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In Vivo Expression of β-Galactosidase in Hippocampal Neurons by HSV-Mediated Gene Transfer

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ABSTRACT

Stereotactic inoculation of a herpes simplex virus (HSV) gene transfer vector into the hippocampus and caudate of rat brain resulted in limited and transient viral replication and the establishment of latency. Virus attenuation was achieved by insertional inactivation of a viral gene, Us3. Insertion of a *lacZ* reporter gene, under the control of the HSV glycoprotein C (gC) late gene promoter, allowed viral replication to be monitored *in vivo*. Unlike unattenuated virus, the Us3::pgC-*lacZ* recombinant caused little apparent damage to normal hippocampal morphology. Transient *lacZ* expression was detected in a considerable population of neurons of the dentate gyrus following hippocampal injection, whereas few positively staining neurons were present within the caudate after injection at that site. Latency-associated transcripts, the hallmark of latent infection, were detected in the brain 10 months after injection. This recombinant virus may be useful as a gene transfer vector for long-term expression of foreign genes in the central nervous system.

OVERVIEW SUMMARY

Development of suitable vectors for transfer of genes to the central nervous system (CNS) is an active field of research. Fink *et al.* have determined that disruption of a herpes simplex virus (HSV) protein kinase gene (Us3) results in significant virus attenuation and prevents spread in the CNS, and that this mutant virus can establish long-term latency in the brain. These results suggest that this vector should be useful for expression of foreign genes in neurons localized to precise regions of the brain following stereotactic vector delivery.

INTRODUCTION

HERPES SIMPLEX VIRUS (HSV) is a neurotropic virus, which in natural infection is taken up by axonal terminals and transported retrogradely to the neuronal cell body (Hill, 1985). The virus can establish long-term latency in which the viral genomes persist in a circular or concatameric extrachromosomal state (Rock and Fraser, 1983; Efstathious *et al.*, 1986; Mellerick and Fraser, 1987). Latency normally occurs in peripheral sensory ganglia following primary infection of the skin or mucosal surfaces. In animal models, infection of peripheral sensory ganglia can be achieved by inoculation of the skin, cornea, or the olfactory bulb (Cook and Stevens, 1973; Stroop and Schaefer, 1987). Focal HSV infection of specific brain regions can be achieved by stereotactic injection of small volumes of virus into specific brain regions (Bak *et al.*, 1977; McFarland *et al.*, 1986). The establishment of latent virus infection of very limited brain components has been reported using defective and attenuated virus vectors in site specific infections (Chiocca *et al.*, 1990). It remains to be determined whether direct inoculation of attenuated virus results in latency in a limited brain location. Moreover, the potential pathological effects of attenuated virus injection into specific brain regions have not been studied long term.

Latently infected neurons show an extremely restricted and characteristic pattern of viral gene expression (Hill, 1985;

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Stevens, 1989). The only transcriptionally active region of the viral genome maps to a segment of the inverted repeat sequences of the long unique (U_L) region of the viral genome just downstream of the structural gene for infected cell polypeptide O (ICPO). The detected RNA species are transcribed off of the opposite strand from ICPO and are therefore partially complementary to the ICPO mRNA. The most abundant transcripts are 1.8 to 2.3 kb in size and have been designated collectively as latency-associated transcripts, or LATs (Deatly *et al.*, 1987; Puga and Notkins, 1987; Rock *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987; Wagner *et al.*, 1988). It remains to be proven whether any of the LATs encode a viral protein. Deletions within LAT do not alter the ability of the virus to establish or maintain latency; however, reactivation appears to be impaired among certain LAT^- mutants (Ho and Mocarski, 1989; Leib *et al.*, 1989; Sederati *et al.*, 1989; Steiner *et al.*, 1989).

This set of features—a virus targeted to neuronal tissues that is capable of naturally entering a latent state whereby it expresses a limited portion of its genome—suggests that HSV may potentially represent an ideal shuttle vector for delivery of foreign genes into the central nervous system (CNS) (Breakefield and Geller, 1987; Freese *et al.*, 1990).

Previous attempts to develop an HSV vector for effective gene transfer into mammalian neurons have used two alternative approaches. Packaged defective HSV-1 amplicons have been used to express foreign genes in cultured peripheral nervous system (PNS) (Geller and Breakefield, 1988) and CNS (Geller and Freese, 1990) neurons. However, the ability of these vectors to express foreign genes *in vivo* has not been demonstrated. Alternately, HSV vectors derived from virus defective in an immediate early gene required for viral replication (ICP4) have been demonstrated to be capable of mediating long-term expression of foreign gene products in dorsal root ganglia after sciatic nerve inoculation (Dobson *et al.*, 1990), trigeminal ganglia after corneal scarification (R.L. Hendricks, L.R. Sternberg, W.F. Goins, and J.C. Glorioso, in preparation), and neurons in the hypoglossal nucleus after injection of the tongue (Dobson *et al.*, 1990).

Although these approaches demonstrate the promise of using HSV for the expression of foreign genes in neuronal structures, a completely defective virus may be limited as a gene transfer vector due to the small number of neuronal cells in which the vector *may* establish latency. An alternative approach employs the development of replicating, but highly attenuated, strains of HSV which may be capable of establishing latency in a greater number of neurons without causing neuropathologic damage.

