Purification of a Tat-associated kinase reveals a TFIIF complex that modulates HIV-1 transcription

León F.García-Martínez,
Gopinath Mavankal, John M.Nevu¹,
William S.Lane¹, Dmitri Ivanov and
Richard B.Gaynor²

Divisions of Molecular Virology and Hematology-Oncology,
Departments of Medicine and Microbiology, University of Texas
Southwestern Medical Center, Dallas, TX 75235-8594 and

¹Harvard Microchemistry Facility, Cambridge, MA 02138, USA

²Corresponding author

L.F.García-Martínez and G.Mavankal contributed equally to this work

The Tat protein is a transcriptional activator which is required for efficient human immunodeficiency virus 1 (HIV-1) gene expression. Tat stimulates HIV-1 transcriptional elongation by increasing the processivity of RNA polymerase II. To address whether Tat-mediated effects on HIV-1 gene expression are due to modulation in the phosphorylation of the RNA polymerase II C-terminal domain (CTD), we developed a purification protocol to identify cellular kinases that are capable of binding to Tat and hyperphosphorylating the RNA polymerase II CTD. A 600 kDa protein complex with these properties was isolated, and specific components were identified using peptide microsequence analysis. This analysis indicated that proteins comprising the multi-subunit TFIIF complex, in addition to several novel factors, were associated with Tat using both *in vitro* and *in vivo* analysis. The Tat-associated kinase bound to the activation domain of Tat, and its ability to hyperphosphorylate RNA polymerase II was markedly stimulated by Tat. Furthermore, the addition of the Tat-associated kinase to *in vitro* transcription assays stimulated the ability of Tat to activate HIV-1 transcription. These results define a cellular kinase complex whose activity is modulated by Tat to result in activation of HIV-1 transcription.

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Introduction

The regulation of human immunodeficiency virus 1 (HIV-1) gene expression is dependent on the ability of the viral transactivator protein Tat to stimulate the elongation properties of RNA polymerase II (reviewed in Jones and Peterlin, 1994). Tat increases the number of processive transcripts synthesized from the HIV-1 long terminal repeat (LTR), in contrast to the abundance of short or non-processive transcripts that are seen in the absence of Tat (Kao *et al.*, 1987; Laspias *et al.*, 1989; Ratnasabapathy *et al.*, 1990; Marciniak and Sharp, 1991; García-Martínez *et al.*, 1995). A regulatory element known as TAR, which forms a stable RNA stem-loop structure extending from

the HIV-1 transcription initiation site to +60, is required for Tat activation of HIV-1 transcription (Rosen *et al.*, 1985; Feng and Holland, 1988). Tat (Dingwall *et al.*, 1990), in addition to at least two cellular proteins, RNA polymerase II and TRP-185, bind to TAR RNA (Wu-Baer *et al.*, 1995a,b). Tat, in conjunction with cellular factors, may function to release RNA polymerase II which is paused by its binding to TAR RNA (Wu-Baer *et al.*, 1995a,b). However, TAR RNA does not function as a classical transcription terminator in that deletion of this element does not result in constitutive activation of HIV-1 gene expression (Feinberg *et al.*, 1991). The ability of Tat to associate directly with RNA polymerase II probably directly or indirectly catalyzes a process that increases HIV-1 transcriptional elongation (Keen *et al.*, 1996; Mavankal *et al.*, 1996).

One of the major mechanisms by which promoter clearance and/or elongation is stimulated is by increasing the phosphorylation of the RNA polymerase II C-terminal domain (CTD) (Cadena and Dahmus, 1987; Payne *et al.*, 1989; Weeks *et al.*, 1993; O'Brien *et al.*, 1994). The CTD of the largest subunit of RNA polymerase II is comprised of 52 repeats of a heptad amino acid sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser and is the target for phosphorylation by cellular kinases (Cadena and Dahmus, 1987; Payne *et al.*, 1989; Weeks *et al.*, 1993; O'Brien *et al.*, 1994). An intact CTD is critical for growth in yeast (Koleske and Young, 1995) and for enhancer function in mammalian cell lines (Gerber *et al.*, 1995). The CTD binds a group of cellular factors, known as SRB or mediator factors (Thompson *et al.*, 1993; Kim *et al.*, 1994), which are important for transcriptional activation. During the initiation of transcription, the hypophosphorylated or Ila form of RNA polymerase II associates with the transcriptional pre-initiation complex by binding to TBP (Lu *et al.*, 1991, 1992) and it is subsequently phosphorylated by a kinase present within transcription factor TFIIF to the Ilo form (Usheva *et al.*, 1992). A cellular kinase complex comprised of cdk7, cyclin H and MAT1/p36, designated cyclin-dependent kinase-activating kinase (CAK), is present in TFIIF and is essential for CTD phosphorylation (Drapkin *et al.*, 1994; Feaver *et al.*, 1994; Fisher and Morgan, 1994; Roy *et al.*, 1994; Fisher *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhatair *et al.*, 1995; Tassan *et al.*, 1995a).

A role for phosphorylation of the RNA polymerase II CTD in transcriptional elongation has been shown elegantly in *in vivo* studies of the *Drosophila hsp70* and *hsp26* genes (Weeks *et al.*, 1993; O'Brien *et al.*, 1994). Antibodies that preferentially recognize the Ila and Ilo forms of RNA polymerase II indicate that paused RNA polymerase II on the *hsp70* and *hsp26* genes is predominantly in the unphosphorylated form (Weeks *et al.*, 1993; O'Brien *et al.*, 1994). However, a change in RNA polymerase II into a form that is competent for transcriptional elongation

coincides with phosphorylation of the CTD (Weeks *et al.*, 1993; O'Brien *et al.*, 1994). Studies using the nucleoside analog 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), which inhibits CTD phosphorylation, indicate that this analog also markedly decreases transcriptional elongation using both *in vitro* and *in vivo* transcription assays (Chodosh *et al.*, 1989; Marciniak and Sharp, 1991; Yankulov *et al.*, 1995). In contrast to these studies, studies on the role of phosphorylation of the CTD in regulating basal transcription in *in vitro* assays using highly purified transcription factors has given variable results (Parvin and Sharp, 1993; Serizawa *et al.*, 1993; Akoulitchev *et al.*, 1995; Makela *et al.*, 1995). *In vitro* transcription assays with the adenovirus major late promoter using either the CTD kinase inhibitor H8 or preparations of TFIIF containing mutant cdk7 proteins demonstrate that basal transcription occurs in the absence of CTD phosphorylation (Serizawa *et al.*, 1993; Makela *et al.*, 1995). However, *in vitro* transcription assays with the TATA-less dihydrofolate reductase (DHFR) promoter indicate that the CTD is important for transcription initiation and that CTD phosphorylation is critical for promoter clearance and/or transcription elongation (Akoulitchev *et al.*, 1995). Thus, CTD phosphorylation appears to be critical *in vivo* for promoter clearance and/or transcriptional elongation, although its effects in *in vitro* transcription assays are dependent on the promoter tested.

Given the fact that CTD phosphorylation can be associated with enhanced promoter clearance and/or transcriptional elongation, Tat may function to catalyze increases in RNA polymerase II phosphorylation resulting in stimulation of HIV-1 promoter clearance and/or transcriptional elongation. Tat has been demonstrated to bind a cellular kinase that is capable of hyperphosphorylating the RNA polymerase II CTD (Herrmann and Rice, 1995; Yang *et al.*, 1996). However, the identity of this kinase has not been reported. We fractionated HeLa nuclear extract using multiple chromatographic steps and assayed fractions based on their ability to bind to Tat, hyperphosphorylate the RNA polymerase II CTD and stimulate Tat activation using *in vitro* transcription assays. We isolated a complex of proteins containing components of TFIIF that met these criteria. Tat stimulated the ability of this Tat-associated kinase to phosphorylate RNA polymerase II and associated *in vivo* with components of this kinase complex. These studies demonstrate that the cdk7–cyclin H–MAT1 kinase complex, when present with other TFIIF components, is critical for CTD phosphorylation and stimulation of transcription by Tat.

