under way by a number of groups to determine the elements and structural features of the LAT promoter that allow long-term expression. In addition, work is still under way to increase the levels of expression within the CNS.

The focus of this chapter will be on the exploitation of HSV as a vector for long-term expression within the nervous system. It will be concentrating on important design considerations of vectors for specific uses, as well as methods for evaluating expression within animals. Other aspects such as preparation and growth of viral stocks, as well as reverse transcriptase-polymerase chain reaction (RT-PCR) quantitation of RNA transcripts, are presented elsewhere in this volume. Since much of the evaluation of HSV vectors will ultimately be performed in the animal, basic techniques for testing of the constructs in vivo are presented here Because the specific assays employed will vary greatly depending on the particular application, techniques, such as immunohistochemistry or *in situ* hybridization (ISH), will not be discussed here.

2. Materials

1 Modified Eagle's Medium (MEM) (Life Sciences, Bethesda, MD)· supplemented with 5% calf serum, 250 U penicillin, 250 μg/mL streptomycin, 2 5 μg/mL amphotericin B, and 292 μg/mL L-glutamine/mL.

2 Rabbit skin, Rat-2 (tk-), or Vero cells (American Type Culture Collection, Rockville, MD).
3. Trypsin: 1.25 g trypsin dissolved in 50 mL dH_2O at 37°C Add 0.5 g EDTA, 20 g NaCl, 1 g KCl, 2 5 g dextrose, 0 5 g penicillin, and 0.25 g streptomycin. Bring volume up to 200 mL with dH_2O Filter-sterilize and store at 4°C Dilute 1 10 for working stock
4 T75 flasks and 60-mm dishes
5 Sterile 5-mL falcon tubes
6. TNE· 10 m*M* Tris (pH 7 4), 1 m*M* EDTA, 0.1*M* NaCl. Filter-sterilize and store at room temperature
7 2X HEPES (for 100 mL)· 1.6 g NaCl, 74 mg KCl, 37 mg Na_2HPO_4 $7H_2O$, 0 2 g dextrose, 1 g HEPES (free acid), pH to 7.05. Filter-sterilize, aliquot, and store at –20°C
8. 2.5*M* $CaCl_2$: Filter-sterilize and store at room temperature
9 1X TE. 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA in dH_2O Autoclave
10 10% Sodium dodecyl sulfate (SDS): 100 g in 900 mL dH_2O dissolved by heating to 68°C, pH to 7 2, and add water to 1 L
11. Pronase: 20 mg/mL in dH_2O. Store at –20°C. Self-digest for 2 h at 37°C
12 Viral lysis buffer. 10 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 0 25% NaDOC, 0 5% NP-40
13. Ethidium bromide: 10 mg/mL in dH_2O
14 Tris-borate buffer· 89 m*M* Tris base, 89 m*M* boric acid, 10 μ*M* EDTA (pH ~8 3).
15 Seakem agarose: (FMC, Rockland, ME).
16 2X MEM
17. Sterile, plugged Pasteur pipets
18 96-Well dish
19 6X Loading dye 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll 400.
20 Hybond-N nylon membrane (Amersham, Arlington Heights, IL).
21. Whatman 3MM chromotography paper
22. Dot-blot apparatus
23. 10*N* NaOH
24. 2.5*M* Tris-HCl, pH 7 6.
25 20X SSPE. 3.6*M* NaCl, 0 2*M* NaH_2PO_4, 0.02*M* EDTA, pH 7.0–7.5 with NaOH Autoclave.
26. BLOTTO stock: 5% nonfat dry milk, 0.2% Antifoam A (Sigma, St. Louis, MO) in water. Warm to 42°C and stir well to completely dissolve. May be stored for several months at 4°C
27 DNA probe labeling kit (Boehringer-Mannheim, Indianapolis, IN).
28 Neutral red solution· 3.330 g/L (Life Technologies, Grand Island, NY)
29. 40% Dextrose· 40% dextrose w/v in distilled water. Filter-sterilize, and store at 4°C
30 Equilibrated phenol· Melt molecular-grade phenol at 68°C and add 1*M* Tris-HCl, pH 8 0. Extract 2X with Tris-HCl, pH 8.0; 0.2% β-mercaptoethanol.
31. SEVAG. 24:1 chloroform·isoamyl alcohol.
32. 10% Saline: 10 g/100 mL dH_2O. Filter-sterilize
33. Sodium pentobarbital: Nembutal (Abbott Laboratories, Abbott Park, IL) 7.5 mg/mL in sterile dH_2O

34 Dissection microscope
35 3c Dumont forceps
36 2X overlay agarose: 0 8% SeaKem ME agarose in dH_2O. Autoclave
37 Dissecting scissors
38. Fine dissecting scissors
39. Microdissecting scissors
40 Solution A 0.1*M* NaOH, 1 5*M* NaCl
41 Solution B: 0.2*M* Tris-HCl, pH 7 6
42. Solution C 2X SSPE

3. Methods

3.1. Vector Design

Initial considerations in the design of HSV vectors depend largely on whether the vector's intended use is for cell culture, animal, or human use. The application will dictate the degree of attenuation and duration of the transgene expression required. This section will discuss these specific points and then provide a basic protocol for general construction. It should be pointed out that once the design is made, vector construction is very straightforward and can be performed by anyone trained in basic molecular techniques. Vector construction involves:

1. Construction of a recombination plasmid containing the gene one wishes to express behind a promoter. This plasmid contains recombination "arms" to allow insertion of the promoter/gene construct into the viral genome by homologous recombination,
2 Transfection of the plasmid and viral DNA in cell culture,
3. Plaquing of the progeny, which will contain a mixture of wild type virus and recombinants, and
4 Screening for, and plaque purification of, the recombinants.

3.1.1. Choice of Viral Background

The degree of attenuation of the virus needs to be considered in choosing a parental strain or viral background. If the vector is intended for human use, an extremely attenuated virus will be required, which will probably involve making the virus completely unable to replicate so that a helper cell line is required for propagation. Some deletions that give rise to a nonreplicating virus are *ICP4* (major viral transactivator, which is required for immediate early and early gene functions) or DNA polymerase. The protocol below uses an *ICP4* deletion virus as an example. This mutant has the advantage over the polymerase deletion of being very restricted in overall expression of other viral genes, thus minimizing cytopathic effects. If the virus is not intended for human use and is going to be used in animal models, more choices are available. Consideration must be given to whether the virus will be inoculated into the CNS

or periphery. An advantage of HSV vectors is that a great deal is known about pathogenic parameters. If the virus is to be used in the periphery, HSV1 strain KOS (*see* Note 16) can be used without further attenuation, since this virus is not neuroinvasive and will not spread through the nervous system *(17)*. However, if one is interested in the CNS, then this strain is virulent and will kill if inoculated directly into the brain. HSV can be easily attenuated with respect to virulence by either insertion of the desired gene into the *tk* gene *(18)*, ribonucleotide reductase *(19)*, or dUTPase gene *(20)*. Inactivation of these genes allow the virus to replicate to near-normal levels in rapidly dividing cells (including cell culture), but not within the nonreplicating neurons of the nervous system. In addition, use of the *tk* gene allows one to select for recombinants based on the *tk* phenotype if a *tk* cell line (such as rat-2) is used. Another example is the nonessential glycoprotein, gC *(16,21)*. Figure 1 (p. 374) illustrates the location of a number of the commonly used insertion sites in the HSV genome; Table 1 (p. 375) compares the pathogenic properties of virus with mutations in these genes. The process of transfecting and screening for viral recombinants is illustrated in Fig. 2 (p. 376).

3.1.2. Design of the Recombination Plasmid

Once one has determined which gene will be used as the insertion site, it is relatively easy to design a recombination plasmid. If the desired gene is to be inserted into the *tk* or *ICP4* gene, a number of insertion plasmids are available, or one can be easily generated. This is facilitated by the fact that the entire nucleotide sequence of HSV (stain 17+) is known *(27)*. The only important point to keep in mind is that one needs at least 200 bp on either side of the insert to obtain homologous recombination of the gene onto the viral genome. This is very minimal, and 1 kb on either side is recommended for optimal recombination efficiencies

3.1.3. Engineering the Promoter

The promoter that is used here will depend on the duration of expression that is desired. If in vitro expression for only several days is required, then an *ICP4*– vector using almost any cellular promoter will be sufficient. The same holds true for short-term expression (<14 d) within animals. However, if long-term expression of genes in the animal is required, then the binary LAT-LTR promoter is recommended. A schematic of an *ICP4* recombination plasmid utlizing this promoter is illustrated in Fig. 3 (p. 377).

3.2. Construction of the Viral Recombinant

Once the recombination plasmid has been constructed, the recombinant virus can be constructed. This is performed by cotransfecting HSV-DNA (which is infectious) with the recombination plasmid DNA.

