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Drosophila cdc2 homologs: a functional homolog is coexpressed with a cognate variant

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Using probes obtained by PCR amplification, we have cloned *Drosophila* cDNAs encoding structural homologs of the p34^{cdc2} cell cycle kinase. Southern blot experiments and *in situ* hybridization to polytene chromosomes demonstrated that the isolated cDNAs, were derived from two distinct genes, *Dm cdc2* (31E) and *Dm cdc2c* (92F). Northern blot and *in situ* hybridization experiments revealed that these two genes are coexpressed during embryogenesis and that expression is correlated with cell proliferation. However, despite the similarity in structure and expression, the two gene products differed in functional assays in yeasts. Expression of *Dm cdc2* in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* rescued cell cycle arrest caused by mutations in *cdc2*$^+$ and *CDC28*, the genes encoding the p34^{cdc2} kinase homologs of these yeasts. In contrast, the *Dm cdc2c* gene product did not restore cell cycle progression. Thus, in addition to the identification of a functional homolog in *Drosophila*, our results indicate the presence of a closely related cognate of the p34^{cdc2} cell cycle kinase.

Key words: cdc2/cdc2-cognate/cell cycle/complementation/*Drosophila*

Introduction

Several regulatory circuits controlling cell cycle progression converge on an evolutionarily conserved protein kinase. Activation of this p34^{cdc2} cell cycle kinase late in the G_2 phase appears to trigger entry into mitosis (Arion *et al.*, 1988; Draetta and Beach, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Booher *et al.*, 1989; Labbe *et al.*, 1989a,b; Moreno *et al.*, 1989). This activation is controlled by a number of regulators. It is dependent on the accumulation of cyclin proteins which are known to bind to the p34^{cdc2} kinase (Booher and Beach, 1988; Booher *et al.*, 1989; Draetta *et al.*, 1989; Labbe *et al.*, 1989b; Meijer *et al.*, 1989; Murray and Kirschner, 1989; Pines and Hunter, 1989). In addition to cyclins, the p13^{suc1} protein associates with the p34^{cdc2} kinase and stabilizes its activity (Hayles and Nurse, 1986; Brizuela *et al.*, 1987; Draetta *et al.*, 1987; Booher *et al.*, 1989; Hadwiger *et al.*, 1989; Moreno *et al.*, 1989). Moreover, p34^{cdc2} kinase activity is also controlled by protein phosphorylation: dephosphorylation appears to be the last step required for the activation of its mitosis promoting activity (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Labbe *et al.*, 1989a; Morla *et al.*, 1989). In *Schizosaccharomyces pombe*, this dephosphorylation is dependent on the *cdc25*$^+$ gene function (Russell and Nurse, 1986; Gould and Nurse, 1989).

While entry into mitosis depends on the activation of the p34^{cdc2} kinase, progression beyond metaphase appears to require its inactivation (Murray *et al.*, 1989). The inactivation is thought to be initiated by proteolytic degradation of cyclins during metaphase (Evans *et al.*, 1983; Murray *et al.*, 1989) and subsequently, rephosphorylation of the p34^{cdc2} kinase appears to maintain its inactivity during interphase (Draetta and Beach, 1988; Gould and Nurse, 1989). A putative serine/threonine kinase, the product of the *S.pombe wee1*$^+$ gene, was found genetically to inhibit entry into mitosis (Russell and Nurse, 1987), possibly by directly phosphorylating the p34^{cdc2} kinase. However, additional kinases appear to be involved in the regulation of the p34^{cdc2} activity, since in addition to serine and threonine residues (Potashkin and Beach, 1988; Gould and Nurse, 1989), a tyrosine residue in the ATP binding site of the p34^{cdc2} kinase is subject to phosphorylation (Gould and Nurse, 1989).

Beyond its central role in the regulation of mitosis, the p34^{cdc2} kinase is also crucial for entry into S phase, at least in *Saccharomyces cerevisiae* and *S.pombe*, where such a role has been demonstrated genetically (Reed, 1980; Nurse and Bissett, 1981).

The demonstration that a *S.cerevisiae* (*CDC28*) and a human homolog can substitute for the *S.pombe cdc2* gene (Beach *et al.*, 1982; Booher and Beach, 1986; Lee and Nurse, 1987), provided a striking indication that many of the regulatory interactions that impinge on p34^{cdc2} are evolutionarily conserved. The extensive structural similarity of these functional p34^{cdc2} homologs in *S.cerevisiae*, *S.pombe* and humans has allowed the generation of probes that detect p34^{cdc2} homologs in many species. Moreover, these homologs can be cloned not only by using complementation assays (Lee and Nurse, 1987) but also because of this extensive structural similarity (Krek and Nigg, 1989; this report). Nevertheless, the concordance of these two different approaches using either functional assays or structural criteria has not yet been addressed. This may prove to be an important issue since evolution has a tendency to create families of genes with related structures and functions. It appears plausible that different p34^{cdc2} homologs having distinctive regulatory features will be used at different times or in different tissues to impart specific regulatory features to cell cycle control during development in complex metazoan species. If this issue is to be addressed, *Drosophila* has two important assets: the genetics allows a test of function, and a tremendous base of information and techniques facilitate developmental studies.

Here we report the isolation of cDNA clones from two *Drosophila* genes encoding homologs of p34^{cdc2}. While only one complements defects in p34^{cdc2} in yeasts, both share all the sequence motifs presumed to characterize

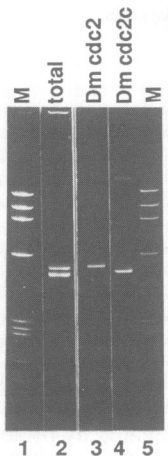

Fig. 1. PCR amplification of *Drosophila* cdc2 homologs. The products obtained in PCR experiments using a pair of primers specific for sequences conserved in all the known $p34^{cdc2}$ homologs were analyzed on polyacrylamide gels and visualized by staining with ethidium bromide. Lanes 1 and 5, mol. wt markers (M), *Hae*III digest of ϕX174 DNA; lane 2, PCR products amplified from total DNA of a λ gt10 cDNA library made from 3–12 h embryos (Poole *et al.*, 1985); lane 3, PCR product amplified from a phage λ containing a cDNA insert of the first class (*Dm cdc2*); lane 4, PCR product from a phage containing a cDNA insert of the second class (*Dm cdc2c*).

$p34^{cdc2}$. Since the two *Drosophila* genes are not the result of a recent duplication, it is likely that these sequences have been independently conserved.

Results

Identification of two structural $p34^{cdc2}$ homologs

We used the polymerase chain reaction (PCR) to identify a *Drosophila* gene encoding a $p34^{cdc2}$ homolog (Saiki *et al.*, 1985). The two primer sequences were chosen to match sequences encoding amino acid stretches particularly well conserved among the known $p34^{cdc2}$ homologs (see regions marked with asterisks in Figure 5). As template, we used total DNA from a phage λ cDNA library derived from early embryonic poly(A)$^+$ RNA (Poole *et al.*, 1985). On polyacrylamide gels, the PCR products were resolved as two closely spaced bands in the size range expected for products derived from cDNA encoding $p34^{cdc2}$ homologs (Figure 1, lane 2). These PCR products were labeled and used to probe the same cDNA library that had been used for the preparation of the PCR template DNA. Of nine positive phages, seven gave rise to a PCR product of the same size as the larger of the two products observed in PCR experiments with total library DNA (compare Figure 1, lanes 2 and 3), and two phages gave rise to a PCR product corresponding in size to the smaller of the two fragments (compare Figure 1, lanes 2 and 4).

