**Journal of NeuroVirology,** 7: 548–555, 2001
 ISSN 1355–0284/01 $12.00+.00

# Helper virus-free HSV-1 vectors packaged both in the presence of VSV G protein and in the absence of HSV-1 glycoprotein B support gene transfer into neurons in the rat striatum

Ju Tang,[1] Tianzhong Yang,[1] Hara P Ghosh,[2] and Alfred I Geller[1,3]

*[1]Division of Endocrinology, Children's Hospital, Boston, Massachusetts, USA; [2]Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada; and [3]Program in Neuroscience, Harvard Medical School, Cambridge, Massachusetts, USA*

**Herpes simplex virus (HSV-1) vectors have potential for gene transfer into quiescent cells, but the gene transfer process could be more efficient. In other vector systems, both the titers and the efficiency of gene transfer have been enhanced by pseudotyping the vector particles with vesicular stomatitis virus (VSV) G protein. In this report, we pseudotyped helper virus-free HSV-1 plasmid vectors with VSV G protein. Packaging was performed in the presence of both VSV G protein and a deletion in an essential HSV-1 glycoprotein, gB. The resulting vector stocks supported gene transfer into both fibroblast and neuronal cell lines. VSV G protein was required for gene transfer because preincubation of these vector stocks with antibodies directed against either VSV G protein or VSV reduced the titer to undetectable levels. Although the titers were lower than those obtained using the unmodified vector system, the titers were not increased by use of chimeric proteins that contain the extracellular domain of VSV G protein and the transmembrane and/or cytoplasmic domains of specific HSV-1 glycoproteins. Also, the titers were not increased by performing the packaging in the presence of deletions in multiple HSV-1 glycoproteins. Nonetheless, pHSVlac pseudotyped with VSV G protein supported gene transfer into striatal neurons in the rat brain. Thus, HSV-1 vectors pseudotyped with VSV G protein may be useful for specific gene transfer studies.** *Journal of NeuroVirology* (2001) **7,** 548–555.

**Keywords:** herpes simplex virus (HSV-1) vector; helper virus-free packaging; vesicular stomatitis virus (VSV) G protein; neurons; gene transfer

## Introduction

Herpes simplex virus type 1 (HSV-1) vectors have potential for gene transfer into neurons and other quiescent or postmitotic cells (Geller and Breakefield, 1988). The use of HSV-1 vectors has been complicated by the cytopathic effects and the inflammatory response associated with gene transfer. These side effects are principally caused by HSV-1 gene expression (Johnson *et al*, 1992a, 1992b, 1994; Wood *et al*, 1994). Therefore, we developed a helper virus-free packaging system for HSV-1 plasmid vectors (Fraefel *et al*, 1996). Upon injection into the rat brain, helper virus-free vector stocks produce substantially less cytopathic effects and inflammatory response than previously observed using helper virus systems (Fraefel *et al*, 1996). Nonetheless, higher titers and more efficient gene transfer would enhance the utility of these HSV-1 vectors.

The titers and gene transfer efficiency of both classical retrovirus vectors and lentivirus vectors

Address correspondence to Alfred I Geller, Division of Endocrinology, Children's Hospital, Boston, MA 02115, USA. E-mail: geller_a@tch.harvard.edu
Received 6 September 2000; accepted 13 June 2001.

have been significantly enhanced by pseudotyping these vectors with vesicular stomatitis virus (VSV) G protein (Burns *et al*, 1993; Naldini *et al*, 1996). In contrast, HSV-1 vectors have relied upon the mechanism that the virus uses to enter cells. An HSV-1 particle contains 11 glycoproteins, of which 5 are essential, and the infection process is complex and involves a number of these glycoproteins (Roizman and Sears, 1993). Thus, pseudotyping helper virus-free HSV-1 vectors with VSV G protein might simplify the mechanism of gene transfer and thereby enhance the titers and/or the efficiency of gene transfer.

We now show that HSV-1 vectors packaged in the presence of both VSV G protein and a deletion in an essential HSV-1 glycoprotein (gB) can support gene transfer into either cultured cells or neurons in the rat striatum. The efficiency of gene transfer in the striatum appears to be higher than that observed using unmodified HSV-1 vectors. VSV G protein pseudotyped HSV-1 vectors may be useful in gene transfer experiments, particularly if the titers can be improved.

## Results and discussion

Pseudotyping HSV-1 vector particles with VSV G protein may make the gene transfer process more efficient. To enable detection of gene transfer events mediated by any VSV G protein that might be present in HSV-1 vector particles, we must first block infection by HSV-1 glycoproteins. Thus, we performed packaging in the presence of VSV G protein and in the absence of an HSV-1 glycoprotein required for the infection process. We characterized the resulting vector stocks, and then used these vector stocks for gene transfer into the rat brain.

To block infection by HSV-1 glycoproteins, we isolated a cosmid that contains a deletion in an essential glycoprotein, gB (Roizman and Sears, 1993). No detectable titer was obtained from pHSVlac that was packaged in the presence of a deletion in HSV-1 gB (Table 1). However, the titers obtained from packaging pHSVlac in the presence of both this deletion in gB and a plasmid that contains the gB gene were similar to the titers obtained from the unmodified packaging system.

