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Transcriptional *trans* Activation by Human Immunodeficiency Virus Type 1 Tat Requires Specific Coactivators That Are Not Basal Factors

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Expression of human immunodeficiency virus type 1 (HIV-1) genes is regulated by the *trans* activator Tat. Tat exerts its effects by increasing the rate of transcription, but the mechanism by which it does so is still unknown. To study the cellular factors required for Tat *trans* activation, we have expressed functional Gst-Tat fusion protein and used it to construct affinity columns. Our findings are as follows. (i) A Gst-Tat affinity matrix depleted HeLa nuclear extracts of a factor(s) required for Tat function. A Tat mutant bearing the missense mutation lysine to alanine at position 41 was incapable of this depletion. (ii) Tat *trans* activation was recovered by addition of unfractionated nuclear extract, the 0.5 M KCl elution fraction from the Tat affinity column, or sedimentation gradient fractions of HeLa extracts. The activity from the gradients sedimented with an apparent molecular mass of 200 kDa. (iii) Tat *trans* activation could not be recovered by use of recombinant human TATA-binding protein or partially purified TFIID. (iv) *trans* activation by Tat was blocked by heating of the nuclear extract under conditions in which basal transcription was not decreased. Our data demonstrate for the first time the existence of unique Tat coactivators distinct from factors required for general basal transcription.

Tat, the transcriptional *trans*-activating human immunodeficiency virus type 1 (HIV-1) protein, has been widely studied (13, 36). Among *trans* activators, Tat presents several uncommon features: it binds an RNA element, the *trans*-activation response element (TAR), to exert its function, and it does not have a classic activation domain. The binding of Tat to TAR and the structural characteristics of the RNA element have been elucidated (3, 11, 17, 18, 21, 22, 31, 34, 53, 60, 61, 63, 78). Tat binds to the RNA bulge (17, 60, 78), but since the Tat-TAR interaction is insensitive to mutations in the TAR loop, which in vivo result in inhibition of Tat-mediated *trans* activation (21), it was postulated that host proteins recognize the loop. The role of these host proteins was suggested to be the stabilization of Tat-TAR binding. In fact, several loop-binding proteins have been described (23, 50, 65, 79), but the biological significance of these proteins remains obscure.

At present, the mechanism by which Tat increases HIV-1 transcription is still unknown. In vitro and in vivo studies have shown effects of Tat that can be interpreted as affecting both the initiation and the elongation phases of transcription (12, 20, 40, 42, 49, 51, 69). Moreover, posttranscriptional effects of Tat have also been described (6, 7). The requirement for the proximity of TAR to the start site of transcription suggests a role for Tat in the transcription initiation complex, and several experiments indicate that the sole function of TAR seems to be to bring Tat (and any cofactor[s]) to the vicinity of the HIV-1 promoter. *trans* activation by Tat is lost when TAR is distanced from the promoter sequences (63). Moreover, by replacement of the sequences of the RNA-binding domain of Tat with those of the HIV-1 Rev protein (70) or those from the coat protein of the bacteriophage MS2 (64) and replacement of TAR with the corresponding target sequence, *trans* activation is retained. Heterologous DNA-binding proteins have been also used to bring Tat to the promoter, although *trans*-activation levels were significantly lower than in the studies with RNA domains (2, 38, 69).

There are now several lines of evidence suggesting that an interaction of Tat with transcription complexes promotes an increase in the otherwise low processivity of the RNA polymerase II (Pol II) complexes. Recently, a model that describes the existence of two different but overlapping promoter elements in the HIV-1 long terminal repeat has been proposed. One of these promoters is responsible for the basal transcription level and is not affected by Tat. The other promoter element is *trans* activated by Tat and is responsible for the dramatic increase of transcription observed in the presence of this viral protein (14, 46, 51).

The DNA *cis*-acting regulatory sites that control HIV-1 gene expression reside in the long terminal repeat. The TATA box and Sp1 motifs seem to be important for Tat-mediated *trans* activation. Alterations within the TATA sequences have little effect on basal transcription but abolished Tat *trans* activation (4, 46, 57). Deletions of the Sp1 sites in the HIV-1 promoter reduce the basal activity and have a more pronounced effect on Tat inducibility (4, 37, 38). In fact, physical interactions between Tat and TATA-binding protein (TBP) (39) and Tat and Sp1 (35) have now been reported. The specificities of these reported interactions in relation to Tat *trans* activation, however, remain unresolved. Despite putative interactions between Tat and transcriptional basal factors, several lines of evidence suggest the presence of Tat-specific coactivators. First, *trans* activation by Tat depends on the cell type assayed. In rodent cells, the level of Tat *trans* activation is very low and this effect is overcome when human chromosome 12 is present in these cells (30, 56). Second, Tat mutants lacking the TAR-binding domain are able to inhibit the *trans* activation by Tat (27, 58). Furthermore, cotransfection analysis carried out with hybrids between the activation domains of HIV-1 and equine infectious anemia virus Tat proteins in combination with the natural or heterologous binding targets squelch HIV-1 Tat-

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dependent *trans* activation (9, 47). It has been suggested that the binding of such coactivators to Tat occurs before recognition of the TAR (47). The work presented in this paper attempts to clarify the relationship of Tat with the transcription complexes that assemble over the HIV-1 promoter and to investigate the presence of Tat coactivators with an in vitro system. The results obtained suggest the existence of a factor(s) distinct from previously recognized general transcription factors that interact with Tat to facilitate its *trans* activation.

MATERIALS AND METHODS

Plasmids and fusion proteins. The adenovirus type 2 major late (AdML) promoter used as a control template in the transcription reactions was the pFLBH plasmid (62) linearized with *Aat*II to give a runoff transcript of 290 nucleotides. pFLBH contains adenovirus type 2 sequences from 14.7 to 17.1 map units. The HIV template was the pBC12/HIV/SEAP plasmid (1) cut with *Bam*HI to give a size fragment of 552 nucleotides. The HIV-1 long terminal repeat spans from −640 to +332 relative to the transcription start site.

Segments of the genes contained in the expression plasmids pcTat (48), pK41A Tat mutant (74), phPTB (for human polypyrimidine tract-binding protein) (24), and pFH15 (for *Fasciola hepatica*) (59) were subcloned into the pGEX-2TK vector (Pharmacia) by standard techniques. The pcTat expression plasmid expresses the entire 86-amino-acid Tat protein. To generate pGEX-2TK-HIVTat, Tat sequence was amplified from pcTat by PCR and directly ligated into the *Bam*HI- and *Eco*RI-digested pGEX-2TK vector. The identity of Tat and the Tat mutant containing a lysine-to-alanine mutation at position 41 (K41A) was confirmed by sequencing.

