Cdk7 is essential for mitosis and for in vivo Cdk-activating kinase activity

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Cdk7 has been shown previously to be able to phosphorylate and activate many different Cdks in vitro. However, conclusive evidence that Cdk7 acts as a Cdk-activating kinase (CAK) in vivo has remained elusive. Adding to the controversy is the fact that in the budding yeast Saccharomyces cerevisiae, CAK activity is provided by the CAK1/Civ1 protein, which is unrelated to Cdk7. Furthermore Kin28, the budding yeast Cdk7 homolog, functions not as a CAK but as the catalytic subunit of TFIIH. Vertebrate Cdk7 is also known to be part of TFIIH. Therefore, in the absence of better genetic evidence, it was proposed that the CAK activity of Cdk7 may be an in vitro artifact. In an attempt to resolve this issue, we cloned the Drosophila cdk7 homolog and created null and temperature-sensitive mutations. Here we demonstrate that cdk7 activity is necessary for CAK activity in vivo in a multicellular organism. We show that cdk7 activity is required for the activation of both Cdc2/Cyclin A and Cdc2/Cyclin B complexes, and for cell division. These results suggest that there may be a fundamental difference in the way metazoans and budding yeast effect a key modification of Cdks.

Key Words: Drosophila; Cdk; CAK; mitosis; cell cycle

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Cdk7 is Kin28. Although Kin28 was shown to be part of the TFIIH transcription factor (Feaver et al. 1994) and to be required for the phosphorylation of the CTD of RNA Pol II, it is not involved in the phosphorylation of Cdc28, the budding yeast Cdc2 homolog (Cismowski et al. 1995). The protein responsible for CAK activity in S. cerevisiae was identified as CAK1/Civ1 (Espinoza et al. 1996; Kaldis et al. 1996; Thuret et al. 1996). Surprisingly, CAK1/Civ1 shares only limited sequence similarity with Cdk7 and other Cdks. The identification of this in vivo CAK in budding yeast and the demonstration that it is not closely related to the vertebrate Cdk7 led to the postulation that Cdk7/Cyclin H may in fact not represent a physiologically relevant CAK activity (Cismowski et al. 1995; Espinoza et al. 1996; Kaldis et al. 1996; Thuret et al. 1996).

Besides the two yeast, Drosophila has become a system of choice for an in vivo analysis of the cell cycle (Edgar and Lehner 1996; Follette and O’Farrell 1997). One of its major values is that it allows the genetic analysis of cell cycle events in a multicellular organism. Like vertebrates, but contrary to the unicellular yeast, Drosophila cells use distinct Cdks at the different cell cycle transitions. Interestingly, although the activity of Cdk7 has been shown in different systems to be constant throughout the cell cycle (Brown et al. 1994; Matsuoka et al. 1994; Poon et al. 1994; Tassan et al. 1994), the level of Cdc2 Thr-161 phosphorylation was shown to oscillate during the late preblastoderm embryonic cycles (Edgar et al. 1994). This indicates that the target site for CAK is regulated at least during some cell cycles.

Here we report the identification of the Drosophila cdk7 gene. By creating null and temperature-sensitive mutations of Dmcdk7 we were able to analyze the in vivo molecular and cellular requirements for Cdk7. Although our analysis does not reveal a Cdk7 requirement for Cdk2/Cyclin E activity, it demonstrates that Cdk7 is required for mitosis and for the activation of Cdc2 in vivo.

Results
Isolation of the Dmcdk7 gene

We isolated a Drosophila melanogaster sequence homologous to the vertebrate cdk7 genes using a degenerate PCR-based approach. This Drosophila cdk7 gene codes for a predicted polypeptide of 353 amino acids with a calculated molecular mass of 39 kD. Drosophila and human Cdk7 proteins share 65% identity over the entire polypeptide (Fig. 1A), a sequence similarity higher than to any other Cdk. A single 1.6-kb Dmcdk7 poly(A+) RNA species is present throughout development and accumulates most strongly in ovaries and young embryos where it is probably maternally deposited (Fig. 1B).

Figure 1. Identification and characterization of the Drosophila cdk7 gene. (A) Comparison of Drosophila and vertebrate Cdk7 proteins. (B) poly(A+) RNA blot showing the developmental profile of accumulation of the Dmcdk7 message. Embryonic stages (E) are in hours. (L1) First instar larvae; (L2) second instar larvae; (L3) early third instar larvae; (L3) late third instar larvae; (ep) early pupae; (LP) late pupae. A single 1.6-kb transcript accumulates predominantly in samples containing the female germ line and in the early embryos where it is contributed maternally. (Bottom) An autoradiograph of the same filter after hybridization with the small ribosomal subunit protein gene DL11.
DmCdk7 is an essential gene

The DmCdk7 gene is located in cytological interval 4F and is separated by ∼0.4 and 3 kb from its proximal neighbors sans fille (snf) and deadhead (dhd), respectively (Fig. 3). snf and Dmcdk7 are oriented head to head. To create a Dmcdk7 null mutation, we took advantage of a P-element insertion at the dhd locus (dhd<sup>P8</sup>). Imprecise excision of this P-element produced a number of lethal mutations (Flickinger and Salz 1994). From this screen we identified a 4.4-kb deletion that removes the entire Dmcdk7 and snf coding regions, as well as part of the dhd gene (Fig. 3). This new deficiency is designated Df(1)J210

