TAR RNA Binding Properties and Relative Transactivation Activities of Human Immunodeficiency Virus Type 1 and 2 Tat Proteins

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Using gel shift assays, we found that the human immunodeficiency virus type 1 (HIV-1) Tat protein (Tat-1) bound both HIV-1 and HIV-2 TAR RNAs with similar high affinities. In contrast, the HIV-2 Tat protein (Tat-2) bound only TAR-2 RNA with high affinity. We conclude that the weak in vivo activity of Tat-2 on the HIV-1 long terminal repeat that has been observed previously is likely the result of low affinity for TAR-1 RNA. Additionally, TAR-2 RNA was found to contain multiple specific binding sites for Tat proteins. GAL4-Tat fusion proteins were analyzed to compare the relative transactivation activities of Tat-1 and Tat-2 in the absence of requirements for binding to TAR RNAs. The GAL4-Tat-2 protein was found to transactivate synthetic promoters containing GAL4 binding sites at levels severalfold higher than did the GAL4-Tat-1 protein.

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are causative agents of AIDS (20, 25). Both HIV-1 and HIV-2 encode a transactivator gene, *tat*, that is essential for high levels of viral replication and whose primary function is to stimulate transcription directed by the viral long terminal repeat (LTR) sequences (reviewed in references 11, 19, and 31). The HIV-1 Tat protein Tat-1 and the HIV-2 Tat protein Tat-2 can stimulate transcription of both the HIV-1 and HIV-2 LTRs, although this cross-transactivation is not completely reciprocal. Tat-1 possesses high transactivation activity for both the HIV-1 and HIV-2 LTRs, while Tat-2 possesses high activity for its own HIV-2 LTR and relatively weak activity for the HIV-1 LTR (1, 4, 15, 17).

Tat-1 function has been more widely studied than has that of Tat-2. The genetic element within the HIV-1 LTR that responds to Tat-1 is an RNA element termed TAR-1. The TAR-1 element extends from +18 to +44 relative to the transcription start site, and it forms a stem-loop structure at the 5' ends of all viral transcripts (18, 30, 34). Tat-1 binds directly to this structure in nascent transcripts and thereby stimulates transcriptional elongation and in some cases initiation through an unknown mechanism (5, 16, 24, 26, 27). The key determinant in TAR-1 RNA for specific binding of Tat-1 is a 3-nucleotide bulge in the stem (9, 13, 21, 35). A basic region of Tat-1, located between residues 49 and 57, recognizes and binds directly to the bulge and a few surrounding base pairs (6, 42). The TAR-2 genetic element is more complex than TAR-1 and consists of two functionally independent subelements (4, 17). The determinants within TAR-2 that specify binding of the Tat-2 protein have not yet been defined, although bulges in each of the stems similar to the TAR-1 bulge are likely to be important.

The Tat-1 protein of the extensively-studied HIV-1 isolate HXB2 is 86 amino acid residues in size, although only 72 residues contained in the first coding exon are required for full activity (10, 40). The Tat-2 protein of HIV-2 isolate ROD is 130 amino acid residues in size, and only 99 residues of the first coding exon are required for full activity (4, 15). Tat-1

and Tat-2 are approximately 80% homologous over a stretch of 39 amino acids in the central regions of both proteins (28). For Tat-1, this region of homology contains the bulk of the transcription activation domain which has been identified by protein fusion experiments (8, 23, 39, 41). In Tat-1, this central region also contains the basic domain which is both the TAR RNA binding domain and the nuclear localization signal (22, 36). Although not yet directly demonstrated, this central region in the Tat-2 protein is also likely to contain some of the transcriptional activation domain, the nuclear localization signal, and the TAR RNA binding domain.

A recent study using exchanges of protein domains between Tat-1 and Tat-2 indicates that structural features unique to the Tat-2 basic domain are involved in the proteins' incomplete cross-transactivation of HIV LTRs (14). For Tat-2, the protein attained high transactivation activity for the HIV-1 LTR when its basic domain was replaced by the basic domain of Tat-1. For Tat-1, the protein became poorly active for the HIV-1 LTR when its basic domain was replaced by the Tat-2 basic domain. These genetic experiments suggest that the transactivation activities of Tat-1 and Tat-2 for the HIV-1 and HIV-2 LTRs may reflect their relative binding affinities for TAR-1 and TAR-2 RNAs.

In this study, we used biochemical and molecular genetic techniques to directly compare the TAR RNA binding and transactivation properties of the Tat-1 and Tat-2 proteins. To compare RNA binding, we performed in vitro binding experiments with purified Tat proteins and TAR RNAs. To compare the relative transactivation activities of Tat-1 and Tat-2 proteins in the absence of requirements for binding to TAR RNA, we analyzed GAL4-Tat fusion proteins for stimulation of synthetic promoters containing GAL4 DNA binding sites.

