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RNA-Targeted Activators, but Not DNA-Targeted Activators, Repress the Synthesis of Short Transcripts at the Human Immunodeficiency Virus Type 1 Long Terminal Repeat

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The human immunodeficiency virus type 1 (HIV-1) promoter directs the synthesis of two types of RNA molecules: full-length transcripts, whose synthesis is activated by the viral activator Tat, and short transcripts, whose synthesis is dependent on the inducer of short transcripts (IST), a bipartite DNA element located in large part downstream of the HIV-1 transcriptional start site. In the absence of Tat, short transcripts constitute the large majority of the RNA molecules synthesized from the HIV-1 promoter. In the presence of Tat, synthesis of the short transcripts is repressed and synthesis of the full-length transcripts is activated. Tat is unique among transcriptional activators in acting through an RNA target, the TAR element. However, Tat has been shown to activate transcription from a DNA target when fused to the appropriate DNA binding domain, raising the question of why Tat has been directed to the RNA. Here we have compared the abilities of Tat and other RNA- and DNA-bound activators to stimulate transcription from the HIV-1 promoter. We show that DNA-targeted activators, including DNA-targeted Tat, activate the synthesis of both short and long transcripts, while RNA-targeted Tat and another RNA-targeted activator activate the synthesis of full-length transcripts but specifically repress that of short transcripts. The unique ability of RNA-targeted activators to down-regulate short transcript synthesis suggests that Tat is directed to the RNA specifically for the purpose of repressing short transcripts.

Human immunodeficiency virus type 1 (HIV-1) provides a fascinating example of an intricately regulated transcription unit. Its promoter can generate two types of RNA molecules, whose relative abundancies are regulated in large part by the viral transactivator Tat. Upon transfection into HeLa, COS, or Jurkat cells, the HIV-1 promoter generates, in the absence of Tat, a large amount of correctly initiated, nonpolyadenylated, short HIV-1 transcripts ending around position +60, as well as a much smaller amount of polyadenylated, full-length transcripts. In contrast, in the presence of Tat, the short transcripts are down-regulated while the full-length transcripts are dramatically up-regulated (21, 35, 44). Consistent with this observation, short transcripts are more abundant in cells infected with viruses that lack an active Tat than in cells infected with wild-type viruses (6), and activation of latently infected T cells (33) or peripheral mononuclear cells from asymptomatic HIV-1-infected individuals (1) results in an increase in the ratio of full-length to short transcripts.

Formation of short transcripts is dependent on a bipartite DNA element referred to as the inducer of short transcripts (IST). IST is largely located downstream of the transcription start site, with the main half-element positioned between positions −5 and +26 and the second half element positioned between positions +40 and +59 (35, 44). Mutations in IST greatly reduce the transcriptional output of the HIV-1 promoter in the absence of Tat by selectively reducing the synthesis of short transcripts. Thus, IST appears to act in concert with the HIV-1 promoter to stimulate the formation of transcription complexes that are incapable of efficient elongation. IST can activate the synthesis of short transcripts when placed downstream of several heterologous promoters and depends only on cellular factors for function (35).

Activation of the full-length transcripts by Tat is dependent on an RNA element known as the transactivation-responsive element (TAR), encoded between positions +18 and +44 downstream of the HIV-1 cap site (7, 13, 16, 38, 41); see reference 17 for a review) and thus contained within the short transcripts. TAR corresponds to the upper part of a stable stem-loop structure formed from the first 59 nucleotides of the RNA (30). Tat binds directly to TAR, probably together with as yet unidentified cellular factors (reviewed in reference 17). Tat has been shown to act at least in part by improving the elongation properties of the transcription complex, both in vivo (6, 23, 24) and in vitro (22, 25, 28, 29, 52). However, the original suggestion that Tat acts as an antiterminator that converts the short transcripts into full-length transcripts is unlikely to be correct because Tat can transactivate full-length transcripts from a mutant promoter incapable of making short transcripts; in this case, TAR sites are probably provided by the low background levels of full-length transcripts synthesized in the absence of Tat (reference 44 and this work; see also the review in reference 5).

The observation that Tat is not an antiterminator similar to, for example, the procaryotic antiterminator N protein (10) suggests that Tat must not necessarily be targeted to the RNA to activate transcription. Indeed, Tat can activate transcription when targeted to the DNA by fusion to an appropriate DNA binding domain. Specifically, Tat increases expression of a chloramphenicol acetyltransferase gene driven by HIV-1 promoter sequences when targeted as a GAL4-Tat fusion protein to multiple GAL4 binding sites inserted in the promoter (8, 19, 45, 46). Activation by DNA-targeted Tat depends on the same promoter elements as activation by RNA-targeted Tat, and mutations in both Tat (8, 19, 45) and TATA-binding protein (32) that debilitate activation from the RNA also debilitate

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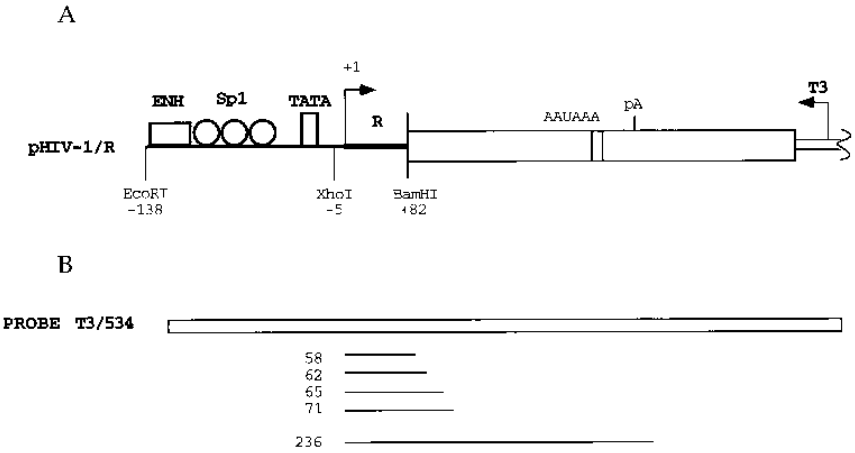