The two phage classes contained inserts derived from two separate *Drosophila* genes. On Southern blots, a probe derived from a phage of the first class hybridized to a set of fragments distinct from that recognized by a probe derived from a phage of the other class (compare Figure 2A and B). Moreover, by *in situ* hybridizations, the first gene (*Dm cdc2*) was localized to the chromosomal region 31E (Figure 2C) and the second gene (*Dm cdc2c*, *c* for cognate) was found to be located in the chromosomal region 92F (Figure 2D).

Sequence analysis of the cDNA clones revealed extended

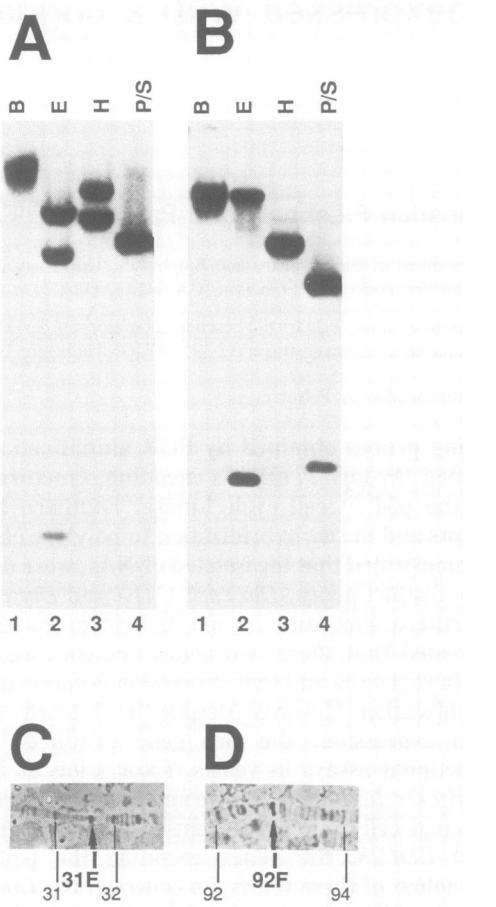

Fig. 2. *Dm cdc2* and *Dm cdc2c* cDNAs are derived from two distinct *Drosophila* genes. Panels **A** and **B**: total genomic DNA from *Drosophila* was digested with *Bam*HI (lanes 1), *Eco*RI (lanes 2), *Hin*dIII (lans 3), *Pst*I and *Sal*I (lanes 4) and probed on Southern blots with a *Dm cdc2* cDNA probe (panel A) or a *Dm cdc2c* cDNA probe (panel B). Panels **C** and **D**: a *Dm cdc2* cDNA probe (panel C) and a *Dm cdc2c* cDNA probe (panel D) were used to localize the corresponding genes on polytene chromosomes from salivary glands of third instar larvae.

open reading frames (*Dm cdc2*, Figure 3; *Dm cdc2c*, Figure 4). The putative proteins encoded by these open reading frames have all the sequence characteristics of $p34^{cdc2}$ kinases (see boxes in Figure 5). The amino acid sequence identities include but extend beyond the kinase consensus residues (Hanks *et al.*, 1988). For example, the PSTAIR motif, a stretch of 16 amino acids that characterizes $p34^{cdc2}$ homologs, is found in all these proteins except that the last residue is different in the *Dm cdc2c* protein (see black dots in Figure 5). Moreover, like other $p34^{cdc2}$ kinase homologs, the two *Drosophila* proteins have a tyrosine residue within the putative ATP biding site (see arrow in Figure 5). This residue is known to be important in *S.pombe* for cell cycle dependent regulation of the $p34^{cdc2}$ kinase activity by phospho-/dephosphorylation (Gould and Nurse, 1989).

