JOURNAL OF VIROLOGY, Aug. 1996, p. 5384–5394 Vol. 70, No. 8
0022-538X/96/$04.00+0

cis-Acting Elements Involved in Transcriptional Regulation of the Herpes Simplex Virus Type 1 Latency-Associated Promoter 1 (LAP1) In Vitro and In Vivo

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Received 28 February 1996/Accepted 25 April 1996

Latency-associated promoter 1 (LAP1) of herpes simplex virus type 1 is required to generate a series of latency-associated transcripts (LATs) in sensory neurons of latently infected animals. Sequence analysis and DNA binding studies have suggested the existence of several *cis*-acting elements within LAP1 that are potentially important for promoter function, although their role in LAT gene expression during latency is largely unexplored. In this report, we present evidence that the LAP1 TATA box is essential for transcription initiation in vitro. A reduction in LAT synthesis measured by in situ hybridization and reverse transcription-PCR (RT-PCR) of rat brain tissue latently infected with a LAP1 TATA substitution virus demonstrated that this sequence was required for full LAP1 activity in vivo. Analysis of additional site-directed and 5′-deletion mutants of LAP1 by in vitro transcription-primer extension assays showed that upstream elements including the USF and cyclic AMP response element (CRE) site specifically contributed to LAP1 function and that sequences beginning at position −620 relative to the transcription start site were essential for full promoter activity. The combination of deleting USF, CRE, and TATA completely abolished LAT expression in the brain, identifying these as essential elements for the neuron-specific functioning of LAP1 during latency. Mutation of the transcription start site did not abolish transcription, suggesting the absence of an initiator element. However, one of the most exciting findings from this study is that the region downstream of the TATA box appears to contain a true enhancer that is not only essential for transcription, but also functional when positioned 1.6 kb downstream of the start site of transcription. It was concluded that (i) the TATA box was essential for full transcriptional activity from LAP1 both in vitro and in vivo, (ii) the USF element and CRE contribute to LAP1 function during latency in combination with the TATA element, (iii) multiple *trans*-acting factors besides the USF- and CRE-binding proteins were required for full promoter activity in vitro, and (iv) sequences downstream of the TATA box enhanced promoter activity in vitro.

One of the distinctive features of herpes simplex virus type 1 (HSV-1) is its ability to persist in a select population of host neurons in a latent state (14, 43). During latency, multiple copies of the viral genome are maintained within the nucleus as nucleosome-bound episomes (9, 31, 44). Upon the establishment of latency within neurons of sympathetic and sensory ganglia, a unique set of HSV-1 transcripts is encoded from sequences within the repeat elements that flank the unique long region of the viral genome (7, 33, 45, 52, 56). Since these overlapping transcripts represent the only HSV-1 genes expressed during latency, they have been referred to as the latency-associated transcripts (LATs).

Although the transcriptional control regions and origins of the different LAT RNAs have not been fully elucidated, the colinear 2-, 1.5-, and 1.45-kb LAT species are believed to be derived via splicing and/or processing of an unstable primary 8.3-kb RNA transcribed from a TATA box almost 700 bp upstream of the 5′ end of the 2-kb LAT (for reviews, see references 16, 54, and 55). Several lines of evidence identify the sequences that span this TATA box, designated latency-associated promoter 1 (LAP1), as being the promoter region responsible for most of the LAT transcription during latency: (i) KOS/29, a virus with a deletion of LAP1 at both loci, does not express LAT during latency (6, 10, 11, 13); (ii) variant 1704, which is similar to KOS/29 but retains some downstream sequence in one LAT locus, not only fails to express the 2-kb LAT but is also negative for the weak in situ hybridization signals specific for the large precursor transcript (33, 34); and (iii) in the recombinant virus KOS/72, in which the genomic copy of the rabbit β-globin gene was cloned downstream of the TATA box, accurate initiation of the β-globin message occurred 28 bp downstream of the TATA sequence with concurrent abolition of LAT expression in latently infected murine trigeminal ganglia (13). However, the inability of recombinant viruses such as KOS/62-3 and KOS/67-7 to express the *lacZ* or *NGF* gene cassettes that were inserted at position +41 of LAP1 suggests that sequences beyond the boundaries of LAP1 that are deleted in these two viruses may also be necessary for expression (28). It was previously shown that the sequences that lie between LAP1 and the 5′ end of the 2-kb LAT, designated LAP2, can express the β-galactosidase reporter gene in trigeminal ganglion neurons even when moved to an ectopic locus in the viral genome (18). Recent studies suggest that LAP1 and LAP2 may function differentially during lytic versus latent infections (6). Accumulation of the 2-kb LAT during lytic infection depends on LAP2 and is independent of LAP1 sequences, while during latency the reverse is true (6, 35).

The LAT loci in HSV-1 and HSV-2 share more than 80%

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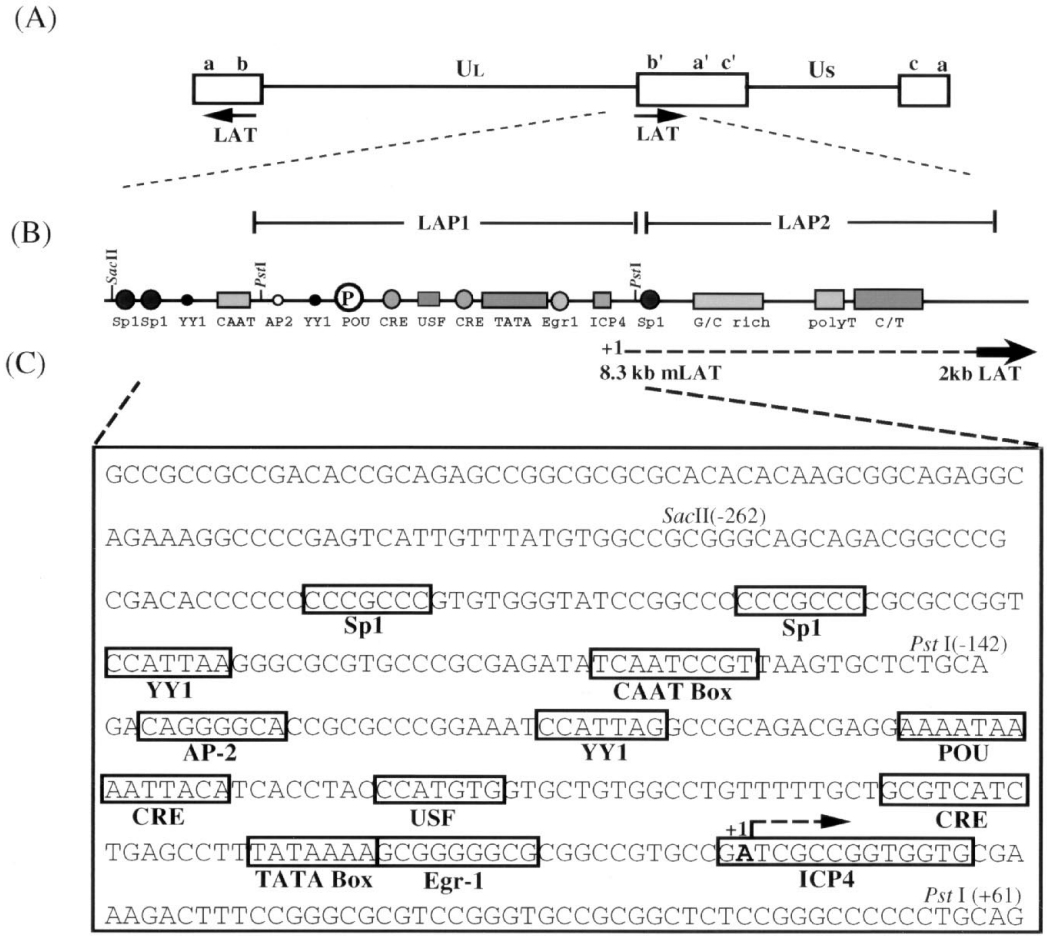

