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The Human Immunodeficiency Virus Transactivator Tat Interacts with the RNA Polymerase II Holoenzyme

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The human immunodeficiency virus (HIV) encodes a transcriptional transactivator (Tat), which binds to an RNA hairpin called the transactivation response element (TAR) that is located downstream of the site of initiation of viral transcription. Tat stimulates the production of full-length viral transcripts by RNA polymerase II (pol II). In this study, we demonstrate that Tat coimmunoprecipitates with the pol II holoenzyme in cells and that it binds to the purified holoenzyme in vitro. Furthermore, Tat affinity chromatography purifies a holoenzyme from HeLa nuclear extracts which, upon addition of TBP and TFIIB, supports Tat transactivation in vitro, indicating that it contains all the cellular proteins required for the function of Tat. By demonstrating that Tat interacts with the holoenzyme in the absence of TAR, our data suggest a single-step assembly of Tat and the transcription complex on the long terminal repeat of HIV.

The human immunodeficiency virus type 1 (HIV-1) encodes a transcriptional transactivator called Tat, which is expressed early in the viral life cycle and is absolutely required for viral replication and progression to disease (9, 23). Tat binds to the transactivation response element (TAR), which forms an RNA stem-loop downstream (positions +1 to +60) from the site of initiation of transcription in the 5′ long terminal repeat (LTR). In the absence of Tat, RNA polymerase II (pol II) terminates transcription of the HIV genome prematurely, resulting in primarily short transcripts. Interactions between Tat and TAR convert pol II into its processive form and lead to the efficient production of full-length viral transcripts (15, 24, 27, 32, 37).

The mechanism by which Tat increases the processivity of pol II is unknown. However, cellular proteins clearly play a critical role in the function of Tat. At least one of these proteins is encoded on human chromosome 12 (hp12) and is required to tether Tat to TAR (1, 2, 18–20). In addition, squelching experiments suggest the existence of other coactivators which mediate interactions between Tat and the cellular transcriptional machinery (6, 35). These include general transcription factors (GTFs) such as the core pol II itself (RPB 1-12) (38), TATA-binding protein (TBP) (26), $TAF_{II}55$ (8), and TFIIH (5), as well as the glutamine-rich activator Sp1 (22). Numerous other proteins whose function in transcription are not known have also been postulated to act as Tat coactivators (10, 39, 50, 52).

In addition to cellular proteins, the carboxy-terminal domain (CTD) of the largest subunit of the core pol II is required for the activity of Tat. We and others have demonstrated that although truncations of the CTD do not affect proximal transcription from the LTR, distal transcription which is induced by Tat is absolutely dependent upon a full-length CTD (40, 49). To this end, a Tat-associated kinase which phosphorylates the CTD has been identified and may play a role in the effects of Tat on pol II processivity (21).

Recently, a large complex of proteins called the pol II holoenzyme has been identified in yeast and mammalian cells (30, 36, 41). The holoenzyme consists of a subset of GTFs, human SRBs (suppressors of mutations in polymerase B) which bind to the CTD and confer responsiveness to activators, and proteins involved in chromatin remodeling (SWI/SNF) and nucleotide excision repair (7, 33, 36, 41, 44, 47). The holoenzyme preassembles independently of DNA, binds the promoter in a single step, and is responsive to transcriptional activators (29). Functional relevance for the preassembly of the transcription initiation complex is provided by experiments demonstrating that a single contact point between a DNA-bound activator and a component of the holoenzyme is sufficient to recruit the preassembled holoenzyme to the template and activate transcription (3, 14).

Since numerous individual components of the holoenzyme have been postulated to interact with Tat, we examined the possibility that Tat binds to the preassembled holoenzyme. In this paper, we report that Tat coimmunoprecipitates with the holoenzyme in the absence of TAR in vivo and that it binds to highly purified preparations of the holoenzyme in vitro. Furthermore, we demonstrate that Tat affinity chromatography purifies the holoenzyme from crude nuclear extracts and that this holoenzyme contains the same GTFs and human SRBs present in the immunoprecipitated holoenzyme preparations. Upon the addition of recombinant TBP and TFIIB, the holoenzyme obtained from Tat affinity chromatography is fully competent for Tat transactivation in vitro.

MATERIALS AND METHODS

Plasmid constructions. Plasmid CMV-TATHA (Tat) was constructed by cloning the wild-type Tat gene (nucleotides 1 to 360) fused to the influenza virus hemagglutinin (HA) epitope tag (3′) into the *Bam*HI-*Eco*RI sites of pCDNAI/Amp (InVitrogen, San Diego, Calif.). A mutation in Tat (C30G) was introduced by PCR-mediated mutagenesis with the oligonucleotides 5′ TCTTGTGAAAC ACGCGTAGCAATGAAAGCAgccCTTTTTACAATAGC 3′ (lowercase letters denote mutations) and 5′ GAGCCAGTAGATCCTAATCTAGAG 3′. The