The extent of amino acid identity determined after pairwise alignments is shown in Table I. The amino acid sequences of the two *Drosophila* proteins were at least 53% identical to any of the $p34^{cdc2}$ kinase homologs, and the highest similarity was found between the human $p34^{cdc2}$ kinase and

```
                                                  1                                  10
                                                  Met Glu Asp Phe Glu Lys Ile Glu Lys Ile Gly Glu Gly Thr Tyr Gly Val Val
GAAATTTAAAAGTCGGTGGCTTGCAAAGAAATAGCTTAATAAATT    ATG GAG GAT TTT GAG AAA ATT GAG AAG ATT GGC GAG GGC ACA TAT GGC GTG GTG

     20                                  30                                  40
Tyr Lys Gly Arg Asn Arg Leu Thr Gly Gln Ile Val Ala Met Lys Lys Ile Arg Leu Glu Ser Asp Asp Glu Gly Val Pro Ser Thr Ala
TAT AAA GGT CGC AAT CGC CTG ACG GGC CAA ATT GTG GCA ATG AAG AAA ATC CGC TTG GAG TCC GAC GAC GAA GGC GTT CCA TCA ACC GCG

     50                                  60                                  70
Ile Arg Glu Ile Ser Leu Leu Lys Glu Leu Lys His Glu Asn Ile Val Cys Leu Glu Asp Val Leu Met Glu Glu Asn Arg Ile Tyr Leu
ATC AGA GAA ATT TCG TTG CTT AAG GAG TTG AAA CAT GAA AAC ATT GTC TGT TTG GAG GAT GTT TTG ATG GAG GAG AAC CGC ATA TAC TTG

     80                                  90                                 100
Ile Phe Glu Phe Leu Ser Met Asp Leu Lys Lys Tyr Met Asp Ser Leu Pro Val Asp Lys His Met Glu Ser Glu Leu Val Arg Ser Tyr
ATC TTT GAA TTC CTA TCG ATG GAC CTC AAG AAA TAC ATG GAT TCG CTG CCA GTT GAT AAG CAC ATG GAG AGT GAA TTG GTC CGT AGC TAT

    110                                 120                                 130
Leu Tyr Gln Ile Thr Ser Ala Ile Leu Phe Cys His Arg Arg Arg Val Leu His Arg Asp Leu Lys Pro Gln Asn Leu Leu Ile Asp Lys
TTG TAC CAA ATA ACT AGC GCC ATT CTT TTC TGC CAT CGT CGG CGA GTA CTT CAC CGT GAT CTT AAG CCG CAG AAC TTA CTA ATC GAC AAG

    140                                 150                                 160
Ser Gly Leu Ile Lys Val Ala Asp Phe Gly Leu Gly Arg Ser Phe Gly Ile Pro Val Arg Ile Tyr Thr His Glu Ile Val Thr Leu Trp
AGT GGC CTC ATA AAA GTC GCC GAC TTT GGA CTT GGC CGA TCC TTT GGC ATT CCG GTG CGC ATT TAT ACG CAC GAG ATT GTT ACC TTG TGG

    170                                 180                                 190
Tyr Arg Ala Pro Glu Val Leu Leu Gly Ser Pro Arg Tyr Ser Cys Pro Val Asp Ile Trp Ser Ile Gly Cys Ile Phe Ala Glu Met Ala
TAC AGA GCG CCG GAG GTG CTA CTG GGT TCA CCC CGG TAT TCC TGT CCC GTC GAT ATC TGG TCC ATT GGA TGC ATA TTC GCG GAG ATG GCA

    200                                 210                                 220
Thr Arg Lys Pro Leu Phe Gln Gly Asp Ser Glu Ile Asp Gln Leu Phe Arg Met Phe Arg Ile Leu Lys Thr Pro Thr Glu Asp Ile Trp
ACG AGA AAG CCG CTA TTC CAG GGT GAC TCG GAA ATT GAC CAG TTG TTT AGA ATG TTT AGA ATT CTG AAA ACA CCT ACC GAA GAC ATT TGG

    230                                 240                                 250
Pro Gly Val Thr Ser Leu Pro Asp Tyr Lys Asn Thr Phe Pro Cys Trp Ser Thr Asn Gln Leu Thr Asn Gln Leu Lys Asn Leu Asp Ala
CCG GGC GTT ACT TCG CTA CCC GAC TAT AAG AAC ACG TTC CCC TGC TGG TCC ACG AAT CAA TTG ACC AAT CAG TTA AAG AAT CTC GAT GCG

    260                                 270                                 280
Asn Gly Ile Asp Leu Ile Gln Lys Met Leu Ile Tyr Asp Pro Val His Arg Ile Ser Ala Lys Asp Ile Leu Glu His Pro Tyr Phe Asn
AAT GGT ATT GAT CTC ATT CAA AAG ATG TTA ATC TAC GAT CCA GTT CAT CGC ATT TCC GCC AAG GAC ATT TTG GAG CAT CCC TAT TTC AAT

    290                         297
Gly Phe Gln Ser Gly Leu Val Arg Asn OC
GGT TTT CAA TCG GGC TTA GTT CGA AAT TAA CGTTCGGTATTCTCGTTTGACTTTAACTAAGAATTTTAAAACAAGAGATCTTGGTATCTAATCTAAAGCAAAATAGCCGTA


AATAAAACTAAGGGTGTAAAACAAAAAAAAAAAAAAAA
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Fig. 3. *Dm cdc2* cDNA sequence and deduced amino acid sequence. The sequence of an apparently full length *Dm cdc2* cDNA (1058 bp) is shown. The deduced amino acid sequence (297 amino acids) of the putative protein (34.4 kd) encoded by the cDNA is shown in three letter code above the cDNA sequence. In addition to the long open reading frame, the cDNA has a polyadenylation signal (underlined) and a poly(A) tail at the 3′ end.

Table I. Sequence identity (%) between $p34^{cdc2}$ kinase homologs

	Hs cdc2	*Sp cdc2*	*Sc CDC28*	*Dm cdc2*	*Dm cdc2c*	*PHO85*
Hs cdc2	100	62	56	69	53	47
Sp cdc2	62	100	60	60	54	45
Sc CDC28	56	60	100	55	53	50
Dm cdc2	69	60	55	100	56	48
Dm cdc2c	53	54	53	56	100	44

The extent of sequence identity (%) obtained after pairwise alignment of the proteins encoded by a human cdc2 homolog, the *S.pombe* $cdc2^{+}$ gene, the *S.cerevisiae* genes *CDC28* and *PHO85*, and the *Drosophila* genes *Dm cdc2* and *Dm cdc2c*, is tabulated. The sequences were according to Lee and Nurse, 1987 (*Hs cdc2*); Hindley and Phear, 1984 (*Sp cdc2*); Lörincz and Reed, 1984 (*Sc CDC28*); Toh-e *et al.*, 1988 (*PHO85*).

the *Drosophila Dm cdc2* protein (69% identity). Recently, sequences encoding putative protein kinases similar to the $p34^{cdc2}$ kinase homologs have been identified in *S.cerevisiae* (*PHO85*: Toh-e *et al.*, 1986; *KSS1*: Courchesne *et al.*, 1989), in humans (PSK-J: Hanks *et al.*, 1988) and in *Drosophila* (8−6: Kalderon and Rubin, 1988). All these kinases, however, are clearly less related to the $p34^{cdc2}$ kinase homologs than the two *Drosophila* sequences reported here. The extent of identity observed for the most closely related of these kinases, the *PHO85* gene product, is significantly less than that observed in the case of *Dm cdc2* and *Dm cdc2c* (Table I).

Interestingly, the two *Drosophila* sequences are not more related to each other (56% identity) than to the $p34^{cdc2}$ kinase homologs (see Table I). This suggests that the evolutionary separation of *Dm cdc2* and *Dm cdc2c* is ancient. Moreover, the major differences between the *Drosophila* homologs are confined to regions that are not conserved among known $p34^{cdc2}$ homologs. This argues that selective pressure conserved similar functions in the two *Drosophila*

