Structural arrangements of transcription control domains within the 5'-untranslated leader regions of the HIV-1 and HIV-2 promoters

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Promoter-proximal downstream regions of the human immunodeficiency viruses (HIV-1 and HIV-2) mediate the action of the viral transcription activator protein, Tat. We demonstrate here that the downstream domain of each virus interacts with two RNA polymerase II transcription factors. One of these, CTF/NF I, is a multifunctional protein associated previously with activation of transcription and DNA replication. The other cellular protein, designated LBP-1 (leader-binding protein-1), recognizes repeated elements within an extended region of DNA corresponding to part of the 5'-untranslated leader. Analysis of clustered point mutants in the HIV-1 leader for DNA-binding and transcription activity in vitro and in vivo suggests a role for LBP-1 as part of the basal promoter. A complex overlapping arrangement is observed between sequences required for the interaction of LBP-1 and CTF/NF I proteins and those defined previously for regulation by the HIV-1 Tat protein.

Recent studies suggest an increasing awareness that diverse classes of regulatory elements may be located within the 5' untranslated leader regions of viral and cellular genes. Although some of these contribute to translation efficiency or RNA stability (Geballe et al. 1986; Rao et al. 1988), other downstream control sequences have been found to enhance general or cell-specific transcription efficiency (Mansour et al. 1985; Hultmark et al. 1986; Stenlund et al. 1987; Theill et al. 1987). Regulation of cellular genes such as c-*myc* apparently requires both downstream promoter elements as well as distinct intron sequences through which transcription antitermination or attenuation mechanisms operate (Bentley and Groudine 1986; Nepveu and Marcu 1986; Yang et al. 1986). The variety of regulatory consequences that may be exerted through these DNA segments, or their corresponding RNA transcripts, suggests a precise control on the design of downstream modulators of gene expression.

The promoter-proximal downstream sequences of the human immunodeficiency viruses HIV-1 and HIV-2 have been shown to communicate the response to a unique viral regulatory protein (Rosen et al. 1985a; Emerman et al. 1987; Guyader et al. 1987), designated Tat (Arya et al. 1985; Sodroski et al. 1985). Transcriptional activation of the HIV-1 promoter by Tat can be readily detected with transient expression experiments

in either lymphoid or nonlymphoid cell lines (Cullen 1986; Peterlin et al. 1986; Wright et al. 1986; Muesing et al. 1987; Rice and Mathews 1988). Evidence implicating the Tat protein in post-transcriptional regulation of HIV-1 gene expression has also been presented (Feinberg et al. 1986; Rosen et al. 1986). Recent data from nuclear run-on experiments suggest that induction is not due to an increased frequency of transcription initiation but, rather, involves events that overcome a block to elongation by RNA polymerase II (Kao et al. 1987). Thus, Tat may act as a sequence-dependent antiterminator of transcription. These results are consistent with observations that the Tat responsive element (TAR) of the HIV-1 promoter functions in an orientation- and position-dependent manner (Rosen et al. 1985a; Peterlin et al. 1986; Muesing et al. 1987), similar to that described for other eukaryotic terminator DNA segments (Zaret and Sherman 1984; Falck-Pedersen et al. 1985; Sato et al. 1986).

Several studies have shown that recombinant plasmids containing the complete HIV-1 leader fused to heterologous promoters retain only fractional inducibility to Tat (Rosen et al. 1985a; Cullen 1986; Peterlin et al. 1986), suggesting a connection between the function of the upstream and downstream regulatory domains of the HIV-1 promoter. The upstream sequences of HIV-1 contain an inducible enhancer that can be activated by phorbol ester tumor promoters in a variety of cell lines (Dinter et al. 1987; Kaufman et al. 1987; Nabel and Bal-

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timore 1987; Tong-Starksen et al. 1987). This enhancer domain overlaps partially with a promoter that contains multiple binding sites for the transcription factor Sp1 (Jones et al. 1986) and at least one other binding site for a distinct positive-acting transcription factor (Dinter et al. 1987; Garcia et al. 1987; Wu et al. 1988; H. Dinter and K. Jones, unpubl.). The TATA homology and associated downstream sequences of HIV-1 also interact with DNA-binding proteins present in crude nuclear extracts, and extensive interactions among proteins bound to various upstream and downstream domains of the promoter have been proposed (Garcia et al. 1987).

To define the downstream regulatory domain in greater detail, we have constructed and analyzed a series of clustered base substitution mutants within the HIV-1 leader region. In vitro transcription and DNA-binding experiments using these mutants identified downstream elements that specify the interaction of two cellular RNA polymerase II transcription factors. One of these proteins, CTF/NF I, has been purified previously and shown to recognize upstream CCAAT elements present in numerous promoters, including those of the human α- and β-globin genes, and the herpesvirus thymidine kinase gene (Jones et al. 1985, 1987). The second factor, designated LBP-1 (for leader-binding protein-1), recognizes reiterated sequence motifs present over a relatively large region of DNA which contribute to basal promoter function in vivo, in either the presence or absence of Tat. The combinatorial association of CTF/NF I and LBP-1 transcription factors was maintained within the downstream region of the distantly related immunodeficiency virus, HIV-2. Comparison of the relative ability of the HIV-1 promoter mutants to respond to Tat activation in vivo (Tong-Starksen et al. 1987; M.J. Selby, P.A. Luciw, and B.M. Peterlin, unpubl.) revealed that sequences required for Tat induction are not limited to those that specify individual protein binding sites. Thus the interaction of these DNA-binding proteins with the downstream region is not sufficient to transmit the effects of Tat to the promoter.

Results

The promoter-proximal downstream region of the HIV-1 promoter is active in vitro

The original design of these experiments was to dissect the downstream boundaries required for basal and regulated HIV-1 promoter activity. A set of clustered point mutations (cpm) that span the RNA initiation site and immediate downstream region was generated by oligodeoxynucleotide replacement mutagenesis using vectors that contain the entire upstream U3 segment and downstream sequences to position +80 (Tong-Starksen et al. 1987). As diagramed in Figure 1, sequences of 3–6 bp were altered in each mutant to cover the region systematically from −17 to +52 of the HIV-1 promoter. Other mutants used in these studies include a TATA substitution mutant, a triple mutant that contains double transversions at three discrete locations in the leader, and three substitution-insertion mutants (BS.ins.) that alter

both the sequence and the spacing between the BglII (+19) and SacI (+38) sites (as described in Materials and methods).

To determine whether any of these sequence alterations influenced the basal HIV-1 promoter, we analyzed the relative transcription efficiency of each mutant using an in vitro reconstituted system derived from human (HeLa) cells. An example of the transcription pattern observed in vitro for the entire series of mutants is shown in the bottom panel of Figure 1. These experiments were carried out using whole-cell extracts of uninfected HeLa cells, which were fractionated to enrich for endogenous RNA polymerase II activity and other general transcription factor activities (Dynan and Tjian 1983). Each reaction was supplemented with affinity-purified fractions of the Sp1 transcription factor, previously shown to be required for HIV-1 promoter function (Jones et al. 1986), and with a heparin–agarose column fraction from HeLa nuclear extracts, h.25, which was determined experimentally to increase overall levels of HIV-1 transcription in this system (data not shown). RNA synthesized in vitro from wild-type or mutant DNA plasmids was analyzed in four independent experiments by primer-extension using a $5'$-^{32}P-labeled oligodeoxynucleotide complementary to position +55 to +70 on HIV-1 mRNA. To standardize the procedure, a separate plasmid containing the human α-globin gene was included in each transcription reaction and RNA transcripts from this control promoter were detected with a globin-specific DNA primer.

The effects on HIV-1 transcription observed with these mutants (bottom panel of Fig. 1) suggests that two classes of basal transcription elements exist within a 25-bp region around the RNA start site. The first class, exemplified by mutants cpm −6/−1, cpm +1/+3, and cpm +1/+5, displayed a reproducible microheterogeneity in the RNA-initiation site. Multiple initiation sites were detected within a 10-bp range of the normal start site for each of these mutants (Fig. 1A). In addition, the overall amount of RNA for mutants cpm −6/−1 and cpm +1/+5 appeared to be reduced relative to the wild-type promoter, although this was difficult to quantitate due to the diversity of initiation sites. The apparent increase in transcriptional efficiency of mutant cpm +1/+3 was not reproduced in repeated experiments. Thus, sequences around the cap site appear to influence the exact number and location of RNA initiation events at this promoter. However, the HIV-1 TATA element (or another upstream promoter element) appears to play a dominant role in positioning the RNA, since insertion of a polylinker DNA fragment immediately downstream of the TATA element caused a concomitant upstream shift in the transcription start site (as indicated in Fig. 2 for mutant P.ins.; data not shown). Furthermore, direct mutation of the HIV-1 TATA element reduced transcription dramatically in vitro (Fig. 1B). Interestingly, the effect in vitro of the TATA mutation did not differ qualitatively from that created by the loss of other upstream promoter elements. We conclude that the HIV-1 TATA element is functional and acts in concert with sequences around

the RNA start site to position RNA initiation events properly.