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PCR fragment (118 bp) was cloned into the *Xba*I-*Afl*III sites of pCMV-TATHA, resulting in the construct pCMV-TAT(C30G)HA (mTat). The mutant construct was sequenced by dideoxy sequencing (U.S. Biochemicals, Cleveland, Ohio) to confirm the integrity of the Tat sequence. The C30G mutation abolished Tat transactivation from the HIV-1 promoter but did not affect the levels of protein expression (31). The hybrid CD8-Nef protein has been described elsewhere (4). Tat was expressed in bacteria with plasmid pET23dSTat (a kind gift from Osvaldo Flores, Tularik, South San Francisco, Calif.). This plasmid contains a streptavidin binding peptide (AWRHPQFGG) fused to the 3′ end of a full-length Tat gene and is cloned into the *Nde*I-*Xho*I sites of pET23d (Novagen, Madison, Wis.). Mutant Tat proteins STat(C30G) and STat(K41A) were made by cloning oligonucleotides with the appropriate mutant sequences into the *Mun*I-*Eco*NI sites of pET23dStat (sequences available upon request). The Stat(C22G) mutation was made by PCR-mediated mutagenesis with primers 5′ GCCTAAAACTGCTggtACCAATTGCTATTGTAA 3′ (lowercase letters denote mutation) and 5′ CGCGGGATCGAGATCTCGGGGAGCGTTGGGG 3′. The PCR fragment (185 bp) was cloned into the *Bgl*II-*Mun*I sites of pET23dStat.

Holoenzyme immunoprecipitations. Approximately 24 h before transfection, 5×10^5 COS-7 cells were seeded onto 100-mm culture dishes. The following day, a mixture of Opti-MEM (2.0 ml), plasmid DNA (5 μg), and Liptofectin (10 μl) was added to the cells as recommended by the manufacturer (Gibco BRL, Gaithersburg, Md.). Typically, two dishes were used for each immunoprecipitation. At 36 h after transfection, the cells were lysed (50 mM HEPES-KOH [pH 7.8], 0.1 M NaCl, 1% Triton X-100, 10 mM EDTA, 5 mM dithiothreitol [DTT], 0.1 mM phenylmethylsulfonyl fluoride, 20 μg of aprotinin per ml, 10 μg of leupeptin per ml) and the supernatants were immunoprecipitated with the indicated antibody. Following binding to protein G-Sepharose (αRAP74 antibodies) (36) or to protein A-Sepharose (αCIITA or αCD8 antibodies) beads, the immunoprecipitates were washed four times in 50 mM HEPES-KOH (pH 7.6)–0.25 M NaCl–0.5% Triton X-100–1% bovine serum albumin–10% glycerol. Washed beads were subjected to gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (5 to 12% polyacrylamide), transferred onto Immobilon-NC membranes (Millipore, Bedford, Mass.), and reacted with the indicated antibodies. The proteins were visualized by enhanced chemiluminescence detection (Amersham, Arlington Heights, Ill.). The anti-HA antibody (12CA5) was purchased from Boehringer-Mannheim (Indianapolis, Ind.), the αTFIIE and αSp1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.), the cyclin H and αTFIIB antibodies were purchased from Upstate Technologies (Lake Placid, N.Y.), and the TBP antibody was purchased from Promega (Madison, Wis.). The αCD8 antibody has been described previously (4), and the αCIITA antibody (46) was from our laboratory.

In vitro binding assays. Wild-type (CMV-TATHA) and mutant Tat [CMV-TAT (C30G) HA] proteins were labeled with L-[^{35}S]cysteine (>600 Ci/mmol; Amersham) and the Promega TnT protein expression system as recommended by the manufacturers. Holoenzyme preparations were immunopurified with the αRAP74 antibody as described previously (36). Purified holoenzyme (0.6 to 0.8 μg) and αRAP74 antibodies (2 μg) (36) were added to the reaction mixtures as indicated. Immunoprecipitations were performed as described above. Immunoprecipitates were resolved by SDS-PAGE (15% polyacrylamide), dried, and exposed to X-ray film. Quantitation of bands was performed with the image analysis system (Alpha Innotech, San Leandro, Calif.).

Tat affinity purifications. Nuclear extracts were prepared as described previously (11). Expression of wild-type or mutant Tat proteins in *Escherichia coli* BL21*lysS* was induced as recommended by the manufacturer (Novagen, Madison, Wis.). The cells were lysed in 50 mM Tris (pH 7.9)–0.1 M KCl–12.5 mM $MgCl_2$–1% Triton X-100–2 mM EDTA–10 mM DTT plus protease inhibitors (see above). Soluble proteins were bound to streptavidin-agarose beads (Gibco BRL) in lysis buffer containing 0.8 M KCl. After extensive washing with lysis buffer, the tat streptavidin-agarose preparations were judged to be >95% pure by Coomassie blue staining (data not presented). Equilibrated Tat streptavidin-agarose beads containing wild-type or mutant Tat were incubated with crude nuclear extracts in binding buffer (25 mM HEPES-KOH [pH 7.7], 0.1 M KCl, 20% glycerol, 0.1 mM EDTA, 10 mM DTT). Pelleted beads were washed three times in binding buffer containing 0.15 M KCl and 10% glycerol. Washed beads were subjected to gradient SDS-PAGE (5 to 12% polyacrylamide) and processed as described in the immunoprecipitation protocol with the antibodies indicated.

