Preface

Viral Vectors for Gene Therapy: Methods and Protocols consists of 30 chapters detailing the use of herpes viruses, adenoviruses, adeno-associated viruses, simple and complex retroviruses, including lentiviruses, and other virus systems for vector development and gene transfer. Chapter contributions provide perspective in the use of viral vectors for applications in the brain and in the central nervous system. *Viral Vectors for Gene Therapy: Methods and Protocols* contains step-by-step methods for successful replication of experimental procedures, and should prove useful for both experienced investigators and newcomers in the field, including those beginning graduate study or undergoing postdoctoral training. The "Notes" section contained in each chapter provides valuable troubleshooting guides to help develop working protocols for your laboratory. With *Viral Vectors for Gene Therapy: Methods and Protocols*, it has been my intent to develop a comprehensive collection of modern molecular methods for the construction, development, and use of viral vectors for gene transfer and gene therapy.

I would like to thank the many chapter authors for their contributions. They are all experts in various aspects of viral vectors, and I appreciate their efforts and hard work in developing comprehensive chapters. As editor, it has been a privilege to preview the development of *Viral Vectors for Gene Therapy: Methods and Protocols*, and to acquire insight into the various methodological approaches from the many different contributors. I would like to thank the series editor, Professor John Walker, for his guidance and help in the development of this volume, and Thomas Lanigan, President of Humana Press. I would also like to thank Danielle Mitrakul for her administrative assistance in the preparation of this volume. Danielle is deeply appreciated for her willingness to help and for her tireless work. I would also like to acknowledge the support of my laboratory members, Ying Bai and Philbert Kirigiti, and thank Dr. Tom Shearer, Associate Dean for Research, for his support of my research program. Special thanks are extended to my wife Dr. Cindy Machida, and my daughter, Cerina, for their support during the long hours involved in

the compilation and editing of this volume. Their understanding of the importance of this work and their support made the development of this volume possible.

Curtis A. Machida

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Construction of Multiply Disabled Herpes Simplex Viral Vectors for Gene Delivery to the Nervous System

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1. Introduction

This chapter aims to describe the main procedures involved in constructing a disabled recombinant herpes simplex virus type 1 (HSV-1) vector. However, before describing the methodology in detail, it is important to briefly consider the structure and life cycle of HSV1 and the modifications that must be made to the virus in order to generate an efficient gene delivery vector.

1.1. HSV1 Biology and Life Cycle

The HSV-1 virion is composed of an electron opaque core containing the viral genome. The linear double-stranded DNA genome of approx 152 kb encodes at least 80 genes (*see* **Fig. 1**). The genome is composed of long and short unique segments flanked by terminal repeat regions. The virion core is surrounded by an icosahedral capsid and an outer lipid envelope. The lipid envelope contains glycoproteins, which interact with cellular receptors in order to facilitate binding of the virus, penetration of the cell, and cell-to-cell spread. Between the capsid and the lipid envelope is the amorphous tegument layer. Following penetration, several viral proteins of the tegument act to recruit cellular resources away from host-cell protein synthesis. These proteins include the virion host shut-off protein (vhs), which indiscriminately degrades mRNA thereby causing a shut-off of host protein synthesis ***(1)***. Virus capsids are transported to the nucleus where the viral DNA and at least some tegument proteins enter the nucleus. Here, the course of the HSV1 infection depends on whether the virus enters the lytic cycle or establishes a latent infection.

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Fig. 1. The organization of the HSV-1 genome. The viral genome is 152 kb and contains unique long and short regions (U_L and U_S, respectively) flanked by the terminal and internal repeats (TR and IR, respectively). The regions marked 'a' contain sequences required for packaging.

1.1.2. The Lytic Life Cycle

In order for the efficient initiation of the lytic cycle, a tegument protein, virion protein 16 (VP16) must interact with cellular factors to transactivate the promoters of the first class of viral genes to be expressed, the immediate early (IE) or α genes. VP16 is dependent on the cellular POU domain protein Oct-1 and at least one other cellular factor, known as host cell factor (HCF) to form a multicomponent complex on the TAATGARAT motifs, which are present in all the IE gene promoters. Following this transactivation, the lytic gene cascade ensues (*see* **Fig. 2**). This cascade is well ordered and tightly regulated and dictates the controlled expression of three classes of genes; the IE or α genes, the early (E) or β genes, and the late (L) or γ genes ***(2)***. There are five IE genes; infected cell polypeptides (ICP) 0, 4, 22, 27, and 47. Four of these IE genes (ICP0, 4, 22, and 27) are involved in the transcriptional and posttranscriptional regulation of the expression of the E and L genes. The remaining IE gene, *ICP47*, is not a regulatory IE protein, but inhibits the transporters of antigen processing (TAP), thereby helping the virus avoid the host's immune surveillance mechanisms ***(3)***. Following the synthesis of the IE genes, the E genes are transcribed. The E-gene products are primarily involved in viral DNA synthesis and encode proteins such as DNA binding proteins, polymerases, and thymidine kinase. Viral DNA synthesis occurs by a rolling circle mechanism, forming head-to-tail concatemers of UL and US separated by the repeat regions ***(4)***.

Expression of the L genes is activated by the IE genes only after viral DNA synthesis has occurred. The L genes encode the structural proteins of the capsid, tegument and envelope. Following the expression of the L genes, the viral DNA concatemers are cleaved into genome length units and packaged into the capsids. It is thought that the capsids then bud through areas of the nuclear envelope that have been modified with viral glycoproteins, thus forming the viral envelope. The newly synthesized virions then pass through the ER and cytoplasm and into the extracellular space. The first newly formed capsids appear in the nucleus within 6 h of infection, the entire lytic cycle taking approx 10 h.

