tat regulates binding of the human immunodeficiency virus *trans*-activating region RNA loop-binding protein TRP-185

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The TAR element extending from -17 to $+80$ in the human immunodeficiency virus long terminal repeat (HIV LTR) is required for activation of gene expression by the *tat trans*-activator protein. TAR RNA forms a stable stem-loop structure, and mutagenesis studies indicate that the stem structure, the primary sequence of the loop, and the bulge element are the major determinants for *tat* activation. RNA gel retardation analysis demonstrates that both *tat* and cellular proteins bind to TAR RNA, but the mechanism by which these proteins increase HIV gene expression is unknown. We have fractionated HeLa cell nuclear extracts in an attempt to identify cellular proteins that bind to TAR RNA and are involved in regulating HIV gene expression. RNA gel retardation and UV cross-linking reveal that a cellular protein of 185 kD, which we designate TAR RNA-binding protein 185 (TRP-185), binds with both high affinity and marked specificity to TAR RNA. RNA gel retardation and competition analyses indicate that TRP-185 binding is strongly dependent on the TAR RNA loop sequences. The binding of TRP-185 is modulated by both a set of cellular cofactors and the *tat* protein. Highly purified preparations of TRP-185 are capable of activating *in vitro* transcription of wild-type, but not mutated, HIV LTR chloramphenicol acetyltransferase (CAT) constructs. These results characterize a positively acting cellular RNA-binding factor, TRP-185, which is involved in the regulation of HIV gene expression.

[Key Words: HIV; TAR RNA; gene expression; cellular factors; *tat*]

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Gene expression of the human immunodeficiency virus (HIV) is regulated by a variety of mechanisms. The long terminal repeat (LTR) is the site of multiple regulatory regions involved in both general and tissue-specific gene expression (Rosen et al. 1985; Jones et al. 1986; Garcia et al. 1987, 1989; Nabel and Baltimore 1987; Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Jones et al. 1988; Wu et al. 1988; Berkhout and Jeang 1989; Harrich et al. 1989; Selby et al. 1989; Ratnasabapathy et al. 1990; Roy et al. 1990a,b). Two regions, including the NFAT motif (Siekevitz et al. 1987; Crabtree 1989) and the NF- κ B motif (Nabel and Baltimore 1987; Tong-Starksen et al. 1987; Harrich et al. 1990), are involved in the regulation of the HIV LTR in activated T lymphocytes. Other elements such as SP1 (Jones et al. 1986; Harrich et al. 1989) and TATA (Garcia et al. 1987, 1989; Jones et al. 1988) are involved in regulating gene

expression in a variety of both lymphoid and nonlymphoid cell lines. HIV contains an additional regulatory element known as the *trans*-activating region (TAR), which extends from -17 to $+80$ in the HIV LTR (Rosen et al. 1985; Garcia et al. 1987, 1989; Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Jones et al. 1988; Berkhout and Jeang 1989; Braddock et al. 1989; Selby et al. 1989; Ratnasabapathy et al. 1990; Roy et al. 1990a,b).

The TAR element is required for activation of gene expression by the viral *tat trans*-activator protein (Dayton et al. 1986; Fisher et al. 1986; Hauber and Cullen 1988; Rice and Mathews 1988; Berkhout et al. 1989; Braddock et al. 1989; Laspija et al. 1989; Marciniak et al. 1990a,b). The structural integrity of TAR RNA is a key element for *tat* activation (Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Berkhout and Jeang 1989; Berkhout et al. 1989; Garcia et al. 1989; Selby et al. 1989; Dingwall et al. 1990; Roy et al. 1990a,b). TAR RNA is capable of forming a stable stem-loop structure, and disruption of stem base-pairing

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results in a marked decrease in *tat* activation. However, compensatory mutations that restore TAR RNA stem base-pairing result in nearly wild-type levels of *tat* activation (Feng and Holland 1988; Jakobovits et al. 1988; Garcia et al. 1989; Selby et al. 1989; Roy et al. 1990b). The loop and bulge regions in TAR are also required for high-level activation by *tat* (Feng and Holland 1988; Berkhout and Jeang 1989; Garcia et al. 1989; Selby et al. 1989; Dingwall et al. 1990; Roy et al. 1990a,b). Substitution of single base pairs in the loop decreases *tat* activation, whereas substitution of multiple base pairs in this region results in even further decreases in *tat* activation (Berkhout and Jeang 1989; Garcia et al. 1989; Roy et al. 1990b). Deletion of the bulge region or substitution for a single U residue at +23 in the bulge region also severely decreases *tat* activation (Berkhout and Jeang 1988; Dingwall et al. 1990; Roy et al. 1990a,b). Thus, three major determinants, the stem, the loop, and the bulge, are required for wild-type activation of the TAR element.

Recently, a number of studies have indicated that the *tat* protein, via its basic domain, is capable of binding to the bulge region in TAR RNA (Dingwall et al. 1989, 1990; Roy et al. 1990a; Weeks et al. 1990). A variety of nuclear proteins are also capable of binding to TAR RNA (Gatignol et al. 1989; Gaynor et al. 1989; Marciniak et al. 1990a,b). In this study HeLa cell nuclear extracts were extensively fractionated and assayed by gel retardation and UV cross-linking in an attempt to characterize cellular proteins that bind to TAR RNA. A cellular protein of 185 kD, which we designated TAR RNA protein 185 (TRP-185), was identified; this protein binds specifically and with high affinity to TAR RNA and requires intact TAR RNA loop sequences for efficient binding. The binding of TRP-185 to TAR RNA is regulated by both a set of cellular cofactors and the *tat* protein. In vitro transcription analysis indicates that TRP-185 stimulates gene expression from a wild-type HIV LTR template but not from an HIV template containing mutations in TAR. Thus, TRP-185 is a likely candidate for a cellular factor

that is regulated by the *tat* protein and is involved in modulating the level of HIV gene expression.

