

In Vivo Epinephrine Reactivation of Ocular Herpes Simplex Virus Type 1 in the Rabbit Is Correlated to a 370-Base-Pair Region Located between the Promoter and the 5' End of the 2.0-Kilobase Latency-Associated Transcript

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A rabbit ocular model of epinephrine-induced herpes simplex virus type 1 reactivation was employed to study the effect of a deletion in the latency-associated transcript domain. A viral construct derived from 17Syn⁺, designated 17ΔSty, has a deletion of 370 nucleotides between genomic positions 118880 and 119250. 17ΔSty has been shown to reactivate with wild-type virus kinetics from explants of trigeminal ganglia from latently infected mice. To determine the behavior of this mutant in an in vivo, inducible reactivation system, rabbit corneas were infected with 17Syn⁺, 17ΔSty, or its rescuer, 17ΔSty-Res. After viral latency was established, transcorneal epinephrine iontophoresis was performed. The rabbits latently infected with 17ΔSty exhibited a significantly reduced ability to undergo adrenergically induced reactivation, i.e., viral shedding in the tears, compared with rabbits infected with either 17Syn⁺ or 17ΔSty-Res. However, quantitative PCR demonstrated similar numbers of viral genomes in the trigeminal ganglia from rabbits latently infected with all three viruses, and all three viruses reactivated in vitro with wild-type kinetics in an explant cocultivation assay. These studies indicate that the 370-bp region deleted in the 17ΔSty construct plays a role in epinephrine-induced reactivation.

An important question regarding reactivation of herpes simplex virus type 1 (HSV-1) relates to the determination of the genomic regions required for efficient viral reactivation and recurrence of clinical disease. The rabbit eye model has been used to assess spontaneous and induced ocular reactivation of numerous HSV strains and genetic constructs (3, 4, 14, 20, 21, 26-28, 31). In vivo ocular studies in mice and rabbits have shown that latency-associated transcript (LAT) promoter deletions (within the 875-bp LAT promoter region located between positions 117927 and 118802; GenBank revision of 25 November 1994) (see Fig. 1) result in significantly decreased viral shedding in both spontaneous (3, 20, 26-28) and induced models (3, 4, 9, 10, 19, 20, 31).

Reactivation phenotypes have been mapped to the LAT domain by using HSV genetic constructs. HSV recombinants constructed with poly(A) addition sites placed at intervals within the LAT primary transcript synthesize a truncated LAT (4). In rabbits, these viral constructs, which lack sequences at the 3' end of the primary 8.3-kb LAT, reactivate at a high frequency, similar to that of the parent 17Syn⁺ (4). This indicates that transcription products of the 3' end of the 8.3-kb LAT, if any, are not essential for reactivation. Also, a recombinant virus with an 8-bp *ClaI* restriction site inserted at genomic location 120469 in the largest open reading frame in the LAT intron reactivates with the same frequency as its parent, 17Syn⁺ (11), demonstrating that this particular open reading frame is not required for efficient adrenergically in-

duced reactivation. Taken together, these results suggest that it is the sequences at or near the 5' end of the LAT domain that influence the ability of HSV to reactivate from latency but that, if a protein is translated from this region, it is not involved in reactivation.

This conclusion was reinforced by results showing that another deletion virus, 17Δ348, has a significantly reduced ability to reactivate following iontophoresis of epinephrine into the cornea in latently infected rabbits (4). This recombinant virus has a 348-bp deletion located between genomic positions 119007 and 119355, 217 nucleotides downstream of the cap site (genomic location 118802) of LAT. Although LAT-negative mutants had been shown to reactivate with reduced efficiency from latently infected rabbits, 17Δ348 was the first LAT-positive recombinant mutant that displayed impaired HSV ocular reactivation in rabbits.

In the current study, we examined the efficiency of adrenergically induced reactivation of another LAT-positive deletion virus, 17ΔSty, which is similar to 17Δ348 in a number of ways. The deletions in 17ΔSty and 17Δ348 share a common 243-bp region (Fig. 1), as well as various physical and biochemical characteristics. In addition, both mutants have the same parent, HSV-1 strain 17Syn⁺. Coculture explant assays of the trigeminal ganglia (TG) from animals latently infected with 17ΔSty (BALB/c mice), 17Δ348 (New Zealand White rabbits), and 17Syn⁺ (mice and rabbits) all exhibited wild-type frequency and rate of in vitro reactivation (4, 24).

