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# Herpes Simplex Virus Genome Replication and Transcription during Induced Reactivation in the Rabbit Eye

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**PCR analysis of herpes simplex virus (HSV) genome replication and productive-cycle transcription was used to examine the role of the cornea in the latency-associated transcript (LAT)-mediated reactivation of HSV type 1 (HSV-1) in the rabbit eye model. The reduced relative reactivation frequency of 17ΔPst (a LAT$^-$ virus) compared to those of wild-type and LAT$^+$ rescuants correlated with reduced levels of viral DNA and transcription in the cornea following epinephrine induction. The timing of virus appearance in the cornea was most consistent with tissue peripheral to the cornea itself mediating a LAT-sensitive step in the reactivation process. Specific results include the following. (i) While viral DNA was found in the corneas of rabbits latently infected with either the LAT$^+$ or LAT$^-$ virus prior to and during the first 16 to 24 h following induction, more was found in animals infected with the LAT$^+$ virus. (ii) A significant increase in levels of viral DNA occurred 20 to 168 h following induction. (iii) The average relative amount of viral DNA was lower at all time points following reactivation of animals infected with the LAT$^-$ virus. (iv) Expression of productive-cycle transcripts could be detected in corneas of some rabbits latently infected with either the LAT$^+$ or LAT$^-$ virus, and the amount recovered and the timing of appearance differed during the reactivation of rabbits latently infected with the LAT$^+$ or LAT$^-$ virus. (v) Despite the reduced recoveries of LAT$^-$ virus DNA and productive-cycle transcripts in reactivating corneas in vivo compared to those of their LAT$^+$ counterparts, such differences were not detected in cultured keratinocytes or in experiments in which relatively high titers of virus were superinfected into the eyes of latently infected rabbits. (vi) A number of LAT$^+$-virus-infected rabbits expressed LAT in corneas isolated from uninduced rabbits. When seen, its amount was significantly higher than that of a productive-cycle (VP5) transcript.**

Latent infection is a hallmark of herpes simplex virus (HSV) infection. Such latent infection and the restricted gene expression characteristic of it have recently been extensively reviewed (25, 34). As documented in these reviews, in humans and in a number of animal models, latent infection is characterized by the presence of viral genomes in neurons of sensory nerve ganglia enervating the sites of initial (primary) viral infection in the epithelium. In neurons during the latent phase, the majority of viral genomes are transcriptionally quiescent while about 10 to 30% serve as the template for the expression of a single transcription unit, the latency-associated transcript (LAT). LAT is encoded within a 9-kb region situated in the long repeat region of the viral genome.

Reactivation from latent infection is sporadic in humans, as well as in guinea pig and rabbit models. Such reactivation is characterized by the ability to recover infectious virus at the site of initial infection with no obvious evidence of pathology in the sensory nerve ganglion innervating that site. Reactivation is triggered by stress and, in the rabbit model, can also be chemically induced by iontophoresis of adrenergic compounds such as epinephrine (1, 11, 12, 14, 17, 18).

In the rabbit, the process of reactivation is facilitated by LAT (2, 15, 24, 33), but the precise mechanistic role of LAT in reactivation is unknown. What is clear is that only a very limited portion (<500 bp) of the 9-kb primary transcription unit is involved in the reactivation phenotype; this critical region maps to the extreme 5′ end of the transcription unit. It is also clear that LAT's mode of action does not involve expression of a LAT-encoded protein during the latent phase of infection; indeed, LAT may well have a *cis*-acting role in reactivation (3, 4, 13). What is not clear is whether LAT expression itself or the presence of critical DNA elements encoding LAT is important in reactivation.

Whatever the role of LAT in reactivation, there is no compelling reason to posit that this role is manifest solely in the trigeminal ganglion—at least in the rabbit model. Although available data, including the time delay between the induction of reactivation with epinephrine and the ability to recover infectious virions from the tear film of the eye, are consistent with a LAT-sensitive step in replication occurring in neuronal or nonneuronal cells in latently infected sensory nerve ganglion, they are equally consistent with the LAT-sensitive step occurring in tissue of or adjacent to the cornea itself.

Indeed, the fact that viral genomes can be detected in corneal tissue in the absence of any evidence of active reactivation, as determined by detection of infectious virions in the tear film, allows the possibility that the source of such virus in reactivating rabbits is not the trigeminal ganglion but rather the cornea itself or tissue associated with it (5, 6, 23). Virions in corneas have some properties seen in latently infected neurons; LAT can be detected and virus can be recovered by explant cocultivation (7), and, as shown here, the ratio of LAT to the productive-cycle VP5 transcript is similar to that seen in latently infected neuronal ganglia. Practically, the ability to isolate virions from human corneas is a very important factor

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in assessing risks associated with corneal transplants and traumatic eye surgery (10, 16, 22).

We have described sensitive, semiquantitative PCR techniques for the study of viral genome replication and productive-cycle viral gene transcription during induced reactivation in the rabbit eye and other animal models (2, 8). In rabbits, transcripts diagnostic of productive-cycle infection can be detected in the trigeminal ganglion within 4 to 8 h following epinephrine iontophoresis, but there is no evidence of an increase in the viral DNA copy number in such tissue. Further, there is no difference between the $LAT^+$ and $LAT^-$ viruses in the level or timing of viral gene expression or the relative amount of viral DNA in trigeminal ganglia of animals induced to reactivate following latent infections. This led us to suggest that the LAT-sensitive step in the reactivation process occurs in one or a few cells peripheral to the neuron (2).

All the observations discussed above lead to a number of questions, subject to experimental analysis, concerning the process of latency and reactivation in the rabbit model, including the following: what tissue peripheral to the neuron mediates the LAT-sensitive step leading to the recovery of infectious virus; i.e., what is the direct source of the infectious virus found in the tear film that designates a reactivation event? This report describes the results of an extensive PCR-based analysis of HSV genome levels and transcription in the corneas of rabbits induced to reactivate by iontophoresis of epinephrine. We concluded that the reduced amount of the $LAT^-$ virus in the cornea compared to the level of the $LAT^+$ virus is responsible for the difference in the relative reactivation frequencies observed for the two viral genotypes.

