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Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA *in vitro*

(trans-activation/RNA-binding proteins/gene synthesis/antisense RNA)

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ABSTRACT **tat, the trans-activator protein for human immunodeficiency virus 1 (HIV-1), has been expressed in *Escherichia coli* from synthetic genes. Purified tat binds specifically to HIV-1 trans-activation-responsive region (TAR) RNA in gel-retardation, filter-binding, and immunoprecipitation assays. tat does not bind detectably to antisense TAR RNA sequences, cellular mRNA sequences, variant TAR RNA sequences with altered stem–loop structures, or TAR DNA.**

Replication of the human immunodeficiency virus 1 (HIV-1) is dependent upon the expression of the trans-activator protein, tat, an 86 amino acid, basic, cysteine-rich, nuclear protein encoded by the *tat* gene of the virus (1–5). Mutational analysis of the viral long terminal repeat (LTR) has identified a cis-acting sequence, called the trans-activation-responsive region (TAR), which is required for tat activity. The TAR sequence is located between residues +1 to +79 and forms part of the 5′ untranslated region of all the mRNAs encoded by HIV-1 (6–8).

The mechanism of action of trans-activation by tat is unknown. The simplest models consistent with the genetic evidence suggest that tat binds directly to the TAR nucleic acid sequence, but these models do not distinguish between DNA or RNA binding, and experimental evidence for tat binding to nucleic acid sequences has not been reported.

Sequences throughout the LTR of integrated proviral DNA, including the TAR region, bind a variety of host proteins (6, 7, 9). Binding of tat either to TAR DNA or to these known transcription factors potentially could regulate transcription. However, several lines of evidence, although not conclusive, suggest that tat may interact with TAR RNA sequences rather than with TAR DNA sequences. The RNA sequence encoded by TAR is capable of forming a stable stem–loop structure (5, 7, 8). Mutations in the sequence which forms the loop or mutations which affect the stability of the loop structure have been reported to abolish trans-activation (7, 8). Additionally, Kao *et al.* (3) have found that tat is required for elongation beyond the TAR site but not for transcriptional initiation. Trans-activation may also have a translational component (4), since the presence of the TAR sequence at the 5′ end of an mRNA molecule inhibits its translation (10) and induces *in vitro* a double-stranded RNA-dependent protein kinase (11), leading to the inhibition of protein synthesis. Here we present evidence that tat is capable of direct binding to TAR RNA, but not to TAR DNA, *in vitro*.

METHODS

Expression Vectors. Two synthetic *tat* genes were prepared to allow expression of tat [strain: LAV_{BRU} (12)] in both *Escherichia coli* (gene a) and mammalian cells (gene b). Oligonucleotides 34–43 residues in length were ligated and cloned in M13 vectors as previously described (13). Codons infrequently occurring in either *E. coli* or mammalian genes were avoided, and where the same amino acid occurred in succession, different codons were chosen. To facilitate later mutagenesis, internal restriction sites were placed at suitable intervals by selection of codons that did not alter encoded amino acid sequences.

The following expression vectors were constructed: pMG627 carries *tat* gene a cloned between the *Bam*HI and *Hin*dIII sites of pUR288 (14), producing a β-galactosidase-tat fusion protein. pMG727 carries *tat* gene b cloned between the *Eco*RI site and *Bam*HI site of pGEM1 (Promega) (15). A synthetic ribosome-binding site (GAATTCAAGGAGGTTTAACCATGG) was inserted between the *Eco*RI site of the vector and *Nco* I site overlapping the initiator codon of the *tat* gene b. pJKC63.4.1 is a Moloney retroviral vector (16) carrying *tat* gene b under the control of the viral LTR and a neomycin phosphotransferase gene under the control of the simian virus 40 early promoter.

Monoclonal Antibodies. Peptides corresponding to residues 2–15 and 73–86, respectively, of tat, were synthesized by a semi-automatic continuous-flow solid-phase method (17). After coupling to keyhole limpet hemocyanin with glutaraldehyde, monoclonal antibodies to the N-terminal (NT3/2D1.1) and C-terminal (NT2/4D5.24) peptide conjugates were prepared. Supernatants were screened after 2 weeks by Western blotting against the β-galactosidase–tat fusion protein.

