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# In Vivo Deletion Analysis of the Herpes Simplex Virus Type 1 Latency-Associated Transcript Promoter

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**During herpes simplex virus latency, transcripts accumulate from a single transcription unit of the viral genome. The promoter for these latency-associated transcripts (LAT) has been located, and a number of studies have documented the specific regions of this promoter which are important in transient assays of neuronal cells in culture. To examine the regulation of this promoter from the viral genome, both in vitro and in vivo, a series of seven promoter deletion viruses which drive the expression of the reporter gene β-galactosidase was constructed. Rabbit skin cells were infected in cell culture with viruses bearing each promoter mutation, and the LAT promoter activity was compared with that obtained by infecting two neuronal cell lines, ND7 cells and C1300 neuroblastoma cells. Mouse dorsal root ganglia were also infected with these recombinant viruses by footpad inoculations, and β-galactosidase activity was measured. Infected neuronal cells lines and dorsal root ganglia exhibit much more LAT promoter activity than infected rabbit skin cells, suggesting that the region upstream of −250 may contain one or several neuronal specific DNA-binding sites. However, a comparison of LAT promoter activities within the deletion series revealed many differences between neurons of the dorsal root ganglia infected in vivo and the two neuronal cell lines infected in vitro. These results suggest that neurons may vary extensively in the quantity or kind of transcription factors they contain.**

Herpes simplex virus (HSV) can infect and remain latent in a variety of peripheral and central nervous system neurons (5, 9, 27). During latency, lytic cycle genes are not expressed and RNA is detectable from a single region of the viral genome (6, 7, 26, 30, 31). We and others (10, 35) have further defined this region as a single transcription unit of 8.3 kb originating in the long repeat and crossing the joint region. The RNAs accumulating from this region appear as two to three bands on Northern (RNA) blots ranging in size between 1.45 and 2.0 kb (32, 33) and are referred to as the latency-associated transcripts (LAT). These RNAs accumulate as stable introns in the nuclei of latently infected cells and are antisense to part of the third exon of the ICP0 mRNA (8, 11, 21, 23). A number of laboratories have shown that recombinant viruses from which the LAT promoter has been deleted are inefficient at reactivating from the latent state compared with wild-type viruses (4, 13, 16). This suggests a biological function for some entity within the LAT transcription unit, and genetic studies are under way in several laboratories to try to define more precisely the function required for reactivation.

The TATA box for the LAT promoter or latency-associated promoter (LAP promoter) is located 688 bp upstream of the 5′ end of the stable LAT intron (10, 24, 34). Deletion of this TATA box and other upstream elements in recombinant viruses causes the virus to be unable to express LAT region transcripts during a latent infection (10, 22). In addition, a recombinant virus containing an insertion of a β-globin gene just downstream of this TATA box synthesizes β-globin RNA and not LAT RNA during latency (10). Transfection experiments with plasmid DNAs locating the LAT promoter to this region are in agreement with the in vivo data (1, 2, 34). Finally, it is clear that the TATA box and other upstream elements control transcription of the entire transcriptional unit during latency, i.e., during latency there is no evidence for internal start sites. For example, removal of a 201-bp *Pst*I fragment containing the TATA box and other upstream elements leaves approximately 600 bases of DNA upstream of the LAT intron; this virus was unable to make the LAT intron, a very stable RNA, during latency (10). Second, insertion of the β-globin gene immediately downstream of the TATA box leaves about 650 bp of DNA contiguous with and upstream of the 5′ end of the LAT intron; this virus also does not make LAT during latency (10). Third, deletion of the TATA box and other upstream promoter elements prevents the accumulation of RNAs outside the intron, the so-called minor LAT RNAs (22).

With these data in mind, we set out to investigate the features of the LAT promoter which cause it to be uniquely active during latency among all of the more than 70 HSV type 1 (HSV-1) promoters. Our approach has been to construct recombinant viruses containing the β-galactosidase gene as a reporter. This approach has the advantage of examining transcription from the viral chromosome in neurons in vivo. During the course of these experiments, a number of reports have been published concerning the activity of the LAT promoter during transient transfections into various cell lines. In vitro studies of the LAT promoter performed by Batchelor and O'Hare have identified regions necessary for constitutive expression as well as enhanced expression in neuroblastomas relative to HeLa cells (1, 2). Leib and colleagues have shown that a functional Cyclic AMP (cAMP)-responsive element exists immediately upstream of the LAT TATA box (16, 24). Zwaagstra et al. (36) have identified a protein, the LAT promoter-binding factor (LPBF), which can enhance transcription in both neuronal and nonneuronal cells. They have also shown that regions farther upstream appear to up-regulate promoter

