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Characterization of a Family of Related Cellular Transcription Factors Which Can Modulate Human Immunodeficiency Virus Type 1 Transcription In Vitro

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LBP-1 is a cellular protein which binds strongly to sequences around the human immunodeficiency virus type 1 (HIV-1) initiation site and weakly over the TATA box. We have previously shown that LBP-1 represses HIV-1 transcription by inhibiting the binding of TFIID to the TATA box. Four similar but distinct cDNAs encoding LBP-1 (LBP-1a, -b, -c, and -d) have been isolated. These are products of two related genes, and each gene encodes two alternatively spliced products. Comparison of the amino acid sequence of LBP-1 with entries in the available protein data bases revealed the identity of LBP-1c to α-CP2, an α-globin transcription factor. These proteins are also homologous to *Drosophila melanogaster* Elf-1/NTF-1, an essential transcriptional activator that functions during *Drosophila* embryogenesis. Three of the recombinant LBP-1 isoforms show DNA binding specificity identical to that of native LBP-1 and bind DNA as a multimer. In addition, antisera raised against recombinant LBP-1 recognize native LBP-1 from HeLa nuclear extract. Functional analyses in a cell-free transcription system demonstrate that recombinant LBP-1 specifically represses transcription from a wild-type HIV-1 template but not from an LBP-1 mutant template. Moreover, LBP-1 can function as an activator both in vivo and in vitro, depending on the promoter context. Interestingly, one isoform of LBP-1 which is missing the region of the Elf-1/NTF-1 homology is unable to bind DNA itself and, presumably through heteromer formation, inhibits binding of the other forms of LBP-1, suggesting that it may function as a dominant negative regulator.

Human immunodeficiency virus type 1 (HIV-1), a member of the retrovirus family, is the causative agent of AIDS. From the early stage of infection, HIV-1-infected $CD4^+$ cells are sequestered in the lymphoid tissues (9, 36), which are the major target of HIV-1 infection. The vast majority of HIV-1-infected cells, however, are latently infected (9). Transcription from the long terminal repeat (LTR) of HIV-1 is regulated by a complex interplay between the viral activator Tat (10, 21, 23, 28, 33) and an array of both positive- and negative-acting cellular transcription factors such as NFAT-1, USF, NF-κB, Sp-1, and LBP-1 (4). In the latent viral state, transcription from the integrated proviral LTR is minimal but can be activated by a variety of stimuli, including various mitogens and superinfecting viruses, that lead to a high level of viral gene expression and replication (42). It seems likely that the induction of transcriptional activators such as NF-κB by these stimuli could activate the dormant HIV-1 provirus. Similarly, repression of negative-acting factors which actively inhibit HIV-1 transcription could, in principle, lead to the same result.

A cellular protein called LBP-1 (UBP-1) has previously been shown to interact in a concentration-dependent manner at two sites in the HIV-1 promoter (19, 22, 50). A high-affinity site surrounding the transcription start site overlaps both an initiator element (7, 43) and an element called the inducer of short transcripts (IST) (40), while a low-affinity site overlaps the TATA box. Mutations in the high-affinity LBP-1 binding site have deleterious effects on HIV-1 transcription in vitro, implying that either LBP-1 or another as yet unidentified binding protein acts positively through that site (19). In contrast, when LBP-1 is bound to the low-affinity site, it specifically represses HIV-1 transcription by preventing the binding of the general initiation factor TFIID to the TATA box (22). To further study the seemingly complex role of LBP-1 in HIV-1 transcription, we have identified and characterized a family of cDNAs encoding LBP-1.

MATERIALS AND METHODS

Plasmid construction. The wild-type and mutant (IS4 in reference 22) HIV-1 templates and pML are described by Kato et al. (22). Plasmids WT4E1b and MT4E1b were generated by the insertion of four copies of oligonucleotides containing wild-type and mutant sequences from −4 to +21 of HIV-1 at the *Sal*I site of pE1bCAT (29). Plasmid pMLI is described by Du et al. (7). Plasmid pL4MLI was constructed by inserting four copies of an oligonucleotide containing the sequence from nucleotides (nt) 271 to 289 of simian virus 40 (SV40) at the *Xba*I site of pMLI.

EMSA. Probes for electrophoretic mobility shift analysis (EMSA) were prepared by end labeling double-stranded oligonucleotides with the Klenow enzyme and α-^{32}P-labeled deoxynucleoside triphosphates (dNTPs). Twenty femtomoles of probe was used in a 20-μl reaction mixture containing 4% Ficoll type 400, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 70 to 80 mM KCl, 1 mM spermidine, 100 μg of bovine serum albumin per ml, 0.03% Nonidet P-40, 1 mM dithiothreitol (DTT), and 2 μg of poly(dI-dC). Reactions were initiated by the addition of protein, and mixtures were incubated for 20 min at 30°C. Protein-DNA complexes were analyzed by a 4% (37.5:1, acrylamide/bisacrylamide) nondenaturing polyacrylamide gel,

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with 0.5× Tris-borate-EDTA and 0.03% Nonidet P-40, after a 45-min prerun.

Purification of LBP-1. HeLa S100 extracts (900 ml, 18 mg of protein) were adjusted to a salt concentration of 250 mM KCl and applied to a 300-ml phosphocellulose column equilibrated with buffer BC (20 mM Tris-HCl [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.03% Nonidet P-40, 0.5 mM phenylmethylsulfonylfluoride, 5 mM DTT) containing 250 mM KCl. The column was washed with buffer BC–275 mM KCl, and proteins were eluted with buffer BC–600 mM KCl. After dialysis to 80 mM KCl, this fraction was applied to a 100-ml DEAE-cellulose column equilibrated with buffer BC–80 mM KCl. Proteins were eluted with buffer BC–300 mM KCl and directly loaded onto a 20-ml phosphocellulose column equilibrated with the same buffer. Proteins were eluted with a linear gradient (3 column volumes) from 300 to 700 mM KCl. LBP-1 eluted at approximately 350 mM KCl. Active fractions were dialyzed to 100 mM KCl and applied to a 3-ml DNA affinity column containing an HIV-1 promoter sequence (nt −17 to +27). The column was washed with buffer BC–200 mM KCl, and proteins were eluted with buffer BC–500 mM KCl. Active fractions were dialyzed against buffer HC (20 mM HEPES-KOH [pH 7.9], 10% glycerol, 0.5 mM EDTA, 5 mM DTT) containing 100 mM KCl and loaded to a Bio-Gel SP-5PW high-pressure liquid chromatography (HPLC) column (7.5 by 75 mm; Bio-Rad) equilibrated with the same buffer, and proteins were eluted with a 30-ml linear gradient from 100 to 500 mM KCl. LBP-1 eluted at 300 mM KCl. All procedures were carried out at 4°C except HPLC, which was run at room temperature. LBP-1 activity was followed by a gel shift assay with an oligonucleotide probe (nt −17 to +27 of HIV-1). HPLC-purified LBP-1 was digested with endoproteinase Lys-C, and some of the derived fragments were sequenced at the Rockefeller Protein Sequencing Facility.