Results

Purification of a Tat-associated kinase

Previous studies indicated that the RNA polymerase II CTD is critical for Tat activation and that Tat can bind a cellular kinase that phosphorylates the CTD (Herrmann and Rice, 1995; Yang *et al.*, 1996). To characterize the properties of this kinase further, a HeLa nuclear extract was fractionated through multiple column chromatographic steps (Figure 1A) and fractions were assayed based on their ability to associate directly with the HIV-1 Tat protein and hyperphosphorylate recombinant CTD (Figure 1B). Approximately 200 l of HeLa cells were

used to prepare nuclear extract which initially was fractionated on a phosphocellulose column. The column was washed with 0.35 M KCl followed by elution of the kinase with 1.0 M KCl at pH 7.9. The 1.0 M KCl eluate from the phosphocellulose column was again chromatographed on a phosphocellulose column and eluted using a 0.1–1.0 M KCl gradient at pH 7.5. Fractions containing the Tat-associated kinase were concentrated on a Q Sepharose column, eluted with 1.0 M KCl and fractionated on a Superdex 200 column with the kinase activity eluting at a mol. wt of ~600 kDa. The kinase activity was then applied sequentially to a heparin–agarose column that was eluted with a 0.1–1.0 M KCl gradient, a mono Q FPLC column which was eluted with a 0.1–1.0 M KCl gradient at pH 7.9, and an additional mono Q FPLC column eluted with a 0.1–0.5 M KCl gradient at pH 7.3. Further chromatographic steps resulted in the loss of kinase activity. The purification scheme outlined in Figure 1A resulted in a >10 000-fold increase in the specific activity of the Tat-associated kinase.

Activity of the Tat-associated kinase correlates with the ability of Tat to stimulate HIV-1 *in vitro* transcription

Fractions from the final mono Q FPLC gradient were analyzed for their ability to phosphorylate the CTD. These fractions were either bound to GST–Tat (Figure 2A) or assayed in solution for their ability to phosphorylate the GST–CTD fusion protein (Figure 2B). Following the kinase reactions, the samples were analyzed by SDS–PAGE and autoradiography. The predominant kinase activity which bound to GST–Tat was present in column fractions 22, 23 and 24, and resulted in small quantities of the hypophosphorylated or I₀ form of the CTD with the majority of the CTD in the hyperphosphorylated or I₅ form (Figure 2A). Kinase activity was also detected with these column fractions in the absence of Tat binding (Figure 2B). The role of Tat in stimulating CTD kinase activity with RNA polymerase II using solution kinase assays is presented below.

Next we determined whether fractions containing the Tat-associated kinase activity correlated with the ability of these fractions to stimulate Tat activation of the HIV-1 LTR using previously described *in vitro* transcription assay conditions (Marciniak *et al.*, 1990). Fractions from the final mono Q column were incubated with HeLa nuclear extract in the presence or absence of bacterially produced Tat. Templates included a wild-type HIV-1 template and an HIV-1 template containing mutation of the TAR RNA loop sequences between +31 and +34 (Wu-Baer *et al.*, 1995). These templates contained different sized G-less cassettes (Zhou and Sharp, 1995) inserted 1100 bp downstream of the HIV-1 transcription initiation site and generated G-less RNAs of 380 nucleotides for wild-type and 110 nucleotides for the loop mutant. Tat-mediated increases in HIV *in vitro* transcription correlated with the presence of Tat-associated kinase activity (Figure 2C). For example, the peak of kinase activity bound to Tat seen in mono Q fractions 22, 23 and 24 (Figure 2A) correlated with increases in Tat stimulation of HIV-1 transcription (Figure 2C). Although there was a 2.7-fold increase in the basal transcription upon addition of the kinase with the wild-type HIV-1 template, there was a 6.5-fold stimulation of

Fig. 1. (A) The purification scheme for the Tat-associated kinase is illustrated and is described in Materials and methods. The fractions that were active for binding to Tat and phosphorylation of the RNA polymerase II CTD from the Ila to the Ilo form were subject to the indicated chromatographic steps. (B) A schematic of the assay system used to isolate the Tat-associated kinase is shown.

Tat-mediated transcription (Figure 2C, compare fractions 20 and 23). Addition of fractions containing the Tat-associated kinase also resulted in a 2.5-fold increase in the level of *in vitro* transcription from the HIV-1 loop mutant template but no Tat activation was detected (Figure 2C). Similar transcriptional stimulation was not seen with the addition of other general transcription factors such as TFIIB (data not shown). These results indicated that the Tat-associated kinase was a limiting component in HeLa nuclear extract and that its addition stimulated the overall level of Tat-mediated transcription.

Identification of proteins comprising the Tat-associated kinase

The protein composition of the chromatographic fractions from the final mono Q FPLC column which contained the peak of Tat-associated kinase and transcriptional activity was then determined. SDS-PAGE followed by silver staining from the peak of kinase and transcriptional activity in fraction 23 demonstrated the presence of proteins of mol. wts 28, 33, 36, 39, 42, 52, 58, 62, 80, 90, a doublet at ~140 and 160 kDa (Figure 3A). The proteins in this fraction were analyzed by SDS-PAGE, transferred to a

PVDF membrane and stained with Ponceau S. After digestion of each excised band with the endoproteinase LysC or trypsin, the resultant mixtures were separated by microbore HPLC, screened for optimum fractions by MALDI-TOF mass spectroscopy and submitted to peptide microsequencing. Comparison of the peptide sequences obtained by this analysis with amino acid sequences in DDBJ/EMBL/GenBank indicated that the 33 kDa protein had sequence identity with the p36/MAT1 assembly factor (Fisher *et al.*, 1995; Tassan *et al.*, 1995a), the 36 kDa protein had sequence identity with cyclin H (Fisher and Morgan, 1994), the 52 kDa protein had sequence identity with the p52 subunit of TFIIF (Marinoni *et al.*, 1996), the 62 kDa protein had sequence identity with the p62 subunit of TFIIF (Fischer *et al.*, 1992) and the 80 kDa protein had sequence identity with the ERCC2 subunit of TFIIF (Weber *et al.*, 1990). The amino acid sequences of the 42 and 90 kDa proteins were not obtained since Western blot analysis confirmed their identity as cdk7 and ERCC3 respectively (see below). The amino acid sequences of the 28, 39, 58, 140 and 160 kDa proteins did not correspond to known components of TFIIF, and a determination of their relevance to TFIIF function is

Fig. 2. CTD kinase and transcriptional activity of column fractions containing the Tat-associated kinase. Fractions (20–27) from the final mono Q FPLC column were analyzed for GST–CTD kinase activity when either (A) bound to GST–Tat or (B) in solution in the absence of Tat. (C) These same fractions (20–27) or HeLa nuclear extract alone (NE) were assayed for *in vitro* transcriptional activity using an HIV-1 wild-type template (100 ng) and an HIV-1 loop mutant template (150 ng) each of which contained different sized G-less cassettes inserted 1100 bp downstream from the HIV-1 transcription initiation site. HeLa nuclear extract (50 µg) and either 100 ng of bacterially produced Tat (+) or GST (–) were included in these assays. Following the *in vitro* transcription reaction, the RNA was isolated and cleaved with RNase T1 to generate different sized G-less cassettes (380 nucleotides for wild-type and 110 nucleotides for the loop mutant). Gel electrophoresis was performed followed by autoradiography. The RNAs transcribed by the wild-type and loop mutant templates are shown in two separate panels because of the distance between these two species following electrophoresis and autoradiography.

currently underway. This data indicated that components of TFIIF, in addition to other cellular factors, comprised the Tat-associated kinase.

Column fractions from the final mono Q FPLC column

assayed in Figure 2 were next analyzed by Western blot to determine whether Tat-associated kinase activity and Tat-mediated increases in transcriptional activity correlated with the presence of TFIIF subunits (Figure 3B). Thus we could correlate the kinase and transcriptional activity of these fractions with the presence of components of CAK, including cdk7, cyclin H and MAT1, in addition to other TFIIF components p89, p80 and p62. The presence of the p34 component of TFIIF was not analyzed by Western blot analysis. Antibodies directed against the RNA polymerase II CTD and cdk8 (Tassan *et al.*, 1995b) were also used in this analysis. The peak levels for different members of the TFIIF kinase complex including cdk7, cyclin H and MAT1 in addition to p89 and p62 correlated precisely with the kinase and transcriptional activity of the Tat-associated kinase (Figure 3B). Western blot analysis indicated that the presence of the TFIIF subunits p80 and p44 (which appeared faintly on the silver-stained gel) also correlated with the activity of the kinase (data not shown). Low amounts of p44 as compared with other TFIIF components were found in three different preparations of the Tat-associated kinase. The reasons for the low quantities of this TFIIF component in our preparations compared with previously described TFIIF preparations may be due to differences in the purification scheme used. Cdk8 (data not shown) and RNA polymerase II (Figure 3B) were not detected in fractions containing the Tat-associated kinase. These results indicated that multiple factors comprising TFIIF were components of the Tat-associated kinase.

