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# The promoter of the latency-associated transcripts of herpes simplex virus type 1 contains a functional cAMP-response element: Role of the latency-associated transcripts and cAMP in reactivation of viral latency

**(protein kinase A/cAMP-response element-binding protein/PC12 cells/viral pathogenesis)**

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ABSTRACT **A 203-base-pair sequence 5′ of the latency-associated transcripts (LATs) of herpes simplex virus type 1 contains a 7-base consensus sequence TGCGTCA that is identical to the cAMP-response element of the proenkephalin gene. This consensus sequence is at −38 relative to the putative 5′ end of the LATs with a TATA box at the −24 position. In transient chloramphenicol acetyltransferase assays in rat pheochromocytoma (PC12) cells, this enhancer region stimulated gene expression up to 3-fold in the presence of dibutyryl cAMP, forskolin, nerve growth factor, or phorbol 12-myristate 13-acetate. Mutation of the cAMP-response element to TGCGCAA resulted in a 4-fold reduction of basal activity and a complete loss of inducible stimulation. In DNA gel retardation assays, purified cAMP-response element-binding protein and a nuclear protein from PC12 cells were shown to bind specifically to this element. Furthermore, it was demonstrated that the reactivation of wild-type herpes simplex virus type 1 from dissociated latently infected murine trigeminal ganglia was significantly accelerated ($P < 0.005$) by the addition of cAMP analogs or adenylate cyclase activators. However, these reagents did not accelerate reactivation of a deletion mutant that lacks the putative cAMP-response element-containing promoter region, transcriptional start site, and 1015 base pairs of the LATs. These studies demonstrate that the promoter region of the LATs contains a functional cAMP-response element and that expression of the LATs is likely controlled by second messenger signal transduction and imply a role for cAMP in triggering viral reactivation.**

Herpes simplex virus (HSV) exhibits a multistage life cycle in humans and experimental animals (1). During acute productive infection, usually in mucosal epithelial cells, immediate-early, early, and late viral genes are coordinately expressed (2, 3). However, during latent infection of sensory neurons, the virus is in a nonreplicating but transcriptionally active state, specifying the abundant latency-associated transcripts (LATs; ref. 4). Four alternatively spliced LAT species have been identified that derive from the DNA strand opposite that which encodes infected-cell protein (ICP) 0, an immediate-early regulatory protein (5). The LATs overlap at least 30% of the 3′ terminus of ICP0. Analogous transcriptional patterns have been observed during latency of bovine herpesvirus type 1 and pseudorabies virus, implying possible conservation of latency-related gene expression patterns among the neurotropic herpesviruses (6–8). Although the LATs are not essential for establishment or reactivation of latency, a number of studies have suggested that they may play a role in promoting the efficiency of reactivation *in vitro* and *in vivo* (9–11). To date, however, there has been no clear demonstration that the LATs encode a protein and their precise function is unclear.

The mechanisms that control the switch between the acute and latent phases of infection are poorly understood. It is likely, however, that a number of reactivation-inducing stimuli in humans and experimental animals including UV irradiation, iontophoresis of epinephrine into the cornea, trauma, and application of retinoic acid may also raise intracellular levels of cAMP either directly or indirectly through prostaglandin release (12–16). Prostaglandins have been strongly implicated in the reactivation of viral latency, and indomethacin, a potent inhibitor of prostaglandin synthesis, has been shown to reversibly inhibit the reactivation of HSV-1 from explanted latently infected ganglia (17–19). cAMP-mediated induction of gene expression is common in organisms from bacteria to humans and is a well-recognized mechanism by which cell-surface receptors may generate intracellular signals (for review, see ref. 20). In mammalian cells, the interaction of a ligand with a cell-surface receptor leads to the activation of adenylate cyclase, which synthesizes cAMP from ATP (21). cAMP binds to the regulatory subunits of cAMP-dependent protein kinase (PKA), releasing active catalytic subunits that can phosphorylate specific transcription factors. Such factors can bind to target sequences known as cAMP-response elements (CREs), and a 43-kDa phosphoprotein that can bind to CREs has been identified in many cAMP-responsive cells and tissues (22). This CRE-binding protein (CREB) can be directly phosphorylated *in vitro* by purified catalytic subunits of PKA (23). CREB, and a family of related proteins, is believed to be involved in regulating many cAMP-inducible genes containing CREs in their upstream regulatory regions (24, 25).

