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Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse

(herpesvirus/pathogenesis/latency/β-galactosidase/expression vector)

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ABSTRACT **During latent infection by herpes simplex virus (HSV), an abundant latency-associated transcript (LAT) that is antisense to a predominant viral α gene (ICP0) is found localized in the nucleus of sensory neurons. We disrupted both copies of the LAT gene in the HSV-1 genome by insertion of the *Escherichia coli lacZ* gene under LAT promoter control. The resulting recombinant virus, RH142, does not express any detectable LAT in either latently or productively infected cells, although β-galactosidase expression is readily detectable in sensory neurons of latently infected mice. Expression was first detectable 3 days postinoculation and continued at approximately the same level for the entire experimental period (56 days). β-Galactosidase expression was not detectable at any time during RH142 replication in Vero cells. Thus, the kinetics of expression and cell-type specificity of the LAT gene are distinct from other HSV-1 genes that are expressed during productive growth. When latently infected trigeminal ganglia were explanted, RH142 reactivated from latency with the kinetics and an efficiency indistinguishable from the parental wild-type virus. These studies argue against any possible antisense regulatory mechanism of LAT in the regulation of viral gene expression or any role of LAT-encoded protein during the establishment or maintenance of latency in the mouse.**

Herpes simplex virus (HSV), a large enveloped animal virus with a 152-kilobase-pair linear DNA genome and a highly regulated replication cycle (1), has long been known to establish latent infection in sensory neurons following natural infection of humans and after peripheral inoculation of a variety of experimental animals (2, 3). Recent efforts from several laboratories have demonstrated that a small region of the viral genome is transcribed in infected neurons during latency in humans (4), mice (5, 6), and rabbits (7). The predominant species of RNA has been termed "latency-associated transcript" (LAT) or "latency-related RNA" and is partially complementary to the prominent α gene ICP0, a gene that apparently plays an important role in the regulation of viral gene expression, particularly in neurons (8). Furthermore, since LAT localizes mainly to the nucleus of latently infected cells and is not significantly polyadenylylated, its function as a regulator of ICP0 expression by an antisense mechanism has been proposed (6). There is a large open reading frame (ORF) within LAT that is conserved among various strains of HSV-1 as well as other small ORFs that are not conserved; however, no protein product from this region has been reported thus far. The abundant expression of LAT during latent infection of human (4) or animal (5–7) hosts strongly implicates the transcript itself or a protein product controlling latency.

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To study the expression of the LAT promoter and the role of LAT in HSV-1 latency, we have constructed a recombinant virus, RH142, in which a *lacZ* indicator gene from *Escherichia coli* was inserted within the LAT gene, disrupting both genomic copies of LAT. RH142 does not produce detectable levels of LAT either in productively infected Vero cells or in latently infected sensory ganglia but produces β-galactosidase (β-Gal) under control of the LAT promoter in latently infected neurons. Despite the absence of LAT, RH142 was capable of latency and reactivation with characteristics indistinguishable from the wild-type parental HSV-1.

MATERIALS AND METHODS

Plasmid and Recombinant Virus Construction. Plasmid pON142 was constructed by the following modifications to clone pON134 (Fig. 1), which carries the HSV-1 (F) *Hpa* I N fragment in the *Hin*cII site of pGEM1 (Promega Biotec). First, a deletion was introduced by BAL-31 digestion (12) from the unique *Kpn* I site of pON134 [at +509 relative to the reported position of the LAT start site (13)] and a *Kpn* I linker was inserted to mark the deletion endpoint (Fig. 1). The region between the *Kpn* I site to the downstream *Hpa* I site was replaced with a full-length *Kpn* I/*Hpa* I fragment (cloned as a *Kpn* I/*Sac* I fragment from pON134) such that a total of 373 base pairs (bp) were removed from the LAT gene. The *Kpn* I site of this resulting plasmid was converted to a *Bgl* II site by addition of a linker, and a *Bam*HI fragment from pON1 (14), containing modified *lacZ* coding sequences with a simian virus 40 (SV40) early polyadenylylation signal, was then inserted at this site to produce pON142 (Fig. 1). The precise endpoints of the deletion in pON142 were determined by dideoxynucleotide sequencing (15). RH142 was isolated by plaque hybridization (16) using *lacZ* as a probe after cotransfection of pON142 (10 μg) and HSV-1 (KOS) DNA (20 μg) into Vero cells and was plaque-purified by standard procedures (17).