In vitro transcription. Transcription reactions (50-μl volumes) were performed essentially as described previously (43); the reaction mixtures contained 300 ng of template DNA, 15 mM HEPES-KOH (pH 7.4), 55 mM KCl, 12% glycerol, 7 mM $MgCl_2$, 0.1 mM EDTA, 150 ng of poly(rI-rC), 250 ng of poly(dI-dC), 5 mM DTT, 10 mM creatine phosphate, 600 μM each rCTP, rGTP, and rATP, 40 μM cold rUTP, and 20 μCi of [α-^{32}P]UTP. The reaction mixtures were incubated at 30°C for 60 min and terminated by the addition of 90 μl of stop solution (1% SDS, 20 mM EDTA, 0.2 M NaCl, 375 μg of tRNA per ml) and 350 μl of 0.3 M sodium acetate (pH 5.1). Following phenol-chloroform extraction, the transcripts were precipitated with ethanol and separated on a 5% denaturing polyacrylamide gel. Wild-type (LTR) and mutated (U23A) HIV-1 promoter constructions yielded specific HIV-1 runoff transcripts of 750 and 620 nt, respectively (24, 48). Recombinant Tat (100 ng) was expressed in *E. coli* and bound to streptavidin-agarose beads as described above. Prior to addition to the transcription reactions, Tat was eluted from the streptavidin-agarose beads in lysis buffer containing 0.8 M KCl and 2 mM biotin and then dialyzed extensively into 25 mM HEPES-KOH (pH 7.6)–0.1 M KCl–20% glycerol–10 mM DTT–0.1 mM EDTA. Crude nuclear extracts (50 μg) were added to the reaction mixtures in lanes 1 to 4 of Fig. 4. In lanes 5 to 12, crude nuclear extracts (500 μg) were incubated with Tat bound to streptavidin-agarose beads or with beads alone and washed three times as described for the Tat affinity purifications. Bound complexes were eluted from the agarose beads in an equal volume of elution buffer (50 mM HEPES-KOH [pH 7.8], 0.1 M KCl, 12.5 mM $MgCl_2$, 0.2 mM EDTA, 2 mM biotin) and added directly (20 μl) to in vitro transcription reaction mixtures (50 μl). In lanes 13 and 14, washed beads (15 μl) were added directly to the transcription reaction mixtures. Recombinant TBP (0.5 μl) and TFIIB (0.5 μl) were obtained from Promega and added to the transcription reaction mixtures in lanes 5 to 14. One-tenth of the reaction products in lanes 1 to 4 were loaded onto the gel. Transcript levels were measured by PhosphorImager analysis (Molecular Dynamics, Inc., Mountain View, Calif.).

RESULTS

Tat binds to the holoenzyme in vivo. Recently, the holoenzyme was purified from mammalian cells by two independent groups. Ossipow et al. used an antibody against a component of TFIIH (CDK7) to purify the holoenzyme from rat liver crude nuclear extracts (41). Their holoenzyme preparations contained all the GTFs necessary for basal transcription but were unresponsive to transcriptional activators. In contrast, we used an antibody against the largest subunit of TFIIF (RAP74) to purify the holoenzyme from HeLa nuclear extracts following initial fractionation on phosphocellulose and DEAE-Sephacel columns (36). This holoenzyme complex had a relative molecular mass of ~2,000 kDa. It was composed of a subset of GTFs (core pol II, TFIIE, TFIIF, and TFIIH), human SRBs (human homologs of yeast SRB7, SRB10 and SRB11), and proteins involved in nucleotide repair (DNA pol ε, RPA, RFC, and HRAD-51). In addition, the holoenzyme contained mediator proteins (29), which bound to the CTD and conferred responsiveness to the acidic activator VP16.

To determine if Tat interacts with the holoenzyme, influenza virus HA-tagged wild-type or mutant (C30G) Tat proteins were expressed in COS cells following transfection by lipofectin. Cysteine at position 30 in the activation domain of Tat is essential for Tat transactivation (31). Cell lysates were immunoprecipitated with the same αRAP74 antibody that we used previously to immunoprecipitate the holoenzyme from crude nuclear extracts (36). The presence of Tat was monitored by Western blotting with the αHA antibody. As demonstrated in Fig. 1A, although both proteins were expressed equivalently in COS cells (lanes 2 and 3), only the wild-type (but not mutant) Tat proteins were coimmunoprecipitated by the αRAP74 antibody (lanes 5 and 6). Moreover, neither protein bound to the αRAP74 antibody or to protein G-Sepharose beads alone (see Fig. 2). Other mutations in the Tat activation domain (K41A) also abolished the binding of Tat to the holoenzyme (data not presented). To control for the possibility that the overexpression of Tat in COS cells resulted in nonspecific interactions between Tat and irrelevant cellular proteins, cells were made to express Tat and a hybrid CD8-Nef protein (4). As demonstrated in Fig. 1A (lane 4), Tat was not immunoprecipitated by the αCD8 antibody. In addition, antibodies against transport and cytoskeletal proteins failed to immunoprecipitate Tat (data not presented). The addition of DNase or RNase to the immunoprecipitation reaction mixtures did not affect our results, suggesting that Tat was not artificially bound to the holoenzyme by contaminating DNA or RNA.