While our data suggest that the cornea could serve as a reservoir for reactivating virus, restriction in replication of the $LAT^-$ virus was not observed in several control experiments. Overall, our results are more consistent with a tissue peripheral to the cornea itself mediating a LAT-sensitive step in HSV replication and serving as the direct source of virions replicating in the cornea and being shed into the tear film.

## MATERIALS AND METHODS

**Virus.** Three HSV type 1 (HSV-1) genotypes described previously were used in these experiments: the wild-type strain 17*syn*$^+$; the $LAT^-$ mutant 17ΔPst, in which a 203-bp portion of the LAT promoter was deleted; and the $LAT^+$ rescuant of this mutant, 17Pr (2, 8, 29).

**Cultured cells.** Virus was propagated on rabbit skin cells and titered on Vero cells grown in minimal essential medium supplemented with 5% fetal calf serum and antibiotics. Virus replication was also assayed on cultured rabbit corneal cells maintained as described above but with 10% fetal calf serum (37).

**Latent infections and induced reactivation.** Unscarified rabbit eyes were inoculated with 25-μl aliquots of $10^5$ PFU of virus. Latently infected trigeminal ganglia and corneas were recovered from rabbits no earlier than 40 days following initial infection. For induced reactivation, each eye received a single course of iontophoresis of 0.01% epinephrine (0.8 mA for 8 min). Animals were sacrificed and tissue was harvested at various times following this procedure. Eye swab specimens were collected daily after iontophoresis and assayed for recovery of infectious HSV. These methods are identical to those described in various previous reports (2, 11, 12, 14, 15).

**Extraction of RNA and DNA from cells and tissues.** Corneas and trigeminal ganglia were kept frozen in the vapor phase of a liquid $N_2$ refrigerator until use. The frozen tissue was minced in a sterile plastic petri dish placed on a block of dry ice. The chopped tissue was suspended in 3 ml of guanidinium isothiocyanate (GITC) solution, containing 100 μg of *Escherichia coli* tRNA, warmed at 65°C (9, 32, 35).

Cultured cells were extracted by our standard methods (36). Plates (60-mm diameter, each containing ca. $10^6$ rabbit skin cells) were rinsed twice with saline containing 50 μg of cycloheximide/ml, soaked in 20 mM HEPES (pH 8.0) containing 50 μg of cycloheximide/ml for 2 min, and scraped into fresh HEPES-cycloheximide. The cells were deposited by centrifugation at 3,000 × *g* for 5 min, resuspended in 3 ml of GITC solution containing 100 μg of tRNA, and sheared by passing the suspension through a 21-gauge needle four times.

Tissue was homogenized with an Ultraturrax homogenizer equipped with a metal probe. The probe was thoroughly cleaned with detergent and bleach and rinsed several times with diethylpyrocarbonate-treated water and ethanol between samples. The homogenized suspension was incubated at 65°C for 5 min and then sonicated (15 bursts of 3 s each) with a Branson sonicator at the 50% maximum setting. Following this step, tissue or infected cells were processed in an identical manner.

**RNA extraction.** The homogenized, sonicated material was then phenol-chloroform extracted at 65°C three times. During this extraction, the aqueous material and interphase were kept in the original tube, and after the third extraction, the aqueous phase was transferred to a fresh tube, the organic phase was discarded, and the interphase was saved for DNA extraction. The aqueous phase was extracted a final time with $CHCl_3$ containing 2% isoamyl alcohol and then precipitated with ethanol at −20°C for 18 h. The ethanol precipitate was harvested by centrifugation, resuspended in 500 μl of diethylpyrocarbonate-treated water and 3.2 ml of GITC, and dissolved at 65°C. The dissolved pellets were layered on a 1.2-ml cushion of 5.7 M CsCl in 5-ml Beckman polyallomer tubes and centrifuged in a Beckman SW41 rotor at 36,000 rpm for 16 to 20 h at 20°C. The resultant RNA pellets were suspended in 400 μl of $H_2O$, adjusted to 0.15 M NaCl, and ethanol precipitated overnight at −20°C. This pellet was rinsed in 70% ethanol containing 0.15 M NaCl, air dried, and resuspended in 360 μl of DNase buffer. Any residual DNA was removed by digestion with 5 U of RNase-free DNase (Promega) in the presence of 0.1 M dithiothreitol and 80 to 120 U of RNasin (Promega) at 37°C for 30 min. The reaction mixture was extracted twice with phenol-chloroform and twice with 1 volume of chloroform and then was ethanol precipitated overnight at −20°C. Control experiments using PCR and alkali digestion consistently showed that no DNA survived this purification scheme.

**DNA extraction.** DNA was extracted from total tissue or from the aqueous layer-organic layer interphase taken during RNA extraction. For total-tissue extraction, tissue was minced in the same way as described for RNA extraction. The chopped tissue was then suspended in 3 ml of DNA extraction buffer (10 mM Tris [pH 7.5], 25 mM EDTA, 100 mM NaCl), 100 μl of 20% sodium dodecyl sulfate and 100 μl of proteinase K (ca. 20 mg/ml) were added, and the tissue was digested at 48°C overnight. The DNA solution was extracted three times with phenol-chloroform and once more with chloroform, and the DNA was then precipitated with ethanol overnight, deposited by centrifugation, and, finally, dissolved in 200 μl of water.

DNA was extracted from the aqueous layer-organic layer interphase of RNA extraction solutions as follows. After three phenol-chloroform extractions, the interphase was precipitated with ethanol, DNA was deposited by centrifugation, and the pellet was suspended in 3 ml of DNA extraction buffer and treated for 10 min with 30 μg of RNase and then for 1 to 2 h with 100 μl (19 μg/μl) of proteinase K (until the pellet was dissolved). The DNA solution was then extracted with phenol-chloroform, precipitated with ethanol, and dissolved as described above.

**Preparation of cDNA.** The DNA-free RNA was dissolved in 60 μl of water by heating it at 65°C for 10 min and then placed on ice for 1 min. Half of each sample (30 μl) was then reverse transcribed by using Pharmacia's Ready To Go You-Prime First-Strand beads and commercial random-hexamer primers (Boehringer Mannheim) at 37°C for 1 h. Following cDNA synthesis, the reaction mixture was diluted to 100 μl with water, and 10% was used for a single PCR reaction.

