Identification of a Cyclin Subunit Required for the Function of Drosophila P-TEFb

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P-TEFb is required for the transition from abortive elongation into productive elongation and is capable of phosphorylating the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II. We cloned a cDNA encoding the large subunit of Drosophila P-TEFb and found that the predicted protein contained a cyclin motif. We now name the large subunit cyclin T and the previously cloned small subunit (Zhu, Y. R., Peery, T., Peng, J. M., Ramanathan, Y., Marshall, N., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997) Genes Dev. 11, 2622–2632) cyclin-dependent kinase 9 (CDK9). Recombinant P-TEFb produced in baculovirus-infected SF9 cells exhibited 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-sensitive kinase activity similar to native P-TEFb. K cell nuclear extract depleted of P-TEFb failed to generate long DRB-sensitive transcripts, but this activity was restored upon addition of either native or recombinant P-TEFb. Like other CDKs, CDK9 is essentially inactive in the absence of its cyclin partner. P-TEFb containing a CDK9 mutation that knocked out the kinase activity did not function in transcription. Deletion of the carboxyl-terminal domain of cyclin T in P-TEFb reduced both the kinase and transcription activity to about 10%. The CDK-activating kinase in TFIIHI was unable to activate the CTD kinase activity of P-TEFb.

The elongation potential of RNA polymerase II is proposed to be controlled by negative transcription elongation factors (N-TEF) and positive transcription elongation factors (P-TEF) reviewed in Ref. 4. All initiated RNA polymerase II molecules enter abortive elongation in which only short transcripts (1–3) reviewed in Ref. 4. All initiated RNA polymerase II molecules enter abortive elongation in which only short transcripts are generated due to function of N-TEF. These negative factors are exemplified by factor 2 (5) and a DRB sensitivity-inducing factor (DSIF) (6). Upon the action of positive factors such as P-TEFb, the RNA polymerase II molecules overcome the promoter-proximal pausing and premature termination to enter productive elongation (2, 3, 7). After the initial switch into a productive mode, the efficiency of elongation can be further increased by other factors such as S-II, TFIIF, ELL, and elongin to generate long transcripts (8).

Phosphorylation of the carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II is a regulatory event in transcription (9, 10). The unphosphorylated RNA polymerase II (IIA) has been found in preinitiation complexes (11) and in early elongation complexes in vitro (3), whereas the hyperphosphorylated polymerase II (IIO) has been observed in productive elongation (11, 12). The phosphorylation state of the CTD is controlled by the action of kinases and phosphatases (9, 10). DmP-TEFb has been identified as a kinase with subunits of 124 and 43 kDa that can phosphorylate the CTD of RNA polymerase II (3). The kinase activity of P-TEFb is very sensitive to the purine analog DRB (3). Consistently, the transition from abortive to productive elongation can be inhibited by DRB in vivo (4). The 43-kDa subunit of DmP-TEFb was cloned and found to be similar to Cdk2, a cyclin-dependent kinase (CDK) (13).

Most known CDKs play a role in regulating the cell cycle, but some CDKs have been found to be involved in other cellular events (14). A CDK can be activated by binding of a cyclin and phosphorylation of a conserved threonine residue in the T-loop of the catalytic subunit. Conversely, a CDK/cyclin complex can be inactivated by phosphorylation of the threonine residue and a tyrosine residue near the ATP binding site or by binding of a family of small proteins termed CKIs (14). The catalytic subunits are well conserved in the Drosophila family, but the cyclins are not conserved except for the cyclin box that is predicted as a helix-rich structure (15). We show here that Drosophila P-TEFb is composed of a CDK/cyclin pair.

EXPERIMENTAL PROCEDURES

Cloning of the Large Subunit of Drosophila P-TEFb—Drosophila P-TEFb was purified as described (3). The large subunit was excised from an SDS gel and used for peptide sequencing (Keck Facility, Yale University, New Haven, CT). Based on the peptide sequences, three degenerate primers were designed to clone a 1.6-kb cDNA fragment with nested PCR reactions. First, a 35-cycle PCR reaction was performed using Taq DNA polymerase, two degenerate primers (5'-GGATTCTGGTATYTYWSNAAYGA and 5'-CGGATCTCCTGRAANGGNCAT, and Drosophila embryonic cDNA from CLONTECH as a template. Second, a 1.6-kb DNA fragment was amplified in another 35-cycle PCR reaction using the total PCR products above as template, and the primer (5'-GGAAATTCTTGTGATYTYWSNAAYGA) and a nested primer (5'-CGGATCCANNGGNCATCC). Third, this 1.6-kb fragment was digested into three fragments of 0.9, 0.6, and 1.0 kb. The 0.9- and 0.6-kb fragments were cloned into the Bluescript II SK' vector (Stratagene). A 1.5-kb cDNA fragment sequence was obtained by combining the 0.9- and 0.6-kb fragments.

