A 437-Base-Pair Deletion at the Beginning of the Latency-Associated Transcript Promoter Significantly Reduced Adrenergically Induced Herpes Simplex Virus Type 1 Ocular Reactivation in Latently Infected Rabbits

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In this study we used a herpes simplex virus type 1 (HSV-1) deletion mutant to identify a segment of the genome necessary for epinephrine-induced reactivation in the rabbit eye model of herpetic recurrent disease. In HSV-1 latently infected neural tissue, the only abundant viral products are the latency-associated transcripts (LATs). At least one promoter of LAT has been identified, and mutations in the LAT domain have been used to investigate HSV-1 reactivation. We used an ocular rabbit model of epinephrine-induced HSV-1 reactivation to study the effects of deleting a 437-bp region beginning 796 bp upstream of the LAT CAP site. Specifically, the 437-bp deletion is located between genomic positions 118006 and 118443 of the parent 17Syn⁴, and the construct is designated 17ΔS/N. This region also controls a portion of the genome encoding two transcripts (1.1 and 1.8 kb) from the LAT domain. A rescuant, 17ΔS/N-Res, was constructed from 17ΔS/N. Following ocular infection, all three viruses produced similar acute dendritic lesions in rabbits. Five weeks after infection, rabbits received transcorneal iontophoresis of epinephrine. The parent, 17Syn⁴, and the rescuant, 17ΔS/N-Res, underwent a high frequency of HSV-1 ocular reactivation as determined by recovery of infectious virus in the tear film. Rabbits infected with 17ΔS/N had a significantly lower frequency of ocular reactivation. Analysis of the trigeminal ganglia from all three groups of latently infected rabbits revealed (i) similar amounts of HSV DNA (genomic equivalents), (ii) accumulation of 2.0- and 1.45-kb LATs, and (iii) explan reactivation at the same high frequency. Therefore, these studies indicate that the 437-bp deleted region in 17ΔS/N is essential for epinephrine-induced reactivation and could implicate the 1.1- and 1.8-kb transcripts in the mechanisms controlling HSV-1 reactivation.

The neurotropic alphaherpesvirus herpes simplex virus type 1 (HSV-1) can establish lifelong latency in the nervous system following an acute infection. A hallmark of herpes latency is reactivation and recurrence of disease (18). Numerous studies have been conducted to determine the viral genomic regions required for efficient viral reactivation and recurrent clinical disease. The rabbit eye model has been used to assess spontaneous and induced ocular reactivation of numerous HSV strains and genetic constructs. The genetic constructs have consisted of (i) deletions and nonsense replacements (14, 17, 20, 23, 25, 28), (ii) frameshift mutations (9), (iii) ectopic recombination (24), (iv) transcriptional terminators (4), and (v) site-directed mutations (5, 6). These studies have shown that latency-associated transcript (LAT) deletion viruses have a significantly reduced ability to undergo reactivation in the rabbit eye model. Deletion viruses such as 17ΔSty and 17Δ348, which are LAT-expressing viruses, lack regions essential for adrenergic reactivation (5, 14). Also, a very small 7-bp alteration in a cyclic-AMP response binding element sequence in the LAT promoter domain has been shown to be required for optimal epinephrine-induced ocular reactivation (6).

17ΔS/N contains a deletion up to the very beginning of the LAT promoter (20). This deletion spares the LAT promoter and allows transcription and accumulation of the 2.0- and 1.45-kb LAT stable introns. However, two transcripts (1.1 and 1.8 kb) previously described by Singh and Wagner (26) are not transcribed during acute infection with 17ΔS/N (20). These are two low-abundance, 5′-collinear transcripts with poly(A) tails. Although their transcription in the acute phase of replication has been described, their function is unknown. We report that rabbits latently infected with 17ΔS/N have significantly reduced reactivation following transcorneal iontophoresis of epinephrine. Therefore, this 437-bp region 796 nucleotides upstream of the CAP site is essential to facilitate induced HSV reactivation.