Cloning of LBP-1 cDNAs. Degenerate PCR primers were designed on the basis of peptide sequences I and III described in Fig. 2. For the first PCR, in which 10^9 PFU of a human Namalwa cDNA library was used as the template, the sense and antisense primers were GA(C/T)ACITT(C/T)AA(A/G)CA(A/G)AA(C/T)GA and CC(A/G)TC(A/C/G/T)GCIGCICC(A/G)CA(A/G/T)AT, respectively. The first-round PCR product was used as the template for the second PCR, for which the primers AA(C/T)GA(A/G)AA(C/T)GG(A/C/T/G)GA(A/G)TA(C/T)AC and AT(C/T)TGIACIAG(A/G)TC(C/T)TC(C/T)TT were used. Two fragments of 470 and 580 bp were obtained and subsequently used to screen cDNA libraries. Nineteen independent clones were isolated. Final positive phages were excised in vivo into pBluescript SK (Stratagene) as instructed by the manufacturer. Following restriction enzyme mapping of inserts, the longest ones of each group were sequenced.

In vitro translation of LBP-1. LBP-1a, -b, and -c cDNAs in pBluescript SK were directly used as DNA templates for in vitro translation of LBP-1. A putative full-length LBP-1d cDNA was created by substituting the *Bsm*I-*Sma*I fragment of LBP-1d cDNA with corresponding sequences from the LBP-1c cDNA. RNA was generated in vitro by transcribing LBP-1 cDNAs with T7 RNA polymerase and NTPs, using a kit from Stratagene. Runoff RNA transcripts were translated by programming rabbit reticulocyte lysates (Promega) in the presence of [^{35}S]methionine as instructed by the manufacturer. The samples were quickly frozen in liquid nitrogen after 20% glycerol was added.

Bacterial expression of LBP-1. An *Nde*I restriction enzyme site was introduced into LBP-1 cDNAs at the initiator ATG codon by oligonucleotide-directed mutagenesis (27). The mutagenized cDNA was subcloned into 6His-pET11d (14). The resulting plasmid was introduced into *Escherichia coli* BL21(DE3) containing pLysS (45). After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), cells were harvested by centrifugation, and the extract was prepared by sonication in buffer BC–500 mM KCl containing 10% glycerol. After centrifugation at 10,000 × *g* for 15 min, the supernatant was adjusted to 5 mM imidazole and loaded onto a Ni^{2+}-nitrilotriacetic acid column (Qiagen). The column was then washed with 10 column volumes of buffer BC–500 mM KCl containing 20 mM imidazole, and proteins were eluted with buffer BC–500 mM KCl containing 150 mM imidazole. The eluate was dialyzed against buffer BC–100 mM KCl, and aliquots were quickly frozen in liquid nitrogen. The specific activities of recombinant LBP-1a, -b, and -c, as measured by EMSA, were similar to that of affinity-purified native LBP-1. To produce the N-terminal half of LBP-1a for use as an antigen, the *Nde*I-*Bam*HI fragment of LBP-1a cDNA was inserted into the 6His-pET11d vector and introduced into *E. coli* BL21(DE3) containing pLysS. After induction with IPTG, cells were lysed in 6 M guanidine-HCl (pH 8.0) with brief sonication. Soluble material was loaded onto a Ni^{2+}-nitrilotriacetic acid column. The column was washed sequentially with 8 M urea–0.1 M sodium phosphate (pH 8.0) and 8 M urea–0.1 M sodium phosphate (pH 6.0) before being eluted with 8 M urea–0.1 M sodium phosphate (pH 4.5). The protein was further purified on sodium dodecyl sulfate (SDS)-polyacrylamide gels before being injected into rabbits.

In vitro transcription. Nuclear extracts were prepared from HeLa cells as described previously (6). In vitro transcription was performed for 1 h at 30°C in a 25-μl reaction with 8 μl (10 mg/ml) of HeLa cell nuclear extract and 10 to 50 ng of supercoiled template DNA. The standard reaction mixtures contained 20 mM HEPES-KOH (pH 7.9), 6 mM $MgCl_2$, 5 mM DTT, and 500 μM rNTPs. Following incubation, 400 μl of stop mix (7 M urea, 10 mM Tris-HCl [pH 7.8], 10 mM EDTA, 0.5% SDS, 100 mM LiCl, 40 μg of tRNA, 300 mM sodium acetate) was added, and the mixture was extracted with phenol-chloroform and ethanol precipitated. An oligonucleotide (5′CAACG GTGGTATATCCAGTG3′) complementary to the coding region of the chloramphenicol acetyltransferase (CAT) gene was end labeled for primer extension, using T4 polynucleotide kinase and [γ-^{32}P]ATP. The transcribed RNA and labeled primer were annealed in 30 μl of 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.6)–0.4 M NaCl–1 mM EDTA–0.2% SDS for 3 h at 37°C. The RNA-DNA duplex was then extended by using 10 U of avian myeloblastosis virus reverse transcriptase for 1 h at 42°C. The products were extracted and analyzed on 6% polyacrylamide gels with 8 M urea.

Antiserum preparation. New Zealand rabbits (3 kg) were initially injected intradermally with 300 μg of antigen emulsified with an equal volume of complete Freund's adjuvant. Rabbits were boosted subcutaneously every 4 weeks with 150 μg of antigen emulsified with an equal volume of Freund's incomplete adjuvant. Blood was collected from dorsal ear veins 10 days after each boost.

Immunodepletion of LBP-1 from HeLa nuclear extracts. To deplete LBP-1 from HeLa cell nuclear extracts, 300 μl of anti-LBP-1 antiserum was incubated with 100 μl of protein A-Sepharose (Pharmacia) for 2 h at 4°C with constant mixing. After four washes with buffer BC–100 mM KCl and two with buffer BC–400 mM KCl, the resin was incubated for 2 h with 300 μl of HeLa nuclear extract adjusted to 400 mM KCl. After centrifugation, the supernatant was dialyzed against buffer

TABLE 1. Purification of LBP-1

Fraction	Protein (mg)	Vol (ml)	Activity (U)[a]	Sp act (U/mg)	Relative purification (fold)	Yield (%)
S100	18,000	900	67,800	3.8	1	100
P11-I	750	110	32,500	43.3	11.4	48
DE52	239	65	23,000	96.2	25.3	34
P11-II	87	15	11,500	132.2	34.8	17
Affinity	0.2	1.8	4,100	20,500	5,395	6.0
HPLC SP-5PW	0.011	2.8	610	55,455	14,593	0.9

[a] One unit is defined as the amount of LBP-1 that retards 10 fmol of the probe in the presence of 2 μg of poly(dI-dC) under the standard gel shift conditions as described in Materials and Methods.

