JOURNAL OF VIROLOGY, Oct. 1996, p. 7190–7197 Vol. 70, No. 10
0022-538X/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Helper Virus-Free Transfer of Herpes Simplex Virus Type 1 Plasmid Vectors into Neural Cells

CORNEL FRAEFEL,[1]* SONG SONG,[1] FILIP LIM,[1]† PHUNG LANG,[1] LINDA YU,[1] YAMING WANG,[1] PETER WILD,[2] AND ALFRED I. GELLER[1,3]

Division of Endocrinology, Children's Hospital,[1] and Program in Neuroscience,[3] *Harvard Medical School, Boston, Massachusetts 02115, and Institute for Anatomy, University of Zurich, CH-8057 Zurich, Switzerland*[2]

Received 20 March 1996/Accepted 14 June 1996

Herpes simplex virus type 1 (HSV-1) plasmid vectors have promise for genetic intervention in the brain, but several problems caused by the helper virus have compromised their utility. To develop a helper virus-free packaging system for these vectors, the DNA cleavage/packaging signals were deleted from a set of cosmids that represents the HSV-1 genome. Following cotransfection into cells, this modified cosmid set supported replication and packaging of vector DNA. However, in the absence of the DNA cleavage/packaging signals, the HSV-1 genome was not packaged, and consequently vector stocks were free of detectable helper virus. In the absence of helper virus, the vectors efficiently infected rat neural cells in culture or in the brain with minimal cytopathic effects. β-Galactosidase-positive cells were observed for at least 1 month in vivo, and vector DNA persisted for this period. This system may facilitate studies on neuronal physiology and potential therapeutic applications.

Genetic intervention in the brain has potential for studying neuronal physiology and for treating neurological diseases. Consequently, vector systems based on adenovirus, adeno-associated virus, and herpes simplex virus type 1 (HSV-1) are being evaluated for gene transfer into neural cells. Each system has limitations. Adenovirus vectors can support limited long-term (2 months) gene expression, but they appear to be gradually lost from neural cells, and moreover, they can cause both cytopathic effects and an immune response (23, 43). Adeno-associated virus vectors cause minimal cytopathic effects and support at least some gene expression for up to 4 months, but gene transfer is inefficient and these vectors can accept only ~5 kb of foreign DNA (13, 22). Vectors based on HSV-1 are attractive for gene transfer into the brain because HSV-1 can efficiently infect neural cells and can persist indefinitely in neurons (38). Both recombinant HSV-1 vectors and HSV-1 plasmid vectors (amplicons) have been used to alter neuronal physiology (5, 8, 18, 41). HSV-1 plasmid vectors contain only ~1% of the 150-kb HSV-1 genome and have been packaged into HSV-1 particles by using a helper virus (11, 36), often a replication-defective mutant of HSV-1 (12, 24). However, the effectiveness of this vector system has been limited by several problems: (i) acute cytopathic effects and an immune response, largely due to gene expression from the helper virus (16, 19, 20, 42); (ii) potential interactions between helper virus and endogenous viruses; (iii) instability of gene expression (5, 18); (iv) potential helper virus-mediated oncogenesis (in contrast, wild-type [wt] HSV-1 is not known to cause tumors); and (v) reversion of the helper virus to wt HSV-1.

To essentially eliminate many of these problems, we developed a helper virus-free packaging system. The DNA cleavage/packaging signals were deleted from a set of cosmids that represents the HSV-1 genome. Cotransfection of cells with this modified cosmid set and vector DNA resulted in the production of vector stocks that are free of detectable helper virus. Vectors which contained the *Escherichia coli lacZ* reporter gene efficiently transduced neural cells with minimal cytopathic effects. Following injection into the rat brain, β-galactosidase-positive cells were observed for at least 1 month, and vector DNA persisted for this period.

MATERIALS AND METHODS

Cosmid mutagenesis. The *a* sequences were deleted by selective digestion at two *Hin*P1I sites which flank the *a* sequences (cos6, nucleotides 783 and 151436; cos48, nucleotides 125585 and 126793) (28), using the RecA-assisted restriction endonuclease cleavage technique (9). Similar methods have been used to delete the large subunit of ribonucleotide reductase from a cosmid set representing the varicella-zoster virus genome (14). Briefly, two oligonucleotides were used to form a region of triple-stranded DNA, resistant to methylation by *Hha*I methylase, at each of the two *Hin*P1I sites. One oligonucleotide is complementary to nucleotides 753 to 812 (cos6) and 125559 to 125618 (cos48) (5′GGCGGCGGC GGTGGGCCGGGCCTCTGGCGCCGACTCGGGCGGGGGCTGTCCGG CCAGTC3′), and the other oligonucleotide is complementary to nucleotides 151409 to 151468 (cos6) and 126764 to 126823 (cos48) (5′CTCAGGTCAGAG ATCCAAACCCTCCGGGGGCGCCCGCGCACCACCACCGCCCCTCGCC CC3′). Ninety nanograms of each oligonucleotide, 1 μg of cosmid DNA, and 10 μg of *E. coli* RecA protein (New England Biolabs) were incubated together for 10 min at 37°C. Ten units of *Hha*I methylase (New England Biolabs) and *S*-adenosylmethionine (120 μM, final concentration) were added, and the reaction mixture was incubated for 50 min at 37°C. After heat treatment (65°C, 10 min) to inactivate the *Hha*I methylase and to dissociate the RecA-DNA complexes, the DNA was digested with *Hin*P1I. The large DNA fragment was isolated by agarose gel electrophoresis, ligated by using T4 DNA ligase, and packaged into bacteriophage lambda particles (Gigapack II Plus; Stratagene). Cosmids were propagated in *E. coli* XL1Blue MR (Stratagene), and cosmids lacking the *a* sequence (cos6Δ*a* and cos48Δ*a*) were identified by restriction endonuclease analysis. This deletion is larger than the HSV-1 fragment in pHSVlac, which contains the DNA cleavage/packaging signal (nucleotides 125770 to 126774).

Helper virus-free packaging of HSV-1 plasmid vectors (pHSVlac/helper-free and pIE1βgalori/helper-free). Cosmids were digested with *Pac*I to excise the HSV-1 inserts and purified by phenol extraction. 2-2 cells (33) were grown in Dulbecco's modified minimal essential medium containing 10% fetal bovine serum and plated at a density of 4×10^5/60-mm-diameter tissue culture dish. The following day, the cells were transfected by the lipofectAMINE procedure (GIBCO) as follows. Four-tenths microgram of either pHSVlac DNA or pIE1βgalori DNA and 0.4 μg of each cosmid DNA were diluted in 100 μl of OPTI-MEM I, mixed with 100 μl of OPTI-MEM I containing 12 μl of lipo-

* Corresponding author. Mailing address: Division of Endocrinology, EN 406, Children's Hospital, 300 Longwood Ave., Boston, MA 02115. Phone: (617) 355-7017. Fax: (617) 730-0741. Electronic mail address: fraefel@a1.tch.harvard.edu.

