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Functional Characterization of a Complex Protein–DNA-Binding Domain Located within the Human Immunodeficiency Virus Type 1 Long Terminal Repeat Leader Region

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Transcriptional *trans* activation of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) by the viral *tat trans* activator is mediated by an LTR-specific sequence located immediately 3′ to the start of transcription initiation. We have used a range of molecular techniques to examine DNA-protein interactions that occur in the vicinity of this *cis*-acting sequence. Our results demonstrate the existence of a sequence-specific DNA-protein interaction involving the HIV-1 leader DNA and map this binding event to between −2 and +21 base pairs relative to the HIV-1 LTR transcription start site. Evidence suggesting that this interaction involves three distinct protein-DNA contact sites extending along one side of the DNA helix is presented. Mutation of these sites was found to ablate protein-DNA binding yet was observed to have no effect on either the basal or *tat trans*-activated level of HIV-1 LTR-specific gene expression. We therefore conclude that this DNA-protein interaction has a function distinct from the regulation of HIV-1 LTR-specific gene expression.

The pathogenic human retrovirus human immunodeficiency virus type 1 (HIV-1) encodes a nonstructural protein, termed *tat*, whose functional expression is required for viral replication in vitro (8, 12). The *tat* protein is localized to the nucleus of expressing cells (15) and acts to greatly enhance the expression of viral or heterologous genes linked to the HIV-1 long terminal repeat (LTR) promoter element (1, 6, 25, 28, 29, 34, 39). Although evidence exists for a posttranscriptional component of this *tat*-mediated *trans* activation (6, 10, 29, 39), the primary effect of *tat* is to enhance the rate of transcription from the HIV-1 LTR (15, 17, 20, 31). This enhancement is in turn mediated by an HIV-1 LTR sequence, termed the *trans*-activation response (TAR) element, which has been mapped to a site located immediately 3′ to the start of viral mRNA transcription (11, 14, 17, 30) (Fig. 1).

Transcriptional *trans* activation is normally mediated by the sequence-specific binding of transcription factors to sites within the affected promoter DNA sequence (22). A good example of this generalization is provided by the HIV-1 LTR U3 region itself, which contains functional binding domains for several transcription factors, including Sp1 and NF-κB (4, 18, 26). A number of promoters have been shown to contain DNA regulatory elements that extend 3′ to the start of transcription (16, 23, 36, 38). It was therefore of great interest when DNase I footprinting and DNase I hypersensitivity analyses (13, 14) revealed the presence of a strong DNA-protein interaction that extended 3′ to the HIV-1 LTR transcription start site to the region of the TAR element.

In this study, we attempted to characterize and precisely identify the target sequences involved in this particular HIV-1 LTR DNA-binding event. Our data demonstrate the existence of three adjacent, highly cooperative protein-binding sites, located between nucleotides −2 and +21 relative to the HIV-1 LTR transcription start site, which interact with constitutively expressed cellular DNA-binding proteins. These binding sites are shown to partly overlap with the HIV-1 LTR TAR element but are also shown to be functionally fully distinct from TAR. We therefore conclude that this HIV-1 LTR DNA-binding event, although highly specific, is not directly involved in the *tat*-mediated *trans* activation of HIV-1-specific gene expression.

MATERIALS AND METHODS

Construction of molecular clones. The HIV-1 LTR-based chloramphenicol acetyltransferase (CAT) gene expression vector pBC12/HIV/CAT has been described previously (3). Cleavage of pBC12/HIV/CAT at the unique HIV-1 LTR *Pvu*II and *Bgl*II sites permitted the introduction of oligonucleotides containing site-specific mutations (Fig. 1). The primary structure of these mutations was confirmed by DNA sequence analysis. The pcTAT/dhfr plasmid expresses both the HIV-1 *tat* gene under the control of a cytomegalovirus immediate-early promoter and a mouse dihydrofolate reductase (*dhfr*) gene under simian virus 40 early-promoter control. pcTAT/dhfr was constructed by insertion of a 1.8-kilobase-pair *Pvu*II-*Bam*HI *dhfr* gene fragment from pSV2-dhfr (37) into the unique *Stu*I site of the *tat* gene expression vector pcTAT (21). Plasmid pTAR contains the *Pvu*II-to-*Hin*dIII (−18 to +81) region of the HIV-1 LTR (Fig. 1) inserted into the polylinker region of pSP68 (Promega Biotech). A probe (−18/+81 probe) derived from this plasmid was used for the methylation interference analysis described below.

