Identification of multiple cyclin subunits of human P-TEFb

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The transition from abortive into productive elongation is proposed to be controlled by a positive transcription elongation factor b (P-TEFb) through phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. *Drosophila* P-TEFb was identified recently as a cyclin-dependent kinase (CDK9) paired with a cyclin subunit (cyclin T). We demonstrate here the cloning of multiple cyclin subunits of human P-TEFb (T1 and T2). Cyclin T2 has two forms (T2a and T2b) because of alternative splicing. Both cyclin T1 and T2 are ubiquitously expressed. Immunoprecipitation and immunodepletion experiments carried out on HeLa nuclear extract (HNE) indicated that cyclin T1 and T2 were associated with CDK9 in a mutually exclusive manner and that almost all CDK9 was associated with either cyclin T1 or T2. Recombinant CDK9/cyclin T1, CDK9/cyclin T2a, and CDK9/cyclin T2b produced in Sf9 cells possessed DRB-sensitive kinase activity and functioned in transcription elongation in vitro. Either cyclin T1 or T2 was required to activate CDK9, and the truncation of the carboxyl terminus of the cyclin reduced, but did not eliminate, P-TEFb activity. Cotransfection experiments indicated that all three CDK9/cyclin combinations dramatically activated the CMV promoter.

[*Key Words:* CTD; cyclin; CDK; RNA polymerase II; transcription; elongation; P-TEFb; DRB]

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The postinitiation processivity of RNA polymerase II is believed to be controlled by both negative and positive (Marshall et al. 1996; Reines et al. 1996; Yamaguchi et al. 1998). Negative factors, such as factor 2 (Xie and Price 1996, 1997, 1998) and a 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)-sensitivity inducing factor (DSIF) (Hartzog et al. 1998; Wada et al. 1998), cause premature stopping and termination of initiated polymerase resulting in the generation of only short abortive transcripts. Positive factors exemplified by positive transcription elongation factor b (P-TEFb) promote the transition into productive elongation (Marshall and Price 1992, 1995; Marshall et al. 1996). After entering productive elongation, the polymerase can be affected further by other elongation factors such as S-II, TFIIF, ELL, and elongin to generate long transcripts (Reines et al. 1996).

P-TEFb is proposed to facilitate the transition from abortive to productive elongation by phosphorylating the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (Marshall et al. 1996; J. Peng, N.F. Marshall, and D.H. Price, in prep). CTD phosphorylation is controlled by various kinases and phosphatases during different transcription stages (Dahmus 1994, 1995). RNA polymerase II has been found to be hypophosphorylated in preinitiation complexes (Laybourn and Dahmus 1989) and in early elongation complexes in vitro (Marshall et al. 1996), but hyperphosphorylated during productive elongation (O'Brien et al. 1994; Laybourn and Dahmus 1989). *Drosophila* P-TEFb has been cloned and identified as a cyclin-dependent kinase pair (CDK9/cyclin T) that can phosphorylate the CTD in vitro (Peng et al., in prep.). The kinase activity of P-TEFb is very sensitive to a purine analog, DRB (Marshall et al. 1996; Peng et al., in prep.). Consistently, the transition from abortive to productive elongation can be inhibited by DRB in vitro and in vivo (Yamaguchi et al. 1998). Removal of the CTD in early elongation complexes abolished P-TEFb function, suggesting that the CTD is the target for P-TEFb function (Marshall et al. 1996).

The kinase activity of CDKs is regulated by phosphorylation and binding of cyclins and CDK kinase inhibitors (CKI) (Morgan 1995). CDKs were first identified as regulators of the cell cycle but have now been implicated in other cellular events (Morgan 1995). Association of a cyclin and phosphorylation of a conserved threonine residue in the T-loop are required to activate a CDK, whereas phosphorylation of specific threonine and tyrosine residues or association of CKIs can inactivate a CDK/cyclin complex (Morgan 1995). Although the sequences of CDKs are well conserved in the CDK family, the cyclins are not conserved except for a helix-rich cyclin box (Bazan 1996).

PITALRE (CDK9) was first cloned and identified as a CDK-like kinase with unknown function (Grana et al. 1994; Garriga et al. 1996). Recently, it was found to be a

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component of human P-TEFb that associated with HIV Tat and was required for HIV-1 Tat transactivation in vitro and in vivo (Mancebo et al. 1997; Yang et al. 1997; Zhu et al. 1997). Here we report the identification and cloning of multiple cyclin subunits of human P-TEFb. All three cyclins have been found associated with CDK9 in HeLa nuclear extract (HNE). Recombinant human P-TEFb possesses strong CTD kinase activity and functions in transcription in vitro and in vivo.

Results

Cloning of three cyclin subunits of human P-TEFb

To further understand the function of P-TEFb in elongation control and in Tat transactivation, it was necessary to clone a human cyclin T subunit. After cloning *Drosophila* cyclin T (J. Peng, N.F. Marshall, and D.H. Price, in prep.), we searched the EST database for homologs. Three human sequences were found that led to the cloning of two related human genes: cyclins T1 and T2 (Fig. 1). Cyclin T2 had two forms termed T2a and T2b that likely arose because of alternative splicing of the primary transcript. Cyclins T2a and T2b shared the first 642 amino acids but had different carboxyl termini. The cyclin box was highly conserved from *Drosophila* to human (Fig. 1B). In the cyclin box region, *Drosophila* cyclin T had 64% identity to either human cyclins T1 or T2, and human cyclins T1 and T2 shared 81% identity. However, the carboxyl terminus was much less conserved. In this region, *Drosophila* cyclin T showed <25% identity to either human cyclin T, whereas human cyclins T1 and T2 shared ~46% identity.

