doi:10.1016/j.ymthe.2005.06.478

ARTICLE

Stable Levels of Long-Term Transgene Expression Driven by the Latency-Associated Transcript Promoter in a Herpes Simplex Virus Type 1 Vector

B. K. Berges,[1] J. H. Wolfe,[2,3,4] and N. W. Fraser[1,*]

[1]*Department of Microbiology,* [2]*Department of Pediatrics, School of Medicine, and* [3]*Center for Comparative Medical Genetics, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*
[4]*Stokes Institute, Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA*

**To whom correspondence and reprint requests should be addressed at the Department of Microbiology, University of Pennsylvania, 319 Johnson Pavilion, Philadelphia, PA 19104-6076, USA. Fax: +1 215 898 3849. E-mail: nfraser@mail.med.upenn.edu.*

Available online 24 August 2005

Previous gene transfer studies of the herpes simplex virus type 1 (HSV-1) using the latency-associated transcript (LAT) promoter have reported a decrease in transgene expression in the brain over time, but the extent of this decrease has not been measured and it is unknown if expression eventually stabilizes. We examined LAT promoter-mediated transgene expression in the mouse brain for 1 year following intracranial injection with a HSV-1 vector expressing human β-glucuronidase (GUSB). The vector genome copy number remained stable from 2 to 52 weeks. Quantitative reverse transcriptase PCR detected a peak of LAT intron expression at 2 weeks (corresponding to the end of the acute phase of viral infection), followed by stable expression during latency (13–52 weeks). The number of GUSB-positive cells also had a peak in the acute phase and then was stable during latency (13–52 weeks). GUSB enzymatic activity was maintained at 11% of normal at 6 and 12 months, indicating that the LAT promoter is capable of driving stable transgene expression in the brain.

Key Words: HSV-1, gene transfer, viral vectors, virus latency, latency-associated transcript, central nervous system, β-glucuronidase

INTRODUCTION

The ability of wild-type herpes simplex virus type 1 (HSV-1) to infect postmitotic neurons and remain transcriptionally active during latency in the nervous system is well established [1,2]. These properties have stimulated interest in developing HSV-1 as a vector for gene therapy of diseases of the nervous system [3]. Although progress has been made in HSV-1 vector development, there are still challenges to be overcome, such as the inability to provide stable transgene expression in the brain [4–10].

For an effective gene therapy treatment of a genetic disorder, long-term expression of the transgene is essential. Some viral vectors (e.g., adeno-associated virus and lentivirus) have been shown to provide stable, long-term transgene expression [11,12]. However, vector integration of retrovirus has been linked to insertional mutagenesis and cancer in humans [13]. HSV-1 does not integrate into the host chromosome [14], but the viral genome is stably maintained in neuronal cells [10,15].

HSV-1 latent infection is accompanied by transcription from the latency-associated transcript (LAT) gene [1,16–18]. LAT is the only actively transcribed gene during latency [16,17]. Introduction of exogenous promoters into HSV-1 vectors results in very little expression during latency [6,7]. Attempts to use HSV-1 promoters other than LAT also have been ineffective for latent expression [4,5]. Thus, most current HSV-1 gene transfer studies use the LAT promoter to drive transgene expression.

Use of the LAT promoter in the brain has resulted in relatively high levels of transgene expression at early time points [19,20]. LAT promoter expression in the brain has been reported to decrease slowly over time when longer time points were examined—up to 6 [10] or 12 months [9]. As the vector DNA levels were stable at longer time

Abbreviations used: GUSB, β-glucuronidase, Q-RT-PCR, quantitative reverse transcriptase polymerase chain reaction, Q-PCR, quantitative polymerase chain reaction, ISH, *in situ* hybridization, LAT, latency-associated transcript, HSV-1, herpes simplex virus type 1, MPS VII, mucopolysacchavridosis type VII.

1525-0016/$30.00

doi:10.1016/j.ymthe.2005.06.478

points, it was concluded that the LAT promoter failed to drive stable expression [10]. However, the evidence was largely based upon semiquantitative methods—the number of positive cells following a histological stain decreased over time. Counting of cells positive for a histological stain does not provide a quantitative measure of gene expression for three reasons: subjectivity must be used when scoring, cells may express below the limit of detection, and histologic or immunologic staining reveals only the presence/absence of positive cells. Thus, while there is evidence that LAT promoter activity decreases over the first few months of infection, there are few quantitative data on the extent of the decrease and it is unknown if LAT promoter activity is eventually lost entirely. HSV-1 gene transfer studies of the sensory ganglia have reported relatively high levels of long-term transgene expression from the LAT promoter [21,22]. However, LAT expression differs in the brain and the sensory ganglia, which may explain the difference in gene transfer results in these two sites [15,18,23].

As most studies of HSV-1 latency have been in the sensory ganglia, relatively little is known about the transcriptional activity of the LAT promoter in the brain. LAT expression has been documented in the brain of the mouse [18,24,25], rat [5,10,15], and dog [26]. However, to date there has been no report of long-term measurement of LAT production in the brain for either wild-type virus or attenuated vector strains. LATs have been detected in the rat brain for up to 10 months [5], but the technique was not quantitative. Quantitative LAT measurements have been done after 8 weeks [15]. A histological analysis of LATs in the brain was performed after 11 weeks using *in situ* hybridization (ISH) [10].

Mucopolysaccharidosis type VII (MPS VII) is a genetic disorder caused by a deficiency in the enzyme β-glucuronidase (GUSB). Lysosomal storage associated with MPS VII affects the brain and results in mental retardation [27], thus we are testing HSV-1 vectors for gene transfer of GUSB in the brain [19,26,28,29]. Since the MPS VII mouse has a shortened life span (averages 5–6 months) [30], for this study we have used a previously described heat-inactivation model to study GUSB production in the unaffected C3H mouse [31,32].

We have examined a HSV-1 vector (1716-LAT-hGUSB) for its ability to drive long-term expression from the LAT promoter in the mouse brain following intracranial injection using quantitative assays for RNA transcription and reporter enzymatic activity. The data show that there is a transient peak of genome, RNA transcription, and GUSB-positive cells during the acute phase of viral infection. GUSB enzymatic activity is stable from 6 to 12 months. After latency is established, LAT intron expression is stable and GUSB levels were well into the therapeutic range [11,33,34]. The data indicate that the LAT promoter is able to mediate stable, long-term expression of transgenes.

