Structure, sequence, and position of the stem-loop in *tar* determine transcriptional elongation by *tat* through the HIV-1 long terminal repeat

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The human immunodeficiency virus (HIV-1)-encoded *trans*-activator (*tat*) increases HIV gene expression and replication. Previously, we demonstrated that *tat* facilitates elongation of transcription through the HIV-1 long terminal repeat (LTR) and that short transcripts corresponding to prematurely terminated RNA are released and accumulate in the absence of *tat*. Here, using a transient expression assay, we tested clustered and compensatory mutations, as well as 3' deletions, in the *trans*-acting responsive region (*tar*) and observed that the primary sequence in the loop and secondary structure in the stem of the stem-loop in *tar* are required for *trans*-activation by *tat*. Insertions in the 5' region of *tar* revealed that *tar* must be near the site of HIV-1 initiation of transcription for *trans*-activation by *tat*. Deletions (3') and an insertion in *tar* demonstrated that an intact stem-loop is required for the recovery of prematurely terminated transcripts. Short and full-length transcripts were observed also with HIV type 2 (HIV-2) in the absence and presence of *tat*, respectively. We conclude that an intact stem-loop in *tar* is essential for *trans*-activation by *tat* and that initiation of transcription by HIV-1 promoter factors and elongation of transcription by *tat* are coupled.

[**Key Words:** Human immunodeficiency viruses; *trans*-acting responsive region; *trans*-activator; transcriptional elongation; RNA structure]

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The human immunodeficiency virus (HIV-1) is the etiologic agent of acquired immune deficiency syndrome (AIDS) (for review, see Ho et al. 1987; Fauci 1988). HIV replication begins after cells are activated and requires the expression of virally encoded *trans*-activator (*tat*) (Arya et al. 1985; Sodroski et al. 1985; Cullen 1986; Dayton et al. 1986; Feinberg et al. 1986; Fisher et al. 1986; Peterlin et al. 1986; Wright et al. 1986; Hauber et al. 1987; Kao et al. 1987; Muesing et al. 1987; Feng and Holland 1988; Jakobovits et al. 1988; Rice and Matthews 1988) and the regulator of expression of virion proteins (*rev*) (Feinberg et al. 1986; Sodroski et al. 1986; Malim et al. 1988; Rosen et al. 1988). *trans*-activations by *tat* and *rev* increase the amounts and lengths of HIV-1 transcripts, respectively (Cullen 1986; Peterlin et al. 1986; Hauber et al. 1987; Kao et al. 1987; Muesing et al. 1987; Malim et al. 1988; Rosen et al. 1988). *tat* and *rev* are essential for viral replication because deletions of or point mutations in these sequences inactivate HIV-1 (Dayton et al. 1986; Fisher et al. 1986). Although these *trans*-acting factors have been characterized biochemically and their target *cis*-acting sequences identified, their precise mechanisms of action remain unknown (for review, see Chen 1986; Varmus 1988).

tat, which is translated from a spliced mRNA, is com-

posed of 86–100 amino acids, depending on the HIV-1 isolate (Muesing et al. 1985; Ratner et al. 1985; Sanchez-Pescador et al. 1985; Wain-Hobson et al. 1985). Localized in the nucleus (Hauber et al. 1987), *tat* contains a series of cysteine residues followed by a stretch of basic amino acids and forms homodimers in the presence of divalent cations (Frankel et al. 1988). *tat* acts on the *trans*-acting responsive region (*tar*) located between positions –17 to +80 in the 5' HIV-1 long terminal repeat (LTR) to increase viral gene expression and replication (Rosen et al. 1985).

It was shown recently that *tar* functions neither as a transcriptional enhancer nor promoter element and that *trans*-activation is observed only when *tar* is placed 3' to the HIV-1 promoter in its native orientation (Peterlin et al. 1986; Muesing et al. 1987). Jakobovits et al. (1988) reported that sequences from positions +19 to +42 in *tar* are required for *trans*-activation by *tat*. Although transcriptional (Hauber and Cullen 1988; Jakobovits et al. 1988) and post-transcriptional (Cullen 1986; Rosen et al. 1986; Wright et al. 1986) effects of *tat* have been reported, it is still not understood how and at what level *tat* interacts with *tar*.

Previously, we observed that *tat* has no effect on the rate of initiation of HIV-1 transcription (Kao et al. 1987).

However, in the absence of *tat*, elongation of nascent transcripts was blocked, resulting in the accumulation of prematurely terminated transcripts in the cytoplasm of transfected cells. These short transcripts, corresponding to untranslated sequences from positions +1 to +57, can form a stable stem-loop in solution (Muesing et al. 1987). In the presence of *tat*, however, full-length HIV-1 transcripts were recovered. To define the primary sequence and secondary structure requirements in *tar* for *trans*-activation by *tat*, we analyzed clustered and compensatory mutations, 3' deletions, and 5' insertions in *tar* in the presence and absence of *tat*. Levels of chloramphenicol acetyltransferase (CAT) RNA and protein were measured in cells transfected with HIV-1 LTR-CAT fusion genes (Gorman et al. 1982). We present evidence that *tat* acts on the predicted stable stem-loop in *tar* but only when it is placed very close to the site of initiation of HIV-1 transcription. These observations are extended to HIV type 2 (HIV-2), a related human lentivirus that also is associated with AIDS (Clavell et al. 1986; Emerman et al. 1987; Guyader et al. 1987).

Results

Transient transfection assay to measure trans-activation by tat

Wild-type and all manipulated LTRs of HIV-1 were placed 5' to the CAT reporter gene (Gorman et al. 1982) in plasmid vectors that contain the SV40 origin of replication and can replicate in COS-7 cells (Gluzman 1981). Constructions in replicating vectors were used because they allowed for higher levels of HIV-1 expression, thus permitting an accurate determination of basal levels of CAT activity and HIV-specific RNA.