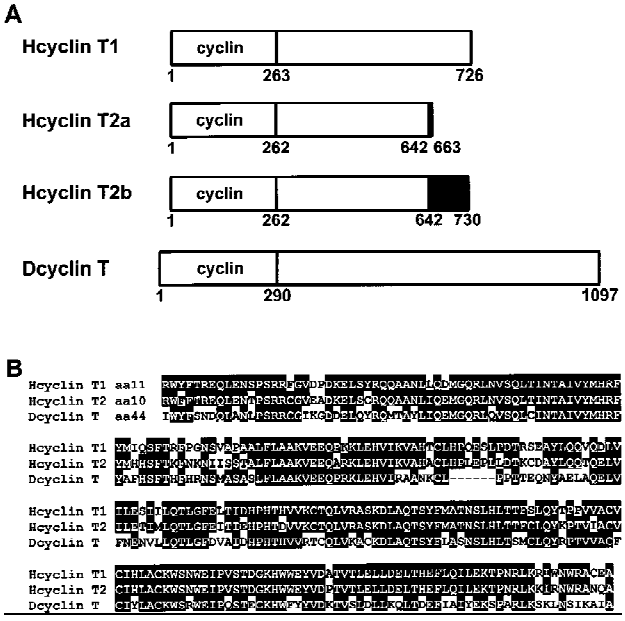

Figure 1. Sequence comparison of multiple human cyclin T's with *Drosophila* cyclin T. (*A*) Diagram of human cyclins T1, T2a, and T2b and *Drosophila* cyclin T. Amino acids are numbered on the bottom of each protein. The cyclin box is indicated. Human cyclins T2a and T2b have 642 amino acids in common but different carboxyl termini (black boxes). (*B*) Sequence alignment of the cyclin boxes. Identity is indicated by reverse shading.

Northern analysis indicated that human CDK9 and cyclins T1 and T2 were expressed in all human tissues examined (Fig. 2). CDK9 had several mRNAs of 2, 2.7, and 3.4 kb, similar to what was reported previously (Grana et al. 1994). Cyclin T1 had a major 8.2-kb mRNA in all tissues and a 9.3-kb mRNA in brain. Cyclin T2 had a major 6-kb mRNA and a heterogeneous pattern of larger RNA species. The 6-kb band was not sharp suggesting the presence of more than one mRNA form. The ubiquitous expression of mRNAs encoding both subunits of P-TEFb was consistent with the general requirement of P-TEFb in transcription elongation.

Multiple cyclin subunits exist and bind to CDK9

cDNAs encoding CDK9 were coexpressed with either cyclin T1, T2a, or T2b in Sf9 cells (Fig. 3A), and recombinant proteins were purified using a nickel column followed by Mono S. The purified proteins were analyzed by SDS-PAGE (Fig. 3B). Only CDK9 was tagged, but the cyclin subunits were quantitatively retained on the nickel column indicating that the interaction of the cyclin and kinase subunit was strong. This was confirmed by the coelution of both subunits on the Mono S column. The interaction between the two subunits is, at least, partly due to the cyclin box region because a truncation mutant containing only the cyclin box of cyclin T2 [CDK9/T2 (1–286)]behaved similarly (Fig. 3).

To validate the cloning of appropriately sized cyclin subunits and to examine the natural abundance of the three potential forms of P-TEFb, antibodies were generated against the carboxyl terminus of either cyclin T1 or T2. Then, HNE was immunodepleted of either CDK9, cyclin T1, cyclin T2, or cyclin T1 and T2 together. GST antibodies were used as a negative control. The immunoprecipitates (IPs) were analyzed by Western blot (Fig. 4A). As expected, CDK9 was present in the IP using antibodies against CDK9, cyclin T1 and T2. Importantly, native cyclin T1, T2a, or T2b proteins with identical mobilities to the recombinant proteins were detected. This indicates that all three forms are expressed in HeLa cells. Cyclin T1 was present in the IPs of CDK9 and cyclin T1 antibodies, whereas cyclin T2a and T2b were present in the IPs of CDK9 and cyclin T2 antibodies. These data strongly argued that CDK9 can bind cyclin T1 or T2 but not both in one complex. Although the samples were loaded to reflect equivalent amounts of starting extract, the CDK9 detected in the IPs of cyclin T1 was about four times that in the IPs of cyclin T2, suggesting that the level of cyclin T1 was significantly higher than that of cyclin T2 in HNE.

