Linker-Scanning Mutational Analysis of the Transcriptional Activity of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat

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We have compared the relative importance of transcription regulatory regions in the U3 and R regions of the human immunodeficiency virus type 1 long terminal repeat (LTR) by using linker-scanning mutational analysis. Twenty-six mutant LTR-chloramphenicol acetyltransferase (CAT) transient expression plasmids were prepared in which consecutive 18-bp regions of wild-type LTR were replaced with an *NdeI-XhoI-SalI* (NXS) polylinker. The mutant LTR-CAT plasmids were transfected into unstimulated Jurkat cells, Jurkat cells stimulated with phytohemagglutinin and tetradecanoylphorbol acetate, and Jurkat cells which constitutively express the human immunodeficiency virus type 1 *trans*-activator protein, Tat. Transcriptional activity was measured by analysis of CAT activity. The activities of these mutants identified one major and several minor transcription control elements in addition to previously identified elements. In addition, this fine-structure analysis identified differences in utilization of regulatory regions between unstimulated, stimulated, and Tat-expressing Jurkat cells. A significant regulatory region was indicated by linker-scanning mutations between nucleotides –183 and –130 (relative to the transcription start site, +1). These mutations caused marked decreases in activity of the LTR in unstimulated and especially in stimulated Jurkat cells but had no effect in Tat-expressing Jurkat cells. DNA mobility shift studies comparing probes of wild-type and mutant sequences in the –183 to –130 region indicated that alterations in specific DNA binding correspond to the altered transcriptional activity of the mutants. The effects of mutations in several regulatory regions, in addition to the –183 to –130 region described above, differ between Tat-expressing and -nonexpressing Jurkat cells. For example, the NF- κ B sites are necessary for transcription in both Tat-expressing and -nonexpressing cells. However, Tat-expressing Jurkat cells primarily require only the 3'-proximal site, while both stimulated and unstimulated Jurkat cells appear to require both sites. Mutants downstream of the TATA element cause a more significant decrease in activity in Tat-expressing Jurkat cells than in the others. Finally, several mutations in the 5' half of the LTR (–453 to –184) show modest increases in transcription (1.5-fold or less) in unstimulated Jurkat cells only, suggesting possible negative regulatory sites. In summary, our studies have identified a control region (–183 to –130) upstream of the NF- κ B sites and have more precisely defined significant differences in the utilization of regulatory regions between unstimulated, stimulated, and Tat-expressing Jurkat cells.

The U3 and R regions of the human immunodeficiency virus (HIV) long terminal repeat (LTR) contain over 450 bp of sequence which is moderately conserved between viral isolates (26, 30, 45). These sequences partially encode the viral protein Nef, serve as a polyadenylation signal in the 3' LTR (44), and function in viral integration and in the 5' LTR principally function in the regulation of viral transcription (Fig. 1). Several transcription regulatory elements have been identified in the HIV LTR (see references 6, 17, 30, and 45 for reviews). The identities and functions of these regulatory elements have been defined by using experimental approaches such as (i) transient expression experiments with the LTR promoting transcription of reporter genes, (ii) *in vitro* transcription experiments, and (iii) mutant virus studies. The best-understood regulatory sequences include the NF- κ B direct repeats, the Sp1 sites, the TATA element, and the *tat*-responsive element, TAR (Fig. 1). In addition, the LTR contains a number of potential regulatory sequences

which have been identified through (i) sequence homologies to known regulatory sites, (ii) protein binding studies, or (iii) mutational analyses.

Despite the extensive studies already performed identifying transcriptional regulatory regions in the LTR, there has been no systematic fine-structure mutational analysis of the entire LTR. The regulatory elements described above, both real and potential, were defined by using not only a variety of techniques but also a variety of different cell types. In this study, we have systematically analyzed the transcriptional activity of linker-scanning mutants which sequentially replace 18 bp at a time across the U3 and R regions of the LTR. The linker-scanning method of mutational analysis (24) provides a more accurate assessment of a promoter's regulatory sequences than does deletion analysis, since the effects of relatively small substitution mutations are analyzed in the spatial context of the remaining wild-type sequences. Deletion mutagenesis, which has been used in many analyses of the HIV LTR, results in the juxtaposition of distant sequences. Such positioning of elements, either from the promoter or from neighboring plasmid sequences, may be neither spatially nor contextually relevant to the promoter

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under study and may result in artifactual transcription activation or inactivation.

