A Deletion Mutant of the Latency-Associated Transcript of Herpes Simplex Virus Type 1 Reactivates from the Latent State with Reduced Frequency

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We have generated and characterized a deletion mutant of herpes simplex virus type-1, dlLAT1.8, which lacks the putative promoter region, transcriptional start site, and 1,015 base pairs of the DNA sequences specifying the latency-associated transcripts (LATs). When tested in a CD-1 mouse ocular model, dlLAT1.8 was replication competent in the eye and in ganglia during acute infection but reactivated from explant cultures of ganglia with reduced efficiency (49%) relative to those of wild-type and marker-rescued viruses (94 and 85%, respectively) despite the fact that levels of mutant viral DNA in ganglia during latent infection were comparable to wild-type levels. The neurovirulence of KO5 was not significantly altered by the removal of sequences specifying the LATs, as judged by numbers of animals dying on or before 30 days postinfection. Examination of ganglia latently infected with dlLAT1.8 by in situ hybridization revealed no LAT expression. The genotype of reactivated virus was identical to that of input dlLAT1.8 virus as judged by Southern blot analysis. These studies suggest that although the LATs are not essential for the establishment and reactivation of latency in our model, they may play a role in determining the frequency of reactivation of virus from the latent state.

Herpes simplex virus (HSV) exhibits two modes of gene expression within the infected host. During the lytic phase of infection, viral genes are expressed in coordinate fashion, according to their temporal class: immediate early (IE or α), early (E or β), or late (L or γ) (6, 7). In contrast, during the latent phase of infection, HSV type 1 (HSV-1) gene expression is almost completely repressed. The only abundant viral gene products detected to date in the neuronal nuclei of latently infected sensory ganglia of mice, rabbits, and humans are the abundant latency-associated transcripts (LATs; 1, 20, 31). Similar patterns of latency-related RNA transcription have been reported during bovine herpesvirus type 1 latency in a rabbit model and pseudorabies virus latency in swine (18, 19). The HSV-1 LATs are derived from the IE strand opposite which encodes the mRNA specifying the IE regulatory protein ICP0 and overlaps 30% of the 3' terminus of the ICP0 gene (Fig. 1). Northern (RNA) blot analysis (27, 28, 31, 38) has demonstrated that the LATs consist of at least three transcripts of 2.0, 1.5, and 1.45 kilobases (kb), which are partially colinear.

Stevens et al. (31) proposed that LATs function to repress the expression of the immediate-early transactivating gene product ICP0 which if active would initiate productive infection. Thus, these investigators hypothesized, LAT expression is the factor responsible for suppressing productive infection during latency. This theory, if correct, would mean that a virus lacking the sequences coding for LATs would be unable to establish or maintain a latent infection. This is consistent with the observation that deletion mutants lacking sequences that code for ICP0 can establish latency but fail to reactivate efficiently (12).

In the present study, we have begun to address the question of the function of the LATs by the isolation and characterization of a mutant specifically deleted in the sequences which encode these transcripts. The mutant was tested in a CD-1 mouse eye model, and its behavior during acute infection was compared with those of the wild-type parent, HSV-1 strain KOS, and FSLAT', a marker-rescued virus. Latently infected trigeminal ganglia were examined for the presence and number of viral genomes by slot-blot DNA hybridization, for LAT expression by in situ RNA hybridization, and for the ability to yield reactivable virus by cocultivation techniques. We found that in spite of the replication competence of the LAT deletion mutant, it was reactivation impaired when compared with wild-type and marker-rescued viruses. It thus appears that the LATs, although not essential for the establishment or reactivation of latency, are required for the efficient reactivation of virus from the latent state.

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MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were propagated as previously described (22). Procedures for the growth and assay of the KOS strain of HSV-1 have been previously described (24), and identical procedures were used for growth and assay of dlLAT1.8 and FSLAT'. One-step growth assays were performed using standard procedures as described previously (25). The growth and assay of the temperature-sensitive mutant tsY46, which contains a mutation in the gene specifying the essential immediate-early regulatory protein ICP27, has been described elsewhere (22).