To further demonstrate the association of CDK9 and the cyclins and quantitate the relative amount of cyclin T1 and T2 in HNE, we performed a Western blot using various immunodepleted HNEs (Fig. 4B). Quantitation of the results was accomplished by loading different

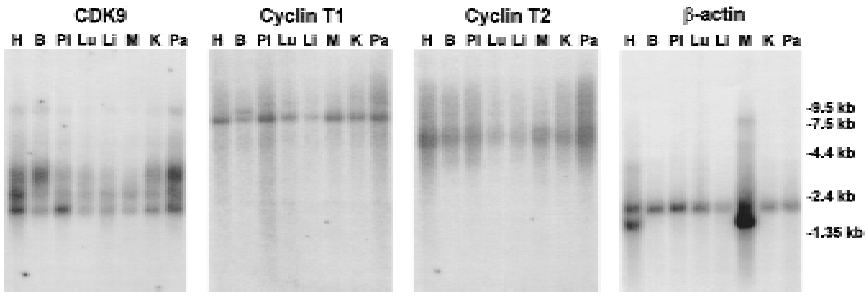

Figure 2. Expression pattern of human P-TEFb subunits. A multiple tissue Northern blot (Clontech) was probed according to the manufacturer. The sequences used for probes are as follows: (CDK9) Total encoding cDNA sequence; (cyclin T1) 0.4 kb encoding the amino terminus; (cyclin T2) 1.35 kb encoding the carboxyl terminus of cyclin T2b; β-actin: 2.0 kb of cDNA. The sizes of molecular markers are at *right*. (H) Heart; (B) brain; (Pl) placenta; (Lu) lung; (Li) liver; (M) skeletal muscle; (K) kidney; (Pa) pancreas.

amounts of the starting HNE from 1× to 10×. Because THIIH was not found to be associated with P-TEFb, p62 (a subunit of TFIIH) was detected in all lanes and the signal correlated with the loading level of HNE (10× for all depleted HNEs). GST antibodies did not deplete any detected protein. In this experiment CDK9 antibodies depleted 85% of CDK9 and 85% of cyclins T1 and T2. T1 antibodies depleted 65% of CDK9, 90% of cyclin T1, but no cyclin T2. T2 antibodies depleted 20% of CDK9, no cyclin T1, and 90% of T2. T1, and T2 antibodies together depleted 75% of CDK9 and 85% of cyclins T1 and T2. These data strongly support that almost all CDK9 is associated with either cyclin T1 or T2. CDK9/cyclin T1 is the major form of the kinase, and no significant amount of free cyclin T or CDK9 exists in HNE. Furthermore, the similar signal detected for cyclins T2a and T2b on the blot suggests that the T2 forms are present in equal amounts.

Recombinant P-TEFb proteins have kinase and transcription activities

Human P-TEFb possesses a CTD kinase activity that is sensitive to DRB (Zhu et al. 1997). To test the kinase activity of recombinant P-TEFb proteins, *Drosophila* RNA polymerase II was incubated with increasing amounts of recombinant proteins in the presence of 10 μM [γ-^{32}P] ATP for 5 min. The products were analyzed by SDS-PAGE followed by autoradiography (Fig. 5A). CDK9/T1 had slightly higher activity compared to both CDK9/T2a and CDK9/T2b, which had similar kinase activity to each other. All three proteins supported hyperphosphorylation of the large subunit of RNA polymerase II to the IIo form. CDK9 alone had very little activity, and CDK9/T2 (1–286) had low activity and could not easily phosphorylate RNA polymerase II and cause a shift to the IIo form. A wide-range titration experiment demonstrated that the kinase activity of CDK9/T2a was 10–20 times higher than CDK9/T2 (1–286), at least 300 times higher than that of CDK9 alone (data not shown). The kinase activity of all three human P-TEFb proteins in the presence of increasing amounts of DRB was quantitated and plotted (Fig. 5B). The 50% inhibition point of CDK9/T1 (0.9 μM) was similar to that of CDK9/T2a (0.4 μM) and CDK9/T2b (0.5 μM).

To analyze the function of recombinant P-TEFb proteins in transcription, they were added back to HNE depleted of CDK9. CDK9-depleted extracts have very little

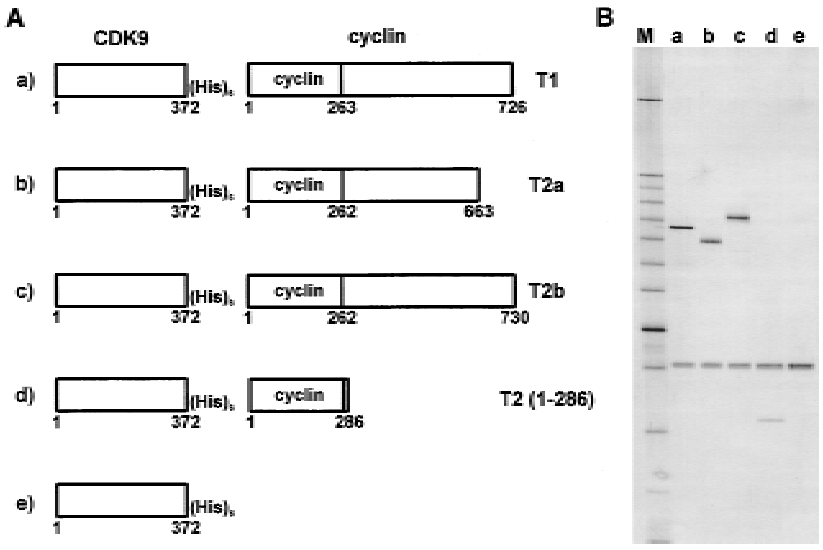

Figure 3. Purified recombinant human P-TEFb proteins. (*A*) Diagram of P-TEFb protein constructs. (*a*) CDK9/T1; (*b*) CDK9/T2a; (*c*) CDK9/T2b; (*d*) CDK9/T2 (1–286); (*e*) CDK9. CDK9 is His-tagged. (*B*) SDS-PAGE (silver stained) of the purified recombinant proteins. (M) 10-kD ladder (10–120 plus 200 kD) with darker 50-kD band.