The role of cAMP in HSV latency and reactivation has not been examined at the molecular level (26). Examination of the DNA sequence of HSV-1 (27) revealed the presence of a CRE consensus sequence in the enhancer region of the LAT promoter. This CRE has complete homology with the CRE of the cAMP-responsive proenkephalin gene (28). A combination of transient chloramphenicol acetyltransferase (CAT) assays, DNA gel retardation assays, and reactivation studies using wild-type and mutant viruses has allowed us to deter-

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Abbreviations: CAT, chloramphenicol acetyltransferase; CRE, cAMP-response element; CREB, CRE-binding protein; $Bt_2$-cAMP, dibutryl-cAMP; HSV, herpes simplex virus; ICP, infected cell protein; LAT, latency-associated transcript; NGF, nerve growth factor; $PGE_2$, prostaglandin $E_2$; PKA, cAMP-dependent protein kinase A; PMA, phorbol 12-myristate 13-acetate.

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mine that the LAT CRE is functional and that the LATs are essential for the cAMP-mediated acceleration of HSV-1 reactivation from latently infected neurons.

## MATERIALS AND METHODS

**Cells and Viruses.** Vero and PC12 cells were propagated as described (29, 30). A126 cells (gift of John Wagner, Harvard Medical School) are a variant of PC12 cells with a defect in PKA II and were propagated as described (29). The generation, growth, and assay of KOS, dlLAT1.8, and FSLAT$^+$ have been described (9, 31).

**Sequences and Plasmids.** The location of HSV-1 sequences used in this study is shown in Fig. 1. The *Pst* I/*Pst* I fragment containing the LAT promoter (positions 118,664–118,866 by the numbering system of McGeogh *et al.*; refs. 5 and 32) was subcloned from pBb1 (9) into the vector pGEM-5Zf(+) (Promega) in which the *Sac* I site had been converted to a *Hin*dIII site to yield the plasmid pPstCRE. Single-stranded DNA was made according to the manufacturer's instructions and oligonucleotide-directed mutagenesis was performed as described (33). Hybridization of the o34FspI oligonucleotide (a 34-mer containing the sequence TGCGCAA in place of TGCGTCA) yielded the plasmid pPstFsp containing a 2-base substitution mutation in the putative CRE with the creation of a unique *Fsp* I site for screening purposes. The presence of the mutation was confirmed by sequencing using the Sequenase system (United States Biochemical). Plasmids pPstCRE and pPstFsp were cleaved with *Sph* I and *Hin*dIII and the resulting 271-base-pair fragments were cloned into the *Sph* I/*Hin*dIII-cleaved CAT vector pLHp5 (C. Dabrowski and J. Alwine, personal communication) to yield pLHp5CRE and pLHp5Fsp, respectively.

**Transfection and CAT Assays.** Transfection of PC12 cells was performed using either calcium phosphate precipitation as described (34) or Lipofectin according to the manufacturer's instructions (Bethesda Research Laboratories). Inducers of cAMP were added 18 hr later and cells were incubated for a further 18 hr prior to harvest and assay as described (34, 35). Inducers added were 1 mM dibutyryl-cAMP ($Bt_2$cAMP), 25 μM forskolin, 50 ng of nerve growth factor (NGF) per ml, or 5 ng of phorbol 12-myristate 13-acetate (PMA) per ml (Sigma).