Because (i) both 17Δ348 and 17ΔSty are LAT-positive constructs, (ii) the region deleted in 17ΔSty significantly overlaps that deleted in 17Δ348, (iii) 17Δ348 was found to undergo adrenergically induced reactivation at a reduced efficiency in rabbits, and (iv) both constructs reactivate with wild-type effi-

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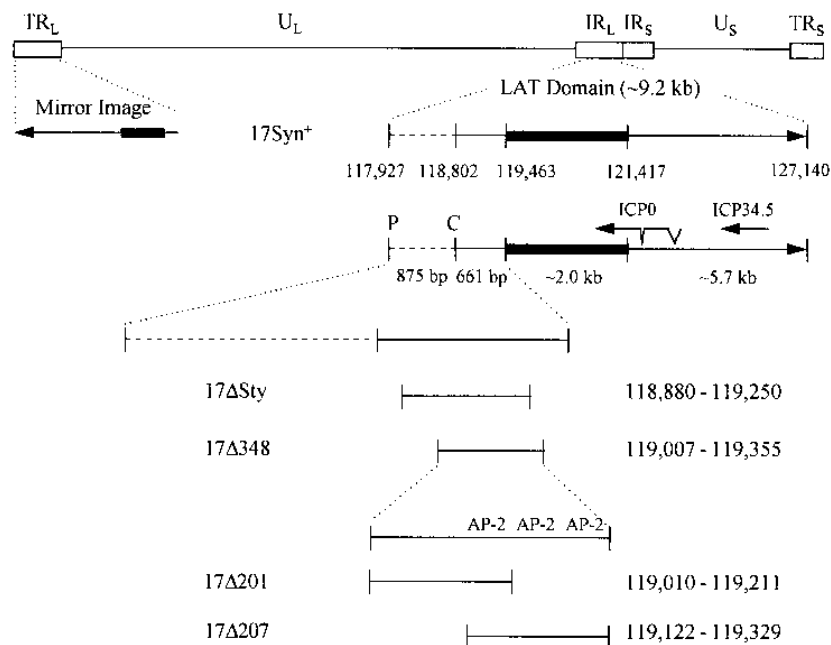


FIG. 1. Location of the deletion in 17 Δ Sty and other HSV constructs. The region encoding the LAT domain is shown with genomic sites numbered according to the 17Syn⁺ parent sequence as published in the GenBank revision of 25 November 1995. Thick lines, major 2.0-kb LAT sequence; dashed lines, LAT promoter region. The locations and directions of two other genes in this area (ICP0 and ICP34.5) are indicated, with introns and direction of transcription. Locations of deletions in relevant viral constructs are given below the parent sequence. AP-2, approximate location of three AP-2 binding sites present in the region deleted in 17 Δ Sty and 17 Δ 348. The consensus binding sequence of AP-2 is GCCN₃GGC. Labeled genomic sites: TR_L, terminal long repeat; U_L, unique long; IR_L, internal repeat long; IR_S, internal repeat short; U_S, unique short; TR_S, terminal repeat short; P, LAT promoter; C, LAT RNA cap site.

ciency in vitro, determination of the reactivation phenotype of 17 Δ Sty after epinephrine iontophoresis could further define the genomic region essential for adrenergically induced HSV-1 reactivation.

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. Rabbit skin and primary rabbit kidney cells were propagated and maintained as previously described (1, 3, 17, 21).

Viruses and plasmids. HSV-1 strain 17Syn⁺ was the parent of the viruses used in this study. 17 Δ Sty, a viral construct having a deletion of 370 nucleotides between genomic positions 118880 and 119250, was isolated from calcium phosphate-mediated transfections of CV-1 cells with genomic 17 Δ BstE and pXho Δ Sty DNAs as previously described (24). Details of all of the plasmids and sequences have been reported (24). pXho Δ Sty is a pGem-derived plasmid having HSV genomic sequences between nucleotide positions 116961 and 123017 and a deletion between the *SlyI* sites at nucleotides 118880 and 119250.

Construction of rescuant virus (17 Δ Sty-Res). Rescuant virus was made by recombination in CV-1 cells transfected with a calcium phosphate precipitant of viral DNA from 17 Δ Sty and the plasmid, pXho, containing 17Syn⁺ sequences between nucleotide positions 116961 and 123017 (25). The rescuant was designated 17 Δ Sty-Res.

