

Transcription elongation factor P-TEFb mediates Tat activation of HIV-1 transcription at multiple stages

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Tat stimulates human immunodeficiency virus type 1 (HIV-1) transcription elongation through recognition of the transactivation response (TAR) RNA stem-loop structure at the 5' end of nascent viral transcripts. Recently, a human transcription elongation factor P-TEFb, consisting of CDK9 kinase, cyclin T and other associated factors, has been shown to interact with Tat to restore Tat activation in HeLa nuclear extract depleted of P-TEFb. Here, we report the purification of a P-TEFb complex fraction containing epitope-tagged wild-type CDK9 or kinase-inactive CDK9 and five tightly associated polypeptides. Only wild-type P-TEFb complex with an active CDK9 kinase was able to hyperphosphorylate the C-terminal domain of RNA polymerase II and mediate Tat transactivation in P-TEFb-depleted HeLa nuclear extract. Tat also stimulated transcription elongation by recruitment of the P-TEFb complex to the HIV-1 promoter through a Tat-TAR interaction. A possible mechanism for P-TEFb to become associated with polymerase elongation complexes and function as a general elongation factor was demonstrated by an interaction of P-TEFb with double-stranded RNA molecules through an 87 kDa subunit. Finally, P-TEFb was found to interact with and phosphorylate Tat-SF1, a Tat cofactor required for Tat transactivation. Our data indicate that the various subunits of the human P-TEFb complex may play distinct roles at multiple stages to mediate Tat activation of HIV-1 transcription elongation.

Keywords: HIV-1 Tat/P-TEFb/Tat-SF1/transcriptional activation/transcription elongation factor

Introduction

Control of eukaryotic transcription at the stage of elongation can be mediated by two classes of elongation factors: general elongation factors, whose functions are to stimulate RNA polymerase II (pol II) elongation on virtually all eukaryotic promoters; and gene-specific elongation factors, whose functions are highly restricted to the expression of specific genes. So far, cellular proteins that can stimulate transcription elongation in a gene-specific fashion have not been identified, though there are many biochemically defined cellular proteins whose activities suggest that they may function as general elongation factors (Reines *et al.*,

1996). Tat, a small regulatory protein encoded by the human immunodeficiency virus type 1 (HIV-1) genome, has emerged as a model system for the study of gene-specific elongation factors. Tat is essential for HIV gene expression and productive viral infection. In the absence of Tat, the HIV-1 long terminal repeat (LTR) generates short or non-processive transcripts. The presence of Tat, however, results in a large increase in the level of transcripts that extend through the entire HIV-1 genome. Tat stimulation of the efficiency of transcription elongation is primarily responsible for this dramatic increase in the level of full-length HIV-1 transcripts (Jones and Peterlin, 1994; Jones, 1997).

Unlike most DNA sequence-specific transcription activators, Tat stimulates polymerase elongation by recognizing the transactivation response (TAR) RNA stem-loop structure located at the 5' end of nascent viral transcripts. Tat activation of HIV transcription is believed to be modulated by cellular cofactors (Jones and Peterlin, 1994; Jones, 1997). Although the apical loop of TAR contributes minimally to the binding of Tat to TAR *in vitro*, it is essential for Tat activation *in vivo* (Feng and Holland, 1988; Selby *et al.*, 1989), suggesting that the loop sequence may have other important functions. A cellular protein has been proposed to form a complex with Tat, and this complex may bind TAR RNA with high affinity and dependent upon wild-type loop sequences (Jones, 1997). Several cellular proteins such as p68 (Marciniak *et al.*, 1990), TRP-185 (Wu-Baer *et al.*, 1995a) and pol II (Wu-Baer *et al.*, 1995b) have been shown to bind TAR RNA dependent on wild-type loop sequences. However, the functional significance of these interactions for Tat activation has not yet been demonstrated.

Using a partially reconstituted *in vitro* transcription reaction that is Tat-specific and TAR-dependent for activation of HIV transcription elongation as a functional assay, a HeLa nuclear factor Tat-SF1 was identified and its cDNA cloned (Zhou and Sharp, 1995, 1996). Tat-SF1 is specifically required for Tat activation of HIV transcription *in vitro* (Zhou and Sharp, 1996). In transcription reactions, Tat-SF1 forms a complex with an unidentified cellular kinase and is a substrate of the kinase. The Tat-SF1-kinase complex binds TAR RNA weakly. However, in transcription reactions, Tat recruits this complex to the HIV-1 promoter through a Tat-TAR interaction.

A possible requirement for a kinase activity in Tat transactivation has been proposed previously by Marciniak and Sharp (1991), who noted that Tat transactivation was highly susceptible to inhibition by the purine nucleoside analog DRB, which is an effective kinase inhibitor. In a recent random drug screen for inhibitors of Tat activation of HIV transcription elongation, Mancebo *et al.* (1997) identified compounds that could block Tat function, all of which turned out to be kinase inhibitors. These results

suggest that protein phosphorylation by a cellular kinase is a critical step in the Tat activation pathway. Tat activation of HIV-1 transcription elongation requires the C-terminal domain (CTD) of the largest subunit of pol II (Chun and Jeang, 1996; Okamoto *et al.*, 1996; Parada and Roeder, 1996; Yang *et al.*, 1996). Hyperphosphorylation of CTD has been shown to correlate closely with the production of highly processive polymerase elongation complexes (Dahmus, 1996). Therefore, Tat activation may be mediated by a cellular kinase whose phosphorylation of CTD is DRB-sensitive and is essential for the generation of highly processive polymerase elongation complexes.

A cellular kinase complex termed TAK that interacts with the activation domain of Tat and phosphorylates CTD was first reported by Herrmann and Rice (1993, 1995). Recently, it was found that a human positive-acting transcription elongation factor complex called P-TEFb is actually equivalent to TAK (Zhu *et al.*, 1997). P-TEFb, a general elongation factor, was first identified and purified from *Drosophila* extracts (Marshall and Price, 1995). It acts to prevent polymerase arrest and contains an associated kinase activity capable of hyperphosphorylating the CTD of pol II (Marshall *et al.*, 1996). Cloning and sequence analysis of the small subunit of the *Drosophila* P-TEFb complex revealed its extensive homology (72% identity) to a previously identified *cdc2*-related human kinase termed PITALRE (now referred to as CDK9; Grana *et al.*, 1994). Immunodepletion of PITALRE (CDK9) from HeLa nuclear extract eliminated basal transcription elongation and Tat transactivation *in vitro*, and the addition of purified *Drosophila* P-TEFb reversed the block to basal elongation but not Tat activation (Zhu *et al.*, 1997). In contrast, Tat transactivation in the depleted HeLa nuclear extract was restored by the addition of a partially purified human P-TEFb activity (Mancebo *et al.*, 1997), suggesting that hyperphosphorylation of CTD by *Drosophila* P-TEFb is necessary but perhaps not sufficient for Tat function, and that an activity unique to human P-TEFb is probably essential for Tat function. In fact, human P-TEFb/TAK was shown to interact with the activation domain of Tat, suggesting that it may be a functional target of Tat. A similar conclusion was also reached from independent research which demonstrated that the inhibition profile of a group of kinase inhibitors on P-TEFb kinase activity closely correlated with their ability to inhibit Tat activation (Mancebo *et al.*, 1997).

Recently, two novel cyclin C-related proteins (cyclin T1 and cyclin T2) were found to be partners of CDK9 in human cells (Peng *et al.*, 1998; Wei *et al.*, 1998). The two cyclins interacted with CDK9 in a mutually exclusive manner, and ~80% of the CDK9 was found to complex with cyclin T1 and 20% with cyclin T2 in HeLa cells (Peng *et al.*, 1998). Recombinant CDK9–cyclin T (T1 or T2) complex produced in Sf9 cells possessed the CTD kinase activity and mediated basal transcription elongation *in vitro*. It is not clear whether this complex can functionally replace the purified human P-TEFb complex to support HIV-1 Tat transactivation. Importantly, Wei *et al.* (1998) have demonstrated that recombinant cyclin T1 interacted specifically with the transactivation domain of Tat and this association mediated the high-affinity, loop-specific binding of the Tat–cyclin T1 complex to TAR RNA, which was not observed with Tat alone.