Previously, we and others have determined that viruses defective in several individual genes in the unique short (U_S) region of the HSV-1 genome show significantly reduced pathogenicity following intracranial inoculation (Weber *et al.*, 1987; Meignier *et al.*, 1988). Previous DNA sequence (McGeoch and Davidson, 1986), immunological (Purves *et al.*, 1987), and mutational (Leader and Katan, 1988) analyses have demonstrated that one of the open reading frames, the Us3 gene, encodes a protein kinase (for review, see Leader and Purves, 1988) whose physiological substrate has recently been identified as HSV-1 UL34 phosphoprotein (Purves *et al.*, 1991). The Us3 gene is dispensable for virus replication in cell culture (Longnecker and Roizman, 1987; Meignier *et al.*, 1988) and for the establishment of latent infection in the murine PNS; however, the ability of a Us3 mutant to establish latency in the CNS has not been evaluated.

In the present report, we demonstrate that this vector can be used to express a foreign gene in a discrete region of rat brain, that it causes limited neuropathologic damage, and that it establishes long-term latency in the CNS following intracranial inoculation. These results suggest that this attenuated mutant may function as an effective gene transfer vector.

MATERIALS AND METHODS

*Construction of the Us3::pgC-*lacZ *recombinant*

The *lacZ* gene was placed under the regulatory control of a well-characterized HSV-1 late gene promoter, that of the glycoprotein C (gC) gene (Homa *et al.*, 1986), ensuring that the β-galactosidase gene product would be expressed at high levels during lytic infection. The reporter gene cassette was constructed in such a way that *lacZ* gene, gC promoter (−144 to +124), and an SV40 polyadenylation signal can be removed as

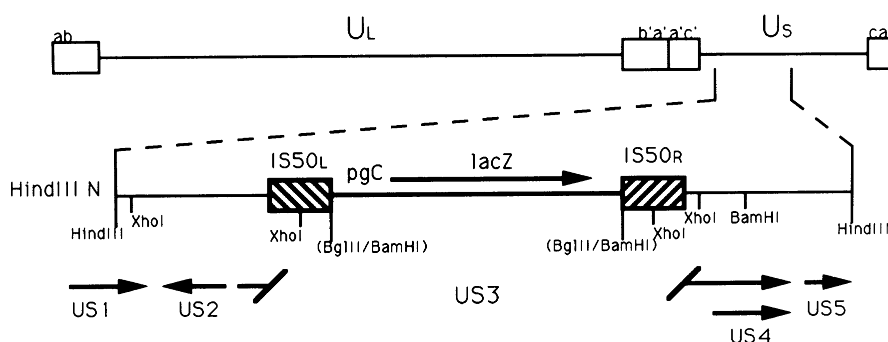

FIG. 1. Schematic representation of HSV Us3::pgC-*lacZ* genome, indicating the location of the pgC-*lacZ* reporter cassette. The pgC-*lacZ* reporter cassette was cloned as a 4.2-kb *Bam* HI fragment into the *Bgl* II sites of the Tn*5* located within the IS*50* repeat elements, replacing the internal Tn*5* sequence. The presence and precise mapping of the Tn*5*::gC-*lacZ* cassette within the Us3 locus (Hendricks *et al.*, 1991) was confirmed by Southern blot analysis (Goins and Stinski, 1986). *In vitro* infection with the recombinant produces blue plaques following X-gal agarose overlay (Ho and Mocarski, 1989).

TABLE 1. GROWTH OF WILD-TYPE HSV-1 AND THE Us3::pgC-*lacZ* RECOMBINANT IN VERO CELLS AND MOUSE CNS

	Vero cells in vitro[a]				*Mouse CNS* in vivo
	moi = 1.0		*moi = 0.1*		
Virus	*Titer (pfu/ml)*[a]	*Fold reduction in yield*	*Titer (pfu/ml)*[a]	*Fold reduction in yield*	LD_{50} *titer in mice*[b]
HSV-1 (KOS)	4.3×10^8	—	4.7×10^8	—	$10^{1.0}$
HSV-1 (Us3::pgC-*lacZ*)	6.0×10^7	7.2	4.7×10^7	10.0	$10^{4.6}$

[a] Titers represent the yield of virus from a 60-mm dish of Vero cells infected at the indicated multiplicity. (moi, multiplicity of infection; pfu, plaque-forming units).
[b] Titers represent the median lethal dose (LD_{50}) of virus after intracerebral inoculation into DBA/2 mice, determined by techniques described in the Materials and Methods.

a single 4.2-kb *Bam* HI restriction fragment, and inserted into a previously generated plasmid (Weber *et al.*, 1987) containing a Tn*5* insertion into the 5′ end of the Us3 open reading frame (McGeoch *et al.*, 1985). The 4.2-kb *Bam* HI fragment was cloned into the *Bgl* II sites located within the IS*50* repeat elements of the Tn*5* insertion within Us3 in a manner so as to replace the internal 2.8-kb *Bgl* II fragment of the transposon (Hendricks *et al.*, 1991). The truncating nature of the Tn*5* mutation is preserved in this construction since the pgC-*lacZ* cassette does not alter the stop codons present within the transposon termini.