BC–100 mM KCl, and aliquots were quickly frozen in liquid nitrogen.

Transfections and CAT assays. Cytomegalovirus-driven expression vectors encoding LBP-1 (pCX-LBP-1) were constructed by subcloning full-length LBP-1 cDNAs between *Xba*I and *Xho*I sites of pCX (12). HeLa cells were cultured in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and antibiotics. HeLa cells (3×10^5/6-cm-diameter dish) were cotransfected with 3 μg of a reporter plasmid and 3 μg of pCX-LBP-1 by the calcium phosphate precipitation method (13) with osmotic shock with 20% dimethyl sulfoxide in phosphate-buffered saline. Cells were harvested 48 h after transfection and processed by three cycles of freezing-thawing. Cell extracts were assayed for CAT activity by incubation at 37°C for 1 h with 0.1 μCi of [^{14}C]chloramphenicol and 1 mM acetyl coenzyme A in 150 μl of 0.25 M Tris-HCl (pH 7.8).

RESULTS

Purification and cloning of LBP-1. To obtain amounts of LBP-1 sufficient for purification and subsequent cDNA cloning, modifications to our previous purification scheme (22) were developed. Crude HeLa S100 extracts were first subjected to phosphocellulose and DEAE-cellulose chromatography. The recovered LBP-1 activity was concentrated on a second P11 column and further purified on a DNA affinity column containing an oligonucleotide extending from nt −17 to +27 of the HIV-1 promoter. HPLC SP-5PW was used for the final purification step (Table 1). Analysis of the purified protein by SDS-polyacrylamide gel electrophoresis (PAGE) revealed two major bands of 63 (doublet) and 68 kDa, consistent with the results of a previous small-scale purification method (22). Renaturation of protein from an SDS-polyacrylamide gel slice which contained these polypeptides regenerated LBP-1 binding activity, as monitored by band shift assay with an oligonucleotide probe containing the high-affinity binding site (data not shown). Eleven micrograms of purified protein was obtained from 18 g of S100 extract protein (Table 1).

This LBP-1 preparation was digested with endoproteinase Lys-C, and amino acid sequences were determined after separation of derived peptides by reverse-phase HPLC. With oligonucleotide primers derived from peptide sequences, the PCR was used to amplify LBP-1 cDNAs from a human Namalwa cell cDNA library. With the PCR products as a probe, two Namalwa cell cDNA libraries were screened. Restriction enzyme mapping of 19 cDNA clones obtained from screening 2 million phage plaques revealed four groups of cDNAs (Fig. 1), which were termed LBP-1a, -b, -c, and -d. Nucleotide sequences of the longest cDNA clones from each group indicated that they were derived from two similar but distinct genes. Thus, LBP-1a and LBP-1b cDNAs are differentially spliced products of one gene, while LBP-1c and LBP-1d are differentially spliced products of a second related gene. Genomic DNA blotting analyses using HeLa and Namalwa cell DNAs were consistent with the presence of more than one gene in the genome (data not shown). LBP-1a, -b, and -c cDNAs encoded open reading frames of 504, 541, and 502 amino acids, respectively. Their translation start sites were assigned on the basis of conformity to the Kozak consensus sequence (26). Compared with the LBP-1c cDNA, the LBP-1d cDNA lacks the first 60 N-terminal amino acid residues. To create a putative full-length LBP-1d cDNA, the missing 5′ end was substituted with corresponding sequences from the LBP-1c cDNA. The putative full-length LBP-1d cDNA has an open reading frame of 451 amino acids.

LBP-1a and LBP-1c are 72% identical in overall amino acid sequence but share higher sequence identity (88%) in the N-terminal halves of the proteins than in the C-terminal halves (52%) (Fig. 2). Comparison of the amino acid sequence of LBP-1 with entries in available protein data bases showed that one isoform of LBP-1, LBP-1c, is identical to the recently cloned human transcription factor α-CP2 (30). The murine α-CP2 binds to the α-globin promoter (extending from −85 to −160) and stimulates transcription in vitro (25). Further sequence comparison revealed significant sequence similarity with the *Drosophila melanogaster* transcription factor E1f-1/NTF-1 (3, 8) in the more conserved N-terminal half of LBP-1. Interestingly, the region of homology in Elf-1/NTF-1 is respon-

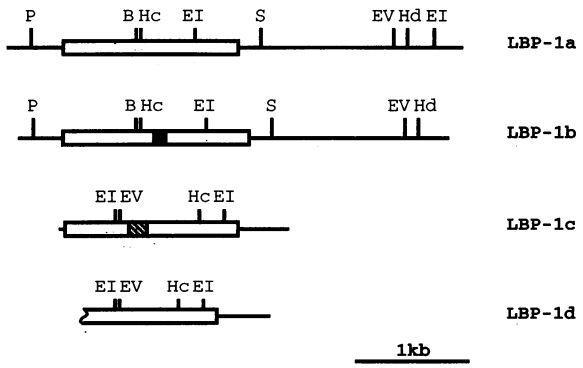

FIG. 1. Restriction enzyme map of LBP-1 cDNAs. The longest LBP-1 cDNAs in each group are shown. Translated regions and untranslated regions are indicated by open boxes and horizontal lines, respectively. Short vertical lines indicate the locations of restriction enzyme sites. Solid and striped boxes represent regions included because of differential splicing. Abbreviations for restriction enzyme sites: P, *Pst*I; B, *Bam*HI; Hc, *Hin*cII; EI, *Eco*RI; S, *Sal*I; EV, *Eco*RV; Hd, *Hin*dIII.