Boundaries of tar: 3' deletions and clustered mutations in tar

To determine the 3' border of *tar* required for *trans*-activation, we made 3' deletions in the HIV-1 LTR. A 3' deletion to position +61 (D1) resulted in wild-type *trans*-activation (Table 1), whereas deletion to position +45 (D2) resulted in a fivefold decreased *trans*-activation (Table 1). A further deletion of only 3 nucleotides to position +42 (D3) additionally decreased *trans*-activation twofold (Table 1). Deletions to positions +20 (D4) and +8 (D5) eliminated *trans*-activation and resulted in three- to fivefold increased basal levels of expression (Table 1). Thus, the 3' border of *tar* required for efficient *trans*-activation lies between positions +45 and +61.

To define the 5' and to refine the 3' borders of *tar* required for *trans*-activation, we analyzed clustered mutations in *tar*, each of which replaced 4 or 5 nucleotides of wild-type sequence with mutant sequence, thereby generating a restriction site (Fig. 1A). The effects on *trans*-activation by clustered mutations from positions -17 to +60 were determined and are presented in Table 1. Clustered mutations in the region between the promoter and the site of initiation of transcription (M1-M3)

Table 1.

A. CAT assay and free energy values for deletions and clustered mutations in *TAR*

Construction	Basal (% of wt)	Fold-TA (% of wt)	ΔG (+1/+57)	ΔG (+14/+45)
D1	93.7	96.9	-35.3	-16.5
D2	124	19.7	-17.2	-16.5
D3	140	9.1	-15.1	-8.1
D4	310	0.85		
D5	490	0.81		
M1	75	79		
M2	121	43		
M3	150	122		
M4	78	84	-32.1	-16.5
M5	124	68	-32.2	-16.5
M6	92	61	-29.0	-16.5
M7	85	54	-29.0	-16.5
M8	60	14	-20.7	-7.8
M9	374	2.2	-28.3	-9.5
M10	270	2.0	-35.3	-16.5
M11	190	3.7	-25.9	-8.8
M12	302	2.7	-16.7	-6.0
M13	458	49.3	-28.0	-12.9
MC1	250	64	-26.9	-11.4
MC2	110	124	-42.4	-16.5

B. Fold-*trans*-activation of HIV-1 and HIV-2 by TAT of HIV-1

Construction	Fold-TA
pHIV-1CATSV	46.7
pHIV-2CATSV	68.7

(A) Basal and *trans*-activated (TA) values of CAT activities expressed as percent of wild type (WT) for cotransfections of COS-7 cells with plasmid constructions (column 1) and pSVTATZX or pSVTAT. (D) Deletions; (M) clustered mutations; (MC) compensatory clustered mutations presented in Fig. 1A. pHIV-1CATSV was *trans*-activated from 54.1- to 74.2-fold. The results represent the average of at least three independent experiments, where the s.e.m. was <30%. (Columns 4 and 5) Calculated free energies (ΔG values expressed in kcal/mole), which were determined according to the method of Zucker and Stiegler (1981). ΔG values are presented for sequences from positions +1 and +57 and from positions +14 to +45. The wild-type free energies are represented by M10 and D1, as their predicted stem-loops are intact. ΔG values are not presented for D4 and D5, as their stem-loops are eliminated. Because the changed nucleotides in M1, M2, and M3 are not transcribed, they do not affect the stem-loop in *tar*. (B) Fold *trans*-activation (TA) values for transfections of COS-7 cells with pHIV-1CATSV and pHIV-2CATSV with pSVTAT are presented.

yielded high levels of *trans*-activation. In the transcribed region, clustered mutations from position +1 to +13 (M4-M7) and +46 to +49 (M13) had a small effect on *trans*-activation. In contrast, clustered mutations from positions +14 to +45 (M8-M12) diminished *trans*-activation significantly, demonstrating that this region is required for *tat* action. These sequences correspond to the top half of the predicted stem-loop in *tar* RNA. Because M8 showed an intermediate phenotype, it is likely that the true 5' border of *tar* is located between positions

Figure 1. (A) Organization of the HIV-1-LTR and the expanded *tar* region. The NF- κ B (nuclear factor κ B) enhancers, Sp1-binding sites, and the TATA box (T) are shown. The first three hatched circles represent consensus sequences that bind leader binding protein (LBP) or untranslated binding protein (UBP) in vitro; the fourth hatched circle represents a potential binding site for CTF/NF-1 (CCAAT transcription factor/nuclear factor-1). *tar* begins at PvuII (−17) and ends at a HindIII site (+80), which is followed by a polylinker and then the CAT gene. The sequences of the HIV-1 LTR and clustered and compensatory mutations from positions −17 to +57 are displayed. The changed sequences were chosen because they introduce unique restriction sites into the HIV-1 LTR sequence. The changed nucleotides are shown for each mutant below the wild-type sequence. (Right) Constructions and the positions of the changed nucleotides. (Top) Locations of the stem and the loop. (B) Secondary structure representations of the *tar* regions of HIV-1 and HIV-2. The most stable structures (Zucker and Stiegler 1981) of the *tar* regions of HIV-1 (left) and HIV-2 (right) are presented.

+14 to +18. CAT assays from cells transfected with the same constructions in nonreplicating vectors yielded similar patterns of expression (data not shown), validating the use of replicating plasmids. Basal levels of expression for M9–M13, like those for D4 and D5, were from two- to fivefold higher than those for wild-type HIV-1 (Table 1). Thus, we have mapped the most important region of *tar* required for *trans*-activation from positions +14 to +45 and identified clustered mutations and deletions that lead to increased basal levels of expression.