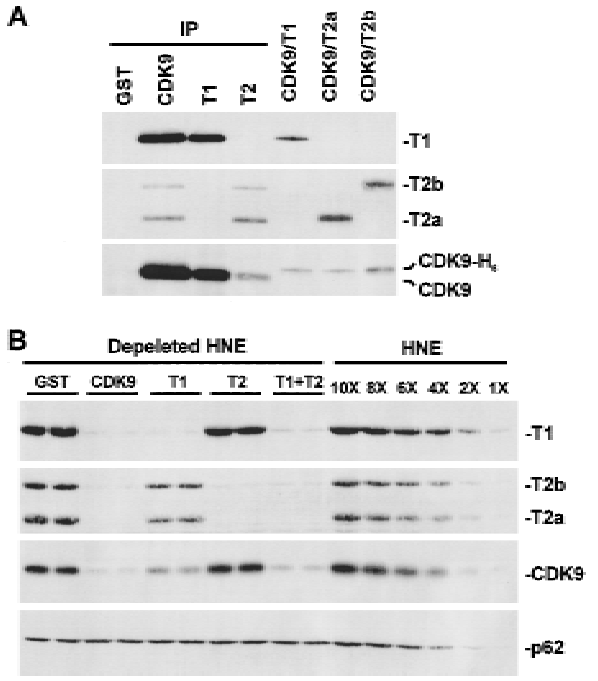

Figure 4. Multiple cyclin subunits are present and associated with CDK9 in HNE. (*A*) Western blot IPs. Human P-TEFb was immunoprecipitated using affinity-purified antibodies against GST, CDK9, cyclin T1, or cyclin T2. The IPs (equivalent to 10 µl HNE) were separated on an SDS gel and subjected to Western blotting using antibodies against cyclins T1, T2, or CDK9. The identity of the proteins is indicated at *right*. Recombinant proteins CDK9/T1, CDK9/T2a, and CDK9/T2b were used as standards. (*B*) Western blot of immunodepleted HNE. HNE was depleted using antibodies against GST, CDK9, cyclin T1, cyclin T2, or cyclins T1 and T2 together. Depleted HNEs equivalent to 2 µl of HNE (10×) were loaded on an SDS gel followed by Western blotting using antibodies against cyclins T1, T2, CDK9, or p62 (a TFIIH subunit as a loading control). (*Right*) Original HNE was titrated as a quantitation standard.

DRB-sensitive transcription unless exogenous P-TEFb is added (Zhu et al. 1997). In this experiment a slight residual DRB sensitivity was detected in the depleted extract (Fig. 6A) that may be attributed to incomplete depletion. Addition of increasing amounts of recombinant P-TEFb proteins to the depleted extract restored DRB-sensitive transcripts to levels higher than that observed with the low level of endogenous P-TEFb in the original extract (Fig. 6). CDK9/T1 gave similar results to CDK9/T2a. Both of them had slightly higher activity than CDK9/T2b. CDK9/T2 (1–286) showed some activity, but CDK9 alone exhibited very little activity. The function of the recombinant proteins in transcription mirrored their ability to phosphorylate the CTD of RNA polymerase II, consistent with the idea that P-TEFb functions through the phosphorylation of CTD.

Overexpression of human P-TEFb activates the CMV promoter in vivo

To examine the effect of overexpression of P-TEFb in vivo HeLa cells were cotransfected with constructs containing the CMV promoter driving luciferase and constructs expressing CDK9 and the three cyclin T's (Fig. 7). Cotransfection with CDK9 increased the expression of CMV sixfold and cyclin T1 increased expression fourfold. These enhancements might be attributlable to the presence of uncomplexed CDK9 and T1 in the cells being driven into complex by increasing the concentration of either partners. It is also possible that either subunit might titrate out an inhibitor of P-TEFb. The most dramatic stimulation of the CMV promoter occurred when both the kinase and a cyclin partner were added together. CDK9/T1 gave the greatest stimulation (38-fold) and CDK9/T2a and CDK9/T2b gave 26- and 16-fold stimulation, respectively. These results strongly suggest that even for one of the strongest promoters known, in vivo expression is limited by P-TEFb activity.

Discussion

We have identified three human cyclin cDNAs encoding proteins T1, T2a, and T2b, which activate CDK9. T1 is the major form of the cyclin, and T2a and T2b are less abundant forms derived from a second gene. All three proteins are expressed in a wide variety of human tissues