**PCR analysis.** PCR amplification of DNA and cDNA samples was accomplished by methods described previously (2, 8). The following primer sets were used (sense strand/antisense strand): (i) 3′-LAT (160-bp product), 5′-GGT GAAACCAACAGAGCACGGC-3′/5′-CCGGGGTACGTCTGGAGGAGCG 3′;(ii) α-27 (283-bp product), 5′-TTTCTCCAGTGCTACCTGAAGG-3′/5′-TCAACTCGCAGACACGACTCG-3′; VP5 (149-bp product), 5′-TGAAC CCCAGCCCCAGAAACC-3′/5′-CGAGTAAACCATGTTAAGGACC-3′; and (iv) rabbit actin (110-bp product), 5′-AAGATCTGGCACCACACCTT-3′/5′-CGAACATGATCTGGGTCATC-3′.

Reactions were carried out in an M. J. Research thermal cycler as follows: denaturation, 94°C for 30 s; annealing, 5°C below the actin primer melting temperature for 30 s; and extension, 60 s at 72°C. When the actin and VP5 primers were used together, annealing was carried out at 5°C below the actin primer melting temperature. The final cycle was terminated with a 10-min extension step. Products were made radioactive for autoradiography and image quantitation by addition of 0.2 μCi of [α-$^{32}$P]dCTP. Generally, 20% aliquots of the amplified products (corresponding to 1 to 2% of the original material) were fractionated on 6% polyacrylamide gels in Tris-borate-EDTA.

The PCR signals were visualized either by scanning a PhosphorImager (Molecular Dynamics) image by use of Phosphorimager software (Molecular Dynamics) or by scanning an appropriately exposed autoradiogram by use of a Deskscan II scanner (Hewlett-Packard). The signals were quantified by densitometry of the PhosphorImager signals or the scanned autoradiogram signals by using IP Lab Gel software (Signal Analysis Corporation) in accordance with operational instructions.

## RESULTS

**Quantitative aspects.** We reported a number of control experiments in order to establish the limits of detection of the PCR methods used in our continuing studies (2, 8). Our stan-

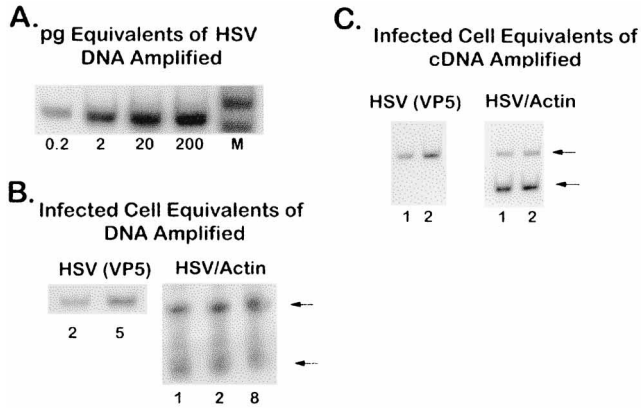


FIG. 1. PCR amplification of DNA and RNA standards. (A) As outlined in reference 8, increasing amounts of cloned HSV fragment *Bam*HI M (0.222 to 0.258 mu) were amplified with the VP5 primer set listed in Materials and Methods. The picogram equivalents of viral DNA were calculated from the ratio of fragment size (5.3 kbp) to genome size (150 kbp). The lane marked M contains 151- and 140-base marker fragments from a *Hin*f1 digest of ϕX174 DNA (Promega) as size standards. (B) Amplification of total DNA isolated from cultures of $2 \times 10^6$ rabbit skin cells infected with 50, 100, or 400 PFU of wild-type HSV-1 strain 17*syn*$^+$. As described in Materials and Methods, 10% of the total isolated DNA was subjected to 30 cycles of PCR amplification with either the VP5 primer set alone or the VP5 and actin primer sets in combination, and 10% of the amplified products were fractionated on our standard acrylamide gels. (C) Amplification of random-hexamer-primed cDNA generated from total RNA isolated from cultures infected with 50 or 100 PFU of virus. Details are as described for panel B. Arrows indicate expected size of PCR product.

dard approach of a 30-cycle amplification suffices to detect 0.02 pg of HSV DNA (>1,000 genomes) when the VP5 primer set is used, which provides the most reliable high-level sensitivity. Further, as shown in Fig. 1A, the signal intensity generated from small amounts of viral DNA has a linear dose dependence over at least a 100-fold variation in concentration (from 0.2 to 20 pg).

In order to establish the limits of detection of viral genomes and transcripts in reactivating tissue, we did a number of reconstruction experiments involving mixing of uninfected rabbit corneas with limited numbers of rabbit keratinocytes infected with 10 PFU of HSV and harvesting them 10 h later. Total DNA and RNA were then isolated from these mixes; DNA was directly amplified, and RNA was used to generate random-hexamer-primed cDNA which was also amplified. Representative data are shown in Fig. 1B and C. With the VP5 primer set, a clear signal was obtained by amplification and gel fractionation of the PCR products produced from viral DNA and cDNA from as little as one infected cell (1 to 2% of the total sample). A similar sensitivity was seen when the α-27 primer set was used (data not shown).

We also investigated the effect of increasing the number of amplification cycles from 30 to 45 in order to increase sensitivity (data not shown). As has been documented in technical reports (30, 31), the effect on sensitivity of increasing the number of cycles is not linear, and the increase in signal strength obtained following 45 cycles of amplification, compared to 30 cycles, depends on a number of factors, including the exact compositions and sources of the buffers utilized for the reactions and the amount of viral sequences in the starting material. Under our conditions, we found that the increase in signal strength in a given sample varied from less than twofold to greater than fivefold in different experimental groups, but this increase appeared to be consistent among samples in any given group. We utilized increased cycling to maximize our ability to detect viral signals but did not attempt to quantitate the signal strength observed.

