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Interaction of Human Immunodeficiency Virus Type 1 Tat with a Unique Site of TFIID Inhibits Negative Cofactor Dr1 and Stabilizes the TFIID-TFIIA Complex

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We have previously reported the direct physical interaction between the human immunodeficiency virus (HIV) type 1 Tat protein and the basal transcription factor TBP/TFIID. Affinity chromatography demonstrated that wild-type Tat, but not a transactivation mutant of Tat, was capable of depleting TBP/TFIID from cell extracts. These experiments represented the first demonstration of a basal transcription factor that binds, in an activation-dependent manner, to Tat. We now report that the Tat-TBP interaction can be detected in HIV type 1-infected cells. The domain of TBP interacting with Tat has been mapped from amino acids 163 to 196 by using deletion and site-specific mutants of TBP. This domain of TBP, which includes the H1 and S2 domains, is distinct from the H2 binding site for other activator proteins, such as E1A. The interaction of Tat with TFIID regulates the binding of accessory proteins to TFIID. Tat stabilizes the interaction of TFIID with TFIIA in a gel shift assay. In addition, Tat competes for Dr1 interaction with TBP. Our results suggest that the basal transcription factor TBP/TFIID represents an important regulatory molecule in HIV transcription.

The Tat protein of human immunodeficiency virus type 1 (HIV-1) plays a key role in virus replication and transformation. Tat-defective proviral clones do not replicate efficiently (5, 6). Transgenic mice harboring the HIV Tat gene develop Kaposi's sarcoma-like lesions, and Tat has recently been shown to synergize with basic fibroblast growth factor in inducing angiogenic Kaposi's sarcoma-like lesions in mice (12, 53). Transcriptionally, Tat has been shown to be a potent transactivator of the HIV promoter. Tat transactivation of the viral long terminal repeat (LTR) is dependent upon the presence of upstream transcription factors and the TAR RNA regulatory element. Tat stimulates both transcription initiation and elongation in vivo (13, 32). In vitro studies of Tat transactivation also support a role for Tat in initiation and elongation (1, 28, 32, 34, 39). Bohan et al. (1) have demonstrated that Tat facilitates the formation of the HIV preinitiation complex. Subsequently, Kashanchi et al. (28) reported that Tat interacts directly with the TATA-binding protein (TBP) subunit of TFIID. In agreement with these findings, Veschambre et al. (52) have recently reported a functional interaction between Tat and human TFIID. In other in vitro studies, Marciniak et al. have shown that Tat stimulates the formation of more-processive elongation complexes (38, 39). Further, Kato et al. (32) have demonstrated that TFIIF, an elongation transcription factor, decreased the requirement for Tat transactivation by increasing polymerase processivity.

The ability of Tat to participate in transcriptional initiation and elongation is perhaps not surprising, given the ability of other transcriptional activators, such as VP16, to function during multiple steps of preinitiation complex assembly and elongation. The physical interaction of VP16 with basal transcription machinery factors such as TBP, TFIIB, TAFII40, and TFIIH has been demonstrated by several independent laboratories (15, 35, 50, 55). VP16 facilitates the recruitment of TFIIB and then, in a second step, facilitates transcriptional initiation through interaction with TBP-associated factors (TAFs) (8). Interestingly, Yankulov et al. (56) have shown that activators such as VP16 and E1A also stimulate RNA polymerase processivity, and they have suggested that this function is as important as the stimulation of transcriptional initiation. Those authors further suggest that the competence of RNA polymerase II to elongate is an integral part of the initiation step that is controlled by activators interacting with the general transcription factors. In this light, it is of interest that the VP16 transcription activation domain is functional when targeted to a promoter-proximal RNA sequence (21).

The functions of the human TBP are complex. This 38-kDa protein was originally identified as a polymerase II transcription factor which interacted specifically with the −25 TATA promoter region (21, 26, 43). Indeed, the purified 38-kDa protein can reconstitute basal TATA-dependent transcription in vitro. More recently, however, it has been demonstrated that the polymerase II TBP complex (TFIID) has a molecular mass of approximately 750 kDa and is composed of TBP and multiple polypeptides referred to as TAFs. Interestingly, while TBP can support basal transcription, TFIID is required for activator-dependent transcription (20, 21, 24, 43, 44, 48). Quite remarkably, in addition to TBP's role in polymerase II transcription, the 38 kDa protein has also been shown to be involved in polymerase I and polymerase III transcription (18). In polymerase I transcription, TBP is found in the SL1 complex, consisting of TBP in tight association with three TAFs of

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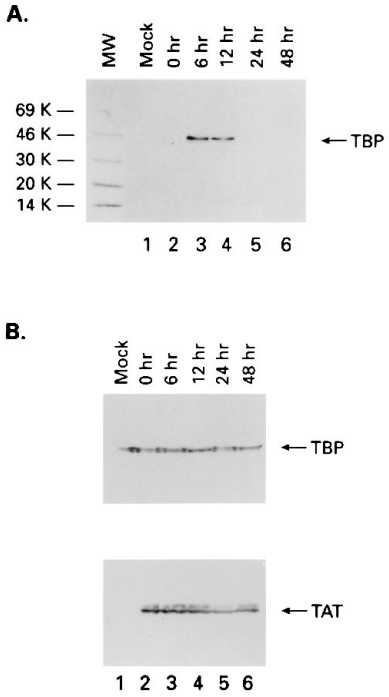


FIG. 1. Immunoprecipitation of Tat-TFIID complex from HIV-1-infected cells by using anti-Tat polyclonal antiserum. Infected cells were cocultivated with uninfected H9 cells at a 1:4 ratio. They were then processed at various times (0, 6, 12, 24, and 48 h) postinfection for immunoprecipitation with anti-Tat immunoglobulin G purified polyclonal serum or anti-orf-1 (30). Samples (100 μg of nuclear preparation) were incubated overnight at 4°C, and immune complexes were collected the next day and subjected to SDS-PAGE (4 to 20% polyacrylamide) and Western blotting with anti-TBP monoclonal serum (Promega). Panel A and the bottom of panel B represent immunoprecipitates fractionated on SDS-PAGE and then subjected to Western blotting with anti-TBP (Promega) (A) or anti-Tat (American Biotechnology) (bottom of panel B) monoclonal antibody. The TBP portion of panel B is a straight Western blot of nuclear extracts (used for immunoprecipitation) with anti-TBP monoclonal antibody. Mock, uninfected H9 cells; MW, molecular weight markers (in thousands [K]).

