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Human immunodeficiency virus type 1 and 2 Tat proteins specifically interact with RNA polymerase II

(transcription factors/gene expression)

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ABSTRACT **The Tat-responsive region (TAR) element is a critical RNA regulatory element in the human immunodeficiency virus (HIV) long terminal repeat, which is required for activation of gene expression by the transactivator protein Tat. Recently, we demonstrated by gel-retardation analysis that RNA polymerase II binds to TAR RNA and that Tat prevents this binding even when Tat does not bind to TAR RNA. These results suggested that direct interactions between Tat and RNA polymerase II may prevent RNA polymerase II pausing and lead to Tat-mediated increases in transcriptional elongation. To test this possibility, we performed protein interaction studies with RNA polymerase II and both the HIV-1 and the closely related HIV-2 Tat protein. These studies indicated that both the HIV-1 and HIV-2 Tat proteins could specifically interact with RNA polymerase II. Mutagenesis of both HIV-1 and HIV-2 Tat demonstrated that the basic domains of both the HIV-1 and HIV-2 Tat proteins were required for this interaction. Furthermore, "far Western" analysis suggested that the largest subunit of RNA polymerase II was the site for interaction with Tat. The interactions between Tat and RNA polymerase II were of similar magnitude to those detected between RNA polymerase II and the cellular transcription factor RAP30, which stably associates with RNA polymerase II during transcriptional elongation. These studies are consistent with the model that RNA polymerase II is a cellular target for Tat resulting in Tat-mediated increases in transcriptional elongation from the HIV long terminal repeat.**

The control of human immunodeficiency virus 1 (HIV-1) gene expression depends on the binding of cellular transcription factors to distinct regulatory elements in the long terminal repeat (LTR) (1). Mutagenesis has demonstrated that at least three elements in the HIV-1 LTR including Sp1, TATA, and Tat-responsive region (TAR) are critical for activation of gene expression in response to the transactivator protein Tat (1). Of these regulatory elements, TAR is especially critical for Tat activation (2–5). TAR, which extends from +1 to +59 relative to the HIV-1 transcription initiation site, forms a stable RNA stem-loop structure that contain distinct loop and bulge sequences (4–6). The bulge binds the Tat protein (7), whereas the loop binds a cellular protein TRP-185 (8, 9). The TAR RNA bulge and the loop elements are also critical for the binding of RNA polymerase II to TAR RNA (10). Thus, TAR may function to activate transcription by the sequential binding of both viral and cellular factors. In contrast to the single TAR element found in the HIV-1 LTR, HIV-2 contains a duplicated TAR RNA structure that is critical for Tat activation (11). However, the bulge and the loop sequences in the HIV-1 and HIV-2 TAR elements are highly conserved (11). Insertion of the TAR element into heterologous promoter constructs can confer Tat-responsiveness to these promoters (12). Thus, an understanding of the mechanism of TAR function is critical for elucidating the pathway leading to Tat activation of the HIV-1 and HIV-2 LTR.

The HIV-1 and HIV-2 Tat proteins have a similar domain structure containing a cysteine, core, and basic domains each of which is critical for Tat function (1). The HIV-1 Tat protein will activate gene expression of the HIV-1 and HIV-2 LTRs to similar levels while the HIV-2 Tat protein activates the HIV-1 LTR to a much lesser degree than the HIV-1 Tat protein (11). However, a single amino acid change between the basic and core domains of Tat2, which converts glutamine to glycine at amino acid residue 77, results in an HIV-2 Tat protein that is able to activate the HIV-1 LTR to similar levels as the HIV-1 Tat (13). Thus, it is likely that HIV-1 and HIV-2 Tat proteins use similar transcriptional mechanisms to activate gene expression from TAR RNA. The results of both nuclear run-on and *in vitro* transcription analysis indicate that Tat markedly increases the transcriptional elongation properties of complexes containing RNA polymerase II that transcribe the HIV-1 and HIV-2 LTRs (14–16). This suggests that Tat either directly or indirectly must alter the activity of cellular factors that compose the transcriptional elongation complex.

One of the potential targets for Tat interaction is RNA polymerase II (10). Recently, we demonstrated that wild-type but not mutant HIV-1 TAR RNA specifically binds RNA polymerase II, suggesting that pausing of RNA polymerase II by TAR RNA may be required for subsequent Tat activation (10). Furthermore, RNA polymerase II was unable to stably bind to TAR RNA in the presence of wild-type Tat but not a Tat basic mutant, and this effect was seen with both wild-type TAR RNA and a mutant TAR RNA that was unable to bind Tat. This result suggested that direct interactions between Tat and RNA polymerase II occur and are involved in stimulating the transcriptional elongation properties of RNA polymerase II. In the current study, we extend these observations and show that RNA polymerase II directly interacts with both the HIV-1 and HIV-2 Tat proteins and that RNA polymerase II is a cellular target for Tat interaction.

