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A LAT-Associated Function Reduces Productive-Cycle Gene Expression during Acute Infection of Murine Sensory Neurons with Herpes Simplex Virus Type 1

DAVID A. GARBER,[1] PRISCILLA A. SCHAFFER,[2] AND DAVID M. KNIPE[1]*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115,[1] and Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104[2]

Received 17 March 1997/Accepted 5 May 1997

Herpes simplex virus (HSV) persists in the human population by establishing long-term latent infections followed by periodic reactivation and transmission. Latent infection of sensory neurons is characterized by repression of viral productive-cycle gene expression, with abundant transcription limited to a single locus that encodes the latency-associated transcripts (LATs). We have observed that LAT$^-$ deletion mutant viruses express viral productive-cycle genes in greater numbers of murine trigeminal ganglion neurons than LAT$^+$ HSV type 1 at early times during acute infection but show reduced reactivation from latent infection. Thus, a viral function associated with the LAT region exerts an effect at an early stage of neuronal infection to reduce productive-cycle viral gene expression. These results provide the first evidence that the virus plays an active role in down-regulating productive infection during acute infection of sensory neurons. The effect of down-regulation of productive-cycle gene expression during acute infection may contribute to viral evasion from the host immune responses and to reduced cytopathic effects, thereby facilitating neuronal survival and the establishment of latency.

Herpes simplex virus (HSV) infection in vivo is characterized by productive infection at peripheral sites followed by viral spread to innervating sensory ganglia. There, transient productive infection or establishment of latent infection may occur in neurons (reviewed in reference 1). This ability of HSV type 1 (HSV-1) to cause both productive and latent infections of neurons in vivo is one of the most fascinating aspects of its interaction with the host. Productive infection by HSV-1 has been studied extensively in cultured cells and involves expression of >80 viral genes in an ordered temporal cascade of immediate-early (IE), early (E), and late (L) genes (30). Latent infection, however, has been studied primarily in animal models, specifically within the sensory ganglia which innervate peripheral sites of virus replication. Within latently infected sensory neurons, the pattern of viral gene expression differs dramatically from that observed in productively infected cells, with abundant gene expression in latent infection being restricted to a single viral locus, resulting in the expression of the latency-associated transcripts (LATs) (35). The mechanisms that down-regulate productive cycle viral gene expression during acute infection of sensory neurons have not been defined, and the lack of evidence for an active role of the virus in establishment of latency has raised the possibility that host cell mechanisms exclusively govern repression of viral gene expression during the establishment of latency (19, 33).

Transcription from the LAT locus results in a ≥8.3-kb transcript that is spliced to give stable, nonpolyadenylated introns of 2.0 and 1.45 kb (Fig. 1) (13), which accumulate in the nuclei of latently infected neurons (26, 38–40, 43). Collectively, these RNA molecules have been called the LATs. While it was postulated at the time of their discovery that the LATs might exert antisense effects on expression of ICP0, a strong activator of viral gene expression (Fig. 1) (35), no experimental evidence exists for effects of the LATs on viral gene expression during productive viral infection. Also, while the stable LATs contain short open reading frames (ORFs), there is no convincing evidence that these ORFs encode a latency-associated protein expressed in vivo. Genetic studies have shown that the LATs are not essential for establishment, maintenance, or reactivation of latent infections (16, 18). However, in a number of animal models, LAT$^-$ mutants display reduced frequency or delayed kinetics of reactivation (15, 23, 37). In addition, LATs may also affect the anatomical site of viral reactivation (42).

In this study, we hypothesized that the LATs may attenuate viral productive-cycle gene expression in sensory neurons. This hypothesis was based, in part, on an observation of increased pathology in trigeminal ganglion sections during latent infection with a LAT deletion virus, *dl*LAT1.8 (23), and on a report that the LATs may actually promote latent infection (32). To determine what modulatory role the LAT locus may play in productive-cycle viral gene expression prior to the establishment of latent infection, we chose to study viral gene expression during the period of acute neuronal infection in vivo.

MATERIALS AND METHODS

Viruses and cells. African green monkey kidney (Vero) cells or murine fibroblasts (L-929 [L] cells) were used for experiments described in this report. Vero cells were maintained in Dulbecco modified Eagle medium supplemented with penicillin, streptomycin, and 10% bovine calf serum. L cells were maintained in spinner culture in S-MEM Joklik medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 10% fetal calf serum. For experiments requiring adherent cells, an L-cell suspension was mixed 1:1 with Dulbecco modified Eagle medium supplemented with 10% bovine serum, penicillin, and streptomycin and plated to yield monolayers on appropriate culture flasks. All viruses in this study were propagated on Vero cells. *dl*LAT1.8, a LAT deletion virus isolated by marker rescue of KOS *ts*Y46 (31), and the corresponding LAT$^+$ rescued virus, FSLAT$^+$, have been described previously (23). Briefly, *dl*LAT1.8 harbors a 1.8-kb deletion in each long repeat region of the HSV-1 genome which removes sequences including the major LAT promoter, LAT transcriptional start site, 1 kb of sequences specifying the LATs, and 100 bp of the sequences encoding the very low abundance 1.8-kb late viral transcript described by Singh and Wagner (34). To construct a new LAT$^-$ isolate, K*dl*LAT, which harbors a LAT deletion

* Corresponding author. Phone: (617) 432-1934. Fax: (617) 432-0223. E-mail: dknipe@warren.med.harvard.edu.