The plasmids used to synthesize wild-type TAR-1 RNA (+1 to +80) and TAR-1 Δ B (+1 to +80 with deletion of bulge nucleotides from +23 to +25) by in vitro transcription have been described previously (35). Plasmid -556/+119 (15) was used as the template in polymerase chain reactions for the construction of plasmids for in vitro transcription of full-length TAR-2 RNA (+1 to +123) and of TAR-2 Δ (+1 to +52); the amplified fragments which contained *Eco*RI and

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*Hind*III sites at the 5' and 3' ends, respectively, were inserted into pGEM3Zf(+) (Promega), and constructs were confirmed by DNA sequencing. The HIV chloramphenicol acetyltransferase (CAT) expression plasmids used contained the HIV-1 LTR (−454 to +80 [40]) and HIV-2 LTR (−556 to +119 [15]). CAT expression plasmids containing promoters with GAL4 DNA binding sites were G6(HIV-1)(−83) (−TAR) (41) and G5(Sp1)(E1B TATA) (23). To construct GAL4-Tat-1 fusion proteins, Tat-1 wild-type and mutant cDNAs were removed from a pGEM-1 vector (33) and inserted into the parental GAL4 (1-147) vector (pSG424 [37]) via 5' *Eco*RI and 3' *Xba*I sites. To construct GAL4-Tat-2, plasmid pROD221 (first exon of wild-type Tat-2 consisting of 99 residues) was used as the polymerase chain reaction template; the amplified fragment (whose identity was confirmed by DNA sequencing) was inserted into pSG424 via *Sma*I (5') and *Xba*I (3') sites. The GAL4/VP16 plasmid (3) and Tat-1 *trans*-dominant 52/57 (29) have been described previously. To produce TAR RNAs by *in vitro* transcription, the pGEM3Zf-TAR RNA plasmid constructs were linearized with the appropriate restriction enzyme to generate sense or antisense transcripts. Transcription reaction mixtures (10 μ l) contained 1 μ g of linearized plasmid; 40 mM Tris-HCl (pH 8.0); 6 mM MgCl₂; 2 mM spermidine; 10 mM dithiothreitol; 50 mM NaCl; 500 μ M ATP, CTP, and GTP; 10 μ Ci of [α -³²P]UTP (3,000 Ci/mmol); and 10 U of RNA polymerase (T7 or SP6) and were incubated at 37°C for 1 h. The full-length RNA transcripts were purified by electrophoresis in 8% denaturing polyacrylamide gels. After elution from gels, purified RNAs were dissolved in 40 μ l of renaturation buffer (100 mM NaCl and 10 mM MgCl₂) and then incubated at 65°C for 10 min and then at room temperature for 20 min. The specific activities of the RNA products were calculated, and 40 nM uniformly labeled RNAs were used in the binding assays. Transcription reactions were scaled up 10-fold to make large quantities of unlabeled RNAs for competition experiments. Unlabeled competitor RNAs were also gel purified.

To purify Tat proteins, Tat-1 and Tat-2 proteins were expressed as glutathione-S-transferase (GST) fusions with a thrombin proteolytic site engineered between Tat proteins and GST (2). After thrombin digestion, the 86-residue Tat-1 protein (isolate HXB2 [28]) contained glycine-serine-threonine preceding the initiator methionine and the 99-residue Tat-2 protein (first exon from isolate ROD [28]) contained glycine-serine-proline-glycine preceding the initiator methionine. A detailed description of purification of Tat-1 and Tat-2 proteins is given elsewhere (32). Briefly, GST-Tat proteins were purified from *Escherichia coli* lysates by selective binding to glutathione-Sepharose beads; while still bound to Sepharose beads, Tat proteins were cleaved from GST by digestion with thrombin and then eluted from the GST-Sepharose bead complex. At this stage in the purification, Tat-1 and Tat-2 were approximately 10 to 25% pure as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the major contaminants were thrombin and GST. For Tat-2 monomer preparations, the eluate from GST-glutathione-Sepharose beads was filtered through a Superdex 75 FPLC column (Pharmacia) and the monomer peak was pooled; monomer Tat-2 preparations were estimated to be approximately 50% pure.