The transcriptional activities of the LTR linker-scanning mutants have been determined by transient transfection analysis in the established human T-cell line, Jurkat. Three growth conditions were used: (i) normal, quiescent, unstimulated Jurkat cells; (ii) Jurkat cells stimulated with phytohemagglutinin (PHA) and the phorbol ester tetradecanoylphorbol acetate (TPA); and (iii) Jurkat cells which constitutively produce the HIV *trans*-activator protein, Tat. The data described below confirm the importance of the NF- κ B, Sp1, and TATA sequences in all three conditions. They also show that sequences between the TATA element and the TAR region are required for optimal expression in unstimulated, in stimulated, and particularly in Tat-expressing Jurkat cells. The fine-structure mutational analyses have identified a previously uncharacterized regulatory region (between -183 and -130), upstream of the NF- κ B sites, which is required for maximal expression in unstimulated and stimulated Jurkat cells but not in Tat-expressing Jurkat cells. In addition, the data indicate regions in the 5' half of the LTR which may mediate very modest negative regulatory effects only in unstimulated Jurkat cells. Finally, the linker-scanning analysis illustrated the differences in the transcriptional utilization of specific regions of the LTR depending on the cellular conditions.

MATERIALS AND METHODS

Cells. Jurkat cells and Jurkat-tat cells (31), the gift of W. Haseltine, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Jurkat cells were stimulated with TPA and PHA as previously described (27) 24 h after transfection. Transfections continued for an additional 24 h after stimulation.

Plasmid constructions. p5'LTR-CAT (a gift from L. Bachlor, DuPont) contains the U3 and R regions (to the *Hind*III site) from the 5' LTR of the HXB2 clone of HIV. These regions are inserted upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene of pSV0-CAT (13). pXLTR-CAT was derived from p5'LTR-CAT by deleting the 5'-flanking plasmid sequences (between the *Acc*I and *Hin*FI sites) and replacing them with an *Xba*I linker at a position 61 bp upstream of the start of the U3 region (Fig. 1).

The linker-scanning mutants, which consecutively replaced 18 bp of wild-type sequence with an *Nde*I-*Xho*I-*Sal*I (NXS) polylinker (CATATGCTCGAGGTCGAC) across the U3 and R regions, were constructed by using a polymerase chain reaction (PCR)-directed technique (49). Briefly, two external primers, common to all mutants, were synthesized; one was complementary to sequences 5' of the *Xba*I site of pXLTR-CAT (in plasmid sequences), and the other was complementary to sequences located 3' of the *Hind*III site (in the CAT-coding sequence). For each mutant, a specific set of internal primers was synthesized. The primer for the 5'-half product included CGC, 12 bases making up *Xho*I and *Nde*I sites, and 17 bases complementary to wild-type coding sequence. The primer for the 3'-half product included CGC, 12 bases making up an *Xho*I site and a *Sal*I site, and 17 bases complementary to wild-type sequence. Using the matched external and internal primers, the two half-products were synthesized via the PCR reaction. The 5'-half product was cleaved with *Xba*I and *Xho*I; the 3'-half product was cleaved with *Xho*I and *Hind*III; each was gel purified. The two fragments were ligated and cut with *Xba*I and *Hind*III, and the products were separated on agarose gels. The appropri-

ate dimer containing both 5' and 3' halves was isolated and ligated into pXLTR-CAT in place of the wild-type LTR sequences between the *Hind*III and *Xba*I sites. The mutations were verified by sequencing.

To examine the effects of mutating larger regions of the LTR, we produced a set of deletion-scanning mutants. These were prepared by recombining appropriate linker-scanning mutants in combinations which replaced 54 bp of wild-type sequence with the NXS polylinker. The consecutive set of eight deletion-scanning mutants is shown in Fig. 1.

Transfections. A modification of the DEAE-dextran method (1) was used. In this method, 600 μ l of Tris-buffered saline (25 mM Tris-HCl [pH 7.4], 137 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM Na₂HPO₄) containing 1 μ g of wild-type or mutant plasmid and 0.5 mg of DEAE-dextran per ml was used to resuspend a washed cell pellet containing 10⁷ cells. The cells were incubated at room temperature for 30 min. The cells were washed with standard phosphate-buffered saline and resuspended in 10 ml of RPMI 1640 medium containing 10% fetal bovine serum. After 48 h the cells were harvested, extracted, and assayed for CAT activity as previously described (13). Acetylated and unacetylated forms of [¹⁴C]chloramphenicol were separated by thin-layer chromatography; radioactive spots were detected and quantitated by using a Molecular Dynamics Phosphorimager. Three to six separate transfection experiments were averaged for each mutant. The activity of each mutant (Fig. 1 and Table 1) is presented as the fraction of wild-type activity. Wild-type LTR plasmid transfections were done in triplicate in each experiment.