Infectious wild-type KOS strain HSV-1 viral DNA and Us3::pgC-*lacZ* plasmid DNA were cotransfected into Vero cells using the calcium phosphate procedure (Shapira *et al.*,

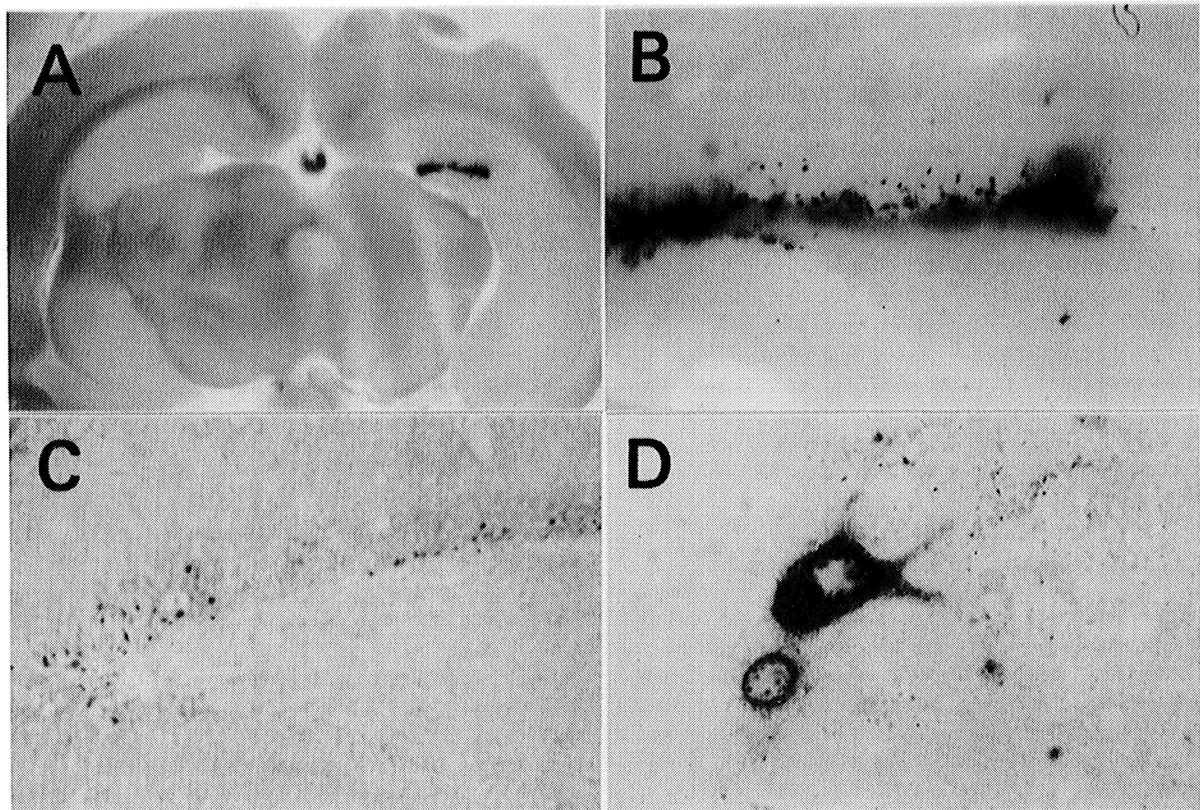

FIG. 2. Transgenic expression of *lacZ* in brain using the Us3::pgC-*lacZ* recombinant. A and B. X-gal staining of 100-μm vibratome sections 2 days after inoculation of 5 μl containing 10^8 pfu/ml of the Us3::pgC-*lacZ* recombinant demonstrates blue cells unilaterally in injected hippocampus. A. Original magnification, 6 ×. B. Original magnification, 50 ×. C. Immunostaining with anti β-gal antibody shows staining confined to neurons in the dentate gyrus of the injected hippocampus (original magnification, 120 ×). D. Sections of 1 μm embedded in plastic show that the vast majority of blue-staining cells had neuronal morphology (original magnification, 1,000 ×).

1987) and recombinants were detected using the blue plaque assay. Briefly, progeny virus from transfections was plated out in suitable dilutions onto 60-mm dishes containing confluent Vero cell monolayers. After virus adsorption, the monolayers were overlaid with methylcellulose supplemented with 2% calf serum and plaques were allowed to develop over 3 days. The monolayers were then washed twice with PBS and overlaid with media containing 0.5% LMP-agarose (2% stock in water; BRL, Gaithersburg, MD) and 150 μg/ml X-gal (2% stock in dimethylformamide; BRL, Gaithersburg, MD). Recombinant viruses could be detected by blue plaque formation within 24 to 48 hrs. These plaques were picked, resuspended in 1 ml of media, and purified by the blue plaque assay three rounds past the last appearance of white plaques in the viral stocks.

Virulence determinations

The median lethal doses (LD_{50}) of viruses after intracerebral inoculation into mice were determined by the methods of Sunstrum *et al.* (1988). Briefly, each of 10 DBA/2 mice (Jackson Laboratories, Bar Harbor, ME) was injected intracerebrally with virus doses ranging from 10^1 to 10^5 pfu in $\log_{10}$ increments. The animals were observed daily for signs of neurologic disease and eventual death due to viral encephalitis.