RESULTS

Characterization of Recombinant Virus 1716-LAT-hGUSB

We made the 1716-LAT-hGUSB vector by homologous recombination of a human GUSB cDNA into the LAT gene of HSV-1 strain 1716 [35]. 1716-LAT-hGUSB is identical to a previously published vector with the exception that the human GUSB cDNA was inserted in place of the rat GUSB cDNA [19]. The human GUSB is resistant to heat denaturation, allowing it to be detected against the mouse endogenous activity [31,32,36]. The insertion site is shown in Fig. 1A. We confirmed the correct genomic structure by the difference in two predicted *Bam*HI genomic fragments between 1716 and 1716-LAT-hGUSB (the LAT gene occurs in two copies): fragments of 10.1 and 8.9 kb in strain 1716 were absent in 1716-LAT-hGUSB and replaced with fragments of 6.7 and 5.5 kb (see Fig. 1B) due to *Bam*HI sites in the human GUSB cDNA. Southern blotting confirmed that the 6.7- and 5.5-kb fragments contained the human GUSB cDNA sequence (Fig. 1C).

We used a murine fibroblast GUSB-deficient cell line (3521) [37] to measure GUSB production by 1716-LAT-hGUSB *in vitro*. We infected 3521 cells with 1716-LAT-hGUSB or 1716, or mock infected them, at an m.o.i. of 3 ($n = 6$ separate infections). After 20 h, we harvested the cells and performed a GUSB fluorescence enzymatic activity assay (data not shown). GUSB levels after infection with 1716-LAT-hGUSB were significantly higher ($P < 0.05$) than infection with 1716 or mock infection.

In Situ Hybridization Indicates a Drop in LAT Expression over Months

We injected mice unilaterally in the right caudate putamen with 1716-LAT-hGUSB or 1716 and analyzed them during acute and latent phases of viral infection. We performed ISH with a LAT-specific riboprobe on tissues from various time points (2, 8, 26, 52 weeks; $n = 4$) to detect cells that expressed LATs during latency. We detected LAT expression at 2 and 8 weeks but not at 26 or 52 weeks (Fig. 2). We detected no LAT expression in uninjected tissues (Fig. 2I). Most LAT-positive cells were found in the midbrain, cerebral cortex, and brain stem. Very few LAT-positive cells were detected at the injection site (data not shown) as has been reported previously with injection of HSV-1 vectors into the caudate putamen [8,10]. Both the duration of expression and the distribution of LAT-positive cells were very similar comparing 1716-LAT-hGUSB to 1716.

Vector Genome Levels Remain Stable over Time

We used a real-time PCR assay to measure the vector genome levels in the midbrain (Fig. 3A), as this was consistently the site of highest transduction at all time

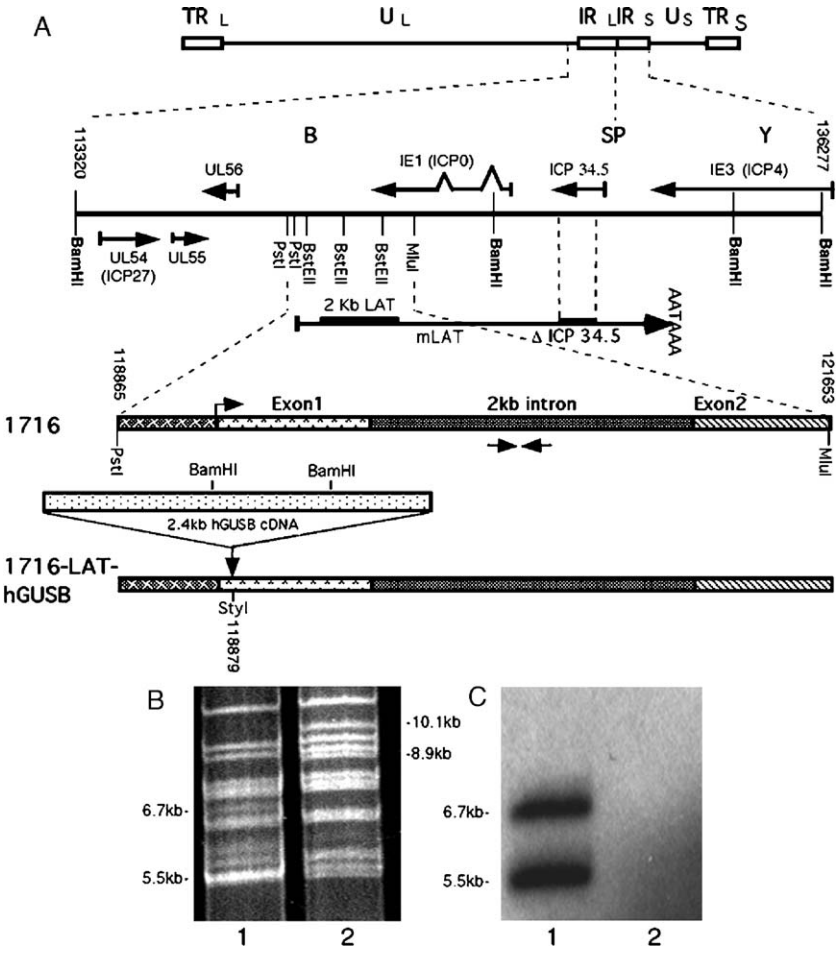

FIG. 1. Analysis of the genome structure of the 1716-LAT-hGUSB vector. (A) The entire HSV-1 genome is represented, with the unique long and short regions (U_L and U_S) and the long and short repeat regions (TR_L, IR_L, IR_S, TR_S) labeled. Below, a detailed map of the genes in the *Bam*HI B, SP, and Y fragments is shown (the LAT gene lies within a repeat region and is diploid). A *Pst*I/*Mlu*I fragment of the LAT gene (118,865–121,653) is shown in greater detail, along with the insertion site of the 2.4-kb human GUSB cDNA into the first *Sty*I site of LAT exon 1 (118,879). Sites of primers used for Q-RT-PCR of LAT intron are also indicated. (B) A *Bam*HI digest was performed on purified viral DNA (2 μg) from 1716-LAT-hGUSB (lane 1) and 1716 (lane 2), then fragments were separated on a 0.8% agarose gel. (C) DNA was transferred to a nitrocellulose membrane and probed with a 1.0-kb nonradioactive fluorescein-labeled human GUSB probe specific to the 5′ end of the human GUSB cDNA. The probe hybridized to the fragments predicted to contain the 5′ end of human GUSB (6.7 and 5.5 kb).