**MATERIALS AND METHODS**

**Cells and medium.** CV-1 and Vero cells (American Type Culture Collection, Rockville, Md.) were maintained at 37°C in 5% CO₂ as monolayers in an Eagle medium (E-MEM; Life Technologies, Gaithersburg, Md.) supplemented with 5% fetal bovine serum (FBS). Rabbit skin and primary rabbit kidney (PRK) cells were propagated and maintained as previously described (2, 5, 15, 18).

**Virus and plasmids.** HSV-1 strain 17Syn⁴ was the parent of the viruses used in this study. 17ΔPst is a construct having a deletion of 202 nucleotides between genomic locations 118664 and 118866 (4). Details of the construction of 17ΔPst have been reported (4). 17ΔS/N, a viral construct having a deletion of 437 nucleotides between genomic locations 118006 and 118443, was isolated from calcium phosphate-mediated transfections of CV-1 cells with genomic 17ΔBstE

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and pXhoΔS/N DNAs as previously described (20). Details of all of the plasmids and sequence locations have been reported (20, 21). pXhoΔS/N is a pGEM-derived plasmid containing HSV genomic sequences between nucleotide positions 116961 and 123017 and a deletion between the SwaI and NotI sites at nucleotides 118006 and 118443, respectively (Fig. 1).

Construction of rescuant virus (17ΔS/N-Res). Rescuan virus was made by recombination in CV-1 cells transfected with a calcium phosphate precipitant of viral DNA from 17ΔS/N and the plasmid, pXho, containing 17Syn1 sequences between nucleotide positions 116961 and 123017. The rescuan was designated 17ΔS/N-Res.

Southern analysis of DNA. DNA was extracted from trigeminal ganglia (TG) as described by Block et al. (2) and digested to completion with the restriction endonucleases SalI and NotI per the directions of manufacturer (Bethesda Research Laboratories, Bethesda, Md.). Digest products were resolved by agarose gel electrophoresis through 0.8% gels, denatured, neutralized, and transferred to nylon membranes (Southern blotted). Nylon membranes were hybridized with 32P-labeled probes made by random priming of pH-H 0.180, a plasmid that contains HSV-1 sequences from 120295 to 120469 (20). The random priming, incubation, and washing conditions of these blots were as described by Block et al. (3).

Northern analysis of RNA. RNA was isolated from TG, resolved by electrophoresis through 1.2% agarose gels, and transferred to GeneScreen (NEN Research Products, Amherst, Mass.) membranes (22). Radiolabeled 32P probes were made by random priming of a plasmid containing the LAT region; hybridization and washing conditions were as previously described (20, 21).

Ocular inoculation of rabbits and SLE. The corneas of New Zealand White rabbits (2 to 2.5 kg) were topically inoculated with 2 × 10^5 to 5 × 10^5 PFU of plaque-purified virus. Corneal infection was monitored by slit lamp examination (SLE) from 3 to 7 days postinoculation (p.i.). At least two masked examiners scored the eyes each day (17). Three weeks p.i., all eyes had completely healed as determined by SLE.

Quantitation of acute ocular infection. Eye swabs were taken beginning 1 day after inoculation and continuing for 8 days. The tear film was eluted from the swabs and quantitated by plaque assay on CV-1 cells.

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Quantitation of HSV-1 in cornea and TG. On p.i. days 3 and 8, at least three rabbits in each group were sacrificed. Corneas and TG were removed rapidly, frozen, pulverized, homogenized, and quantitated by plaque assay on CV-1 cells.

Adrenergically induced ocular HSV-1 reactivation. Five weeks after infection, rabbit eyes underwent transcorneal iontophoresis of 0.01% epinephrine (0.8 mA for 8 min) once a day for 3 days (10, 16). Eyes were swabbed daily for 7 days after iontophoresis, and the swabs were assayed on PRK cells monolayers for detection of infectious virus (13, 15).