Results

TRP-185 and TRP-140 bind to TAR RNA

The fractionation scheme for HeLa cell nuclear extracts containing proteins that bound to TAR RNA is indicated in Table 1. Gel retardation analysis was performed to assay different column fractions for their ability to bind TAR RNA. As shown in Figure 1A, lane 2, one major gel-retarded species was detected following fractionation on heparin agarose. Gel-retardation analysis with unfractionated nuclear extract yielded a number of additional retarded species, but competition analysis indicated that they bound to a variety of nonspecific RNA templates (data not shown). Further chromatography on Sephacryl S-300, Mono S fast protein liquid chromatography (FPLC), and hydroxylapatite columns revealed two major gel-retarded species (Fig. 1A, lanes 3–5). These two species could be separated following preparative sucrose gradient centrifugation (Fig. 1A, lanes 6–8).

UV cross-linking in the absence of RNase with each of the column fractions shown in Figure 1A was also performed (Fig. 1B). The results were consistent with the slower-mobility gel-retarded species migrating on SDS-polyacrylamide gels at 200 kD and the faster-mobility gel-retarded species migrating at 155 kD (Fig. 1B). Treatment of each of these species with RNase T1 following UV cross-linking revealed a decrease in their molecular masses to 185 kD (TRP-185) (Fig. 1C) and 140 kD (TRP-140) (data not shown), respectively. UV cross-linking, gel electrophoresis, and autoradiography of the gel slice containing these gel-retarded species also revealed the presence of either the 185- or the 140-kD species (data not shown). It was noted that in the absence of RNase T1, UV cross-linking of wild-type TAR RNA and TRP-185 resulted in two species, whereas in the presence of RNase, only one species was detected (Fig. 1C). It is thus

Table 1. Partial purification of TRP-185

Procedure	Volume (ml)	Total protein (mg)	Total binding activity (ng)	Sp. act ^a (ng/mg)	Yield (%)
HeLa cells (60 g)	60	3000			
Nuclear extract	60	590			
Heparin-agarose	66	300	1050	3.5	100
Ammonium sulfate (ppt.)	4.9	252	932	3.7	89
Sephacryl S-300	40	33	370	11.2	35
FPLC Mono S	35	14	342	24.4	33
HTP Bio-gel	5.6	3.6	168	46.8	16
Sucrose gradient	10	0.61	154	252	15

Maximal binding conditions at each step were determined, and RNA gel retardation was performed at optimal conditions. Appropriate gel-retarded species were excised and counted by liquid scintillation.

^aSpecific activity is defined as nanograms of labeled (+1/+80) HIV wild-type RNA bound per milligram of protein in the binding reaction.

Figure 1. Assay of TRP-185 and TRP-140 fractionation. Fractions from chromatography that were active for binding to TAR RNA in gel retardation (A) or UV cross-linking (B) assays are shown. (Lane 1) Probe alone; (lane 2) heparin-agarose; (lane 3) Sephacryl S-300; (lane 4) Mono S FPLC; (lane 5) hydroxylapatite; (lane 6) sucrose gradient TRP-185 pool; (lane 7) sucrose gradient TRP-140 pool; (lane 8) sucrose gradient TRP-185 pool and cofactor fraction; (lane 9) cofactor fraction alone. (C) Lanes 2 and 3 show UV cross-linking of TRP-185 from the sucrose gradient TRP-185 pool and cofactor fraction in the absence (lane 2) or in the presence (lane 3) of RNase.

possible that TRP-185 was capable of binding more than one TAR RNA molecule.

Cofactor proteins modulate TRP-185 binding to TAR RNA

To determine the binding properties and the native size of TRP-185, we further characterized preparative sucrose gradient fractions containing TRP-185 by analytical sucrose gradient centrifugation (Fig. 2). Molecular mass markers were included in parallel gradients to determine the position of TRP-185 in the gradient. None of the fractions from the analytical sucrose gradient, including fractions that contained proteins migrating between 180 and 200 kD gave rise to gel-retarded species (Fig. 2A). Prolonged exposures of this autoradiogram did not result in detectable gel-retarded species (data not shown). This result suggested that TRP-185 may require several components for binding to TAR RNA as suggested previously (Fig. 1A, lanes 7–9).

To determine whether this possibility was the case, we added individual fractions from the original preparative sucrose gradient to fractions from the analytical sucrose gradient. The combined factors were then analyzed by gel-retardation analysis (Fig. 2B). Fractions from the analytical sucrose gradient that comigrated at 9.5S (near the 200-kD molecular mass marker) were active in gel retardation only when combined with an additional fraction that sedimented at ~100 kD in the preparative sucrose gradient (Fig. 2B). This 100-kD cofactor fraction was purified further (data not shown) and did not result in UV cross-linked or gel-retarded species either alone (Fig. 1A,B, lanes 9) or when combined with other sucrose gradient fractions. Heat lability, trypsin sensitivity, and further fractionation indicated that this cofactor fraction contained proteins. These results were consistent with a model in which the binding activity of TRP-185 is modulated by cofactors that are themselves not capable of binding TAR RNA directly.

TRP-185 binding requires both the TAR RNA loop sequences and secondary structure

To determine whether TRP-140 and TRP-185 bound spe-

cifically to TAR RNA, we performed RNA gel retardation and competition analyses. Each of the TAR RNA species illustrated in Figure 3 was placed downstream of the T7 promoter and transcribed in vitro in either the presence or the absence of labeled nucleotides. These constructs include the wild type, a substitution of the loop sequences (+31/+34), a mutation of the TAR RNA primary sequence (TAR-sense), disruption of the stem

Figure 2. Analytical sucrose gradient analysis of TRP-185. (A) TRP-185 binding was assayed gel retardation with fractions 5–19 obtained by analytical sucrose gradient centrifugation. (B) These same fractions were also assayed in the presence of cofactor fractions. The positions of the migration of molecule mass markers in the sucrose gradient are shown.

