

A novel RNA polymerase II-containing complex potentiates Tat-enhanced HIV-1 transcription

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The HIV-1-encoded Tat protein controls transcription elongation by increasing processivity of RNA polymerase II (Pol II). Here, we have identified a Tat stimulatory activity (Tat-SF) as a novel RNA Pol II-containing complex. Remarkably, Tat-SF contains the previously identified Tat cofactors Tat-SF1, P-TEFb and hSPT5/Tat-CT1, in addition to RNA Pol II and other unidentified polypeptides, but none of the SRB/MED proteins or other factors found associated with the previously described RNA Pol II holoenzyme complex. Tat-SF supports basal, Sp1-activated and Tat-activated transcription in a reconstituted system, and a Tat-SF-derived fraction lacking RNA Pol II can complement non-responsive RNA Pol II complexes for Tat-enhanced HIV-1 transcription, indicating that Tat-SF contains factors that are critical for Tat function. Both Tat-SF and RNA Pol II holoenzyme are present in HeLa nuclear extracts and each can be recruited to the HIV-1 promoter. Our results indicate that Tat-SF is a Tat cofactor-containing RNA Pol II complex whose recruitment to the promoter provides elongation factors important for Tat-enhanced HIV-1 transcription following TAR RNA synthesis.

Keywords: nucleolin/P-TEFb/SPT5/Tat-activated HIV-1 transcription/Tat-SF

Introduction

HIV-1 gene expression requires virally encoded Tat protein, which potentiates the formation of full-length HIV-1 transcripts by enhancing the processivity of RNA Pol II. In contrast to conventional DNA-binding activators, Tat binds to a stem-loop structure (TAR) located at the 5' end of nascent HIV-1 transcripts. Tat may function, in part, by recruiting a cellular cofactor(s) to RNA Pol II via cooperative binding to TAR RNA in a loop sequence-dependent manner (for reviews see Cullen, 1993, 1998; Jones and Peterlin, 1994).

Hyperphosphorylation of the carboxy-terminal domain (CTD) of the large subunit of RNA Pol II correlates closely with productive transcription elongation (Dahmus, 1996). As Tat function requires the CTD (Chun and Jeang, 1996; Okamoto *et al.*, 1996; Parada and Roeder, 1996; Yang *et al.*, 1996) and is sensitive to kinase inhibitors that can block CTD phosphorylation (Marciniak and Sharp, 1991; Parada and Roeder, 1996; Garcia-Martinez *et al.*, 1997b; Mancebo *et al.*, 1997; Zhu *et al.*, 1997), it was

proposed that a CTD kinase activity was critical for Tat function. Although the TFIIF kinase has been implicated in CTD phosphorylation and Tat function (see below), other studies have shown that a distinct Tat-associated kinase, TAK, can also hyperphosphorylate the CTD of RNA Pol II (Herrmann and Rice, 1993, 1995; Yang *et al.*, 1996) and is involved in Tat function. TAK is comprised, at least in part, of a cdc2-related kinase (PITALRE/CDK9) (Yang *et al.*, 1997; Zhu *et al.*, 1997) and an associated cyclin (cyclin T) (Peng *et al.*, 1998; Wei *et al.*, 1998). CDK9/cyclin T are also subunits of P-TEFb, an elongation factor previously identified in *Drosophila* (Marshall and Price, 1995) and mammalian (Mancebo *et al.*, 1997; Zhu *et al.*, 1997) cells.

The kinase activity of CDK9 (P-TEFb) is required for Tat-activity (Mancebo *et al.*, 1997; Zhu *et al.*, 1997; Fujinaga *et al.*, 1998; Gold *et al.*, 1998; Zhou *et al.*, 1998), and a key role for cyclin T in Tat function is indicated by the observation that recombinant cyclin T1, which interacts with Tat, enhances the affinity and specificity of Tat-TAR interactions in a loop sequence-dependent manner (Fujinaga *et al.*, 1998; Wei *et al.*, 1998; Zhou *et al.*, 1998). Importantly, overexpression of human cyclin T1 in mouse cells rescues the block to Tat-enhanced HIV-1 transcription (Wei *et al.*, 1998), which can also be achieved by single amino acid change (tyrosine to cysteine at residue 261) in the mouse cyclin T (Bieniasz *et al.*, 1998; Garber *et al.*, 1998). Although overexpression of either CBP or P-CAF also facilitates Tat function in mouse cells (Benkirane *et al.*, 1998), the mechanism by which these two histone acetyltransferases lead to enhanced HIV-1 transcription elongation in the absence of human cyclin T remains to be determined. While the cellular factor TRP-185 (Wu-Baer *et al.*, 1995a) and RNA Pol II (Wu-Baer *et al.*, 1995b) also have been reported to interact with TAR RNA in a loop-dependent manner, their functional relationships to P-TEFb remain unclear.

Although the identification of cyclin T1 as a critical Tat cofactor provides insights into the mechanism of Tat action, this process remains complex and poorly understood. We and others have shown that Tat also can interact with TFIIF and enhance CTD phosphorylation by the TFIIF-associated CDK-activating kinase (CAK), which closely correlates with enhanced RNA Pol II processivity (Parada and Roeder, 1996; Garcia-Martinez *et al.*, 1997b). It was shown further that Tat can specifically bind to the CAK kinase through CDK7, and that CDK7 kinase is also required for Tat function (Cujec *et al.*, 1997b). How Tat coordinates the action of TFIIF and P-TEFb is still unclear. However, sequential Tat-enhanced functions of distinct CTD kinases and/or Tat-enhanced synergism between distinct CTD kinases might be involved in enhanced HIV-1 transcription elongation. Tat can also interact with RNA Pol II (Mavankal *et al.*, 1996), RNA

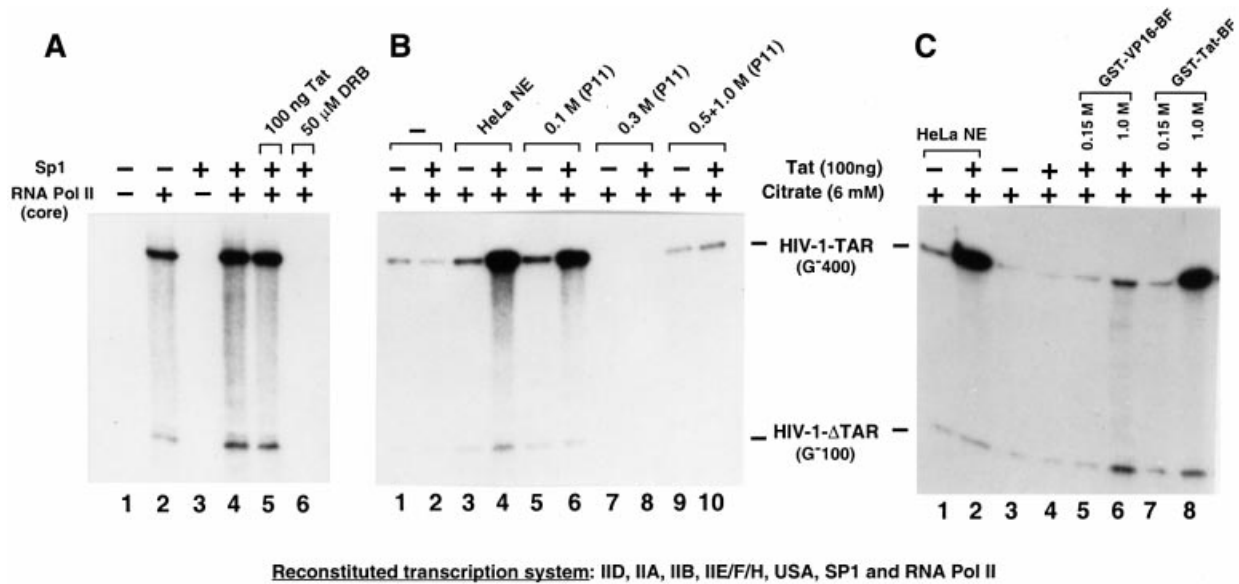


Fig. 1. Identification of Tat stimulatory activity (Tat-SF) in phosphocellulose and GST-Tat selected fractions from HeLa nuclear extract. (A) Basal and Sp1-activated HIV-1 transcription in the reconstituted system. The standard reconstituted transcription system (indicated at the bottom of the figure) lacking RNA Pol II and Sp1 was complemented with core RNA Pol II (0.25 μ l), Sp1 (0.25 μ l), Tat (100 ng) and the kinase inhibitor, DRB, as indicated. (B) Tat-SF in phosphocellulose fractions. The complete Sp1-containing transcription system was treated with citrate and complemented with 12 μ g of HeLa nuclear extract (NE) or with 1 μ l each of P11 fractions, as indicated. The + and - symbols refer to Tat and citrate additions, as in (C). (C) Tat-SF in a GST-Tat selected fraction. Tat function was tested, as indicated, in citrate-treated HeLa nuclear extract (lanes 1 and 2) or in the complete Sp1-containing system complemented with comparable amounts (300 ng) of GST-VP16 (lanes 5 and 6) and GST-Tat (lanes 7 and 8) fractions. RNA transcripts shown in (A), (B) and (C) represent the reaction products derived from wild-type (400 bp G-less cassette) or mutated TAR RNA (100 bp G-less cassette) HIV-1 promoters. These G-less cassettes are located ~1000 bp downstream from the start site for transcription on the HIV-1 promoter (Zhou and Sharp, 1995).

Pol II holoenzyme (Cujec *et al.*, 1997a) and other cellular factors (Yu *et al.*, 1995; Zhou and Sharp, 1996; Sune *et al.*, 1997; Xiao *et al.*, 1998). The human homologue of yeast SPT5, which acts in conjunction with SPT4 and SPT6 to regulate yeast genes (Hartzog *et al.*, 1998), has also been implicated in Tat function (Wu-Baer *et al.*, 1998). hSPT5 and hSPT4 comprise a factor termed DRB sensitivity-inducing factor (DSIF) that (depending on the conditions) regulates transcription elongation both positively and negatively, and it has been proposed that DSIF might be relevant for Tat function (Wada *et al.*, 1998). Overall, these results strongly suggest that factors other than TFIIF and P-TEFb components may also be involved in Tat-enhanced HIV-1 transcription. Whether there is synergism between these factors and/or a temporal order to their functions remains to be determined.