Explant (cocultivation) assay. After adrenergic induction, rabbit TG were removed, the outer sheaths were discarded, and the tissues were separated into three or four pieces and placed in E-MEM containing 10% FBS. Beginning 7 days after removal of the ganglia, the culture supernatants were assayed daily for virus on PRK cells. Culture assessment was done daily for up to 21 days postexplantation. Viruses obtained from these explant assays were tested to confirm that the recovered virus was the same viral construct used to infect the rabbit.

Quantitation of HSV DNA by PCR. DNA was extracted from homogenized TG by a standard procedure (27). The procedures of Coen (7, 8) as modified by Hill et al. (11, 12, 18) were employed for the quantitation of rabbit alpha actin (124 bp) and HSV-1 ribonucleotide reductase (243 bp). These were coamplified from each DNA sample to quantitate the HSV DNA copy number per 100 cells. The primer pair sequences and the details of the PCR have been given previously (13, 14, 19). The PCR products were analyzed by dot blotting and hybridization with 32P-labeled probes (12). Quantitation was performed densitometrically with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

### RESULTS

Acute ocular infections and quantitation of virus in corneas and TG. Figure 2 shows the results of SLE, and no differences exist among the four viruses during p.i. days 3 through 8 for the acute infection of the rabbit corneal epithelium. Figure 3 is the quantitation of HSV from the ocular tear film and shows no differences among the four viruses during p.i. days 1 through 8. Table 1 is the quantitation of HSV in corneas and TG homogenates at p.i. days 3 and 8. No differences in infectious HSV-1 were detectable among the four viruses regardless of the tissue or p.i. day.

Epinephrine-induced ocular reactivation profile of 17ΔS/N from latently infected rabbits. To determine the in vivo reactivation efficiency of the deletion mutant 17ΔS/N, rabbits were inoculated via the cornea with either wild-type 17Syn⁺, 17ΔS/N, 17ΔPst, or 17ΔS/N-Res. Five weeks after inoculation, rabbit eyes received transcorneal iontophoresis of epinephrine. We found that 17ΔS/N and 17ΔPst were significantly (P < 0.004) impaired in their ability to undergo in vivo reactivation induced by corneal iontophoresis of epinephrine, compared with the parent and the rescuant. Overall, 17ΔS/N and 17ΔPst reactivation was approximately 75% less efficient than parent or resulant reactivation regardless of the parameter assessed (Table 2).

Verification of virus genotypes by Southern blot analysis. 17ΔS/N virus recovered from TG of epinephrine-treated rabbits was expected to have the same genotype as the 17ΔS/N virus used in the inoculum. The genomes of virus recovered following reactivation were compared to those used in the inoculum by Southern blot hybridization. Figure 4 shows an image of an autoradiograph of a Southern blot of SalI-NorI-

### TABLE 1. Quantitation of HSV-1 in acute phase of infection

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean PFU</th>
<th>Mean PFU</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cornea</td>
<td>Cornea</td>
</tr>
<tr>
<td></td>
<td>TG</td>
<td>TG</td>
</tr>
<tr>
<td>17Syn⁺</td>
<td>0.85 × 10³</td>
<td>5.1 × 10³</td>
</tr>
<tr>
<td>17ΔS/N</td>
<td>0.78 × 10³</td>
<td>5.8 × 10³</td>
</tr>
<tr>
<td>17ΔPst</td>
<td>0.92 × 10³</td>
<td>4.9 × 10³</td>
</tr>
<tr>
<td>17ΔS/N-Res</td>
<td>0.81 × 10³</td>
<td>4.8 × 10³</td>
</tr>
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</table>

Corneas and TG were homogenized, and the supernatant was quantitated for HSV-1 on CV-1 cells. Values were determined from at least five tissue samples in each group.

Standard errors of the means are indicated.

At least one TG from each group had no detectable infectious HSV-1.

![Graph](https://via.placeholder.com/150)

**FIG. 2.** Scores derived from cornal SLE of rabbits following ocular inoculation of HSV. Rabbits were inoculated on the cornea and examined at daily intervals for the period shown. The data are mean scores for the eyes.

**FIG. 3.** Virus present in the tear film of rabbits following ocular inoculation. Rabbits were inoculated on the cornea, and samples of tear film were taken and titrated for virus on the days indicated. The data are mean PFU recovered from six eyes inoculated with virus.

