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Synergistic action of *Drosophila* cyclins A and B during the G_2-M transition

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A variety of different cyclin proteins have been identified in higher eukaryotes. In the case of cyclin B, functional analyses have clearly demonstrated an important role in the control of entry into mitosis. The function of cyclin A is more complex. It appears to function in the control of both S- and M-phase. The results of our genetic analyses in *Drosophila* demonstrate that cyclin A has a mitotic function and that it acts synergistically with cyclin B during the G_2-M transition. In double mutant embryos that express neither cyclin A nor cyclin B zygotically, cell cycle progression is blocked just before the exhaustion of the maternally contributed cyclin A and B stores. BrdU-labeling experiments indicate that cell cycle progression is blocked in G_2 before entry into the fifteenth round of mitosis. Expression of either cyclin A or B from heat-inducible transgenes is sufficient to overcome this cell cycle block. This block is also not observed in single mutant embryos deficient for either cyclin A or B. In cyclin B deficient embryos, cell cycle progression continues after the apparent exhaustion of the maternal contribution, suggesting that cyclin B might not be essential for mitosis. However, mitotic spindles are clearly abnormal and progression through mitosis is delayed in these cyclin B deficient embryos.

Key words: cyclin A/cyclin B/*Drosophila*/G_2-M transition/mitosis

Introduction

Cyclin proteins act as regulatory subunits of protein kinase complexes involved in the control of eukaryotic cell cycle progression (for a recent review see Lew and Reed, 1992). Cyclin proteins are encoded by a diverse gene family. In higher eukaryotes, cyclin proteins have been classified as A, B, C, D or E-type according to structural comparisons. All these different cyclin types have been found in humans and *Drosophila* (Whitfield *et al.*, 1989; Lehner and O'Farrell, 1989, 1990; Pines and Hunter, 1989, 1990; Lahue *et al.*, 1991; Leopold and O'Farrell, 1991; Koff *et al.*, 1991; Lew *et al.*, 1991; Motokura *et al.*, 1991; Xiong *et al.*, 1991; H.Richardson and R.Saint, personal communication; R.Finley and R.Brent, personal communication). This evolutionary conservation suggests that the different cyclin types have independent functions.

In the case of the B-type cyclins, the functional analysis is well advanced. Biochemical and genetic analyses in a variety of organisms have uncovered the role of cyclin B in the control of entry into mitosis. Cyclin B accumulates during interphase and associates with the $p34^{cdc2}$ kinase. The formation of the cyclin B/$p34^{cdc2}$ kinase complex is accompanied by phosphorylation of the $p34^{cdc2}$ kinase subunit on several sites. Phosphorylation on a Thr residue (Thr161 in human $p34^{cdc2}$) appears to be required for the activity of the kinase (Ducommun *et al.*, 1991; Gould *et al.*, 1991; Krek and Nigg, 1991a; Lorca *et al.*, 1992b; Solomon *et al.*, 1992). Phosphorylation on another Thr and on a Tyr residue (Thr14 and Tyr15 in human $p34^{cdc2}$) inhibits kinase activity (Gould and Nurse, 1989; Solomon *et al.*, 1990; Krek and Nigg, 1991b; Norbury *et al.*, 1991). Some of the kinases and phosphatases controlling these phosphate modifications have been identified. Tyr15 and possibly Thr14 appear to be phosphorylated by the wee1 and mik1 kinases, and these inhibitory modifications are reversed by the cdc25 phosphatase (Russell and Nurse, 1986; Dunphy and Kumagai, 1991; Featherstone and Russell, 1991; Gautier *et al.*, 1991; Lundgren *et al.*, 1991; Millar *et al.*, 1991; Parker *et al.*, 1991; Strausfeld *et al.*, 1991). The cyclin B/$p34^{cdc2}$ kinase complex is activated immediately before mitosis. This activation process presumably involves a positive feedback loop in which cyclin B/$p34^{cdc2}$ kinase activity reduces Thr14/Tyr15-kinase activity and enhances cdc25 phosphatase activity (Kumagai and Dunphy, 1992). The resulting abrupt activation triggers entry into mitosis. During metaphase, the cyclin B subunit is rapidly degraded. This leads to the inactivation of the $p34^{cdc2}$ kinase, a step required for progression beyond metaphase (Ghiara *et al.*, 1991; Glotzer *et al.*, 1991).