DNA fragment mobility shift assays. DNA probes were prepared by PCR. Primers that flank the 149-bp region of the LTR between -231 to -82 were used to amplify the region from the wild-type plasmid pXLTR-CAT and from linker-scanning mutant plasmids which affected this region (33). The synthesized fragments were gel purified, ethanol precipitated, redissolved in 10 mM Tris-HCl (pH 7.5)-1 mM EDTA (TE), and quantitated spectrophotometrically. Then 50 ng of either the wild-type or mutant fragment was labeled by using T4 polymerase replacement synthesis (34). The labeled fragments were isolated from a polyacrylamide gel, ethanol precipitated, and dissolved in TE. Nuclear extracts were prepared essentially as described previously (37) from stimulated Jurkat cells. A 1- μ g sample of nuclear extract protein, 10⁴ cpm of labeled fragment, and 200 ng of poly(dI-dC) · poly(dI-dC) were used with the high-ionic-strength modification (5, 42) of the DNA fragment mobility shift assay (10, 12).

RESULTS AND DISCUSSION

Mutants of the HIV LTR. The wild-type test plasmid, pXLTR-CAT (Fig. 1), contains the U3 and R regions of the LTR from HIV strain HXB2 placed upstream of the CAT gene. An *Xba*I site has been placed 61 bp upstream of the 5' end of the LTR. This site and the *Hind*III site, joining the LTR to the CAT gene, were used in the PCR-directed construction of the linker-scanning mutants (see Materials and Methods). The set of linker-scanning mutants consecutively replaces 18 bp of wild-type sequence with an 18-bp NXS polylinker (Fig. 1). The mutants begin at the 5' end of the LTR (nucleotide -453 with respect to the transcription start site) and continue consecutively to nucleotide +15. The mutants are named *n/m* NXS, where *n* is the LTR nucleotide replaced by the 5'-end nucleotide of the NXS polylinker and *m* is the LTR nucleotide replaced by the 3'-end nucleotide of

FIG. 1. Map of the U3 and R regions of the HIV-1 LTR as they appear in pXLTR-CAT. Nucleotide numbers are relative to the transcription start site, +1. Known and potential transcriptional control regions are indicated (see also Table 1). The *Xba*I and *Hind*III sites were used in construction of the linker-scanning mutants. Positions of the linker-scanning and deletion-scanning mutations are diagrammed under their respective positions in the LTR.

the NXS polylinker (Fig. 1 and Table 1). For example, mutant -453/-436 NXS has LTR nucleotide -453 replaced with the 1st (5') base of the polylinker and LTR nucleotide -436 replaced with the 18th, and last, nucleotide of the polylinker. Figure 1 indicates the positions of various mutations with respect to known or putative regulatory regions. Table 1 lists all of the LTR mutants used in these studies along with known or putative regulatory sequences affected by each mutant.

Two mutant plasmids were used specifically to examine the NF- κ B sites: (i) mutant κ B, a plasmid in which both NF- κ B sites contain point mutations (the GGG residues are mutated to CTC; 27), and (ii) -105/-81 XS+, a plasmid in which the NF- κ B direct repeats have been completely altered. This was accomplished by PCR mutagenesis, changing the wild-type sequence, AGGGACTTTCCGCTGGG GACTTTCC, to CCTCATAGCTCCAGTCTCGTCGATC.

To examine the effects of mutating larger regions of the LTR, we produced a set of deletion-scanning mutants. These were prepared by recombining appropriate linker-scanning mutants in combinations which replaced 54 bp of wild-type sequence with the NXS polylinker. The set of eight consecutive deletion-scanning mutants spanning the LTR is shown in Fig. 1. Table 1 indicates the various known and putative control elements affected by each of the deletion-scanning mutants.

Transient expression analysis of the transcriptional activity of wild-type and mutant LTRs. The transcriptional activities of wild-type and mutant LTRs were determined by CAT transient expression analysis (13). In these experiments, 1 μ g of wild-type or mutant LTR plasmid was transfected, using the DEAE-dextran procedure, into unstimulated Jurkat cells, PHA-TPA-stimulated Jurkat cells, and Jurkat cells

which constitutively express the HIV-1 Tat protein (Jurkat-tat cells; 31). After 48 h, CAT activity was determined. The activities of mutant LTRs are expressed relative to the activity of the wild-type LTR (wild-type activity is designated 1.00) for each cellular condition. Table 2 shows the relative activities of the linker-scanning mutants, with standard deviations based on the results of three to six experiments. Figures 2A to C are graphic representations of the relative activities of the linker-scanning mutants, and of the specific NF- κ B mutants (mutant κ B and -105/-81 XS+), under the three cellular conditions; Fig. 2D to F show the data for the deletion-scanning mutants. Figure 3 further summarizes the results showing generalized LTR regions which, when mutated, decreased LTR expression by 30 to 40, 40 to 60, 60 to 80, or greater than 90%. Regions that caused a modest increase in activity when mutated are also shown. Figure 3 allows comparison of the relative importance of regulatory regions under the different cellular conditions used.