† Present address: Centro Biología Molecular, Facultad de Ciencias, Universidad Autónoma de Madrid, E-28049 Cantoblanco, Spain.

fectAMINE, and incubated at room temperature for 45 min before addition of 0.8 ml of OPTI-MEM I. The cells were washed once with OPTI-MEM I, the DNA-lipofectAMINE solution was added, and the cells were incubated at 37°C for 5.5 h. After the cells were washed three times with OPTI-MEM I, 3 ml of Dulbecco's modified minimal essential medium containing 2% fetal bovine serum was added, and the cells were incubated at 37°C for 2.5 days. To harvest vector particles, the cells were scraped into the medium, the suspension was frozen and thawed three times and sonicated, and cell debris was removed by centrifugation (10 min, 1,400 × *g*). 2-2 cells were used because they transfect at a high efficiency; however, this procedure has been successfully performed with either Vero cells or BHK-21 cells, although the titer of pHSVlac was lower. For use in gene transfer experiments, vector stocks were purified and concentrated as previously described (24).

Packaging of HSV-1 plasmid vectors by using a helper virus (pHSVlac/helper). pHSVlac was packaged by established procedures (12, 24), using the immediate-early 2 gene (*IE2*) deletion mutant HSV-1 KOS 5*dl*1.2 (27) and 2-2 cells (33). This helper virus caused cytopathic effects similar to those caused by *IE3* deletion mutants, and the reversion frequency to wt HSV-1 was $<10^{-7}$ (24).

Analysis of vector stocks. (i) Electron microscopy. The vector particles were adsorbed onto carbon-coated Parlodion films, mounted on 300-mesh copper grids, negatively stained for 30 s with 2% sodium phosphotungstic acid (pH 6.6), air dried, and immediately examined at 100 kV in a Philips CM12 electron microscope.

(ii) Immunocytochemistry. One day after infection, Vero cells were fixed with 4% paraformaldehyde and then incubated overnight with a mouse anti-ICP0 antibody (1:1,000 dilution of ascites fluid) (7). Immunoreactivity was visualized by using an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibody (1:2,000 dilution; Boehringer Mannheim) and the 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium substrate (Sigma).

(iii) Southern analysis. DNA isolated from pHSVlac/helper-free particles was either not digested or digested with *Hin*dIII, displayed on a 0.4% agarose gel, and transferred to a nylon membrane (35). To prepare the probe, pHSVlac was digested with *Hin*dIII and *Eco*RI, and the 3.3-kb fragment containing the *lacZ* gene was labeled by random priming with digoxigenin-11-dUTP (DIG DNA Labeling and Detection kit; Boehringer Mannheim). Hybridization and immunological detection were performed as instructed by the manufacturer. To compare the relative intensities of the individual bands, the blot was scanned with an Ultrascan XL laser densitometer (Pharmacia, LKB), and the data were processed on a personal computer with Gel Scan XI software.

Stereotaxic injection into the adult rat brain. Virus stocks were delivered by stereotaxic injection (3 μl per site over 10 min) into the right midbrain (MB) (AP 3.5, ML 4.0, DV 6.8; 20° angle for needle toward midline) and the left striatum (ST) (AP 0.5, ML 3.0, DV 6.0) of male Sprague-Dawley rats (100 to 125 g). The coordinates indicate millimeters relative to bregma and bregma-lambda (29). Four days or 1 month later, the rats were anesthetized with chloral hydrate (400 mg/kg intraperitoneally) and transcardially perfused with 50 ml of phosphate-buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde in PBS. The brains were cryoprotected, and 30-μm coronal sections were cut with a freezing microtome and stored in PBS at 4°C. Sections were stained with either cresyl violet or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (6), and cell counts were performed at a magnification of ×30.

PCR analysis of vector DNA. DNA was extracted from brain sections (15) adjacent to those used for staining (0.5 mg of tissue per μl), and aliquots were subjected to nested PCR using primers (5 pmol) derived from the *E. coli lacZ* gene (21). Conditions for the first reaction were as follows: 10 ng of DNA in 100 μl; primers, nucleotides 1802-1826 (5′TCTGTATCAACGGTCTGGTCTTGC 3′) and complementary to nucleotides 2882-2905 (5′CATCAGTTGCTGTTGA CTGTAGC3′); 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 4 min. Conditions for the second reaction were as follows: 2 μl of the first reaction mixture in 100 μl; primers, nucleotides 2034 to 2057 (5′GTTGATTGAACTGC CTGAACTACC3′) and complementary to nucleotides 2594 to 2616 (5′CACT TCAACATCAACGGTAATCG3′); 40 cycles of 94°C for 1 min; 55°C for 1 min, and 72°C for 4 min. The reaction products were electrophoresed on a 1.2% agarose gel.

RESULTS

Deletion of the DNA cleavage/packaging signals from a HSV-1 cosmid set. To facilitate manipulation of the large genomes of herpesviruses, sets of cosmids which contain overlapping clones that represent the entire genomes of pseudorabies virus (40), varicella-zoster virus (2), Epstein-Barr virus (39), or HSV-1 (3) have been isolated. Infectious virus can be generated by transfecting permissive cells with the clones of a cosmid set (cosmid backbones excised); a virus genome is reconstructed via homologous recombination between the overlapping sequences in the different clones. We developed a helper virus-free packaging system by modifying a set of five cosmids that represents the HSV-1 genome (cosmid set C) (3).

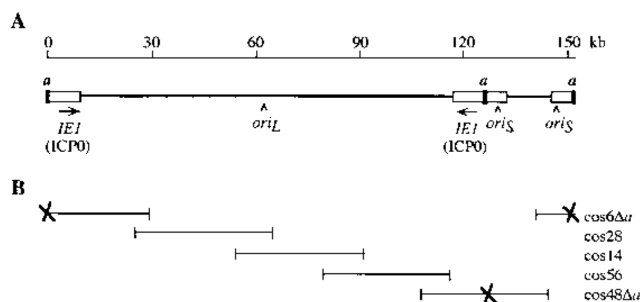


FIG. 1. Development of a helper virus-free packaging system for HSV-1 plasmid vectors. (A) The HSV-1 genome (~150 kb) is composed of unique long and unique short segments (horizontal lines) which are both flanked by inverted repeats (open rectangles). The *IE1* gene, ori_S, and ori_L are shown. The *a* sequences (solid rectangles), which contain the DNA cleavage/packaging sites, are located at the junction between the long and short segments and at both termini. (B) Schematic diagram of the HSV-1 clones from cosmid set C6Δ*a*48Δ*a* (cos6Δ*a*, cos28, cos14, cos56, and cos48Δ*a*). The deleted *a* sequences in cos6Δ*a* and cos48Δ*a* are indicated (X).