Cyclin A is found in mammalian tissue culture cells in a complex with the cdk2 kinase, a cdc2-related kinase (Pines and Hunter, 1990, 1991; Tsai *et al.*, 1991). A number of observations have implicated this cyclin A/cdk2 complex in the regulation of cell proliferation and entry into S-phase. The cyclin A/cdk2 kinase is activated at the G_1-S transition and is found in the cell nucleus throughout S-phase (Pines and Hunter, 1991; Pagano *et al.*, 1992; Rosenblatt *et al.*, 1992). The cyclin A/cdk2 kinase has also been found in a larger complex containing the product of the retinoblastoma tumor suppressor gene pRB (or the related protein p107) and the transcription factor E2F which is released from this complex when the adenoviral oncoprotein E1A is expressed (Devoto *et al.*, 1992; Shirodkar *et al.*, 1992).

Recently, direct evidence for an involvement of cyclin A in the regulation of S-phase has been obtained. Antibodies against cyclin A, or antisense plasmids, inhibit entry into S-phase if injected into tissue culture cells during the G_1-phase (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992). Observations in *Drosophila* and *Xenopus*, on the other hand, have suggested that cyclin A is not essential for entry into S-phase. The arrest of cell cycle progression that is observed after the exhaustion of the maternally

contributed cyclin A stores in epidermal cells of cyclin A deficient *Drosophila* embryos occurs after entry into S-phase (Lehner *et al.*, 1991). In *Xenopus* egg extracts, cell cycle progression does not seem to require cyclin A, although the cdk2 kinase is clearly required for S-phase (Fang and Newport, 1991). Differences in the regulation of entry into S-phase in the growth-independent, early embryonic cycles lacking G_1-phases and the growth-regulated, later cycles with G_1-phases are expected and might explain these contrasting observations.

Cyclin A is found not only in association with the cdk2 kinase, but also in a complex with the $p34^{cdc2}$ kinase. Cyclin A/$p34^{cdc2}$ and cyclinB/$p34^{cdc2}$ complexes have similar but not identical properties *in vitro*. The cyclin A/$p34^{cdc2}$ complex is not efficiently phosphorylated on Tyr15, and is therefore activated faster than cyclinB/$p34^{cdc2}$ (Clarke *et al.*, 1992). In contrast to the B-complex, cyclin A/$p34^{cdc2}$ is not effective in triggering cyclin degradation (Luca *et al.*, 1991; Lorca *et al.*, 1992a). Moreover, the two complexes also have different effects on microtubule dynamics and endosome fusion (Buendia *et al.*, 1992; Thomas *et al.*, 1992). Based on these observations, the cyclin A/$p34^{cdc2}$ kinase complex has been suggested to act as a starter kinase regulating prophase events and the activation of the cyclin B/$p34^{cdc2}$ kinase complex which in turn controls metaphase events (Minshull *et al.*, 1990; Buendia *et al.*, 1991).

We have started a genetic approach in *Drosophila* in order to compare the functions of cyclins A and B. The identification of mutations in the cyclin A gene and the characterization of the phenotypic consequences have been described previously (Lehner and O'Farrell, 1989). Here, we report the isolation of deficiencies that delete the cyclin B gene. Surprisingly, no cell cycle block was observed in homozygous deficient embryos even after the apparent exhaustion of the maternally derived cyclin B stores. Mitoses continue in cyclin B deficient embryos presumably due to the function of another cyclin. Our double mutant analysis demonstrates that cyclin A and B are overlapping in function.

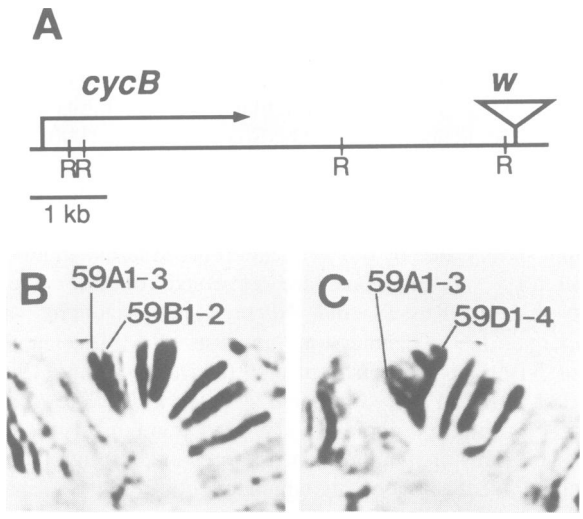

Fig. 1. Deficiencies deleting the cyclin B gene. **A**: The map illustrates the position of the P-element insertion in the *Drosophila* line *A2 2nd 27*. The P-element (triangle) that is marked with w^+ is located downstream of the cyclin B gene (*cycB*) in the chromosomal region 59A. R = *Eco*RI. **B** and **C**: Cytological analysis of the deficiencies *Df(2R)59AB* (panel B) and *Df(2R)59AD* (panel C) isolated as w^- revertants of *A2 2nd 27*.