Purification of tat. *E. coli* strain JM101 carrying pMG627 was grown to late logarithmic phase at 37°C in the presence of ampicillin at 100 μg/ml and isopropyl β-D-thiogalactoside (IPTG) at 50 μg/ml. Inclusion body pellets containing β-galactosidase–tat protein were purified by using nonionic detergents as described (18). The pellet was dissolved in 70% (vol/vol) formic acid, and cyanogen bromide (CNBr) was added to a concentration of 100 mg/ml. After 4 hr, the digest was diluted 10-fold with water and lyophilized. Peptides were resuspended in 50 mM Tris·HCl, pH 8.0/100 mM NaCl/8 M urea and applied to a 25-ml Accell CM column (Waters) equilibrated with the same buffer and eluted with a gradient from 0.1 to 1.1 M NaCl. The tat peptides were redissolved in 0.1% trifluoroacetic acid and applied to a C_4 reversed-phase column (Beckman 5μ Ultrapore, 10 × 250 mm), and tat was eluted from the column with a gradient of acetonitrile,

Abbreviations: HIV-1, human immunodeficiency virus 1; LTR, long terminal repeat; TAR, trans-activation-responsive region; IPTG, isopropyl β-D-thiogalactoside; CAT, chloramphenicol acetyltransferase.

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typically 0–60%. The peak fractions were concentrated by lyophilization and redissolved in buffers suitable for binding assays.

Alternatively, pMG727 was transfected into *E. coli* BL21, (19). After induction by IPTG, tat from the soluble supernatant fraction was precipitated by addition of 1% Polymin P (BASF). The pellet was dissolved in 6 M guanidine isothiocyanate and applied to a Sephadex G-25 column equilibrated with 20% (vol/vol) acetic acid (pH 2.5). The void peak containing tat was applied to a SP-Sephadex column (1 × 10 cm) equilibrated with 20% acetic acid. tat was eluted from the column in 100 mM Tris·HCl, pH 8.0/1 M NaCl/0.1 mM $ZnCl_2$/10% (vol/vol) glycerol.

Estimates of the concentration of tat by amino acid analysis or spectrophotometry, assuming A_{280} (1 mg/ml) = 1.3 (20), were in close agreement.

RNA- and DNA-Binding Assays. TAR RNA was prepared by two methods: (*i*) SP6 RNA polymerase was used to transcribe plasmid pGEM1.TAR, which was constructed by using oligonucleotides corresponding to HIV-1 [strain: LAV_{BRU}: (12)] residues +1 to +57 cloned as a *Hin*dIII to *Pst* I fragment in pGEM1 (15); (*ii*) T7 RNA polymerase was used to transcribe an oligonucleotide template corresponding to HIV residues +1 to +57 plus a 5′-extension complementary to the T7 promoter annealed with a small primer carrying the T7 promoter and the single-stranded extension (26). The TAR RNA prepared by transcription of the pGEM plasmids includes an additional 8 nucleotides derived from plasmid linker sequences at its 5′ end, whereas TAR RNA prepared from oligonucleotide templates does not. TAR RNA prepared by either method behaves identically in protein binding assays. Antisense RNA complementary to the sense RNA was prepared by T7 RNA polymerase transcription of pGEM1.TAR2, a plasmid from which the polylinker sequence in pGEM1.TAR, bounded by *Eco*RI and *Pst* I sites, was removed. A 69-nucleotide 5′ noncoding RNA was produced by SP6 RNA polymerase transcription of the *Xenopus* nucleoplasmin cDNA (22) after cleavage of the pSP65 cDNA clone with *Hin*dIII. Similarly, a 65-nucleotide fragment of a *Xenopus* neurofilament protein cDNA clone (23) was prepared after cleavage of a pSP65 clone with *Sac* I.

Binding reaction mixtures (15 μl) contained 20 pg of probe (DNA or RNA), 0–4 μg of tat, 25 mM Tris·HCl (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$, 5 mM spermidine, 0.5 mM dithiothreitol, 1 μg of denatured salmon sperm DNA, 0.4 μg of yeast carrier tRNA, and 40 units of RNasin (Promega). After incubation at 30°C for 20 min the reaction products were applied to 6% polyacrylamide gels (24). Alternatively, products of reactions in 500 μl of 10 mM Tris·HCl (pH 8.0) and 50 mM NaCl were spotted on 0.45-μm-pore Millipore filters under reduced pressure and dried, and radioactivity was measured as described (25). Products were also immunoprecipitated from reaction mixtures by using the NT3/2D1.1 antibody and staphylococcol protein A-Sepharose, and the labeled RNA was released from the precipitate with 0.1% SDS and its radioactivity was measured by liquid scintillation counting.

Nuclear extracts from HeLa cells or from cells infected with retroviral vectors carrying *tat* genes were made as described (26, 27).

