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Competitive Quantitative PCR Analysis of Herpes Simplex Virus Type 1 DNA and Latency-Associated Transcript RNA in Latently Infected Cells of the Rat Brain

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Competitive quantitative PCRs were used to examine the consequences of stereotactically injecting a highly attenuated herpes simplex virus type 1 mutant into rat brains. This mutant virus, designated RR1*CAT*/RR2*lacZ*, was engineered so that coding sequences of the genes U_L39 and U_L40 specifying the subunits of the viral ribonucleotide reductase were replaced by the chloramphenicol acetyltransferase (*CAT*) and the *lacZ* gene coding sequences, respectively. Stereotactic injection of this virus into the hippocampal region of the rat brain resulted in a localized infection. Viral gene products were visualized by immunochemical, cytochemical, or in situ hybridization techniques in the injected hippocampal region at 2 days postinjection. Viral genomes, represented by glycoprotein B (*gB*), latency-associated transcript (*LAT*), and *lacZ* sequences could be amplified by PCR from templates obtained by scraping hippocampal tissue off single 10-μm frozen sections. Both gB message and LAT could be detected by reverse transcriptase (RT)-PCR. At day 7 postinjection, neither CAT message, gB message, nor β-galactosidase activity could be visualized by the same techniques, although viral DNA was detected by PCR and LAT could be detected by RT-PCR. A similar pattern was seen at 8 weeks, suggesting that latency was established by the mutant virus in cells of the injected hippocampus. By competitive quantitative PCR, hippocampal sections were determined to contain 2.6×10^5 genome equivalents (represented by the *gB* gene) on day 2, 6.2×10^4 on day 7, and 8.3×10^4 at 8 weeks. By competitive quantitative RT-PCR, the numbers of LAT molecules at the same time points were 3.2×10^6, 1.3×10^6, and 1.2×10^6, respectively. The numbers of LAT molecules per genome equivalent were 12.5, 20.3, and 14.5, respectively, being approximately the same for each of the three time points. The data permit the conclusion that the RR mutant virus establishes latency in the rat brain with the persistence of the viral genome and the production of LAT molecules. Once latency is established, the numbers of viral genomes and LAT RNA molecules remain constant. Thus the competitive quantitative PCR and RT-PCR techniques provide very sensitive and reliable methods to quantitate viral DNA and RNA present in infected tissue.

Herpes simplex virus type 1 (HSV-1) is a neurotropic virus which is capable of establishing a lifelong latent state in the nervous system (22a, 29, 51, 59). During primary infection of skin or mucosal surfaces, virus is taken up into axonal terminals and transported to the neuronal cell bodies in sensory ganglia where the viral genome may persist in a circular or concatameric extrachromosomal form (18, 42, 48) condensed into a chromatin-like structure (14). Expression from the latent viral genome is extremely limited (29, 59), with all the HSV-1 genes transcriptionally silent except for two identical regions which map to the inverted repeat sequences of the long unique (U_L) segment of the viral genome downstream of the *ICP0* gene. A family of overlapping transcripts, the latency-associated transcripts or LATs, are transcribed from the strand opposite to that of the *ICP0* gene in this region and are partially complementary to ICP0 mRNA (12, 46, 49, 57, 60, 63). These RNAs are mostly intranuclear and nonpolyadenylated (15, 57, 64). The most obvious LAT is approximately 2 kb in length, with less abundant 1.5- and 1.45-kb species also detectable in latently infected ganglia (12, 43, 57, 63, 64, 67). As yet, no protein product has been ascribed to the *LAT* gene. LATs are not required for the establishment or maintenance of latency, since viruses deleted for *LAT* are not affected in their ability to establish latency (30, 54, 58). However, some LAT$^-$ mutants appear to be impaired in the ability to reactivate from latency (5, 35).

HSV infection of specific brain regions can be achieved by stereotactic injection of small volumes of virus directly into brain tissue (3, 22, 40) or by peripheral injection of sites with direct projections from brain tissue. Latent infection in brain tissue has been observed by using defective and attenuated viruses in site-specific infections (9, 12). Previously, we reported on experiments in which a virus defective in the U_S3 gene was injected into the hippocampi of adult rats (22). The U_S3 gene encodes a protein kinase (for a review, see reference 34), the substrate of which is the HSV-1 U_L34 phosphoprotein gene product (47). The U_S3 gene product is dispensable for virus replication in cell culture (36, 41) and for the establishment of latent infection in the mouse peripheral nervous system. Virus mutants with mutations in the U_S3 gene show significantly reduced neurovirulence following intracranial inoculation (22, 41). We demonstrated that the infection was limited to the injected hippocampus, accompanied by transient

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expression of the reporter β-galactosidase gene in neurons of the dentate gyrus which could not be detected beyond 5 days postinoculation. Despite the absence of foreign gene expression after 7 days, both viral DNA and LATs, the hallmarks of latent infections, could be detected by PCR as long as 10 months after inoculation, suggesting that long-term latency in the central nervous system (CNS) had been established.

HSV-1 mutants with mutations in the ribonucleotide reductase (RR) genes, *U_L39* and *U_L40*, which code for the viral RR subunits, are significantly less neurovirulent following intracranial injection into mice (6) and are less able to reactivate from ganglionic latency than wild-type virus (32). Having used a highly attenuated virus with mutations in both reductase genes, we report here the quantitation of latent virus in specific brain regions following intracranial inoculation into the rat hippocampus. Competitive quantitative DNA and RNA PCRs were used on extracts from the injected hippocampal regions to demonstrate that both the amount of viral genomes and the number of LAT molecules remain constant up to 8 weeks after inoculation once latency is established.

MATERIALS AND METHODS

Cells and viruses. The virus used is a mutant derivative of HSV-1 KOS, designated RR1*CAT*/RR2*lacZ* (13). In this virus, the chloramphenicol acetyltransferase (*CAT*) gene coding sequence replaced the *RR1* gene coding sequence and that of the *lacZ* gene replaced the *RR2* gene coding sequence, so that the RR1 promoter drives *CAT* expression and the RR2 promoter drives the *lacZ* gene. Stocks of the virus were prepared by low multiplicity passage in Vero cells. All cell culture work reported here was carried out with Vero cells grown in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum.

