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Genetic analysis of the *Drosophila cdc2* homolog

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SUMMARY

We have identified mutations in the *Drosophila cdc2* gene. The recessive lethality of these mutant alleles was rescued after P-element-mediated transformation with a genomic *cdc2* fragment. Sequence analysis of amorphic alleles revealed non-conservative exchanges in evolutionary conserved positions. These alleles caused lethality at the larval-pupal interphase due to the absence of imaginal tissues. Embryonic lethality resulted when the maternal *Dm cdc2* contribution was reduced through the use of a temperature-sensitive allele. *Dm cdc2* function, therefore, is essential for cell proliferation throughout development. *Dm cdc2* function is clearly required for mitosis, but no evidence for a requirement in S-phase was obtained. The reversible block of the mitotic proliferation which was observed in the PNS of mutant embryos occurred exclusively in the G_2-phase. Moreover, while the mitotic proliferation of imaginal cells was blocked in the amorphic mutant larvae, non-imaginal larval cells continued to grow and endoreplicate their DNA. The *Dm cdc2* mutant phenotype could neither be rescued with *Dm cdc2c* (encoding a cdc2-like kinase) nor enhanced by a reduction of the *Dm cdc2c* gene dose. These results indicate that the Dm cdc2- and Dm cdc2c-kinases control different processes.

Key words: *cdc2*, *cdc2c*, cell cycle, *Drosophila*, proliferation

INTRODUCTION

The $p34^{cdc2}$ kinase of *Schizosaccharomyces pombe* and the homologous $p34^{CDC28}$ kinase of *Saccharomyces cerevisiae* regulate the progression through the cell cycle at two important control points. They are required for progression through START (G_1/S-transition) and for entry into mitosis (G_2/M-transition) (Hereford and Hartwell, 1974; Nurse and Thuriaux, 1980; Nurse and Bissett, 1981; Piggott et al., 1982). Analyses in *Drosophila*, *Xenopus*, and humans have not only revealed cdc2 homologs that are able to complement efficiently mutations in the homologous yeast genes but also have revealed additional cdc2-related genes which do not or only inefficiently complement despite extensive similarity (Lee and Nurse, 1987; Lehner and O'Farrell, 1990a; Paris et al., 1991; Elledge and Spottswood, 1991; Milarski et al., 1991; Ninomiya-Tsuji et al., 1991; Tsai et al., 1991). The different cdc2-related kinases of vertebrates appear to be specialized for either the G_1/S- or the G_2/M-transition. While depletion of the $p34^{cdc2}$ kinase from *Xenopus* extracts prevents M-phase, depletion of the cdc2-related kinase $p33^{cdk2}$ prevents S-phase (Fang and Newport, 1991). Observations in humans are also consistent with the idea that the G_1/S transition is controlled by the $p33^{cdk2}$ kinase in higher eukaryotes and the G_2/M transition by the $p34^{cdc2}$ kinase (Riabowol et al., 1989; Th'ng et al., 1991; Hamaguchi et al., 1992; Pagano et al., 1992; Rosenblatt et al., 1992; G. Draetta, personal communication).

For entry into M-phase in all eukaryotes, the $p34^{cdc2}$ kinase has to be activated. This activation of the $p34^{cdc2}$ kinase is controlled by an evolutionary conserved network of regulators (for reviews see Nurse, 1990; Pines and Hunter, 1991). In a first step, $p34^{cdc2}$ associates with the regulatory subunit cyclin B and is modified by phosphorylation on different sites. Phosphorylation of Thr167 in *S. pombe* (and of the corresponding Thr in higher eukaryotes) appears to be required for kinase activity (Ducommun et al., 1991; Gould et al., 1991; Krek and Nigg, 1991a; Solomon et al., 1992). In contrast, phosphorylation of Tyr15 in *S. pombe* (and of both Tyr15 and Thr14 in higher eukaryotes) inhibits kinase activity (Gould and Nurse, 1989; Solomon et al., 1990; Krek and Nigg, 1991b; Norbury et al., 1991). While the kinase that phophorylates Thr167 has not yet been identified, Tyr15 is presumably phosphorylated by the kinases encoded by *wee1*$^+$ and *mik1*$^+$ in *S. pombe* (Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren et al., 1991; Parker et al., 1991). In a second step, immediately before entry into mitosis, the inhibitory phosphate modifications are removed by the cdc25-phosphatase (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991; Strausfeld et al., 1991). The resulting activation of the cyclin B/$p34^{cdc2}$ kinase initiates mitosis. The

mitotic, ubiquitin-dependent degradation of cyclin B results in the inactivation of the associated $p34^{cdc2}$ kinase and in the completion of mitosis (Glotzer et al., 1991).

The regulation of the kinase activity required for the G_1/S-transition is less well understood, but it involves distinct cyclin proteins. Genetic approaches in *S. cerevisiae* have led to the identification of several, functionally overlapping G_1-cyclins (CLN1,2,3), which are involved in the regulation of the $p34^{CDC28}$ activity required for progression through START (Richardson et al., 1989). Several potential G_1-cyclins have also been identified in higher eukaryotes. In humans, cyclin A, which is also abruptly degraded during mitosis, is found not only in complexes with the $p34^{cdc2}$ kinase but also in association with the $p33^{cdk2}$ kinase. Cyclin A accumulation starts prior to S-phase and the cyclin A/$p33^{cdk2}$ complex is active throughout S-phase (Pagano et al., 1992; Rosenblatt and Morgan, 1992). Recent observations have implicated the cyclinA/$p33^{cdk2}$ complex in the regulation of the G_1/S-transition and oncogenesis (D'Urso et al., 1990; Wang et al., 1990; Devoto et al., 1992; Girard et al., 1991; Mudryj et al., 1991; Pagano et al., 1992).

Similarly, cyclin C, D and E, which have been identified in higher eukaryotes, have also been implicated in these processes (Koff et al., 1991; Lew et al., 1991; Matsushime et al., 1991; Motokura et al., 1991; Xiong et al., 1991). These cyclins were identified because of their ability to complement *S. cerevisiae* mutants deficient for G_1-cyclins, but it remains to be demonstrated that these cyclins have the same function in higher eukaryotes as the G_1-cyclins in *S. cerevisiae*. Moreover, the kinases that are regulated by cyclin C, D and E have also not yet been identified. The number of candidate kinases is increasing. In humans, an extensive search has revealed eleven cdc2-related kinases, including seven novel kinases apart from $p34^{cdc2}$, $p33^{cdk2}$ and two other previously identified kinases (Meyerson et al., 1992). A large family of cyclin/cdc2-related kinase complexes can therefore be anticipated in higher, multicellular eukaryotes where they may participate in the complex regulation of cell proliferation.

In order to dissect this complexity, we started with a genetic approach in *Drosophila melanogaster*. The genes encoding cyclins A, B, C, D and E as well as cdc2-related kinases have been identified in this organism (Lehner and O'Farrell, 1989, 1990a,b; Whitfield et al., 1989; Jimenez et al., 1990; Lahue et al., 1991; Leopold and O'Farrell, 1991; H. Richardson and R. Saint, personal communication; R. Finley and R. Brent, personal communication; K. Sauer and C.F.L., unpublished observation), and mutations in the cyclin A and the cyclin B gene have been isolated (Lehner and O'Farrell, 1989, J. Knoblich and C.F.L., unpublished observation).

Here we describe the identification and characterization of mutant alleles in the *Dm cdc2* gene. Among the cdc2-related kinases identified in *Drosophila*, the *Dm cdc2* kinase is most similar to the yeast kinases ($p34^{cdc2}$ and $p34^{CDC28}$). Complementation experiments have shown that *Dm cdc2* can complement mutations in these yeast homologs. In yeast, therefore, *Dm cdc2* appears functional at both the G_1/S and the G_2/M transition. In *Drosophila*, however, our phenotypic analyses reveal only a G_2/M function which cannot be complemented by the closely related *Dm cdc2c* gene.

MATERIALS AND METHODS

Drosophila stocks

Abbreviations of genetic loci are used according to Lindsley and Zimm (1992).

The isolation of recessive lethal mutations in the chromosomal region 31 is described in detail elsewhere (Clegg et al., 1992). The lethal mutations were placed into complementation groups based on inter se complementation analyses. The complementation group corresponding to the *Dm cdc2* gene comprised 10 alleles, 8 of which are characterized here. The alleles *Dm* $cdc2^{216A}$, *Dm* $cdc2^{B47}$, *Dm* $cdc2^{D57}$, *Dm* $cdc2^{E10}$, *Dm* $cdc2^{E1\text{-}9}$, *Dm* $cdc2^{E1\text{-}23}$ and *Dm* $cdc2^{E1\text{-}24}$ were isolated after mutagenesis with ethyl methanesulfonate (EMS). The allele *Dm* $cdc2^{216P}$ was isolated after mutagenesis by P-M dysgenesis. The chromosome with the allele *Dm* $cdc2^{216A}$ acts as a dominant suppressor of position-effect variegation [*Su(var)*], which appears to be due to a closely linked second site mutation (Sinclair et al., 1992).

The deficiency *Df(2L)J27*, which deletes the *Dm cdc2* gene (C. F. L., data not shown), was isolated by Sandler (1977). The deficiency *Df(3R)H81*, which deletes the *Dm cdc2c* gene (C. F. L., data not shown), was isolated and kindly provided by A. Preiss, University Basel. The enhancer trap line A37, which directs lacZ expression to the cells of the PNS, has been isolated and characterized previously (Ghysen and O'Kane, 1989).