The proteins were expressed as glutathione *S*-transferase (Gst) fusions under the conditions suggested by the manufacturer. In the case of the Tat proteins, the fusions were highly insoluble. To solubilize the proteins from inclusion bodies, we used a modification of the Sarkosyl procedure of Grieco et al. (28). Briefly, 1 liter of transformed BL-21 cells was grown to an optical density at 600 nm of 0.5 to 0.8, and expression of the fusion proteins was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 0.1 mM for 3 h. Cells were pelleted and resuspended in 10 ml of phosphate-buffered saline (PBS) containing 2 mM of EDTA, phenylmethylsulfonyl fluoride (PMSF) at 100 μg/ml, leupeptin at 1 μg/ml, and pepstatin at 1 μg/ml. The bacterial pellet was lysed on ice by mild sonication and centrifuged at 12,100 × *g* (R_{max}) for 10 min at 4°C. The supernatant was mixed with Triton X-100 to 1% and kept on ice. The pellet was resuspended with 8 ml of 1.5% (vol/vol) sodium *N*-lauroylsarcosinate solution, 25 mM triethanolamine, and 1 mM EDTA (pH 8.0), mixed, and placed on ice for 30 min. The suspension was then centrifuged at 18,900 × *g* (R_{max}) for 20 min at 4°C. Triton X-100 and dithiothreitol (DTT) were added to the new supernatant to final concentrations of 2% and 1 mM, respectively, and the solution was mixed with the supernatant resulting from the first centrifugation. The new solution was then rocked with 0.5 ml of glutathione-agarose beads for 1 h at 4°C and washed extensively with 1% Triton X-100, 1% Tween 20, 1 mM DTT, and 100 μg of PMSF per ml in PBS. The release of the Gst fusion proteins and the cleavage to yield free Tat were carried out as described by Smith and Johnson (68).

Nuclear extract preparation and in vitro transcription. Nuclear extracts were prepared by the method of Dignam et al. (16). Heat-treated nuclear extracts were prepared as described by Nakajima et al. (54). The in vitro transcription reactions were carried out at 30°C for 30 min in 25 μl of a mix containing 10 μl (10 to 20 mg of protein per ml) of HeLa cell nuclear extract, 14 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-KOH (pH 7.9), 14% (vol/vol) glycerol, 68 mM KCl, 15 mM NaCl, 7 mM $MgCl_2$, 4 mM sodium citrate (pH 6.7) (40), 250 ng of poly(I) · poly(C) (Pharmacia), 300 ng of poly(dI) · poly(dC) (Pharmacia), 1 mM DTT, 10 mM creatine phosphate, 0.1 mM EDTA, 4 U of RNasin (Promega), 625 μM (each) ATP, CTP and GTP, 40 μM UTP, and 10 μCi of [α-^{32}P]UTP (3,000 Ci/mmol, 1 μl). The AdML and HIV templates were used at 250 and 100 ng per reaction mixture, respectively. After the incubation time, 100 μl of 200 mM NaCl–300 mM sodium acetate (NaOAc)–10 mM EDTA–0.75% sodium dodecyl sulfate (SDS)–25 μg of yeast tRNA per ml–100 mM Tris-HCl (pH 7.5) and 50 μl of 7 M urea—100 mM LiCl–50 mM EDTA–0.5% SDS–100 mM Tris-HCl (pH 7.5) were added, and transcription products were extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform and then precipitated with 50 μl of 7.5 M ammonium acetate and cold ethanol. Pellets were resuspended in formamide buffer and resolved by electrophoresis on 6% (19:1) polyacrylamide–8 M urea gels that were dried and exposed. The specific bands were quantified in all figures by using a PhosphorImager (Molecular Dynamics) and a densitometer (LKB), and the specific *trans* activation was calculated as the ratio of the level of the HIV-1 transcript in the presence of Tat to the level of the same transcript in the absence of Tat, with normalization of these values to the level of the AdML transcript in those reactions (49). Recombinant basal transcription factors (recombinant TBP [rTBP] and rTFIIB) were purchased from Upstate Biotechnology Inc. Purification of the phosphocellulose fraction of HeLa TFIID has been described previously (62). Recombinant Sp1 was obtained from Promega.

Affinity chromatography. Gst fusion proteins were bound to 0.5 ml of glutathione-agarose beads and packed in disposable Poly-Prep chromatography columns (Bio-Rad). The final concentration of the immobilized protein was determined by Bradford assay (Bio-Rad) after elution of the fusion protein from the beads with free glutathione. These concentrations were in the range of 1 to 2 mg/ml of bed volume. The affinity columns were equilibrated with 20 bed volumes of buffer D (20 mM HEPES [pH 7.9], 20% [vol/vol] glycerol, 0.2 mM EDTA, 0.5 mM DTT, and 100 mM KCl) containing bovine serum albumin at 1 mg/ml and PMSF at 100 μg/ml. Samples (1 ml) of HeLa cell nuclear extract adjusted at 100 mM KCl in buffer D were loaded on the columns. The number of reloading cycles was normally between one and three at a flow rate of approximately 0.05 ml/min. After extensive washing, the column was eluted in two steps with 3 bed volumes of buffer D containing respectively 0.5 and 1 M KCl. The 0.5 and 1 M KCl eluates were collected and dialyzed against buffer D without glycerol containing 100 mM KCl. The fractions were concentrated approximately sevenfold by centrifugation through Amicon filters, the protein concentration was determined by Bradford assay, and the activity was assayed in the in vitro transcription system.

Sedimentation analysis. HeLa cell nuclear extracts were dialyzed against buffer D containing 10% (vol/vol) glycerol and 200 μl was loaded onto a 1.8-ml gradient of 10 to 40% (vol/vol) glycerol in buffer D containing PMSF at 100 μg/ml. Centrifugation was carried out in a TL55 rotor (Beckman) at 55,000 rpm for 5 h at 4°C, and 100-μl fractions were collected. Gradient fractions were dialyzed against buffer D containing PMSF and analyzed in the in vitro transcription system and by Western blot (immunoblot) procedures. Control gradients were prepared in the same conditions and loaded with a mixture of five molecular-weight markers (Bio-Rad) that were visualized in the fractions by SDS-polyacrylamide gel electrophoresis (PAGE) procedures.

Western blotting. Aliquots of gradient and column fractions were subjected to SDS-PAGE and transferred onto Immobilon affinity membranes (Millipore). The proteins were probed with polyclonal rabbit antibodies against TBP and TFIIB (Upstate Biotechnology Inc.) and Sp1 (kindly provided by J. Horowitz, Duke University). After the incubation with a peroxidase-conjugated secondary antibody (Amersham), blots were developed by the ECL system (Amersham).