FIG. 1. Schematic diagram of the insert in the reporter construct pHIV-1/R. (A) pHIV-1/R (35) contains HIV-1 sequences extending from positions −138 to +82, a region that includes (i) the HIV-1 promoter with the enhancer (ENH), the three Sp1 binding sites, and the TATA box and (ii) R sequences from positions +1 to +82 (thick line), which contain the IST and TAR elements. Downstream is an adenovirus type 2 fragment (large open rectangle) containing the L3 polyadenylation site (pA), followed by a fragment carrying the bacteriophage T3 promoter. (B) Complementary RNA probe T3/534 synthesized from the indicated bacteriophage T3 promoter is protected over 58 to 71 nucleotides by short transcripts and over 236 nucleotides by full-length transcripts.

activation from the DNA. These results suggest that Tat uses the same mechanism to activate transcription from the DNA and from the RNA. Why, then, has HIV-1 evolved a so far unique mechanism of transcription activation in which the activator Tat is targeted to the RNA?

Here, we have compared Tat and conventional, DNA-targeted activators with respect to their effects on short and full-length transcripts. We show that DNA-targeted activators, including Tat targeted to the DNA by fusion to the GAL4 DNA binding domain, activate the synthesis of both short and long transcripts. In sharp contrast, Tat targeted to TAR RNA selectively represses the synthesis of short transcripts. This effect is dependent on the Tat activation domain, as mutations in Tat that debilitate activation of the long transcripts also debilitate repression of the short transcripts. Remarkably, the effect is not specific to the Tat activation domain per se; a VP16 activation domain directed to the RNA by fusion to the Rev RNA binding domain also represses the short transcripts. Moreover, when the HIV-1 promoter is activated by both Tat and DNA-targeted activators, the short transcripts are generally still repressed, suggesting that short transcript repression by Tat is dominant over short transcript activation by DNA-targeted activators. Together, these results suggest that of the two transcription functions of Tat, activation of the full-length transcripts and repression of the short transcripts, the second one specifically requires targeting to the RNA.

MATERIALS AND METHODS

Plasmid constructions. pHIV-1/R has been described previously (35). p(4×G17)pHIV-1/R was constructed by insertion of an *Asp*718 fragment containing four copies of a 17-bp GAL4 binding site (51) into an *Asp*718 site located 140 bp upstream of the HIV-1 transcription start site in pHIV-1/R. The GAL4 sites are in the same orientation as the HIV-1 transcription unit. To generate the p(4×G17)HIV-1/R+34Δ4 and p(4×G17)HIV-1/R+34Δ4-FAS constructs, the p(4×G17)HIV-1/R and p(4×G17)HIV-1/R+34Δ4 constructs were partially digested with *Sac*I, which cleaves three times in the construct, the 3′ overhangs were removed with Klenow enzyme, and linear molecules resulting from a single cleavage were isolated by gel electrophoresis and religated. The correct constructs, which were checked by sequencing, had lost the *Sac*I site at position +34 downstream of the HIV-1 transcription start site due to a 4-bp deletion. To construct pHIV-1/R/SLIIB, a *Sca*I/*Afl*II fragment of pSLIIB/CAT (a kind gift from B. R. Cullen) was ligated to a vector created by cutting pHIV-1/R at the *Asp*718 site, filling in that site, and then cutting with *Afl*II. pCGN-Tat was constructed by subcloning Tat-encoding sequences from pCG-Tat (35) into the mammalian expression vector pCGN (48), which adds a 15-amino-acid hemagglutinin (HA) epitope tag to the amino terminus of the expressed protein. To construct pCGN-GAL(1-94)Tat, a *Sty*I/*Xba*I fragment from pUC119sma::GAL (1-94) (a kind gift from M. Tanaka) containing GAL(1-94)-encoding sequences was ligated into pCGN-Tat cleaved with *Xba*I, which cleaves just 5′ of the sequences encoding the amino terminus of Tat. To construct pCGN-GAL(1-94) Tat mutants Tyr-47, Pro-18IS, Δ3/6, and Pro18, PCR with oligonucleotides containing *Bam*HI and *Xba*I restriction sites was used to amplify and isolate the mutant Tat sequences from their pSVL and pGEM expression plasmids (kind gifts from A. Rice or M. Green). *Xba*I/*Bam*HI fragments carrying the mutant Tat genes were subcloned into the *Xba*I and *Bam*HI sites of pCGN-GAL(1-94)Tat. pcRev, pcRev-VP16, and pcRev-VP16m442 were kind gifts of S. Ghosh and B. R. Cullen.

Transfections. HeLa cells were transfected by electroporation. Cells were grown to a density of 80 to 90% in Dulbecco minimal essential medium supplemented with 10% fetal calf serum. For each sample, the cells from one 10-cm-diameter dish were trypsinized and resuspended in 250 μl of medium. A mixture of plasmids containing 7 μg of the test plasmid, 1 μg of pα1×72, a plasmid carrying the human α-globin gene, 0.4 μg of pCGN or pCGNTat, and salmon sperm DNA carrier to a total amount of 20 μg in 30 μl of TE (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) was added to the cells. The mixture was incubated at room temperature for 5 min, transferred to a Gene Pulser cuvette (0.4-cm-wide electrode gap; Bio-Rad), and electroporated with a pulse of 260 V and 960 mF in the Gene Pulser (Bio-Rad). The cells were then transferred to 10-cm-diameter dishes containing 10 ml of medium and collected 24 h later. RNase T_1 protection analyses were performed on RNA extracted from half of each sample, while Western blot analyses were performed on protein extracts collected from the other half of each sample with an antibody (12CA5) directed against the HA epitope tag to determine Tat expression levels.