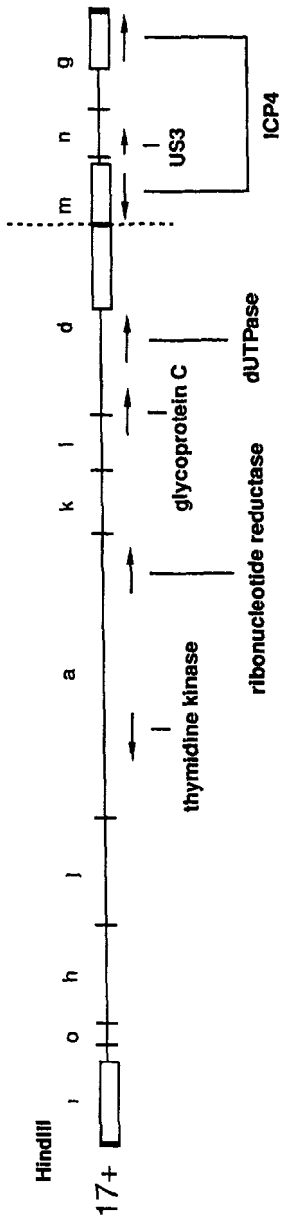

Fig. 1 Location of common insertion sites in HSV-1 strain 17+

Table 1
Comparison of the Virulence Properties of HSV-1 Mutants Commonly Used as Vectors

Viral insertion site	LD_{50}/PFU		Essential gene, helper cell line required	Refs.
	IC	Peripheral		
ICP4 (IE175) transcriptional activator	$>10^8$	$>10^8$	Yes	*7,22*
Thymidine kinase (UL23)	1×10^5	$>10^8$	No	*23*
Ribonucleotide reductase (UL39)	10^5	n.d.	No	*8,19,24,25*
dUTPase (UL50)	3×10^2	3.5×10^6	No	*20*
Protein kinase (US3)	10^4	n.d.	No	*8*
Glycoprotein C (UL44)	10^3	$>10^8$	No	*12,21*
17+ (wild-type)	4	10^4	N/A	*26*
KOS (wild-type)	25	$>10^8$	N/A	*26*

3.2.1. Preparation of HSV Transfection DNA

1. Trypsinize five confluent T75 flasks of rabbit skin cells, and resuspend each flask in a total of 15 mL of MEM. Seed 10- to 150-mm dishes with 7 mL of this cell suspension by adding the cells to 20 mL of supplemented media in each dish Incubate overnight at 37°C.
2. On the following day (the dishes should be approx 90% confluent at this point) the media are removed, and the cells are infected by adding 5 mL of media containing 2×10^6 PFU of HSV The virus is allowed to adsorb to the cells for 60 min at 37°C The dishes are rocked gently halfway through the incubation.
3. After 1 h, 25 mL media are added to the cells, and the dishes incubated until all of the cells have rounded and detach easily when the dish is swirled This usually takes 2–3 d
4. Harvest the cells by pipeting the cells off the bottom of the dishes. Transfer the cell suspension to Sorvall bottles, and centrifuge at 10,000 rpm at 4°C for 40 min
5. Pour off supernatant, and resuspend the pellet in hypotonic lysis buffer (10 mL) and transfer to a conical 15-mL falcon tube. Vortex vigorously and incubate for 5 min on ice. Vortex again briefly
6. Centrifuge at 800*g* for 10 min at 4°C (this pellets the nuclei)
7. Transfer the supernatant to a new conical tube, and add 1 mL 10% SDS and 0 5 mL 20 mg/mL pronase
8. Incubate for 1 h at 50°C
9. After 1 h, add another 0 5 mL of 20 mg/mL pronase, and incubate overnight at 37°C
10. Phenol extract 2X
11. Phenol/SEVAG (1 1) extract 1X.
12. SEVAG extract 1X.
13. Dialyze vs 1X TE overnight at 4°C (with 2 changes of buffer).

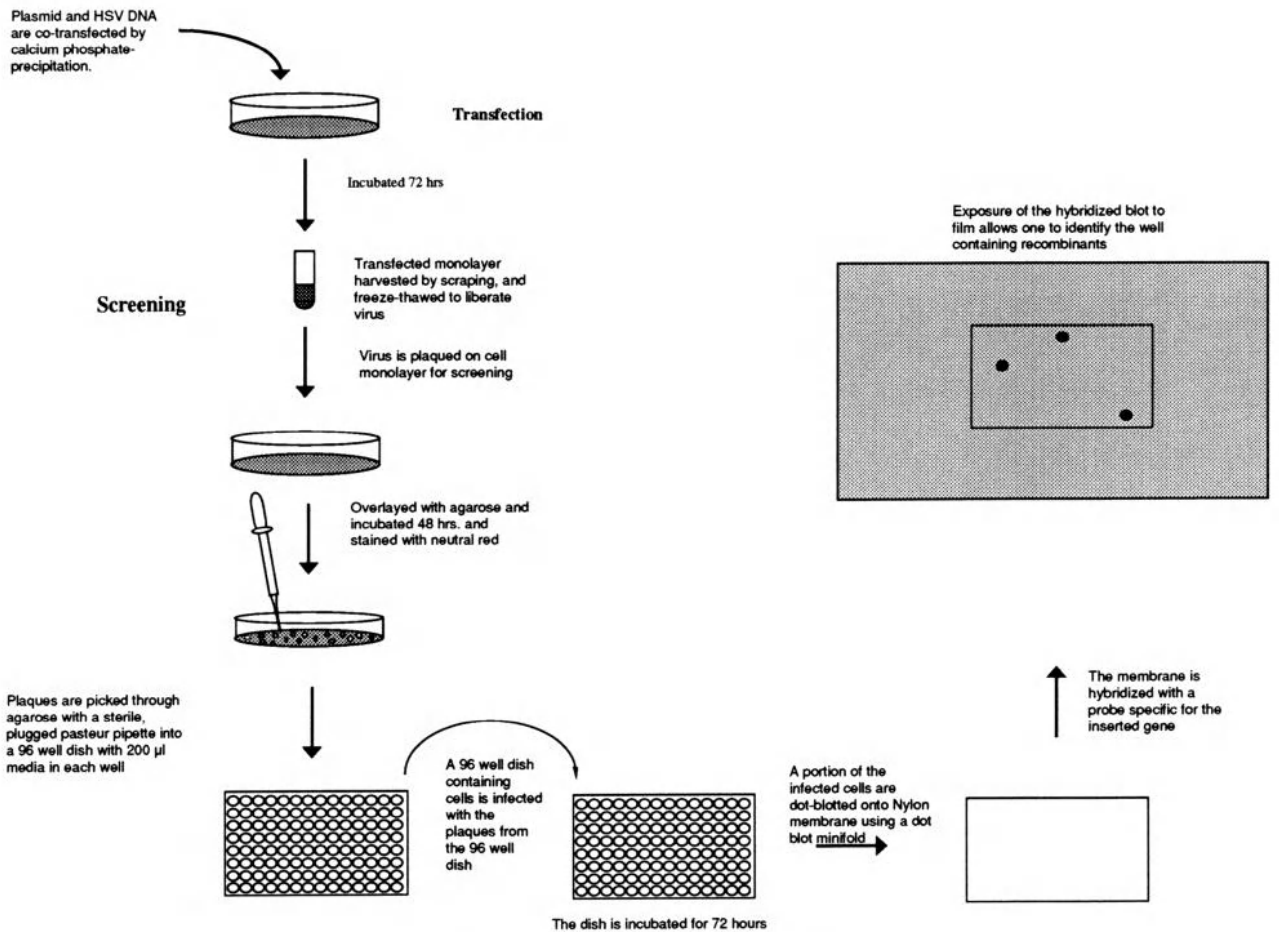

Fig. 2. Transfection and screening of recombinants.

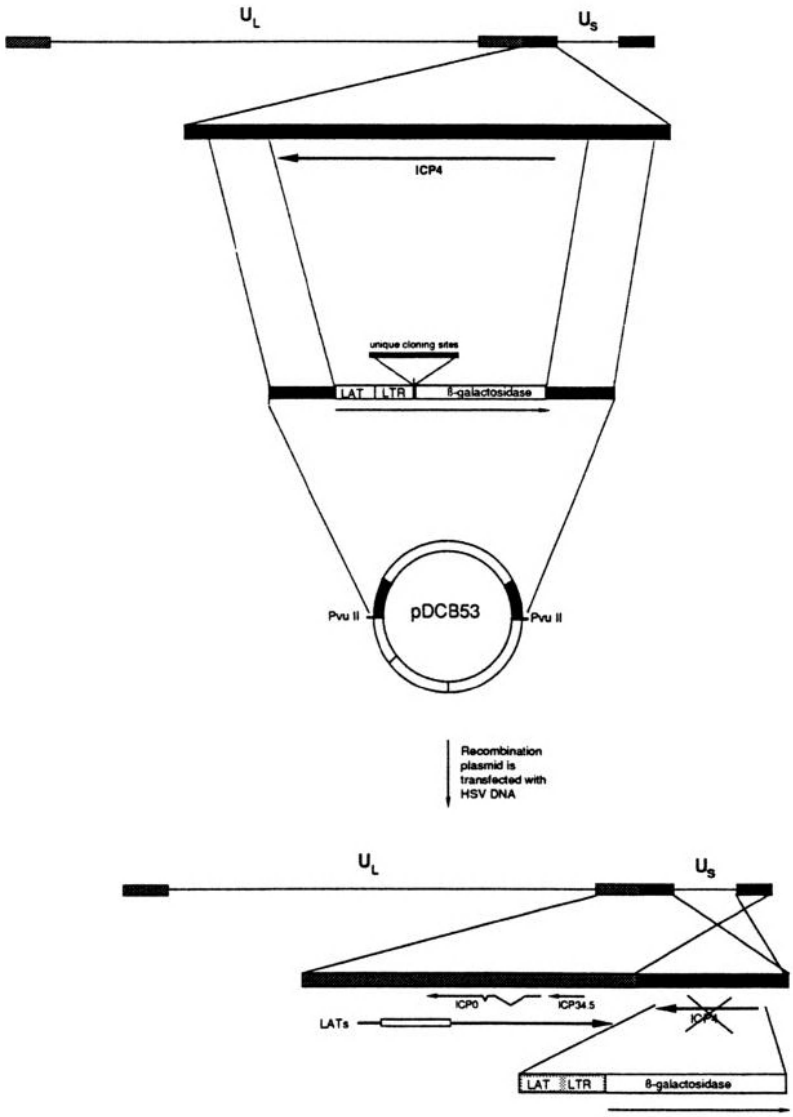

Fig. 3. *ICP4* insertion vector.

14. Determine the concentration of DNA spectrophotometrically by determining the absorbance (A_{260}).
15. Digest 1 μg of DNA with *Hin*dIII, and run on an agarose gel along with uncut DNA to determine the purity and quality of the preparation. There will be some cellular contamination, but the viral DNA should be the predominant form, and there should be little evidence of smearing.

