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Utilization of the Herpes Simplex Virus Type 1 Latency-Associated Regulatory Region To Drive Stable Reporter Gene Expression in the Nervous System

ROBIN H. LACHMANN AND STACEY EFSTATHIOU*

Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom

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The ability of herpes simplex virus type 1 (HSV-1) to establish a lifelong, transcriptionally active, latent infection in neurons has led to much interest in developing HSV-based vectors for gene delivery to the nervous system. A prerequisite of such vectors is that they should be capable of directing long-term transgene expression in latently infected neurons. The continued transcription of HSV-1 latency-associated transcripts (LATs) during neuronal latency suggests that regulatory sequences which mediate expression of LATs could be utilized for long-term expression of heterologous genes in the mammalian nervous system. In addition to upstream regulatory elements which are important for LAT promoter-mediated transcription during neuronal latency, there is growing evidence that sequences downstream of the LAT transcription start site play an important role in facilitating long-term latent-phase transcription. In order to maintain the integrity of both upstream and downstream regulatory elements of the LAT promoter, we constructed viruses which contained the *lacZ* and *lacZ-neo* reporter genes linked to the encephalomyocarditis virus internal ribosomal entry site (IRES) (viruses LβA and LβB, respectively) inserted approximately 1.5 kb downstream of the LAT transcription start site. These viruses expressed low levels of β-galactosidase in lytically infected Vero cells and in cervical dorsal root ganglion neurons during the acute stage of infection in vivo. In contrast, at later times postinfection and consistent with the establishment of latency, increases both in the numbers of neurons expressing β-galactosidase and in the intensity of staining were observed. Examination of the brain stems and spinal cords of animals latently infected with LβA, sampled at time points from 72 to 307 days postinfection, revealed the stable expression of β-galactosidase within neurons located in facial and hypoglossal nerve nuclei and the upper cervical spinal cord. We conclude that the insertion of an IRES linked to a reporter gene 1.5 kb downstream from the LAT transcription start site does not disrupt elements of the LAT promoter necessary for long-term gene expression and, in both the peripheral and central nervous systems, facilitates β-galactosidase expression in a wide variety of neurons.

An important aspect of the natural life cycle of herpes simplex virus (HSV) is its ability to establish latency within neurons that innervate the site of primary infection (6, 31). During latency, the virus genome is maintained in a nonlinear, nonintegrated, nucleosome-bound state (8, 16, 32, 37) and transcription is restricted to a single region, encoding latency-associated transcripts (LATs), which maps to repeats flanking the unique long region of the viral genome (40).

HSV-encoded LATs consist of a series of nuclear transcripts comprising two highly abundant, nonpolyadenylated species of 2.0 and 1.5 kb, termed major LATs, which map in an antisense direction to part of the ICP0 gene. The precise mechanism of synthesis of major LATs is unclear, although there is evidence to support the view that these transcripts may represent stable introns derived from a less abundant 8.5-kb polyadenylated precursor RNA, termed minor LAT (reviewed in reference 18). There is no evidence of viral protein expression in latently infected neurons in vivo, and the expression of LATs is not required for the establishment or maintenance of latent infections. However, these transcripts have been implicated in virus reactivation (21) through an as-yet-uncharacterized mechanism.

The ability of HSV to establish transcriptionally active, lifelong latent infection in neurons of both the peripheral and central nervous systems has led to considerable interest in the potential use of replication-defective HSV-based vectors for neuronal gene delivery (reviewed in reference 19). An important consideration in the development of such vectors is the identification and characterization of promoter and regulatory elements which can drive the stable long-term expression of foreign genes during neuronal latency. The majority of heterologous promoters tested to date have led to either transient gene expression or low-level, long-term gene expression in a small proportion of transduced cells (4, 14, 25, 27). The mechanism of such promoter silencing during HSV latency is unclear, although it has been suggested that this is a consequence of nucleosomal repression (4, 27).

The continued transcription of LATs in latently infected neurons has resulted in a number of studies directed toward understanding their mechanism of synthesis and transcriptional control (2, 3, 10, 13, 26, 44, 45), as well as attempting to utilize elements of the LAT promoter to drive long-term foreign-gene expression in both peripheral and central nervous system neurons. The TATA box and basal transcriptional regulatory sequences, which constitute the core LAT promoter, reside approximately 700 bp upstream of the 2-kb major LAT. Insertion of the core LAT promoter (extending 865 bp upstream of the cap site) into an ectopic genomic locus is, however, unable to drive prolonged reporter gene expression during latency (11, 27). Nonetheless, pairing the core LAT promoter with the Moloney murine leukemia virus (MoMLV)

* Corresponding author. Mailing address: Division of Virology, Dept. of Pathology, Tennis Court Rd., Cambridge CB2 1QP, United Kingdom. Phone: (1223) 336915. Fax: (1223) 336926. E-mail: se@mole.bio.cam.ac.uk.

long terminal repeat (LTR) results in long-term transcription in the peripheral nervous system of mice for at least 42 days postinfection, suggesting that the MoMLV LTR can functionally substitute for HSV-encoded long-term expression elements (27).

The precise nature of such HSV-encoded elements is unclear, but several studies have indicated that sequences downstream of the LAT transcription start site may play an important role in facilitating stable gene expression during latency (9, 11, 20, 29). Furthermore, recent studies (35) have demonstrated that the insertion of a DNA fragment encompassing the core LAT promoter and the first 1.5 kb of transcribed LAT sequences facilitates the transcription of truncated LATs during latency when it is inserted at an ectopic site within the virus genome. This observation suggests that HSV long-term expression elements reside within a 1.5-kb region downstream of the LAT transcription start site.

In this communication, we describe the characterization of HSV type 1 (HSV-1) recombinants containing reporter gene constructs inserted downstream of all currently recognized LAT regulatory sequence elements. Reporter genes linked to the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) were inserted approximately 1.5 kb downstream of the LAT transcription start site, corresponding to a locus which we have previously shown to accommodate large foreign DNA inserts without influencing LAT promoter-mediated transcription during latency (25). After peripheral infection, the recombinant viruses described here were capable of driving stable, long-term reporter gene expression in the peripheral nervous system of mice for at least 190 days postinfection. Stable β-galactosidase expression in discrete regions of the brain stems and spinal cords of animals latently infected with one of the recombinants was also observed. We conclude that the insertion of an IRES-linked reporter gene 1.5 kb downstream from the LAT transcription start site does not disrupt elements of the LAT promoter necessary for long-term gene expression, thus facilitating the stable expression of β-galactosidase in a wide variety of latently infected neurons.

MATERIALS AND METHODS

Cells and viruses. Viruses were grown and assayed on Vero cells. Cells were grown in Glasgow's modified Eagle's medium (28) containing 10% newborn calf serum and 10% tryptose phosphate broth.

All viruses were made on an HSV-1 strain SC16 background (23). Viruses were propagated at a multiplicity of infection of 0.01 PFU/cell, and virus titers were determined by suspension assays.