Through exploitation of a biochemical complementation assay for Tat function, the present study describes the isolation of a Tat-stimulatory activity (Tat-SF) related to that described by Zhou and Sharp (1995). This complex contains not only the single Tat-SF-derived polypeptide (Tat-SF1) reported by Zhou and Sharp (1996), but also Tat cofactors described more recently and polypeptides as yet uncharacterized, in association with RNA Pol II. The complex mediates both Sp1 and Tat-activated HIV-1 transcription in a reconstituted system; and whereas Zhou and Sharp (1996) showed that ectopic Tat-SF1 represses HIV-1 transcription in a Tat-reversible manner, Tat-SF1 is shown to act positively in mediating efficient Tat-stimulated transcription in the reconstituted system and, in agreement with a recent report (Li and Green, 1998), to act at the level of elongation. The prior formation of

an RNA polymerase II-elongation factor complex may facilitate both recruitment of the components to the promoter and consequent enhanced elongation in response to TAR-bound Tat.

Results

Identification of a Tat-SF in HeLa nuclear extract

To search for cellular factors involved in Tat-enhanced HIV-1 transcription, an *in vitro* transcription system was complemented with HeLa nuclear extract fractions purified by either standard or affinity chromatography methods. The standard system (Ge *et al.*, 1996) contained recombinant TFIIA and TFIIB, an affinity-purified f:TFIID, a fraction containing TFIIE/TFIIF/TFIIH and P-TEFb (data not shown), and a fraction (USA) containing general coactivators. This system efficiently supported basal transcription from the HIV-1 promoter upon addition of core RNA Pol II (Figure 1A, lane 2 versus lane 1) and activator-dependent transcription upon further addition of Sp1 (lane 4 versus lane 2). Under these conditions, Sp1-activated transcription was unaffected by addition of Tat (Figure 1A, lane 5 versus lane 4), whereas all transcription was sensitive to the kinase inhibitor DRB (lane 6). The latter result suggests a role for both the CTD and its phosphorylation by cognate kinases, TFIIF and/or P-TEFb, in this reconstituted system (see Introduction).

To test for Tat function, we restricted the intrinsically high Sp1-activated HIV-1 transcription elongation levels seen both in reconstituted systems and in HeLa nuclear extracts by pretreatment with citrate. Under this restricted condition, which is selective for elongation relative to

initiation (Kato *et al.*, 1992), Tat overcame the inhibition (in a TAR-dependent manner) in HeLa nuclear extract (Figure 1C, lane 2 versus lane 1), but not in the reconstituted system (Figure 1B, lane 1 versus lane 2 and Figure 1C, lane 3 versus lane 4). These results suggested that, in contrast to HeLa nuclear extract, the reconstituted system lacked factors other than TFIIF and P-TEFb that are important for Tat function. Therefore, to screen for Tat cofactors, we monitored specific Tat-dependent HIV-1 transcription in this citrate-restricted reconstituted system by complementation with various nuclear extract-derived fractions. Amounts of either HeLa nuclear extract or a nuclear extract-derived phosphocellulose (P11) 0.1 M KCl fraction (Ge *et al.*, 1996) that alone showed no activity (data not shown) were able to restore Tat function, whereas the P11 0.5 M KCl and P11 1.0 M KCl fractions could not (Figure 1B, lanes 3–10). Notably, the P11 0.3 M KCl fraction inhibited basal transcription and Tat could not overcome this effect (Figure 1B, lanes 7 and 8). A potential relationship of this inhibitory activity with the negative elongation factor DSIF which also fractionates in P11 0.3 M KCl (Wada *et al.*, 1998), has not yet been determined. Importantly, a HeLa nuclear extract fraction bound to and eluted (1.0 M KCl) from a GST–Tat affinity column, but not an analogous fraction from a GST–VP16 affinity column, also facilitated strong TAR-dependent Tat function in this transcription system (Figure 1C, lanes 5–8). Overall, these results suggest that the P11 0.1 M KCl and GST–Tat fractions contain potential Tat cofactors (hereafter called Tat-SF).

Purification of Tat-SF and molecular identification of specific components

The Tat-complementing activity in the P11 0.1 M KCl fraction was further purified by chromatography on DEAE–Sepharose and Q–Sepharose (Zhou and Sharp, 1995). This activity peaked in Q–Sepharose fractions #8 and #9 (Figure 2A, lanes 10–13) but was variably dependent on the presence of TAR RNA loop sequences (compare Figures 3A and C, 4C, 7C and 8B, below). This contrasts with the consistent wild-type TAR RNA-dependent Tat function observed in unfractionated nuclear extract (Figures 1C and 6A; Parada and Roeder, 1996) or in the reconstituted system complemented either with HeLa nuclear extract (Figures 1B and 3C) or with the GST–Tat bound Tat-SF (Figure 1C). Although the mutated HIV-1 promoter (HIV-1- Δ TAR) lacks the TAR RNA loop sequences, it nonetheless retains the wild-type Tat binding site (bulge) (Zhou and Sharp, 1995); moreover, the wild-type TAR RNA-independent Tat activity is HIV-1 promoter-specific (see Figure 3A) and also has been observed by others with partially purified reconstituted systems (Zhou and Sharp, 1995, 1996; Wu-Baer *et al.*, 1998). Although it is unclear why the reconstituted system can variably bypass, in part, the TAR RNA loop sequence requirement for Tat-enhanced transcription, this may reflect a dose-dependent interaction of Tat with natural (protein) targets in the absence of TAR RNA-mediated Tat recruitment. This would be analogous to the function of some DNA-binding activators, at elevated concentrations, in the absence of DNA recognition sites (reviewed in Ptashne and Gann, 1997).

A further characterization of the Tat-responsive

Q–Sepharose fractions revealed phosphorylation of several specific polypeptides (p200, p160, p140, p110, p35 and p32) by an endogenous kinase (Figure 2B, lanes 4–9; data not shown). Remarkably, similarly sized proteins were also phosphorylated by an endogenous kinase in the Tat-responsive 1.0 M KCl GST–Tat fraction (Figure 2B, lanes 2–3), but not in the corresponding 1.0 M KCl GST–VP16 fraction (lane 1) that does not support Tat function (Figure 1C, lanes 5–6), thus suggesting that some or all of these phosphoproteins could be relevant to Tat function. The lack of a correlation of the polypeptides indicated with Tat stimulatory activity in the Q–Sepharose fractions could reflect substantial (but not complete) separation of the endogenous kinase(s) from the substrates during fractionation. Moreover, a further analysis of the Q–Sepharose fractions by SDS–PAGE and Coomassie staining (Figure 2C) revealed a number of polypeptides (denoted by arrows) that corresponded in size to the phosphoproteins (lanes 9–14) and/or in elution position to the Tat stimulatory activity described above. Consequently the corresponding p300, p160, p140 and p110 proteins, as well as p35 and p32, were subjected to peptide microsequencing. These analyses identified p300 and p160 as novel proteins (data not shown), p200 as the large subunit of RNA Pol II, p140 as the Tat cofactor Tat-SF1 (Zhou and Sharp, 1996), p110 as nucleolin, a protein with potential functions in transcription activation (Hanakahi *et al.*, 1997), p35 as 60S ribosomal protein L5, and p32 as the splicing factor SF2 (data not shown).

Immunoblot analyses (Figure 2D) confirmed the presence of Tat-SF1 and nucleolin in these Q–Sepharose fractions. They also revealed the presence of RNA Pol II, with the p200 immunoreactive band corresponding to the hypophosphorylated CTD form of the large subunit of RNA Pol II (data not shown); XP-E, a subunit of a UV-damage DNA-binding complex (Takao *et al.*, 1993); PSTAIRE, a cdc2-like kinase (Lee and Nurse, 1987); CKII; the CDK9 and cyclin T components of P-TEFb; and human homologues of yeast SPT5 (also designated Tat-CT1, Wu-Baer *et al.*, 1998) and SPT4. Notably, the elution of Tat-SF1 and nucleolin correlated tightly with Tat stimulatory activity. There was a less tight correlation of RNA Pol II (also present in higher salt fractions) and hSPT5 (peak skewed to lower salt fractions) with Tat stimulatory activity, although these components were present in all fractions containing Tat stimulatory activity. The P-TEFb components CDK9 and cyclin T showed even broader distributions that did not correlate with Tat stimulatory activity although they also were present in all fractions containing Tat stimulatory activity. The lack of strict coelution of the various components could reflect chromatographic resolution of natural subcomplexes and/or partially dissociated components. The capping enzyme (Shuman, 1997) and the CTD-specific phosphatase FCP1 (Archambault *et al.*, 1998) were both present in the DEAE–Sepharose Tat-SF fraction, but while the bulk of these enzymes coeluted in Q–Sepharose fractions #3 and #4, small amounts were also present in those fractions (#8 and #9) that actively support Tat function (data not shown).