Stereotactic injection and preparation of brain sections. Sprague-Dawley rats weighing from 175 to 225 g were used in the experiments to be described. Approximately 10^7 PFU in a 5-μl volume was stereotactically injected into one hippocampus (45) with a Hamilton syringe as described elsewhere (22). At day 2, day 7, or 8 weeks after inoculation, the animals were sacrificed by decapitation and 10-μm cryostat sections were placed on baked glass slides. PCR, reverse transcriptase PCR (RT-PCR), and in situ hybridization for expression of CAT mRNA were performed on these sections. Other animals were perfused with 4% paraformaldehyde, the brains were postfixed for 4 h in the same solution and for 2 days in 30% sucrose, and 50-μm sections were cut on a sliding microtome. β-Galactosidase activity was demonstrated by reacting floating sections in X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; GIBCO BRL, Gaithersburg, Md.) in 0.1 M phosphate-buffered saline (pH 7.6), 53% $K_4Fe(CN)_6$, 0.14% $K_3Fe(CN)_6$, and 0.1 mM $MgCl_2$ for 3 h at 37°C. Alternate sections were stained with cresyl violet for histologic analysis or immunostained for HSV proteins by using a polyclonal antiserum.

PCR amplification of viral DNA and RNA. (i) DNA extraction. The hippocampal region was removed from individual 10-μm brain sections at −20°C with a prechilled, sterile razor blade and transferred to a sterile Eppendorf tube containing 1.0 ml of absolute ethanol at room temperature. The mixture was vortexed for 1 min, pelleted in a microfuge at 10,000 × *g* for 2 min, and resuspended in absolute ethanol, and the procedure was repeated twice. The final pellet was dried and resuspended in 100 μl of a solution containing 400 μg of proteinase K per ml, 50 mM Tris HCl (pH 8.0), 2 mM EDTA, and 0.5% Tween 20. The mixture was incubated at 37°C for 1 to 12 h and then boiled for 15 min, and 10 μl of the extract was taken directly for PCR.

(ii) RNA extraction. Tissue scraped from the slide was transferred to a sterile Eppendorf tube containing 0.8 ml of TRI (Total RNA Isolation reagent; Molecular Research Center, Inc., Cincinnati, Ohio), and RNA was extracted according to the manufacturer's instructions. Extracted RNA was dissolved in 50 μl of distilled water.

(iii) Amplification of DNA. Samples (10 μl) of the DNA extract were amplified in a final volume of 100 μl containing 10 μl of 10× PCR buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 4 μl of deoxynucleoside triphosphates (dNTPs) (10 mM [each] dCTP, dGTP, dATP, and dTTP), and 2 μl (100 ng each) of specific primer pairs, and 2 μl of *Taq* polymerase (gift of D. Engelke, University of Michigan). For amplification of *gB* and *lacZ* DNA, 10 μl of deionized formamide was included. The primer pairs used were (i) for *gB* DNA, 5′ primer ATT-CTC-CTC-CGA-CGC-CAT-ATC-CAC-CAC-CTT and 3′ primer AGA-AAG-CCC-CCA-TTG-GCC-AGG-TAG-T; (ii) for *lacZ* DNA, 5′ primer TTG-CTG-ATT-CGA-GGG-GTT-AAC-CGT-CAC-GAG and 3′ primer ACC-AGA-TGA-TCA-CAC-TGC-GGT-GAT-TAC-GAT (22); (iii) for *LAT* DNA, 5′ primer GAC-AGC-AAA-AAT-CCC-GTC-AG and 3′ primer ACG-AGG-GAA-AAC-AAT-AAG-GG (38); (iv) for glyceraldehyde phosphate dehydrogenase (*GAPDH*) DNA, 5′ primer ATT-GGG-GGT-AGG-AAC-ACG-GAA and 3′ primer ACC-CCT-TCA-TTG-ACC-TCA-ACT-A; (v) for carbonic anhydrase II (*CAII*) DNA, 5′ primer GGA-ATT-CGG-CCA-GTC-CAT-CAG-GTT-GCT and 3′ primer GGA-ATT-CAG-TGC-TCA-AGG-GAG-GAC-CCC-TG (62).

Amplification was carried out in a PTC 100 Programmable Thermal Controller (MJ Research, Inc.) utilizing the following cycle conditions: (i) for *gB* and *lacZ*, 30 cycles at 95°C for 15 s, 54°C for 15 s, and 71°C for 1.5 min; (ii) for *LAT* and *GAPDH*, 30 cycles at 95°C for 1 min and 60°C for 1 min followed by a single extension at 72°C for 10 min; (iii) for *CAII*, 95°C for 1 min, 55°C for 2 min, and 72°C for 3 min for 35 cycles. After completion of amplification, 10 μl of the reaction mixture was loaded on a 4% NuSieve GTG agarose gel (FMC BioProducts, Rockland, Maine) and electrophoresed at 150 V and 150 mA for 3 h at room temperature in 1× Tris-borate-EDTA (TBE) buffer.

(iv) Amplification of RNA. RNA templates were amplified by a modification of the combined reverse transcription-DNA amplification method (66), carried out in a single tube, in a single buffer system. The reaction mixture consisted of 10 μl of 10× PCR buffer-II (Perkin-Elmer Cetus), 8 μl of dNTPs (2 μl each from 10 mM stocks of dCTP, dGTP, dATP, and dTTP), 5 mM $MgCl_2$, 4 μl of primer pairs (100 ng of each primer), 8 μl of RNA template extract, 2 μl of *Taq* polymerase, 1,000 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL), and distilled water to 100 μl. The reaction mixture was incubated at 37°C for 30 min to permit reverse transcription, and then DNA was amplified by using the same conditions described above.

(v) Competitive quantitative DNA PCR (4, 25). Mutated internal standards were created to determine the amount of DNA in the original scraped samples. On the basis of use of the gB primers described above, the amplified *gB* DNA is 191 bp long. A mutant 191-bp *gB* PCR fragment was constructed with an *Hpa*II site 29 nucleotides from the 3′ end by using the wild-type *gB* PCR product as template for amplification with the wild-type *gB* 5′ primer and a new 3′ primer (5′ AGA-AAG-CGC-CCA-TTG-GCC-AGG-TAG-TAC-TCC-GGC-TG3′) in which nucleotide 29 was changed from G to C, introducing an *Hpa*II site internal to the original 3′ primer. The mutant *gB*

product was gel purified and quantified by optical density. Since both the wild-type and mutant fragments are of equal size, they both should be amplified equally well. When a mixture of the original and mutant fragments is digested with *Hpa*II, two DNA fragments of 191 and 162 bp, representing the original and mutant fragments, respectively, could be distinguished, and the ratio of wild-type to mutant internal standard in the original sample was determined.