48, 63, and 110 kDa (9). In polymerase III transcription, TBP is part of the transcription factor TFIIIB. In *Saccharomyces cerevisiae*, TFIIIB consists of three proteins of 69 kDa (yeast BRF1), 38 kDa (TBP), and 90 kDa (31). At this point, it appears that most TBP in a cell is found either in TFIID, SL1, or TFIIIB complexes (18). TBP is also the target of positive transcription factors (TFIIA), viral activators (E1A and T antigen), and negative cofactors (Dr1) (2, 14, 16, 33, 54). Obviously, this basal transcription factor, through complex interactions with a number of cellular proteins, plays a pivotal role in eukaryotic gene regulation.

We have previously reported that Tat interacts with the basal transcription factor TFIID (28). Using peptide competition and binding analysis, we demonstrated that Tat amino acids 36 to 50 were important for this interaction. Importantly, a mutation at lysine 41 which abolishes Tat transactivation also abolished interaction with TFIID. In this report, we extend the initial observations on the Tat-TFIID interaction by showing that (i) the Tat-TFIID complex can be seen in vivo by immunoprecipitation from HIV-1-infected cells; (ii) the domain of TBP required for interaction with Tat includes the H1-S2 domain, which is distinct from the E1A activator binding site; (iii) Tat stabilizes the TFIID-TFIIA complex on the HIV-1 TATA box; and (iv) Tat can compete for Dr1 binding to TBP.

MATERIALS AND METHODS

Virus infection and preparation of nuclear extract. H9 cells were infected with HIV_{IIIB} by the cell-to-cell mode of transmission with cells persistently infected with HIV_{IIIB} (H9/IIIB cells) as previously described (47) with some minor modifications. Both infected and uninfected cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum at an initial density of 5×10^5 cells per ml 2 days prior to infection. For infection, 5×10^6 H9/IIIB cells were mixed with 2×10^7 H9 cells and cocultivated in fresh medium at a density of 10^6/ml in T75 flasks. At 0, 6, 12, 24, and 48 h postinfection, a total of 5×10^7 cells per time point were harvested by centrifugation. For the mock infection, the same number of H9 cells were harvested. Pelleted cells were washed once with cold phosphate-buffered saline and once with buffer A (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9 at 4°C], 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol [DTT]) and resuspended in 0.5 ml of buffer A containing 0.1% Nonidet P-40 (NP-40). After incubation on ice for 10 min, the cell lysate was vortexed briefly and nuclei were pelleted by centrifugation in a Sorvall tabletop centrifuge (MC 12V; Dupont) at 4°C.

Extracts from nuclei were prepared as described by Dignam et al. (10), and one-third of the extract (250 ng) was used for immunoprecipitation. The immunoprecipitation with anti-Tat (polyclonal, 100 μg) was done at 4°C overnight in the binding buffer (10 mM HEPES [pH 7.9], 50 mM KCl, 2.5 mM $MgCl_2$, 50 μM $ZnCl_2$, 50 μM EDTA, 1 mM DTT, and 8.5% glycerol). Immune complexes were precipitated with protein A-Sepharose, washed with binding buffer–0.1% NP-40, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4 to 20% polyacrylamide) and to Western blotting (immunoblotting) with a monoclonal anti-Tat or anti-TBP antibody.

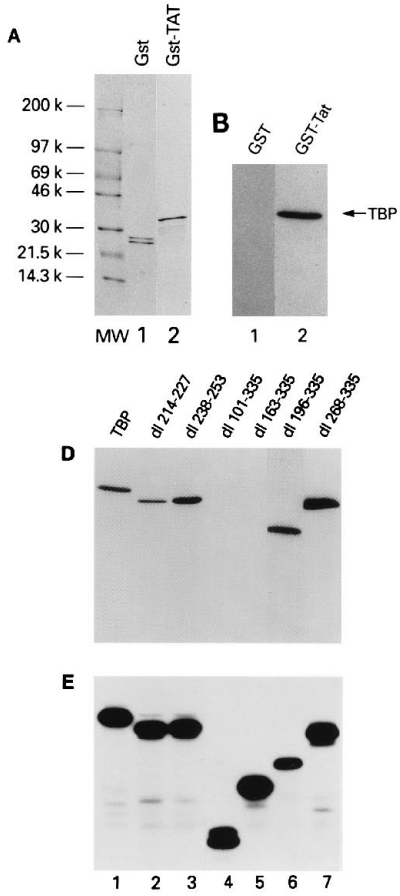
Preparation of glutathione *S*-transferase (GST) proteins. Tat of HIV-1 was subcloned from the pSynTat vector (27) by using PCR. A 5′ oligonucleotide containing a *Bam*HI site and a 3′ oligonucleotide containing an *Xho*I site were used for PCR with Tat72 and Tat86. The fragments were then cut with restriction enzymes *Bam*HI and *Xho*I, purified, and subcloned into the polyclonal site of the pGEX-20T vector. The polyclonal site contained a thrombin-cut site followed by *Bam*HI, *Eco*RI, *Xho*I, *Cla*I, *Spe*I, and *Xba*I restriction sites. Fragments were ligated and transformed into *Escherichia coli* XA-90. GST-Tat86 dl 2-36, GST-Tat72, GST-Tat72 cys22, GST-Tat48, and GST-Tat48 cys22 were generous gifts of A. Rice, Baylor College of Medicine. Recombinant proteins were grown in 1 liter of NZCYM (28) to an A_{600} of 0.6 and induced with fresh 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Cells were harvested 3 h later and washed with phosphate-buffered saline without Mg^{2+} and Ca^{2+}. The pellet was resuspended in 10 ml of extraction buffer containing 50 mM HEPES (pH 7.4), 250 mM NaCl, 1 mM EDTA, 5 mM NaF, 1 mM DTT, 5 μg each of aprotinin, leupeptin, and pepstatin A per ml, 100 μg of phenylmethylsulfonyl fluoride per ml, and 1% NP-40 and sonicated to break the cells.