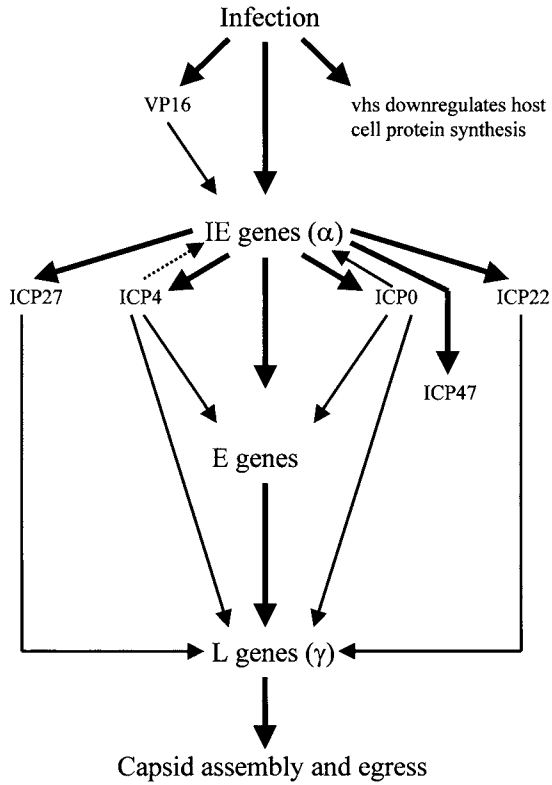

Fig. 2. The HSV-1 lytic gene regulatory cascade. The virion protein VP16 interacts with cellular factors to transactivate the IE genes. Expression of IE genes is required for the timely expression of E and L genes. Positive regulatory effects are indicated with thin solid arrows, whereas repressive effects are indicated with dashed arrows. There are a number of interactions between the regulatory IE genes which are also known to mediate a regulatory effect. For clarity, these are not indicated on this diagram.

1.1.3. The Latent Life Cycle

HSV-1 usually enters sensory neurons innervating the cells of the mucosal membranes, most commonly those around the mouth. Viral capsids are then transported to the neuronal cell bodies where the genome is able to persist in an episomal state for the lifetime of the host. Periodic reactivation of HSV-1 can occur, whereby viral nucleocapsids are transported back to the periphery, leading to lytic infections in the same dermatomal distribution as the initial infection. Reactivation can occur spontaneously or can be induced by stress, either directly to the neuron or to the whole organism.

Analysis of viral gene expression during latency has revealed that no genes of the lytic cycle are expressed. Transcription is limited to a single region within the long repeats of the HSV-1 genome, giving rise to the production of the latency associated transcripts (LATs) ***(5)***. The major LATs are 2-kb and 1.5-kb nonpolyadenylated RNA species that remain nuclear. These appear to be stable introns spliced from an unstable 8.3-kb primary LAT, which is transcribed antisense and complementary to the coding sequence for ICP0. The promoter regions controlling the expression of the LATs are named the latency associated promoters 1 and 2 (LAP1 and LAP 2). LAP1 is a TATA box containing promoter located 700–1300 bp upstream of the primary 8.3-kb LAT. LAP1 is active in most cell types, but contains an enhancer element giving it increased activity in neurons ***(6,7)***. LAP2 is a TATA-less sequence with promoter activity located downstream of LAP1 and approx 750 basepairs upstream of the stable 2-kb LAT intron ***(8)***.

1.2. Advantages of HSV-1 as a Vector for Delivering Genes to Neurons

HSV-1 has often been suggested as a vector for neuronal gene transfer as it has a number of characteristics, which suggest that it might be inherently suitable for this purpose. HSV-1 is naturally neurotrophic; following infection of epithelial cells, the virus enters the terminals of local peripheral nerves and is retrogradely transported to the neuronal cell bodies in the sensory ganglia. In the ganglia, HSV-1 establishes an asymptomatic latent infection that can persist for the lifetime of the host. During latency, gene expression from the vast majority of the HSV genome is silenced, but a small part of the viral genome continues to be transcribed, generating the population of RNA species collectively known as the latency associated transcripts (LATs) ***(5)***. This ability of the HSV-1 genome to persist in the nucleus of infected cells whereas maintaining a basal level of transcription has suggested that novel methods of obtaining long term or even life-long transgene expression after a single application might be developed. In addition, HSV-1 has a very large genome, and is, therefore, not subject to the packaging constraints of some other viral vector systems. Furthermore, HSV-1 remains episomal and does not integrate into the host chromosome. This removes the possibility of insertional mutagenesis of the host genome, a potential risk associated with AAV or lentiviral-based gene transfer systems that integrate at probably random sites in the host genome. HSV-1 also has several practical advantages as a gene delivery vector; the viral genomes are easily manipulated and the virus can be propagated to high titers in culture.

1.3. Disabled HSV-1 Vectors Versus Amplicons

Disabled HSV-1 vectors are generated by recombining the transgene and its regulatory sequences directly into the HSV-1 genome. This is achieved by cotransfection of plasmid and infectious viral DNA into a cell line that complements the deleted viral function(s). The recombination site is dictated by homologous HSV-1 sequences in the plasmid 5′ and 3′ of the transgene and its regulatory sequences. Essential IE genes can be "knocked out," rendering all the recombinant progeny replication-deficient. Disabled vectors utilize promoter systems that exploit the ability of the virus to continue to express the LATs through latency (*see* **Subheading 1.4.2.**).

