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Repression of the HSV-1 latency-associated transcript (LAT) promoter by the early growth response (EGR) proteins: involvement of a binding site immediately downstream of the TATA box

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During herpes simplex virus (HSV) latency, in neurons of the nervous system, a single family of viral transcripts (the Latency-Associated Transcripts or LATs) are synthesized. Within the LAT promoter region, we have identified a consensus sequence for the EGR proteins in an unusual position immediately downstream of the TATA box. The early growth response (EGR) proteins are rapidly induced in cells by stimuli which also induce HSV to reactivate from latency. In order to determine if EGR proteins play any role in control of LAT transcription, we have analyzed the interactions between EGR proteins and the LAT promoter. Gel retardation and DNase I protection assays demonstrated that EGR1 zinc finger protein bound specifically to the LAT promoter region EGR consensus sequence. To determine if EGR proteins could modulate transcription through the LAT promoter, cotransfection assays were performed using chloramphenicol acetyltransferase (CAT) reporter constructs driven by either the wild-type LAT promoter or a LAT promoter with a mutated EGR binding site. Cotransfection of the wild-type LAT promoter construct with EGR expression plasmids resulted in inhibition of the basal level of CAT activity with EGR-2 but not EGR-1 or 3. However, normal levels of CAT activity were observed in cotransfections using the mutant LAT promoter CAT construct suggesting that repression was mediated by the binding of EGR-2 proteins to the LAT promoter. Furthermore, data from combination binding assays using EGR1 and TATA binding protein (TBP) *in vitro* support the hypothesis that binding of EGR proteins to the LAT promoter prevents binding of TBP and thus suppresses transcription. These results may provide a link between stress responses in neurons of the CNS which activate the EGR family of proteins and HSV reactivation from latency due to the same stress response.

Keywords: HSV-1; LAT; EGR; viral latency; gene regulation

Introduction

Following primary infection in man, herpes simplex virus (HSV) establishes and maintains a latent infection in the peripheral and central nervous systems and persists for the lifetime of the individual (for review see: Fraser *et al*, 1991; Roizman and Sears, 1987; Stevens, 1989). However, in response to certain stimuli (eg stress, fever, UV irradiation) the virus may reactivate in the latently infected neuron, be transported back to the primary site of infection at the periphery, and begin a lytic cycle of infection.

Using animal models of HSV-1 latency, several groups have demonstrated that in latently infected tissues, viral transcription occurs from a single gene located within the inverted repeat regions bounding the unique long (U_L) region of the viral genome (Figure 1a; Deatly *et al*, 1987; Dobson *et al*, 1989; Spivack and Fraser, 1987; Steiner *et al*, 1988; Stevens *et al*, 1987). The family of stable transcripts that are detected has been designated the Latency-Associated Transcripts (LATs) (Krause *et al*, 1988;

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Rock *et al*, 1987; Spivack and Fraser, 1987; Steiner *et al*, 1988; Stevens *et al*, 1987). No protein product has yet been identified and the function of the LATs remains unclear.

Analysis of the HSV DNA sequence in the region of the LAT gene has revealed a putative promoter region approximately 700 bp upstream of the 5′ end of the 2 kb LAT (Figure 1b). Within this region lies several SP1 binding sites, a CAAT box homology, and a TATA box (Wechsler *et al*, 1989). Genetic and biochemical evidence suggests that the LAT promoter region is important for the regulation of the LAT gene during latent infection. LAT transcripts cannot be detected in tissues latently infected with viruses in which the LAT promoter region has been deleted (Hill *et al*, 1990; Leib *et al*, 1989; Steiner *et al*, 1989). Likewise, recombinant viruses containing foreign genes inserted downstream of the LAT TATA box have been constructed and these genes are expressed during latency (Dobson *et al*, 1990; Ho and Mocarski, 1989). Also, when a 203 bp fragment containing the LAT TATA box is used to drive the chloramphenicol acetyltransferase (CAT) gene in tissue culture transfection studies, a significant level of basal activity can be measured (Batchelor and O'Hare, 1990).

Binding sites for other cellular factors are also found within the LAT promoter region. A cAMP response element (CRE) lies downstream of the LAT promoter TATA box (Leib *et al*, 1991), and a CRE site-specific mutant virus reactivated with slightly

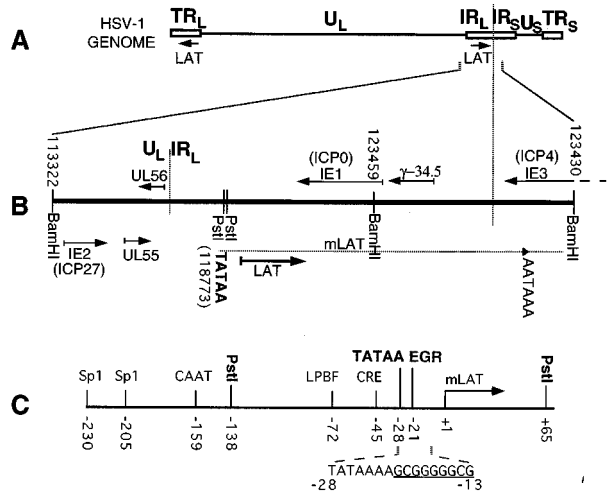

Figure 1 The LAT gene promoter region of HSV-1. (a) The LAT gene is located in terminal (TR_L) and internal (IR_L) inverted long repeat regions of the HSV-1 genome. (b) The LAT promoter TATA box is found between 2 *Pst*I sites approximately 700 bp upstream of the 5′ end of the 2 kb LAT. The positions of the RNAs from other viral genes near the LAT promoter are indicated. (c) Positions of binding sites for several cellular transcription factors within the LAT promoter region. The EGR consensus sequence lies immediately downstream of the TATA box and is underlined. Positions are numbered according to the proposed transcription start site (Dobson *et al*, 1989; Zwaagstra *et al*, 1990). The sequence is described in Perry and McGeoch (1988).

reduced frequency and slower kinetics suggesting that the CRE functions in reactivation (Rader *et al*, 1993). Using DNase I footprint analysis and gel-shift competition assays, Zwaagstra *et al*. (1991) demonstrated that a factor found in extracts of neuronal and non-neuronal cells bound to the LAT promoter at nucleotide positions −72 to −65 relative to the proposed transcription start site (Dobson *et al*, 1989; Zwaagstra *et al*, 1990). Deletion of this region resulted in loss of binding of the Latency Promoter Binding Factor (LPBF) and a reduction in LAT promoter activity in cotransfection experiments.