Southern analysis of DNA. Virion DNA was purified as described by Block et al. (1) and digested to completion with the restriction endonucleases *SalI* and *BglII* per the manufacturer's directions (Bethesda Research Laboratories, Bethesda, Md.). Digest products were resolved by agarose gel electrophoresis through 0.8% gels, denatured, neutralized, and trans-

ferred to nylon membranes (Southern blotted). Nylon membranes were hybridized with ³²P-labeled probes made by random priming of pSwaI-NotI, a plasmid that contains HSV-1 sequences from position 118006 to 118443. The random priming, incubation, and washing conditions of these blots were as described by Block et al. (2).

Northern analysis of RNA. RNA was isolated from TG, resolved by electrophoresis through 1.2% agarose gels, and transferred to GeneScreen membranes (24). Radiolabeled ³²P probes were made by random priming of a plasmid containing the LAT region; hybridization and washing conditions were as previously described (24).

Ocular inoculation of rabbits. The corneas of New Zealand White rabbits (2 to 2.5 kg) were topically inoculated with 1 × 10⁵ to 2 × 10⁵ PFU of plaque-purified virus. Corneal infection was monitored by slit lamp examination (21). The corneas of all rabbits developed dendrites 3 to 7 days postinoculation. Three weeks postinoculation, all eyes had completely healed as determined by slit lamp examination.

Adrenergically induced ocular HSV-1 reactivation. Four to 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 (18). Eyes were swabbed daily for 7 days after iontophoresis, and the swabs were assayed on primary rabbit kidney cell monolayers for detection of infectious virus.

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, which were placed in E-MEM containing 10% fetal bovine serum. Beginning 7 days after removal of the ganglia, the culture supernatant was assayed daily for virus on rabbit skin or primary rabbit kidney cells. Culture assessment was done daily for up to 21 days following explantation. Viruses obtained from

TABLE 1. Ocular reactivation in HSV-1 latently infected rabbits^a

Virus	No. positive (%)		
	Rabbits	Eyes	Swabs
17Syn ⁺	7/11 (64)	12/22 (55)	48/154 (31)
17ΔSty	2/9 (22)	2/18 (11) ^b	4/126 (3) ^b
17ΔSty-Res	5/8 (63)	10/16 (63)	35/112 (31)

^a Cumulative results from three separate experiments.

^b Significantly different from the values with 17Syn⁺ and 17ΔSty-Res ($P < 0.004$; chi-square test).

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 (25, 30). The procedures of Coen (7, 8) as modified by Hill et al. (15, 16, 21) 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 (15, 16, 21). The PCR products were analyzed by dot blotting and hybridization with ³²P-labeled probes (16). Quantitation was performed densitometrically with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Epinephrine-induced ocular reactivation profile of 17ΔSty from latently infected rabbits. To determine the in vivo reactivation efficiency of the deletion mutant 17ΔSty, rabbits were inoculated via the cornea with either wild-type 17Syn⁺, 17ΔSty, or 17ΔSty-Res. Four to five weeks after inoculation, rabbit eyes received transcorneal iontophoresis of epinephrine (Table 1). We found that 17ΔSty was significantly ($P < 0.004$) impaired in its ability to undergo in vivo reactivation induced by corneal iontophoresis of epinephrine, compared with the parent and the rescuant. The decrease ranged from 30 to 90%, depending on the parameter assessed (Table 1).

Verification of virus genotypes by Southern blot analysis. 17ΔSty virus recovered from TG of epinephrine-treated rabbits had the same genotype as the 17ΔSty virus used in the inoculum. Figure 2 shows the hybridization of radioactively labeled pSwaI-NotI, a plasmid containing HSV DNA sequences adjacent to the deletion region (24), to TG DNA extracted from coculture-reactivated TG digested with *Bgl*II and *Sal*I. These blots revealed that 6.6- and 9.3-kb fragments were present in the samples derived from wild-type- and rescuant-inoculated rabbits whereas 7.0- and 4.3-kb fragments were present in the samples from 17ΔSty-inoculated rabbits. The same results were obtained with virus recovered by eye swabs (data not shown).

Abundance of 17ΔSty DNA in TG of latently infected rabbits. The reduced efficiency of epinephrine-induced reactivation of 17ΔSty 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 (15, 16, 21). We found that 17ΔSty established latency in the rabbit TG with approximately the same number of HSV genomes as 17Syn⁺ and 17ΔSty-Res. The mean genome copy numbers per 100 cells for all three groups in all three experiments were 21.0, 19.7, and 23.8 for 17Syn⁺, 17ΔSty, and 17ΔSty-Res, respectively. Therefore, the 370-bp deletion in 17ΔSty had no significant influence on the establishment and/or maintenance of latency relative to the HSV DNA copy number.