To study the effects of lack of Dmcdk7 alone, the two other genes disrupted by Df(1)J210, snf and dhd, were reintroduced into this deficiency background by P-element-mediated transformation (Pw<sup>"snf",dhd"</sup>; Fig. 3). To show that the Pw<sup>"snf",dhd"</sup> transgene is active, we tested its ability to complement the lethal phenotype associated with Df(1)J210, a 3.0-kb deletion with the same proximal breakpoint as Df(1)J254 but that affects only the snf and dhd genes (Fig. 3; Flickinger and Salz 1994). Pw<sup>"snf",dhd"</sup> fully rescues the lethal phenotype of Df(1)J210 (not shown), but fails to rescue the lethality associated with Df(1)J254, indicating that this chromosome lacks at least one additional essential function. This function is Dmcdk7 because the introduction of a wild-type Dmcdk7 gene (Pw<sup>"Dmcdk7"</sup>) into the Df(1)J254-Pw<sup>"snf",dhd"</sup> background results in complete rescue of the lethality. These results demonstrate that Df(1)J254 disrupts the Dmcdk7, snf, and dhd genes, that the defects caused by lack of snf and dhd are rescued by Pw<sup>"snf",dhd"</sup>, and that the absence of Dmcdk7 results in zygotic lethality. Therefore, we refer to animals carrying the Pw<sup>"snf",dhd"</sup> transposon in a homozygous Df(1)J254 background as cdk7<sup>null</sup> mutants.

DmCdk7 exhibits CAK activity in vitro

To be able to analyze the biochemical activity of DmCdk7, we raised antibodies against the DmCdk7 protein and used them to isolate the active enzyme from tissue homogenates. On immunoblots prepared from fly tissues, monoclonal antibodies raised against the full-length recombinant DmCdk7 recognize with high affinity a single polypeptide species, with a relative mobility of ∼40 kD (Fig. 2A). This is the first indication that these anti-DmCdk7 antibodies react specifically with DmCdk7. The second one is that Drosophila Cdc2 and Cdk2 proteins, which both share a high degree of similarity with DmCdk7, cannot be detected in immunoprecipitates performed with anti-DmCdk7 antibodies (not shown).

To demonstrate that the identified Drosophila protein possesses CAK activity, we used DmCdk7 immunoprecipitated from embryos 0–4 hr old to activate recombinant human HA–Cdk2/Cyclin A complexes (Fisher and Morgan 1994). HA–Cdk2/Cyclin A is strongly phosphorylated when incubated with a DmCdk7 immunoprecipitate, indicating that DmCdk7 can act as a CAK (Fig. 2B). The DmCdk7-mediated phosphorylation of Cdk2 seems to occur specifically at threonine residue 160. This is demonstrated by the ability of DmCdk7 to phosphorylate the wild-type Cdk2 but not the Cdk2<sup>T160A</sup> mutant protein (Fig. 2B). DmCdk7 also acts as a CAK as it can stimulate the histone H1 kinase activity of Cdk2/Cyclin A (Fig. 2B). These results confirm that DmCdk7 codes for a protein that exhibits CAK activity in vitro and likely represents a functional homolog of the vertebrate cdk7 genes.
A temperature-sensitive allele of Dmcdk7

To be able to study the requirements for cdk7 activity in different tissues and at different times during development, we created a temperature-sensitive allele. Temperature-sensitive mutations in the related cdc2 and Mek (Carr et al. 1989; Hsu and Perrimon 1994; Sigrist et al. 1995) suggested that substituting the conserved proline residue 140 for a serine was likely to confer temperature sensitivity to cdk7. Therefore, we introduced this mutation into cdk7 by site-directed mutagenesis, and reintroduced the modified gene into flies. Animals carrying a cdk7+ transgene in the cdk7null background were found to be 100% viable at all temperatures tested, whereas those carrying the cdk7P140S allele in the null background proved to be fully viable at 18°C but not viable at 27°C or above. These results demonstrate that the P140S mutation results in a temperature-dependent inactivation of cdk7. Flies carrying only the cdk7P140S transgene as a source of Cdk7 protein therefore will be referred to as cdk7ts mutants.

After transfer of mutant mothers to the restrictive temperature, many of the embryos laid during the first 36 hr will eclosed as larvae. Most of the embryos that fail to eclose during that period exhibit defects later in development. Mutant embryos exhibit gradually earlier developmental arrest to a point where the embryonic nuclear division program fails to be initiated (data not shown).