RNase T_1 mapping. RNA was collected and RNase T_1 protections were performed as described previously (14). To generate the riboprobes, each different construct was cleaved with *Eco*RI and used as a template for bacteriophage T3 RNA polymerase. Gels were quantitated with a Bio-Imaging Analysis System (Fuji), with normalization to the α-globin signal from the internal reference.

RESULTS

Repression of the short transcripts requires a functional Tat activation domain. Upon activation by Tat, the ratio of short and long transcripts synthesized by the HIV-1 promoter is dramatically altered. Tat activates the synthesis of full-length transcripts and concomitantly represses the synthesis of short transcripts. To determine whether, like activation of the full-length transcripts, repression of the short transcripts requires the Tat activation domain, we tested different mutations in Tat that debilitate activation of the long transcripts.

We transiently transfected HeLa cells with a mixture of three constructs. The HIV-1 reporter construct, pHIV-1/R (35), is diagrammed in Fig. 1A and 2A. This construct contains

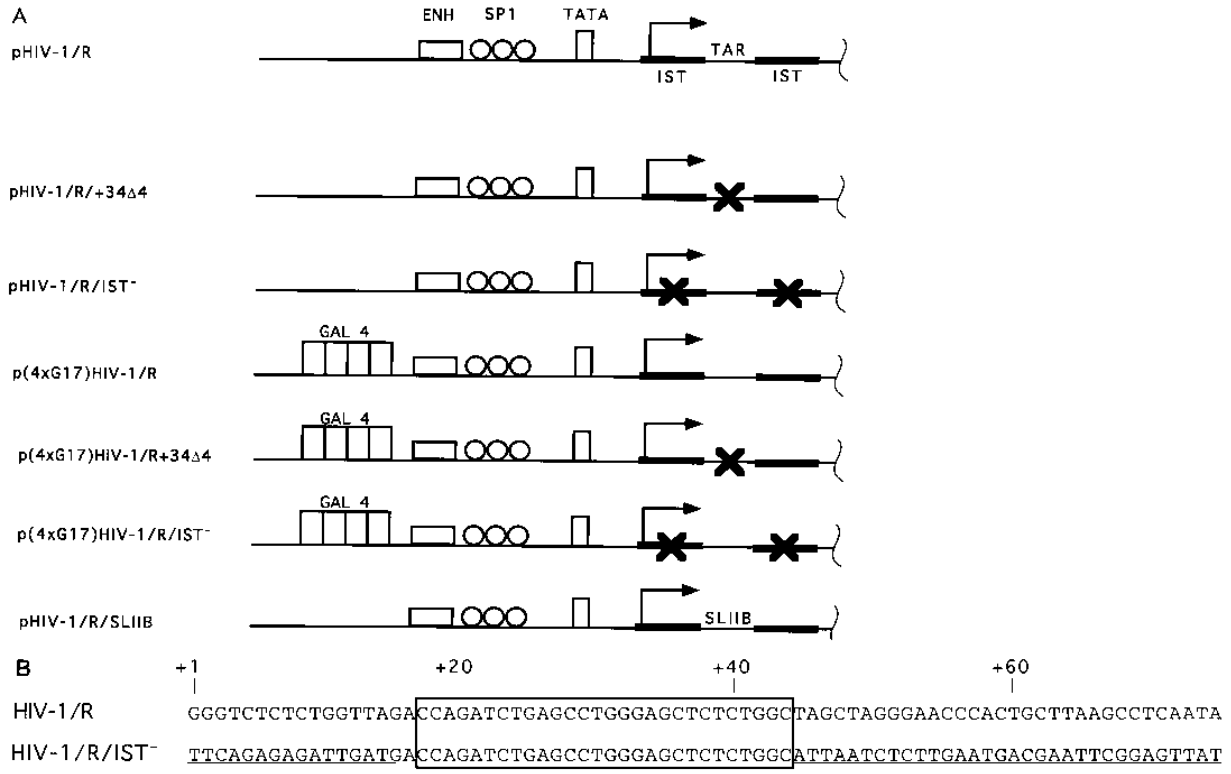

FIG. 2. Structure of the promoter and R regions of the different reporter constructs used in this work. (A) Mutations in TAR or IST are schematically indicated with crosses. The +34Δ4 construct has been described previously (35). The IST-mutation is shown in panel B. The SLIIB mutation is as described in reference 50. (B) Sequence in the repressor region of pHIV-1/R/IST$^-$ compared to that of the wild-type construct pHIV-1/R. Altered base pairs are underlined. Note that the TAR element (boxed) is unchanged.

HIV-1 sequences from −138 to +82 relative to the transcriptional start site, spanning the HIV-1 promoter as well as the downstream IST element and the sequences encoding the TAR RNA element (positions +18 to +44). The HIV-1 sequences are followed by (i) a fragment derived from adenovirus type 2 that contains the L3 polyadenylation signal and (ii) an antisense bacteriophage T3 promoter which serves to generate perfectly complementary RNA probes that map simultaneously the 5′ and 3′ ends of short and long transcripts (Fig. 1B). The second plasmid was either an expression vector (pCGNTat) producing wild-type or mutant Tat proteins tagged at their amino termini with the HA epitope (31) or a control vector (pCGN) lacking Tat coding sequences. The third plasmid (pα1×72) carried the human α-globin gene, whose expression is not affected by Tat and which served therefore as an internal control for transfection efficiency and RNA recovery. We quantitated the accumulation of short and long HIV-1 transcripts as well as α-globin transcripts by RNase T_1 protection analysis. For the HIV-1 transcripts, we used the probe (designated T3/534) diagrammed in Fig. 1B. This probe is protected over 58 to 71 nucleotides by short transcripts and over 236 nucleotides by full-length transcripts polyadenylated at the L3 site.