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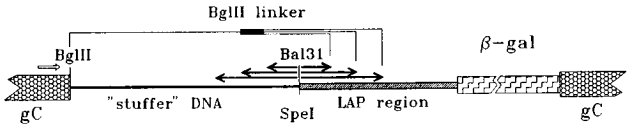


FIG. 1. Plasmid constructions. LH7, a parental plasmid containing a piece of stuffer DNA, was opened at a unique *Spe*I site and digested with the bidirectional exonuclease BAL 31. The remaining stuffer DNA was removed by digestion with *Bgl*II, the free ends of the DNA were blunted with T4 DNA polymerase, and a *Bgl*II linker was ligated into the plasmid deletion site. Dideoxy sequence analysis was performed at the 5′ end of the promoter fragment from an oligonucleotide (open arrow) within the glycoprotein C gene. β-gal, β-galactosidase.

activity in neuronally derived cells but down-regulate it in nonneuronal cells (35).

In order to examine the role of various regions of the latency-associated promoter, we constructed a series of 5′ promoter-deletion viruses which drive the expression of the reporter gene β-galactosidase. These promoter-reporter constructs were placed at the glycoprotein C locus to remove them from the possible influence of the repeat region or potential enhancer elements. We reported previously that the constructs used here are active in neurons which are phenotypically latent during acute ganglionic infection, in that the LAT promoter is active, but they do not express viral antigen (20). The latent-specific activity of the LAT promoter allows us to examine a promoter deletion series in vivo and compare the results with those from infection of commonly used neuronal cells in vitro. LAT promoter activity in dorsal root ganglia was different from infection of commonly used neuronal cell lines. LAT promoter activity also differed significantly from what has been observed previously in plasmid transfection of neuronal cell lines (2). These results suggest that neurons of the dorsal root ganglia may have a unique and different set of transcription factors compared with those in cell lines routinely used to assay LAT promoter function in vitro.

## MATERIALS AND METHODS

**Construction of plasmids and viral recombinants.** The recombinant virus KOS/1 has been described previously (20). It was derived from the plasmid JA1, which contains the β-galactosidase reporter gene construct (from pCH110; Pharmacia) located within the glycoprotein C locus. The promoter consists of a *Sma*I-to-*Sac*II DNA restriction fragment which extends to bp −863 relative to the LAT transcription start site. The deletion series was constructed as outlined in Fig. 1. Six constructs were selected according to the size of the promoter fragment by agarose-gel electrophoresis. Sequencing of alkali-denatured plasmids using the Sequenase version 2.0 kit (U.S. Biochemical Corp.) was performed according to the manufacturer to determine the 5′ extent of the deletion. A primer (5′-AGAACCCCGATTCCAATCC-3′) which annealed to DNA immediately upstream of the deleted region, within the glycoprotein C sequence, was used. These constructs were then cotransfected with KOS(M) viral DNA, and recombinant viruses were isolated and plaque purified as previously described (9). Southern blot analysis (Fig. 2) was performed to confirm their structure (Fig. 3). All of the recombinant viruses are derivatives of the parental strain KOS(M); however, they have been named according to the size of the promoter fragment relative to that of the viral strain 17+ (16). KOS/1 has been renamed KOS863. The remainder of the deletion series were named as follows: KOS593, KOS506, KOS388, KOS252, KOS124, and KOS57 were derived from plasmids ATD120, ATD113, ATD114, ATD121, ATD116, and ATD117 respectively. The enzymes used in this study were obtained from Bethesda Research Laboratories, Boehringer Manheim Biochemicals, and New England Biolabs. *Bgl*II and *Spe*I linkers were obtained from Pharmacia. Isotopes were obtained from New England Nuclear and ICN Biomedicals, Inc.

**Cells and viruses.** Viral stocks were replicated in all cells maintained in minimal essential medium supplemented with fetal calf serum (0.5%), penicillin (250 μg/ml), and fungizone (0.25 mg/ml). Viral stocks were stored at −70°C prior to use. Recombinant viruses and KOS(M) replicated equally well in vitro and readily achieved titers ranging from $1 \times 10^9$ to $5 \times 10^9$ PFU/ml. The parental strain, KOS(M), typically replicated to somewhat higher titers within the ganglia of mice. This is presumably a result of its expression of the wild-type glycoprotein C status.

**Animals and inoculations.** Six-week-old female BALB/C or Swiss Webster mice were inoculated with $4 \times 10^7$ PFU of virus per footpad as described previously (15). Photographs were taken of ganglia from the Swiss Webster mice and are representative of all experiments. All other data was collected by using the BALB/C mice.