Next, to pseudotype HSV-1 vector particles with VSV G protein, we packaged pHSVlac in the presence of both the deletion in gB and a plasmid that expresses VSV G protein. These vector stocks contained biologically active vector particles (infectious vector particles (IVP); Table 1), suggesting that VSV G protein was incorporated into the vector particles and could mediate gene transfer. However, the titers were ~1000-fold lower than those obtained from the unmodified packaging system. In a study on pseudotyping recombinant HSV-1 vectors with VSV G protein, only a 50-fold reduction in the titer was observed (Anderson *et al*, 2000); this difference could reflect the use of a mutated gD rather than gB, differences in the packaging procedures, or differences in the titering assays. To compare the numbers of vector genomes to the biological titer, we performed PCR on DNA extracted from purified stocks of pHSVlac that had been packaged either under standard conditions or in the absence of gB and presence of VSV G protein (Figure 1). The physical titer (vector genomes) and the biological titer (IVP) were similar for each vector stock (Table 2).

To directly show that gene transfer required the activity of VSV G protein, we preincubated these vector stocks with antibodies directed against either VSV G protein or VSV virus, and antibody-vector particle complexes were removed by centrifugation. Using vector stocks packaged in the presence of VSV G protein, this treatment reduced the titers below detectable levels (Table 3). In contrast, this treatment had minimal effect on the titers of vector stocks prepared using standard conditions.

One possible explanation for the low titers is that VSV G protein might not be efficiently incorporated into the vector particles either because VSV G protein is targeted predominately to the plasma membrane (Odell *et al*, 1997), whereas HSV-1 particles are initially assembled at the nuclear membrane (Roizman and Sears, 1993) or because the available VSV G protein at the nuclear membrane may not be efficiently incorporated into vector particles. We have previously reported chimeric proteins that contain the

**Table 1** The titers obtained from packaging pHSVlac in the presence of VSV G protein or chimeras of VSV G protein and HSV-1 glycoproteins

| *Packaging condition* | [a] *Titer, IVP/ml* *BHK cells* | *PC12 cells* |
|---|---|---|
| wt | $2.1 \times 10^6$ | |
| ΔgB | <20 | |
| ΔgB, pUC18gB | $1.3 \times 10^6$ | |
| ΔgB, pXMG | $9.1 \times 10^2$ | $7.2 \times 10^2$ |
| ΔgB, pXMGgB3G | 30 | <20 |
| ΔgB, pXMGgDG | 30 | <20 |
| ΔgB, pXMGgDgD | 30 | <20 |
| ΔgB, pXMGtmgB | <20 | <20 |
| ΔgB, pXMGGgB | <20 | <20 |
| ΔgB, pXMGGgD | 30 | <20 |
| ΔgB, ΔgC | <20 | |
| ΔgB, ΔgC, pXMG | 170 | |
| ΔgB, ΔgC, pXMGgB3G | <20 | |
| ΔgB, ΔgC, pXMGGgB | 30 | |
| ΔgB, ΔgD | <20 | |
| ΔgB, ΔgD, pXMG | 200 | |
| ΔgB, ΔgD, pXMGDG | <20 | |
| ΔgB, ΔgD, pXMGgDgD | 60 | |
| ΔgB, ΔgC, ΔgD | <20 | |
| ΔgB, ΔgC, ΔgD, pXMG | <20 | |
| ΔgB, ΔgC, ΔgG,J,D,I,E | <20 | |
| ΔgB, ΔgC, ΔgG,J,D,I,E, pXMG | <20 | |

[a]The units of the titers are infectious vector particles (IVP)/ml. pHSVlac was packaged using the indicated conditions. BHK cells were infected with 50 $\mu$l of the resulting vector stocks. One day later, the number of cells expressing $\beta$-galactosidase was determined using X-gal. Each value is the average from 2 packagings.

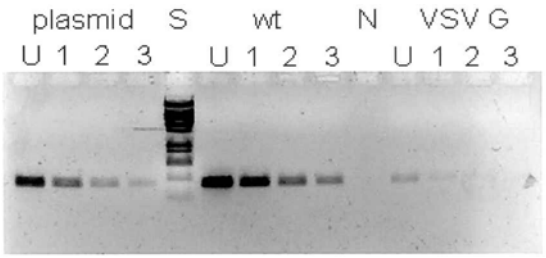


**Figure 1** Detection of *Lac Z* sequences in samples isolated from pHSVlac packaged either under standard conditions or in the absence of gB and the presence of VSV G protein. The linear range for PCR was identified by analyzing 3-fold serial dilutions of each DNA sample and the standard DNA. The standard was linearized pNFHlac plasmid (one *BamH I* site) and added to a sample obtained from a stock of pTH-NFHpkcΔ. The amount of DNA in each sample was determined by comparing the signals produced using the dilutions to those produced using the dilutions of the standard. The predicted size of the PCR products is 583 bp. Lanes: plasmid, pNFHlac plasmid DNA standard; S, bacteriophage λ *BstE II* size standards; wt, pHSVlac packaged using standard conditions; N, no DNA; VSV G, pHSVlac packaged in the absence of gB and the presence of VSV G protein; U, undiluted; 1, 2, and 3 indicate 3-, 9-, and 27-fold dilutions, respectively. The lane of standard undiluted (plasmid, U) represents the signal from $7.3 \times 10^{-8}$ μg of pNFHlac, which corresponds to $6.7 \times 10^3$ molecules of pNFHlac (10.2 kb).