Plasmids. pSLAT 1 contains a 4.6-kb *Pst*I-to-*Bam*HI fragment derived from the HSV-1 SC16 *Bam*HI B region and cloned into pBluescribe M13$^-$ (Stratagene). This cloned region represents nucleotides 118867 to 123460, based on the nucleotide sequence of HSV-1 strain 17 (30, 36).

pCA1 (15) is a pBABE-based plasmid. A cassette consisting of the EMCV IRES linked to a *lacZ-neo* fusion gene (β*geo*), so that the AUG of the *lacZ* gene replaced AUG 11 of the IRES, was inserted between the *gag* and 5′ LTR of pBABEpuro (33) to replace the simian virus 40 promoter and *puroR* genes.

pSLAT 1 β*geo* contains an EMCV IRES linked to β*geo* and cloned into a 168-bp *Hpa*I deletion corresponding to nucleotides 120301 to 120469. The EMCV IRES-β*geo* construct was excised from pCA1 as an *Xba*I insert, which includes the first 270 bp of the MoMLV LTR and 70 bp of upstream sequence from pBABEpuro at its 3′ end, end repaired with the DNA polymerase Klenow fragment, and cloned into the *Hpa*I locus in pSLAT 1 by standard protocols. A recombinant plasmid containing the EMCV IRES-β*geo* cassette in the same orientation as that of LATs was generated.

pLacZ4 contains a 3.6-kb *Bam*HI fragment containing the *lacZ* coding sequence from pMV10 (42) cloned into pBS (Stratagene) so that the 3′ end of *lacZ* lies adjacent to the T7 promoter.

pSLAT 2 contains a 1.6-kb *Pst*I-to-*Hpa*I fragment derived from the HSV-1 SC16 *Bam*HI B region and cloned into pBluescribe M13$^-$ (Stratagene). This cloned region represents nucleotides 118867 to 120301, based on the nucleotide sequence of HSV-1 strain 17 (30, 36).

Construction of recombinant viruses. Viruses SC16 C3b and SC16 C3b$^+$ are recombinant viruses containing the *lacZ* gene driven by the cytomegalovirus (CMV) immediate-early (IE) promoter and inserted in each orientation into a 168-bp *Hpa*I deletion within the major LAT locus of HSV-1 strain SC16. Their structures are shown in Fig. 1, and their construction has been described previously (25).

Virus LβA. pSLAT1 β*geo* was linearized by *Sca*I digestion, and 3 μg of this plasmid DNA was cotransfected into Vero cells with 10 μg of high-molecular-weight SC16 C3b$^+$-infected cell DNA by a $CaCl_2$-dimethyl sulfoxide boost method (41). After 3 days of culture, infected cell monolayers were harvested and sonicated, and recombinant progeny were selected by overlaying Vero cell monolayers infected at a low multiplicity with 1% agarose in medium containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as previously described (17). β-Galactosidase-negative "white" plaques were picked and subjected to three rounds of plaque purification.

Virus LβB. Virus LβB was constructed in the same way as described above for virus LβA, except that high-molecular-weight SC16 C3b DNA was used in the transfection.

Recombinant viruses were examined by restriction digestion and Southern blot hybridization by using standard protocols to confirm their predicted structures.

Mouse infections. Groups of 5- to 6-week-old female BALB/c mice (Harlan Ltd.) were infected with 5×10^6 PFU of LβA in a volume of 20 μl of Glasgow's modified Eagle's medium by subcutaneous injection into the pinna of the left ear for analysis of β-galactosidase expression in ganglia and brain stems. Another group was infected with LβB. At acute (4 to 6 days postinfection) and latent (1, 3, and 6 months postinfection) time points, mice were terminally anesthetized with sodium pentobarbitone and cervical dorsal root ganglia CII through CIV were dissected and pooled prior to being processed for either histochemical staining for β-galactosidase activity or for in situ hybridization (ISH).

If brain stem tissue as well as ganglia was required, the brain was removed and fixed in 4% paraformaldehyde at room temperature for 3 h prior to equilibration with 10, 20, and 30% sucrose (in phosphate-buffered saline [PBS] containing 2 mM $MgCl_2$) on ice. Once they were equilibrated with sucrose, brains were stored at −70°C prior to being sectioned.

Mice from which ganglia were not harvested were terminally anesthetized and then perfuse fixed with 20 ml of 4% paraformaldehyde. Their brains were dissected and fixed for 1 h in 4% paraformaldehyde at room temperature. Then they were equilibrated with sucrose and stored as described above.

Histochemical β-galactosidase detection. (i) Whole-ganglia staining. Ganglia were fixed in 2% paraformaldehyde–0.2% glutaraldehyde in PBS for 1 h on ice. Then they were transferred to detergent solution (0.01% sodium deoxycholate, 0.02% Nonidet P-40, 2 mM $MgCl_2$ in PBS) and incubated for 30 min on ice, after which they were transferred to X-Gal solution (detergent solution containing 4.5 mM potassium ferricyanide, 4.5 mM potassium ferrocyanide, and 1 mg of X-Gal per ml) and incubated at 37°C for 3 h. After being stained, ganglia were rinsed in PBS and clarified by immersion in 20% glycerol in PBS for 2 h at 4°C and then in 50% glycerol overnight. Ganglia were mounted under a coverslip and evaluated as whole mounts. After "blue" neurons were counted, ganglia were embedded in paraffin and serial 5-μm sections were cut. Then these sections were examined under a microscope, and the blue neuronal profiles per ganglionic section were enumerated.

(ii) Brain stem staining. Serial transverse sections of brain stems and upper cervical spinal cords were made either by using a vibratome (150- to 300-μm sections) or by frozen sectioning on a sledge microtome (60-μm sections). Sections were collected in detergent solution in 24-well plates and incubated on ice for 1 to 2 h. Then sections were transferred to X-Gal solution (in some staining reaction mixtures, 0.5 mg of bluogal (Gibco BRL) per ml was used instead of X-Gal) and incubated at 37°C for 3 to 7 h. Then sections were mounted on polylysine-coated slides and air dried at 37°C overnight. They were then defatted by immersion in xylene for 2 min prior to rehydration through graded ethanols and counterstaining with either hematoxylin or cresyl violet.

ISH. Probes to detect LATs were made by T7 polymerase transcription of *Hin*dIII-linearized pSLAT 2 with a digoxigenin (DIG) detection system as described previously (1). One microgram of plasmid was transcribed with Stratagene T7 polymerase under the manufacturer's recommended conditions. After transcription, the reaction mixtures were ethanol precipitated and the product was resuspended in 100 μl of 10 mM Tris (pH 8)–1 mM dithiothreitol with RNase inhibitor.

Probes to detect *lacZ* transcripts were made by T7 polymerase transcription of *Pst*I-linearized pLacZ4 as described above.

ISH was performed as previously described (1). Pooled ganglia were fixed for 1 h in periodate-lysine-paraformaldehyde at room temperature, transferred to 50% ethanol, and then embedded in paraffin. Sections (5 μm) were collected onto glutaraldehyde-activated, 3-aminopropyl-triethoxysilane-coated slides and dewaxed in xylene before use.

Sections were digested with 100 μg of proteinase K per ml for 8 min at 37°C. Overnight hybridization was carried out at 25°C below theoretical melting temperature (72°C for the LAT-specific probe and 60°C for *lacZ*-specific probes). One to three microliters of DIG-labelled riboprobe was used in each 100 μl of hybridization solution. A stringent wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–30% formamide–10 mM Tris-HCl (pH 7.5) was carried out at 10°C below melting temperature (75°C for the LAT-specific probe and 63°C for *lacZ*-specific probes) for 30 min. Bound probe was detected with alkaline phosphatase-conjugated anti-DIG Fab fragments according to the manufacturer's (Boehringer Mannheim) instructions.