Further analyses indicated that the Tat-responsive Q–Sepharose fractions did not contain any RNA Pol II general transcription factors, RNA helicase A (RHA),

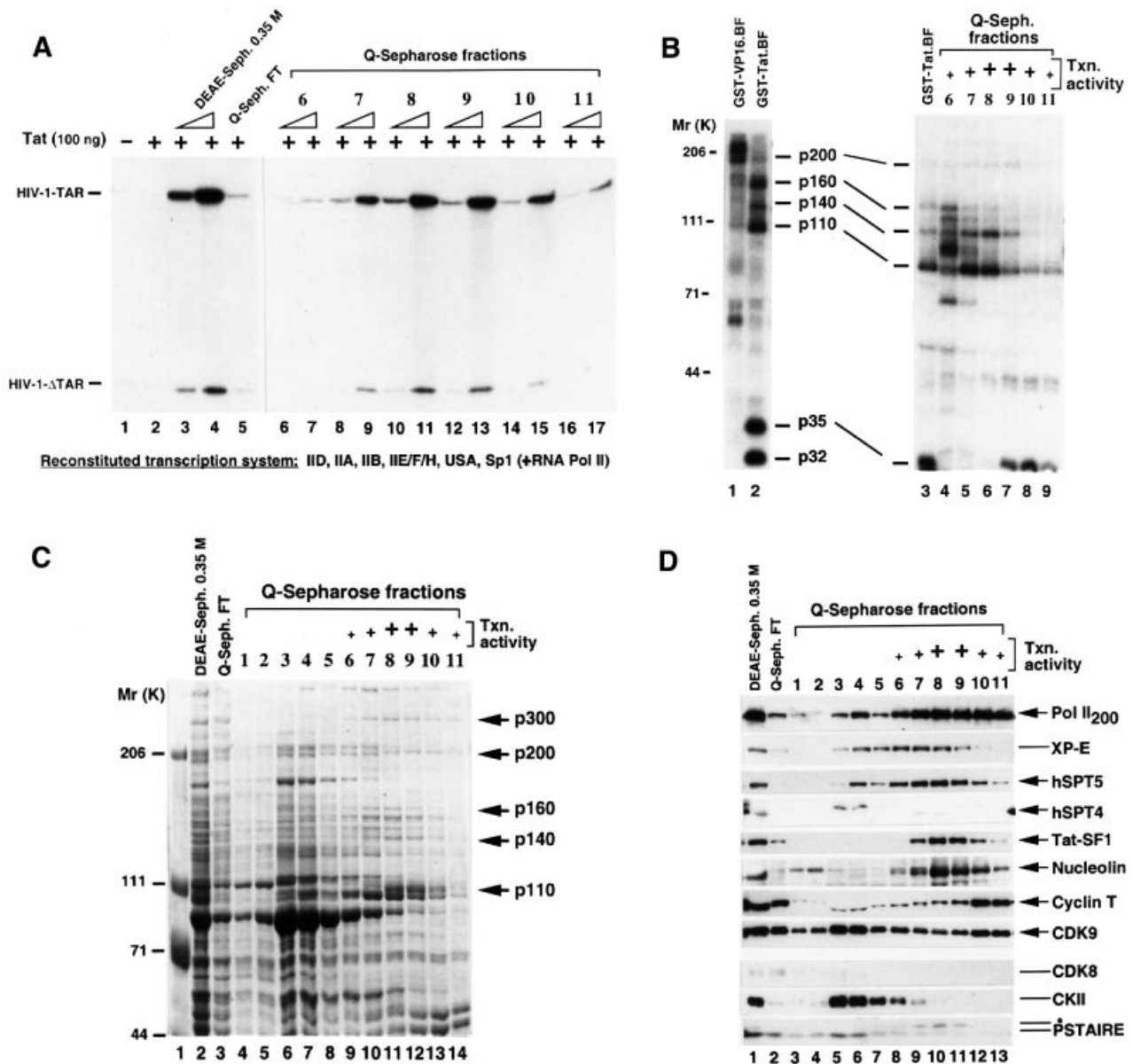


Fig. 2. Purification and characterization of Tat-SF. (A) Purification by ion exchange chromatography. Tat-SF was purified from nuclear extract through phosphocellulose (0.1 M KCl fraction), DEAE-Sephacrose (0.35 M KCl fraction) and Q-Sephacrose (0.3–0.8 M KCl salt gradient fractionation). The citrate-treated reconstituted system was complemented with 0.1 and 0.25 μ l aliquots of indicated Q-Sephacrose fractions (6–11) for Tat-enhanced transcription. (B) Phosphorylation of common polypeptides by endogenous kinase in Tat-SF-containing and GST-Tat-eluted fractions, but not in GST-VP16 fractions. Aliquots (1 μ l) of the fractions indicated were incubated with [γ - 32 P]ATP and the reaction products analyzed by 10% SDS-PAGE. Specific phosphoproteins are indicated by numbers that reflect their apparent size (kDa). (C) Specific polypeptides identified by SDS-PAGE and Coomassie stain correlate with Tat-SF activity. Twenty microliters of the DEAE-Sephacrose and Q-Sephacrose fractions indicated were run on 7.5% SDS-PAGE. Polypeptides corresponding to phosphoproteins identified in (B) are indicated by arrows on the right. (D) The co-elution of hSPT5/hSPT4, Tat-SF1, nucleolin and RNA Pol II (Pol II₂₀₀) in Q-Sephacrose fractions. Aliquots (10 μ l) of the fractions indicated were subjected to Western blot analyses with antibodies to polypeptides indicated on the right. Molecular weight markers (kDa) are shown on the left (B and C) and relative levels of Tat-enhanced transcription activity in Q-Sephacrose fractions (#6 to #11) are indicated with variably-sized + symbols in (B), (C) and (D).

CBP, CDK8/cyclin C (human homologues of yeast SRB10/SRB11), SRB7 or MED7 (data not shown; Figure 6B, lane 2 versus 3), all of which have been reported to be present in various RNA Pol II complexes (reviewed by Greenblatt, 1997; Parvin and Young, 1998). Collectively, these results suggested the possibility (tested further below) that Tat-SF could be a Tat cofactor-containing RNA Pol II complex that is structurally distinct from the RNA Pol II holoenzyme complex.

Tat-SF potentiates HIV-1 transcription in a reconstituted transcription system lacking core RNA Pol II

Although the Tat-SF assays described above used a reconstituted system containing purified core RNA Pol II, the most active fractions (Q-Sephacrose #8 and #9) also contained RNA Pol II. This led us to test whether the Tat-cofactor associated RNA Pol II would suffice as the sole source of RNA Pol II in the assay. Indeed, when reassayed

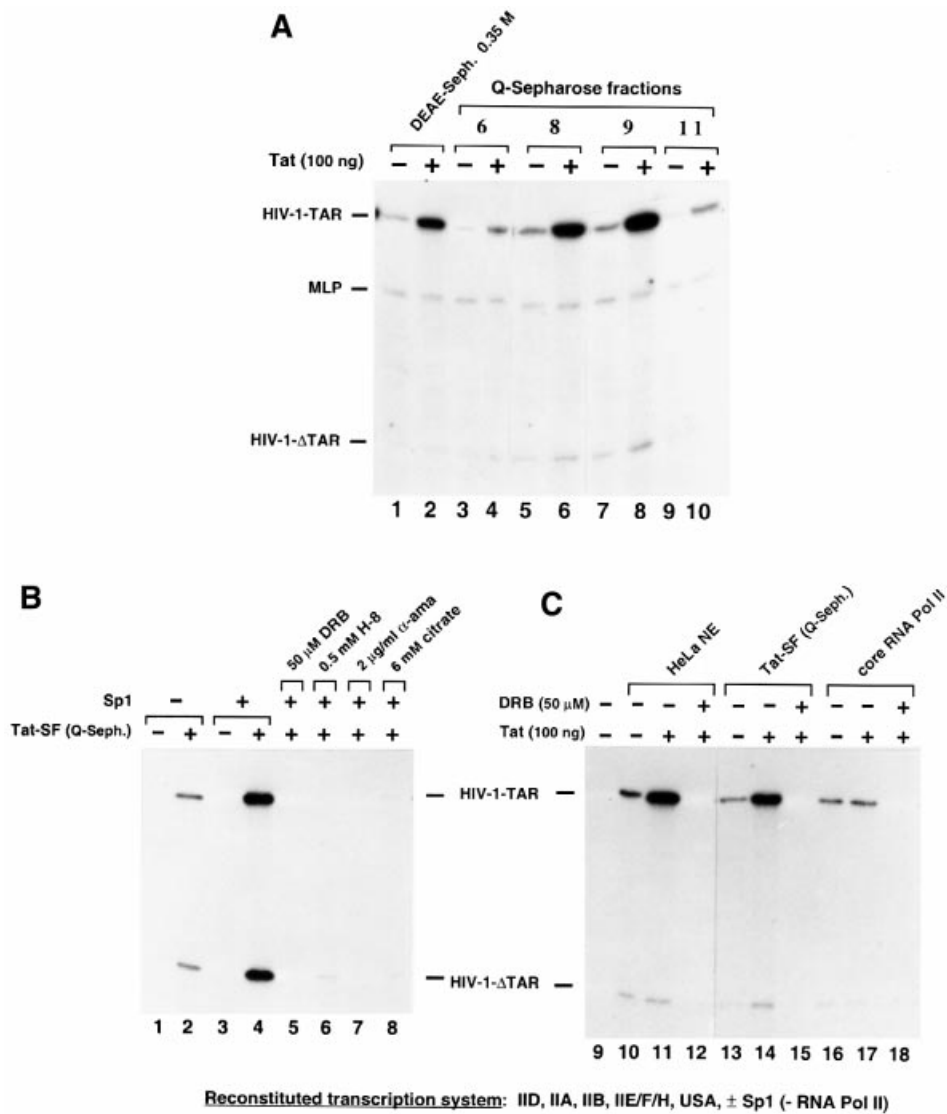


Fig. 3. Basal, Sp1-dependent and Tat-dependent transcription functions are mediated by RNA Pol II in Tat-SF. (A) HIV-1 promoter-specific and TAR RNA sequence-dependent Tat function. The citrate-treated reconstituted system containing Sp1 but lacking core RNA Pol II was complemented with 0.5 μ l of the Tat-SF fractions indicated in either the presence or absence of Tat. Templates included the adenovirus ML promoter (MLP) as well as the HIV-1-TAR and HIV-1- Δ TAR templates. (B) Basal and Sp1-activated HIV-1 transcription. The reconstituted system without core RNA Pol II and Sp1 was complemented with Sp1 (0.25 μ l) and Tat-SF (0.25 μ l of Q-Sepharose fraction #8) and with additions of DRB, H-8, α -amanitin and citrate, as indicated. (C) Tat-enhanced transcription. A citrate-treated reconstituted system without core RNA Pol II was complemented with 12 μ g of HeLa nuclear extract (NE), 0.25 μ l of Tat-SF (Q-Sepharose fraction #8) or 0.25 μ l of core RNA Pol II, and with addition of DRB and Tat, as indicated.