TABLE 1. Recovery of HSV DNA from corneas of latently infected rabbits 0 to 16 h following epinephrine induction[a]

| Virus (relevant phenotype) | Rabbit | Time of sacrifice (h postinduction) | Relative level of viral DNA[b] |
|---|---|---|---|
| 17ΔPst (LAT$^-$) | D56 | 0 | 0.05 |
| | D63 | 0 | 0.06 |
| | D64 | 4 | 0.06 |
| | D58 | 8 | <0.01 |
| | D59 | 8 | 0.02 |
| | D60 | 8 | 0.05 |
| | D54 | 12 | 0.09 |
| | D44 | 16 | 0.01 |
| | D45 | 16 | 0.02 |
| 17*syn*$^+$ (LAT$^+$) | D23[c] | 0 | 0.1 |
| | D21[c,d] | 0 | 0.25 |
| | D27[c] | 0 | 0.19 |
| | D7d[e] | 4 | 0.2 |
| | D7s[f] | 4 | 0.25 |
| | D8 | 8 | <0.01 |
| | D3 | 16 | 0.01 |
| 17Pr (LAT$^+$) | D82 | 0 | 0.48 |
| | D88 | 4 | 0.09 |
| | D83 | 8 | 0.01 |
| | D77 | 12 | 0.08 |

[a] Animals were subjected to a single iontophoresis of epinephrine at time 0.
[b] Ratio of HSV DNA band intensity to that of cellular DNA. Data are based on PCR of DNA samples with both VP5 and actin primers (see text and Fig. 4).
[c] Rabbit was not treated with epinephrine.
[d] Virus was isolated from tear film. Tear film was harvested prior to sacrifice and incubated with indicator cells for 8 days. A positive result indicates virus-induced cytopathic effects.
[e] Right cornea.
[f] Left cornea.

**Viral DNA synthesis in rabbit corneas following epinephrine-induced reactivation.** In order to facilitate handling, groups of four to six corneas isolated from rabbits at various times following epinephrine induction were extracted. We measured the relative level of HSV DNA in the corneas harvested from rabbits by methods identical to those reported previously (2). In essence, we generated PCR products via 30-cycle reactions with the VP5 primer set to amplify viral sequences and with the actin primer set to amplify cellular signals. Densitometry was employed to measure the ratio of the signals generated in a sample amplified with both primer sets. DNA levels in 37 corneas thus measured were grouped into two sets, the first for corneas taken during the first 16 h following induction and the second for those taken from 20 to 168 h following the epinephrine treatment. These data are tabulated in Tables 1 and 2, and representative gels are shown in Fig. 2A and B.

The HSV-specific DNA signals observed in uninduced corneas and in induced corneas during the first 16 h following epinephrine treatment were generally low, with an overall average ratio (± standard error of the mean) of viral to cellular band intensity of 0.1 ± 0.03 (Table 1). Within this time group, there was a higher relative amount of viral DNA in corneas from rabbits latently infected with LAT$^+$ virus than in corneas of rabbits infected with the LAT$^-$ mutant (0.15 ± 0.043 versus 0.04 ± 0.01). A one-way analysis of variance suggested that this difference was significant ($P = 0.035$) (21). Despite this, however, there were a number of corneas from the LAT$^+$ group with ratios of viral to cellular DNA as low as the lowest seen

TABLE 2. Recovery of HSV DNA from corneas of latently infected rabbits 20 to 168 h following epinephrine induction[a]

| Virus (relevant phenotype) | Rabbit | Time of sacrifice (h postinduction) | Relative level of viral DNA[b] |
|---|---|---|---|
| 17ΔPst (LAT$^-$) | D61 | 36 | 0.4 |
| | D62 | 36 | 0.4 |
| | D41 | 48 | 0.07 |
| | E65[c] | 48 | 0.8 |
| | E58 | 72 | 0.17 |
| | E59 | 72 | 0.16 |
| | E61 | 168[d] | 0 |
| | E62[c] | 168[d] | 3.5 |
| | E63 | 168[d] | 0.23 |
| 17*syn*$^+$ (LAT$^+$) | D1 | 20 | 0.7 |
| | D84 | 24 | 1 |
| | E52 | 48 | 1.5 |
| | E44[c] | 72 | 0.8 |
| | E45[c] | 72 | 0.8 |
| | E49[c] | 168[d] | 3.4 |
| | E50[c] | 168[d] | 0.18 |

[a] Animals were subjected to a single iontophoresis of epinephrine at time 0.
[b] Ratio of HSV DNA band intensity to that of cellular DNA. Data are based on PCR of DNA samples with both VP5 and actin primers (see text and Fig. 4).
[c] Virus was isolated from tear film. Tear film was harvested prior to sacrifice and incubated with indicator cells for 8 days. A positive result indicates virus-induced cytopathic effects.
[d] The 168-h time point followed three rounds of epinephrine induction at 24-h intervals (see text).

with LAT$^-$ corneas (Table 1, cf. D3, D8, and D83). It was also noted that the level of viral DNA in a cornea from an uninduced rabbit (D21) that had infectious virions in its tear film at the time of sacrifice was higher than the average value (HSV/actin band intensity ratio, 0.25) but was significantly less than that found in one rabbit (D82) sacrificed immediately after induction from which no infectious virions were isolated (HSV/actin band intensity ratio, 0.48).

The value for the ratio of LAT$^-$ HSV signal to actin signal was higher in corneas harvested later than 20 h after iontophoresis than in those isolated during the first 16 h ($0.64 \pm 0.37$; $P = 0.087$ from two-way analysis). In addition, there was a correlation between the recovery of infectious virions in the tear film and high ratios of HSV signal to actin signal strength (Table 2). It should be noted that low values for ratios were observed in some corneas from rabbits latently infected with the LAT$^-$ virus in this time range (HSV/actin band intensity ratios of 0.07, 0.17, 0.16, and $<0.01$ for D41, E58, E59, and E61, respectively). The increase in the relative viral DNA level was somewhat greater in the corneas isolated from rabbits latently infected with the LAT$^+$ virus ($P = 0.005$). Here, the average ratio of viral to cellular DNA was found to be $1.2 \pm 0.4$ compared to the value of $0.14 \pm 0.04$ for the 0- to 16-h time period.

A total of six of the seven corneas from the LAT$^+$ rabbits isolated within the late time window had relative viral DNA levels appreciably higher than the average value seen in the 0- to 16-h window. In contrast, with the LAT$^-$-virus-infected rabbits, only four of nine corneas had similarly high levels. Despite this, although the average value for the ratio of viral to cellular DNA from corneas latently infected with the LAT$^+$ virus was greater than that measured in corneas of rabbits latently infected with the LAT$^-$ mutant, analysis of the variance suggested that the difference was not as significant as the difference in the early time window average values ($P = 0.32$ versus $P = 0.035$).

