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Molecular Analysis of Herpes Simplex Virus Type 1 during Epinephrine-Induced Reactivation of Latently Infected Rabbits In Vivo

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Infectious virus assays and PCR amplification of DNA and RNA were used to investigate herpes simplex virus (HSV) DNA replication and gene expression in the rabbit corneal model for virus reactivation in vivo. We used carefully defined latency-associated transcript-negative (LAT^-) and LAT^+ promoter mutants of the $17syn^+$ strain of HSV type 1. In agreement with earlier studies using a more extensive LAT^- deletion mutant, the 17ΔPst(LAT^-) virus reactivated with extremely low frequency upon epinephrine induction. In contrast to our findings with murine latency models, amounts of viral DNA recovered from rabbit ganglia latently infected with either LAT^+ or LAT^- virus were equivalent. Also in contrast with the murine models, no net increase in viral DNA was seen in latently infected rabbit trigeminal ganglia induced to reactivate in vivo by iontophoresis of epinephrine. Despite this, transcription of lytic-phase genes could be detected within 4 h following induction of rabbits latently infected with either LAT^+ or LAT^- virus; this transcription diminished by 16 h following induction. These results are discussed in relation to models for the mechanism of action of HSV LAT.

General features of herpes simplex virus type 1 (HSV-1) latent infection in and reactivation from sensory ganglia are described in the introduction to the accompanying report (3). With respect to trigeminal ganglia, initial infection of areas supplied by branches of the trigeminal nerve results in a productive infection in which virus enters associated nerve axons, migrates to the ganglia, and establishes a latent infection. Virus gene expression is highly restricted during the latent phase of infection; only the latency-associated transcripts (LATs) are expressed (see references 7 and 17 for reviews). Reactivation of HSV from ganglia results in the appearance of infectious virus at the site of initial primary infection.

The rabbit eye model has been extensively studied with respect to the pathogenesis of HSV. In this model, latent infection of the trigeminal ganglia can be established with some convenience, especially with the $17syn^+$ and McKrae strains of virus, and epinephrine-induced viral reactivation can be monitored by the ability to recover virus in tear film (6, 9–11). In previous studies with an extensive deletion of the promoter and 5′ regions of the LAT unit resulting in a LAT^- virus (mutant X10-13), we found that efficient epinephrine-induced reactivation of HSV in this model requires the expression of LATs (12). This observation has been confirmed by others (19), and recently it has been reported that the HSV-2 homolog of LAT is also strongly correlated with in vivo reactivation in the guinea pig model (12a). It is not yet fully known which portion of the latent-phase transcription unit is involved in reactivation; however, it is quite clear that the active area lies outside the open translational reading frame within the stable introns generated by processing of the 8.5-kb primary LAT during latent infection (6).

Irrespective of the specific mechanism by which HSV LATs facilitate reactivation, extensive expression of viral genes is an obvious result of the process. Consequently, it is important to assess the qualitative and quantitative features of transcription and replication of the viral genome during reactivation. This report describes initial studies concerning the molecular analyses of HSV-1 DNA levels and viral gene expression during in vivo reactivation in the rabbit model, using the defined LAT expression mutants of strain $17syn^+$ previously used in the murine system (3).

Rabbits latently infected with the precisely defined LAT promoter mutation 17ΔPst exhibited significantly reduced frequencies of both epinephrine-induced and spontaneous ocular shedding of virus compared to both wild-type (*wt*) $17syn^+$ and LAT^+ rescuant viruses. This confirms finding and extends previous conclusions that the HSV LATs have a clearly defined role in reactivation in vivo (12).

A number of lytic-phase HSV transcripts could be detected in reactivating ganglia within 8 h after induction; the levels of these transcripts subsequently declined, while in contrast, viral DNA replication was not detectable. These results suggest that virus is produced but does not persist and/or does not initiate appreciable productive infection in the nerve ganglia during the period of reactivation. No significant differences in levels or timing of appearance of productive-cycle transcripts were seen following induction of rabbits latently infected with either LAT^+ or LAT^- virus. Such observations are not consistent with in vivo reactivation models hypothesizing a total restriction of virus gene expression and replication in neurons transitorily overcome by LAT expression or a LAT-encoded product. Some appropriate models for in vivo reactivation in the rabbit eye model and their relationship to the in vitro process studied in murine systems are discussed in relation to these data.

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MATERIALS AND METHODS

Virus. HSV-1 strains 17*syn*$^+$ and McKrae were used along with the 17ΔPst(LAT$^-$) mutant of strain 17*syn*$^+$ and the 17Pr(LAT$^+$) rescuant of this deletion described in the accompanying report (3). The general use of these viruses in latency studies has been described previously (5, 16, 20).

Latent infections and induced reactivation of rabbit trigeminal ganglia. Methods used for establishing and reactivating latent infections in rabbit trigeminal ganglia have been described elsewhere (2, 9, 10, 12, 20). Each unscarified eye was inoculated with 2×10^6 PFU of the McKrae strain or 10^5 PFU of the 17*syn*$^+$ strain of HSV-1 in 25 μl. The virus suspension was gently rubbed into the eye; all eyes exhibited acute HSV keratitis. Beginning 18 days after inoculation, slit lamp examination was continued until no eyes showed evidence of keratitis (usually by 20 days following inoculation). Latently infected ganglia were taken from animals 40 to 120 days following initial infection.

Spontaneous shedding was determined by swabbing eyes daily from 20 to 45 days following inoculation (10, 12), and after 45 days, transcorneal iontophoresis of epinephrine was used to induce ocular shedding (in vivo reactivation). Each eye received iontophoresis of 0.01% epinephrine (0.8 mA for 8 min) once a day for 3 consecutive days. Eye swabs were taken daily for up to 9 consecutive days after the first iontophoresis.

Extraction of DNA and RNA from cells and tissue. DNA was extracted from single rabbit ganglia by digesting tissue with 250 μg of proteinase K (Sigma) in 1% sodium dodecyl sulfate containing buffer comprising 150 mM NaCl, 10 mM Tris, and 25 mM EDTA (pH 7.4) as described previously (12). RNA was isolated by extraction of tissue with guanidinium isothiocyanate, and RNA was purified by centrifugation for 16 h at 36,000 rpm in a Beckman SW41 rotor through a 5.7 M CsCl cushion; pelleted RNA was then extensively digested with RNase-free DNase (4, 18).

