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Drosophila Cdk8, a Kinase Partner of Cyclin C that Interacts with the Large Subunit of RNA Polymerase II

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A number of cyclins have been described, most of which act together with their catalytic partners, the cyclin-dependent kinases (Cdks), to regulate events in the eukaryotic cell cycle. Cyclin C was originally identified by a genetic screen for human and *Drosophila* cDNAs that complement a triple knock-out of the *CLN* genes in *Saccharomyces cerevisiae*. Unlike other cyclins identified in this complementation screen, there has been no evidence that cyclin C has a cell-cycle role in the cognate organism. Here we report that cyclin C is a nuclear protein present in a multiprotein complex. It interacts both in vitro and in vivo with Cdk8, a novel protein-kinase of the Cdk family, structurally related to the yeast Srb10 kinase. We also show that Cdk8 can interact in vivo with the large subunit of RNA polymerase II and that a kinase activity that phosphorylates the RNA polymerase II large subunit is present in Cdk8 immunoprecipitates. Based on these observations and sequence similarity to the kinase/cyclin pair Srb10/Srb11 in *S. cerevisiae*, we suggest that cyclin C and Cdk8 control RNA polymerase II function.

INTRODUCTION

First named after its cyclical accumulation during early embryonic cell cycles, cyclin B is now the prototype of a growing family of structurally related cyclin molecules that are involved in specific association with and activation of serine-threonine protein kinases termed Cdks (for cyclin-dependent kinases) (for review see Morgan, 1995). The function of these cyclin/Cdk pairs has been particularly well established in the control of the eukaryotic cell cycle where several of these activities trigger the major transitions of the cell cycle via the phophorylation of a series of cellular targets (reviewed in King *et al.*, 1994; Nurse, 1994; Sherr, 1994; Nigg, 1995).

Cyclin/Cdk pairs do not only function in the control of the cell cycle; a growing number of studies implicate Cdks in other cellular processes. In yeast, the Pho80-Pho85 cyclin-Cdk complex is involved in a pathway that senses inorganic phosphate in the cell (Hirst *et al.*, 1994; Kaffman *et al.*, 1994). In high phosphate conditions, the Pho80-Pho85 kinase phosphorylates Pho4, a transcriptional activator of the *PHO5* gene. This phophorylation correlates with negative regulation of the *PHO5* gene and is itself modulated by an additional factor, Pho81, which shows structural similarities with the mammalian Cdk inhibitor $p16^{ink4}$ (Schneider *et al.*, 1994).

Another cyclin-kinase pair has been recently identified as a component of the basal transcription factor TFIIH. Cdk7 (previously called Mo15) and cyclin H, two subunits of a kinase complex previously identified as CAK (Cdk-activating kinase) (Fesquet *et al.*, 1993; Poon *et al.*, 1993; Solomon *et al.*, 1993; Fisher and Morgan, 1994; Makela *et al.*, 1994; Tassan *et al.*, 1994) were shown to co-purify with other TFIIH subunits.

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They are associated with the kinase activity of TFIIH that phosphorylates the C-terminal domain (CTD) of the RNA polymerase (Pol) II large subunit (Feaver *et al.*, 1994; Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995).

More recently, three different genetic approaches have led to the characterization of a new cyclin-kinase pair involved in transcriptional control in yeast. *SRB10* and *SRB11* (Liao *et al.*, 1995) are part of a group of genes identified as suppressors of partial truncations of the CTD that lead to a cold-sensitive phenotype (Koleske *et al.*, 1992; Thompson *et al.*, 1993). Alleles of these genes were isolated in two other searches for genes involved in transcriptional repression: *SRB10* (alias *ARE1*) as a repressor of a-specific genes in α-cells (Wahi and Johnson, 1995) and *SRB10* and *SRB11* (alias *SSN3* and *SSN8*) as repressors of genes in response to glucose (Kuchin *et al.*, 1995). Srb10 and Srb11 proteins are part of a large complex called holoenzyme, consisting of the RNA Pol II core enzyme, a subset of general transcription factors and the other members of the SRB group of proteins (Hengartner *et al.*, 1995; Liao *et al.*, 1995; for review see Koleske and Young, 1995). Genetic and biochemical results indicate that Srb10 and Srb11 form a kinase-cyclin pair whose function is essential for proper transcriptional control in vivo and for CTD phosphorylation in vitro (Liao *et al.*, 1995). The *SRB10* gene encodes a Cdk and is identical to the *UME5* gene that was previously implicated in transcriptional control of meiosis-specific genes (Surosky *et al.*, 1994). *SRB11* encodes a cyclin-like protein that is most similar to human and *Drosophila* cyclin Cs.

Cyclin C was initially suspected to function as a regulator of the G1/S transition in metazoa, because the human and *Drosophila* cDNAs were originally isolated in screens for clones capable of rescuing yeast deficient in the G1 cyclins (Lahue *et al.*, 1991; Léopold and O'Farrell, 1991; Lew *et al.*, 1991). Three metazoan cyclins, cyclin C, D, and E were found to complement a defect in the yeast *CLN* genes. Numerous laboratories have now demonstrated that cyclin E and cyclin D with their kinase partners indeed function in the transition to S phase (for review see Sherr, 1994). In contrast, the function of cyclin C has not yet been elucidated.