Results

Isolation and phenotypic characterization of cyclin B deficiencies

Deficiencies that delete the cyclin B gene were isolated as w^- revertants of a P-element insertion marked with w^+ and located in the vicinity of the cyclin B gene (Figure 1A; see Materials and methods for details). Cytological analysis indicated that the two deficiencies, *Df(2R)59AB* and *Df(2R)59AD*, deleted part of the 59A region including the cyclin B gene (Figure 1B and C).

The development of embryos homozygous for the smaller

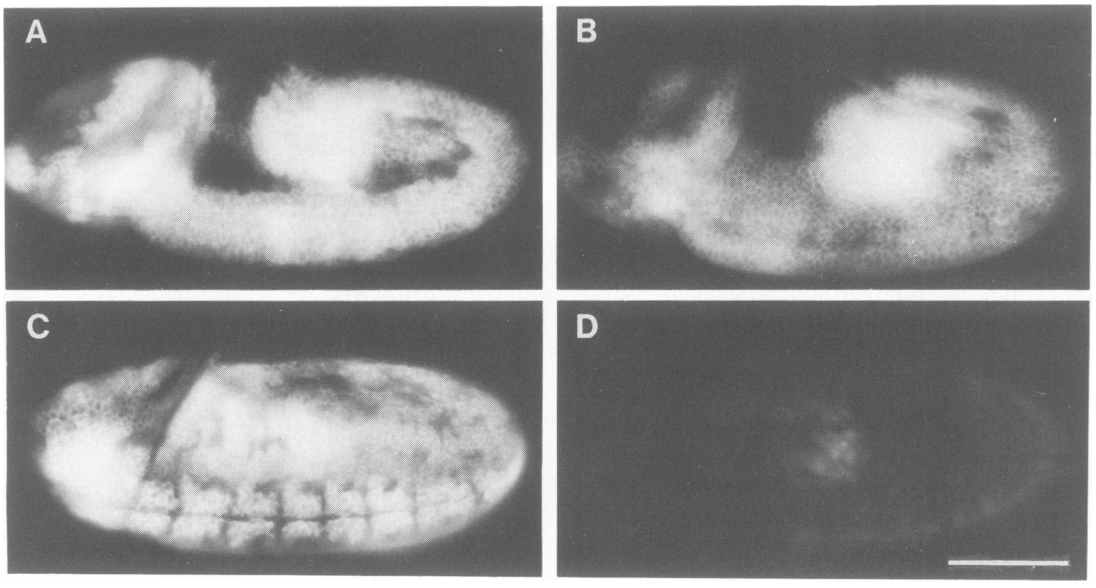

Fig. 2. Cyclin B expression in homozygous deficient embryos. Wildtype embryos (A and C) and embryos homozygous for the cyclin B deficiency *Df(2R)59AB* (B and D) were fixed at the time of mitosis 14 (A and B) or mitosis 15 (C and D), and labeled using a monoclonal antibody against cyclin B for indirect immunofluorescence. Bar in D = 100 μm.

deficiency, *Df(2R)59AB*, was analyzed in immunofluorescence experiments. No abnormalities were detected during the syncytial stages. During these stages, cyclin B is expressed in homozygous deficient embryos due to the presence of maternally contributed mRNA stores (Lehner and O'Farrell, 1990). The exhaustion of this maternal contribution was analyzed by immunolabeling with a monoclonal antibody against cyclin B (Figure 2). Before mitosis 14, signal intensities were already clearly lower in homozygous deficient embryos than in wildtype embryos (compare Figure 2A and B). After the degradation of cyclin B during mitosis 14, cyclin B accumulation was hardly detectable in homozygous deficient embryos before mitosis 15 (compare Figure 2C and D), and no signals above background were seen before mitosis 16 (Figure 4B′) except in the pole cells. The maternally provided cyclin B mRNA is known to be enriched in the pole cells (Whitfeld *et al.*, 1989; Lehner and O'Farrell, 1990). These germ line cells are mitotically quiescent during germ band extension. The maternally derived cyclin B protein is therefore presumably not degraded in these cells.