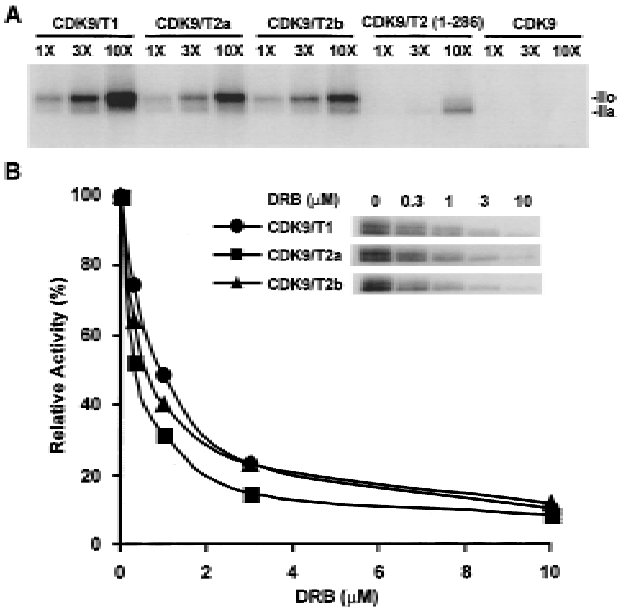

Figure 5. Recombinant human P-TEFb proteins have DRB-sensitive CTD kinase activity. (*A*) CTD kinase assay using *Drosophila* RNA polymerase II and the indicated amount of recombinant proteins. Labeled RNA polymerase molecules were analyzed on a 6%–15% SDS–polyacrylamide gel by autoradiography. (1×) Approximately 10 fmoles. (IIo and IIa) Shifted and unshifted largest subunit of RNA polymerase II, respectively. (*B*) DRB inhibition. P-TEFb proteins (5×) were used in the kinase assay with the addition of the indicated amount of DRB. Radiolabeled RNA polymerase II was quantified using a Packard InstantImager and normalized to the starting level (100%).

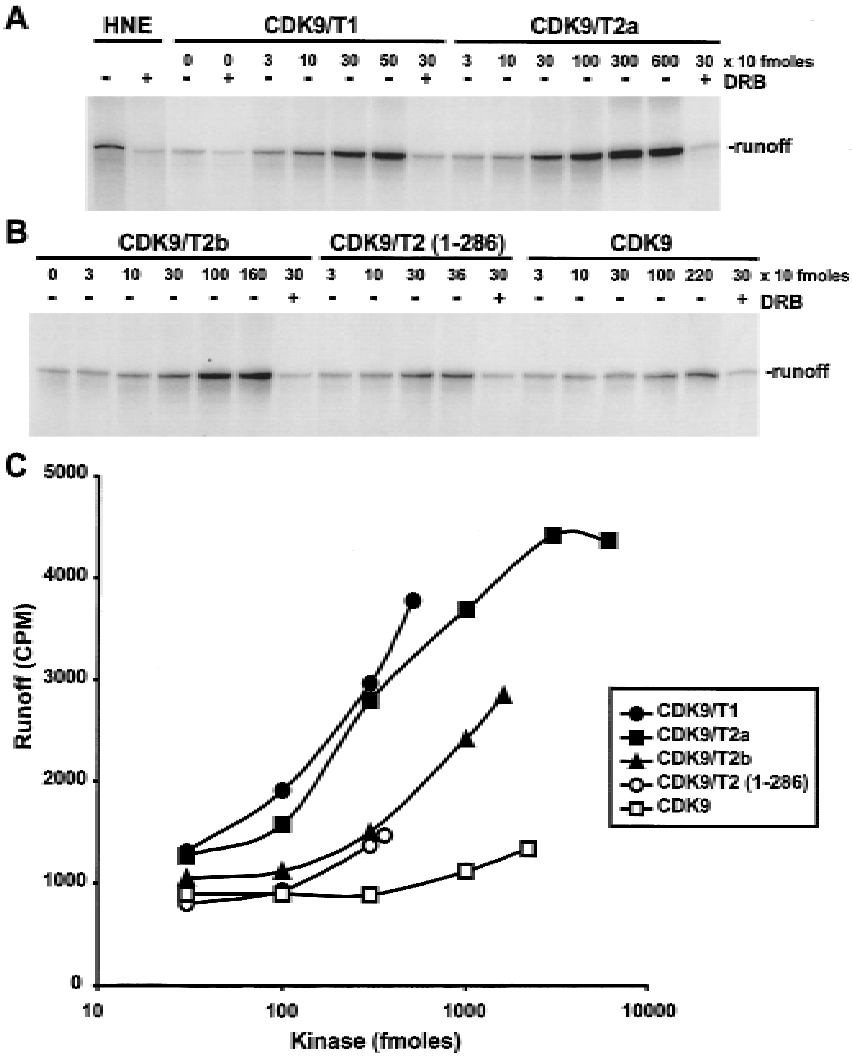

Figure 6. Recombinant human P-TEFb proteins function in transcription. (*A*,*B*) Continuous labeling transcription using a CMV promoter was performed with HNE or CDK9-depleted HNE with addition of the indicated amount of recombinant proteins. DRB (50 μM) was added as shown. (*C*) Plot of runoff. The radioactivity in the runoff transcripts was quantitated using a Packard InstantImager.

and are found complexed with CDK9 in HeLa nuclear extract. Roughly 80% of the CDK9 is complexed with cyclin T1, 10% with T2a, and 10% with T2b. Any of the three cyclin proteins complexed with CDK9 form an active P-TEFb molecule that can phosphorylate the CTD of RNA polymerase II and cause the DRB-sensitive transition from abortive elongation into productive elongation.