Cell culture and transfection. HeLa and Jurkat cells were maintained as previously described (4, 6). For preparation of the HeLa/cTAT cell line, HeLa cells were transfected with a mixture of 250 ng of pSV2neo (35) and 5 μg of pcTAT/dhfr (both linearized by cleavage with *Pvu*I), as well as 5 μg of high-molecular-weight human carrier DNA, using a calcium

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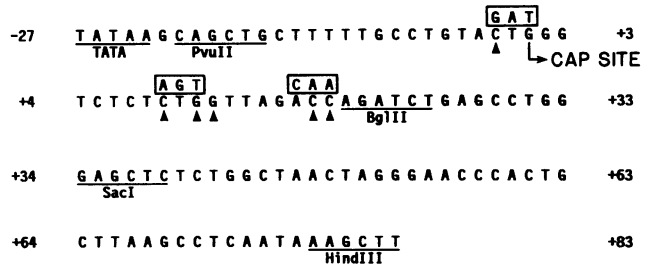

FIG. 1. Sequence of the HIV-1 LTR around the site of transcription initiation. The core TAR element, as defined by mutational analysis (14, 17), extends from ~+20 to ~+44 nucleotides relative to the cap site. Also shown are locations of the TATA box and relevant restriction sites. For simplicity, this entire element is referred to in the text as the HIV-1 leader region. Boxed sequences indicate the mutations introduced into the HIV-1 leader region by oligonucleotide substitution between the *Pvu*II and *Bgl*II sites, termed Δ1, Δ2, and Δ3, respectively, in order from the 5′ to 3′ direction. These mutations were designed to eliminate bases shown by methylation interference analysis to be important for factor binding to the HIV-1 leader DNA (▲).

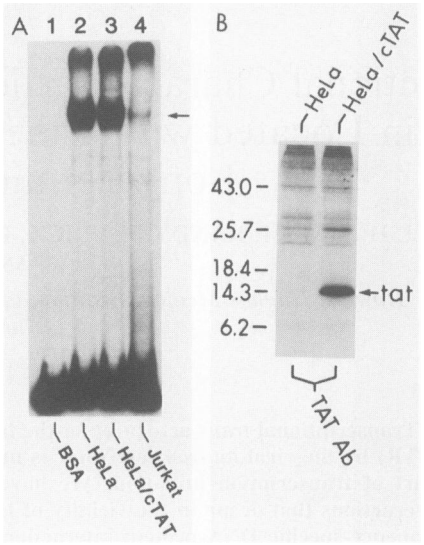

FIG. 2. Gel retardation analysis using an HIV-1 LTR leader region probe. The assay shown in panel A used 10 μg of bovine serum albumin (negative control) or 10 μg of nuclear proteins derived from HeLa, HeLa/cTAT, or Jurkat cells. These proteins were preincubated with 2 μg of poly(dI-dC) competitor before addition of ~0.5 ng of the radiolabeled −18/+81 probe. All nuclear protein samples tested yielded a single retarded band of similar mobility (←). The HeLa/cTAT line, which was shown to express high levels of the 15.5-kilodalton HIV-1 *tat trans* activator by immunoprecipitation analysis (21), yielded the same binding phenotype as did the parental HeLa cell line (panel A, lanes 2 and 3).