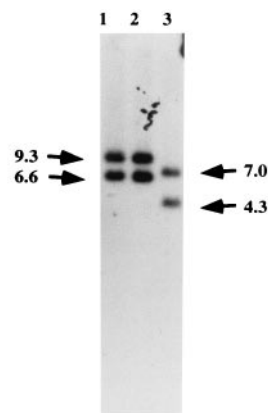


FIG. 2. Southern blot analysis of DNA isolated from virus following in vivo reactivation or explant reactivation. TG from rabbits latently infected with either wild-type, 17ΔSty, or rescuer virus were explanted. The DNA from reactivated virus was isolated, digested with restriction enzymes *Bgl*II and *Sal*I, resolved through 0.8% agarose gels by electrophoresis, and transferred to Nytran membranes by Southern blotting. The blots were hybridized to radioactively labeled pSwaI-NotI, which contains sequences upstream from the LAT promoter. Samples from explants derived from rabbits latently infected with wild-type Syn⁺ (lane 1), rescuer (lane 2), and 17ΔSty (lane 3) virus are shown. The same results were obtained from virus-positive eye swabs. The sizes of the detected restriction fragments are indicated (in kilobases) and were determined by comparison with the mobility of molecular weight markers.

LAT production in TG from latently infected rabbits. The reduced efficiency of 17ΔSty epinephrine-induced reactivation could have been associated with an inability to accumulate 1.45- 2.0-kb LAT. 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 either wild-type Syn17⁺, 17ΔSty, or 17ΔSty-Res, resolved through agarose gels, Northern blotted, and hybridized to a radioactive LAT probe. We found that TG latently infected with 17ΔSty accumulated 1.45- 2.0-kb LATs (lane 2), as did the wild-type virus (lane 1) and the rescuer virus (lane 3) (Fig. 3A). Figure 3B shows the ethidium bromide-stained gel used in the Northern blot in Fig. 3A and

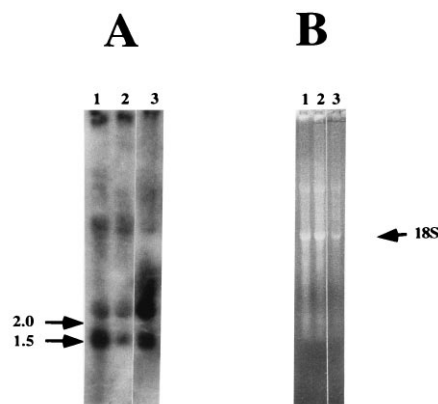


FIG. 3. HSV LAT RNA in TG derived from latently infected rabbits. Rabbits were infected via the eye, and latent infections were established as described in the text. After 6 to 7 weeks, rabbits were sacrificed and total RNA was isolated from the TG, resolved through 1.2% agarose gels, and transferred to membranes (Northern blots). (A) Blots were hybridized with a radioactively labeled plasmid that contained LAT region DNA (see the text). Samples were from rabbits that were latently infected with either wild-type (lane 1), 17ΔSty (lane 2), or rescuer (lane 3) virus. The positions of the 1.45- 2.0-kb LATs are indicated. (B) Ethidium bromide-stained gel.

provides evidence that the lanes were loaded with approximately equal amounts of RNA. Although the sample of 17 Δ Sty shown in this figure appears to contain less LAT RNA than the wild-type and rescuing samples, our analysis of many samples demonstrated variations from sample to sample regardless of the strain. Overall, analysis of more than four different TG per virus suggests that 17 Δ Sty, Syn17⁺, and 17 Δ Sty-Res induce similar amounts of LAT in latently infected rabbits (data not shown).

Reactivation of 17 Δ Sty, 17Syn⁺, and 17 Δ Sty-Res from explants of latently infected rabbit TG. Although 17 Δ Sty reactivated from latently infected rabbits less efficiently than wild-type virus following in vivo epinephrine iontophoresis, it was possible that it would reactivate normally from TG explants. This was important to determine, because 17 Δ Sty has been shown to reactivate from explants derived from latently infected mice with kinetics similar to those of 17Syn⁺ (24). At 6 to 8 weeks after infection, rabbits were sacrificed and the TG were cultured in E-MEM with 10% fetal bovine serum and maintained at 37°C. The supernatant was tested for infectious HSV-1 as described above. All three viruses exhibited virtually the same frequency (75 to 100%) and kinetics (14.0 to 15.2 days) of infectious-virus recovery. Therefore, the 370-bp deletion in 17 Δ Sty had no influence on HSV recovery from TG of latently infected rabbits in the cocultivation explant assay.