![Graph](https://via.placeholder.com/150)
digested viral DNA probed with radiolabeled pH-H 0.180. As described by Maggioncalda et al. (20), pH-H 0.180 hybridizes to a single 2.5-kb fragment of wild-type 17Syn DNA, following SalI and NorI digestion. This is the pattern observed in each of the lanes containing viral DNA isolated from the eyes of wild-type-infected rabbits. The pH-H 0.180 probe hybridizes to a pair of 6.0- and 9.0-kb fragments in the lanes containing SalI-NorI-digested DNA from virus isolated from 17ΔS/N-infected rabbits. These fragments are characteristic and specific for the 17ΔS/N mutant (20) and therefore demonstrate that (i) the virus reactivating from the rabbits contains the same genome as that used in the inoculum and (ii) there have been no major structural changes in the LAT region in the genomes of the mutants during latency.

Quantitation of HSV DNA in TG of latently infected rabbits. The reduced efficiency of epinephrine-induced reactivation of 17ΔS/N and 17ΔPst from latently infected rabbits could be due to a reduced number of viral genomes in the TG. This hypothesis was tested by measuring the amount of viral DNA present in the TG of latently infected rabbits by quantitative PCR, as previously described (11, 12, 18). We found that 17ΔS/N and 17ΔPst established latency in the rabbit TG with approximately the same number of HSV genomes as 17Syn and 17ΔS/N-Res. The mean genome copy numbers per 100 cells were 19.2, 24.1, 21.4, and 22.7 for 17Syn, 17ΔS/N, 17ΔPst, and 17ΔS/N-Res, respectively. The number of TG in each group was at least 7. Therefore, the 437-bp deletion in 17ΔS/N and 202-bp deletion in 17ΔPst had no significant influence on the establishment and/or maintenance of latency relative to the HSV DNA copy number.

LAT production in TG from latently infected rabbits. The reduced efficiency of 17ΔS/N epinephrine-induced reactivation could have been due to an inability to accumulate 1.45- and 2.0-kb LATs. This possibility was tested by detecting LAT production in latently infected rabbits by Northern blot analysis. Total RNA was isolated from TG derived from rabbits latently infected with wild-type Syn17+, 17ΔS/N, or 17ΔS/N-Res, resolved through agarose gels, Northern blotted, and hybridized to a radioactive LAT probe (data not shown). We found that each of the TG isolated from 18 different TG latently infected with either 17Syn, 17ΔS/N, or 17ΔS/N-Res accumulated 1.45- and 2.0-kb LATs at approximately the same average value. These results show that the 17ΔS/N virus was fully capable of synthesizing and accumulating 1.45- and 2.0-kb LATs during latency. However, variation did exist from rabbit TG to rabbit TG and from virus to virus.

Reactivation profile of 17ΔS/N, 17Syn+, 17ΔPst, and 17ΔS/N-Res from explants of latently infected rabbit TG. Although 17ΔS/N reactivated from latently infected rabbits less efficiently than wild-type virus following in vivo epinephrine iontophoresis, it was considered likely that the latent virus would reactivate normally from TG in explant cocultivation. This was significant, because 17ΔS/N has been shown to reactivate from explants derived from latently infected mice with a time to positivity similar to that for explants from mice infected with 17Syn+ (20). At 6 to 8 weeks after infection, rabbits were sacrificed and the TG were cultured in E-MEM with 10% FBS and maintained at 37°C. The supernatant was tested for infectious HSV-1 as described above. All four viruses exhibited virtually the same frequency (80 to 100%) and time to positivity (12 to 18 days) of infectious-virus recovery. Therefore, the 437-bp deletion in 17ΔS/N had no apparent influence on HSV recovery from TG of latently infected rabbits in the cocultivation explant assay.

**DISCUSSION**

The results of in vivo studies of 17ΔS/N complement and extend the results reported for 17Δ448 and 17ΔSty, indicating that the 5' region of the LAT domain is very important for adrenergically induced reactivation and that expression of the 2.0- or 1.45-kb LAT transcripts is not solely sufficient for reactivation.