phosphate transfection technique (7). After 48 h, the culture was subjected to selection in 0.8 mg of G418 per ml. This eventually resulted in the appearance of ~100 independent Neo^r colonies. These were pooled and subjected to a second selection with 5×10^{-8} M amethopterin. We had previously observed that our HeLa cell cultures only very rarely (<1 in 10^7) are able to spontaneously give rise to colonies resistant to this level of amethopterin. However, pooled populations of preselected Neo^r cells cotransfected with a *dhfr* expression vector readily yield viable colonies under these conditions. Experiments using a number of model genes have demonstrated that these doubly selected cells express a high level not only of *dhfr* but also of any linked gene of interest. This level is ~100-fold higher than that obtained by simple coselection with the *neo* gene alone (data not shown). The resultant *dhfr*$^+$ Neo^r cells were pooled and termed HeLa/cTAT. High-level, functional expression of the HIV-1 *tat* gene was confirmed by immunoprecipitation analysis with an anti-*tat* antipeptide antiserum (15, 21) (Fig. 2B) and by transfection with the indicator construction pBC12/HIV/CAT (3) (data not shown).

The expression phenotypes of site-specific mutants of the HIV-1 LTR in the pBC12/HIV/CAT background were tested by calcium phosphate-mediated cotransfection (7) of HeLa cells with the *tat* expression vector pgTAT or the negative control vector pBC12/CMV (6, 21). CAT expression levels were determined at 60 h posttransfection by the method of Neumann et al. (27).

Radiolabeling of restriction fragments and oligonucleotides. The DNA probes used for gel retardation studies were normally generated by cleavage of pBC12/HIV/CAT at the unique *Hin*dIII site (Fig. 1) and filling in with Klenow DNA polymerase I in the presence of α-^{32}P-labeled deoxynucleotide triphosphates (6). In some cases, these probes were prepared by cleavage of pBC12/HIV/CAT with *Pvu*II (Fig. 1), followed by sequential treatment with alkaline phosphatase and T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (6). Probes were isolated by preparative polyacrylamide gel electrophoresis after specific secondary cleavage as indicated. Similarly, the probes used for methylation interference were prepared by digestion of pTAR with *Hin*dIII, followed by dephosphorylation with alkaline phosphatase. The positive strand was labeled with Klenow DNA polymerase I in the presence of [α-^{32}P]dATP; the negative strand was labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The labeled DNA was then cleaved at a pSP68-derived *Hae*III site, and the resultant 135-base-pair probe fragment was isolated as described above. Synthetic oligonucleotides were isolated and annealed as previously described (2, 4).

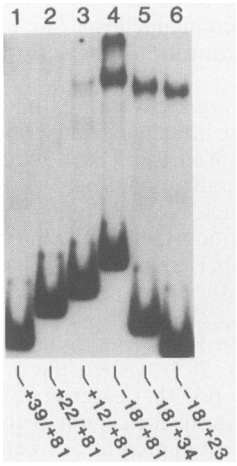

FIG. 3. Mapping of the HIV-1 leader region protein-binding site. End-labeled probes extending across the indicated nucleotide positions were prepared by restriction endonuclease cleavage of the full-length −18/+81 probe at sites indicated in Fig. 1. Equivalent levels of all probes were then tested for protein binding by gel retardation analysis. This experiment demonstrated that the HIV-1 leader region protein-binding site was contained between nucleotides −18 to +23.