X-gal staining

Male Sprague Dawley rats (200–250 grams) were anesthetized with chloral hydrate and 1–10 μl of media containing 5×10^8 pfu/ml of virus was injected stereotactically unilaterally into the hippocampus or caudate using the coordinates in the atlas of Paxinos and Watson (1982). At the time of sacrifice the animals were reanesthetized and perfused with 4% paraformaldehyde, 0.4% glutaraldehyde. The brains were removed, post-fixed 2 hr at 4°C in the same solution, and 100-μm vibratome sections cut and reacted with 0.015% X-gal (BRL, Gaithersburg, MD) in 1 *M* Tris pH 8.0, 12.5% DMSO, 53% $K_4Fe(CN)_6$, and 0.14% $K_3Fe(CN)_6$ for 21 hr at 37°C.

In some experiments the animals were anesthetized, and the brains were removed, frozen at −70°C, and 10-μm cryostat sections post-fixed for 10 min with 4% paraformaldehyde followed by X-gal staining as described above. These sections were counterstained with neutral red or cresyl violet as indicated.

Immunocytochemistry

The specificity of the X-gal reaction product was confirmed by immunocytochemistry. Floating 100-μm vibratome sections, fixed as for X-gal reaction, were reacted with a monoclonal antibody against β-galactosidase (Boehringer Mannhein, Indianapolis, IN) at dilutions of 1:50 to 1:500 and developed with avidin-biotin-peroxidase staining (Zymed Labs, San Francisco, CA).

Electron microscopy

Vibratome sections, stained with X-gal as described above, were further fixed overnight in 2% paraformaldehyde, 2% glutaraldehyde, dehydrated with a series of alcohol washes, and embedded in epon araldite. Semithin (1 μm) and ultrathin sections were examined.

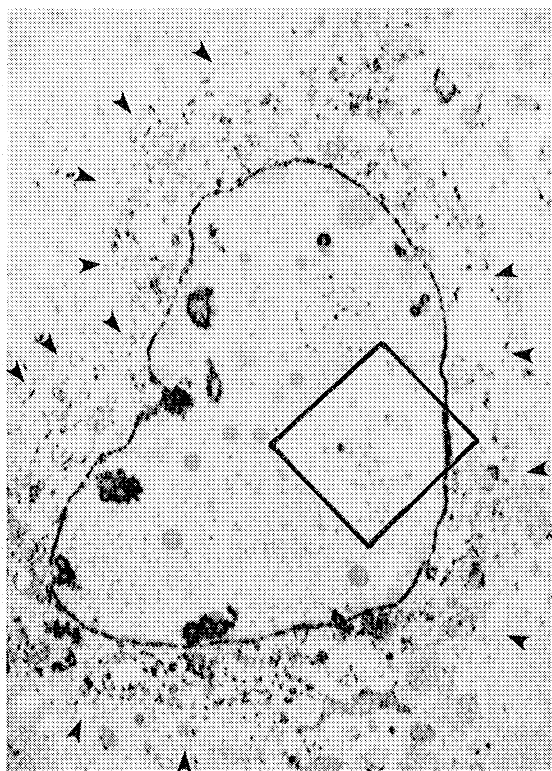

FIG. 3. Subcellular localization of X-gal reaction product and viral recombinant particles. Electron microscopy of hippocampus 2 days post-inoculation with Us3::pgC-*lacZ* shows electron-dense X-gal reaction product along the nuclear membrane and diffusely throughout the cytoplasm of this neuron (outlined with arrowheads). Nonenveloped viral particles are recognizable in the nucleus (enlarged in inset), but are not readily visible within the cytoplasm of the infected cell (original magnification, 14,400 ×).

Nucleic acid isolation

Nucleic acid samples were isolated from brain paste created from minced cerebral hemispheres of infected animals. DNA samples were isolated using the Stratagene DNA Extraction Kit, (Stratagene, La Jolla, CA), according to the protocol provided. Precipitated DNA was resuspended in sterile TE. RNA samples were isolated using the RNAzol B RNA extraction kit (CINNA/BIOTECX Labs, Friendsword, TX) according to the protocol provided. Precipitated RNA was additionally treated with RNase-free DNase (BRL, Gaithersburg, MD), boiled, reprecipitated, and then resuspended in DEPC-treated H_2O. The same animals served as source for both DNA and RNA at a given time point.

PCR analyses

DNA and RNA PCR analyses were performed using three separate Us3::pgC-*lacZ* specific primer pairs. They were (5′ to