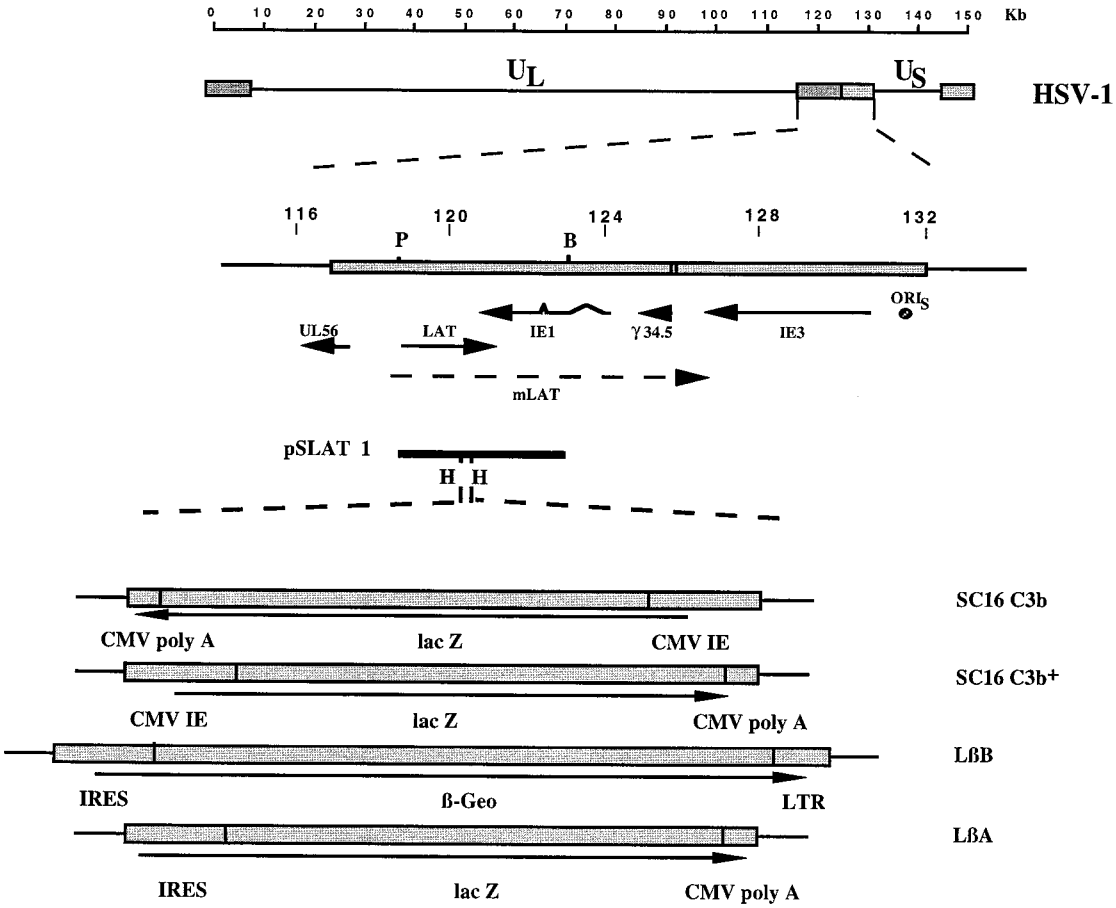


FIG. 1. Genomic structures of recombinant viruses. The overall structure of the HSV-1 genome is shown, with unique long (U_L) and unique short (U_S) regions flanked by inverted terminal repeats. The LAT region, located in terminal repeats, has been expanded, and the locations of LATs and surrounding transcripts are shown. The LAT region insertion contained within plasmid pSLAT 1 is indicated, as is the site of the *Hpa*I (H) deletion used to make the insertions into recombinant viruses. The structures of the insertions made into this deletion in all the recombinant viruses, SC16 C3b, SC16 C3b$^+$, LβB, and LβA, are shown.

RESULTS

Construction of recombinant viruses. In order to maintain the integrity of both upstream and downstream regulatory elements of the LAT promoter, reporter genes were inserted between nucleotides 120301 to 120469 of the HSV-1 genome. The reporter gene cassettes therefore replaced a 168-bp *Hpa*I fragment deleted from the major LAT locus. We have previously shown that DNA insertions at this locus, which lies approximately 1.5 kb downstream of the LAT transcription start site, result in LAT promoter-driven synthesis of transcripts, which are exported to the cytoplasm of latently infected neurons (25). In order to facilitate translation of a reporter gene from such a transcript, which would be expected to include a 1.5-kb 5′ LAT-derived leader containing 10 AUG codons, a cassette in which the reporter gene was preceded by an EMCV IRES was utilized to enable cap-independent ribosomal access to coding sequences. We have previously demonstrated that the EMCV IRES element can function efficiently in the context of the HSV genome during lytic-phase transcription (25).

Two recombinants were constructed with different parental SC16-based viruses. LβB was made by cotransfecting linearized pSLAT 1 β*geo* with SC16 C3b DNA (Fig. 1). A "white" recombinant virus was selected by plaque picking under an X-Gal agarose overlay, and Southern blotting confirmed that the EMCV IRES-β*geo* cassette had replaced the CMV IE-*lacZ* cassette from SC16 C3b (data not shown). SC16 LβA virus was made with SC16 C3b$^+$ as the parental virus. SC16 C3b$^+$ (Fig. 1) contains the CMV IE-*lacZ* cassette in the opposite orientation to that in SC16 C3b, that is, in the same sense as LATs. Hence, the *lacZ* genes in the parental SC16 C3b$^+$ virus and the cotransfected pSLAT 1 β*geo* were colinear with each other. This complementarity allowed recombination between *lacZ* gene sequences in pSLAT 1-β*geo* and SC16 C3b$^+$. Again, a "white" progeny virus was selected by plaque picking. Southern blot analysis of the flanking regions to the left and right of the insert confirmed that the left-hand flank was identical to LβB and that the right-hand flank was the same as that in the parental virus, SC16 C3b$^+$ (data not shown). Hence, the downstream recombinatorial crossover had occurred within the *lacZ* gene, generating a virus with the EMCV IRES linked to *lacZ* and the CMV poly(A) site (Fig. 1).

In order to determine whether β-galactosidase expression occurred during lytic infection of nonneuronal Vero cells, monolayer assays of both LβA and LβB were undertaken. Monolayers containing virus plaques were fixed and stained with the chromogenic substrate X-Gal. Consistent with the known neuronal specific activity of the LAT promoter (3, 45), both viruses produced essentially "white" β-galactosidase-negative plaques. This expected phenotype had been exploited in the selection of these recombinant viruses from parental vi-

ruses SC16 C3b and SC16 C3b$^+$, which express high levels of β-galactosidase during productive infection of Vero cells, which is readily detectable in infected monolayers. A closer examination of LβA and LβB plaques, however, revealed small numbers of infected cells which stained faintly blue, giving plaques a characteristically speckled appearance (data not shown). Thus, it would appear that at least in a proportion of lytically infected Vero cells, low-level transcription through the LAT region occurs, an observation consistent with the detection of major LATs at late time points after the infection of nonneuronal cells in culture (10, 39).