To confirm that the holoenzyme immunoprecipitated from COS cell lysates by the αRAP74 antibody was similar in composition to the one that we had purified previously (36), these immunoprecipitates were also probed with antibodies directed against GTFs which are known components of the holoenzyme (Fig. 1B, lane 3). As a negative control, class II transactivator

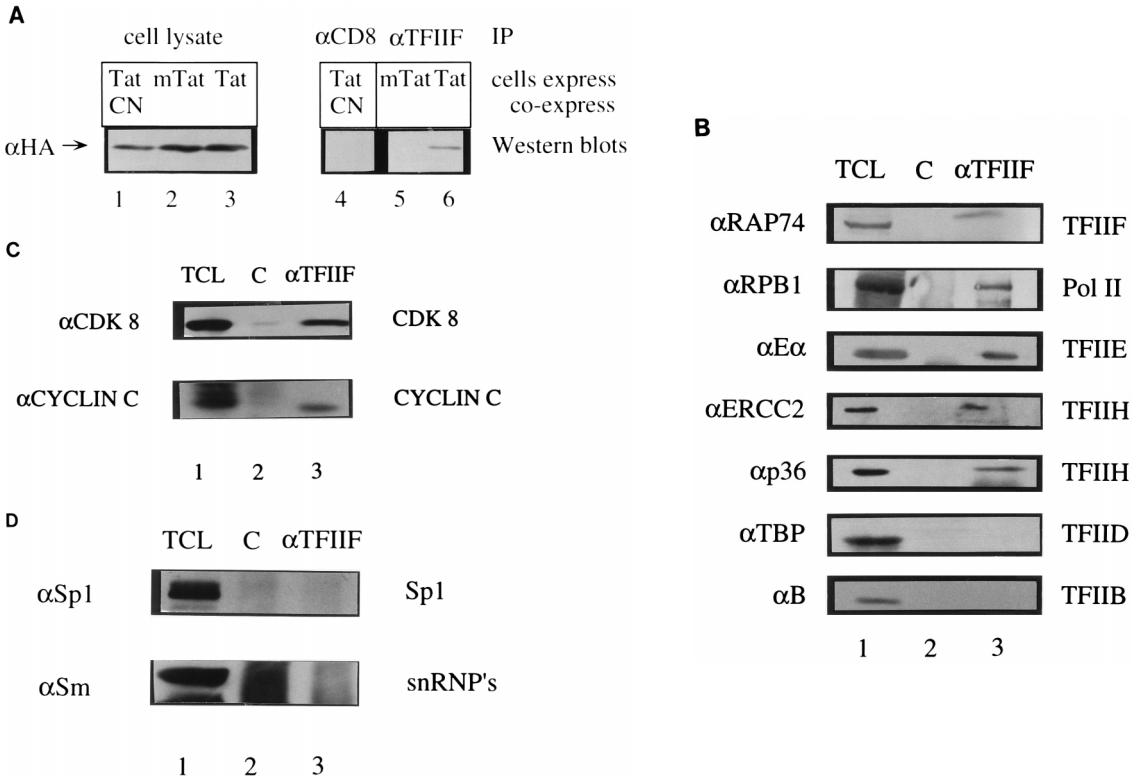

FIG. 1. Tat is coimmunoprecipitated with components of the holoenzyme by using a monoclonal αRAP74(TFIIF) antibody. (A) The αRAP74 antibody immunoprecipitates the wild-type but not mutant Tat proteins from COS cells. COS cells expressed HA-tagged wild-type Tat (Tat), mutant Tat (C30G = mTat), or both wild-type Tat and a hybrid CD8-Nef protein (CN) (4). One-quarter of the cell lysate was loaded as the input control (lanes 1 to 3), and the remainder was immunoprecipitated with using either the αRAP74 antibody (lanes 5 and 6) or the αCD8 antibody (lane 4). Samples were separated by SDS-PAGE, transferred to membranes, and probed with the αHA antibody (12CA5). Arrow indicates HA-tagged Tat. (B) The αRAP74 antibody immunoprecipitates GTFs associated with the holoenzyme from the total cell lysate of COS cells. Total-cell lysates (TCL) from COS cells were immunoprecipitated with the αRAP74 antibody (lane 3) or the αCIITA antibody (46) as the control (C) (lane 2). One-third of the cell lysate was loaded in lane 1. Lysates and immunoprecipitates were separated by SDS-PAGE and transferred to membranes as in panel A. Antibodies used in the subsequent Western blotting are indicated on the left, and the corresponding GTFs are shown on the right. (C) The αRAP74 antibody immunoprecipitates human SRB proteins (suppressors of mutations in RNA polymerase B) associated with the holoenzyme from the total-cell lysate of COS cells. Total-cell lysates from COS cells were immunoprecipitated and processed as in panel B. Antibodies used in the subsequent Western blotting are indicated on the left. Human CDK8 and cyclin C are the human homologs of the yeast SRB10 and SRB11 proteins, respectively. (D) The αRAP74 antibody does not immunoprecipitate Sp1 or snRNPs involved in RNA splicing from the total cell lysate of COS cells. Cell lysates were immunoprecipitated and processed as described in panel B. Antibodies used in the subsequent Western blotting are indicated on the left, and the corresponding proteins are shown on the right. The snRNP recognized by the αY12 antibody contains the conserved Sm motif and is approximately 92 kDa (42).

(CIITA) (46) immunoprecipitates were analyzed in parallel (lane 2). As expected, the αRAP74 antibody recovered at least 50% of the TFIIF protein from total cell lysates of COS cells (lanes 1 and 3). In addition, the αRAP74 antibody precipitated a significant amount of core pol II, TFIIE, and TFIIH (lanes 1 and 3). As previously reported (36), TBP(TFIID) and TFIIB were not part of the holoenzyme precipitated by the αRAP74 antibody. To characterize further the immunoprecipitated complex, Western blotting with antibodies directed against additional nuclear proteins was performed. As demonstrated in Fig. 1C, CDK8 and cyclin C (human homologs of yeast SRB 10 and SRB 11, respectively) were also present in the holoenzyme immunoprecipitated by the αRAP74 antibody. In contrast, neither the glutamine-rich activator Sp1 nor small nuclear ribonucleoproteins (snRNPs) involved in RNA splicing and containing the conserved Sm motif (42) were present (Fig. 1D). Together, these results suggest that the holoenzyme immunoprecipitated from COS cell lysates by the αRAP74 antibody has the same composition as that previously purified from nuclear extracts (36). Furthermore, our results suggest that Tat interacts with the holoenzyme in vivo and that this interaction can occur in the absence of TAR.

