**In vivo** characterization of site-directed mutations in the promoter of the herpes simplex virus type 1 latency-associated transcripts

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Transient expression assays in PC12 cells showed that the cAMP response element (CRE) and the TATA box of the herpes simplex virus type 1 latency-associated transcripts (LATs) promoter are essential for basal expression. Recombinant viruses were generated containing site-specific mutations in these motifs. The abilities of these recombinants to replicate, express LATs and reactivate from latency were compared with wild-type and marker-rescued viruses in a murine ocular model. The acute replication of these TATA and CRE mutant viruses was at a level equivalent to their respective marker-rescued viruses. The reactivation of virus was unaffected by mutation in the TATA box as compared with wild-type or marker-rescued viruses. In situ hybridization of TATA box mutant virus-infected ganglia, however, showed threefold fewer LAT-positive neurons than wild-type virus-infected ganglia, with consistently weaker hybridization signals. Thus, this TATA box is required for normal expression of the LATs but not for efficient reactivation. The LATs CRE mutant reactivated with slightly but reproducibly reduced frequency and delayed kinetics relative to marker-rescued virus. By in situ hybridization, however, the percentage and intensity of LATs-positive neurons were found to be comparable for the CRE mutant- and wild-type virus-infected ganglia, suggesting that the CRE is dispensable for abundant LATs expression but that a reactivation function of the LATs may depend upon the presence of the CRE. Finally, using a modified assay for examining the timing of reactivation, we showed that the induction of viral reactivation by addition of exogenous cAMP can occur independently of the LATs.

**Introduction**

Many neurotropic alphaherpesviruses are capable of establishing transcriptionally active latent infections within neuronal tissues (Croen et al., 1987, 1988, 1991; Rock et al., 1987, 1988; Stevens et al., 1987). The functional significance of latency-related transcription, however, remains obscure (Fraser et al., 1992). A number of studies have shown that the latency-associated transcripts (LATs) of herpes simplex virus type 1 (HSV-1) are important either for efficient establishment of latency, or for reactivation from the latent state in vivo and in vitro (Hill et al., 1990; Leib et al., 1989a; Sawtell & Thompson, 1992; Steiner et al., 1989; Trousdale et al., 1991). In addition, a recent study has indicated that the LATs may play a role in the efficient egress of virus from infected cells (Block et al., 1993). Conversely, other mutational studies have indicated that the LATs play no role in reactivation at least not in explant cultures of latently infected ganglia (Ho & Mocarski, 1989; Izumi et al., 1989; Javier et al., 1988).

The LATs consist of at least four alternatively spliced transcripts (Stevens et al., 1987; Wagner et al., 1988a, b; Wechsler et al., 1988; Zwaagstra et al., 1990) which derive from the strand opposite to that which encodes the immediate early infected cell protein 0 (ICP0 or Vmw110), a potent activator of all classes of viral genes (Everett, 1984; Gelman & Silverstein, 1985; O'Hare & Hayward, 1985a, b; Quinlan & Knipe, 1985). As shown in Fig. 1, the LATs overlap with ICP0, as well as the neurovirulence gene product ICP34.5 (Chou et al., 1990) and possibly the 3' end of the immediate early regulatory protein ICP4 or IE175 (Preston, 1979; Watson & Clements, 1980). The abundant 2 kb LAT species has been shown to exist as a stable intron which can down-regulate ICP0 function in cell culture (Farrell et al., 1991). This 2 kb LAT and another abundant spliced 1·3 kb species with shared 5' and 3' ends are believed to be processed from a large unstable 8·3 kb transcript that spans the junction between the long and short repeat sequences of the HSV-1 genome (Mitchell et al., 1990; Wagner et al., 1988a, b; Wechsler et al., 1988). In

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addition, an unstable 6.3 kb LAT has been observed during acute infection which is believed to result from removal of the 2 kb intron from the 8.3 kb transcript (Wagner et al., 1988 b; Zwaagstra et al., 1990). Furthermore, one group has demonstrated a latency-associated antigen derived from the LATs in latently infected neuronal cell cultures (Doerig et al., 1991) although the functional significance of this finding remains to be determined.

