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Biochemistry

Specific interaction of Tat with the human but not rodent P-TEFb complex mediates the species-specific Tat activation of HIV-1 transcription

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ABSTRACT **Tat stimulation of HIV-1 transcriptional elongation is species-specific and is believed to require a specific cellular cofactor present in many human and primate cells but not in nonpermissive rodent cells. Human P-TEFb, composed of Cdk9 and cyclin T1, is a general transcription elongation factor that phosphorylates the C-terminal domain of RNA polymerase II. Previous studies have also implicated P-TEFb as a Tat-specific cellular cofactor and, in particular, human cyclin T1 as responsible for the species-specific Tat activation. To obtain functional evidence in support of these hypotheses, we generated and examined the activities of human–rodent "hybrid" P-TEFb complexes. We found that P-TEFb complexes containing human cyclin T1 complexed with either human or rodent Cdk9 supported Tat transactivation and interacted with the Tat activation domain and the HIV-1 TAR RNA element to form TAR loop-dependent ribonucleoprotein complexes. Although a stable complex containing rodent cyclin T1 and human Cdk9 was capable of phosphorylating CTD and mediating basal HIV-1 elongation, it failed to interact with Tat and to mediate Tat transactivation, indicating that the abilities of P-TEFb to support basal elongation and Tat activation can be separated. Together, our data indicated that the specific interaction of human P-TEFb with Tat/TAR, mostly through cyclin T1, is crucial for P-TEFb to mediate a Tat-specific and species-restricted activation of HIV-1 transcription. Amino acid residues unique to human Cdk9 also contributed partially to the formation of the P-TEFb–Tat–TAR complex. Moreover, the cyclin box of cyclin T1 and its immediate flanking region are largely responsible for the specific P-TEFb–Tat interaction.**

Tat stimulation of HIV-1 transcription is unique because of its specificity for an RNA target, TAR, and its ability to increase the efficiency of elongation by RNA polymerase II (pol II). Tat recognizes the TAR RNA stem-loop structure at the 5′ end of the nascent viral transcripts. Transactivation by Tat requires specific sequences in both the bulge and apical loop regions of TAR (for a review, see 1, 2). However, binding of Tat to TAR *in vitro* is primarily dependent on the bulge and is not significantly affected by mutations in the TAR loop. Previous reports showed that Tat transactivation can be specifically inhibited when cells or nuclear extracts were treated with synthetic TAR RNAs (3, 4). This inhibition cannot be overcome by the addition of exogenous Tat protein, suggesting that Tat may first form a complex with a cellular cofactor and that this complex interacts with TAR RNA with high affinity and in a loop-dependent manner (reviewed in refs. 1 and 2).

Tat activity has been shown to be species-specific. Tat transactivates the HIV-1 long terminal repeat efficiently in many human and primate cell types but not in cells of other species (e.g., yeast, *Drosophila*, and rodent cells; see ref. 2). Studies with chimeric Tat proteins have demonstrated that the defect in Tat activation in rodent cells is at the level of TAR RNA recognition (5). These observations have led to the proposal that the hypothetical cellular cofactor for Tat may also be a species-restricted protein that interacts with Tat and confers on Tat the ability to bind to TAR RNA in a loop-dependent manner (6, 7). Further evidence in support of this hypothesis came from the observations that Tat activation can be enhanced 5- to 10-fold in Chinese hamster ovary (CHO) cells carrying human chromosome 12, and this enhancement requires wild-type TAR loop sequences (6, 7).

In search of the cellular Tat cofactor, a human transcription elongation factor complex known as P-TEFb was found to interact with the transactivation domain of Tat (8–10). P-TEFb contains a kinase subunit Cdk9, which is capable of hyperphosphorylating the C-terminal domain (CTD) of pol II (11). The importance of P-TEFb and its kinase activity in Tat transactivation was suggested by the observation that all compounds that were identified in a random drug screen to block Tat activation *in vitro* also blocked P-TEFb kinase activity (12). Furthermore, by using dominant-negative Cdk9 (12) as well as a mutant P-TEFb complex with a kinase-defective Cdk9 (13), the kinase activity of Cdk9 was shown to be required for P-TEFb to mediate basal as well as Tat-activated HIV-1 transcription *in vivo* and *in vitro*.

Recently, cyclin T1 (CycT1) was identified as a major partner of Cdk9 in human cells (14, 15). Importantly, recombinant CycT1 was shown to interact with Tat, and this association mediated the high-affinity, loop-specific binding of the Tat–CycT1 complex to TAR RNA (14). This function of CycT1 fits the criteria established previously for the species-specific Tat cofactor (6, 7). Additional evidence in support of this hypothesis came from the observations that the human CycT1 gene maps to chromosome 12 and overexpression of human CycT1 enhanced Tat transactivation in rodent cells (14).