RESULTS

Recombinant Tat and Gst-Tat proteins *trans* activate the HIV-1 promoter. To examine Tat function in detail, we expressed HIV-1 Tat protein as a fusion protein with Gst of *S. japonicum*. The fusion protein was purified from crude bacterial lysates by affinity chromatography to greater than 95% homogeneity (72). To determine whether HIV-1 Tat and Gst-Tat were biologically active, we examined the effects of both proteins on transcription directed by the HIV-1 promoter in a cell-free transcription system. In the experiment shown in Fig. 1A, Tat (lanes 2 to 5), Gst-Tat (lanes 7 to 10), and Gst (lanes 12 to 15) proteins were assayed in vitro in the presence of the HIV-1 and AdML promoter sequences. Tat and Gst-Tat were able to specifically *trans* activate the HIV-1 promoter. The *trans* activation of the HIV-1-specific transcription was calculated as described in Materials and Methods, and it ranged from 7- to 10-fold unless otherwise noted. Transcription directed by the AdML promoter was used as an internal control for the in vitro reactions. The behavior in response to Tat of a mutant with a deletion in the loop region of TAR was indistinguishable of that from the AdML template (data not shown). Therefore, we concluded that *trans* activation by Tat was TAR dependent, which is fully consistent with in vivo data and previous in vitro results (49). The slight increase in the transcription of the HIV-1 template with Gst protein (Fig. 1A, lanes 12 and 13) was general in those reactions, but no specific *trans* activation from this promoter was observed (Fig. 1A). We next examined the transcriptional activity of a Tat mutant (K41A). The integrity of this residue at position 41 has been shown to be essential for Tat function in vivo (74). We expressed and purified the K41A Tat mutant and the Gst-K41A Tat protein, and both were tested in the in vitro transcription system. Figure 1B shows that the K41A and Gst-K41A proteins were unable to *trans* activate the HIV-1 promoter. As a con-

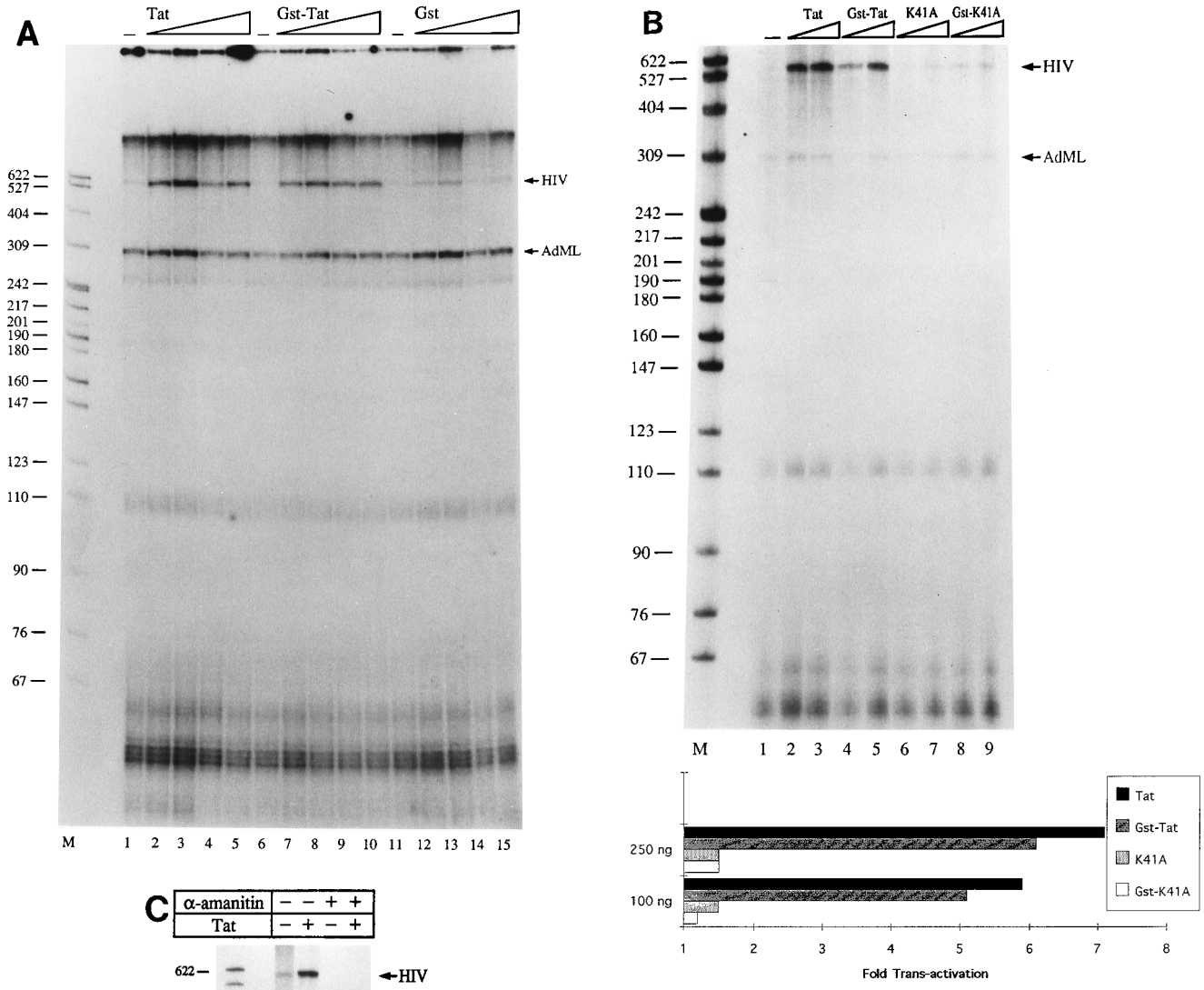


FIG. 1. In vitro *trans* activation. (A) The transcriptional activity of Tat and Gst-Tat proteins was assayed in an in vitro transcription system. Transcription reactions were carried out in the presence (or absence [lanes 1, 6, and 11, respectively]) of 100, 250, 500, and 1,000 ng of recombinant Tat (lanes 2 to 5), Gst-Tat (lanes 7 to 10), or Gst alone (lanes 12 to 15). The positions of the HIV-specific and the AdML transcripts are indicated by arrows. Molecular size markers (lane M; in base pairs) were the pBR322 DNA-*Msp*I digest (New England Biolabs) labeled with [α-^{32}P]dCTP. (B) The experiment described for panel A was carried out with 100 and 250 ng of recombinant Tat (lanes 2 and 3), Gst-Tat (lanes 4 and 5), K41A Tat mutant (lanes 6 and 7), and Gst-K41A Tat mutant (lanes 8 and 9) and in the absence of all of these (lane 1). Experimental data quantified with the PhosphorImager are shown in graphic form (bottom). (C) As a control, α-amanitin was included in the reaction mixture (lanes 3 and 4) at a concentration of 4 μg/ml. Transcription reactions were assayed in the absence (−) or presence (+) of 250 ng of recombinant Tat.

trol, we performed the in vitro *trans* activation in the presence of α-amanitin. Basal and activated HIV-1 transcription was completely eliminated by 4 μg of α-amanitin per ml, which indicates that RNA synthesis was carried out by RNA Pol II (Fig. 1C). Thus, we established that the *trans* activation was driven by RNA Pol II and was dependent on the TAR-Tat axis.

