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Analysis of the Promoter and *cis*-Acting Elements Regulating Expression of Herpes Simplex Virus Type 2 Latency-Associated Transcripts

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In latently infected neurons, herpes simplex virus type 2 (HSV-2) expresses one abundant family of transcripts, the latency-associated transcripts (LATs). We demonstrate here that the sequence lying about 700 bp upstream of the 5′ end of the HSV-2 major LAT acts as a very strong promoter in transient expression assays in both neuronal and nonneuronal cells. Transcription starts about 27 to 32 bp downstream of a functional TATA box. The proximal fragment from −102 to +34 includes the basal promoter and accounts for constitutive transcriptional activity in various cell lines. The distal region from −392 to −103 contributes to particularly strong promoter activity in neuronal cell lines and involves multiple *cis*-acting elements. A functional activating transcription factor/cyclic AMP (cAMP) response element binding protein motif lies just upstream of the TATA. By DNase I footprint and methylation protection assays, we identified several additional protein-binding sites upstream of the activating transcription factor/cAMP response element binding protein motif. A GC-rich element, termed LAT-3, was located between bases −128 to −102. A 2-bp substitution in LAT-3 markedly reduced promoter activity and abolished protein-binding ability in vitro. Gel retardation assay showed no competition for protein binding to LAT-3 by other GC-rich elements. LAT-3 appears to be a novel *cis*-acting element that may contribute to the neuronal responsiveness of the HSV-2 LAT promoter.

Herpes simplex virus type 2 (HSV-2) causes genital infections (12, 28) that recur by virtue of reactivation of virus that persists in sensory neurons from the time of first infection (1, 6). The mechanisms by which HSV-2 or other alphaherpesviruses establish and maintain latent infection and reactivate periodically are not fully understood.

In latently infected human or animal sensory neurons, HSV-1 and HSV-2 express a single, abundant family of transcripts (7, 8, 9, 35, 36). These latency-associated transcripts (LATs) map to the long genomic repeats and arise from the strand complementary to and overlapping that which encodes a major viral immediate-early transcriptional regulatory protein, ICP0 (7, 13, 14, 19, 26, 36, 37).

The most abundant, or major, HSV-2 LATs are about 2.2 kb in length (7, 19, 26). A minor, less abundant, population of HSV-2 LATs of about 8 to 9 kb in length initiates about 700 bp upstream of the major LATs (Fig. 1), extends beyond the ICP0 gene through the gene encoding a neurovirulence determinant, ICP34.5, and terminates near the 3′ end of the ICP4 gene (19, 26).

Since the discovery of these LATs, their properties, biological function, and regulation have been the subject of considerable work and speculation. LATs are not proven to encode protein products. Nevertheless, much of the accumulated evidence suggests that they facilitate reactivation of latent virus (4, 20, 22, 31, 34, 38). As the only abundant viral transcripts detected in latency, the LAT's regulation must differ from that of all other HSV genes.

Most knowledge concerning the LAT promoter derives from studies of HSV-1. An active polymerase II promoter was identified upstream of the HSV-1 minor LAT (10, 39). The transcription start site and TATA box (10, 41), *cis*-acting elements such as the activating transcription factor/cyclic AMP (cAMP) response element binding protein motif (ATF/CREB) (18, 23), LAT promoter-binding factor/upstream stimulating factor (LPBF/USF) (40), and neuron-responsive regions (2, 3, 41) have been localized within the region as well. Recently, an atypical TATA-less promoter was identified just upstream of the major LAT start site (15, 29).

Unlike the major LATs themselves, the upstream untranscribed regions of the HSV-1 and HSV-2 LAT genes are highly conserved (19, 25). Sequence comparisons showed that there are two *cis* elements within the putative HSV-2 LAT promoter (2LATP) region identical to those found in the HSV-1 LAT promoter (1LATP) region, namely, the TATA box and the ATF/CRE, but no LPBF/USF sequence. By transient expression assay and primer extension, Krause et al. identified an active polymerase II promoter domain upstream of the 5′ end of the major LAT (19) and later more precisely localized it to a *Not*I-*Not*I fragment in that region (19a). Deletion of both copies of this fragment from the virus did not appear to alter its in vitro and in vivo growth or virulence but markedly impaired spontaneous virus reactivation in the guinea pig model of genital herpes (20).

To further understand the nature and transcriptional regulation of the HSV-2 LAT gene, we generated and evaluated a series of truncation, deletion, and site-specific mutations of the 2LATP domain, with which we were able to characterize its basal promoter elements, constitutively active region, and neuronal responsiveness. By footprint, methylation protection, site-specific mutation, and gel mobility shift assays, we identified eight protein-binding sites within the 2LATP, including a novel GC-rich *cis*-acting element, which we termed LAT-3.

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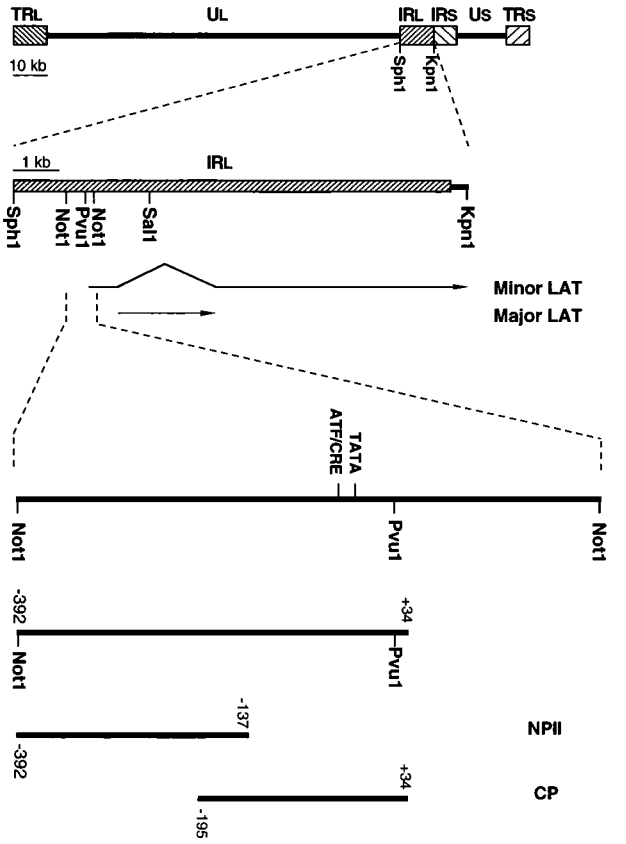