Compensatory mutations: investigation of structural features of *tar*

To determine the contributions of primary sequence and secondary structure in *tar* on *trans*-activation by *tat*, we analyzed two compensatory mutations that restore base pairing in the predicted stem (Fig. 1A,B). Compensatory mutation MC1, located in the middle of the stem, is a composite of mutations M8 and M12, each of which displayed greatly reduced *trans*-activation. The ability to *trans*-activate was recovered mostly with MC1 (Table 1), supporting the notion that secondary structure, but not primary sequence, in the middle of the stem is essential. Because clustered mutations 5' of position +14 and 3' of position +45 displayed slightly lower levels of *trans*-activation than wild-type, we tested a compensatory mu-

tation in this region of the stem. MC2, which restored base pairing of M5 at the base of the stem, *trans*-activated to wild-type levels, suggesting that secondary structure in the lower portion of the stem also contributes to *trans*-activation. However, the effects of clustered mutations that disrupt base pairing in the middle of the stem have a more significant effect (Table 1).

Stability of the stem-loop in *tar*

To define the relationship between *trans*-activation and predicted RNA stem-loop stabilities, we examined predicted free energies (ΔG) for the clustered mutations (Zucker and Stiegler 1981). Because *tar* from positions +14 to +45 is essential for *trans*-activation, we examined ΔG values for sequences from positions +14 to +45 and from +1 to +57 (Table 1). For M8–M12, reduced levels of *trans*-activation are paralleled by decreased predicted stabilities of the stem, especially from positions +14 to +45 (Table 1). The restoration of *trans*-activation and the corresponding recovery of predicted stability in the stem (ΔG) by MC1 support this correlation. Because M10, located in the loop, does not alter the stability of the stem yet abolishes *trans*-activation, the primary sequence of the loop also must be important for *trans*-activation.

The predicted stabilities of RNA structures that might

form in transcripts from 3'-deletion templates in *tar* correlate with *trans*-activation. Deletions to position +61 (D1) showed wild-type *trans*-activation and had no effect on the secondary structure of *tar* (Table 1). However, deletions to positions +45 (D2) and +42 (D3) demonstrated reduced *trans*-activations and correspondingly reduced stabilities of the stems. These data indicate that there is a direct relationship between the predicted stability of the stem-loop in *tar* RNA and *trans*-activation by *tat*.

RNA analyses

Previously, we demonstrated that short transcripts recovered in the cytoplasm reflect premature termination of transcription observed in a nuclear run-on assay of transiently transfected cells and that *trans*-activation by *tat* leads to the recovery of full-length HIV-1 RNA (Kao et al. 1987). The high degree of secondary structure in HIV-1 RNA from positions +1 to +57 required careful optimization of hybridization conditions to allow for quantitative detection of these prematurely terminated transcripts. Best conditions for the detection of short transcripts were 200 mM NaCl at 37°C (Fig. 2). Recovery of full-length RNA was not affected in this range of experimental conditions. Control RNase protection assays using untransfected COS-7 cell RNA showed no protected species (data not shown).

To determine how the clustered and compensatory mutations affect the generation of short and full-length transcripts, we analyzed RNA from cells transfected with these mutant constructions (Fig. 3 and data not shown). In all mutants, we found that changes in the levels of full-length transcripts correlated with *trans*-activation. For instance, with M9 (Fig. 3B, lanes 5 and 6), M10 (Fig. 3A, lanes 1 and 2), and M11 (Fig. 3B, lanes 7 and 8), which could not be *trans*-activated, the levels of full-length transcripts were not increased in the presence of *tat*. With the exception of M5 (Fig. 3B, lanes 1 and 2), M6 (Fig. 3B, lanes 3 and 4), and MC2 (Fig. 3A, lanes 7 and 8), short transcripts were detected in the absence of *tat* with both wild-type and mutant constructions. In MC2, small RNA species of ~21 nucleotides near the bottom of the gel probably correspond to a partial digestion of the probe (Fig. 3A, lanes 7 and 8); additional GC base-pairing in MC2 increased the predicted stability ($\Delta G = -42.4$ kcal/mole) of the lower stem, which might have made hybridization with the probe more difficult. In M5 and M6, the loss of base-pairing in the lower stem probably allowed for degradation of prematurely terminated transcripts (Fig. 1A). The requirement for an intact base of the stem for the recovery of the short transcripts was confirmed with studies on 3' deletions and 5' insertions (see below). Results for M13 hybridized with long (100 nucleotides) and truncated (60 nucleotides) probes confirmed that prematurely terminated transcripts originate from the 5' end of mutant (Fig. 3B, lanes 9–12), as was found with wild-type transcripts (Kao et al. 1987). In Figure 3, protected full-length RNA species from MC1 (Fig. 3A, lanes 5 and 6), MC2

(Fig. 3A, lanes 7 and 8), and M11 (Fig. 3B, lanes 7 and 8) migrated more slowly in the gel, probably as a result of nucleotide changes affecting secondary structure. This notion was confirmed when probes identical in length, but containing distinct RNA sequences, migrated differently in denaturing polyacrylamide gels (Fig. 3D).