Cdk7 not associated with TFIIF fails to associate with Tat and phosphorylate the CTD

Previous data have demonstrated that cdk7, cyclin H and MAT1 can migrate in two major complexes of 600 and 200 kDa in gel filtration chromatography (Fisher *et al.*, 1995; Drapkin *et al.*, 1996; Reardon *et al.*, 1996). The 600 kDa complex contains additional components of the TFIIF complex, while the 200 kDa complex contains cdk7, cyclin H and MAT1 (Fisher *et al.*, 1995), although ERCC2 can also associate with this complex (Drapkin *et al.*, 1996; Reardon *et al.*, 1996). In nuclear extracts, the predominant amount of cdk7 is associated with TFIIF, while in cytoplasmic (S100) extract, most of the cdk7 migrates at 200 kDa and is not associated with TFIIF components (Fisher *et al.*, 1995). To determine whether CAK alone or in conjunction with other components of TFIIF is sufficient for Tat association and phosphorylation of the CTD, we fractionated both HeLa nuclear and cytoplasmic (S100) extract on a Superdex 200 column (Figure 4). Following fractionation of either HeLa nuclear extract or S100 on Superdex 200, the Tat-associated kinase activity was correlated with Western blot analysis of these fractions in the absence of their binding to GST–Tat for the presence of the TFIIF components p89, p62 and cdk7.

As expected, Superdex 200 fractionation of HeLa nuclear extract followed by binding of these fractions to GST–Tat resulted in Tat-associated kinase activity correlating with the 600–650 kDa peak of the TFIIF containing p89, p62 and cdk7 (Figure 4A and B). There was no detectable Tat-associated kinase activity present in fractions from HeLa nuclear extract migrating between 150 and 170 kDa although cdk7 was present (Figure 4A

Fig. 3. TFIIF components comprising the Tat-associated kinase. (A) Fraction 23 from the final mono Q FPLC column containing the peak of kinase and transcriptional activity was subjected to 10% SDS-PAGE and silver staining. The positions of proteins of mol. wts 28, 33, 36, 39, 42, 52, 58, 62, 80, 90, 140 and 160 kDa are indicated. (B) Western blot analysis was performed on fractions from the final mono Q FPLC column using antibodies to the TFIIF subunits p89, p62, cdk7, cyclin H, MAT1/p36 and RNA polymerase II CTD, and detected using ECL.

and B). Superdex 200 fractionation of the S100 extract followed by binding of these fractions to GST-Tat also resulted in Tat-associated kinase activity peaking between 600 and 650 kDa and there was no detectable Tat-associated kinase activity in the 150–170 kDa fraction (Figure 4C). The Tat-associated kinase activity correlated with the presence of TFIIF components including a minor component of cdk7 (Figure 4D), while fractions containing the majority of cdk7 which migrated between 150 and 170 kDa did not have detectable kinase activity (Figure 4C). Western blot analysis of these column fractions indicated that the amount of cyclin H and MAT1 correlated with the distribution of cdk7 (data not shown).

Finally, we analyzed whether the inability of S100 fractions (Figure 4C and D) migrating between 150 and 170 kDa to phosphorylate the GST-CTD was due to a failure of cdk7 to associate with GST-Tat. Portions of S100 fractions which either resulted in CTD phosphorylation (fractions 30 and 32) or failed to phosphorylate the CTD (fractions 40 and 42) were bound to glutathione-agarose beads containing either GST or GST-Tat (Figure 5). Both the unbound and bound proteins from these fractions, as well as the purified Tat-associated kinase, were analyzed by Western blot analysis with antibodies directed against cdk7 (Figure 5A) or p89 (Figure 5B). This analysis indicated that cdk7 present in fractions 30 and 32, which was capable of phosphorylating GST-CTD, could bind efficiently to Tat as compared with GST (Figure 5A, lanes 3–6). In contrast, cdk7 present in fractions 40 and 42 to result in phosphorylation of the GST-CTD was unable to bind to either Tat or GST (Figure 5A, lanes 7–10). The p89 present in fractions 30 and 32 also bound to Tat but not GST (Figure 5B, lanes 3–6), while fractions 40 and 42 lacked other TFIIF components including p89 (Figure 5B, lanes 7–10). These results indicate that a high molecular weight complex containing TFIIF components could

Fig. 4. CAK alone fails to associate with Tat and phosphorylate the RNA polymerase II CTD. HeLa nuclear extract (A and B) or cytoplasmic fractions (S100) (C and D) were fractionated on a Superdex 200 column. The position of the molecular weight markers eluted from the Superdex 200 column and the numbers of each of the column fractions are indicated. (A and C) The ability of these fractions following their binding to GST-Tat to phosphorylate the GST-CTD was assayed. (B and D) Western blot analysis of these same fractions prior to their binding to GST-Tat was performed using antibodies directed against p89, p62 and cdk7 followed by ECL detection.

Fig. 5. CAK alone fails to associate with Tat. HeLa cytoplasmic (S100) fractions 30, 32, 40 and 42 which were isolated by Superdex 200 chromatography and analyzed in Figures 4C and D were bound to agarose beads containing either GST-Tat (+) or GST (-). Both the free and bound proteins were then analyzed by Western blot analysis with antibodies directed against (A) cdk7 or (B) p89, followed by ECL detection. The purified Tat-associated kinase (TAK) was also incubated with either GST-Tat or GST and also analyzed by Western blot analysis.

associate efficiently with Tat and hyperphosphorylate the CTD.

Components of TFIIF directly associate with Tat

Components of TFIIF were present in column fractions containing the Tat-associated kinase and the Tat transcriptional stimulatory activity so that it was important to determine whether these proteins were able to associate directly with Tat. Column fraction 23 from the final mono Q FPLC column that contained the peak of the kinase activity was bound to glutathione beads containing either GST or GST-Tat (Figure 6). Following extensive washing of the beads, proteins that either bound to the beads or remained in the supernatant were assayed for Tat-associated kinase activity, and Western blot analysis was performed with antibodies directed against different TFIIF subunits. Following binding to glutathione beads containing GST-Tat, the majority of the kinase activity was associated with the beads (Figure 6A, lane 3) and was not detected in the supernatant (Figure 6A, lane 1). In contrast, the kinase activity was present primarily in the supernatant following binding to glutathione beads containing GST (Figure 6A, lane 2) rather than being bound to the beads (Figure 6A, lane 4). Both the supernatant and the bound fractions from the GST and GST-Tat beads of the Tat-associated kinase were next assayed using antibodies directed against components of the TFIIF complex including p89, p62 and cdk7. All three of these TFIIF subunits were present predominantly bound to the GST-Tat beads (Figure 6B, lane 2) rather than in the supernatant (Figure

6B, lane 1), while these components were present almost exclusively in the supernatant (Figure 6B, lane 3) rather than bound to the GST beads (Figure 6B, lane 4). These results indicated that the kinase activity that bound to Tat correlated with the association of several components of TFIIF.

Next we determined whether the binding of the Tat-associated kinase complex required domains of Tat that were critical for its ability to activate HIV-1 transcription. The kinase was assayed using wild-type Tat, GST or Tat mutants that were defective for HIV-1 activation. These Tat mutants either substituted glycine for eight arginine residues in the Tat basic domain or substituted serine for four critical cysteine residues in the Tat activation domain (Mavankal *et al.*, 1996). These proteins were expressed as fusions with GST, bound to glutathione-agarose beads, and assayed for their ability to bind the Tat-associated kinase and phosphorylate the CTD. The similar levels of kinase activity bound to wild-type Tat (Figure 6C, lane 1) and the Tat basic mutant (Figure 6C, lane 3) indicated that the basic domain is not essential for Tat interaction with the kinase. However, there was a 10-fold decrease in CTD phosphorylation when the kinase was bound to a Tat protein containing mutations of four cysteine residues (Figure 6C, lane 5) as compared with wild-type Tat (Figure 6C, lane 1). There was no significant association of the kinase with beads containing GST alone (Figure 6C, lane 7). The GST and GST-Tat proteins were subject to Coomassie staining to demonstrate that equivalent amounts of these proteins were used in the kinase assays (Figure 6D). It should be noted that the substitution of the amino acids in the Tat basic domain resulted in slower migration of this protein as compared with wild-type Tat (Figure 6D). Western blot analysis confirmed the weak association of components of the Tat-associated kinase activity with the Tat cysteine mutant as compared with wild-type Tat (data not shown). These data suggest that the Tat activation domain is critical for the association of the cellular kinase.