Cell lysates were centrifuged in a Sorvall RT6000D centrifuge (5,000 rpm) for 10 min at 4°C. The supernatant was mixed with one ml of glutathione-agarose beads (50% slurry) and kept at 4°C for 2 h on a rotator. The beads were centrifuged and washed five times with the extraction buffer, and GST proteins were eluted with 25 mM reduced glutathione (in extraction buffer). Peak fractions containing the proteins were dialyzed against 20 mM HEPES (pH 7.4)–150 mM NaCl–1 mM EDTA–10% glycerol–0.1% NP-40. Aliquots of 1 mg/ml were stored at −70°C.

GST binding assays. The amounts of all GST proteins were normalized according to protein concentration, and the amount bound to beads was confirmed by SDS-PAGE followed by Coomassie staining. GST proteins (approximately 5 μg) and 2.5 to 10 μl of the TNT lysate (Promega) containing ^{35}S-labeled Tat or TBP were incubated in either TNE100 (100 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.1% NP-40) or buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 0.1 mM DTT, and 0.2% NP-40) in a final volume of 200 μl and with a total of 50 μl of beads. Binding reaction mixtures were incubated for 2 h at 4°C with rotation. The complexes were centrifuged for 30 s and washed twice with 1 ml of buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, and 0.2% NP-40. The bound labeled proteins were denatured, subjected to SDS-PAGE (4 to 20% polyacrylamide), dried, and autoradiographed.

For Tat and Dr1 bindings, GST-TBP (wild type) or the version containing amino acids 1 to 163 (GST-TBP 1-163) was used at 10 μg. These proteins were mixed with 100 μl of whole-cell extract (WCE) (described in "In vitro transcription" below), incubated overnight on a rotator at 4°C, and washed three times the next day with binding buffer (used for immunoprecipitation) containing 0.1% NP-40. Bound complexes were denatured and subjected to SDS-PAGE (4 to 20% polyacrylamide) and to Western blotting to detect the presence of Tat or Dr1.

In vitro transcription. HeLa (WCEs) were prepared according to a previously established procedure (37) and added (7.5 μl) to the in vitro transcription reaction mixture at a concentration of 12 μg/ml. pAdML and a HIV-LTR chloramphenicol acetyltransferase (CAT) plasmid were linearized with *Bam*HI and *Eco*RI, respectively, and added to the in vitro transcription mixture at a concentration of 30 ng per reaction mixture. The in vitro transcription buffer contained 10 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1.5 mM DTT, 6.25 mM $MgCl_2$, and 8.5% glycerol. Each reaction mixture also contained nucleoside triphosphates ATP, GTP, and CTP at a final concentration of 50 μM



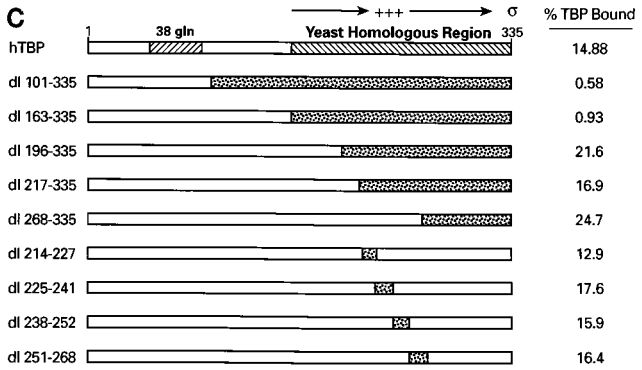


FIG. 2. Mapping of TBP domain involved in binding to Tat. (A) GST and GST-Tat86 were raised in *E. coli*, induced, purified, and subjected to SDS-PAGE (4 to 20% polyacrylamide). Lanes 1 and 2, 100 ng of GST and GST-Tat protein, respectively, stained with Coomassie blue. Lane MW, molecular weight markers (in thousands [K]). (B) Amounts of GST proteins were normalized according to protein concentration, and the amount bound to beads was confirmed by SDS-PAGE followed by Coomassie staining. GST proteins (approximately 5 μg) and 5 μl of the TNT lysate (Promega) containing ^{35}S-labeled TBP were incubated in TNE100 (100 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.1% NP-40) in a final volume of 200 μl and with a total of 50 μl of beads. Binding reaction mixtures were incubated for 2 h at 4°C with rotation. The complexes were centrifuged for 30 s and washed twice with 1 ml of buffer containing 50 mM Tris [pH 7.9], 150 mM NaCl, and 0.2% NP-40. The bound labeled proteins were denatured, electrophoresed on an SDS-PAGE gel (4 to 20% polyacrylamide), dried, and autoradiographed. ^{35}S-labeled TBP was incubated with GST (lane 1) or GST-Tat (lane 2). The arrow represents where hTBP migrates. (C) Results of TBP mapping with various deletion mutants. All bindings were performed as described in Materials and Methods. The percent TBP bound was determined from analyses presented panels D and E. (D) ^{35}S-labeled wild-type TBP and deletion mutants (5 μl) were incubated with GST-Tat as described in Materials and Methods. Following incubation, complexes were centrifuged, washed, analyzed by SDS-PAGE (4 to 20% polyacrylamide), dried, and autoradiographed. (E) Five-microliter portions of ^{35}S-labeled TBPs were electrophoresed on an SDS-PAGE gel (4 to 20% polyacrylamide), dried, and autoradiographed.