Analysis of the region between LTR nucleotides -111 and +15. This region of the LTR contains the two adjacent NF- κ B sites, the three adjacent Sp1 sites, the TATA element, a putative initiator element, and the binding sites for LBP-1 and UBP-1 (Fig. 1 and Table 1). Mutations in this region had the greatest effects on activity, as expected from previous results. However, some mutations in this region displayed different effects depending on the cellular condition used.

(i) **The Sp1 and TATA regions.** In all three cellular conditions, mutants -39/-22 NXS (a mutation which replaces the TATA element), -75/-58 NXS (which replaces the two 5'-proximal Sp1 sites), and -93/-76 NXS (which replaces the 3'-proximal NF- κ B site and the first two bases of the

TABLE 1. Mutations in the HIV LTR

Mutant	Known or putative site in mutated region ^a	Reference(s)
Linker scanning		
-453/-436 NXS		
-435/-416 NXS		
-417/-400 NXS		
-399/-382 NXS		
-381/-364 NXS	Site A	29
-363/-346 NXS	AP-1	9
-345/-328 NXS	AP-1, site B, NRE	9, 29, 32
-327/-310 NXS	NRE	32
-309/-292 NXS	NRE	32
-291/-274 NXS	NRE, NFAT-1	32, 38
-273/-256 NXS	NRE, NFAT-1, IL-2	32, 38, 39
-255/-238 NXS	NRE, IL-2	32, 39
-237/-220 NXS	NRE, IL-2	32, 39
-219/-202 NXS	NRE	32
-201/-184 NXS	NRE	32
-183/-166 NXS	NRE, IL-2R α , negative, regulatory factor, USF-1	11, 22, 32, 40
-165/-148 NXS		
-147/-130 NXS		
-129/-112 NXS		
-111/-94 NXS	NF- κ B, HIVEN86A, EBP-1	4, 9, 20, 27, 39, 43, 47
-93/-76 NXS	NF- κ B, HIVEN86A, EBP-1 (Sp1—1st 2 bases)	4, 9, 20, 27, 39, 43, 47
-75/-58 NXS	Sp1	14, 18
-57/-40 NXS	Sp1	14, 18
-39/-22 NXS	TATA	19, 21, 28, 32
-21/-4 NXS	LBP-1, UBP-1	11, 19
-3/+15 NXS	LBP-1, UBP-1 (initiator)	11, 19
NF-κB		
-105/-81 XS+	NF- κ B (total mutant)	
Mutant κ B	NF- κ B (GGG to CTC)	27
Deletion scanning		
Δ -435/-382		
Δ -381/-323	Site A, AP-2, site B, NRE	
Δ -327/-274	NRE, NFAT-2	
Δ -273/-220	NRE, NFAT-2, IL-2	
Δ -219/-166	NRE	
Δ -165/-112	NF- κ B, HIVEN86A, EBP-1, Sp1	
Δ -111/-58		
Δ -57/-4	Sp1, TATA, LBP-1, UBP-2	

^a NRE, Negative regulatory element; IL-2, interleukin-2; IL-2R α , interleukin-2 receptor α

5'-proximal Sp1 site) had the greatest effects on LTR activity. These data suggest that Sp1, TFIID (the TATA-binding factor), and possibly NF- κ B (see below), or similar factors recognizing these binding sites, are required for maximal expression under all conditions. Under all three cellular conditions, mutant -57/-40 NXS, which affects the 3'-proximal Sp1 site, had less effect than mutant -75/-58 NXS, which affects the other two Sp1 sites. However, mutation of the 3'-proximal Sp1 site (-57/-40 NXS) had a greater relative effect in Jurkat-tat cells than in the stimulated and unstimulated cells (Fig. 2 and 3).