Plasmids. The locations of HSV-1 DNA sequences in
plasmids used for the production of the mutant in this study are shown in Fig. 1. Plasmid pBBl containing the BamHI B fragment in pBR325 was subcloned into pUC8 as two pieces whose limits are the following restriction sites: BamHI to PstI (pUPB1) and PstI to BamHI (pLPB1). Plasmid pΔ1.8LPB1 was derived from pLPB1 by cutting the plasmid with HpaI, adding PstI linkers, and cutting to completion with PstI, followed by religation.

Plasmid pΔLAT1.8 was produced by cutting pUPB1 with BamHI, filling in the ends with the Klenow fragment of DNA polymerase I, ligating HindIII linkers to the plasmid, and cutting with HindIII and PstI. The resultant 5.3-kb fragment was separated by agarose gel electrophoresis, electroeluted, purified over an Elutip-d column (Schleicher & Schuell, Inc., Keene, N.H.), and ligated to pΔ1.8LPB1 which had been cut with HindIII and PstI. The deletion in pΔLAT1.8 removes the putative promoter region (39), transcriptional start site, and 1,015 base pairs of the sequences specifying the LATs. In addition, the limits of the deletion were selected so as not to affect ICP0 (15) or the 0.9- and 1.1-kb transcripts described by Spivack and Fraser (29). Plasmid pPH was constructed by cutting pLPB1 with PstI and HpaI. The resultant 1.4-kb fragment was separated, electroeluted, and purified as described above and ligated to pTZ18R (U.S. Biochemical Corp., Cleveland, Ohio) which had been cut with PstI and SmaI. All enzymes and linkers utilized in the construction of the plasmids were obtained from New England Biolabs, Beverly, Mass., and used according to the instructions of the manufacturer.

**Nucleic acid isolation.** Bacterial plasmid DNA was isolated and purified as previously described (13). Infectious HSV-1 DNA was purified as described by Goldin et al. (4).

**Generation of mutants by transfection.** Mutant dLAT1.8 was introduced into the HSV genome by rescuing the ts mutation in tsY46. For this purpose, plasmid pΔLAT1.8 was linearized and mixed with infectious tsY46 DNA and Vero cells were transfected with the mixture as described previously (22). Viral DNA was obtained from plaque isolates (2), cut with PstI and BamHI, and analyzed by Southern blot hybridization (26) using pLPB1 as the probe for detecting deleted LAT sequences (Fig. 1). The deletion mutant was plaque purified three times, and a high-titer stock was prepared.

**Generation of marker-rescued virus.** The deletion mutation in dLAT1.8 was repaired by cotransfection of pRFS (Fig. 1) with infectious dLAT1.8 DNA into Vero cells as previously described (22). Plasmid pRFS (Fig. 1) does not overlap with sequences that encode ICP0 (15), ICP27 (14), or the 20-kilodalton transcript described by Spivack and Fraser (29), although there is a small region of overlap with the 22-kilodalton transcript (29). Viral DNA was obtained from plaque isolates (2) and screened by DNA hybridization using a slot-blot apparatus (Schleicher & Schuell) according to the instructions of the manufacturer, using pIPH as the probe for inserted sequences. Plaques which hybridized with pIPH were further tested by Southern blot hybridization (26), using pLPB1 as the probe for repaired LAT sequences (Fig. 1). The marker-rescued virus was plaque purified three times, and a high-titer stock was prepared.

**Animal procedures.** Seven-week-old randomly bred CD-1 mice (Charles River Laboratories, Kingston, N.Y.) were anesthetized with sodium pentobarbital, corneas were scarified, and 20 μl of virus at the appropriate titer per eye was added as previously described (35). Assays of acute and latent infection were performed as previously described (12). To test for non-latent infectious virus on day 30, ganglia were removed and immediately frozen, thawed, homogenized, and assayed on Vero cells. Reactivated viruses were retained for analysis by Southern blot hybridization (26) for comparison of their genotypes with those of input virus.

**Slot-blot hybridization.** Slot-blot DNA hybridization, performed to assess levels of viral DNA within individual latently infected ganglia, was done as previously described (12).