Cyclins T2a and T2b are derived from the same gene but differ from each other after amino acid 642. This difference is likely due to alternative splicing, a process known to produce variety in other cyclins. Two splice variants of cyclin E have been described. Cyclin Es lacks 49 amino acids within the cyclin box compared to cyclin E and cannot activate CDK2 (Sewing et al. 1994). Cyclin ET is missing 45 amino acids outside the cyclin box but, like Es, may not be functional (Mumberg et al. 1997). A cyclin C splice variant has also been described that is truncated in the cyclin box (Li et al. 1996). The two forms of cyclin T2 are both able to activate CDK9 and to function in transcription. Although it is possible that the

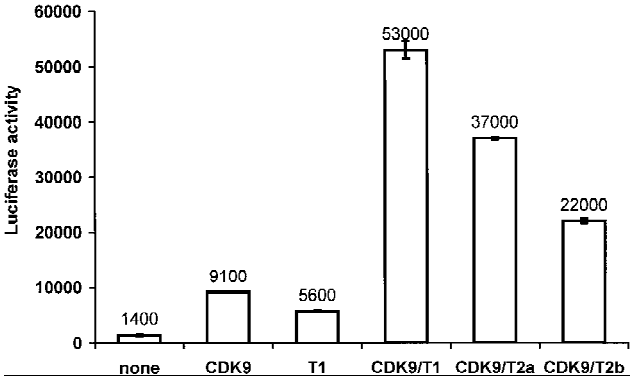

Figure 7. Overexpression of human P-TEFb in HeLa cells stimulates transcription from a CMV promoter. Transfections contained 1 million HeLa cells with 0.5 μg of a plasmid with the CMV promoter-driving luciferase as a reporter and 0.25 μg of each plasmid with the CMV promoter-driving CDK9 or the indicated cyclin. Error bars are derived from comparing results from duplicate transfections. This experiment was performed a number of times and the results are typical.

two forms play different roles in vivo, we have been unable to functionally differentiate the two forms in vitro.

We showed here that T1, T2a, and T2b individually form a complex with CDK9, but it is not clear whether other proteins are also functionally associated with CDK9 in vivo. Earlier results suggested the presence of a number of proteins associated with CDK9 (Grana et al. 1994; Zhu et al. 1997). Silver staining of CDK9 (PITALRE) immunoprecipitates showed proteins of 87, 105, 133, 140, and 207 kD (Zhu et al. 1997). The 87-kD protein has now been identified as T1. T2a and T2b are present in the immunoprecipitates (see Fig. 4) but are difficult to detect by silver staining the IPs because of their low abundance. The identity of the other proteins remains a mystery. Because the combination of CDK9 and either T1, T2a, or T2b forms an active P-TEFb protein, the potential role of the other proteins is not clear.

Our results extend the general finding that multiple cyclin subunits are found for a single CDK subunit (Morgan 1997); however, different roles for the three cyclins have not been found. If the three cyclins do not have redundant functions it is possible that they have specific roles during the transcription of different genes. The CTDs of the T1 and T2 cyclins are significantly different from each other, and this domain in other cyclins has been found to interact with the proteins utilized as kinase substrates (Morgan 1997). Supporting the role of the CTD in substrate binding we found that removal of this region of the T2 cyclin significantly reduced the ability of the CDK9/T2 cyclin box protein to phosphorylate the CTD of RNA polymerase II and function in transcription. Besides the three cyclins, it is possible that there are other minor cyclin species in HeLa nuclear extract that can associate with CDK9.

The cloning and production of recombinant human P-TEFb should facilitate understanding the mechanism of DRB-sensitive transcription by contributing to the generation of a more defined system. It is becoming clear that controlling the elongation phase of transcription involves the interaction of a number of positive and negative factors with the elongation complex (Yamaguchi et al. 1998). P-TEFb is required for Tat transactivation of the HIV promoter both in vitro and in vivo (Mancebo et al. 1997; Zhu et al. 1997). Once a more defined system that exhibits elongation control has been developed it will be possible to examine the mechanism of Tat transactivation in detail, and this in turn may lead to enlightened approaches to the blocking of Tat function.

It is possible that expression of human cyclin T1 (or T2) in rodent cells will allow more efficient Tat transactivation. CHO cells support only very low levels of Tat transactivation (Alonso et al. 1992, 1994). However, Tat transactivation is 10-fold higher in a CHO 12-cell line carrying human chromosome 12 (Alonso et al. 1992, 1994). It is very likely that cyclin T1 is present in the CHO 12-cell because one of the ESTs with high identity to T1 (yd48c03.s1) was mapped to 12q12. As human P-TEFb is required for Tat transactivation (Mancebo et al. 1997; Zhu et al. 1997), it is possible that expression of cyclin T1 in CHO cells will fulfill the chromosome 12 requirement and allow efficient Tat transactivation in the rodent cells. Furthermore, the expression of the appropriate cell surface receptors CD4 (Deng et al. 1997) and CCR5 (McNicholl et al. 1997) in rodent cells, along with cyclin T1, may allow the cells to be infected by HIV. If this is the case, it opens the exciting possibility of developing a mouse model for HIV infection using transgenic mice expressing human cyclin T.

Materials and methods

Materials

[α-^{32}P]CTP (3000 Ci/mmole) and [γ-^{32}P]ATP (6000 Ci/mmole) were purchased from ICN. Ribonucleoside triphosphates were from Pharmacia–LKB Biotechnology. DRB (Sigma) was dissolved in ethanol to 10 mM and stored at −80°C. All other chemicals were reagent grade.