FIG. 1. Schematic representation of HSV-2 genome and LAT promoter (2LATP) region studied. A 632-bp *Not*I-*Not*I fragment, bases 5476 to 6106 according to McGeoch et al. (25), was subcloned, as was its left-most two-thirds derivative, a 426-bp fragment spanning bases −392 to +34 relative to the transcription start site. The later (construct p-392) was used for extensive transient expression studies. Further cleavages of the −392 to +34 segment provided fragments NPII (−392 to −137) and CP (−195 to +34) for footprinting and methylation protection studies.

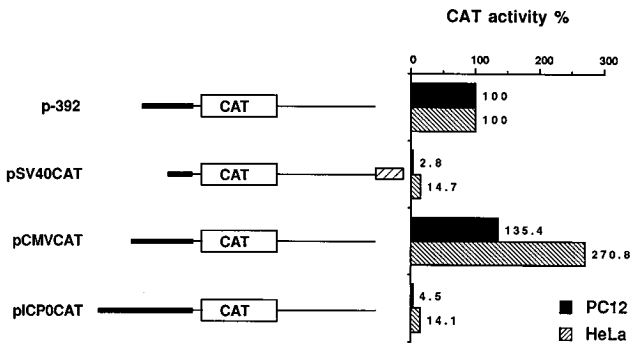


FIG. 2. Comparison of 2LATP activity with that of other promoters in transient expression assays. The promoter activities of p-392 in PC12 and HeLa cells were compared with those of the SV40 early promoter plus enhancer, the CMV immediate-early promoter, and the HSV-1 ICP0 promoter. The percent CAT activity was normalized relative to that for the p-392 construct in PC12 cells (solid bar; 21.7% average acetylation in four experiments) and in HeLa cells (hatched bar; 19.1% average acetylation in four experiments). To the left, the solid bars represent the promoter sequences placed upstream of CAT, the thinner bars indicate the plasmid sequences, and the hatched box shows the SV40 enhancer placed downstream of CAT.

MATERIALS AND METHODS

Plasmid constructs. A 632-bp DNA fragment encompassing the putative 2LATP region from −392 to +240 relative to the transcription start site (see below) was released by *Not*I cleavage (Fig. 1) from a larger *Sph*I-*Sal*I plasmid clone of the inverted long repeat region of HSV-2 strain 333 genomic DNA. This *Not*I fragment was subcloned into the plasmid vector pCAT · Basic (Promega, Madison, Wis.) between *Sal*I and *Xba*I sites upstream of the chloramphenicol acetyltransferase (CAT)-coding sequence and designated pCAT · NN. A derivative of this fragment from −392 to +34 (Fig. 1) relative to the start site of transcription was also cloned into pCAT · Basic and designated p-392. A series of 5′-end deletions of p-392 from base −392 were generated by PCR with pCAT · NN as the template and cloned into the pCAT · Basic vector at its *Pst*I and *Xba*I sites. The names of the plasmids are based on the positions of their 5′ ends relative to the transcription start site (see Fig. 4).

Site-specific point mutations were introduced into the wild-type plasmid p-392 with the Mut-a-gene kit (Bio-Rad, Richmond, Calif.) following the manufacturer's instruction.

pCAT · Control (Promega) is a plasmid in which the CAT gene is driven by the simian virus 40 (SV40) early promoter and enhancer; for convenience, we refer to it here as pSV40CAT. To make pICP0CAT, a DNA fragment spanning the HSV-1 ICP0 gene promoter region from −807 to +120 was released from p110pKS (a gift from Liyanage P. Perera) and inserted into pCAT · Basic at the *Hin*dIII and *Xba*I sites. pCMVCAT was created with a DNA fragment encompassing the human cytomagalovirus (CMV) major immediate-early promoter from −582 to +7 released from pBK · CMV (Stratagene, La Jolla, Calif.) with *Nsi*I and *Nhe*I and cloned into pCAT · Basic at the *Pst*I and *Xba*I sites (Fig. 2) after the ends had been blunted with T4 DNA polymerase.

All sequences of the fragments generated from PCR and mutagenesis were confirmed by chain termination sequencing with a Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, Ohio). All of the plasmids used for transient expression assays were purified as described previously (30) with two cycles of ultracentrifugation in cesium chloride.

Cells. The rat pheochromocytoma cell line PC12 (American Type Culture Collection, Rockville, Md.) was cultured in RPMI 1640 medium supplemented with 10% horse serum (all sera were heat inactivated), 5% fetal calf serum, and 1% GASP (a combination of glutamine, aureomycin, streptomycin, and penicillin). The human neuroblastoma cell line IMR32 (American Type Culture Collection) was cultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum and 1% GASP. HeLa cells were cultured in MEM with 10% fetal calf serum and 1% GASP. The mouse neuroblastoma cell line, C1300 (American Type Culture Collection), was cultured in MEM-199 medium (1:1) with 10% fetal calf serum and 1% GASP.

Transfection and CAT assay. Two days before transfection, cells were plated onto 60-mm-diameter tissue culture dishes and maintained at 37°C in 5% CO_2. Either 5 μg of DNA (for PC12, IMR32, and HeLa cells) or 1 μg of DNA (for C1300 cells) was used for transfection by the calcium phosphate coprecipitation method of Graham and Van Der Eb (16). Two days after transfection, cells were harvested and extracted in 0.25 M Tris-Cl (pH 7.4) with three cycles of freezing on dry ice and thawing at 37°C.