Here we report that cyclin C is a nuclear protein that participates in a large multiprotein complex. It interacts in vivo with *Dm*Cdk8, a new protein kinase of the Cdk family (Tassan *et al.*, 1995), structurally related to the yeast Srb10 kinase (Liao *et al.*, 1995). We also provide evidence for an in vivo interaction between *Dm*Cdk8 and the large subunit of RNA Pol II and we suggest that the Cdk8/cyclin C pair is structurally as well as functionally related to the Srb10/Srb11 pair identified in yeast (Liao *et al.*, 1995).

MATERIALS AND METHODS

Preparation of Extracts

Nuclear and cytoplasmic extracts were prepared from 0- to 24-h collections of *Drosophila* embryos as previously described (Yoshinaga and Yamamoto, 1991; Sprenger *et al.*, 1993). For cell-cycle analysis, whole extracts from single embryos were prepared after gentle fixing, staining with a DNA dye, and selecting the embryos according to their position in the cell cycle (Edgar *et al.*, 1994).

Sucrose Gradient Fractionation

Fifteen to thirty percent sucrose gradients (5 ml) in HEMK buffer (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-K^+, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol, 1 mM sodium bisulfite, 1 mM benzamidine, 2 mM phenylmethylsulfonyl fluoride, pH 7.6) were run at 26K rpm, at 4°C for 16 h in a Beckman SW50.1 rotor. Fractions (250 μl) were collected and 10 μl of each was analyzed by Western blotting.

Immunoblotting

Rabbit anti-cyclin C antibodies were raised against a soluble glutathione *S*-transferase (GST)-cyclin C fusion and purified by affinity using a different gel-purified full length cyclin C produced in *Escherichia coli*. A monoclonal anti-cyclin C antibody (Mab11CD) was obtained after mouse injection of this gel-purified full length cyclin C. Anti-Cdk8 antibodies were prepared as described (Tassan *et al.*, 1995). Mouse monoclonal anti-CTD antibody was directed against five copies of the consensus CTD heptapeptide (a gift from M. Vigneron and C. Kedinger, University of Louis Pasteur, Strasbourg, Austria) (Besse *et al.*, 1995). In our hands, it recognizes the IIa but not the IIo form of *Drosophila* RNA Pol II large subunit. Mouse monoclonal ARNA3 antibody (Kramer *et al.*, 1980) was a gift from C.H. Winter and E.K. Bautz (University of Heidelberg, Germany). Anti-*Dm*Cdk7 antibodies will be described elsewhere (Leclerc and Léopold, unpublished data). Western blots were developed using chemoluminescence with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL).

Polymerase Chain Reaction (PCR) Cloning of DmCdk8

An 800-bp PCR product corresponding to the fly CDK8 sequence (*Dm*CDK8) was isolated using oligonucleotide primers corresponding to conserved regions between the human Cdk8 protein (Tassan *et al.*, 1995) and the yeast Ume5 protein (Surosky *et al.*, 1994) (marked with arrows in Figure 3). This DNA fragment was then used to screen an embryonic cDNA library (Brown and Kafatos, 1988) and three independent cDNA clones, similar in size, were isolated. The sequence of the largest clone was obtained and confirmed by comparing with the two other clones in the coding region. Sequence comparison and alignments were made using the Bisance service at the CITI2 server, Paris, France (Dessen *et al.*, 1990) and the Clustal program, respectively.

In Vitro Expression and Interactions of Cdks and Cyclins

*Dm*Cdk8 and *Dm*Cdc2 messenger RNAs were produced using SP6 and T3 RNA polymerases, respectively, and translated in a reticulocyte lysate system (Promega, Madison, WI) in the presence of [^{35}S]methionine. GST, GST-cyclin C, and GST-cyclin B were produced using pGEX-2T (Pharmacia, Piscataway, NJ) and purified from *E. coli* as described (Smith and Johnson, 1988). For protein interactions, 0.5 to 2 μl of labeled proteins were incubated with 3 μg of GST, GST-cyclin C, or GST-cyclin B in the presence of 10 μl of GSH-agarose beads, for 3 h at 4°C in NP buffer (HEMK, 1% NP-40). After washing in NP buffer, the beads were resuspended in sample

buffer, boiled, and the samples were subjected to SDS-PAGE and revealed by fluorography.

Immunoprecipitations

Immunoprecipitations were performed on nuclear extracts, using either nonimmune serum or anti-Cdk8 serum (rabbit). Typically, 100 μl of nuclear extracts (15 mg/ml) were preincubated with gentle rocking for 1 h at 4°C with 50 μl of protein A-Sepharose pellet, completed to 1 ml with NP buffer. The supernatant was then used for both control and anti-Cdk8 immunoprecipitations, by adding to 25 μl of protein A-Sepharose pellet and 1 μl of either nonimmune or immune serum and incubating another 2 h at 4°C. After four washes with NP buffer, beads were resuspended in sample buffer, boiled, and supernatants were loaded on 5 or 10% SDS-polyacrylamide gels. For kinase assays, immunoprecipitates were washed again three times with HEMK buffer, resuspended in 10 μl of HEMK containing 20 mM [γ^{32}P]ATP (10 μCi), with or without 5 μg of a CTD heptapeptide repeat (kindly provided by M. Dorée, CRBM, Montpellier, France). After a 30-min incubation at room temperature, reactions were stopped after adding 10 μl of 2× sample buffer and boiling for 3 min.