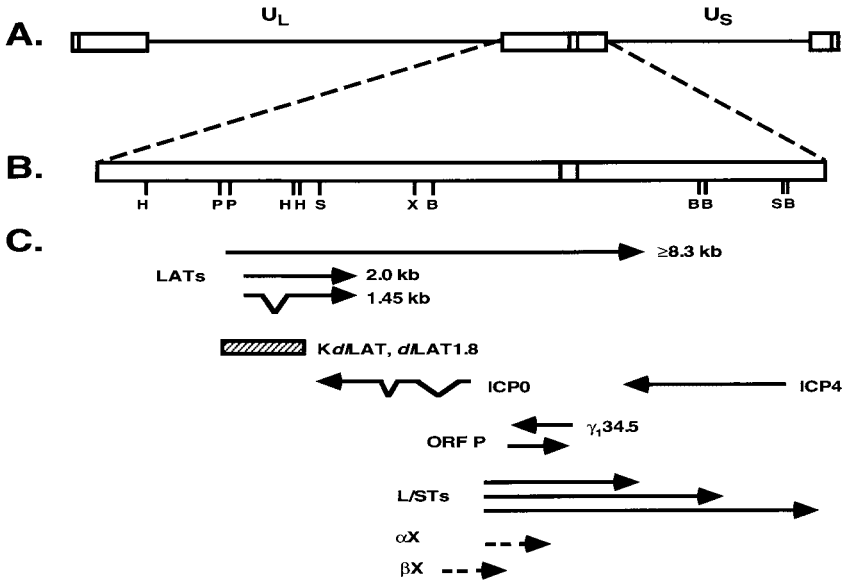

FIG. 1. Map of the HSV-1 genome showing relevant transcripts and the LAT deletion in K*dl*LAT and *dl*LAT1.8. (A) HSV-1 genome in the prototype orientation: U_L and U_S denote the unique sequences of the long (L) and short (S) components of the genome, respectively; the open boxes represent repeat sequences. (B) Expanded view of the L-S junction region with restriction endonuclease cleavage sites (B, *Bam*HI; H, *Hpa*I; P, *Pst*I; S, *Sal*I; X, *Xho*I). (C) Locations and orientations of transcripts of the L-S junction region are denoted by solid arrows and include the LATs, ICP0, ICP4, $\gamma_1$34.5 (9, 10), ORF P (22), L/STs (41), and partially mapped transcripts (dashed arrows) αX and βX (5, 6). The hatched bar represents the region deleted in K*dl*LAT and *dl*LAT1.8.

identical to that in *dl*LAT1.8 (Fig. 1) but is derived directly from KOS wild-type (wt) virus, plasmid pΔLAT1.8 (23) was digested with *Bam*HI and cotransfected with infectious KOS (wt) HSV-1 DNA into Vero cells. Virus isolates were plaque purified three times and screened by Southern blot analysis for introduction of the deletion into both LAT loci within the KOS genome (data not shown). The sequences deleted from K*dl*LAT were restored by marker transfer to generate a rescued virus, KFSLAT. Marker rescue was accomplished by cotransfection of infectious K*dl*LAT DNA and *Sal*I-digested pRFS (23) into Vero cells. Virus isolates were screened for restoration of the LAT loci within the viral genome by diagnostic Southern blot analysis (data not shown), and those with the desired genotypes were plaque purified four times.

Kinetics of viral growth. Viral growth was measured in Vero and L cells. Briefly, cells were plated into flasks at a density of 1×10^6 to 4×10^6/25 cm^2. Cells were trypsinized and counted prior to viral infection by using a hemocytometer. Following infection at stated multiplicities, samples were frozen at the indicated times postinfection. Cells were thawed and disrupted in medium by sonication, and progeny virus was quantified by plaque assay on Vero cells.

Animal inoculation. In vivo characterization of HSV-1 mutants was performed in 3-week-old female CD-1 mice following bilateral inoculation of 2.0×10^6 PFU of virus (in a 5-μl volume) per eye onto scarified corneas. All animal experiments were conducted in accordance with institutionally approved animal welfare protocols.

ISH analysis. Prior to hybridization, individual ganglia were serially cryosectioned (8 μm) and placed on slides such that sections included in a given sample were taken at ≥64-μm intervals to ensure equal representation of neurons occurring throughout the ganglion, as well as to minimize double scoring of in situ hybridization (ISH)-positive cells, thereby eliminating the need for cell count correction factors. Within a given experiment, all hybridizations using a given probe were conducted simultaneously. Methods used for ISH have been described previously (14, 20, 36) and are based on protocols obtained from J. Stevens. Briefly, slides were processed as follows prior to hybridization: 0.2 M HCl, 20 min; two rinses in water, 5 min each; 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 70°C, 30 min; two rinses in water, 5 min each; 0.2 M Tris-HCl (pH 7.4)–2 mM $CaCl_2$–1 μg of proteinase K per ml, 37°C, 15 min; two rinses in water, 5 min each; optional nuclease treatments; 2× SSC, 5 min; 5% paraformaldehyde (pH 7 to 8), 2 h; three washes in 2× SSC, 5 min each; two rinses in water, 5 min each; dipped in 0.1 M triethanolamine–0.25% (vol/vol) acetic anhydride; two rinses in water, 5 min each; dehydrated in 70, 90, and 95% ethanol. For RNase treatment, sections were incubated with RNase A (100 μg/ml) and RNase T_1 (10 U/ml) in 2× SSC at 37°C for 30 min followed by rinsing (five times) in 2× SSC (5 min/rinse). For DNase treatment, sections were incubated with 24 U of DNase (RNase free)/slide in 20 mM Tris-HCl (pH 7.4)–10 mM $MgCl_2$ at 37°C for 1 h followed by rinsing (five times) in 2× SSC (5 min/rinse). The following plasmids were used as probes for viral gene expression: pK1-2 (11) for detection of IE (ICP4) transcripts, pBH27 (28) for detection of IE (ICP27) transcripts, ptkLTR16 (kindly provided by M. F. Kramer and D. M. Coen) for detection of E and L (TK, VP16, and gH) transcripts, and pIPH (23) for the detection of LATs. DNA probes were generated by radiolabeling plasmids with [α-^{35}S]dATP and [α-^{35}S]dCTP (Amersham, Arlington Heights, Ill.) via nick translation (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and hybridized to tissue sections in 50% formamide–10% dextran sulfate–10 mM Tris-HCl (pH 7.4)–2× SSC–1 mM EDTA–1× Denhardt's solution–denatured salmon sperm DNA (100 μg/ml)–tRNA (500 μg/ml)–poly(A) (10 μg/ml) at 45°C for 72 h. Following hybridization, slides were washed in three to four changes of 50% formamide–0.3 M NaCl–10 mM Tris-HCl (pH 7.4)–1 mM EDTA at room temperature over 72 h, followed by a higher-stringency wash of 50% formamide–2× SSC–10 mM Tris-HCl (pH 7.4)–1 mM EDTA at 45°C for 2 h. Following the high-stringency rinse, tissue sections were dehydrated in graded alcohols (70 and 95%) containing 0.3 M ammonium acetate, and slides were dried overnight at room temperature prior to autoradiography. For autoradiography, slides were dipped in Kodak NTB-2 emulsion, previously diluted 1:1 with 0.6 M ammonium acetate, and stored at 4°C for 3-day exposures. Slides were developed in 0.5× D19 developer (Kodak) at 15°C for 3 min and fixed (Kodak fixer) for 4 min at room temperature, after which sections were stained with Giemsa stain (Sigma). Following ISH, quantification of positive neuronal cells was performed by scoring sections under bright-field illumination at a magnification of ×250 to ×630. On average, 10 sections per ganglion times n ganglia per virus per time point were scored for ISH-positive neurons (Fig. 6, $n = 4$; Fig. 7 and 8, $n = 10$ to 12).