To inhibit packaging of the HSV-1 genome, the *a* sequence, which contains the DNA cleavage/packaging signal, was deleted from two cosmids (cos6 and cos48) as described in Materials and Methods. This modified cosmid set (C6Δ*a*48Δ*a* [Fig. 1]) was then tested for its capability both to support the replication and packaging of cotransfected vector DNA and to generate infectious HSV-1.

Cosmid set C6Δ*a*48Δ*a* directs production of vector stocks that are free of detectable helper virus. pHSVlac, the plasmid vector used in this experiment, contains the HSV-1 origin of DNA replication (ori_S) and an *a* sequence to support packaging, and it expresses the *E. coli lacZ* gene from the HSV-1 *IE4/5* promoter (11). Following cotransfection of cells with a mixture of cosmid set C6Δ*a*48Δ*a* and pHSVlac, no detectable helper virus was generated, as determined by standard plaque assays, but the cosmids provided all *trans*-acting functions required for packaging of pHSVlac into HSV-1 particles (pHSVlac/helper-free) (Table 1). Examination of pHSVlac/helper-free particles by electron microscopy revealed nucleocapsids (Fig. 2) with a structure comparable to that of wt HSV-1 (32). Transfections performed with only cosmid set C6Δ*a*48Δ*a* or only pHSVlac produced neither packaged vector nor helper virus, whereas transfections performed with pHSVlac and a cosmid set containing either only one *a* sequence or two *a* sequences (unmodified cosmid set C) supported the packaging of both the vector and the HSV-1 genome (Table 1).

Because of the limited sequence homology between pHSVlac and both cos6Δ*a* and cos48Δ*a* (1.05 kb containing the ori_S and the *IE4/5* promoter), homologous recombination between the vector and either cos6Δ*a* or cos48Δ*a* could produce a helper virus. In addition to the plaque assay (Table 1), we performed immunocytochemistry using an ICP0 antibody to determine whether any recombinants between pHSVlac and either cosmid were packaged. ICP0 is encoded by the *IE1* gene present in both cos6Δ*a* and cos48Δ*a* (Fig. 1) and is absent from HSV-1 particles. ICP0 immunoreactivity was not detected in cells infected with pHSVlac/helper-free but was observed in the nuclei of cells infected with pHSVlac packaged by using a replication-defective helper virus (pHSVlac/helper) (Table 1). To further screen for putative recombinants and to determine whether pHSVlac was properly packaged into HSV-1 particles, we performed Southern analysis using a probe homologous to the *lacZ* gene (Fig. 3). DNA isolated from pHSVlac/helper-

TABLE 1. Properties of pHSVlac stocks obtained by using different packaging protocols

Packaging condition[a]	Vector[b] (IVP/ml)	Helper virus[c] (PFU/ml)	Vector/helper (IVP/PFU)	ICP0 immunoreactivity[d] (positive cells/10^5 IVP)
pHSVlac;cos 6Δ*a*, 28, 14, 56, 48Δ*a*				
Expt 1	4.0×10^5	<1	>4.0×10^5	<1
Expt 2	2.0×10^5	<1	>2.0×10^5	<1
Expt 3	1.5×10^5	<1	>1.5×10^5	<1
;cos 6Δ*a*, 28, 14, 56, 48Δ*a*	<1	<1		ND
pHSVlac;	<1	<1		ND
pHSVlac;cos 6Δ*a*, 28, 14, 56, 48	5.2×10^5	4.0×10^1	1.3×10^4	ND
pHSVlac;cos 6 , 28, 14, 56, 48Δ*a*	7.0×10^5	1.4×10^2	5.0×10^3	ND
pHSVlac;cos 6 , 28, 14, 56, 48	7.0×10^5	1.6×10^3	4.4×10^2	ND
pHSVlac;HSV-1 5*dl*1.2 helper virus	5.9×10^6	6.0×10^6	0.98	1.4×10^4[e]

[a] pHSVlac was packaged by using either the helper virus-free system or a helper virus, KOS 5*dl*1.2.
[b] Titers of pHSVlac were determined by using BHK-21 cells and cytochemical staining with X-Gal.
[c] 2-2 cells were inoculated with 1 ml of vector stock, and titers of helper virus were determined by standard plaque assays.
[d] The *IE1* gene product was detected by using an anti-ICP0 antibody. ND, not done.
[e] The sensitivity of the ICP0 antibody was ~14% (1.4×10^4 ICP0 immunoreactive cells/9.8×10^4 PFU of HSV-1 KOS 5*dl*1.2).

free particles yielded a band at ~150 kb, the size of the HSV-1 genome; this band was faint because such large fragments transfer inefficiently. Digestion with *Hin*dIII produced a 8.1-kb band, the size of linearized pHSVlac DNA isolated from *E. coli*, and a minor 4.5-kb band, a fragment expected from the copy of pHSVlac located at one of the termini of the vector genome. On the basis of the size of pHSVlac, we expected that the genome of pHSVlac/helper-free would contain 19 copies of pHSVlac and that digestion with *Hin*dIII would yield the 8.1-kb band and the 4.5-kb band in a molar ratio of 18:1. Consistent with this expectation, quantitative densitometry revealed a ~15-fold-higher intensity of the 8.1-kb band. The very faint band visible at slightly above 8.1 kb in the undigested lane may be unpackaged pHSVlac DNA that copurified with the vector particles. Additional bands representing recombinants between pHSVlac and cosmid set C6Δ*a*48Δ*a* were not detected.