Figure 3. Mutations in the HIV TAR region. The stem-loop structure of the HIV LTR extending from +1 to +62 is shown for a series of constructs that extend to +80 (*Hind*III) in the LTR. The (+1/+80) TAR DNA fragments were ligated to a linker encoding the T7 RNA polymerase promoter, the linker-TAR fragments were cloned into pUC19, and RNA transcription from these constructs was performed in vitro with T7 polymerase. The shaded areas indicate the nucleotides substituted and/or deleted in each construct. The constructs include (1) wild-type, (2) +31/+34, (3) TAR-sense, (4) +19/+22, (5) (+19/+22)/(+40/+43), (6) +23, (7) Δ (+23/+25), (8) +30, (9) +32, and (10) +34.

structure (+19/+22), restoration of the stem structure [(+19/+22)/(+40/+43)], a point mutation in the bulge region (+23), deletion of the bulge region [Δ (+23/+25)],

and mutations in the loop sequences at positions +30, +32, and +34. Gel retardation with labeled wild-type TAR RNA and competition with unlabeled RNAs were

Figure 4. Competition analysis for TRP-185 and TRP-140 with mutant TAR RNAs. Gel retardation analysis was performed with internally labeled wild-type TAR RNA and either hydroxylapatite (A) or analytical sucrose gradient (B) purified TRP-185 with added cofactors. Lanes include probe alone (lane 1), extract (lane 2), or competition with a 30-fold excess of unlabeled RNA for wild type (lane 3), +31/+34 (lane 4) TAR-sense (lane 5), +19/+22 (lane 6), (19/+22)/(40/+43) (lane 7), +23 (lane 8), and +23/+25 (lane 9). Binding reactions were performed by mixing various amounts of competitor RNA (0–50 ng) with 1.5 ng of wild-type RNA probe and the added extract. (C) Experimental data were plotted, and competition curves and relative competition efficiencies were determined as described (Baker et al. 1986).

performed with both TRP-140 and TRP-185 eluted from the hydroxylapatite column and with TRP-185 plus a cofactor fraction obtained from sucrose gradient centrifugation (Figs. 4 and 5). Table 2 reveals the relative activity of each of these constructs in the context of the HIV LTR when transfected into HeLa cells in the presence of a *tat* expression vector. Thus, we could correlate *in vitro* binding assays with the *in vivo* activity of these templates.

Gel retardation with the hydroxylapatite column fraction revealed two species corresponding to TRP-140 and TRP-185 (Fig. 4A, lane 2). TRP-185 was competed by a 30-fold excess of unlabeled wild-type TAR RNA, whereas TRP-140 was not competed by using a similar amount of competitor (Fig. 4A, lane 3). Larger quantities of wild-type competitor resulted in the loss of TRP-140 binding, but all other TAR RNAs tested also competed at similar concentrations, indicating minimal binding specificity for TRP-140 (data not shown). TAR RNA species containing substitutions of the loop sequences (+31/+34) resulted in only minor levels of competition for TRP-185, indicating a critical role for the loop sequences in TRP-185 binding (Fig. 4A, lane 4). A mutation of the TAR RNA primary sequence (TAR-sense) resulted in levels of competition for TRP-185 similar to that found with wild-type TAR RNA (Fig. 4A, lane 5). Disruption of the TAR stem structure (+19/+22) resulted in decreased competition of TRP-185 binding (Fig. 4A, lane 6), whereas restoration of the TAR stem structure [(+19/+22)/(+40/+43)] resulted in near wild-type levels of competition (Fig. 4A, lane 7). A point mutation in the bulge (+23) resulted in near wild-type competition

(Fig. 4A, lane 8), but deletion of the entire bulge region [Δ (+23/+25)] resulted in minimal competition, being nearly as defective as the loop substitution mutant (+31/+34) (Fig. 4A, lane 9). A similar series of gel retardation and competition assays for TRP-185 were performed with sucrose gradient-isolated TRP-185 and cofactor fractions (Fig. 4B). The pattern of competition for TRP-185 was similar to that seen with fractions from the hydroxylapatite column (Fig. 4B), indicating that TRP-140 was not required for the binding properties of TRP-185. Competition curves and relative competition efficiencies for each of these constructs are shown in Figure 4C.

Because the loop sequences appeared critical for the binding of TRP-185, we used both hydroxylapatite column and sucrose gradient fractions containing TRP-185 in gel retardation and competition analyses with wild-type TAR RNA and several loop point mutants (Fig. 5A,B). With the hydroxylapatite column fractions, the wild-type TAR RNA again resulted in marked competition for TRP-185 binding (Fig. 5A, lane 3), whereas the loop substitution mutant (+31/+34) resulted in only minimal competition (Fig. 5A, lane 4). Mutation of a nucleotide (+30) in the loop resulted in only slight competition of TRP-185 binding, similar to the results obtained with the loop substitution mutant (+31/+34), indicating the importance of this nucleotide in the loop for TRP-185 binding (Fig. 5A, lane 5). Mutation of another nucleotide (+32) in the loop resulted in significant competition for TRP-185 binding (Fig. 5A, lane 6) although less than what was seen with wild-type TAR

Figure 5. Competition analysis for TRP-185 and TRP-140 with TAR RNA loop mutants. Gel retardation analysis was performed with internally labeled wild-type TAR RNA and either hydroxylapatite (A) or analytical sucrose gradient (B)-purified TRP-185 with added co-factors. Lanes include probe alone (lane 1); with extract (lane 2), or competition with a 30-fold excess of unlabeled RNA for the wild type (lane 3), +31/+34 (lane 4), +30 (lane 5), +32 (lane 6), and +34 (lane 7). (C) Experimental data were plotted, and competition curves and relative competition efficiencies were determined as described (Baker et al. 1986).