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                                                                 1                                   10
                                                                 Met Thr Thr Ile Leu Asp Asn Phe Gln Arg
GACCAAAGAAGAAGAATTTCCCTCCAAAATATTAGTTTATTTTGCCAACATCGATCTCAAGGATTGTTTACGAGGTT   ATG ACC ACC ATT CTA GAT AAC TTT CAA CGC

                                    20                                      30                                      40
Ala Glu Lys Ile Gly Glu Gly Thr Tyr Gly Ile Val Tyr Lys Ala Arg Ser Asn Ser Thr Gly Gln Asp Val Ala Leu Lys Lys Ile Arg
GCC GAA AAG ATT GGC GAG GGC ACC TAC GGT ATA GTT TAC AAA GCG CGT AGC AAC TCC ACC GGC CAG GAT GTG GCC CTC AAA AAG ATT CGG

                                    50                                      60                                      70
Leu Glu Gly Glu Thr Glu Gly Val Pro Ser Thr Ala Ile Arg Glu Ile Ser Leu Leu Lys Asn Leu Lys His Pro Asn Val Val Gln Leu
CTA GAA GGC GAA ACG GAG GGT GTT CCT TCG ACG GCC ATT CGA GAG ATC TCC CTG CTG AAG AAC CTT AAG CAC CCA AAT GTG GTC CAA CTA

                                    80                                      90                                     100
Phe Asp Val Val Ile Ser Gly Asn Asn Leu Tyr Met Ile Phe Glu Tyr Leu Asn Met Asp Leu Lys Lys Leu Met Asp Lys Lys Lys Asp
TTT GAC GTA GTC ATT TCC GGC AAC AAT CTG TAC ATG ATA TTC GAG TAC CTG AAC ATG GAT CTA AAG AAG CTG ATG GAT AAG AAA AAA GAC

                                   110                                     120                                     130
Val Phe Thr Pro Gln Leu Ile Lys Ser Tyr Met His Gln Ile Leu Asp Ala Val Gly Phe Cys His Thr Asn Arg Ile Leu His Arg Asp
GTG TTC ACC CCT CAG TTG ATA AAG AGC TAT ATG CAT CAG ATA TTA GAT GCC GTC GGC TTT TGC CAC ACG AAT CGT ATC CTG CAT CGC GAT

                                   140                                     150                                     160
Leu Lys Pro Gln Asn Leu Leu Val Asp Thr Ala Gly Lys Ile Lys Leu Ala Asp Phe Gly Leu Ala Arg Ala Phe Asn Val Pro Met Arg
CTC AAG CCC CAG AAC CTT CTC GTA GAC ACG GCG GGC AAA ATA AAG TTG GCT GAC TTT GGC CTA GCA AGG GCC TTC AAC GTG CCT ATG CGG

                                   170                                     180                                     190
Ala Tyr Thr His Glu Val Val Thr Leu Trp Tyr Arg Ala Pro Glu Ile Leu Leu Gly Thr Lys Phe Tyr Ser Thr Gly Val Asp Ile Trp
GCG TAC ACA CAC GAA GTC GTC ACC CTC TGG TAC CGA GCT CCA GAG ATT CTG TTG GGC ACG AAA TTC TAC TCC ACG GGC GTG GAC ATC TGG

                                   200                                     210                                     220
Ser Leu Gly Cys Ile Phe Ser Glu Met Ile Met Arg Arg Ser Leu Phe Pro Gly Asp Ser Glu Ile Asp Gln Leu Tyr Arg Ile Phe Arg
AGT CTA GGC TGC ATT TTC TCT GAA ATG ATT ATG CGC CGC TCC TTG TTT CCT GGA GAC AGC GAG ATC GAT CAA CTT TAT AGG ATT TTC CGT

                                   230                                     240                                     250
Thr Leu Ser Thr Pro Asp Glu Thr Asn Trp Pro Gly Val Thr Gln Leu Pro Asp Phe Lys Thr Lys Phe Pro Arg Trp Glu Gly Thr Asn
ACC TTA AGC ACA CCT GAT GAA ACA AAT TGG CCT GGT GTG ACG CAG CTG CCA GAC TTT AAG ACC AAG TTC CCT AGA TGG GAG GGA ACT AAC

                                   260                                     270                                     280
Met Pro Gln Pro Ile Thr Glu His Glu Ala His Glu Leu Ile Met Ser Met Leu Cys Tyr Asp Pro Asn Leu Arg Ile Ser Ala Lys Asp
ATG CCA CAA CCC ATA ACC GAA CAC GAG GCG CAC GAA CTC ATA ATG TCA ATG CTG TGC TAT GAT CCC AAC CTG CGC ATC TCA GCC AAG GAC

                                   290                                     300                                     310
Ala Leu Gln His Ala Tyr Phe Arg Asn Val Gln His Val Asp His Val Ala Leu Pro Val Asp Pro Asn Ala Gly Ser Ala Ser Arg Leu
GCA CTG CAG CAC GCT TAC TTC CGC AAT GTG CAG CAT GTT GAC CAT GTA GCC CTG CCT GTA GAT CCC AAT GCC GGC AGC GCT TCG CGT CTA

            314
Thr Arg Leu Val OP
ACG CGG CTC GTC TGA TCGTGTCCAATAGCCCCAGATCTAGCAAATTACTTGTGTTTTCTAGTATACTGCCACCTCATTATCATGCATTCCCCATCCTCACTCGCACGTAACATACGA

TAAATACACGATCATCCATAAGTAAAAAAAAAAAAAAA
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Fig. 4. *Dm cdc2c* cDNA sequence and deduced amino acid sequence. The sequence derived from the two *Dm cdc2c* cDNAs is shown (see Materials and methods for differences between the two cDNAs). The deduced amino acid sequence (314 amino acids) of the putative protein (35.9 kd) is shown in three letter code above the cDNA sequence. The putative polyadenylation signal preceding the poly(A) tail is underlined.

homologs and their functions are related to those of the other p34^{cdc2} homologs. While *Dm cdc2* is somewhat more homologous to other p34^{cdc2} sequences (average 60% identity), the homology of *Dm cdc2c* (average 54% identity) is comparable with that of *CDC28* (average 54.8% identity).

Expression of the Drosophila p34^{cdc2} homologs parallels cell proliferation

The spatial and temporal expression of *Dm cdc2* and *Dm cdc2c* mRNAs are similar and are correlated with developmental periods of cell proliferation. Northern blots revealed a single band of ~1.1 kb whether hybridized with probes specific for *Dm cdc2* or *Dm cdc2c* (Figure 6, lanes 1 and 2). The intensity of the *Dm cdc2* signals (Figure 6, lanes 2–5) and the *Dm cdc2c* signals (not shown) changed in parallel in RNAs from different developmental stages. Relatively high levels of transcripts were detected in the extremely rapid mitotic cycles preceding cellularization (Figure 6, lane 2), and during the post-blastoderm cell divisions following cellularization (Figure 6, lane 4). In contrast, mRNA levels were somewhat lower during the short quiescent interval accompanying cellularization (Figure 6, lane 3), and much lower in late embryos when only a few cells continue to divide (Figure 6, lane 5). The Northern blot experiment also indicated that the transcripts present in early embryos (Figure 6, lanes 1 and 2) are maternally derived, since zygotic transcription has not yet started in these early embryos (Zalokar and Erk, 1976).