each and 20 μCi (2 μl) of [^{32}P]UTP (400 Ci/mmol; Amersham). Transcription reactions were terminated by the addition of 20 mM Tris-HCl (pH 7.8)–150 mM NaCl–0.2% SDS. The quenched reactions were extracted with equal volumes of phenol-chloroform and precipitated with 2.5 volumes of ethanol and 0.1 volume of 3.0 M sodium acetate. Following centrifugation, RNA pellets were resuspended in 12 μl of a formamide denaturation mixture containing xylene cyanol and bromophenol blue, heated at 90°C for 3 min, and electrophoresed at 400 V in a 4% polyacrylamide (acrylamide/bisacrylamide ratio, 19:1) gel containing 7 M urea (prerun at 200 V for 30 min) in 1× Tris-borate-EDTA. Gels were exposed to Kodak X-Omat XR5 film at −70°C with intensifying screens for autoradiography.

Gel shift assay. The gel shift assay conditions and TFIID-TFIIA purification have been described previously (28, 29). Purified Tat (200 ng) was added to the gel shift assay mixtures as indicated in the legend to Fig. 4A. Sarkosyl was precipitated three times in 95% chilled ethanol to remove impurities. After the third precipitation, the pellet was dried to remove the alcohol and kept as a powder at room temperature. A 10% stock was made fresh and filtered before use. A final concentration of 0.03% Sarkosyl was used to dissociate the TFIID-TFIIA complex. Binding was done at room temperature according to previously published procedure (28).

RESULTS

Tat-TBP interaction occurs both in vitro and in vivo. We initially tested whether an interaction between HIV-1 Tat and TFIID could be detected in HIV-1-infected cells. When chronically infected H9 cells are cocultivated with uninfected H9 cells, a synchronized infection cycle lasting approximately 60 to 72 h is readily established (47). In this system, viral DNA synthesis is detectable by Southern blot analysis between 2 and 4 h postinfection, viral protein production is detected between 8 and 12 h by immunoblotting, and release of progeny virus into the medium, as measured by a reverse transcriptase assay, is detected between 16 and 20 h postcocultivation. Fresh, uninfected H9 cells were cocultivated with infected H9/IIIB cells (4:1 ratio), and nuclear extracts were prepared at 0, 6, 12, 24 and 48 h postinfection. Following immunoprecipitation with either anti-Tat polyclonal serum or a control serum (30), the complexes were fractionated by SDS-PAGE and TBP was detected with anti-human TBP antibody by Western blotting. TBP was detected in the anti-Tat immunoprecipitates from HIV-infected cells at 6 and 12 h postinfection (Fig. 1A, lanes 3 and 4). In contrast, TBP was not detected in anti-Tat immunoprecipitates from the 12- and 24-h infected-cell extracts (Fig. 1A, lanes 5 and 6) or from mock-infected cells (Fig. 1A, lane 1) or in immunoprecipitates from either HIV-infected or mock-infected cells with a control immune serum (data not shown). The inability to detect the Tat-TBP complex at the 24- and 48-h time points is not due to a problem with immunoprecipitation of Tat protein from the extracts. Equivalent amounts of the Tat protein were immunoprecipitated at each time point (Fig. 1B, lower panel, lanes 2 to 6). Furthermore, we demonstrated by Western blot analysis that equivalent amounts of TBP were present in the cell extracts at each time point. These results demonstrate that the Tat-TBP complex is present in infected cells.

Mapping of the domain of TBP required for interaction with Tat. To identify the domain of TBP necessary for interaction with Tat, GST or GST-Tat86 was produced in *E. coli* XA-90 and purified by binding to a glutathione bead matrix. After extensive washes, a portion of coupled beads was loaded onto a gradient SDS-PAGE gel (4 to 20% polyacrylamide) to determine the purity and concentration of bound GST or GST-Tat86 protein. As can be seen in Fig. 2A, the GST-Tat86 fusion