Previously, results from a series of TAR-binding experiments led us to propose that Tat stimulates HIV-1 elongation by interacting with P-TEFb and recruitment of P-TEFb to the HIV-1 promoter through a Tat–TAR interaction (13). P-TEFb may subsequently phosphorylate pol II CTD and/or other components of the polymerase elongation complex to stimulate the processivity of elongation. To obtain functional evidence in support of the recruitment model and to examine the role of human CycT1 as a species-specific Tat cofactor, we generated human–rodent "hybrid" P-TEFb complexes and tested their abilities to phosphorylate CTD, interact with Tat

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Abbreviations: pol II, RNA polymerase II; CTD, C-terminal domain; CycT1, cyclin T1; HA, hemagglutinin; CHO, Chinese hamster ovary; GST, glutathione *S*-transferase.
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and TAR, and mediate Tat transactivation. Our data indicated that the specific interaction of Tat with the P-TEFb complexes containing human but not rodent CycT1 mediates the species-specific Tat activation. We conclude that the defect of TAR recognition by Tat in rodent cells is probably attributed to the failure of Tat binding to rodent P-TEFb.

MATERIALS AND METHODS

DNA Constructs and Antibodies. Different hemagglutinin (HA)-tagged hCycT1 cDNA fragments (except HA-CycT1Δ5) were generated by using PCR and subcloned into the *Bam*HI and *Eco*RI sites of pcDNA3 (Invitrogen). HA-CycT1Δ5 expression vector (pCMV-HA-CycT) was kindly provided by K. A. Jones (The Salk Institute) (14). Anti-Cdk9 antibodies were purchased from Santa Cruz Biotechnology, and rabbit anti-hCycT1 antibodies were raised against a fragment of hCycT1 (amino acids 402–701).

Expression and Purification of P-TEFb Complexes. To obtain the four different P-TEFb complexes (hCdk9-HA/hCycT1, hCdk9-HA/rCycT1, hCdk9/hCycT1-HA, and rCdk9/hCycT1-HA), stable cell lines were established by cotransfecting HA-tagged human Cdk9 cDNA (pRc/CMV-PITALRE-HA; ref. 16) or HA-tagged hCycT1 construct (pCMV-HA-CycT; ref. 14) together with pBabe-puro that confers puromycin resistance into human 293 and rodent CHO cells in all four combinations. Stably transfected 293 and CHO cells were selected by 0.5 and 5 μg/ml puromycin, respectively. Individual colonies were expanded into cell cultures and analyzed for the expression of hCdk9-HA or hCycT1-HA by immunoblotting with mAb 12CA5. Cell lysates were prepared from positive clones and used for the affinity-purification of P-TEFb as described (13).

For P-TEFb complexes containing hCycT1 deletion mutants, HA-tagged hCycT1 constructs with different C-terminal truncations were transiently transfected into 293T cells. Cells were harvested 48 hr later, and P-TEFb complexes were purified (13).

Tat-Binding Assay. P-TEFb complexes were incubated with wild-type glutathione *S*-transferase (GST)-Tat(1–48) or mutant GST-Tat(1–48, C22G) bound to glutathione–Sepharose beads at room temperature for 20 min. The binding buffer contains 20 mM Tris·HCl (pH 8.0), 20% glycerol, 500 mM KCl, 0.5% Nonidet P-40, 0.05% SDS, 0.2 mg/ml BSA, 0.2 mM $ZnCl_2$, 1 mM DTT, and 0.5 mM PMSF. After washes in the same buffer, the bound proteins were analyzed by using Western blotting with antibodies specific for Cdk9 and CycT1.

TAR RNA Gel Mobility Shift Assay. ^{32}P-labeled TAR RNA was synthesized by T_7 RNA polymerase from templates pT7TAR (WT) and pT7TAR(+31/+34) as described (17). Gel mobility shift assays were carried out essentially as described (13, 14), except that 0.2 mM $ZnCl_2$ was included in the reactions and gel running buffer. The presence of $ZnCl_2$ enhanced the formation of P-TEFb–Tat–TAR complexes.

***In Vitro* Kinase Assay.** *In vitro* kinase reactions with purified calf thymus RNA pol II (18) as a substrate were performed as described (13). Kinase substrate GST-CTD was prepared from *Escherichia coli* and used in kinase reactions as described (24).

Transcription Assay. *In vitro* transcription reactions containing HeLa nuclear extract depleted of P-TEFb (13), immunopurified P-TEFb complexes, HIV-1 promoter templates (23), and Tat were carried out exactly as described (13).

RESULTS

Efficient Phosphorylation of RNA pol II CTD by Stable Human–Rodent Hybrid P-TEFb Complexes. Because Tat transactivates poorly in CHO cells, we stably transfected these cells with HA-tagged human Cdk9 (hCdk9-HA) or human cyclin T1 (hCycT1-HA) cDNA and obtained cell lines designated A1 and AB1, respectively. As controls, two human 293-derived cell lines (B4 and G3) that express hCdk9-HA or hCycT1-HA also were generated. hCdk9-HA and hCycT1-HA, as well as their associated polypeptides, were affinity-purified from cell lysates of the four different cell lines by immunoprecipitation with immobilized anti-HA (12CA5) mAb followed by elution with HA epitope peptide (13). In addition to the hCdk9-HA/hCycT1 heterodimer that constitutes the mature and active form of P-TEFb and supports Tat activation, the anti-HA immunopurified fraction from human B4 cells also contained two other complexes that consist of hCdk9-HA/Hsp90/Cdc37 and hCdk9-HA/Hsp70, which function as precursors of the active P-TEFb complex (unpublished data).