A second type of transcriptional effect was observed with five substitution mutants: cpm +4/+8, cpm +4/+9, cpm +10/+13, cpm +14/+18, and the triple mutant (Fig. 1A, B). Three of these mutants (cpm +4/+8, cpm +14/+18, and the triple mutant) reproducibly displayed the lowest transcription efficiency of the group, which was approximately eightfold below the wild-type level. The other two mutants (cpm +4/+9
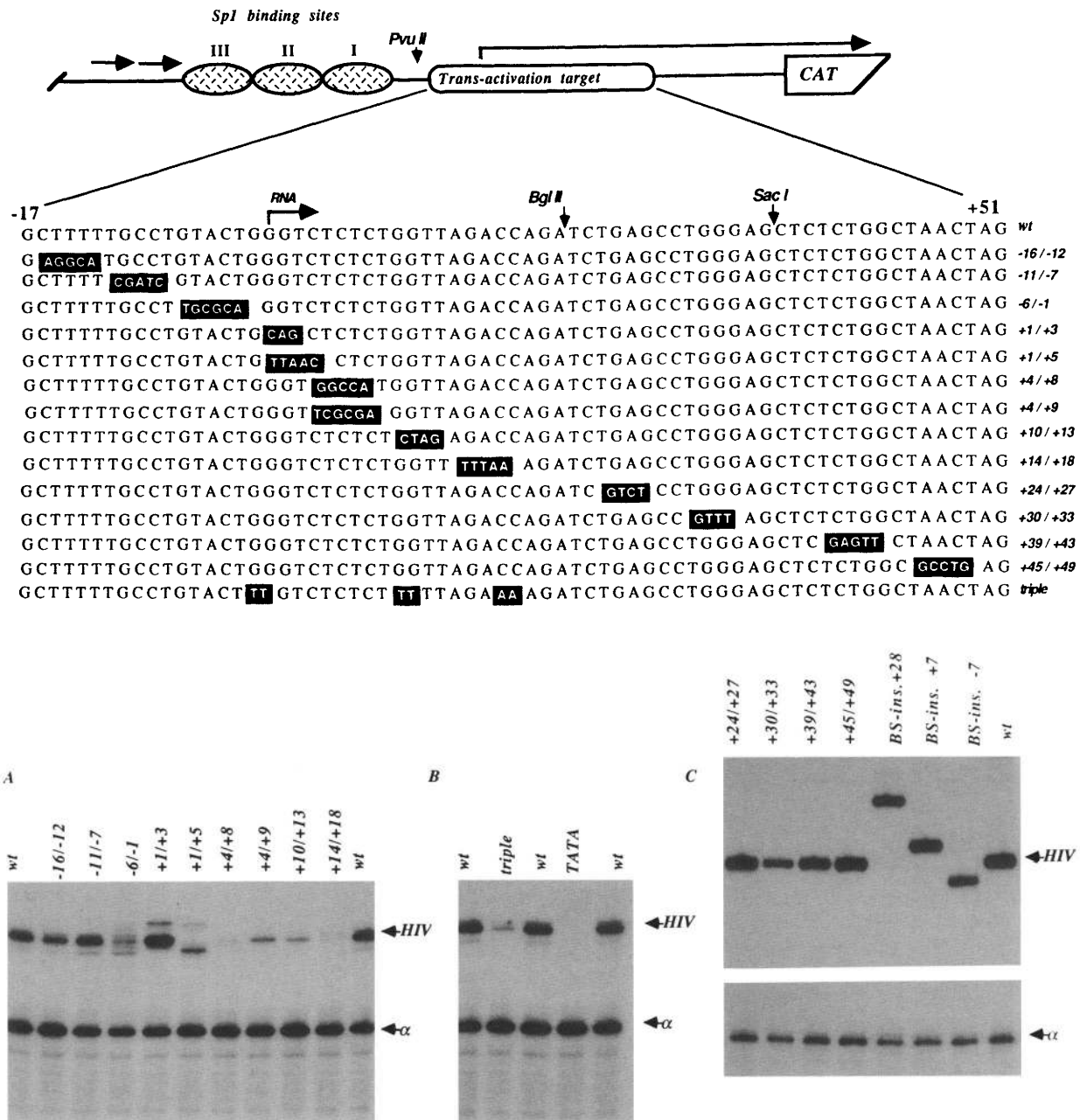

Figure 1. The HIV-1 downstream regulatory domain contributes to promoter efficiency in vitro. (Top) Clustered base-substitution mutants in the HIV-1 promoter. Listed below the wild-type promoter sequence (shown at the top) are 14 substitution mutants with sequence alterations indicated in black boxes. Each mutant was designed to generate a unique restriction enzyme site within the 5'-untranslated leader of HIV-1, and is designated by the position of the mutation relative to the RNA start site (+1). (Bottom) Effect of downstream sequence mutations on HIV-1 transcription in vitro. The transcription reactions each contained 80 ng of human α-globin DNA template as an internal control, and 160 ng of HIV-1 wild-type (wt) promoter or mutant promoters, as indicated above each lane in A–C. Transcription extracts (derived from uninfected HeLa cells) and conditions are as described in Materials and methods. HIV-1 RNA synthesized in vitro was detected by primer-extension using a primer that anneals at position +55 to +72, and globin RNA was detected with a separate primer. Arrows designate the positions expected for correctly initiated α-globin or HIV-1 RNA transcripts.

and cpm $+10/+13$) were each expressed at a fourfold reduced efficiency relative to the unaltered promoter. In contrast, substitution and insertion mutations downstream of position $+24$ did not reproducibly influence promoter activity under these extract conditions (Fig. 1C). Thus, sequences from $+4$ to $+18$ are required for optimal HIV-1 promoter activity in vitro.

Because this segment of the promoter lies within the transcribed region, and within an area of extensive RNA secondary structure (Muesing et al. 1987), it occurred to us that these mutations exerted their effect through destabilization of the RNA. To test directly for RNA stability differences, equivalent amounts of wild-type or triple mutant RNA transcripts were isolated and incubated in the transcription extract in the presence of α-amanitin (1 μg/ml) to prevent reinitiation of RNA synthesis by RNA polymerase II. No difference in the stability of each RNA was detected for incubation periods as long as 3 hr at 30°C (data not shown). Thus, an intact RNA structure appears not to be required for HIV-1 RNA stability in vitro, consistent with the observation that mutations downstream of position $+24$, but still within the putative RNA hairpin, were expressed as efficiently as the wild-type promoter in vitro (Fig. 1C). Moreover, we have not detected prematurely terminated HIV-1 transcripts in this transcription system, using either run-off or RNase-protection techniques, suggesting that the transcription phenotypes did not arise from differential termination of mutant RNA transcripts. Finally, the effects of the downstream mutations were negated at high DNA concentrations and were not detected at low extract concentrations (data not shown) in a manner that suggested the required interaction of a sequence-specific DNA-binding protein that activates transcription.

Two cellular DNA-binding factors recognize HIV-1 downstream sequences

To determine whether the dependence on sequences downstream of the RNA start site was due to cellular DNA-binding transcription factors, we analyzed the transcriptionally active extract supplement by means of DNase I footprint experiments. As shown in Figure 2A, the nuclear h.25 fraction generated two protein-binding sites within the immediate downstream region (Fig. 2A, lane 3). One footprint covered an extensive 62-bp region of DNA (-35 to $+27$; designated site I), and a weaker binding interaction (designated site II) was detected from approximately $+32$ to $+52$. For comparison, a nuclear h.35 column fraction was tested and found to contain equivalent levels of site II DNA-binding activity but lower activity for binding to site I (Fig. 2A, lane 2). All of the downstream elements required for HIV-1 promoter activity in vitro map within the site I binding domain.