In the absence of helper virus, HSV-1 plasmid vectors can efficiently infect neural cells in vitro or in vivo. For gene transfer into either cultured cortical cells or the rat brain, vector stocks were purified and concentrated to achieve a titer of ~10^7 infectious vector particles (IVP) per ml. First, cultured rat cerebral cortical cells (1) were infected with pHSVlac/helper-free or pHSVlac/helper and stained with X-Gal (6) after 2, 4, or 8 days. Cultures infected with pHSVlac/helper displayed a gradual decline in the number of β-galactosidase-positive cells (Fig. 4A, B, and G) during this time period. In contrast, following infection with pHSVlac/helper-free, the number of β-galactosidase-positive cells remained relatively constant (Fig. 4C, D, and G), and those cells displayed either neuronal or glial morphology (Fig. 4E and F). Next, equal amounts (9×10^3 IVP) of either pHSVlac/helper or pHSVlac/

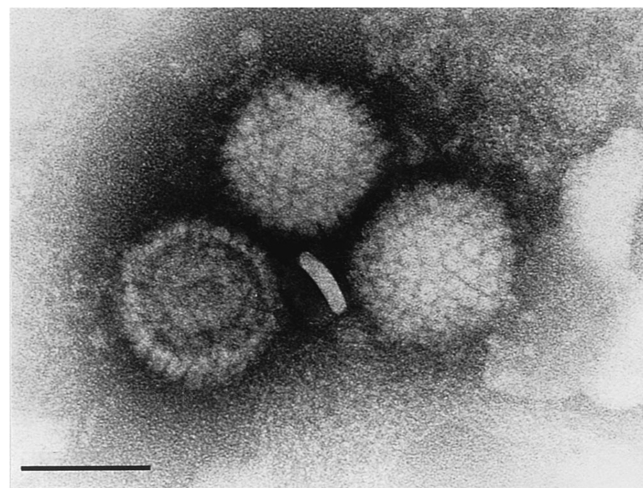


FIG. 2. Electron micrograph showing nucleocapsids of pHSVlac/helper-free. Scale bar, 100 nm.

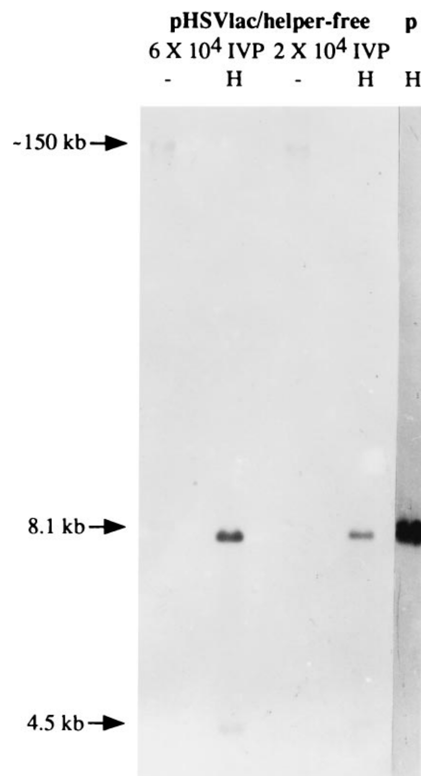


FIG. 3. Southern analysis of the structure of pHSVlac DNA. DNA isolated from 6×10^4 or 2×10^4 IVP of pHSVlac/helper-free was either not digested (−) or digested with *Hin*dIII (H), resolved on a 0.4% agarose gel, transferred to a nylon membrane, and hybridized with a probe homologous to the *lacZ* gene. Control lane (p), pHSVlac DNA isolated from *E. coli* and linearized by digestion with *Hin*dIII (H). The sizes of the bands are indicated at the left.

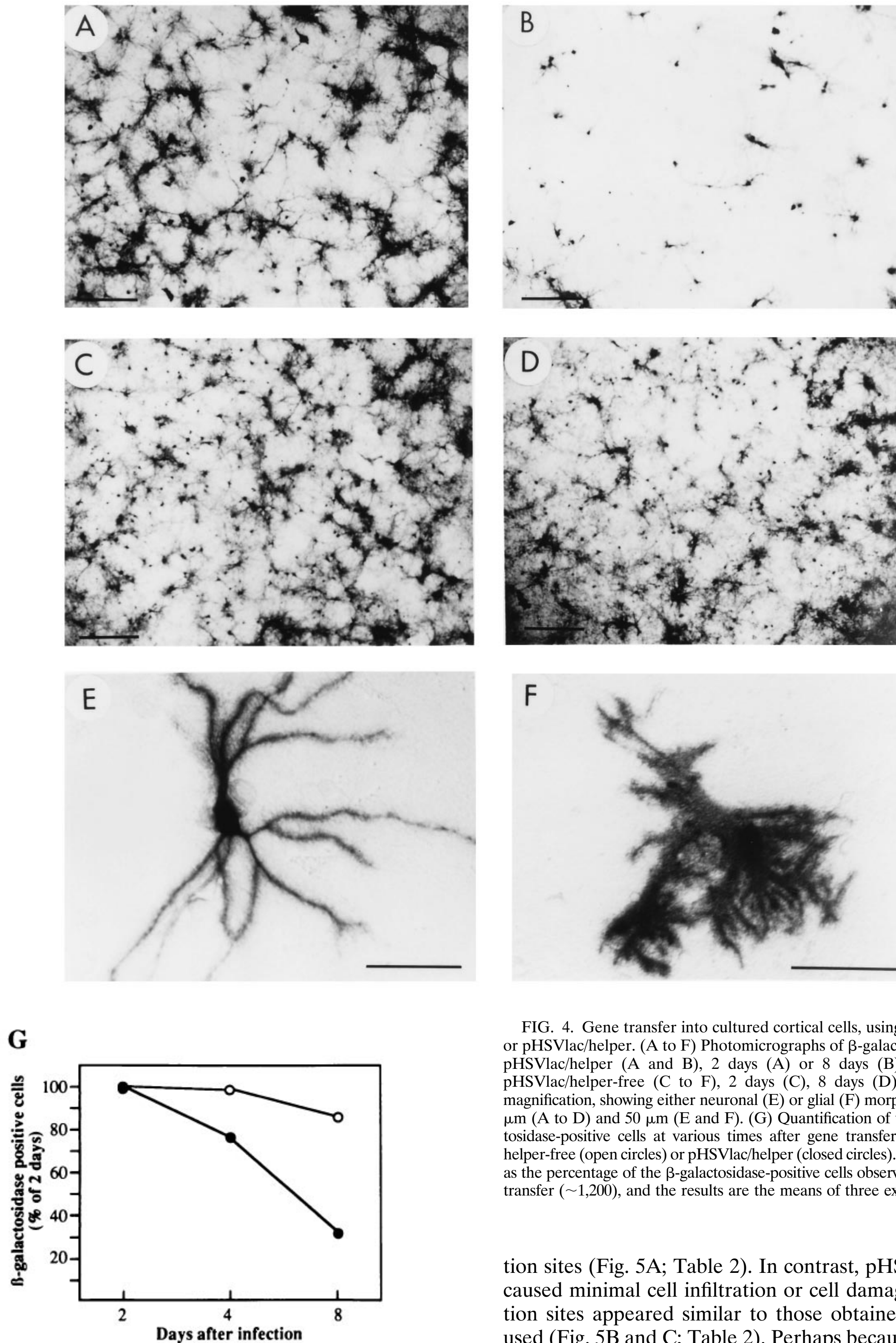
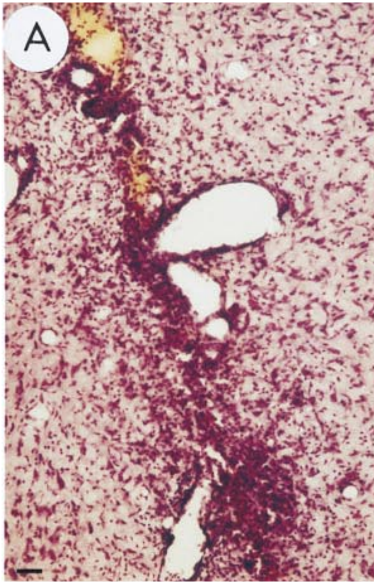
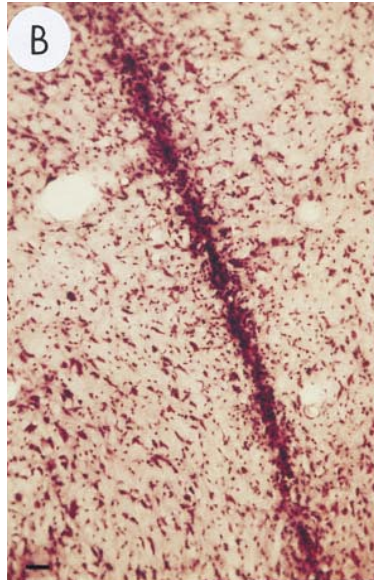
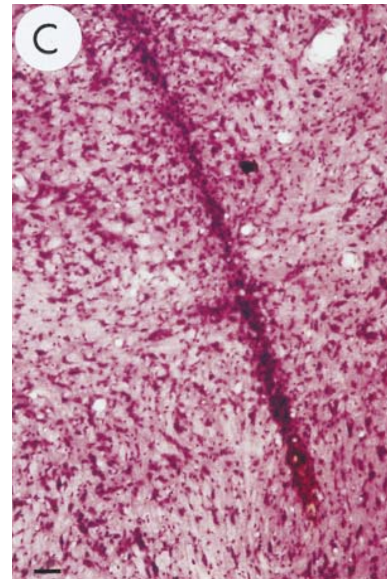