in a reconstituted system lacking core RNA Pol II, the DEAE-Sepharose and Q-Sepharose Tat-SF fractions showed a clear Tat-responsive effect (Figure 3A). Moreover, under the assay conditions of Figure 3A, the Tat response was both completely dependent upon an intact TAR RNA and promoter specific (showing no response in the adenovirus ML promoter). In a further analysis, we also found that a purified Tat-SF fraction (Q-Sepharose #8) could functionally substitute for core RNA Pol II in supporting both basal and Sp1-activated HIV-1 transcription (Figure 3B, lanes 1-4). Since this transcription activity was sensitive to α -amanitin (Figure 3B, lane 7), the transcription functions are mediated by RNA Pol II in Tat-SF. Importantly, and in contrast to core RNA Pol II (Figure 3C, lanes 16 and 17), Tat-SF (lanes 13 and 14)

was as efficient as a limited amount of nuclear extract (lanes 10 and 11) in complementing the transcription system for Tat-enhanced HIV-1 transcription. As both Sp1-activated and Tat-activated transcription are sensitive to the kinase inhibitors DRB and H-8 (Figure 3B, lanes 5 and 6 and 3C, lanes 12, 15 and 18), it appears that phosphorylation functions are as essential for processive elongation in this purified transcription system as they are in HeLa nuclear extract (see Introduction). Overall, these results show that Tat-SF is able both to substitute for core RNA Pol II in mediating activation by Sp1 and to mediate activation by Tat. They further suggest that Tat-SF contains factors that are important for Tat-enhanced HIV-1 transcription but not present in the core RNA Pol II or holoenzyme (see below).

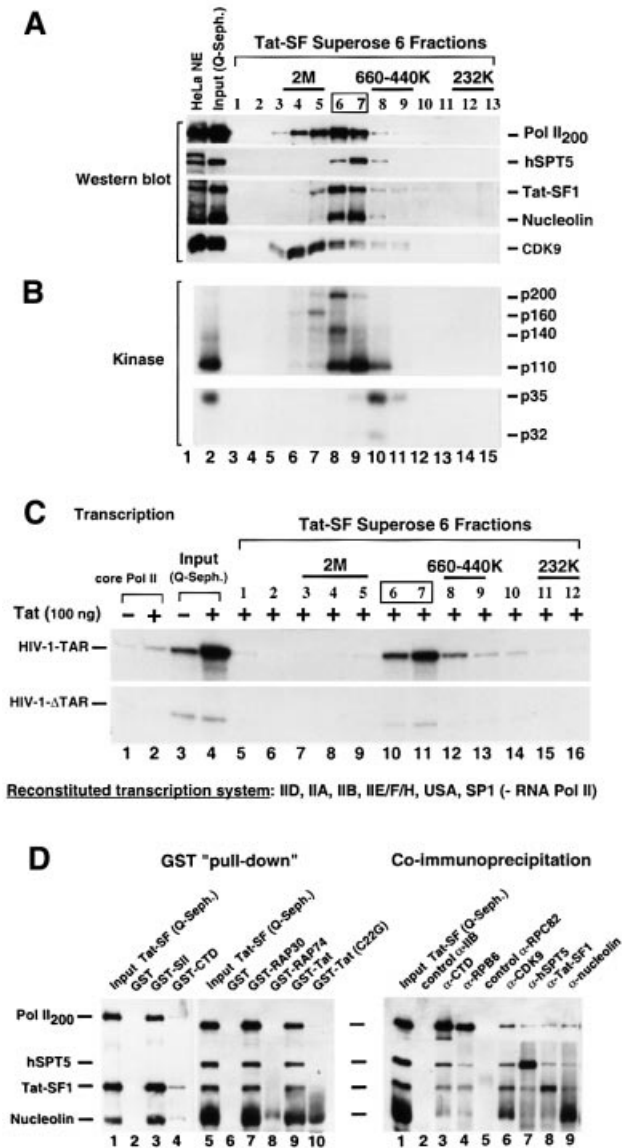


Fig. 4. Tat-SF is a functional RNA Pol II-containing coactivator complex. (A) Co-elution of Tat-SF polypeptides with RNA Pol II during gel filtration. Tat-SF (Q-Sepharose fraction #8) was subjected to gel filtration on Superose 6 (SMART) as described in the Materials and methods. Western blot of 60 μ g of HeLa nuclear extract (NE), 10 μ l of the input (Q-Sepharose) and 10 μ l each of Superose 6 fractions 1–13. (B) Elution of phosphoproteins during gel filtration. Superose 6 fractions were incubated with [γ - 32 P]ATP and phosphoproteins resolved by SDS–PAGE and detected by autoradiography. (C) Elution of Tat-SF activity during gel filtration. The citrate treated-reconstituted system was complemented with core RNA Pol II (0.25 μ l), input (0.25 μ l of Q-Sepharose), Superose 6 fractions (1 μ l of 1–12), and Tat, as indicated. The Dextran-blue (2 MDa), thyroglobulin (660 kDa), ferritin (440 kDa) and catalase (232 kDa) are indicated. The rectangle over Superose 6 fractions 6 and 7 reflects the presence of Tat-SF activity. (D) The hSPT5, Tat-SF1, CDK9 and nucleolin are physically associated with RNA Pol II in Tat-SF. Western blot (10% SDS–PAGE) analyses of polypeptides in Tat-SF (Q-Sepharose fraction #8) bound to the GST or GST-fusion proteins (100 mM KCl) as indicated (lane 1–10) or co-immunoprecipitated (100 mM KCl) with the antibodies indicated (lanes 1–9). Polypeptides of RNA Pol II and Tat-SF are shown on the left.

Tat-SF is an RNA Pol II-containing complex

As the above results raised the possibility that Tat-SF might be a Pol II-containing complex, the native mass of Tat-SF was estimated by subjecting the Q-Sepharose

fraction (#8) to gel filtration at 0.3 M KCl. Western blot analyses (Figure 4A) revealed coelution of RNA Pol II and Tat-SF1, with a peak in fraction #6 corresponding to a size of ~1.2 kDa, and coelution of nucleolin and hSPT5, with a peak in fraction #7. The analysis further showed that most of the CDK9 polypeptide elutes with a small fraction of RNA Pol II in a broad region centered at ~2 MDa. Significantly, most of the Tat stimulatory activity is found in fractions #6 and #7, with trailing activity in later fractions (Figure 4C). Thus, whereas there is not perfect coelution of RNA Pol II, Tat-SF1, hSPT5 and nucleolin with Tat stimulatory activity, these components might still have resided in a single functional complex that was partially dissociated and resolved by the chromatographic conditions. Again, all fractions with Tat stimulatory activity contained these components as well as CDK9. The fractions with Tat stimulatory activity also showed an endogenous kinase activity that phosphorylated p200 and p140 to the greatest extent in the fraction (#6) containing the highest levels of RNA Pol II and Tat-SF1 polypeptides, and p110 to the greatest extent in the fraction (#7) with the highest level of nucleolin (Figure 4B). Overall, these results are consistent with the possibility that Tat-SF could be an RNA Pol II-containing complex and that phosphorylation of Tat-SF components, possibly via P-TEFb, may be relevant for transcriptional functions of Tat-SF.

We next investigated whether Tat-SF polypeptides were physically associated with RNA Pol II. As RNA Pol II has been shown to interact with the transcription factor SII (Agarwal *et al.*, 1991), TFIIF (McCracken and Greenblatt, 1991) and Tat (Mavankal *et al.*, 1996), we investigated whether Tat-SF could bind to these factors. A GST 'pull-down' analysis with a Q-Sepharose fraction (#8) (Figure 4D, left) shows that RNA Pol II, hSPT5, Tat-SF1 and nucleolin, as well as P-TEFb (data not shown), were efficiently retained by GST–SII, GST–RAP30 and GST–Tat affinity columns (lanes 3, 7 and 9), but not by GST, GST–CTD, GST–RAP74 and GST–Tat mutant (C22G) columns (lanes 2, 4, 6, 8 and 10). These results are consistent with Tat-SF binding to SII, RAP30 and Tat via known RNA Pol II interactions. However, to distinguish between direct or indirect interactions of Tat-SF components with RNA Pol II we also carried out a coimmunoprecipitation analysis. This study (Figure 4D, right) showed that antibodies directed against the CTD and a small RNA Pol II subunit (RPB6) specifically co-immunoprecipitated RNA Pol II, hSPT5, Tat-SF1 and (to a lesser extent) nucleolin (lanes 3–4), whereas control anti-TFIIB and anti-RPC82 antibodies did not (lanes 2 and 5). Antibodies against the RNA Pol II CTD, the RPB6 subunit of RNA Pol II, CDK9, hSPT5, Tat-SF1 and nucleolin generally precipitated significant and roughly comparable relative amounts of the assayed Tat-SF components, as well as excess amounts of the specific antigen (Figure 4D, lanes 3, 4, 6–9). The main exceptions are the anti-CDK9 antibodies, which did not appear to precipitate an excess of antigen (Figure 4D, lane 6; data not shown), and the anti-SPT5 and anti-Tat-SF1 antibodies, which precipitated little to no nucleolin (lanes 7 and 8). These variations may reflect the fact that most of the antibodies employed were polyclonal, such that extensive antigen interactions may have partially and variably dissociated