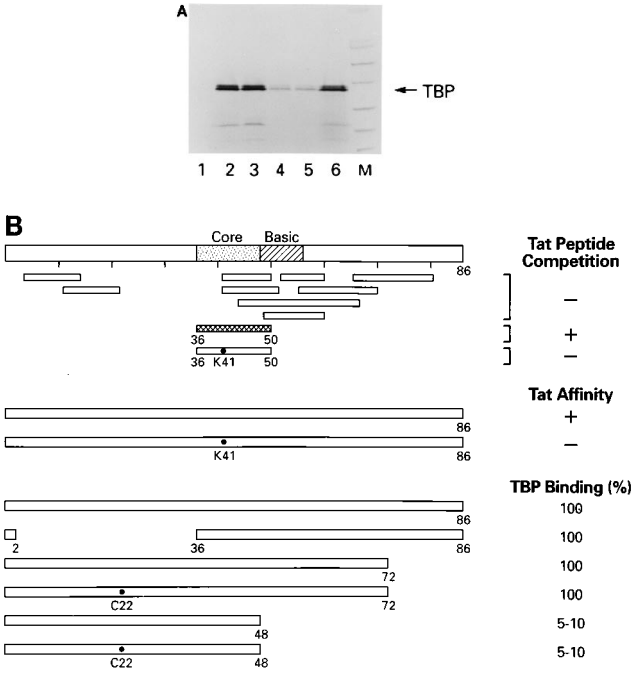


FIG. 3. Interaction of TBP with Tat mutants. (A) Amounts of GST proteins were normalized according to protein concentration, and the amount bound to beads was confirmed by SDS-PAGE followed by Coomassie staining. GST proteins (approximately 5 μg) and 5 μl of the TNT lysate (Promega) containing ^{35}S-labeled TBP were incubated in buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, 0.1 mM DTT) with 0.2% NP-40 in a final volume of 200 μl and with a total of 50 μl of beads. Binding reaction mixtures were incubated for 2 h at 4°C with rotation. The complexes were centrifuged for 30 s and washed twice with 1 ml of buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, and 0.2% NP-40. The bound labeled proteins were denatured, electrophoresed on an SDS-PAGE gel (4 to 20% polyacrylamide), dried, and autoradiographed. Lane 1, GST (4 μl); lane 2, GST-Tat86 (20 μl); lane 3, GST-Tat86 dl 2-36 (2 μl); lane 4, GST-Tat48 (10 μl); lane 5, GST-Tat48 cys22 (10 μl); lane 6, one-fifth of input TBP; lane M, protein molecular weight markers. (B) Schematic representation of Tat wild-type and mutant peptides and their abilities to bind TBP. Results of the peptide competition and Tat affinity column assays were previously published (28), and results of GST binding assays are presented as percent TBP binding to wild-type Tat.

protein is greater than 95% pure and migrates with an apparent molecular mass of 35 kDa. Importantly, when the GST-Tat86 protein was eluted from the Sepharose column, it retained transcriptional activity following electroporation with a HIV-CAT reporter plasmid into CEM cells (data not shown). Upon incubation of ^{35}S-labeled TBP with GST-Tat, we observed a specific binding in the presence of 0.2% NP-40 and 0.2 M NaCl (Fig. 2B, lane 2). The GST protein, in contrast, failed to bind the ^{35}S-TBP under these conditions (Fig. 2B, lane 1).

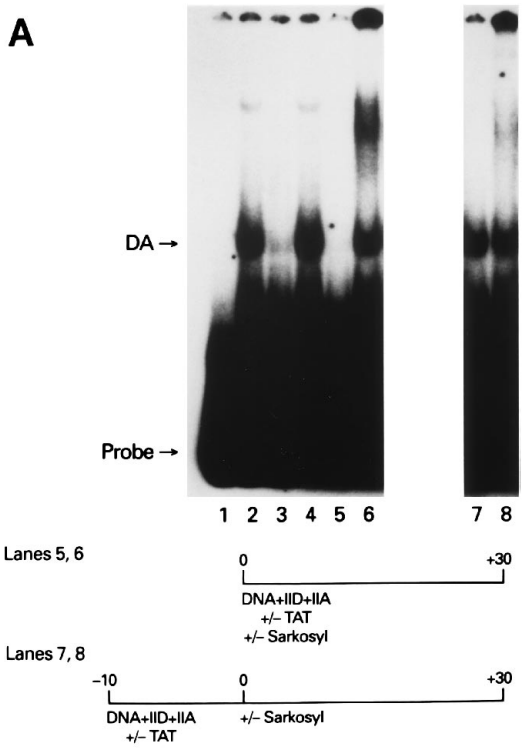
Utilizing a series of N- or C-terminal deletion mutants, we have mapped the TBP domain involved in binding to Tat from amino acid 163 to 196. The quantitative results of the binding assays are presented in Fig. 2C, and an example of the results of a typical TBP binding assay is seen in Fig. 2D. GST-Tat binds to wild-type hTBP (Fig. 2D, lane 1) but not to a TBP mutant with a deletion of amino acids 101 to 335 or 163 to 335 (dl 101-335 and dl 163-337, respectively) (Fig. 2D, lanes 4 and 5). Tat binding was observed, however, with a TBP mutant containing the first 196 amino acids (dl 196-337) (Fig. 2D, lane 6). Consistent with the results presented above, wild-type or mutant TBP failed to bind to the GST control protein. The data shown in Fig. 2E demonstrate that equal amounts of wild-type and mutant TBPs were added to the binding assay mixtures.

The fact that the Tat-TBP binding site was different from that of other activators, such as E1A, was further demonstrated by experiments showing that Tat was found to bind efficiently to mutants dl 214-227, dl 225-241, and dl 238-252 (Fig. 2C). These mutants cover the S5 and H2 domains of TBP and decrease binding with other activator proteins, such as adenovirus E1A (22) (unpublished results). TBP mutant dl 251-268, which overlaps the S1′ domain, did not affect binding of TBP to either Tat or E1A.

In an effort to more directly localize the TBP binding site, we have analyzed the interaction of a mutant with a three-amino-acid H1 substitution (amino acids 169 to 171) with Tat. As a control for the binding studies, we also included a mutant with a three-amino-acid substitution in the TBP H2 domain. The results of these studies demonstrate that the H1, but not the H2, mutation abolished the interaction with Tat. Interestingly, the H1 mutation also abolished the interaction of TBP with other proteins, such as p53, which are known to bind to the H2 or H2′ domain of TBP. The most straightforward interpretation of these results is that the H1 domain may play an important role in the structural conformation of TBP. Mutation within the H1 domain might alter the overall structure of TBP such that protein binding sites in distinct domains of the protein would be altered.