Tat binds to the purified holoenzyme in vitro. Previously, we also purified the holoenzyme by conventional chromatography (36). Nuclear extracts were fractionated on a phosphocellulose column followed by DEAE-Sephacel and DEAE-5PW columns (13, 16). Fractions containing pol II were identified by Western blotting and loaded onto an S-Sepharose column (36). Transcriptionally active fractions eluting at 0.2 M KCl were loaded on an AcA22 gel filtration column and then fractionated on a Mono S column. The holoenzyme complex eluted at 0.18 M KCl and was further fractionated by gel filtration on a Sepharose CL-4B column. The highly purified holoenzyme preparation contained the same GTFs, human SRBs, and nucleotide repair proteins present in the holoenzyme preparations immunopurified with the αRAP74 antibody. Similar to the immunopurified holoenzyme, the holoenzyme purified by conventional chromatography was also responsive to the VP16 activator protein (36).

To determine the ability of Tat to bind to the purified holoenzyme in vitro, holoenzyme fractions were combined with ^{35}S-labeled wild-type and mutant Tat proteins. The resulting complexes were immunoprecipitated using the αTFIIF antibody. As presented in Fig. 2, wild-type Tat bound to the puri-

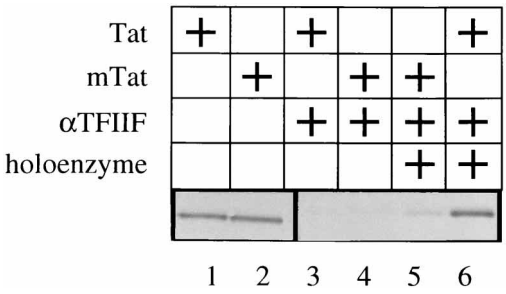

FIG. 2. Tat interacts with the purified holoenzyme in vitro. Wild-type (lane 6) or mutant (C30G = mTat [lane 5]) ^{35}S-labeled Tat proteins were mixed with the purified holoenzyme and immunoprecipitated with the αTFIIF antibody. Lanes 1 and 2 represent a fraction of the total labeled Tat used in the binding experiments; lanes 3 and 4 represent binding of wild-type and mutant Tat proteins to αTFIIF antibody alone.

fied holoenzyme with 50-fold-greater affinity than the mutant (C30G) Tat did. No significant binding occurred between these Tat proteins and the αTFIIF antibody or the protein G-Sepharose beads (Fig. 2, lanes 3 and 4). Identical amounts of labeled wild-type and mutant Tat proteins were used in these experiments (lanes 1 and 2). Similar experiments with the ^{35}S-labeled Nef protein failed to detect an interaction between this negative control and the holoenzyme (data not presented).

Tat purifies the holoenzyme from crude nuclear extracts. Although the above results strongly suggest that Tat interacts with the holoenzyme, they do not rule out the possibility that a binary complex between Tat and TFIIF dissociated from the holoenzyme. Therefore, we tested whether Tat itself could purify the holoenzyme from crude nuclear extracts. Recombinant Tat was bound to streptavidin-agarose beads and incubated with HeLa nuclear extracts (Fig. 3A). Complexes that bound to the Tat-streptavidin beads (Fig. 3A, lane 3) or the streptavidin beads alone (lane 2) were then subjected to Western blotting. Tat affinity purification of crude nuclear extracts yielded core pol II, TFIIE, TFIIF, and TFIIH (lane 3). Similar to the holoenzyme immunoprecipitated by the αRAP74 antibody, TBP and TFIIB were not present in the Tat affinity-purified complex (lane 3). Under these conditions, none of the holoenzyme components bound to streptavidin beads alone (lane 2). To address further the specificity of the Tat-affinity chromatography, crude nuclear extracts were incubated with mutant Tat-streptavidin beads. These mutations in the Tat activation domain (C22G, C30G, and K41A) were previously demonstrated to abolish Tat transactivation in vivo (31) and in vitro (data not presented). Western blotting (Fig. 3B) demonstrated that the core pol II, TFIIF, CDK8, and cyclin C all failed to bind to the mutant Tat proteins. Interestingly, Sp1 was retained on the wild-type Tat-streptavidin beads. We do not know if the retention of Sp1 on the Tat beads was due to an interaction between Tat and Sp1 which occurred independently of the holoenzyme (22) or if Sp1 and the holoenzyme simultaneously bound distinct regions of Tat. Consistent with the immunoprecipitations with the αRAP74 antibody (Fig. 1), snRNPs bound to neither the wild-type nor mutant Tat proteins (Fig. 3). Taken together, these results suggest that Tat interacts with a holoenzyme complex consisting of the core pol II, a subset of GTFs, and human SRBs in vivo and in vitro.

The Tat affinity-purified holoenzyme supports transcription from the LTR. To determine if the holoenzyme obtained from Tat affinity chromatography was competent for transcription, eluates from Tat-affinity columns were added to transcription reaction mixtures containing the wild-type and mutated HIV-1 promoter templates. By using crude nuclear extracts, the addition of the recombinant Tat protein to transcription reaction mixtures containing the wild-type promoter increased the rates of transcription ninefold (Fig. 4A, lanes 1 and 2; Fig. 4B). In sharp contrast, Tat did not affect the rates of transcription from a promoter containing a mutation in the bulge of TAR (U23A), which prevents the binding of Tat to TAR (48) (Fig.