The 5' region of the full-length cDNA sequence was obtained using a Life Technologies, Inc. 3' RACE kit with three gene-specific primers based on the 1.5-kb sequence (5'-ATACAGACACACACAGAGCTTTA, 5'-CGAAATTCAGATCGTAGAACCGA and 5'-CGAATTCGGCGCTTAG- CATT). The 5' region of cDNA sequence was obtained using a Life Technologies, Inc. 5' RACE kit with three gene-specific primers (5'-GAAAGGGCTGAAACCGA, 5'-GCTGACCATTTTCTGTATACAG- TAG and 5'-GGAATTCCTCCTGCGGACATT). The entire coding sequence of the large subunit was cloned by reverse transcription PCR. First, the cDNA of the large subunit was generated in a reverse transcription reaction, secondly, a 35-cycle PCR amplification was performed using Taq DNA polymerase, and a primer (5'-GGAAATTCTTGTGATYTYWSNAAYGA) and a nested primer (5'-CGGATCCANNGGNCATCC) using the template above. Third, 0.9-kb fragments were digested into three fragments of 0.9, 0.6, and 1.0 kb. The 0.9- and 0.6-kb fragments were cloned into the Bluescript II SK' vector (Stratagene). A 1.5-kb cDNA fragment sequence was obtained by combining the 0.9- and 0.6-kb fragments.
transcription using a primer (5′-GGGAATTCGAGGTTCTCATACAT) and Drosophila embryonic mRNA. Second, a 4-kb DNA fragment containing the coding sequence was produced in a 35-cycle PCR reaction using Expand polymerase (Boehringer Mannheim), two primers (5′-CCCGGGTCATATGAGTCTCCTAGCC and 5′-GGGAATTCGAGGTTCTCATACAT), and cDNA as template. Third, the 3.3-kb encoding sequence was amplified in another PCR reaction using Vent DNA polymerase, the 4-kb fragment as template and two primers (5′-TATACATAT). Finally, the coding sequence was digested with SmaI and EcoRI and cloned into a pET21a-GST vector (3) to produce pET21a-GST-DmCDK9.

Generation of P-TEFb Constructs for Expression—The coding region of the small subunit of Drosophila P-TEFbs (DmCDK9) was amplified by reverse transcription PCR and cloned into a pET21a-GST-DmCDK9 vector to generate pET21a-GST-DmCDK9. The DmCDK9 coding region was amplified by Vent DNA polymerase using pET21a-GST-DmCDK9 as template and two primers (5′-GAATTCGGGATCCATAATGGGCACATTCCCCA and 5′-ATCCACACCCTGGCAACACCGGCATTTCAATCT). The amplified 1.2-kb fragment was digested with XhoI and XhoI and cloned into the pBAC4X1 (Novagen) to generate a plasmid (pBAC-DmCDK9) for expression of a His-tagged DmCDK9. The coding region of Dmcyclin T was amplified by Vent DNA polymerase using pET21a-GST-Dmcyclin T as template and two primers (5′-GCTCTAGATATCCATATGGTCCTCCTAGCTGGCATCTTAAAGGCGTGC), and pBAC-DmpTEFb as target plasmid. The mutations were confirmed by sequencing. Because extra mutations in the plasmid could have been generated, a short sequence containing the mutation site was cut from the mutated plasmid and recloned into the original plasmid, yielding a mutated plasmid pBAC-DmpTEFb (D199N).

To make the kinase knockout of DmCDK9 the same mutation used to knockout the kinase activity of the human homologue, PITALRE (D199N) was used (16). Site-directed mutagenesis was performed according to the CLONTECH manual (Transformer site-directed mutagenesis kit) using a selection primer (5′-CTCGAGTTCAACTGCGGATATGGTTCTCATACAT), a mutagenic primer (5′-CTCGAGTTCAACTGCGGATATGGTTCTCATACAT), a mutagenic primer (5′-GGCATCTTAAGGTGCCTGGGCGTGC), and pBAC-DmpTEFb as target plasmid. The mutations were confirmed by sequencing. Because extra mutations in the plasmid could have been generated, a short sequence containing the mutation site was cut from the mutated plasmid and recloned into the original plasmid, yielding a mutated plasmid pBAC-DmpCDK9 (D199N-Dmcyclin T).