These data support previous findings that the TATA element is critically important for LTR function under several conditions (19, 21, 28). In addition, the data support observations that the Sp1 sites are important for LTR-

TABLE 2. LTR transcriptional activity obtained by using NXS mutants in Jurkat cells

Mutant	Relative CAT activity (mutant wild type; mean $\pm$ SD)		
	Unstimulated	Stimulated	Tat expressing
Linker scanning			
-453/-436 NXS	0.77 $\pm$ 0.05	0.81 $\pm$ 0.15	0.89 $\pm$ 0.07
-435/-418 NXS	0.82 $\pm$ 0.22	0.88 $\pm$ 0.09	0.96 $\pm$ 0.09
-417/-400 NXS	0.80 $\pm$ 0.18	0.61 $\pm$ 0.12	0.93 $\pm$ 0.07
-399/-382 NXS	0.91 $\pm$ 0.05	0.64 $\pm$ 0.09	0.89 $\pm$ 0.05
-381/-364 NXS	0.98 $\pm$ 0.06	0.82 $\pm$ 0.23	0.91 $\pm$ 0.05
-363/-346 NXS	1.30 $\pm$ 0.18	0.74 $\pm$ 0.23	0.83 $\pm$ 0.07
-345/-328 NXS	1.14 $\pm$ 0.13	0.86 $\pm$ 0.27	0.90 $\pm$ 0.06
-327/-310 NXS	1.27 $\pm$ 0.13	0.83 $\pm$ 0.11	0.91 $\pm$ 0.09
-309/-292 NXS	1.46 $\pm$ 0.31	0.78 $\pm$ 0.01	0.93 $\pm$ 0.03
-291/-274 NXS	1.05 $\pm$ 0.14	0.84 $\pm$ 0.26	0.91 $\pm$ 0.07
-273/-256 NXS	1.04 $\pm$ 0.13	0.72 $\pm$ 0.17	0.85 $\pm$ 0.17
-255/-238 NXS	0.94 $\pm$ 0.04	0.66 $\pm$ 0.23	0.95 $\pm$ 0.14
-237/-220 NXS	1.17 $\pm$ 0.11	0.96 $\pm$ 0.33	0.78 $\pm$ 0.15
-219/-202 NXS	0.96 $\pm$ 0.01	0.81 $\pm$ 0.21	0.89 $\pm$ 0.21
-201/-184 NXS	1.43 $\pm$ 0.09	0.69 $\pm$ 0.19	0.75 $\pm$ 0.20
-183/-166 NXS	0.55 $\pm$ 0.09	0.42 $\pm$ 0.07	0.80 $\pm$ 0.18
-165/-148 NXS	0.52 $\pm$ 0.09	0.46 $\pm$ 0.09	0.86 $\pm$ 0.13
-147/-130 NXS	0.56 $\pm$ 0.19	0.25 $\pm$ 0.05	0.85 $\pm$ 0.17
-129/-112 NXS	0.79 $\pm$ 0.17	0.65 $\pm$ 0.16	0.95 $\pm$ 0.12
-111/-94 NXS	0.34 $\pm$ 0.26	0.03 $\pm$ 0.02	0.52 $\pm$ 0.10
-93/-76 NXS	0.20 $\pm$ 0.16	0.04 $\pm$ 0.02	0.10 $\pm$ 0.03
-75/-58 NXS	0.21 $\pm$ 0.09	0.21 $\pm$ 0.08	0.07 $\pm$ 0.02
-57/-40 NXS	0.58 $\pm$ 0.11	0.84 $\pm$ 0.05	0.37 $\pm$ 0.21
-39/-22 NXS	0.21 $\pm$ 0.06	0.08 $\pm$ 0.03	0.01 $\pm$ 0.01
-21/-4 NXS	0.63 $\pm$ 0.05	0.35 $\pm$ 0.26	0.22 $\pm$ 0.09
-3/+15 NXS	0.53 $\pm$ 0.03	0.43 $\pm$ 0.04	0.06 $\pm$ 0.03
NF-κB			
-105/-81 XS+	0.31 $\pm$ 0.17	0.02 $\pm$ 0.01	0.23 $\pm$ 0.09
Mutant κ B	0.34 $\pm$ 0.04	0.01 $\pm$ 0.01	0.07 $\pm$ 0.03

mediated transcription (14, 18) and that the 5'-proximal Sp1 sites may be the most important for LTR activity (18). Our finding that in unstimulated and stimulated Jurkat cells the mutation of the 3'-proximal Sp1 had modest to no effect is at variance with a previous report suggesting that mutation of all of the Sp1 sites is required to markedly effect LTR transcriptional activity (14). However, those studies may not be directly comparable, since they used different cells, different transfection conditions, and an LTR truncated at nucleotide -177.

