JOURNAL OF VIROLOGY, Jan. 1992, p. 139–149
0022-538X/92/010139-11$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 66, No. 1

Functional Roles for the TATA Promoter and Enhancers in Basal and Tat-Induced Expression of the Human Immunodeficiency Virus Type 1 Long Terminal Repeat

BENJAMIN BERKHOUT† AND KUAN-TEH JEANG*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 17 July 1991/Accepted 10 October 1991

We have analyzed the contributory role of the human immunodeficiency virus type 1 (HIV-1) promoter and enhancers in basal and Tat-induced transcription. We found that a minimal promoter competent for basal expression is contained within sequences spanning nucleotides −43 to +80. Basal expression from this HIV-1 promoter was boosted more by the additional presence of the NF-κB elements than by the Sp1 elements. The minimal long terminal repeat promoter (−43 to +80), while having an intact TAR sequence, was not Tat inducible. However, the simple addition of short synthetic enhancer motifs (AP1, Oct, Sp1, and NF-κB) conferred Tat responsiveness. This ability to respond to Tat was in part dependent on the presence of the HIV-1 promoter. Changing the HIV-1 TATA to other eucaryotic TATA or non-TATA initiators minimally affected basal expression but altered Tat inducibility. Our findings suggest a specific context of functional promoter and enhancer elements that is optimal for Tat *trans* activation of the HIV-1 long terminal repeat. Our results do not allow conclusions about whether Tat acts at the level of initiation or at the level of elongation to be drawn.

The biochemical mechanism by which RNA polymerase II initiates transcription is incompletely understood (reviewed in reference 36). One of the earliest steps is the binding of the TFIID protein (11, 46, 57) to the TATA motif (49, 58), a highly conserved AT-rich region positioned 25 to 30 bp upstream from the start of transcription (9). Mutational analyses have demonstrated that for many promoters the TATA sequence is necessary to initiate transcription efficiently and accurately (6, 26–28, 64, 70, 73, 74). In contrast, the rate of initiation is determined primarily by protein factors that recognize DNA enhancers that are usually found upstream of TATA (36). These activator proteins influence events at TATA so as to promote either the binding of TFIID or the subsequent formation of transcription complexes. Other observations indicate that the expression of a typical eucaryotic gene is governed by the complex interactions between many DNA-binding factors (reviewed in reference 18). These DNA-binding factors can presumably affect either the initiation of transcription complexes or the propensity of these complexes to elongate once initiated.

The long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1) is a prototypic enhancer-promoter unit. This LTR contains a standard TATA box (21) and upstream enhancers (NF-κB and Sp1) that are commonly found in many viral and cellular genes (30, 37, 48). The R region of the LTR contains an element, TAR, that is found only in HIV and simian immunodeficiency virus. TAR forms the recognition site for the virally encoded *trans*-activator protein, Tat. Tat is used by the virus to dramatically increase expression from the LTR (14, 35, 39, 51, 63). This function is essential for HIV viability. Viruses with deletions in either Tat or TAR do not replicate normally (15, 29, 40).

The mechanism of action of Tat is controversial. Besides a transcriptional function, posttranscriptional effects have been documented (8, 62). A basis for conflicting perspectives arises from the fact that TAR is located downstream of the start of transcription (5, 20, 31, 32, 55, 60). Thus, the TAR sequence is represented simultaneously in both viral DNAs and viral RNAs. Because of this, phenotypes resulting from mutational changes in TAR could be interpreted as resulting from either a DNA or an RNA effect. This often results in confusing and seemingly contradictory molecular models. Experimentally, however, TAR RNA has been shown to be the Tat-responsive target (5, 13, 16, 17, 19, 47, 56, 71). While this could be viewed as being more consistent with a posttranscriptional role than with a transcriptional role for Tat, recent findings indicate that TAR RNA is functionally used as a tether to bring Tat into the transcriptional apparatus (3, 61, 68). TAR, therefore, acts primarily to facilitate the interaction between Tat and the DNA promoter. As such, promoter-enhancer elements may constitute the critical sites at which the Tat effect is manifested.

Three observations are consistent with the proposal given above. First, TAR activity is lost rapidly when it is distanced from the promoter (60). This critical spacing requirement for TAR RNA is expected for a Tat feedback interaction with upstream DNA. Second, Tat is variably efficient when TAR is linked to different promoters. This suggests that Tat function is influenced by the specific composition of upstream DNA (3, 4). Third, Tat is able to work when it is directed to the promoter in a TAR-independent manner (3, 61, 68). Thus, one may conclude that the physical interaction at the promoter is a more important element in *trans* activation than the process by which Tat is delivered to the promoter.

In this study, we made many LTR promoter changes designed to analyze the different roles of upstream elements in basal and Tat-induced transcription. One salient finding is that a given DNA motif in the LTR does not contribute equivalently in basal and Tat-induced expression. For instance, we found that basal transcription was modulated

* Corresponding author.
† Present address: Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands.