Within the LAT promoter region, we have identified the presence of a consensus sequence (5′-GCGGGGGCG-3′) for the Early Growth Response (Sukhatme, 1991) or EGR proteins (Figure 1c). The EGR proteins (also known as Krox 24, Zif268, TIS-8, and NGF-1A; Cao *et al*, 1990; Christy and Nathans, 1989; Herdegen *et al*, 1990; Lemaire *et al*, 1990; Lim *et al*, 1987; Milbrandt, 1987; Sukhatme *et al*, 1988) are members of the immediate early gene family, whose synthesis is regulated by extracellular stimuli such as stress, growth factors, differentiation factors, and cell-cell interaction (reviewed in Rauscher, 1993; Sukhatme, 1990). To date, a family of five EGR proteins have been described: EGR1-4, and the Wilm's tumor susceptibility gene product WT1 (Madden and Rauscher, 1993). All five proteins contain a conserved DNA binding zinc finger region in their C-termini and modulate transcription when bound to target genes via their N-terminal effector regions which are rich in proline, glutamine, and serine. Cotransfection experiments have shown that EGR1 expression will activate a minimal promoter containing an upstream EGR consensus sequence whereas WT1 expression will repress the same promoter (Madden *et al*, 1991). Recently, Gashler *et al*. (1993) identified a negative regulatory domain within the N-terminus region of the EGR1 protein which alone repressed a minimal promoter containing an upstream EGR consensus sequence in cotransfection experiments. Thus, each protein may have the potential to activate or repress transcription.

The LAT promoter EGR consensus sequence lies in a unique position immediately downstream of the HSV-1 LAT promoter TATA box (Figure 1c). Similar to HSV-1, immediately downstream of the HSV-2 LAT promoter lies a possible binding site for the EGR proteins (McGeoch *et al*, 1991). This binding site differs from the HSV-1 binding site by the presence of a cytosine in place of the first guanine in the canonical EGR consensus sequence (5′-CCGGGGGCG-3′). In many published reports of EGR consensus sequences located within eucaryotic promoters, the consensus sequence lies upstream of the TATA box (Ackerman *et al*, 1991; Brand *et al*, 1990; Chavrier *et al*, 1990; Drummond *et al*, 1992). Because of the unusual location of the EGR consensus sequence immediately downstream

of the LAT promoter TATA box, we have examined the interactions between the EGR proteins and the LAT promoter *in vitro*. Since the EGR proteins are synthesized in response to stimuli which induce reactivation of HSV from the latent state (Morgan and Curran, 1989; Sukhatme, 1990), we ultimately would like to examine the role of the EGR proteins in LAT transcription and HSV latency. In this report, we demonstrate that the EGR proteins will bind to the HSV-1 LAT promoter EGR consensus sequence *in vitro*. Furthermore, expression of EGR-2, but not EGR-1, or EGR-3 in cotransfection experiments with the LAT promoter region results in repression of this promoter.

Materials and methods

Plasmids

pPst13 contained the 203 bp HSV-1 *Pst*I–*Pst*I fragment encoding the HSV-1 strain F LAT promoter region cloned into the *Pst*I site of pGEM-3Zf(+) (Promega Corp.). p225.2 contained the 200 bp HSV-2 *Asc*I–*Asc*I fragment encoding the HSV-2 strain 333 LAT promoter region cloned in the *Asc*I site in pNEB193 (New England Biolabs). pLAT-EGR/CAT was constructed by cloning the 203 bp HSV-1 *Pst*I–*Pst*I fragment containing the LAT TATA box and EGR consensus sequence into the *Pst*I site of the promoter-less CAT vector pBASIC (Promega Corp.). The orientation of the insert was confirmed by restriction enzyme analysis and sequence analysis.

The mutant plasmid pLAT/CAT in which the HSV-1 LAT promoter EGR consensus sequence was abolished was created using PCR mutagenesis. Briefly, a 213 bp region was amplified from pPst13 using a T7 primer and the mutagenic primer 5′-CACGGCCCGTAAAGCTTATTTTATAAAGGCTGA-3′ which hybridized to the LAT promoter region. Likewise, a 179 bp product was amplified using the oligomer complementary to the mutagenic oligonucleotide and the SP6 primer. The amplification conditions were as follows: 25 cycles of 97°C for 1 min, 55°C for 2 min and 72°C for 3 min. Aliquots of each product were mixed and subjected to a second round of amplification under the conditions described above using T7 and SP6 primers. The resultant 361 bp product was purified, restricted with *Pst*I, and the 203 bp mutant fragment was cloned into the *Pst*I site of pBASIC. Incorporation of the mutation was confirmed by the presence of a new *Hin*dIII site in place of the EGR consensus sequence (5′-GCGGGGGCG-3′ mutated to 5′-TAAGCTTTA-3′). The orientation and sequence of the insert was determined by sequence analysis.

The expression vectors containing full-length cDNAs for EGR1, EGR2 and EGR3 (Patwardha *et al*, 1991) were a kind gift of Drs V Sukhatme and S Patwardhan. The genes were expressed from a vector (pCMV-CB6$^+$) which contained a human cytomegalovirus immediate-early gene promoter.

EGR gel retardation assay

The 245 bp *Hin*dIII–*Bam*HI fragment containing the LAT promoter region was excised from pPst13. Likewise, the 200 bp HSV-2 *Asc*I–*Asc*I fragment containing the HSV-2 LAT promoter region was excised from p225.2. The LAT promoter probes were synthesized by endlabeling 100 ng of the fragment with SuperScript II RNase H$^-$ Reverse Transcriptase (Life Technologies) and [α-^{32}P]dATP (NEN).

The zinc finger region of mouse EGR1 (Sukhatme *et al*, 1988) encompassing amino acids 321–427 was expressed as a histidine fusion protein and purified from *E. coli* using nickel chelate affinity chromatography (Rauscher *et al*, 1990). The binding reactions consisted of 4 μl of various concentrations of an EGR zinc finger protein, 1 μl of poly(dI-dC) (2 mg/ml) and 5 μl of 2× binding buffer (400 μg/ml poly(dI-dC), 50 mM HEPES-KOH pH 7.5, 100 mM KCl, 20 μM $ZnSO_4$, 0.2% NP40, 400 μg/ml BSA, 10% glycerol, and 2 mM dithiotheitol (DTT)). After 15 min incubation at room temperature, 1 μl of radiolabelled probe (30000 cpm) was added and the tubes were incubated an additional 10 min at room temperature. Tracking dye was added and the mixes were then loaded on a 5% native polyacrylamide gel and electrophoresed at 14.5 V/cm in 0.5 Tris-borate-EDTA buffer (Maniatis *et al*, 1982). The gel was subsequently dried and autoradiographed.

For competition assays, various concentrations of competitor double stranded DNA oligonucleotide (1 μl) were added along with the radiolabeled probe. The names and sequences of the oligonucleotides are as follows: EGR (5′-CCCGGCGCGGGGGCGAGGGCG-3′) and *Pac*I (5′-GTAACCTTAATTAATCTAGAGTCGACTTAATTAAG-3′).