Identification of tat in Mammalian Cells. Cells (10^7) infected with retroviral vectors carrying *tat* were starved for 1 hr in cysteine-free medium and labeled with 500 μCi of [^{35}S]cysteine (Amersham, >1000 Ci/mmol; 1 Ci = 37 GBq) for 4 hr. Labeled tat was immunoprecipitated with the ND3.2D.1.1 antibody from cell extracts prepared in RIPA buffer (1% Nonidet P-40/1% sodium deoxycholate/0.1% SDS/150 mM NaCl/10 mM Tris·HCl, pH 8.0/20 μg of phenylmethylsulfonyl fluoride per ml), bound to protein-A Sepharose, and analyzed by SDS/PAGE.

RESULTS

Purification of tat from *E. coli*. The tat sequence includes a cysteine-rich region of 7 conserved cysteine residues within 16 residues, which resembles the metal-binding domain of metallothionein. It is not known whether these cysteines are involved in forming disulfide bridges, binding metal as proposed by Frankel *et al.* (20), or a combination of both. Tat also has a basic region carrying 2 lysine and 6 arginine residues within 9 residues. These unusual sequence features make purification and refolding of tat expressed in *E. coli* difficult. Because preparations of tat have a tendency to oligomerize due to intermolecular disulfide bond formation (Fig. 1*C*), care must be taken to maintain the protein under reducing conditions during purification. However, such conditions would almost certainly result in removal of associated metal ions, leading to protein denaturation.

In our experiments, tat was produced either by transcription of a synthetic gene from the T7 promoter (pMG727) or from a β-galactosidase–tat fusion protein (pMG627). Extracts from *E. coli* strains carrying pMG727 showed a single band on SDS/PAGE that was reactive to tat-specific antisera, but purification from this strain was difficult. Precipitation of tat by polyethyleneimine and resolubilization in high salt as

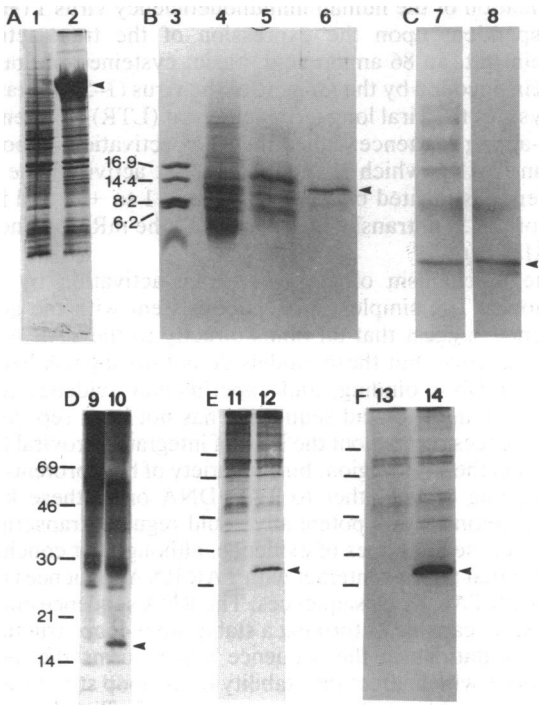

FIG. 1. Expression and purification of tat from *E. coli* and mammalian cells. (*A*) SDS/11% polyacrylamide gel electrophoresis of insoluble fractions from *E. coli* strains carrying pMG627. Lanes: 1, noninduced; 2, induced with IPTG at 50 μg/ml for 6 hr. (*B*) SDS/16–22% polyacrylamide gel electrophoresis of tat protein at various stages of purification. Lanes: 3, M_r markers (indicated × 10^{-3}); 4, CNBr digest of β-galactosidase–tat fusion; 5, tat fraction after chromatography on Accell CM; 6, tat after purification by C_4 reversed-phase chromatography. (*C*) Immunoblots of tat from pMG726. Lanes: 7, nonreduced; 8, reduced with 10 mM dithiothreitol. (*D–F*) Autoradiographs of SDS/16–22% polyacrylamide gel electrophoresis of [^{35}S]cysteine-labeled tat, immunoprecipitated from RIPA extracts of mammalian cells infected with JKC63.4.1 retrovirus by monoclonal antibody NT3/2D1.1. Numbers and dashes at left of panels indicate mobilities of M_r markers (M_r × 10^{-3}). Lanes: 9, control COS*ts* cells (28); 10, infected COS*ts* cells; 11, control HuT-78 cells (ATCC TIB161); 12, infected HuT-78 cells; 13, control CCRF-CEM cells (ATCC CCL119); 14, infected CCRF-CEM cells. Arrowheads identify monomeric tat protein (*B–F*) or β-galactosidase–tat fusion protein (*A*).