Much recent work has focused upon the regulation of the LATs and the identification of cis-acting sequences that confer activity and tissue-specific expression. This work has resulted in identification of several putative transcriptional elements upstream of the LATs (Batchelor & O’Hare, 1990, 1992; Dobson et al., 1989; Leib et al., 1991; Zwaagstra et al., 1989, 1990, 1991). In addition, one study (Dobson et al., 1989) has shown that deletion of the sequences in the viral genome from positions 7504 to 7707 and 118665 to 118866 (numbering system of McGeoch et al., 1991) containing the LAT promoter-binding factor motif, cAMP response element (CRE) and TATA box, resulted in reduction of LAT
expression to undetectable levels. This demonstrated that this 203 bp diploid region is required for LAT expression. The specific roles of these individual motifs in LAT expression from the viral genome, however, have not been assessed. The LAT TATA box and CRE are completely conserved in HSV-1 and HSV-2 (McGeoch et al., 1991) and in this study we wanted to address the role of these specific elements in the expression and function of the LATs during viral latency and reactivation.

Methods

Cells and viruses. African green monkey kidney (Vero) cells were propagated in a humidiﬁed incubator at 37 °C with 5% CO2 in Dulbecco’s modiﬁed Eagle’s medium (DME) (Sigma) supplemented with 2 mm-L-glutamine and 10% fetal calf serum (Gibco–Bethesda Research Laboratories). Rat pheochromocytoma (PC12) cells (kindly provided by Jeffrey Milbrandt, Washington University) were propagated in a humidiﬁed incubator at 37 °C with 12% CO2 in DME supplemented with 2 mm-L-glutamine, 5% newborn calf serum and 5% horse serum (Gibco–Bethesda Research Laboratories). Procedures for the growth and assay of the KOS strain of HSV-1 and the LAT-deletion mutant dlLAT1.8 have been described previously and all mutants generated in this study were grown and assayed in an identical fashion (Leib et al., 1989a).

Plasmids. Site-directed mutagenesis was performed on the LAT promoter plasmid pPstCRE as previously described (Kunkel, 1985). The generation and characterization of pPstFsp, a plasmid containing a two base substitution in the putative TATA box with the creation of a novel PstI site to screen for orientation (Fig. 1). Linearized pREXBcl was used to yield the plasmid pPstBcl containing a two base substitution in the putative TATA box through replacement of the sequences between the PstI and the EcoRI sites of pSP72 (Promega). The absence of PstI sites outside of the LAT promoters thus allowed simple introduction of these mutant promoter fragments into plasmids for use in chloramphenicol acetyltransferase (CAT) assays was performed using standard procedures as described previously (Leib et al., 1991).

Transfection and CAT assays. Transfection of PC12 cells was performed using calcium precipitation as described (Gorman et al., 1982). Cells were incubated for 18 h prior to harvest and assay (Leib et al., 1991).

Generation of mutant viruses. The plasmid pREX was used as a homologous recombination shuttle plasmid in these studies (Fig. 1). pREX was derived from the plasmid pRFS (Leib et al., 1989a) by removing the HSV-containing fragment between the EcoRI to XhoI sites and cloning into the same sites of pSP72 (Promega). The absence of PstI sites outside of the LAT promoters thus allowed simple replacement of the sequences between the PstI sites. The 203 bp mutant PstI fragment from pPstBcl was isolated and ligated in place of the analogous fragment from the wild-type plasmid pREX to yield pREXBcl, using the novel SnaAI site to screen for insertion and the SacI site to screen for orientation (Fig. 1). Linearized pREXBcl was cotransfected with infectious KOS DNA into Vero cells. The resultant virus was plated onto Vero cells under a methyl cellulose overlay which was then stained with neutral red. Plaques were picked and used to infect Vero cells directly in Falcon Multiswell plates (Becton-Dickinson). Following complete c.p.e., viral DNA was obtained from individual wells as described (DeLuca et al., 1984), and the Multiswell plates were frozen at −80 °C to serve as a future source of virus for plaque purification. DNA was cut with BclI and analysed by Southern blot hybridization (Southern, 1975) using nick-translated pREX as a probe for the novel bands. The mutant virus was termed LATBcl. Marker-rescue and screening were performed essentially as above following cotransfection of infectious LATBcl viral DNA with linearized wild-type pREX. Mutant and marker-rescued (termed Bcl+) viruses were plaque-purified three times prior to preparation of a high titre stock. The CRE mutant virus LATFsp was generated and marker-rescued (termed Fsp+) as described above except that a novel FspI site was used to screen for insertion of the mutant CRE and its subsequent repair.