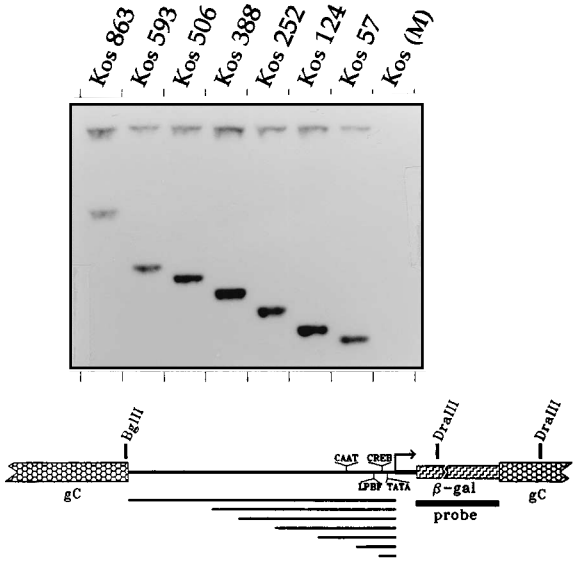


FIG. 2. Southern blot analysis of the viral recombinants. Viral DNA was digested with *Bgl*II and *Dra*III, electrophoresed on a 1% agarose gel, and blotted onto Magnagraph Nylon (MSI). A random-hexamer-primed probe from β-galactosidase (β-gal) was hybridized to the nylon membrane at 65°C according to the method described by Maniatis et al. (18). Two bands hybridized as expected: a standard-size fragment exclusively from within β-galactosidase and a second containing the deleted promoter region.

**Histochemical analysis for β-galactosidase.** Histochemical analysis was carried out as described previously (9). Briefly, the animals were sacrificed by $CO_2$ inhalation and perfusion fixed, and their dorsal root ganglia (L3-S1) were removed. Histochemical staining of whole ganglia was performed with the reagent 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Sigma).

**CPRG assay for β-galactosidase.** Cellular extracts from either infected mice or transfected cell cultures were prepared as follows. (i) Following inoculation of mice, animals were sacrificed on day 4 by $CO_2$ inhalation and perfused with phosphate-buffered saline (PBS). Dorsal root ganglia were removed, ground on ice in a 0.1-ml Dounce homogenizer, and stored in aliquots at −70°C in a volume

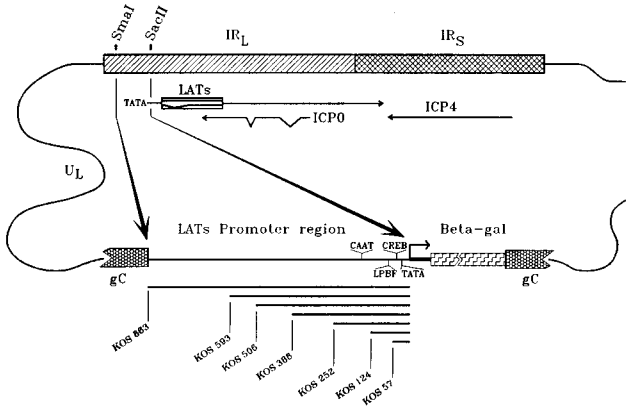


FIG. 3. Structures of the viral recombinants. As can be seen in the upper portion of the figure, a *Sma*I-to-*Sac*II restriction fragment containing the LAT promoter was moved to the glycoprotein C locus cloned in front of a β-galactosidase (beta-gal) reporter gene construct (pCH110; Pharmacia). The deletion series is illustrated in the lower portion of the figure; the deletions are named according to their positions within strain 17+. Several of the promoter elements are also shown. $IR_L$, inverted repeat long; $IR_s$, inverted repeat short; $U_L$, unique long region.

of one set of mouse ganglia per 100 μl of PBS. Prior to use, the samples were microfuged to pellet cellular debris. (ii) Rabbit skin cells and neuronal cell lines were infected with each virus at a multiplicity of infection of approximately 1, in 60-mm tissue culture plates. Cells were harvested at 18 h, resuspended in 200 μl of PBS, and stored at −70°C. Prior to use, the samples were freeze-thawed three times and microfuged to pellet cellular debris. For both the ganglionic and cell culture extracts, 10 μl of supernatant was reacted with a solution containing 5 mM chlorophenol red–β–D-galactopyranoside (CPRG; Sigma), 50 mM potassium phosphate (pH 7.8), and 1 mM magnesium chloride in a total volume of 100 μl at 37°C for 2 to 4 h. $A_{573}$ was measured. Transfections were normalized by determining protein content with the Bio-Rad protein assay solution according to the manufacturer's instructions.