Amplicons are defective HSV-1 based viruses, incapable of replicating in the absence of helper functions. Amplicon vectors are created from plasmids that contain the transgene, an HSV-1 packaging signal, and an HSV-1 origin of replication ***(9)***. Despite the significant recent improvements in the amplicon technology discussed elsewhere in this volume, several potential limitations remain. Most importantly, amplicons do not take advantage of the ability of HSV-1 to establish a latent infection and remain in the host cell nucleus for many years (whereas amplicon DNA can be maintained in the nucleus for extended periods of time, it is likely to eventually be degraded by cellular enzymes). Second, on a more practical note, the yield obtainable from helper-free amplicon systems is low (approx 10^5 pfu/mL) ***(10)*** and the fact that cotransfection is still required means that the system is somewhat cumbersome and not as yet readily amenable to scale-up. However, the potential advantages of the amplicon system are that the only manipulation required is that of the amplicon plasmid and also that each replication-defective vector contains multiple copies of the gene of interest, which may increase expression levels. Furthermore, in contrast to disabled HSV vectors, the choice of promoter driving the expression of the transgene in amplicon vectors is not crucial. This is because the lack of significant HSV-1 DNA in the plasmid means that amplicons are not subject to the same control mechanisms that shut down gene expression at the onset of latency in wild-type virus and are also apparent in disabled vectors. This flexibility in promoter choice has enabled reasonably long-term and cell-type-specific expression to be obtained from amplicons ***(11)***, the latter having not yet been achieved with disabled HSV-1 vectors.

1.4. Constructing Disabled HSV-1 Vectors

There have been two main limitations that have hindered the use of disabled HSV-1 as a vector for gene delivery to neurons. First, as HSV-1 does not

integrate into the host genome, and the majority of HSV-1 and heterologous promoters are switched off at the onset of latency, expression of transgenes has mainly been transitory. Second, the wild-type virus is highly pathogenic; cerebral injection causes a fatal encephalitis. The virus must, therefore, be disabled prior to its use as a gene delivery vector. However, even in a replication-incompetent virus, expression of HSV-1 proteins can still be highly cytotoxic. Advances toward overcoming these two limitations are discussed below.

1.4.1. Reducing Cytotoxicity

HSV-1 rarely enters a lytic replication cycle in neurons during natural infections. However, following stereotaxic injection directly into the central nervous system (CNS), or infections of primary cultures at high multiplicities, lytic replication does occur. Such lytic replication rapidly destroys cells in culture or causes encephalitis and death of a test animal. In contrast, latent infections of neurons do not cause any detectable detrimental effects to the physiology of the cell. It is therefore clear that wild-type HSV-1 must be disabled such that it is incapable of expressing any of the genes of the lytic cycle and the virus is forced to enter latency.

The cascade nature of HSV-1 gene expression suggested that the IE genes were a sensible target for disablement if replication-incompetent and less toxic vectors were to be developed. A number of studies demonstrated that the expression of all the regulatory IE genes would need to be prevented or significantly reduced in order to generate a virus that was completely noncytotoxic ***(12–18)***. Several laboratories have developed disabled HSV-1 vectors that contain deletions in some or all of the IE genes ***(15,17)***. However, the efficient propagation of viruses that express no IE genes requires that all these gene products are provided by the complementing cell line. This presents a potential problem as the toxicity of the IE gene products means that cell lines directing their stable expression are hard to generate. This problem can be partially overcome by the using the promoters which normally control the expression of the IE genes to drive their expression in the cell line, because these are relatively inactive in the absence of viral infection. This approach has been successfully used to generate cell lines expressing single or up to three IE genes ***(12,16,19,20)***. However, the toxicity of the IE proteins means that cell lines that simultaneously express multiple IE genes do not offer full and consistent complementation ***(16)*** and no cell line expressing all four of the regulatory IE genes has yet been generated.

An alternative approach to the individual deletion or inactivation of all the IE genes (and the resulting necessity to complement them all individually from a cell line), is to inactivate the gene encoding VP16 (*see* **Fig. 2**). VP16 is a virion component that enters the nucleus with the viral genome and serves to

transactivate the promoters of the IE class of genes. VP16, therefore, represents an attractive target for disablement as it offers the potential to reduce or prevent the expression of all the IE genes. However, VP16 cannot be deleted from the virus as it also encodes an essential structural component of the virion ***(21)***. Therefore, if VP16 was deleted from the virus and provided *in trans* from a complementing cell line (as for the IE genes), the virions would package the cellularly derived VP16 into their tegument and carry it over to target cells, where it would transactivate the IE gene promoters. This problem was overcome by the identification and subsequent exploitation of the fact that distinct domains in VP16 control the transactivation of IE gene transcription and the assembly of virions ***(22)***. A small insertion made into the transactivation domain of VP16 resulted in a virus, which was severely impaired in its transactivating function, but still able to fulfill its essential structural role ***(23)***. Subsequent work has led to the construction of a virus with the entire transactivation domain of VP16 deleted, further attenuating the virus by almost completely compromising its ability to transactivate the IE gene promoters ***(24,25)***

A mutation to VP16 have been combined with a temperature sensitive mutation in ICP4 and a deletion of part of the ICP0 gene, generating viruses that are conditionally replication-incompetent and which can be propagated on parental cell lines under permissive conditions at 32°C ***(26)***. Furthermore, a VP16 mutation has been combined with deletion of the *ICP4* and *ICP27* genes to generate viruses that are completely incapable of replication under any condition and which do not express significant amounts of any of the IE genes, including *ICP0*, *ICP22*, and *ICP47*, which have not been specifically deleted ***(27)***. In contrast to viruses in which each IE gene has been individually inactivated (for which a fully complementing cell line has yet to be produced), this virus can be propagated on a cell line containing ICP27, ICP4, and the equine herpesvirus homolog of VP16, thereby enabling efficient complementation of all of the deleted functions ***(28)***. The low toxicity and high levels of gene delivery achievable with this virus are demonstrated in **Fig. 3**, which shows gene delivery to cultured adult rat DRG neurons and the rat brain in vivo.