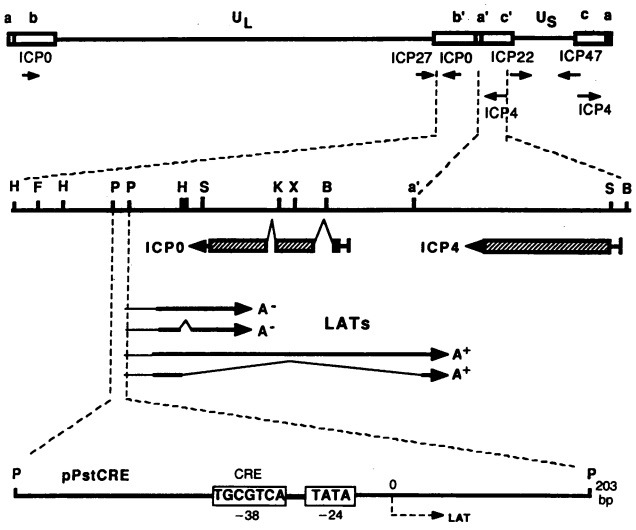


FIG. 1. Physical map of the HSV-1 genome showing the positions of the immediate-early genes and the direction of their transcription (*Top*). Below this map, the positions of transcripts encoding ICP0, ICP4, and the LATs are shown relative to selected restriction sites. Hatched bars indicate known open reading frames and angled lines indicate regions of transcript splicing. Four transcripts are shown for the LATs, although uncertainty exists regarding the locations of the 5′ ends of the poly(A)$^-$ transcripts and the splicing of the poly(A)$^+$ transcript (5). The expanded promoter region (*Bottom*) shows the relative positions of pertinent cis-acting sequences relative to the predicted 5′ start sites of the LATs. B, *Bam*HI; F, *Fsp* I; H, *Hpa* I; K, *Kpn* I; P, *Pst* I; S, *Sal* I; X, *Xho* I. A second copy of the region encoding ICP0 and the LATs (*Hpa* I to a′) is also present in the b repeat sequence at the left end of $U_L$. Correspondingly, sequences specifying ICP4 (*Bam*HI to a′) are also present in the c repeat sequence at the right end of $U_S$. bp, Base pairs.

**Gel Retardation Assays.** Nuclear extracts from PC12 cells were prepared and DNA binding reactions and electrophoresis were performed as described (23). Eight nanograms of affinity-purified CREB (a gift of Marc Montminy, Salk Institute) or 10 mg of PC12 cell extract was added to 1 ng of $^{32}$P-end-labeled pPstCRE probe (Fig. 1) at 2–4 × $10^4$ cpm/ng together with 50 ng of poly(dI.dC) as nonspecific competitor. Various amounts of 17-base-pair LAT promoter oligonucleotides (positions −47 to −31) containing wild-type or mutant CRE were added as specific competitors. To confirm the identity of CREB–CRE complexes, 1 μl of 1:50–1:100 dilution of a CREB-specific antibody (a gift of Marc Montminy) was added to binding reaction mixtures under standard conditions (23).

**Animal Procedures and Reactivation Assays.** Seven-week-old randomly bred CD-1 mice (Charles River Laboratories) were anesthetized and inoculated as described (36). Trigeminal ganglia were removed on day 30; placed immediately into serum-free medium containing either no drug, 1 mM $Bt_2$cAMP, 30 μM 5′-deoxyadenosine, or 30 μM prostaglandin $E_2$ ($PGE_2$) (all supplied by Sigma); and dissociated with trypsin and collagenase as described (36). Cells from each ganglion were seeded into individual wells of six-well dishes (Corning) containing 5 × $10^5$ Vero cells in the presence or absence of 1 mM $Bt_2$cAMP, 30 μM 5′-deoxyadenosine, or 30 μM PGE2. Culture medium containing the appropriate reagent was changed every second day. The day of first appearance of virus-specific cytopathic effects was recorded.