## RESULTS

**Structure of the recombinant viruses.** To facilitate in vivo analysis of the LAT promoter, an 863-bp region consisting of the LAT TATA box and elements upstream of this was placed in front of the β-galactosidase reporter gene construct and inserted into HSV, disrupting the glycoprotein C gene. The choice of β-galactosidase as a reporter was based on the ease of histochemical visualization of the signal, as shown by Ho and Mocarski (14). The decision to move an upstream DNA element to the glycoprotein C locus represented an attempt to isolate the promoter from the repeat region to determine if this piece of DNA could function to promote latent transcription.

A series of LAT promoter deletion viruses were subsequently constructed. A stuffer DNA fragment was inserted between the 5′ end of the LAT promoter and the 3′ end of the glycoprotein C gene (Fig. 1). BAL 31 bidirectional deletions were performed on this DNA, which was subsequently digested with *Bgl*II to remove the 5′ part of the stuffer DNA. Thus, each of these deletions should resemble each other except at the 5′ end of the LAT promoter. A series of plasmids was isolated. Following analysis by restriction digestion and sequencing, six plasmids were chosen for recombination into the viral strain KOS(M) at the glycoprotein C locus. Recombinant viruses were screened by hybridization for the presence of the β-galactosidase gene, plaque purified at least three times, and analyzed by Southern blotting (Fig. 2). The structures of the resultant viral recombinants are illustrated in Fig. 3.

**Histochemical analysis of dorsal root ganglia from mice sacrificed 4 days postinoculation.** Previous studies performed by Margolis et al. (20) and Sawtell and Thompson (28) using the recombinant virus KOS/1 (KOS863) revealed distinct populations of neurons early in infection. Dual immunofluorescence analysis of ganglionic sections of mice infected 4 days previously revealed that as many as 90% of the neurons expressing β-galactosidase were not expressing any detectable viral antigen; conversely, most cells expressing viral antigen were not expressing β-galactosidase. It was also noted that the number of neuronal profiles expressing LAT did not significantly decrease between acute and latent time points (20, 28). This suggested that by day 4 postinoculation, the great majority of neurons had separated into two distinct populations: those in an acute phase of viral replication and those in the latent phase of viral infection. By combining in situ hybridization for the LAT with immunofluorescence for viral antigen, Speck and Simmons (29) were able to provide strong evidence for early divergence into acutely and latently infected neurons using wild-type HSV-1.

In order to assay LAT promoter activity, histochemical analysis of dorsal root ganglia from mice sacrificed 4 days postinoculation was performed. Promoter activity was measured by two methods. In the first method, whole mount ganglia were reacted with the chromogenic substrate X-Gal, and neurons that expressed β-galactosidase turned blue (Fig. 4). With the aid of a dissecting microscope, blue neurons for each mouse were counted independently by two observers. A significant decrease in the number of blue neurons between infections with the virus KOS388 and KOS252 occurred. Thus, the DNA between bp −388 and −252 clearly specifies one or more elements important for in vivo transcription. A more careful inspection of the ganglia photographed in Fig. 4 reveals more intensely stained neurons in KOS863-infected than in KOS593-infected ganglia. Although the numbers of blue sites in each infection are similar, there appears to be more β-galactosidase activity in the KOS863-infected neurons than in those infected with KOS593, which suggests that the additional region of DNA contained within KOS863 is also important for LAT transcription in vivo. This is seen more clearly by the second method of measuring β-galactosidase activity.

In the second method, neuronal extracts at 4 days postinoculation were reacted with CPRG, which forms a soluble product that can be measured by spectrophotometry. This method has the advantage of allowing quantitation of the average amount of β-galactosidase activity per animal. As can be seen in Fig. 5 (graph A), infection of mice with the largest promoter construct virus, KOS863, gives the greatest amount of β-galactosidase activity. Extracts from mice infected with KOS593, the next virus in the series, have an activity that is 40% of that of KOS863, a 2.5-fold decrease. KOS252, the fifth virus in the series, has only 2% of the activity of KOS863. The drop in X-Gal activity between KOS593 and KOS252 appears to be largely due to the 136-bp difference in promoter size between KOS388 and KOS252, consistent with the X-Gal data in Fig. 4. The drop in X-Gal activity between KOS388 and KOS252 represents a 15-fold decrease in activity. The activities of the extracts from the last two viral infections after KOS252 are too low to measure accurately. Therefore, we were unable to verify in vivo the apparent neuronal specificity which others (1, 35) have noted in this region by transient assays.