EGR/TATA binding protein (TBP) gel retardation assays

Double-strand DNA 30-mer oligonucleotides spanning the wild-type or mutant LAT promoter TATA box and EGR binding site were endlabeled for use as probes. A DNA fragment derived from the Adenovirus major late promoter (AdML promoter, positioned at −18 to −42) was used as a control. Human TBP was expressed from plasmid pDS56-hTBP (a gift from T Kerpolla and T Curran, Roche, Institute of Molecular Biology) as a histidine fusion protein and purified from *E.coli* using nickel chelate affinity chromatography (Rauscher *et al*, 1990). Five ng of TBP was incubated with various concentrations of probe in a reaction buffer containing 20 mM HEPES-KOH pH 7.9, 25 mM KCl, 10% glycerol, 0.025% NP-40, 100 μg/ml BSA, 0.5 mM DTT, 0.8 mM spermine, 0.1 mM EDTA and 2 mM $MgCl_2$ to make a final volume of 10 μl. After incubation at 30°C for 30 min, DNA/TBP complex and free DNA probe were separated by electrophoresis in a 4% native polyacrylamide gel as described above. For

the combination binding assay, the incubation time was extended to 60 min.

DNase I protection assay
For probe preparation, a 221 bp *Sph*I–*Xba*I fragment containing the LAT promoter region was excised from pLAT-EGR/CAT or pLAT/CAT. The probe was 5′ endlabeled. The probe mixes contained radiolabeled probe (30000 cpm), 4% polyvinyl alcohol, and poly(dI-dC) (200 μg/ml).

Various concentrations of EGR zinc finger protein (1 μl) diluted in 1 × binding buffer (25 mM HEPES/KOH pH 7.5, 50 mM KCl, 12.5 μM $ZnSO_4$, 10% glycerol, 5% NP40, and 2 mM DTT) were incubated in 25 μl of 2 × binding buffer on ice for 5 min. Control tubes contained no protein. Probe mix (25 μl) was added and the tubes were incubated an additional 15 min on ice. Fifty microliters of 10 mM $MgCl_2$/5 mM $CaCl_2$ was added and the tubes were incubated at room temperature for 1 min. Two microliters of DNase I diluted in 1 × binding buffer was added and tubes were incubated at room temperature. The concentration of DNase I used was that which would cut 50% of the probe in 2 min at room temperature. The reactions were stopped by the addition of 90 μl of stop solution (20 mM EDTA pH 8.0, 1% SDS, 200 mM NaCl and 250 μg/ml yeast transfer RNA). Following phenol : chloroform extraction and ethanol precipitation, the nucleic acid pellets were resuspended in 1 μl of RNase A (500 μg/ml) and incubated at room temperature. A sequencing reaction was used to locate the footprint. Five microliters of formamide loading dye (Maniatis *et al*, 1982) was added, the tubes were incubated at 90°C for 5 min, quenched on ice, and electrophoresed on a 7% polyacrylamide/7 M urea gel. The gel was subsequently dried and autoradiography was performed using an intensifying screen.

Transfections and CAT assays
SY5Y and C1300 cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% calf serum. Cells (1.4×10^5) were plated in 100 mm tissue culture dishes one day before transfection. The medium was replaced with fresh medium 4 h prior to transfection. Calcium phosphate precipitates were prepared (Graham *et al*, 1973) containing 2 μg of the reporter construct, and 10 μg of the EGR expression plasmid per dish. The precipitate was added directly to the medium and incubated for 5 h. Following a one minute 15% glycerol shock, the cells were incubated an additional 48 h. The cells were washed twice with phosphate-buffer saline (PBS) and resuspended in 100 μl of 0.25 M Tris buffer pH 7.5. Cell extracts were prepared by 3 rounds of freeze-thaw lysis. The supernatants were normalized for protein using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories). CAT activity in the normalized supernatants was assayed by the liquid scintillation method of Sankaran (1992).

For CV-1 cells the following modifications were made: (1) they were maintained in Dulbecco's modified Eagle's Medium containing 10% calf serum, 25 mM HEPES and high glucose. (2) Cells (6×10^5) were plated in 100 mm tissue culture dishes one day before transfection. (3) Calcium phosphate precipitates were prepared (Graham *et al*, 1973) containing 2 μg of the reporter construct and either 0 μg, 0.5 μg, 1 μg, 2 μg, 5 μg or 10 μg of the EGR expression plasmid per dish. (4) pBluescript SK(−) was used as a filler such that total DNA per dish was 14 μg. (5) The precipitate was added directly to the medium and incubated for 8 h prior to glycerol shock.

Results

The zinc finger region of the EGR1 protein binds to the wild-type LAT (WT-LAT) promoter EGR consensus sequence in vitro
The HSV-1 LAT promoter region containing the TATA box homology and the EGR consensus sequence lies within a 203 bp *Pst*I–*Pst*I fragment approximately 700 bp upstream of the 5′ end of the 2 kb LAT (Figure 1b and c). Likewise, the HSV-2 LAT promoter region lies within a 200 bp *Asc*I–*Asc*I fragment. These fragments were subcloned as described in the Methods and then isolated for use in the following experiments.

Gel retardation experiments were performed to determine if the EGR proteins were capable of binding to the HSV-1 and HSV-2 wt-LAT promoter probes *in vitro*. An EGR protein expressing the zinc finger region and a histidine tag was used for these experiments. This protein was considered to represent binding of all EGR proteins since (1) >95% sequence identity exists between the zinc finger regions of all known EGR proteins and (2) the zinc finger regions of all EGR proteins recognize the identical DNA consensus sequence (Madden and Rauscher, 1993).

The 245 bp fragment containing the wild-type HSV-1 LAT promoter region was radiolabelled and incubated in the presence or absence of the EGR1 zinc finger protein. The reaction mixes were electrophoresed on a 5% native polyacrylamide gel. A single band was observed when the HSV-1 wild-type LAT (wt-LAT) promoter probe was incubated in the absence of any protein (Figure 2a, lanes 1 and 6). Slower migrating probe-protein complexes were observed only when the HSV-1 wt-LAT promoter probe was incubated in the presence of the EGR1 zinc finger protein (lanes 2 and 7). Competition experiments were carried out to confirm the specificity of the probe-protein complex formation. For these experiments, a non-radiolabeled 21-mer containing an EGR consensus sequence (EGR oligomer) or a non-radiolabeled 35-mer which did not contain an EGR binding site (*Pac*I oligomer) was incubated with the radio-

labeled HSV-1 wt-LAT probe and EGR1 zinc finger proteins. Promoter-protein complex formation was inhibited when the LAT probe was incubated in the presence of the EGR1 zinc finger fusion protein and the competitive EGR oligomer (lanes 3–5). The amount of competition was proportional to the concentration of the EGR oligomer. As expected, no competition was observed when the *Pac*I oligomer was used as the competitor (lanes 8–10). Similar results were obtained using the HSV-2 wt-LAT promoter probe (Figure 2b). These results show that the EGR proteins will bind to the HSV-1 and HSV-2 wt-LAT promoter regions *in vitro*.