To investigate whether rodent Cdk9 (rCdk9) and cyclin T1 (rCycT1) can form stable P-TEFb complexes with the transfected HA-tagged human P-TEFb subunits in CHO-derived cell lines A1 and AB1, the anti-HA immunopurified proteins from these two cell lines were analyzed by using Western blotting with antibodies specific for Cdk9 and CycT1 (Fig. 1*A*). These two antibodies were raised against the two human proteins and were found to recognize rCdk9 and rCycT1 efficiently in Western blotting (Fig. 1*A* and data not shown). The two human–rodent hybrid P-TEFb complexes displayed a ratio of Cdk9 to CycT1 similar to their respective human P-TEFb counterparts hCdk9-HA/hCycT1 (a control for hCdk9-HA/rCycT1, Fig. 1*A*, compare lanes 1 and 2) and hCdk9/hCycT1-HA (a control for rCdk9/hCycT1-HA, compare lanes 3 and 4), indicating the existence of stable cross-species interactions between Cdk9 and CycT1 in these complexes.

The stable cross-species interactions of CycT1 with Cdk9 also activated the kinase activities of both hCdk9 and rCdk9 to a similar extent (Fig. 1*B*). In kinase reactions containing purified RNA pol II as a substrate, hCdk9-HA, associated with either rCycT1 or hCycT1, displayed a similar activity in autophosphorylation and phosphorylation of cyclin T1 and pol II CTD (Fig. 1*B*, lanes 3–8). Likewise, binding of hCycT1-HA to either hCdk9 or rCdk9 generated two P-TEFb complexes with similar kinase activities (lanes 9–14). As negative controls, anti-HA immunoprecipitates derived from the parental 293 and CHO cell lysates displayed no kinase activity (Fig. 1*B*, lanes 1 and 2).

Human but Not Rodent Cyclin T1 Allows for Tat Activation of HIV-1 Transcription *in Vitro*. With the demonstration that all four P-TEFb complexes were able to phosphorylate pol II CTD efficiently, we decided to compare their abilities to mediate Tat activation of HIV-1 transcription *in vitro* (Fig. 2). As shown previously (13), immunodepletion of P-TEFb from HeLa nuclear extract with immobilized anti-Cdk9 antibodies eliminated both basal HIV-1 transcriptional elongation as well as Tat transactivation (Fig. 2, lanes 1 and 2). The addition of affinity-purified hCdk9-HA/hCycT1 into the depleted extract resulted in significant levels of basal elongation from both templates pHIV+TAR-G400 and pHIVΔTAR-G100 (23) present in the same reaction (Fig. 2, lane 3). Importantly, this complex also mediated a Tat-specific and TAR-dependent activation of HIV-1 transcription (Fig. 2, lanes 3 and 4 showed 7-fold Tat-activation when normalized to the ΔTAR-G100 signals). In contrast to hCdk9-HA/hCycT1, the CHO-derived hCdk9-HA/rCycT1 was able to mediate basal but not Tat-activated HIV-1 transcription (Fig. 2, lanes 5 and 6 showed 0.9-fold Tat-activation; no activation observed in other experiments), suggesting that human but not rodent CycT1 is specifically required for Tat activation.

To further test this hypothesis and also to examine whether human Cdk9 is uniquely required for P-TEFb-mediated Tat activation, we compared the transcriptional activities of hCdk9/hCycT1-HA and rCdk9/hCycT1-HA. In reactions containing P-TEFb-depleted HeLa nuclear extract, both com-