In situ hybridization (data not shown) detected uniform levels of both *Dm cdc2* and *Dm cdc2c* mRNAs in early embryos but the signals became gradually restricted to the developing nervous system after germband retraction. At these later stages, proliferation is restricted primarily to the nervous system (Hartenstein and Campos-Ortega, 1985). Thus, the expression of the two genes appears to be correlated both temporally and spatially with cell proliferation.

Dm cdc2 but not Dm cdc2c provides p34^{cdc2} function in yeasts

In order to test whether *Dm cdc2* and *Dm cdc2c* complement mutations in the genes encoding the p34^{cdc2} kinase homologs in *S.cerevisiae* (*CDC28*) and *S.pombe* (*cdc2*$^+$),

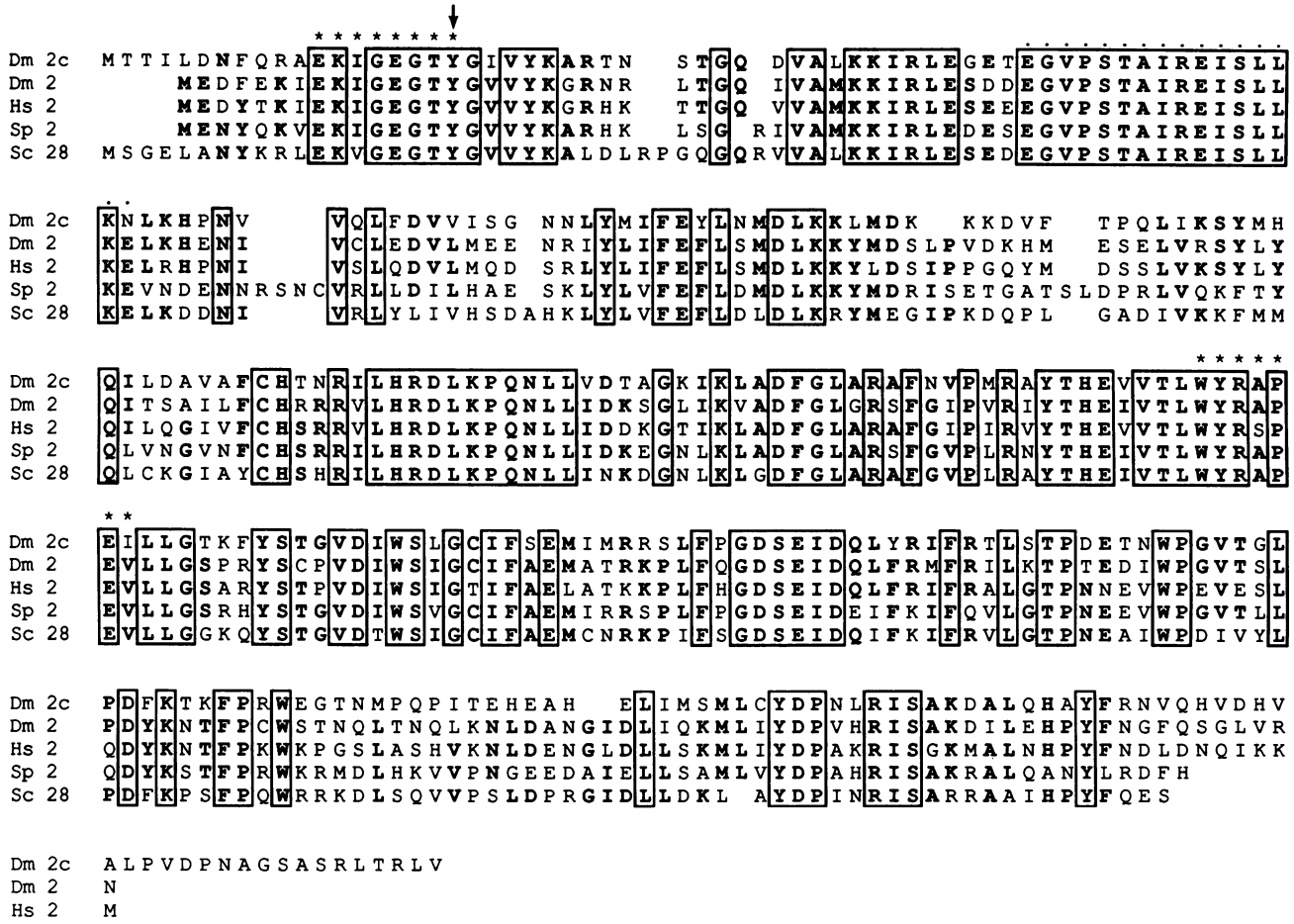

Fig. 5. Comparison of the amino acid sequences of the *Drosophila Dm cdc2* and *Dm cdc2c* protein with $p34^{cdc2}$ homologs from humans, *S.pombe* and *S.cerevisiae*. The proteins encoded by the *Drosophila* genes (*Dm cdc2c* (Dm 2c) and *Dm cdc2* (Dm 2), a human homolog (Hs 2), the *S.pombe* gene $cdc2^{+}$ (Sp 2) and the *S.cerevisiae* gene *CDC28* (Sc 28) are aligned. Positions identical in all five sequences are boxed. Bold letters indicate identity in at least three of the five sequences. Black dots designate the PSTAIR region, a highly conserved stretch of amino acids that so far has been found only in $p34^{cdc2}$ kinase homologs. The arrow indicates a conserved tyrosine in the putative ATP binding site. The asterisks designate the regions that were selected for the design of the cdc2 PCR primers.

we transformed temperature sensitive mutant yeast strains with plasmids allowing the expression of the *Drosophila* proteins in these yeasts (see Materials and methods). In both fission (data not shown) and budding yeast (see below), expression of *Dm cdc2* but not of *Dm cdc2c* allowed the transformed mutant cells to grow at the restrictive temperature.

Since $p34^{cdc2}$ is required for both the transition from G_1 phase to S phase and from G_2 phase to M phase, complementation of the yeast mutations requires that the foreign gene rescues both processes. In *S.cerevisiae*, bud formation provides a convenient marker for cell cycle progression from G_1 to S, so that complementation of this step can be scored independent of G_2 to M.

A *S.cerevisiae* strain carrying the temperature sensitive cdc28-4 allele was transformed with constructs allowing regulated expression of either *Dm cdc2* or *Dm cdc2c* from the *GAL1* promoter (Johnston and Davis, 1984). Following a shift to the restrictive temperature in the absence of galactose (no expression from the *GAL1* promoter) large unbudded cells accumulated (Figure 7a, e, i) as expected for arrest prior to S phase (Hartwell *et al.*, 1974; Reed, 1980). Addition of galactose induces the transcription from the *GAL1* promoter and restores cell cycle progression in cells transformed with *Dm cdc2*. After 1 h 50% of these cells had small buds (Figure 7b), after 2 h nearly all cells had large buds (Figure 7c), and after 3 h unbudded cells and cells with small buds reappeared (Figure 7d).

Although it restored cell cycle progression, the expression of the *Drosophila Dm cdc2* protein did not fully restore wild-type behavior. Many daughter cells apparently failed to separate completely yet budding continued (Figure 7d, arrow). This effect appeared to be dominant: induction of *Dm cdc2* expression at the permissive temperature caused a significant reduction of the growth rate, which was not observed in the presence of glucose or with cells transformed with vector alone (not shown).

Surprisingly, in contrast to *Dm cdc2*, expression of *Dm cdc2c* did not restore cell cycle progression at the restrictive temperature. Cells transformed with *Dm cdc2c* did not produce buds after induction with galactose (Figure 7i–l), and cells merely enlarged to produce abnormal shapes as also observed with cells transformed with *Dm cdc2* when grown in the presence of glucose (no expression from the *GAL1* promoter) (Figure 7e–h), and with cells transformed with control plasmids (not shown).

Immunoblots confirmed that the *Dm cdc2c* protein was expressed after induction with galactose. A mouse antiserum

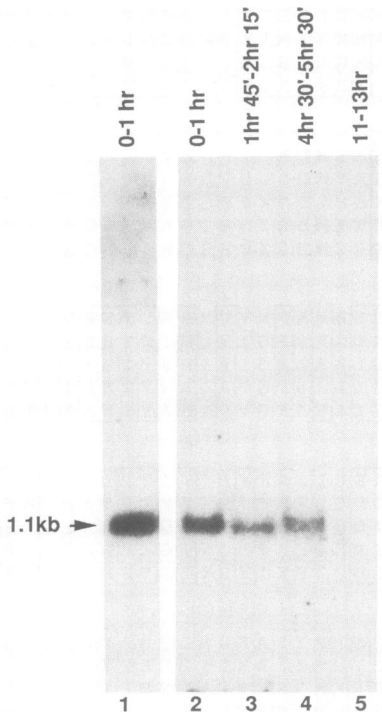

Fig. 6. Expression of *Dm cdc2* and *Dm cdc2c*. Total RNA from different developmental stages were probed on Northern blots with a *Dm cdc2c* cDNA (lane 1) or a *Dm cdc2* cDNA probe (lanes 2–5). The estimated size of the transcript is indicated on the side. RNA was isolated from embryos aged at 25°C for the times indicated above the lanes.

specific for *Dm cdc2c* protein (see Materials and methods) revealed a band at the expected position in extracts from induced but not uninduced cells (Figure 8a, lanes 3 and 4). No cross reaction was seen in extracts from cells carrying the *Dm cdc2* construct (Figure 8A, lanes 1 and 2). Immunoblotting with an anti-peptide antiserum (see Materials and methods) directed against the PSTAIR region common to both *Dm cdc2* and *Dm cdc2c* indicated that induction with galactose caused overexpression of both proteins to similar levels (compare lanes 2 and 4 in Figure 8B). Therefore, we conclude that the different potential to rescue at the restrictive temperature indicates a functional difference between the *Dm cdc2* and *Dm cdc2c* proteins.