16. For long-term storage of the DNA, it is advisable to aliquot the DNA into small fractions and freeze Repeated freeze–thawing should be avoided

3.2.2. Transfection of HSV-DNA

Transfections are performed in 60-mm dishes on subconfluent monolayers of rabbit-skin (RS) cells. The RS cells are propagated in MEM supplemented with 5% calf serum and glutamine, Penn/Strep Unit-length HSV-DNA is cotransfected with the desired plasmid at various ratios using a modified calcium phosphate precipitation procedure The transfections are generally allowed to proceed until all all of the cells are rounded, i.e., 100% cytopathic effect (CPE) is evident (usually 3–4 d), though the dishes may be harvested earlier if one wishes to prevent amplification of siblings.

1. 60-mm Dishes are seeded from a flask of actively growing RS cells at a ratio that will produce a cell density of approx 50% confluence the following day (typically, $1/30$ of a T75 flask/60-mm dish) The dishes are incubated O/N at 37°C, 5% CO_2
2 On the following day, the media are removed from the dishes (which should be at 50% confluence) and replaced with MEM supplemented with 1 5% fetal bovine serum (FBS) The dishes are then incubated O/N at 31 5°C, 5% CO_2 This is to serum-starve the cells
3 The transfection mix is prepared by diluting the desired amount DNA (typically, 1–10 µg of HSV/dish, and a 10-fold molar excess of the linearized plasmid DNA) in a final volume of 225 µL of TNE buffer After the dilutions have been made, 25 µL of a 2 5*M* $CaCl_2$ is added to each tube (*see* Notes 1 and 2)
4. The DNA is precipitated by adding 250 µL of 2X HEPES buffer to the above sample while bubbling air into the solution with a mouth aspirator connected to a sterile-plugged Pasteur pipet
5 The solution is then incubated for 20 min at room temperature
6. Aspirate the 1 5% FBS MEM from the 60-mm dishes, and pour on the transfection mix. Incubate the dishes at room temperature for 20–30 min
7 Add 5 mL of 1 5% FBS MEM, and incubate for 4 h at 37°C. Do not remove the DNA solution.
8. After 4 h, aspirate the media, and wash the monolayer with media two times Then hypertonic shock the cells briefly (<1 min) by adding 1–2 mL of shock buffer (1X HEPES, 20% dextrose solution).
9. Aspirate the shock buffer, and wash 2X with media After the last wash, add 5 mL of MEM 5% calf serum to the dishes, and incubate 3–4 d at 37°C, 5% CO_2
10 The transfections are harvested by scraping into the media with a rubber policeman Recombinants are screened by plaquing the cells on RS cells, and picking the plaques into 96-well dish to which media have been added to the wells These dishes are frozen, and 50 µL of each well used to infect 96-well dishes of confluent RS cells (*see* Note 5). These dishes are then dot-blotted and probed with the desired insert Typical transfections yield 2–10 positives/96-well dish.

3.2.3. Plaquing of Transfections for Recombinants

1. Transfection mixes are plated onto confluent monolayers of rabbit skin cells in 60- or 100-mm dishes Generally, from a transfection that was performed in a 60-mm dish that was allowed to go to 100% CPE, dilutions of 10^{-5} or 10^{-6} yield well-isolated plaques that are suitable for picking The infected monolayers are overlayed with 0.4% (final) agarose in 1X supplemented media, and incubated for 2 d
2. On the morning of the third day, the dishes are counterstained with neutral red to aid in the visualization of the plaques A 1·30 dilution of the Neutral red-stock solution is made in unsupplemented media. An equal volume of the neutral red overlay is then added to the dishes on top of the agar overlay (for 60-mm dishes, 5 mL of diluted neutral red are added to each dish), and the dishes are incubated at 37°C until the monolayers are stained red For RS cells, this is approx 6 h.
3. After the monolayers are stained, the liquid overlay is aspirated and the plaques are picked using a sterile Pasteur pipet. The plaques are picked by applying slight pressure to the bulb of the pipet, then coring the plaque straight down, and twisting the pipet The bulb is then released, and the plaque aspirated partially into the pipet. The plaque is then expelled into a well of a 96-well dish that has been filled with two to three drops of media.
4. After all of the plaques are picked, the dish is frozen at –70°C, and then thawed in the incubator.
5. The plaques are then amplified by plating onto a 96-well dish of confluent RS cells. The media is "flicked" off the dish, and using a multichannel pipeter, 50 µL of the wells with the plaques are transferred to the 96-well plate with the RS cells. The virus is then allowed to adsorb for 1 h at 37°C. At the end of the adsorption period, two drops of supplemented media are added to each well, and the dishes incubated until the wells show 100% CPE (usually 3 d)

3.2.4. Screening for Recombinants

1 After cells in the wells of the 96-well dishes have reached full CPE (usually 3–4 d), they are ready to be dot-blotted.
2 Set up the Millipore dot-blot apparatus with one piece of blotting paper underneath a piece of nylon membrane (Hybond-N™ or Nytrans™) Wet the blotting paper and membrane completely with 2X SSPE before clamping the apparatus together
3. After clamping the apparatus together, apply vacuum Using a multichannel pipeter, transfer 50 µL of the infected cells from each well of the 96-well dish to the apparatus (pipet the wells up and down several times to mix before transferring)
4. After the media have filtered through the apparatus, add 200 µL of solution A to each well of the apparatus
5 Likewise, after solution A has filtered through all of the wells, add 200 µL of solution B.
6. Finally, after all of solution B has filtered through the apparatus, add 200 µL of solution C.

7. Remove filter from apparatus, label the filter (remember to mark orientation), and bake at 80°C for 1 h The blot is now ready for hybridization (*see* Note 3).
8 Freeze the 96-well dish at –70°C for later use.

3.2.5. Confirmation of Viral Recombinants

After the virus stocks have been plaqued-purified for at least three rounds, a small stock of several "clones" can be grown up for confirmation. Typically, the following tests are performed using the standard techniques of viral genome and RNA analysis presented elsewhere in this volume.

1. Southern blot analysis: The goal is to determine that the gene of interest is inserted into the proper location within the viral genome If the recombination site is within the viral repeat sequences, it is important to determine that the virus is "double-sided" or that two copies of the gene are present This is critical in that single-sided viruses tend to be unstable, and the inserted gene may recombine out. In addition, if the construct has been inserted into the ICP4 gene, both copies must be deleted in order to attenuate the virus
2 Northern blot analysis· This is performed in order to determine that the transgene is transcribed properly in the context of the viral genome.

3.3. In Vivo Testing of Recombinant Virus

If the goal is to utilize the recombinant virus to express a transgene in vivo, there is no reliable in vitro method for determining whether a particular construct will express the desired gene during latency and determining levels of expression in the desired cells, so it is necessary to test recombinants in vivo. Presented below is the methodology for evaluating the ability of an HSV recombinant to express a gene within the spinal ganglia of mice.

3.3.1 Testing for Expression in Mouse Sensory Ganglia

For studies involving expression within the peripheral nervous system, establishment of a latent infection within the mouse lumbosacral ganglia provides an efficient model for assessing the expression and/or evaluating the biological effects of a given recombinant. Mice can be easily infected by footpad inoculation. Even nonreplicating vectors can be evaluated in this manner, because a productive infection is not required for the establishment of a latent infection in these ganglia. In fact, all that is needed is to expose the virus to peripheral nerve termini, which can be performed by lightly abrading the rear footpads. In general, a period of 2–3 wk is required prior to assessing latent expression, since this is the amount of time that it takes for all traces of acute infection to subside (*see* Notes 8 and 9). After this period of time, the mice can be sacrificed, and the ganglia removed and subjected to a number of different histologic or biochemical analyses.

3.3.1.1. Footpad Inoculation of Mice

3.3.1.1.1 Saline Pretreatment

1. Six-week-old outbred Swiss-webster mice (16–19 g) are anesthetized one at a time briefly in an ether (or Halothane) jar (just until heavy breathing ensues) (*See* Note 10.)
2. The mice are injected with a sterile 10% saline solution under the footpad of each foot with a 1-cc tuberculin syringe with a 28-gage needle Approximately 0.1 cc/foot is injected (until it is swollen).
3. The mice are returned to their cages and left for at least 4 h (but no longer than 6).
4. The mice are now ready for infection.

3.3.1 1.2. Inoculation

1. The mice are injected intraperitoneally with 0.2 mL of a 7.5 mg/mL solution of sodium pentobarbital in groups of 10, and placed in their cages until they are completely asleep (*see* Note 11).
2. Mice are removed from their cages and laid on their backs.
3. With an emery board, the feet are lightly abraded with 1–2 strokes only to remove the surface layer of skin.
4. With a pipetman, 25–50 µL are applied to each foot and rubbed over the surface with the side of the pipet tip The animals are then arranged on their backs feet up and flat in the cage with the bedding material to prop then up.
5 An additional 25–50 µL are applied to each foot. Care should be taken so that the feet are level and the inoculum stays on the feet.
6 This is allowed to adsorb for 30–60 min (when the mice begin waking) (*see* Note 12).
7. Watch the mice to ensure that they are awakening
8 Return them to the vivarium for 2–3 wk.

3.3.1.1.3. Removal of Spinal Ganglia. The ability of the constructs to express the desired transgene is assessed following removal of the spinal ganglia. Typically, expression is assessed at acute times (4 d) and latent times (21 d or longer). The procedure presented here is for the preparation of the tissue for immunohistochemistry.

1. Mice are killed by ether inhalation.
2. The feet of the mice are then quickly pinned to a styrofoam board.
3. Two incisions are made from the sternum anteriorly to open up the rib cage and expose the heart.
4. The sternum is reflected back to the left of the head, and pinned to provide easy access to the heart.
5. A 28-gage needle on a 12-mL syringe filled with PBS is inserted into the apex of the left ventricle.
6. Pressure is applied, and saline is injected into the heart at a slow rate (3 mL/min). An incision is made immediately at the right atrium to provide a release.