Several probe-protein bands were always observed in the gel retardation assays. Increasing the concentration of the protein did not result in the resolution of the shifted species into a single band (data not shown) suggesting that a second binding site exists in the probes. However, because no other canonical EGR consensus sequences exist within the probes, it is unlikely that the protein binds to a different region of the probe.

The EGR1 zinc finger protein binds to a single consensus binding site within the HSV-1 LAT promoter

In order to determine where the EGR1 zinc finger bound to the HSV-1 LAT probe in gel retardation assays and if any non-canonical EGR binding sites exist, DNase I protection assays were performed. A 221 bp probe containing either the wild-type or mutated HSV-1 LAT promoter region was 5′ end-labeled, incubated in the presence or absence of various concentrations of the EGR1 zinc finger protein, and treated with DNase I as described in the Methods. Regions where the protein binds to the probe are protected from nucleolytic cleavage. The reaction mixes were electrophoresed on a 7% polyacrylamide/7 M urea gel.

Using the HSV-1 wt-LAT promoter probe, no protected regions were observed in the absence of EGR zinc finger protein (Figure 3, lane 1). However, a protected region was observed corresponding to the EGR consensus sequence in the presence of EGR1 zinc finger protein, proportional to the concentration of the EGR1 zinc finger

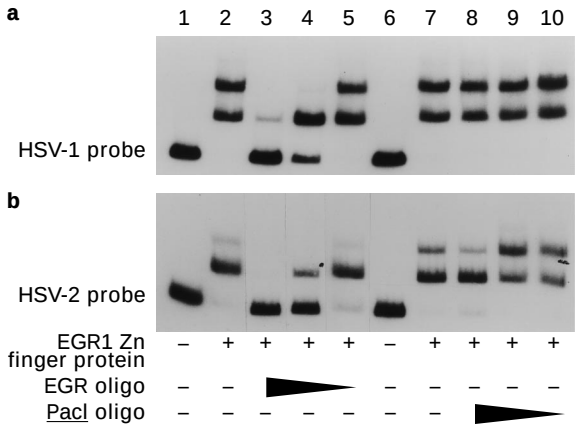

Figure 2 Gel retardation analysis of LAT promoter probe and the EGR1 zinc finger protein binding. The radiolabelled HSV-1 (a) or HSV-2 (b) LAT promoter probes were incubated in the presence or absence of the EGR1 zinc finger protein (160 ng) as indicated below the appropriate lanes. For competition studies, 100 ng (lanes 3 and 8), 30 ng (lanes 4 and 9) or 3 ng (lanes 5 and 10) of competitive EGR oligomer or non-competitive *Pac*I oligomer were also included. The reaction mixtures were electrophoresed on a 5% polyacrylamide gel and detected by autoradiography.

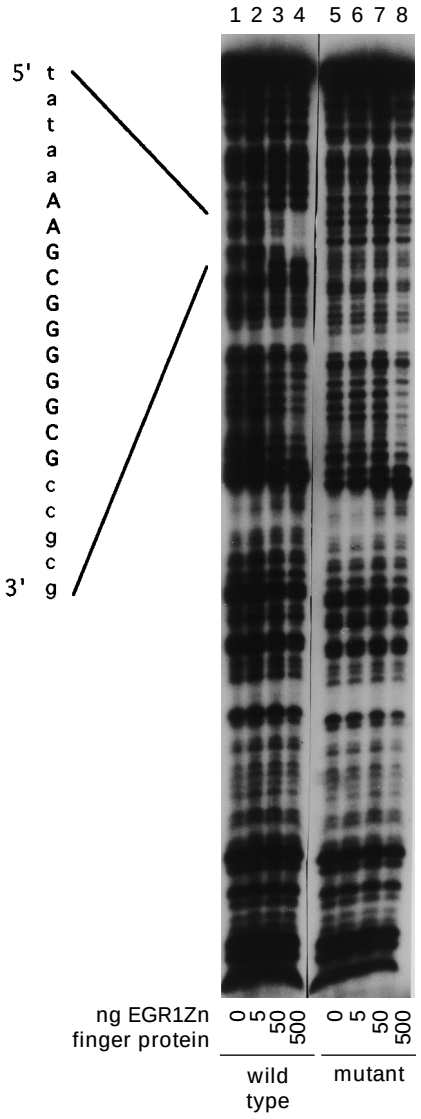

Figure 3 DNase I protection assay of the LAT promoter probe and the EGR1 zinc finger protein. The 5′ endlabeled wild-type or mutant LAT promoter probes were incubated in the presence or absence of the indicated amounts of the EGR1 zinc finger protein. The reaction mixtures were treated with DNase I and electrophoresed as described in the Materials and methods. The protected region corresponds to approximately 11 bases as indicated by the upper case letters.

Table 1 Regulation of the LAT promoter-CAT reporter constructs by EGR proteins in various cell lines

	Relative CAT activity[a] *Cell line*		
Expression plasmid	*C1300*	*SY5Y*	*CV-1*
–	1.00[b]	1.00	1.00
EGR1	0.57	0.50	0.46
EGR2	0.17	0.25	0.12
EGR3	0.25	0.03	0.12

[a]SY5Y, C1300 and CV-1 cells in 100 mm dishes were transiently transfected with 2 μg of wild-type pLAT-EGR/CAT reporter construct, and 10 μg of the EGR expression vector. Supernatants normalized for protein were used for measurement of CAT activity
[b]The basal CAT activity of the CAT construct alone was assigned a relative value of 1.00. Cotransfection data is expressed as percent activity as compared to the CAT construct alone. Values represent the average of a single assay performed in duplicate. Assays were repeated at least twice. Student's *t* test was applied and results found significant in each case ($t \leqslant 0.01$)

Table 2 Mutation of the LAT promoter EGR consensus sequence abolishes repression of CAT activity in cotransfected CV-1 cells

	Relative CAT activity[a]	
Expression plasmid	*pLAT-EGR/CAT*	*pLAT-CAT*
–	1.00[b]	1.00
EGR2	0.13	1.44
EGR1 Zn finger	0.33	6.61