CAT assays were performed as described by Shaw (33). Briefly, cell extracts were mixed with 2 μl (50 nCi) of [^{14}C]chloramphenicol (Amersham, Arlington Heights, Ill.)–20 μl of 4 mM acetyl coenzyme A and 0.25 M Tris-Cl (pH 7.4) to make a volume of 150 μl. The mixtures were incubated at 37°C for 1 h. Reactions were stopped by extraction with 1 ml of cold ethyl acetate, and the organic phase was recovered and lyophilized. The pellets were resuspended in 30 μl of ethyl acetate and subjected to thin-layer chromatography. The radioactivity on the thin-layer chromatography sheet was quantitated by a System 200 imaging scanner (Bioscan, Inc., Washington, D.C.). To insure that the CAT assays were conducted within the linear range of the test, in each set of CAT assays the amounts of cell extracts were adjusted so that the acetylation of the p-392 construct was about 20 to 50%. The percent acetylation in each experiment was then converted into percent CAT activity relative to that of the construct p-392 in the same experiment. The data presented in Fig. 2, 4, and 7 represent the averages of the results of three or four independent transfections.

DNase I footprint assay. Nuclear extracts used for the footprint assay were prepared as described by Montminy and Bilezikjian (27). The DNA fragments NPII and CP shown in Fig. 1 were 3′ end labeled with Klenow enzyme and [α-^{32}P]deoxynucleoside triphosphates or 5′ end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. The footprint assay was conducted as described by Jones et al. (17). Briefly, 16 to 32 μg of nuclear extracts was mixed with 2×10^4 cpm of DNA probes and 1 μg of nonspecific competitor poly(dI-dC)-poly(dI-dC) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) in a 45-μl volume with a final concentration of 25 mM Tris-Cl (pH 7.9)–6.25 mM $MgCl_2$–50 mM KCl–0.5 mM EDTA–0.5 mM dithiothreitol–10% glycerol and incubated on ice for 15 min. Samples were then equilibrated in a 22°C water bath for 2 min, and 5 μl of DNase in 5 mM $CaCl_2$ was added to each tube. After a 90-s incubation, the reaction was stopped with 100 μl of stop buffer (200 mM NaCl, 1% sodium dodecyl sulfate, 20 mM EDTA, 100 μg of tRNA per ml), and the reaction mixture was subjected to phenol-chloroform extraction and ethanol precipitation. The pellet was resuspended in 2 μl of TE (10 mM Tris-Cl [pH 8.0], 1 mM EDTA) and 3 μl of sequencing stop buffer (United States Biochemical), denatured at 90°C for 3 min, and resolved on an 8% acrylamide sequencing gel.

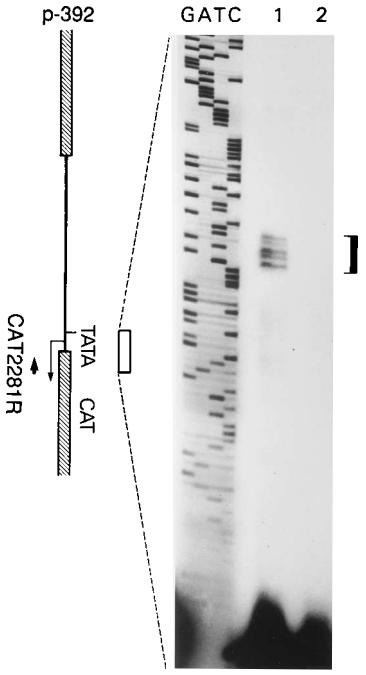


FIG. 3. HSV-2 LAT start site as determined by primer extension assay. (Left panel) Structure of the p-392 construct with its putative TATA box and transcription start site. The primer CAT2281R used in the assay is indicated as a bold arrow facing upstream. Total cellular RNA was purified from C1300 cells transfected with p-392 as well as from untransfected C1300 cells. Extension products from the reaction were loaded on lanes 1 and 2, respectively. Lanes labeled as GATC reveal the sequencing ladders generated with p-392 as the template and CAT2281R as the primer. The open bracket on the right indicates the primer-extended products.

Methylation protection assay. DNA probes (2×10^4 cpm per reaction) were mixed and incubated with nuclear extracts and other reagents as in the footprint assay but in a volume of 45 μl. The mixtures were then subjected to DNA methylation and either cleavage at G residues or strong cleavage at A and weak cleavage at G (A>G) residues as described by Maxam and Gilbert (24). The final products were dissolved in 2 μl of TE and 3 μl of sequencing stop buffer, denatured at 90°C for 3 min, and resolved on an 8% acrylamide sequencing gel. DNA sequencing ladders were generated by the Maxam and Gilbert method (24) with the same probe used in the methylation protection assay.

Gel mobility-shift assay. Synthesized oligonucleotides spanning the regions of footprint 3 (Ft3) were used as probes. To serve as a probe, two complementary oligonucleotides were annealed and end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP to a specific activity about 0.0025 to 0.005 pmol/2×10^4 cpm. Labeled probes were purified on 6% acrylamide nondenaturing gels. For each reaction, 2×10^4 cpm of the probe was mixed, with or without specific unlabeled oligonucleotide competitors as required, in 17 μl of reaction buffer [0.5 μg poly(dI-dC)-poly(dI-dC), 25 mM Tris-Cl (pH 7.9), 6.25 mM $MgCl_2$, 50 mM KCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol] with 1 μl (about 4 μg of protein) of nuclear extract, and the mixture was incubated on ice bath for 15 min. The reactions were resolved on 1.5-mm-thick, 6% acrylamide–0.25× TBE gels, run at 12.5 V/cm at 4°C about 1.5 h while the buffer was recirculated. The gels were dried and autoradiographed. The specific oligonucleotide competitors were synthesized on a Gene Assembler Plus (Pharmacia LKB, Piscataway, N.J.), purified on 15% denaturing acrylamide–urea gels, and quantitated by measuring their absorbance at 260 nm with a DU-40 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The names and sequences of the oligonucleotide competitors are shown in Fig. 8b.

Primer extension. To prepare RNA for primer extension, C1300 cells were transfected, as described above, but 100-mm-diameter dishes and 12 μg of DNA per dish were used. Two days after transfection, cells were harvested, and total cellular RNAs were purified with RNAzol (Tel-Test, Inc., Friendswood, Tex.) following the manufacturer's instruction.