Drosophila strains with transgenes were obtained by P-element-mediated transformation (Spradling, 1986). *Drosophila* strains with a genomic *Dm cdc2* fragment (*P[w*$^+$*,Dm cdc2]*) were obtained after transformation with a pCaSpeR 4 (Pirrotta, 1988) construct containing a 5.8 kb *Hin*dIII fragment, which includes the *Dm cdc2* gene (see Fig. 1A). This fragment was derived from the lambda phage PS-5, which had been isolated from a genomic library by Cronmiller et al. (1988) for the molecular analysis of the *daughterless* (*da*) gene. The *Dm cdc2* transcription unit is upstream of *da* and separated from *da* by a single transcription unit encoding a 0.8 kb transcript.

Drosophila strains harbouring transgenes with a heat-shock promoter controlling the expression of either *Dm cdc2* (*hs-cdc2*) or *Dm cdc2c* (*hs-cdc2c*) were obtained after transformation with pCaSpeR-hs (Pirrotta, 1988) constructs containing cDNA of either *Dm cdc2* or *Dm cdc2c* (Lehner and O'Farrell, 1990a).

Genetic crosses were done using standard *Drosophila* techniques.

Identification of mutant progeny

For the analysis of larval phenotypes, the *Dm cdc2* alleles and the deficiency *Df(2L)J27* were balanced with *In(2LR)Gla* carrying the dominant marker *Bc*. Mutant larvae were identified based on the lack of the *Bc* marker.

For the analysis of the *Dm cdc2* function during embryonic proliferation, we analyzed the progeny of *Dm* $cdc2^{216P}$, *Tft*/*Dm* $cdc2^{E1\text{-}24}$ females crossed with *Df(2L)J27*/*CyO, P[w+, ftz-lacZ]* males. The lacZ-expressing progeny, which were phenotypically wild type, were analyzed as controls. The phenotypically abnormal class of progeny observed after development at 25°C was identified as *Dm* $cdc2^{216P}$/*Df(2L)J27* based on the following observations (data not shown). An aberrant pattern of cyclin A expression was found in 25% of the progeny only. Based on the results of double-labeling with anti-lacZ antibodies, we concluded that these 25% were either *Dm* $cdc2^{216P}$, *Tft*/*Df(2L)J27* or *Dm* $cdc2^{E1\text{-}24}$/*Df(2L)J27*. Analysis of the lethal phase revealed embryonic lethality for about 25% of the progeny and late pupal lethal-

ity for another 25% of the progeny. Based on the absence of the *Tft* marker in the terminal pupae, we concluded that the class of progeny that was affected by embryonic lethality and the aberrant cyclin A expression was *Dm cdc2*^{216P}/*Df(2L)J27*. This interpretation was fully confirmed by the observation that, after incubation at high temperature, aberrant cyclin A expression and embryonic lethality was found to affect both the *Dm cdc2*^{216P}/*Df(2L)J27* class and also the *Dm cdc2*$^{E1\text{-}24}$/*Df(2L)J27* class, which had the temperature-sensitive allele *Dm cdc2*$^{E1\text{-}24}$.

Phenotypic characterizations

For labeling with bromodeoxyuridine (BrdU), third instar larvae were fed with *Drosophila* food containing BrdU (1 mg/ml) as described by Truman and Bate (1988), and embryos were permeabilized and incubated in Schneider's medium containing BrdU (1 mg/ml) as described by Edgar and O'Farrell (1990). Labelling was done for 12 hours with larvae and for 105 minutes with embryos.

For fixation, the anterior one-third of the larvae was cut off, inverted and incubated in PEM buffer (2 mM $MgSO_4$, 1 mM EDTA, 100 mM PIPES, pH 6.9) containing 4% paraformaldehyde and 1% Nonidet NP-40. After 10 minutes, fixation was stopped by several washes in PBS, 0.1% Tween 20, and the larvae were treated with proteinase K (50 μg/μl) for 10 minutes. After several washes in PBS, 0.1% Tween 20, 2 mg/ml glycine, fixation was repeated. Subsequent immunocytochemical staining with a monoclonal antibody against BrdU (Becton-Dickinson) was done as described by Truman and Bate (1988).

Embryos were fixed and analyzed after immunofluorescent labeling with antibodies against cyclin A (Lehner and O'Farrell, 1989), lacZ (Promega), β-tubulin (Amersham) and BrdU as described previously (Lehner and O'Farrell, 1989; Edgar and O'Farrell, 1990; Lehner et al., 1991). Hoechst 33258 (1 μg/ml in PBS) was used for DNA labeling.

Microscopic analyses were done on a Zeiss Axiphot microscope with epifluorescence, differential interference and phase-contrast equipment. Kodak Technical Pan film was used for photography.

Complementation experiments with heat-shock promoter transgenes

In order to induce periodic expression from *hs-cdc2* or *hs-cdc2c*, we reared the progeny from the appropriate crosses (see Fig. 8C) in a simple thermocycler: *Drosophila* bottles were incubated in a water bath placed in a cold room at 4°C which was continuously heated by a first thermostat set to 27°C and periodically heated by a second thermostat set to 37°C. The second thermostat was switched on for 10 minutes at 70 minutes intervals. The resulting temperature measured by a sensor in the fly food was found to cycle between 33°C and 27°C. This temperature cycling was found to be necessary because *hs-cdc2* expression during continuous incubation at 29°C was not sufficient for rescue of *Dm cdc2* mutants, and continuous incubation at temperatures above 29°C was lethal.

Molecular characterization of *Dm cdc2* alleles

All sequence analysis was done with a Sequenase kit (USB) and double-stranded plasmid DNA templates.

To determine the insertion site of the P-element associated with the allele *Dm cdc2*^{216P}, we isolated DNA fragments flanking the insertion site by polymerase chain reaction (PCR). The sequence of the first primer (5′-GCA GGT ACC ACC TTA TGT TAT TTC ATC ATG-3′) was derived from the sequence of the inverted repeat present at the P-element ends (O'Hare and Rubin, 1983). The sequence of the second primer (5′-GCA GTC GAC AT NGC NGT NGA NGG NAC NCC (TC)TC-3′) was derived from the antisense strand of the *Dm cdc2* cDNA in the region of the PSTAIR motif (Lehner and O'Farrell, 1990a). PCR was done as described previously (Saiki et al., 1985; Lehner and O'Farrell, 1990a). Both primers contained restriction sites at their 5′-ends (*Kpn*I and *Sal*I respectively), which allowed an efficient cloning of the PCR product obtained with template DNA isolated from *Dm cdc2*^{216P}/SM6a flies. The insertion site was determined after sequence analysis of the cloned PCR fragment.

For the sequence analysis of EMS-induced *Dm cdc2* alleles, a DNA fragment containing all of the coding sequence of the mutant allele was isolated by PCR. As a template, we used genomic DNA isolated from flies that had the mutant *Dm cdc2* allele over the deficiency *Df(2L)J27*. This strain also contained the genomic 5.8 kb *Dm cdc2* transgene, which was required to rescue the lethality of the hemizygous *Dm cdc2* mutants. The choice of the first primer assured that the transgene sequence was not amplified during the PCR. This first primer (5′-CCG CCT TGA TGG GCA CAT GGT AA-3′) was derived from the DNA sequence immediately upstream of the *Hin*dIII fragment present in the *Dm cdc2* transgene. The sequence of the second primer (5′-TTT GCT TTA GAT TAG ATA CCA AG-3′) was derived from the DNA sequence immediately upstream of the polyadenylation signal of the *Dm cdc2* gene (Lehner and O'Farrell, 1990a). The PCR product obtained with these primers was digested with *Hin*dIII and *Bgl*II. The resulting *Hin*dIII-*Bgl*II fragment was cloned into the corresponding sites of a Bluescript KS$^+$ vector (Stratagene) and sequenced with specific primers spaced at 350 bp intervals. For each allele, at least three different clones obtained from independent PCR reactions were analyzed to exclude PCR artefacts. All the changes shown in Fig. 1B were found in all of the respective clones.

RESULTS

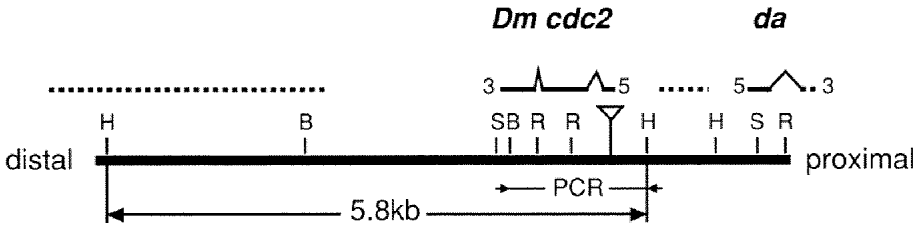
Identification of mutant *Dm cdc2* alleles

Mapping by in situ hybridization to polytene chromosomes assigned the *Dm cdc2* gene to the chromosomal region 31D/E (Lehner and O'Farrell, 1990a). This region was saturated for EMS-induced recessive lethal mutations (Clegg et al., 1992). In order to determine whether any of these lethal mutations were in the *Dm cdc2* gene, we constructed transgenic *Drosophila* lines. A genomic 5.8 kb fragment containing no other complete transcription unit except *Dm cdc2* (Fig. 1) was introduced into the germ line by P-element-mediated transformation. This fragment was able to rescue the lethality of a complementation group with multiple alleles (Table 1), demonstrating that this complementation group represents the *Dm cdc2* gene.