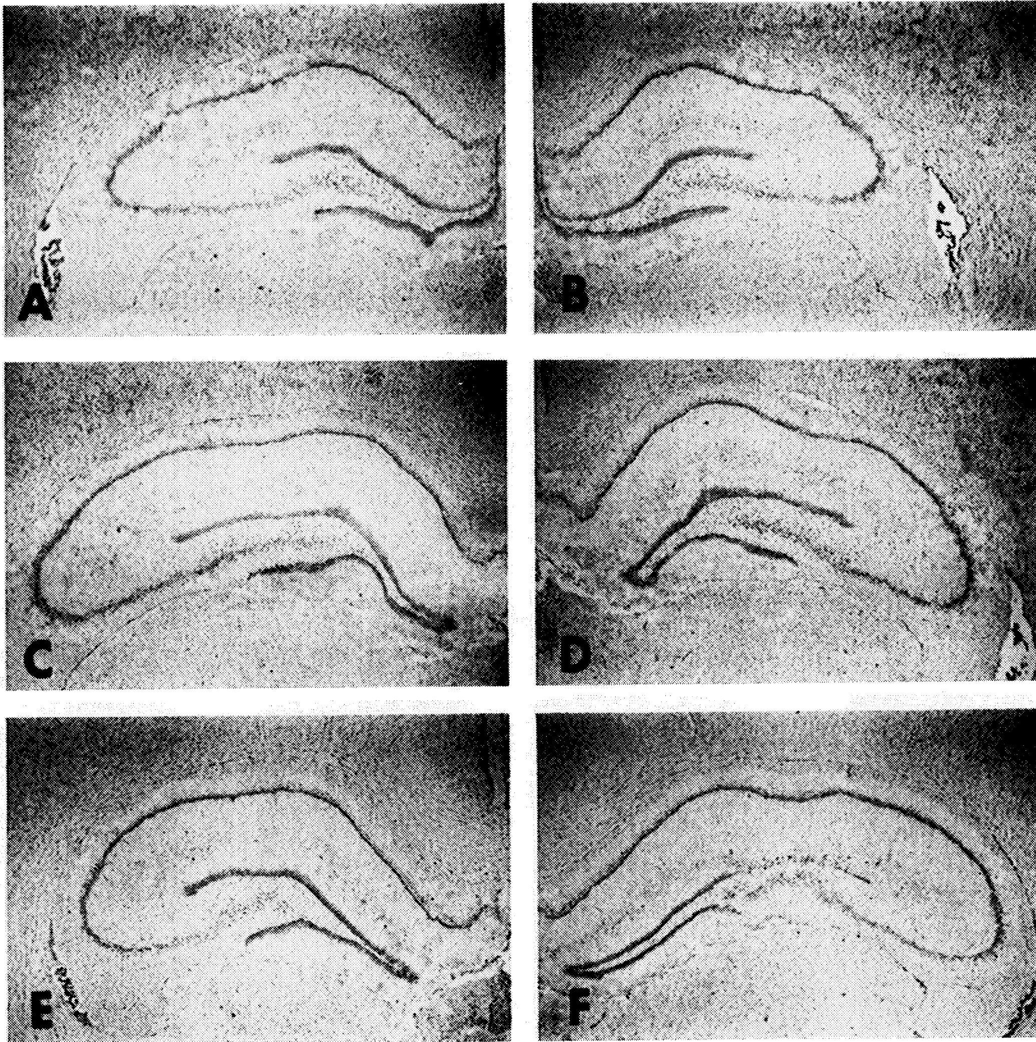

FIG. 4. Assessment of vector neuropathogenicity. Sections of injected (B,C,F) and contralateral (A,D,E) hippocampus 4 weeks after inoculation with 1 μl of 10^8 pfu/ml of the Us3::pgC-*lacZ* recombinant virus. A small area of neuronal cell loss in the dentate gyrus is seen at the point of the needle track in one brain (F), but neuronal architecture is well preserved. These 10-μm sections from the brains of 2 animals, stained with X-gal, and counterstained with neutral red, showed no blue reaction product (original magnification, 12.5 ×).

3′): LAT primer 1 (5′ primer), GAC-AGC-AAA-AAT-CCC-GTC-AG; LAT primer 2 (3′ primer), ACG-AGG-GAA-AAC-AAT-AAG-GG (Lynas *et al.*, 1989); *lacZ* primer 1 (5′ primer), TTG-CTG-ATT-CGA-GGG-GTT-AAC-CGT-CAC-GAG; *lacZ* primer 2 (3′ primer), ACC-AGA-TGA-TCA-CAC-TGC-GGT-GAT-TAC-GAT (Itakura, 1982); gB primer 1 (5′ primer), ATT-CTC-CTC-CGA-CGC-CAT-ATC-CAC-CTT; gB primer 2 (3′ primer), AGA-AAG-CCC-CCA-TTG-GCC-AGG-TAG-T (D.J. Dorney and J.C. Glorioso, unpublished data). PCR analyses were performed using the GeneAmp PCR Core Reagent kit (Perkin Elmer Cetus, Norwalk, CT) and a COY thermocycler (COY Inc., Ann Arbor, MI) according to protocols provided. For DNA PCR amplifications, the specific reaction conditions used were: (i) initial denaturation; 95°C, 4 min; (ii) [anneal 50°C, 1 min, extend 72°C, 1 min denature 94°C, 1 min] × 40 cycles; (iii) final extension 72°C, 6 min; (iv) hold at 4°C until gel electrophoresis. For RNA PCR analyses, the cDNA synthesis step was performed with the appropriate 3′ primer of each primer set (primer #2 of each respective set) and AMV reverse transcriptase (Promega Corp, Madison, WI) using the Promega cDNA synthesis kit (Promega, Madison, WI).

PCR products were analyzed on ethidium bromide stained 3% NuSieve GTG agarose gels (FMC INC, Rockport, ME). Specificity of the bands generated by PCR was confirmed following rapid alkaline transfer (Hatcher *et al.*, 1990) to nylon membranes (Schleicher & Schuell, Keene, NH) using ^{32}P-labeled probes. Riboprobes capable of detecting each PCR product were generated using the Riboprobe Gemini System (Promega, Madison, WI). Template DNAs used in the synthesis were produced by PCR amplification of Us3::pgC-*lacZ* viral DNA using three additional 3′ primers identical in sequence to the LAT primer #2, gB #2, and *LacZ* #2, but which

also included the bacteriophage Sp6 promoter (Haggi *et al.*, 1988).