***trans* activation was suppressed in nuclear extracts that had been passed through Gst-Tat columns.** To study the effect of Tat in the transcription from nuclear extracts, we used a Gst-

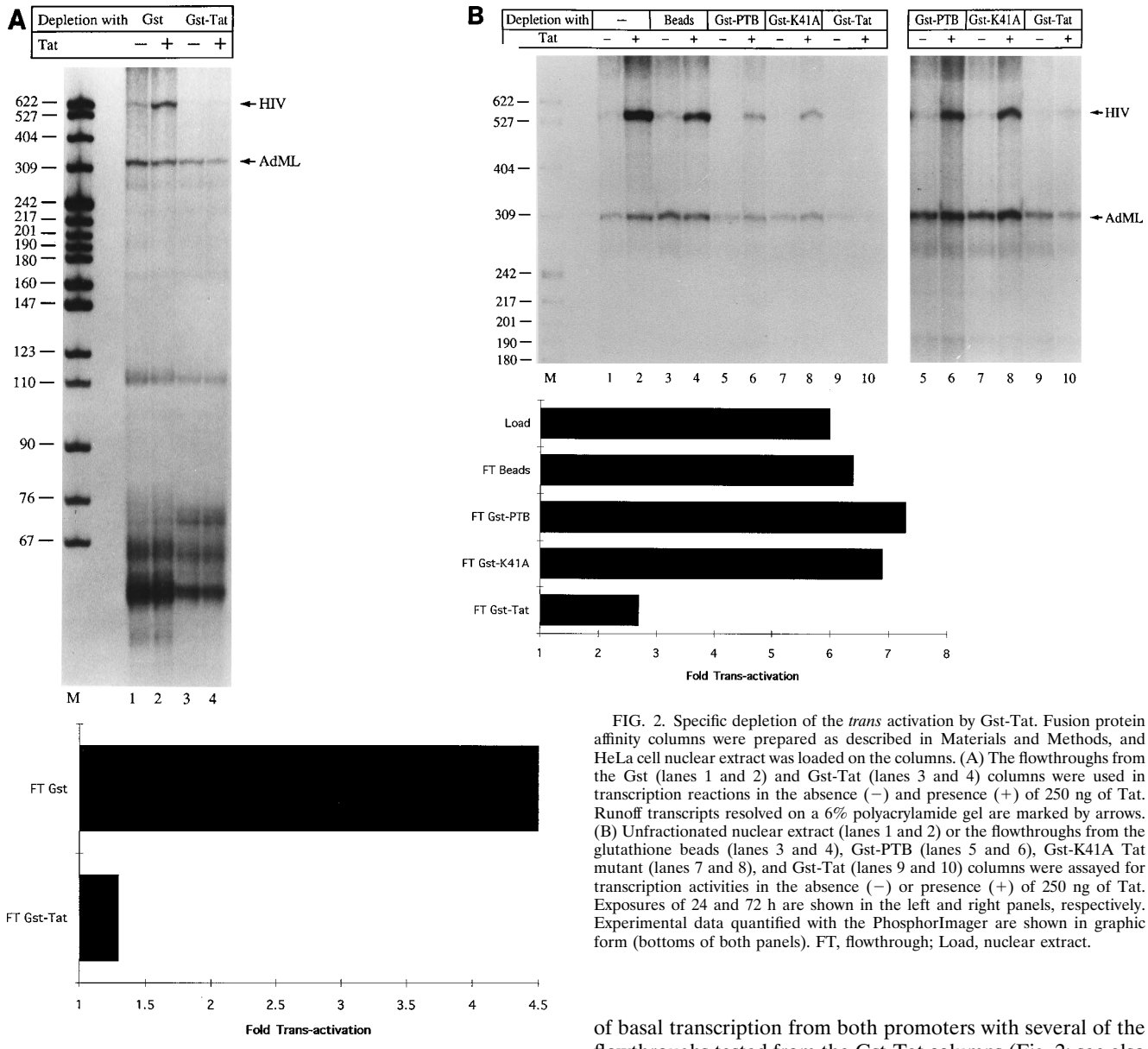


FIG. 2. Specific depletion of the *trans* activation by Gst-Tat. Fusion protein affinity columns were prepared as described in Materials and Methods, and HeLa cell nuclear extract was loaded on the columns. (A) The flowthroughs from the Gst (lanes 1 and 2) and Gst-Tat (lanes 3 and 4) columns were used in transcription reactions in the absence (−) and presence (+) of 250 ng of Tat. Runoff transcripts resolved on a 6% polyacrylamide gel are marked by arrows. (B) Unfractionated nuclear extract (lanes 1 and 2) or the flowthroughs from the glutathione beads (lanes 3 and 4), Gst-PTB (lanes 5 and 6), Gst-K41A Tat mutant (lanes 7 and 8), and Gst-Tat (lanes 9 and 10) columns were assayed for transcription activities in the absence (−) or presence (+) of 250 ng of Tat. Exposures of 24 and 72 h are shown in the left and right panels, respectively. Experimental data quantified with the PhosphorImager are shown in graphic form (bottoms of both panels). FT, flowthrough; Load, nuclear extract.

Tat affinity column. Chromatography on Gst-Tat columns was used to specifically deplete HeLa cell nuclear extracts of transcription activity from the HIV-1 promoter. Transcription-competent nuclear extracts were loaded on the affinity columns, and the flowthroughs were analyzed for basal and Tat-activated transcription by the in vitro system described above. The flowthrough from the Gst control column supported *trans* activation by Tat (Fig. 2A, lanes 1 and 2), whereas the flowthrough from the Gst-Tat column did not (Fig. 2A, lanes 3 and 4). The same result was obtained with four different nuclear extract preparations. The level of the HIV-1 *trans* activation decreased three- to fivefold, as measured with a PhosphorImager. Similar experiments were carried out with the K41A Tat mutant, the unrelated Gst-hPTB and Gst-FH15 proteins, Gst, and glutathione-agarose beads. Neither the Tat mutant, the other unrelated proteins, nor the beads alone were able to deplete the extract of the *trans*-activation activity (Fig. 2B) (72). We noted, however, a decrease in the absolute levels of basal transcription from both promoters with several of the flowthroughs tested from the Gst-Tat columns (Fig. 2; see also Fig. 3 and 6) and also with the flowthroughs from two of the control columns (Fig. 2B, lanes 5 to 8). This could indicate the retention of general transcription factors in the affinity columns (see below). From the experiments described above, we concluded that an activity necessary for the *trans* activation of the HIV-1 promoter was specifically removed from the nuclear extract after its passage through the Gst-Tat column. This flowthrough fraction will be called the depleted nuclear extract.

The Gst-Tat column retained an activity necessary for *trans* activation by Tat. To verify that the activity necessary for the *trans* activation by Tat was retained in the Gst-Tat column, the material bound to the Gst-Tat columns was eluted into two fractions with buffer D containing respectively 0.5 and 1 M KCl. Both fractions were collected and assayed in the in vitro transcription system. As shown in Fig. 3, 0.5 M KCl (lanes 5 and 6) was able to restore *trans* activation to the depleted nuclear extract. The 1 M KCl fraction had no activity (data not shown). The 0.5 M KCl eluate was a complex fraction contain-