**Specificity of expression within the ganglia.** We have previously shown with KOS863 (KOS/1) that at 4 days postinfection, the infected neurons segregate into two distinct populations. Of interest here is that more than 90% of the neurons which express the β-galactosidase gene from the LAT promoter do not express any HSV-1 antigens and are thus phenotypically latent (20, 28). This is apparently due to the facts that the blue neurons are latently infected and that the productively infected neurons shut off the LAT promoter through autoregulation by the ICP4 protein (1, 3, 12). Although the LAT promoter in this construct shuts off sometime during a latent infection, β-galactosidase activity can still be observed at 21 days postinfection (our unpublished observations). We have consistently observed by immunofluorescence staining that the productively infected neurons are cleared by day 8; thus, the neurons staining at 21 days are clearly latently infected. Therefore, we attempted to verify that the expression that we observed for each of the viruses at 4 days postinfection was also similar at 21 days postinfection. Whole-mount ganglia were reacted with X-Gal, and the number of blue neurons was counted as described previously. There was an overall apparent decrease in the number and intensity of blue neurons at latent times postinoculation such that we were unable to quantitate β-galactosidase activity at 21 days postinoculation by CPRG assays. However, as measured by number of sites, the pattern of expression through the deletion series at 21 days postinoculation was similar to that at acute times on the basis of CPRG activity (Fig. 5, graph B). These data strongly suggest that the LAT promoter expression observed at day 4 for each promoter mutant was occuring in cells which had already established a latent infection.