Mouse Infection, *in Situ* Detection of β-Gal, and Recovery of Virus in Infected Tissues. Corneal inoculation and *in situ* detection of β-Gal were performed as described (17). To recover infectious virus, individual ganglia were placed into 2 ml of medium and sonically disrupted using a probe-type sonicator, and the resultant lysate was titered on Vero cells. To recover latent virus, individual ganglia were first incubated in medium at 37°C for 4–5 days before being sonicated, serially diluted, and plaque-assayed on Vero cell monolayers.

RNA Extraction, RNA Blot, RNA Probes, and RNase Protection. Total RNA was harvested from Vero cells by standard procedures (12). To isolate RNA from latently infected cells, trigeminal ganglia from 8–10 mice were solubilized in

Abbreviations: LAT, latency-associated transcript; HSV, herpes simplex virus; ORF, open reading frame; SV40, simian virus 40; β-Gal, β-galactosidase; nt, nucleotide; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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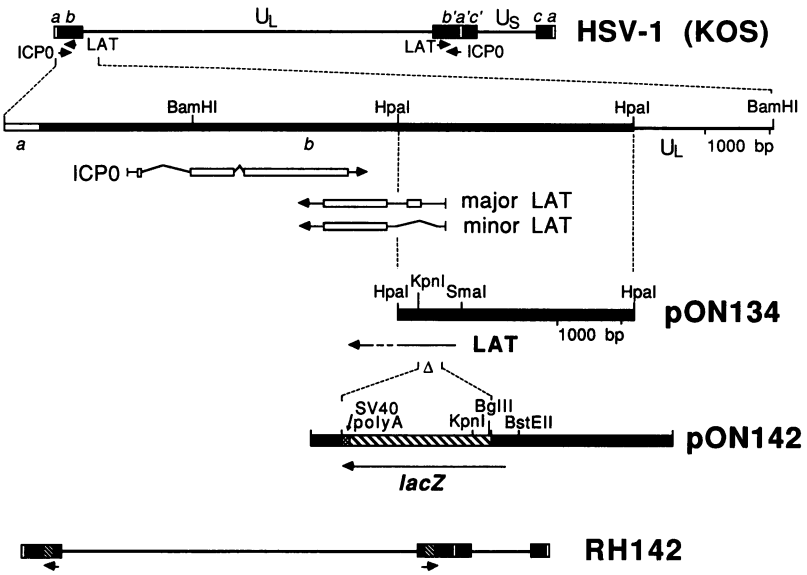

FIG. 1. Structure of RH142. The HSV-1 (KOS) genome is depicted on the top line with *b* and *c* inverted repeat sequences indicated by solid boxes flanking the unique long (U_L) and unique short (U_S) regions and with the terminal repeat *a* sequence indicated by open boxes. The positions of the LAT and ICP0 genes, within the *b* repeats, are indicated by small opposing arrows (9, 10, 11). The *Bam*HI S and E fragments of the viral genome are expanded below. The relative positions and structures of the ICP0 transcript as well as the major and minor LAT are shown. Open boxes represent ORFs and solid horizontal lines represent untranslated regions. One copy of the HSV-1 (F) *Hpa* I N fragment (cloned as pON134) is shown in the expanded region with an arrow indicating the position and direction of transcription of the LAT gene (9, 10, 11). The plasmid pON142 carries a *lacZ* (hatched box) insert placed under control of the LAT transcriptional signals and SV40 polyadenylylation signals (cross-hatched box). The triangle represents a deletion of 373 bp introduced into the LAT gene in the process of making pON142 as described in the text. The recombinant virus RH142, shown on the bottom line, carries *lacZ* inserted within both copies of the LAT gene.

guanidinium isothiocyanate with a Polytron homogenizer, followed by sedimentation through cesium chloride (12). The procedure for RNA blot hybridization has been described (18), except that ^{32}P-labeled RNA probes were used and a wash with RNase A (5 μg/ml) was included.