1.4.2. Promoters for Long-Term Gene Expression

Exploiting the long-term transcriptional activity of the LAT promoters has long been thought of as an obvious means by which continuing expression of a transgene might be achieved. The expression of the LATs is controlled by two promoters, LAP1 and LAP2. Early attempts to take advantage of the LAT promoters to drive expression of exogenous genes concentrated on the core LAT promoter, LAP1. However, a number of studies demonstrated that elements downstream of LAP1 (within and/or surrounding LAP2) are necessary to facilitate the long-term expression of transgenes from disabled HSV-1

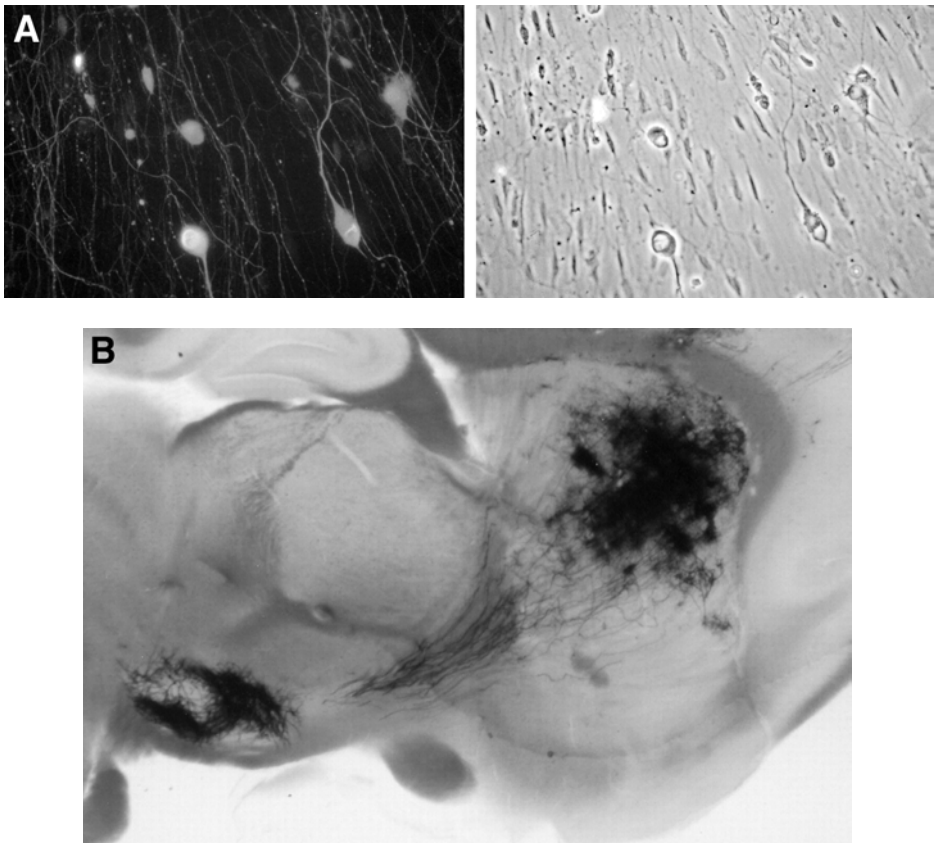

Fig. 3. Gene delivery to cultured neurons and the rat CNS in vivo. A) DRG neurons prepared from an adult rat were infected at an MOI of 10 with a *GFP*-containing virus, which does not express significant amount of any of the HSV IE genes. The primary cultures were photographed 1 wk postinfection under fluorescence and phase contrast microscopy. B) An adult rat was stereotaxically injected into the striatum with 2.5×10^5 PFU of a *lacZ*-containing virus which does not express significant amounts of any of the HSV IE genes. A 100-μm parasaggital section stained for *lacZ* expression is shown 1 wk postinjection.

vectors ***(29–32)***. Furthermore, these downstream elements (collectively known as LAT P2) can be used to confer long-term expression characteristics on heterologous promoters such as CMV ***(33)***. This suggests that the role of the LAT regions in facilitating long-term expression might primarily be a structural one, maintaining local areas of the genome in an "open" form and thus allowing continued access of transcription factors during latency.