FIG. 1. Schematic representation of the LAT region of HSV-1. (A) Diagram of the prototypic arrangement of the HSV-1 genome indicating the location and direction of transcription of the LAT loci within the repeats (open boxes) that flank the unique long (U_L) segment of the genome. The LAT region in the internal repeats is expanded below to show the LAT promoter region, which is structurally and functionally divided into the TATA-containing LAP1 and TATA-less LAP2 regions (6, 17). U_S, unique short region. (B) Line drawing of the LAT promoter region depicting the structural organization of putative *cis*-acting transcriptional elements of LAP1 and LAP2. The location of the predicted start site of the primary 8.3-kb LAT species (dashed line) and the 5′ end of the 2-kb LAT (boldface arrow) are as shown. (C) Nucleotide sequence of LAP1 in the KOS strain of HSV-1 (57) showing the spatial relationship of the putative *cis*-acting elements relative to the TATA box. The consensus binding sites of potential *trans*-acting factors of neuronal and nonneuronal origin (boxes), predicted start site of the 8.3-kb primary LAT (+1), and the direction of transcription from LAP1 (bent arrow) are indicated. All restriction enzyme sites relevant to this study are numbered with respect to the A residue at +1 of LAP1.

sequence identity and contain similarly organized groups of putative *cis*-acting elements (25, 30). Although much information has been inferred from transient-transfection and gel retardation assays using neuronal and nonneuronal cell lines (2, 3), the relevance of most of these findings to LAT expression during latency has yet to be evaluated in the context of viruses with mutations or deletions in the LAT loci. Besides the TATA box, the LAP1 region contains a CCAAT box homology and potential recognition sequences for several transcription factors, including Sp1, AP-2, YY1, CREB, LPBF/MLTF/USF, Egr-1, a POU-domain protein, and the HSV-1 immediate-early transactivator, ICP4 (3, 4, 15, 24, 42, 58–60, 62) (see Fig. 1). In this study, we have used an in vitro transcription system to characterize *cis*-acting elements of LAP1 required for promoter activity. Mutations that affect LAP1 function in vitro have also been tested for their contribution to LAT expression during latency.

MATERIALS AND METHODS

Plasmid constructions. The sequences and positions of known and putative *cis*-acting elements in the LAP1 promoter of KOS strain of HSV-1 are as shown in Fig. 1 (57). A series of chloramphenicol acetyltransferase (CAT) reporter gene constructs containing progressive 5′ deletions (see Fig. 4A) and site-directed mutations of putative *cis*-acting elements in the LAP1 promoter (see Fig. 2, 3, and 5) were constructed by using standard cloning techniques and oligonucleotide-directed mutagenesis (48).

pUC.CAT and pGem.CAT, promoterless control templates, were constructed by cloning a 1.6-kb *Bgl*II-*Bam*HI fragment containing the CAT gene and simian virus 40 early polyadenylation site from pSVOCAT into *Bam*HI-linearized pUC19 and pGem7Zf(−), respectively. The simian virus 40 promoter in pSV2CAT was replaced by the adenovirus type 5 major late promoter (positions −270 to +33 relative to the transcription start site) to create pML.CAT, the positive-control plasmid. pLAP1[−142/+61]CAT and pGemLAP1CAT were constructed by cloning the 203-bp *Pst*I restriction fragment (nucleotides 118664 to 118866) (30) into pUC.CAT and pGemCAT, respectively. pLAP-R contains this sequence in reverse orientation relative to the CAT gene in pUC.CAT.

The *Hin*cII-*Sph*I LAP fragment (nucleotides 117012 to 119290) subcloned into pTZ18U (United States Biochemical, Cleveland, Ohio) was subjected to oligonucleotide-directed in vitro mutagenesis using the Muta-Gene phagemid kit (Bio-Rad, Hercules, Calif.). The sequences of the oligonucleotides used are as follows: rT, (−2) 5′-CGGCACGGCCGCGCCCCCGCGGTACCGGCTGAGATGACGCAGCAAA-3′ (−50); ΔUC, (−18) 5′-CCGCTTTTATAAAGGCTGAGGGTACCAGGTGATGTAATTTTATTTT-3′ (−94); and ΔUCT, (−2) 5′-CGGCACGGCCGCGCCCCCGCGGTACCAGGTGATGTAATTTTATTTT-3′ (−94). The positions of the 5′ and 3′ ends of the oligonucleotides relative to position +1 of LAP1 (Fig. 1C) are indicated in parentheses. The unique restriction sites created for screening purposes are underlined. The prefix "r" indicates

replacement of a specific transcription element with a unique restriction site such that spacing is maintained, while the Δ's in ΔUC and ΔUCT indicate deletions of 43 and 53 bp, respectively. The site-directed mutant plasmids pLAP1[rT]CAT, pLAP1[ΔUC]CAT, and pLAP1[ΔUCT]CAT were then derived from the corresponding mutagenized *Hin*cII-*Sph*I subclones by cloning the respective 203-bp *Pst*I restriction fragment (positions −142 to +61) into pUC.CAT. The presence of the mutation was confirmed by DNA sequencing. pLAP1[rU]CAT, pLAP1[rC]CAT, pLAP1[rINR]CAT, and pLAP1[rINRKO]CAT were derived by oligonucleotide-directed mutagenesis of single-stranded DNA derived from pGEM.LAP1CATpA. The sequences of the oligonucleotides used are as follows: rU, (−88) 5′-AATTACATCACCTAC**GAAGCTT**GTGCTGTGGCCTGTT-3′ (−64); rC, (−60) 5′-GTGGCCTGTTTTTGC**GAAGCTTG**CTCAGCCTTTATA AAAGC-3′ (−20); rINR, (−17) 5′-GGCGCGGCCGTGCC**C**AT**TCCGGA**TG GTGCG-3′ (+14); and rINRKO, (−17) 5′-GGCGCGGCCGTG**GAGGCCTG**G G**A**TGGTGCG-3′ (+14). The *Hin*dIII site in the multiple cloning site of the parent vector was used to derive plasmids pLAP1[−42/+61]CAT and pLAP1[−72/+61]CAT from pLAP1[rU]CAT and pLAP1[rC]CAT, respectively, by removing the intervening upstream LAP1 sequences by *Hin*dIII digestion and religation.

The *Eag*I site at position −11 of LAP1 was converted to a *Bgl*II site by filling in with Klenow fragment and ligation of *Bgl*II linkers to produce pLAP1[−142/Bg*/+61]. pLAP1[−142/Bg*CAT/+61] was constructed by inserting the CAT gene at the newly created *Bgl*II site at position −11 (denoted Bg*) rather than at the downstream *Bam*HI site of the vector. This strategy effectively places the 5′ end of the CAT gene at −11 and places LAP1 sequences from −11 to +61 downstream of the entire CAT gene cassette. pLAP1[−142/Bg*]CAT was constructed from pLAP1[−142/Bg*CAT/+61] by replacing the Bg*-*Bam*HI fragment spanning LAP1[−11/+61]CAT sequences with the 1.6-kb *Bgl*II-*Bam*HI CAT gene fragment used before. pLAP1[−11/+61]CAT was created by subcloning the Bg*-*Bam*HI restriction fragment from pLAP1[−142/Bg*/+61]CAT into *Bam*HI-linearized pUC19 vector. pLAP1[−79/+61]CAT was constructed by insertion of a T4 DNA polymerase blunt-ended 140-bp *Hph*I-*Pst*I restriction fragment into the *Eco*RV site of pBSIISK$^-$ followed by insertion of the CAT reporter gene at the downstream *Bam*HI site. This cloning strategy is expected to add an extra 3 bases to the size of the primer extension product. pLAP1[−262/+61]CAT was constructed in two steps: (i) the Bg*-*Bam*HI fragment from pLAP1[−142/Bg*/+61]CAT containing LAP1[−11/+61]CAT was inserted into *Bam*HI-linearized pBSIISK$^-$, and (ii) sequences between the *Sac*II site in the multiple cloning site of the vector and the *Sac*II site at position +41 of the LAP1 sequence were replaced with a 303-bp *Sac*II fragment of LAP1 corresponding to positions −262 to +41. Similarly, pLAP1[−620/+61]CAT was constructed as follows: the Bg*/*Bam*HI fragment containing LAP1[−11/+61]CAT sequences from pLAP1[−142/Bg*/+61]CAT was inserted into *Bam*HI-linearized pUC19. Next, sequences between the *Ban*II site in the vector and the *Ban*II site at position +53 of LAP1 in p*Ban*II/Bg*CAT were replaced with a 673-bp *Ban*II fragment of LAP1 corresponding to positions −620 to +53 to give pLAP1[−620/+61]CAT.

Cell culture and nuclear extract preparation. Spinner cultures of HeLa cell strain S3 (ATCC CCL 2.2), grown in Joklik's minimal essential medium (ICN Biomedicals, Aurora, Ohio) supplemented with 5% fetal calf serum (Gibco-BRL), were harvested in the exponential growth phase at a density of 2.8×10^5 cells per ml, and nuclear extract was prepared as described by Dignam et al. (12).