RNA probes labeled with [^{32}P]CTP were prepared as follows. A probe of 1057 nucleotides (nt), antisense to a region from −196 to +838 of the LAT gene, was generated from the SP6 promoter of pON134 after restriction digestion by *Sma* I. A probe of 470 nt was generated from the SP6 promoter of pON4, a pGEM2-derived clone that contained the complete leader and protein coding sequences of *lacZ*, as well as the SV40 polyadenylylation signal. This probe was antisense to the 3′ end of *lacZ* (307 nt) plus SV40 sequences (135 nt). Two RNA probes were generated from the SP6 promoter of pON144S, a pGEM1-derived clone that carried a *Kpn* I/*Bst*EII fragment from pON142 (Fig. 1). One probe was antisense to 253 nt of *lacZ* sequences and the other was antisense to 708 nt of both *lacZ* and LAT sequences. The RNA probes were prepared and RNase protection was according to the manufacturer's protocol (Promega Biotec), except that 750 units of RNase T1 was added to each sample and RNase A was omitted. For each sample, 30 μg of total RNA was used and hybridization was performed at 49°C.

RESULTS

Construction of RH142. RH142 was constructed by cotransfecting intact HSV-1 (KOS) DNA and plasmid pON142, which contains the *Hpa* I N fragment from HSV-1 (F) with the *lacZ* gene inserted within the LAT gene (Fig. 1). Recombinant viruses carrying inserts were identified and plaque-purified by plaque hybridization (16) with pON1, a *lacZ*-specific probe (14). After isolation and plaque purification, the genome structure of candidate recombinant viruses was analyzed by Southern blot hybridization using *Sal* I, *Xho* I, and *Hpa* I digests (data not shown). Based on this analysis, we concluded that the *lacZ* gene was inserted into both copies of the LAT gene as depicted in Fig. 1.

The *lacZ* gene was fused to putative LAT promoter-regulatory sequences 136 bp downstream of the transcription start site (9). Even though the precise position of the LAT promoter has not been determined and could be further upstream [the closest TATA box consensus has been identified 687 bp upstream of this apparent start site (10)], RH142 would be expected to place *lacZ* under LAT promoter control. Furthermore, the expected *lacZ* transcript would terminate at a polyadenylylation signal derived from SV40, an arrangement that has been used in recombinant herpesvirus constructs (17, 19, 20).

The region from +137 to +509 relative to the putative start site was deleted in RH142 (Fig. 1). This deletion was within the intron mapped in a minor fraction of LAT (13, 21). As a consequence, both the potential splice donor and the splice acceptor, which are mapped to regions from nucleotides +21 to +106 and +837 to +1005, respectively, were retained (10). Furthermore, the first 83 nt of the small ORF from +430 to +637 in HSV-1 (KOS) (9), which is not conserved in either strain F or 17 syn+ (13), was deleted as a result of the insert. The largest ORF that is conserved among HSV-1 strains (9, 13) is intact but positioned downstream of the *lacZ* insert in RH142.

LAT Expression by RH142. To determine whether the insertion had disrupted transcription of the LAT gene, we performed RNA blot analysis with an RNA probe made from pON134 (Fig. 1) that was antisense to LAT. Although LAT was readily detected in Vero cells productively infected with HSV-1 (KOS) or RH105 (17), a HSV-1 (F)-derived recombinant virus with *lacZ* inserted in the thymidine kinase gene under the ICP4 promoter control, no transcripts homologous to the LAT probe of any size were detected in RH142-infected cells (Fig. 2*A*). A size difference of LAT expressed by HSV-1 (KOS) and (F) (2.2 and 2.0 kilobases, respectively) was observed in our analysis. Furthermore, when RNA from pooled latently infected trigeminal ganglia was analyzed,