RESULTS

To assess the potential utility of a Us3 mutant virus as a gene transfer vector for the CNS, a virus recombinant designated Us3::pgC-*lacZ* was constructed in which the *lacZ* gene under control of the viral late gene promoter of glycoprotein C (gC) was introduced into the Us3 locus thereby interrupting the production of a functional Us3 gene product (Fig. 1). This virus recombinant produced large amounts of β-galactosidase as a late gene product during lytic infection and virus plaques in cell culture stained blue following X-gal agarose overlay (data not shown). Therefore, expression of *lacZ* following intracranial inoculation should demonstrate the extent of virus spread and define the types of cells permissive for viral replication. Failure to detect β-gal upon introduction of virus into the brain should indicate that infected cells could not support viral late gene expression and consequently infectious progeny could not be produced. Continued expression of *lacZ* would indicate a persistent active infection. If *lacZ* expression were transient and limited to the site of inoculation, latency might also be limited to a particular set of neurons at that site. Our previous studies have demonstrated the ability of wild-type HSV-1 (KOS) to replicate in neuronal cells of the limbic system with rapid development of encephalitis following intracerebral inoculation of mice (Sunstrum *et al.*, 1988; Chrisp *et al.*, 1989). Failure to disrupt the well-defined neuronal architecture of the hippocampus would therefore provide a convenient, easily defined measure of the pathogenic effects of the virus following intracranial inoculation.