β-Galactosidase expression in the peripheral nervous system of mice infected with LβA and LβB. In order to determine whether LβA expressed β-galactosidase during latency, BALB/c mice were infected with 5×10^6 PFU of LβA in the left ear. Dorsal root ganglia (CII through CIV) were dissected from animals during the acute phase of infection (4 days after infection) and during latency (at 26, 82, and 190 days after infection). The ganglia from 5 to 6 mice at each time point were pooled and stained histochemically for β-galactosidase activity with the chromogenic substrate X-Gal for 3 h before termination of the color reaction. Whole ganglia were mounted in glycerol, and blue neurons were enumerated (selected ganglia are shown in Fig. 2). The raw data from pooled ganglia sampled at each time point are shown in Fig. 3a and are summarized in Table 1. At 4 days postinfection, the majority of ganglia examined contained only small numbers of detectable β-galactosidase-positive blue neurons (Fig. 2a) and only 2 of 15 ganglia contained >10 β-galactosidase-positive neurons (Fig. 3a). However, as mice entered the latent phase of infection (for example, at 26 days after infection), a greater proportion of ganglia contained detectable blue neurons, with 9 of 15 ganglia containing >10 β-galactosidase-positive neurons. Furthermore, the intensity of staining observed within neurons at this later time was greater than that observed during the acute phase of infection (Fig. 2b) and axonal tracking of β-galactosidase enzyme was readily detected. A similar intense pattern of neuronal staining was observed in sensory ganglia sampled 82 days after infection, and ganglia containing large numbers of blue neurons expressing β-galactosidase were readily detected at 190 days postinfection (Fig. 2c and d).

An examination of the raw data in Fig. 3a shows a wide variation in the numbers of blue neurons detected in individual ganglia. Since the ganglia from mice were pooled prior to being stained, it is unclear from these data whether this skewed distribution is due to latency being established most efficiently at one of the three spinal levels examined or is a result of mouse-to-mouse variation. Nonetheless, as summarized in Table 1, despite the wide range of positively stained cells within individual ganglia, no obvious decrease in the average number of β-galactosidase-expressing cells was evident throughout the time course of these experiments.

Counting β-galactosidase-positive blue neurons in whole mounted ganglia could be subject to at least a degree of inaccuracy, particularly in ganglia containing heavily stained cells, since this method of counting involves enumeration in a number of different focal planes. For this reason, after the enumeration of β-galactosidase-positive neurons in whole mounted ganglia sampled at 82 and 190 days postinfection, tissues were embedded in paraffin and sectioned on a microtome. This allowed an assessment of the number of blue neuronal profiles within ganglionic sections to be made (Fig. 2f). At 82 days after infection, 261 blue neuronal profiles were detected in a total of 271 ganglionic sections (an average of 0.96 blue neuronal profiles per ganglionic section). At 190 days after infection, 354 blue neuronal profiles were detected in a total of 261 ganglionic sections (an average of 1.36 blue neuronal profiles per ganglionic section). From these data, there was no obvious decrease in the numbers of neurons expressing β-galactosidase in ganglia removed from mice at between 82 and 190 days postinfection.

β-Galactosidase expression in the peripheral nervous system of mice infected with LβB was also examined. Figure 3b shows the numbers of blue neurons from whole mounted ganglia removed from animals at various times after infection. As observed for LβA, despite the wide variation in the numbers of blue neurons detected in individual ganglia, we were able to demonstrate long-term β-galactosidase expression within latently infected sensory neurons at up to 140 days after infection. However, through the course of this experiment, we noted that the numbers of β-galactosidase-positive cells per ganglion (Table 1) and the intensity of the staining of cells (Fig. 2e) with this virus appeared to be decreased from those observed with LβA. Whole mounted ganglia sampled from LβB-infected animals at 140 days after infection were embedded in paraffin, and sections were scored for blue neuronal profiles. We observed 59 positive blue neuronal profiles of a total of 612 ganglionic sections (an average of 0.1 blue neuronal profiles/ganglionic section), a figure approximately 10-fold-lower than that obtained from LβA material sampled and analyzed in a similar manner at 82 and 190 days postinfection. In order to determine whether these observed differences between LβA and LβB were due to differences in the efficiencies of latency establishment by these viruses and to confirm ongoing transcription of *lacZ* during latency, we next performed ISH analyses.

ISH detection of *lacZ*-specific RNA in neurons latently infected with LβA and LβB. Cervical ganglia (CII through CIV) were dissected and pooled from mice latently infected with LβA and LβB at various time points and processed for ISH. ISH was performed with a DIG-labelled riboprobe specific for the detection of *lacZ* mRNA. The data from these experiments are summarized in Table 1. At 82 days postinfection with LβA, in 167 ganglionic sections examined, 44 *lacZ* RNA-positive neuronal profiles were detected (an average of 0.26 *lacZ*-positive neuronal profiles per ganglionic section); at 190 days postinfection, in 162 ganglionic sections examined, 42 *lacZ* RNA-positive neuronal profiles were detected (an average of 0.26 *lacZ*-positive neuronal profiles per ganglionic section). In each case, the signal obtained was predominantly cytoplasmic (Fig. 4a and b) and the intensities of signal and the mean numbers of *lacZ*-positive neuronal profiles detected per section were similar at both time points. These data indicate the stable, continued transcription of *lacZ*-specific RNA in sensory ganglia latently infected with LβA.

ISH analyses with a LAT-specific probe also resulted in the detection of transcripts within the cytoplasm of latently infected neurons (Fig. 4c), which is in sharp contrast to the characteristic nuclear localization of LATs observed during latency with wild-type viruses. The localization of both LAT- and *lacZ*-specific signals in the cytoplasm of neurons supports the view that hybrid transcripts are generated and transported to the cytoplasm of latently infected neurons, although this has yet to be confirmed by RNA mapping.

Interestingly, ISH detection of *lacZ*-specific RNA in ganglia latently infected with LβB revealed a pattern of signal different from that observed with LβA. Instead of the uniform cytoplasmic *lacZ*-specific signal observed with LβA (Fig. 4b), animals latently infected with LβB demonstrated a predominantly nuclear, punctate signal (Fig. 4e), with a cytoplasmic signal observed in only some of the more intensely stained cells (Fig. 4f). At 140 days postinfection with LβB, ISH analyses revealed