Animal procedures and reactivation assays. Randomly bred CD-1 mice (21 to 25 g, from Charles River, Kingston, N.Y., U.S.A.) were anaesthetized with xylazine and ketamine, their corneas were scarified, and 5 μl of medium containing 2 × 10^6 p.f.u. of KOS, LATBcl, Bcl+, LATFsp, LATFsp* or dlLAT1.8 was added bilaterally. Eye swab and trigeminal ganglion assays of acute infection (days 1 to 7 post-infection) and explant culture assays of latent infection (days 28 post-infection) were performed as previously described (Leib et al., 1989b). For explant cultures, ganglia were cut into eight pieces and placed onto Vero monolayers for 5 days prior to harvesting the entire culture. Harvested explants were then frozen, thawed, sonicated and homogenized prior to assay on fresh Vero cells. The dissociation of trigeminal ganglia was performed using trypsin and collagenase as previously described (Kennedy et al., 1980; Leib et al., 1991). In contrast to the previous methods, the timing of reactivation was determined by daily plating of dissociated culture supernatants onto fresh Vero monolayers in 24-well plates (Falcon) and then observing daily for c.p.e. Reactivated viruses were retained for analysis by Southern blot hybridization for comparison of their genotypes with those of input virus.

In situ hybridization. In situ hybridization was performed on 5 μm paraﬁn-embedded sections of 28 day post-infection trigeminal ganglia to assess the abilities of the viruses to express the abundant LATs. Double-stranded pPH DNA (Fig. 1) was labelled by random priming (Multi-Prime kit, Amersham) using [32P]dCTP (New England Nuclear). Hybridization was performed for 12 to 20 h at 40 °C in a solution containing 50% formamide, 2 × SSC, 10% dextran sulphate, 10 mM-dithiothreitol, 1 × Denhardt’s solution, and 200 μg per ml boiled salmon testis DNA. Washes were performed four times for 15 min at 37 °C in 4 × SSC, once for 30 min in 2 × SSC at 37 °C, and once for 30 min in 0.1 × SSC at 43 °C. Exposure to Kodak NTB-2 emulsion was for approximately 19000 count days (count days = c.p.m./μl of the probe × the number of days of exposure). In situ positive cells were counted and compared with the total number of neurons.

Results

Activity of LAT promoter mutants in CAT assays

Cloning of the TATA mutant LAT promoter into the CAT plasmid pLHp5 yielded the construct pLHp5Bcl. The activity of this CAT construct was compared with the activity from the CRE mutant pLHp5Fsp and the wild-type promoter pLHp5CRE in PC12 cells. PC12 cells are neural crest-derived and are therefore a useful transfectable cell line for the study of neuron-specific promoters. As judged by two independent transfections, CAT activity (Fig. 2) from the TATA mutant pLHp5Bcl was eightfold less than activity from the wild-type pLHp5CRE-transfected cells. Consistent with previously published work (Leib et al., 1991), the activity of the CRE mutant pLHp5Fsp was sixfold less than that of the
Fig. 2. Effect of site-directed mutations of the LAT's promoter on CAT activity in PC12 cells. PC12 cells were transfected with 5 μg of wild-type (■; pLHp5CRE), CRE mutant (■; pLHp5Fsp), or TATA mutant (■; pLHp5Bcl) LAT promoter cloned in front of CAT. CAT assays were performed at 18 h post-transfection. Results shown are combined from two independent transfection experiments. Numbers shown above bars correspond to the percentage of activity relative to the wild-type promoter.

Introduction of LAT promoter mutations into the viral genome

Having shown that the mutations described above significantly affected CAT activity, we then assessed the activities of these mutations in the context of the viral genome. Plasmids containing mutant LAT promoters were cotransfected with infectious KOS DNA into Vero cells. No phenotypic selection was used for the construction of either the mutant or marker-rescued viruses. Following transfection, virus recovery was approximately 5% in most cases, consistent with previously observed recombination frequencies using comparably sized flanking homologous sequences (D. A. Leib, unpublished). The one exception was Bcl and isolated with a frequency of less than 1%. These findings are at variance with the findings of Block et al. (1990) who isolated a LAT+ rescuant at a very high frequency (70%) and the reason for this discrepancy is unclear.