described by Frankel and co-workers (20, 29) gave less than 10% yields of tat as judged by immunoassays. Preparative isoelectric focusing experiments showed that the tat fraction had heterogenous isoelectric points from pH 5.5 to 11 even after treatment with RNase and DNase. However, we were able to purify a limited amount tat from this strain by chromatography on SP-Sephadex (pH 2.5).

tat contains no internal methionine residues, and tat expressed in mammalian cells appears to lack an N-terminal methionine since the protein is not labeled *in vivo* by [^{35}S]methionine (data not shown). We therefore used cleavage with CNBr of the β-galactosidase–tat fusion protein as a route for preparation of milligram amounts of tat free from nucleic acids. The released tat was purified to near homogeneity by cation-exchange chromatography on Accell CM, followed by C_4 reversed-phase chromatography (Fig. 1) yielding approximately 1 mg of purified tat per liter of induced culture. The identity of the purified protein was established by immunoblotting using antibodies raised to both the N- and C-terminal peptides of tat, and the N-terminal sequence of the product matched the expected tat sequence (i.e., Glu-Pro-Val-Asp-Pro. . .).

Purified tat, prepared by either method, was soluble in low-ionic-strength buffers and, as shown below, a fraction of the protein appeared to renature and bind specifically to TAR RNA sequences.

Trans-Activation by the Synthetic *tat* Gene and tat Expressed in *E. coli*. To confirm that the synthetic *tat* gene encoded a functional tat protein, *tat* gene b was cloned in a retroviral expression vector, pJKC63.4.1 (16). Virus was recovered from the amphotrophic packaging line PA317 and used to infect human T cells (HuT-78, ATCC TIB161; CCRF-CEM, ATCC CCL119) and monkey fibroblasts (COS*ts*) (29). In each cell type, a product of the expected molecular weight was immunoprecipitated by the monoclonal antibody NT3/2D1.1 (Fig. 1 *D–F*). Trans-activation assays were performed in COS*ts* cells (29) carrying an integrated pJKC63.4.1 retrovirus. These tat-expressing cells were transfected with pLC2R, a plasmid carrying a chloramphenical acetyltransferase (CAT) reported gene under the control of the HIV LTR (from the *Xho* I site at −642 to the *Nar* I site at +185; gift of M. Boidot Forget, Bouchet, France). CAT activity in these cells increased 360-fold over basal levels after transfection (data not shown).

Purified tat expressed in *E. coli* from the fusion protein or directly was also demonstrated to be capable of trans-activation after chloroquine-stimulated uptake of protein (28, 30) by COS*ts* cells carrying a integrated derivative of pLC2R (carrying a neomycin phosphotransferase gene under the control of simian virus 40 early promoter). In several experiments using different tat preparations, CAT activity in COS*ts* cells exposed to tat increased 3- to 10-fold over basal levels.

tat Forms a Discrete Complex with TAR RNA. Incubation of a 79-nucleotide TAR RNA molecule with purified tat produced complexes which could be seen in a gel retardation assay (Fig. 2). Low tat concentrations produce a single complex, but a second, slightly larger, complex is also formed when more than 1 μg of tat is added to the reaction mixture (Fig. 2*A*). The antisense RNA, complementary to the TAR sequence, was unable to form a complex with tat (Fig. 2*C*). Complexes also could not be formed with TAR RNA in which the double-helical region of TAR was changed by incorporation of inosine into the sequence in place of guanosine (Fig. 2*D*). RNA transcripts of the 5′ noncoding region of *Xenopus* nucleoplasmin cDNA (22) and the internal coding region of a *Xenopus* intermediate filament cDNA (23) also failed to bind specifically to tat (Fig. 2 *E* and *F*).

At high concentrations of tat, aggregates were formed which failed to enter the acrylamide gels used in the gel-