2. Materials

1. Vero (ATCC CCL81), BHK (ATCC CCL10), or other cell line permissive for HSV-1 growth. Cell lines engineered to complement any essential gene functions which have been or are to be deleted from the virus ***(15–17,28)***. Selection agent(s) appropriate to the complementing cell line (*see* **Notes 1** and **2**).
2. Growth media (Dulbecco's modified Eagle's media [DMEM] containing 100 U/mL penicillin and 100 µg/mL streptomycin and supplemented with 10% fetal calf serum (FCS) and 5% tryptose phosphate broth) and serum-free growth media (DMEM only).
3. Standard tissue culture solutions and apparatus: 1X PBS (137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* Na_2HPO_4, 1.4 m*M* KH_2PO_4); dimethylsulphoxide [DMSO], herring sperm DNA (phenol/chloroform extracted); 96- and 6-well tissue culture plates; T25, T75, and T175 tissue culture flasks; 245 × 245 × 245 tissue culture plates; 2 *M* $CaCl_2$ (filter sterilized); disposable filters (0.45 and 0.2 µm); 20-mL and 50-mL disposable plastic syringes; 15-mL and 50-mL polypropylene tubes; trypsin; versene; Hank's balanced salt solution (HBSS).
4. X-gal stain: 5 m*M* $K_3Fe(CN)_6$, 5 m*M* $K_4Fe(CN)_6$, 1 m*M* $MgCl_2$, 150 µg/mL X-Gal [in DMSO] in 1X PBS.
5. Rotary roller apparatus and roller bottles (optional).
6. HEBES transfection buffer: 140 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 5.5 m*M* D-glucose, 20 m*M* HEPES. pH to exactly 7.05 with NaOH (the pH is critical) and filter sterilize through a 0.2-µm filter. Store at 4°C.
7. 1.6% carboxymethylcellulose (CMC) made up in water and autoclaved.
8. DNAzol solution (Helena Biosciences).
9. Hexamethylbisacetamide (HMBA). Used at a final concentration of 3 m*M* in the growth media of any virus with an inactivation in VP16 (HMBA transactivates the IE gene promoters in the absence of functional VP16).

3. Methods

3.1. Preparation of Infectious Viral DNA

1. Seed a T75 tissue culture flask with BHK or Vero cells (for viruses with only nonessential gene deletions) or the appropriate complementing cell line (if one or more essential genes have been deleted).
2. When the monolayer is approx 90% confluent, discard the growth media and add the virus in 10 mL of fresh growth media. The cells should be infected at an MOI of approx 1 (where MOI is defined as the amount of virus in PFU divided by the number of cells to be infected). Incubate the flask at 37°C/5%CO_2.
3. Allow the infection to proceed until almost every cell is rounded up but few are detached from the flask (this will occur approx 24–48 h postinfection, but the exact timing will depend on the level of disablement of the virus used).
4. Remove and discard the growth media.
5. Add 5 mL DNAzol solution and gently mix until all the cells have been lysed.

6. Transfer the lysed cells/DNAzol solution to a 50-mL polypropylene tube and gently layer on 0.5 vol of 100% ethanol.
7. Mix the two layers by gentle swirling until a DNA precipitate is clearly visible at the interface. Swirl and then gently invert the tube several times until the layers are completely mixed.
8. Use a cutoff 1000-µL pipet tip to transfer the precipitated DNA to a new 50-mL polypropylene tube containing approx 15 mL 70% ethanol.
9. Swirl the tube until the DNA precipitate has formed a tight ball.
10. Repeat this 70% ethanol wash twice more and then transfer the DNA into a microcentrifuge tube.
11. Pellet the DNA for 10 min at 3000*g*.
12. Carefully remove the excess 70% ethanol and air-dry the pellet for 10 min (*see* **Note 3**).
13. Add 500 µL of freshly prepared 8 m*M* NaOH and leave the DNA to resuspend on a blood wheel at 4°C for 1–2 h. Adjust the volume of 8 m*M* NaOH if required (the resuspended DNA should appear viscous, but be easy to pipet, sticking to the tip only slightly).
14. Neutralize by adding 115 µL 0.1 *M* HEPES for every 1 mL of solution and freeze the DNA at –20°C.

This stock of DNA is a mixture of cellular and infectious viral DNA and is of a suitable quality to be used for transfection to generate a recombinant virus (*see* **Subheading 3.2.**). This viral DNA can also be used for Southern blot analysis to confirm the presence or absence of specific sequences in the HSV-1 genome ***(34)***. A Southern blot to confirm predicted genome structure should be carried out each time a new virus is constructed.

3.2. Construction of Recombinant Virus

HSV-1 has a highly active recombinational machinery, allowing efficient recombination into the viral genome to occur following cotransfection of infectious viral DNA and a plasmid cassette containing regions of DNA homologous to sequences in the HSV-1 genome. The plasmid and viral DNA can be introduced into cells cheaply and efficiently using the calcium phosphate method of transfection ***(35)***.

1. Construct a “shuttle plasmid” containing a reporter gene such as *lacZ* or *GFP* between regions of HSV-1 DNA homologous to those flanking the desired insertion site in the viral genome. These flanking regions should ideally be 1–2 kb in length on either side of the reporter gene cassette.
2. Linearize the plasmid using a restriction site, which only cuts in the plasmid backbone and not the HSV-1 flanking regions or the reporter gene expression cassette.

3. Seed BHK or Vero cells (if the gene to be deleted is nonessential) or the appropriate complementing cell line (if one or more essential genes have been or are to be deleted) in a six-well dish. (*See* **Note 1**).
4. When the cells are 70–80% confluent, prepare two microcentrifuge tubes for each transfection to be carried out. Into tube A, aliquot 400 µL of HEBES transfection buffer. Into tube B, aliquot 31 µL 2 *M* $CaCl_2$, 0.5–1 µg plasmid DNA, 2 µg herring sperm DNA (phenol/chloroform extracted) and approximately 30 µL of the infectious viral DNA (the exact volume should be predetermined by performing a number of test transfections without plasmid DNA in order to establish the quantity of viral DNA which produces at least 100 plaques per well of a six-well plate by 3 d posttransfection).
5. Gently mix the contents of tube B and add them slowly (over about 30 s) to tube A whereas it is being continually vortexed on the slowest setting. Leave the mixture for 20–40 min at room temperature. The DNA should form a fine precipitate. If the precipitate is too thick (the solution appears viscous or large white flakes are visible) then too much viral DNA has been used, or the pH of the transfection buffer is wrong.
6. Remove the media from the cells and then slowly add the precipitated DNA mixture in a dropwise manner.
7. Incubate the cells for 20–40 min at 37°C.
8. Add 1 mL of growth media per well of the six-well plate and incubate the plate for 6–7 h at 37°C.
9. Remove the media/transfection mix and wash the cells twice with 2 mL of growth media. Add 1 mL of ice-cold 25% (v/v) DMSO in HEBES transfection buffer per well and leave on the cells for exactly 2 min. Remove the DMSO solution and immediately and quickly wash the cells twice with 3 mL per well of growth media.
10. Add a final 2 mL of growth media per well and incubate the cells at 37°C.
11. When a large number of individual plaques (for more disabled viruses) or complete CPE (for less disabled viruses) is seen (usually between 3 and 5 d posttransfection), transfer the six-well plate to –80°C.