FIG. 4. Gene transfer into cultured cortical cells, using pHSVlac/helper-free or pHSVlac/helper. (A to F) Photomicrographs of β-galactosidase-positive cells: pHSVlac/helper (A and B), 2 days (A) or 8 days (B) after gene transfer; pHSVlac/helper-free (C to F), 2 days (C), 8 days (D), and 2 days at high magnification, showing either neuronal (E) or glial (F) morphology. Scale bars: 500 μm (A to D) and 50 μm (E and F). (G) Quantification of the number of β-galactosidase-positive cells at various times after gene transfer with either pHSVlac/helper-free (open circles) or pHSVlac/helper (closed circles). The data are expressed as the percentage of the β-galactosidase-positive cells observed at 2 days after gene transfer (~1,200), and the results are the means of three experiments.

helper-free, or PBS, were stereotaxically injected into the right MB and the left ST of adult rats. Four days after injection of pHSVlac/helper, cresyl violet staining revealed many infiltrating cells and considerable cell damage proximal to the injection sites (Fig. 5A; Table 2). In contrast, pHSVlac/helper-free caused minimal cell infiltration or cell damage, and the injection sites appeared similar to those obtained when PBS was used (Fig. 5B and C; Table 2). Perhaps because of the reduced cytotoxicity, pHSVlac/helper-free produced more β-galactosidase-positive cells than pHSVlac/helper (Fig. 5D and E; Table 2). Injection of ~3-fold more pHSVlac/helper-free (3×10^4 IVP) resulted in an approximately proportional increase in the number of β-galactosidase-positive cells with minimal cell damage (Fig. 5F). PCR analysis demonstrated that both


A

B

C

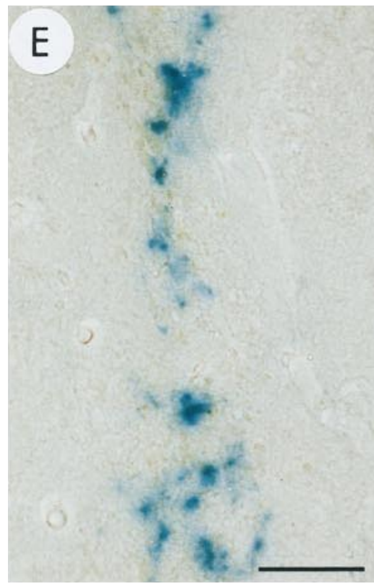
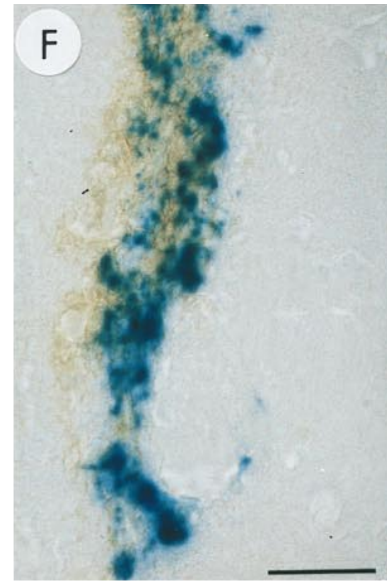
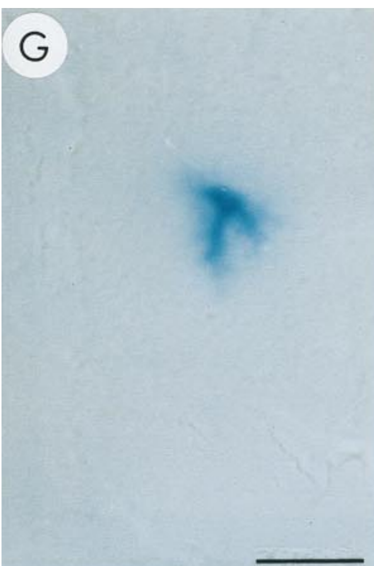
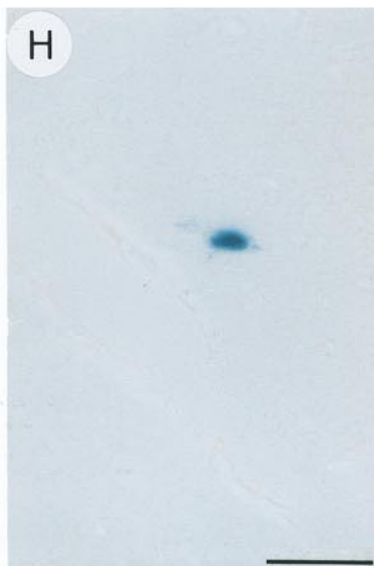
D