The amount of *gB* DNA in the extracts was first approximated by titration against a 10-fold dilution series of the mutant *gB* fragment, followed by a more precise quantitation by titrating the extracts against a 2-fold dilution series spanning the first determination. In the first step, 10 μl of extract was added to samples of a 10-fold dilution series of the mutant *gB* fragment ranging from 100 pg to 1 fg. The mixtures were amplified for 30 cycles by using the original primer pair in the presence of 1 μl of [α-^{32}P]dCTP (400 Ci/mM; Amersham Corp, Arlington Heights, Ill.) as described above. To eliminate heterodimer formation (4, 25), reaction products were diluted 200-fold and amplified again for 2 cycles with radioactive tracer in a 20-μl final volume containing 2 μl of 10× PCR buffer, 4 μl of dNTPs (10 μM each), 2 μl of deionized formamide, 2 μl of *Taq* polymerase, and 7 μl of autoclaved distilled water. After amplification, 20 units of *Hpa*II was added, and the mixture was incubated at 37°C overnight. The entire volume was electrophoresed on a 4% NuSieve GTG agarose gel in 1× TBE buffer at 150 V and 150 mA for 4 h. The gel was dried and exposed to X-ray film (Hyperfilm-MP; Amersham) at −70°C. Visual comparisons of the wild-type and mutant bands allowed for a rough approximation of the amount of viral DNA in the extract. In the second step, 10 μl of extract was added to a twofold dilution enzyme series of mutant *gB* fragments from 100 to 3.1 fg and amplified as described above. Following restriction digestion and electrophoresis, the amount of radioactivity in each band was determined with a radioanalytic imaging detector (AMBIS Inc., San Diego, Calif.). Linear regression curves were prepared by plotting net counts per minute for each sample in the dilution series against the amount of input mutant *gB* DNA in the series, by using the Graph PAD INPLOT software program (Graph PAD Software, San Diego, Calif.). At the point of equivalence, the amount of *gB* DNA in the original extract is equal to the amount of mutant *gB* DNA added to the sample.

To adjust for differences in the amount of tissue in the scraped samples, 10-μl aliquots of each extract were amplified by using primer pairs for either the cellular *GAPDH* or *CAII* gene in the presence of [α-^{32}P]dCTP as tracer. The reaction products from each extract were electrophoresed on a 1% agarose gel and dried, and the net counts per minute per reaction product band was quantified. The relative amounts of initial extracts were than normalized by using these data.

(vi) Competitive quantitative RT-PCR. Competitive quantitative RT-PCR (4, 25) was carried out to determine the amount of LAT RNA in the tissue. Mixtures of target and standard RNA were exposed to reverse transcriptase, and the resulting DNA templates were coamplified in the presence of the wild-type LAT primers. The LAT primers amplify a 195-bp fragment from the 2-kb family of stable LAT transcripts. The amplified DNA fragments were then processed as described above for DNA quantitation. Essential to this analysis was the construction of an RNA template which, on reverse transcription and DNA amplification, produces a mutant fragment which can serve as a standard. Such an RNA template was produced in the following manner.

The normal *LAT* PCR product is 195 bp long and has a *Bsa*HI restriction enzyme site located 21 nucleotides from the 3′ end. A mutant *LAT* PCR product in which the cytosine at residue 22 from the 3′ end was converted to thymine, resulting in the loss of the *Bsa*HI site, was constructed. The 195-bp mutant and 174-bp wild-type *LAT* fragments can be electrophoretically distinguished from one another following *Bsa*HI digestion. The mutant and wild-type templates can be coamplified by using the *LAT* primer pair previously described. The need for cloning into a transcription vector was eliminated by directly attaching a 23-base-long oligonucleotide coding for the T3 RNA polymerase promoter (53) to the 5′ terminus of the 5′ *LAT* PCR primer, by using a 12-base bridging oligonucleotide with 6 bases complementary to the promoter oligonucleotide and 6 bases complementary to the 5′ *LAT* PCR primer (Fig. 1). Five micrograms of *LAT* 5′ primer was treated with 10 U of T4 polynucleotide kinase, as recommended by the manufacturer (GIBCO BRL). The reaction products were extracted with phenol-chloroform, precipitated in ethyl alcohol, dried, and resuspended in 10 μl of autoclaved distilled water. The T3 promoter oligonucleotide was ligated to the *LAT* 5′ oligonucleotide in the presence of the bridging oligonucleotide (5 μg of each) by using 30 U of T4 DNA ligase (Gibco BRL) in a final volume of 30 μl for 12 h at 14°C. The T3 promoter-5′ *LAT* oligonucleotide ligation product (5 μl of the ligation reaction mixture) was used in combination with the 3′ *LAT* mutant primer (5′ ACG-AGG-GAA-AAC-AAT-AAG-GGA-TGC-C-3′) to amplify the wild-type *LAT* fragment, creating a T3 promoter-mutant *LAT* DNA fragment which had lost the *Bsa*HI site 22 nucleotides from the 3′ end of the PCR product. This was electrophoresed on a 1% low-melting-point agarose gel (GIBCO BRL) in 1× TBE buffer, the corresponding band was excised, and the presence of the mutation was confirmed by *Bsa*HI digestion. The identity of the T3-mutant *LAT* construct was confirmed by PCR amplification using (i) the T3 oligonucleotide as the 5′ primer and the 3′ *LAT* primer and (ii) the original primer pair. Both primer sets amplified the T3-mutant *LAT* construct correctly.

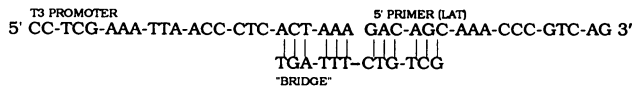

FIG. 1. Schematic of the use of a 12-bp bridge oligonucleotide to stabilize the T3 RNA polymerase promoter and the 5′ *LAT* primer for ligation.

Five microliters of the T3-mutant *LAT* DNA template was subjected to in vitro transcription by using a T3 RNA polymerase kit (Promega, Madison, Wis.) in a final volume of 200 μl at 37°C, according to the directions of the manufacturer. After 60 min of incubation, 20 more units of RNA polymerase was added, and the reaction was continued for an additional 60 min, when 10 U of RNase-free DNase (Boehringer Mannheim, Corp., Indianapolis, Ind.) was introduced to digest the DNA template. The reaction products were extracted with phenol-chloroform, precipitated with ethyl alcohol, dried, and resuspended in 100 μl of autoclaved distilled water. RNA was quantitated by measuring optical density.