To generate the C-terminal truncations of Dmcyclin T, pBAC-DmpTEFb was digested with EcoRV and EcoRI followed by filling in of the cohesive ends and religation to generate pBAC-DmpCDK9-Dmcyclin T (1–543). The plasmid pBAC-DmCDK9-Dmcyclin T (1–714) was made in the same way except that PstI and EcoRI sites were used.

Expression and Purification of Recombinant P-TEFb Proteins—Five plasmids, pBAC-DmCDK9, pBAC-DmpTEFb, pBAC-DmCDK9 (D199N)-Dmcyclin T (1–543), and pBAC-DmCDK9-Dmcyclin T (1–714), were used to cotransfect SF9 cells with BaculoGold DNA (PharMingen) to generate recombinant baculoviruses. The diluted fraction was loaded onto a 1-ml Mono Q column. Proteins bound to the Mono Q column were eluted with a linear gradient from 0.1 M to 0.5 M HGKEDP and then packed into three columns. Kc cell nuclear extract (K_N in 150 mM HGKEDP) was passed over the three columns in succession. The flowthrough of the third column was used as depleted K_N.

In Vivo Transcription—Transcription assay was carried out using a continuous labeling procedure (7). The actinAct5C template linearized with HpaI (15 μg/ml) giving a 520-nucleotide runoff transcript was used. Transcription reactions (15 μl) contained either K_C or depleted K_N, 20 mM HEPES, pH 7.6, 5 mM MgCl₂, 55 mM KCl, 600 μM each of ATP, GTP, and UTP, 30 μM CTP, and 4 μCi of [α-32P]CTP and were incubated at 23 °C for 20 min. P-TEFb proteins and DRB were included as indicated. Labeled transcripts were analyzed on 6% denaturing gels.

RESULTS

To advance our understanding of the function of P-TEFb as a CTD kinase controlling the elongation phase of transcription, it was necessary to obtain cDNAs encoding the two subunits. We recently cloned a cDNA encoding the small subunit and found that it was a kinase that might require a cyclin subunit for activity (13). Our next goal was to clone the large subunit, determine if it had homology to other cyclins, and generate recombinant P-TEFb by co-expressing both subunits.

Cloning of the Large Subunit of Drosophila P-TEFb—We cloned a full-length cDNA encoding the large subunit of Drosohila P-TEFb from sequence information obtained from the pure protein. The large subunit from purified P-TEFb was excised from an SDS gel and subjected to a peptide sequencing protocol. Based on the peptide sequence information, we cloned a 4.3-kb cDNA from Drosophila embryonic mRNA (Fig. 1). The open reading frame was determined by knowing the size of P-TEFb large subunit and by finding a consensus sequence for the translational start site (17). The cDNA encodes a 118-kDa protein (1097 amino acids) with an isolectric point of 9.5 and a putative nuclear localization signal from amino acids 872–876 (18). The 5′-untranslated region is relatively long, and the 3′-untranslated region is terminated by a poly(A) tail. Northern analysis indicated embryonic and adult flies contained mRNAs 5, 5.5, and 7 kb in length (data not shown).

Comparison of the new Drosophila sequence to those in the data bases using a Blast search (19) indicated that the ammino-terminal region had highest similarity to Drosophila cyclin C
(20) and cyclin H² proteins. Although the amino-terminal region of the large subunit of DmP-TEFb showed only 14% identity to that of cyclin C and 12% identity to that of cyclin H, it is predicted to have two 5-helix folds (Fig. 2) that are conserved in cyclin proteins (15, 21). The cyclin-CDK interaction is partly dictated by the first cyclin fold in which helix 3 forms the hydrophobic core as illustrated by the crystal structure of cyclin A and CDK2 (21). The first five helices, especially helix 3, are the most conserved sequences in the cyclin box of the large subunit of DmP-TEFb. The carboxyl terminus of the large subunit of Drosophila P-TEFb did not show high similarity to any other protein in the data bases. All these data suggest that the large subunit is a novel cyclin that we name cyclin T. Because the kinase subunit of P-TEFb associates with a cyclin subunit and shows similarity to other cyclin-dependent kinases, we call it CDK9.