RNA; mutation of yet another nucleotide (+34) resulted in intermediate competition for TRP-185 binding (Fig. 5A, lane 7). None of these RNAs containing point mutations in the loop region resulted in significant competition for TRP-140 at the concentrations tested (Fig. 5A). Similar results were seen with sucrose gradient-purified TRP-185 (Fig. 5B). Competition curves and relative competition efficiencies for each of these constructs are shown in Figure 5C.

Table 2. Relative activity of TAR mutant constructs with *tat*

Construct	Relative CAT activity
1. wild type	1.0
2. (+31/+34)	0.04
3. TAR sense	0.41
4. (+19/+22)	0.08
5. (+19/+22)/(+40/+43)	0.96
6. (+23)	0.22
7. Δ(+23/+25)	0.25
8. (+30)	0.16
9. (+32)	0.18
10. (+34)	0.22

Transfections of each of these constructs in the context of the HIV LTR (−170/+80) fused to CAT in the presence of a *tat* expression vector were performed, and the percent CAT conversion determined (Gorman et al. 1982). The results were normalized to the percent CAT conversion of the wild-type construct and reflect the average of three independent experiments.

The results indicated that individual nucleotides in the loop region were critical for TRP-185 binding, but different nucleotides had somewhat variable effects on its binding. Although a point mutation in the bulge did not greatly alter TRP-185 binding, deletion of the bulge resulted in marked decreases in its binding. In addition to determining regions of TAR RNA required for efficient TRP-185 binding, we also calculated the binding affinity of TRP-185. The disassociation constant (K_d) of TRP-185 for TAR RNA was calculated to be 3.15×10^{-10} M while that for nonspecific RNA was calculated to be 2.11×10^{-5} M (data not shown). Thus, TRP-185 bound to TAR RNA with both high affinity and marked specificity.

Characterization of wild-type and mutant *tat* protein binding to TAR RNA templates

We next determined whether *tat* regulated the binding of TRP-185 to TAR RNA. The possibilities that we addressed were whether both *tat* and TRP-185 bound simultaneously to TAR RNA or whether these proteins competed for binding to TAR RNA. Both wild-type *tat* and mutant *tat* (*tat* 52/57), with a substitution of six neutral amino acids (glycine and alanine) in the basic domain of *tat* between amino acids 52 and 57 (Modesti et al. 1991), were produced as fusion proteins with glutathione *S*-transferase (Smith and Johnson 1988). These *tat* fusion proteins were purified by multiple passages on glutathione-agarose affinity columns. Authentic *tat* proteins were liberated by removal of the glutathione

S-transferase moiety following cleavage with thrombin at a recognition site engineered into the fusion proteins. Both wild-type *tat* and *tat* 52/57 were judged to be >95% pure, and each preparation yielded a single species of ~9 kD following gel electrophoresis and Coomassie staining. The purification of wild-type *tat* protein under these conditions eliminated harsh elution and denaturation procedures used in several other purification schemes, resulting in both higher binding affinity for and higher binding activity of TAR RNA than reported previously (Dingwall et al. 1990; Roy et al. 1990b).

To first characterize the binding properties of these *tat* proteins, we performed gel retardation analysis with labeled TAR RNA derived from the wild type, a loop mutant (+31/+34), a bulge point mutant (+23), or a bulge deletion mutant (+23/+25) (Fig. 6). All TAR RNA probes were labeled to the same specific activity. The wild-type *tat* protein bound to wild-type TAR RNA with a K_d of 6.4×10^{-10} M. Scatchard analysis indicated that one *tat* molecule bound to each TAR RNA molecule and that all the binding sites of *tat* were active (Fig. 6A, lanes 1–5). *tat* 52/57 bound poorly to wild-type TAR RNA be-

cause the majority of basic amino acids in the basic domain were substituted with neutral amino acids (Fig. 6A, lanes 6–10). These results were consistent with previous results, implicating the basic domain in *tat* for binding to TAR RNA (Roy et al. 1990a; Weeks et al. 1990; Calnan et al. 1991).

We then tested the binding of these *tat* proteins to the TAR RNA loop substitution mutant (+31/+34). The wild-type *tat* protein bound to this mutant TAR RNA with fivefold lower affinity than it did to wild-type TAR RNA (Fig. 6B, lanes 1–5). Similar results have been noted previously with *tat* peptides in gel retardation analysis with a similar TAR RNA loop mutant (Calnan et al. 1991). *tat* 52/57 did not bind to this mutant (Fig. 6B, lanes 6–10). TAR RNA containing either a point mutation in the bulge (+23) (Fig. 6C, lanes 1–10) or a deletion of the bulge region (+23/+25) (Fig. 6D, lanes 1–10) did not result in detectable binding of either wild-type *tat* or *tat* 52/57. These results were consistent with previous studies, which indicated a predominant role for the bulge region in *tat* binding (Dingwall et al. 1990; Roy et al., 1990a; Weeks et al. 1990; Calnan et al. 1991), but also suggested that wild-type loop sequences influenced the binding affinity of *tat* for TAR RNA.