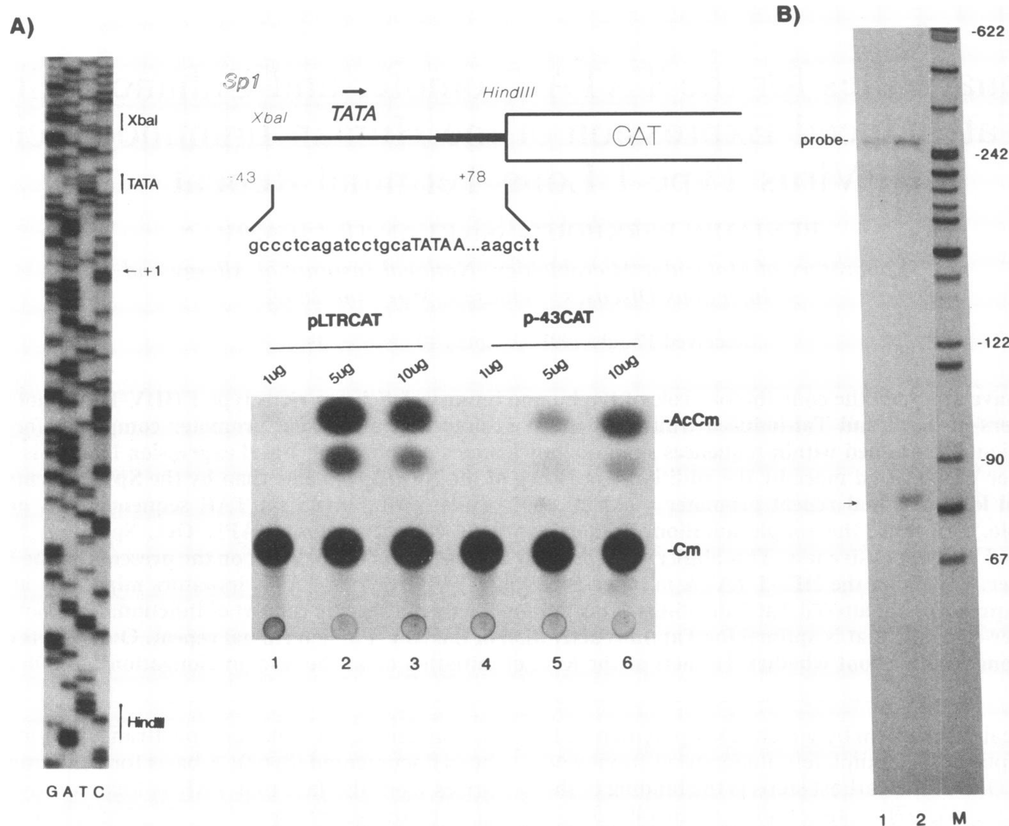


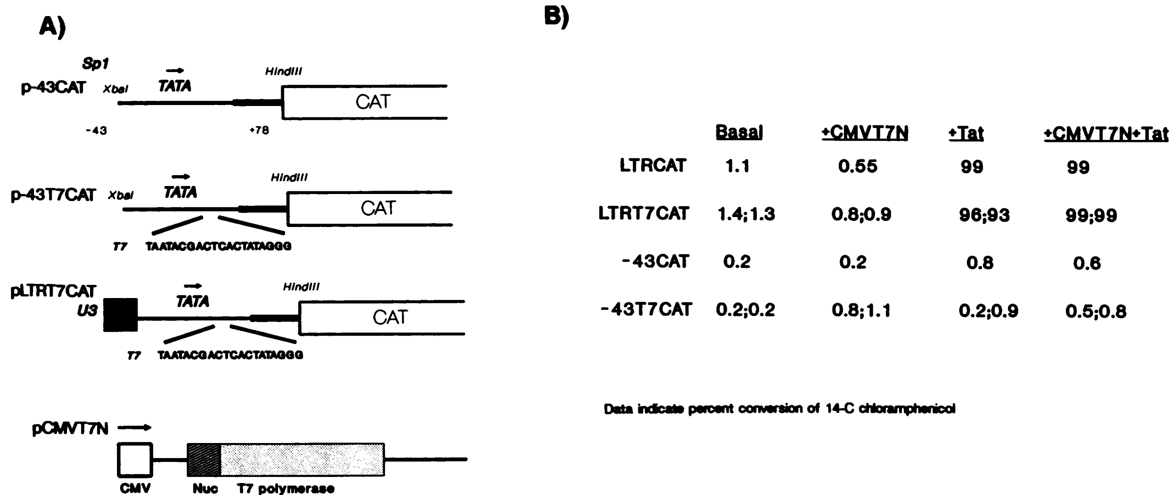
FIG. 1. Comparison of basal promoter activities from pLTRCAT and p−43CAT. (A) The upper right portion of the panel is a schematic representation of the p−43CAT construction. An *Xba*I restriction site has been placed at position −43 relative to the start site of transcription (+1); all additional upstream HIV-1 sequences have been removed. Thus, p−43CAT has no NF-κB or Spl motifs. At the left is a sequencing ladder demonstrating the exact demarcation points of p−43CAT. Below the diagram is a titration series comparing relative CAT expression from transfected pLTRCAT and p−43CAT. Subconfluent HeLa cells were transfected with the indicated amounts of plasmid DNA, and acetylation of [^{14}C]chloramphenicol was resolved by thin-layer chromatography. AcCm, acetylated chloramphenicol; Cm, chloramphenicol. (B) S1 nuclease mapping of transcripts produced from p−43CAT. Poly(A)$^+$ RNA was isolated from 10^7 cells that were mock transfected (lane 1) or transfected with 25 μg of p−43CAT (lane 2). Nuclease protection using an LTR-specific S1 probe yielded a single distinct RNA start site which maps to the authentic +1 position (arrow). Lane M, molecular size markers (in base pairs).

more by the NF-κB enhancers than by the Spl elements. In our experiments, the opposite (i.e., Spl has a greater role than NF-κB) was seen for Tat induction. Mutations engineered into the HIV-1 TATA also affected basal and Tat-induced expression differently. Whereas changes in TATA did not greatly affect basal expression, Tat-dependent transcription was perturbed. A second finding was that a transcription unit consisting of an intact HIV-1 TATA and TAR RNA but lacking upstream enhancers was basally active but was not inducible by Tat. For a Tat response to occur, it was necessary to provide upstream enhancers, even those that do not greatly alter basal expression. Although different enhancers work, albeit with different efficiencies, we found that the Spl sequences were particularly effective.

MATERIALS AND METHODS

Plasmid constructions. Plasmids were generated by standard techniques. Nucleotide numbers are based on the wild-type LTR RNA, with the start site as position +1. p-43CAT contains HIV-1 LTR sequences from position −43, where a unique *Xba*I site was introduced, to position +78 (*Hin*dIII), which immediately precedes the bacterial *cat* gene. p-43T7CAT and pLTRT7CAT were generated with synthetic oligomers such that 20 bp of the HIV LTR sequence (between TATA and +1) was replaced with a T7 promoter sequence (see Fig. 2A). For the addition of heterologous enhancers, synthetic oligonucleotides were ligated into the *Xba*I site. The sequences for the tandem NF-κB and the triple Spl motifs are as they naturally appear in HIV-1 (LAI). The NF/Sp plasmid (see Fig. 4, 5B, 6, and 7) contains the HIV-1 sequence from position −105 to +77. NF/Sp has the natural complement of NF-κB (two copies) and Spl (three copies). Sequences in NF/Sp upstream of +1 were reconstructed with synthetic DNA whose exact sequence is diagrammed in Fig. 6A. Several unique restriction sites in NF/Sp (upstream of NF-κB [*Aat*II], between NF-κB and Spl [*Ava*I], and between Spl and TATAA [*Xba*I]) were engineered to facilitate subsequent insertions of DNA cassettes. pNFSp (Fig. 5A) is virtually identical to pNF/Sp except that the former was made from p−43CAT by cloning into the *Xba*I site a synthetic oligonucleotide that contained the two NF-κB motifs and the three Spl motifs. All plasmids were checked directly by sequencing.

Cell culture, transfection, and RNA-protein assays. Conditions for tissue culture and DNA transfection have been described elsewhere (5). Total cellular RNA or poly(A)$^+$ selected RNA was isolated (4) and analyzed by S1 nuclease



	Basal	+CMVT7N	+Tat	+CMVT7N+Tat
LTRCAT	1.1	0.55	99	99
LTRT7CAT	1.4;1.3	0.8;0.9	96;93	99;99
-43CAT	0.2	0.2	0.8	0.6
-43T7CAT	0.2;0.2	0.8;1.1	0.2;0.9	0.5;0.8

Data indicate percent conversion of 14-C chloramphenicol

FIG. 2. Increased expression of an enhancerless HIV-1 promoter does not confer Tat response. (A) Plasmid constructs used in cotransfection experiments. In p−43T7CAT and pLTRT7CAT, a 20-base oligonucleotide containing the T7 promoter sequence was substituted for 20 bases of the HIV-1 LTR. The placement of the T7 oligonucleotide was such that the last three Gs coincided with (and replaced) the first three Gs found at the HIV-1 +1 mRNA start site. pCMVT7N included a T7 RNA polymerase gene containing an SV40 large T nuclear localization signal (Nuc) (42) positioned downstream of the CMV immediate-early promoter (34). (B) Behavior of plasmids in response to T7N and Tat. The indicated plasmids were transfected into cells alone (Basal) or cotransfected with pCMVT7N (+CMVT7N), Tat (+Tat), or both (+CMVT7N+Tat). The data shown are CAT activities, which were measured 48 h after transfection. Two values are given in some cases because parts of this experiment were performed in duplicate.

protection (5, 33). The single-stranded DNA probe used in the nuclease protection assays contains sequences complementary to the +77 to −119 sequence of the HIV-1(LAI) LTR. This probe was made by cloning the appropriate restriction fragment into an M13 vector and then synthesizing a uniformly labeled DNA probe in vitro with universal primer and Klenow enzyme in the presence of [^{32}P]dCTP (33). Primer extension analysis of RNA and chloramphenicol acetyltransferase (CAT) assays were performed as previously described (4). CAT activities were quantitated in the linear range of acetylation. A Rouse sarcoma virus β-galactosidase plasmid or a simian virus 40 (SV40) β-globin plasmid was used as an internal control for transfection efficiencies. All transfections were performed between 3 and 12 times.

RESULTS

Upstream enhancers are needed for efficient Tat induction. The important role of TAR RNA in Tat *trans* activation has been well documented (reviewed in references 14, 51, and 63). It is, however, not entirely clear whether TAR is the only element that specifies Tat responsiveness (16). A role for DNA elements in the complete Tat-responsive target has been suggested previously (3). Elucidation of the latter proposition is complicated by the need to dissociate the role of upstream DNA motifs in basal promoter function from their independent contribution (if there is any) to Tat induction. For example, if the removal of enhancers resulted in a "dead" promoter, then it would be futile to use this approach to assess enhancer function in Tat induction. A critical element in this type of analysis is the definition of an LTR promoter with basal activity that is not dependent on the enhancer motifs of interest. The ability or inability of this promoter to be induced by Tat is then a reflection of the contributory role of the enhancers.

In systematic mutagenesis of the HIV-1 LTR, we found that a truncation at position −43 (with +1 being the start of transcription) produced a minimal promoter with good basal activity. This abbreviated LTR encompasses the sequence from −43 to +80 so as to contain the HIV-1 TATA with 16 additional upstream bases and the intact TAR element (sequencing gel at left in Fig. 1A). We linked this sequence to *cat* to create p−43CAT (Fig. 1A). Upon introduction of this plasmid into HeLa cells, we found that p−43CAT has a distinct promoter activity at a level fourfold lower than that of the entire LTR (titration series in Fig. 1A). The start site of transcription from p−43CAT was measured by S1 nuclease protection. Using poly(A)$^+$-selected mRNA, a single discrete species whose start maps to the authentic +1 position (Fig. 1B, lane 2) was detected. This result is consistent with transcription in p−43CAT being directed from the HIV-1 TATA box. (Note that p−43CAT has no Sp1 or NF-κB motifs.) The promoter activity of p−43CAT is in part augmented by the 16 nucleotides upstream of TATA. Deletion of these bases eliminated basal activity (data not shown).