Several of these alleles were characterized at the molecular level. The allele *Dm cdc2*^{216P}, which had been isolated after P-element mutagenesis, was found to have a P-element insertion in the 5′ untranslated region of the *Dm cdc2* gene (Fig. 1A, for details see Materials and methods).

For the analysis of the EMS-induced alleles, *Dm cdc2*^{216A}, *Dm cdc2*^{D57}, *Dm cdc2*$^{E1\text{-}9}$, *Dm cdc2*$^{E1\text{-}23}$ and *Dm cdc2*$^{E1\text{-}24}$, the genomic region containing the complete coding sequence was amplified by polymerase chain reactions (PCR) (see Materials and methods). All mutant alleles that were sequenced proved to be missense mutations. The DNA sequence of the genomic region and the changes causing these missense mutations are shown in Fig. 1B. With the exception of *Dm cdc2*^{216A}, all mutations result in

A



B

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AATTTAAAAGTCGGTGGCTTGCAAAGAAATAGCTTAATAAATT ATGGAGGATTTTGAGAAAATTGAGAAGATTGGCGAGGGCACATATGGCGTGGTGT
                                               M  E  D  F  E  K  I  E  K  I  G  E  G  T  Y  G  V  V

ATAAAGGTCGCAATCGCCTGACGGGCCAAATTGTGGCAATGAAGAAAATCCGCTTGGAGTCCGACGACGAAGGCGTTCCATCAACCGCGATCAG GTAA
Y  K  G  R  N  R  L  T  G  Q  I  V  A  M  K  K  I  R  L  E  S  D  D  E  G  V  P  S  T  A  I  R

AATGCCGCGGCTTGGACGCCCAAGCCTCAAAATGTTTCATGTTCTTTCGCGACTTTTCCATTCAAATGGCTTATATGCTTAATTGAAAGTATGTTCCTT
GACATCTTATGGGTTGGTTTGAAGATTTTGCAATATTGTTTTTAATTTATACTGTGGAAGTCTAGCATAATTATGTTACGGCTTATGTTCATCATACAT
ATGTGTGTGTATGTACAATCCTATATGCGTACTTATGCTCATGTGTACATCATCATACTTTCTATTTATGTTTATTAATTGGAAGCCTGCGAATACTTT
CTGGTTCAACTGATAGACCAATAACTAAAATACTTTAATCACATGTTTTTTCTATAATGTAGTAATATTTAATATCCATTACAG AGAAATTTCGTTGC
                                                                                          E  I  S  L

TTAAGGAGTTGAAACATGAAAACATTGTCTGTTTGGAGGATGTTTTGATGGAGGAGAACCGCATATACTTGATCTTTGAATTCCTATCGATGGACCTCA
L  K  E  L  K  H  E  N  I  V  C  L  E  D  V  L  M  E  E  N  R  I  Y  L  I  F  E  F  L  S  M  D  L

AGAAATACATGGATTCGCTGCCAGTTGATAAGCACATGGAGAGTGAATTGGTCCGTAGCTATTTGTACCAAATAACTAGCGCCATTCTTTTCTGCCATC
K  K  Y  M  D  S  L  P  V  D  K  H  M  E  S  E  L  V  R  S  Y  L  Y  Q  I  T  S  A  I  L  F  C  H

                                                                    216A: T  D57: A
GTCGGCGAGTACTTCACCGTGATCTTAAGCCGCAGAACTTACTAATCGACAAGAGTGGCCTCATAAAAGTCGCCGACTTTGGACTTGGCCGATCCTTTG
R  R  R  V  L  H  R  D  L  K  P  Q  N  L  L  I  D  K  S  G  L  I  K  V  A  D  F  G  L  G  R  S  F

GCATTCCGGTGCGCATTTATACGCACGAGATTGTTACCTTGTGGTACAGAGCGCCGGAGGTGCTACTGGGTTCACCCCGGTATTCCTGTCCCGTCGATA
G  I  P  V  R  I  Y  T  H  E  I  V  T  L  W  Y  R  A  P  E  V  L  L  G  S  P  R  Y  S  C  P  V  D

                E1-24: A                                E1-23: A
TCTGGTCCATTGGATGCATATTCGCGGAGATGGCAACGAGAAAGCCGCTATTCCAGGGTGACTCGGAAATTGACCAGTTGTTTAGAATGTTTAG GTAA
I  W  S  I  G  C  I  F  A  E  M  A  T  R  K  P  L  F  Q  G  D  S  E  I  D  Q  L  F  R  M  F  R

CCACAGGTAATTTACTTTCTATTCCCTGTGATACTCACACTCATTGATTGCAG AATTCTGAAAACACCTACCGAAGACATTTGGCCGGGCGTTACTTC
                                                        I  L  K  T  P  T  E  D  I  W  P  G  V  T  S

                E1-9: T
GCTACCCGACTATAAGAACACGTTCCCCTGCTGGTCCACGAATCAATTGACCAATCAGTTAAAGAATCTCGATGCGAATGGTATTGATCTCATTCAAAA
  L  P  D  Y  K  N  T  F  P  C  W  S  T  N  Q  L  T  N  Q  L  K  N  L  D  A  N  G  I  D  L  I  Q  K

GATGTTAATCTACGATCCAGTTCATCGCATTTCCGCCAAGGACATTTTGGAGCATCCCTATTTCAATGGTTTTCAATCGGGCTTAGTTCGAAATTAA C
  M  L  I  Y  D  P  V  H  R  I  S  A  K  D  I  L  E  H  P  Y  F  N  G  F  Q  S  G  L  V  R  N

GTTCGGTATTCTCGTTTGACTTTAACTAAGAATTTTAAAACAAGAGATCTTGGTATCTAATCTAAGCAAAATAGCCGTAAATAAAA
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Fig. 1. *Dm cdc2* gene structure of wild-type and mutant alleles. (A) Molecular map of the *Dm cdc2* gene region illustrating the position of the *Dm cdc2* transcription unit (*Dm cdc2*), of uncharacterized transcription units flanking *Dm cdc2* (dashed lines, Cronmiller et al., 1988; H. Vässin, C.F.L., unpublished results) and of the *daughterless* transcription unit (*da*). The insertion site of the P-element associated with the *Dm cdc2*^{216P} allele is indicated by a triangle. The 5.8kb *Hin*dIII fragment was used for germ line transformation and rescued the lethality of *Dm cdc2* alleles. The region amplified from *Dm cdc2* alleles for subsequent sequence analysis is indicated (PCR). B, *Bgl*II, H, *Hin*dIII, R, *Eco*RI, S, *Sal*I. Some of these restriction sites were found to be polymorphic in different stocks. (B) The sequence of the transcribed region of the *Dm cdc2* gene. The mutant changes (bold) identified in the different *Dm cdc2* alleles (*Dm cdc2*^{216A}, *Dm cdc2*^{D57}, *Dm cdc2*$^{E1\text{-}24}$, *Dm cdc2*$^{E1\text{-}23}$, *Dm cdc2*$^{E1\text{-}9}$) are indicated above the wild-type sequence. The conceptual translation (one letter code) is shown under the wild-type sequence. Non-translated regions are represented in italics. The arrow marks the site of the P-element insertion in the *Dm cdc2*^{216P} allele. The polymorphisms that were identified in some of the *Dm cdc2* alleles and which do not change the predicted protein sequence are not included.

non-conservative exchanges at positions that are completely invariant in all the known cdc2 homologs (Fig. 2).

Phenotypic characterization of *Dm cdc2* alleles

Males containing mutant *Dm cdc2* alleles were crossed to females containing a deficiency of the *Dm cdc2* gene, *Df(2L)J27*, and the lethal phase of the mutant progeny was determined (Table 1). Mutants with the *Dm cdc2* alleles over the deficiency died after pupariation at the larval-pupal interphase except in the case of *Dm cdc2*$^{E1\text{-}24}$. Analyses at different temperatures indicated that the *Dm cdc2*$^{E1\text{-}24}$ is a temperature-sensitive allele. At 18°C *Dm cdc2*$^{E1\text{-}24}$/*Df(2L)J27* flies were fully viable, whereas at 29°C they did not develop beyond the larval-pupal interphase. At 25°C, *Dm cdc2*$^{E1\text{-}24}$/*Df(2L)J27* flies died during eclosion. In the case of *Dm cdc2*^{D57}/*Df(2L)J27*, embryonic instead of larval/pupal lethality was observed if the reciprocal cross was analyzed. Thus, the mutant Dm cdc2^{D57} product appears to have a weak dominant negative effect.

Mutant larvae that reached the wandering stage were completely wild type in appearance in all cases, but in general they reached this stage 1-2 days after the non-mutant

Fig. 2. Missense mutations in *Dm cdc2*. The missense mutations predicted from the sequence analysis of mutant *Dm cdc2* alleles (*Dm cdc2*^{216A}, *Dm cdc2*^{D57}, *Dm cdc2*$^{E1\text{-}24}$, *Dm cdc2*$^{E1\text{-}23}$, *Dm cdc2*$^{E1\text{-}9}$) are indicated (shaded). Except for *Dm cdc2*^{216A}, all missense mutation are non-conservative exchanges in positions that are identical (boxed) in *Dm cdc2* (Dm 2), *Dm cdc2c* (Dm 2c), the human cdc2 homolog (Hs 2), *S. pombe cdc2*$^{+}$ (Sp 2) and *S. cerevisiae* CDC28 (Sc 28).

siblings. Dissection of mutant larvae hemizygous for strong alleles, however, revealed clear defects in imaginal tissues. Imaginal discs were missing or rudimentary and the larval brains were reduced in size. The lower numbers of imaginal cells observed in the imaginal ring of salivary glands of wandering stage larvae are shown in Fig. 3. The number of cells present in this region varied with different *Dm cdc2* alleles (Table 1). The most extensive reduction in cell number was observed with the alleles *Dm cdc2*^{B47}, *Dm cdc2*^{D57}, *Dm cdc2*^{E10}, *Dm cdc2*$^{E1\text{-}9}$ (Fig. 3H) and *Dm cdc2*$^{E1\text{-}23}$. These alleles appear to be amorphic, since the same reduction was also observed in mutant larvae homozygous for *Dm cdc2*$^{E1\text{-}9}$ or *Dm cdc2*$^{E1\text{-}23}$ (data not shown). The hypomorphic alleles *Dm cdc2*^{216P} (Fig. 3F) and *Dm cdc2*^{216A} (Fig. 3G) caused less extensive defects and the defects observed with *Dm cdc2*$^{E1\text{-}24}$ were temperature-dependent (compare Fig. 3I and J). The results of interallelic complementation tests agreed with the phenotypic evaluation of allele strength (Table 1).