7 After the saline has been injected, the barrel of the syringe is removed from the needle, and another syringe that has been filled with 4% paraformaldehyde is placed on the needle. The mouse is perfused with the formaldehyde at the same rate as the saline.
8 The spine is then removed by first wetting the fur with 70% ethanol.
9 A dorsal incision is made starting near the tail and proceeding up the midline anteriorly to the base of the neck The skin is folded back and the spine is exposed.
10 The spine is removed by making two lateral incisions on either side of the spine, starting at the tail and moving anteriorly
11 The spine is grasped with a pair of forceps and lifted up The vessels and adhering tissue are trimmed away as the spine is removed from the carcass
12. The spine is placed in a Petri dish, ventral side up It is grasped firmly on its side with a pair of forceps. Using small scissors, a medial incision is made exactly on the midline cutting through the vertebrae
13 Using two pairs of forceps, the back is pried open to expose the spinal cord and the dorsal root ganglia on the sides The ganglia are small round white-clear translucent buttons located in small lacunae on the sides of the spinal column They are attached to the roots, which connect them to the spinal cord The ganglia are removed by clipping with corneal scissors (*see* Fig 4)
14 They are then placed in 4% paraformaldehyde solution, and incubated for 12 h at 4°C to postfix
15 The ganglia are then placed in 40% sucrose solution overnight
16 The ganglia are then blotted dry on a paper towel, combined, and rolled into a ball using forceps
17 The ganglia are then placed on a bead of O C T compound and flash-frozen in liquid nitrogen.
18 The tissue can then be stored in Eppendorf tubes at –70°C or colder until sectioning
19. The ganglia are sectioned on a cryostat and mounted on slides The tissue can then be processed for immunohistochemistry or *in situ* hybridization

4. Notes

1 Probably the most important single parameter in determining the efficiency of transfection is the quality of the transfecting viral DNA. In order to work, the HSV transfection DNA needs to be unit length—that is, not sheared or degraded Care should be taken at all steps after the SDS/Pronase digestion not to vortex or pipet the DNA vigorously
2 The exact amount of HSV-DNA used/transfection is generally in the range of 1–10 μg/60-mm dish of cells The optimal amount for a given DNA prep should be determined empirically by transfecting dilutions of the stock DNA and determining the concentration that yields the highest efficiency. Once this is determined for a particular stock of DNA, the proper amount of DNA should not vary appreciably from experiment to experiment
3 Although the DNA can be crosslinked to nylon membranes by UV irradiation, we have found that the baking method is more efficient when performing dot-blots
4 When neutral red staining, it is important not to leave the neutral red on too long,

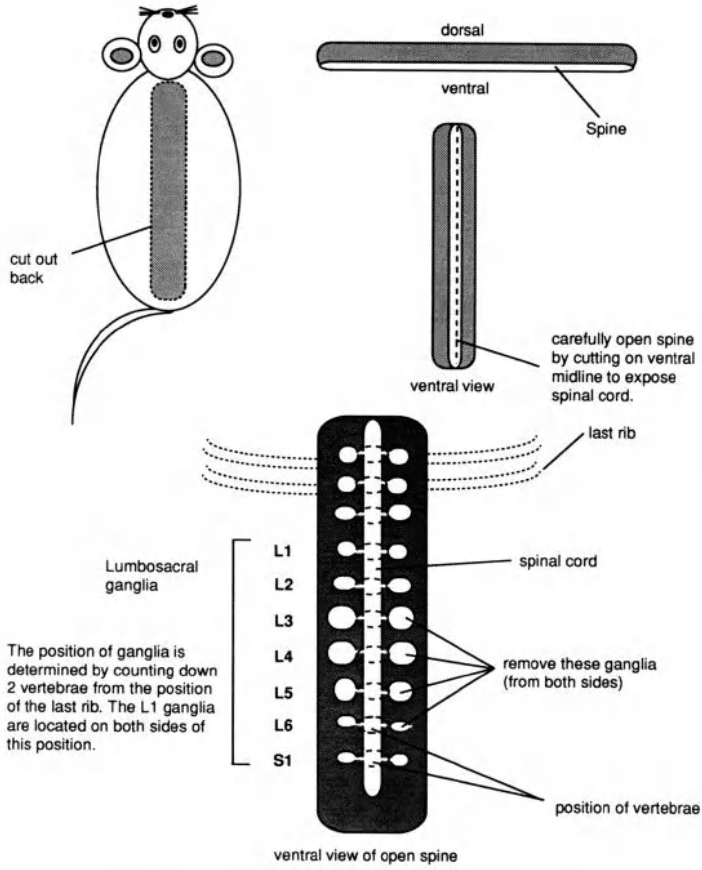

Fig. 4. Removal of mouse spinal ganglia.

since it will eventually kill the cells (rendering the plaques invisible).

5. Plaques can be screened by limiting dilution, but, in our hands, picking the plaques and screening them directly speeds up the screening process and has allowed us to identify some recombinants that we were unable to detect by limiting dilutions. This may be owing to the fact that wild-type virus may have a significant growth advantage over some recombinants.
6. Transfections can be performed using Lipofectin™ (Life Technologies, Gaithersburg, MD) or similar reagent. This method is quicker and yields results comparable the $CaPO_4$ method presented here. However, we have noted greater variability in the transfection efficiencies with Lipofectin. It should also be noted that the optimal amount of transfection DNA should be determined separately for the specific transfection protocol employed.
7. The plasmid DNA used for transfection should be from a CsCl preparation, since impurities from minipreps seem to reduce transfection efficiencies.

8 Saline pretreatment of the feet increases the efficiency of uptake of the virus by some 100-fold. Nonreplicating viruses can be inoculated by directly injecting the virus into the sciatic nerve. Although this route is more efficient, only 10 µL can be injected. In contrast, if the virus is applied to saline-treated feet directly (80 µL), the establishment of latent infection is comparable to that obtained following sciatic nerve infections In addition, the footpad method of inoculation is much easier to perform

9 Technically, a nonreplicating virus has no acute phase though low level transcription of some immediate early genes can be detected for several days after inoculation in mice *(3)* For this reason, a waiting period is still required prior to evaluation of latent-phase gene expression.

10 It is important to infect a large enough number of mice to make the assay statistically significant. Remember—with animals, there is always some degree of variation to be expected Therefore, one should plan to infect at least 4 animals/assay point It is often helpful to infect some additional ones in case some die. Also remember, since it takes 2 wk to establish a latent infection, it is useful to plan ahead

11 The mice should be completely anesthetized—not quivering or moving One wants them to remain under anesthesia to allow the inoculum to stay on the feet long enough for it to adsorb efficiently If the animal is quivering or rolls over, give it 0 1 mL more Nembutol

12. If the animals look as though they are under too deeply (shallow), a heat lamp placed 3 ft from them to keep the cages warm during recovery is often helpful

13 You want just to abrade the foot surface enough to be able to peel the layer of skin off. Do not do it so forcefully that the feet bleed, however.

14 The LTR from the MoMuLV was removed from the plasmid pBAG obtained from Connie Cepko *(28)*.

15 Depending on the procedures used, the ganglia can simply be "fresh-frozen" instead of perfused. In general, if the desired antigen survives the fixation process, the tissue morphology is much better in fixed tissue. Even greater preservation of cell morphology can be obtained by paraffin embedding of the tissue.

16 It should be noted that some variation in the virulence properties of KOS strains used in different laboratories has been reported This is likely because of repeated passage of these stocks outside of the animal. Care should be taken in selecting a source for KOS that has demonstrated the desired virulence characteristics.

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26

Investigation of the Anti-HSV Activity of Candidate Antiviral Agents

Derrick J. Dargan

1. Introduction

Herpes simplex virus (HSV) is a human pathogen that causes diseases ranging in medical importance from herpes labialis, through genital herpes and herpes keratitis, to herpes encephalitis—a life-threatening disease. HSV types 1 and 2 have the ability to enter a latent phase during *in vivo* infection, during which time the virus is able to evade immune surveillance, and from which state it is able to escape from time to time to cause disease, especially in immunocompromised individuals. This characteristic makes antiviral chemotherapy an indispensable weapon in the management of recurrent herpesvirus infection.

The search for antiviral compounds falls into two patterns: first, random screening of large numbers of naturally occurring compounds, usually of plant or fungal origin; second, use of molecular knowledge of a particular antiviral target to design specific inhibitors, e.g., short peptides to inhibit essential protein–protein interactions *(1,2)*, or antisense RNAs to inhibit virus gene expression selectively *(3–5)*.

The aim of this chapter is to assist workers new to the field of antiviral research by providing a series of experimental procedures through which the anti-HSV activity of candidate antiviral compounds may be evaluated in vitro, the potency of the compound assessed, and the antiviral mechanism investigated. Because antiviral research draws heavily on a wide range of techniques that are generally applied in other fields of virology, some experimental protocols mentioned, but not given, here are crossreferenced to other chapters of this book.

The most potent antiviral agents currently available for the treatment and management of herpesvirus infections are nucleoside analogs. The discovery of acycloguanosine (Acyclovir; ACV), was an important milestone in the his-

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tory of antiviral chemotherapy, since it demonstrated that selective antiviral activity was obtainable. Acyclovir has now become the benchmark compound against which candidate antiherpes agents are evaluated. The highly selective antiviral activity of ACV derives from the fact that ACV is activated only in virus (HSV, or varicella zoster virus [VZV]) infected cells; there is little appreciable activation of ACV in uninfected cells. The HSV (or VZV) specified thymidine kinase (TK) activates ACV by phosphorylation to ACV monophosphate. Thereafter, cellular enzymes complete the processing to ACV triphosphate, in which form the drug is incorporated into growing DNA strands resulting in virus DNA chain termination, and freezing of the virus DNA polymerase molecule to the terminated DNA strand (*6*; *see* ref *7* for review).