We used p−43CAT to test the role of NF-κB and Sp1 elements in the Tat response. In 12 independent transfections using different amounts of p−43CAT and cotransfected Tat-expressing plasmid, we detected no inducing activity by Tat (see examples in Fig. 2B and Fig. 3B, lanes 1 and 2). In these experiments, we carefully documented the basal expression from p−43CAT and performed parallel control trials in which the fold *trans* activation of pLTRCAT by Tat was consistently around 100. One interpretation of these results is that the basal synthesis of TAR RNA alone does not confer complete Tat response and that upstream DNA elements (absent in p−43CAT) play a role.

Because the basal activity of p−43CAT was fourfold lower than that of pLTRCAT, it was formally possible that Tat required a higher level of ongoing transcription of TAR RNA for optimum function. To explore this possibility, we boosted transcription from p−43CAT in an enhancer-independent manner (Fig. 2). A 20-bp T7 promoter sequence was

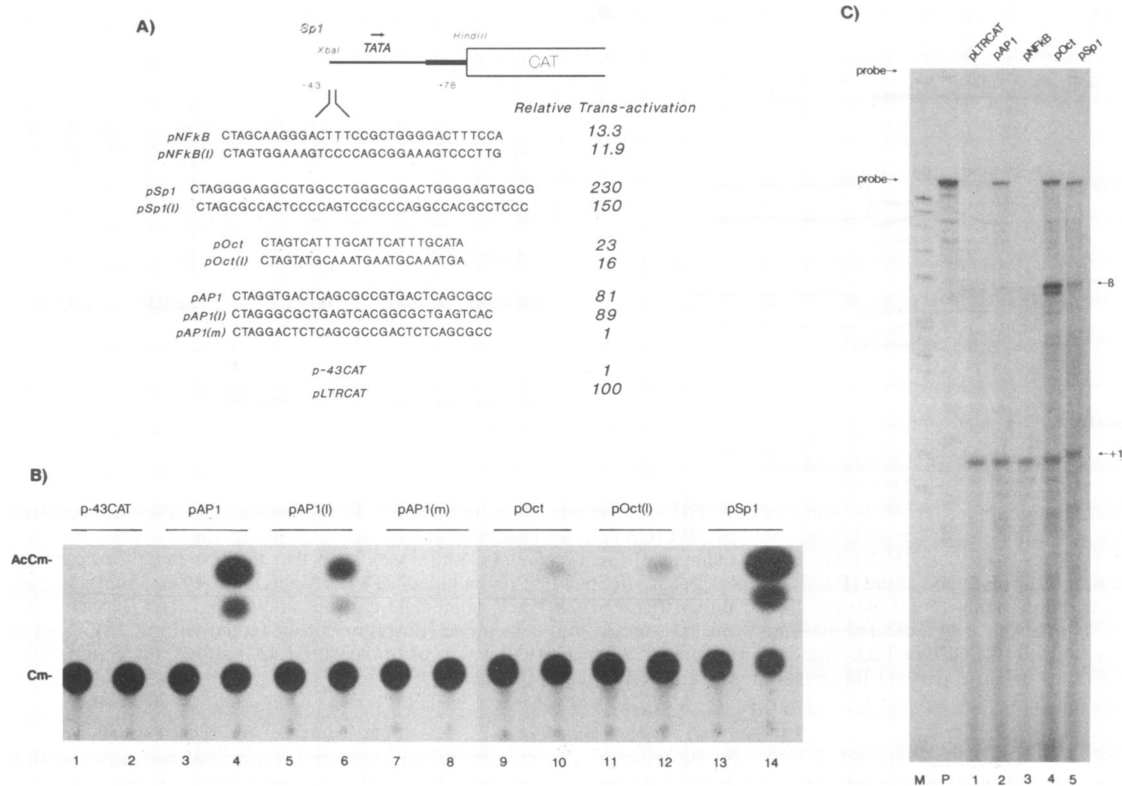


FIG. 3. Heterologous DNA enhancers respond to Tat. (A) Relative *trans*-activation responsiveness of different constructions containing repeated DNA motifs. Each motif was inserted into p−43CAT at the *Xba*I site in either the sense or the inverted (I) orientation. Relative activities after cotransfection with a Tat plasmid were normalized to the activities of p−43CAT and pLTRCAT. (B) A representative example of thin-layer chromatography showing the *trans*-activation responsiveness of pAP1, pAP1(I), pAP1(m), pOct, pOct(I), and pSp1 in transfected HeLa cells. Odd-numbered lanes represent basal activities, and even-numbered lanes represent cotransfections with Tat. AcCm, acetylated chloramphenicol; Cm, chloramphenicol. (C) S1 nuclease protection assay demonstrating that transcription from each of the heterologous enhancer constructs occurred at the authentic +1 position. Each of the indicated plasmids was cotransfected into cells with a Tat-producing plasmid and an SV40 β-globin plasmid. The β-globin-specific transcript (β) was used as a normalization control for LTR-specific transcripts (+1). Lane M, molecular size markers; lane P, input probes.

substituted base for base into p−43CAT such that in the presence of T7 RNA polymerase, the transcription start coincided with HIV-1 nucleotide +1 (p−43T7CAT [Fig. 2A and data not shown]). A control construction was made with pLTRCAT (pLTRT7CAT [Fig. 2A]). In cotransfections into cells with a cytomegalovirus (CMV) promoter (34)-driven T7 polymerase gene (42) (pCMVT7N), p−43T7CAT was found to transcribe at efficiencies comparable to those of pLTR CAT (Fig. 2B, +CMVT7N) in the absence of Tat. However, upon the further addition of Tat, p−43T7CAT remained unresponsive while pLTRCAT and the control pLTRT7CAT were activated 100-fold (Fig. 2B, +CMVT7N+Tat). These results are consistent with an intrinsic role for upstream DNA sequences, beyond their effects on augmenting basal transcription, in Tat *trans* activation.

Quantitation of the contributions of different enhancers to the Tat response. We inserted various DNA enhancers into p−43CAT. Four different motifs were tested. Multimerized copies (two or three) of the NF-κB, Sp1, octamer (Oct), or AP1 sequences were placed into p−43CAT in a sense or an inverted orientation (Fig. 3A). These chimeric plasmids were then compared in parallel with p−43CAT and pLTRCAT for relative Tat responsiveness. The results are shown in Fig. 3B.

In general, we found that an intact enhancer function restored Tat response to p−43CAT. Simple insertion of nonfunctional DNA did not work. For example, the AP1 motif worked well in both the sense orientation and the inverted orientation (Fig. 3B, lanes 3 to 6; 81- and 89-fold induction [Fig. 3A]). However, an inactivating point mutation in the inserted AP1 sequence [Fig. 3A, AP1(m)] completely eliminated this function (Fig. 3B, lanes 7 and 8). Although each of the tested enhancers was effective, we found that the magnitude of rescue varied. One explanation is that each influences the RNA polymerase II complex in a different way, which in turn affects the extent of subsequent interaction with Tat.

To verify that the enhancer-p−43CAT constructions were responding appropriately to Tat, we measured the positions of the respective starts of transcription after *trans* activation. Using S1 nuclease protection, we found that each chimeric LTR promoter used the same +1 nucleotide (Fig. 3C). Thus, qualitatively all transcribed TAR RNA of the same length. Differences in their relative Tat induction (quantitated against a cotransfected β-globin construct [Fig. 3C]) cannot be explained by differences in TAR RNA and are likely due to the inherent characteristics of the individual enhancers.

Sp1 motifs are important for Tat induction. In many different cell types, the HIV-1 LTR is efficiently *trans*-

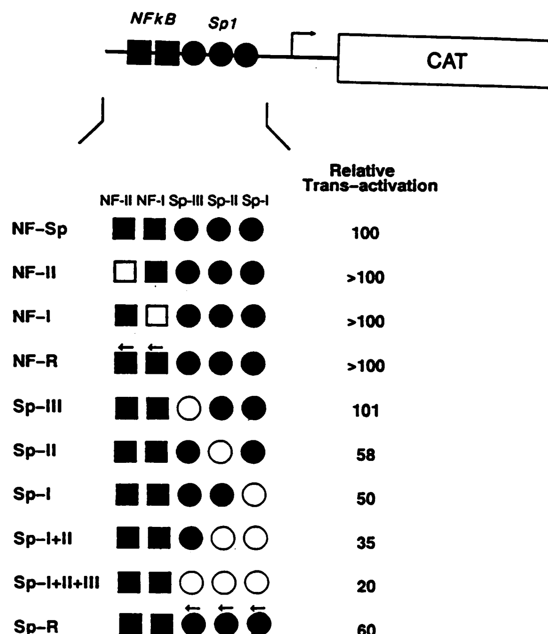


FIG. 4. Relative contributions of the Spl and NF-κB motifs to Tat *trans* activation. The starting plasmid, NF/Sp, has a complete set of upstream activating sequences: two NF-κB sites (filled squares) at positions −105 to −94 and −91 to −80 and three Spl sites (filled circles) at positions −77 to −67, −65 to −56, and −54 to −44. The exact sequence of this promoter region and the mutations introduced into the NF-κB and Spl motifs are shown in Fig. 6A. Inactivated NF-κB and Spl elements are shown as open squares and open circles, respectively. NF-R has both NF-κB elements in a reversed orientation. Sp-R has all three Spl sites inverted relative to the RNA start. Promoter activities were assayed on the basis of transfection of these plasmids into HeLa cells in the absence or in the presence of a cotransfected Tat-producing plasmid. The level of *trans* activation of plasmid NF/Sp by Tat was set at 100.