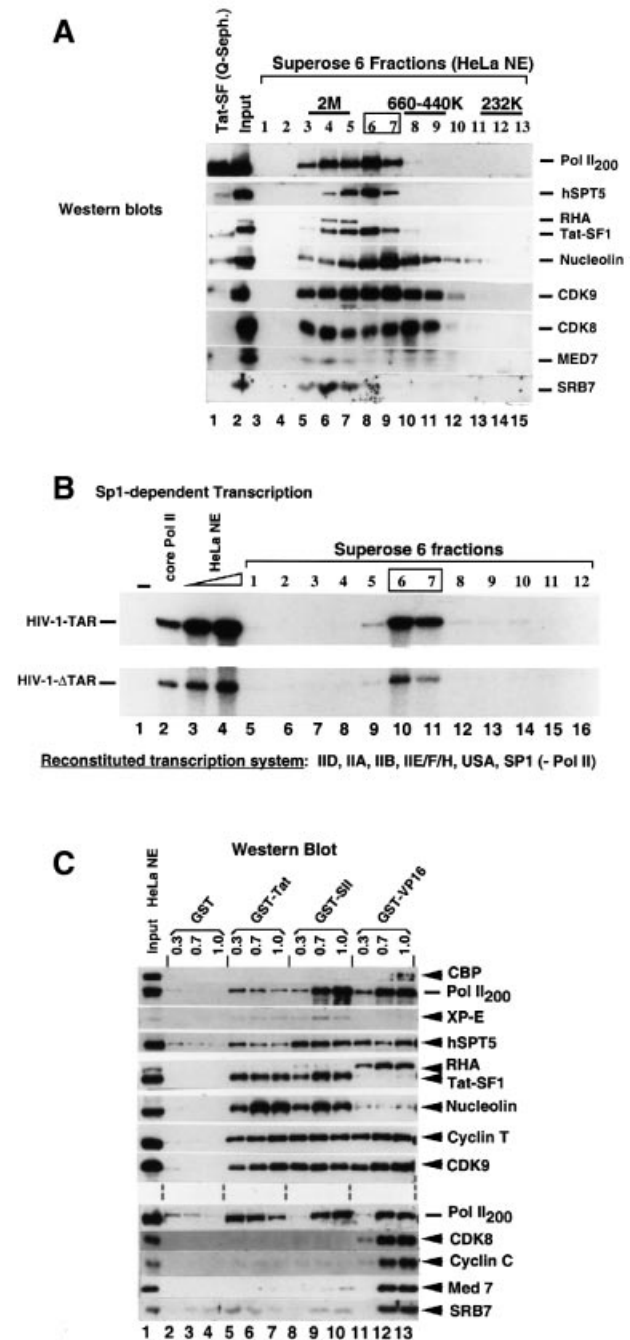
certain Tat-SF components. Along with the failure of control anti-TFIIB and anti-RPC82 antibodies to immunoprecipitate any Tat-SF components (Figure 4D, lanes 2 and 5), these results provide more direct evidence for the suggestion from the gel filtration analysis that Tat-SF is a complex containing RNA Pol II and various Tat cofactors.

Tat-SF is a bona fide RNA Pol II complex that is present with RNA Pol II holoenzyme in HeLa nuclear extract

Gel filtration chromatography was employed to resolve the putative Tat-SF and RNA Pol II holoenzyme complexes directly from HeLa nuclear extract. Western blot analyses of the Superose 6 fractions (Figure 5A) showed two apparent, but overlapping peaks of elution for RNA Pol II (fractions #4 and #6). About half of the RNA Pol II (fractions #3–#5) coeluted with RHA, a peak of CDK8/SRB10, MED7 and SRB7 at a position corresponding to a native mass of 2 MDa. The additional RNA Pol II that peaked in fraction #6 coeluted with hSPT5, Tat-SF1 and much of the nucleolin, but not with RHA and SRB/MED components, at a position corresponding to a mass of ~1.2 MDa. The CDK9 showed a broad distribution over fractions containing both RNA Pol II complexes, whereas CDK8 was resolved into a fraction overlapping the RNA Pol II holoenzyme and a fraction in the 600 kDa range. Functional studies (Figure 5B) indicated that the Superose 6 fractions (#6 and #7) containing Tat-SF (~1.2 MDa complex) efficiently supported Sp1-activated (lanes 10 and 11) and Tat-activated (data not shown) HIV-1 transcription in the reconstituted system. Interestingly, however, the Superose 6 fractions (#3–#5) that contained comparable amounts of RNA Pol II in association with RHA and SRB/MED proteins (~2 MDa complex) failed to support Sp1-activated transcription (Figure 5B, lanes 7–9). As both core RNA Pol II and Tat-SF can mediate this function in the transcription system, our preliminary results suggest that the RNA Pol II holoenzyme in both crude (Figure 5) and highly purified (Figure 8) fractions is in a repressed state that is incapable of efficient transcription elongation (C.A.Parada and R.G.Roeder, unpublished).

Fig. 5. Chromatographic separation of structurally distinct RNA Pol II-containing complexes from HeLa nuclear extract. (A) Gel filtration of HeLa nuclear extract (NE) on Superose 6 (SMART). Tat-SF (10 μ l of Q-Sepharose fraction #8), nuclear extract input (60 μ g), and 10 μ l of Superose 6 fractions (1–13) were subjected to 10–12.5% SDS-PAGE and Western blot with antibodies to proteins indicated on the right. (B) Superose 6 fractions containing Tat-SF support efficient Sp1-activated transcription. The reconstituted system lacking RNA Pol II was complemented with 0.25 μ l of core RNA Pol II, 3 and 12 μ g of HeLa NE and 2 μ l of Superose 6 fractions 1–12 as indicated. Transcriptionally active Superose 6 fractions (#6–#7), indicated by a rectangle, correlated with the presence of Tat stimulatory activity (data not shown) and specific Tat-SF polypeptides (A). (C) Distinct RNA Pol II complexes in HeLa nuclear extract bind to GST-Tat and GST-SII versus GST-VP16. The RNA Pol II-containing complexes were purified by affinity chromatography of HeLa NE on GST-fusion proteins as described in the Materials and methods. Western blot of 60 μ g of input HeLa NE (lane 1) and either 5 μ l each of eluted fractions from GST and GST-fusion proteins (lanes 2–13) (upper panel) or 5 μ l of eluted fractions from GST and GST-Tat (lanes 2–7) and 1 μ l of eluted fractions from GST-SII and GST-VP16 (lanes 8–13) (bottom panel). Samples were eluted from the GST columns at 0.3, 0.7 or 1.0 M KCl and subjected to 7.5–12.5% SDS-PAGE followed by Western blotting with antibodies to the Tat-SF and holoenzyme components shown on the right.

We further examined the presence of Tat-SF and RNA Pol II holoenzyme complexes in HeLa nuclear extract by affinity chromatography methods. It was reported previously that the human RNA Pol II holoenzyme binds both Tat and SII (Cujec *et al.*, 1997a; Pan *et al.*, 1997). However, our observation that fractions bound by GST-Tat, but not those bound by GST-VP16, support Tat-enhanced HIV-1 transcription (Figure 1C) strongly suggested that Tat binds the RNA Pol II-containing complex Tat-SF. Furthermore, since a GST-VP16 affinity column retains the yeast holoenzyme (Hengartner *et al.*, 1995), we expected that it would also retain the human RNA Pol II holoenzyme. In a test of this prediction, Western blot analyses of salt-eluted (0.3, 0.7 and 1.0 M KCl) fractions from affinity columns showed that GST-Tat and GST-SII (Figure 5C, lanes 5–7 and 8–10) specifically



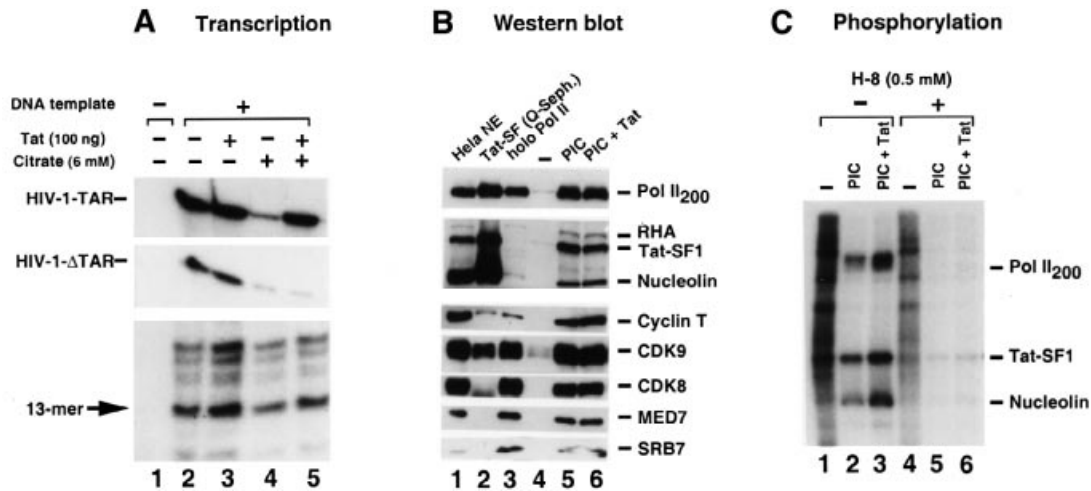


Fig. 6. Tat-SF and RNA Pol II holoenzyme components in HeLa nuclear extract are recruited to PICs on the HIV-1 promoter. (A) Tat-enhanced transcription (top) was tested in HeLa NE without and with citrate treatment and either in the presence or absence of HIV-1 promoters as indicated. Short (13mer) transcripts (bottom) represent accurately initiated transcripts derived from HIV-1 PICs (wild-type and mutant TAR RNA-containing HIV-1 templates) that were isolated and incubated with dATP, [α - 32 P]UTP, GTP and CTP for 10 min at 30°C, as described previously (Parada *et al.*, 1995). (B) Western blot of HeLa NE (60 μ g), Tat-SF (10 μ l of Q-Sepharose fraction #8), RNA Pol II holoenzyme (10 μ l), and HIV-1 PICs formed in the absence (lane 5) or presence (lane 6) of Tat. Samples were run on 7.5–12% SDS-PAGE and Western blotted with antibodies to the Tat-SF and RNA Pol II holoenzyme components indicated on the right. (C) Tat stimulates phosphorylation of Tat-SF components: CTD of RNA Pol II, Tat-SF1 and nucleolin. Phosphorylation of isolated HIV-1 PICs formed in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of Tat was performed in the presence of [γ - 32 P]ATP and cold NTPs to allow transcription elongation as described previously (Parada and Roeder, 1996). Indicated 32 P-labeled phosphoproteins (on the right) were isolated by immunoprecipitation with anti-CTD, anti-Tat-SF1 and anti-nucleolin antibodies as indicated (Parada and Roeder, 1996) and analyzed on 7.5% SDS-PAGE.

bound Tat-SF components that included RNA Pol II, hSPT5, XP-E, Tat-SF1, nucleolin and P-TEFb (CDK9 and cyclin T), whereas GST-VP16 specifically retained holoenzyme components that included RNA Pol II, CBP and RHA (Figure 5C, top). When the eluates were normalized on the basis of RNA Pol II content, Western blot analysis (Figure 5C, bottom) clearly indicated that holoenzyme components (MED7, SRB7, CDK8/SRB10 and cyclin C/SRB11) (lanes 11–13) bind preferentially to GST-VP16. Therefore, in contrast to GST-VP16, which preferentially binds the RNA Pol II holoenzyme complex, the elongation factors Tat and SII appear to interact specifically with a novel RNA Pol II complex, Tat-SF, that exhibits potent elongation activity. Since hSPT5 and P-TEFb components (CDK9 and cyclin T) were also retained by GST-VP16 affinity columns, these transcription elongation factors appear not to be Tat-specific cofactors.