Figure 6. Binding of *tat* proteins to wild-type and mutant HIV TAR RNAs. Both bacterially produced and purified wild-type *tat* (lanes 1–5) and a mutant *tat* protein, *tat* 52/57 (lanes 6–10) were used in gel retardation assays with labeled TAR RNAs corresponding to the wild-type (A), loop substitution mutant +31/+34 (B), bulge point mutant +23 (C), bulge deletion mutant +23/+25 (D). The amount of *tat* protein added to each reaction was as follows: 0 (lanes 1,6); 1.8 ng (lanes 2,7); 3.6 ng (lanes 3,8); 7.2 ng (lanes 4,9); 14.4 ng (lanes 5,10).

tat competes with TRP-185 for binding to TAR RNA

TRP-185 was demonstrated to require an intact TAR RNA stem-and-bulge structure and wild-type loop sequences for efficient binding. We determined whether the addition of either wild-type *tat* or *tat* 52/57 influenced the binding of TRP-185 to TAR RNA. Gel retardation analysis was performed with a constant amount of TRP-185 and increasing amounts of wild-type *tat* protein added to labeled wild-type TAR RNA. As increasing amounts of *tat* bound to TAR RNA, binding of TRP-185 to TAR RNA decreased (Fig. 7A, lanes 1–5). No evidence for the simultaneous binding of both *tat* and TRP-185 to TAR RNA was noted. This result suggested that *tat* was able to compete with TRP-185 for binding to TAR RNA. This result was further substantiated by gel retardation analysis with wild-type TAR RNA and similar amounts of *tat* 52/57 and TRP-185. This *tat* mutant, which was unable to bind efficiently to TAR RNA, did not reduce the binding of TRP-185 to TAR RNA (Fig. 7B, lanes 1–5). The addition of purified glutathione S-transferase protein obtained during *tat* purification also did not reduce the binding of TRP-185 to TAR RNA (Fig. 7C, lanes 1–5). Finally, the wild-type *tat* protein did not decrease the binding of TRP-185 when a TAR RNA bulge point mutant template (+23) was used in gel retardation analysis (data not shown). These results suggested that *tat* competed with TRP-185 for binding to TAR RNA.

TRP-185 stimulates gene expression from the HIV LTR

The role of TRP-185 in stimulating *in vitro* transcription of the HIV LTR was then assayed. *In vitro* transcription assays were performed with a concentration of magnesium (5 mM), which favored the synthesis of full-length

Figure 7. Tat protein competes with TRP-185 for binding to TAR RNA. TRP-185 and cofactor fractions were used in gel retardation assays with wild-type TAR RNA in the presence of increasing amounts of wild-type *tat* (A), mutant *tat* 52/57 (B), or glutathione *S*-transferase (C). The amount of added *tat* or *tat* 52/57 was 0 (lanes 1), 50 ng (lanes 2), 200 ng (lanes 3), 1000 ng (lanes 4), and 3000 ng (lanes 5). The amount of glutathione *S*-transferase used (C) was a 20-fold molar excess over corresponding lanes with *tat* (A) and *tat* 52/57 (B).

transcripts from the HIV LTR. As shown in Figure 8A, a wild-type HIV LTR chloramphenicol acetyltransferase (CAT) template, when restricted with *Nco*I, generated a runoff transcript of 620 bp that was inhibited by a α -amanitin at a final concentration of 2 μ g/ml.

Both wild-type and a TAR mutant containing a deletion of the bulge (+23/+25) were restricted with *Nco*I. The addition of a HeLa cell nuclear extract alone resulted in low-level synthesis of the 620-bp RNA species from both the wild-type and mutant (+23/+25) templates (Fig. 8B, lanes 1,5). There was minimal stimulation of transcription with the addition of the cofactor fraction to the wild-type but not the mutant (+23/+25) template (Fig. 8B, lanes 2,6). However, the addition of either TRP-185 alone or TRP-185 and the cofactor fraction resulted in approximately a fourfold increase in in vitro transcription from the wild-type construct (Fig. 8B, lanes 3,4) but not the mutant (+23/+25) construct (Fig. 8B, lanes 7,8).

The wild-type construct and a mutant HIV LTR construct in which the orientation of TAR RNA was reversed (TAR-antisense) (Garcia et al. 1989) were restricted with *Nco*I and *Eco*RI, respectively, and added to the same in vitro transcription assay. As shown in Figure 8C, lane 1, both the 620-bp wild-type and the 290-bp mutant transcripts were present. The addition of TRP-185 alone (Fig. 8C, lane 2) or TRP-185 and the cofactor fraction (Fig. 8C, lane 3) stimulated the expression of the wild-type HIV LTR with minimal effects on the mutant template. The effects of TRP-185 did not appear to result from alterations in the stability of TAR RNA (data not shown).

Discussion

The TAR element extending from -17 to +80 is required for *tat* activation of the HIV LTR (Rosen et al. 1985; Garcia et al. 1987, 1989; Feng and Holland 1988;

Hauber and Cullen 1988; Jakobovits et al. 1988; Jones et al. 1988; Berkhout and Jeang 1989; Braddock et al. 1989; Selby et al. 1989; Ratnasabapathy et al. 1990; Roy et al. 1990a,b). This element is responsible for multiple regulatory properties. TAR DNA serves as a binding site for several cellular binding proteins, including UBP-1/LBP, which likely function in regulating the basal expression of the HIV LTR (Garcia et al. 1987, 1989; Jones et al. 1988; Wu et al. 1988). In addition, the TAR element is responsible for the synthesis of short transcripts, the function of which is unknown, even when located downstream of a variety of heterologous promoters (Ratnasabapathy et al. 1990). A number of studies strongly implicate nascent TAR RNA as a critical target for *tat* activation, with subsequent stimulation of transcriptional initiation and/or elongation (Feng and Holland 1988; Hauber and Cullen 1988; Jakobovits et al. 1988; Berkhout et al. 1989; Berkhout and Jeang 1989; Garcia et al. 1989; Selby et al. 1989; Roy et al. 1990a,b). Analogies of *tat* activation via the TAR element with prokaryotic transcriptional regulation of bacteriophage λ *nut* sites (Lazinski et al. 1988; Selby et al. 1989) suggests that *tat* and/or cellular factors present on nascent TAR transcripts interact with RNA polymerase II to overcome distal blocks to elongation.