Our phenotypic analyses demonstrated that *Dm cdc2* function is required for the mitotic proliferation of the imaginal cells during larval life. In contrast, the larval cells which no longer proliferate mitotically during the larval stages were not affected in the mutants. As in wild type, larval growth in mutants was accompanied by endoreplication and polytenization of larval cells. Salivary glands in wandering stage larvae from wild-type (Fig. 3A) or mutant larvae (Fig. 3C) were comparable in size, and also the intensity of the DNA staining in the polytene cell nuclei was indistinguishable. Pulse labeling with BrdU revealed that endoreplication continued in amorphic mutant larvae. The polytene nuclei were also labeled by BrdU pulses during the last larval stage (Fig. 3D). These results indicate that *Dm cdc2* function is not required for the endoreplication cycles.

The function of the maternal *Dm cdc2* contribution

The presence of maternally derived *Dm cdc2* mRNA and protein in embryos has been described previously (Lehner and O'Farrell, 1990a). The following results demonstrate the functional significance of this maternal contribution. Moreover, they indicate that this maternal contribution permits the embryonic proliferation in mutants with strong *Dm cdc2* alleles over a deficiency. For these analyses, we took advantage of the temperature-sensitive allele *Dm cdc2*$^{E1\text{-}24}$. Unfortunately, however, flies homozygous for the original *Dm cdc2*$^{E1\text{-}24}$ chromosome were not viable due to a recessive, embryonic lethal mutation at a second site. After this second site mutation had been removed by recombination, homozygous *Dm cdc2*$^{E1\text{-}24}$ flies were recovered at 18°C, but proved to be completely sterile according to preliminary experiments. Therefore, we used females transheterozygous for *Dm cdc2*$^{E1\text{-}24}$ and the hypomorphic allele *Dm cdc2*^{216P}. Such flies were fully viable at 18°C and displayed temperature-dependent fertility.

The progeny of *Dm cdc2*^{216P}/*Dm cdc2*$^{E1\text{-}24}$ females developed normally at 18°C and 25°C (not shown). At 29°C, however, the early syncytial divisions occurred

Table 1. Characterization of Dm cdc2 alleles

Allele	Mutagen	Rescue[a]	Lethal phase[b]	Imaginal cells[c]	Interallelic complementation[d]
Dm cdc2^{B47}	EMS	+	l/p	5-11 (16)	2.6
Dm cdc2^{D57}	EMS	+	l/p	7-12 (13)	0
Dm cdc2^{E10}	EMS	+	l/p	5-10 (18)	0
Dm cdc2^{E1-9}	EMS	+	l/p	4-7 (14)	4.6
Dm cdc2^{E1-23}	EMS	+	l/p	5-11 (14)	0.9
Dm cdc2^{216A}	EMS	+	l/p	10-25 (16)	6.3
Dm cdc2^{216P}	P				
29°C		nd[f]	l/p	105-115 (7)	0
25°C		+	l/p	155-170 (8)	58
18°C		nd	l/p	nd	100
Dm cdc2^{E1-24}	EMS				0[e]
29°C		nd	l/p	5-8 (14)	
25°C		+	p	160-170 (4)	
18°C		nd	nl	nd	

[a]By crossing *Dm cdc2* allele/*SM6a,Cy* with *Df(2L)J27/SM6a,Cy; P[w*$^+$*,Dm cdc2]* and scoring for the presence of adult *Dm cdc2* allele/*Df(2L)J27; P[w*$^+$*,Dm cdc2]* progeny, it was determined whether the 5.8 kb genomic *Hin*dIII fragment present in the P element insertion was able to rescue the lethality of the flies hemizygous for *Dm cdc2* alleles.

[b]After crossing *Dm cdc2* allele/*In(2LR)Gla,Bc* males with *Df(2L)J27/In(2LR)Gla,Bc* females, the lethal phase of the progeny hemizygous for the *Dm cdc2* allele was determined after development at 25°C if not otherwise indicated. l/p, lethality during larval-pupal interphase. p, late pupal lethality. nl, not lethal.

[c]After crossing *Dm cdc2* allele/*In(2LR)Gla,Bc* with *Df(2L)J27/In(2LR)Gla,Bc*,the number of putative imaginal cells at the position of the imaginal rings in larval salivary glands of the progeny hemizygous for the *Dm cdc2* allele was determined. The number of glands that were analyzed is given in brackets. The putative imaginal precursor cells were identified after Hoechst 33258 labeling based on the small size of their nuclei. It is possible that the cells with the small nuclei that were observed in larvae with strong amorphic alleles are not imaginal precursor cells, but the equivalent of the neck cells which have been described in the blowfly *Calliphora erythrocephala* (Berridge et al., 1976). The exact number of imaginal precursor cells present in salivary glands at the end of embryogenesis is not known, but it has been estimated to be 9 in *Calliphora erythrocephala* (Berridge et al., 1976). In *Drosophila* at third larval instar wandering stage, we counted 150-190 (4) cells per imaginal ring in wild-type larvae.

[d]After crossing *Dm cdc2* allele/*SM6a,Cy* to *Dm cdc2*$^{E1-24}$/*SM6a,Cy* at 25°C, the percentage of adult escapers (*Dm cdc2* allele/*Dm cdc2*$^{E1-24}$) in the progeny was determined. At least 300 flies were analyzed. 100%, full viability.

[e]The original *Dm cdc2*$^{E1-24}$ chromosome which was used in these complementation tests has a second site mutation which results in embryonic lethality in the homozygous *Dm cdc2*$^{E1-24}$ progeny.

[f]nd, not determined.

abnormally and defective nuclei were observed particularly in the polar regions (Fig. 4B). Such defects were observed in all of the embryos from mutant mothers. In contrast, the progeny of wild-type females did not display such irregularities after temperature shifts to 29°C (Fig. 4A). These observations indicated that the maternally derived Dm cdc2 kinase is required during the early syncytial division cycles.

The function of the maternal contribution was also evidenced by other maternal effects. The lethal period of the *Dm cdc2*^{216P}/*Df(2L)J27* progeny, for instance, was strongly dependent on the maternal genotype. Late larval/pupal lethality was observed if mothers were *Dm cdc2*^{216P}/+. Embryonic lethality, however, was observed if mothers were *Dm cdc2*^{216P}/*Dm cdc2*$^{E1-24}$ and had no wild-type copy of the *Dm cdc2* gene, therefore. Immunofluorescent labeling with anti-cyclin A-antibodies revealed obvious phenotypic abnormalities in *Dm cdc2*^{216P}/*Df(2L)J27* embryos derived from *Dm cdc2*^{216P}/*Dm cdc2*$^{E1-24}$ mothers (see below). The phenotype of such embryos was characterized in detail, and for simplicity such embryos will be designated as mutant embryos in the rest of the text.

Characterization of the cell cycle block in mutant embryos

Mitotic proliferation can be monitored accurately by immunofluorescent labeling with antibodies against the cell cycle regulatory protein cyclin A (Lehner and O'Farrell, 1989). Cyclin A is expressed exclusively in proliferating cells. It accumulates during interphase and is rapidly degraded during each mitosis. At stage 14 of embryogenesis, mitotic proliferation is terminated in the epidermis and in the peripheral nervous system (PNS), but it continues in the developing central nervous system (CNS). Accordingly, cyclin A staining is restricted to the CNS in wild-type embryos at stage 14 (Fig. 5A, region c). However, cyclin A labeling was not restricted to the CNS in mutant embryos (Fig. 5B). In addition to the labeling in the CNS (which was more extensive than in the wild type), mutants displayed labeling also outside the CNS in a segmentally repeated pattern highly reminiscent of the pattern of the PNS. The same observations were also made with antibodies against cyclin B (not shown).

Experiments with the enhancertrap P-element insertion A37 which directs lacZ-expression to the PNS (Ghysen and O'Kane, 1989) confirmed the idea that the persistent cyclin A expression in mutant embryos occurred predominantly in cells associated with the PNS (data not shown). These experiments also demonstrated that the number of cells in the PNS was significantly lower in mutants than in controls. In contrast, cell density in the epidermis appeared normal in mutant embryos and anti-cyclin A labeling did not reveal abnormalities during the embryonic proliferation of the epidermal cells (not shown).