Recombinant P-TEFb Functions in a Similar Manner to Native P-TEFb—CDNAs encoding both subunits of P-TEFb were co-expressed in Sf9 cells (Fig. 3A), and recombinant Drosophila P-TEFb (rDmP-TEFb) was purified using a nickel column followed by Mono Q and Mono S. Both subunits were retained on the nickel column even though only the small subunit was tagged. Both proteins co-eluted from Mono S at the identical salt concentration at which native P-TEFb eluted. These results demonstrate a strong interaction between the two recombinant proteins. On a polyacrylamide gel (Fig. 3B), the cyclin T subunit of rDmP-TEFb (lane a) had the same mobility as the large subunit of purified native P-TEFb (lane S). The CDK9 subunit of rDmP-TEFb had a slightly lower mobility compared with that of native DmP-TEFb due to the presence of the His tag. These results further support our hypothesis that the CDNAs encode the subunits of Drosophila P-TEFb.

The DmP-TEFb possesses a CTD kinase activity that is very sensitive to DRB (3). To examine the kinase activity of rDmP-TEFb, Drosophila RNA polymerase II was incubated with increasing amounts of either native DmP-TEFb or rDmP-TEFb in the presence of 10 μM [γ-³²P]ATP for 5 min. The reactions were analyzed by 6–15% SDS-PAGE. The protein gel was silver-stained (Fig. 4A) and then subjected to autoradiography (Fig. 4B). As expected, increasing the amount of native DmP-TEFb increased the fraction of the hyperphosphorylated IIO form of the large subunit of RNA polymerase II as well as the total amount of label incorporated into the IIO form. The activity of rDmP-TEFb was indistinguishable from that of native
The sensitivity of rDmP-TEFb to DRB was compared with that of native DmP-TEFb using a similar assay. The radioactivity incorporated into the large subunit of RNA polymerase II was quantitated, normalized to the no DRB point (100%), and plotted (Fig. 4C). The 50% inhibition point of rDmP-TEFb (0.5 μM) was similar to that of DmP-TEFb (0.7 μM).

To analyze the function of rDmP-TEFb in elongation control, it was first necessary to generate a P-TEFb-dependent transcription system. P-TEFb allows the generation of long DRB-sensitive transcripts (3, 7), and in a human transcription system. P-TEFb allows the generation of long DRB-sensitive transcripts (3, 7), and in a human transcription system. The starting extract as well as the preimmune depleted extract were analyzed on a 6% gel. Depletion of DmP-TEFb from Kc cell nuclear extract (KcN) was characterized and then used to compare the activity of rDmP-TEFb to the native factor (Fig. 5B). The starting extract as well as the preimmune depleted extract were analyzed on a 6% gel. Depletion of DmP-TEFb from Kc cell nuclear extract (KcN), we first needed to produce antibodies to Drosophila P-TEFb. A glutathione S-transferase fusion protein containing the carboxyl-terminal half of the cyclin T subunit was used to immunize rabbits. A Western blot indicated that the resulting antiserum recognized both the native and recombinant DmP-TEFb. Labeled transcripts were analyzed on a 6% gel.

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The depleted KcN was characterized and then used to compare the activity of rDmP-TEFb to the native factor (Fig. 5B). The starting extract as well as the preimmune depleted extract were analyzed on a 6% gel. Depletion of DmP-TEFb from Kc cell nuclear extract (KcN) was characterized and then used to compare the activity of rDmP-TEFb to the native factor (Fig. 5B). The starting extract as well as the preimmune depleted extract were analyzed on a 6% gel. Depletion of DmP-TEFb from Kc cell nuclear extract (KcN), we first needed to produce antibodies to Drosophila P-TEFb. A glutathione S-transferase fusion protein containing the carboxyl-terminal half of the cyclin T subunit was used to immunize rabbits. A Western blot indicated that the resulting antiserum recognized both the native and recombinant DmP-TEFb. Moreover, the antibodies in the antiserum, but not in the preimmune serum, were able to deplete the cyclin subunit of DmP-TEFb (Fig. 5A).

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assay. An unusual feature of the phosphorylation of RNA polymerase II by the two truncation mutants was that the shift from IIA to IIo did not occur as readily. DmP-TEFb has been shown to preferentially phosphorylate a CTD that has already been phosphorylated (3), and at low levels of the kinase this leads to the hyperphosphorylation of a subset of the polymerases (see Fig. 4A). Evidently, the carboxyl-terminal region of cyclin T is responsible for this preferential phosphorylation of the partially phosphorylated polymerase molecules.