PCR amplification of DNA and RNA. Primer sets for PCR amplification are described in the accompanying report (3). We experimentally confirmed that the HSV primers sets used were able to detect transcripts in cells infected with the McKrae strain of HSV-1. Cycling reactions were performed with an M.J. Research Corp. (Cambridge, Mass.) thermal cycler. Each cycle included denaturation at 94°C for 30 s, annealing at 5°C below the melting temperature of the primers for 30 s, and extension for 60 s at 72°C. The final cycle was terminated with a 10-min extension period. Aliquots of 0.2 μCi of [α-^{32}P]dCTP were included in the reaction mixes for the visualization of products by autoradiography. Aliquots of 20% of the amplification products (corresponding to amplification of 1% of the original DNA sample or 0.05% of the RNA sample) were fractionated on 6% polyacrylamide gels (20 by 16 by 0.15 cm) running in Tris-borate-EDTA buffer. Run times were 1.5 to 2 h at 250 V (87 mA). Densitometry was carried out with a Bio-Rad model 620 video densitometer.

Samples of the total DNA extracted from tissue were used directly in a 100-μl reaction mix. With RNA, amplification of oligo(dT)-primed cDNA provided the least background. Samples were subjected to oligo(dT)-primed cDNA synthesis, using reverse transcriptase and buffers supplied with an Invitrogen (San Diego, Calif.) cDNA cycle kit.

In situ hybridization. Rabbits that had been infected with 2×10^5 PFU of either 17Pr(LAT$^+$) or 17ΔPst(LAT$^-$) virus per eye 40 days earlier were sacrificed, and the trigeminal ganglia were removed and snap frozen in liquid nitrogen. The ganglia were then cryosectioned into 6-μm sections and prepared for in situ hybridization as described elsewhere (5, 13, 18). For LAT-specific signals, the sections were hybridized with a 347-bp tritiated KOS(M) DNA probe specific for the LAT intron (corresponding to bases 119628 through 119975 in the long internal repeat of the HSV-1 genome) excised from plasmid ATD19 with *Nae*I and *Kpn*I restriction enzymes. The probe was radiolabeled by random hexamer-primed synthesis, using methods supplied by the primer manufacturer (Boehringer Mannheim).

Reactivating rabbit trigeminal ganglia were probed for U_L19 (VP5) mRNA expression. Rabbits that had been latently infected with the McKrae strain were induced to reactivate by epinephrine iontophoresis. At 8 h postinduction, the trigeminal ganglia were removed and snap frozen in liquid nitrogen. The ganglia were then cryosectioned into 6-μm sections and hybridized with a tritiated DNA probe specific for U_L19 mRNA isolated from cloned fragment *Sal*I-W (bases 36948 to 39704), using the methods described above.

RESULTS

Lack of observable LAT expression in neurons latently infected with 17ΔPst virus. Although we have previously demonstrated that ΔPst mutants do not express LATs in mice (5), we confirmed by in situ hybridization that the 17ΔPst-(LAT$^-$) recombinant did not express transcripts during latent infection in rabbit trigeminal ganglia. Animals latently infected as described in Materials and Methods were killed, and trigeminal ganglia were removed. Some were cultivated in vitro to establish that latent virus was present; others were processed for detection of LATs by in situ hybridization. Of those ganglia cultivated, 9 of 13 infected with 17ΔPst(LAT$^-$) and 5 of 7 infected with 17Pr(LAT$^+$) rescuant produced virus. Results of in situ hybridization demonstrate that while LATs are readily detectable in neuronal nuclei of animals latently infected with the 17Pr(LAT$^+$) rescuant virus (Fig. 1B), they cannot be observed in ganglia harboring the 17ΔPst(LAT$^-$) virus (Fig. 1A).

17ΔPst(LAT$^-$) and 17Pr(LAT$^+$) mutants replicate equivalently in rabbit tissue. As shown in the multistep growth experiment in rabbit skin cells presented in Fig. 2, there was no difference in replication efficiency following low-multiplicity infection with either *wt* 17*syn*$^+$ or the 17Pr(LAT$^+$) or 17ΔPst-(LAT$^-$) mutant used in these studies.

Comparison of spontaneous reactivation from latent infections with the 17ΔPst(LAT$^-$) mutant and 17Pr(LAT$^+$) rescuant. We compared spontaneous virus shedding in the eyes of rabbits latently infected with *wt*, 17ΔPst(LAT$^-$), and 17Pr(LAT$^+$) viruses. Rabbits were infected with virus in the eye, and latent infections of the trigeminal ganglia were established following acute infection. During latent infection, virus could be detected in the tear film sporadically between 24 and 45 days following infection; animals harboring the LAT$^-$ recombinant demonstrated a reduced frequency of spontaneous shedding compared with animals infected with either the LAT$^+$ rescuant or *wt* virus (Table 1). Two-tailed chi-square analysis of the data for individual rabbits indicated that the differences in frequency of reactivation between either the *wt* or 17Pr(LAT$^+$) rescuant virus and the 17ΔPst(LAT$^-$) mutant were significant (Table 1). This is in contrast to the situation reported for a more extensive deletion of the LAT promoter region (X10-13) compared with its rescuant (XC-20) (12). We do not know whether this slight difference in observed phenotype indicates a basic difference between the two LAT$^-$ mutants, a difference in the rabbits used, or a combination of both, since both mutants are significantly restricted in their ability to reactivate efficiently when induced (12) (see below).

