Identification of *cis*-Acting Sequences in the Promoter of the Herpes Simplex Virus Type 1 Latency-Associated Transcripts Required for Activation by Nerve Growth Factor and Sodium Butyrate in PC12 Cells

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In the absence of detectable viral proteins, expression of the latency-associated transcripts (LATs) is likely regulated by cellular factors during latent infection of neurons with herpes simplex virus type 1. The amounts and activation states of these factors may in turn be regulated by extracellular regulatory factors. Consistent with this hypothesis, we have recently demonstrated that LAT expression is significantly enhanced by nerve growth factor (NGF) and sodium butyrate (NaB) in neurally derived PC12 cells. With the ultimate goal of identifying *trans*-acting cellular factors involved in regulating LAT expression during latency, we have attempted to identify the *cis*-acting elements to which these putative cellular factors bind by characterizing the LAT promoter and a series of 5' promoter deletion mutants in PC12 cells following treatment with the LAT-enhancing agents NGF and NaB. Transient expression assays demonstrated that distinct *cis*-acting sequences mediate basal and induced LAT promoter expression. Basal activity in PC12 cells is mediated by two elements: a negative regulatory element between –435 and –270 and a positive element between –240 and –204. The positive element contains binding sites for the transactivator Sp-1, whereas the negative element bears some resemblance to known neuron-specific silencer elements. In contrast to basal expression, maximum induction of the LAT promoter by NGF and NaB requires sequences between –159 and –81. Using gel mobility shift assays, we have identified three sets of protein-DNA complexes that bind to this 78-bp region and shown by competition analysis that binding is specific. The abundance and mobility of these complexes were altered by treatment with NGF or NaB. The nucleotide sequences to which these complexes bind were fine mapped by competition analysis with oligonucleotide probes containing substitution mutations. The target sequences identified exhibit no homology to binding sites of known transcription factors. These regions were critical for complex formation *in vitro* and for maximum induction of the LAT promoter by NGF and NaB in transient expression assays. The protein complexes that form with target sequences likely participate in the regulation of LAT expression in response to physiological stimuli in neurons *in vivo*.

Herpes simplex virus (HSV) establishes latent infections in sensory neurons *in vivo*. These infections are characterized by the repression of nearly all of the more than 70 viral replication genes, and high-level expression of the latency-associated transcripts (LATs) (reviewed in reference 17). The LATs are a family of transcripts ranging in size from approximately 1.5 to greater than 8 kbp. Mutant viruses that fail to express the LATs are impaired in the ability to establish and reactivate from latent infection (5, 14, 21, 27, 42, 46, 52). The LATs do not appear to code for protein, and the mechanism by which they influence the establishment and reactivation of latency remains unclear (reviewed in reference 17).

The LAT promoter has been the object of intensive study because of its ability to escape the repression that affects all other HSV genes and maintain long-term, high-level expression within latently infected neurons. This promoter contains elements typical of an RNA polymerase II-dependent eukaryotic promoter such as a TATA box, putative binding sites for several cellular transcription factors, a binding site for a factor

important for basal LAT expression that resembles the adenovirus USF, and two binding sites for members of the ATF/CREB family of transcriptional activators (25, 28, 58). In addition, a region of the LAT promoter necessary for efficient activity in neuronal cell types has been identified through studies that compared LAT promoter sequences required for expression in fibroblasts and neuroblastoma cells (1, 2, 59). Fine mapping of this region has not been performed.

In the absence of detectable viral proteins, LAT expression in latently infected cells is likely regulated by external stimuli in a manner similar to that for cellular delayed-response genes. In support of this concept, we have recently demonstrated long-term activation of the LAT promoter in transient expression assays by treatment of PC12 cells with nerve growth factor (NGF) or sodium butyrate (NaB) (18). As described in the accompanying report, (18), NGF and NaB activation of the LAT promoter occurs with late kinetics, is dependent on activation of the GTP-binding protein, Ras, and can also be induced by activation of the serine/threonine kinase, Raf, in PC12 cells. Like the LAT gene, many cellular delayed-response genes are induced by NGF and NaB, but the *cis*-acting regulatory sequences that mediate this activation have not been characterized.

Activation of transcription of cellular genes by NGF has been well characterized in PC12 cells, and activation by NaB,

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although less well studied, has been observed for several promoters. Genes activated by NGF in PC12 cells include immediate-early genes, *c-fos*, *zif268*, *c-jun*, and *junB*, as well as late-response genes such as *transin/stromelysin*, *SCG10*, *peripherin*, and *Na⁺ channel type II* (45). NaB treatment is known to induce *c-fos*, *proenkephalin*, and genes encoding two transglutaminases in PC12 cells, although no systematic effort to identify NaB-activated genes has been carried out in these cells (7, 8, 35).

The apparent similarity between induction of the LAT promoter and induction of the promoters of NGF-induced delayed-response genes suggests that the same factors which regulate expression of these cellular genes may also regulate LAT expression. The identification of such general neuronal regulatory factors is central to efforts to understand the role of gene regulation in the mammalian nervous system as well as to express foreign genes in nervous tissue. In addition, the inverse relationship between LAT expression and expression of lytic viral genes suggests that LAT promoter activity may be an effective probe for measuring the ability of a particular host cell to support viral replication. The expression of factors that activate the LAT promoter may be regulated in parallel with factors that inhibit productive viral gene expression, or the same factors may be involved in both activities. Identification of factors that regulate LAT expression is an important step toward understanding the mechanisms regulating establishment and reactivation of HSV latency.

In this report, we identify a 78-bp region of the LAT promoter required for maximal NGF and NaB induction and characterize specific binding of PC12 cell nuclear factors to three discrete sites within this region. The abundance and mobility of the binding complexes were altered in response to NGF and NaB.

MATERIALS AND METHODS

Cell culture. PC12 cells were the gift of John Wagner (Cornell University Medical College, New York, N.Y.). Cells were grown at 37°C in 10% CO₂ in Dulbecco's modified Eagle medium (DME; Gibco, Gaithersburg, Md.) supplemented with 10% fetal calf serum (Sigma Chemical Company, St. Louis, Mo.), 5% donor horse serum (JRH Biosciences, Lenexa, Kans.), 0.37% bicarbonate, 0.03% glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Asp-17 Ras-expressing PC12-derived cell lines M-M17-26 and Z-M17-5 were a generous gift from Geoffrey Cooper (Dana-Farber Cancer Institute, Boston, Mass.) and were cultured as described above (47). The oncogenic Raf-expressing cell line IC4-*raf* was a generous gift from Thomas Roberts (Dana-Farber Cancer Institute) and was cultured as described above (54). All cells were dispersed by repeated pipetting with a bent Pasteur pipette and passaged or fed every 3 to 4 days. NGF, human recombinant basic fibroblast growth factor, and receptor-grade epidermal growth factor were purchased from Collaborative Biomedical Products (Bedford, Mass.). 8-(4-Chlorophenylthio)adenosine 3':5'-cyclic AMP (cpt-cAMP), butyrate, and retinoic acid were purchased from Sigma.