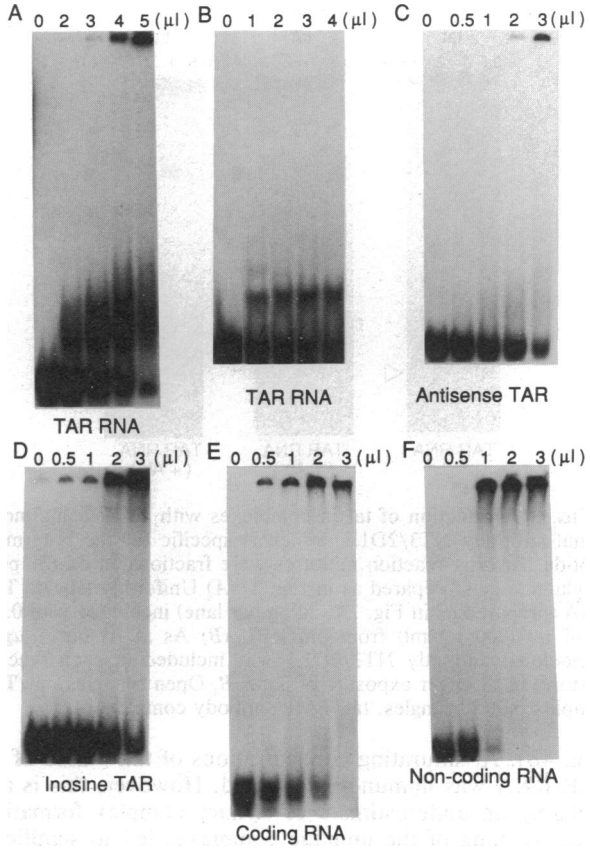

FIG. 2. Autoradiographs of 6% polyacrylamide gels showing discrete complex formation between TAR RNA and tat. (*A*) Uniformly labeled TAR RNA (0.02 ng per lane) prepared by transcription of pGEM1.TAR by SP6 RNA polymerase (15) incubated with 0–5 μl of purified tat (0.4 mg/ml) prepared from pMG627 (β-galactosidase–tat fusion). (*B*) As in *A* but using purified tat prepared from pMG727 (unfused tat). (*C*) Antisense TAR template prepared by transcription of pGEM1.TAR2 by T7 RNA polymerase (15). (*D*) Inosine-substituted TAR template. (*E*) Fragment from the internal coding region of a *Xenopus* intermediate filament cDNA. (*F*) The 5′ noncoding region of *Xenopus* nucleoplasmin cDNA. In *C–F*, tat was prepared from pMG627.

retardation assay and appeared to bind RNA nonspecifically. Analysis of reaction mixtures by electrophoresis on agarose gels indicated that this high molecular weight material was heterogeneous in size. This nonspecific RNA binding by tat is not apparent in filter binding and immunoprecipitation assays (see below).

Complexes between tat and TAR RNA were observed with a number of different highly purified preparations of tat produced by the fusion route (Fig. 2*A*) or by the direct route (Fig. 2*B*). In both cases a large excess of protein was required (see *Discussion*). To demonstrate that the binding activity was due to tat itself rather than a contaminating protein from *E. coli*, the monoclonal antibody NT3/2D1.1 was used to demonstrate the presence of tat in the RNA/protein complexes fractionated on polyacrylamide gels. In the presence of added antibody, a high molecular weight complex composed of tat, TAR RNA, and antibody is formed in addition to the tat/TAR RNA complex (Fig. 3). Control experiments showed that the NT3/2D1.1 antibody did not bind TAR RNA alone, and that tat/TAR complexes could also be detected by immunoblotting (data not shown). In the gel retardation assay, the ternary complex appears to be relatively unstable, and only a low level of ternary complex is detected even in the presence of excess antibody. Ternary complexes are more efficiently detected by immunoprecipitation assays

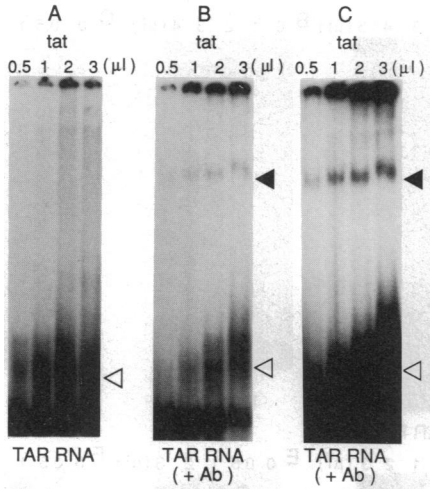

FIG. 3. Detection of tat in complexes with TAR, using monoclonal antibody NT3/2D1.1, which is specific for the N-terminal peptide. Binding reaction mixtures were fractionated on 6% polyacrylamide gels prepared as in Fig. 2. (*A*) Uniformly labeled TAR RNA (prepared as in Fig. 2*A*; 20 pg per lane) incubated with 0.5–3 μl of tat (200 μg/ml) from pMG627. (*B*) As in *A*, but 1 μg of monoclonal antibody NT3/2D1.1 was included in each reaction mixture. (*C*) Longer exposure of panel *B*. Open triangles, tat/TAR complex; solid triangles, tat/TAR/antibody complex.