In addition, we determined whether Tat was able to associate with TFIIF components *in vivo*. Influenza hemagglutinin sequences were fused to the C-terminus of a *tat* cDNA and this construct was cloned downstream of the T7 promoter in the pTM1 expression vector. Following transfection of this construct into HeLa cells, the cells were infected with a vaccinia virus which produced T7 RNA polymerase in order to induce Tat protein expression. Nuclear extract was prepared at 24 h post-transfection and immunoprecipitation of the extract was performed with antibodies directed against components of TFIIF or unrelated antibodies. Following SDS-PAGE of the immunoprecipitation assays, Western blot analysis was performed with the monoclonal antibody 12CA5 which can detect the epitope-tagged Tat protein. Antibodies directed against p89 (Figure 7, lane 3) and cyclin H (Figure 7, lane 6) were able to immunoprecipitate 30–50% of the Tat protein present in the nuclear extract while unrelated antibodies did not result in detectable immunoprecipitation of Tat (Figure 7, lanes 2, 4 and 5). Thus, TFIIF components can associate with Tat using both *in vivo* and *in vitro* assays.

Tat stimulates the phosphorylation of RNA polymerase II

Although we found that components of TFIIF were able to associate with Tat, it was critical to determine whether

Fig. 6. TFIIF subunits directly associate with the Tat activation domain. (A) A fraction with the peak of kinase and transcriptional activity from the final mono Q column was incubated with glutathione-agarose beads containing either wild-type Tat (lanes 1 and 3) or GST (lanes 2 and 4), and the supernatant (lanes 1 and 2) and the bound portions (lanes 3 and 4) were assayed for phosphorylation of the CTD. (B) The supernatants (lanes 1 and 2) and beads (lanes 3 and 4) from (A) were also subjected to Western blot analysis using antibodies against p89, p62 and cdk7, followed by detection with ECL. (C) A fraction from the peak of kinase and transcriptional activity was bound to GST fusions of either wild-type Tat (lanes 1 and 2) or mutant Tat proteins including Tat/basic (lanes 3 and 4) and Tat/cys (lanes 5 and 6) or GST alone (lanes 7 and 8) and assayed for its ability to phosphorylate the CTD (+) or in its absence (-). (D) A Coomassie-stained gel of the proteins used in (C) is also shown.

the association of Tat altered TFIIF kinase activity. The Tat-associated kinase was incubated with purified RNA polymerase II isolated from HeLa cells in the presence of [γ - 32 P]ATP followed by SDS-PAGE and autoradiography. Incubation of the Tat-associated kinase and RNA polymerase II resulted in the generation of both the Ila and Ilo forms of RNA polymerase II (Figure 8A, lane 3). In the presence of wild-type Tat, there was a 7-fold increase in the amount of the hyperphosphorylated or Ilo form of the polymerase (Figure 8A, lanes 4–6). No similar increase in the amount of the Ilo form of RNA polymerase II was noted in the presence of a GST fusion protein with the activation domain of VP16 (Figure 8A, lanes 7–9) or GST alone (Figure 8A, lanes 10–12). Unlike wild-type Tat (Figure 8B, lanes 4–6), a Tat mutant that changed the lysine residue at position 41 to alanine and inactivated Tat transcriptional activation did not stimulate the formation of the Ilo form of RNA polymerase II (Figure 8B, lanes 7–9). In contrast to the results with Tat and RNA polymerase II, Tat did not significantly stimulate the hyperphosphorylation of the GST-CTD (Figure 8C, lanes 3–6). There was no increase in phosphorylation of RNA polymerase II by the addition of TAR RNA or DNA with the Tat-associated kinase (data not shown). These results indicate that Tat markedly stimulates hyperphosphorylation of RNA polymerase II but not recombinant CTD, suggesting that interactions between Tat and RNA polymerase II are potentially critical for this event.

Fig. 7. Tat associates *in vivo* with TFIIF subunits. The 12 amino acid influenza hemagglutinin epitope was attached to the C-terminus of a *tat* cDNA and this construct was cloned downstream of the T7 promoter in the pTMI expression vector. Following transfection of this construct into HeLa cells, expression of Tat was induced by infection with a vaccinia virus that produced T7 RNA polymerase. Nuclear extract was prepared at 24 h post-transfection and immunoprecipitation was performed with 2 μ l of either pre-immune serum (lane 2) or rabbit polyclonal antibodies directed against p89 (lane 3), CREB (lane 4), ILF (lane 5) or cyclin H (lane 6). SDS-PAGE followed by Western blot analysis with 12CA5 monoclonal antibody which recognizes the epitope-tagged Tat protein was then performed. Tat was identified following ECL detection. In lane 1, 20% of the HeLa nuclear extract that was used in each of the immunoprecipitation assays was analyzed directly by SDS-PAGE and Western blot analysis.

Depletion of MAT1/p36 inhibits *in vitro* transcriptional stimulation by Tat and phosphorylation of RNA polymerase II

Since Tat in conjunction with the Tat-associated kinase stimulates the hyperphosphorylation of RNA polymerase

Fig. 8. Tat stimulates the ability of TFIIF to phosphorylate RNA polymerase II. Kinase assays were performed with either (A and B) RNA polymerase II purified from HeLa cells or (C) GST-CTD. The RNA polymerase II or the GST-CTD were either added alone (lane 1), the Tat-associated kinase was added alone (lane 2), or these two components were added together in the absence (lane 3) or in the presence of either 10, 30 or 90 ng of (A) Tat (lanes 4–6), GST-VP16 (lanes 7–9) or GST (lanes 10–12); (B) Tat (lanes 4–6), Tat/K41A (lanes 7–9) or GST (lanes 10–12); (C) Tat (lanes 4–6), GST-VP16 (lanes 7–9) or GST (lanes 10–12).

II, we next addressed whether depletion of the kinase activity decreased *in vitro* transcriptional activation by Tat. Furthermore, we attempted to correlate increases in kinase activity with the ability of Tat to stimulate HIV-1 *in vitro* transcription. Both *in vitro* transcription and kinase assays were performed to address these points. Rabbit polyclonal antibodies to MAT1/p36 were used to determine if they were able to inhibit Tat stimulation of HIV-1 transcription. *In vitro* transcription assays were performed with wild-type and loop mutant HIV-1 LTR templates in the presence of HeLa nuclear extract. Following incubation of these templates with the HeLa nuclear extract for 30 min, 0.5 μ l of either pre-immune serum or the MAT1/p36 antibody was added to the *in vitro* transcription reactions for 15 min. Ribonucleotides and either GST (Figure 9A, lanes 2, 4 and 6) or Tat (Figure 9A, lanes 1, 3 and 5) were then added and the *in vitro* transcription reaction proceeded for 30 min prior to analysis by gel electrophoresis and autoradiography. This analysis demonstrated that MAT1/p36 antibody, but not pre-immune serum, was able to prevent Tat transactivation (Figure

Fig. 9. MAT1/p36 antibody inhibits *in vitro* transcriptional stimulation by Tat and phosphorylation of RNA polymerase II. (A) Run-off *in vitro* transcription assays were performed with HIV-1 wild-type and loop mutant templates in the presence of HeLa nuclear extract (50 μ g) and 100 ng of either Tat (lanes 1, 3 and 5) or GST (lanes 2, 4 and 6). Rabbit serum (0.5 μ l), either pre-immune (lanes 3 and 4) or polyclonal antibody directed against p36/MAT1 (lanes 5 and 6), was added to the *in vitro* transcription reactions after a 30 min pre-incubation of the HIV-1 templates and HeLa nuclear extract followed by the addition of Tat and ribonucleotides and incubation for an additional 30 min. Gel electrophoresis and autoradiography were performed, and the positions of the wild-type and loop mutant transcripts are indicated. (B) Kinase assays were performed with RNA polymerase II purified from HeLa cells added either alone (lane 1), in the presence of the purified Tat-associated kinase (lane 2) or these components were added together with either 0.1 or 0.5 μ l of pre-immune rabbit serum (lanes 3 and 4) or the same amounts of MAT1/p36 polyclonal antibody (lanes 5 and 6). The positions of the Ilo and Ila forms of RNA polymerase II are indicated following SDS-PAGE and autoradiography. (C) Immunoprecipitation of RNA polymerase II was performed following a 30 min pre-incubation of the HIV-1 LTR and HeLa nuclear extract, *in vitro* transcription was performed for 30 min with unlabeled ribonucleotides in the presence of 100 ng of either Tat (lanes 1 and 3) or GST (lanes 2 and 4) and [γ - 32 P]ATP. Immunoprecipitation was then performed by the addition of 3 μ l of monoclonal antibodies directed against either the CTD (lanes 1 and 2) or a flag epitope control (lanes 3 and 4) followed by SDS-PAGE and autoradiography.