Generation of an expression construct for human CDK9

The coding region of the small subunit of human P-TEFb (HuCDK9, also called PITALRE) was amplified by *Vent* DNA polymerase using pET21a–PITALRE (Zhu et al. 1997) as template, and two primers (5′-ACCCCTCCGGATAAATATGGCAAAGCAGTACG and 5′-AATCATGCTCGAGGAAGACGCGCTCAAACT). The amplified 1.2-kb fragment was digested with *Bsp*EI and *Xho*I and cloned into the pBAC4X-1 (Novagen) to generate a plasmid (pBAC–HuCDK9) for expression of a 6-His-tagged human CDK9.

Cloning of multiple cyclin subunits of human P-TEFb

Using BLAST to search the EST database for homologs of *Drosophila* cyclin T, we found three putative human cyclin T EST clones: nc70h05.r1, yd48c03.r1, and zr91f10.s1.

The nc70h05.r1 and yd48c03.r1 clones were found to be derived from the same gene, with the nc70h05.r1 sequence upstream of the yd48c03.r1 sequence. The 5′ cDNA sequence was obtained using a Marathon cDNA Amplification Kit (Clontech), human bone marrow Marathon-ready cDNA (Clontech) as template, and three primers based on nc70h05.r1 (5′-GGAGACAAGTATGTGCTACCTTGATGACA, 5′-GGAATTCGGGCTGCTCCTCCACTTTAG, 5′-GGAATTCGCTGCTGGAGCCACAGAA). Using the same kit and cDNA, the 3′ region was obtained using primers based on yd48c03.r1 (5′-GTGTCACTGAAAGAATACCG, and 5′-GGAATTCAGGTGGAGATAAAGCTGC). The total cDNA sequence was obtained by combining them. According to the length of the PCR products, the whole sequence was 2.8 kb. We sequenced 2.4 kb and identified an intact open reading frame of 2.2 kb designed as cyclin T1. The coding sequence (2.2 kb) was cloned by RT–PCR. cDNA was synthesized using a primer (5′-GGAATTCTTACTTAGGAAGGGGTGGAAGTG) and HeLa total RNA. The coding sequence was amplified using eLONGase (Life Technologies), the cDNA, and two primers (5′-GCTCTAGATAAATATGGAGGGAGAGAGGAA, and 5′-GGAATTCTTACTTAGGAAGGGGTGGAAGTGGTGGAGGAGGTT). Finally, the amplified sequence was cloned into the plasmid pBAC–HuCDK9 to produce a coexpression plasmid pBAC–HuCDK9-T1.

Based on the sequence from zr91f10.s1, the 5′ region of T2 cDNA was obtained using a 5′ RACE kit (GIBCO) with three gene-specific primers (5′-TTCCCACCAATGCTTTCC, 5′-CCATCAGTTGATACAGGGATCT, and 5′-GGAATTCAGAAGGTTGTAAGATGC) and human brain poly(A)$^+$ RNA as tem-

plate. The 3′ region of the total sequence was obtained by using a Marathon cDNA Amplification Kit (Clontech) with three gene-specific primers (5′-ACACACAGATGTGGTGAAATGTACCCA, 5′-GCATCTTACAACCTTCTG, and 5′-GGAATTCATGGAAAGCATTGGTGGGAAT) and a brain Marathon-ready cDNA. The total cDNA sequence obtained was 4.5 kb. The coding region of cyclin T2 was amplified by RT–PCR using Expand polymerase (Boehringer Mannheim) with two primers (5′-GGACTAGTATAAATATGGCGTCGGGCCGTG, and 5′-GGAGATCTTACATGTTCATTCCTTGGG). The cDNA template used in the PCR reaction was synthesized with a primer (5′-CCTCCACTACTGGTTTGCCTGG) from human brain poly(A)$^+$ RNA. Interestingly, two related coding sequences were amplified: 2-kb (for cyclin T2a) and 2.2-kb (for cyclin T2b). These two sequences were also amplified from HeLa total RNA. The 2.2-kb sequence contained a putative intron of 101 bp, which was not found in the 2-kb sequence. Both cyclin T2a and T2b were cloned in an expression plasmid, pBAC–HuCDK9, to generate plasmids pBAC–HuCDK9-T2a and pBAC–HuCDK9-T2b.

To generate a carboxy-terminal truncation of cyclin T2, the coding sequence of cyclin T2a was digested with *Xba*I and *Eco*RI (an internal site in the coding sequence) and cloned into pBAC–HuCDK9 to generate pBAC–HuCDK9-T2 (1–286).