Binding to site I sequences includes both the HIV-1 TATA element as well as adjacent downstream sequences, in a manner strikingly similar to that described by Sawadogo and Roeder (1985) for the interaction of the TATA-binding factor, TFIID, with the adenovirus major

late promoter. In this latter example, binding to adenovirus DNA was dependent primarily on the TATA sequence, even though the footprint extended weakly through approximately 30 bp of downstream sequences. To determine whether binding to site I was due to the interaction of TFIID, we analyzed the footprint protection pattern for mutant P.ins., which contains a DNA polylinker insertion that separates the HIV-1 TATA element from the remainder of the downstream region. As shown in Figure 2B, binding to site I on the mutant promoter was confined within a shorter (44 bp) segment from -17 to $+27$. The binding affinity to the shortened site I sequences was unchanged relative to that of the wild-type promoter, and no binding was apparent over the residual TATA element. Therefore, binding to most of site I is mediated by sequences downstream of the TATA element. Final confirmation that the protein bound to site I is distinct from factor TFIID was obtained by footprint analysis of the TATA substitution mutant, which bound the site I protein at least as well as the wild-type promoter (data not shown). Because the boundaries of site I protection on the TATA mutant (-35 to $+27$) were unaltered relative to the wild-type promoter, it appears that the relatively weak binding of the site I protein to the upstream region (-35 to -17) involves sequences distinct from the TATA box. Thus, the site I protein displays a specificity opposite of that observed for TFIID, in that binding depends on downstream sequences and was not directed by the TATA element. Because the TATA mutant was very poorly transcribed in vitro, we consider it likely that factor TFIID does recognize the TATA element during transcription. It is possible that the binding of TFIID was not of sufficient affinity to be detected under these conditions, or was obscured by the interaction of the site I protein within the upstream region. In subsequent sections of this paper we refer to the cellular protein that binds to site I as LBP-1 (for leader-binding protein-1).

LBP-1 recognizes a repeated element within the HIV-1 leader

We next examined the interaction of LBP-1 with HIV-1 mutant DNAs to define better the sequence requirements for binding and to test the correlation between binding and transcription activation in vitro. For the DNA-binding experiments described below, both crude and affinity-purified LBP-1 protein fractions (M. Schorpp, M. Waterman, and K. Jones, unpubl.) from HeLa nuclear extracts were tested, with equivalent results. An example of the binding to site I mutants obtained in DNase I footprint experiments with purified LBP-1 fractions is shown in Figure 3. Interestingly, none of the clustered point mutations was sufficient to eliminate binding of LBP-1 although two mutants did create small binding sites. The LBP-1 footprint for mutant cpm $+4/+8$ was shortened by 13 bp on the promoter-proximal side, whereas the binding site on mutant cpm $+14/+18$ was shortened by 10 bp on the promoter-distal side. These two mutants were transcribed with re-
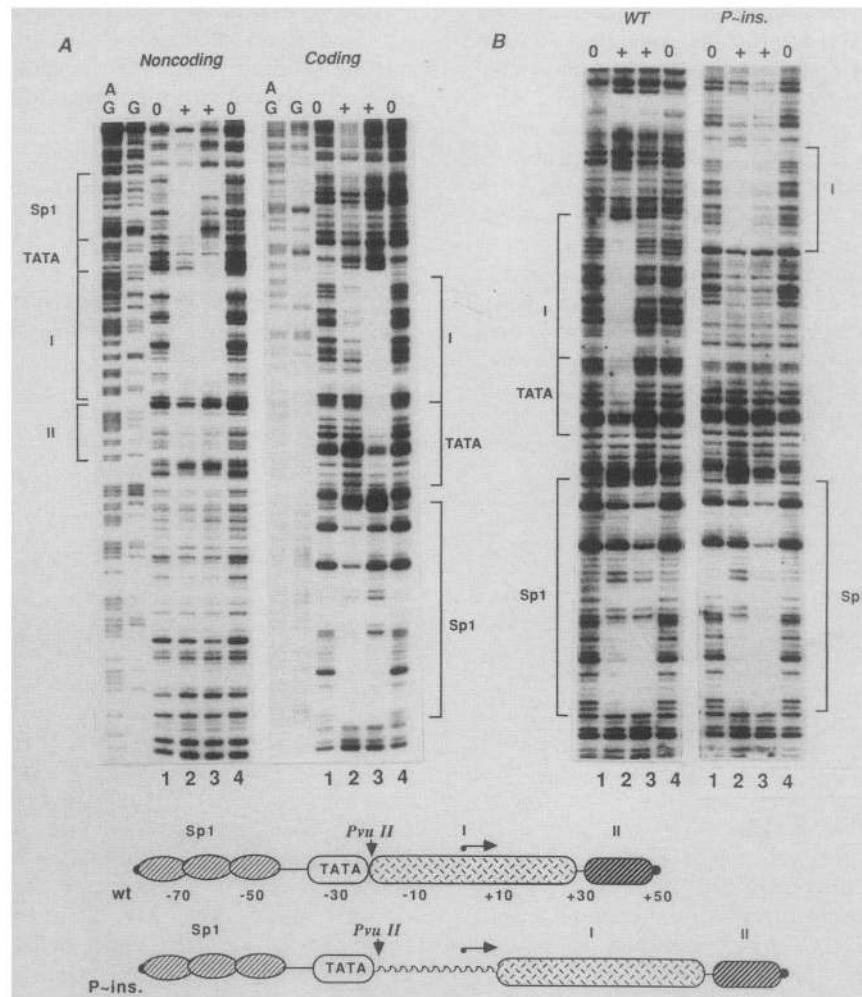
Figure 2. Two distinct proteins recognize HIV-1 promoter-proximal sequences. (A) Specific binding of cellular proteins to the HIV-1 downstream region. Binding was monitored with the DNase I footprint technique using templates 5'-end-labeled at the HindIII site (+80; noncoding strand) or the AvaI site (−160; coding strand). (Lanes 1,4) No protein added; (lane 2) 10 μg of h.35; (lane 3) 10 μg of h.25. A and G refer to sequence markers. (B) Protein recognition of HIV-1 promoter-proximal downstream sequences is not dependent on the adjacent TATA element. DNA templates for footprint analysis were derived from the wild-type (wt) promoter or a PvuII insertion mutant (P.ins.). Both DNA fragments were 5'-end-labeled on the coding strand (AvaI; −160). In each panel, lanes 1 and 4 show the digestion pattern in the absence of added protein; (lane 2) 10 μg h.25; (lane 3) 10 μg h.35. The heparin−agarose protein fractions used in A and B were derived from separate preparations. The RNA start site for mutant P.ins., indicated in the bottom drawing, was mapped in vitro by primer extension (data not shown.). For simplicity, the extension of the site I footprint into upstream sequences overlapping the TATA box (seen at high protein concentrations) is not indicated in the diagram.

duced efficiency in vitro (Fig. 1A). Somewhat surprisingly, the binding pattern on mutant cpm +4/+9, which substitutes a highly overlapping domain to mutant cpm +4/+8, did not involve a shortened binding site (Fig. 3). Instead, this mutant resembled its neighboring mutant, cpm +10/+13, in that binding over the entire site I region occurred with a reduced affinity. Both mutants cpm +4/+9 and cpm +10/+13 were transcribed at moderately reduced efficiencies in vitro (Fig. 1A). No dramatic differences were evident in the binding pattern or affinity for the remaining downstream mutants, including the mutants that generated multiple RNA-initiation sites. Thus, the ability of LBP-1 to bind DNA appears to correlate more closely with the tran-

scriptional effects observed in vitro for mutants cpm +4/+8 through cpm +14/+18 than for·mutants cpm −6/−1, cpm +1/+3, and cpm +1/+5. It is possible that the altered RNA start sites observed in vitro for the latter set of mutants are a consequence of sequence preferences for initiation or of the altered interaction of other transcription factors, like TFIID, that were not detected in these experiments. These data suggest that LBP-1 is a positive-acting transcription factor that resembles other RNA polymerase II activator proteins, with the exception that LBP-1 can activate transcription in vitro from locations downstream of the RNA-initiation site.