Plasmid construction. (i) **pLATCAT2079 and 5' deletion mutants.** Viral DNA in all plasmids was derived from HSV type 1 (HSV-1) strain KOS (Fig. 1A). To generate a plasmid containing the LAT promoter driving expression of the chloramphenicol acetyltransferase (CAT) gene, a 2,371-bp *BspEI-RsrII* (New England Biolabs, Beverly, Mass.) fragment containing the LAT promoter was cloned from the *BamB* fragment of HSV-1 KOS (Fig. 1B). The ends of this fragment were filled in with Klenow fragment (New England Biolabs) and ligated to *XbaI* linkers (New England Biolabs). Following digestion with *XbaI*, the LAT promoter fragment was cloned upstream of the bacterial CAT gene in the *XbaI* site of plasmid pCAT-KB, a derivative of pCAT-Basic (Promega, Madison, Wis.) in which the *EcoRI* fragment containing the simian virus 40 splice site and early polyadenylation signal was replaced with an *EcoRI* fragment containing the simian virus 40 late polyadenylation signal without splicing signals. The structure of the resulting plasmid, pLATCAT2079, was confirmed by restriction enzyme digestion.

A series of 5' LAT promoter deletion mutants for mapping NGF and NaB response elements was created by digestion of pLATCAT2079 with exonuclease III according to the Erase-a-Base protocol (Promega) (Fig. 1C). pLATCAT2079 was cut with *HindIII*, and overhanging ends were filled with thionucleotides to prevent digestion of the vector sequences. The linearized plasmid was then cut with *MluI* and treated for various amounts of time with exonuclease III. Digested plasmid fragments were religated and transformed into bacteria. Locations of 5'

ends of promoter deletion sites were determined by DNA sequencing using a Sequenase 2.0 kit (United States Biochemical, Cleveland, Ohio).

(ii) **pLATCATΔ-156/-83.** To create a plasmid containing the full-length LAT promoter lacking only the NGF and NaB response elements, the 2,371-bp *XbaI* fragment from pLATCAT2079 containing the LAT promoter was cloned into plasmid pUC19 (New England Biolabs) from which the *ApoI* site was removed. This plasmid, pUCLAT, was digested with *NcoI* and *HindIII*, and the ~2,200-bp fragment containing the LAT promoter was isolated. This fragment was digested with *Tsp509I*, the 5' overhangs were filled in with Klenow fragment, and the products were further digested with *XbaI*. The 381-bp *XbaI*-blunt product of these digestions containing LAT sequences from -82 to +294 was isolated. The ~2,200-bp fragment was also digested with *EcoRV* and *XbaI*, and the 1,926-bp product containing LAT promoter sequences from -156 to -2079 was isolated. The 381-bp *XbaI*-blunt and 1,926-bp *EcoRV-XbaI* fragments were ligated, recut with *XbaI*, and cloned into the *XbaI* site upstream of the CAT gene in plasmid pCAT-KB to create plasmid pLATCATΔ-156/-83.

(iii) **pLATCATmABC.** pLATCATmABC contains substitution mutations in the LAT promoter sequences shown to bind proteins in PC12 cell nuclear extracts. Both strands of a 74-bp *Tsp509I*-blunt double-stranded oligonucleotide containing the LAT promoter sequences from -83 to -156 with mutations in the NGF and NaB response elements were synthesized by the Molecular Biology Core Facility, Dana-Farber Cancer Institute. The top strand of this oligonucleotide has the sequence 5' CGACCGAATTTAAGTGTCTGCAGACAGGGG CACCGCGCCCTCCCGACATTAGGCCGACGATCTCCCGCC 3'. This oligonucleotide was ligated to the 1,926-bp *EcoRV-XbaI* fragment containing the LAT promoter sequences from -156 to -2079, and products were religated by digestion with *EcoRV* and *XbaI*. The resulting fragments were then ligated with the 381-bp *XbaI-Tsp509I* fragment containing the LAT promoter sequences from -82 to +294, religated with *XbaI* and *EcoRV*, and cloned into the *XbaI* site upstream of the CAT gene in plasmid pCAT-KB to yield plasmid pLATCATmABC.

(iv) **pLATCATΔ-200/-84 and pLATCATΔ-200/-75.** pLATCATΔ-200/-84 and pLATCATΔ-200/-75 were made to determine whether sequences upstream of -156 or downstream of -83 contributed to NGF- or NaB-dependent induction of LATs. A 183-bp fragment containing LAT promoter sequences from -376 to -201 and a 374-bp fragment containing LAT promoter sequences from -85 to +278 were amplified by PCR (Perkin-Elmer, Norwalk, Conn.), using the pLATCAT2079 deletion mutant plasmid pLATCAT435 as a template. The primers used for the 183-bp fragment were 5' CCCGCCGCGCGGTACC 3' and 5' CAGATCTGGCCGATACCCACACGGCGGG 3'. The primers used for the 374-bp fragment were 5' CAGATCTGAAATTATCATCCTAC CATGTGG 3' and 5' CCCCTCGTCCCGGGCCGT 3'. The primers were designed to create a *BglII* site (underlined) on the end of each fragment bordering the deletion (-201 and -85). Each fragment was ligated to itself and religated with *BglII* to generate complementary 5' *BglII* overhangs. The two fragments were then ligated to each other and digested with *BsaI* and *BsiWI*. The resulting *BsaI-BsiWI* fragment containing LAT promoter sequences from -364 to +258, with sequences including -200 through -84 deleted, was cloned into plasmid pLATCAT914, a deletion derivative of pLATCAT2079, creating plasmid pLATCATΔ-200/-84. Plasmid pLATCATΔ-200/-75 was created by using the same strategy except that the left-hand primer used for amplifying the 364-bp fragment had the sequence 5' CAGATCTGCCTACCCATGTGGTGTGTGGCCT 3'. Structures of the amplified regions were confirmed by DNA sequencing using a Sequenase 2.0 kit (United States Biochemical).

(v) **Viral promoter-CAT plasmids.** Plasmids pWRICP0, pIE3CAT, pL9CAT2.2, pMRICP8, pUL10CAT, pL42CAT, and LST-U-CAT, containing viral promoters representing immediate-early, early, and late genes, were created as described previously (37, 38, 56). Plasmid pMSG-CAT, encoding the mouse mammary tumor virus long terminal repeat driving expression of the CAT gene, was purchased from Pharmacia Biotech (Piscataway, N.J.).

(vi) **pLATLUC.** pLATLUC, containing the LAT promoter driving expression of the firefly luciferase gene, was cotransfected with LAT-CAT deletion plasmids as a transfection efficiency control. The 2,371-bp *XbaI* fragment from pLATCAT2079 containing the LAT promoter was cloned into the *NheI* site of the pGL2-Basic vector (Promega) upstream of the firefly luciferase gene. The structure of the resultant plasmid, pLATLUC, was confirmed by restriction digestion.