MATERIALS AND METHODS

Plasmid Constructs and Antibodies. The HIV-1 Tat (Tat1) from amino acids 1 to 72 was expressed as a fusion with glutathione *S*-transferase (GST; Stratagene) (8). A Tat mutant in the basic domain of Tat between residues 52 and 57, in the first cysteine residue in each of four Cys-Xaa-Xaa-Cys motifs, and a Lys-41 → Ala mutant were used in these studies (8). The wild-type and mutant HIV-2 Tat clones, GST-Tat2 and GST-Tat2Δ 84, were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and contain

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; GST, glutathione *S*-transferase; TAR, Tat-responsive region.

the GST moiety followed by a thrombin cleavage site and either residues 1–99 or 1–83 (17). The Tat2 CL mutant contains a Cys-50 → Ser substitution and a Lys-70 → Ala substitution.

A portion of the largest subunit of RNA polymerase II extending from amino acids 1325 to 1630 (18) and a portion of the second largest subunit of RNA polymerase II extending from amino acids 1 to 550 (19) were fused to GST. The full-length CREB cDNA extending from amino acids 1 to 341 and either wild-type RAP30 extending from amino acid residues 1 to 249 (20) or a RAP30 truncation mutant extending from amino acids 1 to 101 (21, 22) were constructed by PCR and inserted into pGEX-2T for expression in bacteria. Fusion proteins including GST, GST-Tat1, GST-Tat2, GST-p180, and GST-p140 were affinity purified following *Escherichia coli* expression as described (8) and were used as antigens to obtain rabbit polyclonal antisera. The monoclonal antibody 8WG16 (23) directed against the C-terminal domain of RNA polymerase II was purchased from Promega.

Preparation of Immobilized Tat and RNA Polymerase II. RNA polymerase II was isolated from 1000 g of calf thymus, as described (24). Nuclear extract was prepared from HeLa cells by the method of Dignam *et al.* (25) and the C fraction from the phosphocellulose column was obtained by collecting the 0.3–0.5 M KCl eluate of HeLa cells (26).

GST-fusion proteins were prepared and coupled to glutathione agarose as described (8). To prepare immobilized RNA polymerase II, 225 μg of RNA polymerase II was dialyzed against coupling buffer [50 mM Hepes·KOH, pH 7.9/100 mM NaCl/10% (vol/vol) glycerol/0.1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride]. The RNA polymerase II was coupled to 80 μl of Affi-Gel 10 (Bio-Rad) following the manufacturers protocol (27). Glutathione agarose beads containing the fusion proteins previously described were incubated with 5 μg of calf thymus RNAP II or 30 μg of HeLa nuclear extract fraction C at 4°C for 12 hr. The matrix was pelleted and washed three times with 500 μl of binding buffer; the proteins remaining on the matrix were solubilized, resolved on SDS/PAGE, and transferred to nitrocellulose for immunoanalysis. To assay Tat binding to immobilized RNA polymerase II, ≈5 μg of immobilized RNA polymerase II (27) was incubated with 500 ng of the HIV-1 and HIV-2 Tat proteins in 200 μl of binding, and immunoblot analysis was done by using the ECL-protein detection system (Amersham).

Purification and Labeling of HIV-1 and HIV-2 Tat. Tat proteins were isolated on glutathione agarose matrix and cleaved with 5 units of thrombin (Sigma). To remove the thrombin, the native Tat proteins were applied to a heparin-agarose column, the column was washed with buffer containing 200 mM NaCl, and Tat was eluted with buffer containing 800 mM NaCl and dialyzed. Native Tat was of >95% purity as judged by Coomassie staining of an SDS/PAGE gel. To prepare ^{32}P-labeled Tat1, glutathione agarose containing GST fusions with either wild-type or Tat1 basic mutant was labeled with 200 units of the cAMP-dependent protein kinase catalytic subunit (Promega) and 0.33 mCi (7000 Ci/mmol; 1 Ci = 37 GBq) of γ-^{32}P-ATP (ICN) for 1 hr at 25°C. The matrix was washed free of unincorporated label, and the labeled Tat proteins were eluted with 60 mM glutathione. Specific activity was normalized by adding unlabeled protein to a specific activity of 1×10^6 cpm/μg of protein, and the Tat protein at 300,000 cpm/ml was used in "far-Western" analysis as described (28).

RESULTS

Tat Interacts with the Largest Subunit of RNA Polymerase II. To determine whether Tat directly interacted with RNA polymerase II, it was necessary to obtain purified preparations of RNA polymerase II. RNA polymerase II contains 10 subunits with the largest subunit being composed of three species of 240 (IIo), 210 (IIa), and 180 (IIb) kDa, respectively, which are due to differences in the phosphorylation state (IIo vs. IIa) or proteolysis (IIa vs. IIb) (29). The second largest subunit (IIc) of RNA polymerase II is 140 kDa, while other subunits of 34, 25, 22, 18, 19, 16, 14, and 13 kDa have been isolated (29).