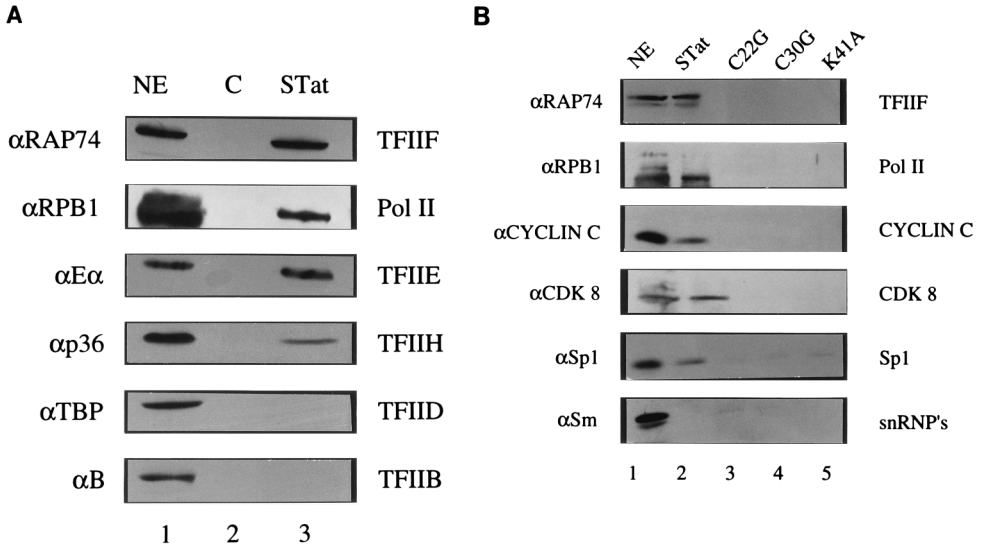

FIG. 3. Tat affinity chromatography purifies the holoenzyme from crude nuclear extracts. (A) Tat affinity chromatography purifies GTFs associated with the holoenzyme from crude nuclear extracts. HeLa nuclear extracts were incubated with Tat bound to streptavidin-agarose beads (STat [lane 3]) or with streptavidin-agarose beads alone as the control (C [lane 2]). After being washed, the streptavidin beads were subjected to SDS-PAGE, transferred to membranes, and reacted with antibodies indicated on the left. Corresponding transcription factors are indicated on the right. Lane 1 represents the input crude nuclear extract (NE). (B) Mutations in the activation domain of Tat abolish the binding of Tat to the holoenzyme. Nuclear extracts were incubated with the wild-type (STat) or mutant Tat proteins (C22G, C30G, and K41A) bound to streptavidin-agarose beads. After being washed, complexes were subjected to SDS-PAGE and processed as in panel A. Western blotting was performed with the antibodies indicated on the left. Corresponding transcription factors are indicated on the right. Lane 1 represents the input crude nuclear extract (NE).