When immunoprecipitated Cdk7P140S is used in CAK assays, a significant amount of activity is still present in embryos from females kept at the restrictive temperature for 1 day. Some activity can still be detected after 2 days at the restrictive temperature (data not shown). Similarly, the Cdk7P140S protein fails to be inactivated rapidly in vivo by incubation at high temperature (Fig. 4A). We observe a similar failure to inactivate the Dm-Cdk7 protein in vitro after immunoprecipitation (data not shown). What then is the basis of the temperature sensitivity of cdk7P140S? Embryos and ovaries isolated from Cdk7P140S animals kept at the restrictive temperature show a progressive reduction of Cdk7 protein levels with time (Fig. 4B, see also Fig. 7, below).

Taken together, these data suggest that the P140S mutation interferes with the stability of the mutant protein synthesized at the restrictive temperature, whereas the mutant protein synthesized at the permissive temperature retains significant activity until turned over. The described properties of the P140S mutation can explain the existence of a lag phase between the shift of temperature-sensitive mutants to the restrictive temperature and the appearance of the mutant phenotype in embryos (see below). Similar lag phases have also been observed, for example, before cell cycle arrest occurs in the cak1-22 mutant of S. cerevisiae (Kaldis et al. 1996).

Loss of either cdk7 or cdc2 causes similar cell proliferation defects

Like their cdk7+ siblings, mutant adults allowed to develop at the permissive temperature can live for >40 days after transfer to the restrictive temperature. The production of gametes, however, stops in cdk7ts animals as females transferred to the restrictive temperature cease to lay eggs after 3–4 days as a result of compromised cell division in the germ line and supporting somatic tissue.

In Drosophila, the ovary consists of a number of tube-like structures called ovarioles at the tip of which two to three mitotically active germ-line stem cells continuously divide. The asymmetric division of a stem cell gives rise to another stem cell and a cystoblast that then goes through four incomplete mitotic divisions, resulting in a cyst of 16 germ cells connected to each other by cytoplasmic bridges (for review, see Spradling 1993). As the 16-cell cyst moves posteriorly down the ovariole, it becomes enveloped by a continuous monolayer of somatic follicle cells that are also supplied by dividing stem cells (Fig. 5A; Margolis and Spradling 1995). When pupae are transferred to the restrictive temperature, viable cdk7ts mutant adults continue to eclosed for up to 3 days.
days after the temperature shift to 29°C. These animals exhibit normal adult viability when kept at the restrictive temperature, but the mitotically active tissues exhibit progressively stronger defects with increasing time spent at 29°C. When females eclose shortly after transfer to 29°C, the first abnormality that is observed in cdk7ts ovaries is the depletion of the somatic follicle cells (Fig. 5C,D). A rapid depletion of follicle cells is predicted to occur if cell division is compromised as each daughter of the follicle stem cells must divide approximately nine times to produce the 1200 follicle cells surrounding each germ-line cyst (Mahowald and Kambysellis 1980; Margolis and Spradling 1995). The depletion of follicle cells in the cdk7ts mutant shows that cdk7 is required for mitotic division of these somatically derived cells. After the reduction in the number of follicle cells surrounding each egg chamber, the effect on the mitotic activity of the germ line becomes apparent when cysts are found that possess fewer than the normal number of 16 germ cells (Fig. 5D,E).

Cyclin E was shown previously to function during endoreplication of polyploid tissues (Sauer et al. 1995; Lilly and Spradling 1996), and its activity is thought to be mediated through the activation of Cdk2 (Knoblich et al. 1994; Sauer et al. 1995). Therefore, we were surprised that although cell proliferation is arrested completely in cdk7ts ovaries, the capability of these cells to endoreplicate their DNA is not affected (Fig. 5E-G). This result may suggest that the activity of Cdk2 is not affected by loss of cdk7 activity.

**Figure 5.** Phenotype of cdk7 during oogenesis. (A) Schematic view of the early stages of oogenesis in one ovariole. The germinal regions 1, 2, and 3 are indicated (1,2,3); region 3 contains the stage 1 egg chamber. (tfc) Terminal filament cells; (inc) inner sheath cells; (gsc) germ-line stem cells; (fsc) follicle stem cells; (dcc) dividing cystocytes; (nc) polyploid nurse cell nuclei; (fc) follicle cells; (Oo) oocyte; (Stg 2) stage 2 egg chamber. The germ-line part of the egg chamber in a 16-cell cyst contains 1 diploid oocyte and 15 nurse cells, which are in the process of becoming polyploid. The cyst is surrounded completely by still dividing somatic follicle cells. (A) Anterior; (P) posterior. Effects of lack of cdk7 in the ovary. Nuclear staining with Hoechst 33258 (B) wild-type egg chambers. (C) Dmcdk7ts animals were transferred to the restrictive temperature shortly (<1 day) before eclosion and ovaries dissected 3 days after eclosion. At this stage, although some follicle cells are present, their number is too low to surround completely each egg chamber. (D,G) Ovaries from cdk7ts females that eclosed 3 days after pupae were transferred to the restrictive temperature and dissected 3 days (D), 5 days (E,F), or 7 days (G) after eclosion. (D) Two cysts with fewer than 16 germ-line cells and no somatic follicle cells. (E) “Egg chamber” with a single polyploid germ-line cell. (F) View of an entire ovary completely populated by large polyploid cells. (G) Ovary containing only seven large polyploid cells. (H–K) Effects of lack of cdc2 activity during oogenesis. Transfer of cdc2ts females to restrictive temperature causes a rapid depletion of somatic follicle cells. (H) cdc2ts ovaries transferred to the restrictive temperature 1 day after eclosion and dissected 2 days later. (I–K) Germarial region from (I) wild-type ovary, (J) Dmcdc2ts, (K) Dmcdk7ts ovaries from female transferred to the restrictive temperature 2 days before and dissected 3 days after eclosion. The region in which mitotic proliferation of the germ line occurs in the wild type is entirely occupied by large polyploid cells in both mutants, resulting in an enlargement of the germarium. cdc2 and cdk7 mutant ovaries eventually empty out of germ line (not shown).
Cyclins in Figure 6.