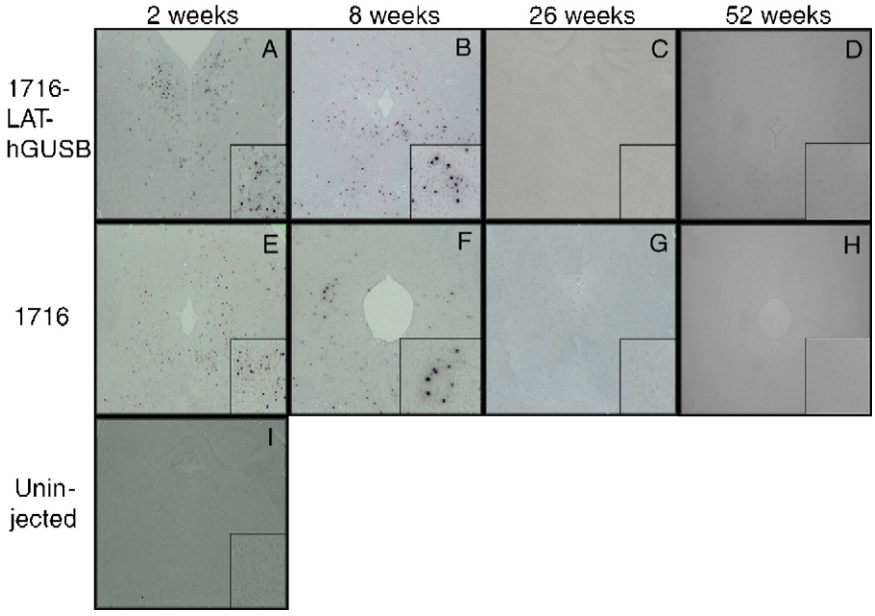

FIG. 2. RNA *in situ* hybridization toward LAT in the midbrain. Cells expressing LATs were detected using a riboprobe specific to LAT exon 1, the intron, and the 5′ end of exon 2. Tissues latently infected with (A–D) 1716-LAT-hGUSB or (E–H) 1716 or (I) uninjected are shown (n = 4). (A, E) 2, (B, F) 8, (C, G) 26, and (D, H) 52 weeks are shown.

doi:10.1016/j.ymthe.2005.06.478

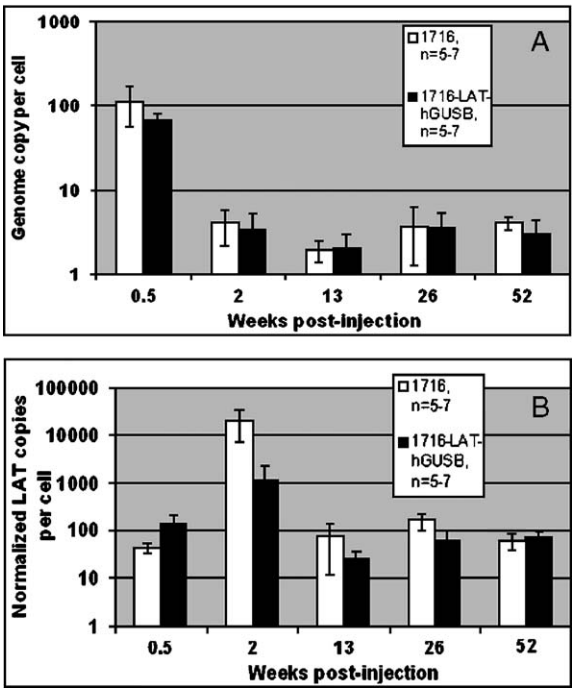

FIG. 3. Quantitation of vector-specific nucleic acids in the midbrain. (A) Vector genome copy number per cell was determined using real-time PCR. A standard curve with known dilutions of vector genome was used to determine the levels in unknown samples, and samples were normalized by total DNA input (100 ng). Primers specific for the viral thymidine kinase gene were used, and nonspecific amplification in uninfected tissues was minimal (0.097 ± 0.02) (±SE shown). (B) Total RNA was extracted and treated with DNase. cDNAs were prepared and real-time PCR was used to detect LAT intron sequence. Samples were normalized by measurement of murine GAPDH. No amplification was detected in uninfected samples or those for which reverse transcriptase was left out of the cDNA synthesis reaction (±SE shown).

points of the study. Infection with 1716-LAT-hGUSB ($n \geq 5$ per time point) resulted in 68.1 ± 14.4, 3.4 ± 1.8, 2.1 ± 0.9, 3.6 ± 1.7, and 2.9 ± 1.4 genome copies per cell (at 0.5, 2, 13, 26, and 52 weeks, respectively). Infection with 1716 ($n \geq 5$ per time point) resulted in 111.9 ± 56.2, 4.1 ± 1.9, 1.9 ± 0.6, 3.7 ± 2.5, and 4.0 ± 0.8 genome copies per cell (at 0.5, 2, 13, 26, and 52 weeks, respectively). We detected nonspecific amplification in uninfected animals ($n = 7$), but this amplification was minimal (0.097 ± 0.02 genome copies per cell).

Quantitative RT-PCR Shows Stable, Latent Phase Expression of LAT Intron in the Brain

To quantify expression of LAT, we isolated total RNA from the midbrain. We used quantitative reverse transcriptase PCR (Q-RT-PCR) to measure LAT expression (Fig. 3B) using primers within the stable 2-kb LAT intron (see Fig. 1 for genomic location). Infection with 1716-LAT-hGUSB ($n \geq 5$ per time point) resulted in 135.1 ± 83.3, 1190 ± 1159, 24.8 ± 11.9, 62.8 ± 40.0, and 70.4 ± 23.7 LAT copies per cell (at 0.5, 2, 13, 26, and 52 weeks, respectively). Infection with 1716 ($n \geq 5$ per time point) resulted in 43.2 ± 10.6, 20,248 ± 13,564, 76.7 ± 64.7, 167 ± 65.5, and 61.1 ± 23.1 LAT copies per cell (at 0.5, 2, 13, 26, and 52 weeks, respectively). We detected no amplification in uninfected animals ($n = 7$). The ratio of LAT copy per vector genome copy was 2, 352, 11, 17, and 15 for 1716-LAT-hGUSB and 0.4, 4725, 31, 18, and 12 for 1716 (at 0.5, 2, 13, 26, and 52 weeks, respectively).