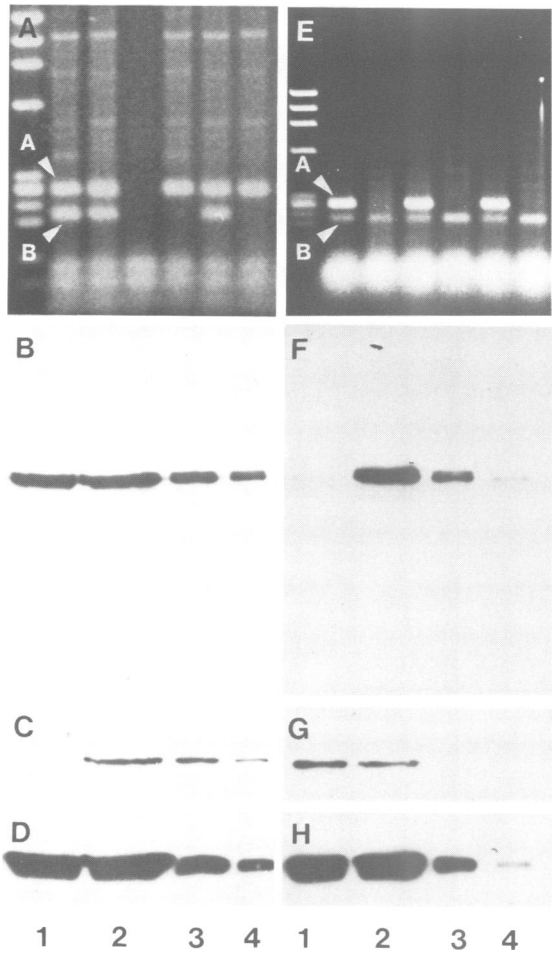

Fig. 3. The levels of cyclin A and B in homozygous deficient embryos. Panels **A** and **E**: Genotype analysis. Single embryos derived from parents heterozygous for either the cyclin B deficiency *Df(2R)59AB*, or the cyclin A deficiency $cycA^3$, were homogenized before mitosis 16. 25% of the homogenate was used in a PCR assay for the identification of homozygous mutant embryos. Homogenates from homozygous cyclin B deficient embryos allowed amplification of a 250 bp fragment derived from the cyclin A gene (arrowhead A) but not of a 210 bp fragment derived from the cyclin B gene (arrowhead B). Homogenates from homozygous cyclin A deficient embryos yielded the converse result. Non-mutant siblings allowed amplification from both cyclin genes. The PCR products obtained with six embryos derived from parents heterozygous for the cyclin B deficiency (panel A) or from parents heterozygous for the cyclin A deficiency (panel B) are shown as examples after resolution by agarose gel electrophoresis. A molecular weight marker (*Hae*III digest of ΦX174) is shown on the left in panels A and E. Panels **B–D** and **F–H**: Immunoblot analysis. The pooled, residual homogenates from cyclin B deficient embryos (panels B–D, lane 1), or from cyclin A deficient embryos (panels F–H, lane 1) and from non-mutant siblings (lanes 2–4) were resolved by SDS–PAGE and probed by immunoblotting with anti-cyclin A (panels B and F), anti-cyclin B (panels C and G), and anti-PSTAIR antibodies (panels D and H). Equal amounts were loaded in lanes 1 and 2. Lanes 3 and 4 contain 3- and 9-fold lower amounts, respectively, than lane 2. Whereas the complete immunoblots are shown in panels B and F, only the relevant regions are shown in panels C, D, G and H.

The exhaustion of the maternal contribution was analyzed quantitatively. Individual embryos at the stage before mitosis 16 were homogenized, and 25% of the homogenate was used in a PCR reaction for the determination of the genotype. An example of such a genotype determination is shown in Figure 3A. Homogenates from embryos which allowed enzymatic amplification of a cyclin A derived fragment but not of a cyclin B derived fragment were pooled and analyzed in immunoblotting experiments (Figure 3B–D, lane 1). For controls, we analyzed serial dilutions of pooled homogenates of siblings that were not homozygous deficient (Figure 3B–D, lanes 2–4). Comparison of signal intensities indicated that cyclin B levels in homozygous deficient embryos were at most 5% of wildtype levels. An analogous analysis in cyclin A deficient embryos which also initially express cyclin A from maternally contributed mRNA stores (Lehner and O'Farrell, 1989), revealed also in this case maximally 5% of the wildtype cyclin A levels (Figure 3E–H).

Previous analyses have indicated that the residual, maternally derived cyclin A is not sufficient for completion of cycle 16 in cyclin A deficient embryos despite the presence of cyclin B. Mitosis 16 and the concomitant, rapid degradation of cyclin B does not occur in the epidermis of cyclin A deficient embryos (Lehner and O'Farrell, 1989, 1990). In contrast, double labeling experiments with antibodies against cyclin A and cyclin B indicated that cyclin A was degraded at the stage of mitosis 16 in cyclin B deficient embryos (Figure 4). The characteristic patterns of stained and unstained cells which reflect progression through mitosis 16 in wildtype embryos (Figure 4A and A′) was also observed in cyclin B deficient embryos (Figure 4B and B′) but only with the antibody against cyclin A (Figure 4B). This observation suggested that mitosis 16 is not blocked in cyclin B deficient embryos despite the apparent exhaustion of the maternal cyclin B contribution.