As mentioned above, the recovery of short transcripts

Figure 2. Optimization of hybridization for RNase protection of HIV-1 transcripts. COS-7 cells were cotransfected with pHIV-1CATSV and pSVTAT or pSVTATZX, plasmids containing functional or nonfunctional *tat*, respectively. (Lanes 1–8) RNA from cells cotransfected with pSVTAT; (lanes 9–16) RNA from cells cotransfected with pSVTATZX. (L) Full-length transcripts; (S) prematurely terminated transcripts. The marker lane (M) contains *Msp*I-digested, end-labeled pUC19 fragments whose sizes are given in base pairs at right. Using 400 mM NaCl, the temperatures of hybridization were: 35°C (lanes 1,9); 37°C (lanes 2,10); 39°C (lanes 3,11); 41°C (lanes 4,12); and 43°C (lanes 5,13). Maintaining the temperature at 37°C, the concentration of NaCl was varied. (Lanes 6,14) 300 mM NaCl; (lanes 7,15) 200 mM NaCl; (lanes 8,16) 100 mM NaCl. The hybridization solution additionally contained 80% formamide, 40 mM PIPES (pH 7.0), and 1 mM EDTA. (Bottom) The probe used and the sizes of the protected RNA species. Uppercase letters represent restriction endonuclease sites: (P) *Pst*I; (H) *Hind*III; (N) *Nar*I. (pA) SV40 polyadenylation sequences.

Figure 3. (A) RNase protection of clustered mutations in *tar*. COS-7 cells were cotransfected with pHIV-1CATSV, clustered or compensatory mutations, and pSVTAT or pSVTATZX. (Lanes 1,3,5,7,9) RNA from cells cotransfected with pSVTAT; (lanes 2,4,6,8,10) RNA from cells cotransfected with pSVTATZX. (Lanes 1,2) M10; (lanes 3,4) M13; (lanes 5,6) MC1; (lanes 7,8) MC2; (lanes 9,10) pHIV-1CATSV. (L and S) Full-length and prematurely terminated transcripts, respectively. (Left) The sizes of *Msp*I-digested pUC19 fragments are given in base pairs. RNA from each clustered mutation was annealed with its corresponding RNA probe. The probes were 220 nucleotides long and extended from *Sca*I (S) to *Hind*III (H) sites. The wild-type probe used and the sizes of the protected RNA are shown in C. (B) RNase protection of M5, M6, M9, M11, and M13. (Lanes 1,2) M5; (lanes 3,4) M6; (lanes 5,6) M9; (lanes 7,8) M11; (lanes 9–12) M13. (Lanes 1,3,5,7,9,12) RNA from cells cotransfected with pSVTAT; (lanes 2,4,6,8,10,11) RNA from cells cotransfected with pSVTATZX. RNA from each clustered mutation was annealed with its corresponding RNA probe. The probes for lanes 1–10 were 100 nucleotides long and extended from *Pvu*II (P) to *Hind*III (H) sites. The probes for lanes 11 and 12 were truncated to 60 nucleotides at *Bgl*III (B) site. The sizes of the truncated probe and protected RNA are shown in C. (C) The probe used and the sizes of the protected RNA species are shown. Uppercase letters represent restriction endonuclease sites: (B) *Bgl*III; (H) *Hind*III; (P) *Pvu*II; (S) *Sca*I. (pA) SV40 polyadenylation sequences. (D) The migration of radiolabeled RNA probes in denaturing polyacrylamide gels. (Lanes 1–3) 220-nucleotide-long *Sca*I–*Hind*III probes to wild-type HIV-1LTR, MC1, and MC2, respectively; (lanes 4–6) 100-nucleotide-long *Pvu*II–*Hind*III probes to wild-type HIV-1 LTR, MC1, and MC2, respectively.

was dependent on an intact base of the stem-loop. Both full-length and prematurely terminated transcripts were recovered with D1 (3' deletion to position +61 in *tar*; Fig. 4, lanes 3 and 4), which displayed wild-type *trans*-activation. In contrast, no short transcripts were recovered with D2 (3' deletion to position +45, Fig. 4, lanes 5 and 6). Thus, stem-loops that are shorter than wild type or contain fewer base pairs at their bases are less stable and therefore more susceptible to degradation by endogenous RNases.

Effect of position of *tar* on *trans*-activation by *tat*

To determine how the location of *tar* relative to the cap site influences *trans*-activation, we examined a series of insertions made in the 5' end of *tar*. Previous studies, which placed *tar* (positions -17 to +80) at various sites relative to the HIV-1 LTR and CAT gene sequences, demonstrated that *tar* functions in a position- and orientation-dependent manner (Peterlin et al. 1986; Muesing et al. 1987). Hauber and Cullen (1988) introduced two insertions between the HIV-1 promoter and the site of initiation of transcription, which changed the cap site and resulted in reduced levels of *trans*-activation by *tat*. We introduced insertions at the 5' end of *tar* which preserved the native HIV-1 cap site. Insertions of defined lengths (multiples of 11 bp or approximately one helical turn) were introduced at an artificial *Xba*I site at position +8 (Fig. 5A), which precedes the functionally important region of *tar* (Jakobovits et al. 1988 and see

above). We found that the levels of *trans*-activation were inversely proportional to the lengths of the insertions (Fig. 5B). CAT activities in the absence of *tat* did not change substantially with these insertions, suggesting that basal transcription was not affected (data not shown). The gradual loss of *trans*-activation suggests that *tat* becomes increasingly less able to function as the target sequence is moved distal to the promoter.