RESULTS

Characterization of a Nuclear Complex Associated with Cyclin C

Antibodies directed against a bacterially expressed fusion of *Drosophila* cyclin C and GST were affinity purified and used in Western blots on embryonic extracts. Cyclin C migrated as a single band at 28 kDa on SDS-PAGE, which was greatly enriched in nuclear extracts (Figure 1A). To determine whether levels of the protein oscillate during the cell cycle, we took advantage of the fact that early embryonic cycles are synchronous nuclear divisions in a syncytial cytoplasm. To stage embryos at precise cell-cycle intervals they were fixed in methanol, stained for DNA, and selected visually according to their position in the cell cycle. Staged embryos were solubilized in SDS and cyclin C detected by Western blots. We detected constant levels of cyclin C protein during the different phases of cell cycle 12 (Figure 1B), while a similar analysis showed clear oscillation in cyclin A and cyclin B levels during this cycle (Edgar *et al.*, 1994).

To determine a possible association between cyclin C and other nuclear proteins, embryonic nuclear extracts were run on 15–30% sucrose gradients and collected fractions were probed with anti-cyclin C antibodies. Cyclin C was present in a high molecular mass complex of ~500 kDa, peaking at fractions n° 9–10 in the gradient (Figure 2). We also used other specific antibodies to examine the distribution of various known cell cycle proteins along the gradient. None of the tested proteins (*Dm*cyclin A, *Dm*cyclin B, *Dm*Cdc2, *Dm*Cdc2c, PCNA, RPA30, RPA70, and DNA-polymerase α) were found to be associated with the cyclin C fractions (our unpublished data). By contrast, antibodies directed against a recently identified human kinase named Cdk8 (Schultz and Nigg, 1993; Tassan *et al.*, 1995) cross-reacted with a 51-kDa *Drosophila* protein that co-sedimented with cyclin C (Figure 2).

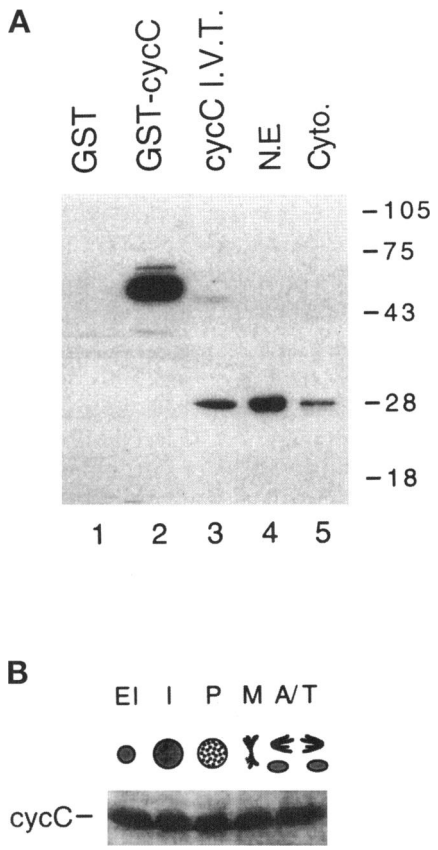

Figure 1. Biochemical characterization of cyclin C protein. (A) Characterization of anti-cyclin C antibodies. Thirty nanograms of purified GST (lane 1), purified GST-cyclin C fusion (lane 2), 3 μl of in vitro–translated cyclin C (lane 3), and an identical amount (5 μg) of nuclear extracts (lane 4) and cytoplasmic extracts (lane 5) from *Drosophila* embryos were subjected to Western blotting using affinity-purified polyclonal antibodies against cyclin C. Cyclin C is primarily present in the nuclear fraction, as a single 28-kDa band that co-migrates with the in vitro translation product. (B) Cell-cycle analysis of cyclin C protein levels. Cell-cycle 12 embryos were selected according to their position in the cell cycle (Edgar *et al.*, 1994). Their protein content was then analyzed by Western blotting using anti-cyclin C antibodies. EI, early interphase; I, interphase; P, prophase; M, metaphase; and A/T, anaphase-telophase.

Isolation of a DmCDK8 Clone

Because the above results suggested that the *Drosophila* homologue of Cdk8 might be associated with cyclin C, we isolated a 1.6-kb cDNA clone of the *Drosophila* homologue using degenerated primers derived from the human Cdk8 and the yeast Ume5 sequences. This clone encodes a 454-aminoacid protein with a predicted molecular mass of 53 kDa, which we named *Dm*Cdk8. *Dm*Cdk8 shares 72% identity with the human Cdk8 homologue (Tassan *et al.*, 1995), 43% with