In vitro reactivation of latent infection. To assess the ability of wt and mutant HSV-1 to reactivate from latency, trigeminal ganglia were dissected aseptically from infected mice 30 days postinfection (dpi). Explanted tissue was cut into eight pieces and cocultivated with Vero cells at 37°C. Cocultivation samples were harvested 5 days after explant, sonicated, and assayed for infectious virus by plaque assay on Vero cells as previously described (24).

RESULTS

Our previous studies on the functions of the LATs used the deletion mutant *dl*LAT1.8 and the LAT^+ rescued virus FS-LAT^+ (23). In some studies, we observed that $FSLAT^+$ virus behaved differently from wt KOS (see below). Therefore, we constructed a new LAT^- virus with the same deletion in the wt KOS background, K*dl*LAT, and a LAT^+ rescuant as described in Materials and Methods. Both sets of LAT^+ and LAT^- viruses were examined in these studies. The phenotype of *dl*LAT1.8 has been described elsewhere (23): (i) it grows similarly to the LAT^+ wt and rescued viruses in cell culture, (ii) no LAT^+ neurons are detected in mouse ganglia latently infected

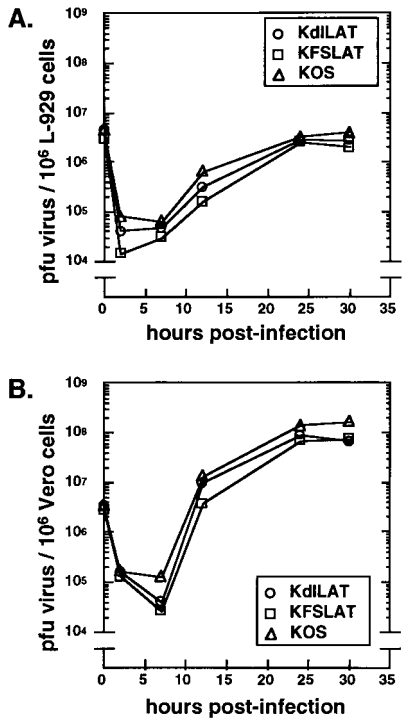

FIG. 2. Growth properties of LAT$^-$ and LAT$^+$ viruses in cultured cells. L cells (A) or Vero cells (B) were inoculated with 3 PFU of K*dl*LAT, KFSLAT, or KOS per cell. At 2, 7, 12, 24, and 30 h postinfection, samples were analyzed for virus yields as described in Materials and Methods. Data points represent the arithmetic means of duplicate samples.

with *dl*LAT1.8, and (iii) *dl*LAT1.8 reactivates approximately 50% as efficiently as FSLAT$^+$ from ganglionic explants. Therefore, we first characterized the phenotypes of K*dl*LAT and KFSLAT for the same parameters.

Phenotypic characterization of K*dl*LAT and KFSLAT. (i) Growth in cultured cells. We first determined if the LAT deletion in the recombinant virus K*dl*LAT affected its ability to grow in cells in culture. Single-step growth experiments were conducted in murine fibroblasts (L cells) or monkey kidney (Vero) cells with the LAT deletion virus K*dl*LAT, its corresponding LAT$^+$ rescued virus KFSLAT, and the wt strain KOS. Figure 2 shows virus yields over time following inoculation of L cells (Fig. 2A) or Vero cells (Fig. 2B) with K*dl*LAT, KFSLAT, or KOS at a multiplicity of 3 PFU/cell. While viral yields in Vero cells were greater than those in L cells by 12 h postinfection, the replication efficiencies of K*dl*LAT, KFSLAT, and KOS were equivalent with respect to production of progeny virus throughout infection in either cell type (Fig. 2). Thus, the LAT deletion of K*dl*LAT had no effect on virus production in cultured nonneuronal cells of murine or simian origin.

(ii) Expression of LAT. We next examined the phenotypes of the new LAT deletion mutant, K*dl*LAT, its corresponding LAT$^+$ marker-rescued virus, KFSLAT, and the wt strain, KOS, with respect to expression of the LATs within murine trigeminal ganglia during acute infection. Following corneal inoculation of mice with 2×10^6 PFU of K*dl*LAT, KFSLAT, or KOS per eye, trigeminal ganglia were removed at 3 dpi and processed for ISH as described above. LAT hybridization was detected within neurons, and ISH silver grain patterns were found to occur with distributions over the nucleus and in some cases the cytoplasm also. Figure 3 shows LAT-specific hybridization in representative sections of trigeminal ganglia following infection with KFSLAT, K*dl*LAT, or KOS or after mock infection. Ganglionic neurons infected with KFSLAT or KOS, each of which have LAT$^+$ genotypes, exhibited hybridization to the LAT probe, pIPH (Fig. 3A and C). In contrast, infection with the LAT deletion virus, K*dl*LAT, resulted in no detectable LAT hybridization signal within ganglia (Fig. 3B), as in mock-infected mice (Fig. 3D). Numbers of LAT$^+$ neurons were determined 3 days after infection with K*dl*LAT, KFSLAT, or KOS (Table 1). These data demonstrate that in trigeminal ganglia of K*dl*LAT-infected mice, the LATs were not detected by ISH during acute infection, whereas LAT$^+$ neurons were readily detected in KFSLAT- and KOS-infected ganglia. Furthermore, KFSLAT-infected ganglia contained the same number of LAT$^+$ neurons as observed during infection with wt virus (KOS). Thus, K*dl*LAT is functionally deficient for LAT expression in neurons, whereas the marker-rescued virus, KFSLAT, exhibits a LAT expression phenotype equivalent to that of KOS.