## RESULTS

**Requirement for CRE in LAT Promoter Activity.** The putative CRE in the LAT promoter was identified at position 118,762 with a TATA box at 118,776 in the sequence of HSV-1 strain 17 (27, 32). The 5′ ends of the LATs have recently been mapped to within 40 base pairs of this CRE (5), some 650 base pairs upstream of its previously reported start. We sequenced the corresponding LAT-promoter region of HSV-1 strain KOS by using the Sequenase system (United State Biochemical) and determined that KOS contains the identical consensus sequences (Fig. 1). To test whether the LAT CRE is functional, we assessed the ability of $Bt_2$cAMP, forskolin, NGF, and PMA to induce activity of wild-type (TGCGTCA) and mutant (TGCGCAA) LAT promoter-CAT chimeras in PC12 cells. PC12 cells are neural crest derived and are cAMP and NGF responsive; they therefore provide an appropriate system for the study of neuronal gene expression. The effects of $Bt_2$cAMP and forskolin are to raise intracellular cAMP concentrations—$Bt_2$cAMP acts directly and forskolin acts through activation of adenylate cyclase. The relationship between NGF and cAMP levels in neurons is not well understood. It has been reported that the effects of cAMP and NGF may be synergistic but are mediated through divergent pathways (37). It has also been shown that cAMP analogs promote survival and neurite outgrowth of neurons in culture independently of NGF (38). Phorbol esters such as PMA serve to activate protein kinase C (PKC) by mimicry of diacylglycerol (39). A number of studies have shown that the PKA- and PKC-mediated transduction pathways interact and that both respond to cAMP (40–43).

The effect of these reagents in a series of three separate transfections and CAT assays was to stimulate expression of CAT from the wild-type CRE-containing LAT promoter 1.5- to 3-fold above uninduced (basal) levels (Fig. 2). None of these reagents was able to stimulate expression from the

mutant CRE promoter above uninduced levels. In addition, the high basal activity of the wild-type promoter was reduced 4-fold by mutation of the CRE. This demonstrated that the increased CAT activity seen in response to these reagents was due to their pharmacologic effects and the presence of the CRE and not merely to mRNA stabilization. These results indicate that the LAT CRE is indeed functional and that the 2-base-pair substitution (TGCGTCA to TGCGCAA) is sufficient to destroy inducible activity. Moreover, the high basal level of activity of this promoter in PC12 cells is also significantly reduced by this mutation, implying that both the basal and inducible activities of this promoter in PC12 cells are dependent on the presence of the CRE. This high basal level of CAT expression may reflect a high level of PKA activity within PC12 cells since we were unable to demonstrate inducible activity from this promoter in PKA-deficient A126 cells in which the basal CAT activity was 2- to 3-fold lower than that found in PC12 cells (data not shown).

**Binding of CREB to the LAT Promoter.** CREB is phosphorylated *in vitro* by the catalytic subunits of PKA and *in vivo* following induction by cAMP and activity of CREB; therefore, *in vivo* phosphorylation and subsequent activation are likely to be mediated by cAMP (23). Recent evidence suggests that a whole family of CREBs may be involved in transcriptional activation of CRE-containing genes (24, 25). To date, however, CREB remains the best characterized of these proteins and we wished to examine the binding of CREB to the LAT CRE in an attempt to correlate CREB binding with CAT activity. We also wished to examine PC12 cells for the existence of further CRE-specific binding proteins.