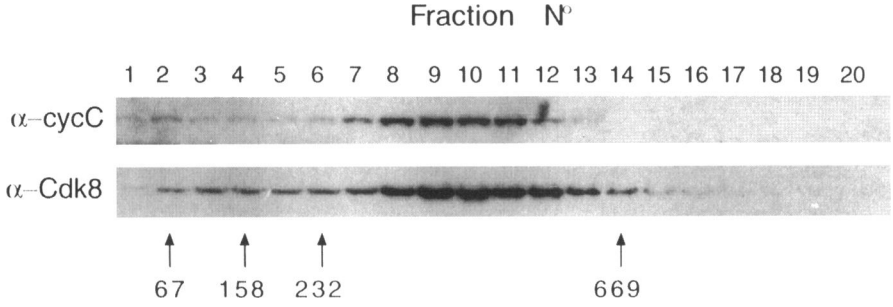

Figure 2. Co-fractionation of cyclin C and a 51-kDa protein antigenically related to Cdk8 along sucrose gradient. Fractions 1 (top) to 20 (bottom) were analyzed by Western blotting using anti-cyclin C and anti-Cdk8 antibodies (Tassan *et al.*, 1995). Arrows correspond to peaking fractions of molecular weight standards run on a similar gradient with corresponding molecular weight. 67, bovine serum albumin; 158, aldolase; 232, catalase; and 669, thyroglobulin.

the yeast Ume5/Srb10 protein kinase (Surosky *et al.*, 1994; Liao *et al.*, 1995), and 32% with the *Drosophila* Cdc2 kinase (Lehner and O'Farrell, 1990) (Figure 3). The homology with Srb10 is particularly interesting considering that Srb10 is itself the kinase partner of Srb11, a yeast cyclin whose closest homologue (28% identity and 58% similarity) is cyclin C (Liao *et al.*, 1995).

In vitro transcription and translation of *Dm*CDK8 cDNA yielded a protein that was recognized by antibodies directed against the human Cdk8 protein and that comigrated in SDS-PAGE with the 51-kDa band detected in Western blots of *Drosophila* embryonic nuclear extracts (Figure 4A). This indicated that the 51-kDa protein detected along the sucrose gradients using anti-human Cdk8 antibodies corresponds indeed to the *Drosophila* Cdk8 homologue. The recombinant *Dm*Cdk8 protein was also shown to bind specifically to a GST-cyclin C fusion in vitro (Figure 4B), indicating that Cdk8/cyclin C forms a kinase/cyclin couple in vitro.

DmCdk8 Interacts In Vivo with Cyclin C and the Large Subunit of RNA Pol II

Immunoprecipitation of nuclear extracts with anti-Cdk8 antibodies co-precipitated *Dm*Cdk8 and cyclin C, suggesting that they are associated in vivo (Figure 5A). We were unable to perform the reciprocal experiment because our anti-cyclin C antibodies did not immunoprecipitate endogenous cyclin C even though they did precipitate in vitro–translated or bacterially expressed soluble cyclin C. Because the endogenous protein was detected by these same antibodies in Western analysis, we attribute the failure to precipitate to epitope masking by other associated proteins in native conditions.

If cyclin C and Cdk8 are productively associated we might expect to see kinase activity in the Cdk8 immunoprecipitates. Indeed, addition of ATP led to phosphorylation of a 240-kDa band present in the immune complex (Figure 5C, lane 2). The size of this phosphoprotein as well as the structural homology between the

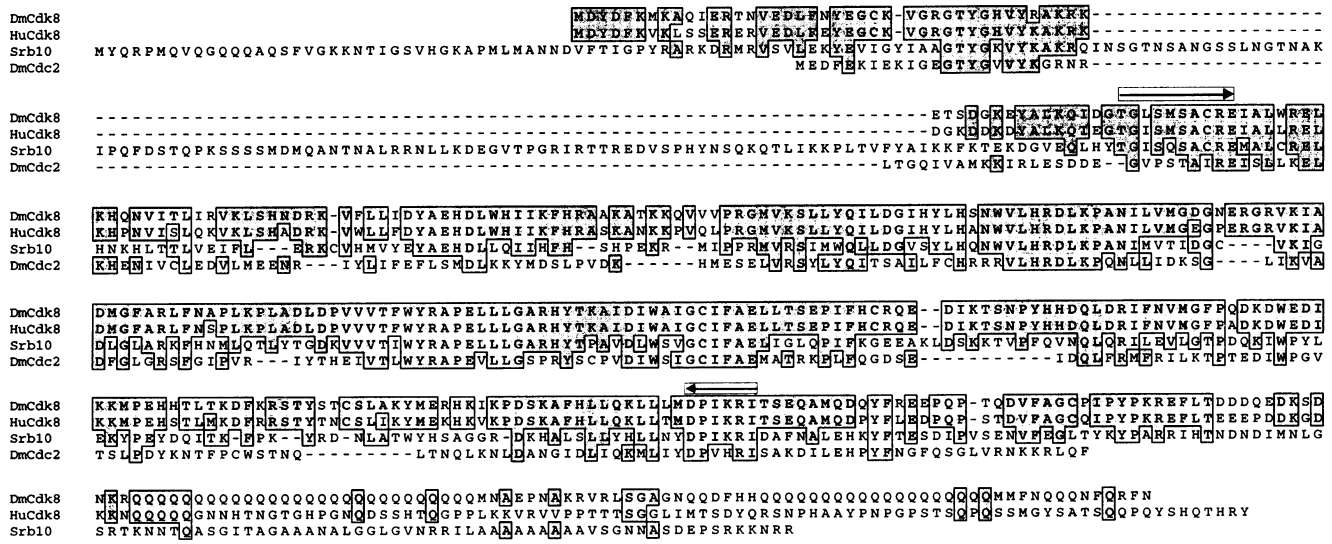

Figure 3. Sequence of *Dm*Cdk8 protein and alignment with *Hu*Cdk8 (Tassan *et al.*, 1995), Ume5/Srb10 (Surosky *et al.*, 1994; Liao *et al.*, 1995), and *Dm*Cdc2 (Lehner and O'Farrell, 1990) sequences. Boxed residues correspond to identities between *Dm*Cdk8 and the other sequences. The nucleotide sequence for *Dm*CDK8 can be found in the EMBL/GenBank databases under accession number U33015.