If the transfection and recombination have been successful, this harvested stock will contain a mixture of recombinant and nonrecombinant virus. The next step is, therefore, to isolate the recombinant plaques away from the nonrecombinant background.

3.3. Plaque Purification of Recombinant Virus

1. Seed BHK or Vero cells or the appropriate complementing cell in a six-well dish.
2. When the cells are approx 80% confluent, defrost the harvested transfection mix (the process of freeze-thawing releases the virus from the cells).

3. Prepare a series of 10-fold dilutions (in 1 mL serum free media) of the harvested transfection (100 µL transfection in 900 µL serum free media is an appropriate starting point for less disabled viruses, this may have to be increased to up to 700 µL of harvested transfection for a very disabled virus).
4. Remove the growth media from the cells and replace with 500 µL of the appropriate serial dilution to each well of the six-well plate and leave the virus to adsorb for 1 h at 37°C.
5. Remove the virus inoculum and add 2 mL of growth media : CMC mix (prepared in a 2 : 1 ratio).
6. Leave the infection to proceed for 2 d at 37°C.
7. Stain the plaques by removing growth media : CMC, washing twice in 1X PBS and adding 2 mL X-gal stain per well (if *lacZ* is being used as a selectable marker) or directly observe under a fluorescence microscope (if *GFP* is being used as a selectable marker).
8. Identify a well-isolated recombinant plaque (*see* **Note 4**) and pick it by removing a few cells in as small a volume as possible (usually about 2 µL) using a sterile narrow pipet tip (suitable for a 10-µL micropipet). Transfer the picked plaque into 100 µL of serum-free media in a microcentrifuge tube and immediately freeze in liquid nitrogen.
9. Defrost the picked plaque and prepare a series of 10-fold dilutions of the 100-µL starting volume (down to 10^{-4} is usually sufficient). Use these to infect 80% confluent cells in a six-well plate as before.
10. Repeat this 2-d cycle of picking plaques and infecting six-well plates until all the plaques are recombinants (the population is pure). Always pick well-isolated plaques from the lowest possible dilution. This purification usually takes approx 3–6 rounds depending on the level of disablement of the virus.

In order to insert the gene of interest, the same procedure is carried out in reverse. Prepare DNA from the virus carrying the reporter gene (*GFP* or *lacZ*) and cotransfect this with a shuttle plasmid containing the gene of interest cloned in place of the reporter gene. This time, recombinant plaques are identified as "white" plaques and purified away from a background of "colored" nonrecombinant plaques. Alternatively, cassettes containing both a marker gene and the gene of interest can be constructed (as in Palmer et al., 2000), enabling the direct generation of vectors expressing the desired gene.

3.4. Producing a Stock of Recombinant Virus

In order to carry out any useful in vitro or in vivo experiments, a concentrated stock of the recombinant virus without contaminating cell debris must be generated.

1. When the virus is pure, use one plaque to infect one well of a six-well plate. Leave the virus to adsorb for 1 h at 37°C, remove the virus inoculum and replace

with 2 mL growth media (no CMC). Harvest the resulting stock (either by scraping the cells off into the media and transferring to a polypropylene tube or by transferring the plate directly to –80°C) when CPE is observed (typically, 2–3 d postinfection). This is the virus master stock.

2. Prepare a series of tenfold dilutions of the virus master stock in 100 µL of serum free media (dilutions ranging from 10^{-2} to 10^{-7} are usually appropriate at this stage).
3. Remove the growth media from an 80% confluent six-well plate of the appropriate cell line and add a minimal volume of serum free media (approx 500 µL/well).
4. Add 100 µL of the appropriate serial dilution of the virus master stock to each well of the six-well plate, leave the virus to adsorb for 1 h at 37°C and replace the virus inoculum with growth media : CMC (prepared in a 2 : 1 ratio).
5. Two days postinfection, determine the titer of the virus master stock (*see* **Note 7**) by counting the number of plaques in an appropriate well of the six-well plate (for example, if there are three plaques in the 10^{-7} well, then this equates to 0.0001 µL of virus master stock containing three plaque forming units (PFU) and therefore the titer of the stock is 3×10^7 PFU/mL).
6. Use the virus master stock to infect a T75 then a T175 tissue culture flask, growing up a working stock of the virus which can be used for large-scale infections. When growing such a stock, each infection should be carried out at an MOI of 0.01.
7. Calculate the titer of the working stock and freeze it in aliquots in liquid nitrogen (*see* **Notes 5** and **7**).
8. Seed 10×850 cm^2 roller bottles or 10×245 mm^2 large tissue culture plates with BHKs, Veros or the appropriate complementing cell line in 100 mL/roller bottle or 50 mL/large plate growth media. If roller bottles are used, they must be gassed with 5% CO_2 in air.
9. When the cells are 80–90% confluent, remove the old growth media and replace with fresh (100 mL/roller bottle or 50 mL/large plate). Add 5×10^6 PFU of virus/roller bottle or 1×10^6 PFU of virus/large plate. This is an MOI of approx 0.01.
10. Incubate the roller bottles or plates at 37°C until complete CPE is seen (all the cells are rounded up but few are detached from the monolayer). This should take between 2 and 4 d, but depends on the level of disablement of the virus (*see* **Note 6**).
11. If the stock has been grown in large plates, transfer the plates directly to –80°C (this ensures all the cells are harvested). If the stock has been grown in roller bottles, harvest the cells by sharply shaking the roller bottles once or twice and transferring the contents to 50-mL polypropylene tubes. Freeze at –80°C.
12. Defrost the harvested virus and cells and pellet the cells at 3000*g* for 45 min at 4°C. Discard the cell pellet.
13. Filter the supernatant through a 0.45-µm filter (*see* **Note 8**) and then decant into sterile centrifuge pots and centrifuge at 35,000*g* for 2 h to pellet the virus.