In vitro transcription-primer extension assay. In vitro transcription reaction mixtures (50 μl) contained 24 μl of HeLa nuclear extract (75 μg of protein), 6 μl of D buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], K^+ [pH 7.9], 20% [vol/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol), 2 μg of supercoiled plasmid template, 6.25 mM $MgCl_2$, 0.5 mM ribonucleoside triphosphates, and 40 U of RNasin (Promega). Where indicated, α-amanitin was added to a final concentration of 8 μg/ml, a concentration low enough to selectively inhibit RNA polymerase II transcription (27). After 1 h of incubation at 30°C, the reactions were halted by addition of 150 μl of stop solution (20 mM EDTA [pH 8.0], 0.2 M NaCl, 5 μl of 2.5-mg/ml proteinase K, 1% sodium dodecyl sulfate [SDS]). The reaction products were extracted once with 200 μl of phenol-chloroform-isoamyl alcohol (25:24:1) and then with an equal volume of chloroform. A 300-μl volume of 0.3 M sodium acetate was added to the resulting aqueous phase, and the reaction products were precipitated with 1 ml of 95% ethanol. The pellet obtained was washed with 70% ethanol, vacuum dried, and dissolved in 10 μl of hybridization solution (10 mM Tris [pH 7.9], 1 mM EDTA [pH 7.9], 0.25 M KCl, and 3 ng of an end-labelled oligonucleotide primer that is complementary to sequences in the CAT gene). Two different CAT primers were employed, depending on the length of the expected extension product from the specific promoter template. The 18-mer CAT primer (5′-AGCTCCTGAAAATCTCGC-3′), complementary to CAT sequence between positions +6 and +24 with respect to the 5′ end of the CAT reporter gene, was used for promoter constructs with LAP leader up to +61. The 24-mer CAT primer (5′-GCCATTGGGA TATATCAACGGTGG-3′), complementary to CAT sequences between +61 and +85, was used to assay transcription activity specifically from constructs pLAP1[−142/Bg*]CAT and pLAP1[−142/Bg*CAT/+61], in which the CAT gene was placed at position −11 relative to the normal LAP1 initiation site (see Fig. 5C). Following hybridization at the appropriate temperature (55°C for the 18-mer CAT primer or 65°C for the 24-mer CAT primer) for 1 h, 25 μl of reverse transcription mix (20 mM Tris [pH 8.7], 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.33 mM (each) deoxynucleoside triphosphate, 10 U of avian myeloblastosis virus reverse transcriptase [RT], and 100 μg of actinomycin D per ml) was added, and the primer was extended at 42°C for 1 h. The products were ethanol precipitated and dissolved in 9 μl of formamide loading buffer–0.1 M NaOH (2:1, vol/vol). The solution was heated at 90°C for 3 min and analyzed on a 6% polyacrylamide–7 M urea sequencing gel. The gel was dried and exposed to X-ray film at −70°C, and the intensity of the signals was measured in terms of relative optical density with a Molecular Dynamics Optical Imaging system.

Construction of mutant viruses. To create recombinant viruses with some of these site-directed mutations, we used the double mutant 4:27, which is a cross between *ts*Y62, an ICP27 temperature-sensitive mutant virus (47), and *d*120, an ICP4 deletion virus (8). The point mutation in the ICP27 coding sequence of *ts*Y62, which renders it unable to produce a functionally active ICP27 protein at the nonpermissive temperature of 39°C, can be rescued by homologous recombination with the *Bam*HI B fragment of HSV-1, which contains a functional copy of ICP27. Rescue of the temperature-sensitive phenotype in the double mutant thus provides selection for introduction of mutations into the adjacent LAT region, yielding a virus lacking only ICP4.

The 203-bp *Pst*I fragments from the respective pTZ18U-based site-directed mutant plasmids were cloned in place of the analogous fragment from the wild-type plasmid containing the HSV-1 strain KOS *Bam*HI-B sequence and cotransfected with DNA prepared from the 4:27 double mutant virus into E5 cells, an ICP4-complementing cell line (8). Each virus was subjected to three rounds of limiting dilution in 96-well trays of E5 cells and characterized by Southern blot analysis (51) to verify that each mutant virus was homozygous for the site-directed mutation in both LAT loci.

In situ hybridization studies. Male Sprague-Dawley rats (250 to 300 g) (Harlan, Indianapolis, Ind.) were anesthetized with ketamine and rompun, and 250 to 500 nl (2×10^9 PFU/ml) of virus was stereotactically injected into the hippocampus with a glass micropipette by using coordinates from the atlas of Paxinos and Watson (36). At 2 weeks postinjection, the animals were sacrificed by perfusion of the heart with 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4). The brains were postfixed for 2 h and cryoprotected with 30% sucrose, and 50-μm sections were cut on a sliding microtome. The sections were rinsed three times in PBS and treated with 1% HCl in PBS for 5 min and then with acetic anhydride for 20 min. After dehydration through a graded series of ethanol concentrations, the sections were prehybridized in 50% formamide–10% dextran–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.01% salmon sperm DNA–0.01% tRNA–0.02% SDS at 56°C for 4 h and hybridized with a digoxigenin-labelled probe complementary to LAT at 56°C overnight. The probe was synthesized from a pGEM-4Z subclone of the LAT region spanning the *Pst*I to *Mlu*I sites (nucleotides 118862 to 121447) (53). After hybridization, the sections were rinsed three times in 50% formamide–1× SSC at 56°C for 15 min, two times in 1× SSC at room temperature for 15 min, and three times in Tris-buffered saline for 5 min at room temperature. The bound digoxigenin-labelled probe was localized with an alkaline phosphatase-conjugated antidigoxigenin antibody (1:250 dilution; Boehringer Mannheim) by using BCIP-NBT (5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium) detection (Vector Laboratories, Inc., Burlingame, Calif.).

PCR analysis of DNA and RNA. Following sacrifice of each rat by decapitation, the hippocampus was removed and homogenized immediately in 100 μl of TRI reagent (Total RNA Isolation reagent; Molecular Research Center, Inc.). DNA and RNA were extracted from each sample according to the manufacturer's instructions and dissolved in 200 μl of diethylpyrocarbonate-treated distilled water.

(i) DNA PCR. A 2-μl volume of DNA extracted from each sample was amplified with PCR primers for the HSV-1 glycoprotein B (gB) and cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, as previously described (41), in the presence of 0.5 μl of [α-^{32}P]dCTP (800 Ci/mmol; Amersham), in a DNA thermal cycler 480 (Perkin-Elmer). For gB, the viral DNA control, the PCR conditions were 30 cycles of 95°C for 1 min and 60°C for 1 min followed by a single extension at 72°C for 10 min. For GAPDH, the cellular DNA control, PCR conditions included 30 cycles of 95°C for 1 min and 58°C for 1 min followed by a single extension at 72°C for 10 min. A 20-μl sample of PCR product was then subjected to electrophoresis on a 1% agarose gel in 1× Tris-borate-EDTA buffer. The gel was dried and exposed to X-ray film at room temperature, and the signal was quantitated by using a Molecular Dynamics Phosphorimaging system.

(ii) RT-PCR. A 2-μl volume of the RNA extracted from each sample was digested with DNase I at 37°C for 1 h and then reverse transcribed in a final volume of 20 μl, containing 1 μl of SuperScript II (Gibco-BRL), 4 μl of 5× SuperScript buffer, 1 μl of either 3′ LAT primer or the 3′ GAPDH primer, and 1 μl of RNasin inhibitor, at 42°C for 1 h. Following heat inactivation at 95°C for 5 min, the template RNA was digested with 2 U of RNase H (Boehringer Mannheim) at 37°C for 45 min. The RNase H was heat inactivated at 95°C for 5 min, and then the entire 20 μl was amplified as described previously (41) with either LAT or GAPDH primers and 0.5 μl of [α-^{32}P]dCTP (800 Ci/mmol; Amersham) in a final volume of 100 μl under the same conditions described above. A 20-μl sample was then electrophoresed on a 1% agarose gel. The gels were dried and processed as described above for DNA PCR.