GUSB-Positive Cells Are Detectable for up to 12 Months

A previously described heat-inactivation model allows for transduction with human GUSB-expressing vectors in

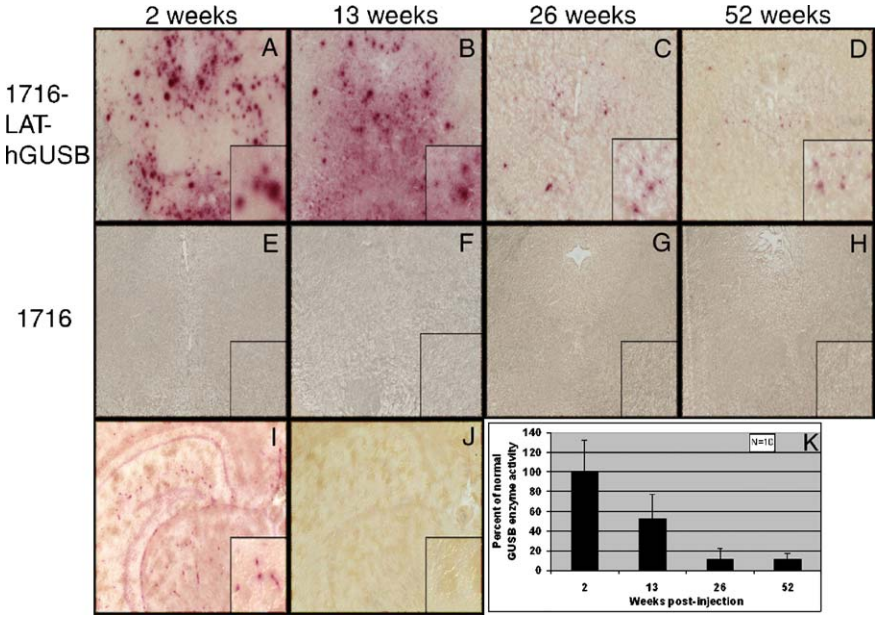

FIG. 4. Analysis of GUSB activity in the midbrain. (A–H) Cells expressing GUSB activity were detected by histochemistry. Tissues latently infected with (A–D) 1716-LAT-hGUSB or (E–H) 1716 were assayed at 2 weeks (A, E), 13 weeks (B, F), 26 weeks (C, G), and 52 weeks (D, H) ($n = 4$). (I, J) Untreated brains ($n = 5$) were also stained either (J) with or (I) without prior heat treatment. (K) Midbrain homogenates were heat inactivated and assayed for GUSB enzymatic activity. Percentage of normal corrected GUSB activity is shown. GUSB activity was significantly higher ($P < 0.05$) than background at all time points (±SE shown).

mice that express endogenous murine GUSB activity [31]. The C3H strain produces low levels of GUSB activity, lives a normal life span, and has unaffected lysosomal storage [38]. In previous studies, it was shown by a histochemical stain that postmortem heat inactivation of sectioned C3H HeOuJ brain tissues resulted in undetectable GUSB activity [32,36]. We have confirmed this result with the C3H HeJ mouse, for which staining is detectable in the normal brain expressing GUSB but is undetectable after heat inactivation (Figs. 4I and 4J).

We performed a histochemical stain to detect GUSB-positive cells in infected animals. Following injection with 1716-LAT-hGUSB, GUSB-positive cells could be found at every time point examined, up to 52 weeks (Figs. 4A–4D). Wild-type C3H GUSB activity is shown in Fig. 4I for comparison purposes. Tissues injected with 1716 did not exhibit GUSB-positive cells at any time point (Figs. 4E–4H). The most positive cells were found in the thalamus, hypothalamus, and brain stem; positive cells were detected in all of these regions through 52 weeks (data not shown). We noted that individual positive cells had a smaller area of staining at later time points (see Figs. 4C and 4D). A comparable distribution of GUSB staining has been noted previously with a similar HSV-1 vector injected into the same site [19] and was attributed to axonal transport of vector since these regions are connected via axonal pathways to the injection site.

We counted GUSB-positive cells in the midbrain (Table 1). Significantly higher ($P < 0.001$) numbers of cells were found at 2 weeks (142 ± 9.5) compared to all later time points (42.5 ± 10, 41.3 ± 7.3, and 43.7 ± 10 at 13, 26, and 52 weeks, respectively). This may represent the difference in LAT gene activity from acute to latent infection. The number of GUSB-positive cells counted at 13, 26, and 52 weeks was not statistically different ($P > 0.6$).

GUSB Enzymatic Activity Level is Stable in Long-Term Transduced Brains

We dissected out midbrains for an assay of GUSB enzymatic activity. We measured GUSB activity in the untreated C3H mouse brain without heat inactivation to determine normal brain expression levels (13.8 nmol/h/mg; $n = 7$). With heat inactivation, levels of GUSB activity after infection with 1716 were not significantly different from those found in untreated animals (data not shown). GUSB activity in tissues after infection with 1716-LAT-hGUSB ($n = 10$) was significantly higher than background (1716-injected animals) at all time points ($P < 0.05$). Percentage of normal GUSB activity is shown in Fig. 4K. At 2 weeks, 100.9% of normal GUSB activity was present, and 52.3, 11.4, and 11.6% of normal GUSB activity was present at 13, 26, and 52 weeks, respectively.

Discussion

In this study, we have demonstrated that the HSV-1 LAT promoter is capable of mediating long-term transgene expression in the mouse brain at the level of both RNA transcripts and enzymatic activity. The LAT intron, the hallmark of HSV-1 latency, was detectable by Q-RT-PCR at stable levels. The level of reporter enzyme expression found during latency stabilized at >10% of wild-type levels.

The Vector Genome Is Stably Maintained

A reduction in LAT expression occurred from 2 to 13 weeks, and a reduction in GUSB activity was seen from 2 to 26 weeks. However, we found that the vector genome was stably maintained at these time points (Fig. 3A), consistent with previous reports [10,15]. There was no significant difference between genome levels of 1716 or 1716-LAT-hGUSB at any time point. Thus, elimination of transduced cells (e.g., by apoptosis or a host immune response toward latent vector or foreign human GUSB) did not explain the reduction in expression of either LAT or GUSB.