Much has been (and continues to be) done to develop further the ACV family of compounds by improving the bioavailability (e.g., Valacyclovir and Famciclovir/Penciclovir; refs. *8–10*) or extending the antiviral range (e.g., Gangciclovir for the treatment of human cytomegalovirus; ref. *11*). However, HSV mutants resistant to ACV have been isolated from both in vitro and in vivo infections. The commonly perceived threat to health management, especially of the immunocompromised patient, represented by the spread of such ACV-resistant HSV ensures that there is likely to be an enduring need for new anti-HSV compounds, particularly those directed against targets other than the viral DNA polymerase.

Figure 1 shows the replication cycle of HSV and identifies stages at which virus replication might be blocked by an antiviral compound. Antiviral targets will usually be virus-encoded proteins or nucleic acid molecules, but it is conceivable that some cellular function, more critically required for viral than cellular growth, could possibly also serve as an antiviral target, e.g., posttranslational processing of a viral protein

Treatment of virus particles with a virucidal compound results in failure of the virus to infect cells. Entry of HSV into cells is a complex, two-step process of adsorption, followed by penetration and involving interactions between different glycoproteins on the virus envelope *(12–14)* with cell-surface proteoglycans that serve as the receptors for the virus *(15,16)* Virucidal compounds may block, or render nonfunctional, one or more of the essential virus glycoproteins involved in entry. Compounds that render the cellular receptors for the virus inoperative can also be expected to prevent infection of the cells.

Amantidine and Rimantidine (*see* ref. *17* for review), which inhibit uncoating of influenza type A *(18,19),* have no activity against HSV, and, as yet, compounds specifically inhibiting HSV uncoating have not been reported.

Following uncoating of the virus particle, the HSV genome is delivered to the nucleus of the infected cell, and transcription and replication of the HSV genome then begin. Transcription of the HSV genome is organized into three

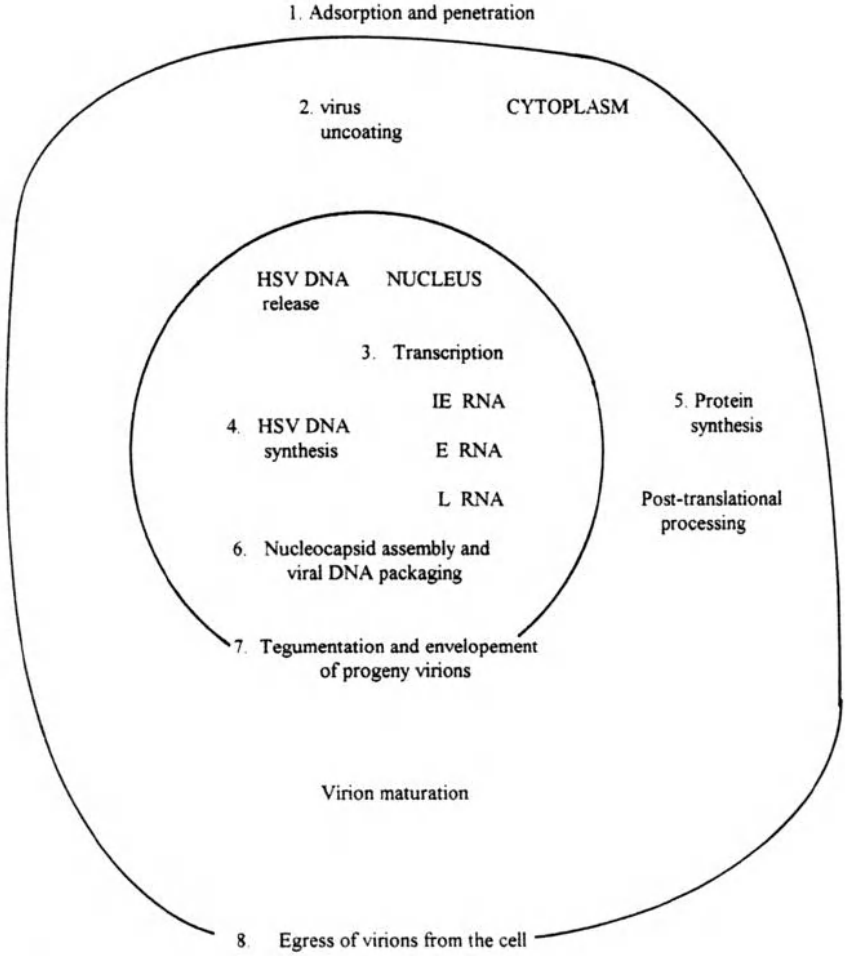

Fig. 1. Stages in the HSV replication cycle that might be blocked by antiviral agents.

phases, immediate early (IE), early (E), and late (L) (*see* Chapter X). HSV transcription has recently been demonstrated to be an accessible anti-HSV target by the finding that antisense RNAs can specifically block HSV-1 IE1, IE4, and IE5 gene transcription *(5)* and by the observation that the anti-HSV effect of the biflavone (Ginkgetin) operates through a strong inhibition of IE transcription *(20)*.

HSV-DNA synthesis represents the most commonly used anti-HSV target: Several compounds licensed for use in humans (e.g., ACV and derivatives, Vidarabine, triflouorothimidine, Foscarnet) operate by blocking viral DNA synthesis. Using a plasmid-based system, Wu et al. *(21)* have identified seven

HSV genes that are necessary, and sufficient, for HSV-DNA origin-dependent DNA synthesis; these include HSV-1 genes UL9 (the origin binding protein), UL5, 8, and 52 (a protein complex having DNA helicase/primase activity), UL30, and 42 (the DNA polymerase complex) (*see* ref. *22* for review) Clearly, compounds that interfere with the expression of one or more of these genes or the function of their protein products (including protein–protein and protein–DNA interactions) can be expected to have anti-HSV activity.

HSV-DNA synthesis generates long head-to-tail concatemers of DNA molecules, which must be cut to genome length for packaging into nucleocapsids. Although HSV-1 mutants unable to package the virus DNA have been described *(23–27)*, no antiherpes agent specifically targeting DNA packaging is yet known.

Translocation of the HSV virus particle to the cytoplasm of infected cells depends on genome encapsidation. Although coating of the nucleocapsid by tegument proteins is required for virion maturation, and presumably full infectivity, the mechanism of tegument assembly is not yet fully understood, nor has the site at which tegumentation occurs been unambiguously identified Envelopment of the particle occurs at the inner nuclear membrane and/or at a cytoplasmic site. The particle may lose the nuclear membrane-derived envelope and acquire another derived from Golgi apparatus or post-Golgi vesicles. Interaction with the Golgi apparatus or post-Golgi vesicles is required for complete processing of the HSV envelope glycoproteins. Various inhibitors of glycoprotein processing (tunicamycin, castanospermine) or of Golgi apparatus function (e.g., monensin and brefeldin A) impair the infectivity of the HSV yield and some also inhibit the release of HSV from infected cells. These compounds, however, are considered too toxic for in vivo use.

2. Materials

1. Phosphate-buffered saline (PBS)· 170 m*M* NaCl, 3 4 m*M* KCl, 10 m*M* Na_2HPO_4, 2 m*M* KH_2PO_4 Store at 4°C
2. Trypsin/EDTA (20% [v/v] trypsin/EDTA) 0 25% w/v Difco trypsin dissolved in phosphate-buffered saline (PBS) 0 6 m*M* EDTA dissolved in PBS Store trypsin at –20°C and EDTA at 4°C.
3. Saline· 0.14*M* NaCl (sterile filter), and store at room temperature
4. Acidic glycine-(HCl): 0.1*M* glycine in saline, pH 3.0 (sterile filter). Store at room temperature.
5. Trypan blue dye· 0.5% (w/v) trypan blue (Sigma, St Louis, MO) prepared in PBS and passed through a 0 22-μm filter Store at room temperature. During long-term storage (>1 mo), a precipitate may form, and the solution should be refiltered before use Trypan blue dye is a suspected carcinogen and must be handled carefully
6. Neutral red dye· 0 4% (w/v) neutral red (Sigma) prepared in PBS and passed through a 0.22-μ*M* filter. Store in the dark at room temperature. Neutral red dye is a hazardous chemical and should be handled carefully

7 Tris-buffered saline. 25 m*M* Tris-HCl, pH 7.4, 137 m*M* NaCl, 5 m*M* KCl, 5.5 m*M* glucose, 0.7 m*M* Na_2HPO_4.
8. Reticulocyte standard buffer· 10 m*M* Tris-HCl, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$.
9 Cell lysis buffer: 20 m*M* Tris-HCl, pH 7.5, 2 m*M* EDTA, 1 2% sodium dodecyl sulfate (SDS) (w/v).
10. Proteinase K: 1 mg/mL in water.
11. 20X SSC· 3*M* NaCl, 300 m*M* sodium citrate.
12 Denaturing solution· 1.5 *M* NaCl, 500 m*M* NaOH.
13. Neutralization buffer· 3*M* NaCl, 500 m*M* Tris-HCl, pH 7 2
14 Prehybridization buffer· 5X SSC, 0.5% SDS, 5X Denhardt's buffer, containing 100 μg/mL calf thymus DNA. The calf thymus DNA must be boiled immediately before use.
15. Hybridization buffer: Prepare as for prehybridization buffer except that the HSV DNA (100 μg/mL) probe is also included (both calf thymus and HSV probe DNAs should be boiled immediately before use).
16. 50X Denhardt's buffer· 1% Ficoll 400, 1% bovine serum albumin, 1% Polyvinyl pyrrolidone prepared in water
17. Wash solution 1. 2X SSC, 0.5% SDS
18 Wash solution 2. 1X SSC, 0.1% SDS.

