Analysis of a Herpes Simplex Virus Type 1 LAT Mutant with a Deletion between the Putative Promoter and the 5' End of the 2.0-Kilobase Transcript

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A herpes simplex virus type 1 strain 17 mutant with a deletion between genomic nucleotides 118880 and 119250 was constructed and called 17ΔSty. The deletion removes most of a putative secondary LAT promoter (called LAPII) as well as 370 of the first 449 nucleotides of the proposed 8.5-kb transcript believed to be the precursor of 2.0-kb LAT. 17ΔSty was shown to produce major 2.0-kb LATs in tissue culture. Moreover, trigeminal nerves from latently infected mice contained an intact 1.45- to 2.0-kb LAT as well as the minor LATs which are recognized by probes specific for regions downstream of the 2.0-kb LAT. Finally, 17ΔSty reactivated with normal kinetics from the trigeminal ganglia of latently infected mice in the explant cocultivation assay and egressed from tissue culture cells as efficiently as wild-type virus. These results clearly show that the region deleted in 17ΔSty is dispensable for intact 2-kb LAT production, viral egress in tissue culture, and normal reactivation from latently infected neurons in mice.

The herpes simplex viruses (HSVs) establish productive and latent infections in animals (30, 34). Productive infections are those in which more than 70 different viral gene products are synthesized and progeny virus is produced, resulting in the death of the infected cell. Productive infection occurs in many cell types and is conveniently studied in tissue culture. Latent HSV infections occur in few cell types and are those in which virus gene expression is very limited and the host cell is presumably spared. In latently infected cells of the peripheral nervous system, transcription of only one region of the viral genome has been detected (12, 31-33). This region is called LAT, an abbreviation for latency-associated transcripts (31).

The LATs are a family of transcripts. The 2.0-kb LAT, which maps to genomic nucleotides (nt) 119461 and 121416 within the long internal repeats of the viral genome, is produced in both latent and productive infections (12). Latently infected neurons also accumulate 1.45-kb LAT transcripts, which are believed to be splice products from the 2.0-kb species. Since the 1.45- to 2.0-kb LATs accumulate to very high abundance in latently infected neurons and can be detected by Northern (RNA) blot analysis, they are called the major 2.0-kb LATs. However, there is some evidence that the 2.0-kb LAT is an intron, derived from an 8.5-kb precursor (8, 11). The 5'-terminal nucleotide of the 8.5-kb transcript has been mapped to nt 118801, with a possible polyadenylation signal at nt 127167 (8; reviewed in reference 12). Moreover, latently infected neurons also accumulate transcripts downstream and upstream of the 2.0-kb LATs, as detected by *in situ* hybridization studies (23). The downstream hybridization signals, called minor LAT1 (mLAT1), may in part represent detection of the 8.5-kb transcript. The origin and polarity of the upstream signal (called mLAT2) is unknown.

Since the LATs (major and minor) are the only HSV gene products detected during latent infection, they have been

extensively studied. LAT synthesis appears to be under the control of viral genomic sequences called the latency-associated promoter (LAPI) (18, 35, 36). Viral mutants with deletions of the LAT locus have been constructed and tested for the ability to reactivate from ganglia of latently infected animals in both *in vivo* and *in vitro* experiments. Since the same LAT mutants may behave differently in separate systems (see Discussion), comparisons made between different viral mutants must consider the system in which they have each been tested. One unifying feature of the mutants studied in the mouse model suggests that in general, mutants with deletions that remove LAPI alone (7, 26) or LAPI and surrounding sequences, such as 17ΔN/H (2), 1704 (29), and KOS *dl*1.8 (19), reactivate aberrantly from latently infected mice in the explant cocultivation assay compared with their parental, LAT-competent viruses. HSV mutants with deletions of regions downstream of LAPI, such as TB-1 (3), RH142 (15), 17ΔBstE (6), and KOS 8117 (16), reactivate normally.