levels in wild-type embryos. These observations indicate parents during stages when they are also present at high their DNA is not affected by the loss of stops but the capacity of the germ-line cells to replicate mutant ovaries, mitotic proliferation of the germ line (Fig. 5H). This depletion of follicle cells is identical to the one observed in cdk7<sup>ts</sup> ovaries. Also, as noted for the cdk7<sup>ts</sup> mutant ovaries, mitotic proliferation of the germ line only when the mitotic division program is terminated (Fig. 5J). Polyploidization of the germ cells usually occurs and the 16-cell cyst is formed. In both cdc2 and cdk7 mutants the polyploidization of the germ line occurs prematurely (Fig. 5j,K). Because Cdc2 mediates this block of endoreplication in mitotic tissues (Hayashi 1996), these results also suggest that the premature endoreplication observed in cdk7 mutant ovaries may be attributable to lack of Cdc2 activity.

Cyclin levels are not reduced in cdk7<sup>ts</sup> mutant embryos

Because Cdk7 has also been implicated in the phosphorylation the CTD of RNA Pol II as part of the TFIIH complex (Roy et al. 1994; Akoulithchev et al. 1995; Serizawa et al. 1995; Shiekhattar et al. 1995), the cell cycle arrest observed in the cdk7 mutants could be an indirect result of reduced RNA Pol II activity, which in turn would result in low cyclin levels (Cismowski et al. 1995). To determine whether reduced cdk7 activity results in a decrease in cyclin levels, the amount of the different cyclins present in the mutant and wild-type embryos was determined by immunoblot (Fig. 6). The level of all three cyclins is high in embryos from cdk7<sup>ts</sup> parents during stages when they are also present at high levels in wild-type embryos. These observations indicate that the lack of cdk7 does not cause a noticeable reduction in cyclin levels. Cyclins A and B are expressed uniquely in mitotically active cells (Lehner and O’Farrel 1990). In total lysates from embryos at different stages of development, the level of mitotic cyclin proteins is greatly reduced in later development (Lehner and O’Farrell 1989; Edgar et al. 1994). The high level of Cyclins A and B still observed in cdk7<sup>ts</sup> embryos aged >6 hr (Fig. 6) is attributable to the fact that these embryos arrested development at a stage when mitotic cyclin levels are still high. Another observation suggesting that transcription in general is not disrupted is the fact the germ-line cells can still endoreplicate their DNA in mutant cdk7<sup>ts</sup> ovaries. Endoreplication has been proposed to require the pulse transcription of cyclin E in embryonic tissues (Sauer et al. 1995), and presumably also in the ovary (Lilly and Spradling 1996).

cdk7 mutant embryos are deficient in physiological CAK activity

To test whether cdk7 is essential for CAK activity, we incubated cdk7<sup>ts</sup> animals at the restrictive temperature for different amounts of time and measured the CAK activity in total cell lysates from their embryos. A gradual reduction of CAK activity, down to background level, was observed (Fig. 7A,B). This indicates a genetic requirement for cdk7 for most or all of the cellular CAK activity that can be measured in vitro. Because immunodepletion of Cdk7 protein from embryonic homogenates can effectively eliminate CAK activity from wild-type extracts (Fig. 7C,D), it can be concluded that the Cdk7 protein itself provides all of the measurable CAK activity in Drosophila embryos.

If Cdk7 also acts in vivo as a CAK, we would expect levels of Cdk T-loop phosphorylation and Cdk activity to be reduced in cdk7 mutant tissues. Therefore, we isolated different Cdk/Cyclin complexes from mutant and wild-type embryos using antibodies directed against Cyclins A, B, and E. In Drosophila, Cyclin A (as well as Cyclin B) associates uniquely with Cdc2 and not with Cdk2 (Knoblich et al. 1994; data not shown). Although Cyclin A can be precipitated equally from either wild-type or mutant embryos, the amount of Cdc2 protein recovered in the Cyclin A immunoprecipitates from mutant embryos is severely decreased (Fig. 8A). In both wild-type and mutant embryos, only the fast migrating isoform of Cdc2 can be found associated with Cyclin A in a stable complex (Fig. 8B). This indicates that Cdk7 activity is required for the formation of a stable Cdc2/Cyclin A complex in vivo. On the contrary, Cdc2 can still form a stable complex with Cyclin B in cdk7 mutant embryos, but the amount of Thr-161 phosphorylated isoform of Cdc2 associated with Cyclin B is reduced (Fig. 8B). The addition of recombinant Cdk7/Cyclin H to the mutant extracts before immunoprecipitation results in an increase in the amount of fast migrating isoform of Cdc2, confirming its identity as T-loop phosphorylated (Fig. 8B).