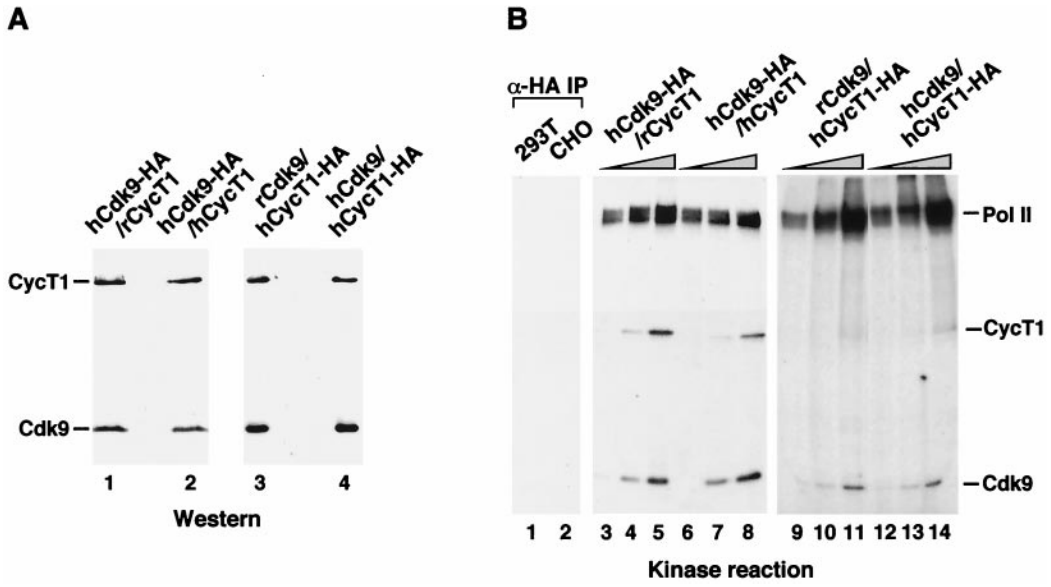

FIG. 1. Efficient phosphorylation of RNA pol II CTD by stable human-rodent hybrid P-TEFb complexes. Cell lysates were prepared from two human 293-based cell lines and two CHO-derived cell lines expressing hCycT1-HA or hCdk9-HA. Four different P-TEFb complexes (hCdk9-HA/hCycT1, hCdk9-HA/rCycT1, hCdk9/hCycT1-HA, and rCdk9/hCycT1-HA) were affinity-purified from these lysates by anti-HA immunoprecipitation followed by HA peptide elution from the antibody column. (*A*) Western blotting with antibodies specific for hCdk9 and hCycT1 was carried out to examine the levels of Cdk9 and CycT1 in these complexes. (*B*) After normalization by Western blotting, equal amounts of the four P-TEFb complexes were analyzed in *in vitro* kinase reactions containing purified calf thymus RNA pol II as a substrate. The abilities of increasing amounts (two-fold increase at each step) of the four complexes to phosphorylate pol II as well as Cdk9 and CycT1 endogenous to the complexes were examined. In control reactions (lanes 1 and 2), anti-HA immunoprecipitates prepared from the parental 293 and CHO cell lysates were analyzed.

plexes were capable of supporting basal as well as Tat-activated HIV-1 transcription (Fig. 2, lanes 7 and 8 showed 5.8-fold and lanes 9 and 10 showed 9.2-fold Tat activation when normalized to internal controls; the two complexes mediated a similar level of Tat activation in other experiments). These results strongly argue that hCycT1, but not hCdk9, is a Tat-specific cofactor that mediates Tat activation in a species-restricted manner. Moreover, the general elongation activity of P-TEFb can be separated from its Tat-specific transcriptional activity.

Human but Not Rodent Cyclin T1 Mediates a Specific and Efficient Interaction of P-TEFb with Tat. To determine whether the abilities of the four P-TEFb complexes to interact with Tat correlate with their abilities to mediate Tat activation, we tested the four complexes for binding to the immobilized GST-Tat(1–48) protein (Fig. 3). After extensive washes, Western blotting with antibodies specific for hCycT1 (Fig. 3A) or hCdk9 (Fig. 3B) was performed to determine the levels of the Cdk9/CycT1 dimers bound to wild-type GST-Tat(1–48). An equal amount of a mutant GST-Tat(1–48, C22G) that contains a point mutation at position 22 in the cysteine-rich activation domain and is completely inactive in transactivation (19) was used as a control. Importantly, while all three complexes containing hCycT1 interacted with the wild-type but not the mutant Tat transactivation domain, virtually no hCdk9-HA/rCycT1 was found to associate with the GST-Tat column (Fig. 3*A*, lane 5). Therefore, the presence of hCycT1 in P-TEFb was essential for its stable interaction with Tat. A comparison of the Tat-binding activities between hCdk9/hCycT1-HA and rCdk9/hCycT1-HA also suggested a partial contribution of hCdk9 to the efficiency of the Tat–P-TEFb interaction (Fig. 3*B*, ≈60–70% reduction by rCdk9).

Requirement of hCycT1, and to a Lesser Extent hCdk9, for Formation of a TAR Loop-Dependent P-TEFb–Tat–TAR Ribonucleoprotein Complex. Previous genetic studies predicted that the species-specific Tat cofactor would confer on Tat the ability to bind TAR RNA in a loop-dependent manner (6, 7). With the demonstration of hCycT1 of P-TEFb as the species-specific Tat cofactor, we carried out electrophoretic mobility shift assay (EMSA) with ^{32}P-labeled TAR RNA to examine the abilities of the four P-TEFb complexes to interact with Tat and TAR to form TAR loop-dependent ribonucleoprotein complexes. As shown in Fig. 4, recombinant Tat protein bound to wild-type TAR (lane 3′, w), and the TAR loop substitution +31/+34 (17) reduced Tat-binding by approximately 5-fold (lane 4′) as reported previously (14, 20). Unlike Tat, the human P-TEFb complexes alone did not bind TAR in reactions containing nonspecific competitors poly(I)–poly(C) and tRNA (only hCdk9-HA/hCycT1 is shown in Fig. 4, compare lanes 1′, 3′, and 5′; also see ref. 13). Instead, they interacted with Tat, and these interactions mediated the binding of the Tat–P-TEFb complexes to wild-type TAR RNA but not to the TAR loop mutant (Fig. 4, lanes 7, 8, 15, and 16). The slow-mobility complex observed in lane 7 indeed contained P-TEFb, as antibodies specific for Cdk9 were able to supershift the complex (compare lane 21 with lane 22) and anti-CycT1 antibodies inhibited the formation of the complex (data not shown).