HIV-1 Tat has also been shown to interact with transcription factor IIIH (TFIIH) and stimulate phosphorylation of the CTD tail of pol II by the TFIIH kinase (Parada and Roeder, 1996; Garcia-Martinez *et al.*, 1997). Recently, Cujec *et al.* (1997) have shown that Tat directly binds the CDK-activating kinase (CAK) of TFIIH. It has been proposed that both TFIIH and P-TEFb may act sequentially and in a concerted manner that promotes hyperphosphorylation of CTD and increase polymerase processivity (Jones, 1997).

In order to identify all the subunits of the human P-TEFb complex, and to study their roles in pol II CTD phosphorylation, Tat interaction, TAR-binding and other aspects of the Tat activation pathway, we extensively purified P-TEFb from human cells by immunoaffinity purification. Purified P-TEFb complex was used to clarify the mechanisms by which a gene-specific elongation factor Tat and a general elongation factor P-TEFb cooperate to stimulate polymerase elongation from the HIV-1 LTR. We have also examined the relationship between P-TEFb and the previously identified Tat cofactor Tat-SF1 in Tat transactivation. Our results suggest that the various subunits of the human P-TEFb complex may play distinct roles at multiple stages to mediate Tat activation of HIV-1 transcription elongation.

Results

Purification of P-TEFb complexes containing wild-type or kinase-deficient CDK9

The inhibition profile of a group of kinase inhibitors identified by Mancebo *et al.* (1997) on P-TEFb kinase activity closely correlated with the ability of these compounds to inhibit Tat activation. To prove unambiguously that the kinase activity of CDK9 is indeed essential for P-TEFb to mediate Tat transactivation, we wanted to obtain a highly purified, multisubunit P-TEFb complex that contained either wild-type or a kinase-inactive CDK9 subunit with a point mutation changing Asp167 to Asn. DNA constructs expressing an HA epitope-tagged CDK9 (CDK9-HA; Garriga *et al.*, 1996) were transfected into 293T cells. P-TEFb complexes containing CDK9-HA and its associated factors were then purified from cell lysates using a column with immobilized monoclonal antibody 12CA5, which recognizes the HA epitope appended to the C-terminus of CDK9. After extensive washes in buffers containing high salt and detergent, P-TEFb complex was eluted from the antibody column with a solution containing synthetic HA epitope peptide (Zhou *et al.*, 1992).

The affinity-purified wild-type and kinase-deficient P-TEFb complex fractions were analyzed by SDS–PAGE and silver staining (Figure 1, lanes 3 and 4). These fractions contain the epitope-tagged CDK9 (CDK9-HA) and five other major polypeptides with mol. wts of ~87, ~68, ~55, ~49 and ~32 kDa. These fractions also contained several minor polypeptides (~61 and ~51 kDa, and polypeptides smaller than 29 kDa) that are visible on the original silver-stained gel but are difficult to reproduce in this photograph. None of these polypeptides co-migrated in SDS–polyacrylamide gel with a small number of non-specific polypeptides in a control fraction prepared in parallel from 293T cells transfected with an empty vector (Figure 1, lane 2, a band of ~86 kDa in this fraction

migrated to a position clearly different from that of the 87 kDa protein in the P-TEFb fraction on a different percentage SDS-polyacrylamide gel). Consequently, this set of polypeptides was eluted from the antibody column as a result of direct or indirect association with CDK9-HA. The 87 kDa protein in the P-TEFb fraction contained cyclin T1 as indicated by immunoblotting with anti-cyclin T1 antibodies (see below) and may also contain cyclin T2b,

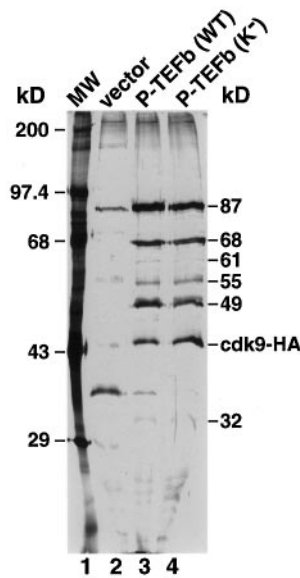


Fig. 1. Kinase-deficient CDK9 associates with the same set of polypeptides as wild-type CDK9. 293T cells were transfected with constructs expressing HA epitope-tagged wild-type CDK9 (WT) or kinase-deficient CDK9 (K⁻). Cell lysates from the transfected cells were applied to an mAb 12CA5 column. Wild-type and kinase-deficient P-TEFb complexes containing HA-tagged CDK9 (CDK9-HA) and associated proteins were eluted using HA peptide and analyzed by SDS-PAGE and silver staining. Polypeptides eluted from the two columns with HA epitope peptide are shown in lanes 3 and 4. A control fraction prepared in the same way as the samples in lanes 3 and 4 from 293T cells transfected with an empty vector is shown in lane 2. Lane 1 contains molecular weight markers.

an alternatively spliced form of cyclin T2. These two cyclins recently were identified to be the cyclin partners of CDK9 and were found to migrate as 87 kDa proteins on an SDS gel (Peng *et al.*, 1998; Wei *et al.*, 1998). Importantly, the kinase-inactive CDK9-HA with a point mutation changing Asp167 to Asn (Garriga *et al.*, 1996) interacted with an identical set of major and minor polypeptides as wild-type CDK9-HA. Because the quantity of the purified material is relatively low, the number of distinct CDK9-containing complexes in these fractions is difficult to ascertain. For the simplicity of presentation, the epitope peptide-eluted fractions will be referred to collectively as the P-TEFb complex fractions.

We next tested the ability of the purified P-TEFb complex fractions to phosphorylate the CTD of pol II and to autophosphorylate *in vitro*. Western blotting using mAb 12CA5 indicated that equivalent amounts of wild-type and kinase-deficient CDK9-HA were present in affinity-purified P-TEFb complexes isolated from transfected cells (Figure 2A). In kinase reactions using purified pol II as a substrate, P-TEFb with wild-type CDK9, but not with the kinase-deficient CDK9, could phosphorylate the CTD of pol II into both hypo- and hyper-phosphorylated forms (pol IIA and IIO; Figure 2B). In an autophosphorylation reaction (Figure 2C), CDK9 in the wild-type P-TEFb complex fraction was able to phosphorylate itself and an 87 kDa protein (p87), which bound tightly to CDK9 and is likely to be the CDK9-associated cyclin T (T1 and T2b). As expected, the kinase-inactive CDK9 in the mutant P-TEFb complex could not phosphorylate itself or the associated p87.

The kinase activity of CDK9 is essential for both basal transcription elongation and Tat transactivation

The affinity-purified wild-type and mutant P-TEFb complex fractions were tested for their ability to mediate Tat activation of HIV-1 transcription in HeLa nuclear extract depleted of the CDK9 subunit of P-TEFb (Figure 3). In

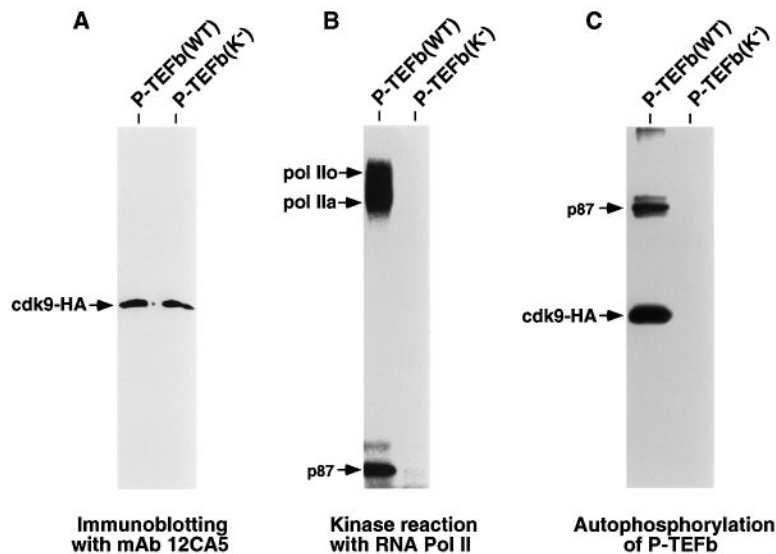


Fig. 2. RNA polymerase II CTD is phosphorylated by the CDK9 subunit of P-TEFb. Wild-type and kinase-deficient P-TEFb complexes containing HA-tagged CDK9 (CDK9-HA) and associated proteins were eluted using HA peptide and analyzed by: (A) immunoblotting using mAb 12CA5 to compare their levels of CDK9-HA; (B) kinase assay for their abilities to phosphorylate RNA polymerase II CTD into hypo- and hyperphosphorylated forms (IIa and IIO); and (C) kinase assay for their abilities to autophosphorylate.