activated by Tat. Because the LTR contains NF-κB and Spl motifs positioned upstream of the TATA box (Fig. 4), HIV-1 expression can vary depending on the levels of DNA-binding proteins present in the host cell (50). As described above, we analyzed the contribution to Tat function of enhancers that were artifactually attached to the HIV-1 TATA without consideration for spatial conservation (Fig. 3). In a complementary approach (described below), we examined the influence of individual motifs within the natural spacing of the HIV-1 LTR. This was done by engineering point mutations into the HIV-1 enhancers. Starting with the NF/Sp sequence (Fig. 4 and 6), mutations were individually introduced into the NF-κB and Spl motifs. We also inverted the relative orientations of the NF-κB and Spl elements to each other (Fig. 4, plasmids NF-R and Sp-R).

In transient transfections, mutation of either of the two NF-κB motifs had little effect on the magnitude of Tat response (Fig. 4, NF-II and NF-I). In fact, the level of induction of these plasmids by Tat was consistently greater than that of NF/Sp. This was due to decreased basal transcription in HeLa cells from these mutated NF-κB promoters (see below) (3). The relatively neutral influence of NF-κB, in the setting of adjacent Spl sequences, for Tat induction is also consistent with the observed phenotype for NF-R (Fig. 4). This plasmid responded in a wild-type fashion to Tat.

In contrast to NF-κB, inactivating changes in Spl did affect Tat induction. When the three individual Spl sites (Sp-III, Sp-II, and Sp-I) were compared for *trans*-activation efficiency, it was evident that the promoter-proximal Spl motifs were quantitatively more important than the one farthest from the promoter (Fig. 4). Whereas mutation in Sp-III had little effect, changes in Sp-II or Sp-I halved *trans* activation (Fig. 4). Simultaneous inactivation of the two proximal Spl sites (Sp-I+II [Fig. 4]) decreased activity further to 35% of wild-type activity. The triply mutated Sp-I+II+III maintained only a 20% level of Tat response compared with the wild type. This residual activity is likely contributed through the remaining NF-κB elements (Fig. 3). We also found that the orientation of the Spl sites relative to TATA did not matter greatly for Tat activation (Sp-R [Fig. 4]). This is similar to the finding for NF-κB (NF-R [Fig. 4]) and is consistent with the orientation-independent but enhancer function-dependent activities illustrated in Fig. 3. Taken together, the results are compatible with a symmetrical interaction between enhancer factors, the initiating polymerase complex, and Tat.

Complex interactions between enhancer motifs regulate basal promoter strength. A notable aspect of the expression of HIV-1 is its low basal LTR activity. Although the HIV-1 LTR is rather typically organized (with NF-κB, Spl, and TATAA), its basal expression in many cells (especially HeLa cells) is considerably lower than those of other viral LTRs (e.g., the LTRs of Rouse sarcoma virus and murine leukemia virus [data not shown]). In the presence of Tat, the expression of the LTR reaches a level comparable to that of strongly constitutive promoters (e.g., the SV40 early and CMV immediate-early promoters). Since unstimulated HIV-1 promoter activity is minimal, induction of the LTR by Tat is reflected in an impressive (often 100- to 500-fold) increase in promoter strength. We investigated the possibility that a suppressive effect on basal expression exists in the organization of the LTR. Using hybrid enhancer-promoter constructs (Fig. 3) and mutations in the NF-κB and Spl binding sites (Fig. 4), we tested this hypothesis.

We assayed for changes in basal expression. From many transfections, two points emerged. First, in the natural context of the LTR (Fig. 5B), each of the NF-κB sites contributed significantly to basal expression. For example, mutation of the distal NF-κB site (NF-II [Fig. 5B]) reduced expression by fivefold. A change in the proximal NF-κB site yielded an even more dramatic phenotype (NF-I [Fig. 5B]). Expression in this instance was reduced by 20-fold. In comparison, individual alterations in Spl motifs had small effects on promoter activity (Sp-III, Sp-II, and Sp-I [Fig. 5B]). However, combined changes in two or more Spl sites (Sp-I+II and Sp-I+II+III) did influence basal expression. These results verified a greater role for NF-κB compared with Spl in the expression of the LTR in the absence of Tat. This contrasts with the preeminence of Spl in Tat-induced expression (Fig. 3 and 4).

A second observation suggests that the organization of NF-κB and Spl motifs in the LTR mitigates a synergistic effect. This is illustrated by the results shown in Fig. 5A. Comparing pLTRCAT, p−43CAT, pNFSp, pNFκB, and pSpl, we found that removal of the sequence upstream of nucleotide −43 (compare p−43CAT with pLTRCAT [Fig. 5A]) reduced expression by fourfold (see also Fig. 1). When two copies of the NF-κB sequence were added back to the −43 promoter (pNFκB [Fig. 5A]), expression was elevated by 80-fold. A much smaller increase in activity was seen

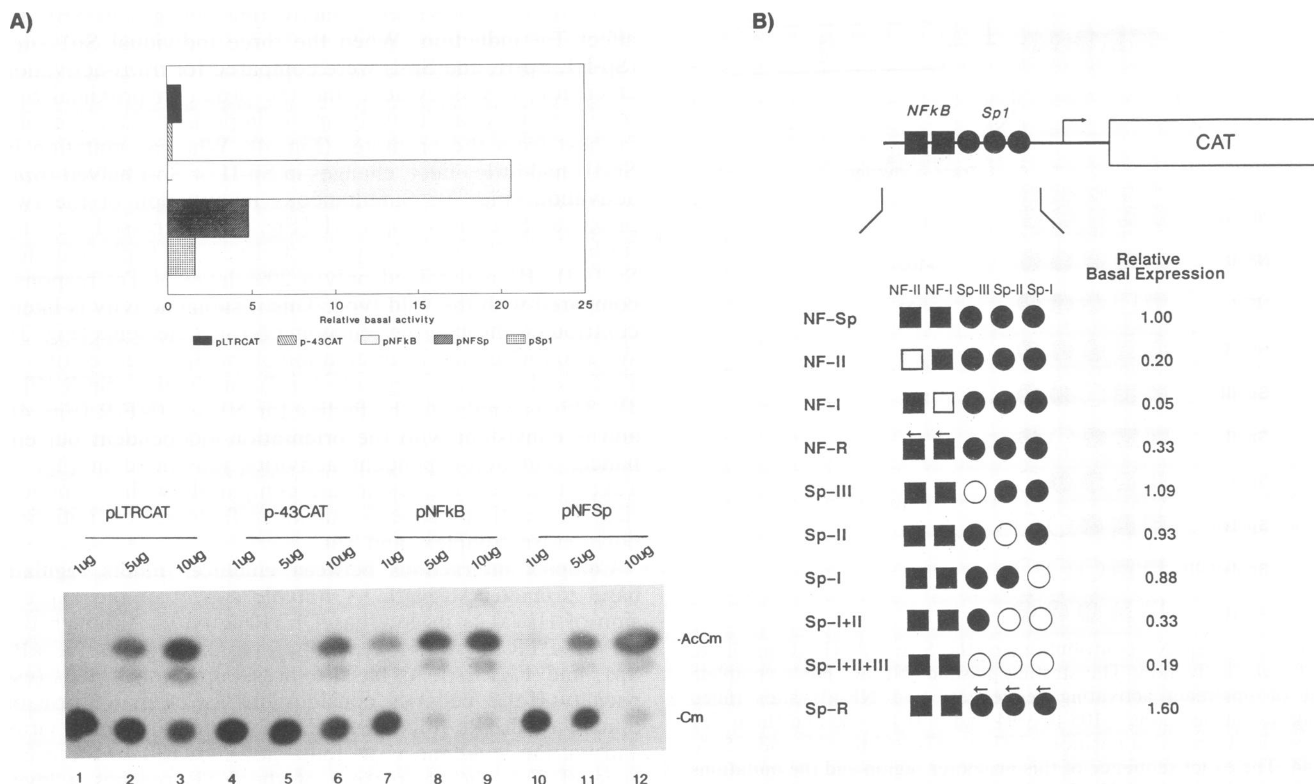


FIG. 5. Contribution of Spl and NF-κB to basal expression of the HIV-1 LTR. (A) (Top) Schematic comparison of the basal promoter activities of pLTRCAT, p−43CAT, pNFκB, pNFSp, and pSpl. (Bottom) representative example of thin-layer chromatography showing a titrated comparison of increasing amounts of transfected pLTRCAT, p−43CAT, pNFκB, and pNFSp. The results for pSpl are not shown. AcCm, acetylated chloramphenicol; Cm, chloramphenicol. (B) Contributions of Spl and NF-κB to basal expression within the natural context of the LTR. NF/Sp and the plasmids mutated in the NF-κB or Spl motifs were individually transfected into HeLa cells. CAT enzyme activities were measured by the phase-extraction method. The relative activity for NF/Sp was set at 1.00; in a typical assay, absolute acetylation (after subtracting for background counts) of [^{14}C]chloramphenicol for NF/Sp-transfected cells (with 25 μg of cell extract in a 1-h incubation) was approximately 150 cpm. Symbols are defined in the legend to Fig. 4.