Our observations suggested, therefore, that the sum of the maternally and zygotically derived *Dm cdc2* function was sufficient for the early embryonic proliferation in

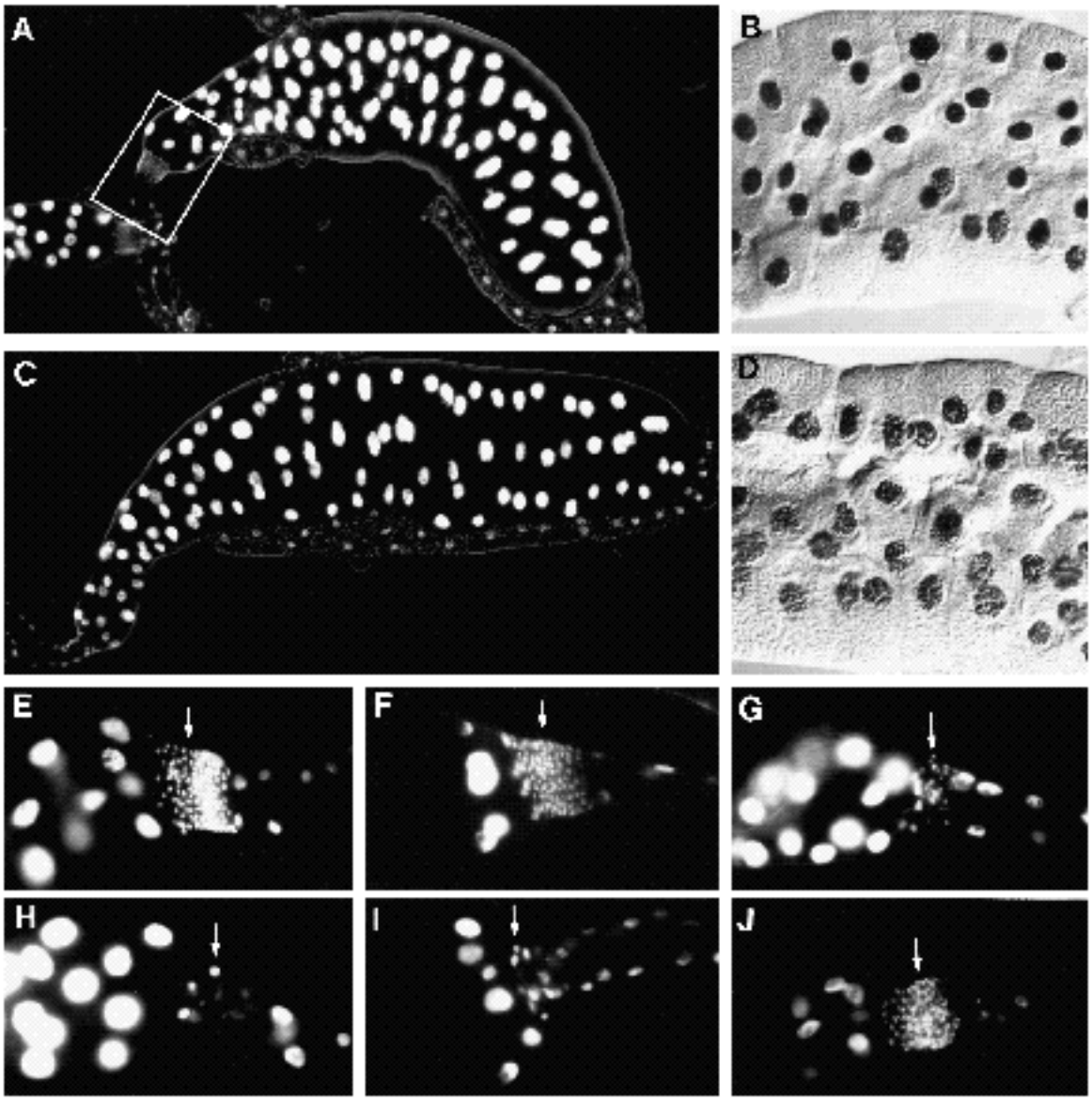

Fig. 3. Polytene and imaginal cells in salivary glands of wild-type and *Dm cdc2* mutant larvae. Salivary glands from wandering third instar larvae were fixed and stained with the DNA stain Hoechst 33258 (A,C,E-J). For the analysis of endoreplication, larvae were fed during the third instar with a BrdU-containing diet and incorporated BrdU was visualized immunocytochemically (B,D). (A,B) Salivary gland from wild-type larvae; (C,D) salivary gland from *Dm cdc2*^{B47}/*Df(2L)J27* larvae; (E-J) high magnification views of the region of the imaginal rings (see white box in A). The position of the imaginal cells is indicated by arrows. (E) Wild type; (F) *Dm cdc2*^{216P}/*Df(2L)J27*; (G) *Dm cdc2*^{216A}/*Df(2L)J27*; (H) *Dm cdc2*$^{E1\text{-}9}$/*Df(2L)J27*; (I,J) *Dm cdc2*$^{E1\text{-}24}$/*Df(2L)J27* grown at 29°C (I) and at 25°C (J).

mutant embryos, but not for the late embryonic proliferation in the PNS and CNS. Accordingly, the persistence of cyclin A expression in the PNS of mutant cells would reflect a block of cell cycle progression. In order to test this notion, we crossed a transgene with a heat-shock promoter controlling expression from a *Dm cdc2* cDNA (*hs-cdc2*) into the mutant embryos. As expected, the embryonic lethality of the mutants was prevented when the expression of *hs-cdc2* was induced with a heat pulse (45 minutes, 37°C) in 7 hour old embryos. Moreover, the persistent expression of cyclin A was no longer observed in the PNS region of mutant embryos after a heat pulse (Fig. 5C). In a control experiment, we confirmed that the cyclin A persistence was not abolished by an identical heat treatment in mutant embryos that did not have the *hs-cdc2* transgene (Fig. 5D). The heat pulse alone, therefore, did not destabilize cyclin A.

A heat pulse applied in 12 hour old embryos also abolished the persistence of cyclin A expression in mutant embryos containing the *hs-cdc2* transgene. In order to determine the dynamics of the cyclin A disappearance, embryos were fixed at different times following the heat shock. A significant reduction in the number of cyclin A-positive cells was observed after only 15 minutes of recovery (Fig.

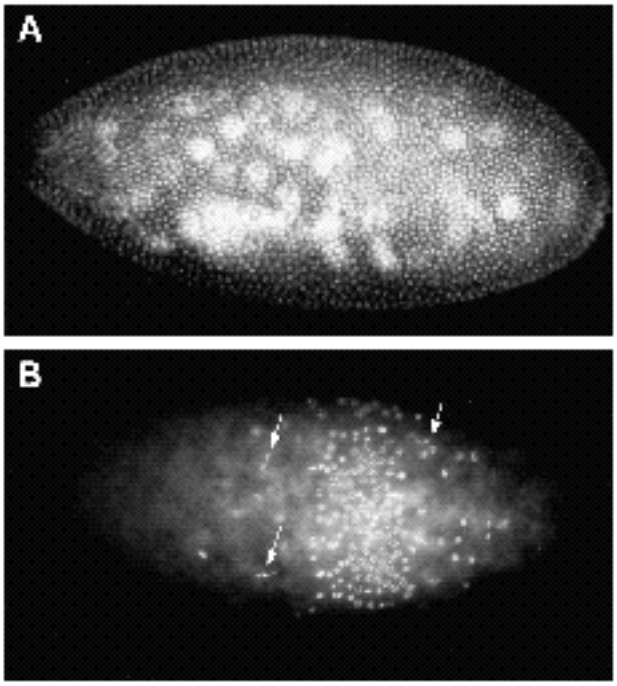

Fig. 4. The function of the maternally contributed Dm cdc2 kinase during the syncytial division cycles. Eggs were collected during 1 hour at 25°C, aged for 1 hour at 29°C and fixed and stained with Hoechst 33258. Eggs were either from (A) wild-type or (B) mutant mothers (*Dm cdc2*$^{E1\text{-}24}$/*Dm cdc2*^{216P}). The arrows in (B) indicate disrupted chromatin.

5E) and, after 45 minutes of recovery, only very few cells were cyclin A-positive in the region of the PNS (Fig. 5F). This experiment demonstrated that a heat pulse caused in the great majority of cells in the PNS region, within 45 minutes, a complete disappearance of cyclin A expression.

Longer recovery periods after a heat pulse at 12 hours did not lead to a further reduction of cyclin A-positive cells. The fact that the cyclin A persistence was not completely abolished if the heat pulse was applied at 12 hours was consistent with the observation that such late heat pulses were also unable to rescue the embryonic lethality of the mutants.

In order to investigate whether the disappearance of cyclin A observed after the heat-induced expression of *Dm cdc2* in mutant embryos was accompanied by progression through the cell cycle, we first performed pulse-labeling experiments with BrdU. The base analog BrdU is incorporated into DNA during replication and can be visualized with a monoclonal antibody.

In non-heat-pulsed mutant embryos containing the *hs-cdc2* transgene, BrdU incorporation was never observed in the cells of the PNS region after labeling pulses during stage 14 (Fig. 6A,B). The absence of BrdU incorporation in the PNS region of mutant embryos with the characteristic persistent cyclin A expression is consistent with the presumed cell cycle block. In contrast to the PNS region, BrdU incorporation was readily detected in the endoreplicating midgut cells (arrowhead in Fig. 6B). Interestingly, BrdU incorporation was also observed in cells of the CNS of mutant embryos. BrdU incorporation in the CNS reflects, at least in wild-type embryos during these embryonic stages, mitotic proliferation and not endoreplication. This observation therefore suggested that some mitotic cell proliferation was still proceeding in the mutant CNS (see discussion).