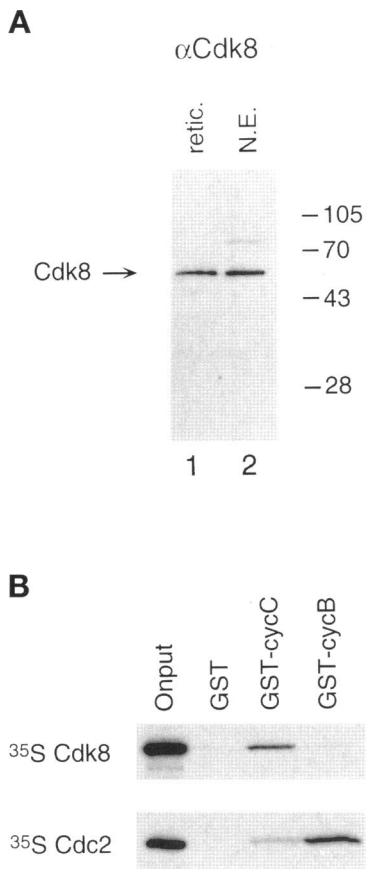

Figure 4. In vitro interactions between cyclin C and Cdk8. (A) In vitro–translated *Dm*Cdk8 (retic.) is recognized by anti-Cdk8 antibodies in Western blot and co-migrates in SDS-PAGE with *Dm*Cdk8 present in nuclear extracts (N.E.). (B) Cyclin C and Cdk8 interact in vitro. [^{35}S]methionine-radiolabeled Cdk8 or Cdc2 proteins were produced in vitro and incubated with either GST, GST-cyclin C, or GST-cyclin B proteins bound to GSH-agarose beads. Retained labeled proteins were subjected to SDS-PAGE and detected by autoradiography.

Cdk8/cyclin C and the Srb10/Srb11 couple suggested that this substrate might be the large subunit of RNA Pol II. The large subunit of RNA Pol II in *Drosophila* is present in two major forms: a nonphosphorylated form, Pol IIa, which migrates with an apparent molecular mass of 215 kDa in PAGE, and a form with multiple phosphates on the CTD, Pol IIo, which migrates at ~240 kDa (Lee and Greenleaf, 1989).

To test whether RNA polymerase associates with *Dm*Cdk8 we probed Western blots of the anti-Cdk8 immunoprecipitations with antibodies directed against the CTD of the large subunit of RNA Pol II (anti-CTD, see Figure 5B). Although the large subunit of RNA Pol II was specifically co-precipitated in our conditions, only a small portion (~10%) of the total RNA Pol II was found to interact with *Dm*Cdk8 in vivo. This observation is consistent with the original description of the yeast holoenzyme (Koleske and Young, 1994) where only 6% of the total RNA Pol II in yeast cells was found to be associated in a holoenzyme form with the SRB proteins. In our case, it suggests either that RNA Pol II is in excess or that it participates with different complexes in the cell, or that the association between *Dm*Cdk8 and RNA Pol II is weak and partly disrupted by our experimental procedure.

We then probed the sucrose gradients fractions with the same anti-CTD antibodies. The vast majority of Pol II peaked at fraction n° 6 (~230 kDa), probably corresponding to a monomeric form of the large subunit. Although the resolution of the gradients did not allow a detailed analysis of the polymerase peaks, it is clear that a small portion of the polymerase co-fractionated with Cdk8 up to fraction n° 12 (Figure 6). This confirmed the results of our anti-Cdk8 immunoprecipitations and supported further an association of a portion of Pol II with Cdk8 and cyclin C in our extracts.

To pursue the identification of the 240-kDa phosphoprotein labeled after ATP addition to the immune complex, we used another monoclonal antibody directed against an internal motif of RNA Pol II (ARNA3; Kramer *et al.*, 1980), which allowed us to detect form IIa as well as form IIo of RNA Pol II from whole embryo extracts. The low sensitivity of ARNA3 antibody did not allow us to detect IIo forms directly in the kinase assay, but we found precise coincidence of the phosphorylated band and the Pol IIo band detected by antibody probing of a parallel lane on the same gel (Figure 5C, lanes 2 and 4). As a further test, we depleted the nuclear extracts of RNA polymerase using anti-CTD antibodies, precipitated the Cdk8 from these depleted extracts, and examined the phosphorylation of proteins in the Cdk8 precipitate. Anti-CTD antibodies were able to deplete the vast majority of the RNA Pol II large subunit without precipitating the Cdk8 and cyclin C proteins (Figure 5C, lanes 5 and 6). We suggest (see DISCUSSION) that Cdk8 and cyclin C do not precipitate with the polymerase because the anti-CTD antibodies displace these proteins from a complex with the polymerase. Precipitation of Cdk8 and cyclin C and addition of [γ^{32}P]ATP did not lead to labeling of the 240-kDa band when the extract had been depleted of polymerase (Figure 5C, lane 3). Loss of this band upon displacement of Pol II from the immune complex can be most simply explained if Pol II, which in its IIo form migrates to this position, is the substrate of the kinase. Further supporting this conclusion, Cdk8 immunoprecipitates efficiently phosphorylate a synthetic CTD heptapeptide repeat in vitro (Figure 5D). None of the other exogenous kinase substrates we tried (histone H1, β-casein, phosvitin, HMG-I, myelin basic protein, or GST-Rb) were phosphorylated in these conditions (our unpublished data).