9A, lanes 3–6). For example, the 5.3-fold level of Tat transactivation (Figure 9A, lanes 3 and 4) decreased to 1.2-fold with the addition of MAT1/p36 antibody (Figure 9A, lanes 5 and 6). There was also a 1.5-fold decrease in

Fig. 10. The Tat-associated kinase activity prevents DRB inhibition of Tat transcriptional activation. (A) A portion of the active kinase fraction from the final mono Q column was assayed following its binding to glutathione-agarose beads containing GST-Tat in the absence (lane 1) or in the presence of 25 (lane 2), 50 (lane 3) or 100 μM (lane 4) of DRB. (B) A Coomassie-stained gel of the GST-Tat beads is shown. (C) *In vitro* transcription analysis of the HIV-1 wild-type template and the loop mutant template was performed with HeLa nuclear extract (50 μg) in the presence of 100 ng of GST protein (-) or Tat (+) in the absence or in the presence of 25, 50 or 100 μM of DRB. (D) *In vitro* transcription analysis was performed with HeLa nuclear extract (50 μg) in the presence of an HIV-1 wild-type template and a loop mutant template with either 100 ng of Tat (lanes 1, 3, 5 and 7) or GST (lanes 2, 4, 6 and 8). The *in vitro* transcription reactions were performed in the absence (lanes 1–4) or the presence (lanes 4–8) of DRB (50 μM) with the Tat-associated kinase either present (lanes 3, 4, 7 and 8) or absent (lanes 1, 2, 5 and 6). Following the *in vitro* transcription reactions, the RNA was isolated and cleaved with RNase T1 to generate different sized G-less cassettes (380 nucleotides for wild-type and 110 nucleotides for the loop mutant) followed by gel electrophoresis and autoradiography.

basal HIV-1 transcription (Figure 9A, lanes 2, 4 and 6) and a 2.4-fold decrease in transcription from the HIV-1 loop mutant in the presence of the MAT1/p36 antibody (Figure 9A, lanes 1–6). In results obtained from three separate experiments, MAT1/p36 antibody prevented the 5- to 6-fold stimulation of transcription by Tat while resulting in an ~2-fold decrease in HIV-1 basal transcription. These data suggest that components of TFIIF are essential for Tat transactivation, although it is evident that these components are also important for regulating basal levels of HIV-1 transcription.

Next we attempted to correlate the depletion of MAT1/p36 with changes in the ability of the Tat-associated kinase to phosphorylate RNA polymerase II. Kinase assays were performed with purified RNA polymerase II isolated from HeLa cells and the purified Tat-associated kinase (Figure 9B). The addition of 0.5 μl of MAT1/p36 antibody (Figure 9B, lane 6), but not a similar quantity of pre-immune rabbit serum (Figure 9B, lane 4), resulted in inhibition of the RNA polymerase II phosphorylation by the Tat-associated kinase. The ability of Tat to increase hyperphosphorylation of RNA polymerase II using *in vitro* transcription conditions was next assayed. *In vitro* transcription of the HIV-1 LTR in the presence of HeLa nuclear extract was performed following a 30 min pre-incubation period and the addition of either Tat (Figure 9C, lanes 1 and 3) or GST (Figure 9C, lanes 2 and 4) and unlabeled ribonucleotides. At this time, [γ - 32 P]ATP was also added to the *in vitro* transcription assay. Following a 30 min *in vitro* transcription reaction, immunoprecipitation of the HeLa nuclear extract was performed with monoclonal antibodies directed against either the CTD (8WG16) or the flag epitope as a control (Figure 9C, lanes 1–4). This analysis demonstrated, using *in vitro* transcription conditions, that the presence of Tat led to the hyperphosphorylation of RNA polymerase II (Figure 9C, lanes 1 and 2). These results suggest that Tat activation is dependent upon the presence of TFIIF components and that Tat activation of HIV-1 transcription is associated with increased phosphorylation of RNA polymerase II.

DRB inhibits both CTD kinase activity and Tat transcriptional stimulation

DRB is a nucleoside analog that markedly inhibits the ability of Tat to stimulate HIV-1 transcriptional elongation and also inhibits the kinase activity present in TFIIF (Chodosh *et al.*, 1989; Marciniak and Sharp, 1991; Yankulov *et al.*, 1995). Since the Tat-associated kinase has a number of components in common with TFIIF, we next determined whether this kinase was sensitive to inhibition by DRB. The addition of DRB to the peak activity from the final mono Q FPLC column inhibited the CTD kinase activity in a concentration-dependent manner (Figure 10A, lanes 1–4). The DRB concentrations used in this experiment (25–100 μM) were similar to those reported to inhibit the kinase activity present in TFIIF (Yankulov *et al.*, 1995). To confirm previous results that DRB inhibited Tat transactivation (Marciniak and Sharp, 1991), DRB was added to *in vitro* transcription analysis with the HIV-1 wild-type and loop mutant templates in the presence and absence of Tat. There was marked inhibition of the ability of Tat to activate HIV-1 transcription in the presence of increasing concentrations of DRB (Figure 10C). There was a smaller fold decrease in the basal level of HIV-1 transcription in the presence of DRB, which is consistent with the observation that the primary effect of DRB was due to the inhibition of transcriptional elongation (Figure 10C) (Chodosh *et al.*, 1989; Marciniak and Sharp, 1991). Thus DRB inhibited both Tat-associated kinase activity and Tat transactivation.

A fraction containing the peak of kinase and Tat stimulatory activity from the final mono Q FPLC column was assayed to determine whether its addition could overcome the effects of DRB on inhibiting Tat transactivation. When this fraction was added to the *in vitro* transcription assay in the absence of DRB, there was an increase in the level of Tat-induced transcription from the HIV-1 wild-type template in addition to stimulation of basal transcription (Figure 10D, lanes 1–4). There was also a 2-fold stimulation of transcription from the HIV-1 loop mutant, although this template could not be transactiv-

ated by Tat (Figure 10D, lanes 1–4). Next we tested whether the addition of the Tat-associated kinase could stimulate *in vitro* transcription from HeLa extract in the presence of 50 μ M DRB. DRB nearly eliminated Tat activation reducing it to 1.2-fold (Figure 10D, lanes 5 and 6), and this inhibition was relieved by the addition of the Tat-associated kinase resulting in a 3.4-fold stimulation of transcription by Tat (Figure 10D, lanes 7 and 8). Similar findings were seen in three independent experiments (data not shown). These results indicated that the addition of the Tat-associated kinase could prevent the inhibitory effects of DRB on Tat-induced transcriptional elongation.

Discussion

The primary effect of Tat is to activate HIV-1 gene expression by stimulating the degree of promoter clearance and/or transcriptional elongation (reviewed in Jones and Peterlin, 1994). Several possibilities exist to explain the effects of Tat on increasing the processivity of RNA polymerase II. Tat may interact with specific transcriptional elongation factors to increase the elongation properties of RNA polymerase II (Zhou and Sharp, 1995). Alternatively, Tat may associate with or stimulate the activity of cellular kinases that increase the phosphorylation state of the RNA polymerase II CTD leading to increases in its transcriptional elongation properties (Herrmann and Rice, 1995; Yang *et al.*, 1996). Finally, Tat-mediated effects on increasing CTD phosphorylation may lead to the association of specific transcription factors with the CTD (Thompson *et al.*, 1993; Kim *et al.*, 1994). In the current study, we purified a complex of cellular proteins that interact with Tat, hyperphosphorylate the RNA polymerase II CTD and stimulate Tat transactivation. Furthermore the association of Tat with this kinase complex occurs under both *in vitro* and *in vivo* conditions. This protein complex, which contains multiple components of transcription factor TFIIF, is a strong candidate for cellular co-factors that interact with Tat to increase the elongation properties of RNA polymerase II.