Calf thymus has been used as a source to obtain abundant quantities of purified RNA polymerase II for biochemical characterization (24). RNA polymerase II was purified from calf thymus according to standard methods and analyzed by SDS/PAGE followed by Coomassie staining (Fig. 1*A*). The 210-kDa species in addition to the predominant 180-kDa form of the largest subunit were noted (Fig. 1*A*). The 180-kDa species, which is generated by proteolysis of the C-terminal domain (CTD) of the 210-kDa subunit, has been demonstrated to be the predominant species isolated from calf thymus during purification of RNA polymerase II. No detectable IIo form of RNA polymerase II is found in calf thymus preparations (24). However, RNA polymerase II preparations containing the 180-kDa form of the largest subunit are transcriptionally active in reconstituted *in vitro* transcription assays (24). The 140-kDa second largest subunit of RNA polymerase II (19, 29), in addition to the 34- and 25-kDa polymerase subunits, were also detected (Figs. 1*A* and 2*A*). The 68-kDa species was due to a small amount of bovine serum albumin that was added to our RNA polymerase II preparations to maintain activity while the 43-kDa species is a contaminant that has previously been noted (24) (Fig. 2*A*). This calf thymus preparation of RNA polymerase II was active in both α-^{32}P[UTP] incorporation into calf thymus DNA and *in vitro* transcription assays with the HIV-1 LTR (data not shown).

Immunoblot analysis was next done with antibodies directed against the largest and second largest subunits of RNA polymerase II to demonstrate its immunologic properties. Monoclonal antibodies directed against the C-terminal domain in the

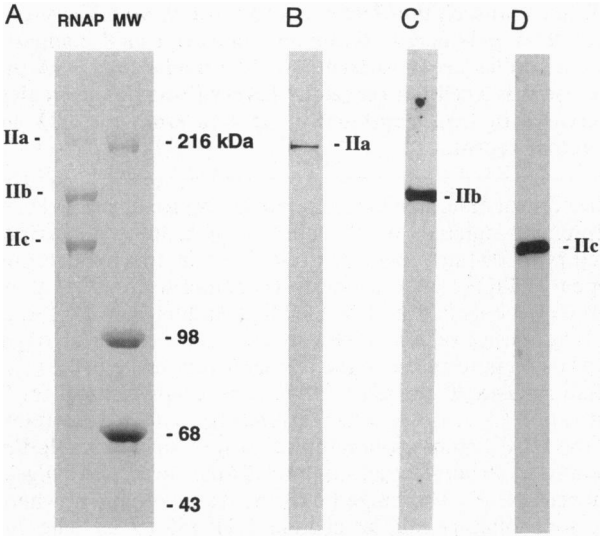

FIG. 1. Characterization of calf thymus preparations of purified RNA polymerase II. (*A*) SDS/PAGE was done with 10 μg of RNA polymerase II purified from calf thymus followed by Coomassie staining of the 5% SDS/PAGE. Positions of the full-length (IIa) and proteolytic form (IIb) of the largest subunit and the second largest subunit (IIc) of RNA polymerase II are indicated. Immunoblot analysis of this preparation of RNA polymerase II was done with a monoclonal antibody directed against the C-terminal domain of the largest subunit of RNA polymerase II (*B*), a rabbit polyclonal antibody directed against an internal portion of the largest subunit of RNA polymerase II (*C*), or a rabbit polyclonal antibody directed against the second largest subunit of RNA polymerase II (*D*). Positions of the 210-kDa (IIa) (*B*), 180-kDa (IIb) (*C*), and 140-kDa (IIc) (*D*) forms of RNA polymerase II are indicated.

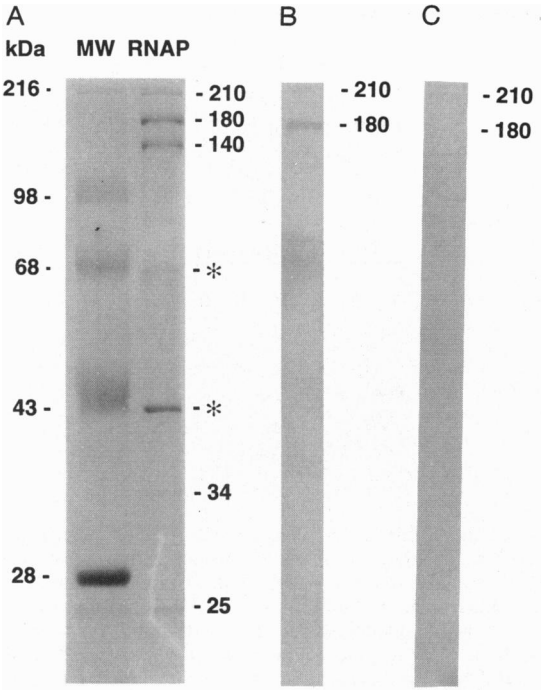

FIG. 2. HIV-1 Tat interacts with the largest subunit of RNA polymerase II. (*A*) Purified RNA polymerase II isolated from calf thymus was subjected to SDS/PAGE followed by Coomassie staining; positions of the 210-, 180-, 140-, 34-, and 25-kDa polymerase subunits and the 68-kDa and 43-kDa contaminant proteins are shown. The polyacrylamide gels were transferred to nitrocellulose filters, and the filters were blocked with 1% milk and probed with either a wild-type Tat1 (*B*) or a Tat1 basic mutant (*C*) that was labeled with ^{32}P at a kinase site engineered into their carboxyl termini. Filters were then washed extensively and subject to autoradiography.