For gel shift assays, we used the conditions described by Dingwall et al. (13). Binding reaction mixtures (10 μ l) contained 0.4 pmol (16 ng or 40 nM) of uniformly labeled RNA probe, 0.5 μ g of poly(I · C), 0.2 μ g of yeast tRNA, 50 mM Tris-HCl (pH 7.6), 20 mM KCl, and 0 to 12 pmol (0 to

1,200 nM) of Tat proteins. After incubation at room temperature for 10 min, the reaction mixtures were applied to a 6% nondenaturing polyacrylamide gel (acrylamide-bisacrylamide ratio of 75:1) in 50 mM Tris-glycine buffer (pH 8.8) which was run at room temperature. In competition experiments, ³²P-labeled RNA and unlabeled RNAs were added simultaneously to Tat proteins and binding assays were carried out as described for ³²P-labeled RNA alone. Gels were dried and exposed to X-ray film with an intensifying screen at −80°C. Only the region of the gels with free ³²P-labeled TAR RNAs and Tat-TAR RNA complexes are shown in the figures.

For transactivation assays, transfections were carried out in HeLa cells by the calcium phosphate method. Culture dishes (6-cm diameter) were transfected with 5 μ g of CAT reporter plasmids plus the indicated amounts of activator plasmids. Cell extracts were harvested 48 h posttransfection, and CAT enzyme assays were performed under conditions in which conversion of chloramphenicol to its acetylated forms was always less than 50%. CAT assays were quantified with a Betagen Betascope 603 scanner.

Tat-1 and Tat-2 binding to ³²P-labeled TAR-1 RNA. We first analyzed binding of Tat-1 and Tat-2 proteins to ³²P-labeled wild-type TAR-1 RNA (Fig. 1A). Tat-1 formed a weakly detectable complex at a 1:1 molar ratio of protein/RNA, indicating that a large portion of Tat-1 in our protein preparations is active in the gel shift assay (also see Fig. 5A and B). The protein-TAR-1 RNA complex seen in Fig. 1A at molar ratios of <30:1 is specific for Tat-1, as no complexes were observed with these amounts of GST or Tat-2 protein. To verify that the Tat-1-TAR-1 RNA complex is dependent upon the wild-type TAR-1 RNA structure, we performed a binding assay with a mutant TAR-1 RNA (TAR-1 Δ B) containing a deletion of the 3-nucleotide pyrimidine bulge at +23 to +25 (Fig. 1B). Several groups have shown that deletion of this bulge largely abolishes specific binding of Tat-1 to TAR-1 (6, 9, 13, 35). As expected, deletion of the bulge in TAR-1 abrogated binding of Tat-1.

We assayed two types of Tat-2 protein preparations for TAR RNA binding properties. One Tat-2 preparation (analyzed next to Tat-1 lanes in Fig. 1 to 4) is similar to the Tat-1 preparation. These types of preparations of Tat-1 and Tat-2 are approximately 10 to 25% pure and contain Tat proteins after thrombin cleavage and elution from GST-glutathione-Sepharose beads; the major contaminants in these preparations are GST and thrombin. The preparation designated Tat-2 monomer in Fig. 1 to 4 is a more highly purified preparation; it is approximately 50% pure and consists of the monomer peak of Tat-2 preparations after gel filtration. Because of extensive aggregation of Tat-1 during gel filtration, it was not possible to purify a monomer peak of Tat-1.

In contrast to Tat-1, the Tat-2 protein did not form gel shift complexes with ³²P-labeled TAR-1 RNA at molar ratios of <30:1 (Fig. 1A). At a molar ratio of 30:1, however, Tat-2 did form weakly discernible complexes (visible in the original X-ray film, near the top of the region of the gel shown in Fig. 1A). The amount of ³²P-labeled TAR-1 RNA remaining as a free probe in the gel shown in Fig. 5A was quantified with a Betagen Betascope scanner. For Tat-1, 50% of the TAR-1 RNA probe remained unbound at a molar ratio (protein:RNA) of 5:1. For Tat-2, 50% of the TAR-1 RNA probe remained unbound at a molar ratio of 23:1. These results suggest, therefore, that the apparent dissociation constant (K_d) of Tat-2 for TAR-1 RNA in the gel shift assay is approximately fivefold higher than that of Tat-1. It is likely that this relatively low affinity of Tat-2 for TAR-1 RNA is

FIG. 1. Gel shift assays with TAR-1 RNA. The indicated molar ratios of GST, Tat-1, and Tat-2 proteins were incubated with ^{32}P -labeled TAR-1 RNA (+1 to +80) (A) or ^{32}P -labeled TAR-1 ΔB (deletion of bulge nucleotides +23 to +25) (B) and analyzed on 6% native polyacrylamide gels.

involved in the low transactivation activity of Tat-2 for the HIV-1 LTR.

Tat-1 and Tat-2 binding to ^{32}P -labeled TAR-2 RNA. We next analyzed Tat-1 and Tat-2 protein binding to the wild-type TAR-2 RNA (+1 to +123) which has been predicted to form two stem-loop structures (4, 17, 38). For Tat-1, a single Tat-1-TAR-2 RNA complex was detected at a 1:1 molar ratio of protein to RNA (Fig. 2A). As the amount of Tat-1 protein was increased in the binding assay, a second distinct Tat-1-TAR-2 RNA complex was detected (molar ratios of 3:1 and 5:1). These results indicate that multiple specific protein-RNA complexes form between the Tat-1 protein and TAR-2 RNA.