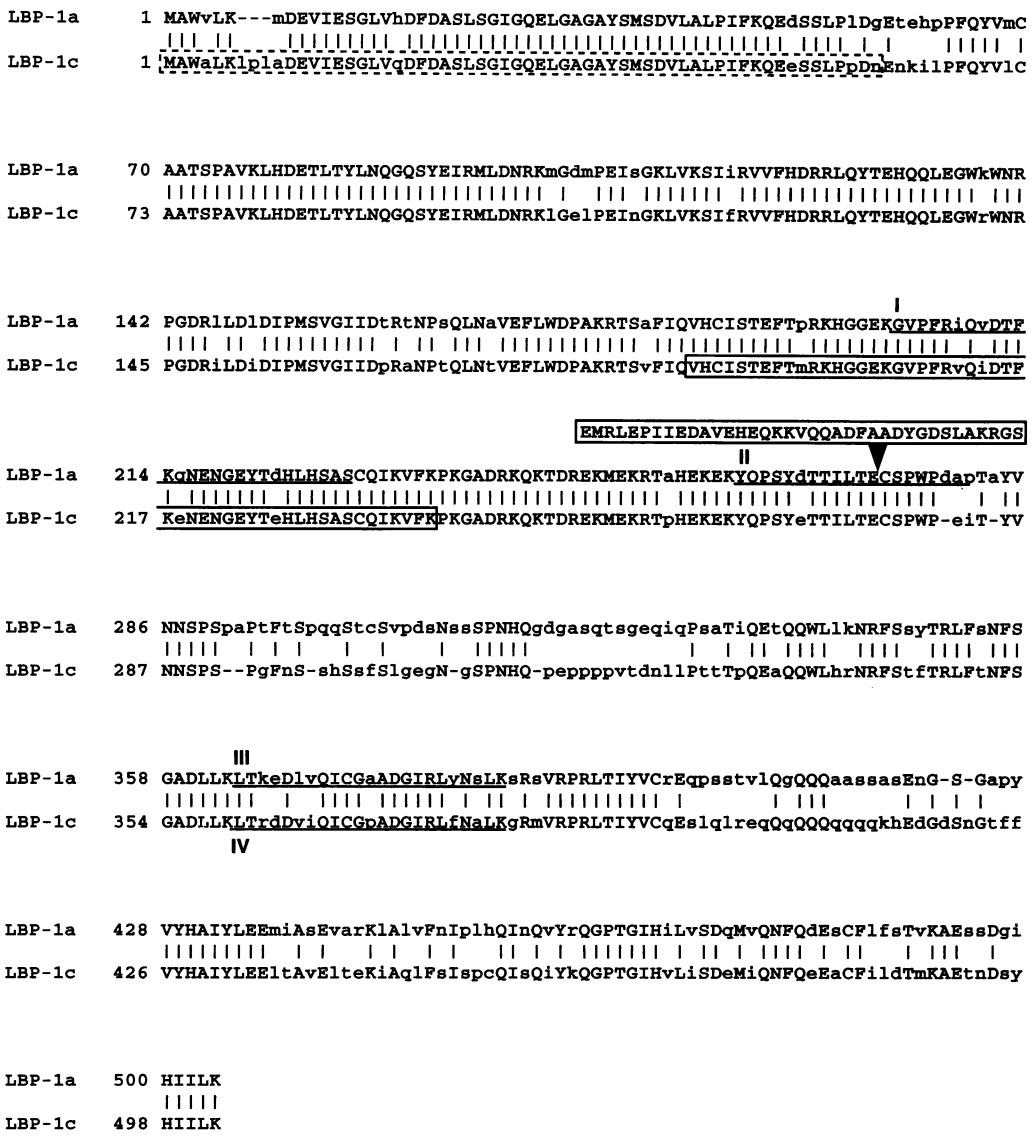

FIG. 2. Alignment of predicted amino acid sequence of LBP-1a and LBP-1c. Amino acids identical in LBP-1a and LBP-1c are denoted by capital letters and short vertical lines between the two sequences. N-terminal amino acids boxed with dashed lines are missing in the longest original LBP-1d, presumably because of partial cloning. The boxed amino acid sequence in LBP-1c is not present in LBP-1d, presumably as a result of alternative splicing. The boxed amino acid sequence above the line is present in LBP-1b at the position indicated by the arrowhead. Peptides derived from natural LBP-1 used for sequence analysis and cloning are underlined and indicated by roman numerals.

sible for DNA binding, implying that the region of homology in LBP-1 may be the DNA binding domain. However, this domain does not appear to be related to any previously defined DNA binding motifs, such as zinc finger, helix-loop-helix, leucine zipper, or homeobox domains (17).

Characterization of recombinant LBP-1. To determine whether these LBP-1 cDNAs were capable of encoding functional LBP-1, they were cloned downstream of the T7 RNA polymerase promoter, and in vitro-transcribed mRNAs were translated in a rabbit reticulocyte lysate system. LBP-1a, -b, -c, and -d cDNA constructs generated polypeptides with apparent molecular sizes of 63, 68, 64, and 58 kDa, respectively (Fig. 3A). The sizes of LBP-1a, -b, and -c are comparable to those of affinity-purified LBP-1. The translated proteins were tested in EMSA for their ability to bind to the HIV-1 promoter in a sequence-specific fashion. LBP-1a, -b, and -c, but not LBP-1d, formed DNA-protein complexes which could be competed for by wild-type but not mutant oligonucleotides (Fig. 3B). The absence of DNA binding by LBP-1d is consistent with the fact that this cDNA has a deletion, generated by alternative splicing, in the region homologous to the presumed DNA-binding domain of E1f-1/NTF-1.

To establish a relationship between native LBP-1 and the recombinant proteins, rabbit antiserum was prepared against the well-conserved N-terminal half of LBP-1a. This anti-LBP-1a antiserum recognized all four different forms of recombinant LBP-1 in Western blot (immunoblot) experiments (data not shown), as well as the natural LBP-1 species which form the DNA-protein complexes in EMSA (Fig. 4). When the immune serum was incubated with HeLa nuclear extract prior

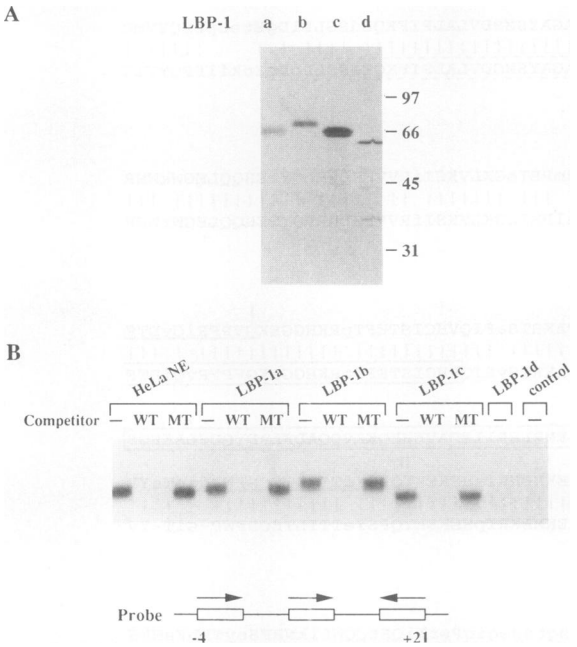

FIG. 3. Recombinant LBP-1 binds to DNA in a sequence-specific fashion. (A) SDS-PAGE of in vitro-translated LBP-1. cDNA clones encoding different forms of LBP-1 were transcribed with T7 RNA polymerase, and the RNA was subjected to in vitro translation with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Labeled LBP-1 analyzed on an SDS–10% polyacrylamide gel is indicated above each lane. (B) Mobility shift assay using in vitro-translated LBP-1. EMSA was performed with 2 μg of HeLa nuclear extract (NE) or 1 μl of in vitro translation mixture. LBP-1 and cold competitors (50-fold molar excess over probe) added to the reaction mixture are given above each lane. Control indicates that no RNA was added to the reticulocyte lysate. WT and MT indicate competitor oligonucleotides with the wild-type and mutant (IS4 in reference 22) LBP-1 sites, respectively. The DNA probe contains the sequence from −17 to +27 of HIV-1.