Further examination of DNA sequence requirements

for LBP-1 binding suggested the importance of a 5-bp motif, 5'-(T/A)CTGG-3' [or 5'-CCAG(A/T)-3'] present in three copies spaced at approximately half-helical (5 bp) distance intervals within the HIV-1 binding site. Mutants cpm +4/+8 and cpm +14/+18 alter the sequences of repeats two and three, respectively, and both generated smaller binding sites. Mutant cpm +4/+8 is particularly interesting in that the upstream boundary for site I changed from position −35 (in the wild-type promoter) to −17 (in the mutant), a striking alteration that lies entirely outside the site of the mutation. Although we do not completely understand this binding pattern, it is possible that sequence changes in mutant

cpm +4/+8 altered the arrangement of individual recognition elements to allow a different binding configuration for promoters of LBP-1. Because none of the mutations eliminated the binding of LBP-1, we designed a mutant that introduces G to T transversions at each G residue in the three unit repeats: 5'-(T/A)CTGG-3'. This mutant, designated triple, was consistently expressed poorly in vitro (Fig. 1B), and completely lacked the ability to bind LBP-1 in DNase I footprint experiments, as shown in Figure 4A. Thus, either one or both of the G residues within each of the repeats outlined in Figure 4A form a critical part of the LBP-1 binding site on HIV-1 DNA.
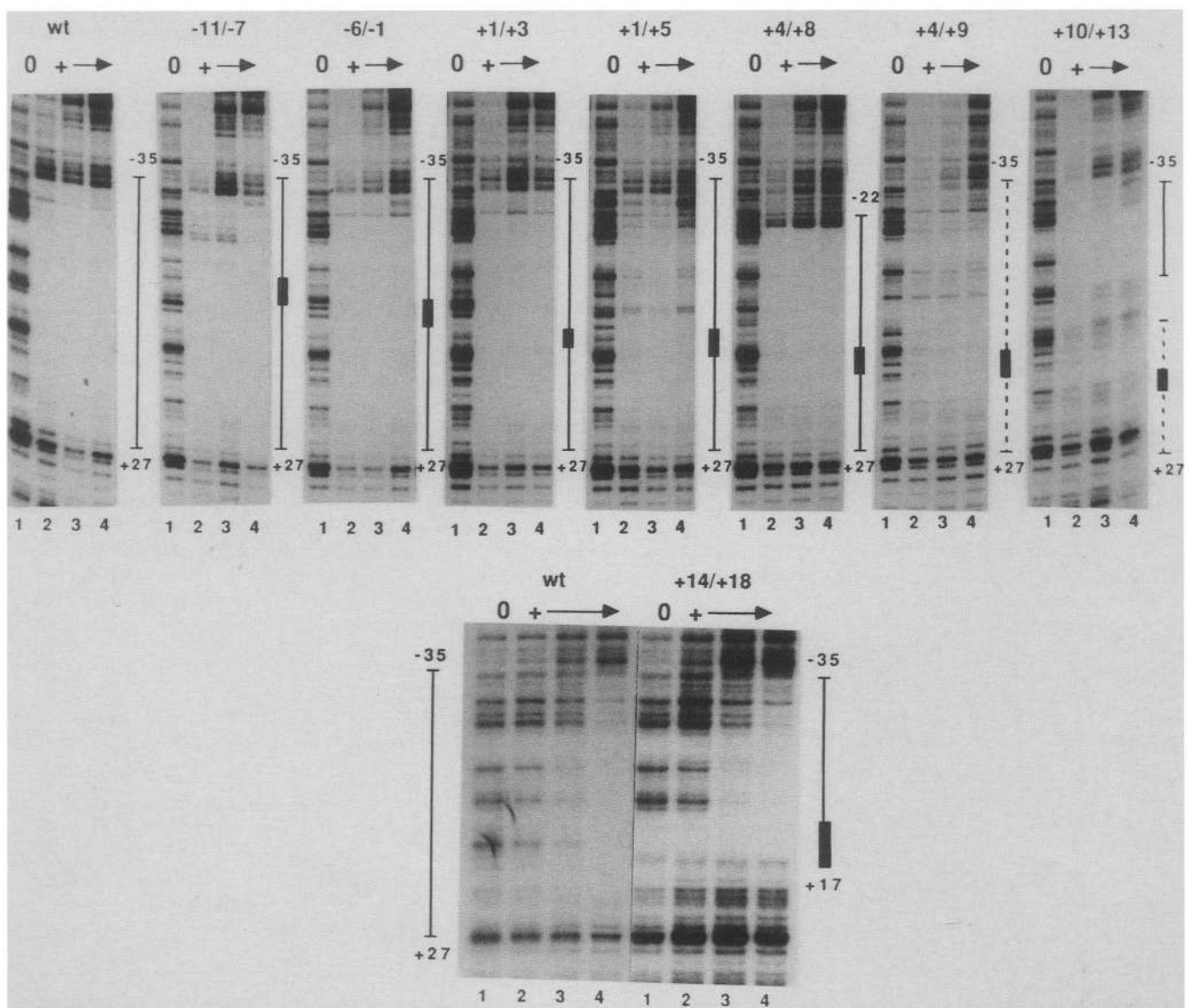

Figure 3. Interaction LBP-1 with HIV-1 promoter mutants. Binding of LBP-1 to different substitution mutants in the HIV-1 promoter was analyzed by DNase I footprint experiments. Each DNA template was 5'-end-labeled on the noncoding strand (HindIII; +80) and incubated without protein (lane 1), or with approximately 12 ng (lane 2), 20 ng (lane 3), or 30 ng (lane 4) of affinity-purified LBP-1 (M. Schorpp, M. Waterman, and K. Jones, unpubl.). Brackets and numbers indicate areas of observed protection for wild-type (wt) or mutant DNAs, as listed on the top of each panel. The position of base substitutions in each mutant are designated with black boxes.
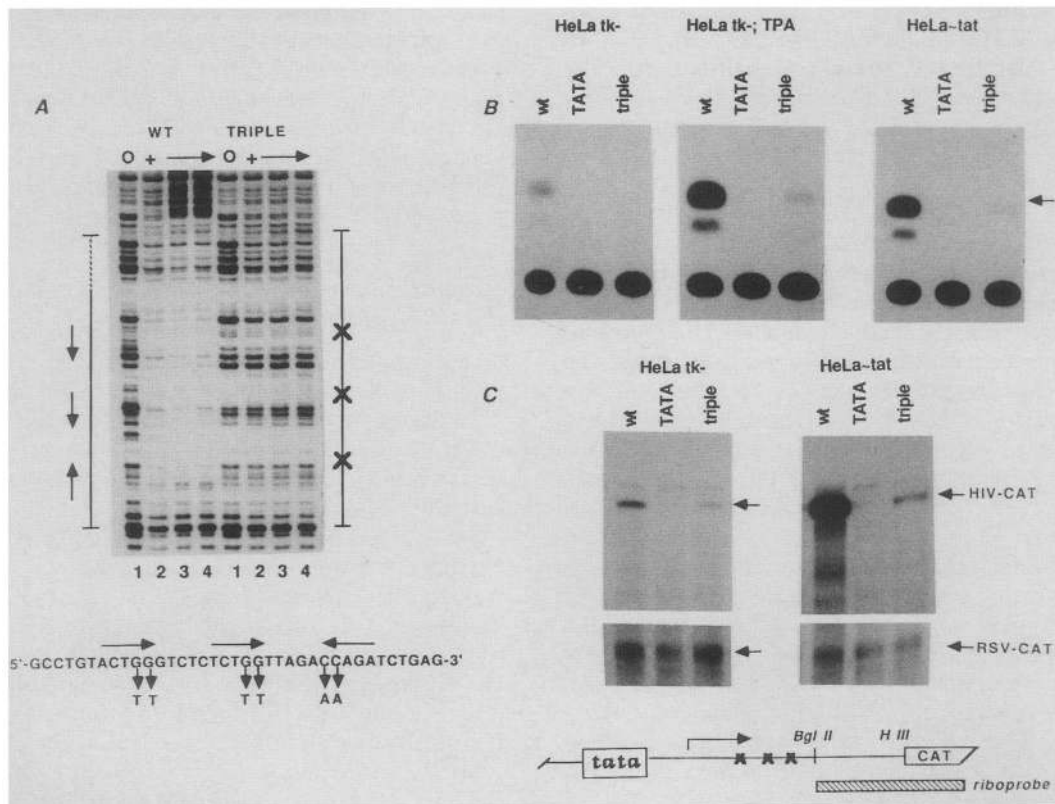
Figure 4. LBP-1 recognizes a repeated motif in the HIV-1 downstream region that contributes to promoter activity in vivo. (*A*) A triple-site base substitution eliminates binding of LBP-1 to the HIV-1 promoter. DNA fragments of the wild-type (wt) or triple-site mutant (triple) were 5'-end-labeled on the noncoding strand (*Hind*III; +80) for footprint analysis and incubated without protein (lane 1), or with 16 ng (lane 2), 32 ng (lane 3), or 48 ng (lane 4) of affinity-purified fractions of LBP-1. The sequence changes in the triple mutant are listed in the figure, and three potential recognition elements are indicated with arrows. (*B*) The triple mutation reduces HIV-1 basal promoter function in vivo in HeLa cells. Shown is a comparison of the relative levels of chloramphenicol acetyltransferase (CAT) activity produced from the wild-type promoter, the TATA substitution mutant, or the triple-site mutant following transfection into the cell lines indicated above each panel. Extracts were normalized for luciferase activity as described in Materials and methods. (*C*) Comparison of RNA levels in vivo by means of RNase-protection experiments. The T7 riboprobe (330 nucleotides) used in this experiment is illustrated schematically in the bottom drawing. Expected fragments from the wild-type, triple, or TATA mutant transcripts (320 nucleotides), and from the complementary portion of pRSV–CAT RNA (140 nucleotides) are indicated with arrows.