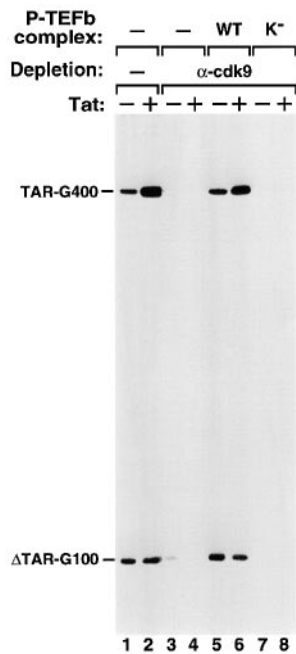


Fig. 3. The kinase activity of CDK9 is essential for both basal transcription elongation and Tat transactivation. Transcription reactions containing both templates pHIV+TAR-G400 and pHIVΔTAR-G100 were performed in the absence (-) or presence (+) of Tat and mock-depleted HeLa nuclear extract (lanes 1 and 2) or HeLa nuclear extract immunodepleted of the CDK9 subunit of P-TEFb (lanes 3–8). Affinity-purified P-TEFb complex containing either wild-type CDK9 (WT) or a kinase-deficient CDK9 mutant (K⁻) was added to CDK9-depleted reactions as indicated.

transcription reactions containing mock-depleted nuclear extract, Tat specifically increased the number of transcripts elongating beyond 1000 nucleotides from a HIV-1 promoter containing the wild-type TAR element (pHIV+TAR-G400; Zhou and Sharp, 1995), but not from an internal control promoter with a mutant TAR (pHIVΔTAR-G100; Figure 3, compare lanes 1 and 2). Removal of the endogenous P-TEFb complex from HeLa nuclear extract using anti-CDK9 antibodies immobilized on protein A-Sepharose beads eliminated both basal transcription elongation and HIV-1 Tat transactivation (Figure 3, lanes 3 and 4). Significantly, addition of the purified P-TEFb complex fraction containing wild-type CDK9 into the depleted HeLa nuclear extract resulted in a complete recovery of both basal transcription elongation and Tat activation of HIV-1 transcription (Figure 3, lanes 5 and 6). In contrast to wild-type P-TEFb, the mutant P-TEFb complex containing the kinase-deficient CDK9 and its associated factors failed to mediate Tat activation (Figure 3, lanes 7 and 8). Therefore, the kinase activity of CDK9 is required for P-TEFb to support both basal transcription elongation and Tat transactivation.

Wild-type Tat protein recruits P-TEFb to the HIV TAR RNA through a Tat-TAR interaction

The results described above strongly argue that P-TEFb supports Tat activation dependent on the CDK9 kinase activity. However, it is not clear how CDK9 in the P-TEFb complex reaches the vicinity of the HIV-1 LTR prior to its phosphorylating the CTD and stimulating the polymerase processivity. Human P-TEFb has been shown to interact

with the activation domain of Tat (Herrmann and Rice, 1993; Zhu *et al.*, 1997). We wanted to investigate whether the interaction between Tat and P-TEFb could recruit the P-TEFb complex to the HIV-1 TAR RNA element through a Tat-TAR interaction. Purified wild-type P-TEFb complex was first incubated alone or together with Tat protein. P-TEFb or P-TEFb associated with Tat was then collected on protein A-Sepharose beads containing immobilized anti-CDK9 antibodies. After a brief incubation, ³²P-labeled wild-type TAR RNA, a TAR mutant with four nucleotide substitutions changing UGGG to CAAA in the apical loop (+31/+34) or a TAR mutant containing a small deletion just below the loop (Δ+35/+38, Figure 4B), was introduced into the reaction together with non-specific RNA competitors. After extensive washes, the amount of ³²P-labeled TAR RNA retained on protein A-Sepharose beads was determined by scintillation counting (Figure 4A).

In reactions containing neither Tat nor P-TEFb (Tat-, P-TEFb-), essentially no TAR RNA remained on anti-CDK9/protein A-Sepharose after washes. The addition of P-TEFb into the reaction (Tat-, P-TEFb+) resulted in a consistent and significant increase in the amount of TAR retained on the beads (an increase from 5 to 280 c.p.m. for wild-type TAR probe and a relatively smaller increase for TAR mutants), suggesting that P-TEFb was able to interact with TAR RNA on its own (see below). Incubation of Tat with the antibody beads also brought down a small amount of TAR RNA (Tat+, P-TEFb-), probably due to a low level non-specific interaction between Tat and antibody beads. Importantly, compared with reactions containing either Tat alone or P-TEFb alone, reactions containing both Tat and P-TEFb together (Tat+, P-TEFb+) showed a dramatic increase in the amount of wild-type TAR associated with the Tat-P-TEFb complex on antibody beads. This increase was a direct result of the interaction between Tat and TAR RNA, because the immobilized Tat-P-TEFb complex precipitated only a small fraction of the TAR deletion mutant Δ+35/+38 (Figure 4A), which has been shown previously to be incapable of forming a complex with Tat (Roy *et al.*, 1990; our unpublished result). Taken together, these results indicate that although P-TEFb alone interacted weakly with TAR RNA, Tat was able to recruit P-TEFb to TAR to significantly increase its local effective concentration through a Tat-TAR interaction. Similar results were obtained when the mutant P-TEFb complex containing a kinase-inactive CDK9 subunit was used in place of wild-type P-TEFb in the above reactions (data not shown). Therefore, the kinase activity of CDK9 is not required for the Tat recruitment of P-TEFb to TAR.

The Tat activation domain, including several important cysteine residues between amino acids 22 and 37, was shown to be required for the interaction of Tat with P-TEFb (Herrmann and Rice, 1993, 1995; Zhu *et al.*, 1997). To determine whether the activation domain of Tat is essential to recruit P-TEFb to TAR, a Tat mutant ΔC, which lacks the entire cysteine-rich activation domain (amino acids 22–37) and is completely defective for transactivation but active in TAR binding (data not shown), was used in place of wild-type Tat in the above RNA pull-down assay (Figure 4A). In contrast to wild-type Tat, TatΔC showed a greatly reduced ability to recruit P-TEFb to TAR. Importantly, this reduction was not due to a