The structure of the plasmid for transfection and the primer are shown in Fig. 3. Primer CAT2281R was used for studies of RNA expressed from cells transfected with plasmid p-392 in that it anneals to the sequence from vector pCAT · Basic at bp 2281 to 2310, which is 74 bp downstream of the putative 2LATP TATA box. Primer NN438R was used for RNA from cells transfected with plasmid pCAT · NN as it anneals to the HSV-2 sequence 73 bp downstream of the putative TATA box (data not shown). The primers were end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified on a 15% acrylamide–urea-denaturing gel. Extension reactions with 100 μg of RNA were carried out as described previously (30), with avian myeloblastosis virus reverse transcriptase. The extension products were digested with RNase, extracted with phenol-chloroform, and precipitated with ethanol. The pellets were resuspended in 2 μl of TE and 3 μl of sequencing stop buffer, denatured at 95°C for 5 min, and resolved on 8% acrylamide sequencing gels. DNA sequencing reaction mixtures, generated with p-392 as the template and CAT2281R as the primer, were loaded side-by-side on the same gel to serve as molecular ladders.

RESULTS

Identification of 2LATP. DNA sequence analysis suggested the presence of a potential polymerase II promoter several hundred bp upstream of the 5′ end of the major HSV-2 LAT. By transient expression, CAT assay, mutagenesis, and in vivo study in the guinea pig, Krause et al. confirmed the presence of an active LAT promoter between two *Not*I enzyme recognition sites (19, 20). To characterize this promoter and its *cis*-acting elements, the 632-bp *Not*I fragment and a 426-bp truncated derivative of it spanning bases −392 to +34 relative to the transcription start site (Fig. 1), as determined below, were cloned into plasmid pCAT · Basic and designated pCAT · NN and p-392, respectively.

When transfected into rat neuronal PC12 cells, p-392 displayed two- to fourfold more CAT activity than pCAT · NN. The quantities of CAT-specific mRNA, however, were the same for each transfection (data not shown), indicating that the pCAT · NN mRNA may be translated less efficiently. Therefore, we chose p-392 as the parental construct for our subsequent analyses.

On transient expression, the p-392 promoter proved to be very strong in neuronal cells. To more precisely define strength of 2LATP, we compared its activity with that of other potent promoters: the human CMV immediate-early promoter, the SV40 early promoter plus enhancer, and the HSV-1 ICP0 gene promoter. The CMV immediate-early promoter is a very active one in many different tissues (5, 32); the SV40 early promoter has high levels of activity in monkey cell lines, less activity in human cell lines, and particularly low levels of activity in neuronal cells (11).

The promoter activities of the CMV, SV40, and ICP0 constructs and the 2LATP construct p-392 were compared in both PC12 and HeLa cells by transient expression and CAT assay. The results of four independent experiments are summarized in Fig. 2. In PC12 cells, the p-392 construct yielded an average of 21.7% acetylation, almost as active as pCMVCAT. In HeLa cells p-392 exhibited an average of 19.1% acetylation, which was about one-half to one-third the activity of pCMVCAT. The activity of the SV40 promoter construct, pSV40CAT, was only 2.8 and 14.7% of that of p-392 in PC12 and HeLa cells, respectively. pICP0CAT exhibited only 4.5 and 14.1% of the activity seen with p-392 in PC12 and HeLa cells, respectively. These studies showed that the 2LATP DNA fragment spanning bases −392 to +34 has very strong transcriptional activity, particularly in neuronal cells.

Identification of transcription start site. The transcription start site of 2LATP was mapped with primer extension. Since transfected C1300 cells afforded even higher levels of CAT activity of p-392 than the other cell lines (see below), we used C1300 cell RNA for these studies. RNA from untransfected as well as p-392-transfected C1300 cells was tested with primer CAT2281R that anneals within the CAT sequence 74 bp downstream of the predicted TATA box in p-392. The labeled primers were hybridized with the RNAs, extended with avian myeloblastosis virus reverse transcriptase, and resolved on a denaturing acrylamide gel. A sequencing ladder was gen-

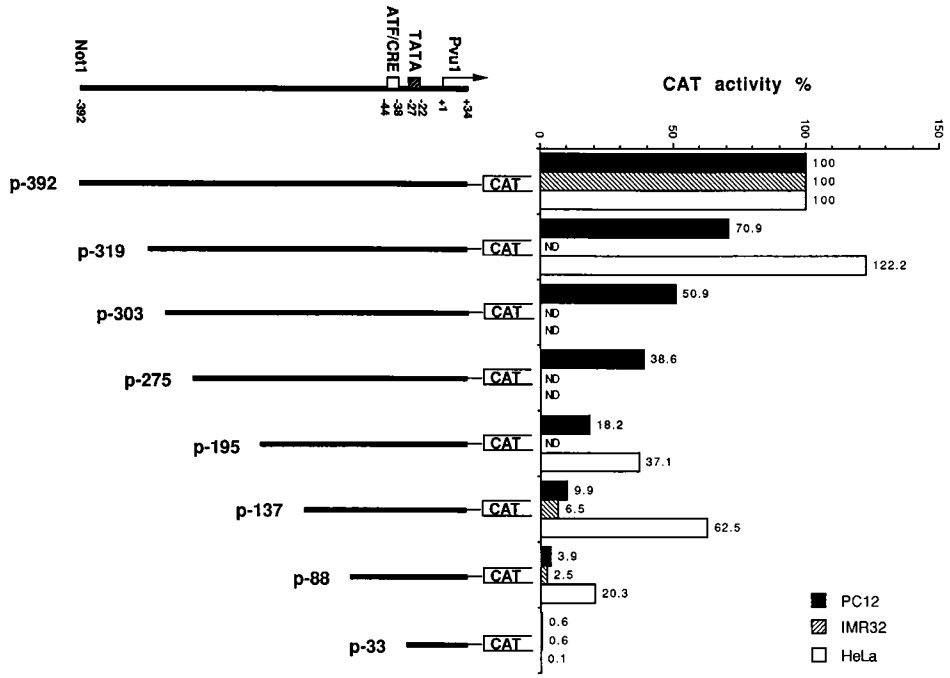


FIG. 4. Schematic representation of structure and CAT activity of p-392 and a series of its 5′-end deletion products in different cell lines. The percent CAT activity is normalized relative to that for the p-392 construct in PC12 cells (21.3% average acetylation in four experiments), in IMR32 cells (11.7% average acetylation in two experiments), or in HeLa cells (15.7% average acetylation in four experiments). The structure of the viral insert in plasmid p-392 is shown on the top. Each plasmid is named according to the base position of its 5′ end relative to the transcription start site (+1). ND, not done.