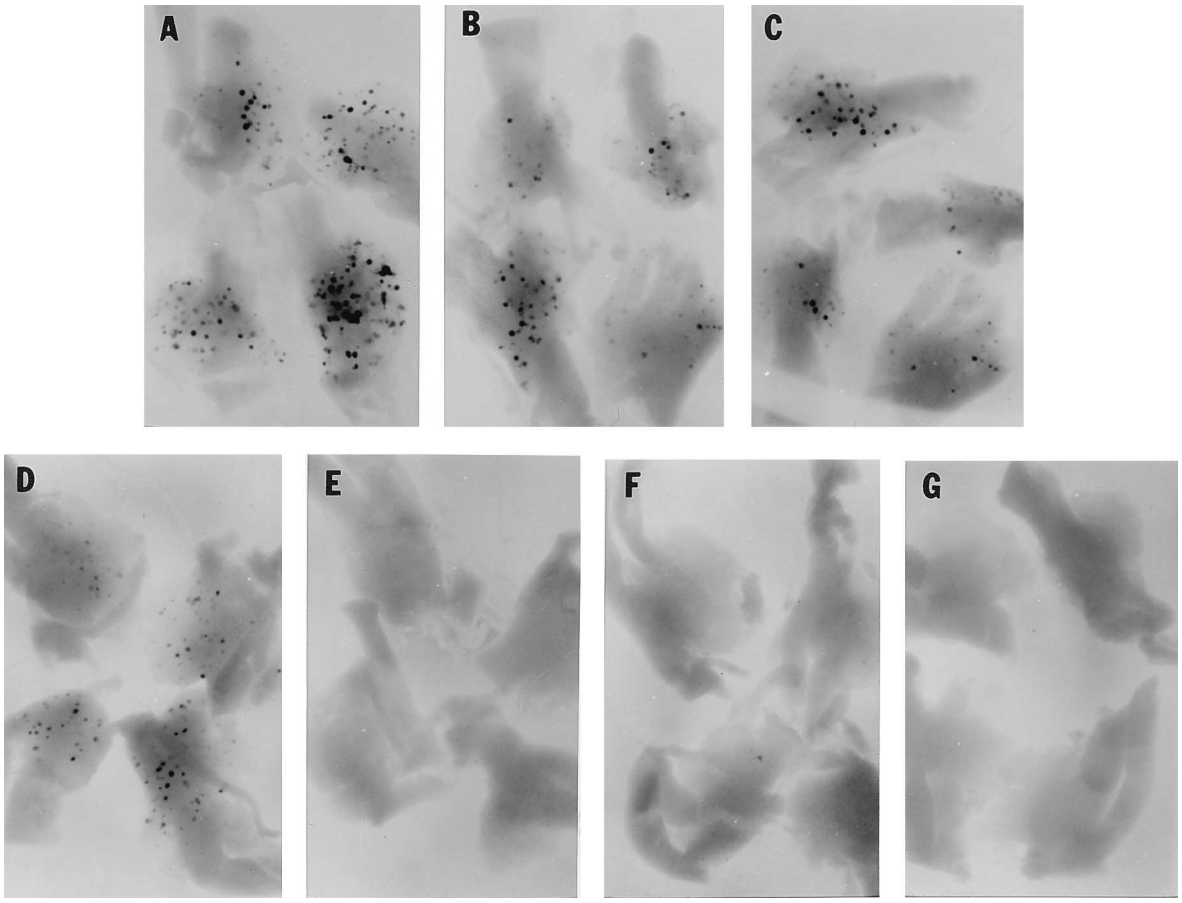


FIG. 4. Photomicrographs of whole mount dorsal root ganglia processed 4 days after footpad inoculation with the viral deletion series. Ganglia were stained with the chromogenic substrate X-Gal. Neurons expressing β-galactosidase appear as dark dots. (A) KOS863; (B) KOS593; (C) KOS506; (D) KOS388; (E) KOS252; (F) KOS124; (G) KOS57. Little to no expression could be seen in KOS252, KOS124, and KOS57, which appeared similar to the negative control [KOS(M)]-infected ganglia (data not shown).

That β-galactosidase expression in vivo is driven by specific promoter elements and is not due to spurious β-galactosidase expression is shown by several pieces of evidence. First, Ho and Mocarski, expressing the same gene from an early viral promoter, reported no β-galactosidase expression at latent times (14). Second, we have previously reported that the neurofilament-L promoter, driving β-galactosidase at gC, does not express β-galactosidase during latency (20). Third, the deletion series itself provides three internal control viruses, all from the same original plasmid in gC, which express little or no β-galactosidase in vivo. Thus, the activity observed in these neurons is dependent on the presence of the upstream LAT promoter elements and is not dependent on the site of insertion into the viral DNA.

**Efficiency of infection.** Because decreases in β-galactosidase activity could also be due to poor ganglionic infection, it was important to assess infection efficiency. This was performed by titrating the ganglionic extract for infectious virus by plaque assays. Table 1 shows that viral titers within the ganglia were equivalent among the recombinant viruses and thus cannot account for any of the decreases in β-galactosidase activity.

**In vitro infection of neuronal and nonneuronal cells in culture.** A number of studies have been reported from other laboratories on the activity of the LAT promoter as measured by transient transfection into various neuronal cell lines. Most of the commonly used neuronal cell lines are not of the same lineage as neurons of the peripheral nervous system in which HSV establishes a latent infection, and thus these cell lines may vary considerably in the quantity and specificity of their transcription factors. In addition, cells in culture are rapidly dividing, as opposed to neurons of the dorsal root ganglia, which are postmitotic. It is possible that dividing cells also vary in the quantity of specific transcription factors compared with postmitotic cells. For these reasons, we decided to analyze the activity of each LAT promoter mutant in three cell lines and compare the results with those obtained from in vivo infections described above. For infection of a nonneuronal cell line, we have used rabbit skin cells, which is the cell line used to propagate the viruses described here. We also infected two different neuronal cell lines. ND7 cells are a fusion between mouse dorsal root ganglia and mouse neuroblastoma cells. This cell line has also been selected for its ability to inhibit the immediate-early gene expression upon infection, and it has been one of the cell lines used to study LAT promoter activity (36). The other neuronal cell line used in these studies is C1300 neuroblastoma cells, a mouse neuroblastoma cell line of a different lineage than neurons of the peripheral nervous system such as dorsal root ganglia. It is important to note that the viral constructs used in these infections retained the true LAT cap, which we and O'Hare have shown to be downregulated by the ICP4 gene product. The consequence of this is that the test promoters will be responsive mainly to the presence or absence