(Fig. 4*B*). At saturating concentrations of tat, ≈25% of the TAR RNA was immunoprecipitated. However, this is also probably an underestimate of ternary complex formation, since washing of the immune complexes led to significant disruption of the tat/TAR RNA complex.

Complexes between TAR RNA and tat were also detected by filter binding (Fig. 4 *A* and *C*). At saturating concentrations of tat, between 40% and 50% of TAR RNA in the reaction mixtures was retained on filters. No appreciable binding of antisense transcripts of TAR RNA by tat was detected by this assay.

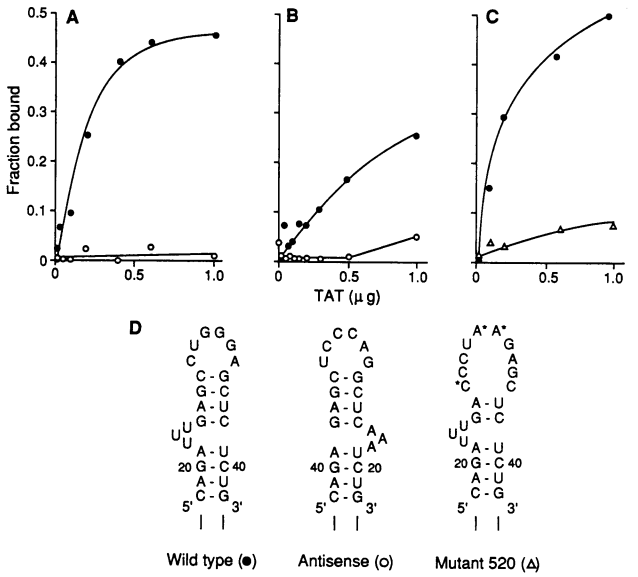

FIG. 4. Filter binding and immunoprecipitation assays to detect complex formation between tat and TAR. (*A*) Filter-binding assay: ●, 20 pg of uniformly labeled wild-type TAR RNA; ○, 20 pg of antisense TAR RNA. RNA fragments were prepared by transcription of fragments cloned in pGEM plasmids and incubated with 0–1.0 μg of tat prepared from pMG627 (β-galactosidase–tat fusion). (*B*) Immunoprecipitation assay using the same templates as in *A*. (*C*) Filter-binding assay: ●, 20 pg of uniformly labeled wild-type TAR RNA; △, 20 pg of mutant 520 TAR RNA. RNA fragments were prepared by transcription of oligonucleotide templates (21). (*D*) Partial sequences of wild-type and mutant TAR RNA stem–loop structures. Numbers refer to the TAR RNA sequence in HIV-1 (LAV_{BRU}). *, Mutated bases in TAR 520.

Nonspecific competitor RNAs do not block formation of the tat/TAR RNA complex. In all the experiments described above a 20,000-fold excess of tRNA was added. Addition of a 10,000-fold molar excess of 16S RNA, which carries numerous hairpin loop structures related to the TAR sequence, or a 100,000-fold molar excess of poly(I·C) did not reduce tat binding to labeled TAR RNA. By contrast, unlabeled TAR RNA is an effective competitor for TAR RNA binding to tat. In gel retardation experiments complex formation was inhibited by addition of a 50- to 250-fold molar excess of unlabeled TAR RNA.

Sequence Requirements for Binding of TAR RNA by tat. Mutant TAR sequences in which U residues were substituted for G residues in the TAR loop were reported by Feng and Holland (8) to abolish trans-activation *in vivo*. We have tested three mutations of this type (1283, U33; 1284, U32; and 1288, U31). These mutations did not significantly reduce tat binding to TAR in immunoprecipitation or gel retardation assays (data not shown). In contrast, mutations in the stem at the base of the loop reduce both trans-activation *in vivo* (8) and tat binding *in vitro*. In mutant 1289, where G35 is mutated to C, tat binding is reduced 25–50%. Mutation of G27 to C is expected to be even more disruptive of the stem–loop structure. Greatly reduced tat binding to TAR was observed in mutant 494 (C27), the double mutant 497 (C27, A32), or the triple mutant 520 (Fig. 4*D*; C27, A31, A32).