We used gel retardation assays to address the question of protein–DNA binding. Fig. 3 shows the results with CREB purified from rat brain. CREB bound to the 203-base-pair wild-type LAT CRE fragment to form one major and three minor complexes. Formation of these complexes was blocked by competition in a dose-dependent fashion 30-fold more efficiently by wild-type competitor (o17CRE) than by mutant competitor (o17Fsp), strongly suggesting specific binding involving the putative CRE. The multiple bands seen in this gel are likely due to existence of the CREB protein in

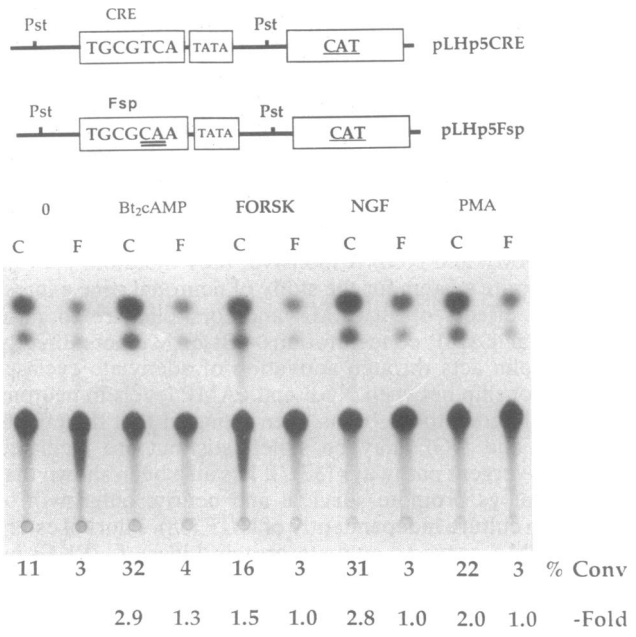


FIG. 2. Effect of 1 mM $Bt_2cAMP$, 25 mM forskolin, NGF (5 ng/ml), PMA (5 μg/ml) on CAT expression from pLHp5CRE (C) and pLHp55Fsp (F) in PC12 cells. Numbers refer to percentage conversion of chloramphenicol to acetylated forms and -fold induction of acetylation over uninduced cells as shown in the two left-most lanes.

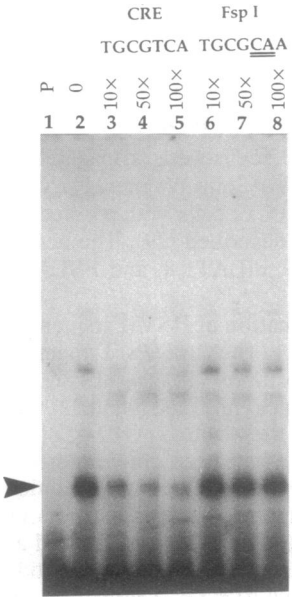


FIG. 3. DNA mobility-shift assay using the 203-base-pair LAT promoter as probe and affinity-purified CREB. Lane 1, probe alone; lane 2, bands obtained with probe and CREB alone; lanes 3–5, effect on CREB binding of a 10- to 100-fold excess of o17CRE, a 17-base-pair unlabeled competitor containing a wild-type CRE as shown above the gel; lanes 6–8, effect of a 10- to 100-fold excess of o17Fsp, a 17-base-pair unlabeled competitor containing a mutated CRE as shown above the gel.

multiple phosphorylated or multimerized forms since these bands were supershifted in the presence of a specific CREB antibody (data not shown). Attempts to obtain CREB–DNA complexes with radioactively-labeled o17CRE oligonucleotide using mobility-shift assays were unsuccessful (data not shown), suggesting that sequences flanking the 17 base pairs may enhance the stability of the protein–DNA complex.

Results of mobility-shift assays with crude nuclear extracts of PC12 cells (Fig. 4) indicate that PC12 cell proteins can form at least one major DNA–protein complex with a 203-base-pair probe. This complex was blocked by competition dose dependently by the addition of wild-type (o17CRE), but not mutant (o17Fsp), oligonucleotide. A second higher-mobility complex seen on this gel is blocked by competition only at very high concentrations (1000-fold excess over probe DNA) of wild-type, but not mutant, competitor. The identity of this protein(s) is not known, but its binding clearly involves sequences within the 7-base-pair CRE motif. The mobilities of both complexes are different from those of complexes formed with purified CREB, indicating that proteins in PC12 cells other than, or in addition to, CREB may be capable of binding to this consensus sequence.