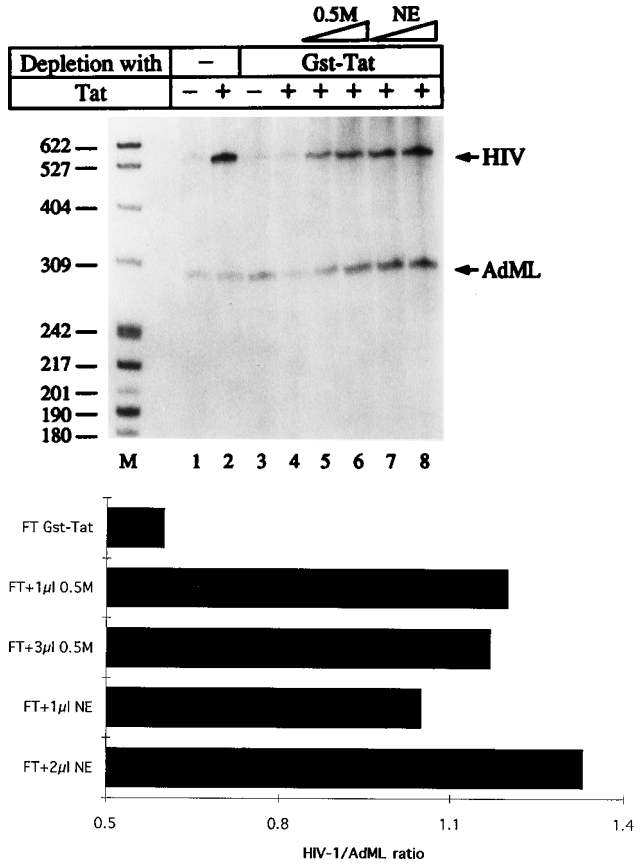


FIG. 3. The Gst-Tat column specifically retains an activity essential for the *trans* activation. A reconstitution of the *trans* activation by using the 0.5 M salt elution fraction is shown (top panel). HeLa cell nuclear extract was chromatographed on the Gst-Tat affinity column, and after being extensively washed the column was eluted with buffer D containing 0.5 and 1 M KCl. Unfractionated HeLa nuclear extract (lanes 1 and 2), the flowthrough from the Gst-Tat affinity column (lanes 3 and 4), and a mixture of the flowthrough plus 1 and 3 μl of the 0.5 M fraction (lanes 5 and 6) or plus 1 and 2 μl of unfractionated HeLa nuclear extract (lanes 7 and 8) were used in the transcription reactions in the absence (–) or presence (+) of 250 ng of Tat. Experimental data quantified with the PhosphorImager are shown in graphic form (bottom). FT, flowthrough.

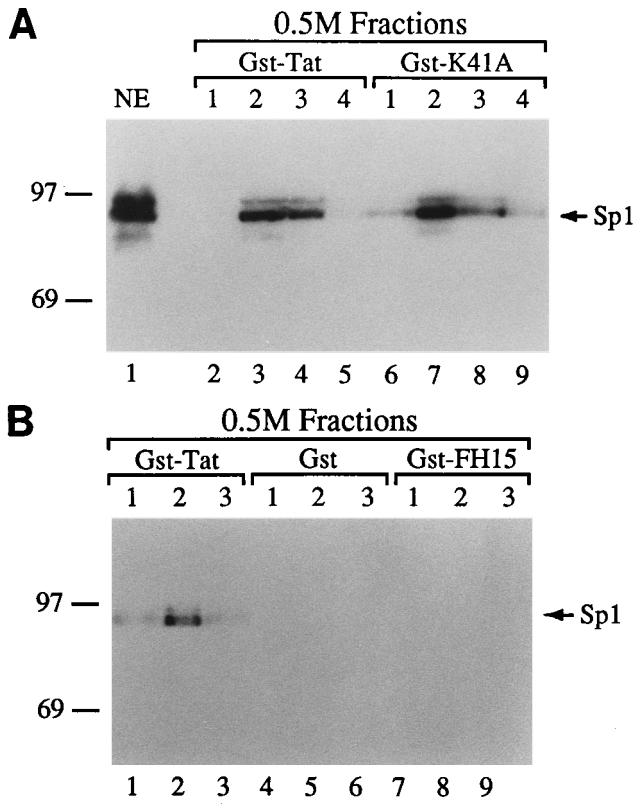


FIG. 4. Binding of Sp1 to the affinity columns. (A) A 4-μl sample of HeLa cell nuclear extract (NE) and 10-μl samples of the 0.5 M KCl eluate (fractions 1 to 4) from the Gst-Tat (lanes 2 to 5) and Gst-K41A Tat mutant (lanes 6 to 9) columns were assayed for binding to Sp1 by ECL Western blotting. (B) The experiment described for panel A was carried out with the 0.5 M KCl eluate (fractions 1 to 3) of the Gst-Tat (lanes 1 to 3), GST (lanes 4 to 6), and Gst-FH15 (lanes 7 to 9) affinity columns. The position of Sp1 is indicated by arrows on the right side of each panel. The molecular masses (in kilodaltons) of the molecular weight markers are shown on the left side of each panel.

ing approximately 7% of the total protein loaded on the column, as demonstrated by Bradford quantification and silver staining (72).

In order to recover the activity that was lost in the nuclear extracts chromatographed through Gst-Tat affinity columns, we also performed complementation experiments adding small quantities of unfractionated HeLa cell nuclear extract to the depleted nuclear extract. We observed a recovery of Tat *trans* activation by addition of 1 or 2 μl of unfractionated extract (Fig. 3, lanes 7 and 8; see also Fig. 6A, lanes 9 and 10). In the absence of Tat, the addition of unfractionated extract had no significant effect on the level of HIV basal transcription (data not shown). Moreover, this small quantity of extract by itself was unable to support transcription under the conditions used (72). The possibility that Gst-Tat affinity chromatography had resulted in unmasking of an inhibitor was made less likely by these experiments.

The transcriptional factors TBP and Sp1 have been implicated in basal and Tat-dependent transcriptional activation (35, 39). The results obtained with the depleted extracts prompted us to investigate whether or not TBP or Sp1 could be detected in the eluates from the affinity columns used in this study. TBP was retained in the GST-Tat column and eluted in the 0.5 M KCl fraction (72). The specificity of this interaction was questionable, however, because we observed nonspecific binding of TBP to most of the affinity matrices used. This was under conditions which resulted in the specific retention of a factor(s) required for Tat *trans* activation in the Gst-Tat affinity column. The 0.5 M KCl fractions from the Gst-Tat and Gst-K41A Tat mutant columns also contained Sp1 (Fig. 4A), whereas it was not detected in other control affinity columns (Fig. 4B). These results suggest that Sp1 protein can interact with some specificity with Tat and that this interaction was not abolished by an alanine at position 41.