After immunoprecipitation from both wild-type and

Figure 6. Cyclins in cdk7<sup>ts</sup> embryos. Before collecting embryos, cdk7<sup>ts</sup> parents were kept at 29°C for 60 hr. Total embryonic extracts from wild-type or cdk7<sup>ts</sup> embryos were analyzed by Western blotting using monoclonal antibodies directed against cyclins A, B, and E. The embryonic age is indicated in hours.
cdk7ts embryos, the kinase activity toward histone H1 of Cdc2/Cyclin A, Cdc2/Cyclin B, and Cdk2/Cyclin E complexes was measured. Although the total amount of Cdc2 associated with Cyclin B is similar in both mutant and wild-type embryos (Fig. 8D), the Cdc2/Cyclin B complex isolated from mutant embryos is less active than the one isolated from wild-type embryos (Fig. 8D). This loss of activity correlates with the observed decrease in Thr-161 phosphorylation of Cdc2 (Fig. 8B). If the reduction in activity of Cyclin B-bound Cdc2 isolated from cdk7ts embryos is attributable uniquely to reduced Thr-161 phosphorylation, normal activity should be restored by treatment of this complex with CAK. To test this, the Cyclin B immunoprecipitates were incubated with active human recombinant Cdk7/Cyclin H after the initial measurement of the histone H1 kinase activity. This treatment results in the restoration of the activity (Fig. 8D) and Thr-161 phosphorylation (Fig. 8B) of the Cdc2 isolated from mutant embryos to a level equivalent to the one isolated from wild-type embryos. Therefore, it appears that the major reason why the activity of the Cyclin B-bound Cdc2 is lower in cdk7 mutant embryos (as compared to the control) is that this Cdc2 is hypophosphorylated on Thr-161. These results indicate that cdk7ts embryos are deficient in physiological CAK activity. The slight delay that is observed between the time at which there is apparently no active Cdk7 protein remaining (Fig. 7) and the loss of Cdc2, Thr-161 phosphorylation (and the appearance of early arrest phenotype) may be attributable to the fact that Cdc2 is phosphorylated maternally starting from mid-oogenesis. Therefore, this pool of active Cdc2 must be used up before the effect of lack of Cdk7 can be clearly observed. In the wild-type situation, Cdc2 Thr-161 does not appear to be significantly dephosphorylated until nuclear cycle 11 (Edgard et al. 1994).

In contrast to the above findings, the histone H1 kinase activity of the Cdk2/Cyclin E complex, as well as the level of Thr-163 (equivalent to Thr-160 in mammalian Cdk2) phosphorylation of Cdk2 are not affected significantly in mutant extracts as compared to wild type (Fig. 8E).

Discussion

Cdk7 has been isolated previously as a Cdk-activating kinase (Fesquet et al. 1993; Poon et al. 1993; Solomon et al. 1993; Fisher and Morgan 1994; Mäkelä et al. 1994), and as a CTD kinase as part of the TFIH basic transcription factor complex (Roy et al. 1994; Aouilichev et al. 1995; Ossipow et al. 1995; Serizawa et al. 1995; Shiekhatar et al. 1995; Adamczewski et al. 1996). Although the
cdk7 is required for mitotic proliferation

In addition to sharing a high degree of sequence similarity with vertebrate cdk7 genes, the Drosophila gene we have isolated in this study codes for a protein that can phosphorylate and activate Cdk2 in vitro. The terminal phenotype of the cdk7null mutant is similar to the strongest loss-of-function cdc2 mutant: death before or during pupation with little imaginal tissue (Stern et al. 1993). A phenotype now considered a hallmark of genes required for mitotic proliferation (Gatti and Baker 1989).

The creation of a temperature-sensitive allele of cdk7 allowed us to further strengthen the possible in vivo link between cdk2 and cdk7. The temperature-dependent inactivation of the cdk7 allele in the ovary results in the rapid depletion of the somatic follicle cells, which need to be replenished continuously from a mitotically active population of stem cells (Margolis and Spradling 1995). Proliferation of the germ line is also disrupted in cdk7 mutant mutants, resulting in the formation of cysts with fewer than the normal complement of 16 cells. We found these phenotypes to be very similar to the temperature-sensitive ovarian cdc2 phenotype we have described in this study. Similar mitotic defects in the ovary were reported recently using a different allele of cdc2 (Reed and Orr-Weaver 1997). These results are consistent with a model whereby Cdk7 acts in the same pathway as Cdc2 in promoting cell division.