Transient expression assays. Cells were transfected by using Lipofectin (Gibco BRL, Gaithersburg, Md.)-mediated transfer. Cells were passaged 1 day prior to transfection and plated in 75-cm² tissue culture flasks with between 6 × 10⁶ and 1.2 × 10⁷ cells per flask; Lipofectin (48 µl) was mixed with serum-free DME (3 ml) in 15-ml polystyrene tubes. DNA (30 to 72 µg) was mixed with serum-free DME in separate 15-ml polystyrene tubes. The DME containing Lipofectin was mixed with DME containing DNA and incubated at room temperature for 10 to 15 min. During incubation, serum-containing medium was removed from PC12 cells and cells were rinsed with serum-free medium. Following incubation, the Lipofectin-DNA-containing DME (6 ml) was added to cells, which were then incubated for 3 to 5 h at 37°C. Following incubation, Lipofectin-DNA-containing DME was removed, serum-containing DME was added back to cells, and cells were allowed to recover overnight. The next day, cells were passaged onto 60-mm-diameter plates at a density of 10⁶ per plate, and medium containing inducing agents was added.

FIG. 1. (A) Diagram of the HSV-1 genome showing unique regions (U_S and U_L) bounded by terminal inverted repeats (TR_L , IR_L , IR_S , and TR_S). (B) Expanded map of the LAT promoter region contained in plasmid pLATCAT2079 indicating the locations of *cis*-acting regulatory sequences. (C) Map of 5' LAT promoter deletion mutants used to map novel regulatory elements. Locations of known *cis*-acting regulatory sequences are indicated.

Cells were harvested at various times posttransfection by scraping into Tris-buffered saline solution (150 mM NaCl, 10 mM Tris [pH 7.5]). Cells in suspension were pelleted by low-speed centrifugation, resuspended in 250 mM Tris, and frozen at -70°C for later assay of reporter enzyme concentration.

CAT enzyme assay. Cell lysates for determination of CAT enzyme activity were prepared by three cycles of rapid freezing and thawing followed by incubation at 65°C for 10 min and high-speed centrifugation to remove cell debris. CAT enzyme activity was assayed by the phase extraction method (44). Briefly, crude cell lysates were incubated with butyryl coenzyme A (375 μ M) and [14 C]chloramphenicol (0.1 μ Ci) in a reaction volume of 100 μ l for 60 min at 37°C. Following incubation, reaction mixtures were placed on ice and extracted with 500 μ l of 2:1 tetramethyl pentadecane (pristane)-xylenes to isolate butyrylated [14 C]chloramphenicol; 350 μ l of the organic extraction mixture was removed for liquid scintillation counting. The volume of cell lysate added to each reaction was adjusted to keep the reaction linear with respect to enzyme activity per microliter of crude lysate per hour. The linear range of the assay was determined through a dilution series of crude extract.

Luciferase assays. Cells were transfected as described above with LAT-CAT deletion plasmids and 5 μ g of pLATLUC per 60-mm-diameter dish as a control for transfection efficiency. Transfected cells to be assayed for luciferase activity were harvested by scraping into Tris-buffered saline followed by centrifugation and resuspension in 100 mM KH_2PO_4 (pH 7.8)–1 mM dithiothreitol (DTT). Cells were lysed by sonication for 230-s bursts, and cellular debris was recovered by centrifugation at $14,000 \times g$. Luciferase enzyme activity in the lysate was assayed by using the Promega luciferase assay system. An aliquot of cell extract at 25°C was mixed with luciferase assay reagent (Promega) for 5 s, and chemiluminescent emission was measured for 1 min with a Pharmacia-Wallac 1251 luminometer (Bio-orbit, Turku, Finland). The linear range of the assay was

determined by dilution of lysate, and the volume of extract used for individual measurements was adjusted to keep the reaction linear with respect to luciferase activity per microliter of crude lysate per minute.

Preparation of nuclear extracts. PC12 cells (10^7 to 10^8) were harvested in cold phosphate-buffered saline (PBS), washed once in cold PBS, collected by centrifugation ($750 \times g$), and resuspended in hypotonic reticulocyte standard buffer (RSB; 10 mM Tris-HCl [pH 7.5], 10 mM NaCl, 5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM DTT). Following a single wash in RSB, cells were resuspended in RSB containing 0.5% Nonidet P-40 and incubated on ice for 5 min. Nuclei were recovered by centrifugation ($750 \times g$), gently resuspended in RSB without Nonidet P-40, and collected by centrifugation ($750 \times g$). After removal of wash supernatant, high-salt extraction buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 25% glycerol, 0.42 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) was added to the pelleted nuclei and the mixture was incubated for 30 min at 4°C with gentle rocking. Following this incubation, the extract was transferred to an Oakridge tube and centrifuged for 30 min in a Sorvall SS-34 rotor at 14,500 rpm ($25,000 \times g$). The supernatant was collected and dialyzed at 4°C for 1 h against dialysis buffer (20 mM HEPES, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) to remove excess potassium. Following dialysis, the protein concentration of the extract was determined by the method of Bradford (6), using the Bio-Rad (Melville, N.Y.) protein assay reagent and bovine serum albumin (BSA) as a standard. Extracts were diluted with dialysis buffer to a final concentration of 2.5 μg of protein per μl of extract and quick-frozen in liquid nitrogen. Frozen extracts were stored at $-70^\circ C$.

Oligonucleotides and probes. To generate probes for use in gel mobility shift assays and competition experiments, single-stranded oligonucleotides corresponding to plus and minus strands of the LAT promoter were synthesized by the

Molecular Biology Core Facility, Dana-Farber Cancer Institute, and by Gibco BRL Life Technologies. The nucleotide sequences of the probes are shown below. Complementary single-stranded probes were annealed by heating to 85°C for 3 min followed by slow cooling to room temperature. Double-stranded probes were separated by polyacrylamide gel electrophoresis to remove unannealed single-stranded oligonucleotides and eluted in elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA [pH 8.0], and 0.1% sodium dodecyl sulfate) (41). Gel-purified double-stranded probes were radiolabeled by incubation with [γ -³²P]ATP (6,000 Ci/mmol; Dupont NEN, Boston, Mass.) and T4 polynucleotide kinase (New England Biolabs) to a specific activity of $\sim 10^8$ cpm/ μ g. Unlabeled double-stranded probes were used as specific and nonspecific competitor fragments in DNA binding assays.

