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Mutational Analysis of the *trans*-Activation-Responsive Region of the Human Immunodeficiency Virus Type I Long Terminal Repeat

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We used site-directed mutagenesis to delineate sequences within the human immunodeficiency virus type I (HIV-I) long terminal repeat (LTR) required for *trans*-activation by the viral *tat* gene product. We demonstrated that sequences 3′ to LTR position +44 are dispensable for *trans*-activation but that almost all of the mutations tested located between positions −17 and +44 greatly reduced *trans*-activation at both the transcriptional and posttranscriptional levels. However, displacement of the HIV-I LTR *trans*-activation-responsive region (TAR) 3′ by insertion of up to 32 base pairs between the LTR TATA box and cap site had little effect on *trans*-activation. An analysis of the DNase I hypersensitivity profile of the HIV-I LTR in transfected cultures suggested the presence of at least two DNase I-hypersensitive sites, including one which extends into the viral TAR element; however, neither of these sites appeared to be significantly affected by *tat* coexpression. These results allow more precise delineation of the sequences important for TAR function and suggest that the TAR may be recognized by a host-specific DNA-binding protein rather than by the *tat* protein directly.

The pathogenic human retrovirus human immunodeficiency virus type I (HIV-I) encodes not only the three structural genes (*gag*, *pol*, and *env*) common to all known replication-competent retroviruses but also at least four other, apparently nonstructural, gene products (4). Two of these, termed *art/trs* and *tat*, have been shown to act in *trans* to increase the level of synthesis of HIV-I-specific proteins (1, 5, 6, 8, 16, 22, 25, 30, 35, 39). In the case of *tat*, evidence has been presented for a bimodal mechanism of action which involves both an increase in the steady-state level of HIV-I-specific mRNA and an increase in the translational utilization of that mRNA (5, 39). More recently, it has been demonstrated that *tat* increases HIV-I mRNA levels by enhancing the rate of transcription rather than by stabilizing HIV-I-specific mRNAs (12). This result is consistent with the predominantly nuclear subcellular localization of *tat* protein (12). The means by which the second, posttranscriptional action of *tat* on HIV-I gene expression is effected remains unknown. However, evidence has been presented suggesting that HIV-I mRNAs are poorly utilized by the cellular translational machinery in the absence of *tat* coexpression (5, 8).

In this study, we attempted to address the mechanism of action of *tat* via mutational analysis of the HIV-I long terminal repeat (LTR) sequence originally identified as the core *trans*-activation-responsive (TAR) region (31). This sequence, which extends from −17 to +80 within the HIV-I LTR, exhibits a number of striking inverted and direct repeats (Fig. 1), and it has indeed been suggested that the LTR sequence from +1 to +59 can form a stable mRNA stem-loop structure important for the posttranscriptional component of HIV-I *trans*-activation (22, 24). Alternatively, direct repeats are frequently observed within transcriptional regulatory sequences such as enhancers or promoters (19), and therefore might also be involved in the transcriptional action of *tat*. By using constructions in which the HIV-I LTR was fused to a reporter gene, the human interleukin-2 (IL-2) gene (5), we measured the effects of different targeted LTR mutations on both the basal and *tat trans*-activated levels of IL-2 protein production in transfected cells. For several mutations, we also quantitated the steady-state level of the reporter gene mRNA and deduced the relative translational efficiencies of the mRNAs encoded by different mutants. These results allow more precise determination of the location of TAR and more accurate assessment of the roles of particular repeat sequences. Finally, we demonstrated the existence of at least two sites of DNase I hypersensitivity within the HIV-I LTR. The location of these sites, including one site which extends into the TAR region, was, however, not markedly affected by coexpression of the *tat* gene product.

MATERIALS AND METHODS

Construction of molecular clones. All molecular clones used in this work were derived from the vector pXF3/ori (5), which contains the simian virus 40 origin of replication inserted into the pBR322 derivative pXF3. The cytomegalovirus immediate-early promoter-based IL-2 expression vector pBC12/CMV/IL-2 and the *tat* expression vector pBC12/CMV/t2 have been previously described, as has the internal control plasmid pBC12ΔI (5). All HIV-I LTR mutants were derived from pBC12/HIV/IL-2, which contains the human IL-2 gene under control of a 728-base-pair (bp) HIV-I DNA fragment (5). This HIV-I sequence is derived from the replication-competent HXB-3 strain of HIV-I (27) and includes the entire LTR U3 region, as well as 84 bp of the LTR R region (Fig. 1). pBC12/HIV-Xho was derived from pBC12/HIV-IL-2 by oligonucleotide-directed mutagenesis by using the procedure described by Morinaga et al. (21). The oligonucleotide used, an 18-mer, introduced a contiguous 2-bp mutation at positions +10 and +11 (TG→GA) and created a unique *Xho*I site (Fig. 1). pBC12/HIV/IL-2 and pBC12/HIV-Xho were then used to