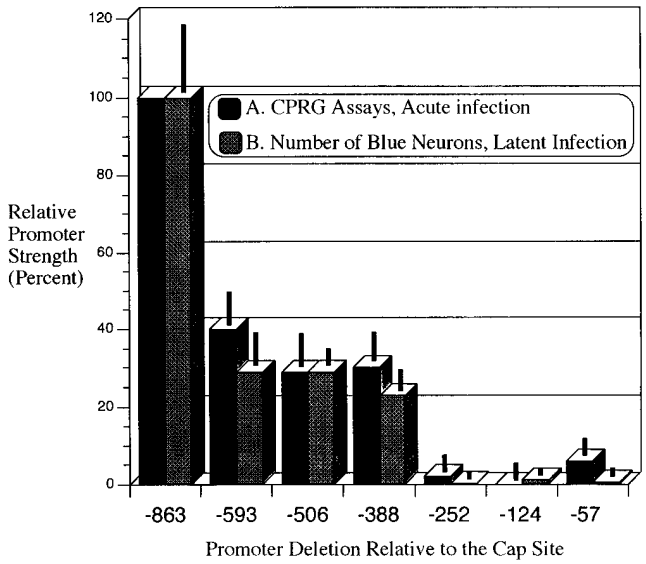


FIG. 5. (■), CPRG assay of ganglionic extracts from mice sacrificed 4 days postinoculation. Values are the means ± standard errors of the means for three separate determinations performed on ganglionic extracts from a total of five mice per group, except for the KOS506 group, which had only three mice. ▨, average number of blue neurons 21 days postinoculation. Values are the means ± standard errors of the means and were determined from a total of five mice per group, except for the KOS124 group, which had only four mice. Values for both were plotted as percentages of the value of the largest promoter construct virus, KOS863.

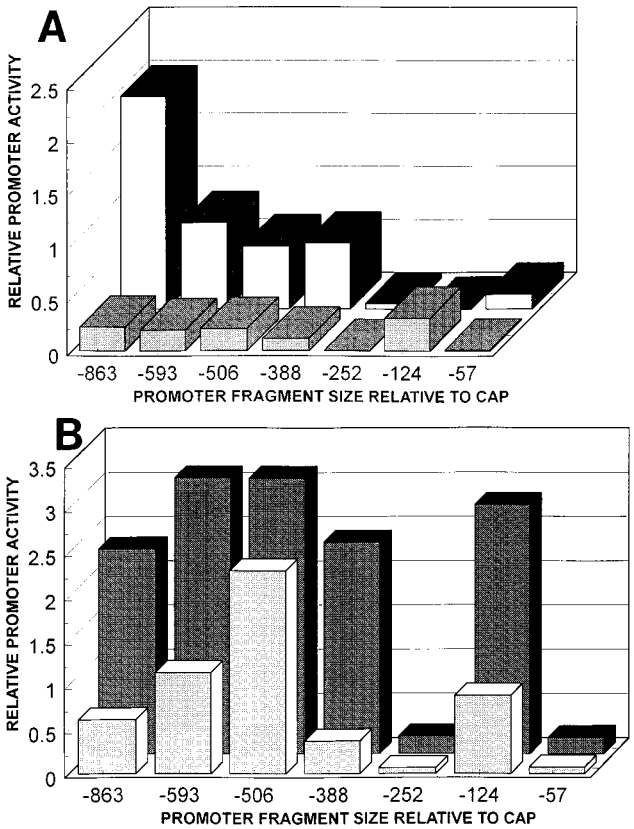


FIG. 6. (A) CPRG assay of ganglionic extracts prepared 4 days following footpad inoculation with the viral deletion series (white graphs). A 2.5-fold decrease in promoter activity is evident when the promoter size is decreased from bp −863 to −593. A 15-fold drop in activity occurs when the promoter is decreased in size from bp −388 to −252. Much less activity for KOS863 and other virus-infected cells occurs upon infection of rabbit skin cells in vitro (shaded graphs). KOS124 has the greatest activity of the promoter deletions. Data were calculated as described in the legend to Fig. 5. (B) The same promoter deletions are used to infect two different neuronal cell lines, ND7 (dark shaded squares) and C1300 (light shaded squares) neuroblastoma cells.

of transcription factors which can bind to the LAT promoter and will minimize the influence of the viral life cycle and viral DNA amplification on the pattern of expression. The results of in vitro infections with the panel LAT promoter mutants are shown in Fig. 6. As can be observed in Fig. 6A, the activity of the LAT promoter mutants in vivo, as measured by CPRG activity, decreases mainly between KOS863 and KOS593, with a more severe decline between KOS388 and KOS250. This is completely different from the pattern observed in infected rabbit skin cells, in which the promoter activity is very low throughout the deletion series and in which KOS124 exhibits the most promoter activity (Fig. 6A). Similar results were obtained by transfection of the promoter deletion plasmids into rabbit skin cells, that is, the total activity was very low, there was no significant decrease between KOS388 and KOS250, and the activity of the KOS124 promoter was relatively high compared with that observed in vivo (data not shown). For these reasons, we would suggest that the region of the LAT promoter from −863 through −388 may contain transcription factor-binding sites for neuron-specific or neuron-enhanced transcription factors.