E

F

G

H

TABLE 2. Efficiency of gene transfer in the rat brain

Condition	pHSVlac IVP (10^3)	Avg no. of β-Galactosidase-positive cells		Efficiency of gene transfer (%)[a]		Area of cell infiltration (mm^2)	
		MB	ST	MB	ST	MB	ST
Helper	9	113	140	1.3	1.6	1.0	1.6
Helper free	9	124	407	1.4	4.5	0.2	0.6
PBS		0	0			0.1	0.6

[a] Average number of β-galactosidase positive-cells per IVP of pHSVlac injected, multiplied by 100.

pHSVlac/helper-free and pHSVlac/helper persisted for at least 1 month after gene transfer (Fig. 6). However, by 1 month after gene transfer, the number of β-galactosidase-positive cells had significantly declined: pHSVlac/helper-free produced an average of ~5% (MB) and ~2% (ST) of the number of the β-galactosidase-positive cells observed at 4 days, and pHSVlac/helper produced no positive cells in either area. To determine if the promoter influences the stability of gene expression, pIE1βgalori/helper-free (which contains the cytomegalovirus *IE1* promoter) (18) was injected into the ST (6×10^3 IVP). At 1 month after gene transfer, the number of β-galactosidase-positive cells (average, 35 positive cells; three rats) was ~15% of the value observed at 4 days (average, 235 positive cells; three rats), and many of those cells displayed neural morphology (Fig. 5G and H). All of the rats injected with pHSVlac/helper-free survived until killed, and no gross neuroanatomical abnormalities, including tumors, were observed.

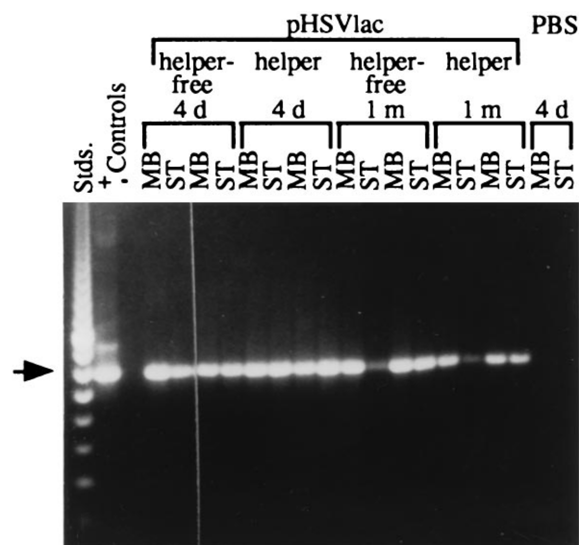


FIG. 6. Persistence of vector DNA in the rat brain. PCR analysis of pHSVlac DNA at 4 days (d) or 1 month (m) after injection of either pHSVlac/helper-free or pHSVlac/helper (two rats at each time point) or PBS (one rat, 4 days). Control reaction mixtures contained either pHSVlac DNA purified from *E. coli* (+) or no DNA (−). Stds., Standards (100-bp ladder). The 583-bp reaction product is indicated by the arrow.

DISCUSSION

Rationale for developing a helper virus-free packaging system for HSV-1 plasmid vectors by transient transfection. Replication and packaging of HSV-1 plasmid or amplicon vectors is dependent on *trans*-acting functions, previously provided by a helper virus, and consequently, vector stocks were contaminated with at least as much helper virus as vector particles. Although those packaging protocols used replication-defective mutants of HSV-1 as helper viruses (12, 24), the safety of these vector systems is limited by several problems, including gene expression from the helper virus, which causes cytopathic effects and immune responses (16, 19, 20, 42), potential interactions between the helper virus and endogenous viruses, potential helper virus-mediated oncogenesis (in contrast, wt HSV-1 is not known to cause tumors), and reversion of the helper virus to wt HSV-1.

With the goal of essentially eliminating many of these problems, we evaluated potential strategies to develop a helper virus-free HSV-1 vector system. Other investigators have developed a cell line that supports the helper virus-free packaging of retrovirus vectors (25). A similar approach for HSV-1 plasmid vectors, however, would require both inducible expression of the ~75 HSV-1 genes and HSV-1 DNA replication to support efficient expression of the true late genes (26). To circumvent these problems, we developed a helper virus-free packaging system by transient transfection. We hypothesized that following transfection into cells, an HSV-1 cosmid set lacking the DNA cleavage/packaging signals could not generate a packageable HSV-1 genome but could provide all the helper functions required for the replication and the packaging of cotransfected vector DNA. Similar systems have also been developed for adeno-associated virus vectors (31) and human immunodeficiency virus type 1 vectors (30).

Properties of the helper virus-free packaging system. We demonstrated that (i) a HSV-1 cosmid set lacking the DNA cleavage/packaging signals can provide all of the *trans*-acting functions required for the replication and packaging of HSV-1 plasmid vectors, (ii) the resulting vector stocks are free of detectable helper virus, (iii) at least one DNA cleavage/packaging signal in a HSV-1 cosmid set is required for the generation of HSV-1 from the cosmid set, (iv) the genome of pHSVlac/helper-free consists of multiple copies of the plasmid vector for up to ~150 kb of DNA, the size of the HSV-1 genome, and (v) nucleocapsids of pHSVlac/helper-free have a structure comparable to that of wt HSV-1.