[a]CV-1 cells in 100 mm dishes were transiently transfected with 2 μg of wild-type (pLAT-EGR/CAT) or mutant (pLAT-CAT) reporter construct, and 10 μg of either the EGR2 expression vector or an EGR1 zinc finger region expression vector. Supernatants normalized for protein were used for measurement of CAT activity
[b]The basal CAT activity of the CAT construct alone was assigned a relative value of 1.00. Cotransfection data is expressed as percent activity as compared to the CAT construct alone. Values represent the average of a single assay performed in duplicate. Assays were repeated at least twice with comparable results. Student's *t* test was applied and the results found significant in each case ($t \leqslant 0.005$)

protein added (lanes 2–4). Similar reactions were carried out using a probe in which the LAT promoter EGR consensus sequence was abolished. By incorporating a *Hin*dIII restriction endonuclease site in to the EGR consensus sequence, five of the six guanine residues critical for binding of the EGR protein to the DNA target were altered (Pavletich and Pabo, 1991). In contrast to the pattern observed with the HSV-1 wt-LAT promoter probe, the EGR1 zinc finger protein did not protect the HSV-1 mutant LAT (mut-LAT) promoter probe from DNase I cleavage even at the highest concentration of protein (lanes 5–8). Also, it is important to note that no other region of protection was observed over the whole length of either probe indicating that the protein does not bind to any other sequences within the 203 bp HSV-1 LAT promoter region.

The EGR proteins repress transcription through the HSV-1 LAT promoter

Since the EGR1 zinc finger protein was capable of binding to the HSV-1 EGR consensus sequence immediately downstream of the LAT TATA box *in vitro*, we next examined the ability of the EGR proteins to modulate transcription through the HSV-1 wt-LAT promoter in various neuronal and non-neuronal cell types. Previous work by others has established that a CAT reporter plasmid driven by the 203 bp *Pst*I–*Pst*I HSV-1 LAT promoter region exhibit significant basal levels of CAT activity in transfected cell extracts (Batchelor and O'Hare, 1990). Therefore, for these studies, the 203 bp wild-type or mutant HSV-1 LAT promoter fragment was cloned into the expression vector pBASIC to generate the plasmids pLAT-EGR/CAT or pLAT/CAT, respectively. The reporter constructs were transfected alone or in combination with EGR1, EGR2, or EGR3 expression plasmids into SY5Y (human neuroblastoma), C1300 (murine neuroblastoma), or CV-1 (African green monkey kidney) cell lines, and CAT activity was measured in various cell extracts as described in the Materials and methods.

The basal level of CAT activity of the wild-type pLAT-EGR/CAT reporter construct in each cell line tested was assigned a relative value of 1.00 (Table 1). At a high level of EGR plasmid (10 μg/dish) expression of EGR1 repressed transcription through the wt-LAT promoter by 43% (57% of basal activity) in cotransfected C1300 neuronal cells. EGR2 and EGR3 consistently inhibited transcription to a greater degree than EGR1 (83% and 75%, respectively). Similar results were observed in extracts from cotransfected SY5Y neuronal and CV-1 non-neuronal cells. Since repression was observed both in neuronal and non-neuronal cell types, the repression appears to be promoter specific rather than cell type specific.

To determine if binding of the EGR proteins to the HSV-1 wt-LAT promoter was necessary for repression, similar assays were performed using the mutant promoter construct, pLAT/CAT, in which the EGR consensus sequence has been abolished. As seen before, EGR2 repressed transcription of the wt-LAT promoter construct pLAT-EGR/CAT by 87% (13% of basal activity) in cotransfected CV-1 cells (Table 2). In contrast, no repression was observed in CV-1 cells cotransfected with the mutant LAT (mut-LAT) promoter reporter construct pLAT/CAT and the EGR2 expression plasmid (144% of basal activity).

These experiments were also performed using an expression plasmid encoding only the zinc finger region of EGR1. As expected, cotransfection of this

plasmid also repressed transcription of pLAT-EGR/CAT by 67% (33% of basal activity). Interestingly, expression of EGR1 zinc finger protein consistently actived transcription of pLAT/CAT which lacks an EGR consensus sequence. The EGR zinc finger protein may transactivate by altering the transcription of a cellular factor which may non-specifically or indirectly activate transcription of the reporter construct.

EGR2 protein represses transcriptional activity of the HSV-1 LAT promoter over a wide variety of concentrations

To show that the pLAT-EGR/CAT repression was not due to high concentrations of EGR transcription factor a range of EGR expression plasmid concentrations was used. Five concentrations of EGR expression plasmid were cotransfected with the reporter constructs into CV-1 cells, and CAT activity measured in the cell extracts as described in the Materials and methods. The assays were repeated three times for each concentration point in combination with both wild-type and mutant reporter constructs.

CAT activity is expressed as a ratio of units of CAT activity/μg protein at a given concentration of EGR relative to units CAT activity/μg protein when the EGR construct is not present in the cotransfection. Figure 4 shows that at lower concentrations of EGR1 and EGR3, statistically significant repression does not occur. For EGR1, there does not appear to be any effect on the *Pst*I–*Pst*I LAT promoter region. On the other hand, EGR2 shows a significant repression by standard deviation of EGR2 on the wild-type LAT promoter construct (pLAT-EGR/CAT) versus the mutant construct (pLAT/CAT). At a concentration of 10 μg of EGR2, both wild-type and mutant reporter constructs exhibit approximately 70% repression (0.3 $\times$ basal level). By halving the concentration of EGR2 to 5 μg a statistically significant repression (0.17 ± 0.049 or 83% repression) of the wild-type reporter is seen. In comparison the CAT activity of the mutant construct is at basal levels. This trend essentially continues through 0.5 μg of EGR2. This indicates that binding of EGR2 protein to the HSV-1 wt-LAT promoter was necessary and sufficient for transcriptional repression in this system.

Binding of the EGR1 zinc finger protein to the LAT promoter prevents binding of TATA binding protein (TBP)

The results of the CAT assays suggested that the repression of the HSV-1 wt-LAT promoter was directly mediated by binding of the EGR2 protein. Since the EGR binding site lies immediately downstream of the LAT promoter TATA box, binding of the EGR proteins may prevent binding of TBP (and hence the general transcription complex). Alternatively, TBP may bind, but movement of the general transcription complex may be sterically inhibited by the bound EGR protein. The following experiments were designed to test these hypotheses.