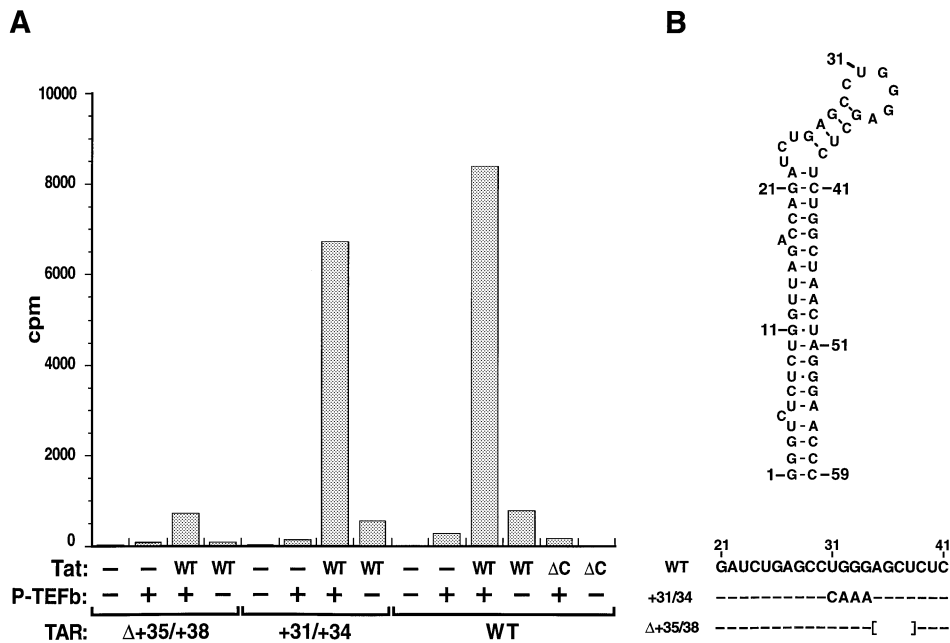


Fig. 4. Tat recruits P-TEFb to the HIV-1 TAR RNA through a Tat-TAR interaction. (A) Immunoprecipitation was carried out using anti-CDK9 antibodies immobilized on protein A-Sepharsose beads. Reactions contained P-TEFb, wild-type Tat protein, Tat mutant ΔC , ^{32}P -labeled and gel-purified wild-type TAR (+1 to +80) or TAR mutants ($\Delta+35/+38$ and $+31/+34$) as indicated. Non-specific competitors tRNA and poly(I)-poly(C) (100 ng each) were present in all reactions. After extensive washes, the amount of labeled TAR RNA (in c.p.m.) retained on Sepharose beads was quantitated by scintillation counting and represented by individual bars in the graph. (B) Computer-predicted secondary structure of the wild-type TAR RNA element is shown. Sequences of wild-type and mutant TAR RNAs between +21 and +41 are also shown.

decreased TAR-binding activity caused by the ΔC deletion. In a gel mobility shift assay with labeled TAR RNA, the amount of Tat ΔC and wild-type Tat protein used in the above RNA pull-down assay produced similar levels of protein-RNA complexes (data not shown). These results demonstrate that an intact activation domain of Tat is essential for Tat to recruit P-TEFb to the TAR RNA element.

Association of cyclin T1 with the P-TEFb complex alters its ability to influence the Tat-TAR interaction

Recently, it has been shown that Tat can interact with isolated recombinant cyclin T1, and this association enhanced the affinity and specificity of the Tat-TAR interaction, and confers a requirement for sequences in the loop of TAR that are not recognized by Tat alone (Wei *et al.*, 1998). Since most cyclin T1 and T2 associate with CDK9 and no significant amounts of free cyclins were found to exist in human cells (Peng *et al.*, 1998), it is important to examine whether the association of Tat with P-TEFb, rather than the isolated cyclin T1 subunit, also allows Tat to recognize TAR RNA in a loop-dependent fashion. We performed a TAR RNA pull-down assay to compare the ability of wild-type TAR and the loop mutant $+31/+34$ containing four nucleotide substitutions in the apical loop (Figure 4B) to associate with the immobilized Tat-P-TEFb complex. When the concentration of Tat used in the binding reaction was the same as that used in an *in vitro* Tat transactivation assay, which produced strong Tat activation from wild-type HIV-1 template but not from the loop mutant ($+31/+34$) template (data not shown),

the loop mutation reduced the association of TAR with the Tat-P-TEFb complex by 23% (Figure 4A). When less Tat protein was tested over a broad range of concentrations in the pull-down assay, wild-type TAR was found to associate with the immobilized Tat-P-TEFb complex with a maximum 2.5-fold higher efficiency than the loop mutant (data not shown). Thus, the loop mutation $+31/+34$ displayed a negative effect on the interaction of TAR with the Tat-P-TEFb complex, but this effect was relatively small (2.5-fold) compared with its strong inhibition of Tat activation (30-fold) both *in vivo* and *in vitro*.

To determine whether cyclin T1 complexed with the P-TEFb complex has the same effect on the Tat-TAR interaction as the isolated recombinant cyclin T1, we performed a gel mobility shift assay with recombinant cyclin T1 and Tat proteins, purified P-TEFb complex, and wild-type and loop mutant TAR RNAs as previously described (Wei *et al.*, 1998). Recombinant cyclin T1 was expressed in bacteria as a GST-cyclin T1 fusion protein, and the GST domain was removed by cleavage with thrombin (Wei *et al.*, 1998). Tat protein, like the GST-Tat fusion protein (Wei *et al.*, 1998), bound to wild-type TAR (Figure 5A, lane 3), and the loop substitution $+31/+34$ reduced the binding of Tat by ~ 4 -fold (Figure 5A, lane 4). A 3- to 5-fold reduction in the Tat-TAR interaction as a result of the TAR loop mutation was also observed previously by Wu *et al.* (1991) and Wei *et al.* (1998). In agreement with the report by Wei *et al.* (1998), recombinant cyclin T1 did not bind to TAR RNA on its own (Figure 5A, lanes 5 and 6), but formed a complex with Tat and strongly enhanced the interaction of Tat with wild-type TAR (Figure 5A, lane 7), but not with the loop

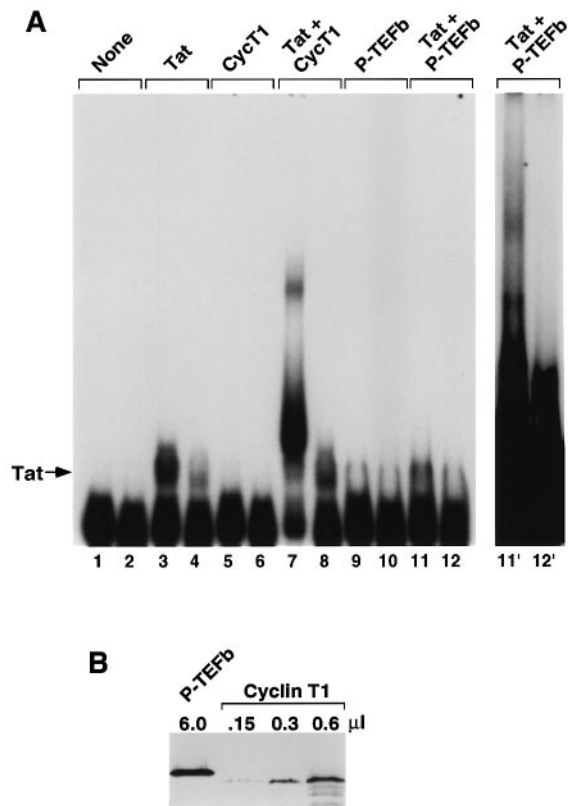


Fig. 5. P-TEFb and free cyclin T1 exert different effects on Tat–TAR interaction. **(A)** Binding of cyclin T1, P-TEFb and Tat proteins to wild-type HIV-1 TAR RNA (odd-numbered lanes) or loop mutant +31/+34 (even-numbered lanes). Where indicated, reactions contained 10 ng of Tat, 30 ng of cyclin T1 (0.6 μ l) or 6 μ l of purified P-TEFb complex fraction. Protein–RNA complexes were separated on a 4.2% Tris–glycine gel. Lanes 11' and 12' represent a seven times longer exposure of lanes 11 and 12. No significant amount of P-TEFb–TAR complex was seen on a longer exposure of lanes 9 and 10 (not shown). Because Tat, rather than GST–Tat, was used here, the Tat–TAR and cyclin T1–Tat–TAR complexes had faster mobilities than the corresponding complexes containing GST–Tat, which forms a dimer (Wei *et al.*, 1998). The slower mobility complex in lane 7 may contain GST–cyclin T1 which was present at a very low level in the cyclin T1 preparation. **(B)** P-TEFb complex fraction (6 μ l) and recombinant cyclin T1 preparation (0.6 μ l) have similar levels of cyclin T1. Immunoblotting with anti-cyclin T1 antibodies was carried out to compare the levels of cyclin T1 in P-TEFb and recombinant cyclin T1 preparations used in the gel mobility shift assay.