Taken together, these results indicate that CREB can bind to the 203-base-pair fragment specifically through the CRE consensus sequence. Mutation of the CRE from TGCGTCA to TGCGCAA leads to loss of the ability to bind CREB, as judged by the ability of o17CRE to compete against probe binding compared with the o17Fsp oligonucleotide. In addition, there is at least one protein in PC12 cells, which appears to be distinct from CREB, that can bind specifically to the LAT CRE.

**The LATs Are Necessary for cAMP-Mediated Acceleration of Reactivation.** We and others have shown that mutant viruses that lack the sequences specifying the LATs reactivate with reduced efficiency (49%) from explant cultures of latently infected ganglia relative to wild-type (94%) or marker-rescued (85%) viruses (9, 10). In this study, wild-type KOS, a LAT-deletion mutant dlLAT1.8, and its marker-rescued recombinant virus $FSLAT^+$ were tested for their ability to reactivate from dissociated latently infected ganglia in the presence and absence of modulators of cAMP. The LAT-deletion mutant dlLAT1.8 lacks the putative CRE-containing promoter region, transcriptional start site, 1015 base pairs of the sequences specifying the LATs, and does not express detectable LATs during ganglionic latency (9).

In contrast to previous results with explant cultures (9), dissociation of ganglia by trypsin-collagenase digestion al-

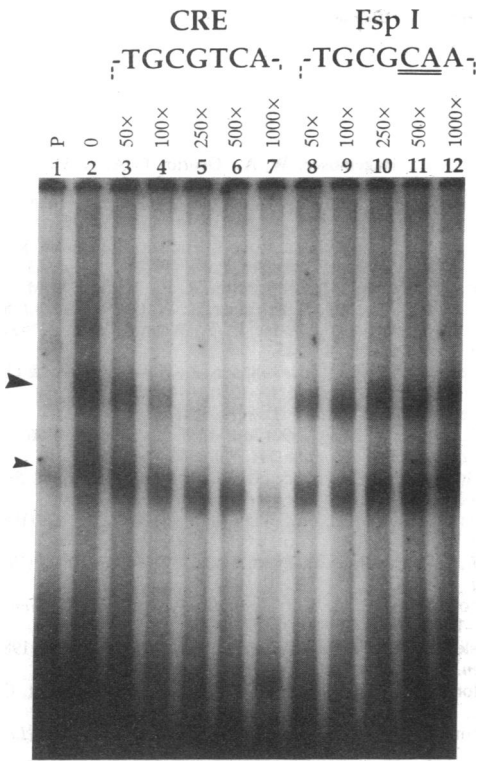


FIG. 4. DNA mobility-shift assay using the 203-base-pair LAT promoter as probe and crude nuclear extracts from PC12 cells. Lane 1, probe alone; lane 2, bands obtained with probe and PC12 extracts alone; lanes 3–7, effect of adding 50- to 1000-fold excess of o17CRE, a 17-base-pair unlabeled competitor containing a wild-type CRE as shown above the gel; lanes 8–12, effect of adding a 50- to 1000-fold excess of o17Fsp, a 17-base-pair unlabeled competitor containing a mutated CRE as shown above the gel. Fifty nanograms of poly(dI.dC) was added to each reaction mixture as a nonspecific competitor.

lowed dlLAT1.8 to reactivate with wild-type efficiency, such that KOS, dlLAT1.8, and FSLAT$^+$ reactivated with nearly equal kinetics from dissociated ganglia under control (untreated) conditions (Table 1). However, addition of 5′-deoxyadenosine, $Bt_2cAMP$, or $PGE_2$ to dissociated ganglia caused a significant reduction ($P < 0.005$; Student's $t$ test) in the mean time for reactivation of KOS compared with untreated controls (Table 1; Fig. 5). The reactivation of FSLAT$^+$ was similarly enhanced by the addition of 5′-deoxyadenosine to the cultures. The reactivation of dlLAT1.8, however, was significantly inhibited ($P < 0.005$) by the addition of these reagents compared with untreated controls. These results suggest that cAMP is important in triggering reactivation of HSV-1 from latency and that the LATs are targets of second messenger signal transduction by cAMP.