largest subunit of RNA polymerase II (23) (Fig. 1*B*) in addition to rabbit polyclonal antibodies directed against domains in either the largest subunit (Fig. 1*C*) or the second largest subunit of RNA polymerase II (Fig. 1*D*) were tested in immunoblot analysis. The monoclonal antibody directed against the C-terminal domain reacted with the 210-kDa species (Fig. 1*B*), the rabbit polyclonal antibody directed against the largest subunit reacted with the 180-kDa species (Fig. 1*C*), while the rabbit polyclonal antibody directed against the second largest subunit reacted with the 140-kDa species (Fig. 1*D*). The failure of the rabbit polyclonal antibody that was directed against the largest subunit of the RNA polymerase II to react with 210-kDa species was due to the fact that this species was present at only 10% of the level as the 180-kDa species and the decreased sensitivity of the rabbit polyclonal antibody as compared to the monoclonal antibody directed against the C-terminal domain. However, with 10-fold more of the calf thymus RNA polymerase II, the 210-kDa species could be detected with the rabbit polyclonal antibody directed against the largest subunit of the polymerase (data not shown).

Our previous observations using gel-retardation assays with TAR RNA and RNA polymerase II demonstrated that wild-type Tat, but not a Tat basic mutant, prevented stable binding of RNA polymerase II to TAR RNA (10). These results suggested that Tat could potentially interact with RNA polymerase II. Wild-type and Tat1 basic mutant proteins were constructed that contained a cAMP-dependent protein kinase A recognition site in their carboxyl termini to facilitate ^{32}P labeling. To determine whether either of these Tat proteins interacted directly with the RNA polymerase II, far-Western analysis was done (28). In this assay, the calf thymus RNA polymerase II was first subjected to SDS/PAGE, transferred to nitrocellulose, and probed with ^{32}P-labeled wild-type Tat1 or a Tat1 basic mutant. Wild-type Tat1 bound predominantly to a 180-kDa form of RNA polymerase II, though a slight degree of binding to the 210-kDa form was noted (Fig. 2*B*) while the Tat1 basic mutant did not bind to these species (Fig. 2*C*). No binding was detected to the 140-kDa second largest subunit or other smaller RNA polymerase II subunits (Fig. 2*A*). Thus, the wild-type HIV-1 Tat protein was able to specifically interact with the largest subunit of RNA polymerase II.

Both HIV-1 and HIV-2 Tat Proteins Specifically Interact with RNA Polymerase II. The specificity of the interaction between Tat and RNA polymerase II was then studied in detail. Furthermore, we wished to determine whether the HIV-2 Tat protein (Tat2) like HIV-1 Tat protein (Tat1) could also interact with RNA polymerase II. It was also important to investigate which domains in the Tat protein were critical for this interaction. A variety of mutants in the cysteine, core, or basic domains of Tat that were defective for activation of HIV-1 and HIV-2 gene expression (1) were used in these experiments to determine the domains which interacted with RNA polymerase II. GST fusions containing either wild-type Tat1 or Tat2 or mutants in different domains of these proteins were coupled to glutathione agarose beads. Other controls such as glutathione agarose beads alone or these beads coupled to either GST or GST-CREB were used to further demonstrate the specificity of Tat interaction with RNA polymerase II. Similar quantities of each of the GST fusion proteins were bound to glutathione-agarose beads and incubated with RNA polymerase II purified from calf thymus. After this incubation, the beads were extensively washed and subjected to SDS/PAGE; immunoblot analysis was done with antibodies directed against the largest or the second largest subunits of RNA polymerase II or the GST moiety.

Wild-type HIV-1 and HIV-2 Tat proteins were each able to specifically interact with RNA polymerase II as reflected by the presence of the largest and second largest subunits of RNA polymerase II that remained bound to the Tat beads after extensive washing (Fig. 3 *A* and *B*, lanes 4–9). Mutants in the cysteine and core domains of both HIV-1 and HIV-2 Tat were also able to interact with RNA polymerase II (Fig. 3 *A* and *B*, lanes 6, 7, and 11). However, substitutions or deletions in the basic domains of Tat1 and Tat2 were unable to bind to RNA polymerase II as reflected in the fact that neither the largest nor the second largest subunits of RNA polymerase II remained bound to these mutant Tat proteins (Fig. 3 *A* and *B*, lanes 5 and 10). CREB, a bZIP protein that contains a region of 12 basic amino acids that facilitates its DNA-binding properties to cAMP-responsive promoter elements (20), was unable to bind to RNA polymerase II, indicating that the polymerase did not bind nonspecifically to any basic amino acid domain (Fig. 3 *A* and *B*, lane 8). There was no interaction of RNA polymerase II with either glutathione-agarose beads or these beads coupled with GST alone (Fig. 3 *A* and *B*, lanes 2 and 3). Immunoblot analysis confirmed that relatively equal amounts of the GST fusion proteins were coupled to the glutathione-agarose beads (Fig. 3*C*). These results indicated that both the HIV-1 and HIV-2 Tat proteins were able to specifically interact with RNA polymerase II as reflected in the binding of the largest and second largest subunits of RNA polymerase II to Tat. These results, in conjunction with the far-Western analysis, suggest that Tat interacts with the multisubunit RNA polymerase II complex upon its direct binding to the largest subunit of the RNA polymerase.