The Cdk in cdk7

In cdk7 mutant embryos, the level of Thr-161 phosphorylation and activity of the Cyclin B-bound Cdc2 is reduced, and both can be restored by incubation with purified Cdk7/Cyclin H (Fig. 8). This indicates that the major difference between Cdc2 isolated from wild-type and cdk7 mutant embryos is the extent of Thr-161 phosphorylation. Therefore, Cdk7 is essential for in vivo CAK activity. Although Cdc2/Cyclin B complexes form normally in cdk7 mutant embryos, Cdc2 and Cyclin A fail to form a stable complex in the cdk7 mutant. This is likely attributable to the fact that this event requires the phosphorylation of Cdc2 on Thr-161, as even in the wild type only the phosphorylated form is associated with Cyclin A (Fig. 8). These in vivo results correlate well with the finding that human Cdc2 needs to be phosphorylated by CAK to form a stable complex with Cyclin A in vitro, whereas stable Cdc2/Cyclin B and Cdk2/Cyclin E complexes can form in the absence of Thr-161 (or 160) phosphorylation (Desai et al. 1995). The Cdc2/Cyclin A complex seems to be more sensitive to a reduction in CAK activity than the Cdc2/Cyclin B complex, as the loss of Cyclin A binding occurs more rapidly than the reduction of Thr-161 phosphorylation of Cyclin B-associated Cdc2.

Because Cdk7/Cyclin H is able to phosphorylate many Cdk/cyclin complexes in vitro, it was postulated that a single CAK could be responsible for the activation of all the different Cdks acting throughout the cell cycle.
CAK activity in But as it appears that all of the measurable embryonic phosphorylation or activity could be detected in cdk7 mutant embryos, it is possible that the threonine-phosphorylated form of Cdk2 is highly stable or requires a lower threshold of CAK activity than Cdc2. Alternatively, Cdk2 may be activated by a CAK different from Cdk7. But as it appears that all of the measurable embryonic CAK activity in Drosophila can be eliminated either genetically by the inactivation of cdk7 or biochemically by immunodepletion of Cdk7, if such a second CAK exists, its biochemical characteristics such as substrate specificity or solubility would have to be very different from that of either Cdk7 or CAK1. Or it would have to be absent from the stages analyzed. Figure 9 summarizes the sum of accumulated data on CAKs and TFIIH kinases in yeasts and metazoans.

CAK in yeast and metazoan: a question of evolution?

It is impossible to demonstrate with absolute certitude that Cdk7 phosphorylates Cdc2 in vivo. But the evidence to that effect is overwhelming: Cdk7 acts as an excellent CAK in vitro and CAK activity can be entirely depleted from a cellular extract with Cdk7 antibodies (this study; Fesquet et al. 1997). The loss of cdk7 gene function results in the abolition of in vivo CAK activity and reduced Cdc2 T-loop phosphorylation and activity (this study) and has a phenotype identical to that of loss of cdc2 (this study). These observations leave little room for an explanation other than that Cdk7 is active as an in vivo CAK.

The basic components of the cell cycle regulatory machinery are, for the most part, shared by both yeast and higher eukaryotes. It has been shown in numerous cases that the mechanisms, as well as molecules, that regulate the cell cycle in yeast are usually also conserved in higher eukaryotes. Therefore, it may come as a surprise that yeast and metazoans would use entirely different molecules, such as Cdk7 and CAK1, to carry out identical enzymatic reactions in such a basic mechanism as the activating phosphorylation of Cdks. Perhaps even more surprising is that a “complex” multicellular organism would use a single enzyme to carry out two very distinct functions, whereas the apparently much simpler unicellular yeast would use two different ones. However, the analysis of all the data obtained in this study and previously with Cdk7, Kin28, and CAK1 clearly point into this direction.

Now that the sequence of the whole genome of S. cerevisiae is known, it is clear that of all yeast proteins Kin28 is the one with the highest sequence similarity to Cdk7. At the functional level, both proteins can be found as subunits of TFIIH and are known to interact physically with related cyclin-like molecules, Cdk7 with Cyclin H (Fisher and Morgan 1994; Mäkelä et al. 1994) and Kin28 with Ccl1 (Valay et al. 1993). Both Cdk7 and Kin28 can use the CTD of RNA Pol II as substrate in vitro. From these data it seems clear that Cdk7 and Kin28 are not only related by sequence, but also carry out similar cellular functions in both organisms. However, there is a major difference between the two molecules; Cdk7 is a very efficient CAK in vitro, whereas Kin28 has no detectable CAK activity either in vitro or in vivo (Cismowski et al. 1995; Valay et al. 1995). The present work underlines another major difference between Cdk7 and Kin28, this time at the level of a genetic requirement. We clearly demonstrate that cdk7 activity is required for the production of CAK activity in vivo, whereas Kin28 is not (Cismowski et al. 1995; Valay et al. 1995). Our failure to detect a defect in transcription in cdk7 mutants could mean that the Cdk7 protein persists longer as part of TFIIH, which may stabilize its activity. Alternatively, these observations may indicate that Cdk7 activity is not essential for transcription under our experimental conditions. For example, it may be that a different protein acts redundantly with Cdk7 as a CTD kinase. At least two other Cks are known to be able to act as CTD kinase in human cells, Cdk8 (Tassan et al. 1995b) and Cdk9 (PITLARE) (Jones 1997).