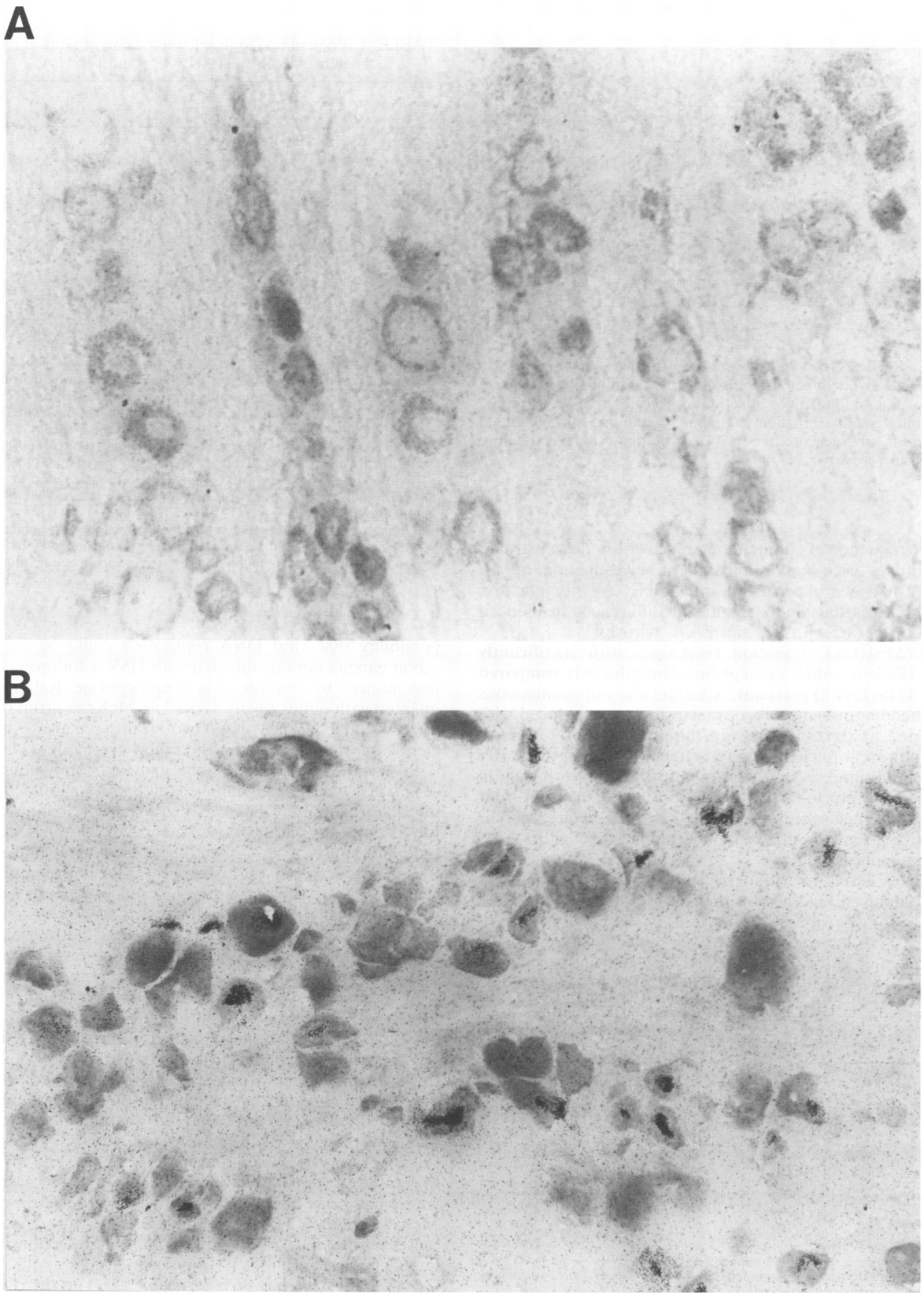

FIG. 1. In situ hybridization with a LAT-specific probe of RNA in rabbit ganglia latently infected with either the 17ΔPst(LAT$^-$) or 17Pr(LAT$^+$) recombinant of HSV-1 with a LAT-specific probe. Rabbits that had been infected with 2×10^5 PFU of either 17ΔPst(LAT$^-$) virus (A) or 17Pr(LAT$^+$) virus (B) per eye 40 days earlier were sacrificed, trigeminal ganglia were snap frozen in liquid nitrogen, and then the ganglia were cryosectioned into 6-μm sections and prepared for in situ hybridization as described in Materials and Methods. LAT-specific signals were assayed by hybridizing the sections with a 347-bp tritiated KOS(M) DNA probe specific for the LAT intron.

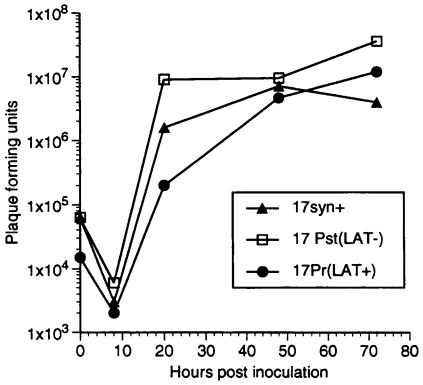

FIG. 2. Recovery of *wt* 17*syn*$^+$, 17ΔPst(LAT$^-$), or 17Pr(LAT$^+$) virus in productively infected rabbit skin cells. Rabbit skin cells were infected with a multiplicity of infection of 0.05 PFU per cell, overlaid with minimal essential medium containing glutamine and 10% fetal bovine serum, and incubated at 38.5°C for the times indicated before assay of virus recovery.

The low frequency of spontaneous reactivation demonstrated by all viruses used makes meaningful investigation of the difference between experiments impossible. At this juncture, we would stress the highly significant differences in induced reactivation between LAT$^+$ and LAT$^-$ viruses.

The 17ΔPst(LAT$^-$) mutant reactivates with significantly lowered efficiency upon epinephrine iontophoresis compared with the 17Pr(LAT$^+$) rescuant. After the assay of spontaneous ocular shedding just described, reactivation was induced with epinephrine at day 45 following initial infection. Virus appeared in the tear film with high frequency beginning 16 h after the first iontophoresis and continuing for 72 to 96 h only in those rabbits infected with LAT$^+$ virus. Data from four experiments are shown in Table 2. Again, chi-square analysis demonstrates that the differences between LAT$^+$ and LAT$^-$ virus are significant.

HSV DNA levels in rabbit trigeminal ganglia during epinephrine-induced reactivation in vivo. We next examined the possibility that viral DNA replication could be detected in rabbit ganglia latently infected with HSV-1 following reactivation induced by iontophoresis of epinephrine. Rabbits latently infected with either the *wt* 17*syn*$^+$ or McKrae strain of HSV-1 were examined, since these two strains reactivate most efficiently and reproducibly in the rabbit eye model (11).

In a typical experiment, rabbit ganglia latently infected with *wt* 17*syn*$^+$ virus were removed at 8, 32, or 56 h following iontophoresis, and DNA was extracted for analysis and subjected to PCR amplification. The specificity and sensitivity of the method used is described in the accompanying report (3). Tissue recovery was normalized by inclusion of a primer set specific for the rabbit actin gene, which generates a 110-bp product (8, 14). This primer set was as follows: sense, 5′-AAGATCTGGCACCACACCTT-3′; and antisense, 5′-CGA ACATGATCTGGGTCATC-3′. These primers are in the equivalent positions as those used for the murine gene in the study described in the accompanying report (3).