The finding that RNA Pol II holoenzyme-containing fractions cannot complement the assay system for Tat function (Figures 1C and 8B; data not shown) is further substantiated by the observation that although a highly purified RNA Pol II holoenzyme interacts with Tat, in this case probably via interactions with TFIID (Cujec *et al.*, 1997a), it fails to support Tat function because of an apparent lack of Tat cofactors. It is noteworthy that our analyses did not reveal interactions of either Tat or SII with an SRB/MED-containing RNA Pol II holoenzyme complex, most likely due to purification procedures different from those of Cujec *et al.* (1997a) and Pan *et al.* (1997).

Tat-SF can be recruited to the HIV-1 promoter in HeLa nuclear extract

As Tat can be recruited to the HIV-1 promoter during preinitiation complex (PIC) formation (Garcia-Martinez

et al., 1997a), we tested whether Tat-SF, in the presence of Tat, could be preferentially recruited (relative to RNA Pol II holoenzyme) to the HIV-1 promoter in HeLa nuclear extract. PICs on the HIV-1 promoters were formed under conditions where Tat activates HIV-1 transcription elongation by 7- to 10-fold in citrate-treated HeLa nuclear extracts (Figure 6A, top, lanes 4 and 5 versus lanes 2 and 3) and isolated by gel filtration (Parada *et al.*, 1995). These PICs are transcriptionally active as they can support the formation of short (13mer) transcripts in a DNA template-dependent manner (Figure 6A, bottom, lanes 2–5). Western blot analysis (Figure 6B) of PICs isolated from reaction mixtures in which Tat stimulates transcription elongation (Figure 6A, lanes 4 and 5) show the presence of both Tat-SF and RNA Pol II holoenzyme components that include RNA Pol II, Tat-SF1, nucleolin, P-TEFb (CDK9 and cyclin T), RHA, and SRB/MED proteins (Figure 6B, lanes 5 and 6). Thus, both Tat-SF (Figure 6B, lane 2) and RNA Pol II holoenzyme (lane 3) can be recruited, most likely independently, to the HIV-1 promoter. Notably, the presence of Tat during PIC formation had no significant effect either on the recruitment of Tat-SF to the HIV-1 promoter (Figure 6B, lane 6 versus lane 5) or on the subsequent formation of a 13mer (Figure 6A, lanes 3 and 5 versus lanes 2 and 4), consistent with the function of Tat at a step following TAR RNA synthesis (Jones and Peterlin, 1994; Jones, 1997).

Since Tat stimulates CTD phosphorylation during transcription elongation (see Introduction), we also examined the phosphorylation state of Tat-SF components after incubation of isolated PICs under transcription initiation and elongation conditions. Interestingly, the presence of Tat during PIC formation led to an enhanced phosphorylation of Tat-SF1 and nucleolin, as well as the CTD (Figure 6C, lane 3 versus lane 2). A kinase inhibitor

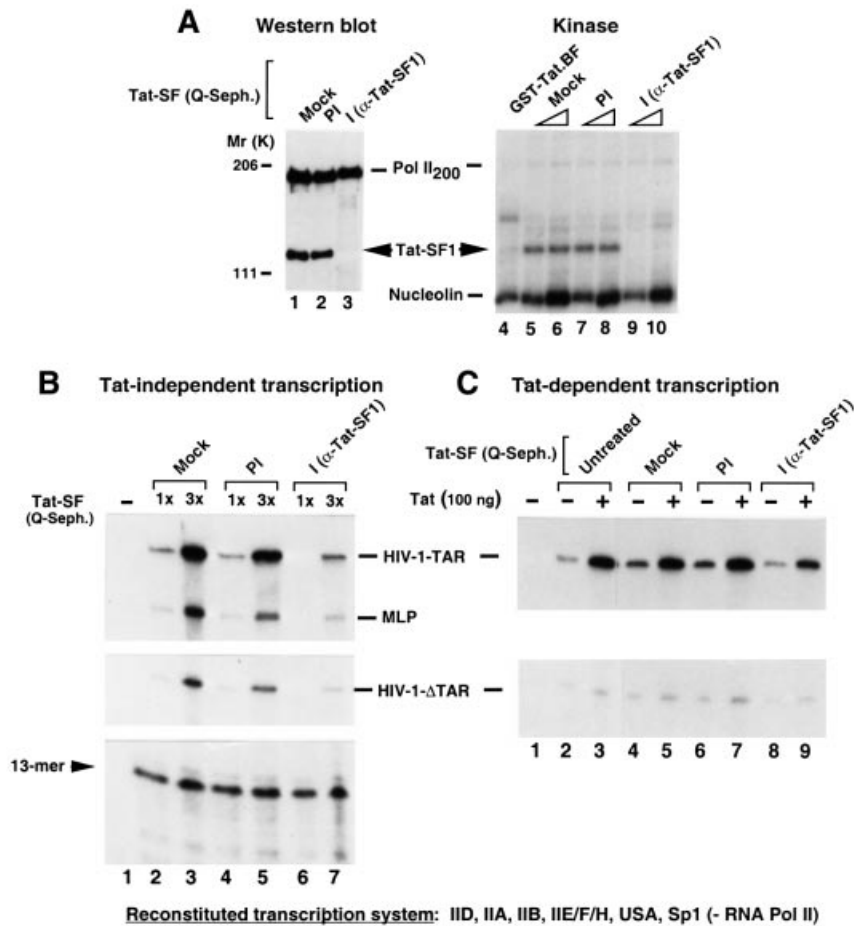


Fig. 7. Immunodepletion of Tat-SF1 from Tat-SF impairs both Tat-independent and Tat-dependent transcription. A Tat-SF (Q-Sepharose fraction #8) was subjected to immunodepletion with no serum (mock), preimmune serum (PI) or anti-Tat-SF1 immune (I) serum. The depletion were done at 700 mM KCl in order to overcome the interactions between RNA Pol II and other Tat-SF components that are observed at lower (100 mM KCl) conditions (Figure 4D). (A) Western blot (7.5% SDS-PAGE) of 10 μ l of the mock, PI and I immunodepleted Tat-SF fractions (lanes 1–3) and kinase assays (7.5% SDS-PAGE) of 1 μ l of 1.0 M GST-Tat fraction and 1 and 2 μ l of mock, PI and I immunodepleted Tat-SF fractions (lanes 4–10). (B) Tat-independent transcription. Transcription from HIV-1 and AdML promoters (top) in the reconstituted transcription system lacking RNA Pol II and complemented with mock, PI and I immunodepleted Tat-SF fractions (1- or 3-fold) as indicated. Short RNAs (13mer) (bottom) reflect transcription products from the HIV-1 promoter formed in the presence of dATP, [α - 32 P]UTP, GTP and CTP for 10 min at 30°C as indicated (Parada *et al.*, 1995). (C) Tat-enhanced HIV-1 transcription. The citrate treated-reconstituted system lacking RNA Pol II was complemented with untreated Tat-SF or with mock, PI and I immunodepleted Tat-SF in either the absence or presence of Tat, as indicated.

(H-8) that blocks transcription elongation also inhibited phosphorylation of these Tat-SF components (Figure 6C, lanes 5 and 6). Collectively, these results suggest that although Tat-SF-associated factors can be recruited to PICs on the HIV-1 promoter in a Tat-independent fashion, they may function more efficiently in response to Tat-induced phosphorylation and TAR RNA synthesis.

A component of Tat-SF, Tat-SF1, is a general elongation factor

Tat-SF1 was reported to be a Tat-specific cofactor (Zhou and Sharp, 1996). However, since Tat-SF efficiently supports both Sp1- and Tat-activated transcription in the RNA Pol II-dependent transcription system (Figure 3), we investigated the contribution of Tat-SF1 to these two Tat-SF functions. The immunodepletion of Tat-SF1 from Tat-SF (Q-Sepharose, fraction #8) at high salt (Figure 7A, see also figure legend) is shown by Western blot (lane 3 versus lanes 1 and 2) and phosphorylation (lanes 9 and 10 versus lanes 5–8) analyses and indicates quantitative

removal of Tat-SF1 with no effect on RNA Pol II and other Tat-SF-associated proteins (data not shown). A functional analysis (Figure 7B, top panel) shows that the ability of Tat-SF1-depleted Tat-SF to support Sp1-activated HIV-1 transcription elongation is impaired 5-fold (lanes 6 and 7) relative to the level observed with intact Tat-SF (lanes 2–5), whereas the production of 13-nucleotide transcripts (13mer) (Figure 7B, bottom panel) is not affected (compare lanes 6 and 7 with lanes 2–5). Tat function is also reduced 5-fold by depleting Tat-SF1 from Tat-SF (Figure 7C, compare lanes 8 and 9 with lanes 2–7). These results show that depletion of Tat-SF1 from Tat-SF impairs transcription elongation, but not initiation, which suggests that Tat-SF1 functions mainly as an elongation factor. Since transcription from the adenovirus major late promoter (MLP) is similarly affected (Figure 7B, top), Tat-SF1 function appears not to be promoter specific. Importantly, the essentially quantitative depletion of Tat-SF1 did not completely impair Tat-SF transcriptional function (Figure 7B, top, lanes 6 and 7;