**In situ hybridization.** The methods used for in situ hybridization have been previously described (5, 34). For probes, double-stranded DNAs were labeled by nick translation using [3H]deoxynucleoside triphosphates. Purified insert DNAs or whole plasmids gave similar results when used as hybridization probes. DNA probes used for this study were pPH, containing a 1.4-kb fragment from within the LAT-encoding region (Fig. 1), and pSG1-ES1 (17), containing the largest EcoRI-SacI fragment from EcoRI J·K of the HSV-1 genome in the L form.

**RESULTS**

**Introduction of the LAT deletion into the viral genome.** In addition to the 1.8-kb deletion in LAT sequences, the plasmid pΔLAT1.8 contains the wild-type gene for ICP27 (Fig. 1) and was therefore used to rescue the ts mutation in
**FIG. 2.** Southern blot showing a comparison of LAT-coding sequences in viruses resulting from cotransfection of Vero cells with pΔLAT1.8 with tsY46 DNA (lanes 1 through 10). Arrows show the positions of wild-type (4.6 kb) and deleted (2.9 kb) bands in lanes containing marker plasmids. Viral DNAs were cut with PstI and BamHI and probed with pLPB1 (Fig. 1). d/LAT1.8 was obtained by three plaque purifications of the virus whose DNA is shown in lane 10.

**tsY46.** This procedure was utilized because demonstration of ts+ virus in transfection progeny would likely reflect the simultaneous transfer of the deletion in LAT-coding sequences into the viral genome. Plaque isolates were screened for the ability to grow at the nonpermissive temperature, and ts+ plaque isolates were selected for further analysis.

The Southern blot in Fig. 2 shows the DNA restriction fragment profiles of the LAT-coding region of KOS, tsY46, and 10 ts+ plaque isolates resulting from cotransfection of Vero cells with infectious tsY46 DNA and pALAT1.8, using parental plasmids as markers for wild-type and deleted fragments. Plaque isolates in lanes 7 and 10 show that the deletion was introduced into both copies of LAT, the sizes of their LAT-encoding fragments being indistinguishable from that of pALAT1.8, but 1.7 kb smaller than the analogous fragments in KOS, tsY46, and pBb1. The plaque isolate analyzed in lane 10 (d/LAT1.8) was plaque purified an additional two times and used for further study.

Southern blot analysis of the marker-rescued virus FSLAT (produced by cotransfection of Vero cells with prFS and infectious d/LAT1.8 DNA) indicated repair of both copies of LAT, the size of the LAT-encoding fragment being indistinguishable from that of the analogous fragment of the wild-type virus (data not shown).

**FIG. 3.** Growth of KOS, d/LAT1.8, and FSLAT during a one-step growth curve experiment in Vero cells infected at a multiplicity of 10 PFU per cell. Total virus yields were measured at 1, 2, and 18 h postinfection. The 0 h time points were determined by back-titration of virus inocula.

**Growth properties of d/LAT1.8 in vitro.** The deletion mutant d/LAT1.8 exhibited growth kinetics and yields of infectious virus comparable to those of wild-type and marker-rescued (FSLAT) virus on Vero cells in a one-step growth assay (Fig. 3), demonstrating that LATs were not required for growth in Vero cells in culture. When plated onto Vero cell monolayers under a methylcellulose overlay, d/LAT1.8, FSLAT, and KOS produced plaques of comparable size.

**Growth properties of d/LAT1.8 in vivo.** Having shown previously that a dose of 2 × 10^6 PFU per eye of KOS routinely produces reactivatable latency in 100% of ganglia while killing less than 30% of the animals (12), we selected 2 × 10^6 PFU per eye as the standard dose for testing d/LAT1.8 and FSLAT.

The mutant d/LAT1.8, FSLAT', and wild-type virus behaved very similarly during acute infection in the eye (Fig. 4) and in trigeminal ganglia (Fig. 5) over a series of time points. Similar results were obtained in three separate experiments. The viruses were almost at the lower limit of detection in eye swabs 3 h following inoculation of 2 × 10^6 PFU per eye but were readily detectable in eye swabs by 24 h (approximately one growth cycle), when peak titers were reached. Levels of virus in eyes declined during the next 6 days postinfection.