after the simple addition of three Spl motifs to p−43CAT (pSpl [Fig. 5A]). These results are consistent with the point mutations in Fig. 5B and support the notion that both NF-κB and Spl can individually contribute to promoter function. Surprisingly, when both NF-κB (two copies) and Spl (three copies) were added together to p−43CAT, an activity level that was only 25% of the pNFκB activity level (pNFSp [Fig. 5A]) was seen. This suggests that the combination of two individually positive elements in the LTR actually resulted in a subtractive, rather than an additive, effect. This effect is explained in part by the spacing (32 bp) caused by Spl sequences separating NF-κB from the TATA. Notably, the point-mutated Sp-I+II+III, which maintains this spacing separation, did not have an elevated basal expression level (Fig. 5B).

Transcriptional effects of TATAA mutations. Recent studies have provided evidence that different classes of TATA exist (7, 41, 64, 65, 69, 72). For instance, the muscle-specific enhancer of the myoglobin gene lost activity when its TATAAAA sequence was converted into the TATTTAT counterpart from the SV40 early promoter (72). We reasoned that an HIV-1-specific TATA function might contribute to basal and Tat-induced transcription.

Using the NF/Sp plasmid (Fig. 6A), we changed the TATAA sequence either to TATTTAT (SV40-TATA) or to a randomly chosen sequence, GTCAC (m-TATA). Alternatively, we deleted TATAA and its immediately flanking 30 nucleotides (d-TATA). These TATA-altered plasmids were transfected into HeLa cells in the absence or presence of a second plasmid encoding Tat. Measuring CAT activities, we found that changing TATAA into TATTTAT had a small effect on the expression of the reporter gene (Table 1). Interestingly, the two constructs without a recognizable TATA element (m-TATA and d-TATA) also maintained basal expression (Table 1). These results suggest that elements within the LTR distinct from TATA could function as surrogates in preserving basal transcription. However, distinctly different results were seen when these promoters were compared in the presence of Tat. We tested otherwise identical promoters containing either SV40-TATA or the HIV-1 TATA. Tat *trans* activation of SV40-TATA was much reduced when compared with that of the HIV-1 TATA (Table 1). Dramatic effects were seen for m-TATA and d-TATA. While these promoters showed little difference from wild-type TATA in basal expression (Table 1), they were completely unresponsive to Tat.

Because the pentanucleotide TATAA is only one component of individual promoters, we examined its role in the context of surrounding sequences. Three additional spatially conserved promoters that maintained the TATAA core were made (human T-cell lymphotropic virus TATA [HTLV-TATA], 5′ HTLV-TATA, and MT1-TATA [Fig. 6A]). These

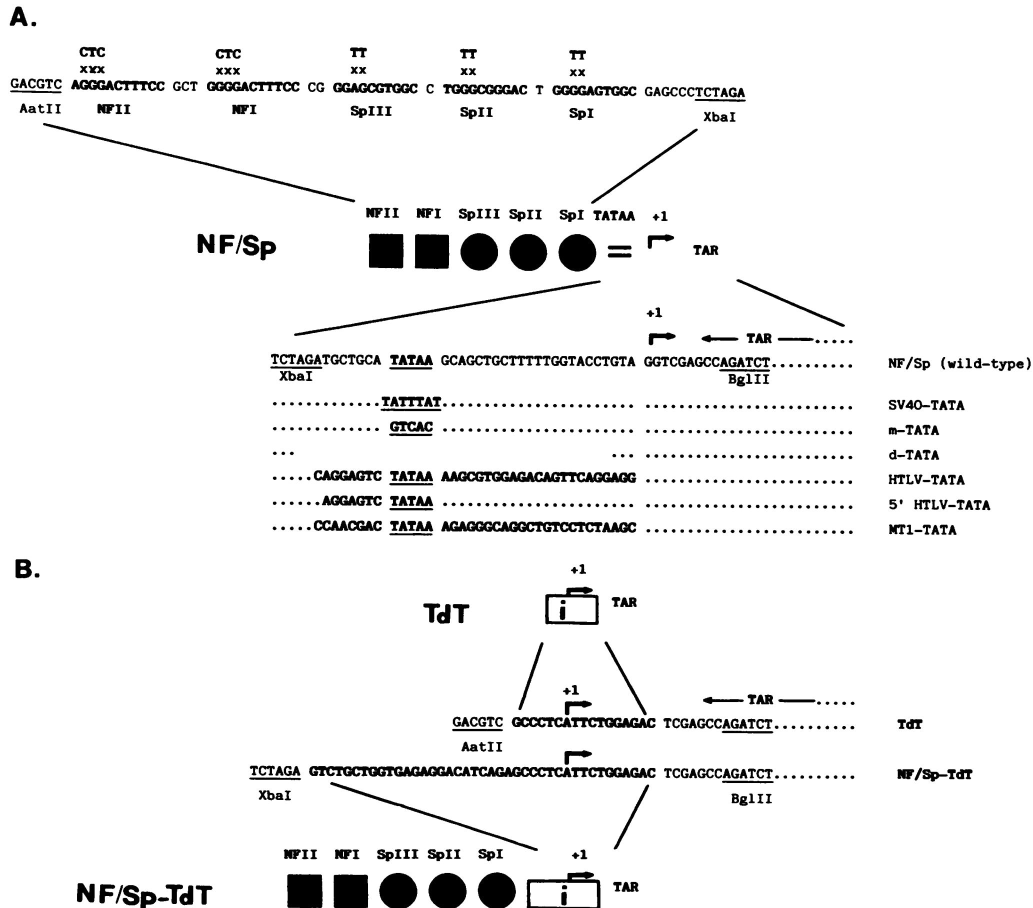


FIG. 6. Nucleotide sequences of the wild-type and mutated LTRs. (A) Nucleotide sequence of the HIV-1 promoter region in plasmid NF/Sp. The upstream sequences of the NF-κB and Spl elements are shown at the top; the TATAA and downstream TAR sequences are shown at the bottom. Unique restriction sites used to generate the mutated constructions are underlined. The mutations introduced into the NF-κB and Spl motifs have been shown previously to render these elements inactive for protein binding and transcription (34, 45). The HIV-1 promoter TATAA (in boldface type and underlined) was deleted (d-TATA) or changed into TATTTAT (SV40-TATA), into the random sequence GTCAC (m-TATA), or into versions of the HTLV type I promoter (HTLV-TATA and 5′ HTLV-TATA) or the mouse metallothionein gene (MT1-TATA) (10). In NF/Sp-TdT, a complete set of HIV-1 upstream activating sequences (two NF-κB and three Spl) was inserted into the *Xba*I site upstream of the TdT initiator (i).

new promoters differ from the wild-type HIV-1 promoter in the sequences 5′ and 3′ to TATA. Their basal activities were similar to that of HIV-1 (Table 1). In the presence of Tat, TATAA in HTLV-TATA or in 5′ HTLV-TATA did not work nearly as well as in MT1-TATA or in the wild-type context (Table 1). Thus, both the nature of the TATAA sequence and its organization within the overall minimal promoter can modulate the response to Tat.

A non-TATA initiator does not respond to Tat. We also examined responsiveness to Tat when transcription was activated not through TATAA but through an alternative initiation element. We used the initiator element from the TATA-less promoter of the TdT gene (66, 67). In TdT, this element spans the RNA start site and encompasses the region between nucleotides −6 and +11. The TdT initiator functions like TATA in having a requirement for the TATA-binding TFIID protein and in its ability to be activated by upstream Spl motifs. Using this information, we explored the role of the TdT initiator in the HIV-1 promoter.