To obtain a first approximation of the amount of LAT RNA in the hippocampal extracts, 5 μl of hippocampal RNA was mixed with a 10-fold dilution series of mutant LAT RNA ranging from 1 μg to 1 fg and subjected to RT-PCR in the presence of 1 μl of [α-^{32}P]dCTP (400 Ci/mM; Amersham). The samples were amplified for 30 cycles, diluted 200-fold, and following reamplification for an additional two cycles, digested with 20 U of *Bsa*HI at 37°C overnight. The entire reaction mixtures were electrophoresed on 4% NuSieve GTG agarose

gels, and the gels were dried and exposed to X-ray film from which visual estimates of RNA quantities were made. Samples (5 μl) were then coamplified with a twofold dilution series of mutant LAT RNA ranging from 50 fg to 3.1 fg, by the same procedure. PCR products were processed, radioactivity was quantitated by using the AMBIS system, and the counts were analyzed by linear regression as described for the viral DNA determinations.

Normalization for differences in the amount of tissue in the scraped samples was carried out similarly to that used for the DNA competitive quantitative PCR. Aliquots (10 μl) of each extract were subjected to RT-PCR using primer pairs for the *GAPDH* gene message. The reaction products from each extract were electrophoresed and dried, and the net counts per minute per reaction product band was quantitated. The relative amounts of RNA in the initial extracts were than normalized.

RESULTS

Gene expression in the stereotactically infected hippocampus region. In the recombinant RR1*CAT*/RR2*lacZ* virus, CAT expression is under the regulatory control of the RR1 promoter and β-galactosidase expression is under the control of the RR2 promoter. Measurable amounts both of CAT and of β-galactosidase activities were observed on infection of Vero cells (13). Following stereotactic injection of 5 μl containing 10^9 PFU/ml of virus into the right hippocampus, all injected animals survived the infection, confirming the attenuated nature of this mutant. Light microscopic examination of infected brains showed limited evidence of virus-induced pathology up to 8 weeks postinjection. Immunochemical staining with the HSV-1-specific polyclonal antiserum demonstrated HSV-positive cells in the injected area on day 2. At day 2 postinjection, expression of the RR1 promoter-driven *CAT* gene was demonstrated by in situ hybridization and expression of the RR2 promoter-driven *lacZ* gene was demonstrated by X-Gal staining. No immunological evidence of HSV antigen, in situ evidence of CAT mRNA, or evidence of *lacZ* expression by X-Gal reaction was seen in the contralateral hippocampus or in other regions of the brain. No β-galactosidase or CAT expression could be detected at day 7 or 8 weeks postinoculation. The time course of transgene expression is similar to that reported by Fink et al. (22) for β-galactosidase expression by the U_S3 attenuated virus.

PCR analysis of viral nucleic acids in stereotactically infected rat brains. To determine the persistence of viral genomes and RNA expression in specific brain regions, we amplified DNA and RNA obtained directly from either the injected or contralateral hippocampal region removed from individual 10-μm tissue sections. The *gB*, *LAT*, and *lacZ* DNA sequences were used as representatives of the viral genome, and gB and LAT RNA were used to measure transcription of lytic cycle and latent genes, respectively. Amplification of the cellular *CAII* and *GAPDH* genes served as internal controls and to normalize the amount of tissue in all experiments.

At day 2, day 7, and 8 weeks postinjection, both *gB* and *lacZ* DNA were detected by amplification of templates from the injected hippocampal region (Fig. 2A, lanes 5 to 10). No gB or LacZ signals were detected in DNA extracted from the contralateral hippocampus of the same sections (Fig. 2A, lanes 2 and 3), even though this region contained amplifiable DNA, as confirmed by the amplification of the cellular *CAII* gene (Fig. 2A, lane 1). Similarly, a signal for the viral *LAT* DNA sequences was detected from the injected hippocampus up to 8 weeks postinjection (Fig. 2B, lanes 2 and 3). These data

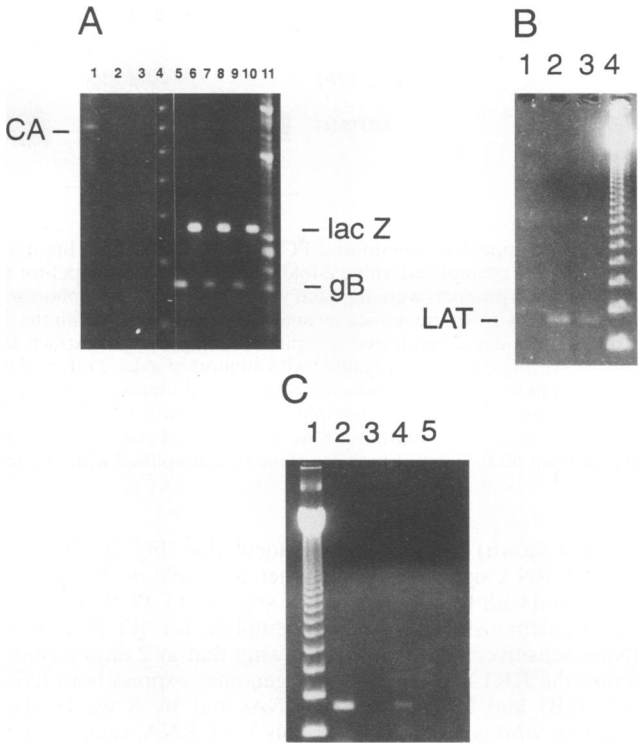

FIG. 2. PCR analysis of viral DNA and RNA in extracts of the hippocampal regions from 10-μm sections of rat brains stereotactically infected with RR1*CAT*/RR2*lacZ* mutant virus. PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. (A) Amplification of viral DNA using *lacZ* and *gB* gene primers and of cellular DNA using carbonic anhydrase II-specific primers. Lane 1, day 2 extract from contralateral region of infected hippocampus amplified with carbonic anhydrase II primers; lane 2, day 2 extract from contralateral region of infected hippocampus amplified with *gB* primers; lane 3, day 2 extract from contralateral region of infected hippocampus amplified with *lacZ* primers; lane 4, 123-bp marker ladder (GIBCO BRL); lanes 5, 7, and 9, amplification products from day 2, day 7, and 8 week infected hippocampal extracts, respectively, using *gB* primers; lanes 6, 8, and 10, amplification products from day 2, day 7, and 8 week infected hippocampal extracts, respectively, using *lacZ* primers; lane 11, ϕX174-*Hae*III molecular weight marker (GIBCO BRL). (B) Amplification of *LAT* DNA. Lane 1, extract from contralateral hippocampus on day 7 postinjection; lane 2, extract from ipsilateral hippocampus on day 7 postinjection; lane 3, extract from ipsilateral hippocampus 8 weeks postinjection; lane 4, 123-bp marker ladder. (C) Amplification of viral DNA and RNA 8 weeks postinjection. Lane 1, 123-bp marker ladder; lane 2, DNA amplification using *gB* primers; lane 3, RNA amplification using *gB* primers; lane 4, RNA amplification using *LAT* primers; lane 5, RNA amplification using *LAT* primers, as in lane 4, but after RNase digestion.

demonstrate that the viral genome persisted in the cells of the injected hippocampus for at least 8 weeks after inoculation. Indeed, we have detected viral DNA sequences in hippocampal cells of frozen sections as late as 6 months after injection using this method (data not shown).