Essentially identical results were obtained when the BrdU labeling was started concomitant with a heat pulse and extended during a 1 hour recovery period (compare Fig. 6B and D). The absence of BrdU incorporation in the region of the PNS demonstrated that the cells did not progress through an S-phase during the time when cyclin A expression disappeared (Fig. 6C). The heat-induced expression of *Dm cdc2* did therefore not trigger entry into S-phase in these cells.

In a subsequent experiment, we monitored mitoses after triple-labeling with anti-cyclin A antibodies, anti-β-tubulin antibodies, and a DNA stain. Mitotic figures were only detected in mutant embryos that had the *hs-cdc2* transgene and had been subjected to a heat shock. In these experiments, mutant embryos were identified based on the persistence of cyclin A expression in the PNS region. Since the disappearance of the persistent cyclin A expression after *hs-cdc2* expression is incomplete (especially after only 25 minutes of recovery at 25°C), mutant embryos could readily be identified. An example of the mitotic spindles and metaphase plates observed in the PNS region of mutants during the period during which the persistence of cyclin A expression disappeared, is shown in Fig. 7. For a quantitative estimation, we counted the mitotic figures in the PNS region of the first four abdominal hemisegments in mutant embryos that had been fixed 25 minutes after the end of the heat shock. We found a total of 68 mitotic figures in 6 mutant embryos with a *hs-cdc2* transgene and no mitotic figures in 6 mutant embryos without a *hs-cdc2* transgene. Our determinations of the number of cells in mitosis provide a lower estimate, since whole-mount preparations do not allow the identification of all mitotic cells. Moreover, as shown in Fig. 5, the hs-cdc2-induced cyclin A disappearance is not completely synchronous. Thus only a fraction of the hs-cdc2-induced mitoses are detected in embryos fixed after 25 minutes of recovery.

All these experiments indicated that the heat-induced expression of *Dm cdc2* caused in the cells of the PNS region of mutant embryos a progression through mitosis during which cyclin A was degraded. Since this division was not preceded by an S-phase, we conclude that these cells were arrested in the G_2-phase.

Independent functions of *Dm cdc2* and *Dm cdc2c*

The *Dm cdc2c* gene encodes a cdc2-related kinase (see Fig. 2) that is essentially coexpressed with *Dm cdc2* (Lehner and O'Farrell, 1990a, J. Knoblich and C.F.L., unpublished observations). In order to evaluate the functional specificity of the *Drosophila* kinases, we analyzed in a first experiment whether a reduction in the *Dm cdc2c* gene dose affected the phenotypic effects of mutations in *Dm cdc2*. Since *Dm cdc2c* alleles have not yet been isolated, we used the deficiency *Df(3R)H81*, which deletes the *Dm cdc2c* gene as determined by in situ hybridizations to polytene chromosomes (not shown). We crossed this deficiency into a *Dm cdc2* mutant background (*Dm cdc2*^{216P}/*Dm cdc2*$^{E1\text{-}24}$; Fig. 8B). This allele combination was chosen because the

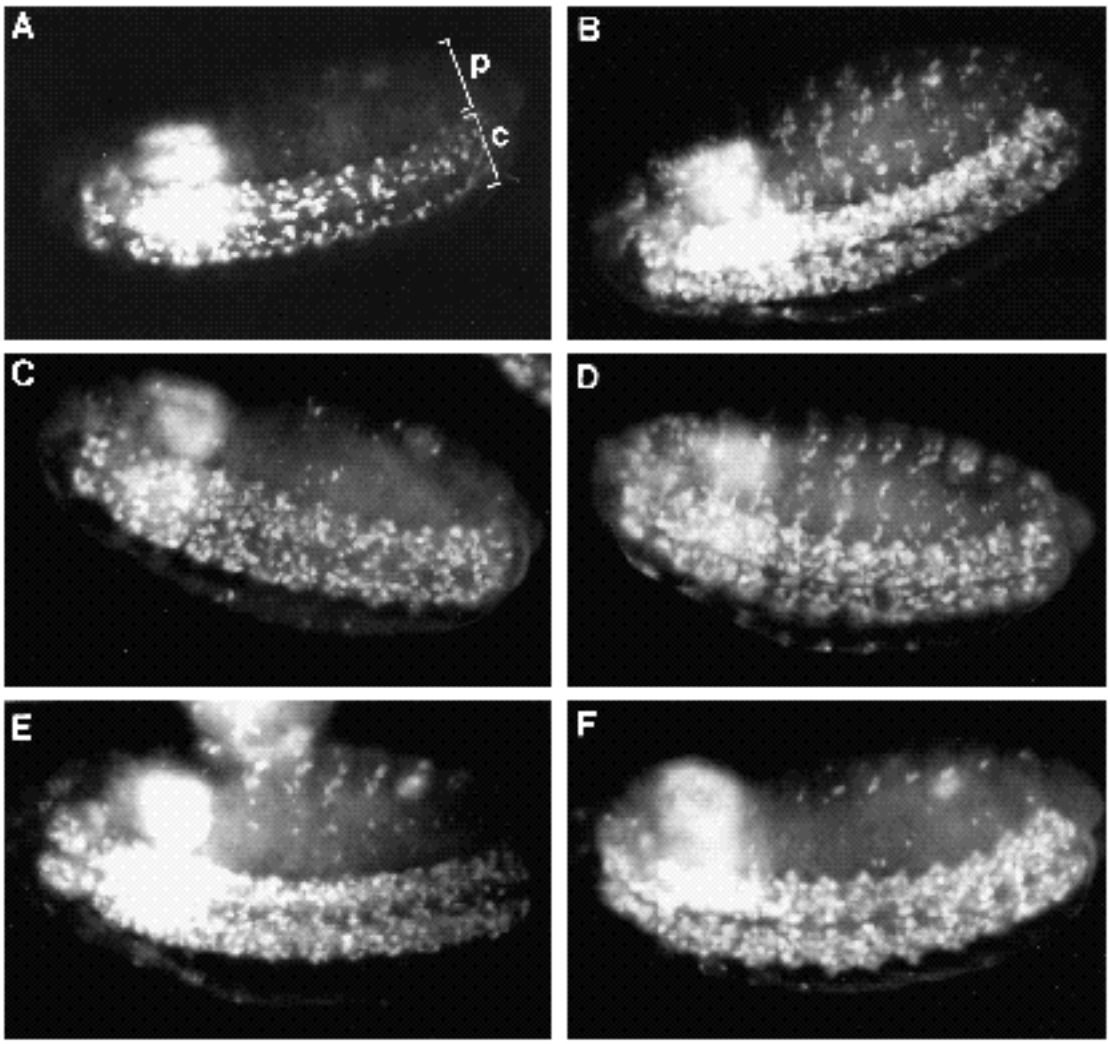

Fig. 5. Persistent expression of cyclin A in mutant embryos. Cyclin A expression was analyzed by immunofluorescent labeling with affinity purified anti-cyclin A antibodies. (A) Cyclin A labeling is present in the CNS region (c) and absent from the PNS region (p) of control embryos at stage 14. (B) Persistent cyclin A expression is observed in the PNS region of mutant embryos (*Dm cdc2*^{216P}/*Df(2L)J27* derived from *Dm cdc2*$^{E1\text{-}24}$/*Dm cdc2*^{216P} mothers). (C) Heat-induced expression of *Dm cdc2* from the *hs-cdc2* transgene prevents the persistence of cyclin A expression in the PNS region of mutant embryos. Eggs were collected during 2 hours and aged for 8 hours at 25°C. They were then subjected to a heat pulse (45 minutes, 37°C) and returned to 25°C for 4 hours before fixation. (D) The heat treatment (same protocol as in C) does not prevent the persistence of cyclin A expression in the PNS region of mutant embryos that do not have the *hs-cdc2* transgene. (E,F) The dynamics of the disappearance of cyclin A expression from the PNS region of mutant embryos was analyzed after expression of *hs-cdc2* (45 minutes, 37°C after 12 hours of development) and (E) 15 minutes or (F) 45 minutes of recovery at 25°C.

resulting phenotype was strongly temperature dependent. At 18°C, *Dm cdc2*^{216P}/*Dm cdc2*$^{E1\text{-}24}$ flies appeared wild type (Fig. 8A, left). At 25°C, however, these flies eclosed with severe phenotypic abnormalities (Fig. 8A, right). Defects were particularily evident in the abdomen. Tergites were severely reduced and segment fusions were frequent. At 29°C, this allele combination was completely lethal. According to these observations, slight changes in Dm cdc2 activity were expected to have profound phenotypic consequences in *Dm cdc2*^{216P}/*Dm cdc2*$^{E1\text{-}24}$ flies. However, no significant differences were observed at 25°C in *Dm cdc2*^{216P}/*Dm cdc2*$^{E1\text{-}24}$ flies with either one or two copies of the *Dm cdc2c* gene indicating that the Dm cdc2c kinase function does not overlap with the limiting Dm cdc2 kinase function.