To verify that transcripts from 5' insertions initiate at the HIV-1 cap site and to assay for prematurely terminated transcripts, we analyzed RNA from cells transfected with the largest insertion, pI4. The size of full-length RNA is consistent with initiation of transcription at position +1 (Fig. 5C, lanes 2 and 3). Smaller RNA species of 41–43 nucleotides were also protected. Because pBR322 sequences from positions +3000 to +3042 were used in the construction of pI4, the small protected RNA species could represent hybridization to transcripts derived from the vector and not the stem-loop in *tar*. This was confirmed by hybridization to RNA from COS-7 cells transfected with vector alone (Fig. 5C, lane 1). Prematurely terminated transcripts could not be recovered with pI4 because of the loss of some base-pairing in the base of the stem. In summary, the introduction of 88 nucleotides in *tar* did not change the cap

Figure 4. RNase protection of 3' deletions in *tar*. COS-7 cells were cotransfected with pHIV-1CATSV or 3' deletions D1 and D2 with pSVTAT or pSVTATZX. The lanes represent RNA from cells transfected with pHIV-1CATSV (lanes 1 and 2), D1 (lanes 3 and 4), and D2 (lanes 5 and 6). (Lanes 1,3,5) RNA from cells cotransfected with pSVTATZX; (lanes 2,4,6) RNA from cells cotransfected with pSVTAT. L, L1, and L2 refer to full-length and S to the prematurely terminated transcripts of wild-type, D1, and D2 protection assays, respectively. L1 and L2 are longer by 150 and 130 nucleotides than L, respectively, because the probes for D1 and D2, but not for pHIV-1CATSV, extend into CAT-coding sequences. Lane M contains *MspI*-digested end-labeled pUC19. The sizes of the DNA fragments are given in base pairs. The probes and the sizes of the protected RNA species are presented diagrammatically to the right of the autoradiogram. (WT) pHIV-1CATSV; (D1) deletion to position +61; (D2) deletion to position +45. Uppercase letters represent restriction endonuclease sites: (S) *ScaI*; (P) *PvuII*; (H) *HindIII*. (pA) SV40 termination and polyadenylation sequences.

site nor reduce basal levels of expression, but resulted in 5.5-fold reduced *trans*-activation.

Prematurely terminated transcripts in HIV-2

Although the genomes of HIV-1 and HIV-2 show only ~50% sequence identity (Emerman et al. 1987; Guyader et al. 1987), the sequences of their predicted stem-loops are very similar, especially in the region that is critical for *trans*-activation in HIV-1 (Fig. 1B; cf. nucleotides 14–45 in HIV-1 and 21–49 in HIV-2). Because CAT assays from COS-7 cells cotransfected with pSVTAT and pHIV-1CATSV or pHIV-2CATSV yielded comparable levels of *trans*-activation (Table 1), we looked for prematurely terminated transcripts in COS-7 cells transfected with pHIV-2CATSV. The hybridization temperature was varied to obtain more efficient recovery of short and full-length HIV-2 transcripts (Fig. 6). In the presence of *tat*, full-length transcripts were observed (Fig. 6; lanes 5–8). However, in the absence of *tat*, the levels of full-length transcripts were reduced, and increased levels of a shorter RNA were observed (Fig. 6; lanes 1–4). This short RNA extends to position +123, which corresponds to the base of the predicted stem-loop in HIV-2 (Fig. 1B). Discrete bands appearing below the full-length bands apparently derive from partial protection of full-length RNA due to incomplete hybridization with the

probe. Thus, in both HIV-1 and HIV-2, short transcripts corresponding to the predicted stem-loops are recovered in the absence of *tat*, whereas full-length transcripts are observed in the presence of *tat*.

Discussion

In this study we defined functionally important regions of HIV-1 *tar*, the relevance of secondary structure in the stem and primary sequence in the loop of the stem-loop in *tar* for *trans*-activation, and the dependence of *tar* on its distance from the HIV-1 cap site. Furthermore, we correlated the stability of the secondary structure in *tar* with *trans*-activation and the recovery of prematurely terminated transcripts. These data extend and support our previous observations that *tat* functions to elongate nascent transcripts through the HIV-1 LTR (Kao et al. 1987).

CAT assays of 3' deletions and clustered mutations in *tar* demonstrated that sequences from positions +14 to +45 are most important for *trans*-activation by *tat*. Our borders of functional *tar* agree with those from positions +19 to +42 mapped by Jakobovits et al. (1988). The sizes of clustered mutations or the actual nucleotides mutated may account for the small differences in the borders between our studies. It should be noted that transfections with nonreplicating (data not shown) and

replicating plasmids yielded equivalent results.

Although studies of clustered mutations alone revealed the borders of *tar* required for *trans*-activation, analysis of clustered and compensatory mutations distinguished between the contributions of primary sequence and secondary structure of the predicted stem-loop in *tar*. Because the mutations in M10 are in the loop exclusively and do not affect the stem, the absence of *trans*-activation and full-length transcripts in M10

demonstrate that the primary sequence of the loop is essential. This notion was confirmed by a study of single point mutations in the loop of HIV-1 (Feng and Holland 1988). MC1, composed of two clusters of mutations in the mid-stem, each of which decreased *trans*-activation greatly (i.e., M8 and M12), restored base-pairing and *trans*-activation. MC1 did not restore *trans*-activation completely to wild-type levels, probably because of less than wild-type stability (three GC and two AU base pairs were replaced by five AU base pairs). MC2 demonstrated that the base of the stem still contributes slightly to *trans*-activation. Because the secondary structure at the very top of the stem is required absolutely for *trans*-activation (Feng and Holland 1988), it appears that the relative contribution of the stem to *trans*-activation decreases with the distance from the loop.