(iii) Efficiency of reactivation from latent infection. The reactivation phenotypes of K*dl*LAT, KFSLAT, and KOS were assessed by explant/cocultivation assays in which latently infected trigeminal ganglia (removed at 30 days postinfection) were explanted and cocultured with Vero cells. At 5 days postexplant, samples were assayed for infectious virus by plaque assay of total culture lysate on Vero cells. K*dl*LAT reactivated from fewer ganglia than did KFSLAT or KOS (K*dl*LAT, 24% reactivation; KFSLAT, 56%; KOS, 60% [Table 2]). Thus, K*dl*LAT reactivated from latency at a relative frequency of 43% compared to KFSLAT and 40% with respect to KOS. In addition, KFSLAT and KOS were found to reactivate with similar efficiencies (Table 2). Thus, the LAT$^-$ virus, K*dl*LAT, exhibited a reduced reactivation phenotype relative to the LAT$^+$ viruses, including marker-rescued virus KFSLAT, as well as wt strain KOS. Moreover, the magnitude of the reduction was similar to that demonstrated for another pair of LAT$^-$ and LAT$^+$ isolates, *dl*LAT1.8 and FSLAT$^+$ (23).

Specificity of ISH for viral RNA. We wished to quantify the number of neurons expressing productive-cycle transcripts during acute infection of trigeminal ganglia. However, viral DNA replication in these neurons produces various viral DNA forms that might be detected by ISH. Therefore, we performed nuclease control experiments to confirm that ISH signals were specific for the detection of RNA and not viral DNA in sections of trigeminal ganglia. Following corneal inoculation of mice with KFSLAT, trigeminal ganglia were removed 5 days after infection and processed for ISH with or without nuclease treatments. Figure 4 shows representative results of ISH with the E/L gene probe following processing of the tissue sections which included no nuclease treatment (Fig. 4A), treatment with RNase (Fig. 4B), or treatment with DNase (Fig. 4C). Treatment of sections with RNase abolished virtually all hybridization signal (Fig. 4B), whereas treatment with DNase (Fig. 4C) had no effect on detection of signal, as the hybridization signal was equivalent to that observed in untreated sections (Fig. 4A). Thus, the hybridization protocol specifically detected viral RNA and not DNA.

LAT$^-$ viruses express productive-cycle genes in greater numbers of trigeminal ganglion neurons than LAT$^+$ HSV-1. We chose to study the period of acute neuronal infection to determine what role the LAT locus might play with respect to modulating productive viral replication in trigeminal ganglia. Following corneal inoculation of mice with LAT deletion virus K*dl*LAT or *dl*LAT1.8, LAT$^+$-rescued virus KFSLAT or FSLAT$^+$, or wt KOS, we removed trigeminal ganglia at daily intervals postinfection during acute infection and processed

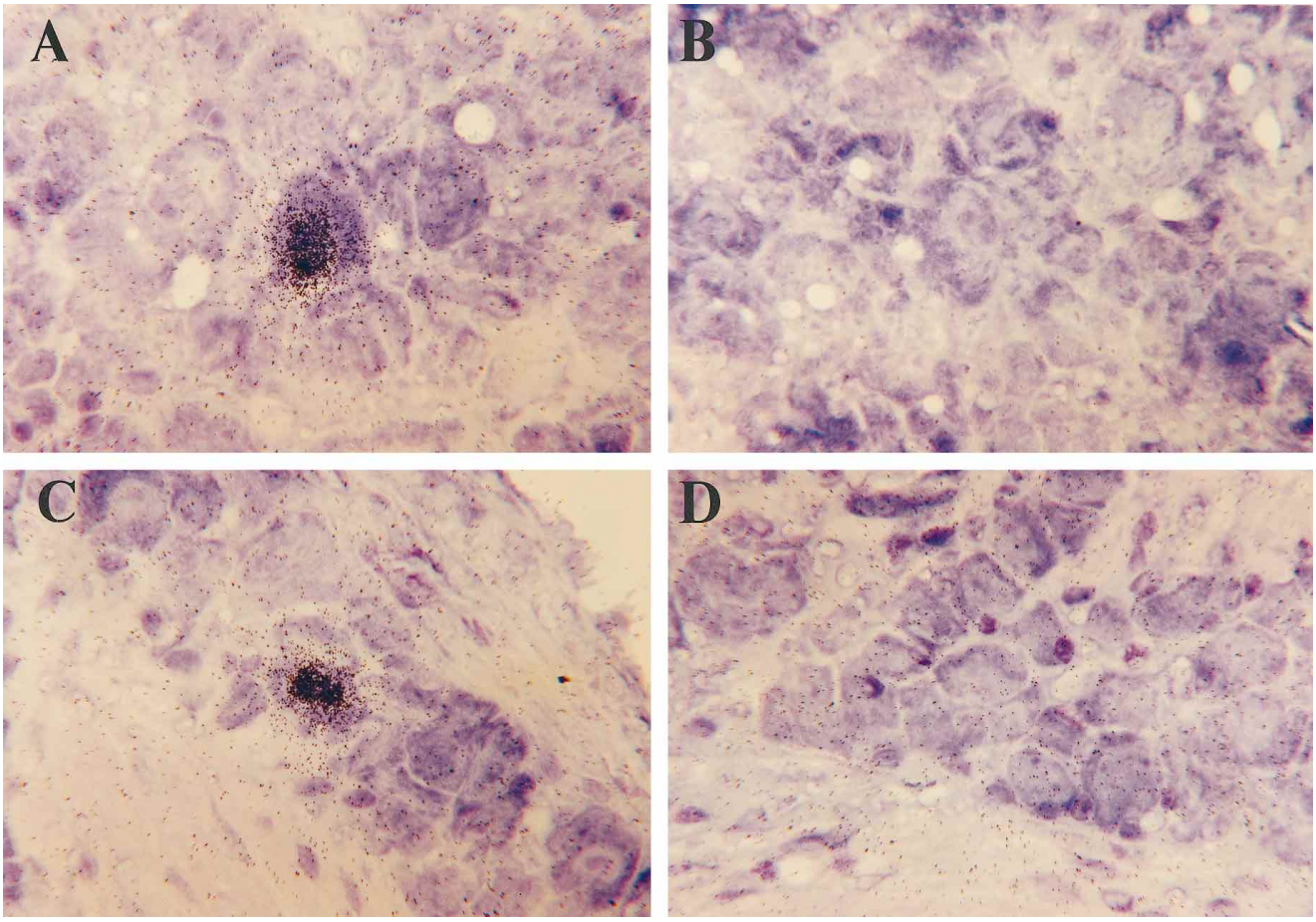

FIG. 3. Bright-field micrographs of HSV-1-infected trigeminal ganglion sections following ISH for detection of the LATs. Trigeminal ganglia were removed at 3 days following ocular infection of mice with KFSLAT (A), K*dl*LAT (B) or KOS (C) or mock infection (D) and processed for ISH with a probe, pIPH, for detection of the LATs.