TFIIF is an essential cellular transcription factor (Gerard *et al.*, 1991; Flores *et al.*, 1992) comprised of at least nine components, some of which are involved in the control of multiple regulatory pathways including DNA repair and cell cycle control, in addition to transcriptional activation (reviewed in Zawel and Reinberg, 1995). TFIIF becomes associated with the transcription pre-initiation complex either as part of the RNA polymerase II holoenzyme (Kim *et al.*, 1994; Koleske and Young, 1994; Ossipow *et al.*, 1995; Chao *et al.*, 1996; Maldonado *et al.*, 1996) or by associating with TFIIE which binds to the basal transcription factors TFIID, TFIIB, TFIIF and RNA polymerase II assembled on the promoter (Drapkin *et al.*, 1994; Maxon *et al.*, 1994; Ohkuma and Roeder, 1994). TFIIF is the only known general transcription factor with enzymatic activities that include an ATPase, helicase and CTD kinase (reviewed in Zawel and Reinberg, 1995). The holo-TFIIF complex contains: cdk7, cyclin H and MAT1, which constitute a kinase complex known as CAK (Drapkin *et al.*, 1994; Feaver *et al.*, 1994; Fisher and Morgan, 1994; Roy *et al.*, 1994; Fisher *et al.*, 1995; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995; Tassan *et al.*, 1995a); p89 (ERCC3), p80 (ERCC2) (Schaeffer *et al.*, 1993; Drapkin

et al., 1994; Schaeffer *et al.*, 1994) and the zinc finger proteins p44 and p34 which are involved in DNA repair (Humbert *et al.*, 1994); p62 (Fischer *et al.*, 1992); and the recently cloned p52 subunit (Marinoni *et al.*, 1996). *In vitro* studies suggest that TFIIF may exist in multiple forms, including core TFIIF which lacks CAK, holo-TFIIF which is associated with CAK or core TFIIF associated with other DNA repair proteins (Svejstrup *et al.*, 1995; Drapkin *et al.*, 1996; Reardon *et al.*, 1996; Sung *et al.*, 1996). Whether additional co-purifying factors such as those identified in our purification scheme are associated with TFIIF and potentially modulate its function remains a possibility. For example, a variety of previously uncharacterized proteins have been noted in different TFIIF preparations (Drapkin *et al.*, 1996; Reardon *et al.*, 1996).

The phosphorylation state of RNA polymerase II is altered during different steps in transcriptional activation. The hypophosphorylated form of RNA polymerase II binds to the pre-initiation complex via interactions with TBP (Lu *et al.*, 1991; Usheva *et al.*, 1992). The transition between the association of RNA polymerase II with the pre-initiation complex and its subsequent transcriptional elongation (Krumm *et al.*, 1995) is mediated, at least in part, by phosphorylation of the RNA polymerase II CTD by a kinase associated with TFIIF (Cadena and Dahmus, 1987; Payne *et al.*, 1989; Usheva *et al.*, 1992; O'Brien *et al.*, 1994). This kinase complex known as CAK can also exist in multiple forms, including the kinase complex alone (Fisher and Morgan, 1994; Tassan *et al.*, 1995a), the kinase complex associated with a single component of TFIIF known as ERCC2 (Drapkin *et al.*, 1996; Reardon *et al.*, 1996) or the kinase complex associated with holo-TFIIF (Feaver *et al.*, 1994; Fisher and Morgan, 1994; Roy *et al.*, 1994; Serizawa *et al.*, 1995). Only CAK associated with TFIIF subunits can efficiently phosphorylate the CTD when this domain is present in RNA polymerase II (Shiekhattar *et al.*, 1995; Drapkin *et al.*, 1996; Reardon *et al.*, 1996). Thus the stable association of CAK with TFIIF may be a critical step in regulating promoter clearance and/or the subsequent elongation properties of RNA polymerase II.

Since Tat binds a cellular kinase that phosphorylates the CTD (Herrmann and Rice, 1995) and the RNA polymerase II CTD is critical for Tat activation (Yang *et al.*, 1996), it was critical to identify and characterize this kinase. We developed a purification strategy to isolate a kinase or kinases from HeLa nuclear extract based on their ability to associate with Tat, hyperphosphorylate the CTD and increase Tat stimulation using *in vitro* transcription assays. During our purification, a variety of different kinases were detected, but only one kinase met our stated criteria. Gel filtration analysis indicated that the Tat-associated kinase was part of a complex with an M_r of ~600 kDa which contained a number of components present within TFIIF, including CAK (Drapkin *et al.*, 1994; Fisher *et al.*, 1995; Reardon *et al.*, 1996). Additional Tat-associated CTD kinase activity was not present in lower molecular weight protein complexes which migrate at ~150–170 kDa and contain cdk7, cyclin H and MAT1 alone (Fisher *et al.*, 1995). Whether CAK associated with ERCC2 may potentially associate with Tat and hyperphosphorylate the CTD remains to be determined

(Drapkin *et al.*, 1996; Reardon *et al.*, 1996). We conclude that CAK, in conjunction with other components of TFIIF or factors co-purifying with this complex, is required for association with Tat and hyperphosphorylation of the CTD. Tat stimulates the ability of the kinase associated with TFIIF to phosphorylate the RNA polymerase II CTD although this kinase does not markedly stimulate the phosphorylation of recombinant CTD. These results suggest that direct interactions between Tat and RNA polymerase II may be required for the ability of Tat to increase CTD phosphorylation (Mavankal *et al.*, 1996). Whether Tat can alter other enzymatic activities of TFIIF similar to the effects of the transcription factor TFIIE on TFIIF remains to be determined (Drapkin *et al.*, 1994; Maxon *et al.*, 1994; Ohkuma and Roeder, 1994).

A variety of transcriptional activators such as VP16, E2F and p53 in addition to Tat activate gene expression when present as GAL4 fusions bound to upstream promoter-binding sites (Southgate and Green, 1991; Xiao *et al.*, 1994; Blau *et al.*, 1996; Yang *et al.*, 1996). Although VP16, E2F and p53 increase transcriptional initiation, nuclear run-on and RNase protection assays indicate that a major component of their ability to activate transcription is due to increases in transcriptional processivity (Xiao *et al.*, 1994; Yankulov *et al.*, 1994; Blau *et al.*, 1996). Furthermore, the activation domain of VP16 associates with transcriptionally active TFIIF present in HeLa nuclear extract, and both the p62 and cdk7 components of TFIIF bind to wild-type but not a transcriptionally defective VP16 mutant activation domain (Xiao *et al.*, 1994; Blau *et al.*, 1996). However, a GST fusion protein containing the VP16 activation domain was unable to increase the ability of the Tat-associated kinase to increase phosphorylation of RNA polymerase II. These results suggest that Tat has properties in common with other transcriptional activators which interact with TFIIF to increase promoter clearance and/or transcriptional elongation, although there are likely critical differences in their mechanism of action.

Herrmann and Rice failed to detect TFIIF components such as cdk7 in HeLa nuclear extract that was bound to Tat was capable of phosphorylating GST-CTD (Herrmann *et al.*, 1996). Our unpublished data confirms that only extremely low levels of cdk7 can bind to Tat in Western blot analysis when incubated with unfractionated HeLa nuclear extract. The reason for this is likely due to a variety of inhibitory proteins present in HeLa nuclear extract that may prevent efficient binding of TFIIF components to Tat. However, our assay conditions and results are similar to data described by Herrmann and Rice (Herrmann and Rice, 1995; Herrmann *et al.*, 1996). Although different kinases can phosphorylate the CTD (Payne and Dahmus, 1993) the only kinase that was isolated in our purification scheme that bound specifically to the Tat activation domain and could hyperphosphorylate the CTD contained multiple components of TFIIF. Recently Zhou and Sharp (1995) described a kinase that was able to phosphorylate a 140 kDa elongation factor. Our unpublished observations indicate that TFIIF will phosphorylate a 140 kDa protein present in a partially purified Tat-SF1 fraction. Studies are currently underway to determine whether in fact this protein is pp140. Further work will be required to determine additional cellular

targets of TFIIF that may be involved in the ability of Tat to activate HIV-1 transcription.

The nucleoside analog DRB prevents Tat from increasing RNA polymerase II processivity in *in vitro* transcription assays but does not markedly alter the level of promoter-proximal transcripts (Marciniak and Sharp, 1991). DRB also inhibits transcriptional elongation by a variety of transcriptional activators and the kinase activity present in TFIIF (Yankulov *et al.*, 1995). Our results confirm these previous data and indicate that the addition of the Tat-associated kinase prevents the inhibition of Tat activation by DRB. Furthermore, DRB inhibits the Tat-associated CTD kinase activity at concentrations similar to those seen for its inhibition of Tat transcriptional activation. The ability of the Tat-associated kinase to prevent the effects of DRB on inhibiting Tat function suggests that TFIIF and Tat may be targeting the same step in transcriptional activation. However, we cannot rule out the fact that DRB inhibition of kinases other than cdk7 may also be involved in regulating Tat activation.