extracellular, fusion domain of VSV G protein and the transmembrane and/or cytoplasmic domains of specific HSV-1 glycoproteins. Cells transfected with plasmids that encode these chimeric proteins exhibit cell–cell fusion (Odell *et al*, 1997), and at least one of these chimeric proteins is targeted to the nuclear membrane (Gilbert *et al*, 1994). Thus, we performed packaging in the presence of both the deletion in gB and these chimeric proteins. The resulting vector stocks exhibited very low, or no detectable, titers (Table 1). We considered the possibility that specific chimeric proteins might be incorporated into vector particles but cannot support gene transfer. Cells that contain specific chimeric proteins exhibit cell–cell fusion at low pH (Odell *et al*, 1997). Thus, we performed the infection for titering at low pH; however, this procedure did not increase the titers (not shown).

We reasoned that perhaps the low titers were due to either competition between VSV G protein and HSV-1 glycoproteins for incorporation into vector particles

**Table 2** The physical and biological titers of pHSVlac stocks that were prepared either in the presence of VSV G protein or under standard conditions

| | *Titers* | |
|---|---|---|
| *Packaging condition* | [a]*IVP/ml* | [b]*VG/ml* |
| wt | $1.0 \times 10^8$ | $8.8 \times 10^7$ |
| ΔgB, pXMG | $1.0 \times 10^5$ | $5.6 \times 10^4$ |

[a]Infectious vector particles/ml.
[b]Vector genomes/ml.
pHSVlac was packaged using the indicated conditions. Purified vector stocks were used to determine the biological titer and the physical titer.

**Table 3** The titers obtained after incubating vector stocks in the presence of antibodies directed against either VSV G protein or VSV

| *Packaging condition* | *Antibody* | [a]*Titer, IVP/ml* |
|---|---|---|
| wt | None | $1.4 \times 10^5$ |
| ΔgB, pXMG | None | $4.0 \times 10^4$ |
| wt | anti-G protein | $3.1 \times 10^4$ |
| ΔgB, pXMG | anti-G protein | <100 |
| wt | anti-VSV | $4.9 \times 10^4$ |
| ΔgB, pXMG | anti-VSV | <100 |

[a]The units of the titers are infectious vector particles (IVP)/ml. pHSVlac was packaged either under standard conditions or in the presence of VSV G protein, and the resulting vector stocks were purified (Lim *et al*, 1996). Aliquots of the purified vector stocks were incubated with antibodies directed against either VSV G protein or VSV (Ghosh and Ghosh, 1999; Wanas *et al*, 1999). The resulting antibody-vector particle complexes were removed by centrifugation, and the supernatants were titered on BHK cells. Each value is the average of 2 independent immunoprecipitations and titerings, and the experiment was repeated 2 times with similar results.

or the chimeras may be incorporated into virions but may not be fusogenic due to interactions with specific HSV-1 glycoproteins (Ghosh and Ghosh, 1999). Thus, packaging in the absence of multiple HSV-1 glycoproteins might increase the titers. We isolated cosmids that harbor deletions in gC, gD, or 5 glycoproteins (gG, gJ, gD, gI, gE). We packaged pHSVlac in the presence of deletions in two, three, or seven glycoproteins and either VSV G protein or specific chimeric proteins. However, the titers obtained using VSV G protein were reduced compared to those obtained from packaging in the presence of only a deletion in gB (Table 1). Moreover, using specific chimeric proteins, we still obtained only very low, or no detectable, titers. Similarly, using recombinant HSV-1 vectors, Glorioso and coworkers recently reported that VSV G protein and specific chimeric proteins can be incorporated into HSV-1 particles, but only VSV G protein supports HSV-1 infection (Anderson *et al*, 2000).

We investigated whether pHSVlac packaged in the presence of a deletion in gB and VSV G protein can support gene transfer into neuronal cells. In an initial experiment, we found that these vector stocks could support expression in PC12 cells (Table 1), a neuronal cell line. One of these vector stocks was microinjected into the striatum, and the rats were sacrificed at 4 days after gene transfer. X-gal-positive cells were observed, and some of these cells displayed neuronal morphology (Figure 2). To directly demonstrate that some of these transfected cells were neurons, we performed double staining using antibodies directed against either *E. coli* β-galactosidase or a neuronal marker, NeuN (Mullen *et al*, 1992). We observed some striatal cells that were positive for both markers (Figure 3). Of 102 β-galactosidase-immunoreactivity (IR)-positive cells that were examined, 80 contained NeuN-IR (78%).