When digested with FspI, DNA from wild-type KOS and marker-rescued Fsp+ viruses gave identical bands of the expected sizes of 12.5 kb and 6.2 kb (Fig. 3, lanes 1, 5). DNA from the LAT deletion mutant dlLAT1.8 gave fragment sizes of 10.7 kb and 4.4 kb (Fig. 3, lane 3) consistent with a 18 kb deletion in both copies of the LAT gene. The CRE mutant LATFsp DNA gave bands of 8.1 kb, 4.1 kb and 2.1 kb, consistent with the introduction of novel FspI sites at positions 7615 and 118756 (Fig. 3, lane 4). When cut with BclI, both KOS and the marker-rescued virus Bcl DNA gave identically sized bands of 21 kb and 5.8 kb (Fig. 3, lanes 2, 7). DNA from the TATA mutant LATBcl gave bands of 17.6 kb, 3.4 kb and 2.4 kb, consistent with the introduction of novel BclI sites at positions 7597 and 118774. The sizes and intensities (indicative of molar ratios) of all DNA fragments shown in Fig. 3 are consistent with the introduction of the designed mutations into both copies of the LAT promoters.

Growth properties of LAT promoter CRE and TATA mutants

Consistent with previously published work regarding viruses with mutations in the LATs, all viruses tested in this study exhibited very similar growth kinetics in Vero cells (data not shown) and in the corneas and trigeminal
HSV-1 LATs promoter mutants

Table 1. Behaviour of wild-type, LAT mutant and marker-rescued viruses in a mouse eye model

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Cornea</th>
<th>Ganglia</th>
<th>Reactivation</th>
<th>LAT+ neurons/section</th>
<th>LAT+ neurons/total neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS</td>
<td>$3 \times 10^5$</td>
<td>$3 \times 10^4$</td>
<td>40/46 (87%)</td>
<td>4/3</td>
<td>223/11802 (1.9%)</td>
</tr>
<tr>
<td>d/LAT1.8</td>
<td>$2 \times 10^4$</td>
<td>$2 \times 10^4$</td>
<td>28/46 (61%)</td>
<td>0/6§</td>
<td>ND§ (0.0%)</td>
</tr>
<tr>
<td>LATBcl</td>
<td>$1 \times 10^4$</td>
<td>$1 \times 10^4$</td>
<td>48/55 (87%)</td>
<td>1/7‡</td>
<td>121/21154 (0.6%)</td>
</tr>
<tr>
<td>Bcl+</td>
<td>$1 \times 10^3$</td>
<td>$6 \times 10^4$</td>
<td>27/29 (93%)</td>
<td>5/5</td>
<td>359/13690 (2.6%)</td>
</tr>
<tr>
<td>LATFsp</td>
<td>$2 \times 10^3$</td>
<td>$1 \times 10^3$</td>
<td>41/56 (73%)</td>
<td>5/7</td>
<td>213/16279 (1.3%)</td>
</tr>
<tr>
<td>Fsp+</td>
<td>$9 \times 10^1$</td>
<td>$8 \times 10^3$</td>
<td>37/40 (93%)</td>
<td>4/4</td>
<td>314/21882 (1.4%)</td>
</tr>
</tbody>
</table>

* Expressed as p.f.u. per ml of eye swab material or ganglion homogenate obtained on day 3.
† Numbers of explanted ganglia yielding virus by day 5 per number tested.
‡ Significant difference from KOS (P < 0.01).
§ Significant difference from KOS (P < 0.001).
¶ Significant difference from Fsp+ (P < 0.05).

3. ganglia of mice (Table 1). Peak titres in trigeminal ganglia were reached on day 3 for each of the viruses and comparable titres were found for all six viruses. Similarly, eye swab assays at this time showed comparable titres for each virus (Table 1).

In situ hybridization analysis

The abilities of wild-type, mutant and marker-rescued viruses to express LATs were compared using in situ hybridization using pIPH as a probe (Fig. 1). A total of 12 ganglia per virus were analysed, four ganglia per virus in each of three independent experiments. In the first and second experiments 5 µm sections of ganglia were examined microscopically and the number of LAT-positive cells per tissue section were counted in a minimum of 100 sections for KOS and each of the mutants. In the third experiment, LAT-positive cells per section were counted, as well as the total number of neurons per ganglia. This additional step was done as a control for any possible inconsistency of section size or neuronal content and to gain information on absolute numbers of LAT-positive neurons. For this experiment the number of sections examined ranged from 57 for KOS up to 72 for LATBcl.