As shown in Fig. 3, lane 1, transfection of the HIV-1 reporter construct in the absence of Tat resulted in accumulation of a large amount of short transcripts and a low amount of full-length transcripts. We have determined previously that both short and full-length transcripts are correctly initiated and that the full-length transcripts are polyadenylated at the L3 polyadenylation site (35). As expected, inclusion of a wild-type Tat expression vector in the transfection resulted in a dramatic increase in the production of full-length transcripts (45-fold) and a 70% decrease in the synthesis of short transcripts (lane 2). This Tat-induced decrease was not observed with an HIV-1 reporter construct carrying a four-nucleotide deletion in the TAR element at position +34 (construct pHIV-1/R+34Δ4 [35] [Fig. 2A]) which debilitates Tat activation (compare lanes 12 and 13 with lane 11, bands labeled ST). This mutation presumably prevents stable association of Tat with TAR, suggesting that Tat must be bound to its target for repression of short transcripts. In addition, the short transcripts are dependent on the IST element, as no short transcripts were observed with the construct pHIV-1/R/IST$^-$ (Fig. 2), which contains a debilitated IST element but an intact TAR (lanes 14 to 16; note that the band labeled with an asterisk is also obtained with RNA from cells transfected only with pα1×72 [lane 17] and therefore does not correspond to short transcripts).

We then tested several Tat mutants originally constructed by Rice and Carlotti (36). These mutants carry single alanine substitutions, deletions, and insertions in the Tat gene. As reported previously (36), the Tyr-47 Tat mutant, in which a tyrosine outside the cysteine-rich region is substituted for an alanine, was able to activate transcription at wild-type levels (39-fold activation, compared with 45-fold activation for the wild type; compare lanes 3 and 4 with lane 2, band labeled FL), whereas mutant Pro-18IS, which contains an insertion of a glutamine and a phenylalanine just after the proline residue at position 18, mutant Δ3/6, in which residues 3 to 6 are deleted, and mutant Pro-18, in which proline 18 is replaced by an alanine, activated transcription only 5- to 6-fold (compare

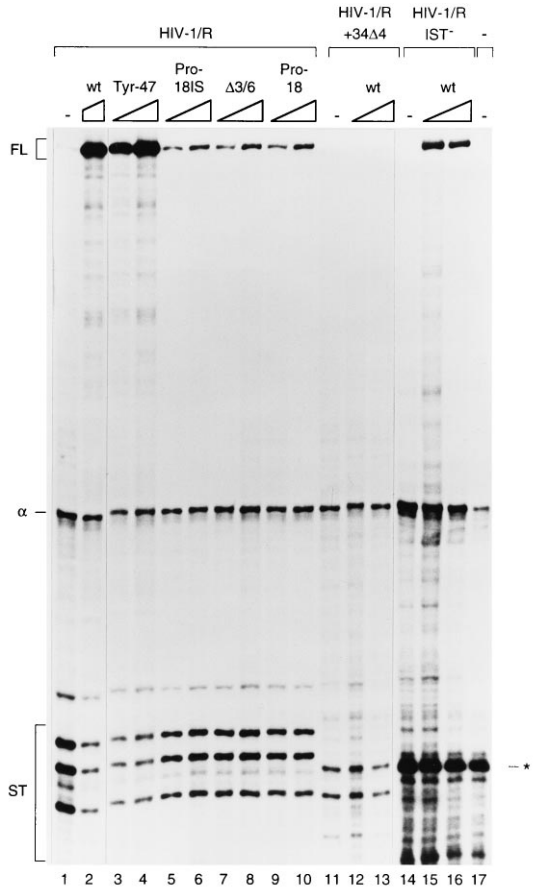

FIG. 3. Repression of the short transcripts requires a functional Tat activation domain. Shown is RNase T_1 protection with RNA isolated from HeLa cells transfected with a plasmid (pα1×72) carrying the α-globin reference gene alone (lane 17) or plasmid pα1×72 and the reporter constructs pHIV-1/R (lanes 1 to 10), pHIV-1/R+34Δ4 (lanes 11 to 13), pHIV-1/R/IST⁻ (lanes 14 to 16), and the expression plasmid pCGN (lanes labeled −), pCGNTat (lanes labeled wt [wild type]), or various derivatives thereof expressing mutated Tat proteins, as indicated above the lanes. The probes used were α98, a probe that protects correctly initiated α-globin RNA over 98 nucleotides, and T3/534. α, correctly initiated α-globin RNA; ST, correctly initiated short transcripts; FL, RNA correctly initiated and polyadenylated at the L3 site; ∗, background bands.