LAT Expression Is Stable from 13 to 52 Weeks in the Mouse Brain

The transcriptional activity from the LAT promoter was determined by both Q-RT-PCR and ISH to detect LATs. LAT intron levels peaked at 2 weeks by Q-RT-PCR (Fig. 3B), which likely represents residual acute phase expression, as strain 1716 is known to replicate in certain cells of the brain, notably ependymal cells [39]. By ISH a LAT signal was detected at 2 and 8 weeks but was undetectable

TABLE 1: Number of GUSB-positive cells in the midbrain at various times postinjection into the caudate putamen

Virus	Weeks postinjection	Brain sections counted	Total GUSB-positive cells	GUSB$^+$/section (mean ± SE)
1716	[a]	40	0	0
Uninjected	N/A[b]	23	0	0
1716-LAT-hGUSB	2	30	4263	142.1 ± 9.5
1716-LAT-hGUSB	13	32	1319	42.5 ± 9.9
1716-LAT-hGUSB	26	41	1653	41.3 ± 7.3
1716-LAT-hGUSB	52	14	612	43.7 ± 9.9

Brains were sectioned, heat-treated, and stained for GUSB activity ($n = 4$). GUSB-positive cells in the thalamus and hypothalamus were counted. N/A, not applicable.
[a] Brain sections from 2, 13, 26, and 52 weeks postinjection were counted.
[b] Uninjected mice were age-matched to the same time points as injected mice.

doi:10.1016/j.ymthe.2005.06.478

at 26 or 52 weeks (see Fig. 2). Detection of LATs at 8 weeks but not at 26 weeks indicates a reduction in LAT expression during the early weeks of the latent phase. However, LAT intron levels could be detected by Q-RT-PCR and were stable from 13 to 52 weeks. These data suggest that the Q-RT-PCR assay is more sensitive than the ISH assay employed. Taken together, the results of Q-RT-PCR and ISH indicate that LAT expression experiences a peak early in latency before later stabilizing (sometime between 8 and 13 weeks) in the mouse brain.

Long-Term Expression at the Level of Reporter Enzyme

Earlier reports examining long-term expression with HSV-1 vectors relied heavily upon histological techniques. Although many positive cells were present at early times postinfection, few were detectable at later time points [9,10]. Similarly, we show that many GUSB-positive cells were detectable at 2 weeks and that numbers dropped by 13 weeks (see Table 1). This is likely because 2 weeks is not a truly latent time point [40] and some residual lytic-phase gene expression is present. However, from 13 to 52 weeks, positive cells were detected at stable levels (at about 30% of the numbers seen at 2 weeks). Our detection of stable numbers of positive cells at late time points may reflect (1) prolonged expression from 1716-LAT-hGUSB compared to other HSV vectors or (2) a difference in sensitivity in the histochemical detection of β-glucuronidase over other reporters (typically β-galactosidase).

Specific brain sites were dissected out to measure GUSB enzymatic activity of an entire brain region, as opposed to activity in individual cells. Stable GUSB activity was found at 26 and 52 weeks, with 11.4 and 11.6%, respectively (Fig. 4K). Our findings are similar to those reported by Puskovic *et al.,* who demonstrated that LAT promoter-driven expression of GDNF was relatively stable through 6 months [41]. Thus, the LAT promoter can exhibit stable, long-term expression at the level of reporter enzyme.

The amount of total GUSB enzymatic activity decreased between 13 and 26 weeks, while the number of GUSB-positive cells was stable during the same period. This suggests that there was a decrease in expression in many transduced cells, as opposed to stable expression by a subpopulation. It was noted that individual positive cells had smaller foci of staining at later time points (see Figs. 4A–4D), providing further support for a global decrease. In terms of an effective gene therapy treatment of a global brain genetic disorder (e.g., MPS VII) a larger distribution of lower levels of transgene activity is advantageous over foci of high levels of activity.

Changes in GUSB Expression Are Delayed Relative to LAT Expression

The time frame of the decrease in gene expression was similar, but not identical, by the assays for reporter enzyme and transcript expression. In sectioned tissues, GUSB-positive cells were detectable at both 26 and 52 weeks, but LATs were undetectable by ISH at these time points, even on serial sections. Since we could detect LATs by Q-RT-PCR, we attributed this finding to differences in sensitivity of the two assays. Although LAT expression had stabilized by 13 weeks, GUSB expression was not stabilized until the next time point (26 weeks). This lag in the stabilization of GUSB expression compared to LAT expression could be attributed to the stability of GUSB. GUSB secreted from gene-corrected cells retains 80% of activity in serum-containing tissue culture media for 2 weeks [42], and infusions of purified GUSB enzyme have been shown to have a half-life of 4.5 days in the brain [43].

Modifications of the LAT Gene Are Not Responsible for a Decrease in LAT Expression

As mentioned above, an apparent decrease in LAT brain expression was found early in the latent period. This pattern of LAT expression differs from that seen in the sensory ganglia, where LAT expression persists in human tissues (likely many years after infection) [44]. LAT exon 1 contains sequences previously shown to promote long-term activity on the LAT promoter [21,45]. 1716-LAT-hGUSB contains a gene insertion in LAT exon 1 (see map in Fig. 1A), which could potentially disrupt long-term expression and explain the decrease in LATs seen early in latency. However, similar levels of LAT intron were detected by Q-RT-PCR in animals infected with either 1716-LAT-hGUSB or 1716 (intact exon 1) (Fig. 3B); thus the insertion did not disrupt promoter function. Also, this was supported by the fact that the ISH data were very similar for 1716 and 1716-LAT-hGUSB (Fig. 2). Although higher levels of LAT intron were seen at 2 weeks with 1716 compared to 1716-LAT-hGUSB, the difference was not significant (P = 0.169). This finding could be attributed to readthrough transcription from the promoter of an upstream lytic-phase gene, as 2 weeks is not considered a latent time point [40]. Readthrough may be more likely to occur with 1716 than with 1716-LAT-hGUSB due to the large (2.4 kb) cDNA insertion into the first exon, which places the intron sequence farther from an upstream promoter. When the LAT promoter is deleted, LATs can be detected at early times postinfection, but not at latent time points [40], supporting the hypothesis that readthrough transcripts containing LAT sequences are made during the acute phase.

The ICP34.5 deletion in both viruses in this study overlaps with the LAT region and could potentially interfere with LAT expression or stability (see map in Fig. 1A). However, the LAT expression levels that we detected in the brain (range of 10.8 to 31.2 LAT copies per genome copy during latency) were similar to those reported by others (14.5 LAT copies per genome copy during latency) for a HSV-1 strain (SC16) that has an

intact LAT region [15]. Further, our LAT ISH results are similar to previous results showing that injection of SC16 resulted in an 87% decrease in the number of LAT-expressing neurons in the rat brain between 2 and 11 weeks [10]. Thus, we conclude that the ICP34.5 deletion did not affect LAT gene activity in the brain.

One model has been proposed to explain the decrease of LAT expression in the brain by a slow down-regulation of LAT promoter activity over time [10]. The measurements of GUSB activity, the GUSB histochemical stain, and the LAT ISH in the present study seem to support that model, with the greatest reduction taking place between 8 and 13 weeks. The stable number of GUSB-producing cells during latency argues that a LAT promoter down-regulation early in latency likely occurs in a global fashion, as opposed to only a subpopulation of latent cells. While our Q-RT-PCR results do not show a slow decrease, the time points of that experiment may not be sufficient to note a gradual decrease if it happens between 2 and 13 weeks. Importantly, our Q-RT-PCR results indicate that LAT expression reaches stable levels by 13 weeks, after which there is no significant change. This finding is strengthened by our finding that measurements of GUSB activity also stabilized by the next time point tested (26 weeks).