There are almost 700 nt between the putative LAPI and the first nucleotide of the major 2.0-kb LAT. The functional significance, if any, of this region is unknown. If this region plays any role in the slow-reactivation phenotype, sequences between the 5' end of the 2-kb LAT and LAPI (Fig. 1A) must be involved, since these are the only sequences within the LAT locus commonly retained in normal reactivators TB-1, 17ΔBstE, and RH142 yet absent in the slow reactivators such as 17ΔN/H. In addition, since the 2.0-kb LAT may be derived from an 8.5-kb precursor (8), it was of interest to know what role, if any, the sequences between LAPI and the 5' end of the 2.0-kb LAT play in influencing the synthesis and accumulation of 2.0-kb major LAT and the mLATs. Since the sequences between LAPI and the first nucleotide of the 2.0-kb LAT have also been shown to have promoter activity, and are called LAPII (Fig. 1A) (13), it is of interest to know what role, if any, LAPII plays in the life cycle of the virus. Therefore, a strain 17 mutant with a deletion between the *StyI* sites at nt 118880 and 119250 was constructed, and its behavior in productively and latently infected cells was studied. This mutant, called 17ΔSty, was shown to synthesize LATs, egress normally from produc-

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FIG. 1. (A) Location of the deletion in 17ΔSty. The region of the HSV-1 genome encoding the major 2.0-kb LAT locus is shown. The relevant part of the internal repeat sequences is presented as the thick line which bifurcates into two thinner lines labeled Bam B and Bam E, since some restriction fragments containing the LAT locus may include sequences derived from the unique region BamHI B and E fragments. The region deleted in 17ΔSty is shown as a hatched box. For comparison, the deletions in strain 17-derived mutants 17ΔN/H and 17ΔBstE are also shown. Probes used in this study are shown as open boxes above the map. Landmark restriction endonuclease cleavage sites and the proposed boundaries of LAPI, LAP I, LAP II, the major 2.0-kb LAT, and the minor 8.5-kb LAT (mLAT1) are also indicated. The *SalI* site shown off the *BamHI* E map is relevant in the Southern analysis shown in panel B, although it is in the middle of UL5 and lies outside *BamHI*-E. 17ΔSty contains a new *BglII* site in place of the deleted sequences, and this is also shown. Abbreviations for restriction cleavage sites are as follows: Bam (*BamHI*), Bg2 (*BglII*), BstE (*BstEII*), Hpa (*HpaI*), Mlu (*MluI*), Not (*NotI*), Pst (*PstI*), Sal (*SalI*), Sty (*StyI*), Xba (*XbaI*), and Xho (*XhoI*). Nucleotide numbers (shown in parentheses) refer to positions along the HSV-1 genome. (B) Southern type blot analysis of 17ΔSty. Briefly, viral DNA was digested with *BglII* and *SalI*, resolved by agarose gel electrophoresis, Southern blotted, and hybridized to radioactively labeled probe pHH-0.180, which contains HSV-1 sequences between nt 120295 and 120469. Lanes: 1, molecular size markers (from top to bottom, 9.4, 6.6, 4.3, 2.3, and 2.0 kb) (see Materials and Methods); 2, strain 17 DNA; 3, 17ΔSty DNA; 4, 17ΔSty' DNA.

tively infected tissue cultures, and reactivate normally from the explanted trigeminal nerves excised from latently infected mice. The results suggest that the slow-reactivation and defective-egress phenotype seen with other LAT locus mutants, such as 17ΔN/H, is due to mutations upstream, not downstream, of LAPI.

MATERIALS AND METHODS

Cells and medium. CV-1 cells (American Type Culture Collection, Rockville, Md.) were maintained at 37°C and 5% CO₂ as monolayers in an Eagle medium (MEM; GIBCO, Inc.) supplemented with 5% newborn calf serum and 0.35% D-glucose.

Viruses and plasmids. HSV-1 strain 17, as described in reference 29, was the parent of all viruses used in this study. 17ΔBstE contains a deletion between nt 119198 and 120095 (6) which is similar to that in KOS 8117 (16) as well as an insertion of the rat β -glucuronidase gene. 17ΔSty was isolated from calcium phosphate-mediated transfections to CV-1 cells of genomic 17ΔBstE and pXhoΔSty DNAs. pXhoΔSty is a pGem-derived plasmid containing HSV genomic sequences between

nt 116961 and 123017 and deleted between the *StyI* sites at nucleotides 118880 and 119250. Transfections were done as in reference 3. Recombinant progeny containing genomes deleted between the *StyI* sites were isolated. The recombinant called 17ΔSty was studied in most detail. Plasmids pHH-0.180 (HSV-1 nt 120295 to 120469), p1-BstE (HSV-1 nt 119195 to 120092), pMlu-Bam (HSV-1 nt 121640 to 123430), and p2-BstE (HSV-1 nt 120092 to 121053) were used. Bacterial cultures were maintained as described elsewhere (22). Plasmids used for transfection of mammalian cells were either CsCl purified as described elsewhere (22) or prepared by the X

Prep. method (X/Gene Corp., Cambridge, Mass.) as instructed by the manufacturer.