A typical experiment is shown in Fig. 3. Relative levels of HSV DNA were measured by densitometric analysis of the levels of viral and cellular DNA as shown in Table 3. Although there was individual variation between samples, no evidence of any consistent significant increase in the levels of DNA compared with the uninduced sample was detected at any time (Fig. 3A). Similar results were obtained with ganglia latently infected with the McKrae strain of HSV-1 (Fig. 3B). In the experiment shown, the intensity of the HSV DNA signal was somewhat greater relative to actin levels at most times for the McKrae infection than for the infection with 17*syn*$^+$ virus; however, other average values for other infections with the latter virus were similar to those seen here for McKrae virus.

LAT expression has no measurable effect on the efficiency of establishment of latent infections in rabbit trigeminal ganglia. Since we have shown that the 17ΔPst(LAT$^-$) mutant of HSV-1 establishes latent infections in murine trigeminal ganglia with measurably reduced efficiency (3), we used PCR to measure viral DNA recovery from trigeminal ganglia latently

TABLE 1. Spontaneous reactivation of virus in rabbit eyes inoculated with 17ΔPst(LAT$^-$) and 17Pr(LAT$^+$)[a]

Virus	Expt no., no. of rabbits[b]	No. of rabbits positive/total	No. of eyes positive/total
17ΔPst(LAT$^-$)	1, 9	1/9	1/18
	2, 10	0/10	0/20
	3, 22	4/22	5/44
		5/41[c]	**6/82**
17Pr(LAT$^+$)	1, 8	3/8	4/16
	3, 20	15/20	22/39[d]
		18/28	**26/55**
wt 17*syn*$^+$	1, 12	9/12	13/24
	2, 10	3/10	3/20
		12/22	**16/44**

[a] *P* values determined by two-tailed chi-square analysis were as follows: 17ΔPst(LAT$^-$) versus *wt* 17*syn*$^+$, 0.005 (rabbits) and 0.001 (eyes); 17Pr(LAT$^+$) versus *wt* 17*syn*$^+$, 0.8173 (rabbits) and 0.5773 (eyes); and 17ΔPst(LAT$^-$) versus 17Pr(LAT$^+$), 0.0011 (rabbits) and 0.00001 (eyes).

[b] Animals in the same experiment were inoculated at the same time with the two viruses. Details are given in the text.

[c] Values in boldface are totals.

[d] One rabbit had a damaged eye which was not swabbed or iontophoresed.

TABLE 2. Epinephrine-induced reactivation of virus in rabbit eyes inoculated with 17ΔPst(LAT$^-$) and 17Pr(LAT$^+$)[a]

Virus	Expt no., no. of rabbits	No. of rabbits positive/total	No. of eyes positive/total
17ΔPst(LAT$^-$)	1,[b] 9	1/9	1/15[c]
	2, 9	1/9	1/17[c]
	3, 22[d]	0/12	0/24
	4, 7	3/7	4/14
		5/37[e]	**6/70**
17Pr(LAT$^+$)	1, 8	8/8	12/15[c]
	3, 20[d]	10/12	14/23[c]
		18/20	**26/38**
wt 17*syn*$^+$	1, 7	7/7	13/14
	2, 8	4/8	6/16
	4, 4	4/4	8/8
		15/19	**27/38**

[a] Animals are the ones scored for spontaneous reactivation in Table 1; those in the same experiment were inoculated at the same time with the two viruses. Details are given in the text. *P* values determined by two-tailed chi-square analysis were as follows: 17ΔPst(LAT$^-$) versus *wt* 17*syn*$^+$, 0.0019 (rabbits) and 0.00001 (eyes); 17Pr(LAT$^+$) versus *wt* 17*syn*$^+$, 0.8165 (rabbits) and 1.000 (eyes); and 17ΔPst(LAT$^-$) versus 17Pr(LAT$^+$), 0.0006 (rabbits) and 0.00001 (eyes).

[b] Iontophoresis was once a day for 3 consecutive days starting at 45 days postinfection. Eye swabs were taken daily for 7 to 9 days following the first iontophoresis.

[c] One rabbit had a damaged eye which was not swabbed or iontophoresed.

[d] Rabbits were sacrificed at various times after induction; only those swabbed at 16 h or longer following iontophoresis were included. See text for details.

[e] Values in boldface are totals.

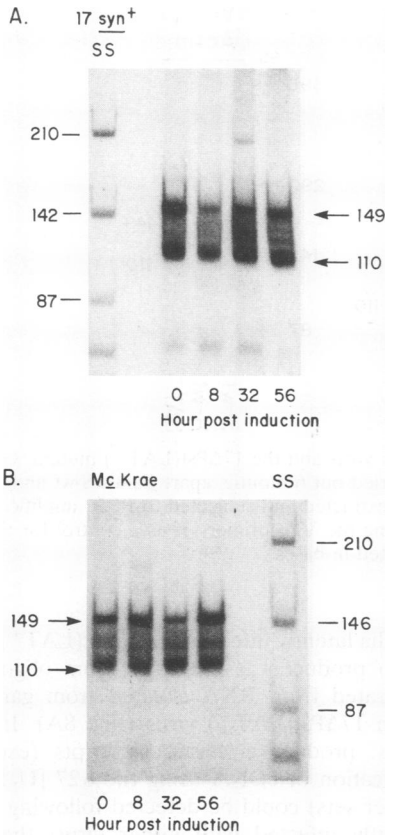

FIG. 3. DNA recovery from epinephrine-induced latently infected rabbit ganglia. DNA was extracted and subjected to PCR amplification with VP5 (U_L19) and actin primer sets in the same reaction. (A) Rabbits latently infected with *wt* 17*syn*$^+$ virus were subjected to iontophoreses, and ganglia were recovered at the times shown. (B) The same experiment was carried out with rabbits latently infected with the McKrae strain of HSV-1. Exposure was overnight without intensifying screens. SS, size standards. Sizes are indicated in bases.

infected with equivalent amounts of either LAT$^+$ or LAT$^-$ virus. In a number of experiments carried out over an extended period, no statistically significant difference in levels of HSV DNA present in latently infected ganglia were observed, although the data cannot exclude a slight reduction in viral DNA recovered from ganglia latently infected with LAT$^-$ virus. Examples of data obtained by using primer sets specific for the HSV α27 and VP5 genes with different groups of rabbits infected with equivalent amounts of viruses at different times are shown in Fig. 4. Using densitometry, we determined the ratios of the signal intensity for the VP5 versus the actin PCR primer products generated in the same samples for the rabbits shown in Fig. 4 and from a number of additional ganglia. The ratios were as follows: ΔPst(LAT$^-$) (n = 5), 1.25 ± 0.11; *wt* (n = 3), 1.33 ± 0.15; and 17Pr(LAT$^+$) rescuant (n = 3), 1.27 ± 0.2.