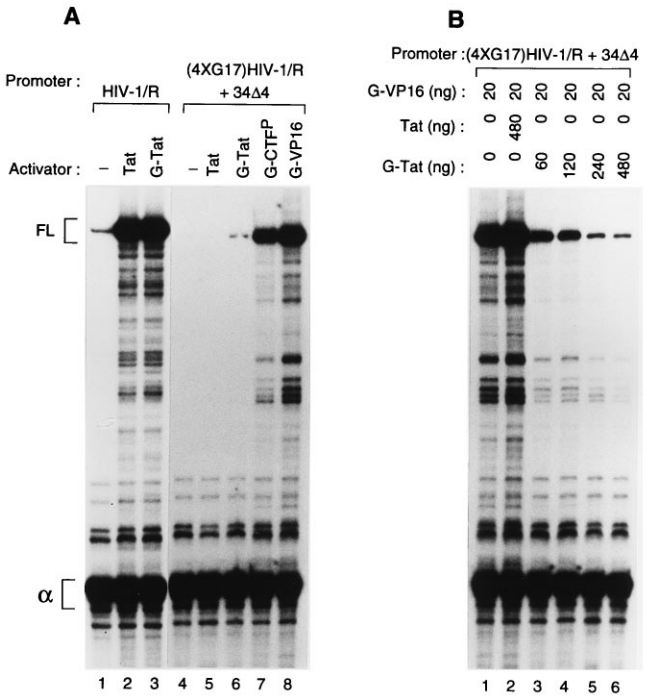

FIG. 4. (A) Tat is a weak activator when targeted to the DNA. Shown is RNase T_1 protection with RNA isolated from HeLa cells transfected with pα1×72, pHIV-1/R (lanes 1 to 3), or p(4×G17)HIV-1/R+34Δ4 (lanes 4 to 8) and the indicated activator expression plasmid. Tat and G-Tat induced full-length transcripts 44- and 48-fold, respectively, from pHIV-1/R (lanes 2 and 3), while Tat, G-Tat, G-CTF^P, and G-VP16 induced full-length transcripts 1.4-, 4.4-, 26-, and 36-fold, respectively, from p(4×G17)HIV-1/R+34Δ4 (lanes 5 to 8). In a second experiment, the same activators induced full-length transcripts 1.1-, 4.9-, 21-, and 39-fold, respectively. (B) The GAL4 DNA binding domain of G-Tat is functional. Shown is RNase T_1 protection of RNA isolated from HeLa cells transfected with pα1×72, p(4×G17)HIV-1/R+34Δ4, 20 ng of pCG-GAL(1-94)VP16(413-490) (expressing G-VP16), and either 480 ng of pCGN-Tat (lane 2) or increasing amounts of pCGN-GAL(1-94)Tat (expressing G-Tat) (lanes 3 to 6). The increasing levels of pCGN-GAL(1-94)Tat reduce activation by pCG-GAL(1-94)VP16(413-490) from 40-fold (lane 1) to 6-fold (lane 6). Bands are labeled as in Fig. 3.

lanes 5 to 10 with lane 2, band labeled FL). Strikingly, the abilities of these different mutant Tat proteins to repress the short transcripts closely paralleled their abilities to activate transcription: Tyr-47 repressed the short transcripts at wild-type levels (61%), whereas the mutants Pro-18IS, Δ3/6, and Pro-18 repressed the short transcripts only 25 to 30% (compare lanes 3 to 10 with lane 2, bands labeled ST).

To exclude the possibility that the effects observed resulted from poor expression of the mutant Tat proteins rather than from the mutations themselves, protein extracts derived from an aliquot of the transfected cells were analyzed by immunoblotting with the monoclonal antibody 12CA5 (31), which recognizes the HA tag at the amino termini of the different Tat proteins. As reported previously (36), we found the levels of wild-type and mutant Tat proteins in transfected cells to be comparable (data not shown). In addition, Rice and Carlotti (37) showed that all of the mutant proteins contain an intact nuclear localization domain, and both the mutants Pro-18IS and Pro-18 show patterns of trypsin resistance similar to that of wild-type Tat, suggesting that their tertiary structure is not drastically altered (37). Together, these results suggest that repression of the short transcripts is dependent on the Tat activation domain.

Tat targeted to the DNA is a weak activator of long transcripts. Tat is, so far, the only transactivator known to act through an RNA target. The question then arises as to why the HIV-1 virus has devised such an unusual way to increase transcription from its promoter. To address this point, we compared RNA-targeted Tat with three activators targeted to the DNA by fusion to the heterologous GAL4 DNA binding domain (residues 1 to 94 [2]): G-Tat, in which the entire Tat protein (residues 1 to 86) is fused to the GAL4 DNA binding domain; G-CTF^P, which contains a single copy of the proline-rich CTF activation domain (49); and G-VP16, which contains a single copy of the acidic VP16 activation domain (49). For this purpose, we inserted four GAL4 DNA binding sites upstream of the HIV-1 enhancer. In addition, we debilitated the TAR element with the +34Δ4 deletion to ensure that the G-Tat fusion protein could transactivate only through its DNA target, the GAL4 binding sites. The resulting construct, p(4×G17)HIV-1/R+34Δ4, is shown in Fig. 2A.

As can be seen in Fig. 4A, lane 5, Tat was incapable of activating the synthesis of full-length transcripts from the construct containing the +34Δ4 TAR deletion, as expected. In