3. Methods

3.1. Cytotoxicity Testing

In order to study the antiviral activity of a new drug, it is important to determine whether antiviral activity can be uncoupled from the confounding effect of cellular toxicity. Cytotoxicity tests define the upper-limit drug concentration, which can be used in subsequent antiviral studies. The vital-staining techniques, whereby cells are treated with trypan blue or neutral red dyes, are among the simplest cytotoxicity tests to perform, need little equipment, and give reliable results *(28–30).* Trypan blue is excluded by live cells, but stains dead cells blue. In contrast, neutral red is taken up by live cells, staining them a brownish-red color, whereas dead cells remain colorless. Cell viability, as determined by vital-staining tests, should be confirmed by additional experiments, e.g., measuring incorporation of radiolabeled amino acids into proteins and/or ^3H thymidine into cellular DNA. Absence of cytotoxicity in in vitro tests does not necessarily exclude toxicity in vivo. Similarly, a moderate level of cytotoxicity in in vitro tests may not necessarily exclude in vivo use of the compound, perhaps as a topical treatment.

3.1.1. Investigating the Effect of Drugs on Cell-Culture Growth and Cell Viability

1 Seed cells sparsely on tissue-culture dishes (e g., 5×10^5 BHK cells on a 50-mm dish), and allow to settle overnight.
2 Replace the growth medium with either drug-free medium or medium containing increasing concentrations of drug (*see* Note 1)

3 At 0, 1, 2, 3, and so forth, days after drug addition, decant the overlay medium into separate centrifuge tubes, and then remove the cells from the culture dishes by two washes with 20% trypsin/versene.
4 Pool the trypsin/versene washes with the overlay medium from each cell culture, and centrifuge at low speed (1000*g* for 10 min at 4°C) Resuspend the cell pellet in 1 mL of tissue-culture medium Return the resuspended cell pellet to the appropriate culture, and use to resuspend the trypsinized monolayer This ensures that cells that may have become detached from the monolayer are not lost to the experiment
5. Mix 0 1 mL of cell suspension with 0.1 mL of a stock solution of trypan blue or neutral red dye.
6. After allowing 5 min for staining, count the number of stained cells and the total number of cells in each sample (in duplicate) using a hemocytometer. Cells mixed with neutral red should be kept in the dark during the staining period. Most cell lines can be kept for up to 30 min in the presence of trypan blue without affecting their viability.
7. Graph the results, and interpolate from the curves the concentration of drug that kills 50% of the cells; this is the 50% inhibitory concentration (IC_{50})
8. Compare the total numbers of cells (live and dead) in drug-treated and drug-free cultures to determine the effect of the drug on cell-culture growth.

It should be appreciated that the same drug might give different results when experiments are performed using exponentially growing or resting cell cultures, different cell types from the same species, primary or established cell lines, or similar cell cultures derived from the same organ, but from different animal species. The cell line eventually selected for antiviral activity assays will, in many cases, be a compromise between drug tolerance and the ability of the test virus to grow adequately in the selected cell line.

3.1.2. Reversibility of Drug-Induced Impairment of Cell-Culture Growth

If the window between antiviral activity and cytotoxicity is narrow, it will be important to investigate whether impairment of cell-culture growth is reversible and to determine the period of time that a particular concentration of drug can be left in contact with cells without affecting their subsequent growth *(28,29)*. Drug-removal experiments (Fig. 2) provide the answer to these questions.

1. Separate sparsely seeded cell cultures into sets (Fig 2), and overlay with either drug-free medium or medium containing increasing concentrations of drug.
2. At 0, 1, 2, 3, and so on, days after drug addition, harvest a control culture and a culture treated with each concentration of drug to determine the percentage cell viability and total cell counts, as described in Section 3 1.1
3. At each time-point, remove the drug from designated sets of cell cultures, by three washes with drug-free medium, and then overlay with drug-free medium and reincubate the cultures at 37°C.

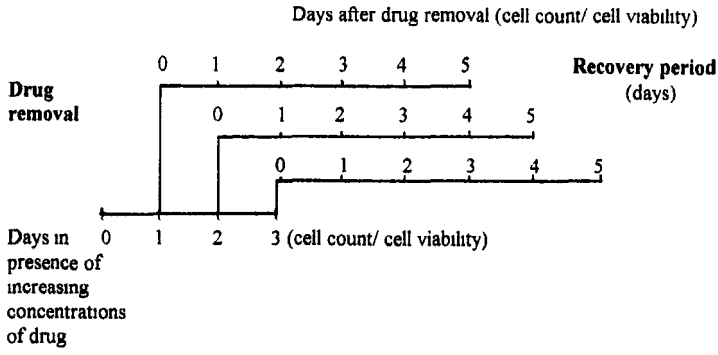

Fig 2 Schematic protocol: reversibility of drug-induced impairment of cell-culture growth

4 To measure the subsequent growth of cell cultures after drug removal (recovery from drug treatment), either harvest (as described in Section 3.1.) sets of cultures daily to monitor recovery, or harvest after a preselected number of days following drug removal
5. To determine the maximum period of time that cell cultures will tolerate contact with a particular concentration of drug, compare the total number of cells in the drug-free and drug-treated cultures following the recovery period

3.2. Measuring Antiviral Activity

3.2.1. Measuring the ED_{50} Concentration

The most commonly used measure of antiviral potency is the ED_{50}, that is, the concentration of drug that eliminates 50% of the virus infectivity.

1. Infect sets of cell monolayers on 50-mm tissue-culture dishes with HSV at 100–200 PFU/dish. After a 1 h absorption period at 37°C remove unbound virus by washing three times with PBS containing 5% calf serum
2 Overlay the infected monolayers with either normal tissue-culture medium containing 1.25% methyl cellulose (to inhibit satellite plaque formation) or with the same medium containing increasing concentrations of drug.
3. Incubate the infected cell layers at 37°C for 48 h or until virus plaques are visible Then fix/stain the cell layers with Giemsa, and count the number of virus plaques.
4. Determine the ED_{50} concentration by interpolation of the graphed data.

This experiment should be performed in duplicate and on several different occasions to test the reliability of the ED_{50} obtained. The ED_{50} drug concentration determined for one cell line may differ from that obtained with another if the uptake of the drug was different. A therapeutic index, derived from the IC_{50}/ED_{50} ratio can be computed. Promising antiviral agents are usually those with a therapeutic index of >10.

3.2.2. Infectious Virus Yield Drug Dose–Response Curves

Plotting the diminishing infectious virus yields from HSV-infected cell cultures treated with increasing concentrations of drug provides another useful way of measuring antiviral activity. Drug dose–response curves allow the kinetics of antiviral activity to be studied, yielding more information than can be obtained from the ED_{50} value alone.

1. Infect sets of cell monolayers with HSV at an MOI of 5 PFU/cell, and allow 1 h at 37°C to absorb the virus Remove unbound virus by washing the cell layers three times with PBS + 5% calf serum
2. Overlay the infected cells with drug-free tissue-culture medium or medium containing increasing concentrations of drug, and incubated at 37°C for 24 h The drug concentrations selected for antiviral studies should have been demonstrated (from Section 3 1 1) to be nontoxic for the cell line used
3. Harvest the total infectious virus yield by scraping the infected cells into the culture medium
4. Sonicate the infected-cell suspension for a few minutes, to break the cell membranes and release the intracellular virus Care must be taken to keep the sonibath cool, since HSV is not stable at temperatures >40°C Alternatively, release the intracellular HSV by three cycles of freeze–thaw In this procedure, the harvest from step 3 is rapidly frozen by plunging into a dry-ice bath (dry-ice granules bathed in ethanol and allowed to equilibrate) and then quickly thawed by immersion in a 37°C water bath Care should be taken when handling dry-ice/ethanol
5. Titrate the virus yields from each culture, and determine the infectivity by plaque assay
6. Plot the virus/drug dose–response curve The dose–response kinetics are characterized by: the concentration that gives the first reduction in infectious virus yield; the rate at which infectivity is lost with increasing drug concentration (i e , the slope of the curve); the greatest reduction in virus infectivity attained, and whether or not the curve plateaus. These important features assist selection of a single (or standard) dose of the drug, which may be used in subsequent experiments

If the compound is suspected to be active at a very early stage in the virus replication cycle (e.g., virus entry or uncoating—*see* Section 3.4.), it will be necessary to pretreat the cells with the drug before infection and to ensure that the drug is present throughout the virus replication cycle, including the virus adsorption period.

The infectious virus yield/dose–response experiment should be repeated several times to confirm the kinetics of the curves. The dose–response kinetics may differ with different cell lines, cell types, or if exponentially growing or resting cell cultures (*see* Note 2) were used.

Separation of the virus yields into cell-associated and cell-released fractions (*see* Note 3) could provide additional information indicating whether drug treatment affects the release of infectious progeny virions *(28,31)* Estimation

of the number of virus particles present in the cell-associated and cell-released fractions by negative-stain electron microscopy allows particle/PFU ratios to be calculated, providing additional information relevant to maturation and release of virus particles produced in the presence of the drug.

3.3. Investigating the Genetic Target of the Drug

The major goal in the investigation of the antiviral mechanism is to identify the virus gene product that is the target of the drug.

3.3.1. Single-Round Selection of Drug-Resistant Virus

To identify the gene that encodes the protein target of the drug, it is first necessary to isolate drug-resistant virus mutants. This should be possible if the target is the product of a single HSV gene, but may be more difficult, if not impossible, if overlapping genes are involved. There are two ways in which such mutants might be isolated: first, by multiple cycles of selection, where HSV is initially passaged at a low (semipermissive) concentration of drug, which is gradually increased at each subsequent passage, second, by a single round of selection in the presence of a concentration of drug that inhibits plaque formation by about 99.999%.