them for ISH. Probes for two of the first genes transcribed during productive infection, ICP4 and ICP27, were hybridized to ganglion sections to measure the number of cells expressing IE transcripts. In addition, a probe that detected E (TK) and L (VP16 and gH) genes was used to assess total numbers of ganglionic cells expressing productive-cycle transcripts (hybridizations with this probe to detect E and L gene expression are referred to as E/L throughout this report). Figure 5 shows representative results from ISH of trigeminal ganglion sections 3 days after inoculation with K*dl*LAT (Fig. 5A) or KFSLAT (Fig. 5B) or mock infection (Fig. 5C), hybridized with the E/L gene probe. Following ISH, tissue sections were analyzed by bright-field microscopy at a magnification of ×250 to ×630, which revealed viral gene expression in both neurons and non-neuronal cells of the trigeminal ganglia and showed that the distributions of positive neurons within ganglia were similar for LAT^- and LAT^+ virus infections.

Our initial studies with the LAT^- virus, *dl*LAT1.8, showed increased acute viral gene expression in trigeminal ganglion neurons compared to the rescued virus, $FSLAT^+$, in which the LAT mutation had been corrected. Quantification of neurons following hybridization with IE probes (Fig. 6) showed that at 3 dpi, 11-fold more ICP4-positive neurons and 8-fold more ICP27-positive neurons were detected in sections of ganglia infected with *dl*LAT1.8 than in sections of ganglia infected with $FSLAT^+$. In addition, quantification of neurons following hybridization with the E/L probe showed that there were 16-fold more E/L-positive neurons in sections from ganglia infected with *dl*LAT1.8 than $FSLAT^+$ (Fig. 6). However, the

TABLE 1. Quantitation of LAT^+ neurons in trigeminal ganglia at 3 days following ocular infection with LAT^- and LAT^+ HSV-1

Virus	No. of sections scored (no. ganglia of assayed)	Mean no. of LAT^+ neurons/section (SEM)
K*dl*LAT	98 (10)	0 (0)
KFSLAT	104 (10)	0.84 (0.37)
KOS	66 (6)	0.76 (0.48)
None (mock infection)	24 (2)	0 (0)

TABLE 2. Reactivation phenotypes of LAT^- and LAT^+ viruses following explant cocultivation of latently infected murine trigeminal ganglia

Virus	No. of ganglia reactivated/no. tested (%)
K*dl*LAT	4/17 (24)
KFSLAT	5/9 (56)
KOS	12/20 (60)

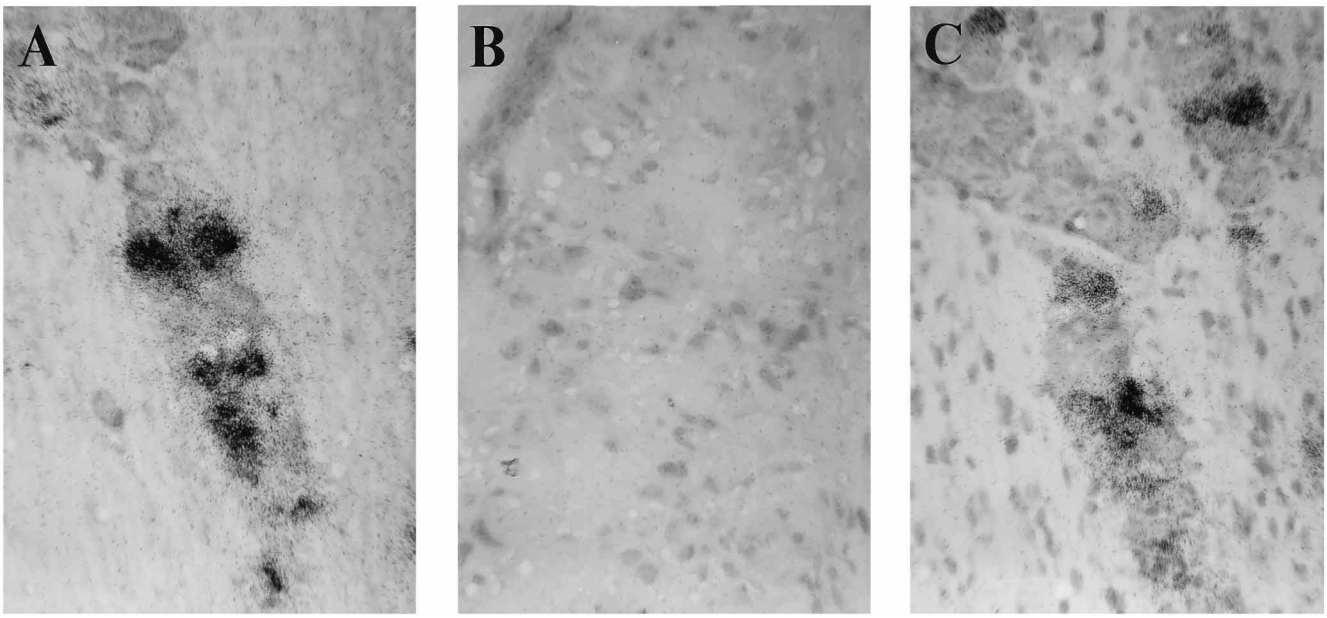

FIG. 4. Effects of nuclease treatments on ISH of tissue sections from HSV-1-infected trigeminal ganglia. ISH for the detection of HSV-1 E/L gene expression within trigeminal ganglia at 5 days following ocular infection of mice with KFSLAT was performed as follows: (A) no nuclease treatment; (B) treatment with RNase; (C) treatment with DNase.

FSLAT$^+$ strain showed fewer cells expressing productive-cycle transcripts than the wt strain KOS (Fig. 6). This observation suggested the possibility that the LAT$^-$ virus *dl*LAT1.8 and/or the rescued virus FSLAT$^+$ contained additional mutations that might affect acute infection of neurons. To confirm the phenotypic difference between LAT$^-$ and LAT$^+$ viruses and to eliminate the contribution of any secondary mutations in the analysis of the *dl*LAT1.8-FSLAT$^+$ virus pair, we constructed a new LAT$^-$ virus by introducing the 1.8-kbp LAT deletion into the LAT loci of wt KOS DNA to generate the mutant strain K*dl*LAT. We then proceeded to isolate from K*dl*LAT a marker-rescued virus, KFSLAT, in which the LAT locus was restored (Fig. 1).