## DISCUSSION

Our results demonstrate a functional CRE in HSV-1 and suggest strongly that this element and cAMP play a significant role in reactivation from latency. The precise functions of the LATs of HSV-1 and other neurotropic herpesviruses are not known. Mutational analysis has suggested a role for the LATs in reactivation *in vitro* and *in vivo*, although the mechanism for this is unclear. To date there is no unambiguous evidence for a LAT-encoded protein, although recent mapping data have suggested that open reading frames other than those examined previously may be significant (5). Our study, however, offers an initial clue as to the mechanism by which LATs may influence reactivation and reveals a potentially important pathway by which HSV-1 may reactivate in response to extracellular stimuli.

Table 1. Mean reactivation times of KOS, dlLAT1.8, and FSLAT$^+$ from dissociated latently infected ganglia in the presence or absence of three modulators of cAMP levels

| Virus | Mean time of reactivation, days | | | |
|---|---|---|---|---|
| | Control | $Bt_2cAMP$ | 5′dA | PGE2 |
| KOS | 5.5 | 4.3* | 4.4* | 4.4* |
| dlLAT1.8 | 5.0 | 6.4* | 6.4* | 6.3* |
| FSLAT$^+$ | 5.2 | ND | 4.3* | ND |

Each of the results with the wild-type and LAT-deleted viruses represents cumulative data from at least 13 ganglia and, in some cases, up to 27 ganglia. 5′dA, 5′-deoxyadenosine; ND, not determined.
*Differs from control cultures ($P < 0.005$) by Student's $t$ test.

We have shown that the LAT CRE is responsive to a variety of modulators of cAMP in CAT assays and that the CRE is also responsible for the high basal level of activity of this promoter in cells of neuronal origin. The induction of expression by $Bt_2cAMP$, forskolin, and PMA is typical of cAMP-responsive genes. The induction of CAT expression by NGF, however, was unexpected since NGF has been shown to inhibit reactivation of HSV-1 from latently infected trigeminal ganglia (44). This may be explained on the basis of differences between PC12 cells and ganglionic neurons or by the fact that NGF induces a large number of divergent metabolic pathways in neuronal cells (45–51).

The core sequence of the LAT CRE (TGCGTCA) has homology with a number of other CREs and complete identity with that of the mammalian cAMP-responsive proenkephalin gene. The CGTCA motif of the CRE is highly conserved and the mutation of this motif leading to a loss of inducible activity is consistent with this conservation. Sequences flanking CREs in the promoters of cAMP-responsive genes, however, are not conserved and the requirements for transcriptional induction are unclear. Our inability to demonstrate CREB binding to a