Cell counts revealed an average of 287 X-gal positive cells/rat ($n = 4$ rats); thus, the efficiency

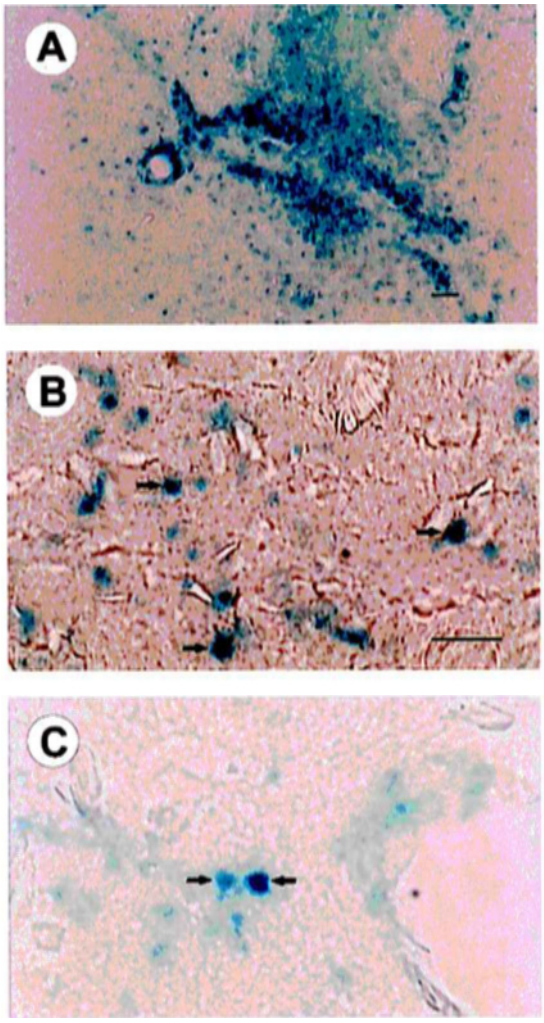


**Figure 2** X-gal-positive cells from rats sacrificed at 4 days after microinjection into the striatum of pHSVlac packaged in the presence of VSV G protein. (**A**) A large X-gal-positive area is visible in a low power view of the striatum. (**B** and **C**) High power views show individual cell bodies. The arrows indicate X-gal-positive cell bodies. Scale bars: (**A**) 25 $\mu$m; (**B** and **C**) 25 $\mu$m.

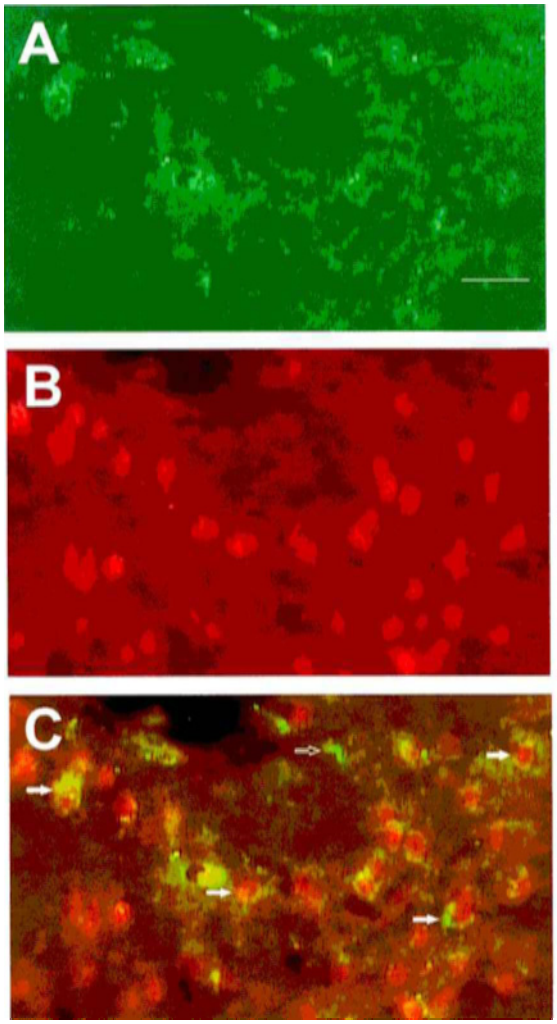


**Figure 3** Some $\beta$-galactosidase-IR-positive cells also contain NeuN-IR from rats sacrificed at 4 days after microinjection of pHSVlac packaged in the presence of VSV G protein. (**A**) $\beta$-galactosidase-IR was detected with a rabbit anti-*E. coli* $\beta$-galactosidase antibody that was visualized using a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. (**B**) NeuN-IR was detected in the same sections with a mouse monoclonal anti-NeuN antibody that was visualized using a rhodamine isothiocyanate-conjugated goat anti-mouse IgG. NeuN is a neuronal marker found in the nucleus (Mullen *et al*, 1992). (**C**) A double exposure shows $\beta$-galactosidase-IR proximal to NeuN-IR. The filled arrows point to 4 cells that contain both $\beta$-galactosidase-IR and NeuN-IR, and the empty arrow points to one $\beta$-galactosidase-IR area that lacks NeuN-IR. Scale bar: 25 $\mu$m.

of gene transfer was 47% (each rat received 600 IVP pHSVlac). In contrast, using pHSVlac packaged with the unmodified vector system, the efficiency of gene transfer was 14% (average of 30 X-gal-positive cells/rat, $n = 2$ rats; each rat received 216 IVP pHSVlac). The difference in the average number of X-gal cells obtained using vectors packaged in the presence of VSV G protein or under standard conditions was significant ($P < 0.01$, $t$-test). In a previous study with pHSVlac, the efficiency of gene transfer was $\leq$5%; however, this study used higher titers and there may be concentration-dependent differences in the efficiency of gene transfer, such as infection of the same cells by multiple vector particles. These results suggest that VSV G protein-pseudotyped HSV-1 vectors enhance the efficiency of gene transfer. Correlatively, VSV G protein may be more stable than the complex of HSV-1 glycoproteins that mediate gene transfer.