Evidently unicellular organisms have continued to evolve just like metazoans did. Maybe it was advantageous for S. cerevisiae to use two distinct proteins to carry out functions for which only one has remained necessary in other organisms. The emergence of CAK1 may then have lead to the evolution of Kin28, a Cdk7 that has lost its ability to act as a CAK. In this context it

### Figure 9

CAK in yeast and multicellular organisms. Simplified representation of TFIIH kinase, Cdk, and CAK activities in the unicellular yeasts S. pombe and S. cerevisiae, and Drosophila incorporating the results of the present study, which provide strong genetic and biochemical evidence that metazoans Cdk7 acts as an in vivo CAK for Cdc2. The well-characterized association of Cdk7 with TFIIH in vertebrates also indicates a role for Cdk7 in transcription. In S. cerevisiae, CAK activity is provided by CAK1/Civ1, whereas the Cdk7-related Kin28 is only active in transcriptional regulation. In combination with our results, those available thus far in S. pombe suggest that the Cdk7 homolog (Mop1/Crk1) may be active in vivo as both a CAK and CTD kinase.
would be interesting to know whether metazoans have a CAK1 homolog. Thus far none have been reported, but whether there is or is not a CAK1 homolog in multicellular organisms will be answered only with its discovery or the completion of the sequencing of a metazoan genome.

Materials and methods

DNA cloning and deficiency analysis

The described Dmcdk7 cDNA was isolated during a screen for protein kinases (Larochelle and Suter 1995). Library screening, nucleic acid hybridizations, and sequencing were done according to standard protocols. Deletions produced by the imprecise excision of the dhdts8 P-element (Flickinger and Salz 1994) were tested by Southern hybridization using a Dmcdk7 cDNA probe. To map precisely the genomic region deleted by the Df(1)B254 deficiency, PCR amplifications were carried out using primers cdk7-7P1 (5'-ACAAACTCATGTTGGGTGGCG-3') in the Dmcdk7 3' UTR region, and dhd-P5 (5'-GGATTGCTGCTTAC- GCCCTC-3') followed by sequencing.

Site-directed mutagenesis and transformation constructs

The mutagenic primer used to create the temperature-sensitive mutation was P1405 5'-GCCGATTGGAAGTCCAACAATT-TGC-3'. The rescue construct for dhd and snf genes was constructed by cloning a 3.7-kb Stu–EcoRI fragment containing 25% of the Dmcdk7 and the entire snf genomic region into pHBluescript KS− Sma–I EcoRI sites. A 4.8-kb Xhol fragment containing the dhd gene was then added at a single genomic Xhol site within the 3.7-kb Stu–EcoRI fragment. A 5.5-kb Xbal–BamHI fragment containing both entire snf and dhd genomic regions was then cloned into the pCaSpeR transformation vector.

Genetics and fly stocks

The P-element excision screen that gave rise to Df(1)B254 is described in Flickinger and Salz (1994). The Dmcdk7Ynull chromosome was constructed by recombining an X chromosomal insert of Pw "snf,dhd" with w Df(1)B254. Dmcdk7Y were obtained by crossing males carrying a Sb Pw+/+, females. The females used for these crosses were either w Df(1)B254 Pw "snf,dhd"/[+] or w Df(1)B254 Pw "snf,dhd"+/+. W(Dmcdk7Y)[P1405] Sb+/+ for analysis of the ovary phenotype or w Df(1)B254 Pw "snf,dhd"/[+] or w Df(1)B254 Pw "snf,dhd"+/+. W(Dmcdk7Y)[P1405]. Pw "Dmcdk7Y" to obtain cdk7+ embryos. The Dmcdk2 alleles B47 and the temperature-sensitive Dmcdk226c; w/w; Pw[cdc2A1717]/+ cdc226c; Pw[cdc2A1717]/+ cdc226c; Pw[cdc2A1717]/+ cdc226c; Pw[cdc2A1717]/+ lines have been described before (Stern et al. 1993; Sigrist et al. 1995). cdc26 flies used in this study were obtained by crossing Dmcdk226c X/+; cdc226c/SMA6; +/+ w/w; Pw[cdc2A1717]; cdc226c; Pw[cdc2A1717]+ were used for phenotypic analysis of the ovary.

Antibodies and protein blotting

Anti-DmCdk2 (Cdc2c) and anti-Cyclin A and B antibodies were provided by C. Lehner (Bayreuth), anti-DmCdk2 and anti-PSTAIR antibodies by P. O’Farrell [University of California, San Francisco (UCSF)] and M. Yamashita (Hokkaido University, Sapporo, Japan), respectively. Recombinant DmCdk7 protein was produced by cloning the entire coding region of the Dmcdk7 cDNA into the NdeI–BamHI sites of the pET-3 (pAR) protein expression vector. BALB/c mice were immunized three times at 5-week interval using 50 µg of protein emulsified in TiterMax synthetic adjuvant (CytRx corp., Atlanta, GA). Screening of the hybridoma lines was carried out by ELISA using a MBP-DmCdk7 fusion protein (New England Biolabs), and immunoblotting using ovarian protein extracts from Drosophila. The antibodies used in this study (19E7 and 20H5) recognize a single polypeptide species with high affinity. For immunoblotting, standard SDS-PAGE was used except for the separation of the Thr-161 phosphorylated and nonphosphorylated form of Cdc2 (Fig. 8A,B). For this purpose the acrylamide stock solution used was 30% T:1.67% C using Piperazine di-acrylamide (BioRad) as crosslinker (Kumagai and Dunphy 1995).