(ii) **The NF- κ B region.** Our results confirm the importance of the NF- κ B sites for LTR-mediated transcription (20, 27, 28, 39). However, mutations in the two NF- κ B sites showed some variation between the cellular conditions used (Fig. 2 and 3). In both unstimulated and stimulated Jurkat cells, mutations -111/-94 NXS (containing the 5'-proximal NF- κ B site) and -93/-76 NXS (including the 3'-proximal NF- κ B repeat and the first two bases of the 5'-proximal Sp1 site) as well as the two specific NF- κ B mutants (mutant κ B and -105/-81 XS+) all decreased activity substantially and similarly. These results emphasize that factors interacting with the direct repeats are required for maximal activity in unstimulated cells, which contain little detectable NF- κ B binding activity, as well as in stimulated cells in which NF- κ B sites are essential for maximal activity.

The NF- κ B region mutations also affected LTR activity in the Jurkat-tat cells; however, the effects of mutations in the two NF- κ B sites were different. Mutant -93/-76 NXS (including the 3'-proximal NF- κ B repeat and the first two

FIG. 2. Transient transfection analysis of the linker-scanning and deletion-scanning mutants. For each experiment, 1 μ g of plasmid was transfected into unstimulated Jurkat cells, stimulated Jurkat cells, or Jurkat-tat cells, using the DEAE-dextran procedure (see Materials and Methods). Cells were harvested after 48 h and assayed for CAT activity. The activity of each mutant is expressed relative to that of the wild type (wild-type activity is set at 1.00 for each set of experiments). It should be noted that the absolute basal activity of the wild-type LTR measured in unstimulated Jurkat cells was increased approximately 6-fold in the stimulated cells and approximately 60-fold in the Jurkat-tat cells. The results shown are the average of three to six experiments; Table 1 shows the means and standard deviations. For each experiment triplicate transfections were done using the wild-type plasmid to ensure accuracy of this measurement. (A to C) Activities of linker-scanning mutants in unstimulated, stimulated, and Tat-expressing Jurkat cells, respectively; (D to F) Activities of deletion-scanning mutants in unstimulated, stimulated, and Tat-expressing Jurkat cells, respectively.

bases of the 5'-proximal Sp1 site) as well as the two specific NF- κ B mutants (mutant κ B and -105/-81 XS+) all decreased LTR activity significantly (as much as 93%). However, mutant -111/-94 NXS, which affects the 5'-proximal NF- κ B site, had much less effect on LTR activity, decreasing expression by only 50%. Hence, while mutation of either or both sites had an equally adverse effect in stimulated and unstimulated cells, mutation of the 5'-proximal site caused a much less severe effect than the mutation of the 3'-proximal site in Jurkat-tat cells. The mutation which affects the 3'-proximal NF- κ B site also affects the first two nucleotides of the 5'-proximal Sp1 site. It could be argued that the effect of this mutation is due to the loss of the Sp1 site rather than the 3'-proximal NF- κ B site. This would indicate that neither NF- κ B site is particularly significant in Jurkat-tat cells. However, this cannot be the case, since the two specific NF- κ B mutants (-105/-81 XS+ and mutant κ B) both severely reduce transcriptional activity in Jurkat-tat cells. Neither of these mutants affects the Sp1 sites. This apparent difference in requirement for the proximal and distal NF- κ B sites suggests, but does not prove, the interesting possibility that factors other than NF- κ B bind here in the Jurkat-tat cells and may function in *tat*-mediated transcriptional activation.

(iii) **Effects of sequences downstream of the TATA element.** The two mutations downstream of the TATA element,

-21/-4 NXS and -3/+15 NXS, caused reduced transcriptional activity under all cellular conditions. However, the mutations had the most severe effects in Jurkat-tat cells, reducing activity as much as mutations in the critical Sp1 sites (Fig. 2 and 3). These mutations affect a putative initiator element (Fig. 1) as well as the potential binding sites for LBP-1 and UBP-1 (11, 19), suggesting that these elements may be necessary for *tat* function. These observations together with the strict position and orientation requirements for TAR function agree with the previous suggestions (2, 3, 23, 36, 41) that interaction may occur between TAR-bound Tat and components of the transcription complex bound close to the initiation site. Mutant -3/+15 NXS may affect TAR. However, strictly defined TAR lies between +19 and +42 (8, 15, 16), and computer analysis of RNA secondary structure indicates that mutant -3/+15 NXS does not disrupt the critical stem-loop-bulge (35, 46) of TAR secondary structure (not shown).

(iv) **Analysis using deletion-scanning mutations through the region between LTR nucleotides -111 and +15.** The results for deletion-scanning mutations (Fig. 1 and Fig. 2D to F) generally confirmed the data for the linker-scanning mutations between nucleotides -111 and +15. The deletion of adjacent regions did not result in unexpected additive, subtractive, or synergistic effects.