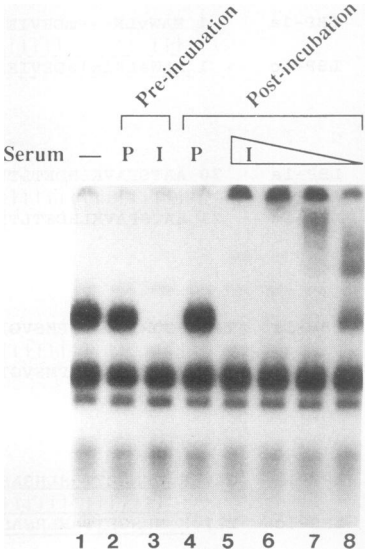

FIG. 4. Antibody to recombinant LBP-1a interacts with natural LBP-1. EMSA was performed with 5 μg of HeLa nuclear extract. Sera (1 μl) were incubated with HeLa nuclear extract before (lanes 2 and 3) or after (lanes 4 to 8) the addition of DNA probe. P and I indicate preimmune and immune sera, respectively.

to the addition of DNA probe, the formation of specific DNA-protein complex was completely abolished (Fig. 4, lanes 2 and 3). When the serum was added to the reaction after complex formation, the immune serum generated antibody-dependent supershifts which were not seen with use of preimmune serum (lanes 4 to 8). These results show that cloned proteins are antigenically related to the native LBP-1 in HeLa cells and that the serum can be used in functional studies.

To test whether recombinant LBP-1 could functionally reproduce the previously observed repression of HIV-1 transcription initiation, full-length LBP-1 was expressed in *E. coli* as a histidine-tagged fusion protein and purified by nickel chelate chromatography (14). The specific activity of recombinant LBP-1 was roughly comparable to that of native HeLa LBP-1, as measured by EMSA. In vitro transcription experiments using HeLa nuclear extracts demonstrated that increasing amounts of recombinant LBP-1a, -b, or -c reduced transcription from the HIV-1 promoter up to 100-fold (Fig. 5). However, no repression was observed with either a mutant HIV-1 template lacking LBP-1 binding sites or with an adenovirus major late promoter lacking LBP-1 sites (Fig. 5). LBP-1d did not repress HIV-1 transcription, suggesting that binding to DNA was required for the observed repression (data not shown).

LBP-1 as a transcriptional activator. LBP-1 binding sites are found both in cellular promoters such as α-globin (30), c-*myc* P2 (18), and DNA polymerase β (49) promoters and in viral promoters, including both SV40 (16, 51) and HIV-1 promoters. Some of the sites are known to be important for the transcriptional activation of the promoters (16, 30, 49). Consistent with the notion that it may act as an activator, LBP-1 contains proline-, glutamine-, and serine/threonine-rich domains characteristic of the activation domains of a number of other transcriptional activators (35). To test directly whether LBP-1 could activate transcription, we constructed reporter genes containing four tandem copies of either wild-type or mutant LBP-1 sites (from the HIV-1 promoter) upstream of the E1b TATA element. LBP-1 cDNAs were inserted into a eukaryotic expression vector, pCX (12), so that the expression of exogenous LBP-1 was driven by the strong enhancer/promoter of cytomegalovirus. Cotransfections into HeLa cells showed that pCX-LBP-1a stimulated CAT activity 30-fold from the promoter containing wild-type LBP-1 sites but not at all from the promoter containing mutant sites (Fig. 6). Similar results were obtained with pCX-LBP-1b and -c (data not shown). Interestingly, pCX-LBP-1d did not affect CAT activity in this assay (Fig. 6), probably because of the intrinsic inability of LBP-1d to bind DNA.

Since the in vivo transfection assays indicated that LBP-1 may work as an activator, depending on the context of the target promoter, we next tested the effect of LBP-1 on transcription from a synthetic promoter, using in vitro transcription assays. The synthetic template pL4MLI contains four copies of an SV40 late promoter LBP-1 site placed just upstream (−45) of the adenovirus major late TATA element. For these analyses, target promoters containing LBP-1 sites were transcribed in vitro, using either control or LBP-1-immunodepleted HeLa nuclear extracts. Depletion of LBP-1 was confirmed by EMSA (Fig. 7A) and Western blot analysis (data not shown). In control HeLa nuclear extract, transcription from pL4MLI was reproducibly fivefold higher than that from the control template (pMLI) lacking LBP-1 sites (Fig. 7B). Upon depletion of LBP-1, transcription from pL4MLI

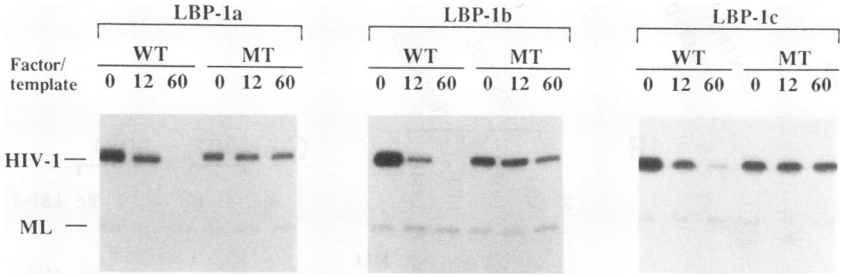

FIG. 5. Recombinant LBP-1 represses HIV-1 transcription in vitro. Transcription reactions were performed as described in Materials and Methods with 600 ng of poly(dI-dC), 30 ng of the HIV plasmid (wild type [WT] or mutant [MT]), and 10 ng of the adenovirus major late plasmid (pML). Recombinant LBP-1 to DNA template molar ratios are given above the lanes. RNA synthesized in vitro was analyzed by the primer extension method. The positions of the HIV-1 and pML (internal control) cDNA products are indicated at the left.

was decreased to the level observed with pMLI, which was not affected by depletion, suggesting that LBP-1 functioned as an activator in a sequence-specific fashion in this context. To test directly whether LBP-1 functions as an activator in vitro, affinity-purified HeLa LBP-1 or recombinant LBP-1 was added to the transcription reactions with depleted HeLa nuclear extracts. Recombinant LBP-1c activated transcription from the pL4MLI but not from the pMLI template and to an extent similar to native LBP-1 (three- to fivefold) (Fig. 7C, lanes 5 and 6). Recombinant LBP-1a and LBP-1b also activated transcription to a similar level (data not shown). These results showed that LBP-1 could function as an activator, in a context-dependent manner, both in vivo and in vitro.