We used ISH to compare the numbers of neurons in trigeminal ganglia exhibiting productive-cycle viral gene expression during acute infection with our new LAT deletion isolate, K*dl*LAT, the marker-rescued virus KFSLAT, and wt strain KOS. Following corneal inoculation of mice with K*dl*LAT, KFSLAT, or KOS, we removed trigeminal ganglia at 3, 5, 7, or 9 dpi. Ganglia were processed for ISH and hybridized with IE (ICP4) and E/L probes. Quantification of neurons with IE (ICP4) hybridization signal (Fig. 7) showed that at 3 dpi, there were fourfold more positive neurons in sections from ganglia infected with K*dl*LAT than with KFSLAT. In addition, K*dl*LAT showed fivefold more IE-positive neurons at 3 dpi compared to KOS. Thus, no difference in numbers of ICP4-positive neurons was observed at 3 dpi between the rescued virus, KFSLAT, and the wt strain, KOS, indicating that the phenotype of K*dl*LAT is due solely to the LAT mutation. The numbers of ganglionic neurons expressing IE (ICP4) transcripts over time are shown in Fig. 8A. While the aforementioned four- to fivefold increases in numbers of IE-positive neurons were observed at 3 dpi in ganglia infected with K*dl*LAT versus KFSLAT (Fig. 8A) or KOS (not shown), by 5 dpi, nearly equivalent numbers of IE-positive neurons were observed in tissue from animals infected with K*dl*LAT or KFSLAT (Fig. 8A). By 7 dpi, the numbers of cells expressing ICP4 transcripts arising from both viruses were greatly reduced (Fig. 8A). Comparison of areas under the curves shown in Fig. 8A revealed a slight increase in day-positive neurons (dpn) for K*dl*LAT (dpn = 12.1) versus KFSLAT (dpn = 10.4). Therefore, IE transcripts accumulated to levels detectable by ISH at earlier times with the LAT$^-$ mutant virus.

Quantification of neurons hybridizing with the E/L probe (Fig. 8B) demonstrated that K*dl*LAT infection resulted in fourfold more E/L-positive neurons than did infection with KFSLAT at 3 dpi. By 5 dpi, nearly equivalent numbers of positive neurons were observed in tissue from animals infected with the two viruses. Therefore, like IE transcripts, E/L transcripts accumulated to levels detectable by ISH at earlier times with the LAT$^-$ mutant virus. Comparison of areas under the curves (Fig. 8B) was used to gauge relative numbers as well as duration of E/L-positive cells. This comparison revealed an approximately 100% increase in dpn for K*dl*LAT (dpn = 44.4) versus KFSLAT (dpn = 22.7). Thus, greater numbers of neurons expressing productive-cycle transcripts were observed with two LAT$^-$ viruses compared to their respective LAT$^+$ rescuants.

The expression of viral productive-cycle genes in an increased number of cells from LAT$^-$ versus LAT$^+$ virus-infected ganglia was occasionally reflected in higher titers of LAT$^-$ versus LAT$^+$ virus production within ganglia. When inoculum doses of 2×10^6 PFU/eye were used, ganglionic titers of K*dl*LAT were 27- or 3-fold greater than those of KFSLAT or KOS, respectively, by 3 dpi in some but not all experiments (results not shown). In some experiments, increased titers of LAT$^-$ versus LAT$^+$ viruses within trigeminal ganglia were observed only following ocular infection at lower doses, e.g., 2×10^4 PFU/eye (results not shown). Thus, the increased numbers of neurons expressing productive-cycle transcripts during LAT$^-$ versus LAT$^+$ virus infections did not necessarily result in higher titers of LAT$^-$ virus within trigeminal ganglia.