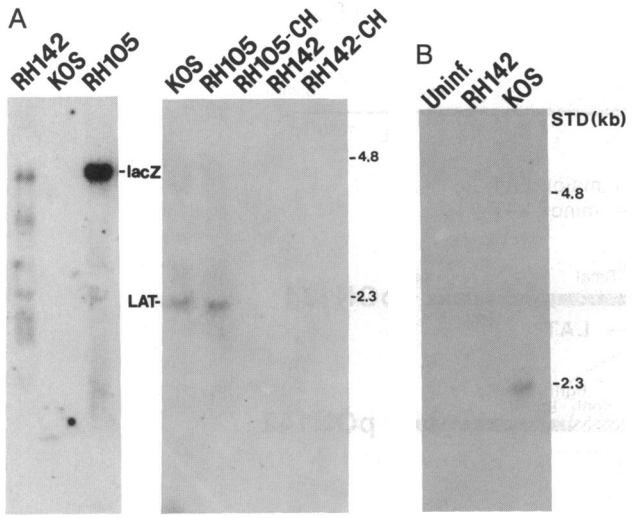

FIG. 2. RNA blot analysis of *lacZ*-specific and latency-associated transcripts expressed by RH142. (*A*) Vero cells were infected at a multiplicity of infection of 10 plaque-forming units per cell in the presence or absence of cycloheximide (50 μg/ml, added from 1 hr before infection) and whole cell RNA was prepared 5.5 hr postinfection. After electrophoretic separation and transfer to nitrocellulose, the blots were hybridized with ^{32}P-labeled RNA probe generated from pON4 to detect *lacZ*-specific transcripts (*Left*) or from pON134 to detect LAT (*Right*). (*Left*) Five micrograms of RNA was loaded in each lane. (*Right*) Ten micrograms of RNA was loaded in the HSV-1 (KOS) and RH105 lanes and 20 μg of RNA was loaded in the RH142 lanes. (*B*) BALB/c mice (4–5 weeks old) were inoculated with 10^7–10^8 plaque-forming units of either HSV-1 (KOS) or RH142 by corneal scarification. At 36 [HSV-1 (KOS)] or 28 (RH142) days postinoculation, total RNA was prepared from pooled ganglia from 8–10 mice. Five micrograms of RNA was loaded in each lane. After electrophoretic separation and transfer, hybridization was performed with a ^{32}P-labeled RNA probe generated from pON134. The positions of large and small ribosomal RNA species are indicated on the right. A 10-fold longer exposure did not reveal any RNA species in the RH142 sample homologous to the LAT probe.

transcripts from the LAT region were detected in HSV-1 (KOS)-infected but not in RH142-infected mice (Fig. 2*B*). Based on the intensity of autoradiographic images, LAT expression was reduced at least 100-fold in RH142-infected neurons. Even though it was LAT deficient, RH142 was found to make similar levels of the ICP0 transcript as compared to the wild-type virus (data not shown).

Expression of a Chimeric *lacZ* Transcript by RH142. To examine expression of *lacZ* transcripts by RH142, we performed RNA blot analysis with an RNA probe that was antisense to the 3′ end of *lacZ*. As shown in Fig. 2*A*, an appropriately sized *lacZ* transcript as well as several smaller sized species were detected in RNA from RH142-infected Vero cells. Transcription of all of these RNA species was blocked by cycloheximide (data not shown). As expected, the *lacZ* transcript from RH105 was abundantly expressed in intact form and no *lacZ* homologous RNA species were detected in HSV-1 (KOS)-infected cells. Using the same probe, we were not able to detect the chimeric *lacZ* transcript in latently infected ganglia of mice, although β-Gal enzymatic activity in these ganglia was readily demonstrated (see below).

RNase protection was used to examine the structure of the chimeric LAT–*lacZ* transcript. RNA probes were generated from pON144S, a clone of the *Bst*EII/*Kpn* I fragment from pON142 (Fig. 1). One probe of 253 nt was generated by digesting pON144S with *Bgl* II. RNA from HSV-1 (KOS)-, RH142-, or RH105-infected Vero cells was hybridized to this probe and subjected to digestion by RNase T1. For the chimeric LAT–*lacZ* transcript from RH142, a 212-nt fragment was protected, while for the ICP4–*lacZ* transcript from RH105, a 207-nt fragment was protected (Fig. 3*A*). Further analysis with a longer probe generated from pON144S by digestion with *Eco*RI and extending to −266 nt relative to the mapped LAT start site (9, 10) identified a 389-nt protected fragment (data not shown). Our data mapped the start site for the chimeric transcript close to the reported position of the start site for LAT. RNase protection analysis was also used to map the 3′ end of the *lacZ* transcript (Fig. 3*B*) with an RNA probe of 470 nt generated from pON4. For the chimeric *lacZ* transcripts from both RH142 and RH105, a fragment of 389 nt was protected, demonstrating successful transcription termination at the SV40 polyadenylylation signals. A minor protected species of 442 nt was also observed; however, this species was absent in RNA samples harvested at early times postinfection (<5.5 hr).

Phenotype of RH142. Although we detected a species of chimeric RNA apparently initiating at the LAT transcription start site in RH142 productively infected Vero cells, we did not detect any β-Gal activity in cells or in cell lysates when highly sensitive assays were used (17, 19). The reason for failure of translation of the chimeric transcript in productively infected cells is not presently understood; however, as described below, RH142 produced readily detectable levels of β-Gal during latent infection in sensory neurons. In Vero cells, RH142 grew to similar high titers with kinetics and plaque size comparable to the parental virus HSV-1 (KOS).