Thus, these results suggest that VSV G protein-pseudotyped HSV-1 vectors may be useful. One explanation for the low titers might be that HSV particles are not stable in the acidic environment of the endosomes where the infected virus particles containing G protein are targeted and uncoated. Alternatively, because VSV G protein can be cytotoxic, the production of high-titer, VSV G protein-pseudotyped retrovirus vectors required inducible expression of

VSV G protein (Burns *et al*, 1993). Thus, perhaps higher titers of VSV G protein-pseudotyped HSV-1 vectors might be obtained either by using a cell line that supports the inducible expression of VSV G protein or by placing VSV G protein under the control of a HSV-1 promoter, particularly a promoter of the late class. The vector stocks used for gene transfer into the rat brain were purified using ultracentrifugation. Nonetheless, higher titers might be obtained by additional centrifugation, as was used in the case of recombinant retrovirus vectors (Burns *et al*, 1993).

## Materials and methods

*Cells*

Baby hamster kidney fibroblast (BHK21) cells and 2-2 cells (Smith *et al*, 1992) were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum, penicillin/streptomycin, and 4 mM glutamine at 37°C in humidified incubators containing 5% $CO_2$. G418 (0.5 mg/ml) was present during the growth of 2-2 cells but was removed before experiments. PC12 cells (Greene and Tischler, 1976) were grown in Dulbecco's modified minimal essential medium supplemented with 5% fetal bovine serum and 10% horse serum.

*Plasmids*

pHSVlac (Geller and Breakefield, 1988) expresses the *E. coli Lac Z* gene from the HSV-1 immediate early (IE) 4/5 promoter. Plasmids that express VSV G protein or chimeras of VSV G protein and HSV-1 glycoproteins (Gilbert *et al*, 1994; Odell *et al*, 1997) are based on the pXM plasmid (Yang *et al*, 1986) and use the adenovirus major late promoter. pXMG expresses VSV G protein; pXMGgB3G contains the VSV G protein extracellular domain, the transmembrane domain of HSV-1 gB (amino acids 775–795), and the VSV G protein cytoplasmic domain; pXMGgDG contains the VSV G protein extracellular domain (Odell *et al*, 1997), the transmembrane domain of HSV-1 gD, and the VSV G protein cytoplasmic domain; pXMGgDgD contains the VSV G protein extracellular domain, the transmembrane domain of HSV-1 gD, and the gD cytoplasmic domain; pXMGtmgB contains the VSV G protein extracellular domain and aa 721 to 795 of gB that allow this chimeric protein to be localized in the nuclear envelope (Gilbert *et al*, 1994); pXMGGgB contains the VSV G protein extracellular and transmembrane domains, and the gB cytoplasmic domain; pXMGGgD contains the VSV G protein extracellular and transmembrane domains, and the gD cytoplasmic domain (Ghosh and Ghosh, 1999). pUC18gB was constructed by inserting a 5.2-kb *BamH I* and *Nde I* fragment [nucleotides 52,588 to 57,747 (McGeoch *et al*, 1988)] into pUC18 that had been digested with *BamH I* and *Nde I*.

Plasmids were isolated that contain deletions in specific HSV-1 glycoproteins. Two fragments that extend 5′ or 3′ from the gB deletion were produced using PCR [template, cos28 (Cunningham and Davison, 1993)]; primers, 5′ fragment, 5′GGGAAGCTTGTCAGCTCGTGATTCTGCAGCTCG3′ (nucleotides 54,185 to 54,208 (McGeoch *et al*, 1988) and 5′GCGGAATTCATCAAGGCGGAGAACACCGATGC3′ (complementary to nucleotides 55,463 to 55,486); 3′ fragment, 5′GGCGAATTCTTATTATCTGTTCTTCTTCGGTTTCGGGTCC3′ (nucleotides 55,576 to 55,600) and 5′GCGGGATCCGACGATCAGATCGAGGCGCTCAT3′ (complementary to nucleotides 56,973 to 56,996). After digestion (5′ fragment, *Hind III* and *EcoR I*; 3′ fragment *EcoR I* and *BamH I*), the fragments were inserted into pUC18 to yield pUC18ΔgB.

Two fragments that extend 5′ or 3′ from the gC deletion were produced using PCR [template, cos56 (Cunningham and Davison, 1993)]; primers, 5′ fragment, 5′GGGGAGCTCATCACCGCAGGCGAGTCTCTTTCC3′ (nucleotides 95,440 to 95,464) and 5′-GGGGTGCACGGTGGAATTCCGAAACCGGCATCG-3′ (complementary to nucleotides 96,737 to 96,760); 3′ fragment, 5′GGGGTGCACGTCATCGGCGGGTAACGCAAGACC3′ (nucleotides 97,832 to 97,855) and 5′GGGTCTAGATGCGGGTCTACGAAAACGTCTGC3′ (complementary to nucleotides 99,042 to 99,065). After digestion (5′ fragment, *Sac I* and *ApaL I*; 3′ fragment *ApaL I* and *Xba I*), the fragments were inserted into pUC18 to yield pUC18ΔgC.