TABLE 3. Normalized recovery of HSV DNA from epinephrine-induced rabbit trigeminal ganglia infected with two different strains of HSV

Virus (*wt*)	Time (h) after induction	Relative DNA signal intensity[a]		Ratio HSV/actin[b]
		HSV	Actin	
17 *syn*$^+$	0	1.19	1.16	1.03
	8	0.65	0.80	0.81
	32	1.15	1.10	1.05
	56	1.13	1.07	1.06
McKrae	0	1.35	1.04	1.30
	8	1.46	1.06	1.38
	32	1.04	1.24	0.83
	56	1.59	1.17	1.36

[a] Data from coamplification with VP5 and actin primer sets in the same sample. Experiment is shown in Fig. 3. Values are arbitrary units.
[b] Ratios varied ±10% in different PCR amplifications of duplicate samples.

A carefully controlled comparison of the amounts of viral DNA recoverable during reactivation from ganglia latently infected with either the 17ΔPst(LAT$^-$) or 17Pr(LAT$^+$) recombinant virus was also carried out. Rabbits were part of those of experiment 3 shown in Tables 1 and 2; latent infections with LAT$^-$ virus showed the expected phenotype: no LAT$^-$ virus-infected rabbits shed virus upon epinephrine induction; on the other hand, all three rabbits infected with the LAT$^+$ rescuant virus shed virus following induction for 48, 72, and 216 h (five of six eyes). As shown in Fig. 5 and Table 4, within the range of experimental variation seen between individual rabbits in the experiments, no consistent difference between the relative amounts of HSV DNA recovered was seen between infections with the LAT$^-$ and LAT$^+$ mutants. While the specific data in Fig. 5 and Table 4 are consistent with the possibility that a small transitory increase in the relative amount of viral DNA occurred at 48 and 72 h after induction, other experiments such as those shown in Fig. 3 and Table 3 do not show such an increase. Finally, the reduced recovery of DNA from ganglia harboring latent LAT$^-$ virus at the zero time point in this experiment was not seen in other experiments.

Analysis of HSV transcription following epinephrine-induced reactivation. We also investigated transcription during in vivo reactivation by analyzing RNA from latently infected rabbit trigeminal ganglia isolated at various times following epinephrine iontophoresis. We have described both the methods and the controls for use of PCR for detection of small amounts of productive-cycle transcripts in the accompanying report (3); in rabbit ganglia, the best ratio of signal to background was consistently found with use of oligo(dT)-primed cDNA. Iontophoresis of latently infected rabbits resulted in the expression of low levels of productive-cycle transcripts immediately after the procedure. The maximum signal intensity was normally around 8 h following induction and declined thereafter. Ganglia latently infected with either the McKrae or 17*syn*$^+$ strain of HSV-1 produced qualitatively similar results, but the signal strength was stronger following reactivation of the McKrae strain.

Examples of the data obtained are shown in Fig. 6. PCR amplification products generated from transcripts encoding the α-U_L54 (α27), β-U_L39 (ribonucleotide reductase, large subunit), and βγ-U_L19 (major capsid protein, VP5) genes could be detected in RNA isolated from ganglia harvested 4 and 8 h following iontophoresis, but less or none could be detected at 16 h following iontophoresis. RNA isolated from latently infected or epinephrine-induced ganglia produced readily detectable signals with the LAT-3′ primer set at all times measured. Finally, as with the in vitro-reactivated murine ganglia, no signal was detected in latently infected ganglia cDNA probed with the LAT-exon primer set, but somewhat surprisingly, a weak signal could be detected in cDNA produced from RNA isolated from ganglia harvested 16 h after iontophoresis of epinephrine. Although these LAT primer sets could detect products of low-abundance late-productive-cycle

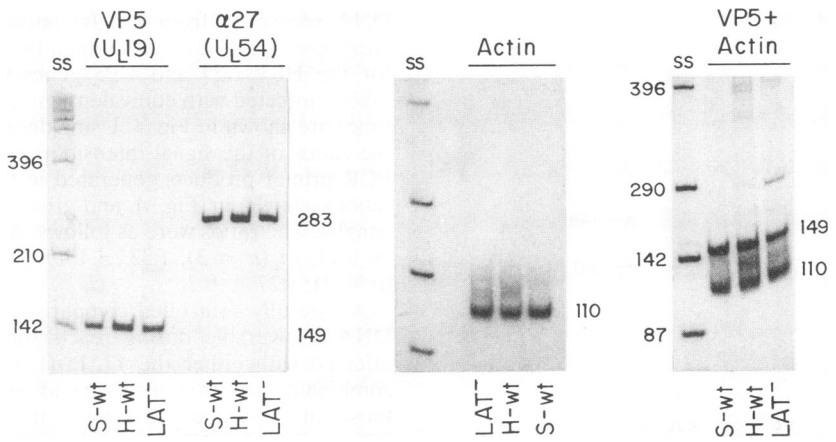

FIG. 4. HSV-1 DNA recovery from rabbit trigeminal ganglia latently infected with *wt* virus and the 17ΔPst(LAT$^-$) mutant. Ganglia latently infected with *wt* 17*syn*$^+$ were removed from rabbits latently infected in experiments carried out 6 months apart (lanes S-wt and H-wt) or from ganglia latently infected with 17ΔPst(LAT$^-$) virus in a different experiment. DNA was extracted and subjected to PCR amplification using the primers indicated. The rabbit actin primer set was used alone and in a sample containing the VP5 primer set as a control for tissue recovery. Exposure was overnight without intensifying screens. SS, size standards. Sizes are indicated in bases.

transcripts coterminal with the 3′ end of LAT (1, 21), data shown below indicate that such transcripts cannot be detected during the in vivo reactivation process; therefore, we can be confident that the signals detected are, indeed, due to LAT expression.