Binding characteristics of LBP-1. Since native LBP-1 consists of several polypeptides with variable activities, it was important to characterize the sequence requirements for LBP-1 binding, using each recombinant LBP-1. On the basis of previous reports (19, 32) which suggested the importance of three (A/T)CTGG repeats for LBP-1 binding, we prepared a series of double-stranded oligonucleotides (Fig. 8A) and tested them for the ability to compete for LBP-1 binding (Fig. 8B). Although oligonucleotides containing the three repeats had a slightly higher affinity for LBP-1 (data not shown), an oligonucleotide (WT [wild type]) with only two direct repeats (−6 to +15 of HIV-1) was sufficient for LBP-1 binding (Fig. 8B, lane 1). LBP-1 was also capable of binding to an oligonucleotide (+5 to +26 of HIV-1) containing 5-bp elements as inverted repeats (data not shown), although with fivefold lower affinity. However, oligonucleotides (MT-1 and MT-2) with mutations (4-bp transversions) in either of the 5-bp repeats did not compete for LBP-1 binding, showing that these repeats are critical for DNA binding (Fig. 8B, lanes 4 to 7). Interestingly, oligonucleotides with mutations (5-bp transversions for MT-3 or 5-bp transitions for MT-4) in the sequence between the two direct repeats competed even better than the oligonucleotide with the wild-type sequence (Fig. 8B, lanes 8 to 11). However, an oligonucleotide (MT-5) with a 3-bp insertion between two repeats did not compete for LBP-1 binding (Fig. 8B, lanes 12 and 13), suggesting that the spacing between the 5-bp repeats is important for binding.

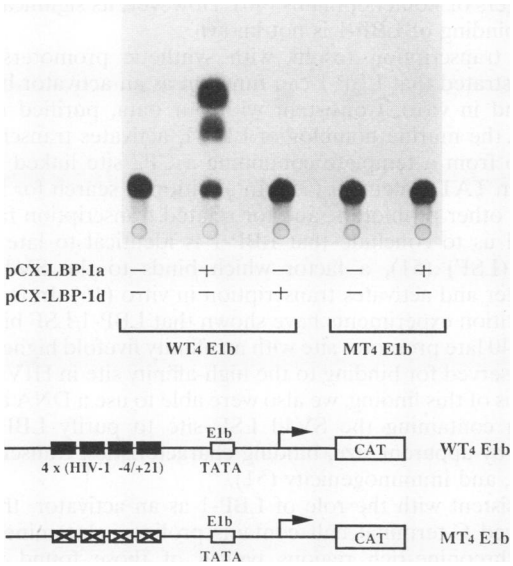

FIG. 6. Ectopic LBP-1 activates a reporter gene in HeLa cells via LBP-1 sites. The reporter templates contain four tandem copies of either the wild-type (WT4E1b) or the mutant (MT4E1b) LBP-1 site upstream of the E1b TATA box linked to the CAT gene as shown in the schematic diagram. The reporter genes were cotransfected into HeLa cells with a control plasmid (pCX) or an LBP-1-producing plasmid (pCX-LBP-1) by a calcium coprecipitation method. Extracts of HeLa cells were prepared at 48 h after transfection and assayed for CAT expression. Typical results are shown.

The requirement for two repeats raised the possibility that LBP-1 binds as a multimer. In such a case, mixing or cotranslation of two forms of LBP-1 should result in the formation of a complex of intermediate mobility. To test this, different forms (a to c) of in vitro-translated LBP-1 were mixed in all possible combinations. Indeed, a/b, b/c, and a/c LBP-1 combinations resulted in complexes migrating between the complexes formed with each protein alone (Fig. 8C), suggesting the formation of heteromers.

Since LBP-1a, -b, and -c can form heteromeric complexes on DNA, we next tested the effect of LBP-1d on the formation of these complexes. As shown in Fig. 8D, increasing amounts of LBP-1d (e.g., 10-fold molar excess over LBP-1a in lane 3) inhibited the binding of LBP-1a to the recognition site. However, an apparent nonspecific complex was unaffected by addition of LBP-1d. LBP-1d also inhibited LBP-1b and -c binding to DNA (data not shown). These results suggest that although LBP-1d may not contain the functional DNA binding domain, it may still interact with other species of LBP-1 to form heteromeric complexes, which in turn cannot bind DNA.

DISCUSSION

LBP-1 was first identified as a sequence-specific DNA-binding factor (11, 19) and subsequently shown to have appar-

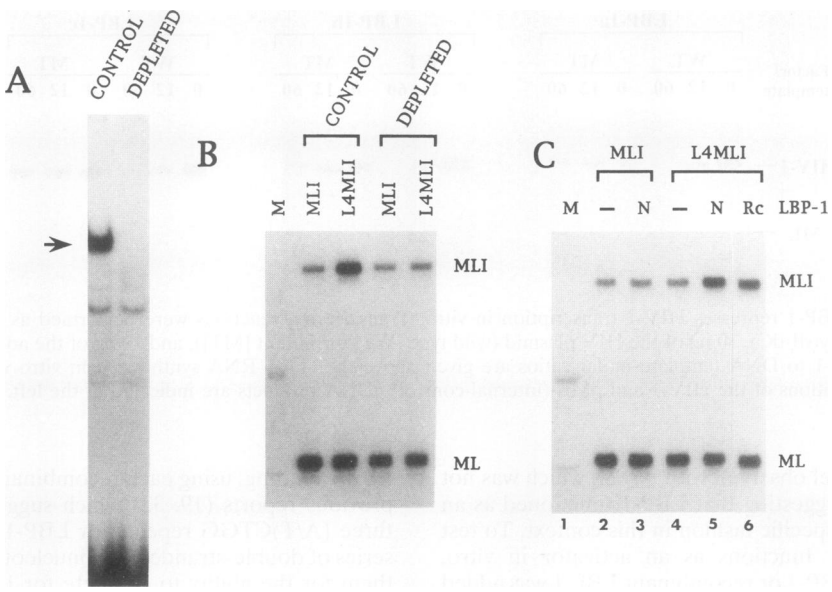

FIG. 7. LBP-1 activates transcription from a synthetic promoter in vitro. (A) Immunodepletion of LBP-1 from HeLa nuclear extract. EMSA was performed with either control or LBP-1-immunodepleted HeLa nuclear extract. The DNA–LBP-1 complex is indicated by the arrow. (B) Primer extension analysis of transcripts from synthetic promoters in either control or LBP-1-depleted HeLa nuclear extract. Transcription reactions were performed as described in Materials and Methods with 30 ng of the test plasmid (pMLI or pL4MLI) and 30 ng of the control plasmid (pML). RNA synthesized in vitro was analyzed by the primer extension method. The templates containing either no site (pMLI) or four tandem copies of SV40-derived LBP-1 site (pL4MLI) are indicated above each lane along with HeLa nuclear extract used in the reaction. The positions of the pMLI and pML (internal control) cDNA products are indicated at the right. Lane M, size marker. (C) Primer extension analysis of transcripts from synthetic promoters in LBP-1-depleted HeLa nuclear extract. Transcription reactions were performed as described in Materials and Methods with 30 ng of the test plasmid (pMLI or pL4MLI) and 30 ng of the control plasmid (pML). A saturating amount of native or recombinant LBP-1 (12-fold molar excess over the DNA template) was added to the reactions as indicated. The templates and factors included in the reaction mixture are indicated above each lane. N and Rc represent native LBP-1 purified from HeLa cells and recombinant LBP-1c, respectively.

ently diverse effects on HIV-1 transcription, depending on the particular site occupied (19, 22). To further characterize these phenomena, we have cloned a family of related members of an LBP-1 gene family.