As shown in Table 1, 19% of KOS-infected neurons were LAT-positive with a mean of 4/3 positive cells per section. As expected, no LAT-positive cells (0 per section) were observed in d/LAT1.8-infected ganglia. Owing to the lack of LAT-positive cells with d/LAT1.8, counting of neurons would have been redundant and therefore was not performed. Examination of LATBcl-infected trigeminal ganglia showed 0/6% of total neurons were LAT-positive with a mean of 1/7 positive cells per section. This represents a significant reduction (P < 0.01 by analysis of variance) in the numbers of LAT-positive cells per section compared to KOS or marker-rescued Bcl+ (2.6% of total neurons, 5/9 positive cells per section). Furthermore, the strength of the LATs in situ signal for LATBcl (arrow in Fig. 4d) latently infected neurons was consistently weaker than for KOS (Fig. 4a) or marker-rescued Bcl+ virus (Fig. 4c) in the majority of LAT-positive cells.

Analysis of LATFsp latently infected ganglia revealed that 1.3% of total neurons were LAT-positive with 5/75 positive cells per section. Mutation of the LATs CRE therefore did not cause any significant alteration in the number of LAT-positive cells compared with marker-rescued Fsp+ (1.4% of total neurons, 4/45 positive cells per section) or wild-type KOS viruses. In addition, the strength of the hybridization signal for positive cells appeared unaltered (Fig. 4e, f). Taken together, these data suggest that in the context of the viral genome, the CRE box, but not the TATA box, is dispensable for abundant LAT expression.

Explant culture reactivation studies

Our initial experiments in this study were performed using standard explant cocultivation reactivation assays. Explants were done on trigeminal ganglia 28 days post-infection and the results described in this study are from three independent experiments (Table 1). The LAT TATA box mutant LATBcl reactivated from 48 of 55 ganglia (87%) which was indistinguishable from either KOS (40 of 46 ganglia or 87%) or the marker-rescued virus Bcl+ (27 of 29 ganglia, or 93%). Southern blot analysis of samples of all reactivated viruses from these explant studies revealed that the genotype of reactivated viruses was identical to that of input virus, eliminating the possibilities of contamination or reversion (data not shown). These data indicate that although the ability of LATBcl to express the LATs is significantly reduced, it is fully reactivation-competent.
As previously reported (Leib et al., 1989a), the 1.8 kb LAT deletion mutant dLAT1.8 reactivated with reduced frequency (28 of 46 ganglia, or 61%). The LAT CRE mutant LATFsp reactivated with moderately reduced frequency (41 of 56 ganglia, or 73%) compared to marker-rescued virus Fsp+ (37 of 40 ganglia, or 93%) or wild-type KOS. This slight reduction of reactivation frequency was consistent over the three independent experiments and the reactivation frequency of LATFsp was statistically significantly different by the χ² test (0.05 > P > 0.01) from marker-rescued Fsp+ although comparison of reactivation frequencies of LATFsp and
KOS yielded $0.1 > P > 0.05$. These data indicated that the LAT CRE may play a role in the LAT-mediated potentiation of HSV reactivation from explant culture, but it was clearly necessary to examine the reactivation kinetics of these viruses more closely by dissociation of latently infected ganglia.

Dissociation culture reactivation studies

Study of the frequency of reactivation of these viruses using explant culture of ganglion fragments allows an overall estimate of the efficiency of reactivation. Owing to the large number of neuronal and non-neuronal cells within a ganglion fragment, however, this method does not give an accurate assessment of the timing of viral reactivation from latently infected neurons. Reactivated virus particles within a ganglion fragment may not be detected until they have undergone several rounds of replication in order to egress from the ganglion fragment itself. By enzymatic dissociation of the trigeminal ganglia, however, direct contact between the latently infected neurons, the permissive Vero feeder layer and the surrounding culture medium can be obtained. Reactivating virus is therefore shed either directly into the supernatant, or if may infect the feeder layer where it may be amplified prior to release into the supernatant. At this point, reactivated virus can be assayed by assessing the timing of appearance of c.p.e. in the feeder layers as previously described (Leib et al., 1991). This system, however, has the problem that low levels of virus cannot be easily detected. A more sensitive method was employed in the present study whereby culture supernatants were sampled daily (100 μl from 1 ml of medium) and plated onto fresh Vero monolayers. The theoretical level of detection of this system is therefore 10 p.f.u. per culture at each timepoints, a concentration of virus which would not be detectable by the system previously employed.