When Tat-2 preparations were analyzed, multiple complexes with TAR-2 RNA were also observed. For both types of preparations of Tat-2, two protein-RNA complexes were observed at a molar ratio of 3:1 to 10:1 (Fig. 2A). In other binding assays, three distinct complexes were sometimes observed over this protein range (see Fig. 3 and 6A). In contrast to Tat-1, none of these Tat-2-TAR-2 RNA complexes appeared preferentially at low protein concentrations.

To assess the specificity of Tat-1 and Tat-2 binding to wild-type TAR-2 RNA, binding assays were carried out with antisense TAR-2 RNA (Fig. 2B). Unlike wild-type TAR-2 RNA, no distinct complexes were observed for antisense TAR-2 RNA with the lower levels of Tat-1 or Tat-2. Some rapidly migrating complexes which may represent relatively

low-affinity binding sites present in the antisense RNA were observed with high levels of protein.

Tat-1 preparations from *E. coli* can be contaminated with proteolytic fragments that contain the Tat-1 basic domain, and these basic domain fragments can bind specifically to TAR-1 RNA (42). To determine whether full-length Tat-2 protein was responsible for gel shift complexes observed in this study, we used a Tat-2 protein containing a large deletion in the amino terminus (residues 8 through 47). This Tat-2 mutant, called $\Delta 8/47$, and wild-type Tat-2 and Tat-1 were analyzed for binding to TAR-2 RNA (Fig. 3). The $\Delta 8/47$ mutant formed complexes with TAR-2 RNA that migrated much more rapidly than complexes formed with wild-type Tat-2. This result suggests that the full-length Tat-2 protein, rather than a fragment containing the basic domain, is present in the protein-RNA complexes observed in this study.

The data in Fig. 2A and 3 indicate that Tat-1 and Tat-2 form complexes with TAR-2 RNA at similar protein concentrations, indicating that the apparent K_d s of Tat-1 and Tat-2 for TAR-2 RNA in the gel shift assay are similar. The amount of ^{32}P -labeled TAR-1 RNA remaining as free probe in the gel shown in Fig. 2A was quantified with a Betagen Betascope scanner. For Tat-1, 50% of the TAR-1 RNA probe remained unbound at a molar ratio (protein-RNA) of approximately 2.4:1. For the less pure Tat-2 preparation, 50% of the TAR-1 RNA probe remained unbound at a molar

FIG. 2. Gel shift assays with TAR-2 RNA. The indicated molar ratios of GST, Tat-1, and Tat-2 proteins were incubated with ^{32}P -labeled TAR-2 RNA (+1 to +123) (A) or antisense ^{32}P -labeled TAR-2 RNA (antisense of +1 to +123) (B) and analyzed on 6% native polyacrylamide gels.

ratio of approximately 3.4:1; this value was 4.0:1 for the monomer Tat-2 preparation.

We next analyzed Tat-1 and Tat-2 binding to a ^{32}P -labeled TAR-2 RNA truncated after nucleotide +52. This mutant TAR-2 RNA, called TAR-2 Δ , contains only the first putative

FIG. 3. Gel shift assay with TAR-2 RNA and Tat-2 deletion mutant. The indicated molar ratios of wild-type Tat-1, wild-type Tat-2, and Tat-2 deletion mutant $\Delta 8/47$ (deletion of residues 8 through 47 in the amino terminus) were incubated with ^{32}P -labeled TAR-2 RNA (+1 to +123) and analyzed on a 6% native polyacrylamide gel.

stem-loop structure. Deletion of the second stem-loop structure in TAR-2 RNA reduces by a few-fold the in vivo response to either Tat-1 or Tat-2 (4, 17). Unlike the wild-type TAR-2 RNA (+1 to +123), the TAR-2 Δ RNA (+1 to +52) formed a single complex with Tat-1 (Fig. 4A). The simplest explanation of this result is that one of complexes seen with Tat-1 and full-length TAR-2 RNA (Fig. 2 and 3) is the consequence of an additional binding site in the second stem-loop structure.

The Tat-2 monomer preparation formed a single predominant complex with TAR-2 Δ RNA, while the less pure Tat-2 preparation formed this complex plus an additional one at molar ratios of 3:1 and 5:1 (Fig. 4A). The explanation for the additional complex seen with the less pure Tat-2 preparation is not known. Similar to results with Tat-1, these results with Tat-2 and TAR-2 Δ RNA suggest that at least one of the complexes seen with Tat-2 and full-length TAR-2 RNA (Fig. 2A and 3) is the consequence of a specific binding site in the second stem-loop structure between +52 and +123.