Two fragments that extend 5′ or 3′ from the gD deletion were produced using PCR [template, cos48Δa (Fraefel *et al*, 1996)]; primers, 5′ fragment, 5′GCCGAATTCCTCAACATACCCCGCTGTTCTCG3′ (nucleotides 137,260 to 137,283) and 5′CGCGGATCCACCCCATGGAGGCCCACTATGACG3′ (complementary to nucleotides 138,463 to 138,486); 3′ fragment, 5′-GGGGGATCCGGGTTGGGATGGGACCTTAACTCC3′ (nucleotides 139,643 to 139,666) and 5′GGGGCTAGCTGGCCGTGGACCTATACCAACAGG3′ (complementary to nucleotides 140,945 to 140,968). After digestion (5′ fragment, *EcoR I* and *BamH I*; 3′ fragment *BamH I* and *Nhe I*), the fragments were inserted into pBR322 to yield pBR322ΔgD.

Two fragments that extend 5′ or 3′ from the gG,gJ,gD,gI,gE deletion were produced using PCR [template, cos48Δa (Fraefel *et al*, 1996)]; primers, 5′ fragment, 5′GCCGAATTCAGGAGATCCTTGCCCAGATGTACG3′ (nucleotides 135,433 to 135,456) and 5′GCCAAGCTTGTGCCAACCCGCAAACAGCACC3′ (complementary to nucleotides 136,683 to 136,706); 3′ fragment, 5′-GGGAAGCTTCGATTCGTCCGTCTTCTGGTAAGG3′ (nucleotides 142,874 to 142,897) and 5′GGGGCTAGCTTTGGTGCGTTTGGGTGTGGGGACC3′ (complementary to nucleotides 144,182–144,206). After digestion (5′ fragment, *EcoR I* and *Hind III*; 3′ fragment *Hind III* and *Nhe I*), the fragments were inserted into pBR322 to yield pBR322 ΔgD,E,G,I,J.

*Cosmids*

Cosmid setC (Cunningham and Davison, 1993) contains 5 overlapping cosmids that represent the HSV-1 genome (cos6, cos14, cos28, cos48, cos56), and the **a** sequence has been deleted from the two cosmids that contain it [cos6Δ**a**, cos48Δ**a** (Fraefel *et al*, 1996)].

Mutations were introduced into the appropriate HSV-1 cosmid(s) by homologous recombination cloning in *E. coli*. Recombination cloning in *E. coli* BJ5183 has been used to manipulate 35-kb adenovirus vectors (Chartier *et al*, 1996), and we have modified this method (Kong *et al*, 1999) to allow the use of up to 6 DNA fragments to reconstruct a HSV-1 cosmid. Each transformation contained 100 $\mu$l *E. coli* BJ5183, 30 ng vector, and the other fragments (1:1 molar ratio relative to the vector). Candidates were analyzed by digestion with several enzymes, including *BamH I* and *EcoR I*, and the presence of each deletion was verified by assaying for a specific altered fragment, as detailed next. Correct cosmids were transferred into SURE cells.

Cosmids containing specific deletions were constructed using the following fragments:

**cos28ΔgB** Vector fragment, 12.9 kb (26,347 to 61,073 (includes SuperCos I; nucleotides refer to HSV-1 sequences (McGeoch *et al*, 1988) except where SuperCos I is specified), *Xho I*); 7.2 kb (56,138 to 63,370, *BspE I*); 24.7 kb (7,887 of SuperCos I to 49,435, *BspH I*); 5.5 kb (49,132 to 54,627, *Ear I*); and 2.7 kb (from pUC18ΔgB, 54,185 to 56,996, *Hind III* and *BamH I*). The construct was verified by digestion with *EcoR I* and *BamH I*; introduction of the deletion removes a 16.1-kb fragment and adds 7.5-kb and 8.5-kb fragments.

**cos14ΔgB** Vector fragment, 8.1 kb (54,826 to 89,654 (includes SuperCos I), *Sal I*); 7.2 kb (56,138 to 63,370, *BspE I*); 22.7 kb (57,747 to 80,467, *Nde I*); 15.6 kb (74,916 to 1 of SuperCos I, *EcoR I*); 2.7 kb (from pUC18ΔgB, 54,185 to 56,996, *Hind III* and *BamH I*). The construct was verified by digestion with *EcoR I*; introduction of the deletion removes a 9.7-kb fragment and adds 8.6-kb and 1.0-kb fragments.

**cos56ΔgC** Vector fragment, 12.7 kb (80,722 to 110,660 (includes SuperCos I), *Spe I* and *EcoR V*); 14.8 kb (98,473 to 113,229, *Nde I*); 17.3 kb (1 of SuperCos I to 96,751, *EcoR I*); 2.6 kb (from pUC18ΔgC, 95,440 to 99,065, *Sac I* and *Xba I*). The construct was verified by digestion with *ApaL I*; introduction of the deletion removes 6.6-kb and 4.6-kb fragments and adds 6.2-kb and 3.9-kb fragments.

**cos48Δ**a**ΔgD** Vector fragment, 9.3 kb (108,008 to 142,839 (includes SuperCos I), *BspE I*); 8.4 kb (140,277 to 3,941 of SuperCos I, *Msc I*); 30.9 kb (107,496 to 138,387, *Pac I* and *Afl II*); 2.6 kb (from pBR322ΔgD, 137,260 to 140,968, *EcoR I* and *Nhe I*). The construct was verified by digestion with *BamH I*; introduction of the deletion removes a 6.5-kb fragment and adds 2.2-kb and 3.1-kb fragments.