Sequences of the top strands of double-stranded oligonucleotides used as probes and competitors in gel mobility shift assays were as follows (substitution mutations are boldfaced):

–200/–171	5' CCC GCC CCG CGC CGG TCC ATT AAG GGC GCG 3'
–170/–141	5' TGC CCG CGA GAT ATC AAT CCG TTA AGT GCT 3'
M1	5' TGC CCG CGA GAT ATC AAT CCT GGC CTT GCT 3'
M2	5' TGC CCG CGA GAT ATA CCG AAG TTA AGT GCT 3'
M3	5' TGC CCG CGC TCG CGC AAT CCG TTA AGT GCT 3'
M4	5' TGA AAT ATA GAT ATC AAT CCG TTA AGT GCT 3'
–155/–126	5' AAT CCG TTA AGT GCT CTG CAG ACA GGG GCA 3'
–140/–111	5' CTG CAG ACA GGG GCA CCG CGC CCG GAA ATC 3'
M5	5' CTG CAG ACA GGG GCA CCG CGC CCT TCC CGC 3'
M6	5' CTG CAG ACA GGG GCC AAT ATC CCG GAA ATC 3'
M7	5' CTT ACT CAA GGG GCA CCG CGC CCG GAA ATC 3'
–125/–96	5' CCG CGC CCG GAA ATC CAT TAG GCC GCA GAC 3'
–110/–81	5' CAT TAG GCC GCA GAC GAG GAA AAT AAA ATT 3'
M8	5' CAT TAG GCC GCA GAC GAG GAA AAG CCC CGT 3'
M9	5' CAT TAG GCC GCA GAA TCT TCA AAT AAA ATT 3'
M10	5' CAG GCT TAC GCA GAC GAG GAA AAT AAA ATT 3'

DNA binding assays. DNA binding assays were carried out as described previously (12). Individual binding reaction mixtures contained 2.5 μ g of nuclear extract, 250 pg of oligonucleotide probe, 100 mM NaCl, 10 mM MgCl₂, 100 mM HEPES, 5% glycerol, 2 mM DTT, 4 μ g of poly(dA-dT) (Pharmacia), 3 μ g of BSA (New England Biolabs), and H₂O to a final volume of 10 μ l. These conditions were based on several preliminary experiments in which concentrations of protein and salt and the type of nonspecific carrier DNA were varied. Protein concentrations of between 1.25 and 12.5 μ g per reaction were tested, and 2.5 μ g per reaction was selected because it provided a clear signal without use of large amounts of extract. Reactions were carried out with 50, 100, and 250 mM NaCl with or without addition of MgCl₂. Optimal formation of DNA-protein complexes was observed in the presence of MgCl₂, and because the binding was not significantly altered in the presence of the three NaCl concentrations, the most physiological concentration (100 mM) was selected. Addition of up to 50 mM KCl had little effect on formation of complexes, and therefore it was omitted from the final reaction conditions. A variety of nonspecific carrier nucleic acids were tested, including dI-dC, poly(dA-dC), calf thymus DNA, and poly(dA-dT). Use of poly(dA-dT) resulted in the clear observation of the complexes that varied in response to treatment with NGF and NaB. Binding reaction mixtures were incubated for 30 min at 25°C, although the complexes were unchanged when incubations were carried out at 4°C, and complexes were separated by electrophoresis in 8% polyacrylamide (37.5:1 acrylamide-bisacrylamide) gels prepared and run in 0.5 $\times$ Tris-borate-EDTA buffer (41). In competition experiments, radiolabeled probe and unlabeled competitor fragment were premixed before addition of buffer and extract. In experiments in which multiple probes were used on the same gel, equivalent picograms of probe DNA were used in each binding reaction and equal amounts of radioactivity from each reaction were loaded onto the gel to correct for differences in specific activity of individual probes. Gels were exposed to PhosphorImager screens overnight and were visualized and quantitated with a Molecular Dynamics (Sunnyvale, Calif.) PhosphorImager.

RESULTS

Localization of sequences required for basal expression of the LAT promoter in PC12 cells. Basal LAT promoter activity has been characterized in a variety of neuronal and nonneuronal cell lines as a part of efforts to identify neuron-specific *cis*-acting enhancer sequences (1, 2, 25, 59). As described in the accompanying report, we have demonstrated that the LAT promoter is activated in response to NGF and NaB in PC12 cells; in this report we identify the *cis*-acting elements that mediate this response (18). We also examined whether the

FIG. 2. Basal activities of LAT promoter 5' deletion mutants. PC12 cells were transfected with a promoter deletion mutant plasmid and plasmid pLATLUC containing the full-length LAT promoter driving expression of the firefly luciferase gene. Following transfection, cells were plated in quadruplicate identical cultures and incubated for 6 days with and without inducing agents. CAT activity and luciferase activity were measured separately, using one pair of duplicate cultures in each assay. CAT activity was normalized to luciferase activity to control for differences in transfection efficiency, and the normalized activity of each deletion plasmid was divided by the normalized activity of the full-length LAT promoter plasmid pLATCAT2079 to obtain the relative promoter activity for each mutant plasmid. Each datum point is the average of three to six separate experiments, and the error bars represent the standard error of the mean.

same elements mediate both basal and activated expression in PC12 cells or whether the two activities are mediated through separate DNA sequences. Since LAT promoter activity has not previously been characterized in PC12 cells, we first set out to identify *cis*-acting sequences required for basal LAT promoter expression in these cells. To locate these sequences, we created a series of 5' deletions in the LAT promoter contained in the pLATCAT2079 promoter fusion plasmid (Fig. 1C). For this purpose, PC12 cells were transfected with individual LAT promoter deletion plasmids and a control plasmid expressing the luciferase gene as a transfection efficiency control. Following transfection, cells were plated in quadruplicate and incubated for 6 days in the absence of inducing agents. For each deletion plasmid, two plates were assayed for CAT activity, and the other two were assayed for luciferase activity. LAT promoter activity was normalized to the activity of the luciferase transfection control.

Systematic deletion of sequence elements in the LAT promoter identified specific sequences that have significant effects on basal LAT promoter expression (Fig. 2). Notably, these sequences exerted both positive and negative effects. First, deletion of the region between –2100 and –914 reduced basal expression by >50%, indicating that this region contains elements which serve to activate basal expression in PC12 cells. Incremental deletion of sequences between –914 and –435 resulted in a gradual and modest increase in basal expression. Deletion of the region between –435 and –270 produced a greater than 2.5-fold increase in basal LAT promoter expression, indicating the presence of a major repressor(s) of basal expression in this region. The presence of upstream silencing

elements is a common feature of a large number of cellular genes with neuron-specific patterns of expression (10, 43). Basal activity remained high when sequences between -270 and -240 were deleted in a stepwise manner but dropped precipitously (~12-fold) upon deletion of the 36-bp sequence between -240 and -204. This sequence likely contains a major activator of basal LAT promoter expression in PC12 cells. Comparison of this sequence with the binding sites of known transcriptional activators revealed the presence of two consensus sequences for the activator Sp1 (Fig. 1C) (16). Further analysis of the region -240 to -204 will be required to identify the specific sequences and proteins that mediate high-level basal LAT promoter expression in PC12 cells. Incremental deletion of sequences between -204 and -54 suggested the presence of a minor repressor element at -69.

Localization of NGF- and NaB-inducible response elements in the HSV-1 LAT promoter. To identify LAT promoter sequences required for regulation by NGF and NaB, we transfected PC12 cells with the same series of deletion constructs used in the studies of basal promoter activity. Following transfection, cells were split into several identical cultures and induced with either NGF alone, NaB alone, or the two agents together. As described in the accompanying report, NGF alone or NaB alone increased full-length LAT promoter activity 8- to 12-fold, and treatment with both agents simultaneously resulted in a 40-fold (after 6 days) to 60-fold (after 8 days) induction (18).