In this study, we have used quantitative methods to measure LAT promoter activity in the mouse brain during latency. We have demonstrated that the LAT promoter is stably active for at least 12 months as measured by quantitation of both LAT intron and reporter enzymatic activity. Further, the stable level of expression produces potentially sufficient GUSB activity to correct a genetic disorder that affects the brain.

Materials and Methods

Cells and viruses. Vero cells were grown in Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MD, USA) containing 5% fetal calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Strain 1716 has been described previously [35]. The 1716-LAT-hGUSB vector is identical to a vector previously described (GUSB JS vector) [19], except that the human GUSB cDNA was inserted. Vector was both grown and titered (by plaque assay) on Vero cells as previously described [1].

Construction of plasmids. Plasmids used to position the human GUSB cDNA into the first *Sty*I site of LAT exon 1 were previously described (pJZ-G8 and pXho-Sal) [19]. pJZ-G8 contains the 5′ portion of the LAT gene from HSV-1 strain F, and a 2.4-kb *Eco*RI human GUSB fragment was obtained from p13-18 [37]. Insertion of the 2.4-kb *Eco*RI human GUSB into the unique *Sty*I site of pJZ-G8 (in the same orientation as the LAT gene) resulted in pBB1, and a 3.0-kb partial *Not*I/*Rsr*II digest fragment of pBB1 was cloned into the *Not*I/*Rsr*II sites of pXho-Sal [19] to create pBB2. pBB2 was linearized by digestion with *Sca*I and *Swa*I and purified by gel electrophoresis to remove plasmid sequence.

Construction of recombinant virus. Homologous recombination was performed between purified viral DNA from strain 1716 (1 μg) and linearized pBB2 (300 ng) by cotransfection into subconfluent Vero cells using Lipofectamine (Gibco BRL). Plaque purification was performed as previously described [46] using a 680-bp fluorescein-labeled human GUSB-specific probe and an anti-fluorescein HRP-conjugated antibody (Perkin–Elmer, Boston, MA, USA). Recombinants were isolated and plaque purified until all plaques were positive, plus one more round to generate 1716-LAT-hGUSB. Virus for brain injection was prepared at an m.o.i. of 0.1 on subconfluent Vero cells and titered to 3.4×10^8 pfu/ml.

Mouse infection and tissue preparation. This study was approved by the Institutional Animal Care and Use Committee of the Wistar Institute and the principles of the care and use of laboratory animals of the NIH were followed. Four- to six-week-old female C3H HeJ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized with a mixture of ketamine/xylazine and inoculated intracranially with 3.4×10^5 pfu (in 1 μl) into the right hemisphere of the caudate putamen by a single site injection using a small animal stereotactic apparatus (Kopf Instruments, Tujunga, CA, USA) as described previously [47]. Injection coordinates were derived from a mouse brain atlas [48]: 2.00 mm right of midline, 0.00 mm rostral/caudal from bregma, 3.00 mm depth from the pial surface. Animals were transcardially perfused with 10 ml cold PBS (DEPC) followed by 10 ml cold 4% paraformaldehyde in PBS (DEPC), then brains were removed by dissection. Brains for sectioning were submersed in OCT compound (Tissue-Tek, Torrance, CA, USA) and immediately frozen on a bed of dry ice. Blocks were stored at −70°C and then sectioned to a thickness of 20 μm. Brains for quantitation of GUSB, DNA, or RNA were immediately frozen at −70°C. Tissues were later thawed and specific sites were dissected out.

Quantitation of vector DNA levels. Genomic DNA was extracted as reported previously [49] from the midbrain. Vector DNA levels were measured using a previously described primer set specific to the thymidine kinase gene [49]. Samples were standardized to 100 ng/μl total DNA and then amplified and measured using real-time PCR (Applied Biosystems). Genome copy levels were determined using a standard curve of known dilutions of viral DNA.

In situ hybridization. An adaptation of a protocol previously described [36] was used to perform *in situ* hybridization. DIG-labeled antisense and sense riboprobes were generated toward a LAT *Pst*I/*Mlu*I fragment (see Fig. 1A) encompassing LAT exon 1, the 2-kb intron, and the 5′ end of exon 2 (nucleotides 118,665–121,653). Hybridization was detected with only the antisense probe during latent time points.

Quantitative RT-PCR of LAT intron. Total RNA was extracted from the midbrain (approx 50 mg) using Trizol reagent (Gibco BRL) as recommended by the manufacturer and then treated with amplification-grade DNase I (Invitrogen). Reverse transcriptase PCR was used to convert 500 ng total RNA into cDNA. Q-PCR was performed on cDNAs with primers specific to the 2-kb LAT intron, GACAGCAAAAATCCCCTGAGTT, corresponding to HSV-1 nucleotides 120,704–120,725, and AAGACACGGGCACCACACA, corresponding to HSV-1 nucleotides 120,761–120,779. The probe was (6FAM)TTAGGGCCAACACAAAAGACCCGCTG(TMAR), corresponding to HSV-1 nucleotides 120,734–120,759. A standard curve was used to determine LAT intron cDNA copy number and was made from viral DNA diluted in mouse DNA in the range of 1000 to 0.001 LAT copies. Q-PCR toward murine GAPDH was used to standardize cDNA input levels.

In situ GUSB staining. Sites of GUSB activity were localized in frozen tissue sections with naphthol-AS-B1-β-D-glucuronide (Sigma) using an established assay [50]. Murine GUSB activity was first inactivated by incubation of tissues at 65°C for 90 min. At least four brains per vector per time point were sectioned coronally and at least 30 sections (a cross section) per animal were stained.

Quantitation of GUSB activity. A previously described assay was performed to measure GUSB enzymatic activity [50] using 4-methylumbelliferyl β-D-glucuronide (Sigma). Tissues were first heat inactivated at 65°C for 90 min. Wavelengths of 360 and 460 nm (excitation and emission, respectively) were read using a CytoFluor fluorometer (Applied Biosystems) to measure nanomoles of substrate cleaved per hour per milligram of total protein added. A standard curve was prepared for each experiment using known concentrations of 4-methylumbelliferylone (Sigma) in the range of 1–100 nmol. Corrected GUSB activity was calculated by subtracting background levels (activity detected with

doi:10.1016/j.ymthe.2005.06.478

1716). Percentage of normal GUSB activity was calculated by dividing corrected values by normal C3H HeJ brain levels (13.8 nmol/h/mg).