erated with the p-392 as the template and CAT2281R as the primer and loaded side-by-side with the primer extension reaction (Fig. 3). No extended bands were detected from untransfected control cell RNA (lane 2). A set of nested bands were detected from the reaction with transfected cell RNA (lane 1), with the largest comigrating with a T, 27 bp downstream of the 5′ end of the putative TATA box.

The result was confirmed by using RNA purified from C1300 cells transfected with the larger 2LATP construct, pCAT · NN, and a primer that anneals to the viral sequence 73 bp downstream of the putative TATA box (data not shown).

The powerful promoter activity and transcription from a specific start site suggested that the putative 2LATP TATA box is functional. It was necessary to prove with site-specific mutation that the putative TATA element is active, but before doing so, we developed a fuller appreciation of the regulatory elements situated further upstream of it.

Characterization of neuron-responsive and constitutive regulatory regions. To detect functional elements upstream of the TATA box, we generated a series of 5′-end deletional derivatives of p-392 and analyzed their promoter activities in both PC12 and HeLa cells. Deletion of the sequence from −392 to −137 reduced the promoter activity by 90% in PC12 but by only 38% in HeLa cells (Fig. 4), implying that some portions of the distal (5′-most) region of 2LATP are particularly neuron responsive. Successive, smaller deletions to positions −319, −303, −275, and −195 reduced CAT activity in PC12 cells by 29, 49, 61, and 82%, respectively (Fig. 4). These data indicate that the whole distal region, extending to position −392, contributes to the promoter activity in PC12 cells, probably because of the presence of multiple *cis*-acting elements.

The further 5′-end deletion of bases −137 to −88 reduced 2LATP activity an additional two- to threefold in both PC12 and HeLa cell lines (Fig. 4), indicating the presence of crucial *cis*-acting elements in this region. Deletion of the sequence from bases −88 to −33, just upstream of the TATA, further reduced the promoter activity in PC12 and HeLa cells to undetectable levels. By analogy to the 1LATP sequence, this region should contain an ATF/CREB element, which may contribute activity to 2LATP (23).

To confirm that differences in promoter activity in rat PC12 and human HeLa cells are tissue specific and not merely species specific, key 2LATP constructs were also tested in the human neuroblastoma IMR32 cell line. The promoter activity pattern was more similar to that of PC12 cells than to that of HeLa cells (Fig. 4).

Localization and characterization of individual 2LATP *cis*-acting elements. We conducted DNase I footprint, methylation protection, and gel mobility shift assays to identify individual protein-binding elements in 2LATP. For the DNase I footprint and methylation protection assays, two overlapping DNA fragments, NPII and CP (Fig. 1), were end labeled and used as probes. Nuclear extracts from multiple cell lines were used, as were purified *trans*-activator proteins AP1 and AP2, because sequences highly homologous to their recognition sites are found in 2LATP. When the labeled NPII probes were incubated with crude nuclear extracts from HeLa, PC12, C1300, or IMR32 cells, bases −305 to −274 were protected from DNase I digestion (Fig. 5a, lanes 5 to 10,) and designated Ft1. When NPII was incubated with purified AP1, a footprint was seen spanning bases −315 to −303 (Fig. 5a, lane 1). AP2 protected two regions of NPII, from bases −380 to −366 and −221 to −201 (Fig. 5a, lane 2). Crude nuclear extracts protected three regions of probes from CP, designated Ft2 (−162 to −144), Ft3 (−128 to −102) and Ft4 (−52 to −30), which spans the putative ATF/CREB site (Fig. 5b, lanes 5 to 12). An AP1-binding region overlapped Ft4. An AP2-binding site was also found within the CP region at −65 to −53 (Fig. 5b, lane 2).