HIV-1 and HIV-2 Tat Proteins Interact with the Immobilized RNA Polymerase II. Next, we investigated whether native HIV-1 and HIV-2 Tat proteins cleaved from the GST moiety by treatment with thrombin could interact with RNA polymerase II purified from calf thymus and immobilized on an Affi-Gel 10 resin (27). The activity of the native Tat proteins was confirmed by *in vitro* transcription analysis with the HIV-1

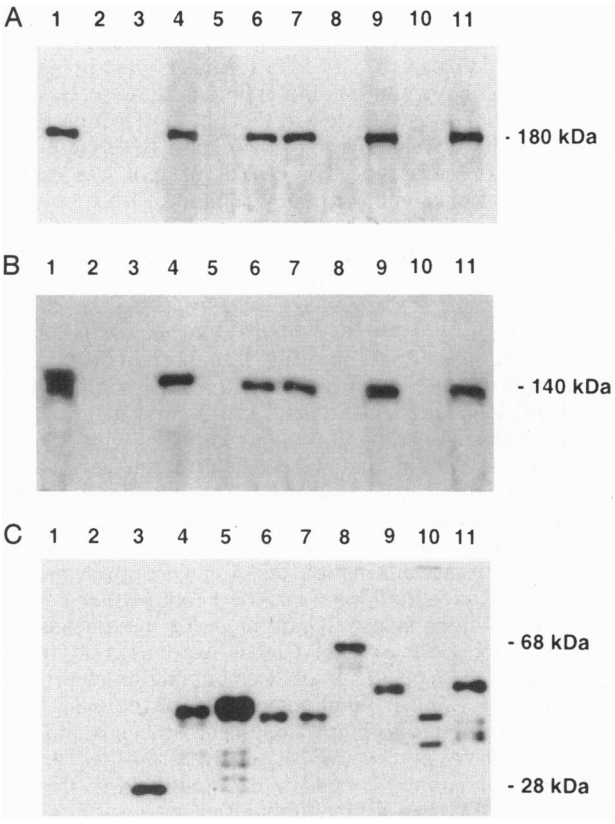

FIG. 3. Immobilized HIV-1 and HIV-2 Tat proteins bind to RNA polymerase II. GST fusion proteins were immobilized on glutathione-agarose beads, and incubated with RNA polymerase II was purified from calf thymus. After extensive washing, proteins that remained associated with matrix were resolved by SDS/PAGE, transferred to nitrocellulose, and probed with antiserum directed against the largest subunit of RNA polymerase II (*A*), the second largest subunit of RNA polymerase II (*B*), or a polyclonal antibody directed against the GST moiety (*C*). The loading order of samples is 30% of the input of RNA polymerase II (lanes 1), glutathione-agarose beads alone (lanes 2), or these beads coupled with either GST (lanes 3), GST–Tat1 (lanes 4), GST–Tat1 basic mutant (lanes 5), GST–Tat1 lysine mutant 41 (lanes 6), GST–Tat1 cysteine mutant (lanes 7), GST–CREB (lanes 8), GST–Tat2 (lanes 9), GST–Tat2 basic mutant (lanes 10), or a GST–Tat2 cysteine and lysine mutant (lanes 11). Positions of the largest (180 kDa) and second largest (140 kDa) subunits of RNA polymerase II and the different GST fusion proteins are indicated.

and HIV-2 LTRs and RNA gel retardation studies with HIV-1 and HIV-2 TAR RNAs (data not shown). As a control, this same resin was also coupled to lysozyme and tested for interactions with HIV-1 and HIV-2 Tat. Wild-type Tat1 and Tat2 proteins were able to bind to RNA polymerase II immobilized on Affi-Gel 10 as determined by immunoblot analysis using specific antibodies directed against either Tat1 or Tat2 (Fig. 4 *A* and *B*, lane 3). However, there was no binding of wild-type HIV-1 and HIV-2 Tat proteins to resin-coupled to lysozyme (Fig. 4 *A* and *B*, lane 2), nor did basic domain Tat mutants bind to the immobilized RNA polymerase II (Fig. 4 *A* and *B*, lane 6). These results demonstrated that native Tat1 and Tat2 proteins could interact with RNA polymerase II. Furthermore, we confirmed that the basic domain mediates HIV-1 and HIV-2 Tat interactions with RNA polymerase II.