In a second experiment, we tested whether expression from a heat-shock promoter *Dm cdc2c* transgene (hs-cdc2c) could rescue the lethality caused by mutations in *Dm cdc2* (Fig. 8C). Immunoblot experiments demonstrated that the *hs-cdc2c* transgene was expressed after heat pulses. Nevertheless, periodic *hs-cdc2c* expression induced in a thermocycler (see Materials and methods) did not rescue the lethality of the *Dm cdc2* mutants, in contrast to the periodic *hs-cdc2* expression, which fully rescued the lethality. In addition, heat-induced *hs-cdc2c* expression was also unable to rescue the persistence of cyclin A expression in

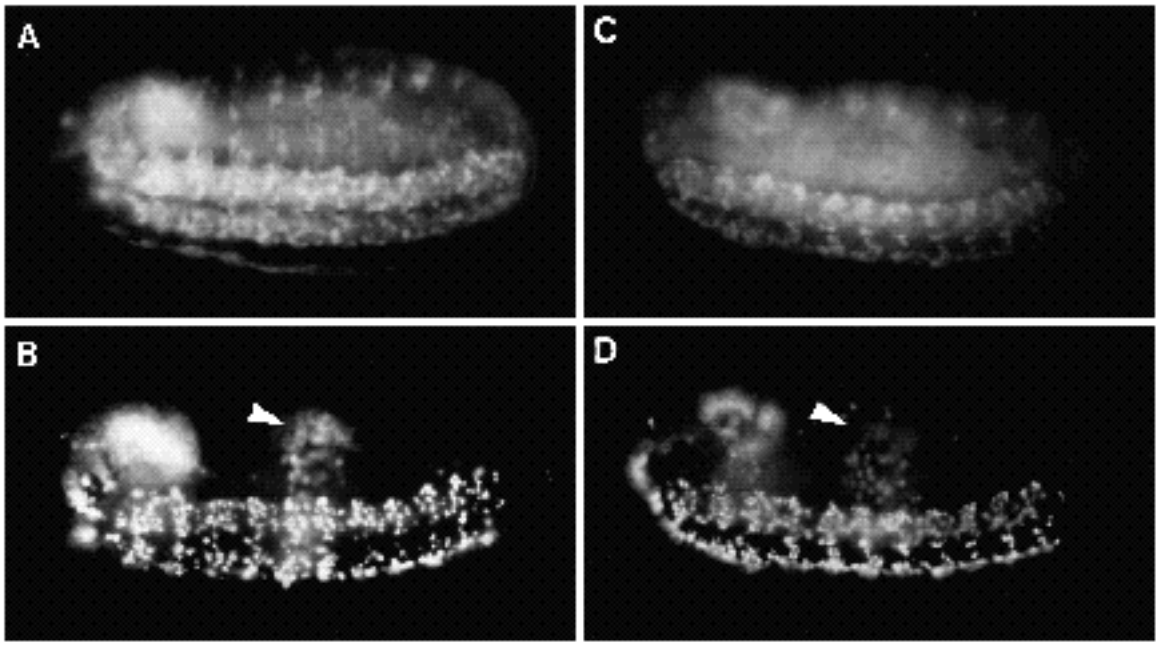

Fig. 6. *hs-cdc2* expression does not induce entry into S-phase in the PNS region of mutant embryos. Mutant embryos harbouring the *hs-cdc2* transgene were either not exposed to a heat pulse (A,B) or exposed to a heat pulse (45 minutes, 37°C) after 12 hours of development (C,D). After labeling with BrdU (1 hour 45 minutes), embryos were fixed and double labeled with anti-cyclin A antibodies (A,C) and with anti-BrdU antibodies (B,D). In the heat-pulsed embryos, BrdU-labeling was started concomitant with the heat pulse and extended during the 1 hour recovery period at 25°C. The arrowheads indicate BrdU incorporation in the endoreplicating midgut. The quality of the anti-cyclin A labeling is strongly impaired by the fixation protocol required for the anti-BrdU labeling.

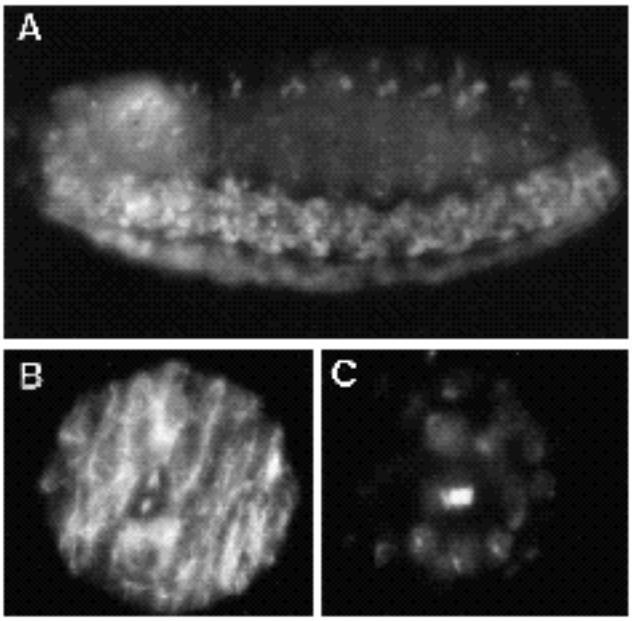

Fig. 7. *hs-cdc2* expression induces entry into M-phase in the PNS region of mutant embryos. Mutant embryos were fixed and double-labeled with anti-cyclin A antibodies (A), anti-β tubulin antibodies (B) and with Hoechst 33258 (C) after the heat-induced expression of *hs-cdc2* (45 minutes, 37°C at 12 hours of development) and 25 minutes of recovery at 25°C. A metaphase spindle (B) and the corresponding metaphase plate (C) from the PNS region of the mutant embryo (A) are shown at higher magnification.

mutant embryos (not shown). These experiments demonstrate that *Dm cdc2c* is unable to complement *Dm cdc2* in *Drosophila*.

DISCUSSION

The cdc2 kinase is of central importance in the regulation of the eukaryotic cell cycle. Initial genetic analyses in fission and budding yeast have established that the $p34^{cdc2}$ kinase (or the homologous $p34^{CDC28}$ kinase, respectively) is required for progression through START (G_1/S-transition) and for entry into mitosis (G_2/M-transition). Interesting differences, however, exist between fission yeast, where the G_2/M-transition has an especially high $p34^{cdc2}$ requirement, and budding yeast, where the G_1/S-transition is characterized by a high $p34^{CDC28}$ requirement. Analyses have therefore emphasized the G_2/M function in fission yeast and the G_1/S function in budding yeast. cdc2 homologs have since been identified in a great variety of organisms ranging from plants to humans, and complementation experiments in yeast have demonstrated their functional conservation. While most of these complementation tests were performed with temperature-sensitive *S. pombe cdc2* strains and therefore assayed primarily the G_2/M function, experiments with the *Drosophila* homolog and most rigorously with the human cdc2 homolog have clearly demonstrated that these homologs are able to complement both the G_1/S and the G_2/M function (Lee and Nurse, 1987; Wittenberg and Reed, 1989; Lehner and O'Farrell, 1990a).