LBP-1 is required for HIV-1 promoter function in vivo

The results of in vitro transcription experiments suggested that LBP-1 may contribute to basal HIV-1 promoter activity as a positive-acting transcription factor that binds downstream of the RNA start site. Alternately, it was possible that our in vitro conditions (which included high levels of LBP-1) exceeded some rate-limiting step for LBP-1 activation in vivo, since previous deletion studies had not detected any component of basal promoter function within the immediate leader region (Rosen et al. 1985a; Peterlin et al. 1986; Wright et al. 1986; Muesing et al. 1987). To test the possibility that LBP-1 contributes to HIV-1 expression in vivo, we compared the relative activity in transient expression experiments of constructs containing the wild-type promoter (pLTR–CAT) or the triple mutant promoter (pTriple–CAT) joined to the bacterial chloramphenicol acetyltransferase (CAT) gene. The triple mutant was

chosen for this experiment because it was the only mutant that completely eliminated the binding of LBP-1 in vitro. Transfection efficiencies were standardized by cotransfection experiments in HeLa cells with plasmids (pRSV–LUC) that express luciferase (Materials and methods).

In three independent experiments, expression of CAT activity from the triple mutant was less efficient than the wild-type construct by a factor of 10-fold to 14-fold (Fig. 4B). This difference was apparent under several diverse cellular conditions, including resting HeLa tk-cells, cells activated by the phorbol ester tumor promoter TPA (which induces HIV enhancer activity in HeLa cells; Dinter et al. 1987), or a HeLa cell line engineered to express the HIV-1 Tat protein (HeLa-Tat cells; Rosen et al. 1985b). Dramatic effects on CAT levels were also observed for the TATA mutant, which was included in this experiment as a control for basal promoter activity. Similar effects on steady-state RNA levels were

observed in RNase protection experiments using a ribo-probe complementary to both the wild-type and mutant promoter RNAs (Fig. 4C). These data suggest that LBP-1 contributes to optimal HIV-1 promoter activity in vivo.

Transcription factor CTF/NFI also binds to HIV-1 promoter-proximal sequences

Immediately downstream of the LBP-1 binding region of the HIV-1 promoter lies a binding site (site II) for a protein distinct from LBP-1 (Fig. 2A). Inspection of the sequence within binding site II as well as the chromatographic properties of the site II protein suggested the possibility that this protein was CTF/NF I, a factor previously purified and shown to activate RNA polymerase II transcription through recognition of a GCCAA motif in the HSV-1 thymidine kinase and human globin genes (Jones et al. 1987). To test this possibility directly, we asked whether affinity-purified fractions of CTF/NF I protein could recognize HIV-1 DNA in a DNase I footprint experiment. As shown in Figure 5, incubation of approximately 15 ng of purified CTF/NF I with the HIV-1 LTR generated a binding site identical to that observed in crude extracts. When mixed in combination with purified fractions of LBP-1, the complete binding pattern observed in crude extracts over the downstream region was reproduced. Analysis of binding to the remaining mutants in the HIV downstream region revealed that only mutant cpm +39/+43 had altered res-

idues essential for binding CTF/NF I (Fig. 5). Thus CTF/NF I appears to recognize the HIV-1 element 5'-AGCCAG-3' (+40 to +45) that is derivative of a higher-affinity half-site CTF/NF I consensus: 5'-AGCCAA-3' (Jones et al. 1987). The orientation of this site on HIV-1 is inverted relative to that found in the upstream regions of the globin genes, and the same as the CTF/NF I site in the HSV-1 tk promoter.

Structural arrangement of downstream binding sites on the HIV-2 promoter

The promoter-proximal downstream region of the distinct viral isolate, HIV-2, also mediates activation by Tat (Emerman et al. 1987; Guyader et al. 1987) and might be expected to contain binding sites for the same transcription factors as HIV-1. This possibility was tested in DNase I footprint experiments using affinity-purified fractions of LBP-1. As shown in Figure 6A, an extended binding domain from position +32 to +71 was observed using a 15 ng (0.3 ng/μl) of LBP-1, and the binding region was extended further to approximately +80 at higher LBP-1 concentrations. Binding to the HIV-2 downstream site was competed with double-stranded oligodeoxynucleotides of the HIV-1 LBP-1 site, but not with binding-site sequences for an unrelated protein (Sp1) in a manner similar to the competition of the HIV-1-binding site (Fig. 6A). The location of the LBP-1-binding site on HIV-2 is substantially down-