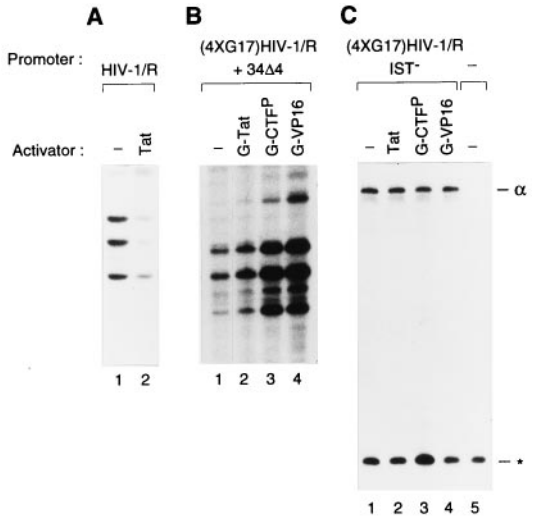

FIG. 5. RNA-targeted Tat represses short transcripts, and DNA-targeted G-Tat, G-CTF^P, and G-VP16 activate short transcripts. (A) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1×72, pHIV-1/R, and either pCGN (lane 1) or pCGNTat (lane 2). Only the short transcripts are shown. Tat represses short transcripts 77%, as quantitated by a phosphorimager. (B) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1×72, p(4×G17)HIV-1/R+34Δ4, and the activator expression plasmid indicated. Only the short transcripts are shown. Short transcripts were activated 2.1-fold by G-Tat 4.5-fold by G-CTF^P, and 6.1-fold by G-VP16, as quantitated by phosphorimaging and normalization to the α-globin internal control. This experiment was repeated twice with similar results. (C) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1×72, p(4×G17)HIV-1/R/IST$^-$, and the activator expression plasmid indicated. Only the α-globin (α) and short transcripts are shown. The band labeled with an asterisk is also detected in RNA from mock-transfected cells (lane 5) and therefore does not correspond to HIV-1 short transcripts.

contrast, G-Tat could activate the promoter (compare lane 6 to lane 4), but to a much lesser extent than G-CTF^P (lane 7) or G-VP16 (lane 8). Since G-Tat was able to activate the HIV-1 promoter very efficiently when targeted to the RNA (compare lanes 3 and 1), the G-Tat fusion protein was not defective for activation. As an additional control, we established that the GAL4 DNA binding domain of the G-Tat construct was functioning in vivo by performing a repression assay, which is shown in Fig. 4B. Transactivation by G-VP16 (lane 1) could be repressed by cotransfection of increasing amounts of the G-Tat expression vector (lanes 3 to 6) but not by cotransfection of the Tat expression vector (lane 2), suggesting that G-Tat was able to bind to the GAL4 binding sites and thereby prevent binding of the more potent activator G-VP16. Together, these results confirm that G-Tat can function from the DNA, even though it appears to be a much weaker activator than other DNA-targeted activators such as G-CTF^P and G-VP16.

Tat must be targeted to the RNA to repress the formation of short transcripts. We then examined whether RNA-targeted Tat differed from G-Tat, G-CTF^P, and G-VP16 in its ability to repress the short transcripts. The results are shown in Fig. 5. In stark contrast to Tat, which repressed the formation of short transcripts (compare lanes 2 and 1 in Fig. 5A), G-Tat, G-CTF^P, and G-VP16 all activated the synthesis of short transcripts (Fig. 5B; compare lanes 2 to 4 to lane 1). The efficiency of short transcript activation paralleled closely the efficiency with which the different fusion proteins activated the synthesis of full-length transcripts (Fig. 4A). G-Tat is a weak activator of both full-length and short transcripts, whereas G-VP16 is a very strong activator of both classes of transcripts. As expected, with the construct carrying a debilitated IST element (construct pHIV-1/R/IST$^-$ [Fig. 2]), only the background band (marked with an asterisk) that is present even with RNA from mock-transfected cells (Fig. 5C, lane 5; see also Fig. 3, lane 17) can be observed. No bands corresponding to short transcripts were obtained even in the presence of DNA-targeted activators (Fig. 5C, lanes 1 to 4). These results indicate that Tat and DNA-targeted activators differ dramatically in their effects on short transcript formation and suggest that Tat may be targeted to the RNA mainly because of its unique effect on short transcript formation.

The VP16 activation domain also represses the short transcripts when targeted to the RNA. The results described above suggest that repression of the short transcripts is dependent on the Tat activation domain and on Tat being targeted to the RNA. We therefore examined whether another activation domain targeted to the RNA could also repress the short transcripts. For this purpose, we replaced the TAR sequences in pHIV-1/R with a 13-bp sequence derived from the Rev response element that constitutes an efficient RNA target for Rev binding in vivo, exactly as described by Tiley et al. (50). The resulting construct, pHIV-1/R/SLIIB, is shown in Fig. 2A. We then used this construct to test the effects of the VP16 activation domain targeted to the RNA by fusion to the Rev RNA binding protein (50), and the results are shown in Fig. 6.

As expected, both Tat and G-VP16 activated full-length transcript synthesis from an HIV-1 reporter construct containing an intact TAR element and GAL4 binding sites (compare lanes 2 and 3 with lane 1, band labeled FL), but only Tat repressed the short transcripts (65% repression; lane 2, bands labeled ST): G-VP16 activated rather than repressed the short transcripts (5.3-fold activation; lane 3, bands labeled ST). The HIV-1/R/SLIIB construct generated longer short transcripts, whose 3′ ends map immediately downstream of the longer stem-and-loop structure predicted for RNA containing the 13-bp Rev response element (50) (Fig. 6B, lane 1, bands labeled LST). This result is consistent with our previous observation that the length of the short transcripts is determined by the length of the stem-and-loop structure (44). In the presence of the Rev protein, a very low level of transcription activation could be observed (2.3-fold activation; compare lanes 2 and 1, LFL band), and interestingly, a low but reproducible repression of short transcripts was also detectable (31% repression; compare lanes 2 and 1, LST bands). This activity may be due to a cryptic activation domain in the Rev protein. In contrast, the Rev-VP16 fusion protein activated synthesis of the full-length transcripts significantly (8.5-fold). Remarkably, it also strongly repressed the short transcripts (66%; lane 3). This effect was dependent on a functional VP16 activation domain, because the Rev-muVP16 fusion protein, in which the VP16 activation domain is debilitated by mutation of the essential Phe-442 to a proline residue, did not activate the full-length transcripts or repress the short transcripts any better than just Rev (compare lane 4 to lane 2). Thus, repression of the short transcripts does not appear to depend on the Tat activation domain per se. Rather, it depends on an activation domain being targeted to the RNA.