Preparation of nuclear protein extracts. The isolation of nuclear proteins from HeLa, HeLa/cTAT, and Jurkat cells was based on the method of Dignam et al. (9). The cells were washed with cold phosphate-buffered saline (Ca^{2+}-Mg^{2+} free; GIBCO Laboratories), harvested, and suspended in ice-cold 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9)–10 mM KCl–1.5 mM $MgCl_2$–200 mM sucrose–0.5 mM dithiothreitol–1× PAL (0.5 mM phenylmethylsulfonyl fluoride [Sigma Chemical Co.], 3 μg of aprotinin per ml [Boehringer Mannheim Biochemicals], 2 μg of leupeptin per ml [Boehringer Mannheim]). The cells were lysed with 10 strokes of a tissue homogenizer, and the nuclei were collected by a low-speed spin (500 × *g*). The nuclei were suspended in (per 10^8 starting cells) 300 μl of ice-cold 10 mM HEPES-NaOH (pH 7.9)–400 mM KCl–1.5 mM $MgCl_2$–0.1 mM EDTA–300 mM sucrose–5% (vol/vol) glycerol–0.5 mM dithiothreitol–1× PAL and agitated for 30 min at 4°C. The suspension was transferred to Eppendorf tubes and centrifuged for 30 min at 4°C, and the supernatant was dialyzed against 20 mM HEPES-NaOH (pH 7.9)–50 mM KCl–0.5 mM $MgCl_2$–0.2 mM EDTA–20% (vol/vol) glycerol–0.5 mM dithiothreitol–1× PAL at 4°C. The extract was transferred to Eppendorf tubes and centrifuged for 15 min at 4°C. The supernatant was then divided into equal portions, frozen in liquid nitrogen, and stored at −70°C. Protein concentrations were assayed by the method of Bradford (5).

Gel retardation assays. Protein-DNA-binding reactions were performed basically as described by Singh et al. (33). For a standard reaction (15 to 25 μl), approximately 10 μg of extracted nuclear proteins was mixed with 2 μg of poly(dI-dC) (Boehringer Mannheim) in extract buffer and incubated at 25°C for 10 to 15 min. Radiolabeled DNA (~0.5 ng at 5 × 10^3 to 20 × 10^3 cpm per reaction) was then added, and the reaction was maintained at 25°C for a further 15 to 20 min. Specific oligonucleotide competitors were added 15 min before the addition of ^{32}P-labeled fragment at an ~100-fold molar excess. Retarded probe samples were resolved by electrophoresis in 5% nondenaturing polyacrylamide gels and were visualized by autoradiography.

Methylation interference assays. A method similar to that of Sen and Baltimore (32) was used to assay methylation interference. A scaled-up binding reaction was performed with 80 μg of nuclear protein extract, 16 μg of poly(dI-dC), and 10^5 cpm of partially methylated (24) radiolabeled probe. The retarded and free DNA fragments were excised from the wet gel and electroeluted onto NA-45 paper (Schleicher & Schuell, Inc.) for 2 to 4 h in 0.25× TBE at 50 V. Fragments were then eluted by heating in 1 ml of 1 M NaCl–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA at 68°C for 2 h, with 2 μg of yeast tRNA added as a carrier. The recovered nucleic acids were cleaved at methylated bases by using piperidine and resolved together with adjacent A+G and G ladders, on an 8% sequencing gel (24). The resultant autoradiographs were quantitatively scanned with an Ultrascan XL densitometer (LKB Instruments, Inc., Rockville, Md.).

RESULTS

Detection of a DNA-protein complex involving the HIV-1 LTR leader region. The impetus for this work was provided by the demonstration by Garcia et al. (13) that an HIV-1 LTR domain extending from −13 to +52 was protected from in vitro DNase I digestion by protein(s) present in a HeLa cell nuclear extract. These investigators proposed that this factor, which they termed the TAR factor, might be importantly involved in mediating the *trans* activation of HIV-1 LTR-specific transcription by the viral *tat* protein. The biological relevance of this DNA-binding interaction was further suggested by the demonstration of an in vivo DNase I-hypersensitive site at this location within the HIV-1 LTR (14). The extraordinary size of the DNase I footprint defined by Garcia et al. (13) suggested the potential for multiple protein-binding events within this sequence.