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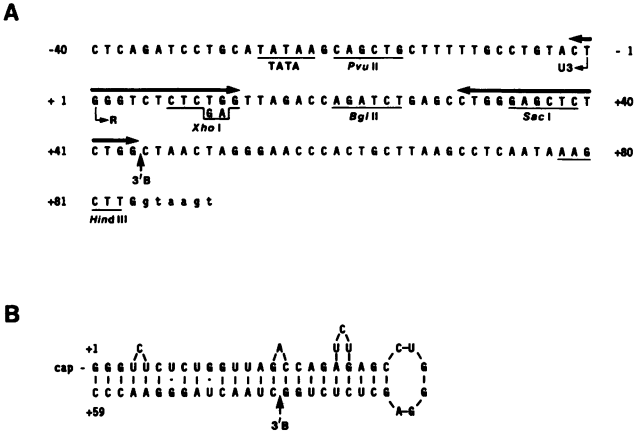

FIG. 1. Sequence of the HIV-I LTR TAR region included in pBC12/HIV/IL-2. (A) A partial sequence of the LTR of the HXB3 strain of HIV-I used in this report is shown (capital letters). This sequence is identical to the HXB3 sequence previously published by Ratner et al. (28), except at position +52, where we observed a G rather than an A. A G has been observed in this position in some other HIV-I isolates (32, 38). Also shown are the locations of relevant restriction sites, including the *Xho*I site introduced by oligonucleotide-directed mutagenesis at position +9. The TATA box and the 3' border of the core TAR region mapped in this work (3'B) are indicated. The location of an imperfect direct repeat which is discussed in the text is also shown (↔). (B) A possible secondary structure involving the first 59 nt of the HIV-I mRNA is shown. This structure has an estimated free energy of ~37 kcal/mol (22, 24) and has been shown to occur in a synthetic SP6 transcript by in vitro enzymatic analysis (22). The residual stem present in the pD+45/+77 mutant, which is deleted to the location indicated by 3'B, has a free energy of ~17 kcal/mol as determined by the procedure of Tinoco et al. (36).

generate series of internal HIV-I LTR deletion (pD) mutants by cleavage at various restriction enzyme sites, followed by blunt ending with Klenow DNA polymerase I and religation. Mutants generated by this procedure include pD−18/+7 (*Pvu*II to *Xho*I), pD−18/+20 (*Pvu*II to *Bgl*II), pD+9/+20 (*Xho*I to *Bgl*II), pD+9/+38 (*Xho*I to *Sac*I), pD+9/+77 (*Xho*I to *Hin*dIII), pD+25/+38 (*Bgl*II to *Sac*I), and pD+35/+38 (*Sac*I site deletion). In each case, the numbers in the plasmid designation describe the inclusive extent of the deletion (Fig. 1). A second set of HIV-I LTR deletion mutants was derived by processive BAL 31 digestion of pBC12/HIV/IL-2 after cleavage with *Hin*dIII. After BAL 31 digestion (International Biotechnologies, Inc.; slow form), the DNA was blunt ended with Klenow DNA polymerase I and ligated to a *Hin*dIII linker (5'-GAAGCTTC-3'). The DNA was then cleaved with *Hin*dIII and with *Mst*II, which cuts at a unique site in the HIV-I DNA at position −526. The resultant fragments of <608 bp were isolated from a preparative 2% agarose gel and recloned into the larger parental pBC12/HIV/IL-2 vector *Hin*dIII-*Mst*II fragment. The resultant clones were characterized as to deletion size and tested for *trans*-activation phenotype. Only the two smallest deletions with a negative phenotype (pD+42/+77 and pD+44/+77) and the two largest deletions with a positive phenotype (pD+45/+77 and pD+46/+77) are described. The final four LTR mutants, termed pI mutants, were derived by insertion of nucleotide sequences at different locations within the LTR. pI4/+7 (a 4-nucleotide [nt] insertion at position +7) was derived by filling in the *Xho*I site in pBC12/HIV-Xho. Similarly, pI4/+20 was derived by filling in the *Bgl*II site in pBC12/HIV/LTR. pI8/−18 and pI32/−18 were derived from pBC12/HIV/IL-2 via a two-step process. Initially, the unique HIV-I LTR *Hin*dIII site was filled in to generate pI4/+78. This clone, which has an *Nhe*I site at position +82 in place of the *Hin*dIII site, exhibits the same *trans*-activation response as the wild-type clone pBC12/HIV/IL-2 (data not shown). Subsequently, one or four of the 8-nt *Hin*dIII linkers were introduced into the unique LTR *Pvu*II site at position −18 to yield, respectively, pI8/−18 and pI32/−18. All of the mutants were sequenced by the dideoxynucleotide sequencing procedure (33) after being subcloned into the vector M13mp9 (20).