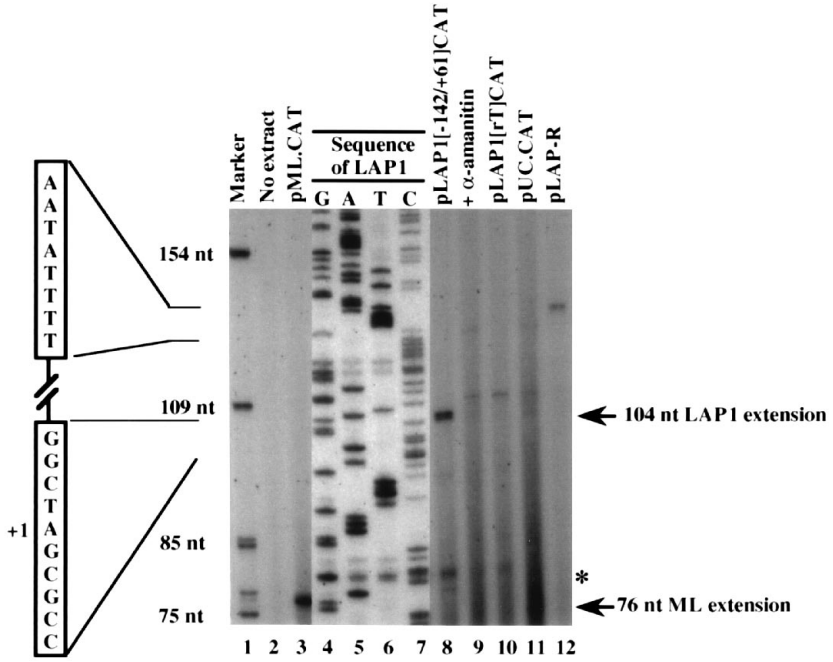

FIG. 2. In vitro transcription of LAP1 of HSV-1, performed with supercoiled template DNA using HeLa nuclear extract. Wild-type or mutant LAP1 fragments used in this experiment correspond to the 203-bp *Pst*I restriction fragment (nucleotides 118664 to 118866) of HSV-1 KOS strain fused to the CAT reporter gene. The level and position of transcription initiation were monitored by primer extension with end-labelled 18-mer oligonucleotide CAT primer (see Materials and Methods). Lane 1, end-labelled *Hin*fI digest of pSV325 as a size standard; lane 2, transcription reaction with Hela nuclear extract excluded; lane 3, in vitro transcription-primer extension of the positive-control template, pML.CAT; lanes 4 to 7, sequencing reactions of the pLAP1[−142/+61]CAT template; lane 8, in vitro transcription-primer extension of the pLAP1[−142/+61]CAT template; lane 9, in vitro transcription of the pLAP1[−142/+61]CAT template in the presence of α-amanitin; lane 10, in vitro transcription-primer extension of the TATA substitution mutant, pLAP1[rT]CAT; lane 11, promoterless CAT construct (pUC.CAT) as a negative control; lane 12, pLAP-R. The extension products from the adenovirus type 5 major late promoter (ML) and LAP1 templates are indicated on the right. The smaller extension product referred to in the text is also shown (asterisk). nt, nucleotide.

RESULTS

Establishment of an in vitro transcription-primer extension assay to identify functional *cis*-acting elements in LAP1. We have used an in vitro transcription-primer extension system to localize *cis*-acting elements within LAP1 (Fig. 1). Our transcription reaction mixtures were standardized by using the pML.CAT template, which contains the adenovirus type 5 major late promoter (positions −279 to +33) fused to the bacterial CAT gene. Accurate transcription initiation at the major late promoter was inferred by the detection of a 76-base primer extension product using the 18-mer CAT primer (Fig. 2, lane 3). The size of this extension product is consistent with accurate initiation 30 bp downstream of the major late TATA box element. To test for LAP1 promoter activity in vitro, we used the pLAP1[−142/+61]CAT template, which contains sequences from positions −142 to +61 relative to the putative start site of the 8.3-kb primary LAT (13). Primer extension of transcripts from the pLAP1[−142/+61]CAT template using the 18-mer CAT primer yielded a 104-nucleotide extension product (Fig. 2, lane 8), the 5′ end of which mapped to an A residue 28 nucleotides downstream of the LAP1 TATA box (Fig. 2, lanes 4 to 7). We also observed a smaller extension product which could represent an additional start site or an incomplete primer extension product of the full-length species. The 104-base extension product was not detected in the presence of low concentrations of α-amanitin in the transcription reaction mixture (Fig. 2, lane 9), indicating that the 104-nucleotide band is an extension product of an RNA polymerase II transcript. Furthermore, the 104-base extension product is not detected when pLAP-R, in which LAP1 sequences are in reverse orientation (Fig. 2, lane 12), or the promoterless pUC.CAT template (lane 11) is used or when HeLa nuclear extract was omitted from the transcription reaction mixture (lane 2). We conclude that the 104-base extension product is LAP1 derived and orientation dependent. The 104-base extension product maps the 5′ end of the LAP1 transcript to the same start site as that of the 8.3-kb LAT in vivo, establishing that the HeLa nuclear extract could be used to reproduce accurate transcription initiation from LAP1 in vitro. This assay was used to identify *cis*-acting elements that mediate accurate LAP1 transcription in vitro.

TATA box dependence of the LAP1 promoter in vitro. To investigate whether transcription initiation at LAP1 is dependent on the TATA box element, we used site-directed mutagenesis to replace the TATAAA sequence with CCGGTA, resulting in a plasmid template (pLAP1[rT]CAT) otherwise identical to the wild-type template (pLAP1[−142/+61]CAT). The substitution mutation of the TATA sequence completely eliminated the 104-nucleotide extension product (Fig. 2, compare lanes 8 and 10). We conclude that the TATA box in LAP1 is essential for in vitro promoter function.

Contribution of USF, CRE, and the TATA box to transcription in vitro. A number of proposed *cis*-acting elements are contained within the pLAP1[−142/+61]CAT construct, including putative binding sites for several transcription factors such as CREB, USF, AP1, YY1, and octamer-binding proteins. In this experiment, we tested the contributions made by the elements closest to the TATA box, a USF binding site and the TATA-proximal CRE (Fig. 3A). In vitro mutagenesis was used to make precise replacements of these elements without

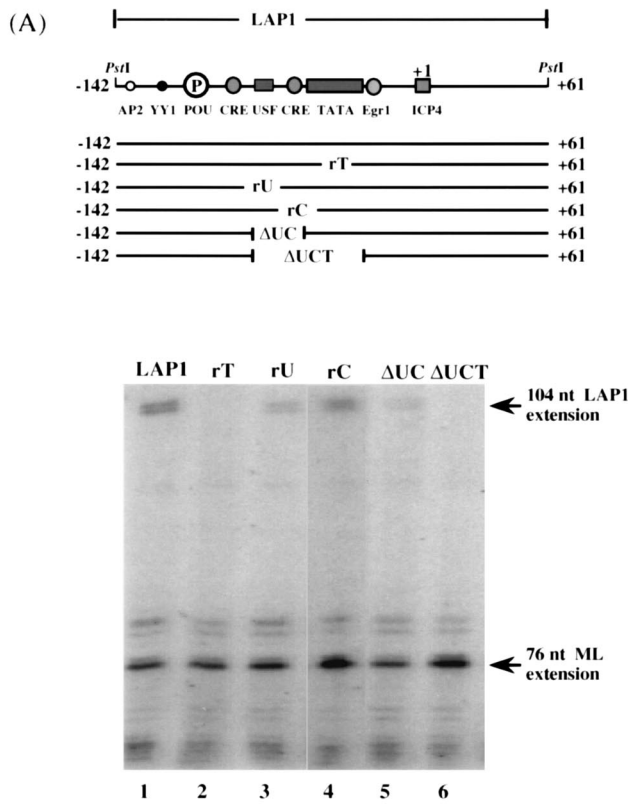

FIG. 3. Functional synergism between the TATA box, USF, and CRE of LAP1 in vitro. (A) Schematic diagram of the putative TATA-proximal *cis*-acting elements of LAP1. Wild-type and mutant templates contain sequences from positions −142 to +61 of LAP1 fused to the CAT gene. rT, rU, and rC are site-directed substitution mutants of the TATA, USF, and CRE binding sites, respectively. ΔUC and ΔUCT are actual deletions that remove sequences spanning the USF element and CRE or the USF element, CRE, and the TATA box, respectively. (B) In vitro transcription-primer extension analysis of wild-type and mutant LAP1 templates with the pML.CAT template included in each transcription reaction mixture as an internal control. Positions of extension products corresponding to accurately initiated transcripts from LAP1 and the adenovirus type 5 major late promoter are shown on the right.

altering the spacing between the remaining promoter elements (see Materials and Methods for details). Each mutant template was then tested for promoter activity in our in vitro transcription assay. As shown in Fig. 3B, mutation of either the USF binding site (lane 3) or the TATA-proximal CRE (lane 4) appreciably reduced the yield of the 104-base extension product. The apparent reduction in promoter activity was not due to variation in the transcription reaction mixtures, since the level of transcription from the control template (pML.CAT) was equivalent in each reaction mixture. Therefore, both elements appear to contribute to LAP1 promoter function in vitro. Two deletion mutants with either USF and CRE (ΔUC) or USF, CRE, and the TATA box (ΔUCT) completely removed were also constructed. Transcription with these templates indicated that these deletions are deleterious for LAP1 function in vitro. The finding that the ΔUC template is more defective (Fig. 3B, lane 5) than the replacement of either element individually suggests that these elements may function in concert, although effects due to changes in promoter spacing cannot be ruled out. The ΔUCT template is completely inactive (Fig. 3B, lane 6), consistent with the finding that the TATA element is essential for LAP1 function in vitro.