Expression and purification of recombinant P-TEFb proteins

All proteins were purified essentially as described (J. Peng, N.F. Marshall, and D.H. Price, in prep.), with the following modifications. Recombinant baculoviruses were generated using BaculoGold DNA (PharMingen) and five plasmids: pBAC–HuCDK9-T1, pBAC–HuCDK9-T2a, pBAC–HuCDK9-T2b, pBAC–HuCDK9-T2 (1–286), and pBAC–HuCDK9. Sf9 cells were infected with the virus, incubated for 2–3 days, and harvested by centrifugation. The cells were lysed for 1 hr in a buffer (10 mM Tris-HCl at pH 7.6, 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, and 0.1% of a saturated solution of PMSF in isopropanol and 0.5 μg/ml of other protease inhibitors, aprotinin, leupeptin, pepstatin, chymostatin, and antipain. The cell lysate was centrifugated at 2000*g* for 10 min and then added with NaCl to 0.5 M and imidazole to 5 mM followed by centrifugation at 250,000*g* for 1 hr. A Ni^{2+}–NTA–agarose column (Qiagen) was loaded with the supernatant, washed with a wash buffer (10 mM Tris-HCl at pH 7.6, 0.5 M NaCl, 5 mM imidazole, PMSF), and eluted in several steps with elution buffers (10 mM Tris-HCl at pH 7.6, 0.5 M NaCl, 20–200 mM imidazole, PMSF). The eluted fractions were analyzed by SDS-PAGE. The fractions containing the desired proteins were pooled, diluted, and loaded onto 1-ml Mono S columns. The columns were eluted with a linear gradient from 0.08–0.5 M HGKEDP (25 mM HEPES at pH 7.6, 15% glycerol, 0.08–0.5 M KCl, 0.1 mM EDTA, 1 mM DTT, PMSF). The recombinant proteins eluted at various salt concentrations: HuCDK9/T1, 180–230 mM; HuCDK9/T2a, 140–190 mM; HuCDK9/T2b, 190–240 mM; HuCDK9/T2, (1–286), 100–150 mM; and HuCDK9, 100–150 mM. The HuCDK9/T2 (1–286) fraction was reconcentrated on a Mono S column using 350 mM step elution. All procedures of purification were carried out at 4°C. The protein concentration of HuCDK9/T2a was determined by a Bio-Rad assay using BSA as a standard. Concentrations of other proteins were obtained by using a Bio-Rad GS 670 scanning densitometer to compare the HuCDK9 subunit in each preparation analyzed on a single silver-stained gel.

Production of cyclin T1 and T2 polyclonal antibodies

Recombinant GST-T1 (402–726) fusion protein was generated using a T7 polymerase-dependent expression system essentially as described (Marshall et al. 1996). A sequence coding the carboxy-terminal portion (amino acids 402–726) of cyclin T1 was amplified and cloned into a GST expression plasmid (pET21a–GST) (Marshall et al. 1996). The fusion protein was expressed in DE3 cells, purified using a glutathione affinity column (Pharmacia), and used to immunize rabbits (Pocono Rabbit Farm). Antibodies against cyclin T1 were affinity-purified. Similarly, antibodies against cyclin T2 were produced, except that GST-T2a (440–663) fusion protein was used as an antigen. Both cyclins T2a and T2b were recognized by these antibodies because they shared the sequence from amino acids 1 to 642.

CTD kinase assay

The assay was performed as described (J. Peng, N.F. Marshall, and D.H. Price, in prep.) in a 20-μl reaction containing purified *Drosophila* RNA polymerase II (~10 ng), various amount of kinases, 20 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 55 mM KCl, 10 μM unlabeled ATP, and 2 μCi of [γ-^{32}P]ATP (ICN) at 23°C for 5 min. Reactions were terminated and analyzed by SDS-PAGE followed by autoradiography.

Immunodepletion of HuP–TEFb

Protein A–Sepharose beads (Sigma) were incubated with affinity-purified antibodies at 4°C for 30 min, washed with a buffer containing 25 mM HEPES (pH 7.6), 0.6 M KCl, 0.5% NP-40, and 1% Triton X-100, followed by 180 mM HGKEDPB (HGKEDP buffer plus 0.1 mg/ml BSA), and packed into three columns. HNE (in 180 mM HGKEDP) was passed over the three columns successively. The flowthrough of the third column was used as depleted HNE. The beads in these three columns were collected and washed with the wash buffer thoroughly. The associated proteins on the beads were eluted with SDS loading buffer and analyzed by western blot using antibodies against PITALRE carboxyl terminus (amino acids 352–372) and cyclins T1 and T2.

In vitro transcription

The transcription assay was performed in a 20-μl reaction with a continuous labeling procedure (Marshall and Price 1995). The CMV template (20 μg/ml, 631-nucleotide runoff) was incubated with either HNE or depleted HNE in the presence of 20 mM HEPES (pH 7.6), 7 mM $MgCl_2$, 55 mM KCl, 200 μM each of ATP, GTP, and UTP, 10 mM CTP, and 4 μCi of [α-^{32}P]CTP at 30°C for 20 min. P-TEFb proteins and DRB (final contribution, 50 μM) were added as indicated. Labeled products were analyzed on 6% denaturing gels followed by autoradiography.

Transient cotransfection assays

A reporter vector containing CMV-driven luciferase was generated by subcloning the CMV transcription templates into pGL2 basic (Promega) vector. All expression plasmids were made in pCDNA3 (Promega) and used to drive the expression of CDK9, cyclin T1, cyclin T2a, or cyclin T2b. Cotransfection assays were performed basically as described in the manual supplied with the lipofectamine reagent (GIBCO BRL). HeLa cells were aliquoted into 12-well plates and incubated at 37°C with 5% CO_2. The dishes containing 1 million cells were ~80% confluent on the day of transfection. Cells were transfected with lipofectamine in serum-free DMEM. Complete DMEM was added to the cells 5 hr after the transfection. Cells were harvested after 42 hr. After lysis the luciferase activity was determined using a Promega luciferase assay system. Transfections and luciferase determinations were both done in duplicate.

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Note added in proof

Another group independently isolated a cDNA encoding cyclin T1 and showed that the cyclin interacted with HIV-1 Tat and increased the affinity of Tat for TAR RNA (Wei et al. 1998).

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