In similar experiments using in situ hybridization, we detected VP5 (U_L19) transcripts in McKrae strain-infected ganglia between 4 and 16 h after iontophoresis (Fig. 7). These positive neurons were relatively rare and represented 1 to 10% of those expressing LAT. No signals could be detected in reactivation of ganglia latently infected with *wt* 17*syn*$^+$ virus, nor could they be detected in uninduced tissue (data not shown).

We next compared transcription in reactivating ganglia which had been latently infected with 17ΔPst(LAT$^-$) or 17Pr(LAT$^+$) virus. As in the DNA recovery experiment represented in Fig. 4, rabbits from experiment 3 of Tables 1 and 2 were used. Ganglia were isolated at 4, 8, 16, and 24 h following induction. Equivalent amounts of the 160-base amplified product obtained by using the LAT-3′ primer set were obtained with all ganglia latently infected with 17Pr(LAT$^+$) virus, but as expected, no product was produced from oligo(dT)-primed cDNA generated from RNA isolated from ganglia latently infected with 17ΔPst(LAT$^-$) virus (Fig. 8A). In contrast to these results, productive-phase transcripts (exemplified by PCR amplification of cDNA using the α27 [U_L54] and VP5 [U_L19] primer sets) could be detected following induction of rabbits latently infected with either virus; the kinetics of expression were essentially the same as observed with induced reactivation of latent infections with *wt* virus.

Although the experimental approach used was not designed to quantitate levels of RNA detected, we did use the actin primer set to amplify cDNA from the tissue samples shown in Fig. 8A. As shown in Fig. 8B, the actin signals are essentially equivalent in all samples, in contrast to the situation with the LAT signals detected with the LAT-3′ primer set. As noted, above, this type of result precludes the expression of some low-abundance lytic-phase transcript interfering with our detection of LATs.

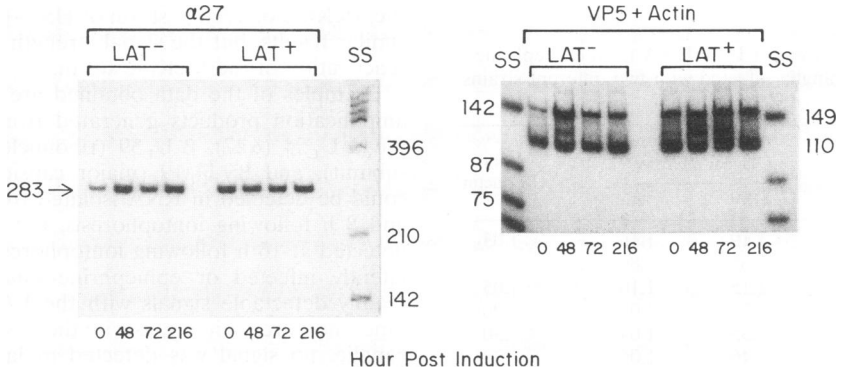

FIG. 5. HSV-1 DNA recovery in reactivating rabbit trigeminal ganglia latently infected with the 17ΔPst(LAT$^-$) or 17Pr(LAT$^+$) recombinant of HSV-1. Rabbits used were part of experiment 3 of Tables 1 and 2. Ganglia were isolated at the times shown following iontophoresis of epinephrine, and DNA was extracted and subjected to PCR amplification with the primers indicated. The actin primer set was included in samples containing the VP5 primer set as a control for tissue recovery. Exposure was overnight without intensifying screens. SS, size standards. Sizes are indicated in bases.

TABLE 4. Relative amounts of 17ΔPst(LAT$^-$) and 17Pr(LAT$^+$) DNAs recovered from latently infected and epinephrine-induced rabbit trigeminal ganglia

Virus	Time (h) after induction	Relative DNA signal intensity[a]		Ratio, HSV/actin
		HSV	Actin	
17Pst(LAT$^-$)	0	0.38	0.54	0.68
	48	1.01	0.73	1.38
	72	0.52	0.57	0.91
	216	0.55	0.67	0.82
17Pr(LAT$^+$)	0	0.65	0.74	0.88
	48	0.77	0.65	1.18
	72	0.87	0.7	1.24
	216	0.84	0.83	1.01

[a] Data from coamplification with VP5 and actin primer sets in the same sample. Experiment is shown in Fig. 5. Values are arbitrary units.

DISCUSSION

Data from use of a well-defined deletion in the LAT promoter presented here confirm our earlier conclusions that LAT plays a major role in facilitating epinephrine-induced reactivation of the virus in the rabbit eye model (12). Also as observed before, there is a level of spontaneous reactivation of LAT$^-$ viruses that occurs in the absence of induction in this model. However, this low-frequency phenomenon is sporadic, making detailed analysis impossible. Consequently, we have concentrated on the induced reactivation, for which LAT expression provides a genetic phenotype.

The results of our analysis of reactivation following epinephrine-induced reactivation of latently infected rabbit ganglia in vivo demonstrate both similarities with and significant differences from the results of in vitro reactivation in murine models. The early productive-cycle transcription of the viral genome following epinephrine induction of latently infected rabbits mirrors the situation in the in vitro reactivation model, yet in contrast to the murine experiments, this wave of transcription disappears (Fig. 6 and 8). The fact that no reproducible net increase in relative levels of viral DNA is seen in the reactivating ganglia in vivo (Fig. 3 and 5) suggests that only a small proportion of stimulated neurons go on to replicate viral genomes and that any infectious virus produced in the ganglia is transported to a peripheral site and reinitiates infection very efficiently. This finding is in agreement with previous studies which have been able to demonstrate little, if any, infectious virus produced by reactivating ganglion tissue (9). Virus-specific host immune responses, possibly combined with other factors, are likely to be responsible for this result.