**cos48Δ**a**ΔgG,gJ,gD,gI,gE** Vector fragment, 15.1 kb (114,517 to 143,481 (includes SuperCos I), *Sal I*); 10.1 kb (113,322 to 123,459, *BamH I*); 20.9 kb (115,246 to 136,151, *Ssp I*); 2.6 kb (from pBR322ΔgD,E,G,I,J, 135,433 to 144,206, *EcoR I* and *Nhe I*). The construct was verified by digestion with *Hind III*; introduction of the deletion removes 10.9-kb and 4.9-kb fragments and adds 6.4-kb and 3.2-kb fragments.

*Packaging vectors into HSV-1 particles*

Vectors were packaged into HSV-1 particles using the helper virus-free packaging system (Fraefel *et al*, 1996) and a modified protocol (Sun *et al*, 1999) that improves the efficiency. Each packaging contained 0.4 $\mu$g of each cosmid, 0.5 $\mu$g pHSVlac, and 0.4 $\mu$g of a small plasmid that expresses VSV G protein (pXMG), or a VSV G protein-HSV-1 glycoprotein chimeric protein, or gB. Lower amounts of pXMG resulted in lower titers, and higher amounts did not increase the titers (not shown). Vector stocks were purified and concentrated as described (Lim *et al*, 1996). Vector stocks were titered by counting the number of X-gal-positive cells obtained at 1 day after infection of either BHK or PC12 cells.

*Titering vector genomes by PCR*

DNA was isolated from purified vector stocks, and *Lac Z* sequences were detected using PCR [for PCR conditions and primers, see (Yang *et al*, 2001)]. For each sample, PCR was performed on 3-fold serial dilutions to identify a concentration range that produced a linear increase in signal with increasing sample. The standard DNA was pNFHlac (Wang *et al*, 1999) plasmid digested with *BamH I* (single site) and added to a sample obtained from a stock of pTH-NFHpkcΔ (expresses a protein kinase C). The reaction products were displayed on an 0.6% agarose gel. The amount of DNA in each sample was quantitated by comparing the signals produced using dilutions of the standard to those produced using dilutions of the sample. The titers of vector genomes were calculated from the DNA concentrations and corrected for packaging pHSVlac as a concatamer similar in size to the HSV-1 genome (8.1 kb, 19 copies; 153.9 kb).

*Incubating vectors stocks with antibodies*

Aliquots of vector stocks were incubated (37°C, 30 min) in the presence of different dilutions (1:10 or 1:50) of antibodies directed against either VSV G protein or VSV (Ghosh and Ghosh, 1999; Wanas *et al*, 1999). Vector stocks were centrifuged at 16,000 $\times g$ for 10 min prior to titering. Five $\mu$l of each supernatant was titered by counting the number of X-gal-positive cells obtained at 1 day after infection of BHK cells; each titering was performed in duplicate.

*Stereotactic injection of HSV-1 vectors into the brain*

Male Sprague-Dawley rats (150–175 gm) were used for these experiments. Vector stocks were

delivered by stereotactic injection (2 sites, 3 $\mu$l/site) into the striatum (anterior-posterior (AP) +0.8, medial-lateral (ML) +2.5, dorsal-ventral (DV) −5.5; AP +0.8, ML −2.5, DV −5.5). AP is relative to bregma, ML is relative to the sagittal suture, and DV is relative to the bregma-lambda plane (Paxinos and Watson, 1986). These studies were approved by the Children's Hospital IACUC.

*Histological analyses*

Four days after gene transfer, the rats were anesthetized with chloral hydrate (300 mg/kg, intraperitoneal) and then perfused with 50 ml phosphate-buffered saline (PBS) followed by 200 ml of 4% paraformaldehyde in PBS. The brains were postfixed in 4% paraformaldehyde in PBS (4 h, 4°C), cryoprotected in 25% sucrose in PBS (2 days, 4°C), and 25 $\mu$m coronal sections were cut on a freezing microtome. Enzymatic staining and immunohistochemistry were performed on free-floating sections. Expression of $\beta$-galactosidase was detected using X-gal (Emson *et al*, 1990); the X-gal reaction was carried out for 3 h at room temperature at pH 7.9.

*E. coli* $\beta$-galactosidase-immunoreactivity ($\beta$-galactosidase-IR; rabbit anti-*E. coli* $\beta$-galactosidase antibody, ICN) and NeuN-IR [mouse monoclonal anti-NeuN antibody (Chemicon; (Mullen *et al*, 1992))] were detected in the same sections as described (Yang *et al*, 2001).

Every fourth section (25-$\mu$m sections) was analyzed for expression of $\beta$-galactosidase and ~10 of these sections contained either the X-gal-positive cells or the $\beta$-galactosidase-IR-positive cells. Counts of positive striatal cells were performed under 40X magnification. Each section was counted twice and the results differed by <10%. To obtain the number of cells/rat, the number of counted cells was multipled by 4, as every fourth section was examined.