As a control for specificity of binding to TAR-2 Δ RNA, binding of Tat-1 and Tat-2 with antisense TAR-2 Δ was analyzed (Fig. 4B). The ^{32}P -labeled antisense TAR-2 Δ did not form distinct complexes with either Tat-1 or Tat-2, indicating that the complexes seen with TAR-2 Δ are the result of specific interactions.

Binding assays with unlabeled competitor RNAs. To further characterize Tat-TAR RNA binding properties, binding experiments with Tat-1 and Tat-2 to ^{32}P -labeled TAR RNAs

FIG. 4. Gel shift assays with TAR-2Δ RNA. The indicated molar ratios of GST, Tat-1, and Tat-2 proteins were incubated with ^{32}P -labeled TAR-2Δ RNA (+1 to +52) (A) or antisense ^{32}P -labeled TAR-2Δ RNA (antisense of +1 to +52) (B) and analyzed on 6% native polyacrylamide gels.

were carried out with an excess of unlabeled competitor RNAs. For Tat-1 binding to ^{32}P -labeled TAR-1 RNA, TAR-1, TAR-2, and TAR-2Δ RNAs, but not tRNA, were effective competitors (Fig. 5A and B). Furthermore, binding assays using an equimolar amount of competitor to labeled RNA indicate that TAR-2 RNA is a slightly more effective competitor than TAR-1 RNA (Fig. 5B). The TAR-2Δ mutant appeared to be similar to TAR-1 in its ability to compete for Tat-1 binding (Fig. 5A). These results likely reflect the presence in TAR-1 and TAR-2Δ RNAs of a single specific Tat-1 binding site and more than one binding site in full-length TAR-2 RNA. This agrees well with the results of the gel shift assays, in which single complexes were observed for Tat-1–TAR-1 RNA and Tat-1–TAR-2Δ RNA (Fig. 1A and 4A), while two distinct complexes were observed for Tat-1–full-length TAR-2 RNA (Fig. 2A).

For Tat-1 binding to wild-type TAR-2 RNA, competition experiments were carried out under conditions that gave two distinct protein-RNA complexes in the absence of unlabeled competitor (Fig. 2A). Unlabeled tRNA showed little ability to compete with ^{32}P -labeled TAR-2 for binding to Tat-1, even at a 40-fold excess (Fig. 5C). In contrast, both unlabeled TAR-1 and TAR-2Δ competed effectively. We note that at low levels of competitor TAR-1 or TAR-2Δ RNAs, the more slowly migrating Tat-1–TAR-2 RNA complex was

preferentially competed with. This is consistent with the results of the gel shift assay in which the faster-migrating complex formed with lower Tat-1 concentrations than the slower complex (Fig. 2A).

Competition experiments were carried out with Tat-2 and ^{32}P -labeled TAR-2 RNA at two concentrations of Tat-2 protein (molar ratios of 3:1 and 10:1). Unlabeled TAR-1 and TAR-2 RNAs both competed for binding, although TAR-2 RNA was a more effective competitor (Fig. 6A; compare equimolar and 10-fold excess of competitor to labeled RNA). This result indicates that Tat-2 has a significantly higher affinity for TAR-2 RNA than for TAR-1 RNA, in good agreement with the results of the gel shift assays (Fig. 1A and 2A). The TAR-2Δ RNA, but not tRNA, was an effective competitor for Tat-2 binding to wild-type TAR-2 RNA (Fig. 6B).

Relative transactivation activities of GAL4–Tat-1 and GAL4–Tat-2 fusion proteins. In order to compare the relative transactivation activities of the Tat-1 and Tat-2 proteins in the absence of requirements for binding to TAR RNAs, we constructed plasmid vectors that express Tat proteins fused to the GAL4 DNA binding domain. A GAL4–Tat-1 fusion protein has been shown to activate synthetic promoters containing GAL4 DNA binding sites (23, 41). Because affinity for the GAL4 DNA binding sites in these artificial

FIG. 5. Tat-1 binding to TAR-1 and TAR-2 RNAs in the presence of unlabeled competitor RNAs. Tat-1 was incubated with ^{32}P -labeled TAR-1 RNA (+1 to +80; molar ratio of protein-RNA of 10:1) (A and B) or ^{32}P -labeled TAR-2 RNA (+1 to +123; molar ratio of protein-RNA of 10:1) (C) in the presence of unlabeled competitor RNAs. Lanes R are ^{32}P -labeled RNA alone. The molar ratios of unlabeled competitor RNA to probe RNA are indicated at the bottoms of the gels.

promoters should be equal for GAL4-Tat-1 and GAL4-Tat-2 proteins, interactions of the Tat moieties in the fusions with the transcription apparatus can be compared.