Previous data indicate that the primary effect of TFIIF is to stimulate promoter clearance and/or the early phases of transcriptional elongation rather than transcription initiation (Parvin and Sharp, 1993; Goodrich and Tjian, 1994; Maxon *et al.*, 1994; Akoulitchiev *et al.*, 1995). Tat also stimulates a similar step to result in HIV-1 transcriptional activation (Kao *et al.*, 1987; Laspia *et al.*, 1989; Marciniak *et al.*, 1990; Marciniak and Sharp, 1991; Kato *et al.*, 1992; Graeble *et al.*, 1993; García-Martínez *et al.*, 1995; Keen *et al.*, 1996). *In vitro* transcription analysis demonstrates that the effect of Tat activation can be detected following the synthesis of transcripts that extended past TAR RNA (García-Martínez *et al.*, 1995). This suggests that TAR RNA may serve as a pause site and that Tat may stimulate promoter clearance, resulting in a more processive RNA polymerase II. The fact that the hypophosphorylated form of RNA polymerase II binds to TAR RNA to a greater extent than does the hyperphosphorylated form would be consistent with a role for Tat in conjunction with TFIIF components in phosphorylating the RNA polymerase to relieve pausing induced by binding to TAR RNA (F.Wu-Baer and R.B.Gaynor, unpublished observations).

Increasing the concentration of TFIIF in *in vitro* transcription assays enhances promoter clearance (Goodrich and Tjian, 1994; Akoulitchiev *et al.*, 1995; Drapkin *et al.*, 1996). For example, the addition of TFIIF components present in the Tat-associated kinase results in a greater enhancement of Tat-stimulated transcription than basal transcription. However, the addition of higher concentrations of TFIIF to *in vitro* transcription assays with the HIV-1 LTR was able to reduce but not totally eliminate Tat stimulation (data not shown). Our preliminary data indicate that the 30 min pre-incubation period of HeLa extract (Marciniak *et al.*, 1990) or citrate treatment of the extract (Kato *et al.*, 1992), which is necessary to detect Tat transactivation in *in vitro* transcription assays, is probably due at least in part to the inactivation of TFIIF (L.F.García-Martínez and R.B.Gaynor, unpublished observations). These results would suggest that one critical step in Tat activation is the enhanced recruitment of limiting concentrations of TFIIF to the HIV-1 transcription complex.

During transcriptional elongation by RNA polymerase

II, most of the basal transcription factors other than TFIIF dissociate from the elongating polymerase (Zawel *et al.*, 1995). For example, TFIIF dissociates from the transcriptional elongation complex between 30 and 68 nucleotides from the transcriptional initiation site. In contrast, Tat remains stably associated with the HIV-1 transcriptional elongation complex (Keen *et al.*, 1996) and in fact can associate directly with RNA polymerase II (Mavankal *et al.*, 1996). Interaction of RNA polymerase II with Tat is mediated by the basic domain of Tat (Mavankal *et al.*, 1996) while cysteine residues in the activation domain of Tat are critical for interaction with components of TFIIF. Thus, it is possible that Tat may act as a bridging factor to anchor TFIIF to the transcriptional elongation complex via interactions with both RNA polymerase II and components of the TFIIF complex. Such effects could maintain RNA polymerase II in the hyperphosphorylated state during transcriptional elongation and could explain the ability of Tat to stimulate transcriptional elongation when delivered to the transcription complex via either DNA or RNA components. In addition, the ability of Tat to maintain RNA polymerase II—the hyperphosphorylated state could help to prevent pausing of transcription complexes which encounter complex RNA secondary structures (Keen *et al.*, 1996). Further studies will be required to understand the specific steps at which Tat catalyzes increases in promoter clearance and/or transcriptional elongation.

Materials and methods

Assay for Tat-associated CTD kinase activity

A fusion of the RNA polymerase II CTD domain with GST (Peterson *et al.*, 1992) (a kind gift of William Dynan) was expressed in *Escherichia coli* BL21 and purified as described previously (Smith and Johnson, 1988). In addition, this protein was cleaved with thrombin to generate the authentic CTD protein. Wild-type Tat, a Tat mutant Tat/cys which changed cysteine residues at position 22, 27, 31 and 34 to serine, or a Tat mutant Tat/basic which changed amino acids between residues 52 and 57 from arginine to glycine were each fused to GST (Mavankal *et al.*, 1996) and these proteins were expressed in *E. coli* BL21 and purified as previously described by binding to glutathione-agarose beads (García-Martínez *et al.*, 1995). The GST fusion with the VP16 activation domain has been described previously (Xiao *et al.*, 1994).

CTD kinase activity assay of cellular proteins bound to Tat was performed as described previously with modifications (Herrmann and Rice, 1995). Briefly, the GST-Tat protein bound to glutathione-agarose beads was quantified using Bradford reagent and standardized to 0.1 µg/µl. Beads were then washed with ECB [50 mM HEPES 7.9, 120 mM NaCl, 0.5% NP-40, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] and resuspended in a 50% slurry. Fractions containing cellular proteins (1/100 of each column fraction) were incubated in a total volume of 200 µl of buffer D (20 mM HEPES pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT and 20% glycerol) and mixed with 20 µl of Tat slurry. After 1 h incubation with rocking at 4°C to maintain the beads in suspension, the samples were spun briefly and beads were washed with 500 µl of 0.3% SDS ECB buffer and then 500 µl of TKB buffer (50 mM HEPES pH 7.5, 5 mM MnCl₂, 5 mM DTT, 1 mM PMSF and 0.5 mM MgCl₂) when needed. Reactions were then divided and incubated in the presence or absence of CTD. Finally, beads were resuspended in 50 µl of TKB which contained 5 mM sodium fluoride, 1 mM sodium orthovanadate and 2 µM ATP. 300 ng of GST-CTD and 0.6 µCi of [γ -³²P]ATP followed by incubation at room temperature for 1 h with rocking. Reactions were stopped by addition of SDS-PAGE loading dye and boiled at 95°C for 2 min before SDS-PAGE on a 10% polyacrylamide gel and autoradiography.

In vitro solution kinase assays with Tat and purified RNA polymerase II

RNA polymerase II was purified from HeLa cells and the activity was assayed as described (Reinberg and Roeder, 1987). For *in vitro* kinase

assays, the purified RNA polymerase II (250 ng) was incubated for 1 h at room temperature in TKB buffer containing 0.6 µCi of [γ -³²P]ATP in the presence of the Tat-associated kinase (100 ng) and either 10, 30 or 90 ng of wild-type or mutant Tat, GST-VP16 or GST. Kinase reactions were then subjected to SDS-PAGE on a 6% polyacrylamide gel and to autoradiography. Similar *in vitro* kinase assays were also performed using these same conditions in the presence of 500 ng of GST-CTD rather than RNA polymerase II.

Purification of Tat-associated kinase activity

Nuclear extracts and S100 fractions were prepared as described previously (Dignam *et al.*, 1983). For chromatography of cellular proteins, the protease inhibitors Pefabloc and pepstatin (Boehringer Mannheim) were added. HeLa nuclear extract (Dignam *et al.*, 1983) prepared from 10¹¹ cells was applied to a cellulose phosphate resin (Whatmann P11) with 15 mg of protein per ml of resin and a flow rate of three column volumes per hour. The column was washed first with 0.1 M KCl, then 0.3 M KCl, followed by elution with 1.0 M KCl, and this protein (70 mg in 70 ml) was dialyzed against buffer D containing 0.1 M KCl at pH 7.5. This protein was again applied to a P11 resin and washed with 0.1 M KCl until the OD 280 was <0.05. The protein was eluted with six column volumes of a gradient from 0.1 to 1.0 M KCl and this activity (11 mg) was dialyzed against buffer D containing 0.1 M KCl at pH 7.0. The protein was concentrated following its binding to a 1 ml Q Sepharose (Pharmacia) column, washed with 0.1 M KCl and eluted using 1.0 M KCl, resulting in 7.0 mg of protein. This activity was then loaded onto an Superdex 200 FPLC gel filtration column which was equilibrated in 0.1 M buffer D containing 0.1 M KCl at pH 7.0 with 5 ml fractions collected. This activity (1.35 mg in 20 ml) eluted with an apparent mol. wt of >600 kDa. The protein was then applied to a heparin-agarose (Gibco BRL) column and eluted with a 0.1–1.0 M KCl gradient at pH 7.3 followed by dialysis against buffer D containing 0.1 M KCl. The activity (234 µg in 1.3 ml) was then subjected to two purification steps on 1 ml mono Q FPLC columns at a flow rate of 0.25 ml/min with 0.5 ml fractions collected. The first mono Q chromatography was resolved using a 0.1–1.0 M KCl gradient in buffer D at pH 7.9. After the first mono Q column, the activity was dialyzed against 500 ml of buffer D containing 0.1 M KCl at pH 7.3 and then re-applied to the mono Q FPLC column which was equilibrated under the same conditions. The column was resolved with a 0.1–0.5 M KCl gradient and the activity (20 µg) was dialyzed against buffer D containing 0.1 M KCl pH 7.0 and stored at –70°C.