TABLE 1. Virus titers from ganglionic extracts processed 4 days after footpad inoculation with the viral recombinants

| Virus | Viral titer (PFU/ml) |
|---|---|
| KOS863 | $9.8 \times 10^3$ |
| KOS593 | $4.5 \times 10^3$ |
| KOS506 | $6.7 \times 10^3$ |
| KOS388 | $5.2 \times 10^3$ |
| KOS252 | $6.8 \times 10^3$ |
| KOS124 | $1.1 \times 10^4$ |
| KOS57 | $7.5 \times 10^3$ |
| KOS(M) | $2.3 \times 10^5$ |

However, a comparison of the apparent neuronal activity in vivo with that observed in two different cell lines in vitro suggests that there are many differences. The LAT promoter activity in infected ND7 cells differs from that observed in vitro in two major areas. In the region between −863 and −593, there is a significant decline in activity in vivo, whereas infected ND7 cells show an increase (Fig. 6B). Secondly, in the region between −252 and −124, there is a small decline in vivo but a very large increase in ND7 cells. However, the severe drop between −388 and −252 suggests the possibility at least that there are common transcription factors which bind to this region. The LAT promoter activity in C1300 neuroblastoma cells is even more different, showing an increase between regions −593 and −506 and a sharp decline between −506 and −388, where none exists in vivo (Fig. 6B). Both neuronal cell lines show a dramatic increase in transcription of KOS124, which is a far larger increase in transcription than that observed in vivo. This may reflect the binding of a factor or factors to this region which are common to dividing cells, since both neuronal cell types as well as rabbit skin cells showed this increase in promoter activity.

In addition to these differences between in vivo and in vitro

expression, the LAT promoter in KOS252 represents a deletion nearly identical to a bp −250 deletion plasmid described by Batchelor and O'Hare (2). They reported this plasmid to have 98% of the activity of their largest promoter construct in IMR-32 neuroblastomas. By contrast, KOS252 expresses only approximately 2% of the activity of our largest promoter construct virus in vivo. Thus, there are many differences between the in vivo and the in vitro systems, indicating that the contribution of specific binding sites to LAT promoter activity should eventually be verified in vivo.

## DISCUSSION

The LAT promoter is the sole promoter active during a latent infection. For this reason, a number of studies have focused on the LAT promoter and on potential sites on the promoter responsible for activity. All of these studies have utilized transfection assays of plasmid DNAs. Thus, Zwaagstra et. al. (36) have identified a protein, LPBF, which enhances transcription in both neuronal and nonneuronal cells. Leib and colleagues have identified a cAMP-responsive element immediately upstream of the LAT TATA box (16, 23). Batchelor and O'Hare (1, 2) have identified specific sites responsible for both constitutive and specific expression in neuroblastoma cells.

The recombinant viruses described here contain regulatory elements composed of no more than 863 bp of LAT promoter, and all of these sequences have been placed into the long unique region. It is apparent that these viruses do not contain the full range of LAT promoter function, because β-galactosidase activity is gradually lost over the course of several weeks. This suggests that the constructs are missing something required for long-term expression, and recently we have obtained evidence suggesting that this region is downstream of the LAT transcription start site. First, other viral constructs not containing the region downstream of the transcription start site turn off during latency (19, 20). Second, the region downstream of the transcription start site does not contribute apparent promoter activity, since in the LAT promoter deletion virus KOS29 this region is intact and no LAT intron is observed in latent infections of mouse dorsal root ganglia (10). Third, the addition of a similar upstream LAT promoter region to the long terminal repeat of Moloney murine leukemia virus was able to promote neuronal expression in vivo, initiating within the long terminal repeat when the LAT TATA box was removed (17). Since the long-terminal repeat itself did not continue expression into latency, this demonstrates that the LAT promoter strength is derived from the upstream LAT promoter sequences, as shown also in the present study.

The present study has addressed transcription from the viral genome in vivo. The results from our studies suggest that two regions of the upstream 863 bases contribute to in vivo function. Of these, the more proximal region, between bp −388 and −252, clearly has neuron-specific elements. Important transcription elements may also be present 3′ of bp −252, but because of the low β-galactosidase levels generated by the constructs in this region, we were unable to assay any differences; in a second set of recombinant viruses will be necessary to examine these sequences in vivo. These results differ significantly from those LAT for promoter activity as measured by infection of two neuronal cells in culture as well as by comparison with transfections of these and other neuronal cell lines reported by others. The differences between our results in vivo and those obtained in vitro most likely can be attributed to the fact that the neuronal cells in culture do not accurately represent the kind or quantity of transcription factors that are found in neurons of the peripheral nervous system. Our results also suggest the possibility that cells in culture contain a level of transcription factors which bind to the proximal part of the LAT promote higher than that found in neurons in vivo.

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