Interaction of Tat mutants with TBP. Utilizing peptides which cover the length of the Tat protein, we have previously shown by peptide binding and competition assays that amino acids 36 to 50 contain the TBP interaction domain (28). Furthermore, we demonstrated that mutation of Lys-41 to Thr-41, which inactivates the transactivation function of Tat, abolished the interaction of Tat protein and peptide with TBP. The results presented in Fig. 3A support those of our peptide binding studies and demonstrate, in the context of a larger protein, that the core domain of Tat is important for interaction with TBP. For example, compared with wild-type Tat, deletion of amino acids 2 to 36 did not affect TBP binding (Fig. 3A, lanes 2 and 3). Similarly, deletion of carboxy-terminal amino acids 73 to 86 did not decrease TBP binding (data not shown). Our results further demonstrate that amino acids 1 to 48 are sufficient for interaction with TBP (Fig. 3A, lanes 4 and 5). The binding efficiency of Tat 1-48 is decreased compared with that of wild-type Tat (Fig. 3A, lanes 2 and 4), a conclusion that was not evident from the peptide competition and binding assays. Lane 6 of Fig. 3A represents one-fifth of input TBP. Interestingly, Veschambre et al. (52) have recently reported that Tat 1-49 interacts with TBP to a level comparable to that of wild-type Tat. These observations may suggest that Arg-49, which is positioned as a transition amino acid between the conserved Tat core domain and the basic domain, may be important for Tat interaction with TBP. Our results are also consistent with earlier results reported by Southgate and Green (49) which demonstrated that a GAL4-Tat construct containing Tat amino acids 1 to 48 was less active than Tat 1-86. Mutation of Cys-22 did not diminish the interaction of Tat with TBP. In view of the fact that the Cys-22 mutation abolished the activation function of Tat and GAL4-Tat, we suggest that Cys-22 may be required for interaction with another protein important for the transcription activation function. The fact that GAL4-Tat constructs containing either amino acids 1 to 36 or 36 to 50 were not active is consistent with this conclusion. A summary of the results of Tat peptide competition, peptide binding, Tat affinity, and GST-Tat binding assays is presented in Fig. 3B.

Effect of Tat on stabilization of TFIID-TFIIA complex. Given that the interaction between Tat and TFIID was observed in vitro and in vivo, we were interested in determining the functional consequence of the Tat-TBP interaction with



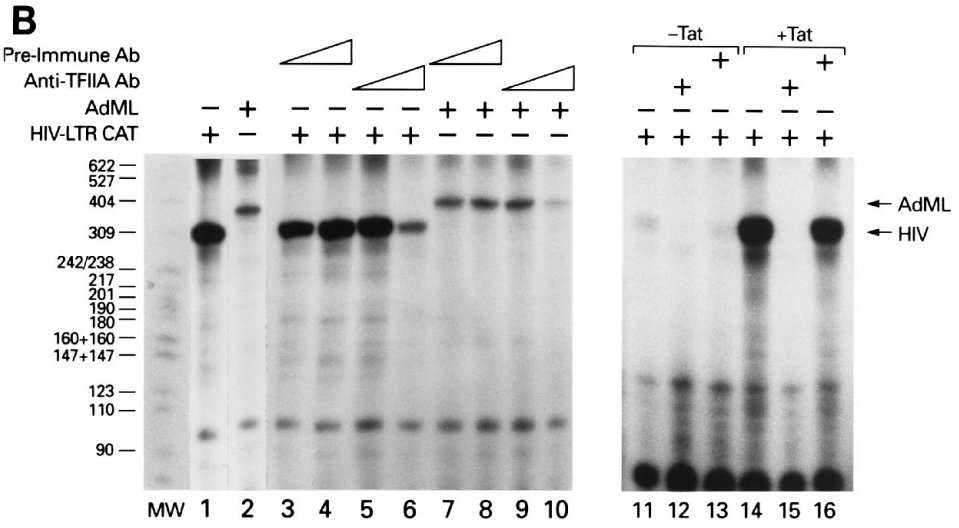


FIG. 4. Electrophoretic mobility shift assay with the HIV-1 TATA oligonucleotide and effect of TFIIA on HIV-1 transcription. (A) The TFIID-TFIIA gel shift analysis was performed as described previously (28). Lane 1, probe alone (HIV TATA oligonucleotide sequence from position −38 to −8) (28). Lanes 2 to 8 all contain TFIID and TFIIA; lanes 5 to 8 all contain 0.03% Sarkosyl during and after preincubation with Tat. Samples (35 μl total) were incubated for the times (minutes) indicated below the gels, electrophoresed on a 4% nondenaturing polyacrylamide gel, dried, and autoradiographed. DA, TFIID-TFIIA complex. (B) Effect of anti-TFIIA antibody (Ab) in transcription with HIV-1 and adenovirus major late (AdML) linear templates. Lanes 1, 3 to 6, and 11 to 16, transcription from an HIV-1 template cut with *Eco*RI within the CAT gene. Lanes 2 and 7 to 10, AdML DNA cut with *Bam*HI at 3′ end of the promoter. A HeLa WCE at 7.5 μl (94 μg) per reaction mixture was used in each lane. Lanes 1 and 2, HIV-1 and AdML transcripts from the HeLa WCE. Lanes 3, 4, 7, and 8, 1 μl of 1:10 diluted or 1 μl of undiluted control serum used in the reaction. Lanes 5, 6, 9, and 10, 1 μl of 1:10 diluted or 1 μl of undiluted anti-TFIIA polyclonal serum (anti-rp55). Lanes 13 and 16, 2.5 μl of undiluted preimmune serum. Lanes 12 and 15, 2.5 μl of anti-TFIIA serum. Lanes 14 to 16, 300 ng of purified Tat protein (28). MW, PBR/Msp1 marker kinase labeled with [^{32}P]dATP. In vitro transcription reactions were carried out as described previously (28). pAdML was linearized with *Bam*HI and HIV-LTR CAT with *Eco*RI. Templates were added to the reaction mixtures at 300 ng (lanes 1 to 10) or 100 ng (lanes 11 to 16).