We made constructions in which just the −6 to +11 TdT initiator sequence or the initiator plus a full complement of NF-κB and Spl was positioned upstream of TAR (plasmids TdT and NF/Sp-Tdt [Fig. 6B]). The former was weakly active and not Tat responsive (Table 1). When its basal activity was boosted by the addition of NF-κB and Spl (pNF/Sp-Tdt), *trans* activation by Tat was still not observed (Table 1). Thus in a setting of intact upstream DNA (NF-κB and Spl motifs) and good basal activity (Table 1) transcribing TAR RNA, an appropriate promoter context is still critical for Tat activation.

We ruled out large positional differences in RNA starts from the different promoters by using a primer extension assay. We found that SV40-TATA used a start site that was shifted only one or two nucleotides upstream of wild-type nucleotide +1 (Fig. 7; compare lanes 3 and 2). Similarly, each of the other constructions differed from the wild type in its major start by no more than ±4 bases. This kind of small change in the mRNA starting position has been previously shown not to affect Tat *trans* activation (60). Thus, we conclude that the differences in Tat response are largely determined by differences in the promoter sequence.

TABLE 1. *trans* activation of TATAA and non-TATAA promoters by Tat

Tat presence or absence and plasmid[a]	CAT activity (cpm)[b]	β-Gal activity (OD, 10^{-3})[c]	Corrected CAT activity (cpm)[d]	Relative expression[e]		Fold induction
				Basal	Tat induced	
−Tat						
NF/Sp	136	239	142	1.0		
SV40-TATA	110	215	128	0.9		
m-TATA	148	216	171	1.2		
d-TATA	203	210	242	1.7		
HTLV-TATA	298	228	327	2.3		
5′ HTLV-TATA	110	241	114	0.8		
MT1-TATA	176	163	232	1.9		
TdT	11	199	14	0.1		
NF/Sp-TdT	477	171	697	4.9		
+Tat (0.1 μg)						
NF/Sp	24,813	279	22,234		100	156
SV40-TATA	1,970	280	1,759		8	14
m-TATA	240	223	269		1	2
d-TATA	428	203	527		2	2
HTLV-TATA	7,198	319	5,641		26	17
5′ HTLV-TATA	1,705	290	1,469		7	13
MT1-TATA	33,733	297	28,394		128	122
TdT	25	203	31		0	2
NF/Sp-TdT	2,478	190	3,261		15	5
+Tat (0.5 μg)						
NF/Sp	56,053	328	42,723		100	301
SV40-TATA	5,388	334	4,033		9	32
m-TATA	398	300	332		1	2
d-TATA	673	207	813		2	3
HTLV-TATA	23,198	225	25,776		60	79
5′ HTLV-TATA	2,468	280	2,204		5	19
MT1-TATA	63,805	330	52,540		123	226
TdT	33	281	29		0	2
NF/Sp-TdT	5,863	217	6,755		16	9

[a] Values in parentheses for +Tat represent amount of Tat-encoding plasmid DNA used.
[b] CAT activities are means from six separate experiments. Variations in values between experiments differed by <15%.
[c] β-Galactosidase (β-Gal) activities are means from two separate experiments. Variations in values between experiments differed by <15%. OD, optical density.
[d] Corrected CAT activities are normalized to β-galactosidase.
[e] Expression values are normalized to NF/Sp (set at 1.0 for basal expression and 100 for Tat-induced expression).

DISCUSSION

To date, the mechanism of Tat action is incompletely understood. Although it is generally agreed that Tat efficiently increases the synthesis of full-length transcripts directed from the HIV-1 LTR, the exact means by which this is accomplished remains to be elucidated (14, 44, 51, 63). A mechanistic model for Tat should elucidate two points. First, the target site(s) for Tat in the transcriptional unit should be defined. Second, the nature of interaction between Tat and polymerase complexes at these sites can be compared and contrasted with other (perhaps better-characterized) *trans* activators. In the present study, we focused on the first issue, using LTR-containing plasmids in transient transfections of HeLa cells. Our results suggest that Tat, like many *trans* activators, requires a specific arrangement of DNA elements (the TATA promoter and enhancers), in addition to TAR RNA, for its transcriptional function.

We examined combinations and permutations of changes in promoter and promoter-upstream elements for their contributions to basal and Tat-induced expression. We found that the context in which DNA motifs are arranged is influential for overall activity. For example, the activity of the HIV-1 LTR is apparently driven by two sets of positive transcriptional elements (NF-κB and Sp1). These elements are sequentially arranged in a manner that is suggestive of synergistic interactions (1, 12, 43). However, compared with basal transcription from other promoters, basal transcription from the HIV-1 LTR is not remarkably different. One possible explanation is that these elements, in the absence of Tat, are arranged in a manner for suboptimal expression. Indeed, we observed that when Sp1 sequences were removed and two NF-κB motifs were located closer (by 32 bases) to TATA, basal expression was actually elevated by 20-fold (compare pLTRCAT with pNFκB [Fig. 5A]). This suggests a spatially downregulatory effect of the Sp1 elements in their natural context with the NF-κB sites. Clearly, other factors such as negative regulatory sequences (55) and negative binding factor (38) must also contribute to the repressed expression of the viral LTR.

When we compared the relative contribution of LTR elements to basal and to Tat-induced transcription, we noticed some differences. For instance, in our system, NF-κB significantly modulated the magnitude of basal expression (Fig. 5B) but had a much smaller influence on Tat-induced transcription (Fig. 3 and 4). A different situation was seen for Sp1. Sp1 motifs functioned more importantly in Tat induction than in basal LTR activity (Fig. 3 to 5). Changes in HIV-1 TATA also affected basal and induced activities differently. Significant alterations in TATA had small effects on basal expression, while the same changes

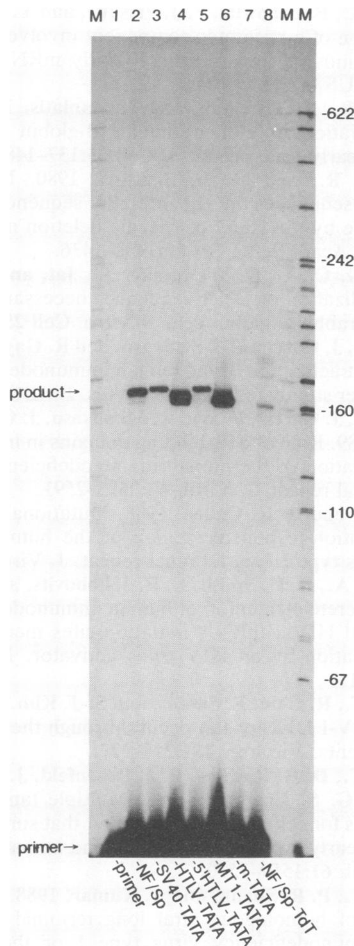


FIG. 7. Primer extension analysis of RNAs transcribed from the different promoter constructions in the presence of Tat. Cells were transfected with 5 μg of the indicated plasmid in the presence of 0.5 μg of a Tat plasmid. A 31-nucleotide primer (lane 2) complementary to the *cat* gene was used in the extension assays. The positions of the primer and the extended product (arrows) are indicated. The molecular size markers (in base pairs) are end-labeled pBR322 *Hpa*II fragments (different amounts loaded in each lane M).

perturbed Tat-induced expression more profoundly (Table 1). In toto, these results suggest that LTR transcription in the presence of Tat uses complex interactions that are different from those used in its absence. It appears that the LTR has evolved some flexibility in tolerating alternative transcriptional initiating events (SV40-TATA, TATA-less, and TdT initiator) for its basal expression. [This ability has also been documented for other viral and cellular promoters (2, 25, 52–54, 59, 67).] Such flexibility is less obvious for Tat-dependent transcription. Thus, conceivably, transcription triggered by Tat may reflect the more highly regulated mode of expression.

Our experiments suggest that optimal Tat induction requires functional enhancer sequences (Fig. 1 to 4). Nonfunctional enhancerlike sequences do not substitute [e.g., AP1(m) (Fig. 3)]. This finding suggests two possible mechanistic scenarios. First, in contributing to Tat-induced expression, enhancers may simply serve to increase basal transcription. In this view, Tat needs a certain threshold of ongoing transcription below which it functions poorly. A second interpretation is that enhancers (and enhancer-binding proteins) interact more directly with Tat. According to this view, it is conceivable that Tat cannot modulate the polymerase complex until it has first interacted with an enhancer function. Different enhancers can modify or process polymerase complexes differently. These differences may then be reflected ultimately in the magnitude of the Tat response (Fig. 3).