At 18 h postinfection, low levels of d/LAT1.8 and FSLAT' were detected in trigeminal ganglia by direct virus assay. KOS, d/LAT1.8, and FSLAT' were all readily detectable in ganglia by 48 h postinfection, reaching peak titers around 72 h postinfection. Because we wished to test ganglia for the presence of reactivatable virus on day 30, we first assayed extracts of homogenized ganglia at this time for the presence of nonlatent infectious virus. None was detected in six ganglia infected 30 days previously with d/LAT1.8, four ganglia infected with FSLAT', or six ganglia infected at that time with wild-type virus.
Measurement of viral DNA levels in ganglia during latency. Although no infectious virus was detected in ganglia on day 30, viral DNA was readily detectable by slot-blot hybridization in ganglia from mice inoculated 30 days previously with KOS or dlLAT1.8 (Fig. 6). The levels of viral DNA in mutant- and wild-type-infected ganglia were comparable, containing between 0.3 and 3.0 copies of viral DNA per cell. The observation that viral DNA levels in ganglia latently infected with the two viruses were comparable has been further confirmed by the polymerase chain-reaction technique (data not shown).

In situ hybridization studies of latently infected ganglia. To compare LAT expression in ganglia latently infected with dlLAT1.8, FSLAT+, and KOS, we performed in situ hybridization studies on 30-day ganglia. Using pSG-ES1 (Fig. 7) or p1PH (data not shown) as probes for LATs, hybridization studies of ganglia latently infected with KOS and FSLAT+ revealed the presence of LATs typically as dense grains over neuronal nuclei. All 24 ganglia from 12 mice latently infected with KOS and 4 ganglia from 2 mice latently infected with FSLAT+ showed strong nuclear hybridization in neurons. In contrast, none of 18 ganglia from nine mice latently infected with dlLAT1.8 or 14 ganglia from seven mock-infected mice demonstrated significant nuclear hybridization with pSG-ES1 (Fig. 7). Thus, no significant level of transcription from this region of the viral genome could be detected in ganglia latently infected with the LAT deletion mutant.

Reactivation and neurovirulence studies of KOS, dlLAT1.8, and FSLAT+. While the growth kinetics in vitro and in vivo of KOS, dlLAT1.8, and FSLAT+ were similar (Fig. 3, 4, and 5) and the levels of viral DNA in latently infected ganglia were found to be comparable for KOS and dlLAT1.8 (Fig. 6), the latency phenotypes of the viruses were significantly different (Table 1). Virus reactivated from 17 of 18 (94%) ganglia latently infected with KOS and 17 of 20 (85%) ganglia latently infected with FSLAT+ after 5 days in explant culture, whereas reactivation from ganglia latently infected with dlLAT1.8 was reduced: only 21 of 43 (49%) ganglia produced virus after 5 days in explant culture.

Whitby et al. (40) have reported that the addition of 200 mM dimethyl sulfoxide (DMSO) to cultures during cocultivation of KOS or dlLAT1.8-infected mice with the reactivation and neurovirulence studies of KOS, dlLAT1.8, and FSLAT+.
vation greatly enhances the reactivation frequency of HSV-1 from latently infected ganglia. In this study, the addition of 200 mM DMSO did not greatly increase the recovery rate of d/LAT1.8 (8 of 14 or 57%) from latently infected ganglia compared with the rate from untreated cultures (21 of 43 or 49%) (Table 1). This is in contrast to our latency studies with the ICP0 deletion mutant dlx3.1, in which the recovery of virus following the addition of DMSO to explant cultures was greatly increased, from 0 of 46 (0%) to 7 of 20 (35%) (12).

The removal of sequences specifying the LATs had little if any effect on neurovirulence, as judged by the relative numbers of animals which died in the 30 days following inoculation of 2 × 10⁶ PFU per eye of KOS (13 of 43 or 30%), FSLAT' (13 of 38 or 34%), or d/LAT1.8 (15 of 55 or 27%).

**Examination of the genotypes of reactivated strains.** Southern blot analysis of the DNAs of five reactivated isolates from ganglia latently infected with dlLAT1.8 demonstrated that the genotype of the reactivated virus was identical to that of the input (deleted) virus with regard to the sequences which specify the LATs (Fig. 8). This experiment eliminated the remote possibilities of contamination or genetic reversion.