Cell culture and DNA transfection. COS cells were maintained as previously described (5) and were transfected with 2.5 μg of plasmid DNA per 100-mm (diameter) culture with DEAE-dextran and chloroquine (5). Cell culture medium and transfected-cell RNA were harvested at 72 h after transfection. Secreted IL-2 levels were measured by using the IL-2-dependent murine T-cell line CTLL (5, 29).

RNA analysis. Total RNA was prepared from transfected cultures (5), and RNA samples, as indicated in the text, were then used to quantitate the level of RNA expression by using S1 nuclease protection analysis (5). The S1 probes used have been previously described (5). The 750-nt 3'-end-labeled probe used (see Fig. 2) rescues a 175-nt fragment derived from the RNA encoded by the IL-2 expression vectors, as well as a 156-nt fragment derived from the RNA encoded by the internal reference vector pBC12ΔI. We have previously shown that the level of RNA encoded by Rous sarcoma virus LTR-based pBC12ΔI is unaffected by *tat* coexpression (5).

The probes used (see Fig. 3) were uniquely 5' end labeled either at position +82 at the wild-type HIV-I LTR *Hin*dIII site or at +84 at the introduced *Nhe*I site of pI32/−18. The probes extend to the HIV-I LTR U3 region *Ava*I site (−159 in pBC12/HIV/IL-2). Transcripts initiating within the HIV-I LTR rescue a probe fragment whose length is determined by the distance of the transcript initiation site from the site of the label (5). The RNA derived from HIV-I-infected H9 cells (26; see Fig. 3) was a gift of M. Dukovich and W. Greene.

DNase I hypersensitivity analysis. Nuclei from transfected cultures were prepared at 72 h posttransfection before digestion with various amounts of DNase I (Worthington Diagnostics; DPFF) (9, 10). Treatment of the nuclear DNA with the restriction enzyme *Bam*HI, agarose gel electrophoresis, Southern blotting, and filter hybridization with the nick-translated probe were carried out as described by Fritton et al. (9, 10). A 261-bp *Xba*I-to-*Bam*HI human IL-2 gene fragment derived from pBC12/HIV/IL2 was used for preparation of the probe used for hybridization.

RESULTS

Effect of TAR mutations on reporter gene protein synthesis. The expression vector pBC12/HIV/IL-2 contains a reporter gene, the human IL-2 gene, under control of a 728-bp HIV-I DNA fragment which includes the entire 456-bp HIV-I LTR U3 region, as well as 84 bp of the LTR R region (5; Fig. 1). The core TAR sequence has previously been shown to be contained within an HIV-I LTR sequence extending from approximately −17 to +80 (31; where the site of transcription initiation is +1). Our initial approach to the determination of the functional significance of different regions of TAR was processive 5' deletion from the HIV-I LTR *Hin*dIII site at +80, with BAL 31 exonuclease, to determine the site of the 3' border of the TAR sequence. It has previously been shown that deletion of sequences 3' to position +37 results

in loss of *tat* responsiveness by the HIV-I LTR (5, 39). Numerous deletion mutants were characterized, and the two smallest deletions that lacked a significant *tat* response (pD+42/+77 and pD+44/+77) and the two largest deletions which demonstrated *tat* responsiveness (pD+45/+77 and pD+46/+77) were sequenced and characterized (Table 1). The results demonstrated that all four BAL 31 deletion mutants exhibited approximately the same level of IL-2 protein expression in the absence of *tat*. However, pD+42/+77 and pD+44/+77 yielded only a small, about two fold *trans*-activation of IL-2 expression in the presence of *tat*. The two mutants pD+45/+77 and pD+46/+77, which differ by only 1 or 2 nt, respectively, from the negative deletion mutant pD+44/+77, in contrast, yielded very significant (~24-fold) *trans*-activation in response to *tat*. This is slightly lower than that observed for the wild-type clone pBC12/HIV/IL-2, thus suggesting some role for sequences 3′ to position +44. Nevertheless, these results allowed placement of the 3′ border of the core TAR element at nt +44 within the HIV-I LTR R region (Fig. 1).

Several other BAL 31 deletion mutants were also examined and yielded the expected *trans*-activation phenotype; i.e., all of the deletions tested extending 5′ to +45 were inactive, whereas all of the deletions terminating 3′ to +44 were active (data not shown). One extensive deletion, pD+9/+77, which removes essentially the entire HIV-I LTR R region, including the sequences proposed to form a stable mRNA stem-loop, was examined in detail (Table 1). As expected, this deletion was not responsive to *tat*. Interestingly, this mutation still demonstrated the same basal level of IL-2 expression seen with the wild-type construction pBC12/HIV/IL-2.