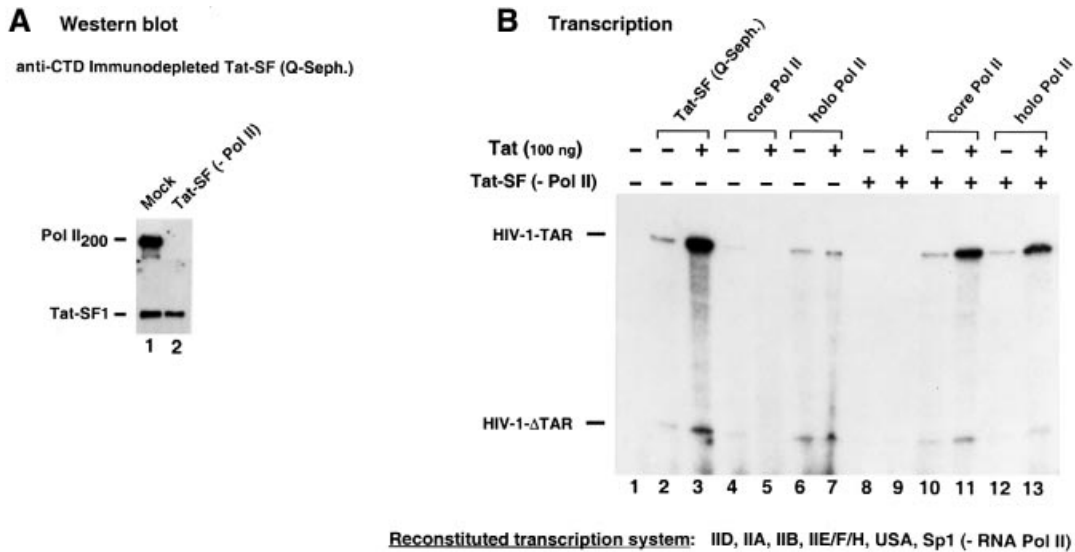


Fig. 8. A Tat-SF-derived fraction lacking RNA Pol II can mediate Tat function via core RNA Pol II or holoenzyme. Tat-SF (Q-Sepharose fraction #8) was subjected to immunodepletion in BC700 with either no serum (mock) or anti-CTD antibodies. (A) Western blot (7.5% SDS-PAGE) of mock or Pol II-depleted Tat-SF fraction. (B) Tat-activated transcription. The citrate treated-transcription system lacking RNA Pol II (lane 1) was complemented with Tat-SF (lanes 2 and 3), core Pol II (lanes 4, 5 and 10, 11) or holo Pol II (lanes 6, 7 and 12, 13) in the absence (lanes 2–7) or presence (lanes 8–13) of Tat-SF (-Pol II), and in the absence or presence of Tat, as indicated.

Figure 7C, lanes 8 and 9), which suggests that Tat-SF contains an additional activity(ies) that is important for both transcription elongation and Tat function (see Discussion).

A Tat-SF-derived fraction lacking RNA Pol II can mediate Tat function via core RNA Pol II and RNA Pol II holoenzyme

We next investigated whether the Tat-stimulatory activity that is physically associated with RNA Pol II in Tat-SF could be separated by immunodepletion of RNA Pol II from Tat-SF. Western blot analyses showed that immunodepletion at high salt (700 mM KCl) removed all RNA Pol II from Tat-SF (Q-Sepharose, fraction #8) but <50% of Tat-SF1 (Figure 8A, lane 2 versus lane 1), probably due to dissociation of Tat-SF1 from RNA Pol II under these conditions. The RNA Pol II-depleted Tat-SF was also tested in a functional assay (Figure 8B). In this assay intact Tat-SF (Figure 8B, lanes 2 and 3), but neither core RNA Pol II (lanes 4 and 5) nor RNA Pol II holoenzyme (lanes 6 and 7), complemented the reconstituted transcription system for Tat function. Interestingly, whereas the Tat-SF-derived fraction lacking RNA Pol II did not support basal activity or Tat function by itself (Figure 8B, lanes 8 and 9), it did complement both core RNA Pol II (lanes 10 and 11) and RNA Pol II holoenzyme (lanes 12 and 13) for Tat-enhanced HIV-1 transcription. Together, these results indicate that a cellular factor(s) that is associated with RNA Pol II in Tat-SF, but not present in RNA Pol II core or holoenzyme complexes, is sufficient to convert a non-responsive RNA Pol II complex to a Tat-responsive complex. The mechanism by which the RNA Pol II-free Tat cofactors are recruited to either preinitiation or elongation complexes on the HIV-1 promoter for TAR RNA-dependent Tat function is not known.

Discussion

Tat-SF as a novel RNA Pol II-associated Tat cofactor complex

We have identified an RNA Pol II-containing complex, Tat-SF, that fully recapitulates Tat function in a reconstituted transcription system. The Tat-SF complex contains previously identified Tat cofactors (Tat-SF1, P-TEFb and hSPT5/Tat-CT1), other known but as yet uncharacterized polypeptides (XP-E and nucleolin), and novel polypeptides, but none of the SRB/MED proteins or other factors found associated with mammalian RNA Pol II holoenzyme complexes (Greenblatt, 1997; Parvin and Young, 1998). Importantly, we found that these cofactors are both separable from RNA Pol II and able to impart Tat responsiveness to both core RNA Pol II and an SRB/MED-containing RNA Pol II holoenzyme, neither of which are normally responsive to Tat (Figure 8). These studies suggest that Tat-SF is physically and functionally distinct from the previously described RNA Pol II holoenzymes and that Tat-SF contains factors important for Tat-activated transcription. The finding that Tat-SF and RNA Pol II holoenzyme are present in HeLa nuclear extract, and can both be recruited (probably independently) to PICs on the HIV-1 promoter (Figures 5 and 6), further indicates that Tat-SF is a bona fide RNA Pol II-containing complex.

Several Tat-SF cofactors are required for Tat function

By using an assay system that is more refined than that used by Zhou and Sharp (1995), we purified Tat-SF as a Tat cofactor-containing RNA Pol II complex. Immunodepletion analysis indicated that a Tat-SF1-depleted Tat-SF complex was impaired, albeit only partially, for Tat function (Figure 7). This result agrees with the findings of Zhou and Sharp (1996), but suggests that Tat-SF may

also contain an activity(ies), in addition to Tat-SF1, that facilitates Tat function. In this regard, Wu-Baer *et al.* (1998) purified a distinct cellular factor, hSPT5/Tat-CT1, using the same semi-purified assay system that had been employed by Zhou and Sharp (1995) for the purification of Tat-SF1. Interestingly, these investigators found that immunodepletion of hSPT5 from a four-column fraction impaired the ability of this fraction to support Tat function; but whereas highly purified hSPT5 could restore the Tat stimulatory activity of this hSPT5-depleted fraction, by itself it could not complement the semi-purified system for Tat function. Our finding of hSPT5 in Tat-SF and an analysis of the contribution of hSPT5 in Tat function by immunodepletion analysis (data not shown) are consistent with this finding, which also implies the existence of multiple cofactors. Our inability to remove nucleolin completely by immunodepletion precluded analysis of its requirement in our assay system for optimal Tat activation. Consistent with other reports implicating CDK9 as a Tat cofactor, depletion of CDK9 from Tat-SF also impaired Tat function in a reconstituted system that lacks P-TEFb (data not shown). Thus, together with past studies, the present results suggest that several cofactors are jointly required for optimal Tat-enhanced transcription.

Tat-SF as an initiation/elongation-competent RNA Pol II complex

RNA Pol II containing an unphosphorylated CTD is more efficient in PIC formation, whereas the elongating form of RNA Pol II contains a hyperphosphorylated CTD (reviewed by Dahmus, 1996). Our study has shown that the CTD of RNA Pol II in Tat-SF is unphosphorylated (Figure 2D), that Tat-SF can be recruited to a PIC on the HIV-1 promoter (Figure 6), and that, as the sole source of RNA Pol II, Tat-SF can support both basal and Sp1-activated transcription (Figure 3B), clearly indicating that Tat-SF mediates initiation functions. Conversely, our study has also shown that Tat-SF is, or has the potential to become, an elongation-competent RNA Pol II complex (Figure 3A and C), consistent with indications that Tat-SF factors P-TEFb and hSPT5/hSPT4 are implicated in transcription elongation in *Drosophila* (Marshall and Price, 1995), yeast (Hartzog *et al.*, 1998) and mammalian cells (Mancebo *et al.*, 1997; Zhu *et al.*, 1997; Gold *et al.*, 1998; Wada *et al.*, 1998). Our observation that both HIV-1 and adenovirus MLP transcription are impaired upon depletion of Tat-SF1 from Tat-SF (Figure 7) agrees with another recent finding, based on a different transcription assay, that Tat-SF1 is a general elongation factor (Li and Green, 1998). Together, these results indicate that Tat-SF contains both transcription initiation and elongation capabilities that are critical for Tat function.

Role of Tat-SF in Tat-enhanced HIV-1 transcription

Even though our reconstituted system contains both TFIID and P-TEFb, it can support Tat-activated transcription only if additional Tat-SF-derived cofactors are present (Figures 3 and 8). Since this Tat function is sensitive to the kinase inhibitor DRB, the results suggest that Tat-SF-derived cofactors may somehow trigger Tat-dependent CTD phosphorylation by these CTD kinases and thus RNA Pol II processivity. How might Tat, in conjunction with Tat-SF-associated factors, link TFIID and P-TEFb

functions for productive Pol II processivity? TFIID functions in transcription initiation/promoter clearance and dissociates from the elongation complex during early elongation (Kumar *et al.*, 1998 and references therein), whereas P-TEFb plays a role in the transition from abortive to productive elongation (Marshall and Price, 1995). Therefore, Tat, in a TAR RNA-dependent manner, may act by coordinating either temporal or synergistic kinase functions on the same and/or different targets (e.g. CTD and/or other Tat-SF components), and thus generate a hyperphosphorylated state of RNA Pol II that, in turn, may be required for the transition to productive elongation. Maintenance of this elongation-competent state of RNA Pol II may require the subsequent association of Tat and cofactors with the elongation complex upon release from TAR RNA (Keen *et al.*, 1997).