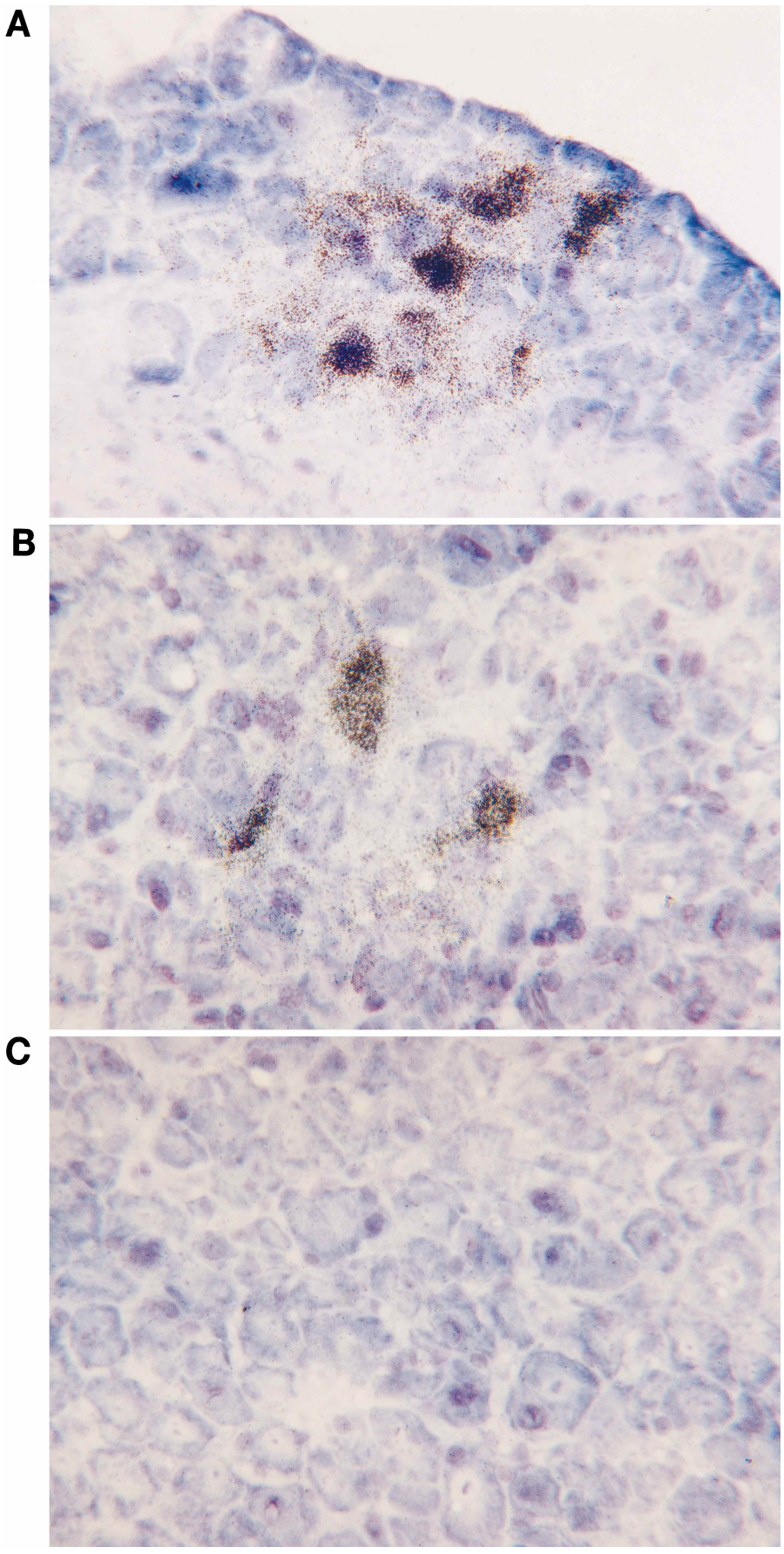
A
B
C

FIG. 5. Bright-field micrographs of HSV-1-infected trigeminal ganglion sections following ISH for detection of viral productive-cycle gene expression. Trigeminal ganglia were removed at 3 days following ocular infection of mice with K*dl*LAT (A) or KFSLAT (B) or mock infection (C) and were processed for ISH with a probe for detection of viral E/L gene expression.

DISCUSSION

These studies demonstrate that in acutely infected trigeminal ganglion neurons, two independent isolates of a LAT$^-$ virus exhibit accelerated expression of IE genes and increased numbers of cells expressing E and L transcripts compared to wt and rescued viruses in which LAT sequences have been restored. Furthermore, infections with LAT$^-$ viruses, which exhibit increased expression of productive-cycle genes within sensory neurons, show decreased frequencies of reactivation from latency. The reduced reactivation may be due to a positive role for the LATs in enhancing reactivation as hypothesized previously (23). Alternatively, at least part of the decreased reactivation phenotype may be due to increased productive-cycle gene expression, enhanced neuronal death, and the subsequent decrease in the efficiency of latent infection and, consequently, of reactivation. For example, if a limited number of neurons in each ganglion can be acutely infected with virus and if expression of productive-cycle genes leads to cell death, then an increase in the number of cells expressing lytic genes could lead to fewer latently infected cells. In addition, an increase in the number of cells expressing productive-cycle genes could result in the increased clearance of infected cells by the host immune response, which would also lead to fewer latently infected cells available for subsequent reactivation. During infection with K*dl*LAT, the observed twofold increase in total cells showing expression of E and L genes is consistent with the twofold reduction in reactivation frequency by viruses bearing this LAT mutation (reference 23 and this report). In addition, Maggioncalda et al. have reported a correlation between reduced numbers of neurons latently infected by a LAT$^-$ versus a LAT$^+$ virus, with reduced frequency of reactivation of LAT$^-$ virus following ganglionic explant (25). While the LATs are clearly not essential for establishment of latency or for reactivation in murine trigeminal ganglia (16, 18, 23), integrity of the LAT locus may serve to delay IE gene expression and reduce the number of cells expressing E and L transcripts during acute infection. In this way, the LATs may contribute to events that lead to repression of gene expression associated with productive infection and to the establishment of latent infection in neurons. Further experiments are in progress to measure the numbers of latently infected neurons in ganglia at various times after infection of mice with LAT$^-$ and LAT$^+$ viruses to test the hypothesis that the LATs contribute to establishment or maintenance of latency through repression of viral productive-cycle gene expression.

In this study, we examined the role of the LATs during acute viral infection in a murine model of infection. During acute infection of mice, LAT$^-$ HSV-1 results in a greater number of cells expressing productive-cycle genes than LAT$^+$ viruses, within trigeminal ganglia. Consistent with our observed effects of the LATs during acute infection are the observations of Chen et al. in the accompanying report (8), showing that the LAT locus exerts an inhibitory effect on the expression of ICP4 and TK gene transcripts during latent infection of murine trigeminal ganglia. These two effects of the LAT locus of repressing productive-cycle gene expression during acute infection and latent infection may be due to the same or different mechanisms. Similarly, we do not know whether the repressive effect of the LAT locus on productive-cycle gene expression reported here is related to the inhibition of IE gene expression in sensory neurons that is relieved by viral DNA replication (20).

Although the LATs appear to exert a limited effect on reactivation and acute viral gene expression in murine systems as observed in this and other studies, the effect of LATs on productive viral gene expression in sensory neurons may be more pronounced in other animal model systems. Studies in rabbits (3, 15) and guinea pigs (21) have shown a more dramatic role for the LATs in promoting spontaneous as well as induced reactivation from latency. Consequently, the effect of the LAT locus in repressing productive-cycle gene expression may also be more dramatic in these species as well as in the human host. Because the LATs are likely to be only part of the process by which HSV gene expression is repressed during the establishment of latent infection, their relative contribution to

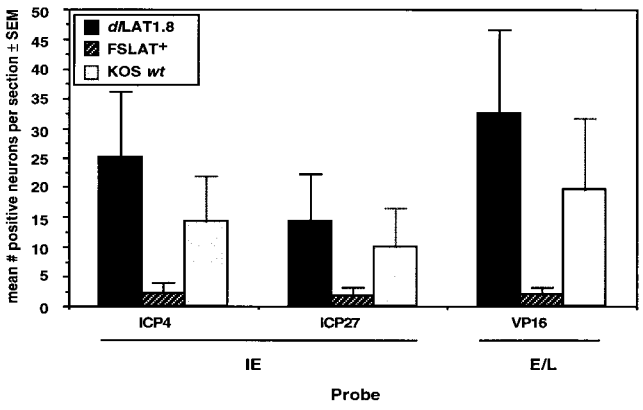

FIG. 6. Numbers of trigeminal ganglion neurons expressing productive-cycle transcripts following ocular inoculation of mice with *dl*LAT1.8, FSLAT$^+$, or KOS. At 3 dpi, trigeminal ganglia were removed and processed for ISH for detection of IE (ICP4 and ICP27) as well as E/L viral gene expression in neurons (n = 4 ganglia per virus).