To determine how viral and cellular factors may interact with TAR RNA to activate HIV gene expression, several experimental approaches have been taken. One approach involves the ability of *tat* fused to heterologous RNA-binding proteins to activate HIV gene expression (Selby and Peterlin 1990; Southgate et al. 1990). Another approach involves a biochemical characterization of viral (Dingwall et al. 1989, 1990; Roy et al. 1990a; Weeks et al. 1990) and cellular (Gatignol et al. 1989; Gaynor et al. 1989; Marciniak et al. 1990a,b) factors that bind to TAR RNA. Fusions of *tat* with a prokaryotic RNA-binding protein activate gene expression of an HIV construct containing heterologous RNA-binding sites inserted into

Figure 8. (A) A wild-type HIV LTR CAT construct was restricted with *NcoI* and used in in vitro transcription assays to generate a 620-bp runoff transcript (lane 1), which was inhibited by α -amanatin (lane 2). (B) A wild-type HIV LTR CAT construct (lanes 1–4) or a similar construct containing a deletion of the TAR bulge region (+23/+25) (lanes 5–8) was restricted with *NcoI*. HeLa nuclear extract alone (lanes 1,5), with the addition of cofactor (lanes 2,6), sucrose gradient-purified TRP-185 (lanes 3, 7, or both cofactor and TRP-185 (lanes 4,8) were added to each reaction. (C) Wild-type or TAR RNA mutant (TAR-antisense) HIV LTR CAT constructs were restricted with either *NcoI* or *EcoRI*, respectively, and combined in in vitro transcription assays with HeLa nuclear extract or HeLa nuclear extract alone (lane 1), with the addition of TRP-185 (lane 2), TRP-185 and cofactor (lane 3), or cofactor alone (lane 4). The positions of the transcripts from the wild-type template (620 bp) and the mutated template (290 bp) are indicated.

TAR (Selby and Peterlin 1990). However, the level of HIV activation seen with these *tat* fusions is markedly decreased compared with that seen in the presence of a wild-type TAR element, suggesting that *tat* binding to TAR RNA is not sufficient to yield high levels of *tat* activation (Selby and Peterlin 1990). This result suggests that cellular factors may be critical for *tat* activation of the TAR RNA element.

We began a characterization of cellular proteins that bind to TAR RNA and may be required for *tat* activation of the HIV LTR. A cellular protein, TRP-185, which binds with high affinity and marked specificity to TAR RNA, was identified. However, multiple cellular proteins may bind to TAR RNA. A previous study used UV cross-linking assays to identify a 68-kD cellular protein that binds specifically to the TAR loop region (Marciniak et al. 1990a,b). Using similar techniques, we also detected a 68-kD cellular protein that binds to TAR RNA. The detection of this protein was dependent on the use of heparin and RNase following the binding reactions. However, when nonspecific RNA or poly(I)–poly(C) was used in gel retardation assays without RNase, we no longer detected this species. Another RNA-binding protein, TRP-140, which binds with high affinity to a variety of double-stranded RNAs, may also potentially have functional significance in regulating HIV gene expression. Determination of the relevance of these RNA-binding proteins in regulating HIV gene expression will require their cloning and detailed biochemical assays. We found that only TRP-185 possesses both high affinity for and marked specificity of binding to TAR RNA in a manner that correlates with in vivo genetic data.

The TAR RNA loop sequences, stem structure, and bulge region are each important in regulating TRP-185

binding. The bulge region in TAR RNA has been demonstrated to serve as a binding site for *tat* via its basic domain (Dingwall et al. 1989, 1990; Roy et al. 1990a; Weeks et al. 1990). Both point mutations in and deletion of this bulge markedly decrease *tat* binding and in vivo *tat* activation (Berkhout and Jeang 1988; Dingwall et al. 1990; Roy et al. 1990a,b). We demonstrate that the binding of TRP-185 to TAR RNA is not altered by point mutations in the bulge. However, a deletion of the bulge region results in a marked decrease in TRP-185 binding. Thus, the binding specificities of TRP-185 and *tat* for the bulge region differ. These results suggest that the bulge region may influence TRP-185 binding by altering the secondary structure of TAR RNA. Bulge-induced kinks in double-stranded RNA structures may alter the tertiary folding of RNA and be important in the potential formation of recognition sites for RNA-binding proteins (Bhattacharyya et al. 1990). Although we note only a small effect of disruption of TAR RNA stem structure on TRP-185 binding, this mutation results in large decreases in *tat*-mediated activation of HIV gene expression in vivo. Dramatic effects on TRP-185 binding are found with several point mutations in the primary sequence of the TAR RNA loop, but individual nucleotide changes in the loop vary in terms of their effect on TRP-185 binding. Multiple-base-pair substitutions in the loop are much more deleterious for TRP-185 binding than are any of the individual point mutations. Further studies will be required to identify points of contact of TRP-185 with TAR RNA, but our results seem consistent with a strong requirement for wild-type loop sequences in the context of an intact stem-and-bulge structure for maximal TRP-185 binding.

Cellular cofactors modulate TRP-185 binding to TAR

RNA. It is possible that these cofactors may be a class of cellular kinases that alter the phosphorylation of TRP-185, with resultant changes in its binding affinity. The observation that protein kinase C expression vectors activate *tat*-mediated expression via the TAR element is consistent with this possibility (Jakobovits et al. 1990). However, we cannot definitively rule out the possibility that TRP-185 may interact weakly with these cofactor species. Similar interactions occur with the multisubunit factor CstF, which is required for mRNA polyadenylation (Takagaki et al. 1990; Wilusz et al. 1990). A 64-kD species is seen upon UV cross-linking of CstF to RNA, but other components of the complex must be present to reconstitute the binding of this species. Both UV cross-linking and sucrose gradient analyses indicate that TRP-185 has a molecular mass of ~185 kD, making the presence of a multicomponent complex unlikely.