The gel-shift activities of the four P-TEFb complexes correlated with their abilities to interact with Tat (Fig. 3) and their abilities to mediate Tat activation (Fig. 2). Again, the presence of hCycT1 but not rCycT1 in the P-TEFb complexes was critical for the loop-dependent assembly of the P-TEFb–Tat–TAR complexes (Fig. 4, compare lane 11 with lanes 7, 15, and 19; the slight shift in lane 11 was caused by Tat alone after a long exposure). In addition to hCycT1, hCdk9 also contributed to the formation of the ribonucleoprotein complex (Fig. 4, compare lane 15 with lane 19). The apparent difference between the normal transcriptional activity of rCdk9/hCycT1-HA (Fig. 2) and its partially reduced ability to interact with Tat/TAR (Figs. 3 and 4) may suggest the existence of additional proteins in HeLa nuclear extract that can stabilize its binding to Tat/TAR during transcription. Together, these results argue that the specific and TAR loop-dependent interaction of human P-TEFb with Tat and TAR plays a direct and major role in mediating the species-specific Tat activation.

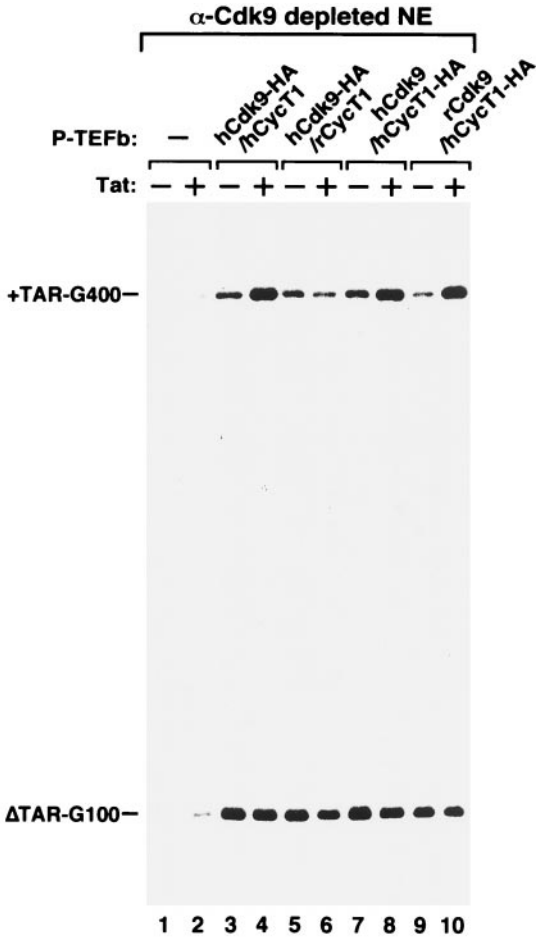

FIG. 2. Human but not rodent CycT1 is required for Tat activation of HIV-1 transcription *in vitro*. Transcription reactions containing P-TEFb-depleted HeLa nuclear extract as well as DNA templates pHIV+TAR-G400 and pHIVΔTAR-G100 (23) were performed in the absence (−) or presence (+) of Tat. Equal amounts of the four P-TEFb complexes were added to transcription reactions as indicated. +TAR-G400 and ΔTAR-G100 represent RNA fragments transcribed from the two G-less DNA cassettes (400 bp and 100 bp) inserted, respectively, into the two DNA templates at a position ≈1 kb downstream of the HIV-1 promoter region.

Cdk9 and Tat Interact with the Cyclin Box and Its Immediate Flanking Sequences in Cyclin T1. With the demonstration of hCycT1 as an important factor for the species-specific Tat function, we decided to map the regions of hCycT1 responsible for its interaction with Tat. A series of C-terminally truncated hCycT1 mutants were generated, HA-tagged at the N termini, and introduced into human 293T cells by transient transfection (Fig. 5*A*). Immunoprecipitation experiments indicated that even CycT1Δ1 (amino acids 1–333) containing only a little more than the cyclin-box region (amino acids 1–303) was capable of forming a stable complex with Cdk9, although the C-terminal regions of hCycT1 also contributed partially to the complex formation (data not shown). A similar result was obtained previously with the cyclin-box region of cyclin T2 and Cdk9 (15). When normalized for the Cdk9 levels, the association of the various hCycT1 deletion mutants (Δ1–Δ5) with Cdk9 activated the kinase activity of Cdk9 to similar levels as judged by its phosphorylation of GST-CTD (Fig. 5*B*, lanes 2–6). As negative controls, GST-CTD was not phosphorylated by anti-HA immunoprecipitates derived from 293T cells transfected with an empty vector (lane 1) or a kinase-defective mutant P-TEFb complex (lane 7).