mutant TAR+31/+34 (Figure 5A, lane 8). In contrast, transcriptionally active P-TEFb complex displayed a much weaker effect on the affinity and specificity of the Tat–TAR interaction (Figure 5A, compare lanes 11 and 12 with lanes 7 and 8), although immunoblotting with anti-cyclin T1 antibodies indicated that similar levels of cyclin T1 were present in 6 μ l of P-TEFb complex fraction and 0.6 μ l of recombinant cyclin T1 preparation used in the binding reactions (Figure 5B). A longer exposure of lanes 11 and 12 of Figure 5A revealed that P-TEFb was able to interact with the Tat–TAR complex to form a high order complex with mobility slower than that of the cyclin T1–Tat–TAR complex (compare lane 11' with lane 7). Furthermore, the TAR loop mutation reduced the formation of the P-TEFb-containing complex by ~6-fold, which was similar to its 4-fold reduction of the Tat–TAR interaction (Figure 5A, lanes 3 and 4), but much smaller than its

>30-fold reduction of the cyclin T1–Tat–TAR complex (Figure 5A, lanes 7 and 8). Taken together, the RNA pull-down assay and gel mobility shift assay suggest that, unlike free cyclin T1, P-TEFb did not significantly alter the affinity and specificity of the Tat–TAR interaction. We noticed that recombinant cyclin T1 was slightly smaller than its counterpart in the P-TEFb complex (Figure 5B), probably due to an incomplete synthesis in *Escherichia coli*. At present, we cannot rule out the possibility that a small deletion in recombinant cyclin T1 may be responsible for cyclin T1 exerting a strong effect on the affinity and specificity of the Tat–TAR interaction.

P-TEFb associates with double-stranded RNA through the 87 kDa subunit

In the TAR RNA pull-down assay, P-TEFb was found to bind TAR RNA weakly in the absence of Tat, and this interaction was not affected significantly by mutations in the apical loop and bulge regions of TAR (Figure 4A). In a gel mobility shift assay, P-TEFb also formed a complex with labeled TAR RNA when no or a low level of competitor RNA was present in the reaction (data not shown). To study this interaction in more detail, we analyzed the effect of various DNA and RNA competitors on binding of P-TEFb to TAR (Figure 6A). Compared with reactions containing non-specific competitors tRNA and poly(I)–poly(C) (Figure 4A, Tat–, P-TEFb+), immobilized P-TEFb complex precipitated a much larger fraction of labeled TAR RNA in a reaction without any competitor (Figure 6A). The P-TEFb–TAR interaction could be inhibited efficiently by a 50-fold excess of cold TAR RNA, double-stranded RNA poly(I)–poly(C) and yeast tRNA, which contains many double-stranded regions (Figure 6A). Partial inhibition of the P-TEFb–TAR interaction was observed with plasmid DNA. In contrast to double-stranded RNA poly(I)–poly(C) and yeast tRNA, no inhibition was seen with single-stranded poly(C) RNA and only a very small effect was observed with single-stranded DNA oligonucleotides in the reaction. These results suggest that P-TEFb has higher affinity for RNA, especially double-stranded RNA, than for DNA. The potential significance of this interaction with regard to the role of P-TEFb as a general elongation factor will be discussed below.

We next performed a UV cross-linking assay (Marciniak *et al.*, 1990) to determine which subunit of the P-TEFb complex mediates the Tat-independent interaction of P-TEFb with TAR. Purified P-TEFb complex was incubated with *in vitro* synthesized TAR RNA, and proteins were cross-linked to RNA by irradiation at 254 nm. After digestion of TAR RNA by nuclease, individual protein–RNA adducts were resolved by SDS–PAGE (Figure 6B). Of all six major polypeptides present in the P-TEFb complex fraction, only the 87 kDa polypeptide was cross-linked to TAR, suggesting a close physical interaction of this polypeptide with TAR RNA. No protein–RNA cross-linking was observed with a control fraction prepared in parallel from 293T cells transfected with an empty vector. Similar to the TAR precipitation results shown in Figure 4A, p87 was also cross-linked to the TAR loop and deletion mutants containing an intact double-stranded RNA stem with an efficiency similar to its cross-linking to wild-type TAR (data not shown).

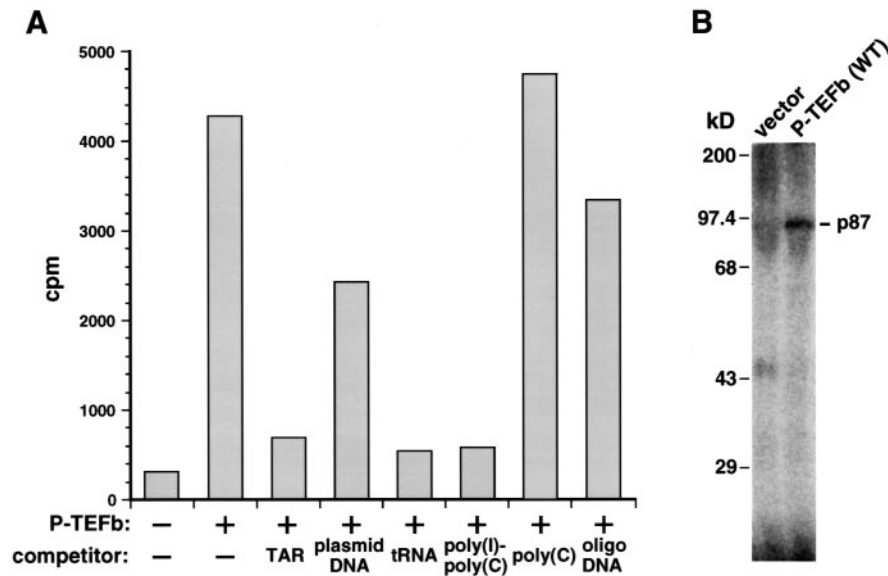


Fig. 6. P-TEFb interacts with double-stranded RNA through an 87 kDa subunit. (A) P-TEFb-TAR interaction can be inhibited by double-stranded RNA. Immunoprecipitation was carried out in reactions containing ^{32}P -labeled TAR, P-TEFb and a 50-fold excess of DNA or RNA competitors as indicated. The amount of labeled TAR RNA retained on Sepharose beads after extensive washes was quantitated by scintillation counting and plotted in the bar graph. (B) The p87 subunit of P-TEFb can be cross-linked to TAR. ^{32}P -labeled TAR RNA was incubated with P-TEFb complex fraction or a control fraction from 293T cells transfected with an empty vector. Reactions were subjected to UV irradiation at 254 nm. After digestion of the TAR RNA by nuclease, individual protein-RNA adducts were resolved by 10.8% SDS-polyacrylamide gel.

P-TEFb associates with and phosphorylates Tat-SF1 in reconstituted transcription reactions

Tat was shown previously to recruit a human Tat cofactor Tat-SF1 and an associated cellular kinase to the HIV-1 TAR RNA through a Tat-TAR interaction (Zhou and Sharp, 1996). Tat-SF1 is a substrate of the kinase and is required for Tat activation of HIV-1 transcription *in vitro* (Zhou and Sharp, 1996). In light of the observation that Tat can also recruit the P-TEFb kinase complex to TAR, we asked whether P-TEFb can interact with and phosphorylate Tat-SF1.