Taken together, these studies indicate that LATs are not required for the establishment or reactivation of viral latency but that they are required for wild-type frequencies of reactivation of virus from the latent state in this model system.

**DISCUSSION**

The discovery that HSV-1 is transcriptionally active in humans and mice during ganglionic latency (3, 34, 36) and that the mRNAs so produced partially overlap and are complementary to the message encoding ICP0 (20, 28, 31)

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**TABLE 1. Behavior of KOS, LAT deletion mutant d/LAT1.8, and marker-rescued virus FSLAT' during acute and latent infection in a CD-1 mouse eye model**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Peak acute titer in³</th>
<th>No. of ganglia reactivated/no. tested³ (%)</th>
<th>No. of deaths/no. infected³</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>8 × 10⁶</td>
<td>4 × 10³</td>
<td>17/38 (94)</td>
</tr>
<tr>
<td>d/LAT1.8</td>
<td>2 × 10⁶</td>
<td>3 × 10³</td>
<td>21/43 (49)</td>
</tr>
<tr>
<td>FSLAT'</td>
<td>7 × 10⁵</td>
<td>5 × 10³</td>
<td>17/20 (85)</td>
</tr>
</tbody>
</table>

³ Peak eye swab and ganglion titers are derived from Fig. 4 and 5. Geometric mean titers are shown for acute infection.

⁴ Explant cultures were performed on day 30 for the assay of latent infection. DMSO was added to the medium of some explant cultures to a final concentration of 200 mM.

⁵ The number of animals which died on or before day 30 postinfection following corneal inoculation of 2 × 10⁶ PFU per eye.

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**FIG. 7.** In situ hybridization of trigeminal ganglia mock infected (B) or infected with KOS (A), d/LAT1.8 (C), or FSLAT' (D). The viral DNA insert in pSG-ES1 (20) was purified and labeled as the hybridization probe for panel C.
has led to much speculation as to the function of these so-called LATs. Three possible roles for the LATs were postulated by Stevens et al. (31). First, the LATs may encode a protein which is important in triggering the reactivation of virus from the latent state, and the nuclear restriction of the LATs prevents their translation, thereby maintaining latency. Second, the LATs may code for a protein which is involved in maintaining the latent state. Third, the LATs may act by antisense repression to suppress the expression of ICP0 during latency.

Examination of the sequence specifying the LATs reveals open reading frames but no convincing evidence that they are likely to code for a protein (14). In addition, the nuclear localization and lack of polyadenylation of these transcripts make it unlikely that a protein is specified by the LATs during latency (38). Wagner et al. (38) used a 14-amino-acid peptide synthesized on the basis of the second HSV-1 LAT open reading frame to raise serum in rabbits. Despite the proven reactivity of this serum with the synthetic peptide, no antigens were detected by immunohistological examination of acutely or latently infected ganglia. Taken together, these data argue against the possibility that the LATs code for a protein, although further characterization of minor poly(A)\(^{\prime}\) transcripts (16, 38) may reveal otherwise.

The idea that the LATs function by antisense repression of ICP0 is an attractive one which has received support by analogy from other recently described antisense repression systems (8, 10, 11, 33, 37). To date, however, there is no direct evidence of such a mechanism for the LATs from studies of HSV-1 latency. There is, however, evidence of an important role for ICP0 in latency. ICP0 is a potent transcriptional transactivator of all three classes of HSV genes. It is one of the first proteins to be synthesized during productive infection and hence is an excellent candidate for an initiator protein in reactivation. Consistent with this hypothesis is the observation that certain deletion mutants that lack ICP0 but specify the LATs are unable to reactivate from latency in a CD-1 mouse ocular model (12; unpublished data). Although it is not absolutely essential for virus replication, ICP0 has been shown to be important for virus growth in cell culture at high multiplicities of infection (23, 32). The small amounts of virus likely produced and the consequent low multiplicities of infection encountered during reactivation from the latent state may explain the importance of ICP0 in the reactivation process.