TAR DNA Is Bound by Cellular Factors But Not by tat. DNA footprinting experiments (6, 7, 9) have demonstrated that cellular proteins can bind to TAR DNA sequences. Complex formation between an end-labeled TAR fragment and DNA-binding proteins present in a HeLa cell nuclear extract (26) is shown in Fig. 5*A*. Two complexes were seen by nondenaturing PAGE, but as more extract was added to the DNA fragment, more of the larger complex was produced and the amount of the smaller complex decreased. This suggests that the protein factor(s) involved in complex formation bind with different affinities and that formation of the larger complex is dependent upon the prior formation of the smaller one. Extracts made from cells constitutively expressing *tat* (following infection with the JKC63.4.1 virus) gave rise to complexes with TAR DNA indistinguishable from those derived from extracts that lack tat (data not shown).

No binding of purified tat protein to TAR DNA has been observed under these conditions (Fig. 5*B*) or under RNA-binding conditions. Furthermore, formation of complexes between TAR DNA and cellular proteins is not affected by addition of tat (data not shown).

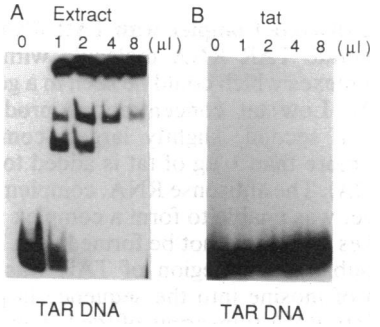

FIG. 5. TAR DNA is bound by cellular factors but not by tat. (*A*) Complex formation between end-labeled TAR DNA fragment (1 ng per lane) and 0–8 μl of a HeLa cell nuclear extract (4 mg/ml). (*B*) Failure of purified tat (200 μg/ml) to form complexes with TAR DNA fragment.

DISCUSSION

Using gel-retardation, filter-binding, and immunoprecipitation assays, we have demonstrated that purified tat binds specifically to TAR RNA and not to TAR DNA. The data suggest that tat recognizes specific features near the junction between the stem and the loop of the TAR RNA stem–loop structure. Antisense transcripts, which are largely homologous to wild-type TAR in the stem region because the TAR sequence is palindromic, but differ from wild-type TAR in their loop sequences and in the placement of the small U-rich bubble (U23 and U25) near the base of the loop, fail to bind tat. Similarly, TAR sequences carrying mutations expected to disrupt the stem-loop structure (1289, 494, 497, 520) show reduced tat binding. Tat also does not bind to TAR RNA in which inosine replaces guanosine or to two other unrelated RNA sequences of size and base composition similar to TAR. By contrast, TAR sequences carrying mutations in the loop itself (1283, 1284, 1288) bind tat with the same affinity as the wild type.

The data suggest that multiple regions of the TAR sequence may be required for trans-activation. Mutations in the stem which fail to bind tat *in vitro* do not trans-activate. However, mutants with altered residues in the TAR loop bind tat efficiently, suggesting that these sequences may be required for other aspects of TAR functions such as binding to host proteins.

Although we have demonstrated specific sequence requirements for tat binding to TAR RNA, the data should not be considered to indicate that the majority of tat in our preparations has a native configuration and is active. A dissociation constant of $\approx 3 \times 10^{-8}$ M has been calculated from the data in Fig. 4*A*, on the assumption that all the tat molecules are competent to bind TAR. However, in these experiments, a 1000- to 10,000-fold excess of tat was required to form complexes efficiently, and it is probable that relatively little of the protein is capable of binding RNA. Thus, the dissociation constant of the complex could be as high as 10^{-12} M. Similarly, although tat prepared in this manner is also capable of trans-activation *in vivo*, after uptake of the protein by cells which carry viral LTR sequences, the specific activity of tat cannot be determined from this assay. Studies of DNA transcription factors have shown that even protein preparations of low specific activity retain binding specificity. For example, experiments with transcription factor TFIIIA, prior to the recognition of the role of metal ions, showed that TFIIIA could bind specifically to DNA and produce a clear "footprint," but a large excess of protein was required (31, 32). It should also be noted that RNA/protein complexes may be intrinsically less stable than DNA/protein complexes. A 400- to 800-fold excess of R17 coat protein was required to obtain 50% retention of R17 in a filter-binding assay (25).

It is also difficult to demonstrate tat binding to TAR RNA, using extracts from mammalian cells as a source of "native" tat, because extracts from cell lines that do not express tat contain other RNA-binding proteins which form complexes with TAR RNA. Nuclear extracts from COS*ts*, U937, or CEM-CCRF cells that constitutively express *tat* from the integrated JKC63.4.1 retroviral vector produced patterns very similar to the relevant control extracts. However, complexes containing tat prepared from extracts of COS*ts* cells could be observed on immunoblots (data not shown).