## Acknowledgements

We gratefully thank Dr. A. Davison for HSV-1 cosmid setC and Dr. R. Sandri-Goldin for 2-2 cells. This work was supported by the Medical Research Council of Canada (HPG), Neurovir, R01AG16777, R01NS34025, and R01NS42016 (AG)

## References

Anderson DB, Laquerre S, Ghosh K, Ghosh HP, Goins WF, Cohen JB, Glorioso JC (2000). Pseudotyping of glycoprotein D-deficient herpes simplex virus type 1 with vesicular stomatitis virus glycoprotein G enables mutant virus attachment and entry. *J Virol* **74:** 2481–2487, 7698.

Burns JC, Friedmann T, Driever W, Burrascano M, Yee JK (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proc Natl Acad Sci USA* **90:** 8033–8037.

Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. *J Virol* **70:** 4805–4810.

Cunningham C, Davison AJ (1993). A cosmid-based system for constructing mutants of herpes simplex virus type 1. *Virology* **197:** 116–124.

Emson PC, Shoham S, Feler C, Buss T, Price J, Wilson CJ (1990). The use of a retroviral vector to identify foetal striatal neurones transplanted into the adult striatum. *Exp Brain Res* **79:** 427–430.

Fraefel C, Song S, Lim F, Lang P, Yu L, Wang Y, Wild P, Geller AI (1996). Helper virus-free transfer of herpes simplex virus type 1 plasmid vectors into neural cells. *J Virol* **70:** 7190–7197.

Geller AI, Breakefield XO (1988). A defective HSV-1 vector expresses *Escherichia coli* beta-galactosidase in cultured peripheral neurons. *Science* **241:** 1667–1669.

Ghosh K, Ghosh HP (1999). Role of the membrane anchoring and cytoplasmic domains in intracellular transport and localization of viral glycoproteins. *Biochem Cell Biol* **77:** 165–178.

Gilbert R, Ghosh K, Rasile L, Ghosh HP (1994). Membrane anchoring domain of herpes simplex virus glycoprotein gB is sufficient for nuclear envelope localization. *J Virol* **68:** 2272–2285.

Greene LA, Tischler AS (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci USA* **73:** 2424–2428.

Johnson PA, Miyanohara A, Levine F, Cahill T, Friedmann T (1992a). Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J Virol* **66:** 2952–2965.

Johnson PA, Wang MJ, 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.

Johnson PA, Yoshida K, Gage FH, Friedmann T (1992b). Effects of gene transfer into cultured CNS neurons with a replication-defective herpes simplex virus type 1 vector. *Mol Brain Res* **12:** 95–102.

Kong Y, Yang T, Geller AI (1999). An efficient *in vivo* recombination cloning procedure for modifying and combining HSV-1 cosmids. *J Virol Methods* **80:** 129–136.

Lim F, Hartley D, Starr P, Lang P, Song S, Yu L, Wang Y, Geller AI (1996). Generation of high-titer defective HSV-1 vectors using an IE 2 deletion mutant and quantitative study of expression in cultured cortical cells. *Biotechniques* **20:** 460–469.

McGeoch DJ, Dalrymple MA, Davison AJ, Dolan A, Frame MC, McNab D, Perry LJ, Scott JE, Taylor P (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.

Mullen RJ, Buck CR, Smith AM (1992). NeuN, a neuronal specific nuclear protein in vertebrates. *Development* **116:** 201–211.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272:** 263–267.

Odell D, Wanas E, Yan J, Ghosh HP (1997). Influence of membrane anchoring and cytoplasmic domains on the fusogenic activity of vesicular stomatitis virus glycoprotein G. *J Virol* **71:** 7996–8000.

Paxinos G, Watson C (1986). *The Rat Brain in Stereotaxic Coordinates*. Academic Press: Sidney.

Roizman B, Sears AE (1993). Herpes simplex viruses and their replication. In: *The Human Herpesviruses*. Roizman B, Whitley RJ, Lopez C (eds). Raven Press: New York, pp 11–68.

Smith IL, Hardwicke MA, Sandri-Goldin RM (1992). Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* **186:** 74–86.

Sun M, Zhang GR, Yang T, Yu L, Geller AI (1999). Improved titers for helper virus-free herpes simplex virus type 1 plasmid vectors by optimization of the packaging protocol and addition of noninfectious herpes simplex virus-related particles (previral DNA replication enveloped particles) to the packaging procedure. *Hum Gene Ther* **10:** 2005–2011.

Wanas E, Efler S, Ghosh K, Ghosh HP (1999). Mutations in the conserved carboxy-terminal hydrophobic region of glycoprotein gB affect infectivity of herpes simplex virus. *J Gen Virol* **80:** 3189–3198.

Wang Y, Yu L, Geller AI (1999). Diverse stabilities of expression in the rat brain from different cellular promoters in a helper virus-free herpes simplex virus type 1 vector system. *Hum Gene Ther* **10:** 1763–1771.

Wood MJ, Byrnes AP, Pfaff DW, Rabkin SD, Charlton HM (1994). Inflammatory effects of gene transfer into the CNS with defective HSV-1 vectors. *Gene Ther* **1:** 283–291.

Yang T, Zhang G, Zhang W, Sun M, Wang X, Geller AI (2001). Enhanced reporter gene expression in the rat brain from helper virus-free HSV-1 vectors packaged in the presence of specific mutated HSV-1 proteins that affect the virion. *Mol Brain Res*. *In Press*.

Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wong GG, Clark SC (1986). Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47:** 3–10.