Cloning of a family of LBP-1 cDNAs. We and others have noted that DNA affinity-purified LBP-1 contains multiple polypeptide species upon SDS-PAGE analyses (22, 25, 50). Our isolation of multiple related LBP-1 cDNAs explains the apparent complexity of purified LBP-1 polypeptides. Thus, in vitro-translated LBP-1 isoforms (a, b, and c) match well in apparent sizes with the three major polypeptides of purified LBP-1. Our effort to show the presence of the non-DNA-binding LBP-1d form in crude HeLa nuclear extract by using Western blot analysis was hampered by the presence of nonspecific background bands. However, the isolation of two independent LBP-1d cDNA clones argues that LBP-1d is an authentic splicing product and the least abundant (2 of 19 clones) of the four LBP-1 isoforms.

Previous studies of structure-function relationships in transcription factors have revealed structural motifs (17, 35) important for DNA binding, transcriptional activation, and other functionally important (e.g., dimerization) domains. Although LBP-1 does not contain any of the well-established DNA binding domains, it is tempting to speculate that the more conserved N-terminal half of LBP-1 might serve as a DNA binding domain, since it shows significant homology with the DNA-binding protein Elf-1/NTF-1 and contains a conserved basic region. Our finding that the non-DNA-binding isoform LBP-1d has a deletion in the region homologous to Elf-1/NTF-1 is consistent with this notion. In addition, LBP-1 contains several copies of the sequence SPXX, which is proposed to contribute to DNA binding when located next to Zn fingers or homeodomains (46). However, its significance in DNA binding of LBP-1 is not known.

Our transcription results with synthetic promoters have demonstrated that LBP-1 can function as an activator both in vivo and in vitro. Consistent with our data, purified mouse α-CP2, the murine homolog of LBP-1, activates transcription in vitro from a template containing a CP2 site linked to the α-globin TATA element (25). In addition, a search for LBP-1 sites in other promoters, and for related transcription factors, has led us to conclude that LBP-1 is identical to late SV40 factor (LSF) (51), a factor which binds to the SV40 late promoter and activates transcription in vitro (16). In fact, our competition experiments have shown that LBP-1/LSF binds to the SV40 late promoter site with an affinity fivefold higher than that observed for binding to the high-affinity site in HIV-1. On the basis of this finding, we also were able to use a DNA affinity column containing the SV40 LSF site to purify LBP-1, as judged by apparent size, binding characteristics, transcription activity, and immunogenicity (51).

Consistent with the role of LBP-1 as an activator, the less conserved C-terminal half contains proline-, glutamine-, and serine/threonine-rich regions typical of those found within activation domains of a number of other transcription factors (35). Interestingly, the exact locations of these domains vary in the two different gene products of LBP-1, which may have important implications for isoform-specific transcriptional regulation and raises the possibility of specific interactions with other cellular factors. LBP-1c and -d each contain a stretch of 10 glutamines. Glutamine tracts are found in transcription factors such as TATA-binding protein (15, 20, 38), glucocor-