Frankel *et al.* (20) have demonstrated that tat may dimerize *in vitro* through chelation of metal ions by cysteine residues and have suggested that the biologically active form of tat is a dimer. We have been able to demonstrate tat binding to TAR RNA under conditions where extensive metal-induced dimerization would not be expected to occur—e.g., in the presence of high concentrations of dithiothreitol and EDTA (data not shown)—but until questions about the specific activity of the tat preparations are resolved, and a reliable method is devised for refolding tat efficiently, one cannot say whether or not the active form of tat requires metal ions.

In spite of these limitations, our experiments provide strong evidence for the specific binding of tat to TAR RNA. Since the same tat preparations do not bind to TAR DNA, the experiments also suggest that trans-activation of the viral LTR involves this specific interaction with RNA.

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1. Arya, S. K., Guo, C., Josephs, S. F. & Wong-Staal, F. (1985) *Science* **229**, 69–73.
2. Dayton, A. I., Sodroski, J. G., Rosen, C. A., Goh, W. C. & Haseltine, W. A. (1986) *Cell* **44**, 941–947.
3. Kao, S. Y., Colman, A. F., Luciw, P. A. & Peterlin, B. M. (1987) *Nature (London)* **330**, 489–493.
4. Hauber, J., Perkins, A., Heimer, E. P. & Cullen, B. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6364–6368.
5. Muesing, M., Smith, D. H. & Capon, D. J. (1987) *Cell* **48**, 691–701.
6. Garcia, J. A., Wu, F. K., Mitsuyasu, R. & Gaynor, R. B. (1987) *EMBO J.* **6**, 3761–3770.
7. Garcia, J. A., Harrich, D., Soultanakis, E., Wu, F., Mitsuyasu, R. & Gaynor, R. B. (1989) *EMBO J.* **8**, 765–778.
8. Feng, S. & Holland, E. C. (1988) *Nature (London)* **334**, 165–167.
9. Jones, K. A., Luciw, P. A. & Duchange, N. (1988) *Genes Dev.* **2**, 1101–1114.
10. Parkin, N. T., Cohen, E. T., Darvae, A., Rosen, C. A., Haseltine, W. A. & Sonenberg, N. (1988) *EMBO J.* **7**, 2831–2837.
11. Edery, I., Petryshyn, R. & Sonenberg, N. (1989) *Cell* **56**, 303–312.
12. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. & Alizon, M. (1985) *Cell* **40**, 9–17.
13. Heaphy, S., Singh, M. & Gait, M. J. (1987) *Protein Eng.* **1**, 425–431.
14. Rüther, U. & Müller-Hill, B. (1983) *EMBO J.* **2**, 1791–1794.
15. Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12**, 7035–7056.
16. Karn, J., Watson, J. V., Lowe, A. D., Green, S. M. & Vedeckis, W. (1989) *Oncogene* **4**, 773–787.
17. Atherton, E., Cameron, L. R. & Sheppard, R. C. (1988) *Tetrahedron* **44**, 843–857.
18. Nagai, K. & Thøgersen, H. C. (1987) *Methods Enzymol.* **153**, 461–481.
19. Studier, F. W. & Moffatt, B. A. (1987) *J. Mol. Biol.* **189**, 113–130.
20. Frankel, A. D., Bredt, S. & Pabo, C. O. (1988) *Science* **240**, 70–73.
21. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. (1987) *Nucleic Acids Res.* **15**, 8783–8798.
22. Dingwall, C., Dilworth, S. M., Black, S. J., Kearsey, S. E., Cox, L. S. & Laskey, R. A. (1987) *EMBO J.* **6**, 69–74.
23. Sharpe, C. R. (1988) *Development* **103**, 269–277.
24. Treisman, R. (1986) *Cell* **46**, 567–574.
25. Carey, J., Cameron, V., de Haseth, P. L. & Uhlenbeck, O. C. (1983) *Biochemistry* **22**, 2601–2610.
26. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
27. Shapiro, D. J., Sharp, P. A., Wahli, W. & Keller, M. J. (1988) *DNA* **7**, 47–55.
28. Frankel, A. D. & Pabo, C. O. (1988) *Cell* **55**, 1189–1193.
29. Rio, D. C., Clark, S. G. & Tjian, R. (1985) *Science* **227**, 23–28.
30. Green, M. & Loewenstein, P. M. (1988) *Cell* **55**, 1179–1188.
31. Sakonju, S. & Brown, D. D. (1982) *Cell* **31**, 395–405.
32. Miller, D., McLachlan, A. D. & Klug, A. (1985) *EMBO J.* **4**, 1609–1614.