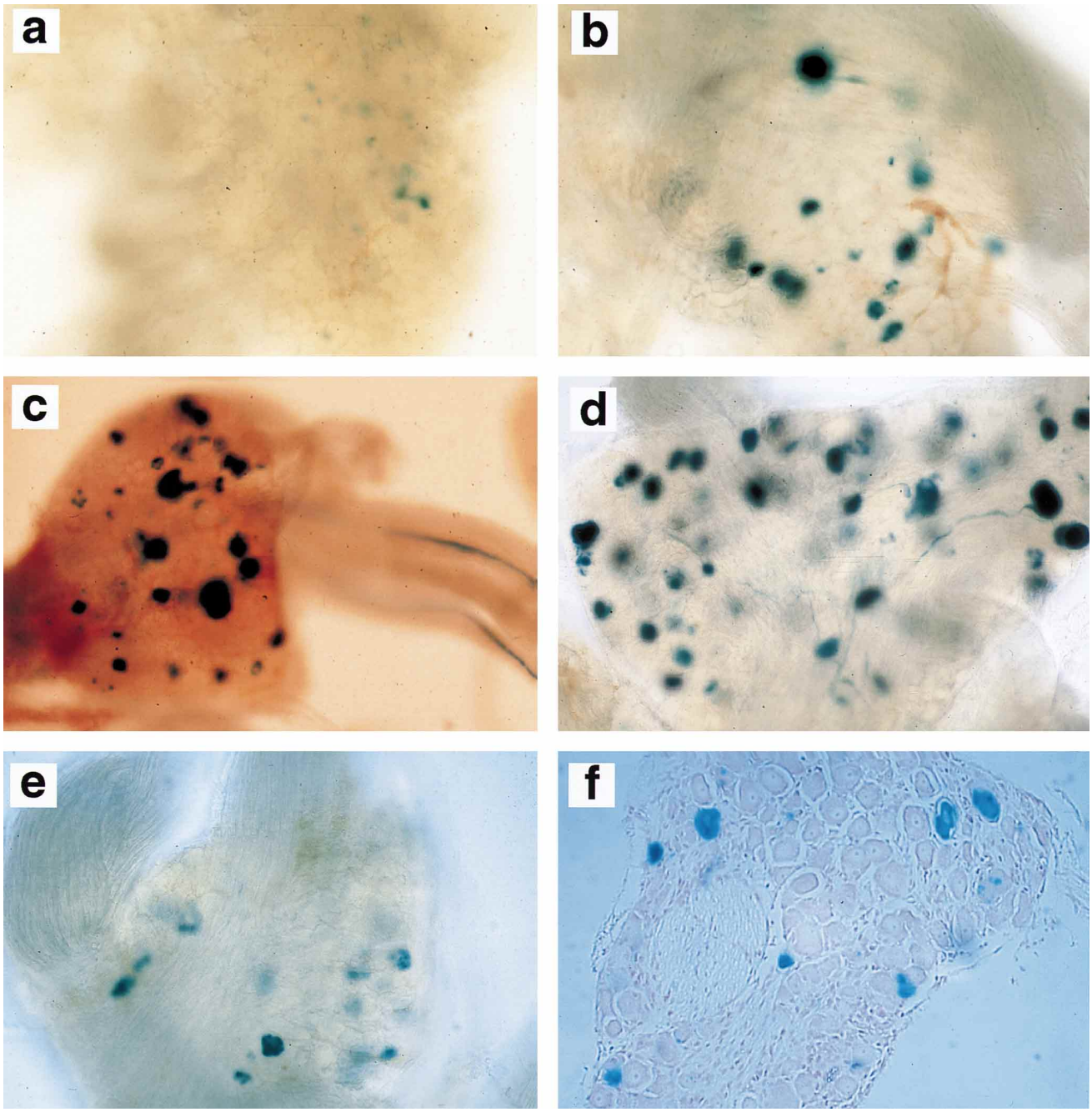


FIG. 2. Light micrographs showing histochemical detection of β-galactosidase expression in cervical dorsal root ganglia from mice infected with LβA and LβB. Mice were infected with 5×10^6 PFU of virus by subcutaneous injection into the left ear. At each time point, whole ganglia were stained with the X-Gal reagent for 3 h prior to microscopic examination. (a) At 5 days postinfection with LβA. There is only weak β-galactosidase expression at acute time points. (b) At 26 days postinfection with LβA. As the virus enters the latent state, β-galactosidase expression increases and is seen in more neurons per ganglion. (c) At 82 days postinfection with LβA. There is continuing high-level β-galactosidase expression, and axonal tracking of the protein is clearly demonstrated. (d) At 190 days postinfection with LβA. β-Galactosidase expression is stably maintained for more than 6 months postinfection. (e) At 140 days postinfection with LβB. This virus also directs long-term, stable β-galactosidase expression, but the intensity of histochemical staining seen is less than that with LβA, with individual neurons showing a more speckled staining pattern. (f) Section of ganglion histochemically stained at 190 days postinfection with LβA and counterstained with neutral red. Both uniform staining and focal staining of neuronal profiles are observed. Magnification: ×88 (a, b, d, and e); ×53 (c); ×141 (f).

23 *lacZ*-positive neuronal profiles in 128 ganglionic sections examined (an average of 0.18 neuronal *lacZ*-positive profiles/ganglionic section); at 257 days postinfection, 102 *lacZ*-positive profiles were observed in 288 ganglionic sections examined (an average of 0.36 *lacZ*-positive neuronal profiles/ganglionic section). It therefore appears that LβB established transcriptionally active latency at an efficiency comparable to that of LβA. However, despite the fact that similar numbers of latently infected neurons harbor transcriptionally active LβA and LβB genomes, fewer neurons latently infected with LβB scored positive for functional β-galactosidase gene expression. Our ISH data suggest that this is the result of relatively inefficient