Recombinant virus was made by transfecting CV-1 cells with calcium phosphate precipitates of genomic viral DNA and the indicated plasmids as described previously (3).

Single-step growth analysis for intra- and extracellular virus progeny. Confluent monolayers of CV-1 cells in T-25 flasks (Nunc) were inoculated with the indicated multiplicity of infection (MOI) of virus and incubated at 37°C for 60 min. Inoculum was removed, the monolayer was washed once with growth medium, and the cells were maintained in growth medium until harvested. At the indicated times postinoculation, the growth medium was harvested from the samples and centrifuged to remove any cellular debris (3,000 × g, 10 min). The titer of the extracellular, egressed virus contained in the medium was then determined on CV-1 cells. Also, at the same indicated times, intracellular virus was measured by harvesting the cells in phosphate-buffered saline (GIBCO BRL) with a rubber policeman, freezing and thawing the virus once, and then subjecting the virus to 1.5 min of sonication. The titer of this intracellular virus was then determined on CV-1 cells. The fraction of the total (intra- and extracellular) virus progeny found in the medium at a given time point was then reported as a percentage. This value was used as a measure of the virus's ability to egress from an infected cell.

Southern analysis of DNA. Virion DNA was purified as described previously (3) and digested to completion with restriction endonucleases as instructed by the manufacturer (Bethesda Research Laboratories). Digest products were resolved by agarose gel electrophoresis through 0.8% gels, denatured, neutralized, and transferred to nylon membranes (Southern blotted) as described elsewhere (22). Nylon membranes were hybridized with probes made radioactive with ³²P by the method of random priming as directed by the manufacturer (GIBCO BRL). Incubation and washing conditions for these blots were as in reference 2.

Northern analysis of RNA. RNA was isolated from infected cells and tissue preparations as described previously (2). Briefly, cells and tissue were dissolved in guanidine isothiocyanate solution and sedimented through CsCl cushions by ultracentrifugation. Pellets were resuspended in sterile water containing RNase inhibitor (RNasin; Promega, Inc.) and treated with glyoxal as described previously (2). RNA was resolved by electrophoresis through 1.2% agarose gels and transferred to GeneScreen membranes (Northern blotted) as described elsewhere (22). GeneScreen membranes (Dupont, Inc.) were hybridized to probes made radioactive by random priming (GIBCO BRL) as instructed by the manufacturer. Hybridization and washing conditions for blots probed with radioactive plasmid and radioactive restriction fragments were as in reference 28.

Infection of mice and explant reactivation assay. Four- to six-week old BALB/c mice (Jackson Laboratory) were inoculated with between 10⁵ and 10⁶ PFU of HSV-1 via the eye following corneal scarification. After at least 4 weeks postinoculation, mice were sacrificed. Trigeminal ganglia were removed and either sectioned for in situ hybridization analysis or placed whole onto monolayers of CV-1 cells growing in culture dishes (GIBCO). Monolayers were observed daily for the appearance of a cytopathic effect. Ganglia were transferred to new monolayers every 5 days. These methods are as described in reference 28.

In situ RNA hybridization. Trigeminal ganglia, removed from latently infected mice, were placed in paraffin and cut in to 4-μm slices with a microtome cutter. The probes for the major 2.0-kb LAT and mLAT were plasmids p1-BstE and

pMlu-Bam, radioactively labeled with [³⁵S] by nick translation. Details of the in situ hybridization protocol are provided in reference 23.