respect to TFIID binding. The results presented in Fig. 4A demonstrate that Tat facilitates the formation of the TFIID-TFIIA complex in the presence of Sarkosyl. Low concentrations of Sarkosyl (0.03%) prevent the assembly of, but do not dissociate, the preinitiation TFIID-TFIIA complex (1, 3, 4, 17, 36, 45, 46). When an oligonucleotide containing the HIV-1 TATA sequence (positions −38 to −8) was incubated with purified TFIID and TFIIA, the TFIID-TFIIA complex was observed (Fig. 4A, lane 2). Incubation of the probe with either TFIID or TFIIA alone did not result in the formation of the gel shift complex (data not shown) (28). The TFIID-TFIIA gel shift complex could be specifically inhibited by excess wild-type oligonucleotide but not a TATA mutant oligonucleotide (Fig. 4A, lanes 3 and 4). The TFIID-TFIIA complex was sensitive to Sarkosyl if the detergent was added to the incubation mixture at zero time but not if the DNA and transcription factors were preincubated for 10 min prior to addition of Sarkosyl (Fig. 4A, lanes 5 and 7). Upon addition of purified Tat to the incubation mixture, the TFIID-TFIIA complex was formed even when Sarkosyl was present in the incubation mixture at time zero (Fig. 4A, lane 6). In the gel shift reaction mixtures containing Tat, we have observed a higher-molecular-weight complex migrating above the TFIID-TFIIA complex in approximately 50% of our experiments. The composition of this unstable gel shift complex is presently under investigation. To date, we have not been able to supershift either complex with Tat antibody. These results raise the possibility that Tat functions catalyti-

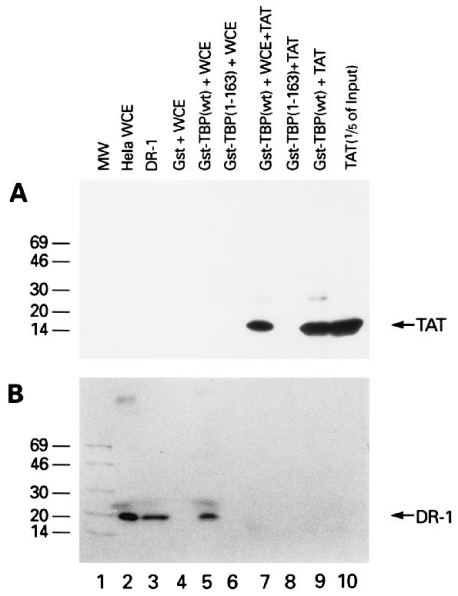


FIG. 5. Competition of Tat for Dr1 binding to TBP. Panels A (Western blot with anti-Tat serum) and B (Western blot with anti-Dr1 serum) represent samples that were split in half and loaded on two SDS-PAGE gels (4 to 20% polyacrylamide). Lane 1, molecular weight markers (in thousands); lane 2, 5 μl (12.5 μg/μl) of HeLa WCE; lane 3, ~50 ng of purified recombinant Dr1; lane 4, GST and HeLa WCE; lane 5, GST-TBP and HeLa WCE; lane 6, GST-TBP 1-163 and HeLa WCE; lane 7, GST-TBP, HeLa WCE, and Tat protein; lane 8, GST-TBP 1-163 and Tat protein; lane 9, GST-TBP and Tat protein; lane 10, Tat protein. Following incubation, GST complexes were pelleted and washed three times, and proteins were separated by SDS-PAGE (4 to 20% polyacrylamide).

cally, in that it may stabilize the TFIID-TFIIA complex without remaining stably bound.

Stabilization of the TFIID-TFIIA complex is important for HIV transcription, since both TFIID and TFIIA are required for HIV transcription. We have previously shown that heat inactivation or affinity depletion of TFIID in HeLa WCEs abolishes HIV transcription (28). The results presented in Fig. 4B demonstrate that TFIIA is also required for HIV basal and Tat-transactivated transcription. When a HeLa WCE was precleared with increasing amounts of anti-TFIIA antibody, but not preimmune serum, a significant decrease in HIV-1 transcription was observed (Fig. 4B, lanes 1 and 3 to 6). As expected, a similar decrease in transcription was observed with the adenovirus major late template (Fig. 4B, lanes 7 to 10). Similarly, when the anti-TFIIA antibody, but not preimmune serum, was added to a transcription reaction mixture containing the Tat protein, the transactivation was inhibited (Fig. 4B, lanes 11 to 16). The decrease in transcription is specific to TFIIA, since the inhibition can be overcome by the addition of purified TFIIA to the depleted extract (11).

HIV Tat interferes with binding of the negative cofactor Dr1 to TBP. In addition to stabilizing the TFIID-TFIIA complex, the interaction of Tat with TFIID may have other functions. Inostroza et al. have identified an activity, designated Dr1, which inhibits the activity of TBP (23). Dr1 inhibition involves a direct physical interaction with TBP which blocks association with other components of the transcriptional machinery, including TFIIA and TFIIB. In preliminary binding assays, the TBP binding domain for Dr1 was mapped to a region downstream of amino acid 163, potentially overlapping the Tat binding site (56a). To determine if Dr1 and Tat competed for binding to TBP, we performed a Tat-Dr1 competition experiment with GST-TBP in the presence of a HeLa WCE. Constant amounts of GST, GST-TBP wild-type, and GST-TBP 1-163 proteins were incubated with the HeLa WCE in the presence or absence of purified Tat protein. Subsequently, glutathione beads were added to the incubation mixture, and the protein complexes were pelleted by centrifugation. The complexes were separated on SDS gels and subjected to Western blotting to detect the presence of Dr1 and Tat. The results presented in Fig. 5A demonstrate that Tat specifically interacts with GST-TBP wild type but not GST-TBP 1-163 (Fig. 5A, lanes 8 and 9). The Tat-TBP interaction was also observed in the presence of the HeLa WCE (Fig. 5A, lane 7). Similarly, GST-TBP, but not GST-TBP 1-163, specifically interacted with Dr1 from the HeLa WCE (Fig. 5B, lanes 5 and 6). Interestingly, when Tat was added to the incubation mixture, the interaction between Dr1 and TBP was inhibited (Fig. 5B lanes 5 and 7). These results provide direct evidence that Tat can inhibit the interaction of Dr1 with TBP.