Immunological characterization of Drosophila p34^{cdc2} homologs

Various immunological probes have been developed to detect p34^{cdc2} in a number of species. These are generally presumed to detect a unique product. We have characterized the immunoreactivity of *Dm cdc2* and *Dm cdc2c*, and examined the expression of p34^{cdc2} homologs in *Drosophila* using several immunological reagents. In addition to the mouse antiserum specific for *Dm cdc2c* and the PSTAIR antiserum described above, we also tested two other antibodies: a monoclonal antibody (J-4) that was made against *S.pombe* p34^{cdc2} and characterized as detecting a conserved epitope (Draetta *et al.*, 1987), and finally, affinity purified antibodies against chicken p34^{cdc2} (a gift of W.Krek and E.A.Nigg). Reactivity with *Dm cdc2* and *Dm cdc2c* protein was compared in immunoblotting experiments using equal amounts of bacterially produced fusion proteins (see Materials and methods). The monoclonal antibody J-4 had a much higher affinity for *Dm cdc2c* protein

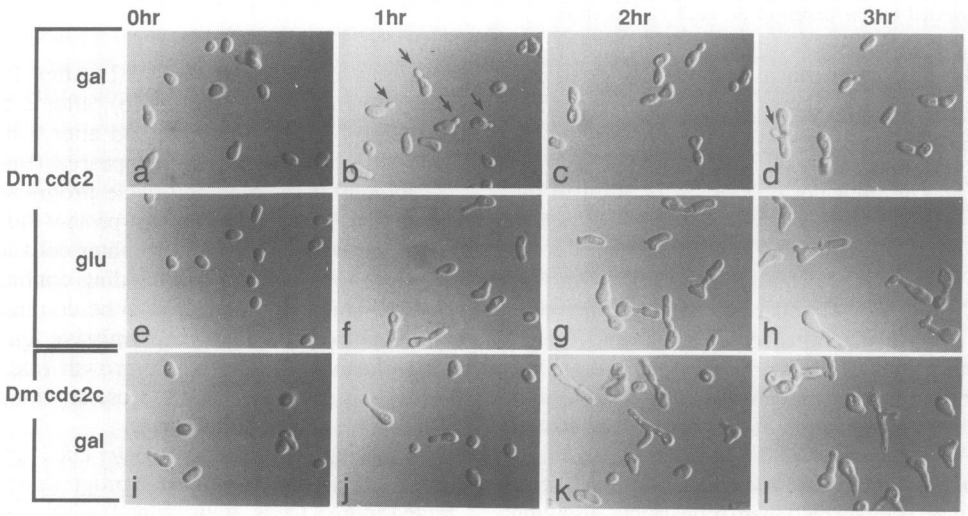

Fig. 7. Complementation of *CDC28* function after *Dm cdc2* and *Dm cdc2c* expression in *S.cerevisiae*. *cdc28-4* cells carrying the plasmids yCpG2[*Dm cdc2*] or yCpG2[*Dm cdc2c*] were arrested at START by a 3 h incubation at the restrictive temperature (36°C). *Dm cdc2* expression was induced by addition of galactose (gal) and aliquots were fixed for microscopic analysis after (**a**) 0 h, (**b**) 1 h, (**c**) 2 h and (**d**) 3 h. As indicated by the appearance of small buds (arrows in b) that subsequently developed into large buds (c), cell cycle progression is rescued by the expression of *Dm cdc2*. Some cells with an aberrant morphology were seen after prolonged incubations (arrow in d) indicating that *Dm cdc2* expression does not restore complete wild-type behavior. (**e–h**) No budding was apparent after addition of glucose (glu) which represses *Dm cdc2* expression. In contrast to *Dm cdc2*, induction of *Dm cdc2c* expression (**i–l**) did not rescue cell cycle progression.

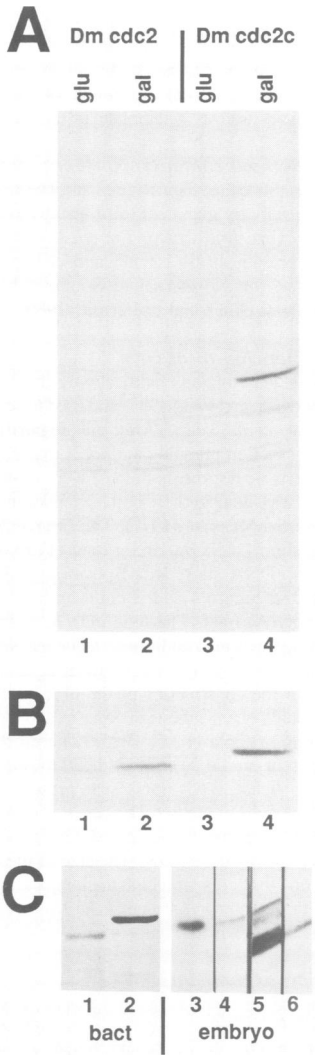

Fig. 8. Immunological detection of *Dm cdc2* and *Dm cdc2c* expression. Panels **A** and **B**: extracts from *cdc28-4* cells transformed with yCpG2[*Dm cdc2*] (lanes 1 and 2) or yCpG2[*Dm cdc2c*] (lanes 3 and 4) and grown in the presence of the repressor glucose (lanes 1 and 3) or the inducer galactose (lanes 2 and 4) were probed on immunoblots with an antiserum against *Dm cdc2c* protein (panel A) or an antiserum against a peptide (PSTAIR region, see black dots in Figure 5) present in all the known $p34^{cdc2}$ homologs (panel B). Only the region around 34 kd is shown in panel B. **C**: bacterial extracts containing equal amounts of either *Dm cdc2* (lane 1) or *Dm cdc2c* fusion proteins (lane 2), or extracts from 0–2 h *Drosophila* embryos (lanes 3–6) were probed on immunoblots with the monoclonal antibody J-4 (lanes 1–3), a mouse antiserum against *Dm cdc2c* (lane 4), an anti-PSTAIR antiserum (lane 5) or affinity purified antibodies against chicken $p34^{cdc2}$ (lane 6). Bound antibodies were visualized using phosphatase coupled secondary antibodies (lanes 1,2,4–6) or for increased sensitivity ^{125}I-iodinated secondary antibodies (lane 3). Only the region around 34 kd is shown.

than for the *Dm cdc2* protein (Figure 8C, compare lanes 1 and 2). On the other hand, the affinity purified antibodies against chicken $p34^{cdc2}$ gave a strong signal with *Dm cdc2* protein and reacted only weakly with *Dm cdc2c* protein (not shown).

In immunoblotting experiments with total protein from 0–2 h embryos, J-4 recognized a single band with the size expected or *Dm cdc2c* (Figure 8C, lane 3). This band was also detected by the mouse antiserum against *Dm cdc2c* (Figure 8C, lane 4). Moreover, a signal at this position was also detected with the PSTAIR antiserum (Figure 8C, lane 5). However, the PSTAIR antiserum gave also a second, more prominent signal at the position expected for *Dm cdc2* (Figure 8C, lane 5). This lower molecular weight band was also recognized by the anti-chicken $p34^{cdc2}$ antibodies (Figure 8C, lane 6). Although we cannot exclude conclusively that other, unidentified $p34^{cdc2}$ homologs contribute to these immunological signals, the immunoblotting experiments suggest that both *Dm cdc2* and *Dm cdc2c* protein are expressed in early *Drosophila* embryos. According to the signal intensities observed with the anti-PSTAIR serum (Figure 8C, lane 5), which reacts with comparable affinities with both *Drosophila* proteins, *Dm cdc2c* appears to be less abundant than *Dm cdc2*. In addition, Northern blots (see above) and immunoblots agree in detected reduced signals in extracts from older (15–17 h) embryos (not shown).

Discussion

Based on molecular homology we have identified two *Drosophila* genes encoding proteins homologous to the $p34^{cdc2}$ kinases of *S.pombe*, *S.cerevisiae* and humans (Hindley and Phear, 1984; Lörincz and Reed, 1984; Lee and Nurse, 1987). One of these homologs (*Dm cdc2*) can substitute for the yeast $p34^{cdc2}$ kinases. While this result suggests a parallel between structural and functional homology, our isolation of a second $p34^{cdc2}$ homolog (*Dm cdc2*) that fails the yeast complementation tests emphasizes three areas of ambiguities that can arise when structural and functional homology are compared.