Contribution of upstream sequence elements to transcriptional activity in vitro. Up to this point in this study, LAP1 promoter activity has been analyzed by using sequences between positions +61 and −142. To determine the upstream boundary of the sequences that contribute to LAP1 activity in vitro, a series of templates were generated so that each contained variable lengths of upstream LAP1 sequences (ranging from positions −620 to −11 relative to the transcription start site) with a common 3′ end corresponding to +61 of LAP1 cloned upstream of the CAT reporter gene cassette (Fig. 4A). Transcription levels normalized to that of the pMLCAT internal control have been expressed in terms of relative transcription (percent) with respect to that of the largest LAP1 fragment tested (−620/+61) (Fig. 4B). Transcription assays with this set of LAP1 templates defined the sequences critical for LAP1 promoter function in vitro as being between positions +61 and −262. Deletion of sequences upstream of −262 had a minimal effect on promoter activity in vitro (Fig. 4B, lanes 6 to 8). However, removal of sequences between position −262 and the start site resulted in progressively lower promoter activities (Fig. 4B, lanes 1 to 6). The largest changes in LAP1 function occurred when sequences between positions −262 and −79 (Fig. 4B, lanes 4 to 6) and those between −72 and −42 (lanes 2 and 3) were deleted. Further deletion to position −11 (Fig. 4B, lane 1) deletes the essential TATA box (Fig. 2, lane 10, and Fig. 3B, lane 2) and thus completely abolished transcription activity. Sequences between positions −142 and −262 include two potential Sp1 binding sites and the CCAAT box homology (Fig. 4A). Since both Sp1 and CTF are abundant in HeLa nuclear extracts, it is likely that these proteins contribute to LAP1 activity in vitro. Together, these results confirmed that the LAP1 TATA box and sequences downstream to position +61 constitute the minimum basal promoter in vitro and that other upstream elements contribute to LAP1 function in vitro.

Importance of sequences at the initiation site of LAP1 to promoter function in vitro. Transcription reactions with a template that lacked LAP1 sequences downstream of position −11 (not shown) suggested that sequences in the region from −11 to +61 contained an essential promoter element(s). Our initial suspicion was that the activity detected in this region was due to sequences in the immediate vicinity of the transcription start site. Initiator elements have been shown to promoter transcription in numerous promoters. The sequence around the LAP1 start site (CCGATCGC, where the start site is underlined) shows some similarity to the consensus sequence for initiator elements [YYAN(T/A)YY] (21). To test whether the LAP1 promoter contained a functional initiator element, we used site-directed mutagenesis to change the sequences at the transcription start site such that they either increased (rINR) or decreased (rINRKO) their match with functional initiator elements (Fig. 5A). Transcription analysis with these constructs indicates that the initiator is not an important functional element in the LAP1 promoter, since mutation of the potential initiator element did not significantly reduce LAP1 function (Fig. 5B, compare lanes 5 and 9). However, the introduction of changes to match the consensus initiator (rINR) caused an increase in LAP1 activity when normalized to the major late internal-control template (Fig. 5B, compare lanes 5 and 7). These results demonstrate that the native LAP1 does not contain a functional initiator element.

Contribution of sequences downstream of the LAP1 start site to promoter function in vitro. Since the initiator element was not contributing to LAP1 function, we considered the possibility that there were as yet unidentified promoter elements in the region downstream of the transcription start site. To test this hypothesis, we generated two templates that con-

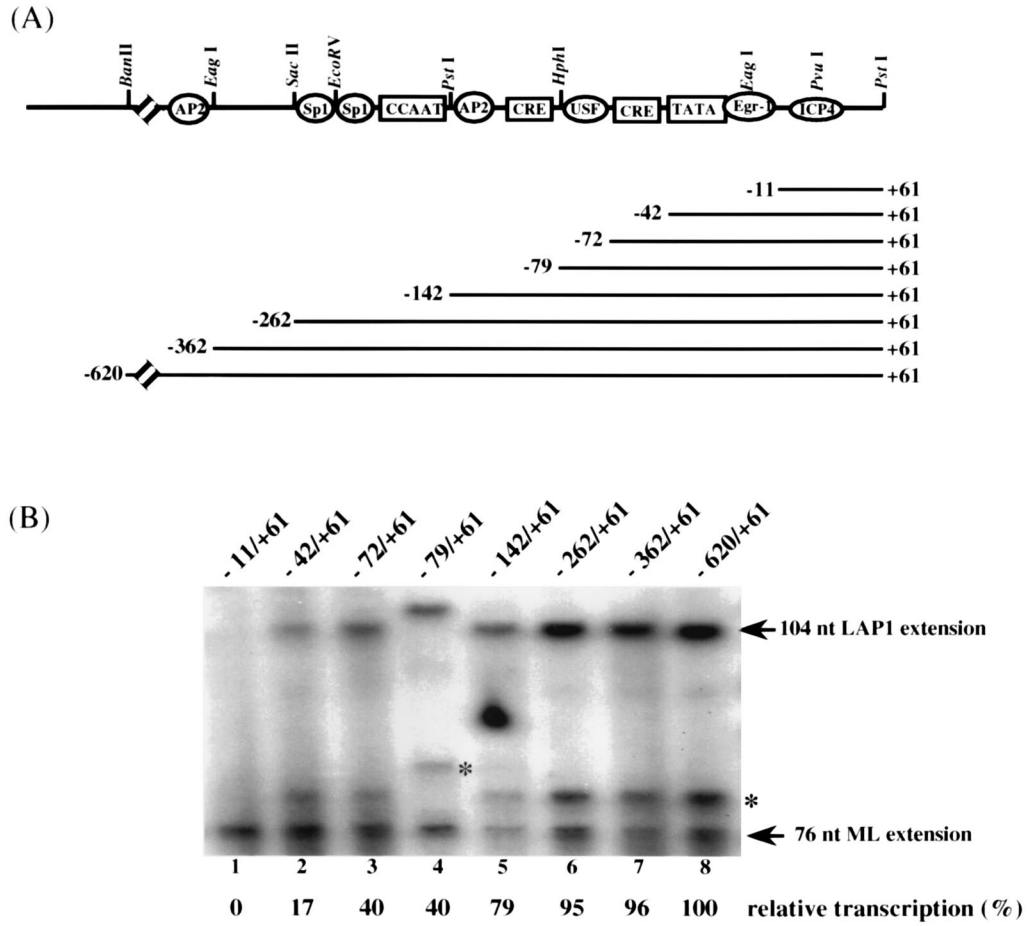

FIG. 4. 5′-deletion analysis: contribution of upstream sequences to LAP1 transcriptional activity. (A) Schematic representation of the putative *cis*-acting elements in the distal and proximal sequences of LAP1. Aligned below this schematic is the set of LAP1-CAT templates that have progressive 5′ deletions from positions −620 to −11. The 3′ limit of LAP1 sequence in all these templates is at +61. The double slash on the line drawing indicates that it is not to scale. (B) In vitro transcription-primer extension analysis of the 5′-deletion templates of LAP1, with pML.CAT included in each transcription reaction mixture as an internal control. The 5′ and 3′ extents of the respective LAP1 fragments are indicated above each lane. The transcription activity of each construct normalized to that of the internal control is expressed as a percentage of transcription relative to that of the −620/+61 construct at the bottom of each lane. The positions of extension products corresponding to accurately initiated transcripts from LAP1 and the adenovirus type 5 major late promoter are indicated on the right. The slight difference in size of the extension product from the −79/+61 LAP1 template (lane 4) is caused by Klenow end filling of a restriction terminus (see Materials and Methods). The position of the smaller extension product referred to in the text (asterisk) is also shown.