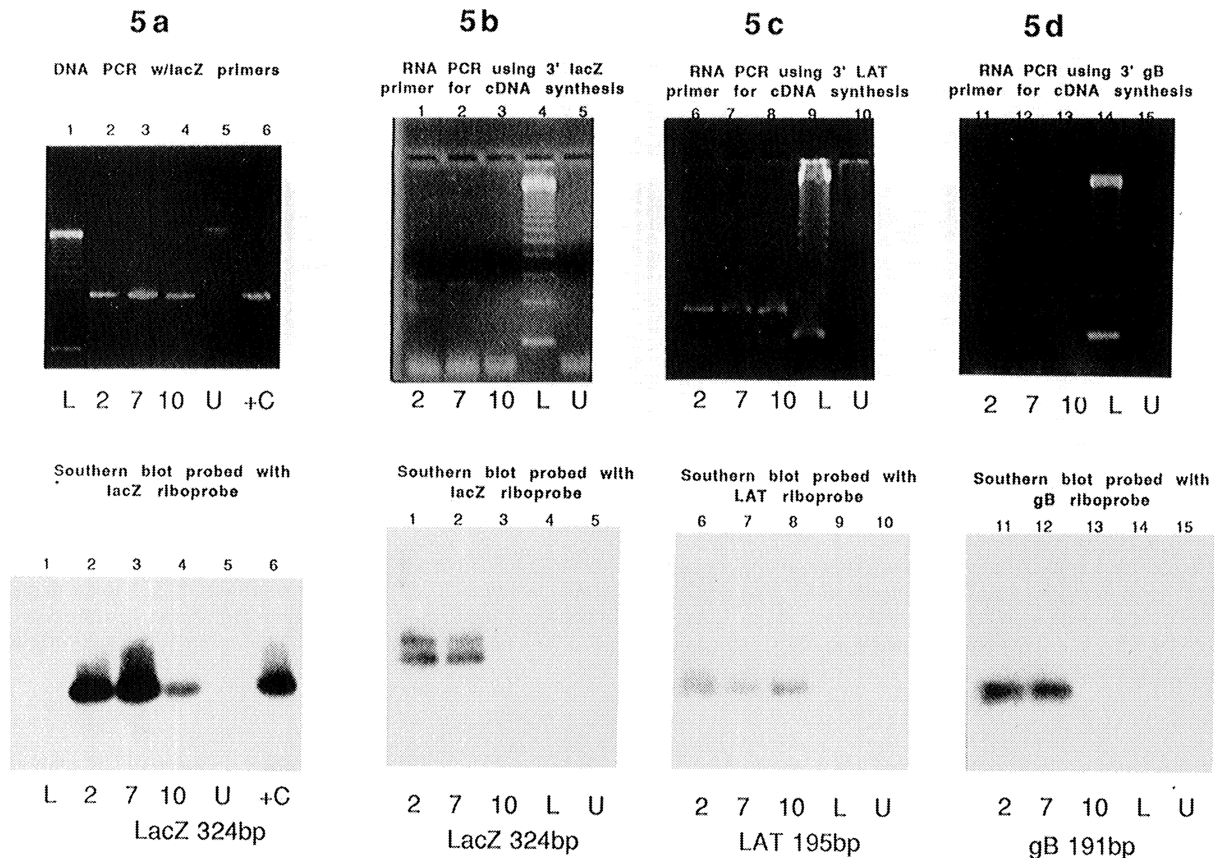

FIG. 5. PCR analysis of viral-specific DNA and RNA isolated from rat CNS following intracranial inoculation with Us3::pgC-*lacZ*. a, top. Gel electrophoresis of PCR products generated from 1.0 μg of total DNA isolated from brain amplified using the *lacZ* primer pair (see Materials and Methods). Legend: 123-bp ladder (L); 2 days p.i. (2); 7 days p.i. (7); 10 months p.i. (10); uninfected control (U); and positive control (+C) using Us3::pgC-*lacZ* viral DNA as template. a, bottom. Southern blot of the same samples probed with ^{32}P-labeled *lacZ*-specific riboprobe. b–d, top. Gel electrophoresis of PCR products generated from 1.0 μg of RNA isolated from brain amplified 40 cycles with the primer pairs for *lacZ* (b), LAT (c), and gB (d). Legend: 2 days p.i. (2); 7 days p.i. (7); 10 months p.i. (10); 123-bp ladder (L); and uninfected control (U). b–d, bottom. Southern blots of the same gels probed, respectively, with ^{32}P-labeled riboprobes for *lacZ*, LAT, and gB. A second, higher-molecular-weight band was reproducibly seen in Southern blots of RNA-generated, but not DNA-generated, *lacZ* PCR amplifications. The origin of this second band has not been determined; however, it may represent spurious hybridization of one primer of the *lacZ* primer pair to a region approximately 50 bp from the actual site of hybridization.

In tissue culture, the Us3::pgC-*lacZ* recombinant replicated virtually as well as wild-type virus, but *in vivo* showed a 1,000-fold increase in LD_{50} by direct intracranial inoculation of DBA/2 mice (Table 1). These findings are consistent with data from another Us3-defective mutant (Meignier *et al.*, 1988). Additionally, Us3::pgC-*lacZ* displayed impaired replication in serum-starved tissue culture cells arrested in G_0 (A.M. Bonneau, J. Chafouleas, J.C. Glorioso, unpublished data). To assess the behavior of this virus in the rat CNS, 1–10 μl of media containing 10^8 pfu/ml of virus was stereotactically injected unilaterally into the hippocampus or into hippocampus and contralateral caudate. A marked blue reaction product indicating β-gal production was seen in the injected hippocampus from 1 to 5 days after inoculation, most prominent at 2 and 3 days (Fig. 2A,B) extending throughout the dorsal hippocampus, at least 3 mm in a rostral-to-caudal direction. Staining disappeared completely by day 10. Few blue neurons were detected in caudate indicating that the virus replicated poorly in this brain region. In cryostat sections counterstained with neutral red, blue cells were confined to the dentate gyrus of the injected hippocampus. Animals injected with an equivalent number of pfu of wild-type virus (KOS) showed an extensive inflammatory infiltrate at 2 days, but no evidence of blue reaction product upon X-gal staining.

The presence of β-gal was confirmed by immunocytochemistry using alternate floating vibratome sections immunostained with a monoclonal antibody specific for β-gal. The immunostaining pattern was comparable to that seen with X-gal (Fig. 2C). This result demonstrates that the cytochemical reaction product results from β-galactosidase gene expression from the virus genome. To determine the identity of the cells supporting β-galactosidase expression, semithin sections prepared from the vibratome sections were examined. The vast majority of cells containing X-gal reaction product were neurons (Fig. 2D) defined by their location in the dentate gyrus and appearance characteristic of granule cells, or by the presence of typical neuronal processes. Electron microscopic examination of ultrathin sections confirmed that the electron-dense X-gal reaction product was localized predominantly in neurons (Fig. 3), This result is in agreement with the fact that a late gene promoter is responsible for driving *lacZ* expression. Thus, both β-gal activity and viral particles are observed in the same cell. Few enveloped particles were observed in the cytoplasm, however, supporting the limited replication of the mutant virus.

Stereotactic inoculation of either 5 μl of media containing 10^7 pfu/ml wild-type HSV-1 (KOS) or a similar dose of a glycoprotein C deletion mutant, gC^- 39, described earlier (Sunstrum *et al.*, 1988; Chrisp *et al.*, 1989) caused death at 2–3 weeks post inoculation in two-thirds of the injected rats. Surviving animals, sacrificed at 3 weeks to 3 months after inoculation, often showed focal encephalomalacia as a result of brain destruction by those viruses of unattenuated pathogenicity. In contrast, the Us3::pgC-*lacZ* recombinant showed only limited disruption of the normal neuronal architecture in animals examined 4 weeks to 20 months after inoculation (Fig. 4).

To determine whether the Us3::pgC-*lacZ* vector persisted in the brain, the PCR (Saiki *et al.*, 1985) was used to amplify viral-specific DNA and RNA isolated from the brain of rats sacrificed from 2 days to 10 months after inoculation. PCR analyses were performed using three different primer pairs (Fig. 5). Using Us3::pgC-*lacZ* viral DNA as template in 40 cycles of PCR amplification, the primer pairs generate bands of the following lengths: LAT = 195 bp; *lacZ* = 324 bp; gB = 191 bp (data not shown). The LAT primer set has been used previously to detect LAT RNA in latently infected trigeminal ganglia (Lynas *et al.*, 1989). Each primer pair generated an appropriate length band using template DNA isolated from animals sacrificed 2 days, 7 days, and 10 months p.i., but not from DNA isolated from uninoculated controls. An example showing amplification of viral DNA from brain with the *lacZ* primer pair is shown (Fig. 5A, top). Specificity of the bands was confirmed by Southern blot analysis (Fig. 5A, bottom) (Haggi *et al.*, 1988).