Because of the limited sequence homology between the vector (pHSVlac, 1.05 kb; pIE1βgalori, 0.2 kb) and both cos6Δ*a* and cos48Δ*a*, homologous recombination between the vector DNA and either cosmid could generate a fragment of the HSV-1 genome that contains both elements required for packaging (ori_S and an *a* sequence). However, to enable replication and packaging, such a DNA molecule would have to be circularized, and the generation of a complete circular HSV-1 genome would require a minimum of six recombination events. Three experiments demonstrated that the vector stocks were free of contaminating helper virus. First, no helper virus was detected by standard plaque assays, even after serial passage of the cell lysates (not shown). Second, ICP0 immunoreactivity (ICP0 is a nonstructural HSV-1 protein encoded by the *IE1*

FIG. 5. Photomicrographs showing cell infiltration and β-galactosidase-positive cells following gene transfer into the rat brain. (A to C) Cresyl violet staining of the injection site in the MB at 4 days after delivery of pHSVlac/helper (A), pHSVlac/helper-free (B), or PBS (C). (D to F) β-Galactosidase-positive cells adjacent to the injection site in the ST at 4 days after delivery of pHSVlac/helper (D) or pHSVlac/helper-free (E and F). (G and H) Individual β-galactosidase-positive cells in the ST at 4 days (G) or 1 month (H) after injection of pIE1βgalori/helper-free. Scale bars, 50 μm.

gene present on both cos6Δ*a* and cos48Δ*a*) was not detected in cells infected with pHSVlac/helper-free but was observed in the nuclei of cells infected with pHSVlac packaged by using a replication-defective helper virus (pHSVlac/helper). Third, Southern analysis of DNA isolated from pHSVlac/helper-free did not reveal bands that represented recombinants between pHSVlac and cosmid set C6Δ*a*48Δ*a*.

Implications for gene transfer into neurons. The two primary, and probably related, problems of the previously developed HSV-1 vector systems are the cytotoxic effects induced by gene expression from the HSV-1 genome (16, 19, 20, 42) and the instability of gene expression. The absence of helper virus from HSV-1 plasmid vector stocks resulted in (i) markedly reduced cytotoxic effects and cell infiltration, (ii) more efficient gene transfer, and (iii) more stable gene expression, indicating that the helper virus may at least in part be responsible for the instability of gene expression. For example, Ho et al. (18) reported that pIE1βgalori, packaged by using a temperature-sensitive mutant of HSV-1 as the helper virus, supported only short-term gene expression in the rat hippocampus; the number of β-galactosidase-positive cells declined to zero by 7 days after gene transfer. In contrast, 1 month after gene transfer with pIE1βgalori/helper-free into the rat ST, the number of β-galactosidase-positive cells was ~15% of the value observed at 4 days.

Another factor that appears to influence the efficiency of long-term gene expression is the promoter, since the cytomegalovirus *IE1* promoter (pIE1βgalori/helper-free) supported more stable gene expression than the HSV-1 *IE4/5* promoter. However, even in the absence of helper virus, the number of β-galactosidase-positive cells at 1 month was significantly lower than at 4 days after gene transfer. The decline in the number of positive cells is probably due at least in part to promoter shutoff and cannot be attributed exclusively to the death of cells containing vector DNA or loss of vector DNA: 2 months after injection of pHSVlac/helper into the ST, injection of helper virus at the same site directed an increase in the number of β-galactosidase-positive cells to ~30% of the levels observed at 4 days after gene transfer, thereby demonstrating both reactivation of the *IE4/5* promoter and the efficient persistence of vector DNA (37). Since vector DNA appears to persist in neural cells, the use of different promoters, such as the HSV-1 latency-associated transcript promoter (4, 17) or neuron-specific promoters, may increase the efficiency of long-term gene expression. Consistent with this latter hypothesis, relatively stable gene expression was achieved, even in a helper virus system, with a vector that expresses the *lacZ* gene from the rat tyrosine hydroxylase promoter (pTHlac/helper): at 6 weeks after injection into the MB, the number of β-galactosidase-positive cells was still ~44% of the value observed at 4 days (34). We are currently examining the efficiency of long-term gene expression from the same vector packaged by using the helper virus-free system (pTHlac/helper-free).

Perspectives. The helper virus-free transient packaging system described in this report produces vector stocks with titers of approximately 4×10^5 IVP per ml of culture medium. By optimizing the packaging procedure, we recently obtained helper virus-free vector stocks with titers (~10^6 IVP per ml) similar to those realized with the helper virus packaging systems (10). Since wt HSV-1 can be grown to titers of up to 10^9 PFU/ml, higher vector titers may be achieved by other improvements to the packaging procedure. Further modifications to the vector system may add additional barriers to the formation of HSV-1 and reduce potential remaining cytopathic effects by mutating specific virion proteins. The helper virus-free packaging system may facilitate studies in neuronal physiology and may eventually allow HSV-1 plasmid vectors to be used for therapeutic applications.

ACKNOWLEDGMENTS

We thank E. Schraner for technical assistance, M. Ackermann and J. Haycock for critical readings of the manuscript, A. J. Davison for the HSV-1 cosmids, T. C. Heineman for advice on the RARE method, R. D. Everett for the anti-ICP0 antibody, and D. Y. Ho for plasmid pIE1βgalori.

This work was supported by the Swiss National Science Foundation (C.F.), Pharmaceutical Manufacturers Foundation (S.S.), National Alliance for Research on Schizophrenia, NIH grant AG 10827, American Health Assistance Foundation, and the Burroughs Wellcome Fund.