Using RT-PCR, both gB mRNA and LAT RNA could be detected in ipsilateral hippocampus extracts 2 days postinjection (data not shown). By day 7 (data not shown) and at 8 weeks postinjection, gB mRNA was not detectable, even though *gB* DNA was present in this tissue (Fig. 2C, lanes 2 and 3). However, LAT RNA was detected in this tissue at 7 days

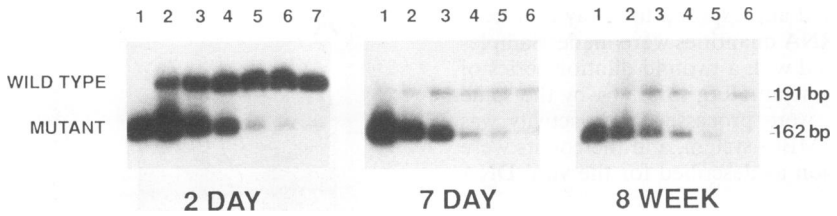

FIG. 3. Competitive quantitative PCR for viral *gB* DNA. Hippocampal region extracts from the tissue scraped off 10-μm sections of infected rat brains were coamplified with a 2-fold dilution series of competitor mutant *gB* DNA by using *gB* primers as described in the text. The radioactive amplified PCR products were digested with *Hpa*II and electrophoresed on 4% NuSieve GTG agarose gels. Gels were dried and exposed to X-ray film. Radioactivity was quantified by analyzing the dried gels with the AMBIS system. (2 DAY) Lane 1, 100 fg of mutant *gB* DNA coamplified with an aliquot of a day 2 uninfected contralateral hippocampal extract; lanes 2 to 7, twofold dilution series of mutant *gB* DNA ranging from 100 fg (lane 2) to 3.1 fg (lane 7) coamplified with aliquots of a day 2 infected hippocampal extract. (7 DAY) Lane 1, 100 fg of mutant *gB* DNA coamplified with an aliquot of a day 7 uninfected contralateral hippocampal extract; lanes 2 to 6, twofold dilution series of mutant *gB* DNA ranging from 50 fg (lane 2) to 3.1 fg (lane 6) coamplified with aliquots of a day 7 infected hippocampal extract. (8 WEEK) Lane 1, 100 fg of mutant *gB* DNA coamplified with an aliquot of an 8 week uninfected contralateral hippocampal extract; lanes 2 to 6, twofold dilution series of mutant *gB* DNA ranging from 50 fg (lane 2) to 3.1 fg (lane 6) coamplified with aliquots of an 8 week infected hippocampal extract.

(data not shown) and 8 weeks postinoculation (Fig. 2C, lane 4). No LAT RNA signal was found when a sample of the extract was treated with RNase prior to the start of RT-PCR (Fig. 2C, lane 5), demonstrating that the template for RT-PCR was RNase sensitive. These results, showing that at 2 days postinjection the RR1 and RR2 mutant genomes express both lytic cycle (gB) and latency (LAT) RNAs but by 8 weeks the persistent viral genomes express only LAT RNA, suggest that viral latency had been established in the infected hippocampus by 8 weeks.

Quantitation of viral DNA and RNA in the hippocampus of the rat brain. To understand the significance of the persistent LAT expression by the RR1-RR2 mutant virus, we quantitated the amount of *gB* DNA and LAT RNA in the injected hippocampal region. The *gB* gene serves as a representative of the HSV-1 genome. Five rats each were sacrificed at day 2, day 7, and 8 weeks postinoculation, 10-μm sections were prepared from each brain, and quantitative DNA and RNA PCR analyses (as described in Materials and Methods) were run on extracts from three independent sections. Figure 3 presents representative data from a single section from a single rat at each time point. The upper row of bands represent the wild-type 191-bp amplified product of the *gB* gene in the hippocampal extract. The more rapidly migrating bands, used as internal standards, represent the *Hpa*II-digested 162-bp fragment of the 191-bp mutant PCR product. The observed decrease in radioactivity reflects the twofold dilution of the mutant *gB* template, ranging from 100 to 3.1 fg, which plots as a straight line (data not shown). For LAT RNA (Fig. 4), the upper set of bands represent the twofold dilution series of standard RNA, ranging from 50 to 3.1 fg. The lower bands represent the amplified target RNA.

The results of linear regression analyses of the average net counts per minute of ^{32}P detected for 5 animals, each measured from three independent sections at each time point, are summarized in Table 1 for both *gB* DNA and LAT RNA. Fifty-five femtograms of *gB* DNA was found in tissue extracted from each 10-μm-thick section of injected hippocampus 2 days postinfection. By day 7, the amount of *gB* DNA in the injected hippocampus had decreased to 13 fg, and 17 fg was found in the same region at 8 weeks postinoculation. Converting these numbers to HSV-1 genome equivalents, the day 2 brain slices contained 2.6×10^5, the 7 day slices had 6.2×10^4, and the 8 week slices had 8.3×10^4. We conclude that the number of viral genomes did not change significantly in the infected hippocampal region between day 7 and 8 weeks postinjection. The average concentrations of LAT RNA were 338, 132, and 125 fg per section on day 2, day 7, and 8 weeks, respectively