The HIV-I LTR contains a number of direct and inverted repeats. An almost perfect 14-bp direct repeat occurs between positions −2 and +12 and again between +30 and +44 (Fig. 1). This particular repeat is of interest because of the above observation that the 3′ border of the second repeat element precisely coincides with the 3′ border of the TAR core sequence. To address the significance of these and other reported repeat elements, we constructed a number of additional mutants between HIV-I LTR positions −17 and +38 within pBC12/HIV/IL-2. Initially, we used oligonucleotide-directed mutagenesis (21) to generate a 2-bp change at positions +10 and +11 within repeat element 1 (Fig. 1). This LTR mutant, termed pBC12/HIV-Xho, demonstrated a *trans*-activation phenotype indistinguishable from that of the parental clone (Table 1). Subsequently, we used pBC12/HIV-Xho and pBC12/HIV/IL-2 to generate a series of deletion mutations between the different restriction enzyme sites present within the TAR region (Fig. 1). All of these deletion mutants yielded an essentially negative *trans*-activation phenotype (i.e., ≤ twofold), except for pD+9/+20, which demonstrated a low but significant *trans*-activation of about fourfold (Table 1). Of particular interest is the small-deletion mutant pD+35/+38, which lost 4 bp from within the 3′ repeat element and which was *trans*-activation negative. All of the deletion mutants retained essentially the same basal level of expression as the parental clone pBC12/HIV/IL-2.

TABLE 1. Levels of expression from the HIV-I LTR mutants in the presence or absence of *tat*[a]

Clone transfected	IL-2 production (U/ml)		Avg *trans*-activation[b]
	−*tat*	+*tat*	
BC12/HIV/IL-2	96	6,148	64
pD+46/+77	192	6,148	24
pD+45/+77	128	3,072	23
pD+44/+77	128	192	2.1
pD+42/+77	96	192	1.8
pD+9/+77	128	192	1.4
pBC12/HIV-Xho	96	6,148	64
pD−18/+7	128	192	2.0
pD−18/+20	96	192	1.5
pD+9/+20	192	768	4
pD+9/+38	192	192	1.0
pD+25/+38	96	96	1.0
pD+35/+38	128	192	1.4
pI4/+7	192	6,148	32
pI4/+20	192	1,536	8
pI8/−18	128	4,096	28
pI32/−18	256	3,072	14

[a] Cultures were transfected with equimolar amounts of each HIV-I construction, together with either the *tat* expression vector pBC12/CMV/t2 or the negative control vector pXF3/ori. Secreted IL-2 levels were determined at 72 h posttransfection, and results obtained in a representative experiment are shown. Mock-transfected cultures yielded no detectable IL-2 activity (<2 U/ml).

[b] The average level of *trans*-activation of HIV-I LTR-driven IL-2 expression due to *tat* coexpression is shown (average of two to six independent transfections).

A final set of mutants was constructed which contained small insertions within the TAR sequence. Insertion of 4 bp at position +7 (pI4/+7) had little effect on the *trans*-activation phenotype; however, insertion of 4 bp at position +20 (pI4/+20) resulted in a significantly reduced level of *trans*-activation (about eightfold). Two mutants, pI8/−18 and pI32/−18, contained different size insertions 5′ to the cap site at position −18 adjacent to the TATA box (Fig. 1). Both of these mutants demonstrated levels of *trans*-activation slightly but significantly lower than that of the wild-type construction. In the case of pI32/−18, in particular, this reduction appeared to result in part from a modestly (two- to threefold) higher level of basal IL-2 expression.

Effect of TAR mutations on reporter gene mRNA levels. In the previous section, we examined the level of reporter gene expression at the protein level for a number of HIV-I LTR mutants in the presence or absence of *tat*. It has been suggested that *tat*-mediated enhancement of HIV-I-specific gene expression is due to both an increase in HIV-I mRNA levels and an increase in the utilization of that mRNA by the cellular translational machinery (5, 39). To examine also the phenotype of the HIV-I LTR mutants at these levels, we performed an internally controlled S1 nuclease protection assay (5) to quantitate the level of HIV-I-specific IL-2 mRNA in cultures transfected with several of the HIV-I mutants described above (Fig. 2). In addition, we examined the parental vector pBC12/HIV/IL-2 and a control, *tat*-nonresponsive construction (pBC12/CMV/IL-2) which contains the IL-2 gene driven by the active human cytomegalovirus immediate-early promoter (5). The results (Fig. 2; Table 2) demonstrated that pBC12/HIV/IL-2, pD+46/+77, and pD+45/+77 all responded to *tat* with a marked increase in IL-2 mRNA steady-state levels. In contrast, pD+42/+77 yielded only a slight increase in IL-2 mRNA, whereas pD+9/+77 was almost nonresponsive to *tat*. The cytomegalovirus immediate-early promoter was, as expected, highly transcriptionally active in both the presence and absence of *tat*.