Multiple-cycle selection will show, relatively quickly, whether drug resistance is possible and indicates that at least a component of the antiviral activity is virus-specific. However, the continuous selective pressure placed on the virus to increase drug resistance with each successive passage is likely to result in an accumulation of point mutations, at best within the target gene and, at worst, within a number of different genes whose products interact with each other. In the latter case, it may be difficult, if not impossible, to map and identify the genes involved.

Single-round selection has the advantage that the stock of drug-resistant virus so generated is likely to differ from the wild-type virus by only a single mutational event and so is suitable for further genetic analysis.

1. Infect 100–200 cell monolayers (on 50-mm tissue-culture dishes) with HSV at a multpilicity of infection (MOI) of 1000/PFU/dish After the absorption period, wash the monolayers to remove unbound virus, and then overlay with culture medium containing, 1 25% methylcellulose, and a concentration of drug sufficient to inhibit plaque formation by about 99 999%. Incubate the cultures at 37°C for 48 h or until plaques develop.
2. Pick virus plaques, that are well separated from each other, into 0 5 mL PBS + 5% calf serum and sonicate to release the intracellular virus.
3. Titrate the virus yield from the plaque, and determine the infectivity in the absence and presence of the drug (concentration as before). Select and pick second-generation plaques from the drug-free cultures of those plaques that have an approximately equal titer in the absence and presence of the drug.

4 Continue this process for at least three generations, always picking from the drug-free cultures and monitoring drug resistance with the drug-treated culture. From time to time, it may be necessary to boost the titers of the plaque-produced virus by a passage in the absence of the drug. Take care to ensure that the final stocks of drug-resistant viruses produced are not clonally related (i.e , they were not picked from the same plate).

3.3.2. Mapping the Drug Resistance Gene

Once drug-resistant virus stocks have been produced, it becomes possible to identify the genetic locus specifying drug resistance by standard marker-rescue techniques (*see* Chapter 3). The salient points are given below.

1. Purify DNA from a preparation of the drug-resistant virus particles (*see* DNA extraction protocol; Chapter 3)
2. Cotransfect intact wild-type HSV-DNA and individual purified DNA fragments, derived from the drug-resistant virus, into duplicate cell monolayers (*see* virus DNA transfections protocols; Chapter 3). Then overlay one set of monolayers with drug-free tissue-culture medium and the other with medium containing the concentration of drug used in the selection of the drug-resistant virus.
3 Virus plaques that grow on the drug-treated cultures arise from recombinant viruses, which have incorporated the drug resistance marker contained within the cotransfected DNA fragment. These plaques should yield approximately equal titers in the presence and absence of the drug.
4 Several controls are required· drug-free transfected cultures to indicate whether transfection has been successful, wild-type HSV-DNA alone to exclude drug resistance in the wild-type DNA stock used, intact drug-resistant virus DNA to confirm the drug resistant phenotype, and each purified DNA fragment alone to detect uncleaved drug-resistant genomes contaminating the stocks of purified DNA fragment.
5. Confirm the location of the drug resistance gene within the identified fragment, by repeating the experiment in mirror image that is cotransfecting cell cultures with uncleaved DNA from drug-resistant virus and purified fragments of wild-type DNA The fragment containing the drug resistance marker will now be identified by HSV recombinant viruses having the drug-sensitive phenotype

To fine-map the genetic locus containing the drug-resistant marker, the above procedure must be repeated, using progressively smaller restriction fragments.

3.3.3. Identification of Drug Resistance Mutation by DNA Sequencing

Restriction enzyme mapping should position the drug resistance locus within a few kilobases of DNA. To identify the virus gene involved and to pinpoint the mutation conferring drug resistance, this DNA fragment and the appropriate parental wild-type DNA fragment must be sequenced (*see* DNA sequencing protocols; Chapter 4). If the gene product is one that has already been

identified as the target of other antiviral compounds (e.g., the DNA polymerase), then it will be useful to check for drug crossresistance, since this could yield insights into the molecular basis for drug inhibition of protein function.

3.4. Investigation of the Antiviral Mechanism

If no drug-resistant variant can be isolated or if the function of the identified drug resistance gene cannot be deduced from its DNA sequence, it will be necessary to investigate the point in the virus replication cycle at which the drug-sensitive function operates in order to focus further investigations. Chemically induced inhibition of virus replication can be separated into virucidal and antiviral activities. Virucidal activity eliminates infectivity when virus particles are mixed with the drug in solution, whereas antiviral activity inhibits the synthesis of mature infectious progeny virions in infected cells.

Virucidal activity may be caused by disintegration of the entire HSV particle, solubilization of the virus envelope, or the chemical modification, degradation, or masking of some essential envelope proteins. HSV particles treated with a virucidal agent are blocked at adsorption and/or penetration, the earliest stage of the virus replication cycle.

3.4.1. Virucidal Activity

1. Prepare a set of HSV aliquots (1×10^6 PFU in 2 mL) in drug-free tissue-culture medium or medium containing increasing concentrations of drug, and incubate at 37°C
2. Withdraw duplicate 0.1-mL samples for titration of residual infectivity at the time of drug addition and at various times thereafter. Titrate each sample without delay or storage, lest the infectivity continue to decrease. The titer in the virus aliquot must be sufficiently high to allow dilution, during titration, of the virucidal agent below the concentration at which cytotoxicity is apparent on the indicator cells. This type of experiment is frequently performed at a range of temperatures, e g., 4°C, room temperature, and 37°C.

3.4.2. Investigating the Effect of Drugs on HSV Adsorption

Two techniques have been used to investigate adsorption of HSV to target cells. The first technique provides an indirect measure of virus adsorption, quantifying the decrease in infectivity, with time, in the inoculum applied to cell monolayers *(20,32)*. Because noninfectious particles might also adsorb to the cells, use virus stocks with low particle/PFU ratios for these experiments (i.e., <50:1). The second technique quantitates HSV particles bound to cell-surfaces by measuring the radioactivity associated with ^{35}S-methionine-labeled HSV particles, accumulating with time and resistant to removal by washing of the cell layer. Both techniques should be performed at 4°C, since at this temperature, HSV binds to, but does not penetrate cells.

1 Prepare radiolabeled HSV by infection of roller bottle cultures of cells (~2 × 10^8/roller) with HSV at an MOI of 1 PFU/300 cells in growth medium containing 1/5 normal concentration of methionine. Incubate for 24 h at 31°C, then add 25 μCi/mL ^{35}S-methionine, and incubate at 31°C until complete CPE is evident.
2. Harvest the extracellular HSV yield, and purify the virus particles by density gradient centrifugation (*see* Chapter 1)
3 Cool confluent monolayers of cells (on 50-mm tissue-culture dishes) to 4°C
4. Prepare a series of radiolabeled HSV inocula (10^6 PFU/mL) using culture medium precooled to 4°C, and containing either no drug or increasing concentrations of drug.
5. Immediately after preparing the virus inocula, decant the growth medium from the cell monolayers, and infect duplicate cultures with 1 mL of virus/drug inoculum
6. At the time of infection and at increasing times thereafter (e.g., 15, 30, 45, 60, 90, 120, 180, and 240 min), remove the inoculum from the appropriate culture dishes, and remove unbound virus by three washes with PBS.
7 Prepare cell extracts from the washed cell layers by adding 300 μL of SDS-PAGE sample buffer.
8 Estimate the amount of radioactivity in each sample by spotting 20 μL of cell extract onto a Whatman no 1 filter paper disk (there is no need to TCA precipitate this material), place in scintillation vial with 5 mL of scintillation fluid, and measure the amount of radioactivity counts/min in each sample using a scintillation counter Plot the percentage virus adsorption with time (100% is the cpm obtained with the drug-free control at the maximum time allowed for virus adsorption).

The important features of the virus adsorption curve are the time at which binding of virus to the cells is first detected, the rate at which virus particles bind to the cells, the time at which the curve plateaus, and the maximum amount of virus binding to the cells. The proportion of the total inoculum binding to cells at any time may be computed by comparing total counts/min in the cell extract with total counts/min in the inoculum at 0 min.

3 4.3. Investigating the Effect of Drugs on HSV Penetration

The effect of drug treatment on the rate of virus entry into cells may be monitored by measuring the infectivity that becomes resistant to low pH inactivation with time *(20,32)*. This experiment is only valid for compounds that can readily be washed from the intracellular compartments of cells. The HSV penetration assay depends on the observation that HSV binds to cells at 4°C, but does not penetrate until the temperature is raised.

1. Seed cells in several sets of 24-well plastic tissue-culture trays (one tray for each time-point), and allow to grow overnight at 37°C
2. Prepare a series of virus inocula (100–200 PFU/cell layer) in drug-free tissue-culture medium and cool to 4°C
3. Cool the cell cultures to 4°C
4. Decant the tissue-culture medium from the monolayers and infect with 100–200

PFU HSV/monolayer in a 200 µL inoculum. Incubate at 4°C for 2 h to allow maximum adsorption of the virus.

5 Wash the monolayers with ice-cold PBS to remove unbound virus, overlay with drug-free medium or medium containing increasing concentrations of drug, and then shift to 37°C to allow virus penetration.

6. At various times (0, 15, 30, 45, 60, 75, and 90 min) after temperature upshift, remove the appropriate 24-well tray from the incubator and inactivate virus particles that have not yet penetrated the cells by treating the infected cell cultures as follows:
   a. Wash once with 1 mL saline.
   b Wash once, for 1 min only, with acidic glycine
   c Wash once with tissue-culture medium to neutralize the acid.
   d. Finally overlay the infected cells with drug-free medium containing 1.25% methyl cellulose

7 Incubate the infected cell cultures for 2 d at 37°C to allow virus plaques to develop. Fix/stain the cultures with Giemsa, count the numbers of plaques on each culture, and plot the data.

The important features of the virus penetration curve kinetics are: the time at which the first virus particles enter the cells; the rate of virus penetration; the maximum number of virus particles entering the cells; and the time at which the curves plateau.