RESULTS

Construction of an HSV mutant deleted between the *StyI* sites of the major LAT region. To study the behavior of an HSV-1 mutant deleted between the LAPI and the 5' end of the major 2-kb LAT, HSV-1 strain 17 mutants containing a deletion between the *StyI* sites at positions 118880 and 119250 were generated and called 17Δ*Sty*. Briefly, 17ΔBstE genomic and plasmid pXhoΔ*Sty* DNAs were cotransfected to permissive CV-1 cells, and progeny virus was harvested as described in Materials and Methods. 17ΔBstE, derived from strain 17 virus, lacks the region between the *BstEII* sites at nt 119198 and 120095 (6). pXhoΔ*Sty*, derived from pXho (3), contains HSV genomic sequences between nt 116961 and 123017 as well as a deletion between the *Sty* sites (see Materials and Methods and Fig. 1A). Screening was done to find recombinant progeny resulting from the transfection which possessed both the HSV-1 sequences absent in 17ΔBstE and a deletion between the *StyI* sites as shown in Fig. 1A. One such isolate was called 17Δ*Sty* and was studied in most detail. As shown in Fig. 1B, restriction endonuclease analysis of the genome of 17Δ*Sty* shows that it has the expected genotype. HSV-1 strain 17 genomic DNA digested with *SalI* and *BglII* yields two bands of 9.1 and 6.1 kb which are detected by the pHH-0.180 probe (Fig. 1B, lane 2). This is because the pHH-0.180 probe is from a fragment located within the repeats (Fig. 1A) and recognizes only *SalI-SalI* fragments which are fusions of the internal repeat and long unique sequences (Fig. 1A). In 17Δ*Sty*, the sequence between the *StyI* sites was removed, and the ends were made blunt and converted to a *BglII* site through linker ligation. Thus, the pHH-0.180 probe recognizes only a single 1.7-kb *SalI-BglII* digest product (Fig. 1B, lane 3), and this novel *BglII* site is therefore diagnostic of the intended deletion and modification. There is no evidence of the 9.1- and 6.1-kb products, which are characteristic of strain 17, or the 3.0-kb product characteristic of the 17ΔBstE virus (data not shown). The digest pattern of 17Δ*Sty*^r (Fig. 1B, lane 4) is identical to that seen with strain 17. 17Δ*Sty*^r is a rescuant of 17Δ*Sty* in which the deletion in 17Δ*Sty* was corrected (see Materials and Methods). Should any phenotypes have been associated with 17Δ*Sty*, rescuants in which the deletion had been repaired would be important. That is, if a phenotype is due to the intended deletion, rescuants in which the deletion had been repaired should behave as wild-type virus.

These results show that 17Δ*Sty* has the expected deletion, as shown in Fig. 1A, and that the deletion was corrected in 17Δ*Sty*^r.

17Δ*Sty* grows normally in tissue culture and during the acute phase of infection in mice. LAT region mutants with deletions removing LAPI and surrounding region, such as 17ΔN/H, 1704, and 17ΔNotI/BstE (unpublished data), replicate normally in tissue culture but egress inefficiently (2). On the other hand, LAT mutants with lesions downstream LAPI replicate and egress normally from tissue culture (3, 10, 15). Therefore, a function that influences viral egress may be located between the 5' end of the major LAT and LAPI surrounding region. It was therefore of interest to know how 17Δ*Sty* would behave in culture. A 100% confluent monolayer of continuous CV-1 cells was infected with an MOI of 1 with either wild-type strains 17, 17Δ*Sty*, or 17Δ*Sty*^r. At various times after infection, samples of cells and culture medium were tested for virus by standard plaque assay. Virus in the culture

medium is evidence of egress. At 10 and 24 h after infection, approximately 8 and 11%, respectively, of the total strain 17 progeny was in the medium (1.7×10^4 and 8.0×10^6 PFU/ml). 17 Δ Not-Hpa, an egress mutant, releases only 1 to 2% of the total virus progeny into the media (2). 17 Δ Sty and 17 Δ Sty^r behaved similarly to wild-type strain 17, with 7 and 13% of the virus progeny egressed into the media at 10 and 24 h, respectively (1.5×10^4 and 1.0×10^7 PFU/ml for 17 Δ Sty and 2.0×10^4 and 7.0×10^6 PFU/ml for 17 Δ Sty^r).

The ability of 17 Δ Sty to replicate in mice during the acute phase of infection was also tested. BALB/c mice were infected in the eye as described in Materials and Methods with either strain 17, 17 Δ Sty, or 17 Δ Sty^r. On day 4, sets of mice were sacrificed, the trigeminal ganglia were removed, and the amount of infectious virus present was assayed. Again, all three viruses behaved similarly, growing to $4.0 \times 10^4 \pm 1.8 \times 10^4$ (wild-type strain 17), $2.3 \times 10^4 \pm 1.7 \times 10^4$ (17 Δ Sty), and $1.1 \times 10^4 \pm 0.70 \times 10^4$ (17 Δ Sty^r) PFU per trigeminal ganglion.