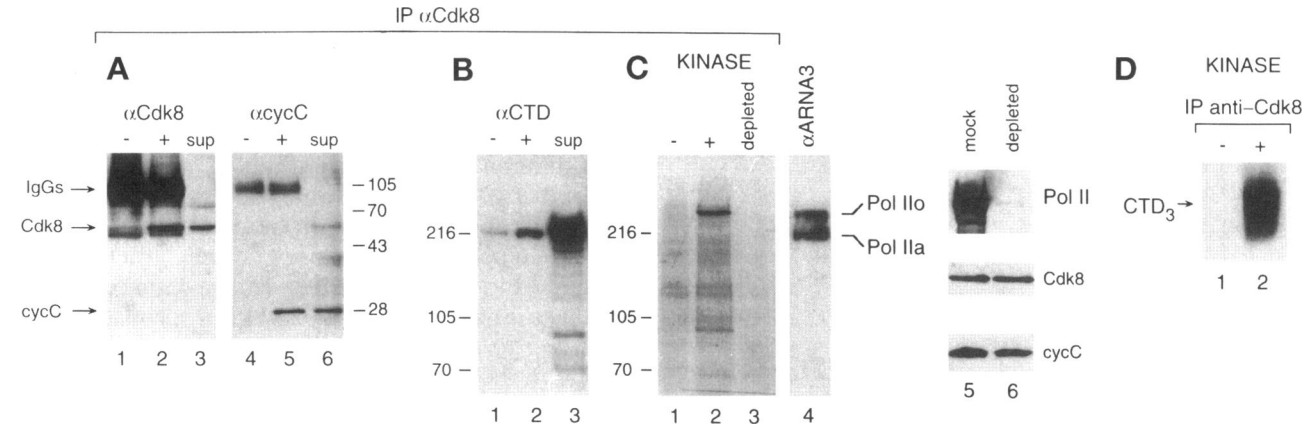

Figure 5. Cdk8 interacts in vivo with cyclin C and RNA Pol II large subunit. (A and B) Co-immunoprecipitation of Cdk8, cyclin C and RNA Pol II from nuclear extracts. Immunoprecipitations were performed on nuclear extracts using either a nonimmune antibody (−) or an anti-Cdk8 antibody (+). Immunoprecipitates as well as supernatants (sup) were analyzed by Western blotting using the following: A, anti-Cdk8 antibodies (polyclonal, rabbit) (lanes 1–3) or anti-cyclin C antibodies (monoclonal, mouse) (lanes 4–6); and B, anti-CTD antibodies, directed against the conserved c-terminal heptapeptide repeat of the large subunit of RNA Pol II (monoclonal, mouse). (C) A kinase activity is associated with anti-Cdk8 immunoprecipitates. Immunoprecipitations were carried out as in panels A and B and the immunoprecipitate was tested for autophosphorylation using [γ^{32}P]ATP (lane 1, nonimmune; and lane 2, anti-Cdk8). For lane 3, the nuclear extract was first depleted twice with anti-CTD antibodies before anti-Cdk8 immunoprecipitation. After in vitro phosphorylation, the immunoprecipitates were separated by SDS-PAGE and revealed by autoradiography. Lane 4, whole embryo extracts were run on the same gel and analyzed by Western blot using anti-Pol II antibodies (ARNA3) that recognize both Pol IIa and Pol IIo forms. Lanes 5 and 6, after mock (lane 5) or anti-CTD (lane 6) depletion, the extracts were analyzed by Western blotting for their RNA Pol II, Cdk8, and cyclin C content. (D) Cdk8 immunocomplexes have a CTD kinase activity. Immunoprecipitations were carried out as in panels A, B, or C and the immunoprecipitate was tested for kinase activity using [γ^{32}P]ATP and a synthetic peptide (CTD_3) corresponding to three copies of the consensus CTD repeat (lane 1, nonimmune; and lane 2, anti-Cdk8).

Our present results do not show whether the kinase activity present in anti-Cdk8 immunoprecipitates is due to the Cdk8/cyclin C pair or to another kinase present in the multiprotein complex with Cdk8 and cyclin C. Recent work from several laboratories has implicated the Cdk7/cyclin H kinase in the in vitro phosphorylation of the CTD. We used affinity-purified anti-*Dm*Cdk7 antibodies to test whether Cdk7 might be present in Cdk8 immunoprecipitates. Analysis of nuclear extracts revealed a single band that co-migrated with in vitro-translated *Dm*Cdk7 protein (Figure 7, lanes 1, 4, and 5), but these same antibodies failed to detect *Dm*Cdk7 in anti-Cdk8 immunoprecipitates (Figure 7, lanes 2 and 3). Thus, although other kinases may be present in our Cdk8 immunoprecipitates, it is unlikely that Cdk7 is responsible for the activity we detected.