Our initial approach to the dissection of this extensive DNA-protein interaction was to examine whether an end-labeled DNA probe, extending from −18 to +81 nucleotides relative to the site of transcription initiation within the HIV-1 LTR (Fig. 1), would demonstrate specific protein binding as determined by gel retardation analysis (33). This experiment demonstrated that proteins present in a HeLa cell nuclear extract yielded only a single retarded complex in the presence of excess nonspecific competitor DNA (Fig. 2A, lane 2). Similarly, a nuclear extract derived from the human T-cell line Jurkat yielded a single retarded band of similar mobility but lower intensity (Fig. 2A, lane 4). To test whether expression of the HIV-1 *tat* gene would affect this DNA-protein interaction, we used gene-linked coamplification (7) to prepare a HeLa cell line (HeLa/cTAT) that expressed highly elevated levels of the *tat* protein as determined either by immunoprecipitation analysis (Fig. 2B) or by phenotype (data not shown). A nuclear extract derived from this cell line yielded a single retarded complex similar in mobility and intensity to the complex observed by using the parental HeLa cells (Fig. 2A, lanes 2 and 3). These results therefore suggested that the *tat* protein is not a component of this DNA-protein-binding event. The single retarded band observed in these initial binding experiments also suggested that this HIV-1 DNA sequence may support the formation of only one major DNA-protein complex.

The HIV-1 LTR leader region contains multiple protein-binding sites. To more closely define the sequence requirements for this HIV-1 LTR DNA-protein interaction, we next truncated the initial −18/+81 probe from either the 5′ or the 3′ end (Fig. 3). These results demonstrated that a fragment extending from −18 to +23 retained full binding activity, whereas fragments extending from +12 to +81 or +22 to +81 retained little or no binding activity, respectively.

To identify specific G residues within the −18/+81 region that participate in binding-site recognition, we performed gel retardation studies that used DNA probes modified by partial G methylation (24, 32). DNAs corresponding to the free and protein-bound forms were eluted from gel slices, cleaved at methylated G residues with piperidine (24), and analyzed on a denaturing polyacrylamide gel. A representative methylation interference gel analysis is shown in Fig. 4A; quantitation of the level of interference due to methylation of individual G residues located on each DNA strand is presented in Fig. 4B. Interestingly, interference with DNA binding at any particular G residue never reached 100%. However, a major area of interference (80 to 90%) was noted at positions +9, +11, and +12. In addition, two regions exhibiting partial interference (50 to 70%) were noted at positions −2 and +18/+19. It is of interest to note that these three sites are separated by ~10 base pairs, i.e., by approximately one turn of the DNA helix, which raises the possibility that this DNA-protein interaction extends along one side of the DNA helix. Overall, these results demonstrated that this HIV-1 leader region protein-binding domain extended from at least positions −2 to +21 and suggested that protein-DNA contact occurred at at least three distinct sites within this larger sequence. DNA footprinting of this protein-DNA complex, using 1,10-phenanthraline copper as

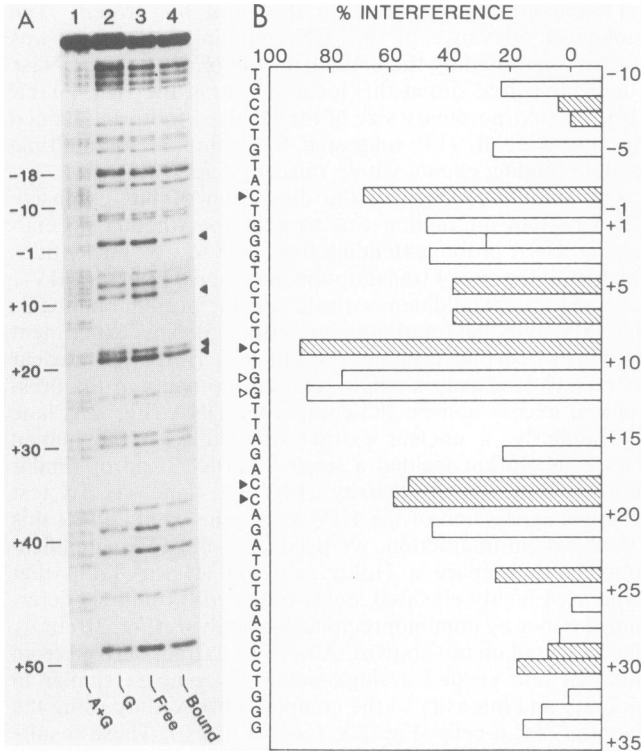

FIG. 4. Methylation interference analysis of the HIV-1 leader DNA protein-binding site. The partially methylated (24) probes used extended from −18 to +81 and contained a 36-base-pair 5′ extension consisting of procaryotic sequences derived from the pTAR vector (see Materials and Methods). Gel retardation and oligonucleotide competition analyses demonstrated that these modified probes displayed the expected protein-binding pattern (data not shown). (A) Representative methylation interference (32) sequencing gel, using the antisense strand. (B) Quantitative representation of the average level of interference with protein binding observed for individual G residues present in the sense (▭) and antisense (▨) strands. ▶, Residues yielding more than 50% interference.