Consistent with predictions from the explant studies, LATBcl reactivated with kinetics which were comparable to KOS (Fig. 5). Also consistent with the explant studies, LATFsp reactivated with delayed kinetics, markedly slower than for either KOS or Fsp+ (Fig. 5). The data shown in Fig. 5 are from two independent experiments each with 20 trigeminal ganglia for each virus. We next examined the effect of cAMP on the reactivation of LATFsp from dissociated explant cultures. It has been demonstrated that cAMP is a potent accelerator of viral reactivation (de la Maza et al., 1989; Leib et al., 1991; Smith et al., 1992). Given the ability of the LATs to potentiate reactivation we tested whether the LATs CRE might be responsible, at least in part, for the effect of cAMP on reactivation. In this independent series of experiments (Fig. 6), ganglia were dissociated and cultured in the presence or absence of 1 mM-N6,O-dibutyryladenosine 3’-5’-cyclic monophosphate (dBcAMP) and supernatants were sampled daily and plated onto fresh Vero monolayers which were examined daily for c.p.e. The data shown in Fig. 6 are from two independent experiments each with 20 trigeminal ganglia for each virus. In the absence of dBcAMP the reactivation kinetics of LATFsp and d/LAT1.8 were slightly delayed relative to Fsp+. In this series of experiments, however, the reactivation of d/LAT1.8 was more profoundly delayed than in the experiments shown in Fig. 5. In the presence of dBcAMP, the reactivation kinetics of LATFsp, d/LAT1.8 and Fsp+ were all accelerated relative to those of the untreated controls. The acceleration of d/LAT1.8 reactivation was particularly unexpected since, in our previous study (Leib et al., 1991), the reactivation of d/LAT1.8 was not accelerated by cAMP. As discussed further below, this apparent anomaly most likely arises as a result of the different assay systems used in these two studies. Taken together, however, consistent with the explant studies, these results using dissociated latently infected ganglia indicate that mutation of the LATs CRE yields a virus which is modestly but reproducibly delayed in its reactivation kinetics relative to wild-type or marker-rescued virus. Furthermore, the HSV cis- or trans-acting elements that are largely responsible for cAMP-mediated acceleration of reactivation must lie outside of the 1.8 kb deletion of d/LAT1.8.
of LAT expression, however, did not cause a loss of reactivation efficiency from either explant cultures or dissociated ganglion cultures. This demonstrates that the reactivation potentiating function of the LATs is unaffected by this TATA box mutation. Loss of the expression of abundantly occurring LATs, however, may not be accompanied by a loss of expression of the other LATs. Indeed, it has been proposed that the larger less abundant LATs may be polyadenylated and may be the biologically active species serving to enhance either establishment or reactivation or both (Devi-Rao et al., 1991; Sawtell & Thompson, 1992; Zwaagstra et al., 1990). Consistent with this hypothesis, Block et al. (1990) generated a virus (termed TB1) with a 166 bp deletion in the abundant LAT which was replaced with 440 bp of bacteriophage λ DNA. TB1 did not express any detectable major LATs during latency but exhibited normal reactivation kinetics, suggesting that the reactivation function of the LATs must lie outside of the abundant LATs. Alternatively, it is possible that only a low or undetectable level of biologically active latency-related transcription is required for potentiation of reactivation. A similar phenomenon has been shown for thymidine kinase whereby only low levels are required for efficient reactivation of latency (Coen et al., 1989). Fine mapping of the transcripts expressed by wild-type and mutant viruses is currently in progress and will be necessary to further confirm or refute these hypotheses and to test whether other upstream TATA elements may in fact be compensating for the mutation in the LATBcl virus.