Affinity-purified P-TEFb complexes containing Cdk9 and the various hCycT1 deletion mutants were normalized for the Cdk9 levels and tested for binding to the GST-Tat column (Fig. 5*C*). CycT1Δ5, with a deletion of the PEST sequence (amino acids 709–726) at the very C terminus of hCycT1, was shown to have wild-type hCycT1 activities in a previous study by Wei *et al.* (14). Compared with Cdk9/CycT1Δ5, further deletions of sequences in the C-terminal half of hCycT1, especially the region between amino acids 634 and 708, resulted in Cdk9/CycT1 complexes that interacted with wild-type Tat with 3- to 4-fold reduced efficiency (Fig. 5*C*), suggesting that the C-terminal region of hCycT1 also contributed to the overall binding affinity. However, this reduction in Tat-binding affinity did not affect the specificity of these interactions, because all complexes showed specific binding to wild-type Tat, which can be disrupted by the point mutation C22G in the Tat activation domain (Fig. 5*C*). Based on these observations, we conclude that the cyclin box of hCycT1 (amino acids 1–303) and its immediate flanking sequences in hCycT1Δ1 play a major role in mediating a specific interaction of P-TEFb with Tat.

DISCUSSION

P-TEFb was first identified and purified from *Drosophila* extracts as a general transcription elongation factor (21). It was also suspected to have a Tat-specific function based on the observation that P-TEFb interacts specifically with the Tat transactivation domain (8–10, 25). We and others have previously shown that HeLa nuclear extract depleted of P-TEFb failed to support both basal and Tat-activated HIV-1 transcription *in vitro* (10, 12, 13). The addition of affinity-purified P-TEFb to the depleted extract efficiently restored both processes (13). Moreover, inhibitors of P-TEFb kinase activity

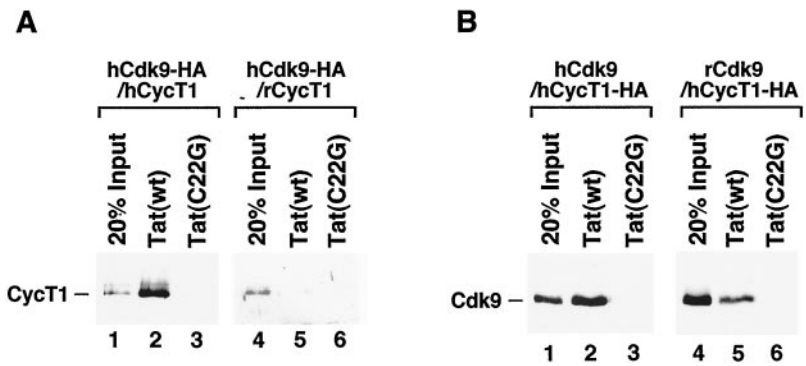

FIG. 3. Human but not rodent CycT1 mediates a specific and high-affinity interaction of P-TEFb with Tat. Four different P-TEFb complexes as indicated were incubated with glutathione-Sepharose beads coupled with equal amounts of wild-type GST-Tat(1–48) or a mutant GST-Tat(C22G, 1–48). After washes, the amount of P-TEFb bound to Tat was examined by Western blotting with anti-CycT1 (*A*) or anti-Cdk9 (*B*) antibodies. For each complex, 20% of the sample used in the binding reaction was shown as input.