Figure 5. A second transcription factor, CTF/NF I, binds downstream of LBP-1 in the HIV-1 promoter. Binding of purified CTF/NF I (Jones et al. 1987) and LBP-1 protein to the HIV-1 wild-type or mutant promoters (as indicated above each panel) was carried out with DNA fragments 5'-end-labeled on the noncoding strand (HindIII; +80). Reactions in each panel were as follows: (lane 1) no protein control; (lane 2) 15 ng of CTF/NF I; (lane 3) a combination of 15 ng each of purified CTF/NF I and LBP-1 fractions; (lane 4) 30 ng each of purified CTF/NF I and LBP-1.
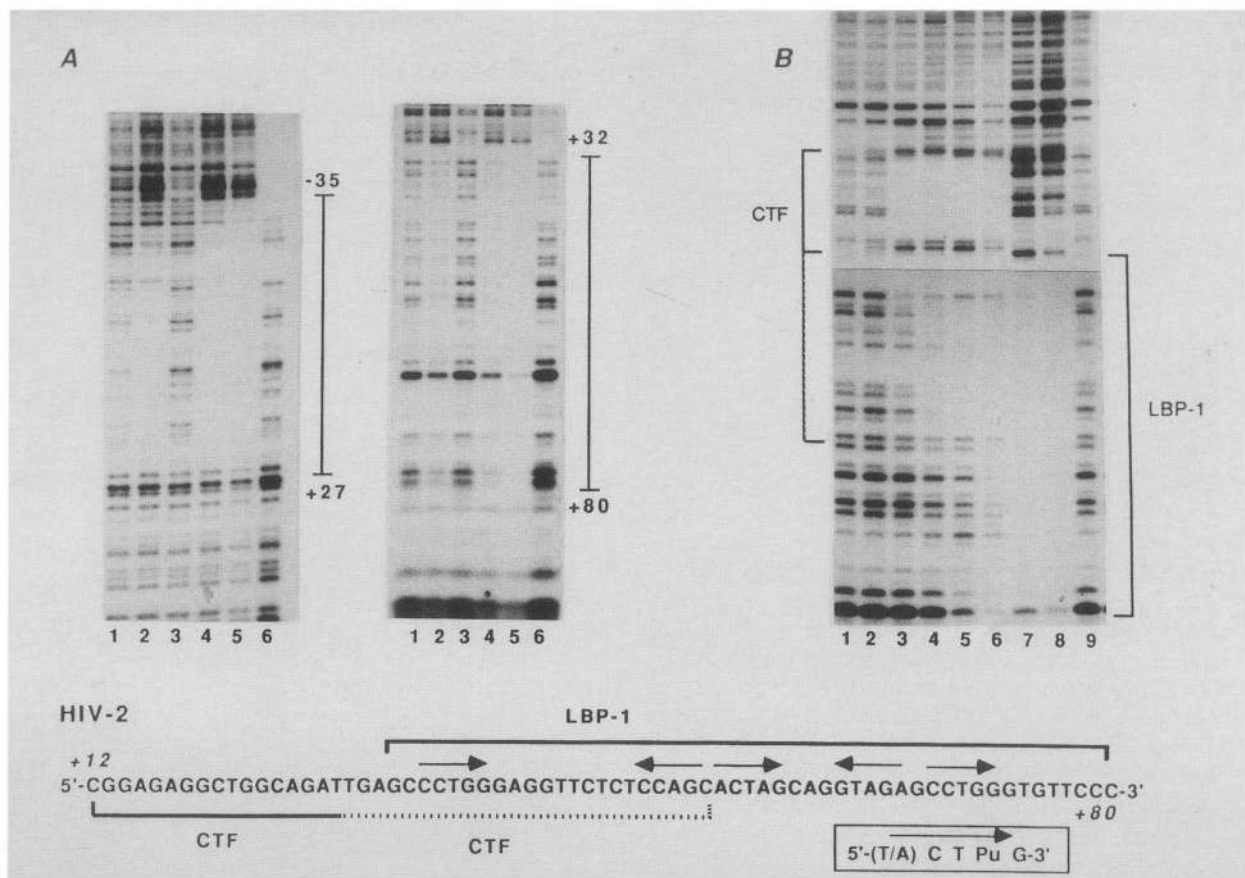
Figure 6. A distinct arrangement of LBP-1 and CTF/NF I sites in the HIV-2 downstream region. (A) Competition footprint analysis of the binding of approximately 25 ng LBP-1 to fragments of HIV-1 (*left panel*, 5'-end-labeled on the noncoding strand at HindIII, +80) or HIV-2 (*right panel*, 5'-end-labeled on the noncoding strand at Eae-1, +105). Concentration of ligated HIV-1 LBP-1 oligodeoxynucleotides (listed in Materials and methods) used in each experiment was as follows: (lane 2) 0.16 nM; (lane 3) 80 nM. Control competitor DNA (for Sp1; as described in Jones et al. 1987) was present at 0.16 nM (lane 4) and 80 nM (lane 5). Reactions without added protein and with 15 ng of LBP-1 are shown in lanes 6 and 1, respectively. (B) HIV-2 DNA fragments (5'-end-labeled on the noncoding strand at Eae-1; +105) were incubated with different fractions of CTF/NF I (lane 3, 10 ng; lanes 4 and 5, 25 ng; lane 6, 40 ng), 30 ng LBP-1 (lanes 7, 8), or without protein (lanes 1, 2, 9) and analyzed by the DNase I footprint procedure. The binding site sequences are listed below the figure, and potential LBP-1 recognition elements are outlined with arrows.

stream of its position on HIV-1, and binding over the TATA domain of HIV-2 was not evident in these experiments. Analysis of the HIV-2-binding site reveals several repeats identical or similar to the motif 5'-(T/A)CTGG-3' [or 5'-CCAG(A/T)-3'] implicated in binding to HIV-1 DNA. Methylation interference will be required to establish the contacts of LBP-1 on both the HIV-1 and HIV-2 promoters with greater certainty.

DNase I footprint experiments with purified CTF/NF I revealed that this factor also interacts with the downstream region of the HIV-2 promoter (Fig. 6B). A promoter-proximal binding site was mapped between +12 and +32, and a second, lower-affinity site was identified between +32 to +48. The first site includes a likely CTF/NF I recognition element, 5'-AGCCAA-3', which occurs in the same orientation as the CTF/NF I site on the HIV-1 promoter and is recognized with a greater affinity than the HIV-1 site. The binding of LBP-1 dominated the second CTF/NF I-binding site in mixing experiments (data not shown) suggesting that this weaker

site is not recognized in the presence of LBP-1. Further experiments are required to determine whether the LBP-1 or CTF/NF 1-binding sites influence basal HIV-2 promoter activity in vitro or in vivo.

Because CTF/NF I and LBP-1 both interact with sequences that span the *tat*-responsive region TAR, it was of further interest to determine whether the binding of these proteins might mediate viral *trans*-activation. The response of each of the HIV-1 promoter mutants (Fig. 1) to Tat has been characterized by cotransfection experiments in HeLa, COS-1, and T lymphocyte cell lines, and critical TAR sequences were localized to the region between +14 to +44 (Tong-Starksen et al. 1987; M.J. Selby et al., unpubl.). These results are consistent with recent findings of other groups (Garcia et al. 1987; Hauber and Cullen 1988; Jakobovits et al. 1988). Thus, the TAR region overlaps only partially with the LBP-1-binding domain, and includes, but is not restricted to, the sequences that specify binding of CTF/NF I. Sequences required for optimal response of the HIV-2 pro-

Figure 7. A complex overlapping arrangement of transcription factor binding sites and Tat regulatory signals. The relative positions of the transcription-factor binding sites within the downstream regulatory regions of HIV-1 and HIV-2 promoters are illustrated. LBP-1 and CTF/NF I-binding sites are described in the text. The lower-affinity LBP-1-binding region from -17 to -35 on the HIV-1 promoter is not indicated in this diagram. Arrows refer to repeats indentical or similar to the sequence, 5'-(T/A)CTGG-3', which are candidates for LBP-1 recognition elements. Sp1 binding sites on the HIV-2 promoter (approximately -40 to -70) were localized using DNase I footprint experiments with 20–40 ng of affinity-purified Sp1 (data not shown). Black boxes designate Tat-responsive (TAR) sequences, which have been defined experimentally for HIV-1 using internal deletion and substitution mutants (Garcia et al. 1987; Tong-Starksen et al. 1987; Hauber and Cullen 1988; Jakobovits et al. 1988; M.J. Selby, P.A. Luciw, and B.M. Peterlin, unpubl.). A short region of HIV-2 promoter with homology to HIV-1 TAR sequences is indicated, and this core region as well as sequences further downstream are required for optimal *trans*-activation in vivo (Emerman et al. 1987; Jakobovits et al. 1988). Possible RNA secondary structures within TAR sequences are shown, configured according to the HIV-1 hairpin structure suggested by nuclease mapping experiments (Muesing et al. 1987).
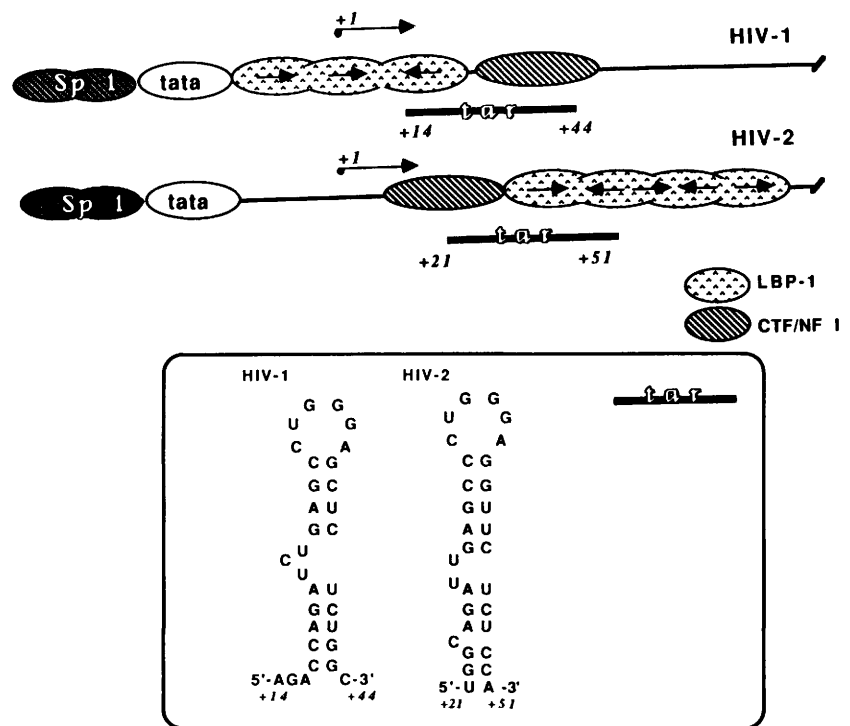
moter to Tat have also been mapped by deletion mutagenesis to a short region with imperfect homology to HIV-1 TAR sequences (Emerman et al. 1987; Jakobovits et al. 1988). The minimal *trans*-activation regions on both the HIV-1 and HIV-2 promoters overlap the junction of LBP-1, and CTF-binding sites on each promoter, as diagramed in Figure 7. Further downstream sequences of the HIV-2 promoter that modulate the response to Tat (Emerman et al. 1987; Jakobovits et al. 1988) span the remainder of the LBP-1-binding sites on the DNA. Interestingly, DNA-binding sites for CTF/NF I and LBP-1 were constructed from nonhomologous regions of the HIV-1 and HIV-2 promoters to create an overall arrangement that is reversed on HIV-2 relative to HIV-1. Thus, the same two cellular transcription factors interact with downstream regions of HIV-1 and HIV-2 DNA in a complex, partially overlapping arrangement with the *trans*-activation region.