14. Discard the supernatant and gently resuspend the pellets in serum free media (the volume depends on the level of disablement of the virus used but typically ranges from approx 500 µL for a very disabled virus to 2 mL for a less-disabled virus).
15. Determine the titer of the virus (*see* **Note 7**), aliquot into cryovials and store in liquid nitrogen.

We have found that this relatively simple purification procedure yields virus of a suitable quality for most laboratory applications. However, the virus can be further purified by spinning through a Ficoll gradient if required (*see* **ref. *36*** for a detailed method).

4. Notes

1. A complementing cell line will be routinely maintained on selective agents such as G418, puromycin, hygromycin, or zeocin. These agents should be omitted from the growth media of cells which are to be infected with virus as there is some evidence to suggest that they inhibit virus entry ***(37)***.
2. If zeocin is used to maintain complementing cell lines, the cells should be routinely split to 25% confluence (as zeocin is only active as a selection agent against untransfected cells at this low confluence).
3. When preparing viral DNA it is important not to over-dry the pellet (for example, by using a vacuum drier) as this will result in a pellet which cannot be resuspended in the 8 m*M* NaOH. After the final centrifugation step, the DNA often appears as a viscous mass rather than a discrete pellet in the bottom of the microcentrifuge tube. Take care not to go too close to the DNA when attempting to remove the excess 70% ethanol. It is better to leave some 70% ethanol behind than risk losing the viral DNA.
4. When plaque purifying a virus it is important to always pick a well-isolated plaque. It is often worth identifying the lowest dilution of the transfection mix, which yields a recombinant plaque and then plating out approx five six-well plates at one log dilution lower than this. This should give one very well-isolated recombinant plaque in every 10 wells examined. This is particularly worthwhile in instances when the nonrecombinant virus has a growth advantage over the recombinant virus (a common situation when recombination results in a viral gene being deleted).
5. In order to avoid excessive serial passage of viruses, it is worth going back to the original master stock or first working stock each time a new high-titer stock is to be grown up.
6. The time of harvest is very important when growing a high-titer stock of the recombinant virus—the media should be orange, but not yellow, and all cells should be rounded up but only a small percentage should have detached from the monolayer.
7. Always read viral titers at the same time post-infection (usually 48 h). This is essential for consistency between experiments. Reading titers later than 48 h

postinfection leads to falsely high titers owing to the formation of secondary plaques. For the same reason, growth media:CMC should always be used to overlay titers (thus limiting the spread of virus within a well).

8. Filtering large volumes of virus by hand is tiring. Prefiltering the virus through a 5-µm filter (prior to the 0.45-µm filtration step) can ease this process. If large volumes of virus are to be filtered routinely appropriate apparatus such as the Proflux M12 tangential flow filtration system (Millipore) can be used.

References

1. Kwong, A. D. and Frenkel, N. (1987) Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. *Proc. Natl. Acad. Sci. USA* **84,** 1926–1930.
2. Honess, R. W. and Roizman, B. (1974) Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* **14,** 8–19.
3. York, I. A., Roop, C., Andrews, D. W., Riddell, S. R., Graham, F. L., and Johnson, D. C. (1994) A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes. *Cell* **77,** 525–535.
4. Jacob, R. J., Morse, L. S., and Roizman, B. (1979) Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J. Virol.* **29,** 448–457.
5. Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L., and Feldman, L. T. (1987) RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons. *Science* **235,** 1056–1059.
6. Zwaagstra, J. C., Ghiasi, H., Slanina, S. M., Nesburn, A. B., Wheatley, S. C., Lillycrop, K., et al. (1990) Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: evidence for neuron specificity and for a large LAT transcript. *J. Virol.* **64,** 5019–5028.
7. Batchelor, A. H. and O'Hare, P. (1990) Regulation and cell-type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. *J. Virol.* **64,** 3269–3279.
8. Goins, W. F., Sternberg, L. R., Croen, K. D., Krause, P. R., Hendricks, R. L., Fink, D. J., et al. (1994) A novel latency-active promoter is contained within the herpes simplex virus type 1 UL flanking repeats. *J. Virol.* **68,** 2239–2252.
9. Spaete, R. R. and Frenkel, N. (1982) The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30,** 295–304.
10. Fraefel, C., Song, S., Lim, F., Lang, P., Yu, L., Wang, Y., et al. (1996) Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. *J. Virol.* **70,** 7190–7197.
11. Kaplitt, M. G., Kwong, A. D., Kleopoulos, S. P., Mobbs, C. V., Rabkin, S. D., and Pfaff, D. W. (1994) Preproenkephalin promoter yields region-specific and long-term expression in adult brain after direct in vivo gene transfer via a defective herpes simplex viral vector. *Proc. Natl. Acad. Sci. USA* **91,** 8979–8983.