Extensive structural homology does not necessarily imply functional homology. The *Dm cdc2* protein appears to possess all the sequence hallmarks of the functional $p34^{cdc2}$ kinases. That is, every region containing two or more contiguous residues that are identical in the four functional $p34^{cdc2}$ sequences (*S.pombe cdc2*, *S.cerevisiae CDC28*, the human *cdc2* homolog and *Dm cdc2*) is also conserved in the *Dm cdc2c* sequence (Figure 5). The structural resemblance of *Dm cdc2c* to the other complementing $p34^{cdc2}$ homologs is further emphasized by the following comparison. At 13 isolated positions where the sequence is invariant among the four functional $p34^{cdc2}$ homologs, the *Dm cdc2c* sequence differs. Surprisingly, the corresponding number of differences unique to the *S.cerevisiae* $p36^{cdc28}$ protein is even higher. Thus the inability of *Dm cdc2c* to restore cell cycle progression in yeast complementation studies is not paralleled by a deficit in any sequences thought to characterize functional $p34^{cdc2}$ kinases, and we expect that the *Dm cdc2c* kinase interacts with similar or with some of the same components that interact with *Dm cdc2*.

If a gene fails to complement defects in the yeast $p34^{cdc2}$ homologs it might nevertheless encode a $p34^{cdc2}$ kinase function in the organism from which it is obtained. Though the human $p34^{cdc2}$ kinase and the *Dm cdc2* kinase complement $p34^{cdc2}$ defects in yeast they do not restore complete wild-type behavior. This is not surprising since complementation in a foreign species is a stringent test of function, and species specific regulatory interactions may exist. The same reasoning would suggest that the activity of some $p34^{cdc2}$ kinase homologs may have an absolute dependence on species specific interactions. Indeed, the evolutionary

divergence of *Dm cdc2c* appears to have been constrained so as to maintain sequences characteristic of functional $p34^{cdc2}$ kinases, while the rest of the sequence has diverged completely. Since the two *Drosophila* proteins are not more related to each other than to the other known homologs, they are not the result of a recent gene duplication. Consequently, the *Dm cdc2c* sequence must be ancient and preserved by functional constraints. The pattern of sequence conservation of *Dm cdc2c* is most easily explained if its functions are similar or overlap those of the complementing $p34^{cdc2}$ homologs.

Molecular probes that are selected for their ability to detect $p34^{cdc2}$ homologs are unlikely to be specific for a unique product. In our characterization, we found that PCR reactions amplified both *Drosophila* homologs equally, and that $p34^{cdc2}$ specific antibodies exhibited unexpected reactivities with *Dm cdc2* and *Dm cdc2c* proteins. Thus, antibodies to the highly conserved PSTAIR peptide react with both homologs. More surprisingly, the monoclonal antibody J-4, which was selected as a probe for an epitope conserved in both $p34^{cdc2}$ from *S.pombe* and $p36^{cdc28}$ from *S.cerevisiae*, preferentially recognizes the *Dm cdc2c* protein in *Drosophila*. Consequently, the human antigen detected by J-4 might represent a human homolog of *Dm cdc2c* rather than the human $p34^{cdc2}$ kinase homolog (Draetta *et al.*, 1987). Finally, an affinity purified antibody against the chicken $p34^{cdc2}$ homolog gave a strong signal with *Dm cdc2* and a weak signal with *Dm cdc2c*. Since the sequences common to *Dm cdc2* and *Dm cdc2c* are also conserved among the $p34^{cdc2}$ kinases from different species, the utility of immunological reagents that detect conserved epitopes is likely to be compromised by cross reaction with proteins related to the $p34^{cdc2}$ kinase.

It is not yet clear how diverse the family of *Drosophila* $p34^{cdc2}$ homologs might be, however preliminary observations suggest that there are not many genes that share the level of homology of *Dm cdc2* and *Dm cdc2c*. An independent screen for *Drosophila* genes capable of complementing $p34^{cdc2}$ kinase fusion in *S.pombe* identified only a single gene, *Dm cdc2* (J.Jimenez, D.Glover and P.Nurse, personal communication). Additionally, immunoblots using *Drosophila* extracts reveal a limited degree of heterogeneity that appears to be accounted for by *Dm cdc2* and *Dm cdc2c* products.

It will be possible to assess the roles of the *Dm cdc2* and *Dm cdc2c* proteins after isolation of mutations in the corresponding genes. Analysis of the *Dm cdc2* and *Dm cdc2c* gene products in *Drosophila* might detect regulatory interactions unique to the development of a multicellular organism where cell cycle progression is not primarily coupled to growth as it is in yeasts but is integrated in the developmental processes of morphogenesis and differentiation.

Materials and methods

PCR experiments

The sequences of the primers used for enzymatic amplification (Saiki *et al.*, 1985) of *Drosophila cdc2* homologs were:

Primer 1: 5′-GAGAAGAT(CT)GG(ACGT)GAGGG(ACGT)AC(TC)TA-3′

Primer 2: 5′-ACTTCGGG(ACGT)GC(ACGT)CG(AG)TACCA-3′

For the isolation of template DNA, a λgt10 cDNA library prepared from poly(A)$^+$ RNA from 3–12 h embryos (Poole *et al.*, 1985) was plated and phage DNA was isolated from the plate stock as described in Maniatis *et al.* (1982). Total library DNA 3 μg was used in a 100 μl reaction containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM dATP, dGTP, dCTP, TTP, 5 μM primer 1, 5 μM primer 2 and 2.5 U Taq polymerase. After an initial 2 min at 94°C, 30 cycles of amplification were carried out (1 min at 94°C, 2 min at 55°C and 3 min at 72°C). Enzymatic amplifications for the characterization of isolated λ phages were done using the same conditions except that 10 μl of phage stock in SM (Maniatis *et al.*, 1982) was boiled for 5 min after adding 10 μl of 10 mM EDTA and used as a template instead of purified λ DNA.

In situ hybridization experiments, Northern and Southern blots

In situ hybridizations to localize the *Dm cdc2* and *Dm cdc2c* gene on polytene chromosomes from salivary glands of third instar larvae, as well as *in situ* hybridizations to localize *Dm cdc2* or *Dm cdc2c* mRNA in embryos were done as described previously by Lehner and O'Farrell (1990).

The methods used for Northern and Southern blots have also been described previously (Lehner and O'Farrell, 1989, 1990).