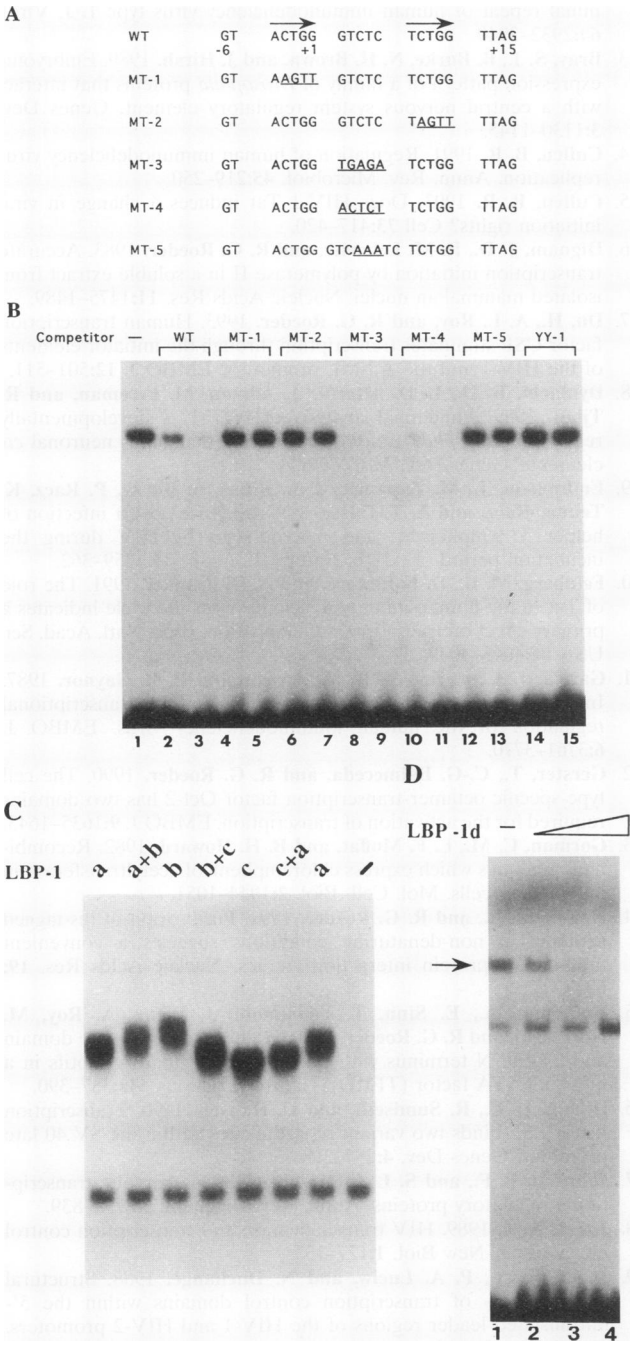

FIG. 8. LBP-1 requires two repeats for binding and binds as a multimer. (A) Wild-type and mutant oligonucleotides used in the competition experiments in panel B. The wild-type (WT) oligonucleotide is derived from nt −6 to +15 relative to the HIV-1 transcription start site. Arrows represent direct repeats. Mutations are underlined. (B) Competition analysis using wild-type and mutant oligonucleotides. Gel shift assays were performed with 1 ng of bacterially expressed LBP-1a and 20 fmol of radiolabeled wild-type oligonucleotide. Unlabeled competitor oligonucleotide indicated above each lane was used at a 10-fold (lanes 2, 4, 6, 8, 10, 12, and 14) or 50-fold (lanes 3, 5, 7, 9, 11, 13, and 15) molar excess over the probe. Nonspecific oligonucleotide YY-1 is derived from the adeno-associated virus P5 promoter (nt −10 to +13). (C) Gel shift assay using combinations of in vitro-translated LBP-1. Different forms of LBP-1 were expressed in reticulocyte lysates and mixed before the assay was performed. The DNA probe contains the sequence from −6 to +15 of HIV-1. LBP-1 (1 μl of lysate containing approximately 1 ng of LBP-1 protein) added to the reaction mixture is indicated above each lane. (D) Gel shift assay using LBP-1a and excess amounts of LBP-1d. EMSA was performed as described in Materials and Methods with 1 μl of reticulocyte lysate containing approximately 1 ng of in vitro-translated LBP-1a protein. Bacterially expressed LBP-1d was added to the reactions in lane 2 (1 ng), lane 3 (10 ng), and lane 4 (100 ng). Final salt concentrations of all the reactions were adjusted to approximately 75 mM KCl.

ticoid receptor (34), and many *Drosophila* homeotic gene products (39, 41). However, the significance of the glutamine stretch is unknown.

Our study of LBP-1 binding indicates that LBP-1 recognizes two half-sites and binds DNA as a multimer, possibly resulting from either multimerization of LBP-1 in solution or highly cooperative DNA binding. Although excess LBP-1d blocks DNA binding of LBP-1a, which may suggest multimer formation in solution, we do not know the efficiency of the formation of LBP-1 multimer in solution.

Function of LBP-1 in HIV-1 transcription. Using recombinant LBP-1, we have confirmed our previous results that LBP-1 represses HIV-1 transcription in vitro at high concentrations. Since our present results have also demonstrated that LBP-1 can either activate or repress transcription, depending on the promoter context, we have tested the effect of LBP-1 on HIV-1 transcription in transient cotransfection assays using reporter constructs containing the HIV-1 promoter fused to the CAT gene (51). However, our preliminary study failed to show any effect of ectopic LBP-1 on CAT expression from the HIV-1 promoter in these assays. The fact that we do not detect HIV-1 repression by LBP-1 through the low-affinity site (see the introduction) could be due to the high copy number of the reporter template introduced during transient transfection.

In vivo, the HIV-1 LTR directs the synthesis of two kinds of transcripts (10, 21, 28, 40): short transcripts (extending from nt +1 to about +60) that account for the majority of HIV-1 transcripts in the absence of Tat and either decrease (21, 40) or remain unchanged (28) in abundance in response to Tat, depending on the experimental system used, and long transcripts whose absolute abundance increases greatly in the presence of Tat (10, 21, 28, 40). Other studies (40, 44) have identified a *cis*-acting DNA element (IST) that specifies nonprocessive transcription of short transcripts (which may be stable derivatives of somewhat longer terminated RNA molecules). This element has been mapped to a region between −5 and +26 in the HIV-1 LTR (44) and appears to overlap perfectly the DNA binding sites of LBP-1. When linked downstream of heterologous promoters, the IST element increases total transcription activity of the promoters and produces both short and full-length transcripts (40). Although ectopic LBP-1 did not alter the level of long CAT-encoding transcripts in our assays, we do not know whether LBP-1 affected the expression of short transcripts. In view of the present observation that LBP-1 functions as an activator in a promoter context-dependent manner, and our recent finding (37) that LBP-1 antagonizes elongation of HIV-1 transcription in a promoter-specific fashion, it seems an intriguing possibility that LBP-1 is at least in part involved in the function of the IST element. Experiments to test this possibility are under way.

Previous transient transfection experiments with an HIV-1 template mutated in the high-affinity (downstream) LBP-1 sites (32) or IST element (40) suggested only a minimal effect of LBP-1 on either basal (long transcripts) or Tat-activated levels of HIV-1 transcription. However, for the reasons mentioned above and since transiently transfected templates sup-

port a basal level of HIV-1 transcription even in the absence of Tat, which may well be enough to generate TAR element-containing long transcripts (5), previous studies do not eliminate a possible involvement of LBP-1 on either basal or Tat-activated transcription of HIV-1. Moreover, we have found (37) that under modified conditions, LBP-1 can still bind specifically both to the upstream LBP-1 sites and to the high-affinity sites bearing the previously tested mutations (32, 40).

We have also tested the effect of LBP-1 immunodepletion on HIV-1 transcription in nuclear extracts. As shown in Fig. 7, in vitro transcription from an artificial promoter with high-affinity LBP-1 sites was reduced either by deletion of the LBP-1 site or by immunodepletion of LBP-1. However, our preliminary data have failed to show any effect of LBP-1 immunodepletion on in vitro transcription from the HIV-1 promoter (51). These results suggest that the function of LBP-1 is highly context dependent and, further, raise the possibility that another transcriptional activator functions through the same or an overlapping binding site.

It is worth noting that whereas both short and long transcripts are generated from the HIV-1 promoter in vivo, short transcripts are not readily detected in the standard in vitro transcription system (47). Among possible reasons for the absence of short transcripts is that nuclear extracts prepared by standard techniques contain unusually high antiterminator activities and/or activator functions that elicit the formation of much more processive elongation complexes than normally assemble on the HIV-1 promoter in vivo (31, 47). Thus, if LBP-1 is involved in formation of short transcripts by both activating transcription initiation and antagonizing elongation from the HIV-1 promoter, it may be difficult to detect this activity of LBP-1 in a standard in vitro transcription assay using crude nuclear extracts.

In summary, our results support the intriguing possibility that LBP-1 has a dual function in either activation or inhibition of HIV-1 transcription, depending on the cellular state and relative abundance of various LBP-1 species (possibly including modified forms). Related, LBP-1 (CP-2) DNA binding activity is modulated during erythroid cell differentiation (1). Moreover, the additional possibility of LBP-1 modifications by herpes simplex virus and cytomegalovirus (2, 48) suggests that overall LBP-1 activity may be governed by the cell in a complex manner. Finally, our recent finding that LBP-1 can inhibit elongation as well as initiation of HIV-1 transcription (37) argues that regulation of HIV-1 transcription by LBP-1 may be an important mechanism of HIV-1 regulation. The availability of recombinant LBP-1 and its antibodies will allow us to further investigate at a molecular level the role of LBP-1 in transcription of HIV-1 and other viral and cellular promoters.

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