DISCUSSION

We have demonstrated a physical interaction between Tat and TFIID in HIV-infected cells. This is an important observation since it directly demonstrates the presence of the protein-protein interaction in the infected cell. Tat has been reported to interact with other transcription factors, including Sp1, TAP, a cellular kinase (TAK), and other, less well characterized proteins or cofactors. Only the interactions with Sp1 and TFIID have been confirmed to occur in an HIV-infected cell (19, 25, 41, 57). It is interesting that Tat interaction with TFIID is detectable at 6 and 12 h postinfection. In contrast, the Tat-TFIID complex was not observed at the 24- and 48-h time points. Several control experiments demonstrate the specificity of this interaction. First, we demonstrated that the polyclonal Tat antibody used in these experiments immunoprecipitates Tat protein from the infected-cell extracts. Further, equivalent amounts of Tat are immunoprecipitated in the 24- and 48-h samples, but no TBP is detected. Second, we demonstrated that the anti-Tat antibody does not cross-react with and immunoprecipitate TBP from uninfected cells. Third, we demonstrated that the level of TBP does not vary at the different time points. Thus, the inability to coimmunoprecipitate TBP is not due to a change in the cellular protein level. Perhaps factors like TAR RNA or a viral or cellular protein may physically sequester Tat's function as a transactivator at later stages of viral infection.

Using in vitro methods, we have mapped the TBP binding domain for Tat from amino acid 163 to amino acid 196, which contains the H1 and S2 domains of TBP. This observation may be of particular significance. First, this is a unique site for the interaction of regulatory or activator proteins with TBP. Other activator proteins, such as TFIIA, p53, VP16, and c-Rel, interact primarily with the H2-S1′ domain of TBP. Second, Tat effectively inhibits binding of a negative cofactor of TBP-mediated transcription, Dr1. Thus, part of Tat's ability to regulate transcription might revolve around its ability to compete with Dr1 binding to TBP. Importantly, in cotransfection experiments we have observed that HIV-1 Tat transactivation is repressed by Dr1 in human lymphocytes (data not shown). Moreover, we have demonstrated that overexpression of TBP in eukaryotic cells will, in fact, reverse the transcription inhibition of Dr1. If Tat interferes with binding of Dr1 to TFIID, why does Tat not activate transcription of many other genes under the control of Dr1? Removal of the negative cofactor is just one step in the transcriptional activation pathway. If other

promoter-specific upstream activators or cofactors are not induced, subsequent steps in the activation pathway would be blocked.

We have demonstrated that Tat stabilizes the TFIIA-TFIID interaction and that TFIIA is required for HIV transcription. This function may be significant, since formation of the TFIID-TFIIA complex is a rate-limiting step in formation of the preinitiation complex. Interestingly, it has also recently been suggested that TFIIA has a direct role in the activation process, stimulating the activity of a TAF(s) in a reconstituted transcription assay (51), and may function as a regulatable TAF (42). It remains to be established whether Tat is an integral part of the TFIID-TFIIA complex. To date, we have not been able to supershift either complex with Tat antibody, opening the possibility that Tat functions catalytically, in that it can stabilize the TFIID-TFIIA complex without remaining stably bound. Given the results of the Dr1 binding and Tat competition analyses, Tat may effectively compete for Dr1 binding to TFIID and stabilize the interaction between TFIID and TFIIA. This model does not rule out the possibility that Tat may first bind to TAR RNA and then interact selectively with TFIID in the initiation complex to modify or facilitate the formation of a distinct, more processive, complex.

Finally, several interesting observations regarding Tat interaction with TBP and TFIID have recently been made. First, using a protein overlay assay, Chiang and Roeder (7) have determined that Tat physically interacts with $TAF_{II}55$ of TFIID. This observation is consistent with our earlier report that the species of TFIID interacting with Tat contains $TAF_{II}55$ (28). $TAF_{II}55$ interacts with a variety of mammalian activators with different activation domains. This interaction may explain the common activation properties of Tat and VP16, which have previously been reported (49). The interactions of Tat with multiple components of the TFIID complex, TBP and $TAF_{II}55$, are not mutually exclusive, but they may represent a synergistic pathway involving interaction with multiple domains of Tat or multiple bound Tat activators. The observation that Dr1, which may negatively regulate HIV transcription, and Tat compete for a common binding site adds additional complexity to understanding the biochemical function of Tat in transactivation. Finally, Zhou and Sharp (58) have reported that in vitro Tat transactivation, primarily a transcriptional elongation effect in their system, could be supported by TBP. Although there are no reports which demonstrate the existence of free TBP in eukaryotic mammalian cells, these data may suggest a unique Tat activation pathway independent of TAFs and negative factors. These multiple interactions may represent the sequential or alternative interactions of a single activator with several transcription factors in the transcriptional initiation and elongation complexes. The combination of the interactions may be responsible for the potent transactivation function of Tat. Our current understanding of the mechanism of Tat transactivation awaits future experiments using purified and reconstituted transcription factors to determine the requirements and roles of these distinct transcription factors in Tat transactivation.

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