In order to confirm the progression through mitosis 16 in cyclin B deficient mutants, we labeled the progeny from flies carrying the cyclin B deficient chromosome (over a balancer chromosome with a *fushi tarazu (ftz)–lacZ* transgene) with a mouse monoclonal antibody against β-tubulin. Since no rabbit antibody against cyclin B was available, we double labeled the embryos with a rabbit antibody against β-galactosidase for the identification of cyclin B deficient embryos. These experiments clearly revealed the presence of mitotic spindles not only in control embryos (Figure 5A, A′ and B) but also in cyclin B deficient embryos (Figure 5C, C′ and D). Mitotic figures were also evident after DNA labeling in cyclin B deficient embryos (see Figure 6).

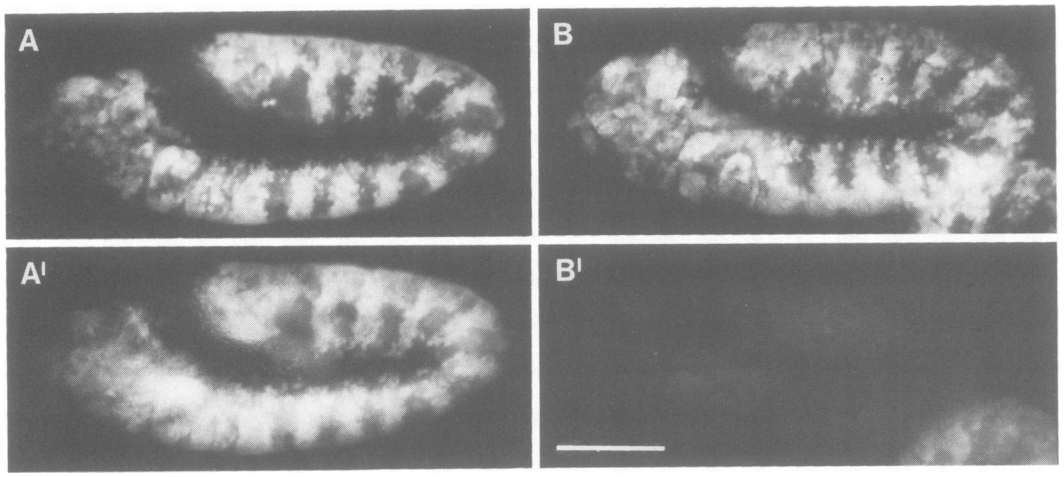

Fig. 4. Cyclin A degradation during mitosis 16 in cyclin B deficient embryos. Wildtype embryos (A,A′) and embryos homozygous for the cyclin B deficiency *Df(2R)59AB* (B,B′) were fixed at the time of mitosis 16 and double labeled with antibodies against cyclin A (A,B) and B (A′,B′). Adjacent to the cyclin B deficient embryo, part of a heterozygous sibling is visible in the right corner in (B,B′). Bar in B′ = 100 μm.