Analysis of the region between -453 and -112: positive-

FIG. 3. Diagram of the relative effects of the linker-scanning mutations in unstimulated, stimulated, and Tat-expressing Jurkat cells. Results of the linker-scanning analyses are summarized with respect to the known and putative transcription control elements of the U3 and R regions. Indicated are the general regions in which mutations reduced the activity, relative to the wild type, by 30 to 40, 40 to 60, 60 to 80, and greater than 90%. In addition, the regions in which mutations caused a modest increase in activity are indicated (UP). IL2, Interleukin-2; IL2R, interleukin-2 receptor; I, initiator; NRE, negative regulatory element.

FIG. 4. DNA fragment mobility shift studies of factor binding in the -183 to -130 region of the LTR. A 149-bp fragment spanning the region between -231 and -82 was synthesized by PCR, using either the wild-type template or a template containing the $-183/-166$ NXS mutation. The fragment was labeled by the T4 polymerase replacement synthesis procedure and used as a probe with nuclear extracts from stimulated Jurkat cells. Lanes: 1, wild-type probe alone; 2, mutant probe alone; 3, wild-type probe plus nuclear extract; 4, mutant probe plus nuclear extract. The two bands lost when the $-183/-166$ NXS probe was used are labeled 1 and 2.

acting elements. Three mutations ($-183/-166$ NXS, $-165/-148$ NXS, and $-147/-130$ NXS) identified a region, from -183 through -130 , that is required for maximal expression from the LTR in unstimulated and stimulated Jurkat cells. Compared with wild type, these mutations decreased expression between 50 to 75%, depending on the cellular condition; the effect was somewhat greater in the stimulated cells. These data are confirmed by results obtained with the deletion-scanning mutants (Fig. 2D and E). Both $\Delta-219/-166$ and $\Delta-165/-112$ significantly reduced activity of the LTR in both unstimulated and stimulated cells. Again the effect of the deletion-scanning mutants, especially $\Delta-165/-112$, was greater in the stimulated cells.

When these same mutants were transfected into the Jurkat-tat cells, there was no apparent effect (Fig. 2 and 3). In fact, none of the mutations between -453 and -112 had a significant effect on LTR activity in the Tat-expressing cells.

The region between -183 and -130 includes areas known to interact with factors present in some nuclear extracts, e.g., negative regulatory factor and USF-1 (11, 22, 48). Previous studies suggested that deletions in this region increased expression approximately threefold in transfection assays and that viruses containing the deletion grew more rapidly (21, 22). These results are at odds with the decreased

activity seen with the linker-scanning mutants. Our data imply that sequences in this region of the LTR are necessary for maximal LTR transcriptional activity in unstimulated and stimulated Jurkat cells, but their effect is apparently unnecessary for the *tat* activation mechanism.

We performed DNA fragment mobility shift assays (5, 10, 12, 42) to determine whether the -183 to -130 mutations which decreased transcriptional activity correlated with effects on specific factor binding. For the binding studies, stimulated Jurkat cell nuclear extracts were used. The DNA probe, generated by PCR, consisted of the 149 nucleotides of the wild-type sequences between -231 and -82 or the same region containing the $-183/-166$ NXS mutation.

The wild-type fragment produced several bands when incubated with the nuclear extract (Fig. 4). Competition experiments using unlabeled wild-type sequences indicated that all of the bands resulted from specific binding (not shown). When the fragment containing the $-183/-166$ NXS mutation was used as the probe, a faint band labeled 1 and a more prominent band labeled 2 did not appear. These data indicate that the loss of LTR transcriptional activity caused by mutations in this region correlated with alterations in factor binding.

One other region between -453 and -112 appeared to have a slight positive regulatory effect on the activity of the LTR in stimulated Jurkat cells only. This was indicated by mutations $-417/-400$ NXS and $-399/-382$ NXS (Fig. 2 and 3). A decrease of 30 to 40% compared with wild-type activity was noted with each mutant.