HIV-1 and HIV-2 Tat Proteins Interact with RNA Polymerase II Present in HeLa Nuclear Extract. Finally, we wished to address whether Tat could interact with RNA polymerase II present in HeLa nuclear extract. In contrast to the presence of the 180-kDa form of the largest subunit of RNA polymerase II that is found in preparations isolated from calf thymus (24), HeLa nuclear extract contains predominantly the 210-kDa

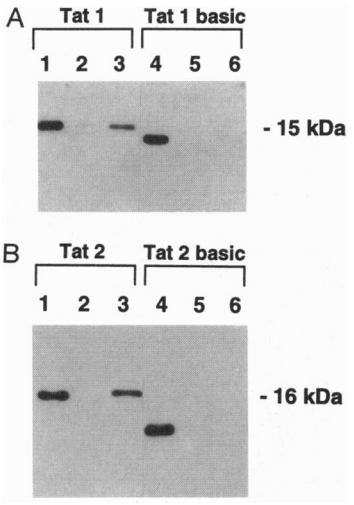

FIG. 4. Immobilized RNA polymerase II binds to Tat1 and Tat2. Either RNA polymerase II purified from calf thymus or lysozyme were covalently coupled to an Affi-Gel 10 matrix and then incubated with native wild-type or mutant HIV-1 and HIV-2 Tat proteins. After extensive washing, proteins that remained bound to the RNA polymerase II or the lysozyme coupled to Affi-Gel 10 were resolved on SDS/PAGE and transferred to nitrocellulose. The Tat proteins in each binding reaction were detected by immunoblot analysis using rabbit polyclonal antisera directed against the entire portion of the native HIV-1 and HIV-2 Tat proteins for either Tat1 (lanes 1–3) and the Tat1 basic mutant (lanes 4–6) (*A*) or Tat2 (lanes 1–3) and the Tat2 basic mutant (lanes 4–6) (*B*). Thirty percent of the input of HIV-1 and HIV-2 Tat proteins (lanes 1 and 4), Tat bound to the lysozyme-coupled matrix (lanes 2 and 5), or Tat bound to the RNA polymerase II-coupled matrix (lanes 3 and 6) are shown.

form of the largest subunit which has an intact C-terminal domain. HeLa nuclear extract was fractionated using phosphocellulose chromatography followed by step elution with 0.1, 0.35, 0.50, or 1.0 M KCl concentrations (26). Our studies indicate that RNA polymerase II is present predominantly in the C fraction of HeLa nuclear extract when eluted with KCl by phosphocellulose chromatography (data not shown). The HeLa fraction C was incubated with glutathione beads containing either wild-type or basic mutants of Tat1 and Tat2 in addition to wild-type or mutant forms of RAP30. RAP30 along with its dimeric partner RAP74 composes the transcription factor TFIIF that functions in both transcriptional initiation and elongation by facilitating the binding of RNA polymerase II to the transcriptional preinitiation complex and also by inhibiting the pausing of the elongating RNA polymerase II (29). RAP30 has been demonstrated to directly associate with RNA polymerase II and the domains that facilitate this interaction have been defined (21, 22, 27). Thus, we could compare the binding of RNA polymerase II to both wild-type and mutant RAP30 and Tat proteins.

Immunoblot analysis was done with monoclonal antibody directed against the RNA polymerase II C-terminal domain. Wild-type Tat1, Tat2, and RAP30 were each able to interact with RNA polymerase II (Fig. 5*A*, lanes 2, 4, and 6). In contrast there was no binding of RNA polymerase II detected with the Tat1 and Tat2 basic mutants nor a RAP30 mutant that was deleted in its binding site for RNA polymerase II (Fig. 5*A*, lanes 3, 5, and 7). The second largest subunit of RNA polymerase II also bound to the immobilized Tat and RAP30 proteins (data not shown). Coomassie staining of an SDS/PAGE indicated that the amounts of wild-type and mutant Tat and RAP30 proteins coupled to GST beads were relatively similar (Fig. 5*B*). Thus Tat, like the well-characterized transcription factor RAP30, binds specifically to RNA polymerase II.

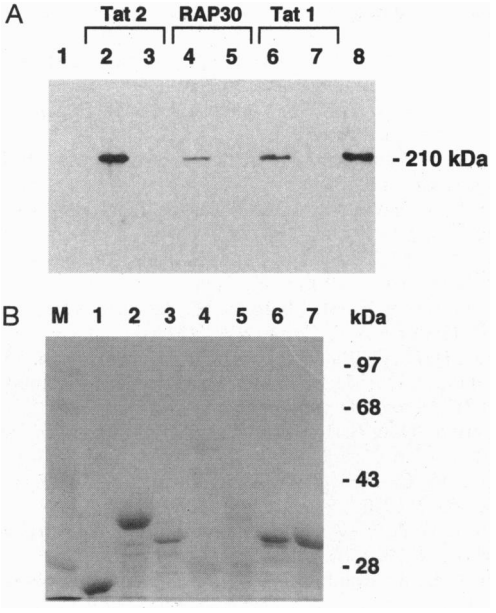

FIG. 5. Immobilized HIV-1 and HIV-2 Tat proteins bind to RNA polymerase II isolated from HeLa nuclear extract. Glutathione-agarose matrices containing either the specified wild-type or mutant Tat or RAP30 proteins were incubated with 30 μg of the phosphocellulose C fraction obtained by fractionation of HeLa nuclear extract. Proteins remaining on beads after extensive washing were resolved by SDS/PAGE and transferred to nitrocellulose; immunoblot analysis was done with a monoclonal antibody directed against the RNA polymerase II C-terminal domain. (*A*) Amount of RNA polymerase II bound to GST (lane 1), GST–Tat2 (lane 2), GST–Tat2 basic mutant (lane 3), GST–RAP30 (lane 4), a GST–RAP30 deletion mutant (lane 5), GST–Tat1 (lane 6), GST–Tat1 basic mutant (lane 7), and the nuclear extract fraction C input (lane 8) are shown. (*B*) A Coomassie-stained polyacrylamide gel containing each of the glutathione-agarose-coupled proteins in *A* is shown.