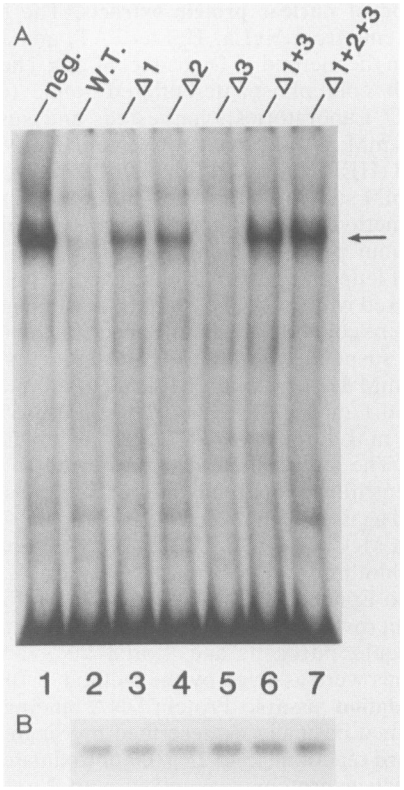

FIG. 5. Oligonucleotide competition analysis using HIV-1 leader-derived oligonucleotides. Gel retardation assays (A) were performed as described for Fig. 2 except that the nuclear proteins were preincubated with synthetic double-stranded oligonucleotides extending from −18 to +24 relative to the HIV-1 LTR. These oligonucleotides either had the wild-type (W.T.) HIV-1 sequence or contained the single or multiple clustered point mutations described in the legend to Fig. 1, as indicated. The equivalent double strandedness of all competitor oligonucleotides was confirmed by treating equal portions of the annealed synthetic DNAs with Klenow DNA polymerase in the presence of a trace amount of α-^{32}P-labeled deoxynucleotide triphosphate, followed by analytical acrylamide gel electrophoresis (B). Unlabeled competitor oligonucleotides were used at an ~100-fold molar excess over the labeled −18/+81 leader DNA probe.

the cleavage reagent, also revealed protection over this same sequence element (data not shown).

To confirm the importance of each of these three protein-DNA interaction sites in the formation of the overall DNA-protein complex, we prepared a series of double-stranded oligonucleotides extending from the *Pvu*II site at position −18 to the *Bgl*II site at position +23 in the HIV-1 LTR. A wild-type oligonucleotide served as the control; other synthetic oligonucleotides contained a triple point mutation at positions −2, −1, and +1 (Δ1), +9, +10 and +11 (Δ2), or +17, +18, and +19 (Δ3) (Fig. 1). Oligonucleotides containing two (Δ1+3) or all three (Δ1+2+3) of these mutations were also prepared. All of the oligonucleotides were annealed under identical conditions. The 4-nucleotide *Bgl*II overhang present in each double-stranded oligonucleotide was then filled in with Klenow DNA polymerase in the presence of either cold deoxynucleotide triphosphates or a trace amount of ^{32}P-labeled deoxynucleotides. Equal amounts of the labeled, annealed oligonucleotides were then analyzed by acrylamide gel electrophoresis. This analysis (Fig. 5B) confirmed that all of the oligonucleotides were of the same, expected mobility and were equivalently labeled by Klenow DNA polymerase, thus demonstrating equivalent double strandedness. The unlabeled, annealed oligonucleotides were then tested for the ability to compete for factor binding to the full-length −18/+81 TAR DNA probe. As expected, a 100-fold excess of the wild-type oligonucleotide essentially completely ablated detectable binding (Fig. 5A, lane 2). In contrast, the same excess of the Δ1 or Δ2 mutant oligonucleotide reduced binding by only ~3-fold (lanes 3 and 4). The Δ3 mutation did not appear to affect binding significantly when present alone, as this oligonucleotide competed effectively for binding to the wild-type sequence at this concentration (lane 5). However, the oligonucleotide containing the Δ1+3 double mutation was reproducibly a less effective competitor than the Δ1 oligonucleotide, and both this competitor and the triple mutation (Δ1+2+3) had no detectable specific effect on binding to the wild-type probe (lanes 6 and 7). These results therefore confirmed the importance of the three sites identified by methylation interference in mediating formation of this leader DNA-protein complex.