Major LAT induced by 17 Δ Sty during productive and latent infection. The major 2.0-kb LAT has been hypothesized to be derived from the processing of an 8.5-kb precursor by a splicing event (11). The 5' end of the 8.5-kb precursor has been mapped to nt 118801 (Fig. 1A) (reviewed in reference 12). Thus, 17 Δ Sty is lacking 370 of the first 449 nt of the 8.5-kb precursor. Moreover, although synthesis of these LATs has been shown to be under the control of LAPI in latently infected cells, a second promoter, located within the region deleted in 17 Δ Sty, has also been shown to be functional (13) and may operate in productively infected cells (24). In *in vitro* transfection assays, removal of the sequences deleted in 17 Δ Sty resulted in the loss of more than 85% of LAPII activity (13). Therefore, it was of interest to know if 17 Δ Sty would synthesize normal abundance and intact major LAT in productive and latently infected cells.

Total cellular RNA was harvested from CV-1 cells 7 h after infection with either strain 17, 17 Δ Sty, or 17 Δ Sty^r at an MOI of 10. RNA was resolved by agarose gel electrophoresis, Northern blotted, and hybridized to a probe which recognizes the major 2.0-kb LAT region. The results in Fig. 2A show that strain 17, mutant 17 Δ Sty, and rescuant 17 Δ Sty^r synthesized equal amounts of 2.0-kb LAT in productively infected cells.

Major 1.45- to 2.0-kb LAT production in latently infected cells was also determined. BALB/c mice were infected via the eye in the usual manner with either wild-type strain 17, mutant 17 Δ Sty, or rescuant 17 Δ Sty^r. Total RNA was prepared from trigeminal ganglia removed from mice 30 days after inoculation. This is at a time when latency has been shown to be established in wild-type infections, and no virus can be recovered from eye washings and tissue homogenates (data not shown). The RNA was resolved by agarose gel electrophoresis, Northern blotted, and hybridized to a LAT-specific probe as described above. The results are shown in Fig. 2B. The 1.45-kb LAT is characteristic of HSV latency, since it is not produced during productive infection. All three virus strains induce the synthesis and accumulation of similar amounts of 1.45- and 2.0-kb LATs.

Therefore, the absence of most of LAPII and 370 of the first 449 nt of the putative 8.5-kb LAT did not have any detectable impact upon 1.45- to 2.0-kb LAT synthesis and accumulation.

Distribution of major and minor LAT in neurons of mice latently infected with 17 Δ Sty, as detected by *in situ* hybridization. Although intact major LAT accumulated in trigeminal neurons of mice latently infected with 17 Δ Sty, the number of neurons expressing major LAT might have been reduced compared with wild-type virus. In addition, neurons latently infected with HSV express viral transcripts both downstream

FIG. 2. Northern blot analysis of major LAT produced by 17 Δ Sty in tissue culture and in latently infected cells. Total cellular RNA was isolated from CV-1 cells (A) or trigeminal ganglia from latently infected mice (B). RNA was resolved by agarose gel electrophoresis, Northern blotted, and hybridized to probe pHH-0.180, which recognizes the major 2.0-kb LAT (see Fig. 1 and Materials and Methods). (A) CV-1 cells were harvested 7 h after infection with an MOI of 10. Lanes: 2, mock infection; 3, strain 17; 4, 17 Δ Sty; 5, 17 Δ Sty^r. (B) RNA was isolated from trigeminal ganglia derived from latently infected mice. Lanes: 2, strain 17; 3, 17 Δ Sty; 4, 17 Δ Sty^r. Molecular size markers (lanes 1) are, from top to bottom, 9.49, 7.46, 4.40, 2.37, and 1.35 kb. Arrow indicates the 2.0-LAT (A) and 1.45- and 2.0-kb LATs (B).

and upstream the major 2-kb LAT (Fig. 1). These have been called mLAT1 and mLAT2, (8, 12), since they are present in insufficient abundance to be detected by Northern blot analysis and have been imaged only by *in situ* hybridization. Moreover, TB-1, which contains a deletion limited to within the 2-kb LAT, does not produce detectable mLATs (3). Thus, mLAT1 production or accumulation appeared to be influenced by mutations downstream of LAPI and within the major 2.0-kb LAT. Therefore, it was of interest to know if a deletion between the start of the 2.0-kb LAT and the first nucleotide of the 8.5-kb precursor would influence mLAT1 production.