Previous studies have indicated that LAT is expressed to highest levels in latently infected neurons. The LAT-deficient phenotype of RH142 did not influence its ability for growth or latency in the trigeminal ganglia of the mouse. As shown

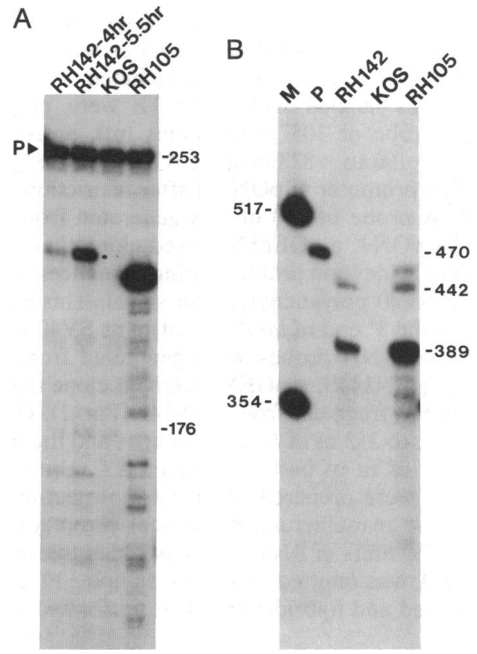

FIG. 3. RNase protection analysis of chimeric LAT–*lacZ* RNA in RH142-infected Vero cells. Cells were infected and RNA was collected as described in Fig. 2, except that in *A*, RNA samples were prepared 5.5 hr postinfection with an additional RH142-infected cell RNA sample prepared 4 hr postinfection; in *B*, RNA samples were prepared 6 hr postinfection. Thirty micrograms of RNA was hybridized with ≈5 × 10^5 cpm of a ^{32}P-labeled 253-nt RNA probe (P) generated from pON144S (*A*), or with a ^{32}P-labeled 470-nt RNA probe generated from pON4 (*B*). After digestion with RNase T1, protected species were resolved after electrophoretic separation on a 5% polyacrylamide gel run under denaturing conditions and subjected to autoradiography. Size markers (lane M) were ^{32}P-end-labeled *Hin*fI fragments from pGEM1.

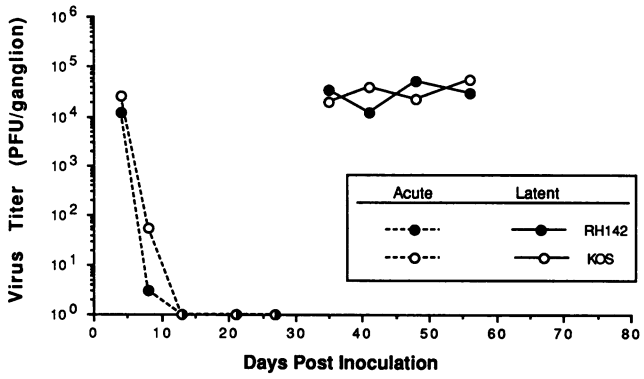

FIG. 4. Growth and latency characteristics of RH142 in the mouse. Sixty BALB/c mice (4–5 weeks old) were inoculated by corneal scarification with 10^7 plaque-forming units (PFU) of either HSV-1 (KOS) or RH142. At the indicated times, two or three mice were sacrificed. One ganglion from each mouse was assayed for the presence of acute or latent virus and the other was used for *in situ* β-Gal detection (see Fig. 5). To assay for acute virus, ganglia were directly sonicated, diluted, and plated on Vero cells; to assay for latent virus, the ganglia were first incubated at 37°C as explants for 4–5 days before being sonicated and plated.

in Fig. 4, mice (4–5 weeks old) infected by corneal scarification with 10^7 plaque-forming units of RH142 or HSV-1 (KOS) had similar levels of infectious virus in the trigeminal ganglia during acute infection (up to 8 days postinoculation). Titers of latent virus reactivated from the ganglia were measured 35, 41, 48, and 56 days postinoculation. RH142 was equal to its parent in establishing latent infections, with all inoculated mice yielding virus on explant and with a level of reactivation similar to wild type. Given the fact that all explanted ganglia yielded similar levels of reactivated virus after 4–5 days, the kinetics of RH142 reactivation appeared similar to HSV-1 (KOS). Thus, a deficiency in LAT expression did not influence establishment or explant reactivation of latency in mice.