Since an RNA Pol II-depleted Tat-SF fraction can complement non-responsive RNA Pol II complexes for Tat function (Figure 8), these results suggest that recruitment of Tat-SF cofactors to preinitiation or elongation complexes on the HIV-1 promoter could be a rate-limiting step for efficient Tat function. Therefore, the finding that Tat-SF1, hSPT5/hSPT4 and P-TEFb (among other polypeptides) associate with RNA Pol II in Tat-SF provides a mechanism by which these Tat cofactors are efficiently recruited to PICs on the HIV-1 promoter (Figure 6). Since P-TEFb is generally required for transcription elongation from various promoters (Marshall and Price, 1995; Peng *et al.*, 1998), as well as for Tat-independent HIV-1 transcription (Mancebo *et al.*, 1997; Zhu *et al.*, 1997; Wei *et al.*, 1998), interactions of P-TEFb with either Tat-SF or RNA Pol II holoenzyme complexes (Figures 4 and 5; data not shown) also provide a general mechanism by which P-TEFb is recruited to promoters (PICs) (Figure 6). Tat, in turn, can also be recruited to these PICs (Garcia-Martinez *et al.*, 1997a), most likely via direct interactions with either Tat-SF (this study) or RNA Pol II holoenzyme (Cujec *et al.*, 1997a). Overall, these results suggest that most of the factors required for Tat function may already be present, along with Tat, in the PIC (prior to RNA synthesis) but that Tat would only function upon TAR RNA synthesis.

At the same time, recent studies suggest a mechanism by which P-TEFb may be recruited to the elongation complex, through the cyclin T component, in a Tat- and TAR RNA-dependent manner (Wei *et al.*, 1998; for a review see Cullen, 1998). Along with previous studies, these results suggest that P-TEFb may be recruited to the HIV-1 promoter by two different (Tat-independent and Tat-dependent) mechanisms. They also suggest that Tat-cyclin T-TAR RNA interactions may provide additional means to enhance occupancy by P-TEFb in early elongation complexes and thus stimulate RNA Pol II processivity, probably via the ability of CDK9 (in P-TEFb) to hyperphosphorylate the CTD.

As both Tat-SF1 and nucleolin phosphorylation correlate with Tat-enhanced CTD phosphorylation during RNA synthesis (Figure 6C), and since purified P-TEFb both associates with and phosphorylates Tat-SF1 (Zhou *et al.*, 1998), phosphorylation also may control the function of these Tat-SF-associated factors in Tat-enhanced processivity.

General role of Tat-SF in transcription

The RNA Pol II holoenzyme complexes isolated from mammalian cells have been reported to contain, in addition to expected yeast SRB and MED homologues, a variety of polypeptides that variably include SWI-SNF components, CBP, RHA and some GTFs (Greenblatt, 1997; Parvin and Young, 1998). Conversely, in our study we have isolated a novel RNA Pol II-containing complex that is devoid of these holoenzyme-associated polypeptides. Recent studies in yeast have indicated an alternative RNA Pol II complex that is both structurally (lacking SRB/MED proteins) and functionally distinct from the holoenzyme (Shi *et al.*, 1997). Similarly, the ability of some genes to be activated by an RNA Pol II that lacks the CTD, and thus the capability to assemble SRB/MED components into a holoenzyme, was also demonstrated (McNeil *et al.*, 1998). These results establish precedent for transcription by alternate RNA Pol II complexes.

Our study strongly suggests that Tat-SF function is not restricted to HIV-1 transcription. Indeed, the Tat cofactors P-TEFb, hSPT5/hSPT4 and Tat-SF1 have been shown to play important roles in transcription from other promoters (this study; Marshall and Price, 1995; Li and Green, 1998; Wada *et al.*, 1998). It would appear, however, that Tat takes advantage of a bona fide RNA Pol II complex containing these factors to relieve the elongation block imposed on HIV-1 transcription. Thus, the results of our study, which describes a novel RNA Pol II complex that mediates Tat function, should have broader implications for other genes whose transcription is regulated at the level of elongation.

Materials and methods

RNA Pol II and transcription factors

Phosphocellulose (P11) 0.1, 0.3, 0.5 and 1.0 M KCl fractions were prepared as described by Ge *et al.* (1996). Bacterially-expressed recombinant TFIIA (p55 plus p12) was purified as described by DeJong *et al.* (1995) and His₆-TFIIB, flag-tagged TFIID, a TFIIE/TFIIF/TFIIH (Mono S) fraction that also contains P-TEFb, USA (Heparin/0.7 M KCl), and RNA Pol II (Mono Q) were purified as described by Ge *et al.* (1996). SP1 was purchased from Stratagene. Recombinant HIV-1 His₆-Tat protein was prepared as described by Parada *et al.* (1995).

The RNA Pol II holoenzyme was purified from a P11 0.5 M KCl fraction, employing the BC buffer (50 mM Tris-HCl pH 7.9 at 4°C, 20% glycerol, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 2 mM dithiothreitol and 0.1% Triton X-100) and the chromatographic steps in the following order: DEAE-Sepharose (Pharmacia), loaded in BC100 (numbers denote millimolar concentration of KCl) and step-eluted with BC350; Q-Sepharose (Pharmacia), loaded in BC100 and step-eluted with BC700; Heparin (Pharmacia), loaded and re-loaded twice in BC300, step-eluted with BC700, dialyzed to BC300 and concentrated with Microcon (30) (Amicon). This RNA Pol II holoenzyme-containing fraction was then subjected to gel filtration on Superose 6 (SMART) in BC300. The purification of the holoenzyme was monitored with antibodies against SRB and MED proteins as well as RNA Pol II.

Purification of Tat-SF

Tat-SF was purified from the P11 flow-through fraction (0.1 M KCl) in BC buffer containing 0.3% Triton X-100 using procedures similar to those described by Zhou and Sharp (1995), but modified as follows. The P11 0.1 M KCl fraction was diluted with BC100 to a protein concentration of 2 mg/ml, applied and re-loaded twice to a DEAE-Sepharose column (Pharmacia) and step-eluted with BC350. This fraction was diluted to BC300 and applied and re-loaded twice to a Q-Sepharose column (Pharmacia). The column was eluted with a step gradient in BC buffer containing 0.3–0.8 M KCl (in 50 mM increments) and fractions were dialyzed against BC100. Tat-SF activity was monitored by comple-

menting the reconstituted transcription system for Tat-activated HIV-1 transcription. The active fraction eluted within 0.55–0.75 M KCl.

In vitro transcription systems and kinase assays

Transcription assays (12.5 µl) with citrate (6 mM) treated-HeLa nuclear extract were as described previously (Kato *et al.*, 1992; Parada *et al.*, 1995). When the reconstituted system was used, either without or with citrate (7 mM) treatment optimal for Tat-activated transcription in a loop sequence TAR RNA-dependent manner, the following protein mixture replaced HeLa nuclear extract: 40 ng of recombinant TFIIA, 20 ng of His₆-TFIIB, 100 ng of f:TFIID, 300 ng of the TFIIE/TFIIF/TFIIH/P-TEFb fraction and 200 ng of USA (heparin fraction). When indicated, 100 ng each of RNA Pol II and Sp1 were also added to the protein mixture. Plasmids containing wild-type HIV-1 (400 bp G-less cassette) (50 ng), mutated TAR RNA HIV-1 (100 bp G-less cassette) (100 ng) (Zhou and Sharp, 1995) and, when indicated, adenovirus MLP (200 nt G-less cassette) (50 ng) promoters served as templates. Reactions (12.5 µl) were preincubated at 30°C for 10 min before transcription was allowed to occur for 60 min at 30°C upon addition of NTPs. RNA products were analyzed as described (Parada and Roeder, 1996). Kinase reactions (15 µl) were performed as described previously (Parada and Roeder, 1996).

Gel filtration

Tat-SF (10 µl of Q-Sepharose fraction #8) or HeLa nuclear extract (100 µg) were fractionated on Superose 6 (SMART, Pharmacia) in BC300/10% glycerol. Fractions were concentrated with Microcon (30) (Amicon), before analyses in transcription, kinase and Western blots.

Affinity chromatography

GST fusion proteins were expressed in *E. coli* strain BL21 and immobilized at 2 mg/ml on glutathione-Sepharose 4B beads (Pharmacia). Affinity-purified fractions were obtained as follows: 100 µg of immobilized GST or GST fusion proteins were mixed with 1 ml of 10 mg/ml HeLa nuclear extract (in BC100/0.3% Triton X-100) at 4°C for 8 h. Both resin-bound fractions were extensively washed with BC buffer containing 100 mM NaCl/0.5% Triton X-100, eluted as indicated, dialyzed to BC100 and analyzed by Western blots or tested in transcription or kinase assays.

Protein-protein interaction assays

GST 'pull-down' and coimmunoprecipitation assays were performed by mixing 2 µg of immobilized GST-GST fusion proteins or the protein A-immobilized antibodies indicated with 50 µl of Tat-SF (Q-Sepharose fraction #8) in BC100/0.3% Triton X-100. After 5 h at 4°C, resin-bound complexes were extensively washed with BC 150 containing 0.5% Triton X-100 and bound proteins were resolved by 7.5% SDS-PAGE and detected by Western blotting.

Immunodepletion of Tat-SF1 and RNA Pol II from Tat-SF

Tat-SF (Q-Sepharose fraction #8) was depleted of Tat-SF1 by incubation with anti-Tat-SF1 (I) polyclonal antibodies in BC700 for 12 h at 4°C. Control immunoprecipitations employed either no serum (mock) or preimmune serum (PI). Tat-SF (Q-Sepharose, fraction #8) was also depleted of RNA Pol II by incubation with anti-CTD monoclonal (8WG16) antibodies in BC700 for 6 h at 4°C. After depletion, Tat-SF-derived fractions were dialyzed against BC100, analyzed by Western blotting and tested in transcription.

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