The distribution of major and minor LAT production was studied by *in situ* hybridization of neurons derived from latently infected mice to probes that recognize either the major 2.0-kb LAT or mLAT1 region. Probe location is shown in Fig. 1A, and the results are shown in Fig. 3 and 4. Figure 3 shows that major LAT accumulates in the neurons derived from mice latently infected with strain 17, 17 Δ Sty, or 17 Δ Sty^r. The number of neurons from 17 Δ Sty-infected mice which were positive for the major LAT was similar to that seen in tissue from strain 17- and 17 Δ Sty^r-infected mice. The actual numbers of neurons counted and scored positive are summarized in Table 1. The large standard deviation between the trigeminal ganglia derived from wild-type-infected mice resulted from the fact that in the sections examined from one of the trigeminal ganglia, no LAT-positive neurons were observed. Figure 4 shows the pattern of hybridization to an mLAT1 probe, with the numbers of positive neurons presented in summary form in Table 2. Neurons derived from the trigeminal ganglia of mice latently infected with strain 17 or 17 Δ Sty accumulate nuclear mLATs. Again, trigeminal ganglia derived from mice latently infected with either strain 17 or 17 Δ Sty contained similar numbers of hybridizing neurons (Table 2). Therefore, the mutation in 17 Δ Sty does not influence the ability of the virus to accumulate major or minor LATs.

17 Δ Sty reactivates normally from trigeminal ganglia explanted from latently infected mice. LAT mutants fall into two

FIG. 3. Major LAT in neurons prepared from latently infected mice are detected by in situ hybridization. Trigeminal ganglia were prepared from BALB/c mice which were left uninfected (A) or were latently infected with either wild-type strain 17 (B), 17 Δ Sty (C), or 17 Δ Sty' (D). Sections of tissue were prepared and hybridized to radioactive probe p2-BstE, which recognizes the major 2-kb LAT (see Materials and Methods). No hybridization signals are detected in tissue prepared from uninfected mice, whereas dense signals are easily detected in tissue from infected mice. See Materials and Methods for details. Bars indicate 50 μ m.

categories with respect to the rate at which they reactivate from the trigeminal ganglia explanted from latently infected mice. Those with deletions of LAPI alone or LAPI and the first several hundred nucleotides of the transcribed region of the

2-kb LAT reactivate slowly (2, 7, 21, 26). Those that possess deletions limited to the region within the 2-kb LAT reactivate normally (3, 6, 15, 16, 20). Since 17 Δ Sty possesses a deletion of the region between the LAPI and the 2-kb LAT, it was

TABLE 1. Number of neurons positive for the major 2.0-kb LAT in trigeminal ganglia from latently infected mice^a

Virus strain ^b	No. of neurons counted ^c	No. of neurons positive ^d	% Positive/trigeminal ganglion $\pm$ SD ^e
17	>10,000	270	2.45 $\pm$ 3.1
17 Δ Sty	>10,000	226	2.26 $\pm$ 1.0
17 Δ Sty ^r	>6,000	144	2.47 $\pm$ 1.5

^a Four trigeminal ganglia were analyzed per virus. Each ganglion was derived from a separate mouse (i.e., four ganglia represent four separate mice).

^b Mice were latently infected, via the eye route, with strain 17, 17 Δ Sty, or 17 Δ Sty^r.

^c Neurons were counted as described in reference 27.

^d At more than 30 days after infection, trigeminal ganglia were explanted and sectioned for analysis by *in situ* hybridization, probing for expression of the major 2.0-kb LAT with the p1-BstE probe, as described in Materials and Methods.

^e Standard deviation refers to the variation of the percentage of neurons positive for hybridization from trigeminal ganglion to trigeminal ganglion. These data are derived from the mice represented in Fig. 3 and 4.

important to know into which category, with respect to reactivation, it would fall.

Therefore, BALB/c mice were inoculated via the eye in the usual manner with either wild-type strain 17, 17 Δ Sty, or 17 Δ Sty^r. At 30 days after infection, at a time when wild-type virus has established latency, mice were sacrificed and the trigeminal ganglia were placed in cultures of confluent monolayers of CV-1 cells. The results of a typical explant experiment are shown in Fig. 5. By day 9 following explantation, 100% of the ganglia removed from strain 17-infected mice reactivated HSV. This is typical of wild-type-infected ganglia (e.g., reference 3). The graph of negative control virus 17 Δ Not-Hpa (2) has been added to define slow reactivation. The trigeminal ganglia explanted from mice latently infected with either 17 Δ Sty or 17 Δ Sty^r behaved similarly to those from strain 17-infected mice: 100% reactivation by day 10. Therefore, the deletion in 17 Δ Sty has no significant influence upon the rate of HSV reactivation from trigeminal ganglia of latently infected mice in this explant assay.