Discussion

We have shown that sequences immediately downstream of the HIV-1 and HIV-2 promoters interact with a combination of two cellular transcription factors. One of these proteins, LBP-1, appears to recognize derivatives of a reiterated motif 5'-TCTGG-3' [or 5'-CCAGA-3'] present within extensive 50- to 60-bp binding sites downstream of each viral promoter. This protein has the interesting property of activating basal HIV-1 transcription from the downstream binding site. Binding studies indicate that multiple repeats promote stable binding to the LBP-1 site; however, it is not clear whether this is due to the interaction of multiple LBP-1 promoters or a single extended protein. Although the LBP-1 binding site on the HIV-1 promoter extends into upstream sequences overlapping the TATA element, binding of LBP-1 to this region is not affected by TATA and is possibly directed by one or more of the CTGG-like repeats surrounding the TATA box. Interestingly, the TATA transcription factor and LBP-1 appear to cooperate in HIV-1 transcription through highly overlapping recognition elements. Because mutations within the LBP-1-binding site influence both transcription initiation and efficiency in vitro, it will be important to examine directly the effect of LBP-1 on HIV-1 transcription by means of extract depletion and reconstitution experiments. The recent purification of LBP-1 (M. Schorpp, M. Waterman, and K. Jones, unpubl.) should assist in these experiments and allow a more complete description of the role of LBP-1 in cellular transcription. It also remains to be established whether LBP-1 is also capable of activating transcription from binding sites upstream of the RNA start site, and from locations distal to the promoter.

LBP-1 binding sites on HIV-1 and HIV-2 DNA are flanked by recognition elements for another positive-acting transcription factor, CTF/NF I. The combined binding pattern of both CTF/NF I and LBP-1 proteins reconstitutes the protection pattern reported previously in partially fractionated extracts (Garcia et al. 1987). Upstream binding sites of CTF/NF I contribute to the basal promoter function of numerous viral and cellular genes (Jones et al. 1987), and have been implicated in regula-

tion of transcription induction by transforming growth factor beta (Rossi et al. 1988). The recent isolation of the gene encoding this family of CCAAT-binding proteins (Santoro et al. 1988) should expedite studies on the tissue- and cell-type distribution of this transcription factor. Because of the extensive overlap of the CTF/NF I-binding site with TAR sequences and the relatively low activity of this protein in vitro, further experiments will be required to determine the influence of this factor on basal promoter function. The binding of LBP-1 and CTF/NF I to HIV downstream sequences suggests that the ability to activate transcription equivalently from sites upstream and downstream of the RNA start site may reflect a general property shared by many cellular DNA-binding proteins, or at least a diverse subset of RNA polymerase II transcription factors.

Analysis of site-directed mutants for DNA-binding and in vitro transcription suggested that LBP-1 is an integral part of the HIV-1 promoter. Although the cell type distribution of this factor is not known in detail, LBP-1 activity is also present in extracts from a variety of lymphoid cell lines including Jurkat, JY, U937, and H9 (Wu et al. 1988; data not shown), and thus does not appear to be a highly restricted transcription factor. To eliminate the possibility that redundancy of LBP-1-binding elements might obscure the effect of this basal promoter element, we generated a triple site mutation that completely eliminated binding of LBP-1. Transient expression experiments with this mutant supported the idea that LBP-1 contributes to basal promoter activity in vivo in HeLa cells. These results were unexpected in light of previous reports that a variety of mutations within HIV-1 5'-untranslated leader sequences had no effect on basal promoter activity (Rosen et al. 1985a; Cullen 1986; Wright et al. 1986; Muesing et al. 1987; Tong-Starksen et al. 1987). Although the basal rate of HIV-1 transcription in vivo is low and may therefore be difficult to quantitate, it is also apparent from our results that deletion mutations remove multiple elements, including the CTF/BF I-binding site, the overlapping TAR, and the LBP-1-binding site. The effects of promoter deletions are further complicated by the possibility that a negative signal may reside in the leader region (see discussion below). Therefore, deletions and large substitutions of downstream sequences could simultaneously remove positive and negative elements that differentially affect HIV-1 transcription. More detailed comparisons of these and other mutants should help define the contribution of downstream sequences to HIV basal promoter activity in vivo.

Recent evidence that purified HIV-1 Tat protein does not interact specifically with either DNA or RNA (Frankel et al. 1988) suggests that the action of this protein may be mediated indirectly through cellular proteins. Detailed comparisons of sequences required for binding LBP-1 and CTF/NF I with those required for the tat activation in vivo (M.J. Selby, P.A. Luciw, and B.M. Peterlin, unpubl.) indicate that the binding of these two cellular proteins to DNA cannot explain the action of Tat on the promoter. For example, specific mutations

between +24 and +33 no longer respond to tat activation in vivo (M.J. Selby, P.A. Luciw, and B.M. Peterlin, unpubl.) but do not influence the binding of either LBP-1 or CTF/NF I in vitro (Fig. 5). We have not been able to eliminate the possibility that CTF/NF I or LBP-1 play an accessory role to trans-activation, however, because mutants such as +39/+43 that are defective for binding CTF/NF I do not respond to tat (Tong-Starksen et al. 1987). Moreover, the triple mutation that eliminates binding of LBP-1 is defective for basal promoter activity, and is also unable to respond to tat activation in vivo (M.J. Selby, P.A. Luciw, and B.M. Peterlin, unpubl.). We have not ruled out the possibility that the recognition properties of these proteins are altered during viral infection or that different proteins may be induced by Tat to bind to the TAR region. Nevertheless, the data available to date suggest that Tat does not follow the mechanism proposed for other activator proteins such as E1A (for review, see Berk 1986; Jones et al. 1988), which act through the specificity inherent in preexisting cellular transcription factors.

A second possibility that we considered was that LBP-1 or CTF/NF I might recognize HIV RNA transcripts in a sequence-specific manner. Specific binding of a transcription factor to intragenic DNA and the corresponding segment of RNA has been reported for factor TFIIIA, a positive-acting transcription factor that recognizes the internal control region of the Xenopus 5S rRNA gene as well as distinct regions of the complex secondary structure of 5S RNA (Sands and Bogenhagen 1987). A stable RNA hairpin proposed for HIV-1 on the basis of solution structure mapping in vitro (Muesing et al. 1987) extends over the immediate downstream portion of the 5'-untranslated leader region. Although a portion of this structure falls outside the of the TAR, a region corresponding to the upper stem and loop is retained in TAR sequences of both HIV-1 and HIV-2, and the predicted sequence of the loop would be identical for both RNAs (see Fig. 7). Although both the stem-and-loop regions of the RNA structures contain elements involved in the binding of LBP-1 and CTF/NF I to DNA, neither LBP-1 nor CTF/NF I demonstrated an affinity for SP6 transcripts (−4 to +80) of the HIV-1 promoter in gel mobility shift experiments (data not shown). Thus, these downstream-binding transcription factors appear to lack the multifunctional nucleic acid recognition properties observed with TFIIA.