The results of these tests indicate that the sequence between -204 and -159 may contain a repressor of NGF-induced activation, as deletion of this sequence reproducibly yielded somewhat higher levels of LAT promoter activity (Fig. 3). Deletion of the 12-bp sequence between -159 and -147 resulted in a significant and reproducible decrease in NGF-induced promoter activity, as did deletion of the 15-bp sequence from -96 to -81, suggesting that these sequences may contain *cis*-acting targets of NGF-induced transactivating factors.

A similar pattern emerged when the promoter deletion plasmids were tested for inducibility by NaB. In these tests, however, the incremental deletion of sequences between -435 and -240 produced a more dramatic but still gradual decrease in promoter activity. As for activation by NGF, the 12-bp sequence between -159 and -147 is apparently also important for activation by NaB. Although deletion of the sequence from -96 to -81 resulted in decreased NaB inducibility, this decrease was not as dramatic as for NGF inducibility. The observed incremental decreases in promoter inducibility following systematic deletion of sequences in this region suggests that multiple regulatory factors bind to this region of the LAT promoter. The similarity in the sequence requirements for NGF- and NaB-mediated activation suggest that some factors may be activated in common by the two agents.

The complex pattern of activation demonstrating cooperative induction of some sequences but not others by NGF and NaB suggests that (i) the activities of some factors involved in cooperative activation differ, (ii) for noncooperative activation, some factors are induced by one agent or the other, and (iii) as for induction by NGF in NaB alone, the sequence -159 to -81 is targeted by factors induced simultaneously by NGF and NaB. In summary, these studies demonstrate that sequences between -159 and -81 are required for high-level activation of the LAT promoter by NGF and NaB in PC12 cells and provide a useful starting point for studies of the cellular factors that mediate this response.

The NGF- and NaB-responsive region of the LAT promoter forms multiple complexes with PC12 cell nuclear proteins. Having identified a region of the LAT promoter required for

FIG. 3. Identification of LAT promoter sequences required for activation by NGF and NaB. PC12 cells were transfected with the indicated LAT promoter deletion mutant plasmids and treated for 6 days with no inducer, NGF (100 ng/ml), NaB (1.25 mM), or NGF (100 ng/ml) and NaB (1.25 mM) simultaneously. CAT activity was measured for each plasmid, and fold induction was calculated by dividing the activity from induced cultures by the activity from uninduced cultures. Relative induction for each inducing agent was calculated by dividing the fold induction by that agent for a particular deletion mutant plasmid by the fold induction for the full-length plasmid pLATCAT2079. Datum points are the average of activities from duplicate or triplicate plates in three to six separate experiments. Error bars represent the standard error of the mean.

activation by both NGF and NaB in PC12 cells in transient expression assays, we characterized this region for its ability to serve as a target for protein-DNA complex formation with proteins in PC12 cell nuclear extracts. For this purpose, we synthesized four contiguous, nonoverlapping oligonucleotides spanning the region from -200 to -81. These oligonucleotides were radiolabeled and incubated with nuclear extracts from untreated PC12 cells or PC12 cells treated with NGF alone, NaB alone, or the two agents together. Protein-DNA complexes were separated by polyacrylamide gel electrophoresis.

As described above, deletion of sequences between -204 and -159 had no major effect on LAT promoter activation by NGF or NaB in transient expression assays. Consistent with

FIG. 4. Binding of PC12 cell nuclear proteins to LAT promoter probes. Radiolabeled LAT promoter probes $-200/-171$, $-170/-141$, $-140/-111$, and $-110/-81$ were incubated with PC12 cell nuclear extracts from untreated cells or cells treated for 6 days with NGF (100 ng/ml), NaB (1.25 mM), or NGF (100 ng/ml) and NaB (1.25 mM) simultaneously. Complexes were resolved on 8% polyacrylamide gels. Complexes formed with each probe are labeled A for $-170/-141$ complexes, B for $-140/-111$ complexes, and C for $-110/-81$ complexes.

this observation, protein-DNA complexes were not detected with an oligonucleotide spanning the region from -200 to -170 and PC12 cell nuclear extracts (Fig. 4). By contrast, the promoter sequences between -159 and -81 were required for high-level activation by these agents (Fig. 3). Consistent with this finding, each of the three oligonucleotide probes spanning this region formed complexes with PC12 cell nuclear proteins, providing a correlation between promoter activity in transient assays and binding of proteins in electrophoretic mobility shift assays. Three sets of protein-DNA complexes were detected by using the three different probes, and the relative abundance of these complexes varied following treatment of PC12 cells with NGF, NaB, or NGF plus NaB (Fig. 4).

Six distinct protein-DNA complexes (A1 to A6) were detected when nuclear extracts from untreated cells were used as the source of protein and the oligonucleotide spanning the sequence -170 to -141 was used as the probe. On the basis of the results of specificity studies demonstrating that complexes A4 to A6 were less specific than complexes A1 to A3 (see below), we further characterized only complexes A1 to A3. In the presence of extracts from NGF-treated PC12 cells, complexes that comigrated with complexes A1 and A2 were greatly reduced, whereas a prominent complex that comigrated with the A3 complex from untreated lysates formed with this probe (Fig. 4, $-170/-141$ probe). This complex may be identical to A3, or it may contain different binding factors which produce a protein-DNA complex of similar mobility. Alternatively, the three complexes A1 to A3 may reflect differential modifications of the same factor or factors. In the presence of nuclear extracts from NaB-treated cells, three complexes with the same mobility as A1 to A3 were also formed. However, the relative amounts of the three complexes differed, favoring formation of A1. In extracts of cells treated with both NGF and NaB simultaneously, the NGF-specific pattern of complex formation predominated.

Three distinct complexes, B1 to B3, formed with the probe spanning the region from -140 to -111 . The relative abundance of these complexes also varied with NGF or NaB treatment. Specifically, B1 was not detected in extracts of cells treated with NGF or NGF and NaB. B3 was evident in cells treated with either of the activating agents and, most prominently, when the two agents were added together.

Interestingly, the pattern of complexes that formed with the $-110/-111$ probe resembled a subset of the complexes formed with the $-170/-141$ probe. Both the sizes of the complexes and the alterations in the relative amounts of the three complexes, C1 to C3, formed on the $-110/-81$ probe were remarkably similar to those of complexes A1 to A3 observed with the $-170/-141$ probe.

Because the three oligonucleotides used in these studies do not overlap, the possibility existed that additional protein-DNA complexes which formed at the junction regions between the three probes might not be detected. To determine whether the same or additional complexes formed on sequences spanning the junctions between the $-170/-141$, $-140/-111$, and $-110/-81$ probes, we synthesized two additional probes containing LAT promoter sequences: $-155/-126$ and $-125/-96$. These probes were radiolabeled and incubated with nuclear extracts from untreated PC12 cells or PC12 cells treated with the two inducing agents. Neither of the two junction-spanning probes bound complexes which were not detected with the nonoverlapping probe set, suggesting that additional LAT promoter DNA complexes are not formed in these junction regions (data not shown).