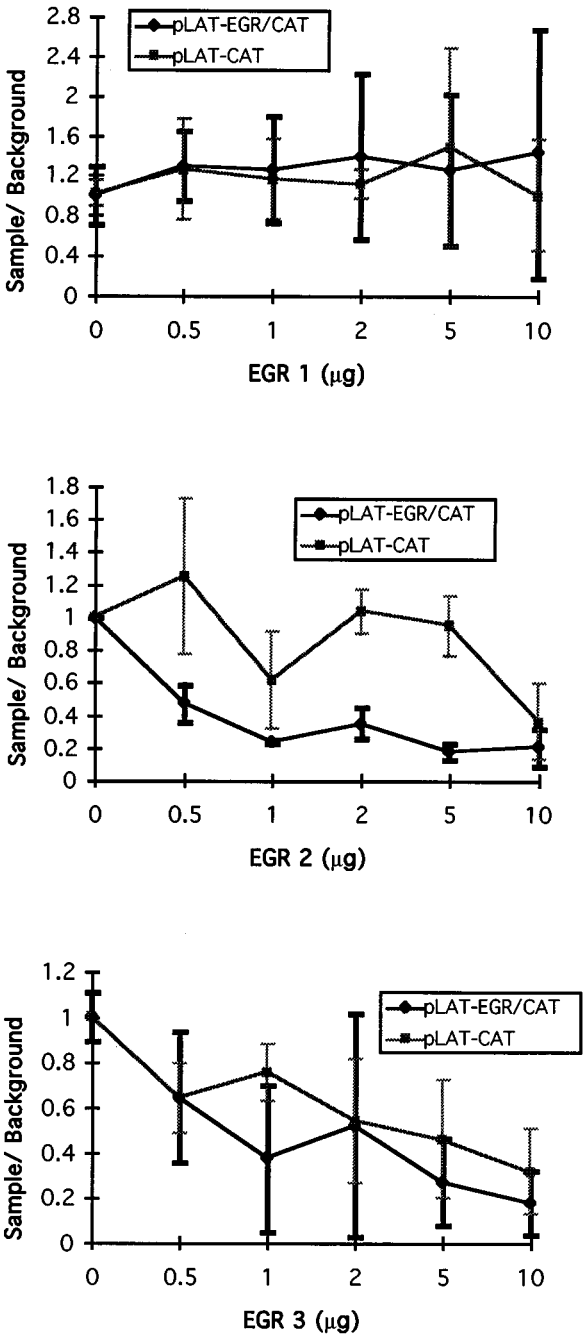

Figure 4 EGR Titration of HSV LAT-promoter-CAT reporter constructs. A fixed amount (2 μg) of wild-type or mutant LAT promoter-CAT reporter construct was cotransfected with various amounts of EGR-1, 2, or 3 expression plasmids (0 μg, 0.5 μg, 1 μg, 2 μg, 5 μg or 10 μg) as described in Materials and methods. Cellular supernatants normalized for protein were used for measurement of CAT activity. The basal level of CAT activity of the reporter construct alone was assigned a relative value of 1.00. Cotransfection data is expressed as a value relative to basal CAT activity. Values represent the average of three assays performed in duplicate.

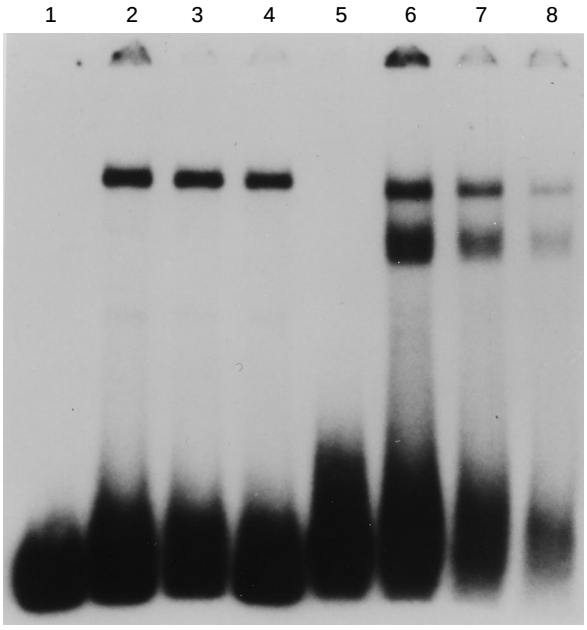

Figure 5 Gel retardation analysis of TBP binding to the LAT promoter region. Radiolabeled Adenovirus major late promoter (AdML) probe (lanes 1–4) or HSV-1 LAT promoter probe (lanes 5–8) were incubated in the presence (lanes 2–4, 6–8) or absence (lanes 1 and 5) of 5 ng of TBP and electrophoresed as described in the Materials and methods. The concentrations of the probes in lanes 1–4 and 5–8 were 4, 4, 2, and 1 nM respectively.

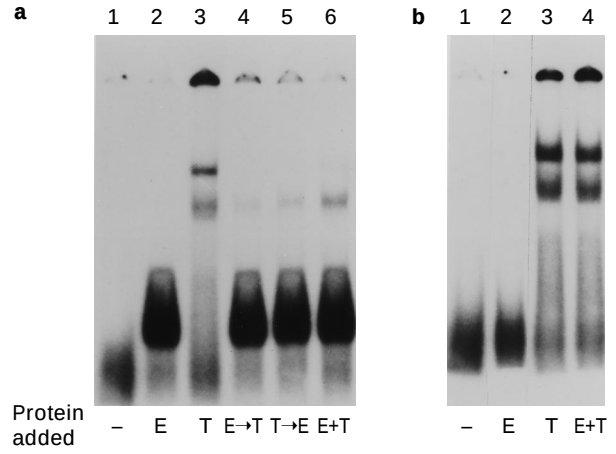

Figure 6 Gel retardation analysis of the binding of combinations of EGR1 and TBP to the LAT promoter region. Wild-type LAT promoter probe (a) or mutant LAT promoter probe (b) was incubated with 4 ng EGR1 and/or 5 ng TBP as described in Figure 5 except that the incubation time was 60 min instead of 30 min. Lane 4 (a) indicates the DNA probe treated with EGR1 for 30 min prior to the addition of TBP. Lane 5 indicates the DNA probe treated with TBP for 30 min prior to the addition of EGR1. Abbreviations: E=EGR1; T=TBP.

TBP gel retardation assays were first performed to establish that the protein could bind to an HSV-1 wt-LAT promoter oligomer probe. A DNA fragment probe derived from the Adenovirus major late promoter (AdML) was used as a positive control. In the absence of TBP, only free AdML probe was observed (Figure 5, lane 1). Shifted species were observed when various concentrations of the AdML promoter probe were incubated in the presence of TBP (Figure 5, lanes 2–4). As expected, LAT promoter/TBP complexes were observed only when the HSV-1 wt-LAT promoter oligomer probe was incubated in the presence of TBP (Figure 5, lanes 5–8).