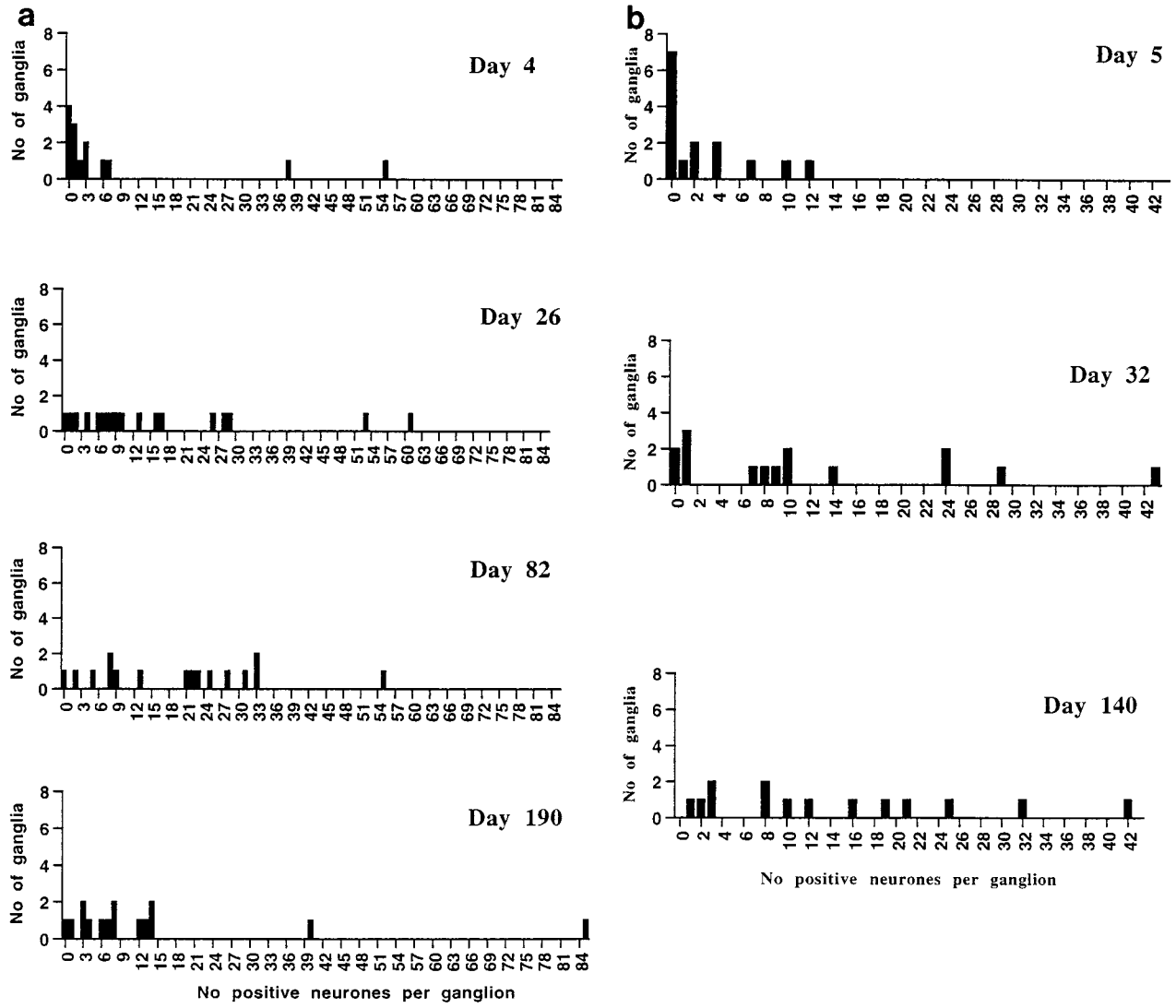


FIG. 3. β-Galactosidase expression in the peripheral nervous system of SC16 LβA (a)- and SC16 LβB (b)-infected mice. At each time point (days 4, 26, 82, and 190 for SC16 LβA and days 5, 32, and 140 for SC16 LβB), cervical dorsal root ganglia CII through CIV were dissected from 5 to 6 mice and pooled for histochemical detection of β-galactosidase activity. After staining for 3 h, the reaction was stopped and the ganglia were clarified in glycerol prior to microscopic examination and enumeration of the number of β-galactosidase-expressing neurons.

translocation of *lacZ*-containing transcripts from the nucleus to the cytoplasm of neurons latently infected with LβB rather than the failure of LAT promoter-mediated transcription.

β-Galactosidase expression in neurons of the central nervous system from mice latently infected with LβA. By using the mouse ear model, it has previously been demonstrated that after the resolution of acute-phase infection, animals harbor latent virus DNA in both brain stem and spinal cord tissues, as well as peripheral sensory ganglia (16). It is also well established that by using this model of infection, virus gains access to the brain stem via the facial nerve, which supplies motor fibers to the ear muscles (22).

Therefore, we were interested in determining whether latently infected animals contained neurons expressing β-galactosidase at these sites. To date, our studies have focused on an examination of brain stems and cervical spinal cords of mice latently infected with recombinant virus LβA and have involved analyses of 3 mice sampled at 2 to 3 months postinfection, 2 mice sampled at 4 months postinfection, 1 mouse sampled at 6 months postinfection, and 11 mice sampled at 9 to 10 months postinfection. As observed previously in our examination of β-galactosidase expression in latently infected sensory ganglia, there was considerable mouse-to-mouse variation in the level of transcriptionally active latency established. However, the anatomical distribution of β-galactosidase expression was maintained and β-galactosidase-expressing neurons were consistently detected in the cervical spinal cord and bilaterally within facial nerve and hypoglossal nerve nuclei. Representative sections from each of these regions at different time points are shown in Fig. 5.

β-Galactosidase expression was detected most frequently in facial nerve nuclei (14 of 17 mice examined), and blue neurons were often observed throughout the nucleus (in up to 13 consecutive 60-μm sections), with some neurons showing tracking of β-galactosidase into the dendritic tree and axon (Fig. 5a and c). The wide distribution of latently infected blue neurons within the facial nerve nucleus would be indicative of virus spread within the nucleus during acute infection. β-Galactosi-

TABLE 1. Detection of β-galactosidase expression by histochemical staining and ISH for mRNA in the peripheral nervous system of mice infected with LβA and LβB[a]

Virus	Days postinfection	Avg no. of blue neurons per ganglion (range)[b]	ISH-positive neuronal profiles per ganglionic section[c]
LβA	4	8.4 (0–55)	ND[d]
	26	17.1 (0–53)	ND
	82	19.8 (0–55)	0.26 (44/167)[e]
	190	14.7 (0–85)	0.26 (42/162)
LβB	5	2.8 (0–12)	ND
	32	11.7 (0–43)	0.36 (68/187)
	140	14.4 (1–32)	0.18 (23/128)
	257	ND	0.36 (102/288)

[a] Dorsal root ganglia CII through CIV were dissected and pooled from 5 to 6 mice for histochemical staining and from 7 to 10 mice for ISH at each time point.
[b] Data are average numbers of β-galactosidase-positive neurons per whole mounted ganglia (for raw data, see Fig. 2).
[c] For ISH, pooled ganglia were embedded in paraffin and 5-μm sections were cut. ISH was carried out with sections representative of the whole block with a DIG-labelled riboprobe specific for β-galactosidase mRNA.
[d] ND, not determined.
[e] The numbers in parentheses are the ratios of the total number of ISH-positive neurons to the total number of ganglionic sections examined.

dase-positive neurons were detected in hypoglossal nerve nuclei in 8 of 17 animals examined. Latently infected blue neurons were again commonly observed throughout the nucleus (in up to 15 consecutive 60-μm sections). Cervical spinal cord sections were not obtained from all mice, but β-galactosidase expression was observed in both anterior and posterior horn neurons in 6 of 14 mice examined. We observed occasional β-galactosidase-expressing neurons in the region of dorsal column sensory nuclei within the posterior caudal medulla of some mice as well as blue axonal profiles traversing this area (data not shown). These likely represent axons projecting from latently infected sensory neurons located in dorsal root ganglia.

DISCUSSION

The viruses described in this study were constructed with the aim of using the endogenous LAT promoter, with all associated upstream and downstream regulatory elements, to drive reporter gene expression during HSV latency. During lytic infection of Vero cells, recombinants LβA and LβB demonstrated similar plaque phenotypes after being stained with X-Gal. Plaques were predominantly white, but a proportion of infected cells expressed low levels of detectable enzyme. This was an unexpected observation, suggesting the nonuniform expression of LATs within any given population of lytically infected cells. The synthesis of such transcripts is unlikely to be the result of LAT promoter-driven transcription since viruses containing deletions of the core LAT promoter are able to synthesize major LATs during lytic infection (5, 34). Furthermore, although sequences located between the core LAT promoter and the start of major LATs have detectable promoter activity in transient assays (20), deletion of this element, LAP2, either alone or in conjunction with the core LAT promoter reduces but does not eliminate production of major LATs during lytic infection (5). We therefore consider it most likely that the β-galactosidase expression observed during lytic infection was a consequence of readthrough transcription that initiated upstream of the LAT promoter.

An examination of recombinants LβA and LβB in vivo revealed that both viruses were able to drive long-term reporter gene expression within sensory neurons that innervate the site of primary infection. Sensory ganglia removed during the acute stage of infection demonstrated detectable but low-level β-galactosidase expression. However, at later time points and consistent with the establishment of latency, increases in both the numbers of cells expressing β-galactosidase and in the intensity of staining were observed. The pattern of β-galactosidase expression we observed with LβA and LβB during lytic infection of Vero cells and acute and latent infection of sensory neurons in vivo appears to closely resemble the pattern of expression expected for a gene placed under authentic LAT promoter control.