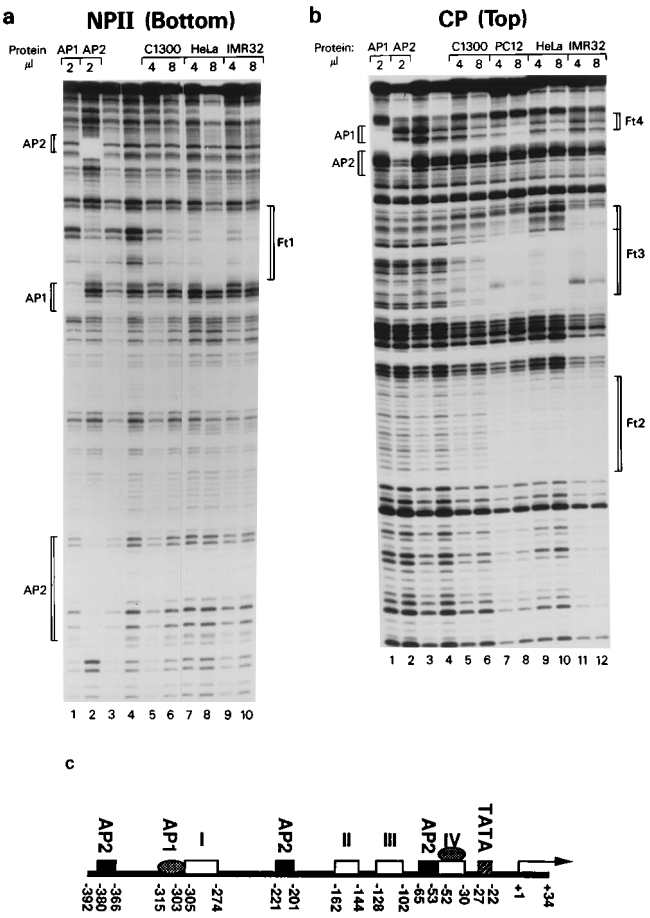


FIG. 5. Results of DNase I footprint assays. (a and b) DNase I protection by binding of 2 μl of purified nuclear proteins AP1 and AP2 or of 4 or 8 μl of nuclear extracts from C1300, PC12, HeLa, or IMR32 cells, as indicated above the lanes. Lanes 3 and 4, probe only (no added binding proteins) digested with various amounts of DNase I. In panel a, the probe, NPII (Bottom), was the NPII fragment (bases −392 to −137) labeled on the 3′ end of the bottom strand. In panel b, the probe, CP (Top), was the CP fragment (bases −195 to +34) labeled on 5′ end of the top strand. Footprints created by binding of AP1 or AP2 are shown as brackets to the left of each panel, while footprints generated by nuclear extracts are bracketed as Ft1 to Ft4 to the right of each panel. (c) Summary of the footprints and their locations in 2LATP.

The above experiments located at least eight *cis*-acting elements which can interact with nuclear proteins or purified AP1 or AP2 in vitro (Fig. 5c). When these data are considered together with the results of the CAT assays using sequential 5′ deletions, it would appear that some of the footprints, Ft3 and Ft4 for example, may be particularly important to 2LATP activity. Since the nuclear extracts from neuronal cells seem to protect a longer sequence at the Ft3 region than extracts from HeLa cells, Ft3 may also contribute to the neuronal responsiveness of 2LATP.

Mutational analyses of footprints. To delineate the functional relevance of each of the above footprints more precisely, it was necessary to create site-specific mutations within them. Because the footprints were large, methylation protection assays were first used to identify the bases in closest proximity to the bound nuclear proteins and, hence, the best targets for mutagenesis.

The results of methylation protection assays are shown in Fig. 6. According to the bases which appeared to be enhanced or protected from methylation, we chose a series of 2- to 3-base stretches to be mutated in each footprint (Fig. 6e). Specifically, bases −284, −283, and −281 in Ft1 were mutated from GGCC to taCg; bases −157 to −155 in Ft2 were changed from GGG to tta; bases −119 and −118 in Ft3 were converted from GG to ta; bases −41 and −40 in Ft4 (ATF/CREB) were mutated from GT to ca; and the bases −26 and −25 in the TATA box were mutated from AT to gg. Each site-specific point mutation was introduced into p-392 and designated p-392Ft1M2, p-392Ft2M1, p-392Ft3M1, p-392CREM1, or p-392TATAM1, respectively.

The promoter activity of each of these constructs was compared with that of the parental p-392 in both PC12 and HeLa cells (Fig. 7). In PC12 cells the activity of p-392Ft1M2 was 57.6% of that of the parental construct; p-392Ft2M1 was 37.2%, p-392Ft3M1 was 19.8%, p-392CREM1 was 20.4%, and p-392TATAM1 was 8% of that of p-392. In HeLa cells their activities proved to be 57.1, 98.5, 54.3, 26.1, and 15%, respectively, of that of p-392. This demonstrated that the TATA box and ATF/CREB element or Ft4 are important in both cell lines. The elements in Ft2 and Ft3 are more important for the promoter activity in neuronal cells than in nonneuronal cells. These data also verified the earlier suggestion that multiple elements contribute to the neuronal responsiveness of the 2LATP.

Since mutational analyses proved the ATF/CREB sequence in 2LATP to be active, its response to cAMP was tested in PC12 cells by using plasmid p-195 and its derivative, p-195CREM1. The mutation in p-195CREM1 was the same as that in p-392CREM1. The basal activity of the mutant was about 10% of that of the parental construct, but the response to 1 mM dibutyryl cAMP in the culture medium was the same as that of the parental DNA. That is, the activities of both plasmids increased two- to fourfold in the presence of 1 mM dibutyryl cAMP (data not shown). This may indicate that the protein binding to this region is an ATF family member which is not responsive to cAMP. That the purified AP1 protein protected this region supports this possibility. Alternatively, the 2LATP sequence between bases −195 to +34 may include a different CRE, as recently suggested for HSV-1 by Kenny et al. (18).

Novel *cis* element in 2LATP. Because Ft3 seemed so important to the overall activity of 2LATP, we sought to characterize it more fully. The sequence of Ft3 is GC rich. A computer search of the database sites from the National Center for Biotechnology Information revealed Ft3 to have a high degree of homology with the binding sites for nuclear proteins SP1, GCF, and EGR-1, as shown in Fig. 8b. We, therefore, used gel mobility shift competition assays to investigate the binding ability of those known elements to the protein binding to Ft3. Two complementary 30-mer oligonucleotides encompassing Ft3 were annealed and termed FP3 (Fig. 8b). When end-labeled FP3 was incubated with nuclear extracts from PC12 cells, a major retarded complex was seen (Fig. 8a, lane 2). This complex could be competed with effectively by 10- to 100-fold excesses of unlabeled FP3. FP3M1, a mutant oligonucleotide containing the same 2-bp alteration as that in p-392Ft3M1, lost the protein-binding activity and no longer competed for the formation of the major complex (Fig. 8a, lanes 16 and 17). This suggests that the major retarded complex is the functional complex formed on Ft3. None of the oligonucleotides specific for binding of SP1, GCF, or EGR-1 efficiently competed for formation of the complex (Fig. 8a, lanes 5 to 10). Thus, Ft3 spans a novel *cis* element, designated here LAT-3.