The LAT promoter region from -170 to -141 competes for complexes formed with the $-110/-81$ probe and vice versa.

The apparent similarities in the complexes formed with the $-170/-141$ and $-110/-81$ probes (Fig. 4) led us to examine whether the formation of these complexes with one probe could be competed for by the other probe. For this purpose, the labeled $-170/-141$ probe was incubated with nuclear extracts from untreated or NGF-treated cells (Fig. 5A). In addition to the radiolabeled probe, we added an excess of either homologous ($-170/-141$) or heterologous ($-110/-81$ and $-140/-111$) oligonucleotide probes to the reaction mixture as competitors. Resolution of the complexes formed in both types of nuclear extract demonstrated that the homologous $-170/-141$ probe competed for binding of all six complexes, heterologous probe $-110/-81$ competed primarily for binding of complexes A1 to A3, and heterologous probe $-140/-111$ competed primarily for binding of complexes A4 to A6. A similar pattern was observed in competition experiments using nuclear extracts of NGF-treated cells as the source of protein. In these tests, however, no competition for complex formation was observed when heterologous probe $-140/-111$ was used as the competitor. Relative to complexes formed with extracts from untreated cells, complex A5 was enhanced two- to threefold and levels of complexes A1 to A4 were reduced in three independently isolated sets of nuclear extracts from cells treated with NaB (Fig. 5B). Competition for formation of complex A5 was greatest with a homologous probe ($-170/-141$) and least (or not at all) with the heterologous probe ($-140/-111$). The precise roles of the multiple complexes formed on the $-170/-141$ probe remains unclear, but the large drop in NGF and NaB inducibility in transient expression assays upon deletion of this region suggests that they may play a role in regulation of LAT promoter activity by these agents (Fig. 3).

In an experiment the converse of that shown in Fig. 5A, the $-110/-81$ probe was radiolabeled and formation of complexes C1 to C3 from untreated and NGF-treated cells was competed for with unlabeled homologous and heterologous ($-170/-141$

FIG. 5. (A) Competition analysis of complexes formed on the $-170/-141$ probe by using the $-110/-81$ probe. The $-170/-141$ probe was incubated with PC12 cell nuclear extracts from untreated cells or cells treated for 6 days with NGF (100 ng/ml) in the presence of a 150- or 600-fold excess of the $-170/-141$, $-110/-81$, or $-140/-111$ probe. (B) Competition analysis of complexes formed on the $-170/-141$ probe in extracts from PC12 cells treated with NaB. The $-170/-141$ probe was incubated with three independently isolated sets of nuclear lysates from untreated and 1.25 mM NaB-treated PC12 cells in the presence of a 600-fold excess of either the $-170/-141$ or the $-140/-111$ probe. (C) Competition analysis of complexes formed on the $-110/-81$ probe by using the $-170/-141$ probe. The $-110/-81$ probe was incubated with PC12 cell nuclear extracts from untreated cells or cells treated for 6 days with NGF (100 ng/ml) in the presence of a 150- or 600-fold excess of the $-110/-81$, $-170/-141$, or $-140/-111$ probe.

and $-140/-111$) probes. Addition of homologous $-110/-81$ probe competed for formation of these complexes, whereas addition of heterologous $-140/-111$ probe did not, demonstrating the specificity of the protein-DNA interaction with positions -110 to -81 (Fig. 5C). The $-170/-141$ probe also competed significantly for binding of C1 to C3 to the $-110/-81$ probe. These studies demonstrate that formation of complexes A1 to and A3 with the $-170/-141$ probe and formation of complexes C1 to C3 with the $-110/-81$ probe are specific and that the complexes formed on the two different probes are likely composed of the same or similar proteins.

The pattern of protein-DNA complexes formed with the $-140/-111$ probe is altered by treatment of PC12 cells with NGF or NaB. The oligonucleotide consisting of LAT promoter sequences from -140 to -111 forms a unique series of complexes with PC12 cell nuclear extracts (Fig. 4). When this probe was radiolabeled and incubated with untreated nuclear extracts, a prominent and reproducible fast-migrating complex, B1/B2, and a less prominent complex, B3, were observed (Fig. 6). Complexes B1/B2 and B3 could be resolved into a doublet by extended electrophoresis (data not shown). The specificities of the B1/B2 and B3 LAT promoter interactions were shown by the demonstration of competition with unlabeled excess homologous $-140/-111$ probe but not with heterologous $-170/-141$ probe. In the presence of NGF-treated nuclear extracts, two major complexes formed with this probe: a complex which comigrated with B1/B2 but appeared to be a singlet (B2) form of this complex as well the higher-mobility complex, B3. B3 formation was enhanced in NGF-treated cells. All three B complexes that formed with NGF-treated extracts were effectively competed for by an unlabeled homologous probe ($-140/-111$) but not by a heterologous probe ($-170/-141$). In NaB-treated cell extracts, B1/B2 formed as a doublet similar to the complex that formed with untreated cell extracts; however, B3 formation was enhanced above the level observed with extracts from untreated and NGF-treated cells. Both com-

plexes bound specifically to $-140/-111$ probe sequences. In nuclear extracts from cells treated with both NGF and NaB, complexes resembling those formed with extracts from NGF-treated cells predominated, although the amount of complex

FIG. 6. Competition analysis and effect of NGF and NaB treatment on PC12 cell nuclear protein binding to the $-140/-111$ probe. The $-140/-111$ probe was incubated with PC12 cell nuclear extracts from untreated cells or cells treated for 6 days with NGF (100 ng/ml), NaB (1.25 mM), or NGF (100 ng/ml) and NaB (1.25 mM) simultaneously in the presence of a 50- or 150-fold excess of the $-140/-111$ probe or a 150-fold excess of the $-170/-141$ probe. Complexes were resolved on 8% polyacrylamide gels. comp, competitor.

B3 was significantly enhanced. Taken together, these studies demonstrate the formation of specific complexes of nuclear proteins with the LAT promoter region from positions -140 to -111 .

Fine mapping of binding sites for the A, B, and C complexes, in the HSV-1 LAT promoter. To fine map sequences in the LAT promoter necessary for binding of the A, B, and C complexes from untreated and NGF-treated extracts, we performed competition analysis with a series of oligonucleotides with 6-bp substitution mutations in LAT promoter sequences. By comparing the efficiencies of competition of mutant probes and wild-type probes, we were able to identify nucleotides important for formation of LAT promoter protein-DNA complexes.