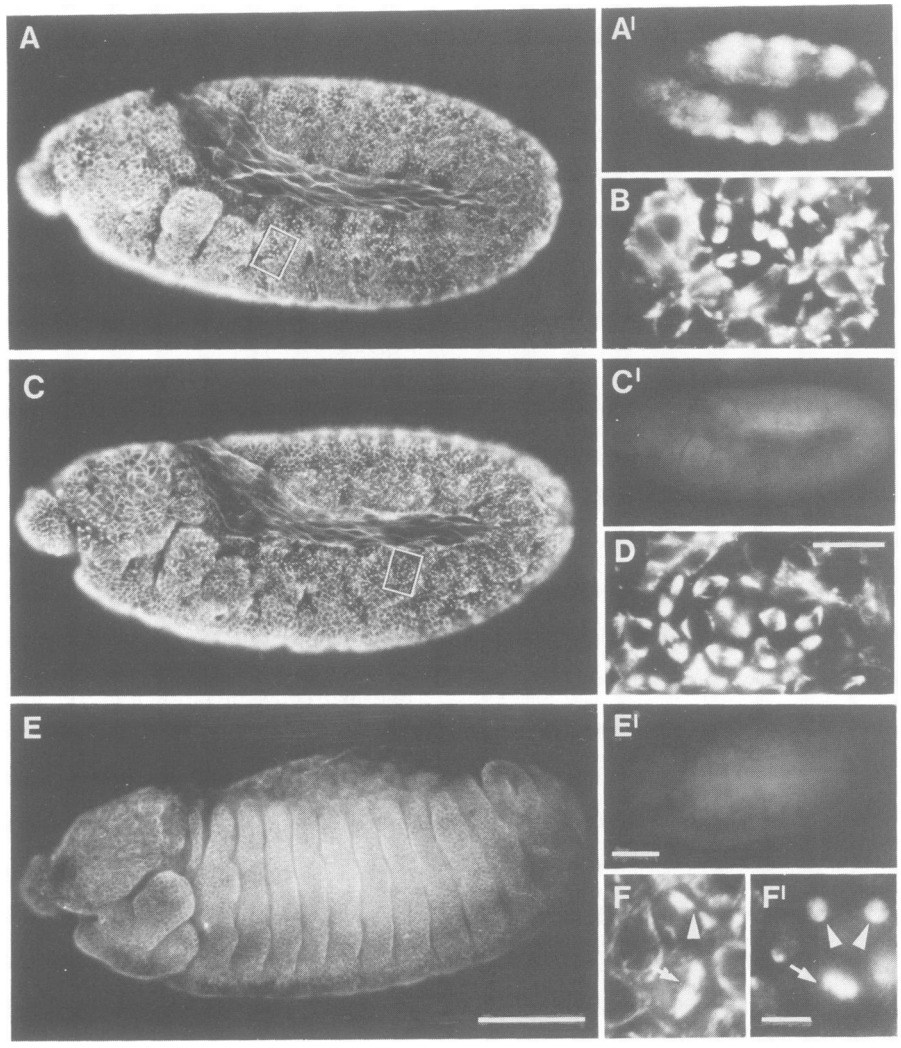

Fig. 5. Mitotic divisions in cyclin B deficient embryos. The progeny of flies carrying the cyclin B deficiency *Df(2R)59AB* over a balancer chromosome with a *ftz-lacZ* transgene were fixed and double labeled with antibodies against β-tubulin (A, B, C, D, E and F) and β-galactosidase (A′, C′ and E′). The regions boxed in (A) and (C) are shown at higher magnification in (B) and (D) respectively, and reveal mitotic spindles in the control embryo (A, A′ and B) and in the cyclin B deficient embryo (C, C′ and D) at the stage of mitosis 16. Mitotic figures are also present in the cyclin B deficient embryos after germ band retraction (E and E′). The arrow in (F) indicates a metaphase spindle, and the arrowhead points to a midbody of a telophase cell. Double labeling with a DNA stain (F′) reveals the corresponding metaphase plate (arrow) and daughter nuclei (arrowheads). Bars in D, E and E′, and F′ = 10, 100, 100 and 5 μm, respectively.

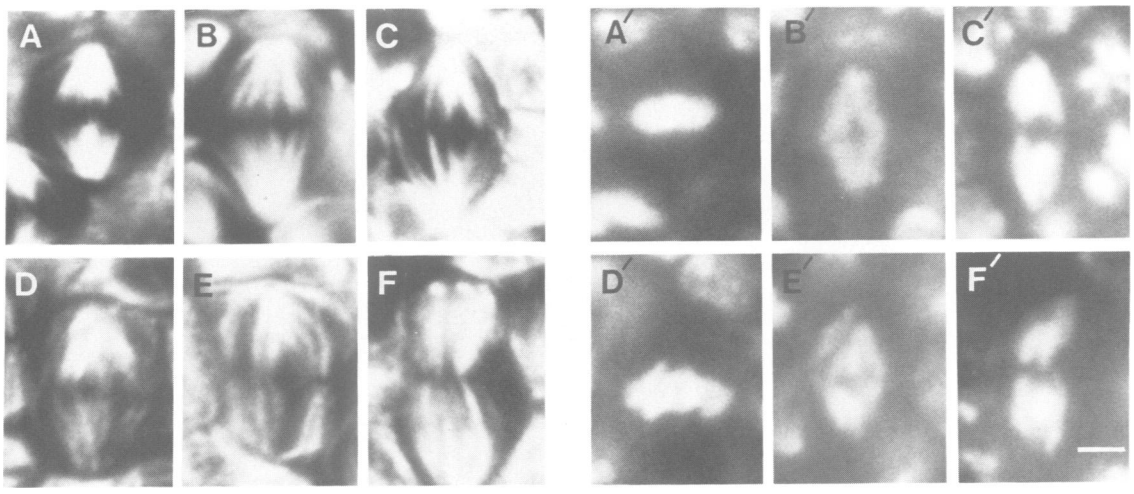

Fig. 6. Spindle abnormalities in cyclin B deficient embryos. Wildtype embryos (A−C and A′−C′) and embryos homozygous for the cyclin B deficiency *Df(2R)59AB* (D−F and D′−F′) were fixed during mitosis 16 and double-labeled with anti-β-tubulin antibodies (A−F) and with Hoechst 33258 (A′−F′). Bar in F′ = 2 μm.