The *tat* protein competes with TRP-185 for binding to TAR RNA in vitro. A direct interaction between TRP-185 and *tat* that is independent of TAR RNA is possible. As a result of the proximity of the bulge-and-loop regions and the fact that the dissociation constants for both of these proteins are similar (between 3×10^{-10} and 6×10^{-10} M), steric effects may be responsible for the fact that only *tat* or TRP-185 can bind in vitro to each TAR RNA template. However, we cannot rule out that high-affinity binding of *tat* to TAR RNA requires the loop sequence in addition to the bulge region, and this binding of *tat* to the loop sequences could potentially inhibit TRP-185 binding. Mutations in the upper portion of TAR RNA, including the loop or bulge region may dramatically influence TAR RNA structure with subsequent effects on the ability of both cellular and viral proteins to bind to TAR RNA. Thus, the in vivo phenotype of the TAR RNA loop-and-bulge mutations may be the result of decreased binding of either TRP-185 or *tat*.

Because *tat* is able to regulate the binding of TRP-185 to TAR RNA, it is critical to determine the function of TRP-185. The addition of TRP-185 to a HeLa cell nuclear extract results in the stimulation of in vitro transcription from the HIV LTR. Although the level of activation is only fourfold, it must be remembered that the HeLa cell nuclear extract contains significant amounts of both cofactor and TRP-185. Thus, it is likely that a much greater level of transcriptional activation may be obtained with a completely reconstituted extract. We were unable to obtain significant additional stimulation by the addition of *tat* protein to in vitro transcription assays, as described previously (Marciniak et al. 1990a,b). The conditions used in our in vitro transcription assays do not address whether TRP-185 is involved primarily in the stimulation of HIV LTR transcriptional initiation or in elongation (Laspias et al. 1989). Furthermore, we do not address the role of *tat* in terms of its role in the in vitro transcription of the HIV LTR.

A model consistent with our data and previous studies would suggest that TRP-185 might be a critical factor for the formation of an efficient cellular transcription complex assembled on the HIV promoter. In this model, TRP-185 would interact with the loop of the nascent

TAR RNA transcribed from the HIV LTR to form a complex that can proceed slowly and, as such, become rate determining. Binding of *tat* to the bulge region as it arises during the formation of TAR RNA would then cause the release of TRP-185 from the loop region with subsequent catalytic effects on the transcriptional initiation or elongation process. Further studies will be needed to address the validity of such a model. The purification and cloning of TRP-185 and its associated cofactor species will be required to address the potential roles of these proteins in mediating *tat* activation of the HIV LTR.

Materials and methods

Plasmid constructs and labeling of mRNAs

Wild-type and mutant HIV mRNAs were constructed by fusing a synthetic linker containing a T7 RNA polymerase promoter to DNA fragments of the indicated TAR constructs from +1 to +80 (Garcia et al. 1989). Transcription of these constructs was performed after they were linearized with *Hind*III (+80) by using T7 RNA polymerase resulting in transcripts consisting of nucleotides +1 to +80 of the HIV LTR. RNA synthesis, labeling, and purification were performed by using the reagents and procedures of the Riboprobe System II (Promega) (Gaynor et al. 1989).

For *tat* bacterial expression, DNA fragments encoding either wild-type *tat* or *tat* 52/57 (Modesti et al. 1991), which contains the amino acids Gly-Gly-Ala-Gly-Gly-Gly in place of the native amino acids Arg-Arg-Gln-Arg-Arg-Arg (amino acids 52–57), were used. By changing the sequence GAAATG encompassing the initiating methionine to AGATCT, a *Bgl*III–*Eco*RI fragment of a *tat* subclone containing the second exon of *tat* was cloned into pGEX-2T (Smith and Johnson 1988). Following cleavage with thrombin at the recognition motif between the GST–*tat* junction, *tat* proteins were generated and purified that consisted of amino acids 2–72 of *tat* preceded by amino acids Arg-Ser contributed by the *Bgl*III sequence.

Purification of HIV TAR RNA-binding protein (TRP-185)

Nuclear extracts were prepared as described previously (Dignam et al. 1983). Nuclear extract prepared from 60 liters of HeLa cells was applied to a heparin-agarose column (2.5 × 9 cm) equilibrated with buffer A [(20 mM Tris (pH 7.9), 20% glycerol (vol/vol), 0.2 mM EDTA)] containing 0.1 M KCl, 0.5 mM DTT, and 0.5 mM PMSF. The column was washed with the same buffer until the A_{280} was almost zero, and bound proteins were eluted with buffer A containing 0.4 M KCl, 0.5 mM PMSF, and 0.5 mM DTT. The 0.4 M KCl buffer A fractions were pooled, precipitated with 70% ammonium sulfate, and applied to a Sephacryl S-300 column (2.6 × 93 cm) equilibrated with buffer A containing 0.1 M KCl and 1 mM DTT. The active fractions were pooled and applied to a 1-ml Mono S FPLC column equilibrated with the same buffer. The active flowthrough fractions were pooled and loaded onto an HTP Bio-Gel column (1.5 × 5 cm) equilibrated with the same buffer. The column was washed with the same buffer and eluted with 0.1 M potassium phosphate (pH 7.0), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, and 1 mM DTT. The active 0.1 M phosphate fractions were pooled and dialyzed against 20 mM Tris (pH 7.9), 5% glycerol, 0.2 mM EDTA, 0.1 M KCl, and 1 mM DTT. One-milliliter sample were each loaded into six tubes (1.4 × 8.9 cm) containing 10 ml of a 5–20% continuous sucrose gradient. Preparative sucrose gradient analysis was performed

with a Beckman SW41 Ti rotor at 28,000 rpm for 40 hr at 4°C. Analytical sucrose gradient analysis was performed with a Beckman SW50.1 rotor at 38,000 rpm for 18 hr. The sucrose gradients were fractionated from the bottom of the tube, assayed, and stored at 4°C. The dissociation constants were determined, and Scatchard analysis was done for TRP-185 as described previously (Baker et al. 1986).