The S1 protection analysis presented in Fig. 2 used a 3′-end-labeled probe specific for the 3′ noncoding region of the IL-2 reporter gene mRNA. This probe has the advantage that it also allows determination of the level of an internal

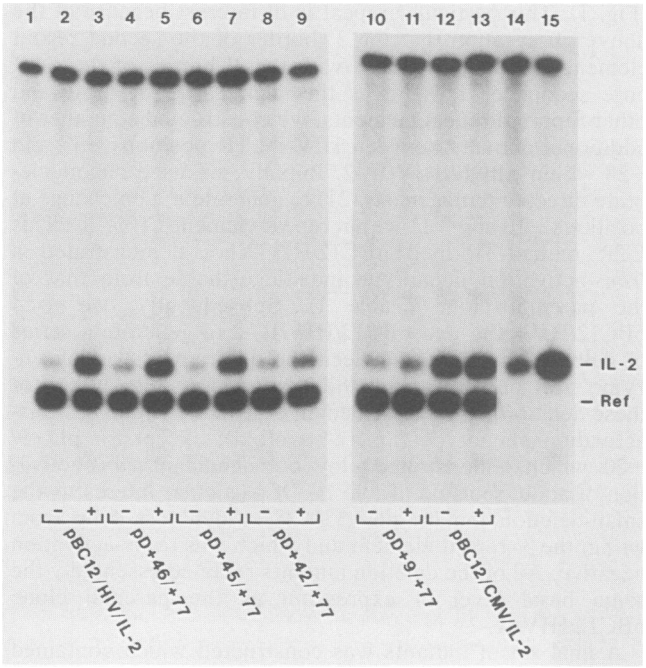

FIG. 2. Analysis of the effect of TAR mutations on the *trans*-activation of HIV-I mRNA expression. COS cells were transfected with the different vectors indicated in the absence (−) or presence (+) of the *tat* expression vector pBC12/CMV/t2. Total RNA was harvested 72 h after transfection, and 4-μg samples were used in the quantitative S1 nuclease protection assay visualized here (except for lanes 14 and 15; see below). Levels of IL-2 mRNA (175-nt protected probe fragment) and reference mRNA (Ref; 156-nt protected probe fragment) in the culture are proportional to the level of rescued probe (5; Table 2). All of the cultures except the negative control (lane 1) were also transfected with equal amounts of the reference plasmid pBC12ΔI. Lanes: 1, cells transfected with pBC12/CMV/t2 alone (negative control); 14, 2 μg of positive control IL-2 mRNA; 15, 20 μg of positive control IL-2 mRNA.

control insulin mRNA encoded by the *tat*-nonresponsive Rous sarcoma virus LTR-based reference plasmid pBC12ΔI (5, 12). We also examined (Fig. 3) the RNA levels encoded by PBC12/HIV/IL-2 and pI32/−18 in the presence or absence of *tat* by using S1 probes 5′ end labeled at the location of the HIV-I LTR *Hin*dIII site (Fig. 1). These probes are able to map transcription initiation (cap) sites used by the HIV-I transcripts (5). These data confirmed the large increase in HIV-I-specific mRNA induced by *tat* cotransfection in pBC12/HIV/IL-2-transfected cells (Fig. 2) and also demonstrated that the cap site used in the pBC12/HIV/IL-2-transfected cells was the same as that in HIV-I-infected H9 cells. In contrast, the site of transcription initiation used in pI32/−18-transfected cells, which contain a 32-bp insertion mutation between the HIV-I LTR TATA box and the normal cap site, was displaced 5′ by ~32 bp into the inserted sequence. In addition, pI32/−18-specific mRNA levels appeared somewhat lower than those encoded by pBC12/HIV/IL-2 in both the presence and absence of *tat* (Table 2). Nevertheless, these results clearly demonstrated that shifting the site of transcription initiation within the HIV-I LTR does not necessarily significantly affect *trans*-activation by the viral *tat* gene product (Table 1; Fig. 3).

Effect of *tat* on reporter gene mRNA translational efficiency. It has been proposed that the reported enhancement in the translational efficiency of HIV-I-specific mRNAs in the presence of *tat* is due to relief of a specific translational

TABLE 2. Effect of HIV-I LTR mutations on indicator gene mRNA and protein levels

Clone transfected[a]	IL-2 mRNA U (%)[b]	IL-2 protein U/ml (%)[c]	IL-2 protein/ mRNA ratio[d]
−*tat*			
pBC12/HIV/IL-2	203 (5.4)	48 (0.4)	0.072
pD+46/+77	360 (9.6)	64 (0.6)	0.054
pD+45/+77	250 (6.7)	48 (0.4)	0.059
pD+42/+77	232 (6.2)	96 (0.8)	0.126
pD+9/+77	237 (6.3)	48 (0.4)	0.062
pI32/−18	127 (3.4)	128 (1.0)	0.31
pBC12/CMV/IL-2	3,749 (100)	12,286 (100)	1.00
+*tat*			
pBC12/HIV/IL-2	2,581 (56)	3,072 (25)	0.45
pD+46/+77	2,491 (54)	1,536 (13)	0.24
pD+45/+77	1,849 (40)	768 (6.3)	0.16
pD+42/+77	794 (17)	192 (1.6)	0.09
pD+9/+77	512 (11)	48 (0.4)	0.04
pI32/−18	521 (11)	1,536 (13)	1.11
pBC12/CMV/IL-2	4,629 (100)	12,286 (100)	1.00

[a] Equimolar levels of the reference plasmid pBC12ΔI were also cotransfected in each culture. In addition, the negative control plasmid pXF3/ori was cotransfected in the −*tat* cultures, and pBC12/CMV/t2 was cotransfected in the +*tat* cultures. A culture transfected with pBC12/CMV/t2 alone yielded no detectable IL-2 mRNA (Fig. 2) or protein (<2 U/ml).