The data generated in this study show that LATs are not essential for the efficient growth of the virus in cell culture or in mice and that establishment or reactivation of the latent state can be achieved in the absence of LATs. This is in agreement with other studies (9, 30), although this is the first mutational study of the LATs to be performed using a defined deletion mutation which completely removes both copies of the LATs and a marker-rescued virus in an isogenic system. It is difficult at this stage to envisage how \(d\)LAT1.8 can become latent in ganglia if ICP0 antisense repression is the sole means by which the latent state is established and maintained following lytic infection. This difficulty is reinforced by the finding that \(d\)LAT1.8 is no more lethal than KOS or FSLAT\(^{\prime}\) in our mouse model. In the absence of LATs, therefore, the suppression of gene expression characteristic of the establishment of latency must be achieved by an alternative pathway, possibly, as suggested by Roizman and Sears (21), involving ICP4. ICP4 is both a negative and positive regulator of viral gene expression and may act in these respective capacities to establish and reactivate latency. This alternative pathway may actually augment the function of LAT in the wild-type virus, serving to further down- and up-regulate gene expression during the establishment and reactivation of latency.

Although the LATs are not essential for the establishment or reactivation of latency, reactivation appears to be more efficient in the presence of LAT sequences. This is consistent with the studies of Steiner et al. (30) but different from the work of Javier et al. (9), in which the lack of sequences specifying the LATs was not associated with any change in reactivation efficiency. The observed reduction in reactivation efficiency of \(d\)LAT1.8 can be attributed specifically to sequences within prf1 (most likely those which specify the LATs), since the marker-rescued virus FSLAT\(^{\prime}\) reactivates with a frequency comparable to that of the wild-type virus.

The removal or restoration of the LATs did not alter the lethality of KOS in our system, as judged by the numbers of mice dying on or before 30 days postinfection with \(d\)LAT1.8 KOS, or the marker-rescued virus FSLAT\(^{\prime}\). This is at variance with the findings of Steiner et al. (30), who found that their LAT deletion mutant (1704) was somewhat less lethal than its wild-type parent strain 17\(^{\prime}\). Although the reason for this difference is not certain at this time, it is possible that the reduced virulence of 1704 is associated with a secondary mutation outside LAT-encoding sequences (30).

Although comparable levels of \(d\)LAT1.8 and KOS DNA were found in ganglia during latency, as judged by slot-blot hybridization, this assay fails to address the question of the structural configuration or biological activity of the latent genomes. Microscopic examination of tissue sections from ganglia latently infected with \(d\)LAT1.8 by in situ hybridization revealed more extensive cytopathic changes than in ganglia infected with KOS or FSLAT\(^{\prime}\) (unpublished results). This is consistent with the finding that \(d\)LAT1.8 tends to reach slightly higher titers than KOS or FSLAT\(^{\prime}\) in eyes and in ganglia at early times during acute infection. One could speculate, therefore, that the repression of gene expression in the absence of the LATs during the establish-
ment of latency may have been less efficient, leading to increased virus-specific damage and possibly fewer viable genome-containing neurons available for reactivation. Under these circumstances, the levels of viral DNA could appear similar, suggesting similar establishment capability, but the levels of biologically relevant DNA may be quite disparate, possibly accounting for the differing rates of viral reactivation upon explant culture. Further studies are needed to determine whether the increased histopathologic changes associated with dFLAT1.8 infection are due to increased spread of the virus during early stages in the establishment of latency.

It is also possible, as suggested by Spivack and Fraser (28), that the LATs may play a more critical role in the maintenance of latency once it has been established. The enhanced cytopathic damage associated with dFLAT1.8 infection of ganglia could therefore be a consequence of limited reactivation events which lead to the destruction of some genome-containing neurons prior to explant culture. However, no free infectious virus was found in ganglia 30 days postinfection, suggesting that if such limited reactivation events occur, the amounts of virus produced are quite small or the virus is rapidly cleared.

From these results it can be stated that the sequences which code for the LATs are not essential for the establishment or reactivation of viral latency in this model system. It is clear, however, that deletion of these sequences is associated with a reduced frequency of reactivation, although the reason for this reduction is not clear at this time.

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