tained LAP1 sequences from positions −142 to −11 fused to the CAT gene. To one template, the LAP1 sequences from positions −11 to +61 were returned to the template but downstream of the CAT gene. The 24-mer CAT primer used in this experiment hybridizes to CAT gene sequences further downstream than did the 18-mer CAT primer used in previous experiments and detects the major late transcript as a 136-base extension product (Fig. 5C). Transcription analysis of these LAP1 templates demonstrates the presence of an essential promoter element within the region from positions −11 to +61 (Fig. 5C, compare lanes 3 and 4). The critical sequence element is not the start site of transcription itself, since LAP1 promoter activity is restored when the region from −11 to +61 is returned to the plasmid downstream of the CAT gene. The 87-base primer extension product in Fig. 5C, lane 4, maps to 27 bp downstream from the LAP1 TATA box. Thus, we conclude that the region from −11 to +61 contains at least one element that is essential for LAP1 function in vitro. Furthermore, this element is capable of promoting LAP1 activity even when present 1.6 kb downstream of the transcription start site. Sequence analysis of this region has not identified any specific elements within this region.

Contribution of the USF, CRE, and TATA box to LAT expression in vivo. To determine whether the *cis*-acting elements that were found to be important for transcription in vitro were also essential for LAT expression in vivo, we constructed viruses with mutations in the LAP1 promoter of both LAT loci. To prevent virus replication and encephalitis, the mutations were recombined into *d*120, a replication-defective ICP4$^-$ virus backbone (8). Each virus was stereotactically injected directly into the rat hippocampus (Fig. 6A). At 2 weeks postinjection, a time consistent with latency in the central nervous system, brains were sectioned and subjected to in situ hybridization using a probe specific for the 2-kb LAT (Fig. 6C). The 2-kb LAT is readily detected when the virus contains a wild-type LAP1 promoter (Fig. 6C, panel i). LAT expression is observed predominantly in granule cells of the dentate gyrus layer of the hippocampus. In contrast, LAT expression is greatly reduced or undetectable in sections of rat brains injected with viruses containing either a substitution mutation of

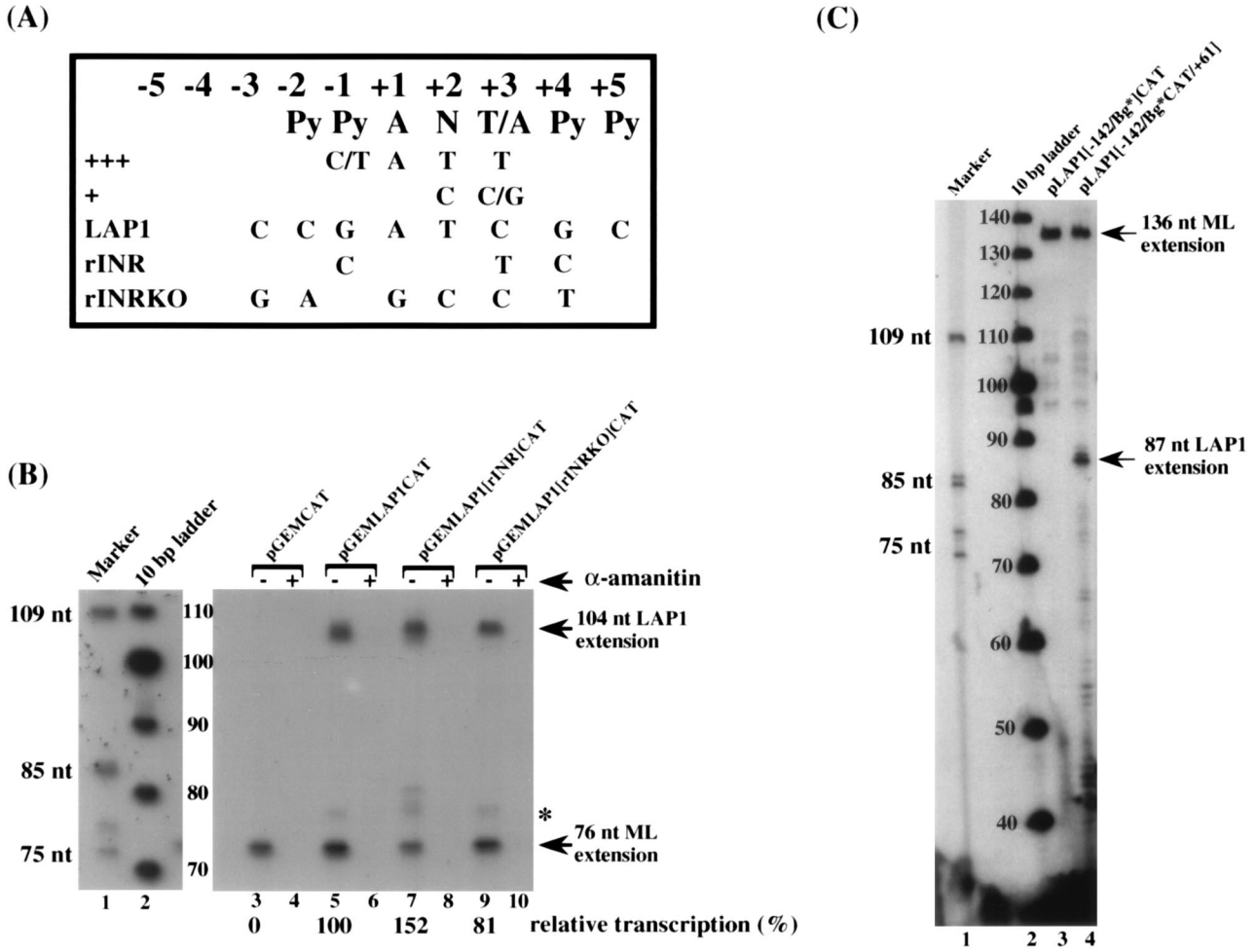

FIG. 5. Contribution of sequences downstream of the TATA box to LAP1 activity in vitro. (A) Consensus sequence of an initiator element. +++, sequence preferences in a strong initiator; +, alternate nucleotides at specific positions relative to +1 that permit accurate, albeit weaker, initiation levels (21). LAP1, nucleotide sequence surrounding the putative start site of the 8.3-kb primary LAT species in strain KOS; rINR and rINRKO, site-directed substitution mutations of sequences around the LAP1 start site which strengthen or weaken the match to the consensus initiator, respectively. (B) In vitro transcription-primer extension analysis of wild-type and initiator mutants of LAP1 with pML.CAT included in each reaction mixture as an internal control. The transcription reactions have been performed in the presence (+) or absence (−) of α-amanitin. Transcription activity of pLAP1[−142/rINR/+61]CAT and pLAP1[−142/rINRKO/+61]CAT templates normalized to that of the adenovirus type 5 major late promoter (ML) has been expressed in terms of percent transcription relative to that of wild-type LAP1 at the bottom of the lanes. The position of the small extension product referred to in the text (asterisk) is shown on the right. (C) Identification of enhancer function associated with sequences between positions −11 and +61 using an in vitro transcription-primer extension assay. Lane 1, end-labelled *Hin*fI digest of pSV325 as a size standard; lane 2, end-labelled 10-bp DNA size standard (Bethesda Research Laboratories); lane 3, in vitro transcription-primer extension of a template containing the CAT gene at position −11 relative to the start site of LAP1; lane 4, in vitro transcription-primer extension of pLAP[−142/Bg*CAT/+61], a template in which the sequence between −11 and +61 is returned downstream of the CAT gene. The positions of extension products obtained with the 24-mer CAT gene primer that correspond to accurately initiated transcripts from LAP1 and ML promoters are indicated on the right (arrows). nt, nucleotide.