[b] IL-2 mRNA levels were determined by scanning of the autoradiographs shown in Fig. 2 and 3 with an LKB soft-laser scanner. The values are corrected for slight differences observed in the intensity of the reference RNA signal. Activity is given in arbitrary units.

[c] Secreted IL-2 protein levels were determined by bioassay (29).

[d] The ratio of IL-2 protein to IL-2 mRNA determined in each culture is expressed relative to pBC12/CMV/IL-2, which is arbitrarily set at 1.00.

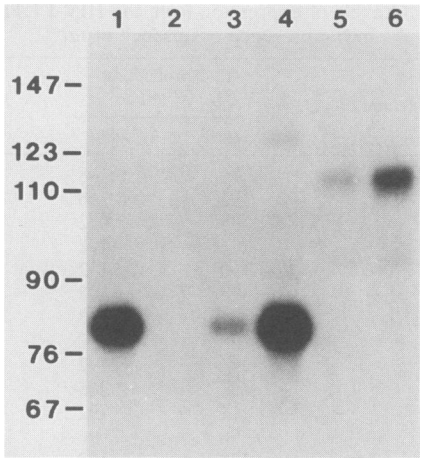

FIG. 3. Analysis of the HIV-I mRNA transcription start sites in transfected cells. RNA from transfected COS cells was harvested at 72 h posttransfection, and 10-μg samples were used in the quantitative S1 nuclease protection assay visualized here. The size of the rescued probe fragment was determined with reference to *Msp*I-cleaved pBR322 DNA size markers and measures the distance of the transcription start site from the site of labeling at position +82 (see Materials and Methods for details). Lanes: 1, 0.5 μg of poly(A)$^+$ mRNA from HIV-I-infected H9 cells (a gift of M. Dukovich and W. Greene); 2, pBC12/CMV/IL-2-transfected COS cells (negative control); 3, pBC12/HIV/IL-2 + pXF3/ori; 4, pBC12/HIV/IL-2 + pBC12/CMV/t2; 5, pI32/−18 + pXF3/ori; 6, pI32/−18 + pBC12/CMV/t2. The numbers on the left indicate molecular size in nucleotides.

inhibition (5, 8). To examine this question, we compared the ratios of IL-2 protein to IL-2 mRNA levels produced after transfection of cultures with several of the HIV-I LTR constructs (Table 2). This ratio is a measure of the efficiency of translational utilization of each mRNA molecule by the cell (5).

As previously described (5), the parental pBC12/HIV/IL-2 construction yielded an IL-2 mRNA which, in the absence of *tat*, was translated much less efficiently (~10- to 25-fold) by the cell than was the IL-2 mRNA encoded by the cytomegalovirus immediate-early vector. All of the deletion (pD) mutants yielded similar low translational efficiencies, including the extensively deleted mutant pD+9/+77. In the presence of *tat*, the three *tat*-responsive clones (pBC12/HIV/IL-2, pD+46/+77, and pD+45/+77) all showed significant increases in mRNA translational efficiency. In contrast, the translational efficiencies of the RNAs encoded by the two essentially nonresponsive clones pD+42/+77 and pD+9/+77 were unaffected by *tat*. This suggests that the transcriptional and posttranscriptional effects of *tat* are tightly linked. The mutant pI32/−18 produced an interesting phenotype. In the absence of *tat*, the mRNA encoded by pI32/−18 was present at a slightly lower steady-state level (Fig. 3) yet encoded a higher level of IL-2 protein than did the other LTR mutants tested (Table 2). In the presence of *tat*, the level of this mRNA increased only modestly, to a level essentially identical to that of the *tat*-nonresponsive clone pD+9/+77. The level of IL-2 protein encoded by pI32/−18 mRNA was, however, ~32-fold higher than that encoded by pD+9/+77 mRNA, thus suggesting a large difference between the translational efficiencies of these two similar mRNA molecules.