**Productive-phase transcription and viral DNA replication in corneas of epinephrine-induced rabbits latently infected with the LAT$^+$ virus occur at different frequencies than in LAT$^-$-virus-infected corneas.** We then asked whether the levels of viral DNA observed in the corneas of induced rabbits correlated in any readily observable way with productive-phase viral transcription. RNA was isolated from corneal tissue, and random-hexamer-primed cDNA was synthesized. Typically, PCR products were generated by 30 and 45 cycles of amplification with primer sets specific for the HSV VP5 and α-27 transcripts, the 3′ region of LAT, and/or the rabbit actin transcript. The latter was utilized as a control for recovery and for the cDNA reaction.

A typical experiment is shown in Fig. 3. Rabbit corneas were isolated at 20 and 36 h following epinephrine iontophoresis. In the experimental group, no LAT was detected in the corneas, but a weak positive signal for α-27 was seen in both corneas from the rabbits latently infected with the LAT$^+$ virus (D73 and D79) and a somewhat stronger signal was seen with the VP5 primer set in the cornea isolated 36-h postinduction.

The results of 30 and 45 cycles of amplification with the VP5 and 3′-LAT primer sets with cDNA generated from RNA extracted from 49 corneas isolated between 0 and 168 h following induction are tabulated in Tables 3 and 4, and representative examples of data obtained following 30 cycles of amplification are shown in Fig. 4.

The VP5 transcript could be detected in some corneas isolated from rabbits latently infected with either the LAT$^+$ or LAT$^-$ virus at all times (including the zero time point) following induction, and as viral DNA levels were measured, the numbers of transcript-positive corneas isolated within early

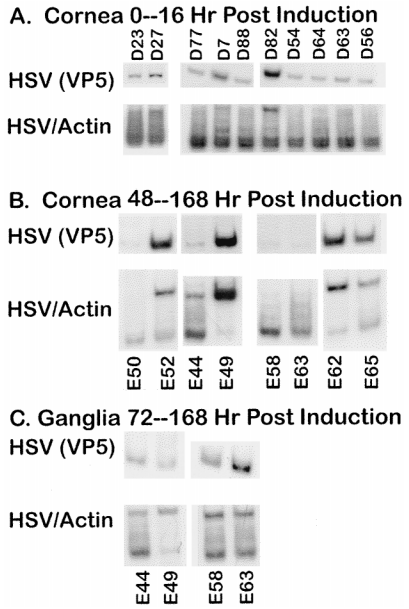


FIG. 2. PCR amplification of total DNA isolated from the corneas and ganglia of epinephrine-induced, latently infected rabbits. Details of DNA extraction and amplification are as described in Materials and Methods and in the legend to Fig. 1B. DNA samples were grouped so that those from rabbits latently infected with the LAT$^+$ virus are on the left and those from rabbits latently infected with the LAT$^-$ virus are on the right. Specific details concerning the time of isolation are summarized in Tables 1 and 2. (A) DNA from corneas of noninduced rabbits or rabbits sacrificed 0 to 16 h following induction with epinephrine. (B) DNA from corneas taken at periods between 48 and 168 h postinduction. (C) DNA from ganglia taken at periods of 72 to 168 h following induction.

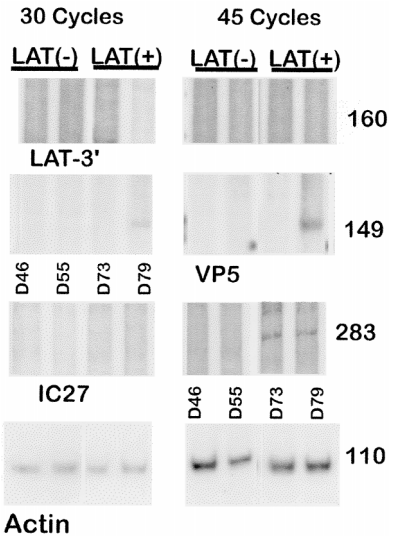


FIG. 3. A typical transcription analysis of RNA isolated from epinephrine-induced rabbit corneas. Corneas were taken from rabbits at 20 (D46 and D73) or 36 (D55 and D79) h following epinephrine induction. Total RNA was isolated, and random-hexamer-primed cDNA was generated. This was amplified with the 3′-LAT VP5, ICP27, or actin primer set for 30 or 45 cycles as described in Materials and Methods, and the products were fractionated by electrophoresis on denaturing acrylamide gels.

time windows following induction (0 to 24 h postinduction) were lower than those seen at later times after induction. Further, there was somewhat more transcriptional activity in corneas from LAT$^+$-virus-infected rabbits than in LAT$^-$ corneas, based on both the number of corneas providing a positive signal and the strength of the signal itself. This difference was manifest at all time intervals examined.

As shown in Tables 3 and 4, based on the ability to detect a positive signal with a 30-cycle PCR amplification of cDNA, 6 of 14 LAT$^+$ corneas isolated within 24 h of induction were positive for VP5 transcripts, compared to 1 of 8 LAT$^-$ ones. Analysis of data obtained using 45 cycles of PCR amplification indicated that some of the differences in the proportion of positive signals for the VP5 transcript seen within this first 24-h period with LAT$^+$ and LAT$^-$ corneas reflected the presence of smaller amounts of transcript in the latter. Thus, while the proportion of positive corneas increased from 6 of 14 to 9 of 14 for LAT$^+$ latent infections, the proportion of corneas increased from 1 in 8 to 4 in 8 for LAT$^-$-virus-infected rabbits.

With 30 cycles of amplification, 8 of 12 LAT$^+$ corneas isolated from 36 to 168 h following induction were positive for the VP5 signal, compared to 8 of 15 LAT$^-$ corneas (Tables 3 and 4). Unlike the situation with corneas isolated in the 0- to 24-h window, there was no difference in the ratios obtained following 45 cycles of amplification. Further suggestive evidence that the signals seen in the later time periods were more intense can be seen in Fig. 4.

**Some corneas from rabbits latently infected with the LAT$^+$ virus express LAT.** In addition to expressing productive-phase transcripts, 5 of 25 corneas from rabbits latently infected with the LAT$^+$ virus expressed significant amounts of LAT as measured by PCR amplification with the 3′-LAT primer pair of cDNA generated from corneal RNA (Table 3 and Fig. 4). Of the 5 LAT-positive corneas, 3 were among the 6 corneas isolated 0 to 4 h following induction and only 2 were among the 19 corneas isolated at later times following induction. Four of the LAT-positive corneas also expressed VP5 mRNA, but in contrast to numerous observations with RNA isolated from productively infected cultured cells, the LAT signal was more intense in all four corneal samples than that obtained with the VP5 primer pair (Fig. 4).