The binding pattern of both TBP and EGR1 zinc finger (zf) on the HSV-1 LAT promoter region was examined by a combination assay run under TBP binding conditions. This experiment was designed to test the effects of the order of addition of proteins into the reaction. Incubation of the wt-LAT promoter oligomer probe with EGR1 (zf) resulted in a probe/protein complex which migrated more slowly than free probe (Figure 6a, lanes 1–2). The wt-LAT promoter/TBP complex migrated more slowly than the wt-LAT probe/EGR1 protein complex (Figure 6a, lane 3). Therefore, the differences in migration patterns of the two complexes could be used to determine which protein was bound to the probe. Incubation of the wt-LAT promoter probe with EGR1 (zf) 30 min prior to the addition of TBP resulted in a shifted species comigrating with the wt-LAT promoter/EGR1 complex (Figure 6a, lane 4). The same result was obtained when the probe was incubated with TBP 30 min prior to the addition of EGR1 (zf) (Figure 6a, lane 5). Therefore, bound EGR1 (zf) was not displaced by TBP, but EGR1 (zf) could displace bound TBP. Following simultaneous incubation of the wt-LAT promoter probe with EGR1 (zf) and TBP for 60 min (Figure 6a, lane 6), the majority of shifted species comigrated with the wt-LAT probe/EGR1 complex.

An HSV-1 mutant LAT (mut-LAT) promoter oligomer lacking the EGR binding site was also tested for its ability to bind EGR1 (zf) and TBP. As expected, no shifted complexes were observed when the mut-LAT promoter probe was incubated in the absence or presence of EGR1 (zf) (Figure 6b, lanes 1 and 2, respectively). Incubation of the mutant probe with EGR1 (zf) and TBP simultaneously (Figure 6b, lane 4) resulted in the formation of a complex which comigrated with a mut-LAT promoter/TBP complex (Figure 6b, lane 3). These results suggest that binding of EGR proteins to the LAT promoter *in vitro* prevents binding of TBP and thus suppresses transcription.

Discussion

This report describes the presence of an EGR consensus sequence in an unusual position immediately downstream of the HSV-1 LAT promoter TATA box. A non-canonical EGR consensus

sequence is located in the same position in the HSV-2 LAT promoter region. EGR consensus sequences usually lie upstream of the TATA box within eucaryotic promoters (Ackerman *et al*, 1991; Brand *et al*, 1990; Chavrier *et al*, 1990; Drummond *et al*, 1992). However, Wang *et al*. (1992) demonstrated that EGR1 could bind to a sequence 50 bp downstream of the PDGF A-chain promoter transcription start site. Analysis of this region revealed a non-canonical EGR consensus sequence. However, the function of this binding site was not determined.

Gel retardation assays demonstrated that the EGR1 zinc finger protein specifically bound to the HSV-1 and HSV-2 LAT promoter probes (Figures 2a and b). Several shifted bands were consistently seen in the LAT promoter probe gel retardation assays. Titration of the protein in the presence of the HSV-1 LAT promoter probe revealed the presence of three shifted bands at low protein concentrations which could only be resolved into the two more slowly migrating bands even at saturating concentrations of the protein (data not shown). This result suggests that two EGR binding sites may exist in the LAT promoter probe. However, DNase I protection assay data demonstrated that no other EGR binding sites exist in the HSV-1 LAT promoter sequence (Figure 3). It is possible that very low affinity EGR binding sites are found within the promoter region which could not be detected by DNase I protection analysis or by sequence analysis. If these very low affinity binding sites exist, their importance *in vivo* is questionable. Alternatively, the EGR1 zinc finger fusion protein may bind to the DNA probe as a multimer. However, this observation has not been reported in other systems.

Using the HSV-1 LAT promoter to drive the CAT reporter gene, a significant level of basal activity is reported here, and elsewhere by others (Batchelor and O'Hare, 1990). Cotransfection of the wild-type LAT-EGR/CAT constructs with EGR expression plasmids consistently resulted in repression of the promoter in both neuronal and non-neuronal cells (Tables 1 and 2). This result was surprising since the EGR proteins were shown to activate a minimal promoter containing an EGR consensus sequence (Madden *et al*, 1991). Wang *et al*. (1992) demonstrated that EGR1 slightly repressed the activity of a PDGF A-chain promoter construct that contained a downstream, non-canonical EGR binding site in cotransfected NIH3T3 fibroblasts. However, a slight activation was measured in cotransfected human embryonic kidney-derived 293 cells. The authors hypothesized that the promoter context of the EGR binding site and cell type cotransfected may determine whether EGR1 is an activator or a repressor of transcription.

In our co-transfection experiments high concentrations (10 μg) of EGR1, EGR2, and EGR3 all repressed transcription from the LAT promoter in both neuronal (SY5Y, C1300) and non-neuronal (CV-1) cell types. However, a detailed titration of each EGR expression vector in the transfection assay revealed that EGR2 was the most efficient at repressing the LAT promoter, in that significant effects were observed at the lowest concentrations (0.5 μg) used. The question arises as to whether these differences reflect a true specificity for EGR2 at this particular EGR binding site in the LAT promoter or whether it is due to experimental variables such as level of expression, protein turnover, etc. A few points are germane to this discussion. First, the DNA binding domains of EGR1, 2, and 3 display greater than 95% amino acid sequence identity. We have carefully compared the DNA recognition specificities of each of these proteins among a spectrum of potential EGR binding sites and have found no significant differences in affinities or specificities. Thus, it is unlikely that the specificity we have observed for EGR and the LAT site is due to preferential recognition *in vivo* of that site by EGR2 zinc finger region. Second, although the DNA binding domain of EGR 1–3 are highly related, the regions outside of this domain are highly divergent. Thus, a region in the EGR2 protein may adopt a conformation (*in vivo*, when bound to the LAT promoter) most efficient at displacing TFIID or any other adjacent complex. Third, we have shown that a plasmid which only expresses the DNA binding domain of EGR1 is sufficient to repress the LAT promoter. Thus, we believe that simple competition and/or displacement of TFIID and/or the basal transcription machinery by the zinc finger region of the EGR proteins is the most likely explanation for LAT promoter repression.

Because of the unique position of the EGR consensus sequence immediately downstream of the LAT TATA box, binding of the EGR2 protein to this site may repress transcription by direct competition between the protein and general transcription factors (Levine and Manley, 1989). Binding of the EGR proteins may prevent binding of the transcription complex to the LAT promoter (Figure 7a). Alternatively, even if the transcription complex binds to the promoter, the presence of the bound EGR proteins may prevent movement of the general transcription complex down the gene (Figure 7b). However, in the absence of an EGR consensus sequence (eg pLAT/CAT), the transcription complex can bind to the LAT promoter region and begin transcription of the CAT gene (Figure 7c).