During the course of our studies, we observed that LβB expressed β-galactosidase at a lower level and in fewer latently infected neurons than did LβA. Although this could reflect the fact that the reporter gene used in LβB constitutes a fusion between β-galactosidase and neomycin phosphotransferase which may be less active than is the native enzyme, the signal pattern of *lacZ* RNA as detected by ISH provides an alternative explanation. In the case of LβA, ISH revealed a cytoplasmic distribution of *lacZ* RNA within neurons. In contrast, LβB synthesized *lacZ* RNA which, although localized to the cytoplasm in some cells, was most frequently observed in neuronal nuclei, with an appearance similar to the focal distribution of minor LATs (1). These observations presumably reflect differences in the nature of the inserts and/or the efficiency of poly(A) site usage in these virus constructs.

An examination of the brain stem and spinal cord tissues of mice latently infected with LβA revealed β-galactosidase-positive neurons in a number of distinct regions of the central nervous system at time points from 72 to 307 days after infection. β-Galactosidase was detected in neurons located in facial nerve nuclei, hypoglossal nerve nuclei, and the upper cervical spinal cord. These observations indicate that HSV is able to establish transcriptionally active latency in these neuronal types, a result consistent with previous descriptions of transcriptionally active latent HSV genomes in a variety of central nervous system neurons (4, 7, 12, 38).

The distribution of β-galactosidase-positive neurons in the brain stems and spinal cords of latently infected animals was striking, and we rarely saw evidence of β-galactosidase-positive neurons in other regions of the brain stem. One possible explanation for this is that during acute infection, after a round of replication within neurons of the facial nerve nucleus which supply the muscles of the left ear, virus is able to gain access to sensory neurons which supply taste sensation to the tongue. Virus could then be transported to the tongue via the corda tympani. A further round of acute replication would allow virus to spread to hypoglossal nerve nuclei and the contralateral facial nucleus, allowing the establishment of latency at these sites. At this time, we cannot exclude the possibility that establishment of latency occurred in other cell types and in other regions of the brain stem in which the LAT promoter regulatory region is inactive or operative below the level of sensitivity of our assays.

Foreign-gene expression mediated by the HSV LAT promoter during latency has been reported previously. Dobson et al. (12) inserted the β-globin gene immediately downstream of the LAT TATA box and upstream of potentially important HSV sequence elements implicated in long-term neuronal expression. This virus resulted in the expression of β-globin RNA during latency, although a reduction in the number of cells expressing this transcript, in conjunction with a reduction in signal intensity of β-globin message detected by ISH, in the transition from acute to latent infection has been reported

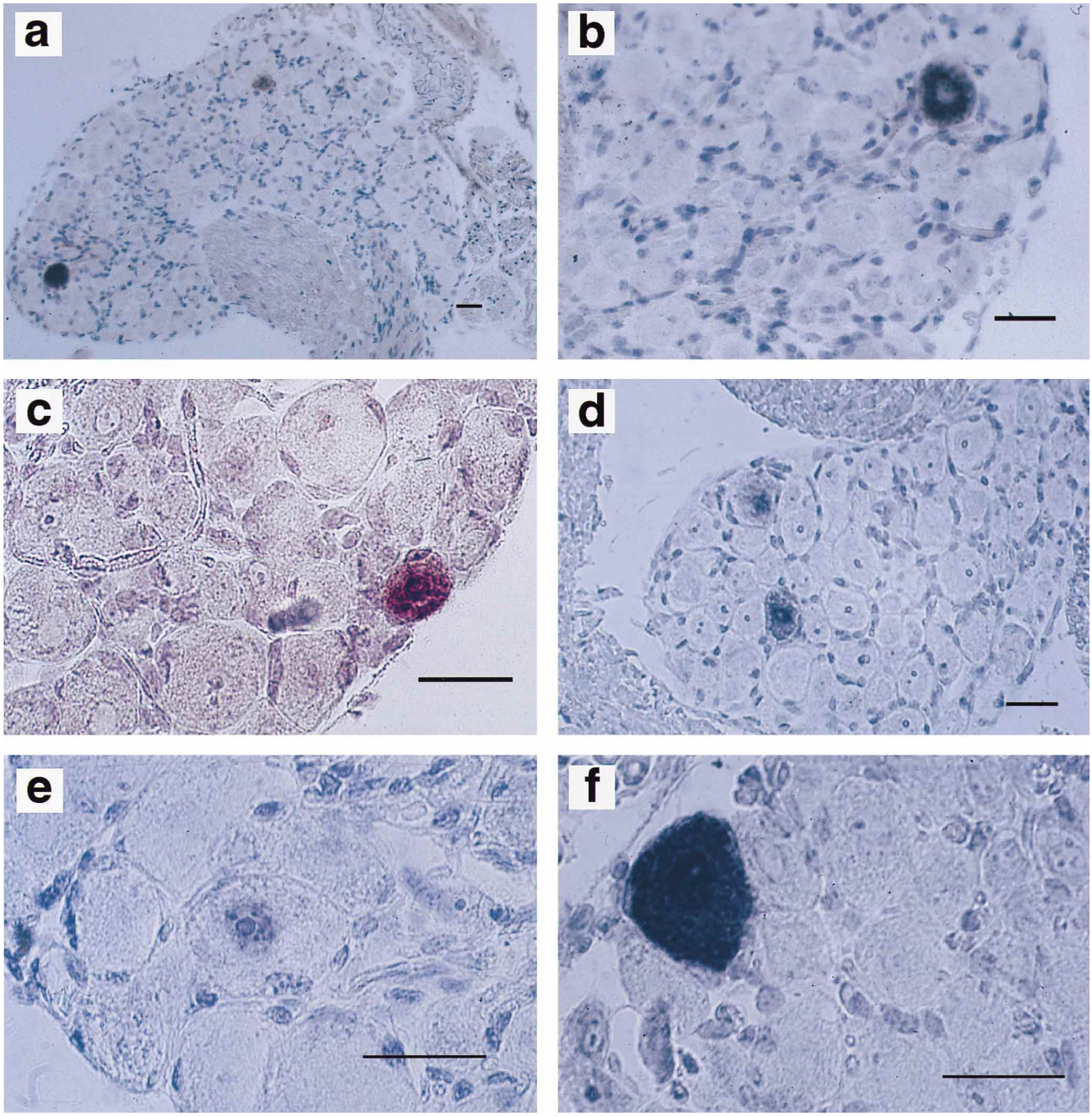
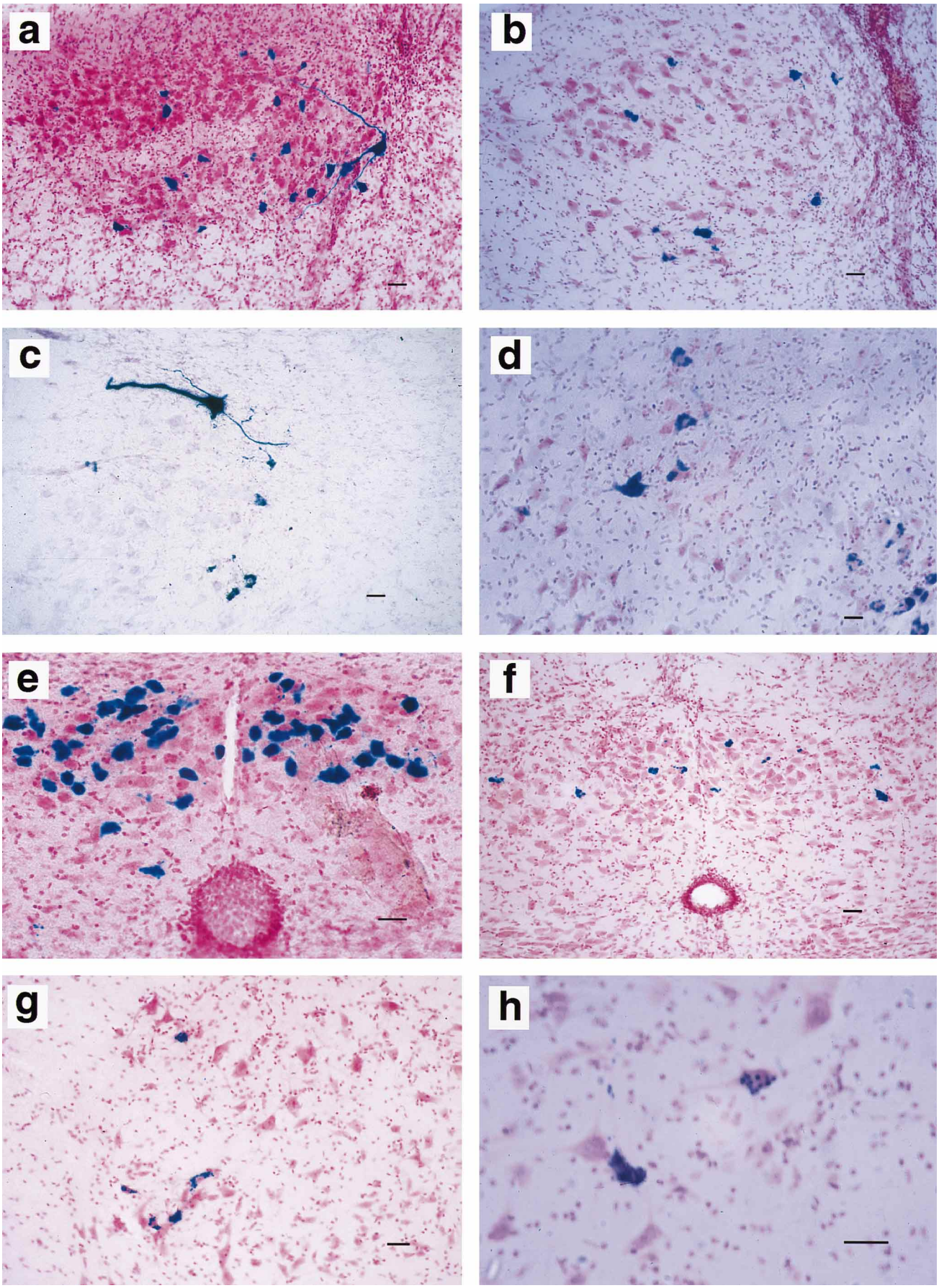