TABLE 3. PCR analysis of LAT$^+$ HSV transcription following epinephrine-induced reactivation in rabbit corneas[a]

| Virus | Rabbit | Time (h) | cDNA detectable with[b]: 30 cycles with primer | | 45 cycles with primer | |
|---|---|---|---|---|---|---|
| | | | VP5 | 3′-LAT | VP5 | 3′-LAT |
| 17*syn*$^+$ | D21[c,d] | 0 | − | − | ± | + |
| 17*syn*$^+$ | D23[c] | 0 | − | ± | ± | ± |
| 17*syn*$^+$ | D27[c] | 0 | − | − | ± | + |
| 17Pr | D82 | 0 | ± | + | + | + |
| 17*syn*$^+$ | D7 | 4 | + | + | + | + |
| 17Pr | D88 | 4 | + | − | + | − |
| 17*syn*$^+$ | D5 | 12 | − | − | − | − |
| 17Pr | D72 | 16 | − | − | − | − |
| 17Pr | D77 | 16 | ± | − | + | − |
| 17*syn*$^+$ | D1 | 20 | ± | − | ± | − |
| 17Pr | D73 | 20 | − | − | − | − |
| 17Pr | D84 | 24 | − | − | − | − |
| 17Pr | D85 | 24 | − | − | ± | − |
| 17Pr | D79[d] | 36 | + | − | + | − |
| 17Pr | D81 | 36 | − | − | + | − |
| 17Pr | D66 | 48 | ± | − | + | − |
| 17Pr | D70 | 48 | ± | − | + | − |
| 17*syn*$^+$ | E46[d] | 48 | − | − | − | − |
| 17*syn*$^+$ | E52 | 48 | + | + | + | + |
| 17*syn*$^+$ | E43[d] | 72 | − | − | − | − |
| 17*syn*$^+$ | E44[d] | 72 | + | − | + | − |
| 17*syn*$^+$ | E45[d] | 72 | + | − | + | − |
| 17*syn*$^+$ | E48[d] | 168 | + | − | + | − |
| 17*syn*$^+$ | E49[d] | 168 | + | − | + | − |
| 17*syn*$^+$ | E50[d] | 168 | − | − | − | − |

[a] Animals were subjected to a single iontophoresis of epinephrine at time 0.
[b] The conditions for amplification and primer sets used are described in Materials and Methods. −, cDNA not detected; +, cDNA detected; ±, barely visible.
[c] Rabbit was not treated with epinephrine.
[d] Virus was isolated from tear film. Tear film was harvested prior to sacrifice and incubated with indicator cells for 8 days. A positive result indicates virus-induced cytopathic effects.

**The effect of LAT expression on patterns of HSV transcription and DNA replication in the trigeminal ganglia of epinephrine-induced rabbits.** We also continued our analysis of the pattern of transcription of VP5 mRNA in the trigeminal ganglia of latently infected rabbits by using PCR amplification. We previously reported that transcription of this mRNA was seen during the first 12 h following epinephrine induction of rabbits latently infected with either the LAT$^+$ or LAT$^-$ virus. The longer times following iontophoresis and the higher sensitivity of the PCR amplification used in the present study demonstrate potential differences in the levels of transcription, however. With one exception (rabbit D40), RNA from ganglia of rabbits latently infected with the LAT$^-$ virus produced a weaker signal, and the signal was not seen as often in ganglia isolated later than 16 h or so following induction. Some representative data are shown in Fig. 5.

Despite the difference in the duration of transcription of VP5 mRNA in ganglia latently infected with the LAT$^-$ virus and that for LAT$^+$-virus-infected ganglia at the later times following induction examined here, there were no significant differences in the amounts of viral genomes present in latently infected tissue. This result is a direct confirmation of our ear-

TABLE 4. PCR analysis of LAT$^-$ HSV transcription following epinephrine-induced reactivation in rabbit corneas[a]

| Rabbit | Time (h) | VP5 cDNA detectable with[b]: | |
|---|---|---|---|
| | | 30 cycles | 45 cycles |
| D56 | 0 | − | − |
| D63 | 0 | − | − |
| D64 | 0 | − | ± |
| D54 | 12 | − | + |
| D43 | 16 | − | + |
| D54 | 16 | + | + |
| D79 | 20 | − | − |
| D46 | 20 | − | − |
| D55 | 36 | − | − |
| D62 | 36 | + | + |
| D61 | 36 | ± | + |
| D40 | 48 | − | − |
| D42 | 48 | − | + |
| D41s[c] | 48 | + | − |
| D41d[d] | 48 | + | ± |
| E60 | 48 | − | − |
| E65[e] | 48 | − | − |
| E57 | 72 | − | − |
| E58 | 72 | + | + |
| E59 | 72 | + | + |
| E56 | 168 | − | − |
| E61 | 168 | + | + |
| E62[e] | 168 | ± | − |

[a] Animals were subjected to a single iontophoresis of epinephrine at time 0.
[b] The conditions for amplification and primer sets used are described in Materials and Methods. −, cDNA not detected; +, cDNA detected; ±, barely visible.
[c] Right cornea.
[d] Left cornea.
[e] Virus was isolated from tear film. Tear film was harvested prior to sacrifice and incubated with indicator cells for 8 days. A positive result indicates virus-induced cytopathic effects.

lier conclusions (2). Representative data are shown in Fig. 2C. The two ganglia from rabbits latently infected with the LAT$^+$ virus (E44 and E49) had viral-to-cellular DNA signal ratios of 0.5 and 1.6, while the ratios in the ganglia from the rabbits latently infected with the LAT$^-$ virus (E58 and E63) were 0.8 and 0.9, respectively. These ratios compare favorably with the range of values of 0.7 to 1.4 determined previously (see Table 4 in reference 2).