RNA-targeted activators can repress the short transcripts even in the presence of DNA-targeted activators. The results above indicate that the RNA-targeted activators Tat and Rev-VP16 can repress the short transcripts, whereas the same activators, when targeted to the DNA, activate the short transcripts. In the natural situation, however, the HIV-1 promoter is most probably activated simultaneously by RNA-targeted Tat and DNA-targeted activators. To determine the effects on the short transcripts in such a situation, we cotransfected the

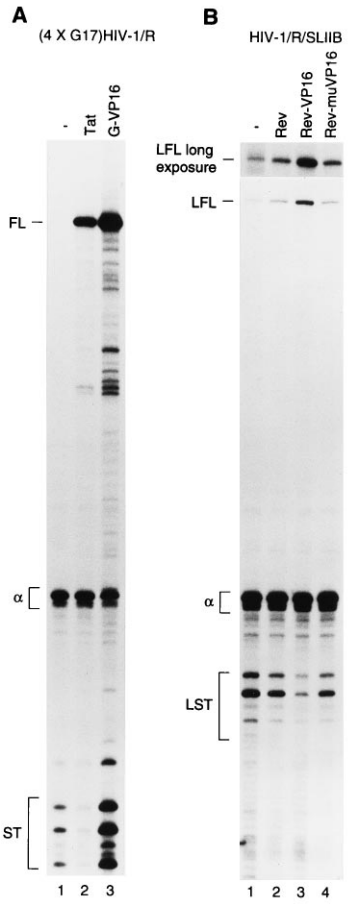

FIG. 6. The VP16 activation domain represses the short transcripts when targeted to the RNA. (A) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1×72, p(4×G17)HIV-1/R, and the activator expression plasmids indicated. (B) RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1×72, pHIV-1/R/SLIIB, and the activator expression plasmids indicated. LFL, long full-length transcripts; LST, long short transcripts derived from the pHIV-1/R/SLIIB reporter construct. The long exposure of the long full-length transcripts was for 12 days, as opposed to 2 days for the rest of panel B and 1 day for panel A. Other bands are labeled as in Fig. 3.

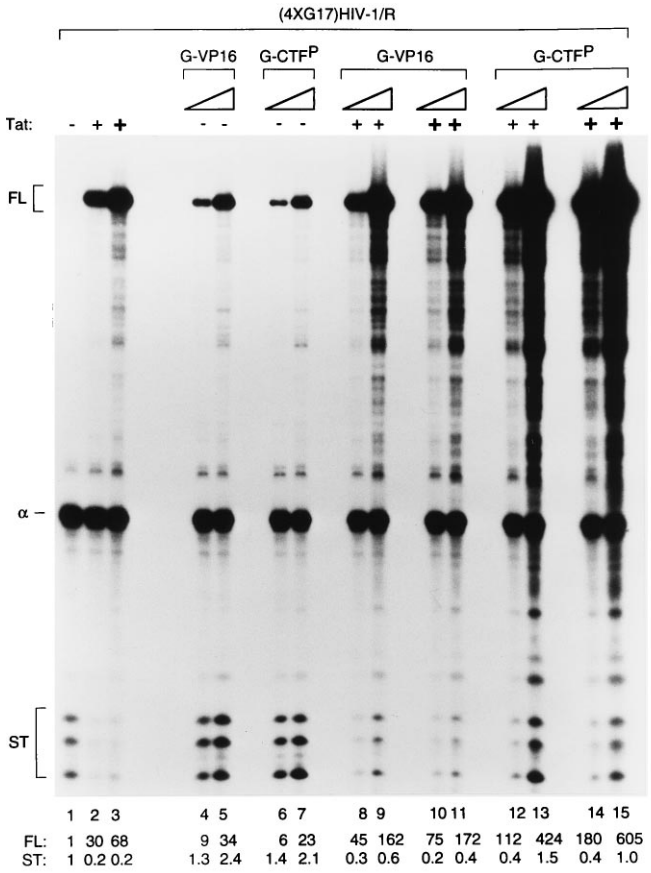

FIG. 7. Repression of the short transcripts by Tat is dominant over activation of the short transcripts by DNA-targeted activators. Shown is RNase T_1 analysis of RNA isolated from HeLa cells transfected with pα1×72, p(4×G17)HIV-1/R, and pCGN (lanes labeled −) or either 20 ng (lanes labeled +) or 200 ng (lanes labeled +) of pCGNTat either alone (lanes 1 to 3) or in combination with expression vectors encoding the indicated DNA-targeted activators (lanes 4 to 15). Below the lanes numbers, the amounts of full-length (FL) and short (ST) transcripts, as quantitated by a phosphorimager after subtraction of background and normalization to the α-globin internal control (α), are indicated.

reporter HIV-1 construct containing both an intact TAR element and GAL4 DNA binding sites [p(4×G17)HIV-1/R (Fig. 2A)] with two expression vectors, one encoding Tat and the second encoding a DNA-targeted activator. The results are shown in Fig. 7.