To better compare the transcriptional activity of the two cyclin truncation mutants with intact DmP-TEFb, a wider range of kinase concentrations was titrated (Fig. 7). We were especially interested in determining if higher levels of the truncation mutants could compensate for their lower kinase activity or if they were in some other way defective. Intact DmP-TEFb functioned as expected, giving rise to a high level of runoff transcripts. Comparison of the amount of runoff at intermediate levels of intact DmP-TEFb (4× to 16×) indicated that addition of 2-fold more kinase had an effect greater than 2-fold (Fig. 7B). A different result was obtained with the two truncation mutants. Neither showed the effect seen at intermediate levels of intact DmP-TEFb (Fig. 7). Even though very high levels were used, the effect of both truncation mutants saturated before reaching the high level of runoff seen with the intact protein (Fig. 7B). We conclude that the carboxyl-terminal region of cyclin T is important for the function of P-TEFb in transcription.

P-TEFb and TFIIH Do Not Affect Each Other’s Ability to Phosphorylate the CTD—Others have implicated the kinase activity of TFIIH in CTD phosphorylation (22) and in elongation control (23). Therefore, we wanted to determine if the CDK-activating kinase associated with TFIIH could affect the CTD kinase activity of P-TEFb. Kinase reactions were set up with RNA polymerase II as substrate, limiting amounts of P-TEFb or lower levels of Drosophila TFIIH. As expected, CTD phosphorylation by P-TEFb but not TFIIH was sensitive to DRB (Fig. 8). In reactions containing both kinases, the signals were apparently the sum of the two activities. The phosphorylation of cyclin T (Fig. 8) and CDK9 (data not shown) was also unaffected by the presence of TFIIH. This suggests that neither kinase has a dramatic effect on the other.

**DISCUSSION**

We have completed the cloning of cDNAs encoding Drosophila P-TEFb, a novel CDK/cyclin pair and we name the subunits CDK9 and cyclin T. Cytogenetic mapping was performed, and we found that the CDK9 and cyclin T mapped to 58F and 74EF, respectively (data not shown). A number of mutations including lethal mutations also map to these areas. We produced fully functional recombinant DmP-TEFb. Furthermore, we showed that the cyclin subunit was required to activate the kinase and that the carboxyl-terminal region of cyclin T was important in this activation. Finally, we found that TFIIH did not affect the ability of P-TEFb to phosphorylate the CTD of pure RNA polymerase II.

Our results here coupled with earlier results suggest that DmP-TEFb is not part of a larger complex and does not stably associate with RNA polymerase II or the elongation complex
Using pure RNA polymerase II as a substrate, TFIIH had no activating P-TEFb, our results do not support this hypothesis. Although it is possible that TFIIH plays a role in activating P-TEFb, it is also possible that P-TEFb purified from eucaryotic cells is already activated. Further work in vitro and in vivo will be required to understand how P-TEFb activity is regulated.

The timing of phosphorylation of the polymerase in an early elongation complex is important because of the termination activity of factor 2 (5). Essentially, there is a functional competition between factor 2 and P-TEFb. During a short period of time after initiation, P-TEFb must phosphorylate the CTD to an appropriate extent to cause the transition into productive elongation. Otherwise factor 2 will cause premature termination. This may be why the cyclin mutants lacking the carboxy-terminal region were able to generate only low levels of runoff transcripts. The reduced ability of the cyclin mutants to recognize partially phosphorylated RNA polymerase II in an early elongation complex would lead to a reduced rate of phosphorylation and, therefore, ultimately a reduced number of hyperphosphorylated polymerases that generate runoff.

As a cyclin-dependent kinase, P-TEFb is likely to be activated by phosphorylation of the catalytic subunit. The TFIIH-associated kinase is a CDK-activating kinase termed CDK7 that has been shown to activate CDC2, CDK2, and CDK4 (14). TFIIH has also been shown to function in transcription elongation (23). Although it is possible that TFIIH plays a role in activating P-TEFb, our results do not support this hypothesis. Using pure RNA polymerase II as a substrate, TFIIH had no effect on the kinase activity of P-TEFb. It is possible that in the context of transcription with other factors involved, TFIIH might play a role in activating P-TEFb. In this case, it is also possible that P-TEFb purified from eucaryotic cells is already activated.