The results of competition and combination gel retardation assays using wild-type and mutant LAT promoter probes, EGR1 zinc finger protein region and TBP (Figure 5; also see Chiang *et al*, 1996, Figure 3) support the hypothesis that repression of transcription is mediated by direct competition of the EGR proteins and TBP for LAT promoter binding sites. Specifically, EGR1 (zf) can displace

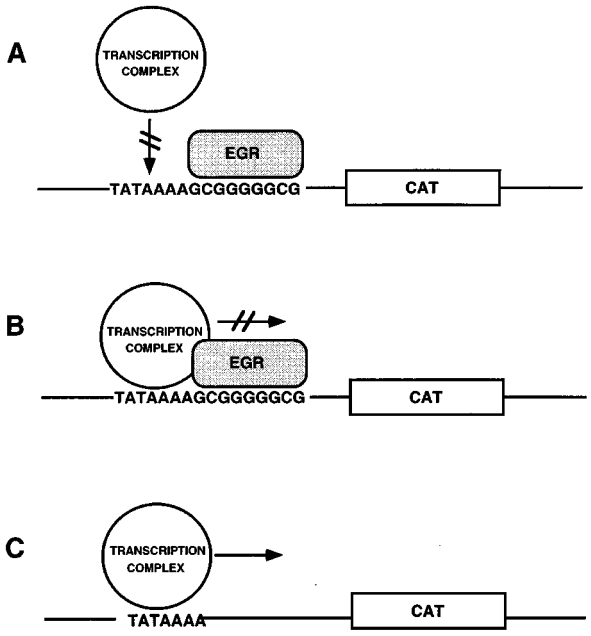

Figure 7 Schematic representation of the possible mechanisms of transcriptional repression of the LAT promoter by the EGR proteins. (a) The binding of EGR2 prevents the transcription complex from binding the LAT promoter resulting in a repression of CAT production. (b) CAT production could alternatively be repressed by EGR2 binding preventing the bound transcription complex from moving down the gene. (c) The bound transcription complex transcribes the CAT gene when EGR2 is incapable of binding the DNA due to the absence of its DNA binding consensus sequence.

TBP which has already bound to the LAT promoter. Also, addition of TBP to LAT promoter/EGR1 complexes could not displace bound EGR1 (zf) (Chiang *et al*, 1996, Figure 3). These data indicate that the binding of EGR1 (zf) to the LAT promoter is stronger than the binding of TBP. Therefore, in addition to the promoter context of the EGR consensus sequence, the position of the EGR binding site in relation to the TATA box may also determine if the proteins activate or repress transcription through a specific promoter.

Additionally, data from cotransfection assays using the expression plasmid encoding only the zinc finger region of EGR1 suggested that the EGR zinc finger region is the only EGR protein domain necessary for repression of the wt-LAT promoter/CAT construct (Table 2). Although a small repressor domain has been described for the EGR1 protein, the dominant activity of the full length N-terminus region of the EGR1 appears to be activation (Gashler *et al*, 1993). It has not been shown whether EGR2 and EGR3 also contain the small repressor domain. The data presented here suggest that a mutant EGR protein with a deleted repressor domain would still repress transcription through the LAT promoter via the model of direct competition.

In other situations, the EGR proteins may also repress transcription by other mechanisms. EGR1 has been shown to repress transcription by competing with Sp1 for binding to overlapping consensus sequences upstream of the murine adenosine deaminase promoter (Ackerman *et al*, 1991). Disruption of the EGR specific region of the overlap resulted in increased promoter activity in cotransfection assays whereas disruption of the Sp1 specific region greatly reduced promoter activity.

It is important to note that considerable sequence homology exists between the HSV-1 and HSV-2 LAT promoters in the region of the TATA box (Krause *et al*, 1988). Since HSV-2 also establishes and reactivates from latency in humans and in animal models and produces latency associated transcripts (Burke *et al*, 1991; Mitchell *et al*, 1990), the presence of EGR binding sites in both HSV-1 and HSV-2 LAT promoters (McGeoch *et al*, 1991) suggests that the EGR proteins play a role in the regulation of both LAT genes.

Recent evidence shows that sequences distal to the region surrounding the LAT TATA box are also capable of regulating expression of the LAT gene. During latent infection in mice, KOS-29, a mutant lacking the 203 bp HSV-1 *Pst*I–*Pst*I fragment which contains the LAT gene TATA box, failed to synthesize any LATs (Dobson *et al*, 1989; Nicosia *et al*, 1993). However, during the acute infection or in infected tissue culture cells, the 2 kb LAT was detected. In another study, a probe specific for the 2 kb LAT hybridized to tissues latently infected with a mutant in which the LAT TATA box had been specifically mutated (Rader *et al*, 1993). These observations suggest that another promoter outside of the *Pst*I–*Pst*I fragment can also drive LAT transcription during the acute infection. Therefore, binding of the EGR proteins to the consensus sequence immediately downstream of the LAT TATA box may specifically affect LAT gene expression during reactivation. Recently, Chen *et al*. (1995) showed that there are two promoters capable of expressing LAT RNA: LAP1, which contains the TATA element, expresses during latency; and LAP2, which does not have a TATA element, expresses during acute infection.

In order for EGR proteins to play a role in HSV infection and pathogenesis, expression of the cellular EGR proteins must be demonstrated in tissues which are latently infected with HSV. EGR expression can be induced by a variety of stimuli in numerous neuronal and non-neuronal cell types (Gupta *et al*, 1991; Kreider and Rovera, 1992; Nguyen *et al*, 1993; Ryseck *et al*, 1988). Recently, Herdegen *et al*. (1993) reported basal expression of EGR2 in the rat dorsal root ganglia, the site of HSV latency following footpad inoculation of mice. EGR expression within the trigeminal ganglia, the site of

HSV latency following ocular inoculation of mice, has not been definitively determined (Herdegen *et al*, 1990).

It is now apparent that the LAT gene is a more complicated transcription unit than previously thought (Chen *et al*, 1995; Dobson *et al*, 1989; Fraser *et al*, 1992; Goins *et al*, 1994; Leib *et al*, 1989; Nicosia *et al*, 1993) and the role that EGR proteins play in the regulation of the LAT gene during reactivation will have to be established. During HSV reactivation, the rapid induction of EGR proteins within latently infected cells may play a role in the induction of viral replication by repressing LAT transcription. In fact, a transient decrease in LAT expression has been observed following synchronous reactivation of bovine herpesvirus in latently infected rabbits (Rock *et al*, 1987). Following reactivation, LAT transcription may also be repressed by a second mechanism. Downstream of the TATA box and overlapping the transcription start site of the 2 kb LAT lies a binding site for the viral protein ICP4, one of the first viral proteins expressed during viral replication. Batchelor and O'Hare demonstrated that, in transient expression assays, ICP4 significantly downregulates gene expression through the LAT promoter (Batchelor and O'Hare, 1990). Finally, EGR proteins may also act on cellular promoters to alter the concentration of factors which either promote or inhibit viral replication.

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