To determine whether RH142 was able to direct the expression of β-Gal in neuronal cells, trigeminal ganglia from infected mice were examined directly by an *in situ* staining procedure (17). Frozen sections were prepared from the ganglia, fixed with 0.5% glutaraldehyde, and stained overnight with the β-Gal substrate X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) (Fig. 5). RH142 expressed detectable levels of β-Gal as blue-staining neurons beginning at 3 days at the same time as peak expression from RH105 (Fig. 5 *A* and *B*); however, the intensity of staining at this time was lower than with RH105. β-Gal continued to be expressed in 0.1–1.0% of neurons in RH142-infected animals during the latent phase of infection until at least 8 weeks postinoculation (Fig. 5 *C–E*). These results clearly demonstrated that the LAT regulatory signals can direct the expression of a functional mRNA.

In RH142-infected mice, X-Gal staining was specific to neuronal cells in sensory ganglia and was not detectable in other tissues that were examined, including various inocu-

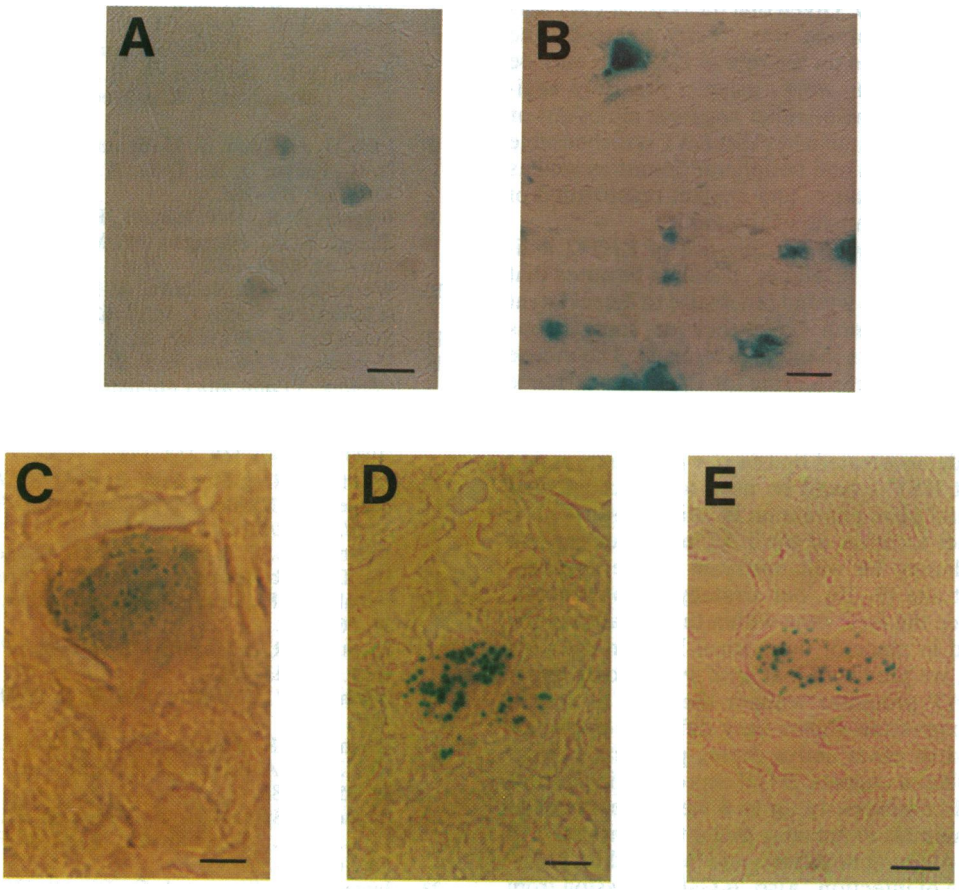

FIG. 5. Long-term expression of β-Gal in RH142-infected trigeminal ganglia at 3 (*A* and *C*), 8 (*D*), and 30 (*E*) days postinoculation. Ganglia stained 4 days postinoculation with a mixture of RH105 (ref. 17; a virus that carries the *lacZ* gene under the HSV-1 α4 promoter-regulator control) and HSV-1 (KOS) are shown for contrast (*B*). Ganglia, removed at the indicated times after inoculation with 10^7 plaque-forming units by corneal scarification, were cut in frozen sections (10-μm sections), fixed, and stained as described (17). In addition to the times shown, RH142 latently infected ganglia expressed a similar distribution of β-Gal 4, 7, 10, 22, 35, 41, and 56 days postinoculation. (*A* and *B*, ×240, bars = 25 μm; *C*, ×2400, bar = 2.5 μm; *D* and *E*, ×960, bars = 6.25 μm.)