**LAT expression has no measurable correlation with virus yield in cultured rabbit keratinocytes infected at low and high multiplicities.** Since the absence of LAT expression has a small but measurable role in the determination of the efficiency of establishment of latent infection in murine trigeminal systems (8, 20, 27, 28), and since there were differences in the amounts of transcripts and viral DNA levels in corneas from induced rabbits that had been infected with the LAT$^+$ virus and in those from LAT$^-$-virus-infected rabbits, a comparison of the replication efficiencies in cultured keratinocytes was done using various multiplicities of infection. Following initial infection with 10 to 10,000 PFU of virus per 60-mm$^2$ dish ($2 \times 10^6$ cells), the total amount of virus produced within a 72-h period showed no relation to the ability of the virus to express LAT. Identical results were obtained with Vero and rabbit skin cells (data not shown).

**LAT$^+$ and LAT$^-$ viruses can infect corneas of latently infected rabbits with equivalent efficiencies.** As a final control, the eyes of rabbits which had previously been latently infected with wild-type HSV-1 (McKrae) were infected with $10^4$ PFU of either the wild-type virus 17*syn*$^+$ or 17ΔPst, the LAT$^-$ mutant virus. Infection was carried out with 25 μl of virus suspension, and special care was taken not to abrade the cornea. Corneas were then harvested at 12 and 24 h following inoculation, and viral DNA and RNA levels were determined by PCR analysis. For DNA, the relative HSV-to-actin band intensity ratio in the corneas of several latently infected but untreated control corneas ranged from undetectable to 0.05. In contrast, the value from corneas infected with the LAT$^+$ virus ranged from 0.47 to 0.74, and the VP5-to-actin signal intensity ratio in the corneas infected with the mutant LAT$^-$ virus ranged from 0.4 to 1 (Fig. 6A).

PCR amplification of random-hexamer-primed cDNA synthesized from RNA isolated from all samples generated weak but readily detectable products when the HSV-specific VP5 primer set was used (Fig. 6B). We interpret these results to indicate that there is no a priori blockage of the ability of the LAT$^-$ virus to replicate in the eye in vivo, even when the animal has developed immunity by virtue of prior infection.

## DISCUSSION

A major conclusion from this study was that HSV productive-cycle transcripts and genome replication were evident in the corneas of reactivating rabbits whether the latent infection was established with the LAT$^+$ virus or the LAT$^-$ virus. However, there was, on average, a greater amount of HSV DNA seen in corneas of rabbits originally infected with the LAT$^+$ virus. This observation suggests that the reduced recovery of the LAT$^-$ virus compared to that of the LAT$^+$ virus during reactivation was due to a restriction in the replication of the former in the cornea or in tissue peripheral to it. In the latter case, the reduced presentation of infectious virions to the cornea would result in lower yields of the LAT$^-$ phenotype. The source of virus for such a presentation could be subclinical replication in another tissue peripheral to the ganglion.

A second conclusion was that the cornea contained sufficient viral genomes to serve as a source of virus fated to be amplified during the reactivation event. Our conclusion that the cornea could serve as a reservoir of reactivating virus came from our ability to readily detect viral genomes in corneal tissue before and at the earliest times following the induction event. The intensity of the PCR signals demonstrated that the amounts of viral transcripts and genomes present in corneal cells during all stages of reactivation were rarely more intense than the signals observed when RNA and DNA from one to two infected cells were subject to amplification (compare Fig. 1, 2A, 2B, and 4). This indicated that either the number of cells in which productive transcription occurred was quite limited (50 to 200 cells) or the relative level of expression of RNA in a given cell undergoing productive infection was significantly less than that seen in infected keratinocytes in culture. Indeed, the recovery of viral DNA and amounts of viral transcription seen during these early time points following reactivation were quite similar to those seen in cultured cells infected with very small numbers of virus particles (8).

Our data also confirm that the restricted expression of the HSV genome leading to the high proportion of LAT expressed compared to productive-cycle transcripts in latency, which is so characteristic of neurons, is not confined to neuronal tissue (9, 36). In agreement with others (7), we observed a positive signal upon PCR amplification of random-hexamer-primed cDNA with the 3′-LAT primer set in RNA from 5 of 25 corneas isolated from rabbits latently infected with the LAT$^+$ virus (Table 3 and Fig. 4). It is notable that the relative number of LAT-positive corneas was higher in tissue harvested from uninduced rabbits or at very early times following induction (0 to

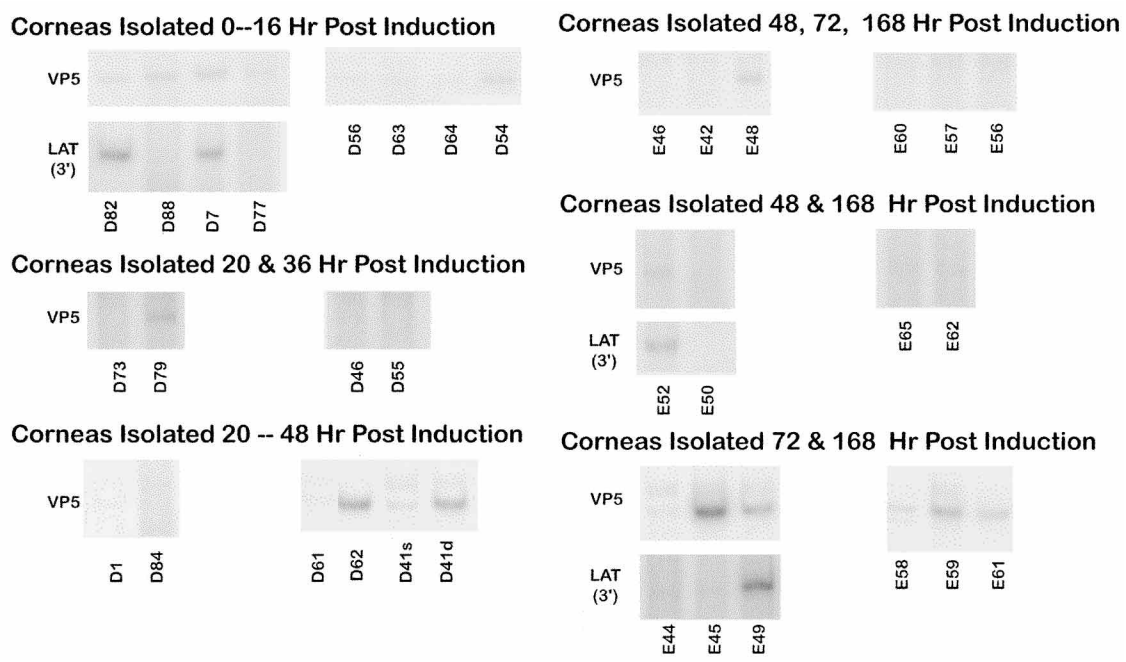


FIG. 4. Summary of transcription data from epinephrine-induced rabbit corneas. Products of amplification of random-hexamer-primed cDNA, generated from RNA isolated from 34 corneas of latently infected rabbits following epinephrine induction, with the VP5 primer set are shown. Also shown are data sets that contained at least one positive signal with the 3′-LAT primer pair. Corneas from rabbits latently infected with the LAT$^+$ virus are on the right in each grouping. Details concerning individual rabbits are shown in Tables 3 and 4.