A partially reconstituted transcription reaction that supports Tat activation requires the presence of Tat-SF1 and a phosphocellulose 0.5–1.0 M KOAc (pc-D) fraction containing the Tat-SF1 kinase (Zhou and Sharp, 1996). We performed immunoprecipitation assays using anti-CDK9 antibodies and tested whether P-TEFb, which was also detected in the pc-D fraction by immunoblotting, is a kinase that phosphorylates Tat-SF1 (Figure 7A). When the pc-D fraction (without Tat-SF1) or a partially purified Tat-SF1 fraction (Q, without the Tat-SF1 kinase) was incubated alone with anti-CDK9, no phosphorylated Tat-SF1 was detected when the immune complex was analyzed in kinase reactions (Figure 7A, lanes 1 and 2). However, when both the pc-D and Tat-SF1 fractions were incubated together with immobilized anti-CDK9, a phosphoprotein of ~140 kDa was found to associate with CDK9 and to co-migrate with Tat-SF1 precipitated from a similar reaction using anti-Tat-SF1 antibodies (Figure 7A, compare lanes 3 and 6). The 140 kDa phosphoprotein in Figure 7A, lane 3, is indeed phosphorylated Tat-SF1, as it can be re-precipitated with anti-Tat-SF1 antibodies after release from the immune complex by SDS-PAGE sample buffer (data not shown). Importantly, no phosphorylated Tat-SF1 was detected in control immunoprecipitation reactions containing antibodies directed against CDK7 (also called

MO15, the kinase subunit of CAK and TFIIF; Figure 7A, lane 4) or p57 (Kip2, lane 5), both of which are relatively abundant in the pc-D fraction. These results indicate that in reconstituted transcription reactions, CDK9 and its associated factors in the P-TEFb complex fraction were able to bind to and phosphorylate Tat-SF1.

Tat-SF1 associates with CDK9 in vivo

We next examined whether Tat-SF1 also associates with CDK9/P-TEFb *in vivo*. 293T cells were transfected with either the plasmid expressing CDK9-HA or an empty vector. CDK9 and its associated factors in P-TEFb were then isolated from transfected cells by immunoaffinity purification and epitope peptide elution. Immunoblotting using anti-Tat-SF1 antibodies (Figure 7B) indicates that Tat-SF1 was indeed a component of the P-TEFb complex fraction isolated from 293T cells. Notably, Tat-SF1 could only be detected in the P-TEFb complex fraction isolated under a relatively low salt condition (0.15 M NaCl and 1% NP-40), and was mostly washed away during purification of the high salt-stable (0.5–0.8 M NaCl, 1% NP-40) P-TEFb complex shown in Figure 1. In agreement with this observation, it was shown previously that the association of Tat-SF1 with its kinase was salt-sensitive (Zhou and Sharp, 1996). These results indicate that Tat-SF1 is in fact a CDK9-associated protein under physiological salt conditions *in vivo*. However, since the number of distinct CDK9-containing complexes in the P-TEFb complex fraction is unknown, it is not clear whether Tat-SF1 and other CDK9-associated factors are all in the same complex or whether only a subset of CDK9 complexes contain Tat-SF1.

Tat-SF1 is phosphorylated by a novel kinase activity associated with P-TEFb

To test whether the CDK9 kinase of P-TEFb was responsible for Tat-SF1 phosphorylation, affinity-purified P-TEFb

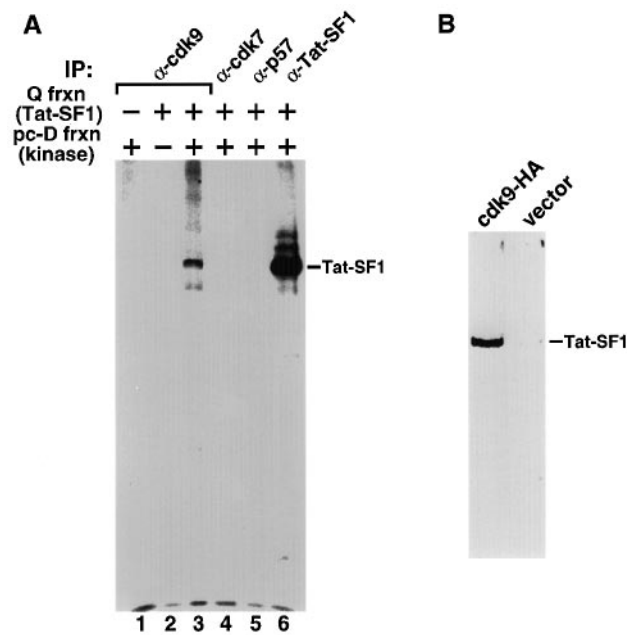


Fig. 7. P-TEFb complex associates with and phosphorylates Tat-SF1. (A) P-TEFb binds and phosphorylates Tat-SF1 in reconstituted transcription reactions *in vitro*. Immunoprecipitation with the indicated antibodies was performed in chromatographic fractions Q (containing Tat-SF1), pc-D (containing a Tat-SF1 kinase) or both together. Fractions Q and pc-D were used to reconstitute a Tat transactivation reaction. The isolated immune complexes were then analyzed in *in vitro* kinase reactions containing [γ - 32 P]ATP. The position of phosphorylated Tat-SF1 is indicated. Because the kinase activity of CDK9 was found to be inhibited by anti-CDK9 antibodies, no phosphorylated CDK9 or p87 was detected in the immune complex (lanes 1 and 3). (B) Tat-SF1 is a CDK9-associated polypeptide *in vivo*. P-TEFb complex fraction was affinity purified under a relatively low salt condition (0.15 M NaCl, 1% NP-40) from 293T cells transfected with a CDK9-HA expression construct. A control fraction was also prepared in parallel from 293T cells transfected with an empty vector. These two fractions were analyzed by immunoblotting with anti-Tat-SF1 antibodies.

complex fraction containing wild-type CDK9 or the kinase-deficient CDK9 was incubated with Tat-SF1 and [γ - 32 P]ATP in kinase reactions (Figure 8). When using pol II as a substrate, only wild-type P-TEFb with an active CDK9 kinase subunit could phosphorylate the CTD of pol II into both hypo- and hyperphosphorylated forms (Figure 8, lane 2, Ila and Ilo). In contrast to pol II CTD, Tat-SF1 was phosphorylated to the same extent by both wild-type P-TEFb and the mutant P-TEFb complex containing the kinase-deficient CDK9 (Figure 8, lanes 4 and 5). Tat-SF1 was not phosphorylated in a kinase reaction containing a control fraction prepared in parallel from 293T cells transfected with an empty vector (Figure 8, lane 6). Furthermore, transcription factor TFIIH, which has a CDK7 (MO15) kinase subunit and is capable of phosphorylating pol II CTD *in vitro*, also failed to phosphorylate Tat-SF1 (Figure 8, lane 8). These results suggest that although CDK9 is the kinase used by P-TEFb to phosphorylate CTD, it is not required for Tat-SF1 phosphorylation. It is possible that a second kinase activity tightly associated with CDK9 in the P-TEFb complex fraction did not phosphorylate pol II CTD, but was responsible for the Tat-SF1 phosphorylation.

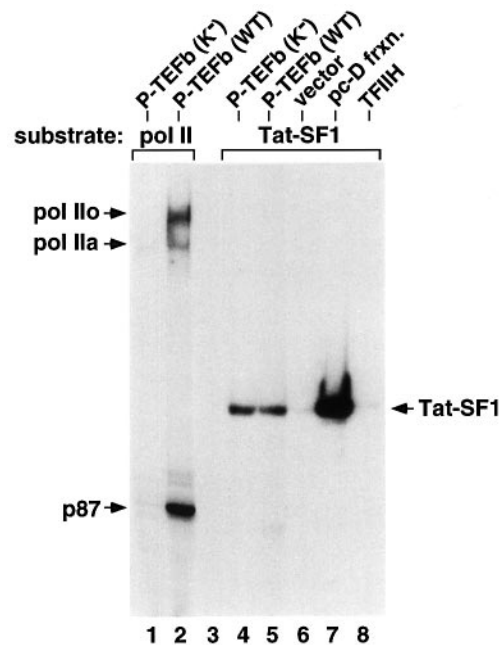


Fig. 8. Tat-SF1 is phosphorylated by a novel kinase associated with P-TEFb. Affinity-purified P-TEFb complex fraction containing wild-type CDK9 (WT) or a kinase-deficient CDK9 mutant (K⁻), a control fraction from 293T cells transfected with an empty vector (vector), extensively purified transcription factor TFIIH or the pc-D fraction was mixed with immobilized Tat-SF1 protein affinity-purified from a partially purified Tat-SF1 fraction. Kinase reactions were initiated by adding [γ - 32 P]ATP. After the reaction, free isotope and proteins (including P-TEFb) not tightly bound to the Tat-SF1 protein beads were washed away with buffers containing 0.8 M KCl. Purified RNA polymerase II was incubated with P-TEFb in kinase reactions shown in lanes 1 and 2, and the entire reaction mixtures were resolved on a 7.5% SDS-polyacrylamide gel. The positions of phosphorylated Tat-SF1, the 87 kDa subunit of P-TEFb, hypophosphorylated pol Ila and hyperphosphorylated pol Ilo are indicated by arrows.