DISCUSSION

Synthesis of LAT in neurons appears to be under the control of a promoter involving HSV genomic sequences between nt 118660 and 118870, called LAPI (1, 9, 25, 33, 35). A second, downstream promoter located between nt 118864 and 119461 has been identified and is called LAPII (13, 24). The possible functional activity of LAPII was reinforced in transfection studies and by the observation that a LAPI-negative virus still induced a 2.0-kb LAT homologous RNA in tissue culture, although the precise nature of this tissue culture RNA was not studied (24). In some circumstances, this LAPI-negative virus even reactivated normally (5). The biological significance of this promoter is unclear. HSV mutants with lesions limited to LAPI or sequences downstream and upstream of LAPI have also been studied. For example, HSV-1-derived mutants 17 Δ N/H (2), 1704 (29), and *d1*1.8 (19) all contain deletions that span LAPI and the first several hundred nucleotides of the major 2-kb LAT. Where studied, they have been shown to be unable to accumulate major and minor LAT species in latently infected neurons and reactivate aberrantly from latently infected animals *in vitro*. On the other hand, HSV mutants TB-1 (3), 17 Δ BstE (6), KOS 8117 (16), and RH142 (15) reactivated normally from the trigeminal ganglia explanted from latently infected mice. These mutants contained lesions well downstream LAPI. Moreover, point mutations within specific se-

quences of LAPI (such as the cyclic AMP response element) have been reported to influence reactivation rates (21). HSV isolates with mutations limited to the LAPI vary with respect to their reactivation rates. Strain 17-derived LAPI mutant 17 Δ Pst reactivated aberrantly in the explant reactivation mouse models from both dorsal root and trigeminal ganglia (7). The strain KOS LAPI mutant was, in our hands, far less defective in reactivation in the mouse system (5), and others have also found that the KOS strain LAPI mutant reactivates normally from dorsal root ganglia explanted from latently infected mice (7). However, another group has reported that lesions within the LAPI of strain KOS marginally influence the reactivation rate (26).

In this regard, the behavior of LAT mutants appears to depend upon (i) the strain of the virus in which the mutation has been created (7), (ii) the anatomical site in which the latent infection has been established (26), and as (iii) the species of animal used and whether or not the reactivation is an *in vivo* or *in vitro* induction (e.g., references 14 and 17). For example, some mutants, e.g., X10-13, reactivate normally in *in vitro* explant assays (17) while reactivating slowly in *in vivo* assays (14). Perhaps the presence of host immune factors in the *in vivo* assay, not found in the *in vitro* explant assay, may account for some of these differences. There are also differences between laboratories using the *in vitro* explant assay when defining slow reactivation, causing different groups studying the same virus to draw different conclusions from the reactivation data (5, 26). Finally, the condition of the ganglia and the permissiveness of the cell monolayer, as well as the explant technique used, may all create variability in an *in vitro* assay which could skew an interpretation of a mutant's reactivation profile. Thus, it is essential that the particular system used be considered when one is drawing conclusions about a virus's behavior and making comparisons between LAT mutants.

Overall, it is still possible to conclude that viruses with lesions such as 17 Δ N/H, which remove or alter LAPI and surrounding regions, are most severely impaired for reactivation and those that have defects limited to within the major 2.0-kb LAT reactivate normally. Therefore, if the major LAT locus played any role in the reactivation process, sequences between LAPI and the 5' end of the 2-kb LAT must be involved. This seemed logical, since the 5' end of the 8.5-kb mLAT was spared in the downstream mutants. There is evidence that the 8.5-kb transcript is polyadenylated, and it is hypothesized to serve as the mediator of LAT function (8).

This report shows that the DNA sequences between LAPI and first nucleotide of the major 2-kb LAT (eliminating 85% of the activity associated with LAPII) are not essential for (i) normal reactivation from trigeminal ganglia of latently infected mice in the explant cocultivation assay, (ii) production of intact 1.45- to 2.0-kb LATs in latently infected cells or production of the 2-kb LAT in tissue culture, (iii) production of mLATs in latently infected neurons, or (iv) proper egress from productively infected cells in culture. Therefore, the region between LAPI and the 2-kb LAT is completely dispensable for all of these functions, and the elimination of LAPII is without detectable phenotypic effect.