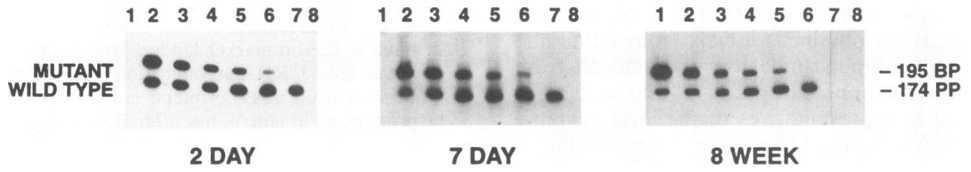

FIG. 4. Competitive quantitative RT-PCR for viral LAT RNA. RNA from hippocampal region extracts from the tissue scraped off 10-μm sections of infected rat brains were reverse transcribed and coamplified with a twofold dilution series of competitor mutant LAT RNA as described in the text. Radioactive RT-PCR products were digested with *Bsa*HI and electrophoresed on 4% NuSieve GTG agarose gels. Gels were dried and exposed to X-ray film. Radioactivity was quantified by analyzing dried gels with the AMBIS system. (2 DAY) Lane 1, RNA from day 2 infected hippocampal extract after RNase treatment; lanes 2 to 6, twofold dilution series of mutant LAT RNA ranging from 50 fg (lane 2) to 3.1 fg (lane 6) coamplified with aliquots of a day 2 infected hippocampal extract; lane 7, RNA from day 2 infected hippocampal extract; lane 8, RNA from a day 2 infected hippocampal extract without addition of reverse transcriptase. (7 DAY) Lane 1, RNA from day 7 infected hippocampal extract after RNase treatment; lanes 2 to 6, twofold dilution series of mutant LAT RNA ranging from 50 fg (lane 2) to 3.1 fg (lane 6) coamplified with aliquots of a day 7 infected hippocampal extract; lane 7, RNA from a day 7 infected hippocampal extract; lane 8, RNA from a day 7 infected hippocampal extract without addition of reverse transcriptase. (8 WEEK) Lanes 1 to 5, twofold dilution series of mutant LAT RNA ranging from 50 fg (lane 1) to 3.1 fg (lane 5) coamplified with aliquots of an 8 week infected hippocampal extract; lane 6, RNA from an 8 week infected hippocampal extract; lane 7, RNA from an 8 week infected hippocampal extract after RNase treatment; lane 8, RNA from an 8 week infected hippocampal extract without addition of reverse transcriptase.

TABLE 1. Quantitation of genome equivalents of HSV-1 and LAT RNA per brain slice

Time postinfection	Amt of *gB* DNA (fg)[a]	No. of genome equivalents[b]	Amt of LAT RNA (fg)[c]	No. of LAT RNA molecules[d]	No. of LAT RNA molecules/no. of genome equivalents
2 days	54.9 ± 0.3	2.6×10^5	338.3 ± 7.4	3.2×10^6	12.5
7 days	13.1 ± 0.3	6.2×10^4	131.8 ± 36.4	1.3×10^6	20.3
8 weeks	17.4 ± 0.3	8.3×10^4	124.7 ± 16.1	1.2×10^6	14.5

[a] Net counts per minute corresponding to amplified wild-type and mutant *gB* DNA were quantified by using the AMBIS Radioanalytic Imaging System, and the amounts of wild-type *gB* DNA were determined from linear regression plots of net counts per minute against amounts of input mutant *gB* DNA. Each value shown is an average from five different animals, each measured from three independent sections, ± standard deviation.

[b] The *gB* PCR product is 191 bp, which corresponds to 1.26×10^5 g/mol. The total number of *gB* molecules was calculated by using Avogadro's number. Since *gB* is a single-copy gene, the number of *gB* molecules is also the number of genome equivalents.

[c] The amount of LAT RNA was calculated in a manner similar to that for *gB* DNA, by using linear regression plots of net counts per minute against amounts of input mutant LAT RNA. Each value shown is an average from five different animals, each measured from three independent sections, ± standard deviation.

[d] The *LAT* PCR product is 195 bp, corresponding to 1.28×10^5 g/mol. As in the case of *gB*, the total number of LAT RNA molecules was calculated from Avogadro's number.

(Table 1). These concentrations correspond to 3.2×10^6, 1.2×10^6, and 1.2×10^6 LAT RNA molecules per section for day 2, day 7, and 8 weeks, respectively. We conclude that the number of LAT RNA molecules decreased by approximately two-thirds between day 2 and day 7 but remained unchanged from day 7 to 8 weeks. This suggests that transcription from the *LAT* region remained constant after the establishment of latency. Using the number of genome equivalents determined from the *gB* measurements and the number of LAT RNA molecules in a section, we calculated that there were 12.5 molecules per genome equivalent at day 2, 20.3 molecules per genome equivalent at day 7, and 14.5 molecules per genome equivalent at 8 weeks. We interpret these data to indicate that the amount of LAT message per viral genome did not change over time.

A number of control reactions were performed (Fig. 4). The day 2 (Fig. 4, lane 1), day 7 (lane 1), and week 8 (lane 7) extracts were treated with RNase prior to RT-PCR. These reaction mixtures failed to produce the wild-type bands seen in Fig. 4 (day 2, lane 7; day 7, lane 7; and week 8, lane 6), in which only extract samples were amplified, proving that the target of RT-PCR was indeed an RNA template. This was substantiated by the absence of amplification if the Moloney murine leukemia virus reverse transcriptase was not added to the reaction mixture (Fig. 4, day 2, lane 8; day 7, lane 8; week 8, lane 8). A single band was observed when the extract alone was amplified and digested with restriction enzyme, indicating that the digestion was complete (Fig. 4, day 2, lane 7; day 7, lane 7; week 8, lane 6).

Although the amplification products of the quantitative PCR reactions migrated in agarose gels as single bands of the expected sizes, the specificity of the amplification products of both DNA and RNA at each time point was confirmed by DNA sequencing (data not shown). In addition, hybridization analyses using oligonucleotides complementary to the amplified sequences between the primers also confirmed the identity of the quantitative DNA PCR product as that of the viral *gB* gene and the identity of the quantitative RT-PCR product as that of the *LAT* gene (data not shown).

Quantitation of the number of genome equivalents per PFU in the RR1*CAT*/RR2*lacZ* virus stock. We also determined the number of genome equivalents per PFU in the suspension of virus used for injections into rat brains. RR1*CAT*/RR2*lacZ* particles were treated with proteinase K to extract viral DNA, and samples equivalent to 10 PFU of virus were coamplified with a 10-fold dilution series of mutant *gB* DNA fragments ranging from 10^5 to 10 molecules, by using the wild-type *gB* primers in the presence of [α-^{32}P]dCTP and the products processed as described above. The number of genome equivalents present in the virus suspension for each PFU was found to be in the range of 50 to 150 molecules. In the second step of the quantitation process, samples of DNA containing 10 PFU were coamplified with twofold dilutions of mutant *gB* DNA, ranging from 1,000 to 10 molecules and processed as before. The average value from three independent determinations was found to be 88.87 ± 11.15 genome equivalents per PFU.