REFERENCES

1. **Choi, D. W., M. Malucci-Gedde, and A. R. Kriegstein.** 1987. Glutamate neurotoxicity in cortical cell culture. J. Neurosci. **7:**357–368.
2. **Cohen, J. I., and K. E. Seidel.** 1993. Generation of varicella-zoster virus (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication *in vitro*. Proc. Natl. Acad. Sci. USA **90:**7376–7380.
3. **Cunningham, C., and A. J. Davison.** 1993. A cosmid-based system for constructing mutants of herpes simplex virus type 1. Virology **197:**116–124.
4. **Dobson, A. T., T. P. Margolis, F. Sedarati, J. G. Stevens, and L. T. Feldman.** 1990. A latent, nonpathogenic HSV-1-derived vector stably expresses β-galactosidase in mouse neurons. Neuron **5:**353–360.
5. **During, M. J., J. Naegele, K. O'Malley, and A. I. Geller.** 1994. Long-term behavioral recovery in parkinsonian rats by an HSV-1 vector expressing tyrosine hydroxylase. Science **266:**1399–1403.
6. **Emson, P. C., S. Shoham, C. Feler, T. Buss, J. Price, and C. J. Wilson.** 1990. The use of retroviral vector to identify foetal striatal neurones transplanted into the adult striatum. Exp. Brain Res. **79:**427–430.
7. **Everett, R. D., A. Cross, and A. Orr.** 1993. A truncated form of herpes simplex virus type 1 immediate-early protein Vmw110 is expressed in a cell type dependent manner. Virology **197:**751–756.
8. **Federoff, H. J., M. D. Geschwind, A. I. Geller, and J. A. Kessler.** 1992. Expression of ngf *in vivo* from a defective HSV-1 vector prevents effects of axotomy on sympathetic neurons. Proc. Natl. Acad. Sci. USA **89:**1636–1640.
9. **Ferrin, L. J., and R. D. Camerini-Otero.** 1991. Selective cleavage of human DNA: recA-assisted restriction endonuclease (RARE) cleavage. Science **254:**1494–1497.
10. **Fraefel, C.** Unpublished data.
11. **Geller, A. I., and X. O. Breakefield.** 1988. Defective HSV-1 vector expresses *Escherichia coli* β-galactosidase in cultured peripheral neurons. Science **241:**1667–1669.
12. **Geller, A. I., K. Keyomarski, J. Bryan, and A. B. Pardee.** 1990. An efficient deletion mutant packaging system for defective HSV-1 vectors; potential applications to neuronal physiology and human gene therapy. Proc. Natl. Acad. Sci. USA **87:**8950–8954.
13. **Halbert, C. L., I. E. Alexander, G. M. Wolgamot, and A. D. Miller.** 1995. Adeno-associated virus vectors transduce primary cells much less efficiently than immortalized cells. J. Virol. **69:**1473–1479.
14. **Heineman, T. C., and J. I. Cohen.** 1994. Deletion of the varicella-zoster virus large subunit of ribonucleotide reductase impairs growth of virus in vitro. J. Virol. **68:**3317–3323.
15. **Higuchi, R.** 1989. Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications **2:**1–3.
16. **Ho, D. Y., S. L. Fink, M. S. Lawrence, T. J. Meier, T. C. Saydam, R. Dash, and R. M. Sapolski.** 1995. Herpes simplex virus vector system: analysis of its *in vivo* and *in vitro* cytopathic effects. J. Neurosci. Methods **57:**205–215.
17. **Ho, D. Y., and E. S. Mocarski.** 1989. Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. Proc. Natl. Acad. Sci. USA **86:**7596–7600.
18. **Ho, D. Y., E. S. Mocarski, and R. M. Sapolsky.** 1993. Altering central nervous system physiology with a defective herpes simplex virus vector expressing the glucose transporter gene. Proc. Natl. Acad. Sci. USA **90:**3655–3659.
19. **Johnson, P. A., A. Miyanohara, F. Levine, T. Cahill, and T. Friedman.** 1992. Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. J. Virol. **66:**2952–2965.
20. **Johnson, P. A., K. Yoshida, F. H. Gage, and T. Friedman.** 1992. Effects of gene transfer into cultured CNS neurons with a replication-defective herpes simplex virus type 1 vector. Mol. Brain Res. **12:**95–102.
21. **Kalnins, A., K. Otto, U. Ruther, and B. Muller-Hill.** 1983. Sequence of the *lacZ* gene of *Escherichia coli*. EMBO J. **2:**593–597.
22. **Kaplitt, M. G., P. Leone, R. J. Samulski, X. Xiao, D. W. Pfaff, K. O'Malley, and M. J. During.** 1994. Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. Nat. Genet. **8:**148–154.

23. **Le Gal La Salle, G., J. J. Robert, S. Berrard, V. Ridoux, L. D. Stratford-Perricaudet, M. Perricaudet, and J. Mallet.** 1993. An adenovirus vector for gene transfer into neurons and glia in the brain. Science **259:**988–990.
24. **Lim, F., D. Hartley, P. Starr, P. Lang, S. Song, L. Yu, Y. Wang, and A. I. Geller.** Packaging defective HSV-1 vectors using an IE2 deletion mutant supports efficient expression in cultured cortical cells. BioTechniques, in press.
25. **Mann, R., R. L. Mulligan, and D. Baltimore.** 1983. Construction of retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33:**153–159.
26. **Mavromara-Nazos, P., and B. Roizman.** 1989. Activation of herpes simplex virus 1 γ2 genes by viral DNA replication. Virology **161:**593–598.
27. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. **63:**18–27.
28. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
29. **Paxinos, G., and C. Watson.** 1986. The rat brain in stereotaxic coordinates. Academic Press, Sidney, Australia.
30. **Richardson, J. H., J. F. Kaye, L. A. Child, and A. M. L. Lever.** 1995. Helper virus-free transfer of human immunodeficiency virus type 1 vectors. J. Gen. Virol. **76:**691–696.
31. **Samulski, R. J., L.-S. Chang, and T. Shenk.** 1989. Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression. J. Virol. **63:**3822–3828.
32. **Schrag, J. D., B. V. Prasad, F. J. Rixon, and W. Chui.** 1989. Three-dimensional structure of the HSV1 nucleocapsid. Cell **56:**651–660.
33. **Smith, I. L., M. A. Hardwicke, and R. M. Sandri-Goldin.** 1992. Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. Virology **186:**74–86.
34. **Song, S.** Unpublished data.
35. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
36. **Spaete, R. R., and N. Frenkel.** 1982. The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell **30:**295–304.
37. **Starr, P., F. Lim, F. D. Grant, L. Trask, P. Lang, L. Yu, and A. I. Geller.** Long-term persistence of defective HSV-1 vectors in the rat brain is demonstrated by reactivation of vector gene expression. Gene Ther., in press.
38. **Stevens, J. G.** 1975. Latent herpes simplex virus and the nervous system. Curr. Top. Microbiol. Immunol. **70:**31–50.
39. **Tomkinson, B., E. Robertson, R. Yalamanchili, R. Longnecker, and E. Kieff.** 1993. Epstein-Barr virus recombinants from overlapping cosmid fragments. J. Virol. **67:**7298–7306.
40. **van Zijl, M., W. Quint, J. Briaire, T. de Rover, A. Gielkens, and A. Berns.** 1988. Regeneration of herpesviruses from molecularly cloned subgenomic fragments. J. Virol. **62:**2191–2195.
41. **Wolfe, J. H., S. L. Deshmane, and N. W. Fraser.** 1992. Herpesvirus vector gene transfer and expression of β-glucuronidase in the central nervous system of MPS VII mice. Nat. Genet. **1:**379–384.
42. **Wood, M. J. A., A. P. Byrnes, D. W. Pfaff, S. D. Rabkin, and H. M. Charlton.** 1994. Inflammatory effects of gene transfer into the CNS with defective HSV-1 vectors. Gene Ther. **1:**283–291.
43. **Yang, Y., Q. Li, H. C. J. Ertl, and J. M. Wilson.** 1995. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. J. Virol. **69:**2004–2015.