The ability of 17 Δ Sty to synthesize intact major LAT and produce mLAT is particularly interesting. If the 8.5-kb transcript identified by Devi-Rao et al. (8) is the precursor of the 2.0-kb LAT, then 370 of the first 449 nt are dispensable. mLATs are transcripts hybridizing to probes specific for regions downstream (mLAT1) and upstream (mLAT2) of the 2.0-kb LAT (23). Since TB-1 (3) bears a deletion (and insertion) only within the 2.0-kb LAT and does not accumulate any

FIG. 4. mLAT1 detected in neurons prepared from latently infected mice by in situ hybridization. Trigeminal ganglia from mice left uninfected (A) or latently infected with either wild-type strain 17 (B), 17 Δ Sty (C), or rescuant 17 Δ Sty' (D) were sectioned and hybridized to radioactive probe pMlu-Bam, which recognizes mLAT1 (see Fig. 1 and Materials and Methods). No specific hybridization to tissue from mock-infected mice is seen, whereas clear specific signals are apparent over cells derived from mice latently infected with the indicated virus. See Materials and Methods for details. Bars indicate 50 μ m.

detectable mLATs, it is curious that 17 Δ Sty is completely mLAT1 competent.

These data suggest that the HSV genomic region responsible for the delayed reactivation phenotype observed in 17 Δ N/H and other LAT null mutants maps either within the

LAPI itself (as implied in reference 7) or upstream of those sequences. KOS 62, which contains a deletion and insertion within the major 2.0-kb LAT between nucleotides 118842 and 120466, was reported to reactivate slowly from latently infected mice and exist in fewer latently infected neurons compared

TABLE 2. Number of neurons positive for mLAT in trigeminal ganglia from latently infected mice^a

Virus strain ^b	No. of neurons counted ^c	No. of neurons positive ^d	% Positive/trigeminal ganglion $\pm$ SD ^e
17	4,940	104	1.8 $\pm$ 0.3
17 Δ Sty	2,393	42	2.6 $\pm$ 0.65
17 Δ Sty ^r	4,040	98	2.1 $\pm$ 0.46

^a Four trigeminal ganglia were analyzed per virus. Each ganglion was derived from a separate mouse (i.e., four ganglia represent four separate mice).

^b Mice were latently infected, via the eye route, with strain 17, 17 Δ Sty, or 17 Δ Sty^r as described in Materials and Methods.

^c Neurons were counted as described in reference 27.

^d At more than 30 days after infection, trigeminal ganglia were explanted and sectioned for analysis by in situ hybridization, probing for expression of the mLAT with the pMlu-Bam probe, as described in Materials and Methods.

^e Standard deviation refers to the variation of the percentage of neurons positive for hybridization from trigeminal ganglion to trigeminal ganglion. These data are derived from the mice represented in Fig. 3 and 4.

with LAT-competent controls (26). The KOS 62 mutant includes the deletion in 17 Δ Sty and extends more than 1 kb downstream, raising the possibility that sequences downstream (not upstream) of the 17 Δ Sty deletion are responsible for slow reactivation. However, the reduced reactivation rate of KOS 62 was modest compared with that seen in other slowly reactivating viruses. The most severe defect of KOS 62 appeared to be the reduced number of neurons harboring latent virus (26). It may be that deletions such as those in KOS 62 have more impact upon the efficiency or maintenance of latency than upon the reactivation rate in vitro. Reactivation rates may be more significantly influenced by sequences im-

FIG. 5. Explant reactivation profiles of trigeminal ganglia derived from latently infected mice. Trigeminal ganglia were explanted from mice latently infected with either strain 17 (open boxes), 17 Δ Sty (diamonds), or rescuant 17 Δ Sty^r (solid boxes) and placed on monolayers of CV-1 cells. The curve of a known slow reactivator, 17 Δ Not-Hpa (open diamonds) (2) has been added as a negative control. The x axis shows the time represented as days postexplantation. The y axis shows the cumulative percentage of trigeminal ganglia which have reactivated virus. The total numbers of trigeminal ganglia used were 12, 18, and 6 for strain 17, mutant 17 Δ Sty, and rescuant 17 Δ Sty^r, respectively. The explant reactivation assay is described in Materials and Methods and reference 28.

mediately upstream of LAPl. Given the different behaviors of the same mutants in different models of latency and reactivation, it will be important to study 17 Δ Sty in the in vivo rabbit eye system. In addition, more work must be done to fine map the regions responsible for these phenotypes and study the viral functions involved.

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