Recent data of Kao et al. (1987) suggest that Tat acts to overcome premature termination of transcription. Alternate proposals for trans-activation that involve subnuclear compartmentalization or stabilization of HIV transcripts would also invoke mechanisms whereby Tat acts to overcome a negative signal in the leader. The data reported here suggest that binding sites for positive-acting transcription factors superimpose this potentially negative region. These results may help explain observations that basal transcription levels remain unchanged for heterologous promoter constructs carrying TAR sequences (Cullen 1986; Peterlin et al. 1986; Wright et al. 1986; Muesing et al. 1987). That these same constructs re-

spond relatively poorly to Tat introduces the possibility that termination efficiency (as well as *trans*-activation efficiency) might be dependent on the promoter, as has been reported for termination of human U1 snRNA transcription (Hernandez and Weiner 1986; Neuman de Vegvar et al. 1986). Although we do not detect premature termination of HIV-1 transcription under the extract conditions used in this paper, it is possible that a cellular termination activity is blocked in nuclear extracts or has been lost during fractionation of the in vitro system. Further efforts will be required to understand this aspect of HIV promoter function and to determine the precise contributions of CTF/NF I and LBP-1 to HIV transcriptional control.

Materials and methods

HIV promoter constructs

Wild-type promoter plasmids HIV-1 pLTR–CAT (Peterlin et al. 1986) and mutants cpm +4/+9, cpm +14/+18, cpm +39/+43, and cpm +45/+49 were described previously (Tong-Starksen et al. 1987). Other clustered point mutants were created by double-stranded oligodeoxynucleotide replacement using appropriate combinations of the unique *Pvu*II (−17), *Bgl*II (+19), *Sac*I (+38), or *Hin*dIII (+80) restriction enzyme sites in the LTR. Each mutant was designed to introduce or alter restriction enzyme sites in the promoter, as follows: cpm −16/−12 (*Sph*I); cpm −11/−7 (*Pvu*I); cpm −6/−1 (*Fsp*I); cpm +1/+3 (*Pst*I); cpm +1/+5 (*Hpa*I); cpm +4/+8 (*Bal*I); cpm +4/+9 (*Nru*I); cpm +10/+13 (*Xba*I); cpm +14/+18 (*Dra*I); cpm +24/+27 (no *Bgl*II); cpm +30/+33 (no *Sac*I); cpm +39/+43 (*Xho*I); cpm +45/+49 (*Nar*I); and the TATA mutant (*Pst*I). The TATA mutant substituted the wild-type sequence 5′-CTGCATATAAG-3′ with 5′-CTGCAGCCCAG-3′, which was generated by multiple fragment replacement of sequences from −16/−120. The triple mutant contains double transversions at −1/+1, +10/+11, and +17/+18. Both the TATA and triple mutants contained HIV-1 downstream sequences to position +172, and were compared to an appropriate wild-type promoter plasmid that contains the equivalent downstream region. Mutant P.ins. contains a 36-bp polylinker insertion at the *Pvu*II site (−17) that introduces sites for *Bam*HI, *Xba*I, and *Sal*I. Each of the B.S.ins. mutants contains differing amounts of a polylinker substitution between the *Bgl*II (+19) and *Sac*I (+38) sites. These different mutants were obtained by deletions of the polylinker DNA, resulting in net spacing changes as listed: B.S.ins.1 (28-bp insertion), B.S.ins.2 (7-bp insertion), and B.S.ins.3 (7-bp deletion). The HIV-2 promoter construct used for footprint experiments (pLAV–2B) contained a 500-bp cDNA insert of the 3′LTR of HIV-2 into a pSP65 vector (Guyader et al. 1987). The riboprobe vector (pHIV1–GEM1) contains the *Pvu*II subfragment of pLTR–CAT (which includes HIV-1 sequences from −17 to +172, 40 bp of polylinker DNA, and 140 bp of the CAT gene) inserted into an *Sma*I and *Hin*cII-digested pGEM-1 vector (Promega Biotec).

In vitro transcription experiments and protein fractions

The reconstituted extract consisted of a mixture of different protein fractions, as listed for each reaction (25 μl): 9 μl (27 μg) of CL.225, 4 μl (2.5 μg) Sp2 (CL.225 and Sp2 were prepared as described by Dynan and Tjian 1983), 5 μl (25 ng) of affinity-purified Sp1 (Kadonaga and Tjian 1986), and 7μl (10 μg) of h.25 (a 0.25 M KCl step-fraction obtained by chromatography of a HeLa

nuclear extract on a matrix of heparin–agarose resin. See Dynan and Tjian (1983). These protein fractions were dialyzed into buffer containing 25 mM HEPES (K⁺) (pH 7.8), 5 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, 20% (vol/vol) glycerol, and 0.1% Nonidet P-40. The protein mixture was incubated with an equal volume of a DNA mixture containing circular HIV-1 wild-type (pLTR–CAT) or mutant plasmids and α-globin templates (amounts listed in the figure legends), and were carried out as described previously (Jones et al. 1985). Sequences for the HIV-1 and α-globin DNA primers were reported by Dinter et al. (1987). Gel autoradiograms were exposed overnight with an intensifying screen. For DNA-binding experiments, CTF/NF I was purified by sequence-specific affinity chromatography as described (Jones et al. 1987). Purified LBP-1 protein fractions used in Figure 3 were derived from a second-pass elution, and protein fractions used in Figures 4, 5, and 6 were derived from a third-pass elution from an HIV-1 DNA-affinity column. Details of the purification of LBP-1 from HeLa nuclear extracts will be presented elsewhere (M. Schorpp, M.Waterman, and K. Jones, unpubl.).

DNA-binding techniques

DNase I footprint experiments were carried out as described previously (Jones et al. 1985). DNA fragments were 5′-end-labeled at sites indicated in each figure legend with [γ-³²P]ATP and polynucleotide kinase, and incubated with different extract or purified protein fractions as indicated in the figure legends. In general, autoradiograms of dried gels were exposed overnight with intensifying screen. Competition experiments with ligated oligodeoxynucleotides were carried out as described previously (Jones et al. 1987) using synthetic LBP-1 binding sites (derived from the HIV-1 promoter) of the sequence: 5′-TCGAGCCTGTACTGGGTCTCTCTGGTTAGACCAGATC-TGAG-3′. The Sp1-binding site sequence was as described (Jones et al. 1987).

Cell culture and transient expression experiments

HeLa tk- and HeLa-Tat (Rosen et al. 1985b) cell lines were cultivated in Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum. For transfection experiments, a total of 15 μg of plasmid DNA was introduced into 10⁶ cells by calcium phosphate precipitation. The DNA mixture was removed after a 12-hr incubation, and cells were shocked for 1.5 min with a solution of 25% glycerol. Cells were harvested after a further 24-hr incubation for RNA extraction (see below), or after a 48-hr incubation for determination of CAT activity. TPA induction was carried out by an 8-hr incubation in medium supplemented with 10% fetal calf serum and 100 ng/ml of TPA at a time point 10 hr following the glycerol shock. Transfections for CAT analysis involved 10 μg of HIV–CAT plasmids and 5 μg of a plasmid carrying the luciferase gene under the control of the RSV promoter (deWet et al. 1987). Extracts were normalized for luciferase activity (10⁴ U). CAT activity was then measured after 3-hr incubations (37°C) for the HeLa tk-or HeLa tk-plus TPA cell extracts, or after 1-hr incubation for extracts from HeLa-Tat cells. Conversion of chloramphenicol was detected by 8-hr autoradiography of thin-layer chromatograms without intensifying screen, and measured by liquid scintillation counting of acetylated and nonacetylated products.

For in vivo RNA analysis, 10 μg of HIV–CAT plasmids and 5 μg of pRSV–CAT were transfected into HeLa tk- or HeLa-Tat cells. Total cellular RNA was extracted according to the modified procedure described by Chomczynski and Sacchi (1987) and

was processed further by RNase-free DNase I (RQ1, Promega) treatment (0.2 U/μg RNA; 10 min at 37°C). For RNase protection experiments, 20 μg of total RNA was annealed overnight at 42°C with 10^6 cpm of a 330-nucleotide T7 riboprobe (BglII-digested pHIV1–GEM1). Samples were treated with 1300 U/ml RNase T1 and 10 μg/ml RNase A for 1 hr (25°C) and visualized after electrophoresis on 6% sequencing gels by 12-hr autoradiography with an intensifying screen.

Acknowledgments

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Structural arrangements of transcription control domains within the 5'-untranslated leader regions of the HIV-1 and HIV-2 promoters.

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