lation sites such as corneal epithelium and abdominal dermal tissues. In contrast, RH105- or RH116-inoculated animals contained blue-staining cells in nervous tissues as well as in peripheral tissues (17). Besides the differences in temporal expression and tissue specificity of expression, the pattern of X-Gal staining in RH142-infected neurons was also distinct. We had previously observed that RH105 and RH116 expressed β-Gal in neuronal cells or other cell types with a uniform staining pattern (Fig. 5*B*). Interestingly, the pattern of staining with RH142 was punctate throughout the latent phase of infection (Fig. 5 *D* and *E*). On day 3 postinoculation, we observed cells with both patterns of staining (Fig. 5*C*). In all recombinants we have studied here and elsewhere (17, 19, 20), β-Gal is made from the same start codon as a transcriptional fusion; thus, the protein product in RH105- and RH142-infected neurons is likely to be the same. This observation suggests that there may be some physiological differences between acutely infected and latently infected neurons, and cells with the change in staining pattern from uniform to punctate may represent a transition from acute phase to latency.

DISCUSSION

The presence of LAT as an abundantly transcribed RNA species in latently infected ganglia suggests a potential role in the regulation of latency (6, 22). Two reports (22, 23), both using fortuitously identified HSV-1 mutants that leave one or both copies of LAT intact (but that do not apparently express this transcript), have previously suggested that LAT plays a subtle role, if any, in latency. Given that the body of LAT was left intact (although expression was clearly reduced), the conclusions of these studies on the role of LAT can only be considered suggestive. Our work, using a precisely engineered mutant virus carrying no other apparent mutations or genome rearrangements, establishes that LAT is dispensable for growth in cultured cells and peripheral tissues as well as for establishment, maintenance, and explant reactivation of latency in the trigeminal ganglia of the mouse.

Although the reactivation characteristics of RH142 indicate that LAT plays no role in latency, it should be noted that explant reactivation, which we and others use to detect latent virus, is very different from recurrence or spontaneous shedding as typically occurs in infected humans. The mouse model system would not detect a role for LAT in recurrence because spontaneous shedding of HSV-1 does not occur in these animals.

We previously demonstrated that *in situ* β-Gal expression from recombinant HSV-1 could be used to detect infected cells and study viral gene expression in animal tissues (17). Although the exact location of the LAT promoter has not been defined and may be well upstream of the apparent transcription start site (9, 10), our strategy in constructing RH142 was to place the *lacZ* gene within the 5′ leader of the transcript inserted in place of the normal LAT in the viral genome. As such, we predicted that *lacZ* would come under control of the LAT promoter without the need to map it precisely. LAT expression shows very different characteristics as compared to genes active during productive infection. In RH142-infected animals, β-Gal expression in neurons could be detected continuously up to 8 weeks postinoculation, whereas in animals expressing β-Gal from viral α or β promoters, X-Gal-staining neurons could be found only during the acute phase of infection. Also, β-Gal expression from RH142 could be detected only in sensory neurons and not in cultured cells or in epithelial or dermal tissues in inoculated mice. At present, we do not know whether β-Gal activity from RH142 would be detected in cultured neuronal cells. Our results clearly point to a tissue specificity of the expression or processing of the chimeric LAT–*lacZ* transcript. Viruses similar to RH142 should prove to be extremely useful to deliver and stably express other genes, in particular neuron-specific genes, in latently infected animals.

The punctate X-Gal staining pattern in RH142-infected ganglia was dramatically different from the uniform staining pattern observed with other viruses expressing β-Gal under α or β promoter control. Such a punctate pattern of staining suggests a cell-specific localization of β-Gal to vesicles or other cytoplasmic structures. Based on the structure of the gene fusion, the β-Gal protein product is expected to be the same in all constructs we have used and should remain a cytoplasmic protein (17, 19, 20). Although we do not understand the basis for the observed histological differences, we believe that they indicate a difference in the physiological state of the set of neurons that have entered latency.

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