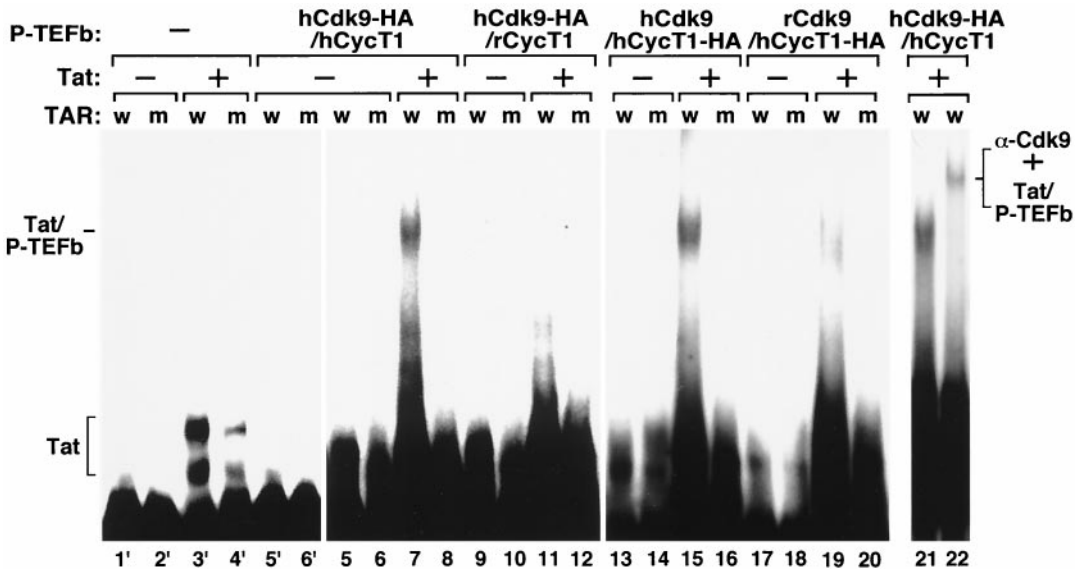

FIG. 4. Requirement of hCycT1 and to a lesser extent hCdk9 for formation of a TAR loop-dependent P-TEFb–Tat–TAR ribonucleoprotein complex. Equal amounts of the indicated four P-TEFb complexes were bound to ^{32}P-labeled wild-type HIV-1 TAR (labeled w) or loop mutant +31/+34 (labeled m) in the presence (+) or absence (−) of Tat. In lane 22, α-Cdk9 antibodies, Tat, TAR, and hCdk9-HA/hCycT1 were present in the binding reaction. The exposure time for lanes 1′–6′ was ≈20% of that for lanes 5–22. Compared with the previous condition (13, 14), the presence of Zn^{2+} in the binding reaction increased the efficiency of the formation of P-TEFb–Tat–TAR complexes. Formation of these complexes, however, was still less efficient than that of the Tat–TAR complex and the hCycT1–Tat–TAR complex containing recombinant hCycT1 (13). The reason is unclear.

(e.g., DRB) were found to block both basal elongation as well as Tat transactivation (10, 12, 22). Because at the time of these studies it was impossible to separate the basal and Tat-specific functions of P-TEFb, a key question to be addressed in the Tat field was whether P-TEFb is really a Tat-specific coactivator or simply a general transcription elongation factor that plays a basic role during polymerase elongation.

A hint that the abilities of P-TEFb to mediate basal elongation and Tat activation can be separated came from studies of *Drosophila* P-TEFb. Zhu *et al.* (10) mentioned (the actual

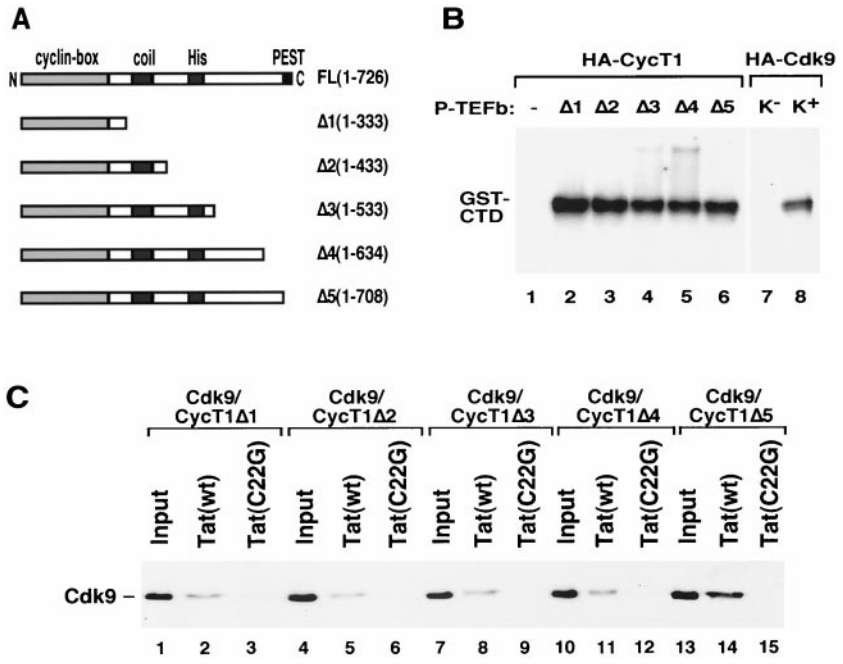

FIG. 5. Cdk9 and Tat interact with mainly the cyclin-box region of cyclin T1. (*A*) A diagram showing the domain structures of the full-length hCycT1 and it C-terminal truncation mutants Δ1-Δ5. (*B*) Different P-TEFb complexes consisting of Cdk9/CycT1Δ1-Δ5 were affinity-purified from human 293T cells transfected with the HA-tagged CycT1Δ1-Δ5 constructs (lanes 2–6). After normalization of their Cdk9 levels, equal amounts of Cdk9/CycT1Δ1-Δ5 were tested for their abilities to phosphorylate GST-CTD (lanes 2–6). Lane 1 contains anti-HA immunoprecipitates derived from 293T cells transfected with an empty vector. P-TEFb complexes containing the HA-tagged wild-type Cdk9 (K^+, lane 8) or a kinase-defective Cdk9 (K^-, lane 7) were also obtained from transfected 293T cells (13) and tested for GST-CTD phosphorylation. These control experiments demonstrated that GST-CTD was indeed phosphorylated by Cdk9 rather than an unknown factor that coimmunoprecipitated with the HA tag. (*C*) Equal amounts of Cdk9/CycT1Δ1-Δ5 were incubated with wild-type GST-Tat(1–48) and mutant GST-Tat(1–48, C22G) bound to glutathione-Sepharose beads. After extensive washes, the bound proteins were analyzed by Western blotting for the levels of Cdk9. For each complex, 20% of the material used in the binding reaction was also shown (input).