As expected, we were readily able to detect LAT expression in ganglia during latent and reactivating infection; this result is the same as that seen in murine explant reactivation. The fact that the LAT promoter deletion mutant did not express any measurable LAT signal at any time following induction confirms the fact that the primer sets used are specific for latent-phase transcripts. Since the PCR signal intensity did not change appreciably during the reactivation period, we conclude that most latently infected cells do not change their ability to express LAT during these processes. We were, however, surprised that we could detect the signal expected from the spliced product of the primary LAT with use of the LAT-exon primer set late in the reactivation process in rabbits, while we could not see it in explanted murine ganglia (3). The weakness of the signal makes direct study of this phenomenon

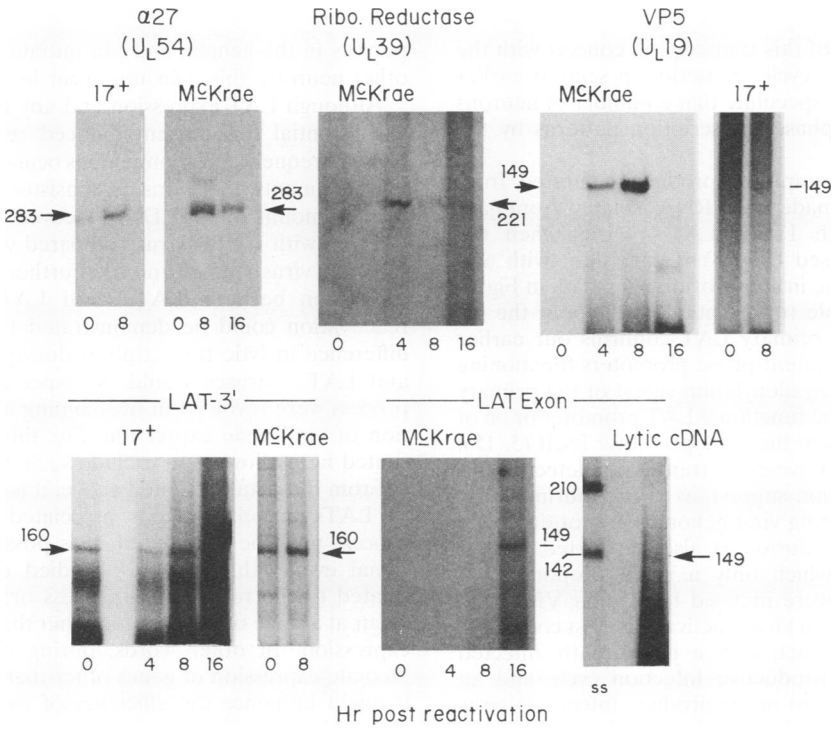

FIG. 6. Detection of HSV-1 transcription by using PCR-amplified cDNA isolated from latently infected and epinephrine-induced reactivated rabbit ganglia. Time of RNA isolation following iontophoresis of epinephrine to induce reactivation is shown. cDNAs generated from RNA extracted from rabbits infected with either the McKrae or 17*syn*$^+$ strain of HSV-1 are shown. Primer sets used for PCR are indicated at the top. Exposure was overnight at −70°C with intensifying screens. Sizes are indicated in bases.

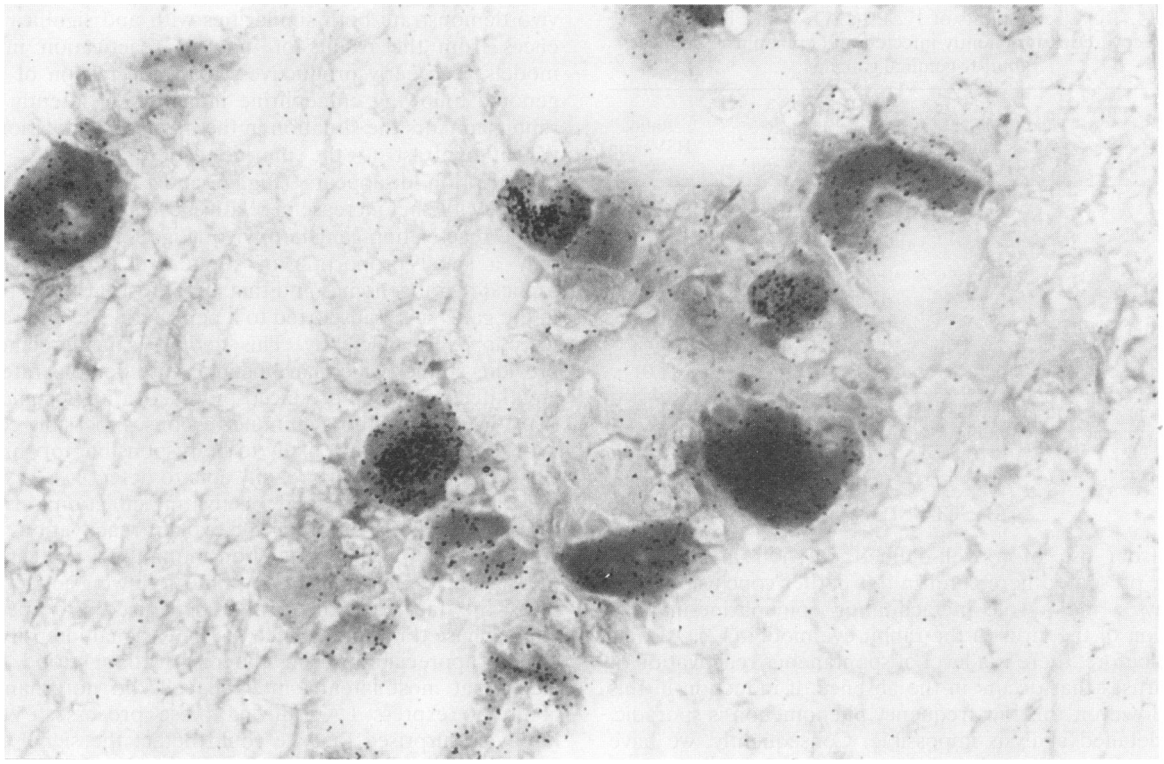

FIG. 7. In situ hybridization of VP5 (U_L19) mRNA-specific probes to RNA from rabbit trigeminal ganglia. Rabbits that had been latently infected with the McKrae strain were induced to reactivate by epinephrine iontophoresis; at 8 h postinduction, the trigeminal ganglia were removed and snap frozen in liquid nitrogen. The ganglia were then cryosectioned into 6-μm sections and hybridized with a tritiated DNA probe specific for U_L19 mRNA (see Materials and Methods). VP5 transcripts were present in three neurons.

difficult, but the presence of this transcript in concert with the limited wave of productive-cycle transcription seen at earlier times makes it tempting to speculate that a number of neurons are reestablishing latent phase transcription patterns by the 16-h time point.

The complete lack of amplified product generated from oligo(dT)-primed cDNA made from RNA isolated from ganglia latently infected with 17ΔPst(LAT^-) virus when the LAT-3′ primer set was used (Fig. 8) is consistent with and extends the sensitivity of the in situ hybridization data in Fig. 1. This absence of measurable transcription throughout the region encompassed by the primary LAT confirms our earlier conclusions that any other latent-phase promoters functioning between the 5′ end and polyadenylation signal of the primary LAT must either require the functional LAT promoter or be of very much lower activity than the LAT promoter itself (5, 15).