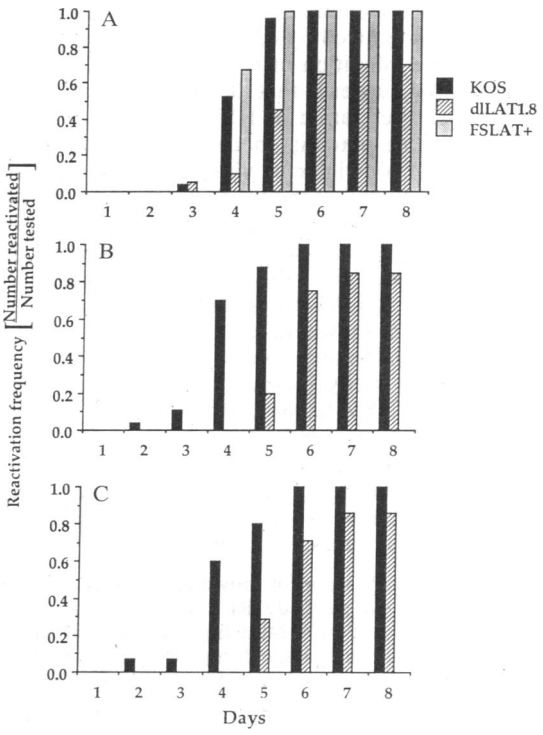


FIG. 5. Comparison of the effect of 30 $\mu$M 5′-deoxyadenosine (*A*), 1 mM $Bt_2cAMP$ (*B*), or 30 $\mu$M $PGE_2$ (*C*) on reactivation kinetics of KOS, dlLAT1.8, and FSLAT$^+$. For each treatment, the cumulative proportion of total ganglia in each virus group that had reactivated on any given day is shown on the $y$ axis.

17-base-pair oligonucleotide containing the LAT CRE may reflect the importance of flanking sequences in the stabilization of transcription complexes and subsequent cAMP-mediated induction of expression.

Interestingly, the reduced frequency of reactivation of the LAT-deleted virus previously reported by us and others (9, 10) was not observed in the control (untreated) cultures in these tests. Indeed, using the dissociated ganglion assay rather than the explant cocultivation assay, dlLAT1.8 appears to reactivate at least as well as KOS and FSLAT$^+$. One possible explanation for this disparity is that dissociation of neurons allows closer contact between the nonpermissive latently infected neurons and the permissive Vero indicator cells than does explant culture of ganglion fragments. Therefore, a virus that reactivates inefficiently, such as dlLAT1.8, will show a more marked phenotype in an explant assay than a dissociated ganglion assay. A more likely explanation is that dissociation of ganglia leads to more rapid diffusion of pharmacologically active agents away from the neurons. These agents, which may be potential cAMP agonists (e.g., prostaglandins), may remain in a more concentrated or active form during culture of explanted ganglion fragments than during culture of dissociated neurons. This hypothesis would predict that the reactivation of dlLAT1.8 (which is inhibited by cAMP) would be more rapid from dissociated neurons than from ganglion fragments unless cAMP levels are experimentally raised. The addition of cAMP to dissociated cultures would therefore mimic the reactivation kinetics of dlLAT1.8 from ganglion explant culture. This prediction is consistent with our observations in this study.

The results of our reactivation tests indicate that agonists of cAMP levels can accelerate the reactivation of wild-type but not LAT-deleted HSV-1. This finding is consistent with the observation by Hill *et al.* (11), who demonstrated inefficient reactivation of a LAT-deleted virus following iontophoresis of epinephrine, a potent cAMP agonist, in a rabbit ocular model. This suggests that a LAT-associated function has a stimulatory effect on reactivation and that overexpression of the LATs induced by cAMP can lead to accelerated reactivation. The inhibition of reactivation of dlLAT1.8 induced by cAMP suggests that the precise mechanism of LAT-mediated acceleration of HSV-1 reactivation may be more complex. One potential model is that cAMP mediates the expression of a cellular or viral repressor and that the LATs, or the product(s) of the LATs, may compete with this repressor to induce reactivation. Therefore, addition of cAMP agonists to ganglia latently infected with dlLAT1.8 leads to overexpression of the repressor, without a corresponding increase in LAT production.

cAMP has been shown to play a pivotal role in gene activation and signal transduction in many other systems and the results of this study indicate that it may also play an important role in HSV latency. It is clear that the cAMP-mediated acceleration of HSV-1 reactivation shown here is dependent on the presence of the LATs. This observation should prove useful in directing future efforts to elucidate the function of the LATs, which are conserved among the neurotropic herpesviruses.

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