4 h). This is consistent with very early data of Rock and collaborators suggesting that, in neurons, LAT expression is inversely proportional to productive-cycle gene expression in methotrexate-induced rabbits which had been latently infected with bovine herpesvirus (26).

Despite the clear evidence for relatively high levels of LAT expression in some uninduced corneas, the ease of detecting VP5 expression in such corneas and those isolated immediately following induction compared to the situation in trigeminal ganglia suggests that there are appreciable numbers of productive-infection events occurring in the cornea compared to neuronal tissue. This could be the source of virus-initiating productive infections leading to recovery of infectious virus following reactivation. Alternatively, the small amount of productive infection occurring in the cornea could be seeded from a peripheral site.

Considering all potential reservoirs—corneas, trigeminal ganglia, and ancillary tissues—a consistent model for reactivation in the rabbit eye model is as follows: upon occurrence of the physiological event triggering reactivation, there is a transitory increase in the amount of infectious virus available for initiating productive infection in the cornea. This LAT-sensitive critical event in reactivation could be the result of an increase in cell permissivity allowing productive-cycle tran-

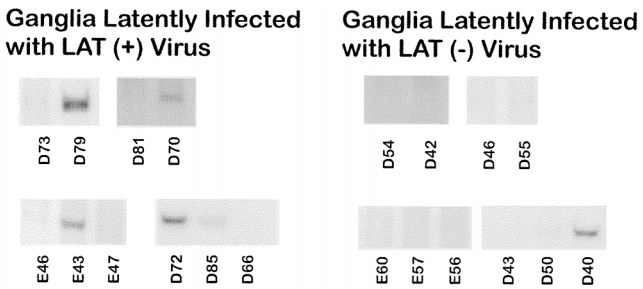


FIG. 5. Summary of transcription data from the corneas of epinephrine-induced rabbit ganglia. Products of amplification of random-hexamer-primed cDNA, generated from RNA isolated from 20 ganglia of latently infected rabbits following epinephrine induction, with the VP5 primer set are shown. Details concerning individual rabbits are shown in Tables 3 and 4.

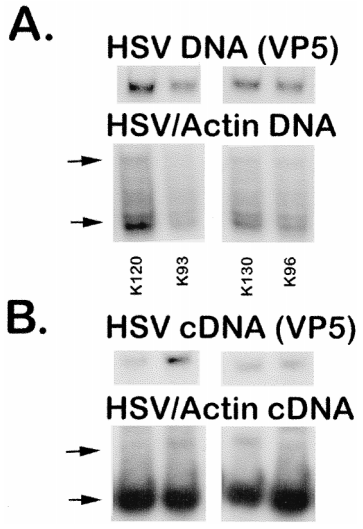


FIG. 6. HSV DNA and transcription in the corneas of latently infected rabbits superinfected with the LAT$^+$ or LAT$^-$ virus. Corneas of rabbits previously latently infected with the McKrae strain of HSV-1 were superinfected with $10^4$ PFU of either the LAT$^+$ virus 17Pr (K130 and K96) or the LAT$^-$ virus 17ΔPst (K120 and K93). Corneas were taken at 12 h (K130 and K120) and 24 h (K93 and K96) following superinfection. Arrows indicate expected size of PCR product.

scription and genome replication in the cornea. Alternatively, the critical event could result from a temporary increase in the mean survival time of extracellular virions produced by limited numbers of persistently infected cells in or near the cornea. In either case, reactivation would ultimately be manifest as a transitory increase in the number of infected cells in the cornea. The timing of the process in the rabbit model suggests that either route must result in the appearance of increased numbers of infectious virions within a 16- to 24-h window; this is only enough time to allow one or, at most, two complete replication cycles under optimal conditions.

It is important to note that while this scenario could allow the cornea itself to serve as a reservoir of infectious virions, it certainly does not exclude the trigeminal ganglion from serving in this capacity. The prolonged productive-cycle gene expression in the ganglia of rabbits reactivating from a $LAT^+$ latent infection (Fig. 5) is consistent with the ganglion being a continuing source of virus. This could be especially important in that viral replication in the cornea is limited under the best of conditions, i.e., even during reactivation with the $LAT^+$ virus.

The strongest argument against a primary restriction of replication of the $LAT^-$ virus in the cornea in vivo under conditions of reactivation comes from our determination that none of the relatively straightforward control experiments involving either infection of cultured keratinocytes or superinfection of latently infected rabbit corneas could demonstrate any evidence of it. This could be a result of the facts that corneal cells are in growth arrest in vivo and that the amount of infectious virus transiently presented to the critical tissue at the initiation of reactivation is very small. Still, it is (at this point, at least) more reasonable to postulate that the reduced delivery of the $LAT^-$ virus to the cornea compared to that of the $LAT^+$ virus is a result of growth restriction of the former in another anatomical site.

Finally, our data suggest that LAT-facilitated replication in the eye or associated tissue is a common site for the action of LAT in the reactivation process in both the murine eye and rabbit eye models, despite the difference between the mouse and rabbit models in the specific location of reactivation-critical *cis*-acting elements within the region of the HSV genome encoding the 5′ region of LAT (3, 19). We suggest that titration of the amount of virus used to initiate latent infections in rabbits may result in a situation in which readily measurable differences in the amount of viral DNA can be observed in the ganglia, with resulting enhancement of the differences between the reactivation rates of the $LAT^+$ and $LAT^-$ viruses. Alternatively, the lack of such a critical multiplicity of infection could provide more compelling evidence against the trigeminal ganglion serving as a major source of virus for reactivation in the rabbit eye.

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