DISCUSSION

The HIV-1 and HIV-2 Tat proteins are critical for the control of HIV gene expression and replication (1). TAR RNA appears to be the key element influencing subsequent Tat activation (1–6) and serves as the binding site for both Tat (7) and a cellular protein TRP-185 (8, 9). In addition, we recently demonstrated that RNA polymerase II also binds to TAR RNA, and the same regulatory elements in TAR RNA that are critical for the *in vivo* function of TAR are also critical for the binding of RNA polymerase II (10). The fact that wild-type, but not a mutant, Tat protein could prevent the stable binding of RNA polymerase II to TAR RNA even when Tat was unable to bind to TAR RNA suggested that Tat could directly interact with RNA polymerase II (10). In the current study, we investigated this possibility using protein interaction studies with wild-type and mutant HIV-1 and HIV-2 Tat proteins. The basic domain of Tat functions in binding to TAR RNA and in nuclear localization of Tat (1, 7, 8). Mutations in this domain ablate Tat activation from both the HIV-1 and HIV-2 LTR (1, 13). Our studies reveal that the basic domain of the HIV-1 and HIV-2 Tat proteins specifically interact with the largest subunit of RNA polymerase II and that this interaction may be a requirement for increasing the transcriptional elongation properties of RNA polymerase II.

TAR may serve as an RNA attenuator element that is capable of pausing the HIV transcriptional elongation complex (30). However, unlike classical RNA attenuator elements, disruption of TAR does not markedly increase HIV transcription (1–6). This may be due to the fact that pausing of the HIV transcriptional elongation complex by TAR RNA could be required for inducing a conformational change in RNA polymerase II or facilitating the association of RNA polymerase II with Tat. Thus, TAR RNA may serve as both a positive and negative transcriptional regulatory element with pausing of the transcriptional elongation complex being required for subsequent Tat stimulation. Tat, like the elongation factor RAP30 (21, 22), may then become stably associated with the transcriptional elongation complex to increase its processivity. Further studies will be necessary to address this point and to characterize specific cellular transcription factors that are able to stably associate with the HIV transcriptional elongation complex.

Tat has also been demonstrated to interact with several other transcription factors including Sp1 (31), TATA-binding protein (TBP) (32), and TAF55 (33). Though detailed mutagenesis was not done in all of these studies to define sites of interaction, Tat may have multiple cellular targets in the HIV transcription complex. For TBP, amino acids 36–50 in the HIV-1 Tat protein were critical for interaction with TBP (32). For example, Tat could become associated with the HIV preinitiation complex where it associates with the TBP/TAF complex and then be delivered to the transcriptional elongation complex after initiation of transcription. The structure of the HIV-1 promoter, especially the sequences of the TATA and Sp1 elements, may assemble transcription complexes able to efficiently recruit Tat. This mechanism may explain why mutations of these elements severely alter the levels of both basal and Tat-induced gene expression. Perhaps passage of the transcriptional complex through TAR leads to the transfer of Tat from components of the preinitiation complex to the transcriptional elongation complex.

The basic domain of the HIV-1 and HIV-2 Tat proteins that interact with RNA polymerase II are similar to an arginine-rich RNA-binding domain found in the λ phage antiterminator protein N (34). N binds to an RNA transcribed from a region of lambda known as the nut site. N forms a complex with four *E. coli* host proteins that include Nus A, Nus B, Nus E, and the ribosomal protein S10 that bind to RNA transcribed from the nut site (34). This complex interacts with the *E. coli* RNA polymerase to prevent its pausing at RNA terminator sites. It remains unclear how N prevents this pausing, but the possibility has been raised that N itself may directly contact the polymerase to stabilize a terminator-resistant polymerase. The fact that RNA polymerase II binding to TAR RNA requires host-cell factors (10) and that the arginine-rich Tat protein interacts with RNA polymerase II has many similarities to the model of N-mediated antitermination. Thus, TAR-induced pausing of RNA polymerase II with subsequent association of Tat may have evolved from a well-characterized model in prokaryotic systems to modulate the transcriptional elongation properties of RNA polymerase (34).

Though the basic domain of Tat interacts with RNA polymerase II, the question arises concerning the role of the cysteine and core domains of Tat that are also critical for its function. Recent data suggest that this activation domain of Tat interacts with a specific cellular kinase that can phosphorylate the C-terminal domain of RNA polymerase II (35). We have recently confirmed this result. Thus, an attractive model to explain the function of Tat would be based on its ability to associate with a cellular kinase that binds to its activation domain and RNA polymerase II that binds to its basic domain. Recruitment of these proteins would result in phosphorylation of RNA polymerase II C-terminal domain with subsequent association of specific cellular factors that increase HIV transcriptional elongation. Further studies to characterize the cellular and viral factors associated with the HIV transcriptional elongation complex will be critical for a better understanding of the mechanism of Tat activation.