DISCUSSION

RR mutant virus was used for these injection experiments because HSV-1 mutants with mutations in either the *RR1* or the *RR2* gene have been reported to be highly attenuated and less neurovirulent following intracranial injection in mice (6) and less capable of reactivating from ganglionic latency (32). Cameron et al. (6) found the virulence of RR mutants to be reduced about 10^6-fold when compared to that of the parental virus, HSV-1 strain 17. The RR1 protein is multifunctional, having protein kinase activity in addition to being a component of the reductase. The protein kinase and RR activities reside in nonoverlapping regions of the *RR1* gene product, with the kinase activity in the amino third and the reductase in the carboxyl two-thirds of the protein (1, 2, 10, 11, 19, 37). It remains to be determined whether neurovirulence is correlated with either the protein kinase or RR function of the protein or with both, although virus mutants with mutations in the *RR2* gene are also greatly attenuated (6), suggesting that the reductase activity is the major factor in neurovirulence.

The work presented in this paper confirms and extends earlier reports that HSV-1 establishes latency in neurons of the CNS, with many of the characteristics of latency in peripheral neurons. Stereotactic injection of the RR1*CAT*/RR2*lacZ* recombinant virus in the hippocampal region of the rat brain resulted in a localized infection which could be detected both at the level of the viral genome and at that of its gene products. HSV-1 genomic sequences from the *gB*, *LAT*, and *lacZ* (replacing the *RR2* coding sequence) genes were amplified from templates obtained by scraping the hippocampal region off single 10-μm frozen sections of injected brain. While these genes are only a small portion of the more than 70 genes of HSV-1, they are from three different physical regions of the HSV-1 genome and we therefore assume that they represent the presence of the entire viral genome. We were able to amplify viral sequences only from the area of the injected hippocampus, indicating that virus does not readily spread to other regions. Visualization of viral gene products by immunochemical, cytochemical, and in situ hybridization techniques

on brain sections confirmed the localization of infected cells to the injected hippocampus region at day 2 postinjection, and gB message and LAT could be detected by RT-PCR. By day 7, neither CAT message, gB message, nor β-galactosidase activity could be visualized by the same techniques, although viral DNA was detected by PCR and LAT could be detected by RT-PCR. This pattern of persistence of viral DNA and LAT, but not gB RNA, was also seen at 8 weeks and 6 months and is similar to that observed following intracranial inoculation with other HSV-1 mutants (22). We used competitive quantitative PCR and RT-PCR to investigate this phenomenon quantitatively.

The injected RR1*CAT*/RR2*lacZ* virus stock contained approximately 4×10^9 PFU/ml, or approximately 2×10^7 PFU in the 5-μl injection volume. With 88.9 genome equivalents for each PFU in this stock, about 1.8×10^9 genome equivalents was injected into each brain. Hippocampal infections with this volume of virus typically extend approximately 3 mm in a rostral-to-caudal direction. Assuming that viral genomes are evenly distributed through this region, the total number of genomes in the hippocampus can be calculated from the number found in individual 10-μm sections (Table 1, number of genome equivalents per 10-μm section multiplied by 300) and corresponds to approximately 7.8×10^7 at day 2, 1.8×10^7 at day 7, and 2.4×10^7 at 8 weeks postinjection. Only 4.3% of the injected genome equivalents were recovered at 2 days. A further loss of genome equivalents occurred between day 2 and day 7 (1% of injected genome equivalents were recovered), after which the number remained constant up to 8 weeks (1.2% of injected genome equivalents were recovered). The approximately 2×10^7 genome equivalents present at 7 days and 8 weeks were equal to the number of PFU originally injected into the brain. It is not clear whether this concordance is fortuitous or represents the establishment of latency by the viable genomes represented by each PFU. The amount of LAT RNA also decreased by about 60% from day 2 to day 7 and then remained constant (Table 1). The number of LAT RNA molecules per genome equivalent was approximately the same for each of the three time points, about 16. The data permit the conclusion that from day 7 to 8 weeks the number of viral genome equivalents and LAT molecules remained constant in the hippocampal region, presumably for the life of the host, without apparent loss of template or transcript.

As far as we are aware there are no quantitative studies of ganglionic latency which address the question of the number of LAT molecules per HSV-1 genome. On the basis of an equivalence of signals for LAT and VP5 (the major capsid protein gene) RNA on Northern blots and in situ hybridizations, Wagner et al. (63) made a rough estimate that LATs are present at 2×10^4 to 5×10^4 molecules per latently infected mouse neuron. Estimates of the number of viral genomes in latently infected neurons vary greatly, from ten to as many as a few hundred being the most reasonable (50, 51, 59). Assuming that each latently infected neuron contains 100 viral genomes on the average, there would be 200 to 500 LAT molecules per genome in these cells. Taken at face value, these calculations suggest that the LAT promoter is less active in brain tissue than in ganglia. We are currently applying the competitive quantitative PCR and RT-PCR techniques described in this paper to an analysis of the number of HSV-1 genomes and LAT molecules in the latently infected rat ganglion.

The net loss of 99% of the input HSV-1 genomes suggests that the RR1-RR2 virus replicated little or not at all following intracranial inoculation. This observation is consistent with the failure of the HSV-1 KOS mutant ICP6Δ containing a 2.9-kbp deletion in the *RR1* gene (27) to actively replicate in the mouse trigeminal ganglia after corneal inoculation or in embryonic mouse cells at 38°C, the mouse body temperature (32). However, the viral genomes in the hippocampus region appear to be transcriptionally active on day 2, as demonstrated by the presence of gB mRNA in the tissue, although it is not clear whether viral DNA replicated.

A number of approaches to using PCR techniques for quantifying HSV-1 DNA in ganglia have been reported. Known amounts of a standard HSV-1 DNA and latently infected ganglionic DNA were amplified in parallel reactions with the same set of primers (33, 52). The amount of HSV-1 DNA in ganglia has been calculated from the standard curve, after normalization with the PCR product of the mouse adipsin gene as an internal standard. This method used large amounts of starting DNA samples and did not control for tube-to-tube variations, which can occur even if the same dilution of template is amplified (25). Others amplified a cellular DNA template of known concentration along with the target DNA in the same tube (8, 23). While this represents an improvement, the kinetics of amplification may vary for different templates (16, 25, 44).