3.4.4. Investigating the Stage of the HSV Replication Cycle Blocked by the Drug

If the drug inhibits virus replication at a point subsequent to virion entry, the stage at which the replication cycle is blocked may be deduced from electron microscope studies (*see* Note 4) or from single-step HSV growth experiments in which a single (standard) antiviral concentration of drug (determined from Section 3.2.2.) is added to infected cell cultures at progressively later times throughout the virus replication cycle.

1 Infect a series of cell monolayers with HSV at an MOI of 5 PFU/cell. Allow the cells 1 h at 37°C to absorb the virus, then wash three times with PBS 5% calf serum to remove unbound virus, and overlay with drug-free culture medium.

2. At various times after infection (e.g., 2, 4, 6, 8, 10, 12, 16, and 20 h post infection), add the drug to the culture medium of the appropriate set of infected monolayers.

3 Similarly, at various times after infection (e g , 0, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h pi), harvest selected cell cultures (as described in Section 3 2.2)

4. Determine the total infectious virus yields by titration, and plot the data.

If the protein target for the drug specifies an early function, late addition of the drug will have no inhibitory effect on the infectious virus yield. Drug-induced inhibition of a protein function that is required late, or throughout the replication cycle, will result in loss of infectivity whenever the drug is present.

The converse experiment where drug is removed from infected cell cultures at progressively later times can also give useful data, as can experiments in which pulses of drug are used. However, such experiments can be difficult to interpret due to the difficulty of ensuring efficient removal of drug from intracellular sites.

3.4.5. Investigating the Effect of Drugs on HSV-DNA Synthesis

In the past, HSV-DNA synthesis has been studied by separation of ^{3}H-thymidine labeled infected-cell DNA into viral and cellular fractions by cesium chloride gradient centrifugation *(33)*. More recently, however, this technique has been superseded by a dot-blot, Southern blotting method. The protocol described here uses a radiolabeled probe to detect HSV-DNA bound to a nylon filter, but a nonradioactive detection system might also be used. To reduce the background HSV-DNA signal, caused by residual inoculating HSV particles, it is advisable to extract DNA from isolated nuclei *(34)*.

3.4 5 1 Preparation of DNA from Nuclei

1. Infect monolayers of cells (~2 × 10^6 cells) on 35-mm tissue-culture dishes with HSV at an MOI of 5 PFU/cell
2. Allow the cells 1 h at 37°C to absorb the virus, and then remove unbound virus by three washes with PBS + 5% calf serum
3. Overlay the infected cell cultures with either drug-free medium or medium containing increasing concentrations of drug, then incubate at 37°C for 24 h.
4. Scrape the infected cells into the medium, and pellet by low-speed centrifugation (1000*g* for 10 min). Carefully remove the supernatant, wash the cells with Tris-buffered saline, and then repellet the cells (1000*g* for 10 min).
5. Raise the cell pellet in 1 mL of reticulocyte standard buffer containing 0 5% NP40, and incubate on ice for 10 min to lyse the cells.
6. Pellet the nuclei by low-speed centrifugation (1000*g* for 10 min)
7. Carefully pipet off the cytoplasmic fraction (supernatant), and resuspend the pelleted nuclei by vortexing in 1 mL of water to which 1 mL of lysis buffer is then added. Incubate at 37°C for 6 h.
8. Dilute the sample with 20X SSC to give a final concentration of 6X SSC

3.4 5 2 Dot-Blot/DNA Hybridization

1. Prepare a series of fivefold dilutions (of 300 µL total volume) of each nuclear extract in 6X SSC
2. Boil for 10 min, and then cool on ice
3. Assemble the dot-blot apparatus according to the manufacturer's instructions, e g , place two sheets of Whatman 2MM chromatography paper presoaked in 2X SSC on the base of the apparatus, and overlay these with a sheet of Hybond N (Amersham, Little Chalfont, Buckinghamshire, UK) nylon membrane presoaked in 2X SSC, then close the apparatus
4. Apply the vacuum to the dot-blot apparatus, and load 200 µL of each sample into

the appropriate well of the apparatus.

5 When all the sample has passed through the membrane filter, break the vacuum and disassemble the apparatus. Lay the nylon membrane (DNA side up) on top of a stack of three Whatman 3MM chromatography paper sheets soaked by partial immersion in denaturing buffer. Incubate at room temperature for 10 min.

6 Transfer the nylon membrane (DNA side up) to a stack of three 3MM chromatography paper sheets soaked by partial immersion in neutralization buffer, and incubate at room temperature for 5 min

7. Allow the membrane to air-dry, then immobilize the DNA on the membrane by baking at 80°C or by UV crosslinking on a transilluminator (as described in the manufacturer's instructions).

8 Incubate the membrane at 65°C for 2 h with prehybridization buffer

9. Prepare the radiolabeled HSV-DNA hybridization probe as described in the random-priming or nick-translation protocols (*see* Chapter 4)

10. Incubate the membrane at 65°C overnight in hybridization buffer containing the radiolabeled HSV-DNA probe.

11. Carefully remove the radioactive hybridization buffer, and then wash the membrane twice in wash solution 1 (1 min at 65°C), twice in wash solution 1 (15 min at 65°C), and finally twice in wash solution 2 (15 min at room temperature)

12. Seal the membrane in a polythene bag, and expose to X-ray film to obtain an autoradiograph

By using a densitometer to measure and comparing the optical absorbance of the dots on the autoradiograph, the effect of the drug on HSV-DNA synthesis can be computed. Alternatively, if HSV-DNA standards of known concentration are included on the same membrane with the test samples, it will be possible to quantitate the amounts of HSV-DNA synthesized.

3.4.6. Investigating the Effect of Drugs on Viral Protein Synthesis and Posttranslational Processing

The HSV-infected cell polypeptide patterns obtained on SDS-PAGE gels are well characterized. Similarly, the temporal classification of most HSV proteins is known from SDS-PAGE studies using temperature-sensitive mutants or from studies with drugs that block HSV replication at defined stages in the virus replication cycle *(35)*.

SDS-PAGE can be used to investigate the effect of drugs on HSV polypeptide synthesis. Drug-induced inhibition of HSV replication is usually reflected in changes in the quantities, or the apparent molecular weights, of HSV-specified polypeptide bands on SDS-PAGE gels. Reduction in the quantity of a particular protein can have a number of possible explanations, including inhibition of the transcription of the affected gene, inhibition of translation of the mRNA, or reduction in the stability (half-life) of the mRNA or the protein product (*see* Chapter 13 for transcription and mRNA stability protocols). If the protein

affected by the drug specifies a regulatory function, the drug may indirectly affect the synthesis of other proteins under its control. Drug-induced changes in the apparent molecular weights of protein bands on SDS-PAGE gels argue for alteration in the normal pattern of posttranslational processing of the affected protein, resulting in accumulation of unprocessed precursor forms, intermediately processed forms, or breakdown products of the affected proteins. The protocols for investigating HSV-infected cell proteins by SDS-PAGE are given in Chapter 7.

Posttranslational processing of HSV proteins can be of several types, including glycosylation, phosphorylation, myristilation, and sulfation (*see* Chapter 7 for labeling protocols).

4. Notes

1. The test compound should be soluble in either aqueous solution, tissue-culture medium, distilled water, and so forth, or organic solvents, dimethylsulfoxide (DMSO), ethanol, and so forth Drug solutions should be sterilized by ultrafiltration (0.22 μ), or if heat-stable, by autoclaving before use Where organic solvents are used, additional controls for the effect of the solvent must be included in experiments If the molecular weight of the test compound is known, then molar amounts should be used; otherwise, weight/volume doses may be used
2. To prepare resting cell cultures, the cells are sparsely seeded (1×10^5 cells/50-mm dish) in tissue-culture medium containing 0.5% serum and incubated at 37°C in a well-humidified incubator After about 7 d, growth of the culture should have stopped. The decrease in cellular metabolism can be monitored by measuring the incorporation of ^{3}H-thymidine into cellular DNA during an overnight labeling period. The growth-factor-depleted (conditioned) medium from resting cell cultures must be retained at the time of infection, so that it can be reused, with drug added as appropriate, to overlay the infected cells
3. Before harvesting the infectious virus yields at step 3 of Section 3 2 2 , there is an option to harvest the total virus yield or to separate the virus yield into cell-associated and cell-released components; the latter can provide information on whether the drug impairs the release of virions from the cell or whether the drug has a strong virucidal effect To obtain the cell-released yield, remove the supernatant tissue-culture medium from infected cells and clarify by low-speed centrifugation (1000*g*/10 min/4°C) To obtain the cell-associated yield, raise the cell pellet (generated by the centrifugation step above) in fresh culture medium, and use this to harvest the cells, which remain attached to the tissue-culture dish, by scraping them into the medium The cell-associated and cell-released volumes are brought to the same volume with fresh medium, and the cell suspension is then sonicated to generate the cell-associated virus fraction
4. The herpesvirus replication cycle has been extensively studied by electron microscopy, and the temporal sequence of morphological events occurring during HSV replication has been examined by time-course experiments *(36)*, experi-

ments in which virus replication is blocked by drugs *(37)*, and experiments in which virus replication is arrested by various temperature-sensitive mutations in the infecting virus *(38–41)*

HSV-infected cells treated with drugs that inhibit IE gene expression show little morphological evidence of infection, except that the nucleus of the infected cell exhibits condensation and margination of the cellular chromatin. HSV infected cells treated with drugs that block HSV-DNA synthesis exhibit margination of chromatin and intranuclear accumulation of large numbers of immature nucleocapsids (i e., both A- and B-type capsids *[42]*). Infected cells treated with drugs that block HSV replication at a stage after viral DNA replication and packaging exhibit, in addition, mature nucleocapsids having an electron-dense DNA-containing core (C-type capsids *[42]*)

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