The GAL4-Tat-1 and GAL4-Tat-2 proteins were first assayed for ability to stimulate the natural HIV-1 and HIV-2 LTRs. In these transactivation experiments, the GAL4-Tat proteins function by binding to TAR RNAs through the Tat basic domains. Two amounts of plasmids expressing the GAL4 parent (DNA binding domain alone), GAL4-Tat-1, or GAL4-Tat-2 were cotransfected with plasmids expressing either the HIV-1 LTR or HIV-2 LTR fused to CAT as a reporter. Cell extracts were prepared 2 days after transfections and assayed for CAT expression. Transactivations by GAL4-Tat-1 and GAL4-Tat-2 were calculated by using CAT expression observed with the GAL4 parent as the basal level

(Fig. 7). For the HIV-1 LTR, the GAL4-Tat-1 activator displayed 28- and 41-fold transactivation at the low and high levels of input plasmid, respectively (Fig. 7A). In comparison, the GAL4-Tat-2 activator displayed 3.5- and 6-fold transactivation for the HIV-1 LTR reporter. For the HIV-2 LTR reporter, the GAL4-Tat-1 activator displayed 34- and 245-fold transactivation at the low and high levels of input plasmid, respectively (Fig. 7B). In comparison, the GAL4-Tat-2 activator displayed 80- and 293-fold transactivation for the HIV-2 reporter. The higher levels of transactivation observed for the HIV-2 LTR were the result of lower basal expression than that from the HIV-1 LTR; the absolute levels of expression from the HIV-1 and HIV-2 LTRs at maximal transactivation were similar.

The transactivation assay results shown in Fig. 7 demon-

FIG. 6. Tat-2 binding to TAR-2 RNA in the presence of unlabeled competitor RNAs. Tat-2 was incubated with ^{32}P -labeled TAR-2 RNA (+1 to +123; molar ratios of protein-RNA are shown at the tops of the gels) in the presence of unlabeled competitor RNAs. The molar ratios of unlabeled competitor RNA to probe RNA are indicated at the bottoms of the gels.

strate that the GAL4-Tat-1 protein has high activity for both the HIV-1 and HIV-2 LTRs, while the GAL4-Tat-2 protein has high activity for the HIV-2 LTR and relatively weak activity for the HIV-1 LTR. The GAL4-Tat-1 and GAL4-Tat-2 proteins, therefore, display the incomplete reciprocal transactivation for the HIV-1 and HIV-2 LTRs that has been documented for the natural Tat-1 and Tat-2 proteins (4, 15, 17).

We next assayed transactivation activities of the GAL4-Tat fusions for synthetic promoters containing GAL4 DNA binding sites. One such promoter [G6(HIV-1)(-83)(-TAR)] contains six GAL4 binding sites positioned 5' to a portion of the HIV-1 LTR extending from -83 to +25; the HIV-1

sequences in this plasmid construct contain the three natural Sp1 sites, a TATA element, and a deletion of the TAR element (41). The other synthetic promoter [G5(Sp1)(E1B TATA)] contains five GAL4 binding sites and three synthetic Sp1 sites positioned 5' to the adenovirus E1B gene TATA element (23).

For the promoter based upon the HIV-1 core sequences, GAL4-Tat-1 displayed 6- and 24-fold transactivation at the low and high levels of input plasmid, respectively (Fig. 8A). In comparison, GAL4-Tat-2 displayed 32- and 83-fold transactivation. For the promoter based upon the adenovirus E1B core sequences, GAL4-Tat-1 showed 1.7- and 3.3-fold transactivation; GAL4-Tat-2 showed 11- and 28-fold transactiva-

FIG. 7. GAL4-Tat-1 and GAL4-Tat-2 transactivation of the HIV-1 and HIV-2 LTRs.

tion (Fig. 8B). The GAL4-Tat-2 protein displayed, therefore, from three- to eightfold greater transactivation than did GAL4-Tat-1 on these two synthetic promoters. Because the GAL4-Tat-1 and GAL4-Tat-2 proteins presumably bind equally to the GAL4 binding sites in these promoters, these results suggest that the Tat-2 protein may interact more efficiently than Tat-1 with the transcription apparatus.