Cloning and sequencing of cDNAs

PCR products were gel isolated, radioactively labeled using the random primer method (Hodgson and Fisk, 1987) and used to screen a λ gt10 3–12 h cDNA library (Poole *et al.*, 1985). After plaque purification, phages were characterized by restriction mapping and PCR experiments. For sequence determination, inserts or fragments thereof were cloned into M13 vectors and the sequence was analyzed in both orientations using the dideoxy sequencing method (Sanger *et al.*, 1977). The *Dm cdc2c* sequence presented is derived from a short (1054 bp) and a long (1750 bp) cDNA that were isolated. The short cDNA starts at the position of amino acid 18 of the sequence presented in Figure 4 and is colinear with the long cDNA until its end after the poly(A) tract. The long cDNA, apart from being longer at the 5′ end (131 bp), has also additional sequences after the poly(A) tract (565 bp) and ends with a poly(G) tract indicating that this 3′ extension is artifactual, since for the construction of the library, oligo(dG) tailing was used after first strand cDNA synthesis in order to allow priming of the second strand with oligo(dC) (Poole *et al.*, 1985). Therefore, poly(C) tracts are expected to be present on the 5′ ends of the cDNAs, and all the analyzed cDNAs had in fact such poly(C) tracts. The presence of the poly(G) tract at the 3′ end on the longer of the two *Dm cdc2c* cDNAs would be explained if an unrelated cDNA fragment was ligated during the linker ligation step, head to tail, onto the *Dm cdc2c* cDN. The size of the *Dm cdc2c* transcript (1.1 kb) detected on Northerns with both the long and the short cDNA probe was consistent with the interpretation that the *Dm cdc2c* mRNA ends after the poly(A) tract, as shown in Figure 4.

Complementation experiments

For the complementation experiments in *S.pombe*, *Dm cdc2* and *Dm cdc2c* cDNAs were cloned into the expression vector pSM2 (Jones *et al.*, 1988) containing the *LEU2* gene as a selectable marker. Spheroplasts prepared from the *S.pombe* strain h^- *cdc2-33 leu1-32* (Nurse *et al.*, 1976) were transformed with the vector or with the constructs containing the *Drosophila* cDNAs as described by Beach *et al.* (1982). Leu^+ transformants were selected by plating cells on minimal plates. In order to assay the potential of these plasmids to rescue growth at the restrictive temperature, half of the transformation was plated at 36°C. The other half of the transformation was grown at the permissive temperature (25°C) in order to control for the success of the transformations.

For the complementation experiments in *S.cerevisiae*, *Dm cdc2* and *Dm cdc2c* cDNAs were cloned into the expression vector yCpG2 (pGAL1 LEU2 URA3 CEN4) (Richardson *et al.*, 1989). Cells from the strain BF264-15D, *leu2-33, 112 cdc28-4* (Reed, 1980) were transformed (Ito *et al.*, 1983) with either vector or constructs containing the cDNAs, and leu^+ transformants were selected. Transformants were grown to mid log phase at room temperature in leucine-free medium containing 2% raffinose as a non-inducing carbon source. Subsequently, cells were shifted to the restrictive temperature (36°C) and cultured for 3 h to arrest cell cycle progression at START (Reed, 1980). Glucose was added to one half of the culture (2% final concentration) and galactose to the other half of the culture (2% final concentration). Aliquots were taken at 1 h intervals, and after brief sonication to disperse cell aggregates, samples were counted or fixed in 70% ethanol for microscopic analysis using a 100× objective on a Nikon optiphot microscope equipped with differential interference contrast (Nomarski) optics.

Immunoblotting experiments

For the generation of a mouse antiserum against the *Dm cdc2c* protein, a fusion protein was produced in *Escherichia coli* as described by Studier and Moffat (1986) after cloning the *Dm cdc2c* cDNA into the *Bam*HI site of the expression vector pAR 3040. *Bam*HI sites immediately before the initiation codon and after the stop codon were introduced by using appropriate

primers in PCR experiments. An analogous construct allowing expression of *Dm cdc2* was used for the characterization of antibody specificities. The fusion proteins produced from these constructs have 11 amino acids from a T7 coat protein fused to either full length *Dm cdc2* or *Dm cdc2c* protein. After gel isolation, *Dm cdc2c* fusion protein emulsified in Freund's complete adjuvant was injected intraperitoneally into mice. After two booster injections using the immunogen in Fruend's incomplete adjuvant, serum was isolated.

Yeast extracts for immunoblotting experiments were made by boiling cells in SDS gel sample buffer for 2 min. Subsequently, an equal volume of glass beads was added, cells were vortexed for 2 min and boiled again for 2 min. Insoluble material was sedimented by centrifugation and the extracts were loaded on SDS polyacrylamide gels (Laemmli, 1970). *Drosophila* embryo extracts were made as described previously (Lehner and O'Farrell, 1989). Protein transfer to nitrocellulose and immunological detection of antigens was done as described previously (Lehner *et al.*, 1987) except that phosphatase-coupled secondary antibodies (Boehringer Mannheim Biochemicals) were used in most experiments. The mouse antiserum against *Dm cdc2c* and the rabbit antiserum against the PSTAIR peptide (a gift of M.Solomon and M.Kirschner, University of California at San Francisco) were used at a dilution of 1:300. Ascites fluid (a gift of G.Draetta and D.Beach, Cold Spring Harbor Laboratory) containing the mouse monoclonal antibody J-4 (Draetta *et al.*, 1987) was diluted 1:500 and the affinity purified antibodies against chicken $p34^{cdc2}$ (a gift of W.Krek and E.A.Nigg, Lausanne) was diluted 1:200.

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