Our experiments suggest that a simple threshold in the number of basally transcribing polymerases cannot directly determine Tat competence. When we increased template-linked transcription from the p−43CAT promoter in an enhancer-independent manner, Tat responsiveness was not restored (Fig. 2). This result suggests that the Tat defect in p−43CAT caused by the removal of enhancers is unlikely to be due to a simple reduction in the threshold of transcription. Similarly, most of the promoter changes (Fig. 5) resulted in higher basal expression (Table 1), yet Tat responsiveness was often lacking (Table 1). These results together support the idea that the nature of the transcriptional complex (which is influenced by promoter and promoter-upstream elements) rather than the number of engaged complexes specifies Tat responsiveness.

Our present study implicates a significant role for DNA elements (TATA promoter and enhancers) in conferring Tat *trans* activation. This conclusion is consistent with results of others' (30) and our (3) previous studies of a role for Sp1 in Tat *trans* activation. An extended finding of the present study is that the contextual arrangement of particular enhancer and promoter sequences is ultimately important. This differs from interpretations reached in previously published studies (47, 55) and suggests that TAR RNA alone insufficiently embodies a complete Tat target. We instead propose a model, which does not exclude others, in which the transcription factors that coalesce at the promoter are the ultimate targets for Tat. TAR RNA brings Tat and cellular proteins (22–24, 45) to these assembled factors (5, 61, 68). The ability of Tat to recognize and to modulate these (formed) factors is determined in part by the inherent nature of TATA and the linked enhancers. For a modular Tat-responsive target, our results suggest a composition that includes Sp1 motifs, an HIV-1 TATA promoter, and a correctly initiated TAR RNA.

ACKNOWLEDGMENTS

We thank Malcolm Martin, Keith Peden, Anne Gatignol, and Yung-Nien Chang for readings of manuscript and for discussions of results. We are grateful to M. Strauss for the gift of the T7N phage polymerase gene.

This work was supported in part by the Intramural AIDS Targeted Antiviral Program of the Office of the Director of the National Institutes of Health.

REFERENCES

1. **Anderson, G. M., and S. O. Freytag.** 1991. Synergistic activation of a human promoter in vivo by transcription factor Sp1. Mol. Cell. Biol. **11:**1935–1943.
2. **Benoist, C., and P. Chambon.** 1981. *In vivo* sequence requirements of the SV40 early promoter region. Nature (London) **290:**304–310.
3. **Berkhout, B., A. Gatignol, A. B. Rabson, and K.-T. Jeang.** 1990. TAR-independent activation of the HIV-1 LTR: evidence that Tat requires specific regions of the promoter. Cell **62:**757–767.
4. **Berkhout, B., A. Gatignol, J. Silver, and K.-T. Jeang.** 1990. Efficient *trans*-activation by the HIV-1 Tat protein requires a duplicated TAR RNA structure. Nucleic Acids Res. **18:**1839–1846.

5. **Berkhout, B., R. H. Silverman, and K. T. Jeang.** 1989. Tat *trans*-activates the human immunodeficiency virus through a nascent RNA target. Cell **59**:273–282.
6. **Boyer, T. G., J. R. Krug, and L. E. Maquat.** 1989. Transcriptional regulatory sequences of the housekeeping gene for human triosephosphate isomerase. J. Biol. Chem. **264**:5177–5187.
7. **Boyer, T. G., and L. E. Maquat.** 1990. Minimal sequence and factor requirements for the initiation of transcription from an atypical TATATAA box-containing housekeeping promoter. J. Biol. Chem. **265**:20524–20532.
8. **Braddock, M., A. Chambers, W. Wilson, M. P. Esnouf, S. E. Adams, A. J. Kingsman, and S. M. Kingsman.** 1989. HIV-1 Tat "activates" presynthesized RNA in the nucleus. Cell **58**:269–279.
9. **Breathnach, R., and P. Chambon.** 1981. Organization and expression of eucaryotic split genes coding for proteins. Annu. Rev. Biochem. **50**:349–383.
10. **Bucher, P., and E. N. Trifonov.** 1986. Compilation and analysis of eukaryotic POL II promoter sequences. Nucleic Acids Res. **14**:10013–10017.
11. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56**:549–561.
12. **Carey, M., Y.-S. Lin, M. R. Green, and M. Ptashne.** 1990. A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives. Nature (London) **345**:361–364.
13. **Cordingley, M. G., R. L. LaFemina, P. L. Callahan, J. H. Condra, V. V. Sardana, D. J. Graham, T. M. Nguyen, K. LeGrow, L. Gotlib, A. J. Schlabach, and R. J. Colonno.** 1990. Sequence-specific interaction of Tat protein and Tat peptides with the transactivation-responsive sequence element of human immunodeficiency virus type 1 *in vitro*. Proc. Natl. Acad. Sci. USA **87**:8985–8989.
14. **Cullen, B. R.** 1990. The HIV-1 Tat protein: an RNA sequence-specific processivity factor? Cell **63**:655–657.
15. **Dayton, A., J. G. Sodroski, C. A. Rosen, W. C. Goh, and W. A. Haseltine.** 1986. The trans-activator gene of the human T-cell lymphotropic virus type III is required for replication. Cell **44**:941–947.
16. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 Tat protein stimulates transcription by binding to a U-rich bulge in the stem of the TAR RNA structure EMBO J. **9**:4145–4153.
17. **Dingwall, C., I. Ernberg, M. J. Gait, S. M. Green, S. Heaphy, J. Karn, A. D. Lowe, M. Singh, M. A. Skinner, and R. Valerio.** 1989. Human immunodeficiency virus 1 Tat protein binds *trans*-activation-responsive region (TAR) RNA *in vitro*. Proc. Natl. Acad. Sci. USA **86**:6925–6929.
18. **Dynan, W. S.** 1989. Modularity in promoters and enhancers. Cell **58**:1–4.
19. **Feng, S., and E. C. Holland.** 1988. HIV-1 tat *trans*-activation requires the loop sequence within TAR. Nature (London) **334**:165–167.
20. **Garcia, J. A., D. Harrich, L. Pearson, R. Mitsuyasu, and R. B. Gaynor.** 1988. Functional domains required for tat-induced transcriptional activation of the HIV-1 long terminal repeat. EMBO J. **7**:3143–3147.
21. **Garcia, J. A., D. Harrich, E. Soultanakis, F. Wu, R. Mitsuyasu, and R. B. Gaynor.** 1989. Human immunodeficiency virus type 1 LTR TATA and TAR region sequences required for transcriptional regulation. EMBO J. **8**:765–778.
22. **Gatignol, A., A. Buckler-White, B. Berkhout, and K.-T. Jeang.** 1991. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. Science **251**:1597–1600.
23. **Gatignol, A., A. Kumar, A. Rabson, and K.-T. Jeang.** 1989. Identification of cellular proteins that bind to the human immunodeficiency virus type 1 trans-activation responsive TAR element RNA. Proc. Natl. Acad. Sci. USA **86**:7828–7832.
24. **Gaynor, R. E., E. Soultanakis, M. Kuwabara, J. Garcia, and D. S. Sigman.** 1989. Specific binding of a HeLa cell nuclear protein to RNA sequences in the human immunodeficiency virus transactivating region. Proc. Natl. Acad. Sci. USA **86**: 4858–4862.
25. **Ghosh, P. K., P. Lebowitz, F. J. Frisque, and Y. Gluzman.** 1981. Identification of a promoter component involved in positioning the 5′ terminus of simian virus 40 early mRNAs. Proc. Natl. Acad. Sci. USA **78**:100–104.
26. **Green, M. R., R. Treisman, and T. Maniatis.** 1983. Transcriptional activation of cloned human B-globin genes by viral immediate-early gene products. Cell **35**:137–148.
27. **Grosschedl, R., and M. L. Birnstiel.** 1980. Identification of regulatory sequences in the prelude sequences of an H2A histone gene by the study of specific deletion mutants *in vitro*. Proc. Natl. Acad. Sci. USA **77**:1432–1436.
28. **Grosveld, G. C., C. K. Shewmaker, P. Jat, and R. A. Flavell.** 1981. Localization of DNA sequences necessary for transcription of the rabbit B-globin gene *in vitro*. Cell **25**:215–226.
29. **Harrich, D., J. Garcia, R. Mitsuyasu, and R. Gaynor.** 1990. TAR independent activation of the human immunodeficiency virus in phorbol ester stimulated T lymphocytes. EMBO J. **9**:4417–4423.
30. **Harrich, D., J. Garcia, F. Wu, R. Mitsuyasu, J. Gonzalez, and R. Gaynor.** 1989. Role of SP1-binding domains in in vivo transcriptional regulation of the human immunodeficiency virus type 1 long terminal repeat. J. Virol. **63**:2585–2591.
31. **Hauber, J., and B. R. Cullen.** 1988. Mutational analysis of the *trans*-activation-responsive region of the human immunodeficiency virus type I long terminal repeat. J. Virol. **62**:673–679.
32. **Jakobovits, A., D. H. Smith, E. B. Jakobovits, and D. J. Capon.** 1988. A discrete element 3′ of human immunodeficiency virus 1 (HIV-1) and HIV-2 mRNA initiation sites mediates transcriptional activation by an HIV *trans* activator. Mol. Cell. Biol. **8**:2555–2561.
33. **Jeang, K.-T., R. Chiu, E. Santos, and S.-J. Kim.** 1991. Induction of the HTLV-I LTR by Jun occurs through the Tax-responsive 21-bp elements. Virology **181**:218–227.
34. **Jeang, K.-T., D. R. Rawlins, P. J. Rosenfeld, J. H. Shero, T. J. Kelly, and G. S. Hayward.** 1987. Multiple tandemly repeated binding sites for cellular nuclear factor 1 that surround the major immediate-early promoters of simian and human cytomegalovirus. J. Virol. **61**:1559–1570.
35. **Jeang, K.-T., P. R. Shank, and A. Kumar.** 1988. Transcriptional activation of homologous viral long terminal repeats by the human immunodeficiency virus type 1 or the human T-cell leukemia virus type 1 tat proteins occurs in the absence of *de novo* protein synthesis. Proc. Natl. Acad. Sci. **85**:8291–8295.
36. **Johnson, P. F., and S. L. McKnight.** 1989. Eucaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58**:799–839.
37. **Jones, K. A., J. T. Kadonaga, P. A. Luciw, and R. Tjian.** 1986. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. Science **232**:755–759.
38. **Kato, H., M. Horikoshi, and R. G. Roeder.** 1991. Repression of HIV-1 transcription by a cellular protein. Science **251**:1476–1479.
39. **Laspia, M. F., A. P. Rice, and M. B. Mathews.** 1989. HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. Cell **59**:283–292.
40. **Leonard, J., C. Parrott, A. J. Buckler-White, W. Turner, E. K. Ross, M. A. Martin, and A. B. Rabson.** 1989. The NF-κB binding sites in the human immunodeficiency virus type 1 long terminal repeat are not required for virus infectivity. J. Virol. **63**:4919–4924.
41. **Li, W.-Z., and F. Sherman.** 1991. Two types of TATA elements for the *CYC1* gene of the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11**:666–676.
42. **Lieber, A., U. Kiessling, and M. Strauss.** 1989. High level gene expression in mammalian cells by a nuclear T7-phage RNA polymerase. Nucleic Acids Res. **17**:8485–8493.
43. **Lin, Y.-S., M. Carey, M. Ptashne, and M. R. Green.** 1990. How different eukaryotic transcriptional activators can cooperate promiscuously. Nature (London) **345**:359–361.
44. **Marciniak, R. A., B. J. Calnan, A. D. Frankel, and P. A. Sharp.** 1990. HIV-1 Tat protein trans-activates transcription in vitro. Cell **63**:791–802.
45. **Marciniak, R. A., M. A. Garcia-Blanco, and P. A. Sharp.** 1990. Identification and characterization of a HeLa nuclear protein that specifically binds to the trans-activation-response (TAR)