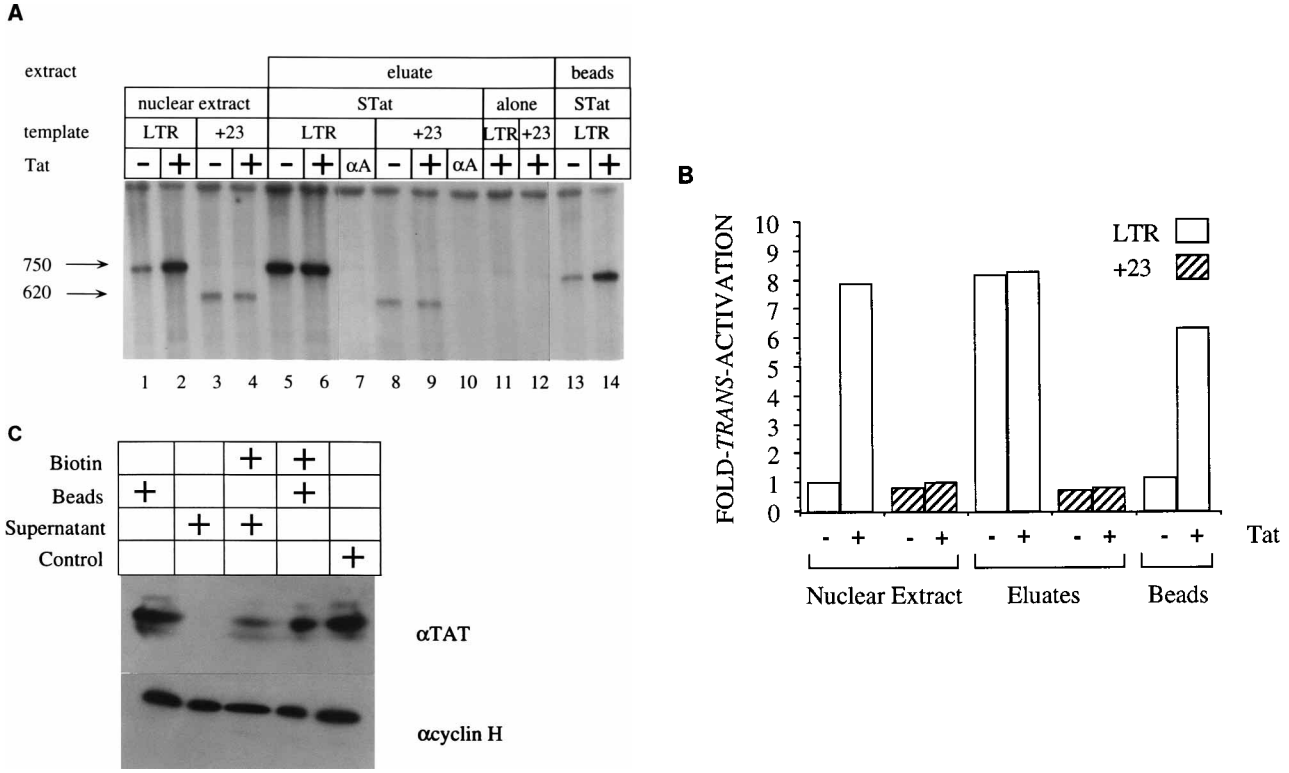

FIG. 4. The holoenzyme obtained from Tat affinity chromatography supports Tat transactivation in vitro. (A) Holoenzyme preparations obtained from Tat affinity chromatography were supplemented with recombinant TBP and TFIIB and added to in vitro transcription reaction mixtures. Linearized DNA templates containing the wild-type (LTR) or mutated (+23) HIV-1 promoter sequences were used. Lanes 1 to 4 display basal (−) and Tat-activated (+) RNA transcripts from these two promoters by using crude nuclear extracts. In lanes 5 through 12, HeLa cell nuclear extracts were first incubated with Tat-streptavidin agarose beads (lanes 5 to 10) or with streptavidin-agarose beads alone (lanes 11 and 12). After extensive washing, biotin was added to elute the bound complexes from streptavidin beads. Supernatants from the biotin elution were then added to transcription reaction mixtures containing the wild-type (lanes 5 and 6) or mutated LTR (lanes 8 and 9) templates in the presence (lanes 6 and 9) or absence (lanes 5 and 8) of exogenously added recombinant Tat. In lanes 13 and 14, streptavidin-agarose beads containing the holoenzyme obtained from Tat affinity chromatography were added directly to transcription reaction mixtures without prior elution with biotin. Reactions were performed in the presence (lane 14) or absence (lane 13) of exogenously added Tat. Lanes 7 and 10 are identical to lanes 6 and 9, respectively, except that transcription reactions were carried out in the presence of α-amanitin (αA). (B) Quantitation of transcript levels presented in panel A by PhosphoroImager analysis. Open and hatched bars represent transcription from the wild-type and mutated (U23A) LTRs, respectively. Reactions were performed in the presence (+) or absence (−) of Tat (A). Fold transactivation was standardized to that in the transcription reaction containing crude nuclear extract in the absence of recombinant Tat (panel A, lane 1), which was set to 1. Transcript levels in reactions containing crude nuclear extracts (panel A, lanes 1 to 4), biotin-eluted complexes (panel A, lanes 5 and 6 and lanes 8 and 9), or beads containing bound holoenzyme (panel A, lanes 13 and 14) were analyzed. (C) Elution of Tat from streptavidin-agarose beads is dependent on the presence of biotin. The wild-type Tat which was bound to streptavidin-agarose beads was incubated with crude nuclear extracts as described for the in vitro transcription reactions. After incubation in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of biotin, eluates and beads were subjected to SDS-PAGE and Western blotting performed with αTat or α cyclin H antibodies. Recombinant STat was used as the control for the αTat antibody, and crude nuclear extracts were used as the control for the α cyclin H antibody.

4A, lanes 3 and 4). Protein complexes that eluted from Tat-streptavidin beads with excess biotin also supported transcription from the wild-type and mutated LTRs (lanes 5, 6, 8, and 9). Although exogenously added Tat did not increase the rates of transcription from the wild-type LTR (compare lanes 5 and lane 6), rates of transcription from this promoter were almost 12-fold greater than those from the mutated LTR (lanes 8 and 9). This result suggests that under these conditions, Tat was present in saturating amounts following the elution of Tat and bound transcription complexes from streptavidin beads.

The transcription from the wild-type and mutated promoters was sensitive to α-amanitin (Fig. 4A, lanes 7 and 10) and depended on the addition of recombinant TBP and TFIIB (data not presented), thus confirming functionally the composition of the holoenzyme purified by Tat affinity chromatography (Fig. 3). In contrast, no transcription from the wild-type or mutated promoters was observed when eluates from streptavidin beads alone or from proteins containing mutations in the Tat activation domain (C30G), which abolished the ability of Tat to interact with the holoenzyme in vivo and in vitro, were used (Fig. 4A, lanes 11 and 12 and data not presented). Interestingly, exogenously added Tat stimulated transcription in reactions where Tat was not eluted from streptavidin beads (lanes 13 and 14), suggesting that Tat was present in limiting amounts under these conditions. To address further this possibility, eluates from the Tat affinity columns used in the transcription reactions were subjected to Western blotting. As demonstrated in Fig. 4C, the release of recombinant Tat from streptavidin beads was dependent upon the presence of biotin. In contrast, roughly equivalent amounts of cyclin H (TFIIH) and the holoenzyme were released from streptavidin beads regardless of the presence or absence of biotin. These results suggest that in the absence of biotin, transcription reactions are responsive to exogenous Tat because the majority of Tat is bound to streptavidin beads and cannot participate in transcription. Our in vitro transcription results are consistent with the binding assays demonstrating an association between Tat and the holoenzyme. Significantly, the activity of the holoenzyme purified by Tat affinity chromatography mimicked that of crude nuclear extracts, and the effects of Tat were dependent

on a functional TAR element in both systems. Thus, Tat purifies a transcriptionally competent holoenzyme, Sp1, other coactivators, and TAR-tethering factors from crude nuclear extracts, all of which function to support Tat transactivation in vitro.