Does *tat* affect the location of DNase I-hypersensitive sites within the HIV-I LTR? The *trans*-acting gene product *tat* enhances HIV-I-specific gene expression in part by increasing the steady-state level of HIV-I-specific mRNAs (5, 22, 25, 39). This increase, which is due to an increase in the rate of HIV-I mRNA transcription (12), must be mediated by the sequences located within the LTR TAR sequence. A property which can be associated with shifts in the level of transcription of a gene is a shift in the pattern of DNase I hypersensitivity (7, 13). This is believed to be due to changes in the components of the chromatin which enclose the gene. We therefore examined the effect of *tat* on the hypersensitivity profile of the HIV-I LTR within transfected cells (Fig. 4). COS cells were transfected with pBC12/HIV/IL-2 in the presence or absence of the *tat* expression vector pBC12/CMV/t2, and permeabilized nuclei were prepared at 72 h after transfection (9, 10). The nuclei were treated with various levels of DNase I, and the nuclear DNA was then isolated and cleaved with *Bam*HI. This restriction enzyme cuts the 4.7-kilobase pBC12/HIV/IL-2 vector at a unique site located at position +765 relative to the HIV-I LTR cap site. The isolated DNA was subjected to electrophoresis on a 1.1% agarose gel and transferred to a nitrocellulose filter. The hypersensitive sites were visualized with a probe prepared against a 261-bp *Xba*I-to-*Bam*HI fragment derived from the IL-2 indicator gene coding region.

Two hypersensitive sites were observed within the HIV-I LTR by this procedure. The location and intensity of these sites were not markedly affected by *tat* coexpression, although the faster-migrating band did appear slightly more diffuse in the presence of *tat* protein (Fig. 4, lane e). No specific bands were seen after probing of DNase I-treated nuclei derived from mock-transfected COS cells or when naked pBC12/HIV/IL-2 DNA was used (data not shown).

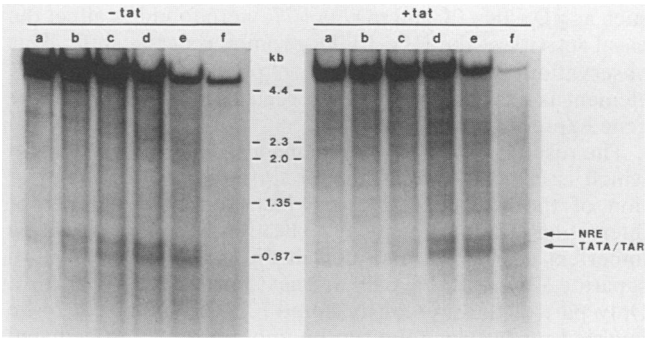

FIG. 4. DNase I-hypersensitive chromatin sites in the HIV-I LTR. The autoradiograph visualizes DNase I-hypersensitive sites located within the HIV-I LTR in COS cells transfected with pBC12/HIV/IL-2 with (+tat) and without (−tat) the *tat* expression vector pBC12/CMV/t2. *Tat* coexpression resulted in a 64-fold increase in IL-2 expression. The slower-migrating band maps to position −175 to −125 in the HIV-I LTR, the site of a possible negative regulatory element (NRE). The faster-migrating band maps to HIV-I LTR position −45 to +25, the site of the TATA box and the TAR element (Fig. 1). Lanes: a, no DNase I; b, c, d, e, and f, 128, 256, 512, 1,024, and 2,048 U of DNase I, respectively. kb, Kilobases.

The location of the two DNase I-hypersensitive sites was carefully mapped in relation to DNA size markers (*Hin*dIII-cleaved phage λ DNA and *Hae*III-cleaved φX174 DNA) which were visualized by reprobing of the blot with the appropriate nick-translated phage DNA probes. This measurement indicated that the larger band was 910 ± 25 bp in size, whereas the smaller band was 775 ± 35 bp in size. This correlates with the HIV-I LTR locations −175 to −125, the possible site of a negative regulatory element (11, 34a), and to −45 to +25 bp, which coincides with the HIV-I LTR TATA box and part of the TAR element.

DISCUSSION

In this study, we used site-directed mutagenesis of the HIV-I LTR, combined with a transient-expression assay in the HIV-I-permissive (8, 18) monkey cell line COS, to examine the roles of specific LTR sequences in *trans*-activation by the viral *tat* gene product. Because this *trans*-activation event occurs at least in part at the transcriptional level (12) and repeat structures have been shown to be important in the regulation of expression from several transcription control regions (19), we focused particularly on the functional significance of inverted and direct repeats present within the core TAR sequence (Fig. 1). Initially, we used processive deletion mutagenesis to define the 3′ border of the core TAR element at position +44 relative to the cap site. These data confirm previous results showing that the 3′ border of TAR lay between +31 and +54 (22). Subsequently, we used deletion and insertion mutagenesis to examine the roles of different areas within the TAR sequence between −18 and +44 (Table 1). These studies revealed that most mutations of this region dramatically reduced the *trans*-activation response. Two minor mutations at about +10, pBC12/HIV-Xho and pI4/+7, which disrupt the first of the two direct repeat elements noted in Fig. 1, were, however, found to have little or no effect. Also of interest is pD+9/+20, which is the largest internal TAR deletion mutation to permit some level of residual *trans*-activation. None of the TAR mutations, including extensive deletions

such as pD−18/+20 and pD+9/+77, were found to affect the basal activity of the HIV-I LTR promoter significantly. This observation argues against the hypothesis that the TAR element is a *cis*-acting negative regulator of HIV-I-specific gene expression.