ACKNOWLEDGMENTS

We are indebted to Aracelis Polesky (Children's Hospital of Philadelphia) and Elsa Aglow (The Wistar Institute) for excellent technical assistance. This research is supported by National Institutes of Health Grant NS29390. B. Berges was supported by Training Grant AI07324.

RECEIVED FOR PUBLICATION MAY 4, 2005; REVISED JUNE 24, 2005; ACCEPTED JUNE 24, 2005.

REFERENCES

1. Spivack, J. G., and Fraser, N. W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection in mice. *J. Virol.* **61:** 3841–3847.
2. Deatly, A. M., Spivack, J. G., Lavi, E., O'Boyle, D. R., and Fraser, N. W. (1988). Latent herpes simplex virus type 1 transcripts in peripheral and central nervous system tissues of mice map to similar regions of the viral genome. *J. Virol.* **62:** 749–756.
3. Glorioso, J. C., and Fink, D. J. (2004). Herpes vector-mediated gene transfer in treatment of diseases of the nervous system. *Annu. Rev. Microbiol.* **58:** 253–271.
4. Huang, Q., Vonsattel, J.-P., Schaffer, P. A., Martuza, R. L., Breakefield, X. O., and DiFiglia, M. (1992). Introduction of a foreign gene (Escherichia coli lacZ) into rat neostriatal neurons using herpes simple virus mutants: a light and electron microscopic study. *Exp. Neurol.* **115:** 303–316.
5. Fink, D. J., Sternberg, L. R., Weber, P. C., Mata, M., Goins, W. F., and Glorioso, J. C. (1992). In vivo expression of beta-galactosidase in hippocampal neurons by HSV-mediated gene transfer. *Hum. Gene Ther.* **3:** 11–19.
6. Wood, M. J., Byrnes, A. P., Pfaff, D. W., Rabkin, S. D., and Charlton, H. M. (1994). Inflammatory effects of gene transfer into the CNS with defective HSV-1 vectors. *Gene Ther.* **1:** 283–291.
7. Bloom, D. C., Maidment, N. T., Tan, A., Dissette, V. B., Feldman, L. T., and Stevens, J. G. (1995). Long-term expression of a reporter gene from latent herpes simplex virus in the rat hippocampus. *Mol. Brain Res.* **31:** 48–60.
8. McMenamin, M. M., *et al.* (1998). Potential and limitations of a gamma-34.5 mutant of herpes simplex 1 as a gene therapy vector in the CNS. *Gene Ther.* **5:** 594–604.
9. Smith, C., Lachmann, R. H., and Efstathiou, S. (2000). Expression from the herpes simplex virus type 1 latency-associated promoter in the murine central nervous system. *J. Gen. Virol.* **81:** 649–662.
10. Scarpini, C. G., *et al.* (2001). Latency associated promoter transgene expression in the central nervous system after stereotaxic delivery of replication-defective HSV-1-based vectors. *Gene Ther.* **8:** 1057–1071.
11. Skorupa, A. F., Fisher, K. J., Wilson, J. M., Parente, M. K., and Wolfe, J. H. (1999). Sustained production of beta-glucuronidase from localized sites after AAV vector gene transfer results in widespread distribution of enzyme and reversal of lysosomal storage lesions in a larger volume of brain in mucopolysaccharidosis mice. *Exp. Neurol.* **160:** 17–27.
12. Passini, M. A., Watson, D. J., Vite, C. H., Landsburg, D. J., Feigenbaum, A. L., and Wolfe, J. H. (2003). Intraventricular brain injection of adeno-associated virus type 1 (AAV1) in neonatal mice results in complementary patterns of neuronal transduction to AAV2 and total long-term correction of storage lesions in the brains of beta-glucuronidase-deficient mice. *J. Virol.* **77:** 7034–7040.
13. Buckley, R. H. (2002). Gene therapy for SCID—a complication after remarkable progress. *Lancet* **360:** 1185–1186.
14. Mellerick, D. M., and Fraser, N. W. (1987). Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. *Virology* **158:** 265–275.
15. Ramakrishnan, R., Fink, D. J., Jiang, G., Desai, P., Glorioso, J. C., and Levine, M. (1994). Competitive quantitative PCR analysis of herpes simplex virus type 1 DNA and latency-associated transcript RNA in latently infected cells of the rat brain. *J. Virol.* **68:** 1864–1873.
16. Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L., and Feldman, L. T. (1987). RNA complementary to a herpes virus gene mRNA is prominent in latently infected neurons. *Science* **235:** 1056–1059.
17. Wechsler, S. L., Nesburn, A. B., Watson, R., Slanina, S. M., and Ghiasi, H. (1988). Fine mapping of the latency-related gene of herpes simplex virus type 1: alternate splicing produces distinct latency-related RNAs containing open reading frames. *J. Virol.* **62:** 4051–4058.
18. Steiner, I., Mador, N., Reibstein, I., Spivack, J. G., and Fraser, N. W. (1994). Herpes simplex virus type 1 gene expression and reactivation of latent infection in the central nervous system. *Neuropathol. Appl. Neurobiol.* **20:** 253–260.
19. Zhu, J., Kang, W., Wolfe, J. H., and Fraser, N. W. (2000). Significantly increased expression of β-glucuronidase in the central nervous system of mucopolysaccharidosis type VII mice from the latency-associated transcript promoter in a nonpathogenic herpes simplex virus type 1 vector. *Mol. Ther.* **2:** 82–94.
20. Lilley, C. E., *et al.* (2001). Multiple immediate-early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in cell culture and widespread gene delivery to the central nervous system in vivo. *J. Virol.* **75:** 4343–4356.
21. Berthomme, H., Lokensgard, J., Yang, L., Margolis, T., and Feldman, L. T. (2000). Evidence for a bidirectional element located downstream from the herpes simplex virus type 1 latency-associated promoter that increases its activity during latency. *J. Virol.* **74:** 3613–3622.
22. Marshall, K. R., Lachmann, R. H., Efstathiou, S., Rinaldi, A., and Preston, C. M. (2000). Long-term transgene expression in mice infected with a herpes simplex virus type 1 mutant severely impaired for immediate-early gene expression. *J. Virol.* **74:** 956–964.
23. Ramakrishnan, R., Levine, M., and Fink, D. J. (1994). PCR-based analysis of herpes simplex virus type 1 latency in the rat trigeminal ganglion established with a ribonucleotide reductase-deficient mutant. *J. Virol.* **68:** 7083–7091.