the LAP1 TATA box (Fig. 6C, panel ii) or a 53-bp deletion spanning the USF, CRE, and TATA box (panel iii). These results confirm that the same *cis*-acting elements critical for LAP1 function in vitro are also required for the accumulation of the 2-kb LAT during latency. To ensure that each virus had established equivalent copies of latent viral genomes in each animal, DNA extracted from the hippocampus was analyzed by PCR. To normalize for the amount of total DNA harvested per tissue sample, PCR was first performed using primers specific to the cellular gene for GAPDH (data not shown). Amplification of HSV-1 DNA sequences with primers specific for the gB locus indicated that each animal contained equivalent amounts of latent viral genomes (Fig. 7A). Similarly, to normalize for the amount of RNA in each sample, RT-PCR was first performed, using primers specific to the cellular transcript for GAPDH (data not shown). Subsequently, RT-PCR using primers specific for the 2-kb species detected LAT RNA in animals injected with the wild-type and TATA mutant viruses (Fig. 7B, lanes 1 and 3) but not in animals injected with the virus containing a deletion of the USF, CRE, and TATA box of LAP1 (Fig. 7B, lane 5). The difference in LAT expression from the rT mutant virus (Fig. 6 and 7) is consistent with (i) the lower level of sensitivity of in situ hybridization itself and (ii) the fact that the section shown is one representative of 100 sections through the hippocampus region with LAT-expressing cells distributed across the plane of sectioning. On the other hand, RT-PCR was performed on total RNA extracted from the entire hippocampus and is capable of amplifying much lower levels of LAT RNA than could be detected by in situ hybridization. Finally, the RT-PCR establishes that even with the degree of amplification afforded by PCR, there is absolutely no LAT RNA expressed by deleting the sequence that

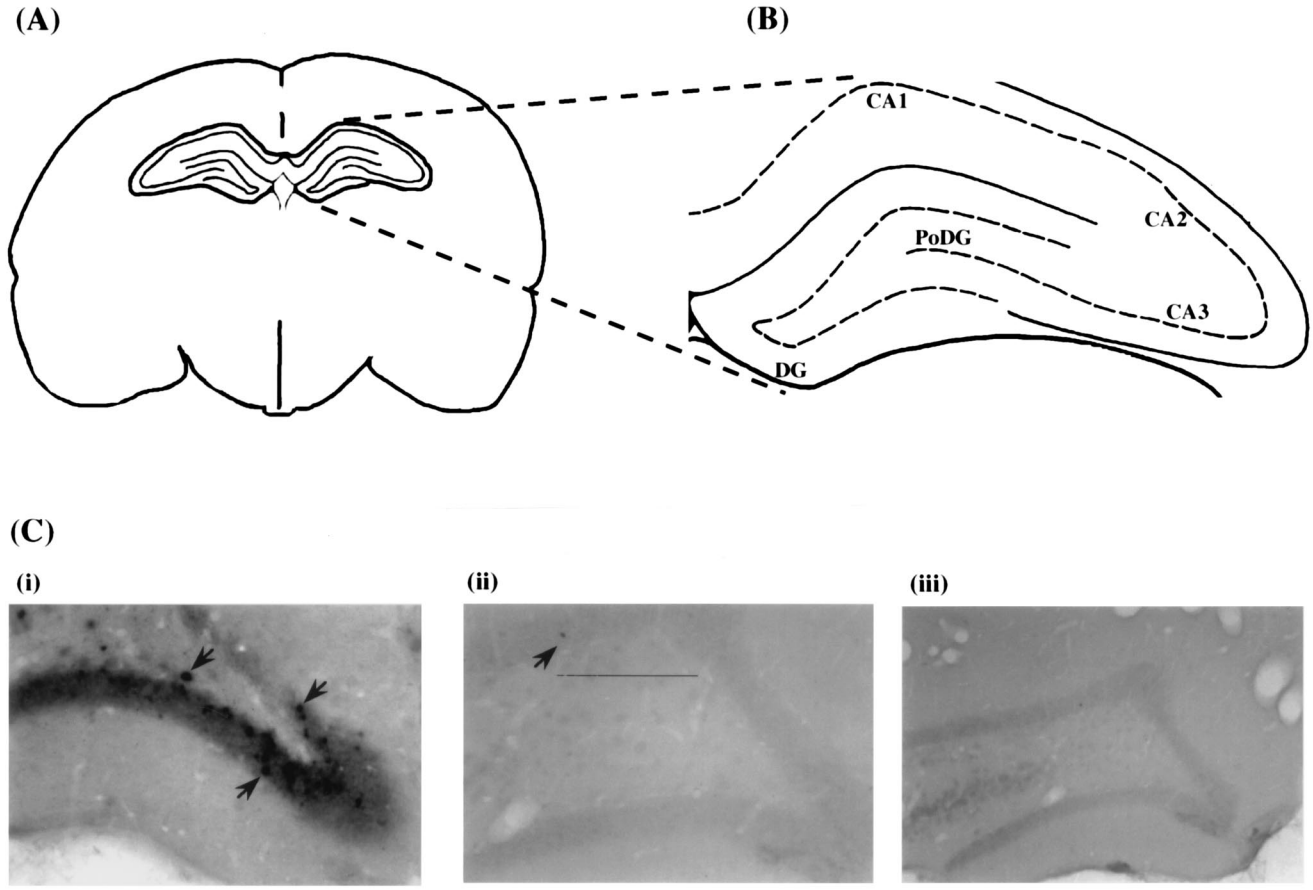

FIG. 6. In situ hybridization to LAT RNA in rat brain injected with site-directed mutant virus. (A) Schematic diagram of the cross section of a rat brain indicating the hippocampal region into which the replication-defective LAP1 mutant viruses are delivered by stereotactic injection. (B) Anatomical organization of the hippocampus. DG, dentate gyrus; PoDG, polymorph layer of the dentate gyrus; CA1 to CA3, different layers of Ammon's horn. (C) Representative tissue sections of rat brain at 2 weeks postinjection, showing in situ hybridization to a LAT-specific riboprobe. The photographs show the hybridization profiles of brains infected with *d*120 (ICP4$^-$ virus with wild-type LAT loci) (i), *d*120:LAP1rT (LAP1 TATA substitution mutant in an ICP4$^-$ virus background) (ii), and *d*120:LAP1ΔUCT (ICP4$^-$ virus with a 53-bp deletion that removes the USF, CRE, and TATA elements in LAP1) (iii) in neurons of the dentate gyrus layer of the hippocampus. Positive hybridization signals, predominantly in the dentate gyrus layer of the hippocampus, are indicated (arrows).

spans the USF element, CRE, and the TATA box. This result refines the limits of sequence elements required for LAT expression by narrowing the 203-bp *Pst*I fragment deleted in KOS/29 to a mere 53-bp sequence encompassing USF, CRE, and TATA box.

DISCUSSION

The goal of this study was to identify and characterize the role of several *cis*-acting elements in transcriptional activity of LAP1 of HSV-1. Our strategy was to study the effects of substitution mutations and 5′ deletions on transcriptional activity, using in vitro transcription-primer extension assays, and subsequently assess the effect of mutation of critical elements on latency-specific expression of the LAT transcript in vivo.

Results from in vitro transcription-primer extension assays provided evidence that the TATA box in LAP1 was essential for accurate transcription initiation. The start site 28 bases downstream of the TATA box was in agreement with previous primer extension data that mapped the 5′ end of CAT mRNA 28 nucleotides downstream of the TATA box (63) and with previous predictions of the start site of the 8.3-kb minor LAT RNA from latently infected rabbit trigeminal ganglia (13, 63). Substitution mutation of the TATA box in LAP1 resulted in loss of transcriptional activity in vitro. Furthermore, in contrast to the TATA box in the adenovirus type 2 major late promoter (5, 49), our results showed that the LAP1 TATA box required other additional downstream sequences to support basal levels of transcription.