To evaluate viral-specific gene expression in brain, RNA isolated at various times post injection was reacted with the appropriate 3′ primer in the presence of AMV reverse transcriptase (RT) to generate cDNA, which was then used as the template for DNA PCR amplifications (Kawasaki *et al.*, 1988). Because the LAT 3′ primer is antisense with respect to LAT but sense with regards to ICPO, this primer will only generate cDNAs to LAT. An additional primer pair, specific for the α2 isoform of the cellular (Na,K)-ATPase gene, included as an internal control, confirmed that RNA isolated was intact (data not shown). Samples lacking AMV RT, included as controls for the efficiency of the DNase treatment, failed to generate PCR product. LAT RNA was detected in brains of animals sacrificed 2 days, 7 days, and 10 months post-inoculation, consistent with expression of LAT during lytic viral replication and from latent viral genomes (Fig. 5C). mRNA for *lacZ* (Fig. 5B) and gB (Fig. 5D) were only detected in RNA from the 2-day and 7-day post-inoculation brains, but were not detected in RNA from the 10-month post-inoculation brains, consistent with the shut-off of lytic gene expression during latency (Stevens, 1989).

DISCUSSION

These experiments were designed to determine whether an attenuated replication-competent HSV vector could be used to express a foreign gene product and establish latency in a specific region of the brain. Previous studies had demonstrated the potential utility of HSV-based vectors to express foreign genes in neurons *in vitro* and *in vivo*. The expression of the human HPRT mRNA in mouse brain from a replicating tk^- derivative of HSV-1 has been reported, although long-term expression and pathogenesis of the vector were not examined (Pallela *et al.* 1989). HSV-packaged amplicons have also been used as gene transfer vectors *in vitro* (Geller and Breakefield, 1988; Geller and Freese, 1990) and replication-incompetent HSV vectors in neurons that project to the periphery (Dobson *et al.*, 1990). Due to the limitations of these systems, they do not represent ideal gene transfer vehicles for the CNS. A prerequisite for a useful gene transfer vector is foreign gene expression in a significant number of neurons in discrete brain regions *in vivo*, which could be achieved through use of a replicating highly attenuated mutant.

Our results indicate that stereotactic injection of an attenuated virus directly into the brain provides a useful means of targeting an HSV-mediated vector to a specific brain region.

With the Us3-defective virus, *lacZ* expression was transient and limited to the injection site. Because *lacZ* expression in this recombinant is driven by a viral late gene (gC) promoter, this result implies that viral replication was transient and limited to the injection site. This might represent a failure of viral transport to distant brain sites, or a failure to replicate following axonal transport. A second correlate of decreased neurovirulence is limited cell-to-cell spread in the hippocampus, resulting in little apparent disruption of normal brain architecture. Although a detailed analysis was not carried out, electron microscopy of the infected hippocampus 2 days post-injection showed an abundance of nonenveloped nucleocapsids in the nucleus with few enveloped viral particles in the cytoplasm. Additionally, infectious particles were recovered from brain paste up to 4 days post-inoculation but not at times greater than 7 days (data not shown). Together, these findings suggest that infection by a Us3-defective virus is self-limited.

The expression of *lacZ* within neurons in the brain shows that the HSV gene transfer vector can be used to express a foreign gene *in vivo* in the rat CNS. The PCR data indicate that the vector persists in the brain and continues to express the LAT transcript, the hallmark of latency. These findings provide evidence for *in vivo* LAT promoter activity in brain in the absence of expression of either viral or recombinant genes driven by lytic cycle promoters after direct intracranial inoculation. In a previous study, Chiocca *et al.* (1990) performed stereotactic CNS inoculations using defective ($ICP4^-$) and attenuated ($ICPO^-$) mutants possessing *lacZ* reporter gene cassettes driven by lytic gene promoters (ICP6 and ICPO, respectively). Foreign gene expression was transient and the attenuated mutant displayed limited virus spread without causing death of the inoculated animal, similar to our results with the Us3 mutant. Neither long-term virus-mediated pathology nor long-term persistence latent virus were determined. Attempts to reactivate the Us3 mutant virus from brain by cocultivation have proven unsuccessful, in agreement with the results of Deatly *et al.* (1988) who were unable to reactivate wild-type virus from the CNS after establishement of latency by peripheral inoculation. Attempts to reactivate the Us3 mutant virus from the trigeminal ganglia of rats infected by corneal scarification have also failed, suggesting that the Us3 gene product may play a role in virus reactivation from the PNS (L. Sternberg, unpublished). The DNA PCR analyses showed that viral sequence representing the LAT, gB, and Us3 (*lacZ*) loci were present in latently infected 10-month animals. Although we have not demonstrated the existence of the entire viral genome in the latent state, the expression of LAT in brain suggests that other genes under the control of the LAT promoter could be expressed long-term with this vector. Recent experiments using the *lacZ* gene under control of an HSV-1 LAT promoter have confirmed these predictions (W.F. Goins, R.L. Hendricks, L.R. Sternberg, D.J. Fink, and J.C. Glorioso, in preparation). Thus, by placing foreign gene expression under the control of the HSV LAT or neuronal cell-specific promoters, this vector may be useful for the transfer and continuous expression of foreign genes in the brain. Similarly engineered vectors are currently in use in our laboratory for introducing a variety of cellular genes into the brain for studies of fundamental aspects of CNS biology and gene expression.

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