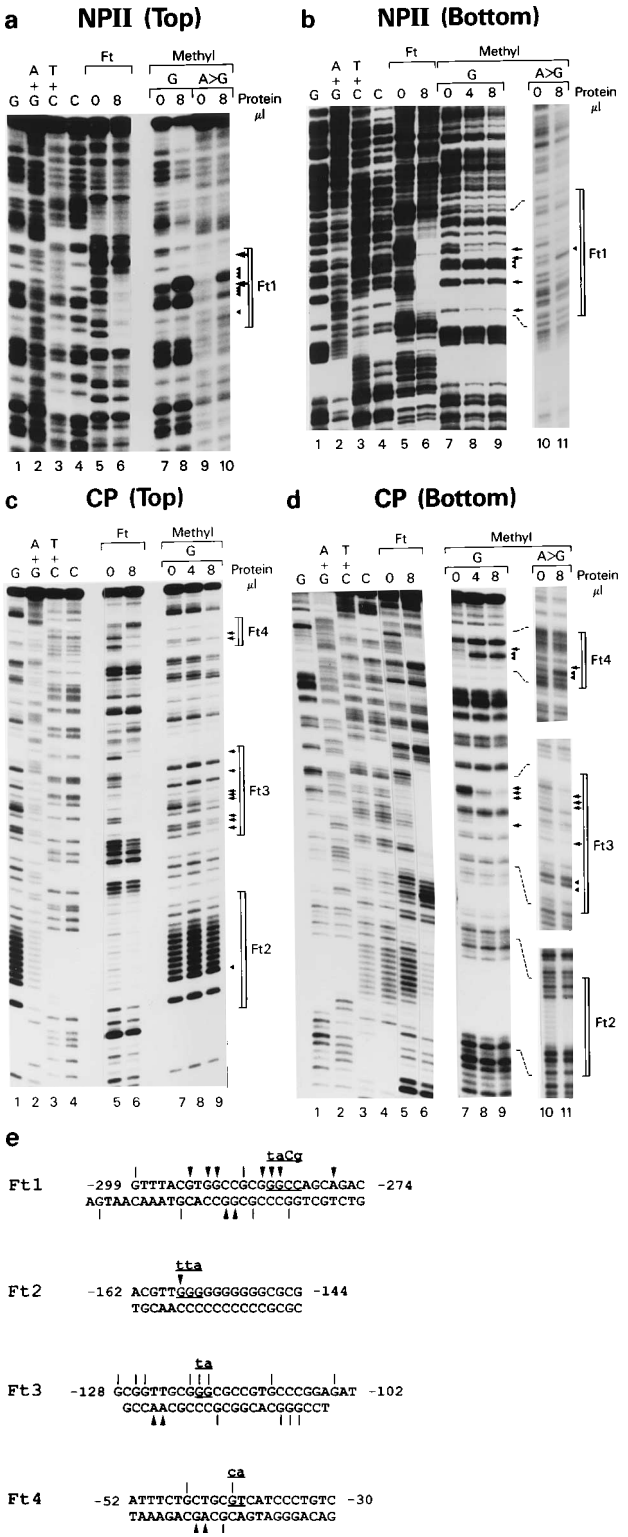


FIG. 6. Methylation protection assays of 2LATP segments. (a to d) Lanes: 1 to 4 DNA sequencing ladders; 5 and 6, DNase I footprints Ft1 to Ft4 generated by binding of 8 μl of nuclear extracts; 7 and above, results of methylation protection assays. Brackets are used to delineate the footprint regions, arrows show bases protected from methylation, arrowheads show areas of enhanced methylation, and dotted lines in panels b and d connect corresponding regions in lanes from different gels. In panel a, the probe, NPII (Top), was the NPII fragment labeled at the 3′ end of the top strand. In panel b, the probe, NPII (Bottom), was NPII fragment labeled at the 3′ end of its bottom strand. In panel c, the probe, CP (Top), was the fragment CP labeled at the 5′ end of its top strand. In panel d, the probe, CP (Bottom), was the CP fragment labeled at the 3′ end of the bottom strand. (e) Summary of all methylation protection data for footprints Ft1 to Ft4. The flanking numbers indicate the positions of the 5′ and 3′ ends of footprints on the top strand. Vertical bars indicate methylation protected bases; arrowheads show bases subject to enhanced methylation. Specific bases mutated in later studies are underlined, and the substituting bases are indicated above each of the sequences.

DISCUSSION

We conducted a detailed study of *cis* elements that may regulate the expression of the HSV-2 LAT gene. By specific mutational and transient expression assays, the TATA sequence of 2LATP was localized and proven to be functional. Transcription starts at about 27 to 32 bp downstream of this TATA box, similar to that seen with the 1LATP. Alteration of the TATA sequence abrogated 85 to 92% of the activity of this promoter. These findings suggest that the basal transcription activity of the LAT gene depends largely on a classical polymerase II promoter. The residual promoter activity of the TATA mutant construct may be due to the remaining activity of an impaired TATA element or, alternatively, initiation without a TATA box.

Upstream of this basal promoter is a very potent enhancer region composed of multiple elements. By direct comparisons with other promoter constructs, the sequence from bases −392 to +34 of 2LATP, which spans both the basal promoter and enhancer regions, displays very strong activity in both neuronal and nonneuronal cell lines. The strength of this promoter seems surprisingly great since the concentration of the minor LAT, the primary product of the gene in latently infected neurons, is very low (19, 41). This suggests either that LAT expression in neurons is actually very low because of the inhibitory influence of some regulatory elements contained in sequences beyond that which we tested here or that the level of LAT expression in vivo is high but the minor LAT is very labile.

The sequence spanning bases −392 to −137 seems particularly important for promoter activity in neuronal cells, similar to the observations of Batchelor and O'Hare (3) and Zwaagstra et al. with 1LATP (40). The progressive reduction in promoter activity with sequential deletions towards the TATA box predicted that there must be multiple *cis*-acting elements in this region. With DNase I footprint and methylation protection assays, we documented multiple footprints generated by binding of proteins in nuclear extracts or purified AP1 and AP2 proteins.

Just upstream of the TATA box, we demonstrated an ATF/CREB binding motif that contributes constitutively to 2LATP activity. Its mutation reduced promoter activity by 76 to 80%, depending on the type of cells transfected. Site-specific alteration of this element, however, did not impair its responsiveness to cAMP, in contrast to the observations of Leib et al. (23), with this element in 1LATP. Nevertheless, we demonstrated that crude nuclear protein extracts or purified AP1, a member of the ATF family, bound to this element and protected it from DNase digestion in vitro. It has been reported that the ATF/CREB binding motif can bind proteins of both the ATF and CREB families, resulting in differential regulation of transcription (21).