One of the most exciting findings of this study is the possibility of an as-yet-uncharacterized downstream enhancer element(s) in LAP1 that not only is essential for promoter function but also is functional when positioned downstream of the reporter gene cassette. That this critical element is not a strong initiator element at the start site of transcription that collaborates with the LAP1 TATA box for transcription initiation is implied by (i) accurate and efficient transcription from a site-directed substitution mutant of the sequence at the start site and (ii) the ability of sequences between positions −11 and +61 to restore accurate transcription initiation even when distanced from the TATA box and upstream elements by as much as 1.6 kb of intervening CAT gene sequence. The latter observation also rules out the possibility that this sequence is required to promote contacts made by TFIID at the TATA box that sometimes extend into the leader (23, 37, 38). The region between positions −11 and +61 displays weak homology to leader sequences of other HSV-1 late genes, such as the UL19 (VP5) and UL44 (gC) genes, which have been shown to play a

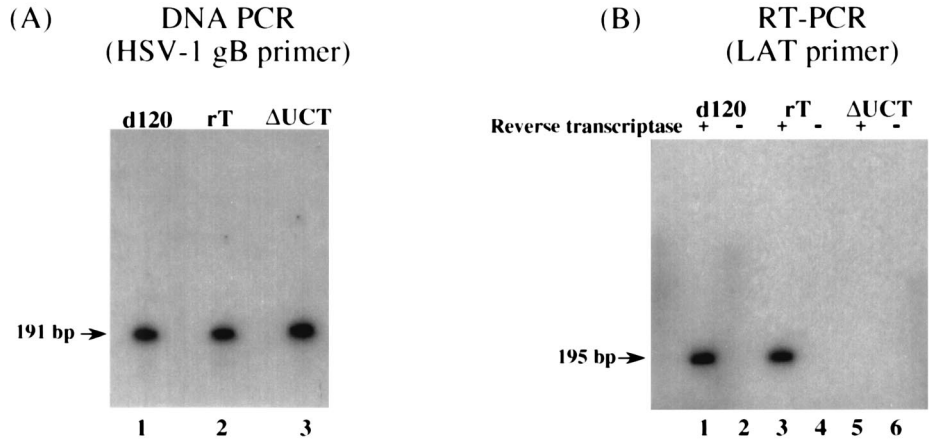

FIG. 7. PCR of DNA and RNA from homogenates of latently infected rat brain tissues. (A) Amplification of viral DNA using primers specific for the gB gene of HSV-1 from rat brains infected with the *d*120 virus (lane 1), *d*120:LAP1rT virus (lane 2), and *d*120:LAP1ΔUCT virus (lane 3). A 191-bp fragment corresponding to the amplified gB product is seen at equivalent levels in all three animals, showing that each of the LAP1 mutant viruses had established an equivalent number of latent genomes. (B) Amplification of viral RNA by RT-PCR using LAT-specific primers. Lanes 1, 3, and 5, RT-PCRs performed on RNA extracted from rat brains injected with the *d*120, *d*120:LAP1rT, and *d*120:LAP1ΔUCT viruses, respectively. A band corresponding to the amplified LAT product (195 bp) is seen only in lanes 1 and 3. Lanes 2, 4, and 6, control reactions of the same RNA extracts without reverse transcriptase added to the reaction mixture to show that the 195-bp PCR product is derived from LAT RNA and not genome contamination in the RNA preparation.

role in transcriptional regulation (20, 61). It is possible that there are *cis*-acting elements within this sequence that collaborate with upstream activating sequences to promote preinitiation complex assembly. The location of this sequence juxtaposed in between the two LAT promoter regions invites speculation as to whether it may be shared between LAP1 and LAP2. A similar situation has been seen with the USF binding site that is located between the divergent IVa2 promoter and the major late promoter in adenovirus (1). The latter is an example not only of a promoter element that is shared between two different promoter motifs, the IVa2 promoter being a TATA-less and the major late a classical TATA-containing promoter, but, more interestingly, of a shared element being a decisive factor in the switch of expression from one promoter to the other.

In vitro transcription-primer extension assays to assess the importance of the USF element, CRE, and the TATA element for LAP1 activity confirmed the predominant role of the LAP1 TATA box and suggested that both the USF element and CRE contribute to the level of in vitro transcription. Deletion of the TATA box in addition to the USF element and CRE (ΔUCT) abolished transcription. These results are in agreement with those of previous studies by Batchelor and O'Hare (3), who have reported that deletion of 40 bp surrounding and including the TATA box reduced expression in transient-transfection assays in HeLa cells to <5% of that of the wild-type promoter construct. In addition, there is evidence that the USF site contributes to LAP1 activity, since addition of a fraction of HeLa cell nuclear extract enriched in USF activity to a reconstituted in vitro transcription system greatly stimulated basal levels of transcription from LAP1 (42). Furthermore, an 8- to 30-fold reduction in promoter activity was observed in a mutant with abolished binding of USF in transient-transfection assays in both neuronal and nonneuronal cells (62). The contribution of USF to LAT expression during latency remains to be investigated in vivo. However, the significance of any effect seen in vitro and its role in determining the level of LAT expression or effects on reactivation can be fully appreciated only in the context of viral latency in the nervous system. For example, although mutation of CRE at positions −79 to −83 resulted in a fourfold reduction in reporter gene activity in transient-transfection assays, and although addition of this element to the HSV-1 immediate-early (IE110K) gene promoter enhanced expression levels in vitro, a virus with a deletion of CRE showed delayed reactivation kinetics but normal LAT expression by in situ hybridization to latently infected trigeminal ganglia (26, 39).

In this study, the predominant role of the TATA box in expression from the LAP1 promoter was also seen in vivo, where substitution of the TATA box resulted in a reduction in LAT expression from the *d*120:LAP1rT virus compared with *d*120, while LAT expression was altogether abolished upon deletion of USF, CRE, and the TATA box. Previous results have shown that although deletion of the TATA-containing *Pst*I fragment in KOS/29 was deleterious to LAT expression in latently infected murine trigeminal ganglia (10, 11, 13), mutation of the TATA box (LAT*Bcl* virus) only resulted in weaker in situ hybridization signals in one-third the number of neurons compared with those for the wild-type virus (39). The effect of mutation of the TATA box on LAT expression in latently infected trigeminal ganglia (39) was not as dramatic as the severely reduced expression in in situ hybridization of latently infected rat brain tissues reported in this study. This difference may be a function of the virus backbone used to construct these mutant viruses or the specific neuronal cell populations in which latency was established. Besides phenotypic differences that have been used to distinguish neuronal cell subpopulations from one another, the fact that not all neurons are equivalent with respect to latency is exemplified by the finding that only a fraction of neurons harboring HSV-1 genomes express LATs (29, 40, 46).

To further identify the upstream boundary of sequences that contribute to the transcriptional activity of LAP1 in vitro, a series of progressive 5′ LAP1 deletion mutants were studied. pLAP[−620/+61]CAT exhibited maximum in vitro transcriptional activity. Progressively lower transcriptional levels were observed with more truncated promoter constructs. This result appears to reflect the contribution of multiple promoter elements to LAP1 activity. Basal transcription was first detected in a template containing the TATA box and downstream sequence up to position +61. While only slight enhancement of transcription was observed when an additional USF binding

site, two CREs, and an AP-2 site were present in the promoter, whether this apparently minimal contribution to LAP1 in vitro reflected their relative importance in vivo remains to be determined. Although several of these cellular factors are ubiquitous, the unique ability of the LAT promoter to remain transcriptionally active in particular populations of neurons that support latency-specific transcription may be due to the existence of neuron-specific homologs of these transcription factors or the relative abundance of these factors in different cell types resulting in a difference in LAP1 activity (19, 22, 32, 50). It has also been suggested that the level of LAP1 activity in Vero, L929, and C1300 cells upon deletion of the USF site may also result from differential regulation of USF function in these different cell types (62).

In conclusion, we have shown that the TATA box in LAP1 directs accurate transcription initiation in vitro and is absolutely essential for transcription from LAP1 as determined by in vitro transcription and in situ hybridization studies during latency in rat brain hippocampal neurons. LAP1 is a complex promoter having multiple elements which control its function, most interesting are those which activate LAP1 during latency in vivo. LAP1 functions as a late gene during lytic infection and does not function in the absence of viral DNA synthesis. In vivo, however, LAP1 is constitutively active in at least a portion of latently infected neurons. Future experiments will be directed at identifying both the *cis*-acting elements of LAP1 and the relevant transcription factors which control its activity under these different circumstances. Finally, the phenotype of the subpopulation of neurons that harbor viral genomes expressing LAT must be determined in order to fully understand the *cis-trans* interactions which control LAP1 function.

ACKNOWLEDGMENTS

We thank L. Brunet for the pML.CAT plasmid, N. A. DeLuca for the *d*120 virus and E5 cell line, S. Person for the 4:27 virus, G. Jiang and W. A. Wojnar for technical assistance, J. G. Spivack for the *Pst*I-*Mlu*I LAT subclone, and W. F. Goins, S. W. French, C. A. Meaney, and S. Rasty for helpful suggestions in preparation of the manuscript.

This work was supported by Public Health Service grants NIH GM34534, NIH AG0947001, NIH NINCOS NS 19608, and NIH1 RC1 DK49095-01.

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