The radiolabeled $-170/-141$ oligonucleotide probe was incubated with nuclear extracts from untreated and NGF-treated cells in the presence of excess homologous probe or mutant probe M1, M2, M3, or M4 (the sequences of the mutant probes are presented in Materials and Methods). Competition with an unlabeled homologous probe or mutant probe M1 or M4 resulted in similar ~ 6 -fold reductions in the formation of complexes A1 to A3 (Fig. 7A). By contrast, competition with mutant probe M2 or M3 resulted in twofold or less reduction in A1, A2, and A3 complex formation, similar to competition demonstrated previously with the nonspecific $-140/-111$ probe. These tests indicate that the 6-bp sequences mutated in the M2 and M3 probes are important for the binding of cellular factors which make up complexes A1 to A3. The importance of these sequences, which correspond to the region from -162 to -151 of the LAT promoter, is corroborated by the decrease in NGF (or NaB)-dependent promoter activation in transient assays upon deletion of sequences from -159 to -147 (Fig. 3), suggesting that this 10- to 15-bp region comprises the binding site for NGF- and NaB-dependent activators. A search of the transcription factor database indicated that this sequence does not match any known transcription factor recognition site.

Similar analysis was performed on the B1/B2 and B3 complexes by using mutant forms of the $-140/-111$ probe. In this analysis, a probe (M5) bearing mutations between -117 and -111 failed to compete effectively for B1/B2 and B3 formation, whereas two other probes bearing mutations between -137 and -132 (M6) or -127 and -122 (M7) competed as efficiently as the wild-type probe for these complexes (Fig. 7B). Similar patterns of competition were observed for both B1/B2 and B3, suggesting that these complexes are formed on the same region of the LAT promoter and that the B3 complex may be a modified form of the B1/B2 complex.

Despite the unexpected similarity in the complexes formed on the $-170/-141$ and $-110/-81$ probes, no obvious nucleotide sequence similarity exists between the two probes. Competition for C1, C2, and C3 binding with wild-type and mutant $-110/-81$ probes identified the region between -82 and -97 contained in mutant probes M8 and M9 as the target site for formation of these complexes (Fig. 7C). A probe mutated at sequences -103 to -108 (M10) competed as effectively as the wild-type probe for formation of complexes C1 to C3, implying that these sequences are not involved in complex formation.

Together, these experiments identify three discrete 10- to 15-bp regions, A, B, and C, which bind PC12 cell nuclear proteins differently in response to NGF and NaB (Fig. 8).

LAT promoter sequences between -156 and -83 are required for maximal induction by NGF or NaB. To determine whether the sequences that bind PC12 cell nuclear proteins in gel mobility shift assays (Fig. 7 and 8) correlate with sequences required for LAT promoter activation in transient expression assays, we constructed a series of plasmids containing internal

FIG. 7. (A) Competition analysis of complexes formed on the $-170/-141$ probe by missense mutant LAT promoter probes. The $-170/-141$ probe was incubated with PC12 cell nuclear extracts from untreated and NGF (100 ng/ml)-treated cells in the presence of 600-fold excesses of mutant probes M1 to M4, homologous $-170/-141$ probe, or heterologous $-140/-111$ probe. The locations of the mutations in each competitor probe (comp) are underlined. (B) Competition analysis of complexes formed on the $-140/-111$ probe by missense mutant LAT promoter probes. The $-140/-111$ probe was incubated with PC12 cell nuclear lysates from untreated and NGF (100 ng/ml)-treated cells in the presence of 150-fold excesses of mutant probes M5 to M7, homologous probe $-140/-111$, or heterologous probe $-170/-141$. The locations of the set of mutations in each competitor probe are underlined. (C) Competition analysis of complexes formed on the $-110/-81$ probe by missense mutant LAT promoter probes. The $-110/-81$ probe was incubated with PC12 cell nuclear lysates from untreated and NGF (100 ng/ml)-treated cells in the presence of 600-fold excesses of mutant probes M8 to M10, homologous probe $-110/-81$, or heterologous probe $-170/-141$. The locations of the mutations in each competitor probe are underlined.

deletions within LAT promoter sequences upstream of the CAT gene (Fig. 9). Deletion of LAT promoter sequences between -156 and -83 in pLATCAT2079 resulted in levels of NGF- and NaB-induced CAT activity that were roughly 75 and

FIG. 8. Sequence of the LAT promoter indicating binding sites for proteins from PC12 cell nuclear extracts. Discrete binding sites were determined on the basis of competition analysis of binding complexes, using missense mutant probes. The boldface sequences represent minimal binding sequences which were unable to compete for complex formation when mutated. Additional sequences flanking these may also bind PC12 cell nuclear proteins.

50% lower, respectively, than the levels achieved with wild-type pLATCAT2079. Larger deletions in this region, from -202 to -85 and -202 to -74, in a plasmid containing 914 bp of the LAT promoter resulted in a significant but slightly less marked decrease in NGF- and NaB-mediated induction. It is possible that these deletions had moved upstream regulatory elements closer to the transcriptional start site, allowing it to compensate for the loss of the deleted sequences. The failure to eliminate all NGF- or NaB-dependent activation of this promoter by deleting specific protein-DNA binding sites implies the presence of redundant elements upstream that are brought closer to the transcriptional start site by the deletions.

To test the relevance of sequences in this region to NGF- and NaB-induced LAT promoter activity without affecting the spacing of upstream elements, we constructed plasmid pLATCATmABC, containing three substitution mutations within each of the three putative protein binding sites identified in gel mobility shift assays. Similar to deletion mutant promoter constructs lacking sequences in this region, the LAT promoter construct containing substitution mutations in the complex A, B, and C binding sites was significantly impaired in its ability to be induced by NGF, NaB, or the two agents together. Activa-

tion by either agent alone was reduced by ~65%, whereas activation by the two agents together was reduced by ~80%. Together, these studies demonstrate that the region between -159 and -83 is required for maximal induction by NGF and/or NaB in PC12 cells. They further suggest that LAT promoter sequences important for maximal induction by NGF and NaB in transient expression assays are also involved in protein-DNA complex formation with factors in PC12 cell nuclear extracts.

DISCUSSION

NGF- and NaB-responsive elements in the LAT promoter.

In this study, we have identified a 78-bp fragment of the LAT promoter between nucleotides -159 and -81 that is necessary for efficient promoter activation by NGF or NaB. Characterization of protein-DNA complex formation with this region indicates that it contains binding sites for three series of complexes of PC12 cell nuclear proteins, that the pattern of complex formation is altered by treatment of cells with NGF or NaB, and that these sequences are required for maximal activation by NGF and NaB.

Previous studies of the LAT promoter have focused on identifying *cis*-acting sequence elements that confer neuron-specific expression (2, 3, 25, 59). These studies compared promoter regions required for basal activity in neuronal cell lines with those required for basal activity in nonneuronal cell lines. Such analysis revealed that the 108-bp LAT promoter region between -251 and -143 is required for efficient basal promoter expression in the neuroblastoma cell line IMR-32 but not in nonneuronal HeLa cells (2). Similarly, in the present study, sequences between -240 and -204 were shown to be critical for maximal basal promoter activity in PC12 cells. In another study, a cAMP response element-like sequence between nucleotides -83 and -74 was necessary for efficient LAT promoter expression in C1300 neuroblastoma cells but not L929 mouse fibroblast cells (25). Nucleotides -83 to -74 had no major effect on basal promoter activity in these studies.