Discussion

The results presented in this study are consistent with a model whereby transcription elongation factor P-TEFb mediates Tat activation of HIV-1 transcription at multiple stages. First of all, the kinase activity of the CDK9 subunit of P-TEFb has been shown here unequivocally to be required for both basal transcription elongation and Tat transactivation. Hyperphosphorylation of the CTD of the largest subunit of pol II has been implicated in the control of polymerase processivity during elongation (Dahmus, 1996). P-TEFb has been shown to hyperphosphorylate CTD *in vitro* (Zhu *et al.*, 1997). Our data indicate that this phosphorylation was mediated directly by the CDK9 kinase. Therefore, phosphorylation of CTD and perhaps other components of the polymerase elongation machinery by CDK9 is likely to be essential for P-TEFb to exert a stimulatory effect on both basal transcription elongation and Tat transactivation.

Our depletion and complementation experiment shown in Figure 3 did not reveal whether the dual effects of human P-TEFb on basal elongation and Tat activation can be separated from each other. However, experiments with *Drosophila* P-TEFb have indicated that these two functions do not necessarily go hand in hand. When highly purified *Drosophila* P-TEFb consisting of CDK9 and cyclin T was added to CDK9-depleted HeLa nuclear extract, only basal

transcription elongation but not Tat activation was restored (Zhu *et al.*, 1997). Like human P-TEFb, *Drosophila* P-TEFb can phosphorylate polymerase CTD efficiently (Marshall *et al.*, 1996). Therefore, CTD phosphorylation is probably necessary and sufficient for basal transcription elongation, but not sufficient for Tat activation. The difference between human and *Drosophila* P-TEFb in their ability to support Tat activation could be the result of CDK9 or cyclin T sequence divergence existing between the two species (72% identity between human and *Drosophila* CDK9, <40% identity between human and *Drosophila* cyclin T; Zhu *et al.*, 1997; Peng *et al.*, 1998), which may result in differences in the substrate specificity of the CDK9 kinase or Tat–P-TEFb interaction. Another possibility is that Tat activation requires other CDK9-associated polypeptides not conserved in flies. The human CDK9-associated proteins may function at stages other than CTD phosphorylation to mediate Tat activation of HIV-1 transcription.

Prior to the phosphorylation of CTD and stimulation of polymerase processivity, P-TEFb must find a way to reach the vicinity of a polymerase elongation complex on the HIV-1 LTR. Our results indicate that an important stage separable from that of CTD phosphorylation is the association of P-TEFb with HIV TAR RNA through Tat as a mediator. The kinase activity of CDK9 is not required at this stage, but is required for the subsequent CTD phosphorylation and polymerase elongation. In order for Tat to recruit P-TEFb to TAR, an intact activation domain of Tat is required to interact with P-TEFb. The RNA-binding domain of Tat then tethers the Tat–P-TEFb complex to TAR through a Tat–TAR interaction. In agreement with this model, Tat was found to recruit P-TEFb to wild-type TAR, but not to a TAR deletion mutant ($\Delta+35/+38$) defective for Tat binding. Notably, a TAR apical loop mutant +31/+34 interacted with the Tat–P-TEFb complex with an efficiency 2- to 3-fold lower than that of wild-type TAR, which may be partially responsible for the observed major reduction of Tat transactivation caused by the loop mutation.

Sequences in the apical loop of TAR are known to be required for Tat transactivation both *in vivo* and *in vitro*, but are mostly dispensable for the Tat–TAR interaction in a pure system *in vitro* (Feng and Holland, 1988; Garcia *et al.*, 1989; Selby *et al.*, 1989). It has been postulated that a cellular factor(s) may bind Tat and the resulting complex may bind TAR RNA with high affinity and dependent on wild-type loop sequences (Jones and Peterlin, 1994; Jones, 1997). Recent experiments with recombinant cyclin T1 suggest that this protein may be such a TAR RNA-binding cofactor for Tat (Wei *et al.*, 1998). Surprisingly, our data suggest that, unlike free cyclin T1 which does not seem to exist in human cells (Peng *et al.*, 1998), a transcriptionally active P-TEFb complex containing cyclin T1 and other associated proteins did not significantly alter the affinity or loop specificity of the Tat–TAR interaction. Although it has been shown that Tat binds directly to the isolated cyclin T1 molecule (Wei *et al.*, 1998), it is not clear whether cyclin T1 can still be recognized by Tat when it becomes part of the P-TEFb complex. Moreover, if cyclin T1 is indeed the Tat-binding subunit of P-TEFb, it remains to be examined whether CDK9 and other subunits of P-TEFb also contribute to the

specificity and affinity of this interaction. The difference between free cyclin T1 and the P-TEFb complex in affecting the Tat–TAR interaction is striking and will be understood better when the exact nature of the interactions among P-TEFb, Tat and TAR is known.

P-TEFb is a general transcription elongation factor; however, little is known about how it becomes associated with the polymerase elongation complex on many cellular and viral promoters. Our observation that P-TEFb has a general preference for double-stranded RNA molecules may provide important clues regarding how this protein complex may function as a general elongation factor. In fact, the interaction between P-TEFb and double-stranded regions of RNA transcripts may be used by P-TEFb to reach the vicinity of stalled pol II elongation complexes along the template. An arrest site that blocks polymerase elongation previously was identified downstream of the adenovirus major late promoter. It has been shown that to prevent polymerase arrest, a double-stranded RNA stem of no specific sequence needs to be present just upstream of the arrest site (Reeder and Hawley, 1996). It is probable that this double-stranded region attracts P-TEFb to the stalled polymerase nearby, which is then hyperphosphorylated by P-TEFb and resumes elongation along the template.

Since P-TEFb was found to interact weakly with double-stranded RNA structure, the recruitment of P-TEFb to the elongation complex is likely to be a rate-limiting step on most non-HIV templates. On the HIV-1 promoter, however, Tat may overcome this barrier through binding to TAR to recruit P-TEFb to pol II and increase its local effective concentration at the promoter. Subsequently, Tat and cellular cofactors may become attached to the transcription elongation complex, from which TAR RNA is released (Keen *et al.*, 1997). It remains to be determined whether Tat may have additional functions such as stimulation of the kinase activity of P-TEFb following its recruitment of P-TEFb to the HIV LTR.

The third function of P-TEFb in mediating Tat transactivation is its ability to bind and phosphorylate a previously identified Tat cofactor Tat-SF1, which was shown to be a substrate of an associated cellular kinase in transcription reactions (Zhou and Sharp, 1996). Here, we show that Tat-SF1 is a CDK9-associated protein both *in vivo* and *in vitro*. Our data also suggest that Tat-SF1 may be phosphorylated by a novel kinase activity tightly associated with CDK9, but not CDK9 itself, in the P-TEFb complex fraction. We have not obtained direct evidence indicating whether phosphorylation of Tat-SF1 by P-TEFb is critical for Tat activation, although a kinase inhibitor DRB that inhibits both Tat activation and CTD phosphorylation by P-TEFb (Zhu *et al.*, 1997) also inhibited phosphorylation of Tat-SF1 by P-TEFb (data not shown). Future experiments will be informative to address the functional significance of Tat-SF1 phosphorylation in Tat activation.