The HIV-1 leader DNA-binding protein is not a transcription factor. To test whether these same leader mutations would have any effect on the basal or *tat trans*-activated level of HIV-1 LTR-specific gene expression, each of the oligonucleotides was introduced into the HIV-1 LTR present

TABLE 1. Levels of expression from the HIV-1 leader DNA mutants in the presence and absence of *tat*[a]

Clone transfected	Relative CAT activity (cpm)[b]		*trans* activation
	−*tat*	+*tat*	
pBC12/HIV/CAT	100	40,790	408
pΔ1	40	12,560	314
pΔ2	40	13,710	343
pΔ3	60	15,740	262
pΔ1+3	60	20,810	347
pΔ1+2+3	90	22,970	255
pD+35/+38	70	110	<2

[a] HeLa cell cultures were transfected (7) with equimolar amounts of the HIV-1 LTR constructions together with either the *tat* expression vector pgTAT or the control vector pBC12/CMV (21). CAT expression levels were determined at 60 h posttransfection by the diffusion assay of Neumann et al. (27).

[b] Corrected for the level of activity observed in a culture transfected with pBC12/CMV alone (i.e., 40 cpm). The small differences observed in this assay were neither reproducible nor significant.

in the previously described CAT gene expression vector pBC12/HIV/CAT, using the *Pvu*II and *Bgl*II sites (Fig. 1). Each of these vectors was then transfected into HeLa cells in the presence or absence of the *tat* expression vector pgTAT and analyzed for the level of CAT expression at 60 h posttransfection. These leader DNA mutations had no significant effect on either the basal or *trans*-activated level of HIV-1 LTR-specific gene expression when compared with the wild-type HIV-1 LTR present in pBC12/HIV/CAT (Table 1). Mutant pΔ1+2+3, which was mutated at all three protein-DNA interaction sites, is particularly notable in that it displayed levels of CAT activity, both in the presence and in the absence of *tat*, that differed by less than twofold from levels of the wild-type HIV-1 LTR construction. In contrast, a previously described (14) deletion mutant lacking nucleotides +35 to +38 (pD+35/+38) was found to be entirely refractory to *tat trans* activation.

DISCUSSION

In this study, we have attempted to delineate the HIV-1 LTR DNA sequences required for binding of the TAR factor initially defined by Garcia et al. (13) and to understand the importance of this interaction in the *trans* activation of HIV-1 LTR gene expression by *tat*. Our results confirm that there is a readily detectable, sequence-specific DNA-protein interaction involving the HIV-1 LTR leader and map this binding event between nucleotide positions −2 and +21 relative to the HIV-1 transcription start site. Despite the large size of this binding site, only a single specific DNA-protein complex was detected by gel retardation analysis. This complex, however, appeared to be divisible into three protein-DNA contact sites by methylation interference analysis (Fig. 4B). In particular, this technique detected a strong core interaction centered on nucleotide +10 as well as two flanking interactions, one located immediately 5′ to the cap site and a second centered on nucleotide +19. Oligonucleotide competition analyses (Fig. 5) support the hypothesis that this interaction represents a single, highly cooperative binding event rather than three distinct protein-DNA interactions. In particular, mutation of only one of these three sites was, in the case of Δ1 and Δ2, sufficient to markedly reduce the ability of oligonucleotides to compete for protein binding to the wild-type leader DNA probe (Fig. 5). In addition, none of the oligonucleotide competition experiments led to the detection of a protein-DNA complex of increased mobility, as might be predicted if one of several binding proteins present in the complex were specifically competed. This finding is particularly noteworthy since recent evidence suggests that the TAR factor, renamed UBP-1 by Wu et al., consists of at least three distinct polypeptide species (40; our unpublished results).