Mitotic divisions in cyclin B deficient embryos occurred not only at the stage of mitosis 16 but also at later developmental stages when the mitotic proliferation is restricted to a few cells predominantly in the nervous system. Figure 5F and F′ show a metaphase cell (arrow) and a telophase cell (arrowheads) from a cyclin B deficient embryo (Figure 5E and E′) after germ band retraction.

So far, we have not detected obvious morphological abnormalities in the cyclin B deficient embryos. Nevertheless, these embryos did not hatch. We do not know whether this embryonic lethality results from the absence of the cyclin B gene or from the deletion of another gene.

The analysis of embryos homozygous for the larger deficiency, *Df(2R)59AD*, was complicated by the fact that it also deleted the *twist* gene which results in characteristic developmental abnormalities already in early homozygous embryos (Leptin and Grunewald, 1990). As in the case of the smaller deficiency, however, mitotic proliferation continued even after the apparent exhaustion of the maternal cyclin B contribution (not shown).

Mitotic abnormalities in cyclin B deficient embryos

The comparison of the spindle appearance in normal and cyclin B deficient embryos revealed subtle, but characteristic differences at high magnification (Figure 6). The mitotic spindles during metaphase and early anaphase appeared more organized in control embryos than in cyclin B deficient embryos. While chromosomes and spindle poles were connected by straight fibers in controls (Figure 6A and B), mutants were characterized by somewhat disheveled spindles (Figure 6D and E). Moreover, the onset of cytokinesis (as indicated by a constriction in the central plane of the spindle) was already apparent in mutants before the chromosomes were fully separated (Figure 6F and F′). In wildtype anaphase cells, with chromosomes separated by a comparable distance, the onset of cytokinesis is never observed (Figure 6C and C′). This difference is consistent with the idea that the disorganized mutant spindles separate the chromosomes only slowly. The fact that the fraction of mitotic cells was found to be elevated in cyclin B deficient embryos (Table I) further argues that mitosis is slow in these mutant embryos.

For a direct demonstration of a mitotic delay, we forced cells into mitosis 16 by inducing *string* expression from a transgene (*hs-string*). Previous experiments have demonstrated that the *string* gene encodes a *Drosophila* cdc25 homolog which is rate limiting for entry into the postblastoderm mitoses (Edgar and O'Farrell, 1990). Heat-induced expression of *string* resulted in a premature and almost perfectly synchronous mitosis 16 in cyclin B-deficient embryos and also in non-mutant siblings which served as controls (Figure 7). However, progression through mitosis was clearly slower in cyclin B deficient embryos. In these embryos, cyclin A was still present in the epidermal cells which were all in pro/metaphase 5 min after the heat shock (Figure 7C′ and C). In contrast, cyclin A was only present in a few epidermal cells in control embryos at the same timepoint (Figure 7A; cells labeled p and m). Whereas these few cells were also in pro/metaphase, all other cells had already progressed beyond the stage of cyclin A degradation and were in telophase (Figure 7A, see for example arrowheads) or early interphase. In the cyclin B deficient embryos, many cells were still in metaphase even 15 min after the heat shock, but anaphase and telophase figures became visible at this timepoint as well (Figure 7D; arrows and arrowheads respectively).

Table I. Mitotic delay in cyclin B deficient embryos

	Wildtype	Cyclin B deficient − *hs-cycB*	Cyclin B deficient + *hs-cycB*
Mitotic cells[a]	20 ± 4	60 ± 11	26 ± 5

[a] Wildtype embryos and cyclin B deficient embryos with or without the *hs-cycB* transgene were heat pulsed and fixed at the time of mitosis 16. After labeling with anti-β-tubulin antibodies, epidermal cells with mitotic spindles were counted in the hemisegments T1 and T2. The numbers given represent the average of at least five different embryos.

Since all these mitotic abnormalities were observed in embryos homozygous for a deficiency that presumably deletes a number of genes in addition to cyclin B, we tested whether the mitotic abnormalities could be rescued by expression of cyclin B. We therefore crossed a transgene with a heat shock promoter controlling the expression of cyclin B into the homozygous deficient embryos. Heat-