Peptide microsequence analysis of components of the Tat-associated kinase

Purified fractions containing the Tat-associated kinase activity were pooled and loaded onto two wells of a 10% SDS-polyacrylamide gel and subjected to electrophoresis in Tris-glycine buffer. Following overnight electrotransfer to PVDF membrane, individual protein bands were excised and submitted to *in situ* digestion with either endoproteinase LysC or trypsin (Fernandez *et al.*, 1994). The amount of each of the different Tat-associated kinase subunits that were present ranged between 2 and 10 pmol. The resulting peptide mixtures were separated by microbore HPLC using a Zorbax C18 1 mm×150 mm reverse-phase column on a Hewlett-Packard 1090 HPLC/1040 diode array detector. Optimum fractions from each chromatogram were chosen based on differential UV absorbance at 205, 277 and 292 nm, peak symmetry and resolution. Peaks were screened further for length and homogeneity by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-MS) on a Thermo Bioanalysis Lasermat 2000 (Hemel, UK); and selected fractions were submitted to automated Edman degradation on an Applied Biosystems 494A or 477A (Foster City, CA). Details of strategies for the selection of peptide fractions and their microsequencing have been described previously (Lane *et al.*, 1991). The 31 kDa protein had the peptide sequences sTq1EmQLEK and vqLFdPt (lower case letters indicate residues observed with less than full confidence) with sequence identity for the p36/MAT1 assembly factor (Fisher *et al.*, 1995b; Tassan *et al.*, 1995a), the 36 kDa protein with the peptide sequence FYLNNsVMEY had sequence identity to cyclin H (Fisher and Morgan, 1994), the 52 kDa protein with the peptide sequences NLQFLGGLSPGVLD_r and AQEESTGLLSG had sequence identity with the p52 subunit of TFIIF (Marinoni *et al.*, 1996), the 62 kDa protein with the peptide sequence tMVS1Gvk had sequence identity with the p62 subunit of TFIIF (Fischer *et al.*, 1992) and the 80 kDa protein with the peptide sequence LLNFYEK had sequence identity with the ERCC2 subunit of TFIIF (Weber *et al.*, 1990). The peptide sequences of the 28, 39, 58, 140 and 160 kDa proteins did not have homology

with known TFIIF subunits, and their functional significance is being characterized further. The 89 and 42 kDa subunits were not subjected to protein microsequence analysis since Western blot indicated that they corresponded to the TFIIF subunits ERCC3 and cdk7 respectively.

Western blot analysis of TFIIF components

Western blot analysis on the TFIIF-containing fractions was performed following electrophoresis on 10% SDS-polyacrylamide gels and transfer to PVDF membranes. The membranes were probed with rabbit polyclonal antibodies (Santa Cruz Biotechnology) against the TFIIF subunit p89 at a dilution of 1:3000, p62 at a dilution of 1:1000, cdk7 at a dilution of 1:2000 and cyclin H at a dilution of 1:1000. The MAT1 antibody raised to a GST fusion protein with p36 was used at a dilution of 1:1000. The RNA polymerase II CTD monoclonal antibody designated 8WG16 (Promega) was used at a dilution of 1:2000. The p80 and p44 rabbit polyclonal antibodies were used at a dilution of 1:500 and were gifts of Dr Danny Reinberg. The cdk8 antibody was used at a dilution of 1:1000 and was a gift of Jean-Pierre Tassan.

In vivo association of Tat with TFIIF components

A *tat* cDNA containing the 12 amino acid C-terminal influenza hemagglutinin sequence (Field *et al.*, 1988) was cloned downstream of the T7 promoter in the pTM1 vaccinia expression vector (Elroy-Stein *et al.*, 1989). Following calcium phosphate transfection of HeLa cells (10^7 cells/100 mm plate), the cells were infected with a vaccinia virus producing T7 RNA polymerase at an m.o.i. of 3 (Wu-Baer *et al.*, 1995b). At 24 h post-transfection, nuclear extract was prepared and 100 µg of the extract was subjected to immunoprecipitation for 30 min at 4°C using 2 µl of rabbit polyclonal antibody directed against either p89, cyclin H, CREB or ILF. The latter two antibodies were directed against transcription factors that have not been demonstrated to regulate HIV-1 gene expression. Protein A-Sepharose (15 µl) was added for 1 h, the beads were harvested by centrifugation and the immunoprecipitates were subjected to SDS-PAGE and Western blot analysis with 12CA5 monoclonal antibody to detect the epitope-tagged Tat protein. ECL detection was then performed.

In vitro transcription with HIV-1 templates containing G-less cassettes

An HIV-1 provirus pHXB2 which extended from -454 to +1587 was restricted with *SpeI*-*ApaI* at nucleotides 1092 and 1587 respectively, and a 380 bp G-less cassette from a construct containing the adenovirus major late promoter (gift from M.Sawadogo) was generated by PCR and inserted between these sites. A similar HIV-1 proviral clone which contained a mutation in the TAR RNA loop sequences between +31 and +34 was also restricted with *SpeI*-*ApaI*, and a 110 bp G-less cassette generated by PCR was inserted between these sites. *In vitro* transcription was also performed with HIV-1 wild-type and loop mutant templates which lacked the G-less cassettes and were linearized with either *XbaI* or *SspI* to generate run-off products of 1100 and 500 nucleotides respectively.

In vitro transcription reactions (García-Martínez *et al.*, 1995) were performed with 50 µg of HeLa nuclear extract (Dignam *et al.*, 1983) and 100 ng of the wild-type and 150 ng of the loop mutant HIV-1 G-less cassette templates. Reactions (30 µl) were incubated at 30°C for 60 min and contained 10 mM HEPES (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.25 mM DTT, 5.0 mM MgCl₂, 0.06 mM ATP, GTP, UTP, 6 µM CTP, 10 µCi of [α -³²P]CTP (3000 Ci/mM) and 10% glycerol. After incubation at 30°C, 10 µl of RNase T1 (Boehringer Mannheim) was added to the reactions and incubated at 37°C for 10 min. Reactions were stopped by addition of 400 µl of stop solution containing 7.0 M urea, 0.1 M Tris-HCl pH 7.4, 0.5 M sodium chloride 0.5% SDS, 10 mM EDTA, 25 µg/ml yeast tRNA. The samples were extracted with phenol/chloroform and precipitated with ethanol. The transcription products were analyzed on a 6% (w/v) polyacrylamide gel and quantitated by either Phosphorimager scanning or densitometry. The strategy followed to observe Tat transactivation was similar to that described by pre-incubation with dATP (Marciniak and Sharp, 1991).

DRB inhibition of *in vitro* transcription was performed by making a 30 mM stock solution in 100% methanol containing 10 mM HEPES (pH 7.9) and adding either 0, 25, 50 or 100 µM to each reaction prior to the addition of proteins. For antibody inhibition experiments, 50 µg of HeLa nuclear extract was incubated with 400 ng of wild-type and 200 ng of loop mutant template for 30 min at 30°C. Two µl of an appropriate dilution of pre-immune or MAT1/p36 antibody (equivalent to 0.5 µl of rabbit serum) was added to the *in vitro* transcription reaction for 15 min. This was followed by the addition of 100 ng of either Tat

or GST, transcription buffer described above and 10 µCi of [α -³²P]GTP in a final volume of 50 µl. The *in vitro* transcription reaction was continued at 30°C for 30 min and processed as described above.

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