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1. Gaynor, R. (1992) *AIDS* **6,** 347–363.
2. Harrich, D., Hsu, C., Race, E. & Gaynor, R. B. (1994) *J. Virol.* **68,** 5899–5910.
3. Rosen, C. A., Sodoroski, J. G. & Haseltine, W. A. (1985) *Cell* **41,** 813–823.
4. Feng, S. & Holland, E. C. (1988) *Nature (London)* **334,** 165–167.
5. Berkhout, B., Silverman, R. H. & Jeang, K. T. (1989) *Cell* **59,** 273–282.
6. Muesing, M. A., Smith, D. H. & Capon, D. J. (1987) *Cell* **48,** 691–701.
7. Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Heaphy, S., Karn, J., Lowe, A. D., Singh, M. & Skinner, M. A. (1990) *EMBO J.* **9,** 4145–4153.
8. Wu, F., Garcia, J., Sigman, D. & Gaynor, R. B. (1991) *Genes Dev.* **5,** 2128–2140.
9. Sheline, C. T., Milocco, L. H. & Jones, K. A. (1991) *Genes Dev.* **5,** 2508–2520.
10. Wu-Baer, F., Sigman, D. & Gaynor, R. B. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6213–6217.
11. Emerman, M., Guyader, M., Montagnier, L., Baltimore, D. & Muesing, M. A. (1987) *EMBO J.* **6,** 3755–3760.
12. Ratnasabapathy, R., Sheldon, M., Johal, L. & Hernandez, N. (1990) *Genes Dev.* **4,** 2061–2074.
13. Elangovan, B., Subramanian, T. & Chinnadurai, G. (1992) *J. Virol.* **66,** 2031–2036.
14. Laspia, M. F., Rice, A. P. & Mathews, M. B. (1989) *Cell* **59,** 283–292.
15. Marciniak, R. A., Garcia-Blanco, M. A. & Sharp, P. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 3624–3628.
16. Kato, H., Sumimoto, H., Pognonec, P., Chen, C. H., Rosen, C. A. & Roeder, R. G. (1992) *Genes Dev.* **6,** 655–666.
17. Rhim, H., Chan, F., Echetebu, C. O. & Rice, A. P. (1993) *Protein Expression Purif.* **4,** 24–31.
18. Wintzerith, M., Acker, J., Vicaire, S., Vigneron, M. & Kedinger, C. (1992) *Nucleic Acids Res.* **20,** 910.
19. Acker, J., Wintzerith, M., Vigneron, M. & Kedinger, C. (1992) *J. Mol. Biol.* **226,** 1295–1299.
20. Yin, M.-J., Paulssen, E. J., Seeler, J.-S. & Gaynor, R. B. (1995) *J. Virol.* **69,** 3420–3432.
21. Tan, S., Conaway, R. C. & Conaway, J. W. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6042–6046.
22. Horikoshi, M., Fujita, H., Wang, J., Takeda, R. & Roeder, R. G. (1991) *Nucleic Acids Res.* **19,** 5436.
23. Thompson, N. E., Steinberg, T. H., Aronson, D. B. & Burgess, R. R. (1989) *J. Biol. Chem.* **264,** 11511–11520.
24. Hodo, H. G., & Blatti, S. P. (1977) *Biochemistry* **16,** 2334–2343.
25. Dignam, J. D., Martin, P. L., Shastry, B. S. & Roeder, R. G. (1983) *Methods Enzymol.* **101,** 582–598.
26. Reinberg, D. & Roeder, R. G. (1987) *J. Biol. Chem.* **262,** 3310–3321.
27. Sopta, M., Carthew, R. W. & Greenblatt, J. (1985) *J. Biol. Chem.* **260,** 10353–10360.
28. Feaver, W. J., Svejstrup, J. Q., Henry, N. L. & Kornberg, R. D. (1994) *Cell* **79,** 1103–1109.
29. Zawel, L. & Reinberg, D. (1995) *Annu. Rev. Biochem.* **64,** 533–561.
30. Bengal, E. & Aloni, Y. (1991) *J. Virol.* **65,** 4910–4918.
31. Jeang, K.-T., Chun, R., Lin, N. H., Gatignol, A., Glabe, C. G. & Fan, H. (1993) *J. Virol.* **67,** 6224–6233.
32. Kashanchi, F., Piras, G., Radonovich, M. F., Duvall, J. F., Fattaey, A., Chiang, C.-M., Roeder, R. G. & Brady, J. N. (1994) *Nature (London)* **367,** 295–299.
33. Cheng-Ming, C. & Roeder, R. G. (1995) *Science* **267,** 531–534.
34. Greenblatt, J., Nodwell, J. & Mason, S. (1993) *Nature (London)* **364,** 401–406.
35. Herrman, C. & Rice, A. P. (1995) *J. Virol.* **69,** 1612–1620.