A comparison of *tar* from HIV-1 and HIV-2 supports our conclusion on the relative contributions of primary sequence and secondary structure of the predicted stem-loop to *trans*-activation. We compared sequences from positions +1 to +57 in HIV-1 to those from positions +1 to +123 in HIV-2 because these sequences correspond to those in the prematurely terminated transcripts. *tar* of HIV-2 is composed of sequences that not only duplicate the upper stem-loop of *tar* from HIV-1 (stem-loop I), but also may contain two shorter stem-loops (stem-loops II and III) on the 3' side of the main stem. The sequences CUGGG (Fig. 1B) in the loop are conserved between HIV-1 and HIV-2 (loops I and II). Deletion analysis demonstrated that predicted stem-loops I and II of HIV-2 are required for *trans*-activation (Emerman et al. 1987; Jakobovits et al. 1988). Between these viruses, the stems most proximal to the conserved loops contain insertions, substitutions, and inversions of nucleotides. Despite variability in sequence, the predicted stability of this portion of the stem is conserved, and the levels of *trans*-activation are comparable. This analysis supports the notion that primary sequence in the loop and secondary structure in the stem of the pre-

Figure 5. (A) Structure of the four insertions, p1–p4, and the parent construction, pX. The insertions in *tar* (positions –17 to +80) at the *Xba*I site (position +8) in pX are shown below the triangle. The name of the plasmid and the length of the inserted DNA are shown to the right of each insertion. (B) The percent *trans*-activation of pX for each insertion is presented. The data shown are from a single transfection performed in duplicate or triplicate. The same pattern was observed with the analysis of three other independent transfections. (Insert) A log–log plot of the percent *trans*-activation of insertion mutants compared with that of pX versus the length of the insertion. (C) RNase protection of p4 transfections. (Lane 1) RNA from COS-7 cells transfected with pSVTAT; (lanes 2,3) RNA from cells cotransfected with p4 and pSVTAT and p4 and pSVTATZX, respectively. (Right) The sizes of *Msp*I-digested end-labeled pUC19 fragments are given in base pairs. (Bottom) The probe used and the sizes of the protected RNA. (L and S) Full-length HIV and short RNA species that result from protection of vector transcripts, respectively. Uppercase letters represent restriction endonuclease sites: (S) *Sca*I; (P) *Pst*I; (X) *Xba*I; (H) *Hind*III.

Figure 6. Optimization of hybridization for RNase protection of HIV-2 transcripts. COS-7 cells were cotransfected with pHIV-2CATSV and pSVTAT or pSVTATZX. (L and S) Full-length and prematurely terminated transcripts, respectively. (Lanes 1–4) RNA from cells cotransfected with pSVTATZX; (lanes 5–8) RNA from cells cotransfected with pSVTAT. The temperatures of hybridization were 39°C (lanes 1,5); 45°C (lanes 2,6); 50°C (lanes 3,7); and 55°C (lanes 4,8). The hybridization solution additionally contained 80% formamide, 40 mM PIPES (pH 7.0), 1 mM EDTA, and 200 mM NaCl. (Bottom) The probe for the HIV-2 LTR and the sizes of the protected RNA species.

dicted stem-loops in *tar* of HIV-1 and HIV-2 are important for *trans*-activation.

The detection of prematurely terminated transcripts represents a means to assess the effects of alterations in *tar* on *trans*-activation by *tat*. The secondary structure of these short RNA species allows for their recovery. Thus, mutants that disrupt base pairing at the base of the stem (M5, M6, pI4; Figs. 3 and 5) and deletions 5' of position +61 (D2 and D3; Fig. 4) yielded no prematurely terminated transcripts. However, even with intact stem-loops, the detection of the RNA can be difficult. Conditions of hybridization must be optimized to demonstrate quantitative recovery of wild-type and mutant RNA species because of competition between inter- and intramolecular base-pairing.

We found that some RNA species migrated with slower or faster mobilities than wild-type full-length or prematurely terminated transcripts (Fig. 3A,B). These bands could represent transcripts that initiated at different sites in the HIV-1 LTR, especially because different cap sites were observed with clustered point mutations from positions –6 to +5 in an *in vitro* transcription system (Jones et al. 1988). However, in our transient expression assay, two slower migrating transcripts con-

tain clustered point mutations 3' to these sequences (M11 and MC1), and wild-type, MC1, and MC2 RNA probes of identical lengths migrated differently in our denaturing polyacrylamide gels (Fig. 3D). Thus, full-length M11, MC1, and MC2 transcripts probably represent appropriately initiated RNA possessing different intrinsic mobilities. Low levels of transcription from cryptic promoters in our plasmid vector were also observed (e.g., see Fig. 5C). However, these transcripts that initiate 5' to the HIV-1 LTR cannot explain the presence of short transcripts in the absence of *trans*-activation. First, prematurely terminated transcripts are not polyadenylated (data not shown). Second, the levels of prematurely terminated transcripts are much higher than those of read-through transcripts. Third, our previous nuclear run-on experiments revealed that short transcripts initiated and terminated in the HIV-1 LTR (Kao et al. 1987).

The requirement of primary sequence in the loop and secondary structure in the stem of the predicted stem-loop in *tar* for *trans*-activation by *tat* is similar to the recognition structures for some RNA-binding proteins. In the replicase gene of bacteriophage R17, the coat protein binds to the loop sequence preceding the coding regions only in the context of a stable stem (Romaniuk et al. 1987). However, *tat* synthesized in bacteria does not bind specifically to *tar* RNA or DNA (Frankel et al. 1988). Because *tat* shares structural and functional features with bacteriophage λ N antiterminator (Franklin and Bennett 1979), which also does not bind to its N-utilization (*nut*) site, but does bind to a transcription complex that pauses at the *nut* site (Barik et al. 1987), it is possible that *tat* will bind only to its target sequence in the presence of RNA polymerase II and associated proteins. In a further analogy with HIV, the *nut* site forms an RNA stem-loop, and a single nucleotide substitution in the loop abolishes antitermination by N (Rosenberg et al. 1978; Salstrom and Szybalski 1978; Das and Wolska 1984).