The second aim of this study was to understand the importance of this leader DNA-binding event for *tat*-mediated *trans* activation of the HIV-1 LTR. The relevant observations are as follows: (i) coexpression of *tat* has no effect on the electrophoretic mobility or the extent of formation of the leader DNA-protein complex (Fig. 2); (ii) deletion of sequences 3′ to position +23, which have been shown to be critical for *tat*-mediated *trans* activation (11, 14, 17), has no effect on this protein-DNA-binding event (Fig. 3); and (iii) mutation of sequences shown by methylation interference analysis (Fig. 4) to have an important role in this DNA-protein interaction results in the predicted loss of this DNA-protein-binding event (Fig. 5). However, insertion of these same mutations into the HIV-1 LTR had no significant effect on either the basal or *tat trans*-activated level of HIV-1 LTR-specific gene expression (Table 1). In conclusion, we believe that the evidence presented here strongly suggests that this HIV-1 LTR DNA-protein interaction, although readily detectable and highly sequence specific, does not have a major role in the *tat*-mediated *trans* activation of HIV-1 gene expression.

The major impetus for this study was the hypothesis that a sequence-specific transcriptional *trans* activation must involve a sequence-specific DNA-protein interaction, yet our data suggest that the only readily detectable interaction in the area of the TAR element is not relevant to this *trans*-activation event. One explanation for this result is that a second, as yet undetected, HIV-1 leader DNA-protein interaction exists. Alternatively, *tat trans* activation might instead be mediated by recognition of TAR as an RNA, rather than a DNA, sequence. Indeed, recent results from a number of laboratories suggest that *trans* activation by *tat* is dependent on the integrity of a predicted RNA stem-loop structure that coincides with the TAR element (11, 17, 25).

Before completion of this work, Jones et al. (19) used a somewhat different approach, i.e., a combination of scanning mutagenesis and DNase I footprinting analysis, to examine the sequence requirements for protein binding to the HIV-1 LTR leader DNA region. In general agreement with our results, they conclude that this leader binding domain extends from −17 to +27 and that it can be further subdivided into three binding sites approximately centered on nucleotides −1, +11, and +18, respectively. They further propose that this interaction results from the presence of three equivalent binding sites for a single protein (leader binding protein, or LBP-1), each having the consensus 5′-$^{T}_{A}$CTGG-3′ (or 5′-CCAG$^{T}_{A}$-3′). Although our results do not directly address the validity of this latter hypothesis, it is of interest to note that the methylation interference patterns at these three sites are not identical; for example, methylation of the G at position +2 appears to have little effect, whereas methylation of the equivalent G at position +12 appears to greatly inhibit protein binding. However, the major distinction between our results and the data of Jones et al. is that we have been unable to confirm any role for this DNA-protein interaction in enhancing either the basal or *tat trans*-activated level of expression from the HIV-1 LTR promoter. In fact, mutagenesis of the HIV-1 LTR sequences responsible for the binding of LBP-1 resulted in no detectable phenotypic change, as determined by transient-expres-

sion assays (Table 1). Nevertheless, the demonstration by Jones et al. (19) that this binding event is fully conserved between HIV-1 and the related but distinct retrovirus HIV-2 does strongly suggest that this interaction has some physiological role. Potential functions include a role in DNA replication, in the organization of proviral chromatin structure (14), or perhaps in some aspect of HIV-1 proviral integration. It will be of interest to examine whether mutations that interfere with this binding event induce any detectable phenotypic change in the in vitro replication of HIV-1.

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