data was not shown) that *Drosophila* P-TEFb complements basal transcription in P-TEFb-depleted HeLa nuclear extract but fails to restore Tat transactivation. The data presented in this study indicated that similarly to *Drosophila* P-TEFb, the human–rodent hybrid P-TEFb complex consisting of hCdk9/rCycT1 supported only basal but not Tat-stimulated HIV-1 transcription (Fig. 2), confirming the separation of the two activities of P-TEFb. Unlike hCdk9/rCycT1, hCdk9/hCycT1 mediates both basal and Tat-activated transcription, arguing that human P-TEFb is both a general transcription elongation factor and a Tat-specific cofactor.

A possible mechanism for P-TEFb to associate with polymerase elongation complexes and function as a general elongation factor was suggested previously by the observation of a weak interaction of P-TEFb with double-stranded RNA stems of no specific sequence requirement (13). This interaction may help P-TEFb reach the vicinity of stalled pol II elongation complexes present immediately downstream of these RNA stems. It remains to be tested whether P-TEFb also binds to other components of the elongation complex. Importantly, the observation that the hybrid P-TEFb complexes containing either human or rodent CycT1 were equally active in basal elongation suggests that they may be equally efficient in carrying out these interactions. With regard to the Tat-specific function of P-TEFb, the data presented in this study indicated a good correlation between the abilities of the hybrid P-TEFb complexes to interact specifically with Tat and TAR to form multicomponent ribonucleoprotein complexes and their abilities to mediate Tat activation. These results provided functional evidence in support of our model that Tat activates HIV-1 transcriptional elongation through recruitment of human P-TEFb to the HIV-1 promoter via cooperative binding to nascent TAR RNA (13), a possible mechanism for human P-TEFb to mediate HIV-1 transcription in a Tat-specific manner.

The ability of human P-TEFb to interact with Tat/TAR is crucial not only for P-TEFb to function as a Tat-specific cofactor but also for it to mediate Tat transactivation in a species-specific manner. Our data indicated that the presence of hCycT1, and to a lesser extent hCdk9, in P-TEFb allowed the complex to interact with Tat/TAR and to mediate Tat transactivation. In contrast, the hybrid P-TEFb complex containing rCycT1 was defective in both processes. Importantly, human P-TEFb confers on Tat the ability to bind to TAR RNA in a loop-dependent manner. This observation, together with the finding that CycT1 is encoded on human chromosome 12, fit several important descriptions predicted for the species-specific cellular Tat cofactor (6, 7). It is likely that hCycT1 contains a few critical amino acid residues that vary in rCycT1, and these human-specific residues contribute directly or indirectly to the specific binding of human P-TEFb to Tat and TAR RNA.

We have identified the cyclin-box and its immediate surrounding region located in the N-terminal half of hCycT1 as a major Tat-binding domain of hCycT1 and the C-terminal region of hCycT1 as a secondary area contributing to the overall binding efficiency. Like the interaction between Tat and hCycT1, the hCdk9–hCycT1 interaction also depends largely on the cyclin-box region (Fig. 5). In agreement with these binding results, Cdk9/CycT1Δ1 with only a little more than the cyclin box of hCycT1 was capable of supporting basal and Tat-activated HIV-1 transcription *in vitro*, although with reduced efficiency compared with wild-type P-TEFb (data not shown). It will be of interest to examine the spatial relationship between Tat and hCdk9 when both are associated with the same general region of hCycT1 and to analyze the effect of these interactions on the affinity and specificity of the Tat-hCycT1 interaction as well as the kinase activity of hCdk9. The weaker binding of rCdk9/hCycT1 to Tat/TAR compared with hCdk9/hCycT1 suggests a role for hCdk9 in facilitating a high-affinity interaction of Tat with hCycT1. However, this weak interaction did not significantly affect the ability of rCdk9/hCycT1 to support Tat activation, probably because other proteins present in HeLa nuclear extract may stabilize this weak interaction during transcription. As other regulatory elongation factors (e.g., Tat-SF1 and DSIF; refs. 26–29) are identified and characterized, it will be interesting to learn whether they may interact with and affect the formation of the P-TEFb–Tat–TAR ribonucleoprotein complex.

Separation of P-TEFb functions may provide an avenue for the development of pharmaceutical agents that disrupt the specific interaction of P-TEFb with Tat without inhibiting the critical functions of P-TEFb in general elongation. Future studies will also reveal whether transgenic mice expressing hCycT1, along with the appropriate cell surface receptors and coreceptors can be infected with HIV and used for the study of AIDS.

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