DISCUSSION

Our results indicate that Tat interacts with the holoenzyme in vivo and that this interaction occurs in the absence of TAR. Several lines of evidence support this conclusion. First, wild-type but not mutant Tat proteins were coimmunoprecipitated with the holoenzyme (Fig. 1) from total cell lysates with an antibody (αRAP74) that immunoprecipitates the holoenzyme from nuclear extracts (36). Second, wild-type but not mutant Tat proteins bound to extensively purified preparations of the holoenzyme in vitro (Fig. 2). Third, wild-type but not mutant Tat affinity columns specifically retained a holoenzyme complex (Fig. 3) which was similar in composition to that immunoprecipitated from total cell lysates by the αRAP74 antibody (Fig. 1). Finally, upon the addition of recombinant TBP and TFIIB, the holoenzyme obtained from Tat affinity chromatography fully supported Tat-activated transcription in vitro (Fig. 4). These results indicate that Tat interacts with a holoenzyme complex which contains a subset of GTFs, Sp1, Tat co-activator(s), and possibly TAR-tethering factors.

Immunoprecipitations and conventional chromatography have demonstrated that the mammalian holoenzyme consists of a restricted set of nuclear proteins which include GTFs, human SRBs, SWI/SNF, and nucleotide repair proteins (36, 41). In this study, holoenzyme preparations which were immunoprecipitated by the αRAP74 antibody or obtained from Tat affinity chromatography contained the same set of previously described nuclear proteins and therefore represent genuine holoenzyme complexes. The selective precipitation of the holoenzyme and not more global nuclear complexes was demonstrated by the absence of TBP, TFIIB, and snRNPs. We used in vivo coimmunoprecipitation experiments, direct in vitro binding assays, and Tat affinity chromatography to demonstrate a direct interaction between the wild-type but not mutant Tat proteins and the holoenzyme. Tat affinity chromatography showed that three different Tat mutants (C22G, C30G, and K41A) did not bind to the holoenzyme.

The holoenzyme obtained from Tat affinity chromatography contained the same GTFs and human SRBs as the holoenzyme immunoprecipitated by the αRAP74 antibody. In addition, affinity chromatography purified proteins which interact with Tat directly. The presence of Sp1 in this holoenzyme preparation explains why transcription reactions containing these holoenzyme preparations did not require the addition of Sp1 (Fig. 4A). Moreover, the absence of Sp1 and other Tat-interacting proteins might be the reason why the holoenzyme purified in the absence of Tat (Fig. 1 and 2) did not support Tat transactivation in vitro (data not presented).

Our results are consistent with recent reports demonstrating direct interactions between Tat and the core pol II (38) and the p62 subunit of TFIIH (5). Nevertheless, functional evidence that these interactions are required for Tat function is lacking. It is also possible that Tat binds to these individual proteins weakly. This situation would be analogous to the bacteriophage λ, where, although individual interactions between the N protein and its cellular targets (NusA, NusB, NusG, S10, and core RNA polymerase) are weak, the assembled complex on RNA (NutB site) is stable (17). Interactions between Tat and TBP were also reported to occur in vitro (26) and in infected cells (25). Despite repeated attempts, we were unable to detect TBP in the holoenzyme obtained from Tat affinity chromatography. Interestingly, the absence of TBP in the holoenzyme might explain the absolute requirement of a functional TATA box, to which TBP can bind independently, for the activity of Tat in vivo (34).

Based on our results, we propose a new model for the entry of Tat into the transcription complex. Tat interacts with the preassembled holoenzyme. The Tat-holoenzyme complex then binds to the LTR with the help of TFIID, Sp1, initiator, and possibly other DNA-binding proteins. Tat then binds to TAR and is modified or repositioned in such a way as to increase the processivity of pol II. This event could be direct, such that Tat remains associated with the elongating pol II (28), or catalytic, such that the pol II is modified, e.g., by phosphorylation of the CTD (40, 49).

A precedent for an association between Tat and the holoenzyme prior to its activation exists with TFIIH. Although TFIIH is part of the holoenzyme, phosphorylation of the CTD by TFIIH occurs only following the assembly of the initiation complex onto DNA (12, 13). Our model simplifies the loading of Tat onto the LTR and might also explain the ability of Tat to activate transcription via DNA (45). Like GAL11, Tat could recruit the holoenzyme when tethered to DNA (3, 14). Moreover, our model does not negate the possibility that nascent TAR also attracts additional free Tat to increase transcriptional processivity (28). Similar to competing step-by-step assembly of the GTFs on the promoter (51) and the single-step binding of the holoenzyme (30), these modes of recruitment of Tat to the LTR might be complementary and additive. Future work on template commitment, the ability of TAR decoys to deplete Tat at different stages of transcription initiation and elongation, and pulse-chase analyses of Tat on initiating and elongating complexes will reveal further mechanistic details of these interactions among Tat, TAR, and the transcriptional machinery.

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