The results of studies of 17 Δ Sty complement and confirm the results reported for 17 Δ 348 (4). A comparison of the deleted sequences common to the two viruses allows further assessment of potentially essential elements that could be involved in HSV reactivation (Fig. 1). One conclusion is that the ATG codon (a possible open reading frame start site) located at positions 119288 to 119290 and the region between positions 119213 and 119329 are not essential for induced reactivation in the rabbit. Bloom et al. (4) described three separate viral constructs (17 Δ 110, 17 Δ 91, and 17 Δ 116) containing smaller nonoverlapping deletions of approximately 100 bp each in the Δ 348 region that are not essential for induced reactivation in the rabbit. By deduction, these results suggest that the 201-bp region at the 5' end of Δ 348 is essential for induced reactivation. Conversely, it is likely that the 207-bp region at the 3' end of the deletion in 17 Δ 348 is not essential for induced reactivation. The 243-bp deletion common to 17 Δ Sty and 17 Δ 348 is located between nucleotides 119007 and 119250. In this region there are two AP-2 sites and one SP-1 site that could be involved in adrenergic reactivation. Two new viral constructs (Fig. 1) will be tested for their abilities to undergo adrenergically induced reactivation. We expect that the virus with the 201-bp (Δ 110 + Δ 91) deletion at the 5' end of Δ 348 will demonstrate impaired reactivation after epinephrine iontophoresis. This will provide additional evidence that this 201-bp region (i.e., 119010 to 119211) is essential for adrenergically induced reactivation, and more focused analysis of this essential region will be conducted in the future.

The deletions in 17 Δ Sty and 17 Δ 348 are within the region referred to as latency-associated promoter 2 (LAP-2) (5, 13). Bloom et al. (4) and Maggioncalda et al. (24) reported no major effects on the production of downstream transcription of LAT during acute or latent infection with 17 Δ Sty and 17 Δ 348. Goins et al. (13) and Chen et al. (5) assessed LAT transcription in cell culture and a mouse ocular model infected with HSV strain KOS deletion constructs. These constructs also had deletions in LAP-2. Although these studies did not assess HSV reactivation, the results suggest that the absence of LAP-2 does not alter the establishment of latency. If reactivation from latency mimics certain aspects of the lytic infection, the results

with LAP-2 deletions and with 17 Δ Sty and 17 Δ 348 complement and confirm each other.

Although rabbits latently infected with 17 Δ Sty exhibited reduced reactivation following epinephrine iontophoresis, the TG from these rabbits reactivated with wild-type kinetics in explant cocultivation. Epinephrine-induced in vivo reactivation in rabbits and in vitro reactivation in mouse explants appear to involve different kinds of genetic regulation. Thus, it is possible that the difference between the behavior of 17 Δ Sty as reported by Maggioncalda et al. (24) and the adrenergically induced reactivation described here is not an indication of a species difference but rather the result of the difference between in vivo and in vitro mechanisms. Finally, it appears that the ability of the virus to spontaneously reactivate from rabbits can be predicted, to the extent demonstrated by dLAT371 and similar mutants, by the explant cocultivation studies with mice. Therefore, we propose at least two distinguishable reactivation phenotypes, namely "spontaneous/explant" and "induced."

In addition to transcriptional activator sites such as those for SP-1 and AP-2 present in sequences essential for adrenergic reactivation, other physical properties in this region could be involved in the mechanisms controlling induced HSV reactivation. Sequence analyses of the deleted regions reveal a CpG island (high CpG-to-GpC ratio). Although the function of CpG's is not fully known, their methylation has been postulated to be involved in mechanisms controlling neuronal latency (6, 12, 22, 29, 32). The ratio of CpG/GpC in these regions was also higher than expected, compared with the ratio in the total HSV genome (23). Experiments assessing methylation of the LAT promoter and other specific regions of the LAT domain are ongoing.

Current experiments involve (i) specific fine mapping of the 201-bp region postulated to be essential for adrenergically induced reactivation, (ii) localization and analysis of other possible regions involved in adrenergically induced reactivation, such as cyclic AMP response element binding sites and AP-2 sites, and (iii) extensive analysis of the structural and regulatory components in the 201-bp segment in the LAT region. This research will allow us to assess the role of the LAT domain in neuronal latency and reactivation, especially the mechanisms involved in adrenergic induction.

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