element of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **87**:3624–3628.

46. **Matsui, T., J. Segall, P. A. Weil, and R. G. Roeder.** 1980. Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J. Biol. Chem. **255**:11992–11996.
47. **Muesing, M. A., D. H. Smith, and D. J. Capon.** 1987. Regulation of mRNA accumulation by a human immunodeficiency virus *trans*-activator protein. Cell **48**:691–701.
48. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326**:711–713.
49. **Nakajima, N., M. Horikoshi, and R. G. Roeder.** 1988. Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. Mol. Cell. Biol. **8**:4028–4040.
50. **Parrott, C., T. Seidner, E. Duh, J. Leonard, T. S. Theodore, A. Buckler-White, M. A. Martin, and A. B. Rabson.** 1991. Variable role of the long terminal repeat Sp1-binding sites in human immunodeficiency virus replication in T lymphocytes. J. Virol. **65**:1414–1419.
51. **Pavlakis, G. N., and B. K. Felber.** 1990. Regulation of expression of human immunodeficiency virus. New Biol. **2**:20–31.
52. **Pellman, D., M. E. McLaughlin, and G. R. Fink.** 1990. TATA-dependent and TATA-independent transcription at the HIS4 gene of yeast. Nature (London) **348**:82–85.
53. **Reynolds, G., S. Basu, T. Osborne, D. Chin, G. Gil, M. Brown, J. Goldstein, and K. L. Luskey.** 1984. HMG CoA reductase: a negatively regulated gene with unusual promoter and 5′ untranslated regions. Cell **38**:275–286.
54. **Rio, D., A. Robbins, R. Myers, and R. Tjian.** 1980. Regulation of simian virus 40 early transcription *in vitro* by a purified tumor antigen. Proc. Natl. Acad. Sci. USA **77**:5706–5710.
55. **Rosen, C. A., J. G. Sodroski, and W. A. Haseltine.** 1985. The location of cis-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell **41**:813–823.
56. **Roy, S., U. Delling, C.-H. Chen, C. A. Rosen, and N. Sonenberg.** 1990. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated *trans*-activation. Genes Dev. **4**:1365–1373.
57. **Samuels, M., A. Fire, and P. A. Sharp.** 1982. Separation and characterization of factors mediating accurate transcription by RNA polymerase II. J. Biol. Chem. **257**:14419–14427.
58. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
59. **Seghal, A., N. Patil, and M. Chao.** 1988. A constitutive promoter directs expression of the nerve growth factor receptor gene. Mol. Cell. Biol. **8**:3160–3167.
60. **Selby, M. J., E. S. Bain, P. A. Luciw, and B. M. Peterlin.** 1989. Structure, sequence, and position of the stem-loop in TAR determine transcriptional elongation by tat through the HIV-1 long terminal repeat. Genes Dev. **3**:547–558.
61. **Selby, M. J., and B. M. Peterlin.** 1990. *Trans*-activation by HIV-1 Tat via a heterologous RNA binding protein. Cell **62**:769–776.
62. **SenGupta, D. N., B. Berkhout, A. Gatignol, A. Zhou, and R. H. Silverman.** 1990. Direct evidence for translational regulation by HIV-1 leader RNA and Tat protein. Proc. Natl. Acad. Sci. USA **87**:7492–7496.
63. **Sharp, P. A., and R. A. Marciniak.** 1989. HIV TAR: an RNA enhancer? Cell **59**:229–230.
64. **Simon, M. C., T. M. Fisch, B. J. Benecke, J. R. Nevins, and N. Heintz.** 1988. Definition of multiple, functionally distinct TATA elements, one of which is a target in the hsp70 promoter for E1a regulation. Cell **52**:723–729.
65. **Singer, V. L., R. Wobbe, and K. Struhl.** 1990. A wide variety of DNA sequences can functionally replace a yeast TATA element for transcriptional activation. Genes Dev. **4**:636–645.
66. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57**:103–113.
67. **Smale, S. T., M. C. Schmidt, A. J. Berk, and D. Baltimore.** 1990. Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. Proc. Natl. Acad. Sci. USA **87**:4509–4513.
68. **Southgate, C., M. L. Zapp, and M. R. Green.** 1990. Activation of transcription by HIV-1 Tat protein tethered to nascent RNA through another protein. Nature (London) **345**:640–642.
69. **Struhl, K.** 1986. Constitutive and inducible *Saccharomyces cerevisiae* promoters: evidence for two distinct molecular mechanisms. Mol. Cell. Biol. **6**:3847–3853.
70. **Wasylyk, B., R. Derbyshire, A. Guy, D. Molko, A. Roget, R. Teoule, and P. Chambon.** 1980. Specific *in vitro* transcription of conalbumin gene is drastically decreased by single-point mutation in TATA box homology sequence. Proc. Natl. Acad. Sci. USA **77**:7024–7028.
71. **Weeks, K. M., C. Ampe, S. C. Schultz, T. A. Steitz, and D. M. Crothers.** 1990. Fragments of the HIV-1 Tat protein specifically bind TAR RNA. Science **249**:1281–1285.
72. **Wefald, F. C., B. H. Devlin, and R. C. Williams.** 1990. Functional heterogeneity of mammalian TATA-box sequences revealed by interaction with a cell-specific enhancer. Nature (London) **344**:260–262.
73. **Williams, G. T., T. K. McClanahan, and R. I. Morimoto.** 1989. E1a transactivation of the human *HSP70* promoter is mediated through the basal transcriptional complex. Mol. Cell. Biol. **9**:2574–2587.
74. **Wu, L., D. S. E. Rosser, M. C. Schmidt, and A. Berk.** 1987. A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promoter. Nature (London) **326**:512–515.