In this study, we identified sequences required for LAT promoter regulation in response to extracellular regulatory

FIG. 9. Effects of internal deletions of LAT promoter sequences on induction by NGF and NaB. PC12 cells were transfected with the indicated plasmids and treated for 6 days with no inducer, NGF (100 ng/ml), NaB (1.25 mM), or NGF and NaB simultaneously. CAT activity was measured, and fold induction was calculated by dividing activity in induced cells by activity in uninduced cells. Results are the average of activities from duplicate plates in two to four separate experiments. For abbreviations, see the legend to Fig. 1.

factors. By comparing expression from promoter deletion mutants with expression from the full-length promoter in the presence and absence of inducing agents, we were able to identify important inducible regulatory sequences while minimizing the effects of sequences required for basal-level expression. We did not determine whether the sequences identified also contribute to neuron-specific LAT expression. However, we believe it likely that the NGF-dependent regulatory factors that bind to the LAT promoter in PC12 cells are also expressed in neurons in vivo and that these factors contribute to LAT expression during latent infection of those neurons. Many cellular-delayed response genes induced by NGF in PC12 cells exhibit neuron-restricted expression in vivo (23, 26, 34, 53, 55). The relationship between NGF inducibility and neuron-restricted expression of these genes is not known, but this observation suggests that NGF responsiveness in PC12 cells may correlate with high-level expression in neurons in vivo and that the same promoter sequences may mediate both effects. The activity of LAT promoter- β -galactosidase reporter gene constructs within the context of the glycoprotein C locus of the HSV-1 genome was recently described, and significant differences were found between previously identified promoter sequences required for efficient expression of reporter constructs in neuronal cell lines and sequences required for expression of a reporter gene during acute or latent infection in mouse dorsal root ganglia (15). Deletion of promoter sequences upstream of -252 resulted in nearly complete elimination of expression of the reporter construct in infected neurons in vivo. Because the constructs used in that study were 5' deletions and all activity was eliminated by removal of sequences 5' of -252, it is difficult to assess the role in vivo of expression of downstream regulatory sequences identified by us and others. The apparent requirement for distal upstream sequences of LAT promoter reporter constructs during infection of mice, but not during transient transfection of cells in culture, is similar to results obtained with the *c-fos* promoter. This promoter has been extensively characterized in many in vitro systems. Recently, however, when a *c-fos* promoter-reporter gene fusion was expressed in transgenic mice, the promoter exhibited much more stringent requirements for a variety of upstream regulatory elements for correct expression in neurons (40). Sequences that were superfluous in cell culture were absolutely required for expression in vivo. Together, these two studies demonstrate the importance of confirming results obtained with LAT reporter constructs in vitro by observing effects of subtle mutations within the promoter in its native locus during infection in vivo. Such experiments using small LAT promoter substitutions are in progress.

The putative neuron-specific silencing element. The presence of negative regulatory elements which inhibit expression in nonneuronal cells is emerging as a common theme in promoters of many genes expressed primarily in neurons (9, 22, 23, 29, 34, 50, 51). A neuron-restricted silencing factor (NRSF) which binds to many of these negative regulatory sequences was recently cloned and characterized (10, 43). In the course of the present study, a region of the LAT promoter which appears to repress basal expression in PC12 cells was identified. Comparison of the nucleotide sequence of this region with the consensus sequence of known neuron-restricted silencer elements (NRSEs) revealed a sequence within the LAT promoter between nucleotides -323 and -303 that bears some resemblance to these elements (Fig. 10). Two observations suggest that this element does not bind the recently cloned negative regulatory factor, however. First, the inhibitory effect of the cloned factor is much stronger than the inhibition observed in the LAT promoter. Second, the cloned factor is apparently not

FIG. 10. Sequence comparison of known and putative (*) NRSEs and LAT promoter sequences. NRSE and consensus sequences were compiled from references 9, 10, 23, 24, 26, 29, 31, 34, 36, 43, 48, and 50. The sequence identified in the synapsin II promoter has not been characterized in detail, and the inhibition mediated by sequences containing this site is significantly lower than inhibition by the other non-LAT sites.

expressed in PC12 cells (10, 43). Despite these differences, the negative element identified in the LAT promoter may bind a factor similar to the NRSF. The promoters of the neuron-specific genes encoding synapsin I and synapsin II show similar four- to sevenfold increases in activity in PC12 cells when negative regulatory sequences are deleted (9, 49). Likewise, expression of the gene encoding the neuronal intermediate filament protein peripherin is regulated by a negative regulatory element in PC12 cells (50). These studies suggest that NRSF-related proteins may be expressed in PC12 cells and in neurons in vivo and that such factors may regulate the expression of specific sets of genes in distinct subpopulations of neurons. In theory, these elements may play a role in determining which neuronal cells express LAT and which do not. Further experiments will be required to determine the significance of the NRSE-like element for LAT promoter expression during latency.

Nuclear protein complex formation with LAT promoter sequences between -170 and -81. By dividing the LAT promoter region that responds to NGF and NaB into three separate fragments for protein binding studies, we were able to simplify the identification of protein complexes that bind to each site and determine more precisely the specific sites of protein-DNA interaction (Fig. 8). Discrete binding sites for three series of protein complexes on the LAT promoter were identified in this way. Analysis of two probes spanning the junctions between the nonoverlapping probes revealed no additional binding sites for protein-DNA complexes not observed on the nonoverlapping probes.

Two sequences, -170 to -141 and -110 to -81, appear to bind similar or identical sets of complexes. Despite the similarity of these complexes, the nucleotide sequences required for binding of the complexes are not recognizably related. Three possible explanations for these disparate binding sites are as follows: (i) the two binding sites may consist of distinct nucleotides that form similar secondary and tertiary configurations, making a single consensus sequence difficult to recognize; (ii) the complexes may be made up of several different factors which multimerize to recognize a compound binding site; or (iii) three factors may multimerize one that binds to site A, a bridging factor, and one that binds to site B. Probes containing either site would result in formation of similar tripartite complexes and would complete for complex formation. This is precisely the result obtained in this study.

As noted in the accompanying report (18), the synergistic activation of the LAT promoter by NGF and NaB may be a consequence of the cumulative activation of similar cellular

factors by the two agents through different mechanisms or by the activation of different factors by each agent.

Whatever the case, the actual mechanism of promoter activation by NGF and NaB is not obvious from studies of *cis*-acting regulatory elements and the factors which bind to them, although the relative abundance of complexes A1 to A3 is clearly altered in response to NGF and NaB. These three complexes may represent differentially modified forms of the same protein or proteins, or they may each be composed of one or more different factors. The same is true of the B1/B2 and B3 complexes. The changes observed in response to the activating agents may simply be the result of increases or decreases in the amounts or phosphorylation states of the same series of proteins, or they may result from addition or subtraction of individual proteins from a larger binding complex. These various possibilities can be addressed through detailed characterization of the proteins which comprise the complex in combination with subtle mutations in individual binding sites, coupled with *in vivo* tests of mutant viruses containing these mutations.

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