In the presence of Tat, we observed activation of full-length transcript synthesis and repression of the short transcripts (compare lanes 2 and 3 with lane 1). In contrast, as described above, the DNA-targeted activators G-VP16 and G-CTF^P activated both full-length and short transcripts, although the activation of short transcripts was low in this experiment (lanes 4 to 7). In the presence of Tat and either G-VP16 or G-CTF^P, we observed a very large increase in full-length transcripts, as large as 600-fold at high concentrations of Tat and G-CTF^P (lane 15; see bottom of Fig. 7 for quantitation). Remarkably, however, we still observed short transcript repression with an activation of full-length transcripts as high as 180-fold (lane 14). At higher activation levels of full-length transcripts (424 and 605-fold; lanes 13 and 15), the amounts of short transcripts were equivalent or slightly higher than those obtained in the absence of any activators (lane 1). Thus, in the presence of Tat, the short transcripts were not activated; in fact, they were repressed even under conditions where the full-length transcripts were activated nearly 200-fold. This finding suggests that the repression of short transcripts exerted by Tat is dominant over the activation of short transcripts exerted by DNA-targeted activators.

DISCUSSION

All cellular activators of transcription described so far are thought to act through a DNA target. This suggests that functions other than transcriptional activation may be associated with the binding of Tat to its RNA target TAR. Here we have compared the effects of Tat and DNA-targeted activators including G-Tat on transcription from the HIV-1 LTR. We find that Tat targeted to the RNA has the unique property of repressing the HIV-1 short transcripts. This observation suggests that repression of the short transcripts may be an important function of Tat.

RNA-targeted Tat versus DNA-targeted Tat. Although Tat is unique among transcription factors in that it is brought to the promoter via an RNA element, it can function from GAL4 DNA binding sites if fused to the GAL4 DNA binding domain

through similar mechanisms (8, 20, 32, 45). We confirm that Tat functions from a DNA target, although in our experiments, as observed by others (7, 19), G-Tat was much less efficient than Tat targeted to the RNA in an identical promoter context. The difference may be due to Tat binding with higher affinity to TAR than G-Tat to GAL4 binding sites in vivo. Southgate and Green (45) obtained comparable activation by G-Tat and G-VP16 in a chloramphenicol acetyltransferase assay, probably because they used six GAL4 binding sites instead of the four used here. Indeed, Ghosh et al. (8) observed that as they increased the number of GAL4 binding sites, activation by G-VP16 saturated earlier than activation by G-Tat.

Unlike DNA-targeted activators including G-Tat, Tat targeted to the RNA represses the synthesis of short transcripts. Although Tat can function from a DNA target, if not optimally, the fact remains that in the natural HIV-1 long terminal repeat, Tat acts through an RNA target. Why does HIV-1 use such an elaborate transactivation mechanism? A unique feature of RNA-targeted Tat is that it represses short transcript production. In sharp contrast, G-Tat and other DNA-targeted activators increase the production of short transcripts. This is consistent with the previous observation that activation of the HIV-1 promoter by E1A results in an increase in the levels of short transcripts (23, 24) and suggests that the repression of short transcripts upon Tat activation through TAR is not simply the result of promoter occlusion by the preferential activation of processive complexes over nonprocessive complexes: Clearly, the HIV-1 promoter has the potential to sustain simultaneously higher rates of both short and full-length transcripts, as when activated by, for example, G-VP16. Rather, it seems that Tat bound to TAR can specifically repress IST. This property appears to depend more on the activator being targeted to the RNA than on the nature of the activation domain, as the VP16 activation domain targeted to a Rev response element also represses the short transcripts. Perhaps an activation domain targeted to the RNA and interacting with the preinitiation complex assembled at the promoter interferes with factor(s) that bind to the overlapping IST DNA element.

Why repress the short transcripts? The observation that Tat activation can occur without IST in transfection experiments (44) argues against a mechanism for Tat activation in which the short transcripts are converted into full-length transcripts. However, since the short transcripts contain TAR, it seems likely that one of their roles is in the regulation, both positive and negative, of Tat transactivation. Under conditions where levels of full-length transcripts are very low, IST-directed short nascent RNAs probably ensure a constant supply of functional Tat binding sites, while once released from the template, the short transcripts are stable and may sequester Tat away from the HIV-1 promoter. Indeed, overexpression of HIV-1 RNAs that contain TAR inhibits HIV-1 replication (47) and Tat activation (9, 26), and Tat-TAR particles have been observed to accumulate in the cytoplasm of infected cells (34). The specific repression of short transcripts by RNA-targeted Tat may, then, simply ensure maximal Tat transactivation, and down-regulation of the short transcripts by Tat may be a consequence of, rather than a cause for, Tat functioning through an RNA target.

Another possibility, however, is that the short transcripts exert another function in a process unrelated to Tat transactivation. For example, there is evidence for nontranscriptional roles of Tat in HIV-1 infectivity and cytopathicity (15) and for cellular proliferation (reviewed in reference 3). Short transcripts may regulate such activities by sequestering Tat or altering its localization. Also, it is interesting that the HIV-1 short transcripts themselves have been implicated in the regulation of both 2′,5′-oligoadenylate synthase (43) and the protein kinase regulated by RNA (PKR [previously referred to as p68, DAI, dsI, P1, or PK_{ds}] [11, 12, 42, 43]), two α-interferon-induced enzymes (reviewed in reference 4), although it is controversial whether the short transcripts activate PKR (27, 39, 42, 43) or prevent activation of PKR (11, 12). If the short transcripts indeed activate PKR, they must stimulate host antiviral defenses and perhaps help establish a latent infection. Strikingly, latency in culture cells and in HIV-1-infected individuals is characterized by a high ratio of short versus long HIV-1 transcripts (1). In this scenario, activation of PKR would then have to be counterbalanced upon Tat transactivation and full-blown viral gene expression. Tat could achieve this by down-regulating the synthesis of short transcripts, by binding and thus sequestering existing short transcripts (34), and perhaps also by a direct effect on PKR, as Tat has been implicated in a direct inhibition of PKR (18, 40).

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