Tat *trans*-dominant mutant inhibition of wild-type Tat function. The GAL4-Tat proteins can function either through a GAL4 DNA element located upstream from the transcrip-

tion start site or through natural TAR RNAs located at the 5' ends of nascent transcripts. It is unclear whether these two means of directing Tat to transcription complexes lead to mechanistically distinct pathways of transcriptional stimulation. Mutant Tat proteins, termed *trans*-dominant mutants, have been shown to block wild-type Tat activity when the wild-type protein operates through either the TAR element or a bacteriophage MS2 RNA operator in place of TAR (29). We assayed one such Tat-1 *trans*-dominant mutant for the ability to inhibit GAL4-Tat-1 and GAL4-Tat-2 function. If

FIG. 8. GAL4-Tat-1 and GAL4-Tat-2 transactivation of synthetic promoters containing GAL4 DNA binding sites. GAL4-Tat activator plasmids were cotransfected with synthetic promoter based upon HIV-1 LTR core sequences (-83 to +25 with TAR element deleted [40]) (A) or a synthetic promoter based upon the adenovirus E1B TATA element (23) (B).

wild-type Tat functions through different pathways for DNA and RNA elements, it is possible that the *trans*-dominant mutant will have differential effects on the two types of promoters.

Plasmids expressing the HIV-1 LTR, the HIV-2 LTR, or a GAL4 binding site promoter [G6(HIV-1)(-83)(-TAR)] were cotransfected with plasmids expressing the GAL4 parent, GAL4-Tat-1, or GAL4-Tat-2 plus increasing amounts of a Tat *trans*-dominant inhibitor called 52/57 (Fig. 9). The 52/57 *trans*-dominant inhibition contains substitutions of glycines and alanine for the wild-type residues in the basic domain (29). The *trans*-dominant inhibitor alone (plus the GAL4 parent) showed low levels of activation of the HIV LTRs and the synthetic promoter (Fig. 9A to C). These results are consistent with those of Modesti and colleagues, who demonstrated that the 52/57 *trans*-dominant protein possesses low levels of wild-type Tat activity (29).

The *trans*-dominant inhibitor effectively blocked GAL4-Tat-1 activity on all three promoters tested. At the largest amount of input plasmid (3 μ g), the *trans*-dominant inhibitor inhibited GAL4-Tat-1 transactivation of the HIV-1 LTR 7-fold, the HIV-2 LTR 4-fold, and the GAL4-based promoter 10-fold. The largest amount of *trans*-dominant inhibitor inhibited GAL4-Tat-2 transactivation of the HIV-2 LTR 10-fold and the GAL4-based promoter 2-fold (Fig. 9B and C). There was little inhibitory effect of the *trans*-dominant inhibitor on GAL4-Tat-2 transactivation of the HIV-1 LTR (Fig. 9A). This result was expected because the *trans*-dominant mutant has some wild-type Tat activity and the GAL4-Tat-2 protein has only weak activity for the HIV-1 LTR. A control experiment using the potent GAL4-VP16 fusion protein as an activator (3) of the synthetic GAL4 promoter demonstrated that inhibition by the *trans*-dominant mutant was specific to GAL4-Tat proteins (Fig. 9D).

We conclude from these experiments that the Tat *trans*-dominant mutant has equivalent inhibitory properties on wild-type Tat-1 or Tat-2 function through either a DNA or TAR RNA element. Thus, our experiments have provided no evidence that Tat functions through mechanistically distinct pathways when directed to promoters via a DNA or RNA element. Rather, our results are consistent with the notion that Tat functions through a common pathway when directed to promoters by either a DNA or RNA element. Previous analyses of GAL4-Tat-1 proteins containing inactivating point mutations in the Tat transcriptional activation domain have shown that these mutations have equal reductions on transactivation of GAL4 promoters and the HIV-1 LTR (23, 41). We also constructed four GAL4-Tat-1 fusions containing point mutations in the Tat activation domain. In the natural Tat-1 protein, these mutations (termed pro18IS, tyr26, lys2829, and phe32 [33]) reduce transactivation activity but do not disrupt Tat-1 protein structure (33). We found that these GAL4-Tat-1 mutants were equivalently defective for transactivation of both the HIV-1 LTR and the GAL4 promoter (data not shown). These results further support the idea that Tat proteins function through a common transcriptional pathway when directed to promoters through either DNA or RNA elements.

We have presented data here that the Tat-1 protein bound in vitro with similar high affinity to both TAR-1 and TAR-2 RNAs. In contrast, the Tat-2 protein bound with high affinity only to TAR-2 RNA. These results offer an explanation for the incomplete cross-transactivation of the Tat-1 and Tat-2 proteins (1, 4, 15, 17). It is probable that Tat-1 has high transactivation activity for both the HIV-1 and HIV-2 LTRs because it binds TAR-1 and TAR-2 RNAs with similar high

affinity. Likewise, it is probable that Tat-2 has poor activity for the HIV-1 LTR because it binds relatively poorly to TAR-1 RNA. Results of a previous genetic study are consistent with those of our biochemical experiments (14). When the basic domain of Tat-2 was replaced by the basic domain of Tat-1, the resulting chimera became fully active on the HIV-1 LTR, suggesting that the basic domain of Tat-2 binds poorly to TAR-1 RNA in vivo.