Our identification of mutant *Dm cdc2* alleles has allowed

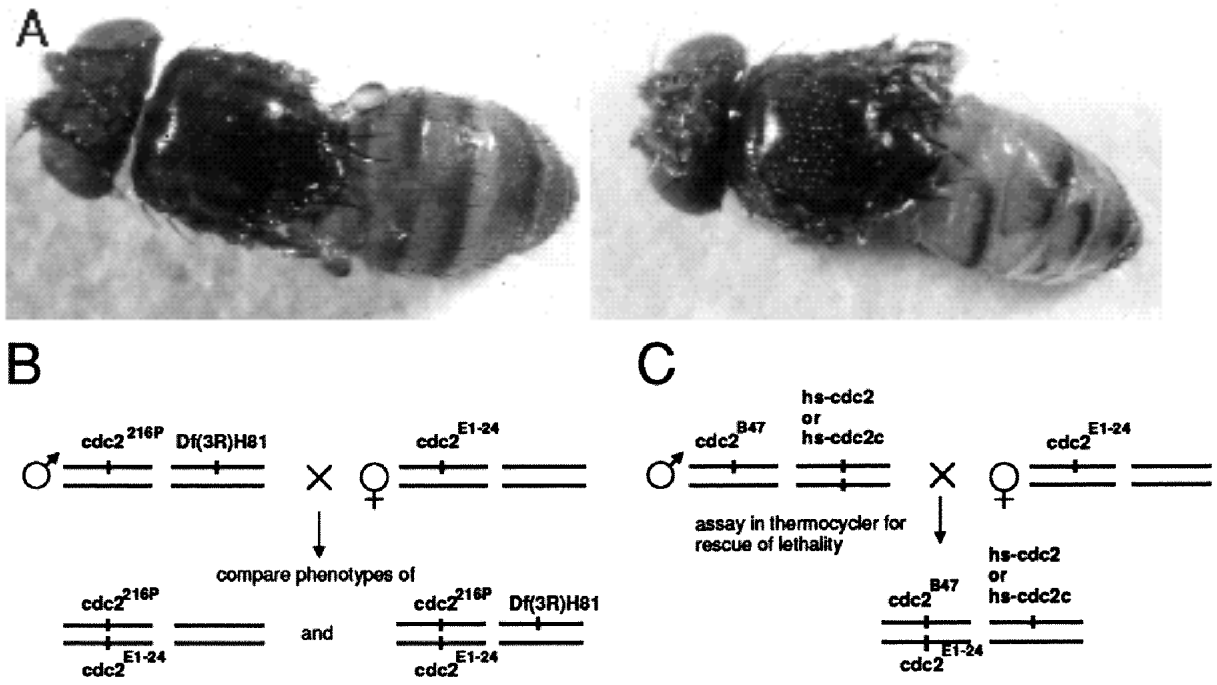

Fig. 8. Genetic assays for functional overlap between *Dm cdc2* and *Dm cdc2c*. (A,B) The consequence of a reduction in the *Dm cdc2c* gene dose on the phenotype of *Dm cdc2*^{216P}/*Dm cdc2*$^{E1-24}$ flies was analyzed. Changes in *Dm cdc2* activity have profound phenotypic consequences as illustrated in A which shows *Dm cdc2*^{216P}/*Dm cdc2*$^{E1-24}$ flies of wild-type appearance after development at 18°C (left) or with strong abdominal defects after development at 25°C (right). The wings were removed before photography in order to obtain a better view on the abdomen. B shows a scheme of the genetic cross allowing the phenotypic comparison of *Dm cdc2*^{216P}/*Dm cdc2*$^{E1-24}$ flies with either one or two copies of the *Dm cdc2c* gene which is deleted in the deficiency *Df(3R)H81*. The following dominant markers allowed the identification of the relevant genotypes: the chromosomes carrying *Dm cdc2*^{216P} or *Df(3R)H81* were marked with *Tft* or *H*, respectively. *Dm cdc2*$^{E1-24}$ was balanced with a *Cy* chromosome in the mothers. (C) Complementation tests with heat-inducible transgenes. Transgenes expressing either *Dm cdc2* (*hs-cdc2*) or *Dm cdc2c* (*hs-cdc2c*) were crossed into *Dm cdc2*^{B47}/*Dm cdc2*$^{E1-24}$ flies as illustrated in the crossing scheme and periodically induced in a thermocycler (see Materials and methods). In contrast to *hs-cdc2*, *hs-cdc2c* was unable to rescue the lethality of the *Dm cdc2*^{B47}/*Dm cdc2*$^{E1-24}$ flies. These flies could be identified because the chromosomes carrying the *Dm cdc2* alleles were balanced with *Cy* chromosomes in the parental strains.

the genetic characterization of *cdc2* function in *Drosophila*, which clearly revealed a requirement for mitosis. Interestingly, our results do not provide evidence for a requirement for entry into S-phase. The cell cycle arrest that was observed in the cells of the developing PNS of mutant embryos with a reduced maternal contribution occurred exclusively during the G_2-phase according to our results. *Dm cdc2* expression from an inducible transgene caused in these mutant cells a rapid entry into a normal mitosis. Entry into S-phase was not observed in these cells after *Dm cdc2* expression as would have been expected in the case of a G_1-arrest.

Similar results were obtained with human and mouse cells and with *Xenopus* egg extracts. Microinjection of antibodies against p34^{cdc2} was found to be ineffective in blocking the G_1/S transition in human tissue culture cells but caused an arrest before entry into mitosis (Riabowol et al., 1989). Furthermore, immunodepletion of the p34^{cdc2} kinase from *Xenopus* egg extract was ineffective in preventing S-phase but completely inhibited M-phase (Fang and Newport, 1991). Finally, the cell cycle arrest in the mouse FT210 cell line, which has a temperature-sensitive mutation in the cdc2 gene, occurred exclusively during the G_2-phase at the restrictive temperature (Th'ng et al., 1991; Hamaguchi et al., 1992).

Although the evidence arguing against a role of the higher eukaryotic p34^{cdc2} homolog in the G_1/S-transition is very strong, the presence of low amounts of residual p34^{cdc2} kinase activity sufficient for entry into S-phase cannot be excluded completely in all these experiments in higher eukaryotes where, similar to *S. pombe*, the cdc2 requirement might also be much lower for the G_1/S- than for the G_2/M-transition. However, the identification and functional characterization of an additional cdc2-related kinase (p33^{cdk2}) in vertebrates has provided further support for the notion that the p34^{cdc2} kinase acts specifically during the G_2/M-transition and not during the G_1/S-transition, since this additional p33^{cdk2} kinase appears to be specialized for the G_1/S-transition. Immunodepletion of p33^{cdk2} from *Xenopus* egg extracts or microinjection of anti-p33^{cdk2}-antibodies into human cells was found to inhibit entry into S-phase (Fang and Newport, 1991; Draetta et al., personal communication).

As yet it is unclear whether the additional cdc2-related kinase (*Dm cdc2c*) that has been identified in *Drosophila* is functionally homologous to cdk2. According to structural

comparisons, vertebrate $p33^{cdk2}$ is only marginally more related to Dm cdc2c- than to Dm cdc2-kinase. Circumstantial evidence, however, argues in favour of a cdc2c-cdk2 homology. Similar to $p33^{cdk2}$, Dm cdc2c-kinase is located predominantly in the nucleus and is not associated with cyclin B. However, it is associated with low amounts of cyclin A according to preliminary observations. Moreover, none of the additional cdc2-related kinases identified in an extensive search in *Drosophila* are more closely related to cdk2 than cdc2 (K. Sauer and C.F.L., unpublished results). Conversely, none of the cdc2-related kinases that have been identified in an extensive search in humans (Meyerson et al., 1992) is more closely related to cdc2c than cdk2. The isolation of mutant *Dm cdc2c* alleles (which is currently in progress) will hopefully clarify this relationship and reveal the functional role of the Dm cdc2c kinase. Our demonstration here that the Dm cdc2c kinase was unable to rescue *Dm cdc2* mutants and that a reduction of the *Dm cdc2c* gene dose did not enhance the phenotype caused by limiting *Dm cdc2* function, indicates clearly that the Dm cdc2c kinase is functionally distinct from Dm cdc2.

During *Drosophila* development, a variety of different cell cycle types are encountered. During the first thirteen cycles, G_1- and G_2-phases, as well as cytokinesis, are omitted. Starting with cycle 14, cell cycles acquire a G_2-phase and entry into M-phase is precisely regulated according to developmental fate. Cell cycle regulation at later stages is even more complex and entry into S-phase also is subjected to developmental regulation. Our experiments involving the temperature-sensitive allele $Dm\ cdc2^{E1\text{-}24}$ indicate that the Dm cdc2 kinase is essential for all these different mitotic cycles, including the early syncytial cycles which are dependent on maternally derived Dm cdc2 kinase.

In contrast to these mitotic cycles, Dm cdc2 kinase does not appear to be required during the process of polytenization during which cells progress through cycles of alternating G- and S-phases (Smith and Orr-Weaver, 1991). This endoreplication, which accompanies the growth of larval cells, continued normally in mutant larvae with amorphic *Dm cdc2* alleles up to the last larval instar. Although we cannot rule out the persistence of very low levels of maternally derived cdc2 activity present even in late mutant larvae, we emphasize that this hypothetical residual activity is insufficient to support the mitotic proliferation of the imaginal cells. Moreover, zygotic expression of *Dm cdc2* was detected exclusively in mitotically proliferating cells and not in polytene tissues (Lehner and O'Farrell, 1990; J. Knoblich and C.F.L, unpublished observation). The Dm cdc2c kinase, however, is present in endoreplicating salivary glands although at 10-fold lower levels compared to diploid imaginal tissues (B.S., unpublished observation).

The presence of a maternal contribution sufficient for all the mitotic proliferation during embryogenesis, but not sufficient for the imaginal cell proliferation during larval life, combined with the independence of larval growth from mitotic proliferation (Shearn et al., 1971; Szabad and Bryant, 1982; Gatti and Baker, 1989), results in a characteristic terminal phenotype in the case of amorphic *Dm cdc2* alleles. Fully grown larvae pupariate but fail to develop further because of the absence of imaginal tissues (imaginal discs, imaginal rings, abdominal histoblast nests). This organismal phenotype was postulated previously to characterize mutations in genes encoding functions specifically required for mitotic proliferation (Szabad and Bryant, 1982; Gatti and Baker, 1989). In the case of null mutations, however, this organismal phenotype can only be expected if the maternal contribution is stable enough to last through all the embryonic proliferation. While this is true in the case of Dm cdc2, this is not true in the case of the other mitotic regulators that have been analyzed so far. Zygotic expression of cyclin A and of *string*, which encodes a cdc25 phosphatase homolog, is required for embryonic proliferation (Lehner and O'Farrell, 1989; Edgar and O'Farrell, 1990). In contrast to Dm cdc2, the regulation of protein levels is crucial for the function of these regulators and necessitates the instability of the maternal contribution.

The reduction of the maternal *Dm cdc2* contribution resulted in an arrest of the mitotic proliferation in mutant embryos. Interestingly, we still observed proliferation in the CNS of mutant embryos while the proliferation in the PNS was completely blocked. Moreover, according to BrdU pulse-labeling experiments, the proliferation in the CNS appeared surprisingly normal, and the pattern and number of labeled cells in mutant embryos were comparable to wild type (data not shown). These findings might be explained by the pattern of residual expression from the $Dm\ cdc^{216P}$ allele, which is characterized by a P-element insertion in the 5′-untranslated region. However, in contrast to the nearly normal pattern of BrdU-incorporation, cyclin A was clearly much more widely expressed in the CNS of mutants compared to controls (compare Fig. 5A and B). This observation is consistent with the idea that exclusively terminal mitoses (i.e. the last divisions before cells withdraw from mitotic proliferation and enter a G_0-phase) are inhibited in these mutants. The consequences of *Dm cdc2* expression induced in the mutant embryos with the help of the *hs-cdc2* transgene are consistent with this idea. In the PNS, cells divided rapidly after *Dm cdc2* expression but never entered an additional cell cycle. In the CNS, the number of BrdU-labeled cells increased only twofold, whereas the number of mitotic figures increased over 20-fold (B. S. and C. F. L, data not shown). All these observations suggest that the imminent change in the proliferation status is forecasted by a higher Dm cdc2 requirement during the terminal mitosis. A further analysis of cell cycle regulators in *Drosophila* is expected, therefore, to reveal regulatory mechanisms specifically involved in the developmental control of cell proliferation in multicellular organisms.

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