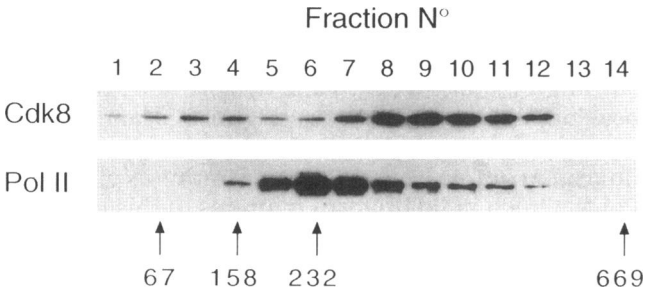

Figure 6. Fractionation of Cdk8 and RNA Pol II from nuclear extracts on sucrose gradient. Fractions 1 (top) to 20 (bottom) were analyzed by Western blotting using anti-Cdk8 (Tassan *et al.*, 1995) and anti-CTD antibodies. Only the 15 first fractions are shown. Arrows indicate the position of molecular weight standards (see legend of Figure 2).

DISCUSSION

Our anti-cyclin C antibodies identified cyclin C as a nuclear protein whose level does not oscillate during the blastoderm cell cycles in *Drosophila* embryos. Because analysis of the same embryos with cyclin A and cyclin B antisera revealed mitotic destruction of these cyclins (Edgar *et al.*, 1994), it appears that cyclin C is not subject to the degradation that drives oscillation of these cyclins. Absence of mitotic destruction was further supported by cyclin C immunolocalization in the embryo: antigen levels were constant throughout interphase nuclei, and at mitosis there was striking localization of the antigen to mitotic chromosomes (Léopold, unpublished observation). The protein, which migrates in SDS-PAGE with an apparent molecular mass of 28 kDa, sediments on native sucrose gradients with an estimated mass of ~500 kDa, which indicates that cyclin C is part of a large multiprotein complex. This unusually large size allowed us to eas-

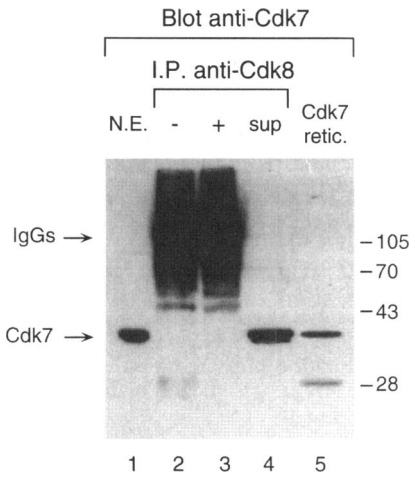

Figure 7. Cdk7 is not present in anti-Cdk8 immunoprecipitates. Control (−, lane 2) or anti-Cdk8 (+, lane 3) immunoprecipitates, as well as supernatants (sup) were tested for the presence of *Dm*Cdk7 using affinity-purified anti-Cdk7 antibodies. *Dm*Cdk7 protein produced in a reticulocyte lysate is detected by anti-Cdk7 antibodies and co-migrates with the band detected in nuclear extracts (lanes 1, 4, and 5).

ily test the co-sedimentation of members of the Cdk family and other cell cycle regulators with cyclin C fractions. Neither *Dm*Cdc2, *Dm*Cdc2c (alias *Dm*Cdk2), cyclin A, nor cyclin B co-sedimented with cyclin C. They were present in complexes in the 100- to 200-kDa range as expected from previous sedimentation results (Léopold, unpublished data; Rosenblatt *et al.*, 1992). Consequently, the co-sedimentation of cyclin C and a unique 51-kDa band recognized by anti-human Cdk8 antibodies (Tassan *et al.*, 1995) strongly suggested in vivo interaction between these two molecules; this was confirmed later by co-immunoprecipitation of *Dm*Cdk8 and cyclin C from nuclear extracts. It also falls in line with the recent finding that human Cdk8 interacts specifically with cyclin C in HeLa cell lysates (Tassan *et al.*, 1995).

Based on sequence data for a human Cdk8 and the yeast Ume5/Srb10 protein (Surosky *et al.*, 1994; Tassan *et al.*, 1995), we amplified *Drosophila* sequences by PCR and isolated and sequenced a cDNA clone for the *DmCDK8* gene. The human and *Drosophila* Cdk8 aminoacid sequences show numerous identities, and comparison to Srb10 and other Cdks leads to a few remarks. The classical PSTAIRE sequence that is involved in Cdk-cyclin interactions (Ducommun *et al.*, 1991; Endicott *et al.*, 1994; Jeffrey *et al.*, 1995) is replaced in both Cdk8 and Srb10 by a S(M/Q)SACRE sequence, whose conservation could reflect the presence of a contact region for related cyclins like Srb11 and cyclin C. The "T-loop" region of the Cdks is regulatory: in the inactive structure it virtually blocks the active site, while the active site is unmasked in the cyclin-bound structure (Jeffrey *et al.*, 1995). Full activation of characterized Cdks requires phosphorylation of the T-loop (for reviews see Clarke, 1995; Morgan, 1995). Although the structure of the phosphorylated enzyme has not yet been solved, based on an analogy to the structure of the cyclic AMP-induced kinase, it has been proposed that phosphorylation introduces new interactions that stabilize the active conformation of the T-loop (Jeffrey *et al.*, 1995). Cdk8 of human and *Drosophila* appear to lack a requirement for this activating phosphorylation because the T-loops of these kinases lack candidate residues for phosphorylation (S, T, or Y). Although Srb10 has a T within its T-loop, it is not clear whether it is analogous to the phosphorylated residue in other Cdks. The ambiguity arises because both Cdk8's and Srb10 have a three-aminoacid insertion within the T-loop, and the level of homology is not sufficient to define the correct alignment. Most likely the change in the T-loop occurred at the site that is usually phosphorylated because structural comparison indicates that adjacent residues are involved in highly conserved structure. We suggest that the Cdk8 kinases as well as the Srb10 kinase do not require activating phosphorylation and that the Asp residue that has been introduced roughly at the position normally occupied by Thr provides a negative charge to substitute for phosphorylation.