In addition to localizing the TATA box and ATF/CREB element, we identified seven other upstream elements in 2LATP, including some that have not been reported for 1LATP. Three AP2, one AP1, and three still incompletely characterized binding sites, termed Ft1, Ft2, and Ft3, were documented. Ft1 and Ft2 were located in the region that was

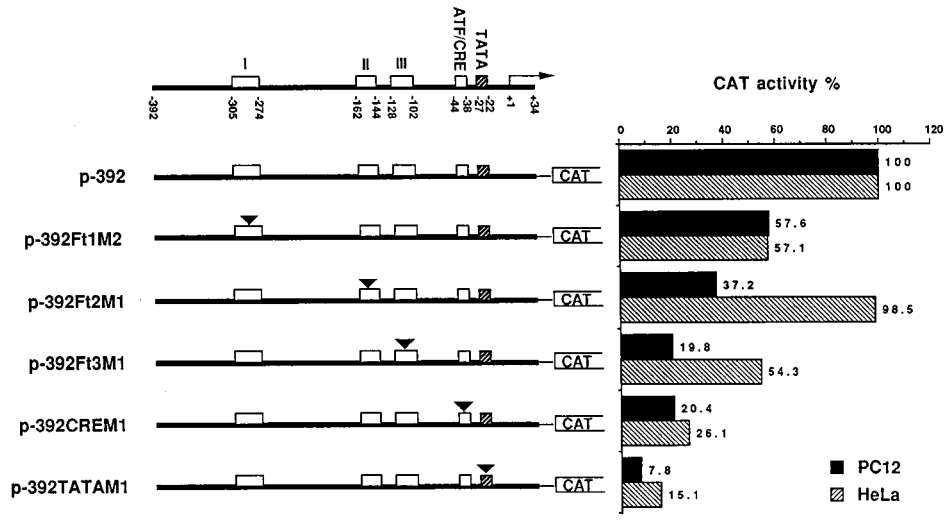


FIG. 7. Schematic representation of structure and CAT activity of the p-392 segment and its derivatives mutated at the specific bases shown in Fig. 6e. The positions of footprints Ft1 to Ft3, Ft4 (ATF/CRE), and the TATA box are shown on the top. Arrowheads indicate the sites of specific base substitutions. The percent CAT activity is normalized relative to that for p-392 construct in PC12 cells (46.6% average acetylation in four experiments) or HeLa cells (25.2% average acetylation in four experiments).

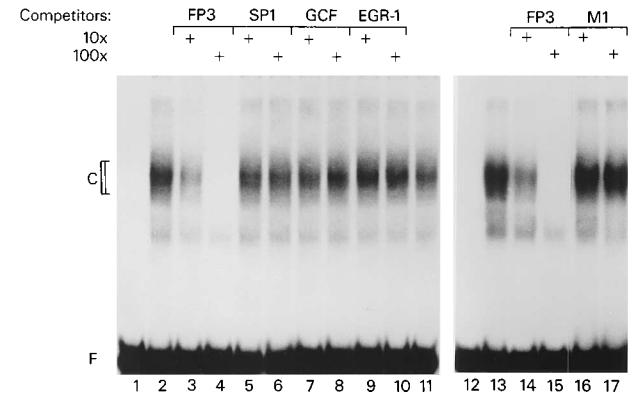
particularly neuron responsive. Site-specific mutation studies found Ft1 to have a modulatory effect on 2LATP activity. Alteration of the Ft2 sequence, however, reduced promoter activity by 63% in PC12 cells but by only 2% in HeLa cells.

Transient expression revealed that Ft3 contributed substantially to the neuronal responsiveness of the −392 to +34 fragment. Its mutation reduced promoter activity in neuronal cells by 80% but only within the context of an intact upstream region. Ft3 proved less able to influence the activity of the promoter constructs in which sequences upstream of it were deleted (Fig. 4). Thus, Ft3 does not directly enhance the neuronal responsiveness of 2LATP. Rather, it may do so in cooperation with elements upstream of it.

More refined analyses showed that a protein recognition site within Ft3 that we termed LAT-3 spans an incomplete palindrome from bases −121 to −107 (CGGGCGCCGTGCCCG). Mutation of two G's in this region at positions −119 and −118 reduced 2LATP activity by 80% in transient assays. Competition gel shift studies showed LAT-3 to be a novel GC-rich element that differs from other GC-rich elements which bind SP1, GCF, and EGR-1.

The present studies uncovered evidence of a complex interplay of *cis*-acting elements, yielding the in vitro phenotype of 2LATP. The roles that these elements play in LAT expression in vivo and their influence on viral latency and reactivation are now being studied by creation of targeted viral mutants and analyses of them in experimentally infected animals.

a



b

FP3	TGCTCTGCGGTTGCGGGCGCCGTGCCCGGA
FP3M1	TGCTCTGCGGTTGCGtaCGCCGTGCCCGGA
GCF	CCCGGCGCAGCGCGGCCGCAGCGC C GCC
SP1	ATTCGATCGGGGCGGGGCGAGC
EGR-1	GATCTCGCGGGGGCGAGGGGGATC

FIG. 8. Electrophoretic gel mobility shift competition analysis of Ft3 region of 2LATP. (a) Radiolabeled probe FP3 encompassing Ft3 was reacted with nuclear extracts from PC12 cells with (+) or without the addition of a 10-fold (10×) or 100-fold (100×) excess of unlabeled oligonucleotides FP3, FP3M1 (a 2-bp substitution mutant of FP3), and binding sites for SP1, GCF, and EGR-1, as indicated above each lane. Lanes: 1 and 12, probe only; 2, 11, and 13, probe plus nuclear extract but no competitor. (b) Sequences of top strands of probe FP3, mutant competitor FP3M1, and the competitors of known GC-rich elements, with GC-rich regions underlined.

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