FIG. 4. Light micrographs showing ISH of sections of ganglia from mice latently infected with LβA and LβB. All sections were counterstained with hematoxylin. Scale bars, 30 μm. (a) Ganglionic section prepared at 190 days postinfection with LβA. ISH was performed with a probe specific for *lacZ* mRNA, which was detected in the cytoplasm of latently infected neurons. (b) Dorsal root ganglion neuron expressing *lacZ* mRNA at 190 days postinfection with LβA. Strong staining is seen throughout the cytoplasm, with relative sparing of the nucleus. (c) Latently infected neuron at 82 days postinfection with LβA. ISH was performed with a LAT-specific probe. As with the *lacZ* mRNA-specific probe, uniform cytoplasmic staining is seen. (d) Ganglionic section prepared at 257 days postinfection with LβB. ISH was performed with a probe specific for *lacZ* mRNA. In contrast to LβA, the distribution of mRNA in latently infected neurons is predominantly nuclear, although cytoplasmic signal is also seen. (e) Latently infected neurons at 257 days postinfection with LβB probed for *lacZ* mRNA. The predominant punctate nuclear staining seen is demonstrated. (f) Latently infected neuron at 140 days postinfection with LβB. A proportion of latently infected neurons show strong cytoplasmic staining for *lacZ* mRNA.

FIG. 5. Light micrographs of brain stem and spinal cord sections from mice latently infected with LβA, histochemically stained for β-galactosidase. (a through d) β-Galactosidase expression in the neurons of facial nerve nuclei of latently infected mice sampled at 80, 120, 272, and 307 days postinfection, respectively. (e and f) β-Galactosidase expression in the neurons of hypoglossal nerve nuclei of latently infected mice sampled at 72 and 120 days postinfection, respectively. (g and h) β-Galactosidase expression in the neurons of the spinal cords of latently infected mice sampled at 130 and 120 days postinfection, respectively. All sections were counterstained with hematoxylin, except for the section in panel c, which was counterstained with cresyl violet. All sections were 60-μm frozen sections, except for the section in panel a, which was a 150-μm vibratome section, and that in panel e, which was a 200-μm vibratome section. Scale bars, 30 μm.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(29). Previous work with HSV recombinant RH142, which contains *lacZ* inserted approximately 0.8 kb downstream of the LAT transcription start site within a 372-bp deletion of major LAT, revealed that this virus expressed detectable levels of β-galactosidase within murine sensory neurons for at least 8 weeks after infection (24). Latent-phase gene expression of β-glucoronidase inserted approximately 0.4 kb downstream of the LAT transcription start site, within a 0.9-kb deletion of major LAT (17/LAT-RGUSB), has also been observed in small numbers of murine sensory and central nervous system neurons for at least 18 weeks after infection (43). In contrast to the observations we made with recombinants LβA and LβB, increasing levels of reporter gene expression were not observed in the transition from acute to latent infection in these studies.

Clearly, a more direct comparison of the viruses, LβA and LβB, described here and both RH142 and 17/LAT-RGUSB is required in order to draw firm conclusions as to the relative efficiencies by which these recombinants can drive stable gene expression during latency. Nonetheless, it is possible that the insertion sites and/or deletions introduced within regions downstream of the LAT transcription start site in RH142 (24) and 17/LAT-RGUSB (9, 43) interfere with elements important for the maintenance of optimal transcriptional activity during latency. In addition, decreases in the translational efficiencies of the reporter genes in these viruses could occur as a consequence of the coding sequences being preceded by a number of translational start and stop codons derived from a minor LAT leader sequence.

The failure to observe long-term latent gene expression from the endogenous LAT promoter with recombinant viruses containing downstream deletions (29) indicates that the integrity of such elements is critical for stable LAT-mediated gene expression. We inserted IRES-linked reporter genes 1.5 kb downstream of the LAT transcription start site in order to minimize disruption of such elements and generated recombinant viruses capable of directing stable reporter gene expression in latently infected neurons. The importance of the precise site of reporter gene insertion and the contribution of the EMCV IRES in mediating the long-term expression of β-galactosidase observed with recombinants LβA and LβB are currently under evaluation.

The frequent detection of β-galactosidase-positive neurons in a variety of sites within the central nervous system of mice latently infected with recombinant LβA suggests that the expression strategy we have adopted could be exploited in the context of replication-defective vectors in the delivery of potential therapeutic genes to the nervous system. Furthermore, the sensitivity and ease of detection of β-galactosidase in latently infected neurons will allow studies of the sites of latency establishment by different routes of infection with phenotypically wild-type virus to be made.

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