Analysis of the region between -453 and -112 : negative-acting elements. Previous studies of the transcriptional activity of the LTR have suggested the existence of negative regulation mediated by sequences in the 5' half of the LTR. The initial report mapping LTR regulatory regions (32) showed that deletion of sequences between -423 and -167 resulted in a 2.5- to 3-fold increase in transcriptional activity in uninfected and infected H9 cells and uninfected HeLa cells. In addition, it was suggested that a fragment between -340 and -185 can suppress expression from a heterologous promoter in a position- and orientation-independent fashion. Other experiments (39) indicated that deletion of sequences from the 5' side of the LTR, up to -278 , caused a fivefold increase in transcriptional activity in unstimulated Jurkat cells, a threefold increase in stimulated Jurkat cells, and a fivefold increase in Jurkat cells in the presence of Tat. These effects became less pronounced when the deletion extended to -176 . In partial agreement, other data (21, 22) suggested that deletion of sequences between -419 and -157 caused a three- to sixfold increase in Jurkat-tat cells, a fivefold increase in stimulated Jurkat cells, and essentially no change in unstimulated Jurkat cells. Two smaller deletions showed either no increase in expression in transfection experiments (-253 to -213 deletion) or about a twofold increase (a -173 to -159 deletion). However, when introduced into viruses, both deletions were reported to cause increased rates of growth.

There have also been many reports which do not support the existence of negative regulation. Two studies (7, 25) deleted sequences from the 5' side of the LTR to -121 and noted either no effect or a modest decrease in transcriptional activity. Another recent study (2), using deletion mutants which removed 5' sequences to -159 , failed to show evidence of negative regulation but indicated a decrease in expression in the absence of Tat and no change in activity in the presence of Tat.

The discrepancies between these results could be due to

differences in cellular conditions, differences in transfection techniques, or differences in the amounts of plasmids transfected. This latter possibility is of specific interest when one considers that transfection of varied amounts of promoter sequences can titrate significant transcription factors. In our experiments we have used a minimal input amount of plasmid in order to avoid such effects.

The linker-scanning mutations spanning -453 to -112 should affect the putative negative regulatory element. The expected result of mutating a negative element would be increased transcriptional activity. In unstimulated Jurkat cells, several rather widely spaced mutations between -453 and -184 produced activities greater than that of the wild type (Fig. 2). Although reproducible, these effects were not dramatic. For example mutants -309/-292 NXS and -201/-184 NXS produced, at best, only 1.4- to 1.5-fold increases over the wild-type activity. The deletion-scanning mutants were tested to determine whether the deletion of several adjacent regions would act synergistically to exhibit the phenotype of a negative regulatory element. There was no significant increase in activity with use of these mutations (Fig. 2D).

The modest evidence for negative regulation was detected only in unstimulated Jurkat cells. In stimulated Jurkat cells and Jurkat-tat cells, the same mutations had no positive effect indicative of negative regulatory elements. Overall, results obtained for our linker-scanning and deletion-scanning mutants suggest that under our experimental conditions there is no single element or region which mediates a substantial negative control of LTR transcription.

Patterns of regulatory site utilization. Figure 3 indicates one of the most interesting findings of the linker-scanning mutant analysis of LTR transcriptional activity. These are the relative activities of several of the mutants under different cellular conditions. The data show distinct regions which became more or less active depending on the cellular condition used. The data indicate that promoter utilization is quite complex. There are differences found not only within the well-studied 3' half of the LTR but also in the less well characterized 5' half.

The overall pattern of regulatory sequence utilization changes notably among the three cellular conditions. In unstimulated cells, major effects were seen with mutants 3' to -112, while more moderate inhibitory effects were seen with mutants in the -183 to -130 region. In addition, there was modest indication of negative regulatory elements with mutants in the region 5' to -183. In stimulated cells, the major effects were again observed 3' to -112, but the general pattern of inhibition differed somewhat from that of unstimulated cells. This was particularly the case with mutations around the NF- κ B and TATA sequences. Stimulated cells also showed moderate inhibitory effects with mutants in the -183 to -130 region, including the somewhat more prominent effects of the -147/-130 NXS mutation. There was no evidence for negative regulatory elements in the stimulated cells.

The presence of Tat changed the pattern of utilization dramatically. None of the mutants 5' to -112 had any effect, including those in the -183 to -130 region. Only mutants in the extreme 3' end had effects on activity, with the most significant being those involving the Sp1 sites, the TATA element, and the sequences 3' to the TATA element.

These changes may suggest the unfolding pattern of regulatory sequence utilization as the virus makes the transition from the quiescent provirus in the resting lymphocyte to active replication. In resting cells, the intrinsically low

activity of the basal promoter, possibly together with the slight inhibitory effects of several sequences at the 5' end, leads to low rates of transcription. After stimulation, NF- κ B (or similar factors) together with auxiliary factors interacting with the -147 to -130 region increases expression from the LTR. When high enough rates of transcription allow for the simultaneous presence of Tat protein and the TAR stem-loop-bulge, the pattern of regulatory sequence utilization changes again. In the presence of Tat only the several regulatory elements closest to the TAR region are required for wild-type activity.

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