An activity of approximately 200 kDa complements Tat *trans* activation in the Gst-Tat-depleted extracts. Tat-dependent *trans* activation could be restored with unfractionated HeLa cell nuclear extracts (Fig. 3; see also Fig. 6). To further examine the activity responsible for the complementation, we subjected the nuclear extract to centrifugation in a 10 to 40% (vol/vol) glycerol gradient. Fractions from the gradient were assayed for their abilities to complement Gst-Tat-depleted nuclear extracts in the in vitro transcription system. An activity capable of reconstituting the *trans* activation by Tat in the depleted extracts was detected as a symmetrical peak around the middle of the gradient (Fig. 5). Each of the protein complexes contained in these fractions is predicted to have an approximate molecular mass of 200 kDa. Analysis of the gra-

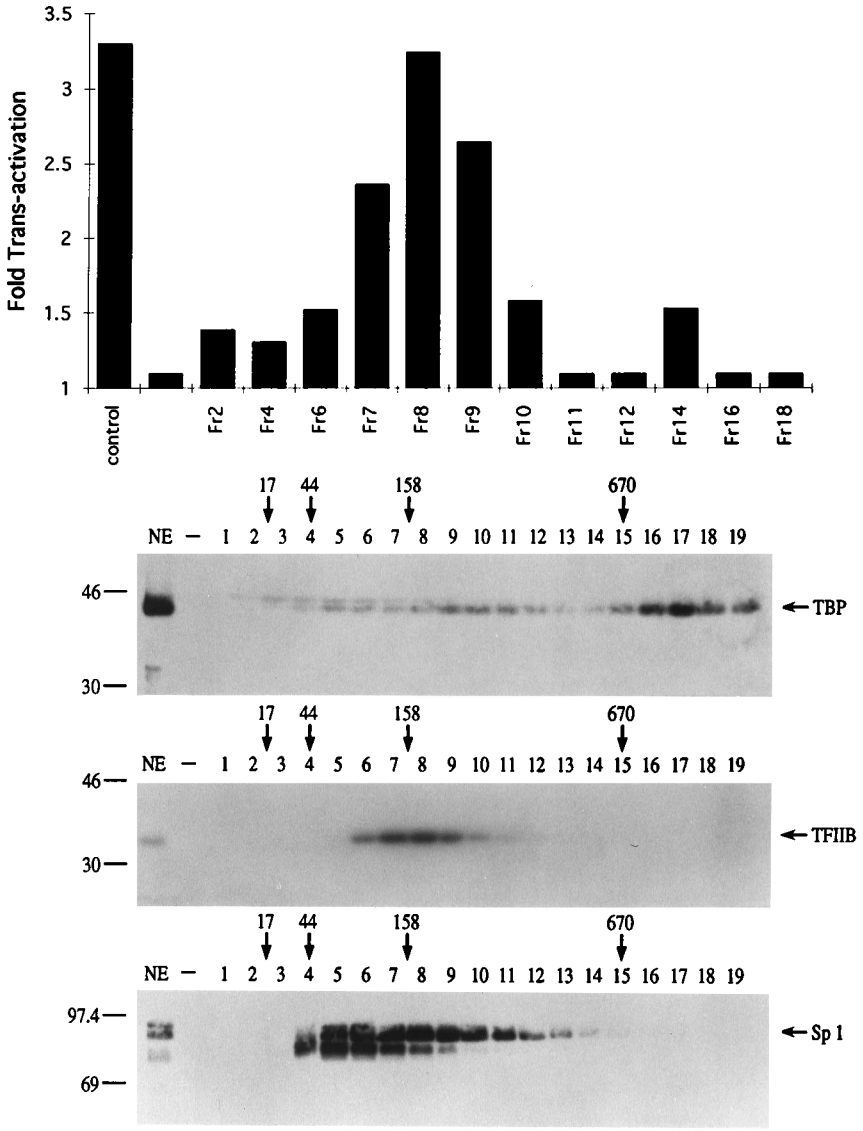
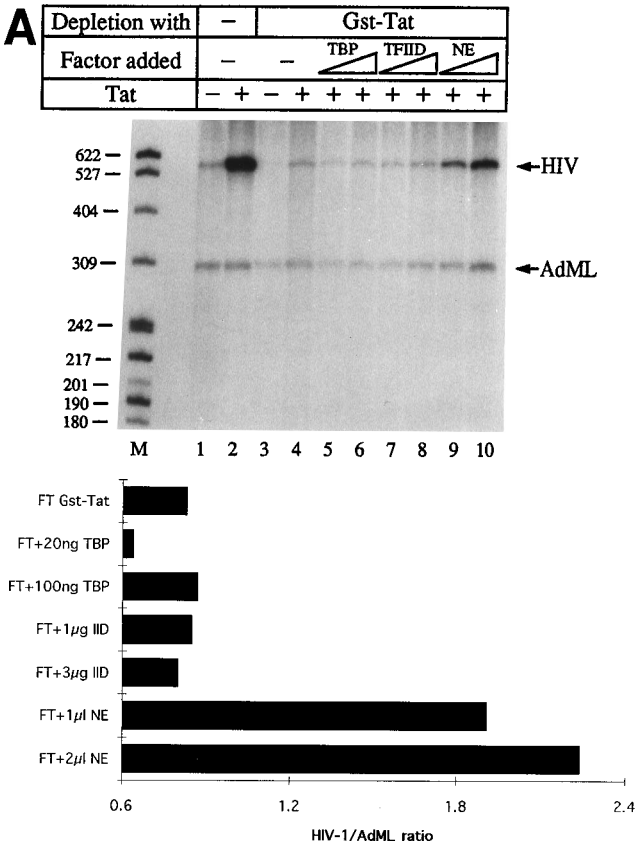


FIG. 5. An activity essential for the *trans* activation by Tat migrates with an apparent molecular mass of 200 kDa in glycerol gradients. A schematic representation of the *trans* activation in the depleted extracts is shown (top panel). Transcription reactions were carried out with the flowthrough from the Gst-Tat affinity column and 10-μl samples of the gradient fractions (Fr) in the presence of 250 ng of Tat. The profile of one experiment is shown. The same result was obtained with three different gradients. The three bottom panels show the Western blot analysis of 10 μl of the gradient fractions (1 to 19) and 4 μl of HeLa nuclear extract (NE) as a control, with antisera specific for TBP, TFIIB, and Sp1 as indicated. The positions and sizes (in kilodaltons) of prestained molecular mass markers and the positions of the proteins are indicated to the left and to the right of the panels, respectively. The arrows above the numbers indicate the positions of the protein standards in the glycerol gradient.

dients by Western blots showed the presence of TBP and Sp1 in the gradient fractions that reconstituted the activity (Fig. 5). It is important to point out that TBP was also present in two other zones corresponding to the first and last fractions of the gradient. The highest levels of TBP were found in the fractions corresponding to a molecular mass of 800 kDa and most likely represent the TFIID transcription complex. Small TBP-containing complexes were found spread along the first fractions of the glycerol gradient. None of these was able to recover the activity of the depleted nuclear extracts. Transcription factor TFIIB precisely copurified with the activity that reconstituted the depleted extract (Fig. 5).

TBP or TFIID did not reconstitute Tat-dependent transcriptional activation in the Gst-Tat-depleted extracts. Because of the finding of TBP and Sp1 in the elution fraction from the Gst-Tat column, we carried out reconstitution experiments of the flowthrough from the Gst-Tat affinity column using these and other purified transcription factors. In the experiment shown in Fig. 6A, rhTBP (lanes 5 and 6), TFIID fraction (lanes 7 and 8), and unfractionated HeLa nuclear extract as a control (lanes 9 and 10) were added to the depleted extract in the presence of Tat protein. rhTBP and TFIID failed to reconstitute *trans* activation of the HIV-1 promoter, whereas the unfractionated extract showed an increase in the *trans* activation by Tat. Addition of recombinant Sp1 or of Sp1 in combination with TFIID failed to complement Tat-dependent *trans* activation in the depleted extract (data not shown).