24. Rodahl, E., and Stevens, J. G. (1992). Differential accumulation of herpes simplex virus type 1 latency-associated transcripts in sensory and autonomic ganglia. *Virology* **189:** 385–388.
25. Kesari, S., Lee, V. M.-Y., Brown, S. M., Trojanoski, J. Q., and Fraser, N. W. (1996). Selective vulnerability of mouse CNS neurons to latent infection with a neuro-attenuated herpes simplex virus-1. *J. Neurosci.* **16:** 5644–5653.
26. Springer, S. L., Vite, C. H., Polesky, A. C., Kesari, S., Fraser, N. W., and Wolfe, J. H. (2001). Infection and establishment of latency in the dog brain after direct inoculation of a nonpathogenic strain of herpes simplex virus-1. *J. Neurovirol.* **7:** 149–154.
27. Levy, B., Galvin, N., Vogler, C., Birkenmeier, E. H., and Sly, W. S. (1996). Neuropathology of murine mucopolysaccharidosis type VII. *Acta Neuropathol.* **92:** 562–568.
28. Wolfe, J. H., Deshmane, S. L., and Fraser, N. W. (1992). Herpesvirus vector gene transfer and expression of beta-glucuronidase in the central nervous system of MPS VII mice. *Nat. Genet.* **1:** 379–384.
29. Deshmane, S. L., *et al.* (1995). An HSV-1 containing the rat beta-glucuronidase cDNA inserted within the LAT gene is less efficient than the parental strain at establishing a transcriptionally active state during latency in neurons. *Gene Ther.* **2:** 209–217.
30. Birkenmeier, E. H., *et al.* (1989). Murine mucopolysaccharidosis type VII: characterization of a mouse with beta-glucuronidase deficiency. *J. Clin. Invest.* **83:** 1258–1266.
31. Moullier, P., Marechal, V., Danos, O., and Heard, J. M. (1993). Continuous systemic secretion of a lysosomal enzyme by genetically modified mouse skin fibroblasts. *Transplantation* **56:** 427–432.
32. Passini, M. A., Lee, E. B., Heuer, G. G., and Wolfe, J. H. (2002). Distribution of a lysosomal enzyme in the adult brain by axonal transport and by cells of the rostral migratory stream. *J. Neurosci.* **22:** 6437–6446.
33. Wolfe, J. H., *et al.* (1992). Reversal of pathology in murine mucopolysaccharidosis type VII by somatic cell gene transfer. *Nature* **360:** 749–753.
34. Taylor, R. M., and Wolfe, J. H. (1997). Decreased lysosomal storage in the adult MPS VII mouse brain in the vicinity of grafts of retroviral vector-corrected fibroblasts secreting high levels of beta-glucuronidase. *Nat. Med.* **3:** 771–774.
35. MacLean, A. R., Ul-Fareed, M., Robertson, L., Harland, J., and Brown, S. M. (1991). Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence-related sequences in Glasgow strain 17+ between immediate early gene 1 and the 'a' sequence. *J. Gen. Virol.* **72:** 631–639.
36. Passini, M. A., and Wolfe, J. H. (2001). Widespread gene delivery and structure-specific patterns of expression in the brain after intraventricular injections of neonatal mice with an adeno-associated virus vector. *J. Virol.* **75:** 12382–12392.
37. Wolfe, J. H., Kyle, J. W., Sands, M. S., Sly, W. S., Markowitz, D. G., and Parente, M. K. (1995). High level expression and export of beta-glucuronidase from murine mucopolysaccharidosis VII cells corrected by a double-copy retrovirus vector. *Gene Ther.* **2:** 70–78.
38. Pfister, K., Paigen, K., Watson, G., and Chapman, V. (1982). Expression of beta-glucuronidase haplotypes in prototype and congenic mouse strains. *Biochem. Genet.* **20:** 519–536.
39. Kesari, S., *et al.* (1998). A neuroattenuated ICP34.5-deficient herpes simplex virus type 1 replicates in ependymal cells of the murine central nervous system. *J. Gen. Virol.* **79:** 525–536.
40. Nicosia, M., Deshmane, S. L., Zabolotny, J. M., Valyi-Nagy, T., and Fraser, N. W. (1993). Herpes simplex virus type I latency-associated transcript (LAT) promoter deletion mutants can express a 2-kilobase transcript mapping to the LAT region. *J. Virol.* **67:** 7276–7283.
41. Puskovic, V., *et al.* (2004). Prolonged biologically active transgene expression driven by HSV LAP2 in brain *in vivo*. *Mol. Ther.* **10:** 67–75.
42. Taylor, R. M., and Wolfe, J. H. (1994). Cross-correction of β-glucuronidase deficiency by retroviral vector-mediated gene transfer. *Exp. Cell Res.* **214:** 606–613.
43. Vogler, C., *et al.* (1993). Enzyme replacement with recombinant beta-glucuronidase in the newborn mucopolysaccharidosis type VII mouse. *Pediatr. Res.* **34:** 837–840.
44. Steiner, I., Spivack, J. G., O'Boyle, D. R. N., Lavi, E., and Fraser, N. W. (1988). Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. *J. Virol.* **62:** 3493–3496.
45. Goins, W. F., *et al.* (1994). A novel latency-active promoter is contained within the herpes simplex virus type 1 UL flanking repeats. *J. Virol.* **68:** 2239–2252.

46. Zhu, J., and Aurelian, L. (1997). AP-1 cis-response elements are involved in basal expression and Vmw110 transactivation of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *Virology* **231:** 301–312.
47. Kesari, S., *et al.* (1995). Therapy of experimental human brain tumors using a neuroattenuated herpes simplex virus mutant. *Lab. Invest.* **73:** 636–648.
48. Franklin, K. B. J., and Paxinos, G. (1997). *The Mouse Brain in Stereotaxic Coordinates.* Academic Press, San Diego. Fig. 31.
49. Mukerjee, R., Kang, W., Suri, V., and Fraser, N. W. (2004). A non-consensus branch point plays an important role in determining the stability of the 2-kb LAT intron during acute and latent infections of herpes simplex virus type-1. *Virology* **324:** 340–349.
50. Wolfe, J. H., and Sands, M. S. (1996). Murine mucopolysaccharidosis type VII: a model system for somatic gene therapy of the central nervous system. In *Gene Protocols for Gene Transfer in Neuroscience: Toward Gene Therapy of Neurologic Disorders* (P. R. Lowenstein and L. W. Enquist, Eds.). Wiley, Essex.