The results presented here define a small, ~60-bp element which is critically important for *tat*-mediated *trans*-activation of the HIV-I LTR and which retains a number of interesting structural features. These include the two 14-bp imperfect direct repeats noted in Fig. 1, which are in turn separated by a 10-bp palindrome (5′-TAGACCAGAT-3′). Only part of the previously noted (22, 24) imperfect 24-bp inverted repeat was found to be important for *tat*-mediated *trans*-activation of HIV-I gene expression; however, this could still form the basis for a 9-bp hairpin loop structure (Fig. 1B). The limited mutational analysis described here appears to focus attention on the second of the two direct repeats (Fig. 1), since all deletions involving this element, including pD+35/+38 and pD+44/+77, which affect this element only slightly, result in almost total abrogation of the *trans*-activation response. It will be of interest to determine whether synthetic copies of this element can confer a *trans*-activation response on heterologous promoters when present in *cis*.

It has previously been shown that the function of the TAR element is both position and orientation dependent (22, 25), thus distinguishing TAR from inducible enhancer elements which have been described for other systems (19). One possible function of TAR, by analogy to other transcription control regions (3, 15, 34), is as a binding site for a virus- or host-specific transcription factor. Occupation of TAR by this protein would, in turn, stabilize binding of transcription factors to LTR U3 region sequences. In simian virus 40 late-gene *trans*-activation by simian virus 40 T antigen, stable binding of transcription factors to T antigen-binding site II and the simian virus 40 72-bp repeat element occurs only when the two binding sites are correctly and closely spaced (3). Insertion of as little as 42 bp totally abrogated functional binding, and even a 4-bp insertion produced a significant effect (3). To test whether the proximity of TAR to the body of the HIV-I LTR transcription control region in the LTR U3 region was critical for function, we changed this distance by insertion of 8 or 32 bp between the TATA box and the core TAR region (pI8/−18 and pI32/−18). These mutations resulted in only a modest decrease in *trans*-activation and therefore suggest that the position of TAR within the HIV-I LTR is flexible to at least some degree.

Because *trans*-activation appears to involve transcriptional activation of the HIV-I LTR (12), we examined the DNase I hypersensitivity profile of the LTR and asked whether this was affected by *tat* coexpression. Our results (Fig. 4) revealed two areas of hypersensitivity within the HIV-I LTR which mapped to −45 to +25 bp (3′ site) and to −175 to −125 bp (5′ site) but were not markedly affected by *tat* coexpression. The location of the 3′ site extends from ~15 bp 5′ to the TATA box into the TAR region sequences and the HIV-I LTR R region. The 5′ site does not accord with any known important structure within the HIV-I LTR; however, deletion of this sequence has been associated with elevated expression from the HIV-I LTR, and this may, therefore, be a negative regulatory element (11, 34a). No signal was noted at the site of the HIV-I LTR Sp1 binding sites (14) (about −50 to −80) or at the location of the inducible HIV-I enhancer element (23) (about −80 to −105). However, because only a subfraction of the introduced DNA may be fully transcriptionally active in this transient-expression assay, it is possible that this assay cannot detect a weak but significant signal at these sites.

It is of interest to compare the in vivo DNase I hypersensitivity analysis presented here with a recent study on the location of DNase I-protected sites (footprints) in the HIV-I LTR after in vitro incubation with a HeLa cell nuclear extract (11). This report identified three protected sites, of which the most 5′, extending from −173 to −159, appears coincident with the 5′ site noted in Fig. 4. A second weakly protected site, located at −97 to −78, was not noted in our data. Of particular interest is a third protected site, extending from −42 to +28 in the HIV-I LTR coding strand and as far as +52 in the noncoding strand (11). This protection of the TAR sequence closely mirrors both the location of the DNase-hypersensitive 3′ site noted in Fig. 4 (−45 to +25) and the location of the sequences required for *tat trans*-activation as defined by the above mutational analysis. The close correlation between these in vivo data and the previously published in vitro DNase I footprint analysis strongly suggests the existence of a cellular TAR sequence-binding protein in cells that do not express the *tat* gene product. Recent reports indicate that a number of viral and cellular *trans*-acting transcriptional activators, including the *trans*-activator (*tatI* or $p40^x$) encoded by the human retrovirus human T-cell lymphotropic virus type I, may act via cellular sequence-specific DNA-binding proteins (2, 13a, 17, 37). The evidence for a constitutive cellular TAR sequence-binding protein may suggest a similar mechanism of action for the HIV-I *tat* gene product.

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