In addition to these factors, we also examined the effect of TFIIB on basal and Tat-activated transcription using the flowthroughs from the Gst (Fig. 6B, lanes 1 to 6) and from the



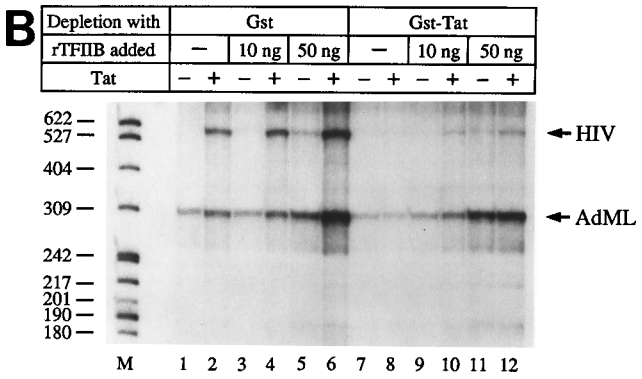


FIG. 6. *trans* activation by Tat was not reconstituted with recombinant factors. (A) Transcription reactions were carried out with unfractionated HeLa nuclear extract (lanes 1 and 2), with the Gst-Tat-depleted extract (lanes 3 and 4), and with 20 and 100 ng (lanes 5 and 6), 1 and 3 μg (lanes 7 and 8), and 1 and 2 μl (lanes 9 and 10) of rhTBP, TFIID fraction, or unfractionated nuclear extract, respectively, added to the depleted extract and with 250 ng of Tat protein where indicated. Experimental data quantified with the PhosphorImager are shown in graphic form (bottom panel). FT, flowthrough. (B) The experiment described for panel A was carried out with the nuclear extract passed through the Gst (lanes 1 and 2) and Gst-Tat (lanes 7 and 8) columns and with 10 (lanes 3, 4, 9, and 10) and 50 ng (lanes 5, 6, 11, and 12) of rhTFIIB added back to the flowthroughs from the Gst and Gst-Tat columns, in the absence (−) and presence (+) of 250 ng of Tat protein. Lanes M, molecular size markers; their sizes (in base pairs) are indicated at the left.

Gst-Tat (lanes 7 to 12) affinity columns. We did not detect a recovery of specific *trans* activation of the HIV-1 promoter with TFIIB. The increase in the basal transcription from both the HIV-1 and AdML promoters when TFIIB was added to the Gst-Tat-depleted extract was also seen with the flowthrough from the Gst control column (Fig. 6B) and with unfractionated nuclear extracts (72) and was probably due to limiting concentrations of this factor in the extracts.

Temperature dependence of Tat *trans* activation. The selective inactivation of TFIID by heating of HeLa nuclear extracts at 47°C has been previously described (54). To further study the role of TFIID and the effect of heating the nuclear extract in Tat-mediated transcriptional activation, we did the experiments shown in Fig. 7. Heat-treated HeLa nuclear extracts (HTNE) showed the expected decrease in basal and Tat-activated transcription (Fig. 7A, lanes 3 and 4). The addition of rhTBP (lanes 5 and 6) or TFIID fraction (lanes 7 and 8) partially or completely recovered the basal transcription from the HIV-1 and AdML promoters, respectively. *trans* activation by Tat was only minimally affected by TBP or TFIID. Untreated nuclear extract was able to activate both basal and Tat-activated transcription (lanes 9 and 10). The heat dependence of Tat *trans* activation was further studied by heating of the nuclear extract at different temperatures (Fig. 7B). We observed a significant reduction in Tat *trans* activation when the extract was heated at 43 and 45°C. Basal transcription from the HIV-1 and AdML promoters was not diminished by this treatment; on the other hand, we observed an increase in basal transcription upon heating at 45°C (Fig. 7B). The decrease in the specific *trans* activation of the HIV-1 promoter by Tat was also obtained by heating of the extracts at 47°C for short periods, a treatment not detrimental to basal transcription (72). In both cases, *trans* activation by Tat could not be recovered by the addition of TFIID, whereas basal transcription could be recovered in this way (72). These results demonstrate the presence of a heat-labile factor required for Tat function that can be differentiated from TBP and TFIID.

DISCUSSION

Despite extensive work, the mechanism of HIV-1 Tat-mediated transcriptional activation remains obscure. In vitro and in vivo studies support a role of Tat in both the initiation and elongation phases of transcription by RNA Pol II. There are now data that indirectly suggest the involvement of cellular factors. Here, we show the first direct evidence for unique Tat coactivators not required for general basal transcription. The studies presented here confirm previous work of Marciniak et al. (49) and others (5, 26, 40, 43) which showed that the *trans* activation by Tat could be studied in vitro with HeLa cell nuclear extracts. Moreover, our studies indicate the potential of fractionation of this system into complementing fractions capable of recapitulating Tat *trans* activation. HeLa nuclear extracts passed through Gst-Tat affinity columns showed a small reduction of basal transcription from the HIV-1 and AdML promoters. Second, and more important, Tat *trans* activation of the HIV-1 promoter was almost completely abolished in the depleted extracts. The depleted extract could be reconstituted with unfractionated nuclear extract or with the 0.5 M KCl eluate from the Tat affinity column. These experiments suggest the retention of a specific coactivator of Tat *trans* activation by the Gst-Tat column.

Interaction of viral regulatory proteins with components of the basal transcriptional machinery has been described elsewhere (8, 29, 44, 45, 67, 71, 80). Those reports and the recent data of Kashanchi et al. (39) describing an interaction between TBP and Tat prompted us to test whether TBP was retained in the Gst-Tat column. In our experimental conditions, TBP bound to many, if not all, of the affinity matrices tested, making