We used the competitive quantitative PCR strategy (4, 25) in our studies. The competitive template used to generate the standard curve was identical in size and sequence with the target *gB* DNA except for a single base change producing a unique *Hpa*II restriction site. The two templates were coamplified in the same tube using the same primers, eliminating both tube-to-tube and template variability. Since the conditions of amplification were identical for the target and competitor DNA, the rates of amplification for both should be similar, if not identical. The two PCR products were distinguished from one another after *Hpa*II digestion and electrophoresis. The amounts of amplified products could be determined by ethidium bromide staining after electrophoresis, although we used incorporation of a radioactive precursor during amplification. The radioanalytic imaging system was employed to determine the number of counts per minute in individual bands, because it is more sensitive and accurate than densitometric scanning.

Two variations of the competitive quantitative PCR are equally effective. (i) Similar quantitative data were obtained by running the amplification reactions in the absence of radioactive precursor followed by hybridization of the amplified products with an appropriate radioactive probe. Using this latter technique, we obtained the values of 50.1 fg of viral DNA and 290 fg of LAT RNA in 10-μm sections of 2 day postinjected rat brains. These numbers correspond well with those in Table 1 on day 2. This strategy is similar to that employed by Mahalingam et al. (39), who have determined that there are between 6 and 31 copies of latent varicella-zoster virus DNA molecules per 100,000 human trigeminal ganglionic cells. (ii) The PCRs were carried out in two stages. In the first stage, samples were amplified for 30 cycles and then the reaction products were diluted 200-fold. In the second stage, the diluted reaction products were amplified for an additional two cycles, thus eliminating heterodimer formation (4). It was not necessary to have radioactivity present during the first 30 cycles of amplification, as the radioactivity incorporated during the last two cycles was sufficient.

When the amount of message available for analysis is low, conventional methods for quantitating RNA, such as Northern hybridization and RNase protection assays, are often of limited utility. LATs have been detected by in situ hybridization (24, 55, 61) and by Northern blotting (60, 63), both of which are relatively insensitive and nonquantitative techniques. Compet-

itive PCR has been used to quantitate rare messages. In one procedure (25), the mRNA of interest was reverse transcribed into cDNA and quantified by coamplification with a mutant DNA, using the same set of primers. The amount of mRNA may be underestimated by this method, as the efficiency of reverse transcription can range from 5 to 90% (28). In another method, target RNA is directly reverse transcribed and amplified with a synthetic RNA standard having the same primer sequences as the mRNA but a different internal sequence (65), giving rise to PCR products of different sizes. However, a problem may arise if the kinetics of the reverse transcription reactions vary between the two templates (56). In the so-called PATTY method (4), competitive DNA is cloned into a transcription vector and RNA equivalents are generated in vitro. The competitive DNA derived from the RNA differed from the target cDNA by one or two nucleotides, resulting in the creation or deletion of a unique restriction site. Subsequently, all the reactions were carried out in the same tube, subjecting both templates to the same reaction conditions. Following reverse transcription, PCR, and digestion with the appropriate restriction endonuclease, the mutant standard and target cDNA can be distinguished. This method gives results more accurately representative of the original RNA populations, and we have utilized it in our analysis of *LAT* expression in infected cells of the CNS.

A time-consuming step in the PATTY method is cloning the mutant standard DNA into a transcription vector for in vitro transcription. We circumvented the cloning step by directly attaching an oligonucleotide coding for the T3 RNA polymerase promoter sequence (53) to the 5′ terminus of the 5′ PCR primer to be used to amplify competitive DNA. The T3 promoter oligonucleotide and the 5′ primer were held in place for ligation by a bridging oligonucleotide which is complementary to both. The composite 5′ primer was used with an appropriate 3′ PCR primer to amplify competitive DNA. The T3 RNA polymerase promoter site at the 5′ end of the amplified DNA was then utilized to transcribe in vitro and produce standard RNA. We believe that the strategy described is a rapid and reliable method of general utility for generating internal RNA standards for competitive RT-PCR, without the need for cloning.

The accuracy of the quantitation of HSV-1 DNA and RNA by PCR is dependent on the reproducibility of the techniques employed. The efficiency of the reaction can be affected by the concentration of the components (Mg, dNTPs, *Taq* polymerase, template DNA, primers) and by the temperatures used for annealing and for extension (for reviews, see references 20, 21, and 31). Thus, reproducible conditions must be established for each PCR reaction. We believe the conditions under which we have carried out our DNA and RNA PCR quantitations are reproducible since the variation between different equivalent samples was small.

The results reported in this paper have a number of important implications for the use of HSV-1 as a vector for gene therapy in the CNS. (i) Mutations in the RR genes did indeed result in attenuation of neurovirulence in the rat brain. All 15 rats employed survived the injections, with five out of five animals surviving up to 8 weeks without apparent ill effects. Primary sympathetic neuronal cultures prepared from superior cervical ganglia of day 21 embryonic rats have been reported to survive infection with an HSV-1 RR1 mutant (7). RR mutations may, therefore, prove valuable in building a nonpathogenic vector. (ii) Viral genomes were stably maintained in the CNS once latency was established, with no evidence for loss over time. Thus, any therapeutic construct incorporated into the HSV-1 genome should persist in the CNS for the life of the host. (iii) The *LAT* promoter would seem to be a good candidate to drive expression of a therapeutic gene in the CNS (12). LAT RNA appears to persist in peripheral ganglia for the lifetimes of mice (12) and humans (58), and reporter genes driven by *LAT* gene regulating sequences have been expressed in these neurons (17, 26, 30). Wolfe et al. (68) observed expression of a β-glucuronidase gene driven by the *LAT* promoter in brain stems of mice lacking this enzyme for up to 4 months postinoculation. We found that steady-state levels of LAT RNA in the rat brain remained constant up to 8 weeks, and they presumably persist for the life of the host, suggesting that the *LAT* promoter might serve to drive a therapeutic gene long term in the CNS. However, as discussed above, the 16 LATs transcribed on the average from each latent genome suggest that the *LAT* promoter is not very strong. It should be remembered that each HSV-1 genome carries two copies of the *LAT* gene. On the other hand, the total number of LATs in the hippocampal region may be significant (2×10^7 viral genome equivalents × 16 LATs per genome equivalent = 3.2×10^8 LAT RNAs) and could be the source of a sufficient amount of a therapeutic protein.

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