Ovary staining

Ovaries were dissected in Ringer’s solution and fixed for 20 min. in 200 µl of 4% paraformaldehyde in PBS + 0.2% Tween 20, 20 µl of DMSO, and 600 µl of n-heptane. Washed several times in PBST, and stained for 1 hr with 10 µg/ml of Hoechst 33258 in PBST.

Kinase assays

For CAK assays on immunoprecipitates, ~50–200 µl embryos (or 20–100 pairs of ovaries) were homogenized in 1.0 ml of H0B buffer (25 mM HEPES (pH 7.4); 150 mM NaCl, 5 mM NaF; 1 mM EDTA, 1 mM DTT; 0.1 mM Na2VO4, 0.1% Triton X-100, 2 µg/ml of aprotinin; 2 mM PMSF, 1 µg/ml of leupeptin). The homogenate was centrifuged at 14,000 rpm for 20 min and the cleared supernatant incubated with 20 µl of protein G plus agarose (CabiBiochem) previously reacted with monoclonal anti-Dmcdk7 19E7. The immunoprecipitate was washed three times with the same buffer and three times with HD buffer (25 mM HEPES (pH 7.4), 1 mM DTT). CAK activity was measured by adding 0.6 µg of recombinant human HA-Cdk2/Cyclin A (a gift from H. Espinoza and D.O. Morgan, University of California, San Francisco) in 20 µl [25 mM HEPES (pH 7.4), 10 mM MgCl2; 50 µM ATP; 10 µCi [γ-32P]ATP (3000 mCi/mmol), 1 mM DTT] and incubated at room temperature for 20 min. The supernatant was removed from the immunoprecipitate and boiled twice in one volume of sample buffer. For direct assessment of HA-Cdk2 phosphorylation the protein was run over a 10% polyacrylamide gel and the dried gel exposed for 2 hr with intensifying screens. For direct detection of Cdk2, samples were transferred to nitrocellulose and the HA-Cdk2 protein detected with anti-HA (12CA5 or HEA.11) antibody (BabCO) and SuperSignal (Pierce).

For histone H1 kinase assays on Cdk/cyclin complexes, 50 µl of control or mutant embryos (2–6 hr old) were homogenized in 300 µl of H0B and incubated 3 hr with 15 µl of protein G–agarose previously reacted with monoclonal anti-cyclins A, B, or E. The immunoprecipitates were washed three times with H0B, three times with HDS buffer [25 mM HEPES (pH 7.4), 1 mM DTT, 150 mM NaCl] and once with HD buffer. The immunoprecipitates were incubated with 50 µl of histone H1 kinase mix (3.0 µg of histone H1 (Boehringer), 25 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM DTT, 50 mM NaCl, 50 µM ATP, 10 µCi [γ-32P]ATP) and incubated at room temperature for 20 min. The reaction mixture was removed from the immunoprecipitate and boiled in one volume of SDS sample buffer. For reactivation by recombinant CAK, the immunoprecipitates from the previous step were washed several times with H0B and HD buffers as above and then incubated with 50 ng of recombinant human
Cdk7/Cyclin H in 20 µl (25 mM HEPES (pH 7.4), 10 mM MgCl₂, 1 mM DTT, 0.1 mM ATP) for 20 min. The beads were subsequently washed with HoB and HDS to remove the CAK. Finally, the histone H1 kinase activity was remeasured as described above.

For CAK assay on total lysates, samples (embryos or ovaries) were homogenized in EB buffer [10 mM Tris (pH 7.5), 80 mM Na⁺-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 2 mM Na₂VO₃, 1 mM benzamidine, 2 µg/ml of aprotinin, 2 mM PMSE, 1 µg/ml of leupeptin] (Edgar et al. 1994). The protein concentration in each sample was measured using Bradford protein assay (Bio-Rad) with BSA as standard, and the protein concentration in the lysates adjusted to ~30 µg/µl. Samples of 10 µl were mixed with 10 µl of assay mix [25 mM HEPES (pH 7.4), 10 mM MgCl₂, 0.1 mM ATP, 10 µCi of [γ-32P]ATP (3000 mCi/mmol), 1 mM DTT, 0.1 µg of HA–Cdk2/Cyclin A]. The reaction was stopped after 25 min by the addition of 500 µl of HoB + 20 mM EDTA. HA–Cdk2 was immunoprecipitated using anti-HA antibodies/protein G–agarose and the activity of the precipitated HA–Cdk2/Cyclin A complexes was assayed on histone H1 as described above. Quantitative data were obtained using a Fuji BASS 2000 TR PhosphorImager.

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Note

The sequence reported in this manuscript has been deposited into GenBank (accession no. U56661).

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