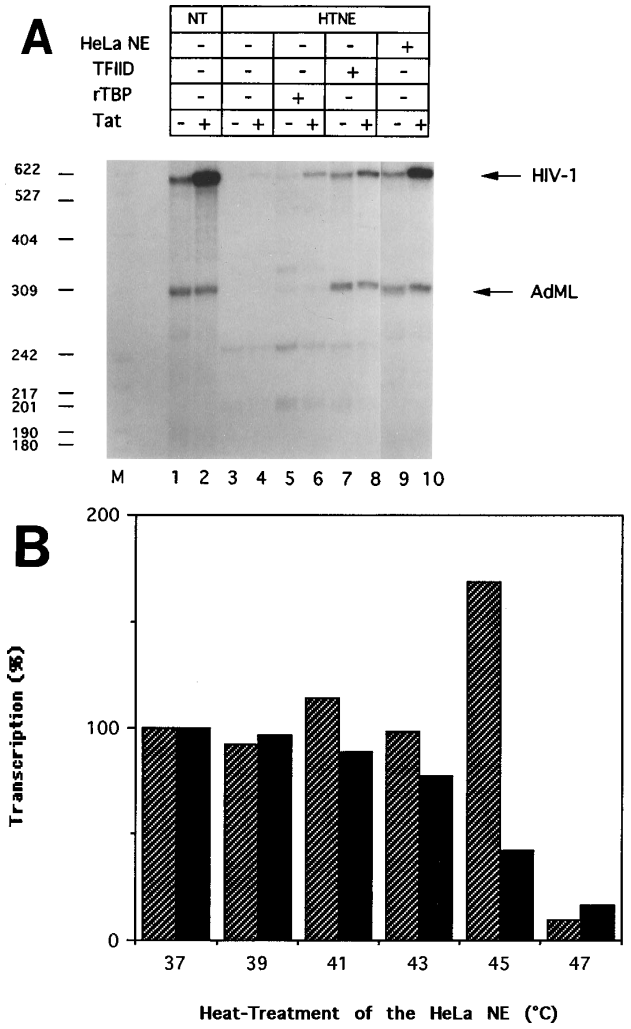


FIG. 7. *trans* activation by Tat in HTNE. (A) TFIID cannot recover *trans* activation by Tat in HTNE. Transcription reactions were carried out with untreated HeLa nuclear extract (NT, lanes 1 and 2) and with nuclear extract heated at 47°C for 15 min (HTNE, lanes 3 to 10). The addition of rhTBP (rTBP; 100 ng), TFIID (3 μg), or untreated nuclear extract (HeLa NE; 2 μl) to the HTNE is indicated above the gel. Lanes 9 and 10 are from a separate experiment. Lane M, molecular size markers; sizes (in base pairs) are shown at the left. (B) Effects of temperature in Tat-mediated *trans* activation. Transcription reactions were carried out as described for panel A, except that the extracts were heated at the indicated temperatures. After analysis by the PhosphorImager, experimental data were represented in graphic form. ▨, transcription directed by the AdML promoter in the absence of Tat. The same profile is obtained with the values for the HIV-1 promoter. ■, the specific increase in the HIV-1 transcription by Tat as described in Materials and Methods. Two independent experiments gave similar results.

it difficult to assess the specificity of a direct interaction between Tat and TBP. Furthermore, rhTBP was unable to restore the *trans* activation by Tat in the Gst-Tat-depleted extracts. Thus, it is very unlikely the activity we were depleting was that of TBP alone. The partial retention of TBP in the affinity matrixes could explain the increase in the basal transcription observed when unfractionated nuclear extract was added back to the depleted extracts. In other systems, the binding of TBP to its recognition sequence is necessary to initiate basal transcription, but the TBP-associated factors (TAFs) bound to TBP in the TFIID complex are required for transcription activation (10, 19, 52, 73, 81, 82). Binding of transcriptional activators to TAFs has now been described; for example, TAF_{110} binds Sp1 and TAF_{40} binds VP16 (25, 33). Moreover, it has been shown that VP16 can activate HIV-1-specific transcription when targeted to a promoter-proximal RNA target sequence (75), arguing that Tat may act through similar mechanisms. For these reasons, TFIID was a likely candidate for a factor that would interact with Tat and mediate *trans* activation. We tested whether or not TFIID could restore the *trans* activation by Tat in the depleted extract. A conventional TFIID fraction was not active in reconstitution of the depleted extract. Moreover, the 800-kDa TFIID-containing fractions in the glycerol gradient were also inactive. The same rhTBP and TFIID preparations were active and able to reconstitute basal transcription from heat-treated nuclear extracts. These data convinced us that the activity of conventional TFIID is not the activity depleted by the Tat affinity column. A TBP-containing complex called B-TFIID (77) would sediment close to the complementing activity in glycerol gradients; however, purified B-TFIID (76) was tested and was not active in the complementation (72).

The activity was shown to copurify perfectly with TFIIB in glycerol gradients, and it was tempting to speculate that a complex including TFIIB was involved. However, TFIIB was not bound by the Tat affinity column (72) and rTFIIB could not specifically activate the depleted nuclear extract. Furthermore, identical fractionation of the depleted extract in the glycerol gradient revealed an identical sedimentation profile for TFIIB (72). These results make it highly unlikely that a complex containing TFIIB is the seat of the activity. It is puzzling that all of the TFIIB in the nuclear extract sedimented as a large complex, given that the molecular mass of the protein is approximately 35 kDa. Molecular mass standards and the sedimentation of other nuclear factors showed that the proteins were separating well and that migration of TFIIB was probably not artifactual. Interestingly, Koleske and Young (41) have reported the purification of a preassembled RNA Pol II transcription complex from yeast extracts that contain TFIIB.

Sp1 sites are important elements in the *trans* activation of the HIV-1 promoter (38, 69), and Sp1 has been shown to bind Tat (reference 35 and this study). Sp1 was found in the active glycerol gradient fractions probably as an Sp1 multimer or as heteromeric complexes with other factors and was also found in the active 0.5 M KCl eluate from the Tat affinity column. Thus, Sp1 was an obvious candidate for the activity missing in the depleted extracts. Nonetheless, several lines of evidence strongly suggest that this activity is not that of Sp1. Sp1 was shown to bind both the wild-type Tat and the K41A mutant affinity columns, whereas depletion was only seen with the former. Sp1 was present in many gradient fractions that did not have activity in the reconstitution of the depleted extract. Moreover, recombinant Sp1 did not have activity either alone or in combination with TBP and TFIID. Previous work suggested that Sp1 can be replaced by other unrelated cellular transcription factors without alteration of an efficient Tat response (69). All of this taken together indicates that Sp1 is not the required factor(s) missing in depleted extracts. Nevertheless, it is a formal possibility that a unique complex containing Sp1 is the source of activity and that a second component of this complex can interact with wild-type Tat but not with the K41A Tat mutant.

Further characterization of Tat-mediated *trans* activation showed the presence of a heat-labile factor required for Tat function but not for basal transcription by RNA Pol II. Previous work has shown the preferential inactivation of TFIID by mild heat treatment of nuclear extract and restoration of basal transcription upon addition of this factor (54). We could heat

the nuclear extract at lower temperatures (or at the same temperature but for shorter times) without losing basal transcription; however, this treatment significantly reduced the *trans* activation of the HIV-1 promoter by Tat. In our experimental conditions, we observed restoration of basal but not Tat-activated transcription when the heat-treated extracts were supplemented with partially purified TFIID. These results argue for the presence of a heat-sensitive factor(s) distinct from TFIID. The heat-sensitive factor was distinct from the factor(s) retained in the Gst-Tat column, because the depleted extract restores Tat *trans* activation in the HTNE (72).

Taken together, our results demonstrate the presence of coactivators of Tat function that can be differentiated in vitro from the factors required for basal transcription. The data presented here are in agreement with the in vivo experiments that indirectly suggest the existence of specific coactivators for Tat *trans* activation (9, 47). At present, four mammalian Tat-binding proteins have been identified (15, 32, 55, 66). Although some of these proteins can modulate the activity of Tat in cotransfection experiments, their role in the activation of HIV-1 genes by Tat is still in question. At this time, the identity of the factor(s) responsible for the complementation of the Gst-Tat depleted extracts is unknown. Purification and identification of the factor(s) are under way and should greatly enhance the understanding of the mechanism of Tat-dependent transcriptional activation.

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