Two results showed that the cloned *Drosophila* Cdk8 is specifically recognized by the antibody to the human Cdk8: in vitro–translated *Dm*Cdk8 protein was recognized by anti-human Cdk8 antibodies and co-migrated on SDS-PAGE with a 51-kDa band seen in extracts. Western blots using this antibody showed that *Dm*Cdk8 co-sediments with cyclin C in sucrose gradients and immunoprecipitations showed that cyclin C co-precipitates with *Dm*Cdk8, suggesting that these proteins might work in association to produce an active kinase complex. Indeed, a kinase activity was found in anti-Cdk8 immunoprecipitate that phosphorylates a 240-kDa band that co-migrates with form IIo of RNA Pol II. We confirmed that this band corresponded to form IIo of RNA Pol II by four independent lines of evidence. 1) RNA Pol II is present in the immunoprecipitates. 2) Depleting the extracts before immunoprecipitation with anti-Cdk8 antibodies suppresses the appearance of the 240-kDa phosphorylated band. 3) The 240-kDa phosphoprotein co-migrates exactly with form IIo of RNA Pol II run on the same gel. 4) Cdk8 immunocomplexes are capable of phosphorylating a CTD peptide in vitro.

Attempts to precipitate the cyclin C/*Dm*Cdk8 complex with two different antibodies to RNA polymerase failed. In the case of the ARNA3 antibodies, the failure can be simply explained because under our experimental conditions we saw no precipitation of the large subunit of RNA Pol II. Anti-CTD antibodies efficiently immunoprecipitated Pol II in the extracts but no trace of Cdk8 or cyclin C were detected in the immunopre-

cipitate. Failure to co-immunoprecipitate both Cdk8 and cyclin C using anti-CTD antibodies might be due to a variety of reasons, but we think it might reflect a competition effect between antibody molecules and the proteins interacting with the CTD epitopes. A similar effect was previously suggested to explain the separation of the RNA Pol II core enzyme and a group of associated proteins called "the mediator" (and among them, Srb10 and Srb11) during affinity purification experiments using anti-CTD antibodies (Kim *et al.*, 1994).

Our experiments do not resolve whether phosphorylation of Pol II is due to the activity of the Cdk8 kinase itself. Other kinase activities might be present in the immune complex. For this reason, we tested for the presence of a kinase previously implicated in phosphorylation of the CTD. Transcription factor TFIIH, which is involved in the phosphorylation of the CTD (Conaway and Conaway, 1989; Feaver *et al.*, 1991; Lu *et al.*, 1992), contains Cdk7 and cyclin H. This pair of proteins is responsible for the in vitro CTD-kinase activity of the TFIIH complex (Feaver *et al.*, 1994; Roy *et al.*, 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995). We did not detect any trace of *Drosophila* Cdk7 protein in the anti-Cdk8 immune complex using affinity-purified anti-*Dm*Cdk7 antibodies. This suggests either that our experimental conditions do not allow the stabilization of a complex comprising Cdk7 and Cdk8, or that the two kinases associate separately with the RNA Pol II enzyme. It indicates, furthermore, that the kinase activity present in the anti-Cdk8 immune precipitate is not due to the Cdk7 kinase.

In conclusion, we have identified *Drosophila* Cdk8, a kinase partner of cyclin C, and demonstrated that it can interact in vivo with the large subunit of RNA Pol II. Furthermore, *Dm*Cdk8 is associated either directly or indirectly with a kinase activity that can phosphorylate the large subunit of RNA Pol II in vitro. The structural identity between Cdk8/cyclin C in *Drosophila* and SRB10/SRB11 in yeast, as well as the in vivo interaction between RNA Pol II and Cdk8 lead us to suggest a possible functional homology between the two kinase/cyclin pairs. Recent results assign a role for SRB10/SRB11 in transcriptional regulation in vivo as well as CTD phosphorylation in vitro (Kuchin *et al.*, 1995; Liao *et al.*, 1995; Wahi and Johnson, 1995). *Drosophila* genetics as well as additional biochemical studies will help to define the precise function of Cdk8/cyclin C. The possible involvement of cyclin C and Cdk8 in transcription suggests that we might re-evaluate the basis of the original isolation of cyclin C as a gene capable of complementing deficiencies in G1 cyclins in yeast. Although it is possible that cyclin C can play a cell cycle role in a foreign context, it is also possible that cyclin C function bypassed the G1 block by disturbing transcriptional controls in yeast.

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