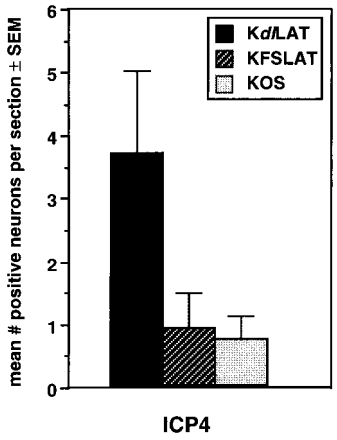

FIG. 7. Numbers of trigeminal ganglion neurons expressing IE transcripts following ocular inoculation of mice with K*dl*LAT, KFSLAT, or KOS. At 3 dpi, trigeminal ganglia were removed and processed for ISH for detection of IE (ICP4) viral gene expression in neurons (n = 10 to 12 ganglia per virus).

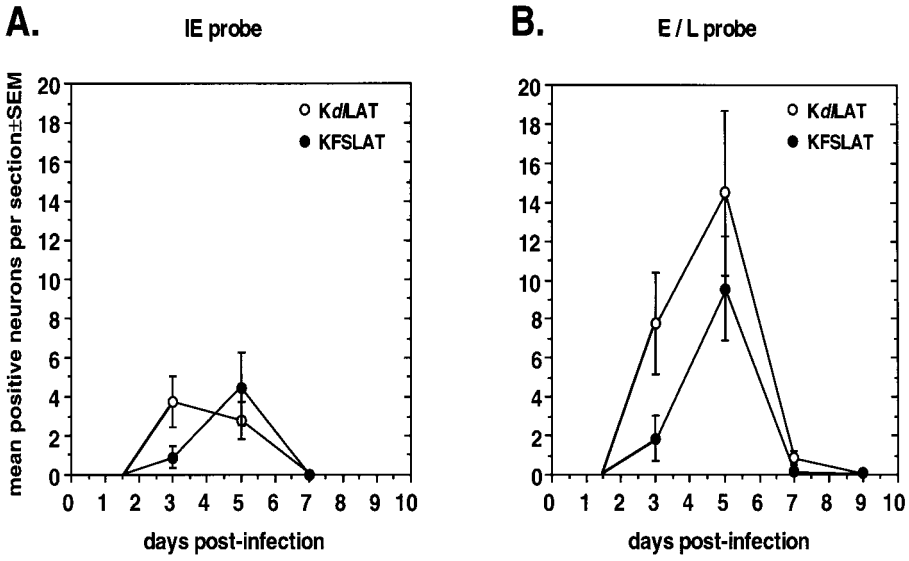

FIG. 8. Numbers of trigeminal ganglion neurons expressing productive-cycle transcripts following ocular inoculation of mice with K*dl*LAT or KFSLAT as a function of time postinfection. Results are shown for IE (ICP4) hybridization (A) and for E/L hybridization (B) (n = 10 to 12 ganglia per virus per time point). Curves are extrapolated to zero at 1.5 dpi because other experiments showed low numbers of positive cells at 2 dpi.

this repression may vary in different species. Thus, studies of the effects of LATs on productive-cycle gene expression should be performed in other species such as rabbits or guinea pigs.

Possible mechanisms of the repressive effects of the LAT locus. The mechanism of repression of productive-cycle gene expression by the LATs has not been elucidated, but several possible mechanisms are conceivable. First, the LATs or other transcripts whose synthesis is up-regulated by the LAT locus may encode a protein(s) that down-regulates productive-cycle gene expression. One group has reported expression in infected cultured cells of a protein from an ORF encoded by LATs (12), but there is no evidence that this protein is expressed during acute or latent infection of neurons in vivo. There is a precedent for a protein product being expressed from an alphaherpesvirus latency-related transcriptional unit in that the bovine herpesvirus 1 latency-related gene encodes a protein when inserted into a mammalian expression vector and transfected into cells (17). However, to our knowledge, there is no evidence that this protein is expressed in bovine herpesvirus 1-infected cells. In addition to the potential for proteins directly encoded by the LATs, the LATs may up-regulate other transcripts that encode proteins. For example, downstream transcripts such as that encoding ORF P, a gene product recently hypothesized to regulate viral gene expression (7), or the transcripts antisense to ICP4 detected by Chen et al. (8) could encode proteins that down-regulate productive-cycle gene expression. Despite the unsuccessful attempts thus far to detect latency-associated proteins in HSV-infected sensory neurons, a virus-encoded protein that down-regulates productive-cycle gene expression remains as a very possible mechanism for this effect.

Second, antisense RNA regulation of expression of viral genes encoding transcriptional activating proteins could provide a potential mechanism for repressive effects observed in this work. The delayed and decreased numbers of cells expressing ICP4 transcripts in neurons infected with LAT^+ viruses could be due to direct antisense effects of LATs or downstream transcripts on ICP4 mRNA. For example, the ICP4 antisense transcripts detected by Chen et al. (8) in latently infected ganglia could exert a negative effect on ICP4 transcripts through antisense down-regulation. Alternatively, LATs could exert an antisense effect on ICP0 mRNA expression as originally proposed by Stevens et al. (35) and as observed by Farrell et al. (13) in a transfection study in nonneuronal cells. ICP0 is an important IE regulatory gene product that could up-regulate ICP4 mRNA expression, and antisense down-regulation could provide an indirect mechanism for LATs to affect expression of ICP4 transcripts. The growth and latency phenotypes of ICP0 mutants (24) are also consistent with this potential explanation for the mechanism of LAT function. Third, the LATs could exert a silencing effect on the viral genome similar to the effect of the XIST RNA on gene expression from the X chromosome (27) as proposed by Bloom et al. (4). Further tests of these different models can now be performed to determine which explains the repressive effects of the LAT locus on productive-cycle gene expression.

In summary, these results provide evidence for a viral function, directly or indirectly specified by the LATs, that contributes to the suppression of the expression of HSV productive-cycle genes during acute infection of sensory neurons. We hypothesize that this effect enables the virus to evade immune recognition and exhibit reduced cytopathogenicity, thereby promoting survival of the infected neuron and the establishment of latent infection by the virus. Finally, these results provide evidence for a potential regulatory loop in which LATs delay and reduce ICP4 gene expression to promote latent infection. ICP4, in turn, may block transcription of the LATs and activate E and L gene expression to promote productive infection (2, 29). This regulatory loop involving HSV-encoded gene products could contribute to the ability of the virus to undergo a productive or a latent infection in individual sensory neurons.

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