Mutagenesis of the LATs CRE in this study resulted in a virus which is modestly reactivation-impaired in explant cultures and dissociation cultures when compared with either wild-type or marker-rescued viruses. These differences in reactivation were not highly statistically significant, but they were reproducible in the two separate reactivation systems that we have used. This observation is also consistent with another recent report of a LATs mutant virus which is capable of LATs expression but is reactivation-impaired (Block et al., 1993). These data provide further evidence that the reactivation function of the LATs probably lies outside of the abundant species and indicates that reactivation does not correlate per se with levels of LATs expression. Consistent with our previous work (Leib et al., 1989a), by slot-blot hybridization, DNA levels within latently infected trigeminal ganglia were comparable for d/LAT1.8, LATFsp, KOS and Fsp+ (data not shown). Furthermore, the percentages of LAT-positive neurons for these three viruses are comparable. The reactivation defect of LATFsp, therefore, does not appear to be at the stage of latency establishment.

Although the reactivation of LATFsp in explant cultures is reduced relative to that of the wild-type, it is
not as drastically impaired as d/LAT1.8, whose reactivation deficiency is highly statistically significant. This may be explained by the fact that d/LAT1.8 is a LAT-null mutant with a diploid 1.8 kb deletion, whereas LATFsp has a relatively subtle 2 bp substitution mutation in each of the LATs CRE. Although the reactivation impairments are reproducible for both viruses, the more important question is whether these impairments are biologically significant. With respect to this question, it is noteworthy that two groups have independently demonstrated reactivation impairment of LATs mutants using in vivo reactivation models (Hill et al., 1990; Trousdale et al., 1991). In both of these in vivo studies the behaviour of the mutants mimicked that seen in explant studies, supporting the idea as suggested by Trousdale et al. (1991) that explant reactivation is a suitable system for the study of the biological behaviour of HSV-1 LATs mutants. Moreover, the delayed reactivation phenotypes of the LATs mutants in the in vivo reactivation studies were more profound than those seen in explant studies, suggesting that explant studies may actually underestimate the degree of reactivation impairment. This suggests that the reactivation deficiency of LATFsp may indeed be biologically significant, although testing of LATFsp using in vivo reactivation models will be necessary for further confirmation.

The results from CAT assays in this and other studies predicted that a dramatic loss of LAT expression should have occurred following mutation of the CRE and TATA box in the virus. This prediction was not consistent with respect to the presumed biological activity or level of expression of the LATs. The results presented here therefore serve to highlight the potential difficulties in extrapolating information regarding promoter activity from transient transfection assays. Moreover, our previous findings indicated that the LATs may be necessary for cAMP-mediated acceleration of reactivation (Leib et al., 1991). In the previous study, the kinetics of reactivation were measured by assessing viral c.p.e. in the Vero feeder layer of the dissociated culture itself. In contrast, in this study, reactivation was measured by a more sensitive and accurate method which can detect the release of low levels of virus (10 p.f.u. per ml) at each timepoint since each supernatant sample is cultured for up to 5 days in a fresh culture. By the method used in the present study it is apparent that both LATFsp and d/LAT1.8 are inducible from reactivation by cAMP and the acceleration of reactivation that is demonstrable by addition of exogenous cAMP is therefore due to a genetic locus or loci outside of the 1.8 kb deletion of d/LAT1.8. The data of Block et al. (1993) suggest a likely explanation for the discrepancy between the two methods in that the amount of virus released from neurons during reactivation may be reduced for LATs-deficient mutants. This implies a possible function for the LATs in efficient egress of virus from latency in dissociated neurons and experiments to test this are in progress.

The exact mechanism by which the LATs affect the outcome of establishment and reactivation of latency still remains obscure. Our data and those of others are consistent with the idea that the LATs serve to increase the efficiency of reactivation, although this does not exclude the possibility that, as proposed by Sawtell & Thompson (1992), the LATs also play a role in the efficient establishment of latency at certain anatomical sites. Testing of the variety of LATs mutants now available in a series of different animal and in vitro models will further our understanding since as seen with ICP0 (Clements & Stow, 1989; Leib et al., 1989b), it is likely that animal model, viral strain, and route of infection can all influence the outcome of latent infection.

We thank Jay Muller and Carl Romano for assistance with photomicrography, and Paul Olivo and Gene Johnson for helpful discussions. This work was supported by Public Health Service Grant EY 09083 from the National Eye Institute and by a grant to the Department of Ophthalmology from Research to Prevent Blindness Incorporated, New York, N.Y., U.S.A.

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(Received 15 March 1993; Accepted 6 May 1993)