The low levels of productive-cycle transcripts detected following epinephrine induction suggest, as in the murine situation, that most cells harboring viral genomes do not enter the productive cycle. This conclusion is also supported by our morphologic studies, in which only a small proportion of neurons expressing LAT were induced to express VP5 transcripts (Fig. 7). A course of in vivo reactivation most consistent with the data is one in which only a few latently infected neurons quickly begin a productive infection cycle and an unknown fraction of these go on to produce infectious virus which is cleared from the ganglia. This course of reactivation is consistent with the murine in vitro models examined previously (3), but as noted above, it diverges in that in the latter situation, some virus produced during these very early events persists in the ganglia and can initiate productive infection in other neurons; this does not occur in rabbits in vivo.

Although LAT expression and any functions encoded by it are essential for efficient induced reactivation and for the highest frequency of spontaneous ocular shedding, in a number of experiments, there was no consistent measurable difference in the amount of viral DNA recovered from ganglia latently infected with LAT^- virus compared with either *wt* or LAT^+ rescuant virus (Fig. 4 and 5). Further, no difference in gene expression between LAT^- and LAT^+ viruses in induced reactivation could be demonstrated (Fig. 8A). A significant difference in lytic transcription during reactivation of LAT^+ and LAT^- viruses would be expected if a LAT-mediated process were involved in overcoming a total neuronal restriction of viral gene expression. For this reason, the data presented here allow us to exclude such models.

From the data presented above, it is clear that the influence of LATs in other events associated with the reactivation process must be considered. It is possible that the transcriptional events that we have studied either are not directly related to the reactivation process or, as seems more likely, occur at a stage of reactivation other than that sensitive to LAT expression. In other words, during reactivation, LAT may mediate expression of genes other than those studied here, or it might influence the efficiency of virus replication in other tissue. For example, it could be posited that there is a restriction of LAT^- virus replication in a critical subpopulation of sensory neurons or in peripheral tissue in which reactivated virus first replicates. In the latter case, sensitivity to

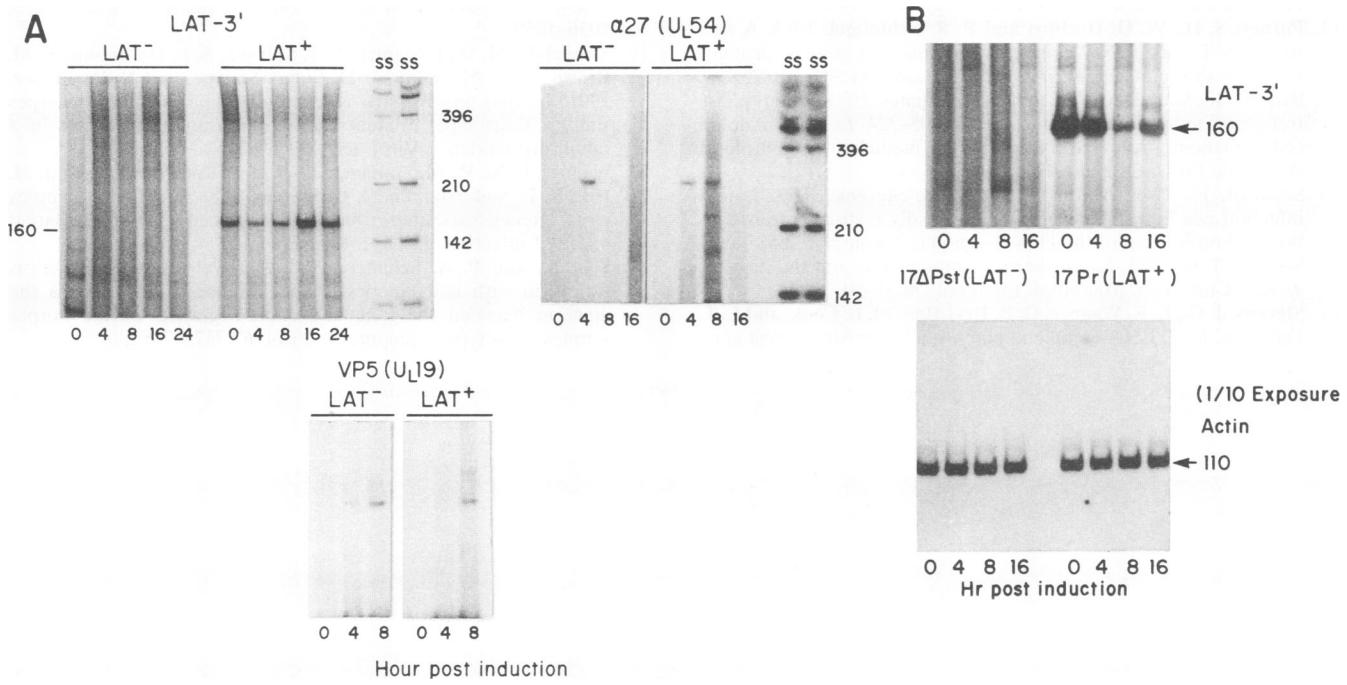

FIG. 8. HSV-1 transcription in reactivating rabbit trigeminal ganglia latently infected with the 17ΔPst(LAT⁻) or 17Pr(LAT⁺) recombinant of HSV-1. (A) Rabbits used were part of experiment 3 of Tables 1 and 2; ganglia were isolated at the times shown following iontophoresis of epinephrine, and RNA was extracted. RNA was used to generate oligo(dT)-primed cDNA, which was subjected to PCR amplification with the primers indicated. Exposure was overnight at −70°C with intensifying screens. (B) Identical samples were amplified with LAT-3′ and actin primers in parallel experiments to confirm the equivalence of tissue recovery in all samples. SS, size standards. Sizes are indicated in bases.

LAT expression could be related to the low multiplicity of virus infection initially involved in the reactivation process.

Whatever the explanation may be, it is clear from our data that the LATs do play a significant role in the reactivation process in the rabbit model. In addition, we have shown that viral gene expression occurs at early time intervals in sensory neurons of rabbits induced to reactivate the virus. The methods and approaches outlined here should be directly applicable to other in vivo models as well.

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