12. DeLuca, N. A., McCarthy, A. M., and Schaffer, P. A. (1985) Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J. Virol.* **56,** 558–570.
13. Johnson, P. A., Miyanohara, A., Levine, F., Cahill, T., and Friedmann, T. (1992) Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J. Virol.* **66,** 2952–2965.
14. Johnson, P. A., Wang, M. J., and Friedmann, T. (1994) Improved cell survival by the reduction of immediate-early gene expression in replication-defective mutants of herpes simplex virus type 1 but not by mutation of the virion host shutoff function. *J. Virol.* **68,** 6347–6362.
15. Krisky, D. M., Wolfe, D., Goins, W. F., Marconi, P. C., Ramakrishnan, R., Mata, M., et al. (1998) Deletion of multiple immediate-early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Ther.* **5,** 1593–1603.
16. Samaniego, L. A., Wu, N., and DeLuca, N. A. (1997) The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. *J. Virol.* **71,** 4614–4625.
17. Samaniego, L. A., Neiderhiser, L., DeLuca, N. A. (1998) Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. *J. Virol.* **72,** 3307–3320.
18. Wu, N., Watkins, S. C., Schaffer, P. A., and DeLuca, N. A. (1996) Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding ICP4, ICP27, and ICP22. *J. Virol.* **70,** 6358–6369.
19. Howard, M. K., Kershaw, T., Gibb, B., Storey, N., MacLean, A. R., Zeng, B. Y., et al. (1998) High efficiency gene transfer to the central nervous system of rodents and primates using herpes virus vectors lacking functional ICP27 and ICP34.5. *Gene Ther.* **5,** 1137–1147.
20. Samaniego, L. A., Webb, A. L., and DeLuca, N. A. (1995) Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. *J. Virol.* **69,** 5705–5715.
21. Batterson, W. and Roizman, B. (1983) Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. *J. Virol.* **46,** 371–377.
22. Ace, C. I., Dalrymple, M. A., Ramsay, F. H., Preston, V. G., and Preston, C. M. (1988) Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. *J. Gen. Virol.* **69(Pt 10),** 2595–2605.
23. Ace, C. I., McKee, T. A., Ryan, J. M., Cameron, J. M., and Preston, C. M. (1989) Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *J. Virol.* **63,** 2260–2269.
24. Mossman, K. L. and Smiley, J. R. (1999) Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 renders expression of the immediate-early genes almost entirely dependent on ICP0. *J. Virol.* **73,** 9726–9733.

25. Smiley, J. R. and Duncan, J. (1997) Truncation of the C-terminal acidic transcriptional activation domain of herpes simplex virus VP16 produces a phenotype similar to that of the in1814 linker insertion mutation. *J. Virol.* **71,** 6191–6193.
26. Preston, C. M., Mabbs, R., and Nicholl, M. J. (1997) Construction and characterization of herpes simplex virus type 1 mutants with conditional defects in immediate early gene expression. *Virology* **229,** 228–239.
27. Lilley, C. E., Groutsi, F., Han, Z., Palmer, J. A., Anderson, P. N., Latchman, D. S., et al. (2001) Multiple immediate early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in culture and widespread gene delivery to the central nervous system in vivo. *J. Virol.* **75,** 4343–4356.
28. Thomas, S. K., Lilley, C. E., Latchman, D. S., and Coffin, R. S. (1999) Equine herpesvirus 1 gene 12 can substitute for vmw65 in the growth of herpes simplex virus (HSV) type 1, allowing the generation of optimized cell lines for the propagation of HSV vectors with multiple immediate- early gene defects. *J. Virol.* **73,** 7399–7409.
29. Berthomme H., Lokensgard J., Yang L., Margolis T., and Feldman L.T. (2000) Evidence for a bidirectional element located downstream from the herpes simplex virus type 1 latency-associated promoter that increases its activity during latency. *J. Virol.* **74,** 3613–3622.
30. Lachmann, R. H. and Efstathiou, S. (1997) Utilization of the herpes simplex virus type 1 latency-associated regulatory region to drive stable reporter gene expression in the nervous system. *J. Virol.* **71,** 3197–3207.
31. Lokensgard, J. R., Berthomme, H., and Feldman, L. T. (1997) The latency-associated promoter of herpes simplex virus type 1 requires a region downstream of the transcription start site for long-term expression during latency. *J. Virol.* **71,** 6714–6719.
32. Marshall, K. R., Lachmann, R. H., Efstathiou, S., Rinaldi, A., and Preston, C. M. (2000) Long-term transgene expression in mice infected with a herpes simplex virus type 1 mutant severely impaired for immediate-early gene expression. *J. Virol.* **74,** 956–964.
33. Palmer, J. A., Branston, R. H., Lilley, C. E., et al. (2000) Development and optimization of herpes simplex virus vectors for multiple long-term gene delivery to the peripheral nervous system. *J. Virol.* **74,** 5604–5618.
34. Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–517.
35. Stow, N. D. and Wilkie, N. M. (1976) An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. *J. Gen. Virol.* **33,** 447–458.
36. Matis, J., Lesso, J., Mucha, V., and Matisova, E. (1975) Purification and separation of enveloped and unenveloped herpes simplex virus particles. *Acta Virol.* **19,** 273–280.
37. Langeland, N., Oyan, A. M., Marsden, H. S., Cross, A., Glorioso, J. C., Moore, L. J., et al. (1990) Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. *J. Virol.* **64,** 1271–1277.