Do Tat-SF1 and the high salt-stable P-TEFb complex (core P-TEFb minus Tat-SF1; Figure 1) work independently or do they function together to mediate Tat activation? When core P-TEFb was removed from HeLa nuclear extract under high salt conditions (Figure 3), Tat-SF1 was left behind (data not shown). Similarly, after depletion of Tat-SF1 from a partially purified Tat-SF1 fraction, core P-TEFb was still present in the reconstituted transcription

reaction (Zhou and Sharp, 1996). Importantly, depletion of either the core P-TEFb alone (Figure 3) or Tat-SF1 alone (Zhou and Sharp, 1996) inhibited Tat activation, suggesting that both are required for Tat function. Due to technical difficulties, we have not been able to obtain recombinant Tat-SF1 protein, which would allow us to ascertain whether and how Tat-SF1 and the core P-TEFb cooperate to mediate Tat activation. Nevertheless, the identification of Tat-SF1 as a CDK9-associated polypeptide in the P-TEFb complex fraction has established an important link between the two cofactors required for Tat function. It is possible that Tat-SF1 and the core P-TEFb interact to assemble a functional P-TEFb complex, which binds Tat and is recruited to the HIV-1 promoter through a Tat-TAR interaction. Since Tat-SF1 has RNA recognition motifs (Zhou and Sharp, 1996), it will be interesting to determine whether Tat-SF1 is the TAR loop-specific factor which will allow the Tat-P-TEFb-Tat-SF1 complex to bind to TAR RNA with more stringent loop sequence specificity than that of the Tat-P-TEFb complex.

The results presented here argue that human P-TEFb supports Tat transactivation at multiple stages. This conclusion rests on the extensive purification of an active P-TEFb complex using a simple epitope tagging and affinity purification strategy. Because the yield of the purified P-TEFb complex fraction is low, we have not been able to find out how many CDK9-containing complexes exist in human cells and what function each complex may have in basal transcription elongation and Tat transactivation. During the purification of P-TEFb activity from HeLa cells by conventional chromatography, Mancebo *et al.* (1997) have noticed the existence of two different CDK9-containing complexes in HeLa nuclear extract, only one of which could support Tat activation. It will be of interest to examine the subunit compositions of these two complexes to see whether they share any common subunits. Future cloning and characterization of all the subunits of P-TEFb will certainly shed more light on the mechanisms by which P-TEFb participates at the various stages of Tat activation of HIV-1 transcription.

Materials and methods

Immunoaffinity purification of P-TEFb

Human 293T cells grown in a 10 cm dish at 50% confluence were transfected with 30 µg of plasmid pRc/CMV-PITALRE-HA or pRc/CMV-PITALRE^(D-N)-HA (Garriga *et al.*, 1996) or an empty vector pcDNA3 (Promega) using the calcium phosphate method. Cells were subjected to lysis 48 h later in high salt lysis buffer [20 mM HEPES pH 7.9; 500 mM NaCl; 1% NP-40; 5 mM EDTA; 1 mM dithiothreitol (DTT); 1 mM phenylmethylsulfonyl fluoride (PMSF)] and supernatant was collected after centrifugation at 16 000 g for 15 min. Cell lysates were pre-cleared with protein A-Sepharose beads and incubated with mAb 12CA5-protein A-Sepharose beads for 2–3 h. After extensive washes with the lysis buffer, P-TEFb complexes were eluted from the antibody column with the elution solution containing 1 mg/ml HA epitope peptide as previously described (Zhou *et al.*, 1992). mAb 12CA5 was coupled directly to protein A-Sepharose with dimethylpimelimidate as previously described (Harlow and Lane, 1988). For the results shown in Figure 7B, cells were lysed in low salt lysis buffer, with 150 mM NaCl in place of 500 mM NaCl in high salt conditions.

Transcription assays

Transcription reactions were carried out essentially as described (Zhou and Sharp, 1995), with some modifications. Reactions (20 µl) contained 13 mM HEPES-KOH (pH 7.9), 9% (v/v) glycerol, 60 mM KCl, 5 mM MgCl₂, 5 mM DTT, 0.1 mM EDTA, 0.025% NP-40, 4 mM sodium

citrate, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM UTP, 6.25 nM CTP, 10 µCi [α -³²P]CTP (800 Ci/mmol; 1 µl), 250 ng polydI-dC, 25 ng pHIV+TAR-G400 (Zhou and Sharp, 1995), 50 ng pHIVΔTAR-G100, 30–50 µg HeLa nuclear extract, and 30 ng of purified Tat protein when indicated. Reactions were assembled on ice and then incubated at 30°C for 60 min. No pre-incubation step was included in the procedure. Following transcription, RNA transcripts were isolated and analyzed as previously described (Zhou and Sharp, 1995).

Immunodepletion of CDK9/P-TEFb from HeLa nuclear extract

HeLa nuclear extract (30 µl) in 0.8 M KCl D buffer (20 mM HEPES pH 7.9, 15% glycerol, 800 mM KCl, 0.2 mM EDTA, 0.1% NP-40 and 1 mM DTT) was incubated with 15 µl of protein A-Sepharose beads pre-bound with anti-CDK9 antibodies (3–5 µg of IgG, Santa Cruz Biotechnology). After repeating the procedure twice, depleted nuclear extract was dialyzed against 0.1 M KCl D buffer and assayed in transcription reactions.

In vitro kinase assay

Kinase assays were performed in ~20 µl reactions containing 20 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, 60–100 mM NaCl, 30–75 µCi of [γ -³²P]ATP, affinity-purified P-TEFb complexes, purified calf thymus pol II (Parvin and Sharp, 1993) or Tat-SF1 affinity-purified from a Tat-SF1 fraction derived from HeLa nuclear extract. The reactions were incubated at 30°C for 20–30 min and analyzed by SDS-PAGE.

In vitro binding and UV cross-linking assay

³²P-labeled TAR RNAs were synthesized from the appropriate DNA templates using T7 RNA polymerase as described (Marciniak *et al.*, 1990). RNAs for direct UV cross-linking were labeled at 6000 Ci/mmol with [α -³²P]ATP. Reaction mixtures containing P-TEFb and labeled TAR RNA (7.5 × 10⁵ c.p.m.) in a 15 µl volume were transferred to ice and irradiated with UV light (254 nm; model UVGL-58 from UVP, San Gabriel, CA) at 2.0 cm from the surface of the lamp for 10 min. The samples were diluted 1:1 with water, followed by the addition of RNase A to 1 mg/ml. After incubation at 37°C for 15 min, the reactions were stopped by the addition of SDS-PAGE sample buffer. The cross-linked proteins were then resolved by 10.2% SDS-PAGE.

RNA pull-down assay

Purified P-TEFb complex fraction (3 µl) and wild-type Tat or Tat mutant ΔC (20 ng each) were incubated together on ice for 10 min. Anti-CDK9 antibodies (0.2 µg, Santa Cruz Biotechnology) immobilized on protein A-Sepharose beads (30 µl of a 50% suspension in 0.1 M KCl D buffer) were then added to the reactions. After incubation on ice for another 30 min with frequent mixing, ³²P-labeled and gel-purified wild-type TAR or TAR mutants (5 × 10⁴ c.p.m., ~10 ng) mixed with non-specific competitors tRNA and poly(I)-poly(C) (100 ng each) were introduced and the reactions were incubated for 10 min at room temperature. Free TAR RNA was washed away with 0.06 M KCl D buffer, and labeled RNA retained on the beads was quantitated by scintillation counting.

TAR RNA gel mobility shift assay

Recombinant cyclin T1 was expressed in bacteria as a GST-cyclin T1 fusion protein, and the GST domain was removed by cleavage with thrombin (Wei *et al.*, 1998). Gel mobility shift reactions were carried out essentially as described (Wei *et al.*, 1998).

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