If *tat* functions through an antitermination mechanism, then one would predict that removal of the termination site would result in constitutively high levels of expression. Although our clustered point mutations in the upper half of the stem-loop in *tar* and 3' deletions to positions +20 and +8 in *tar* yielded higher basal CAT activities, they did not approach the levels of expression observed with the wild-type HIV-1 LTR after *trans*-activation by *tat*. Because we have shown previously that *tar*-containing RNA is extremely stable (Peterlin et al. 1988), one possibility is that while relieving termination, these alterations in the stem-loops also resulted in decreased RNA stabilities. The reduced stability would affect both full-length and prematurely terminated transcripts (Peterlin et al. 1988). However, equivalent levels of prematurely terminated transcripts were observed with M9–M11, as with wild-type HIV-1 LTR, making reduced RNA stability an unlikely explanation. Because some alterations in *tar* resulted in an up-phenotype, there must be at least some negative regulation contributed by *tar* RNA or DNA. Such negative regulation

could be achieved by a sequence or structure where transcription pauses or terminates. Conversely, *tar* could load a factor that causes premature termination, i.e., a terminator. However, this negative regulation cannot account for the difference between wild-type basal and *trans*-activated levels of expression. Thus, other features of HIV-1 LTR must be responsible for premature termination. A strong candidate is the type of transcription complex that is assembled at the HIV-1 TATA-box, especially because only certain heterologous promoters placed 5' to *tar* lead to *trans*-activation by *tat* (Peterlin et al. 1986). One way in which the transcription complex might terminate prematurely is that RNA polymerase II initiates in an unstable conformation. In this model, *tat* or proteins induced by *tat* would create a conformational change in the polymerase, allowing for efficient elongation. Alternatively, a terminator could associate with the transcription complex at the HIV promoter and suppress transcriptional elongation actively. In this model, *tat* would either displace the terminator directly or modify the transcription complex. A prediction of this hypothesis is that wild-type levels of expression can be achieved in the absence of *tat* by depleting the terminator. This might be accomplished by cotransfections with plasmid DNA that contains promoter elements binding the terminator. Alternatively, this protein could be added to in vitro transcription systems and result in premature termination with template DNA from the HIV-1 LTR.

Finally, insertions at position +8, which displaced *tar* by multiples of 11 nucleotides or roughly single helical turns but retained the authentic cap site, led to decreased levels of *trans*-activation (Fig. 5). Lower levels of *trans*-activation might have resulted from the displacement of binding sites for transcription factors that have been mapped 3' of the HIV-1 cap site (Dinter et al. 1987; Garcia et al. 1987; Jones et al. 1988), especially because mutations in these binding sites decreased transcriptional efficiencies in an in vitro transcription assay (Jones et al. 1988). However, neither insertions nor cluster mutations in *tar* led to decreased basal levels of expression in our transient transfection assay. A more likely explanation for our findings is that the stem-loop in *tar* must be near the cap site to allow for efficient interaction between *tat* or factors induced by *tat* and the HIV-1 promoter elements facilitating elongation.

Antitermination of transcription in HIV closely resembles that in some prokaryotic and eukaryotic system (for reviews, see Platt 1986; Roberts 1988; Yanofsky 1988). For example, in bacteriophage λ , N and Q antiterminators act at their utilization sites (*nut* and *qut*, respectively) to modify their operon-specific transcription complexes, thereby allowing efficient elongation through distal termination sites (Grayhack et al. 1985; Barik et al. 1987). The *qut* site, like *tar*, has position and orientation dependency, and Q, like N, requires transcription to pause for efficient loading and/or modification of the transcription complex (Yang et al. 1987). Also, the rapid induction of *c-myc* (Bentley and Groudine 1986; Nepveu and Marcu 1986), *c-myb* (Bender et al.

1987), and *c-fos* (Fort et al. 1987) oncogenes may be a consequence of antitermination. Recently, prematurely terminated transcripts were recovered when the *c-myc* gene was injected into *Xenopus* oocytes (Bentley and Groudine 1988). In *c-myc* and small nuclear (sn) RNA genes, their promoters play important roles in transcription termination (Hernandez et al. 1986; Neuman de Vegvar et al. 1986; Bentley and Groudine 1988; Hernandez and Lucito 1988; Tanaka et al. 1988). Similarly, replacement of the HIV-1 promoter with the SV40 early or thymidine kinase (TK) promoters resulted in greatly reduced levels of *trans*-activation (Rosen et al. 1985; Peterlin et al. 1986). In addition, transcriptional enhancers can inhibit 3'-end formation in snRNA genes (Hernandez and Lucito 1988). Activation of HIV-1 LTR by phorbol esters or by T cell activation also leads to increased levels of viral gene expression, perhaps through an analogous suppression of premature termination (Kaufman et al. 1987; Nabel and Baltimore 1987; Tong-Starksen et al. 1987, 1988; Siekevitz et al. 1987). Finally, stalled transcription complexes at inducible promoters have been observed (Rougvie and Lis 1988). Transcriptional activation of these promoters involves efficient elongation of transcription and not increases in the rates of initiation of transcription. It is possible that in many eukaryotic genes, transcriptional enhancers and promoters function in an analogous fashion.

In summary, these data are compatible with the mechanism of transcriptional elongation by *tat* through the HIV LTR (Fig. 7). We propose that the stem-loop in *tar* provides a signal for *tat* or factors induced by *tat* to associate with and/or modify the transcription complex, thus facilitating elongation of transcription. In the absence of *tat*, the transcription complex remains susceptible to premature termination. How the HIV promoter and enhancer elements, as well as host cellular factors, contribute to *trans*-activation by *tat* is not yet known. Furthermore, the precise termination site(s) has not been mapped. These questions are perhaps best approached by studying *trans*-activation by *tat* in well-defined systems, like in *Xenopus* oocytes, yeast, and in vitro transcription systems.