The sequence of the Tat-1 basic domain is RKRRRQRRR (residues 49 to 57), while the Tat-2 basic domain sequence is RKGRRRRTPKKTK (residues 78 to 90). Analyses of synthetic peptides and mutant Tat-1 proteins have shown that the arginine at residue 52 or 53 in the Tat-1 basic domain is the crucial determinant for specific binding to the bulge in TAR-1 RNA (6, 12). It has been proposed that one of these arginines contacts two adjacent phosphates in the TAR-1 RNA bulge in a complex termed the arginine fork (7). Our results indicate that for some reason none of the arginines in the Tat-2 basic domain are able to form such high-affinity contacts with the TAR-1 RNA bulge. It is possible that specific contacts between Tat-2 and TAR RNA are dependent upon the precise sequence of the Tat-2 basic domain in the context of the entire protein.

In contrast to the single stem-loop structure of TAR-1 RNA, TAR-2 RNA has been predicted by RNA secondary structure considerations and mutagenesis studies to consist of two stem-loop structures (5, 17, 38). Our binding assays demonstrate that multiple distinct gel shift complexes form between Tat proteins and full-length TAR-2 RNA (+1 to +123). For Tat-1, two complexes were observed: a rapidly migrating complex at low protein concentrations and an additional, more slowly migrating complex at higher protein concentrations (Fig. 2A). When the 3' stem-loop structure in TAR-2 RNA was deleted in the TAR-2 Δ RNA mutant (+1 to +52), only a single complex was seen for Tat-1 (Fig. 4A). Likewise, Tat-2 formed multiple gel shift complexes with the full-length TAR-2 RNA and a simpler pattern of complexes with the TAR-2 Δ RNA mutant. These results indicate that both the first and second stem-loop structures of TAR-2 contain Tat binding sites. In agreement with this observation, mutagenesis studies have shown that both stem-loop structures contribute to the Tat response (5, 17). Deletion of the first stem-loop, however, has a more drastic reduction on the Tat response than deletion of the second stem-loop, perhaps because the first stem-loop structure is positioned optimally relative to the transcription start site.

We note that our data cannot determine whether TAR-2 RNA can bind simultaneously to more than one Tat protein. The most rapidly migrating gel shift complexes observed with Tat-1 and Tat-2 could be TAR-2 RNA bound to one Tat protein, while the more slowly migrating complexes could be a single TAR-2 RNA molecule bound to two or more Tat proteins. Conversely, multiple complexes seen with Tat-1 and Tat-2 could represent a single Tat protein bound to different sites on TAR-2 RNA; occupation of different binding sites by a single protein may lead to protein-RNA complexes with distinct migration properties. To address this point, we attempted binding assays with mixtures of Tat-1 or Tat-2 with mutant Tat-2 Δ 8/47 (Fig. 3). It was anticipated that if TAR-2 RNA could bind simultaneously to more than one Tat protein, we would observe novel heteromeric protein-RNA complexes with these mixtures of proteins. However, the resolution of complexes in gels was insufficient to give unequivocal results. In order to determine whether TAR-2 RNA can bind simultaneously to more than one Tat protein, it will be necessary to perform detailed

FIG. 9. Tat *trans*-dominant mutant inhibition of wild-type Tat activity. Reporter plasmids were cotransfected with 1 μ g of GAL4 parent, GAL4-Tat-1, or GAL4-Tat-2 activator plasmids plus the indicated amount of Tat-1 *trans*-dominant inhibitor. The *trans*-dominant mutant, 52/57, contains substitutions of alanine and glycine for wild-type residues in the basic domain (29).

site-directed mutagenesis studies in conjunction with gel shift and protein-RNA footprinting assays.

In this study we also assayed the relative *in vivo* transactivation activities of Tat-1 and Tat-2 proteins fused to the yeast GAL4 DNA binding domain. Similar to the natural Tat proteins, the GAL4-Tat-1 protein had high activity for both the HIV-1 and HIV-2 LTRs, while the GAL4-Tat-2 protein had high activity for only the HIV-2 LTR. This result was expected, as the GAL4 DNA binding domain is irrelevant for function of these fusions on the HIV LTRs. However, when directed to synthetic promoters via 5' GAL4 DNA binding sites, the GAL4-Tat-2 protein displayed severalfold-higher levels of transactivation than did the GAL4-Tat-1 protein. Because GAL4-Tat-1 and GAL4-Tat-2 are likely to have identical binding affinities for the GAL4 binding sites in these promoters, these results suggest that the Tat-2 protein may interact more efficiently than Tat-1 with the transcription apparatus.

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ADDENDUM IN PROOF

Chang and Jeang (Y.-N. Chang and K.-T. Jeang, *Nucleic Acids Res.* 20:5465–5472, 1992) have recently published data and conclusions in agreement with this study.

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