Although rabbits latently infected with 17ΔS/N exhibited reduced reactivation following epinephrine iontophoresis, the TG from these rabbits reactivated with wild-type kinetics in explant cocultivation. Wild-type frequency of reactivation from explant cocultivation has been previously noted with this mutant in latently infected mouse TG (20), although viral induction was not assessed in that study. Based on this and other studies (5, 6, 14, 15), we propose that there are at least two distinguishable reactivation phenotypes, namely the one manifested in explant coculture and the one displayed following in vivo induction.

Analysis of the 437-bp sequence that is deleted in 17ΔS/N reveals that this sequence has the unique feature of a very low (0.56) CpG-to-GpC ratio, compared to the CpG-to-GpC ratio for the entire genome, which is 1.08 (19). The GC content of the 437 bp is 67.11% compared to 68% for the entire genome. No stress-related transcriptional factor consensus sequence such as AP-2 or cyclic-AMP response element (CRE) was found. One Sp-1 site and one early growth response (Egr-1) site were found in the consensus sequence. The Sp-1 sequence

![FIG. 4. HSV DNA from reactivated TG analyzed by Southern blots.](http://jvi.asm.org/)

**TABLE 2. Ocular reactivation in HSV-1 latently infected rabbits**

<table>
<thead>
<tr>
<th>Virus</th>
<th>No. positive/total (%) [Pa]</th>
</tr>
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<tbody>
<tr>
<td>17Syn+</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>17ΔS/N</td>
<td>4/6 (25) [0.014]</td>
</tr>
<tr>
<td>17ΔPst</td>
<td>5/5 (100)</td>
</tr>
</tbody>
</table>

* Statistical comparison of 17Syn+ versus 17ΔS/N, 17ΔPst, and 17ΔS/N-Res. The two-sided P value is from chi-square analysis using the exact method (1).

Pa for two of the rabbits only one eye could be used.

For one of the rabbits, only one eye could be used.

a For two of the rabbits only one eye each could be used.

b For one of the rabbits, only one eye could be used.
is GAGCGAG at genomic location 118299 to 118307. The Egr-1 sequence is CGCCCCGCC at genomic location 118213 to 118221. The general conclusions are that (i) this region is very unlikely to have any methylation due to low CpG-to-GpC ratio and (ii) no other nondegenerate transcriptional activating sites other than the Sp-1 and Egr-1 were detected. Therefore, the possibility exists that a never before described, perhaps unique, control site is present in the 437-bp region. These two transcriptional activating sites have not been shown to be involved in mechanisms controlling RNA synthesis in HSV.

So far, mechanisms by which the LAT region could affect HSV reactivation have not been satisfactorily explained nor proven by antisense mechanisms or postulated LAT protein products. There are several novel means by which the LAT region could influence the reactivation potential of HSV. We (5, 14) have suggested that methylation in the LAT region regulates certain aspects of reactivation. This methylation could be mediated by an unusual pathway. As an intron, 2.0-kb LAT DNA is methylated in the LAT domain. Other experiments involve construction of deletion viruses containing smaller deletions within the 437-bp region. All these experiments involve assessment of protein binding of transcriptional activating factors to sequences in the 437-bp region. A herpes simplex virus type 1 DNA and latency-associated transcripts in rabbit trigeminal ganglia demonstrate a stable reservoir of viral nucleic acids during latency. J. Virol. 70:3137–3141.

Current experiments to determine the mechanism of HSV reactivation involve assessment of protein binding of transcriptional activating factors to sequences in the 437-bp region. Sequencing experiments are being conducted to determine if the HSV DNA is methylated in the LAT region. Other experiments involve construction of deletion viruses containing smaller deletions within the 437-bp region. All these experiments would allow us to more specifically define the sequence essential for adrenergic reactivation. Furthermore, the smaller deleted region could also be assessed for binding of novel transcriptional activating factors.

ACKNOWLEDGMENTS

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