Methods

Plasmid constructions

PHIV-1CATSV, pSVTAT, and pSVTATZX have been described previously in Kao et al. (1987). Clustered mutations in *tar* (from position -17 to +80), which introduced unique restriction sites, were generated in pHIV-1CAT by ligating synthetic oligonucleotides from *Pvu*II (position -17) to *Bgl*III (position +20), from *Bgl*III to *Sac*I (position +38) and from *Sac*I to *Hind*III (position +80). The HIV-1 LTR and CAT sequences from these nonreplicating plasmid constructions were then moved into a vector containing SV40 origin of replication (pSV-T4, P. Luciw, unpubl.). Nonreplicating and replicating plasmid constructions were transfected into COS-7 cells. Replicating plasmids were cotransfected with pSVTAT or pSVTATZX. Nonreplicating plasmids were cotransfected with *tat* transcribed from the RSV LTR or with pUC19. D1, D2 and D3 were made by digesting pHIV-1CAT with *Sac*I and *Hind*III, followed by replacement

Figure 7. Model for *trans*-activation by *tat*. HIV transcriptional enhancer and promoter factors assemble the transcription complex at the site of initiation of transcription. Transcription begins and nascent transcripts are made. By position +57, *tar* secondary structure is formed and the stem-loop becomes accessible to *tat* or factors induced by *tat*. In the absence of *tat*, the transcription complex and the nascent RNA fall off the template. Prematurely terminated RNA is stable and can be recovered in the cytoplasm. In the presence of *tat*, the transcription complex is stabilized, and transcription elongates beyond the stem-loop. Whether *tat* (or *tat*-induced factors) binds to RNA and/or the transcription complex and whether *tat* remains associated or just modifies the transcription complex must be determined.

with synthetic oligonucleotides that resulted in 3' deletions to positions +61, +45, or +42. To construct deletions D4 and D5, pHIV-1 CATSV, and plasmid X (pX) (see below), respectively, were digested with *Hind*III and *Bgl*II and *Xba*I, blunt-ended, and religated. These constructions were subcloned into the pSV-T4 vector. To construct 5' insertions, pX was made by replacing sequences from *Sph*I (position -10) to *Bgl*II sites in M1 with synthetic oligonucleotides containing changed nucleotides at positions +6, +7, and +8, which created an *Xba*I site. Into this *Xba*I site were cloned synthetic oligonucleotides of 88 bp that regenerated only the 3' *Xba*I site and additionally contained *Sma*I, *Cla*I, and *Mlu*I restriction sites. This plasmid is called p14. The sequence of the synthetic oligonucleotide is presented below:

```

"XbaI"      SmaI      ClaI      MluI
CTAGGTCACCGGAGGCATCGATTCCGGCAACAAACCACGGCTCTGAAGCCAGTTACCT
CAGTGGGCCCTCCGTAGCTAAGCCGTTTGTGTTGGTGGCAGACTTCGGTCAATGGA

                                XbaI
TCGGAAGAAAGAGTTGGTAGCTCTTGAT
AGCGTTTTTCTCAACCATCGAGAAGTATC

```

The DNA sequence used between the *Cla*I and *Xba*I sites, excluding the restriction sites, was derived from positions 3000–3042 in pBR322. As only the 3' *Xba*I site remained, p14 was digested and self-ligated sequentially with *Sma*I, *Cla*I or *Mlu*I, and *Xba*I, which resulted in progressively shorter insertions. The sequence of all clustered and compensatory muta-

tions, 3' deletions, and 5' insertions was verified by double-stranded DNA sequencing.

pHIV-2CAT, a generous gift of M. Emerman and L. Montagnier, was digested with *Kpn*I and *Hind*III and subcloned into *Kpn*I–*Hind*III digested pHIV-1CATSV, to generate pHIV-2CATSV.

The RNase protection of HIV-1 was optimized with pLG1 (Kao et al. 1987). Other RNase protection assays used pGEM-1/WT, which was derived by digesting pHIV-1CAT with *Sca*I (position -138) to *Hind*III (position +80) and ligating the HIV-1 LTR containing fragment into pGEM-1. The antisense probes for 3' deletions were made by cloning the HIV-1 containing fragments following digestion by *Pvu*II (from positions -17 to +150 in CAT) into pGEM-2 to generate pGEM-D1 and pGEM-D2. The antisense probe for p14 was derived by subcloning *Sca*I (position -138) to *Hind*III (position +168) from p14 into pGEM-1. The antisense probe for HIV-2 was derived by ligating the *Kpn*I (position -253)–*Hind*III (position +186) fragment into pGEM-2.

Transient transfection assays

COS-7 cells in 10-cm dishes were transfected with 5–10 µg of supercoiled plasmid DNA as described previously (Kao et al. 1987). All transfections were done with the designated plasmid construction and either pSVTAT or pSVTATZX. Cells were harvested for CAT assays as described (Kao et al. 1987; Neumann et al. 1987). The CAT assay data represent the average values of at least two independent transfections performed in duplicate or triplicate. RNA for RNase protection experiments was prepared from the cytoplasmic fractions of transfected cells (Maniatis et al. 1982).

RNase protection assays

Cytoplasmic RNA (2–2.5 µg) was assayed. The assays were done as outlined (Kao et al. 1987), except that the concentration of NaCl was reduced from 400 to 200 mM. The hybridization solution additionally contained 80% formamide, 40 mM PIPES (pH 7.0), and 1 mM EDTA. Unless stated otherwise, all annealings were conducted at 37°C. One half of the samples were electrophoresed in 11% polyacrylamide–urea sequencing gels, together with *Msp*I-digested and end-labeled pUC19 DNA fragment (Maniatis et al. 1982).

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