Purification of bacterially synthesized *tat* protein

Overnight cultures of wild-type or mutant *tat* 52/57 in pGEX-2T were diluted $\frac{1}{100}$ in 500 ml of fresh medium and grown to an OD of 0.6 at 37°C. The *tat* fusion proteins were induced by the addition of IPTG to a final concentration of 0.1 mM. Cultures were grown for an additional 4 hr, and the pellets were harvested and resuspended in 5 ml of $1 \times$ PBS (150 mM NaCl, 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 at pH 7.3, plus 0.5 mM PMSF and 1 mM DTT). Cells were lysed on ice by sonication, spun at 12,000 rpm for 15 min, and loaded on a 1-ml glutathione-Sepharose affinity column (Smith and Johnson 1988). The column was washed with the buffer described above and eluted with said buffer containing 50 mM Tris (pH 8.0), 1 mM DTT, and 5 mM glutathione. Fractions containing the *tat* fusion proteins were pooled, dialyzed extensively against PBS with 1 mM DTT, and loaded for a second passage on a glutathione-Sepharose column. The column was washed with five column volumes of PBS with 1 mM DTT and five column volumes of buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 2.5 mM CaCl_2 , and 1 mM DTT and incubated with the same buffer and 6 μg of human thrombin (Sigma) for 40 min at room temperature with mixing. The flowthrough fractions that contained thrombin-released *tat* were collected, dialyzed extensively against 20 mM Tris (pH 7.9), 0.2 mM EDTA, 100 mM KCl, 20% glycerol, 1 mM DTT, and 0.5 mM PMSF, and stored at -70°C .

Following dialysis to remove the CaCl_2 and after the addition of PMSF, no residual thrombin activity in *tat* preparations was demonstrated, as judged by assays with other proteins containing thrombin recognition motifs. The presence of *tat* was confirmed by Western analysis with *tat* antisera and Coomassie staining of SDS-polyacrylamide gels. The dissociation constants were determined and Scatchard analysis was done for *tat* binding to the TAR RNA wild-type and loop mutants as described previously (Baker et al. 1986).

Gel retardation assay for TRP and *tat* binding

The probe for the binding assay was prepared by *in vitro* transcription of a plasmid directing the synthesis of nucleotides +1 to +80 from the HIV LTR by use of T7 polymerase and [α - ^{32}P]GTP (3000 Ci/mmol). The transcribed RNA was gel isolated, eluted, and used for binding. Approximately 1.5 ng of TAR RNA probe was mixed with extract (0.6–10 μg), poly(I)–poly(C) (0.5–4 μg), and a final concentration of 10 mM Tris (pH 7.4), 0.1 mM EDTA, 50 mM KCl, 1 mM 2-mercaptoethanol, and 10% glycerol in 50 μl total volume. Protein samples from the heparin-agarose column (10 μg), S-300 column (4 μg), Mono S column (3.9 μg), HTP Bio-Gel column (2.6 μg), and sucrose gradient pool (0.6 μg) were used. Binding was performed at room temperature for 30 min, and the samples were loaded onto a 4% polyacrylamide gel containing $1 \times$ Tris-borate-EDTA (TBE) and 2% glycerol and electrophoresed at 180 V in $1 \times$ TBE at room temperature. The gel was dried and exposed overnight with an intensifying screen at -70°C . For sucrose gradient fractions, 10 μl of the cofactor fraction was added to restore activity. For competition analysis, 0–50 ng of each of the unlabeled *in vitro*-transcribed competitor RNAs was mixed with probe and bind-

ing was performed. Binding assays with labeled TAR RNA probes (1.5 ng) were performed in 50 μl total volume as described previously (Roy et al. 1990a). Competition experiments with TRP-185 (0.6 μg), the cofactor fraction (10 μl), and *tat* protein (0–60 ng) were performed under the same conditions. UV cross-linking of TRP-185 TAR RNA was performed under similar binding conditions.

In vitro transcription of the HIV LTR

HIV LTR constructs containing LTR sequences extending from -179 to $+80$ were restricted with *Nco*I to generate a 620-bp runoff transcript. The TAR-antisense construct (Garcia et al. 1989) was restricted with *Eco*RI to generate a 290-bp runoff transcript. Transcription reactions were performed in 10 mM HEPES (pH 7.9), 10 mM Tris (pH 7.9), 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5 mM MgCl_2 , 10 mM creatine phosphate, 0.5 μg of poly(I)–poly(C), 600 mM each ATP, CTP, and UTP, 40 μM GTP, and 1 μl of [α - ^{32}P]GTP (3000 Ci/mmol). HeLa cell nuclear extract (Dignam et al. 1983) (100 μg) was included in each reaction and incubated at 30°C for 1 hr. The final α -amanitin concentration was 2.0 $\mu\text{g}/\text{ml}$. Either 0.6 μg of a TRP-185 fraction from a sucrose gradient preparation or 10 μl of cofactor fractions was included to determine the effects of TRP-185 on activation of the HIV LTR. All reactions were stopped by the addition of 400 μl of 7 M urea, 0.35 M NaCl, 0.01 mM EDTA, 0.1 M Tris (pH 7.4), and 1% SDS. The supernatant was extracted with phenol–chloroform and ethanol-precipitated in the presence of oyster glycogen. Reaction products were electrophoresed on a 5% polyacrylamide sequencing gel containing 8 M urea in $1 \times$ TBE. Gels were exposed overnight at -70°C with an intensifying screen.

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F Wu, J Garcia, D Sigman, et al.

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