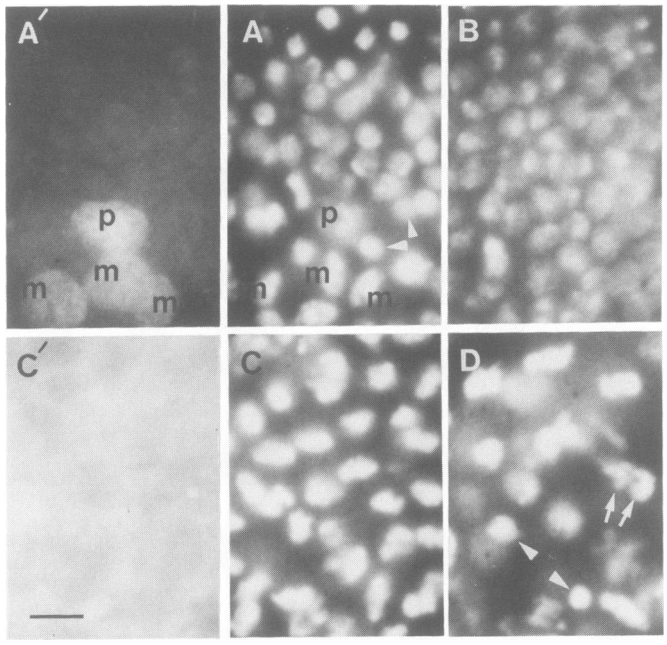

Fig. 7. Mitotic delay in cyclin B deficient embryos. A synchronized entry into mitosis 16 was induced by a heat pulse in the progeny of flies heterozygous for the *hs-string* transgene (Edgar and O'Farrell, 1990) and the cyclin B deficiency *Df(2R)59AB*. Embryos were fixed either 5 min (A, A′, C and C′) or 15 min (B and D) after the end of the heat pulse, and double-labeled with antibodies against cyclin A (A′ and C′), β-galactosidase (not shown) and Hoechst 33258 (A−D). Non-mutant siblings (A, A′ and B) and homozygous deficient embryos (C, C′ and D) were identified based on the anti-β-galactosidase labeling (see Materials and methods). Regions with epidermal cells are shown in A−D. **A** and **A′**: In non-mutant siblings, cyclin A staining is only detected in a prophase cell (p) and in three metaphase cells (m). All other cells have progressed beyond metaphase 5 min after the heat pulse and have degraded cyclin A. White arrowheads indicate a telophase figure. **B**: Interphase cells 15 min after the heat pulse in non mutant siblings. **C** and **C′**: None of the cells has progressed beyond metaphase and none has degraded cyclin A in cyclin B deficient embryos 5 min after the heat shock. **D**: 15 min after the heat shock, anaphase (arrows) and telophase figures (arrowheads) become visible in cyclin B deficient embryos. Bar in C′ = 5 μm.

induced expression of cyclin B completely rescued the spindle abnormalities (not shown) and the mitotic delay (Table I). We conclude that the observed mitotic abnormalities reflect the lack of cyclin B.

Synergistic action of cyclin A and B

The observation that mitotic divisions continued in cyclin B deficient embryos suggested the presence of another cyclin protein providing some of the functions of cyclin B. In *Xenopus* and humans, a pair of closely related B-type cyclins has been described (Minshull *et al.*, 1989; Pines and Hunter, 1989; S.Reed, personal communication). However, in *Drosophila*, we were unable to detect an additional, closely related B-type cyclin by low stringency hybridizations or PCR.

We tested whether cyclin A might functionally overlap with cyclin B. Mitotic divisions were analyzed after anti-β-tubulin labeling in double mutant embryos unable to express either cyclin A or cyclin B zygotically. Double mutant embryos were identified after double labeling with anti-β-galactosidase antibodies (see Materials and methods for details). Interestingly, no mitotic figures were observed in double mutant embryos at the stage of mitosis 15 (Figure 8E, E′ and F). In contrast, mitotic figures were readily observed in siblings expressing either only cyclin A (Figure 8A, A′ and B) or only cyclin B (Figure 8C, C′ and D) zygotically.

It was important to exclude the possibility that the early arrest of mitotic proliferation which was observed in double mutant embryos was due to the absence of an unidentified gene deleted in the deficiency *Df(2R)59AB*. We therefore tested whether expression of cyclin B from the heat inducible transgene was able to rescue the early block of cell divisions in double mutant embryos. Anti-β-tubulin labeling clearly demonstrated that mitosis 15 was rescued by cyclin B expression in double mutant embryos. As expected, mitosis 15 was also rescued in double mutant embryos by heat induced expression of the *hs-cycA* transgene (not shown).

By pulse labeling with BrdU, we analyzed DNA replication in double mutant embryos. BrdU was clearly incorporated during S-phase 15 both in control embryos (Figure 9A) and in double mutant embryos during S-phase 15 (Figure 9B). Progression through S-phase 15 occurs asynchronously in a temporally and spatially defined pattern (Edgar and O'Farrell, 1990). In Figure 9, BrdU incorporation is therefore found restricted to the region of the late replicating, heterochromatic chromocenter in cells that enter S-phase 15 early (see arrowhead in Figure 9), and throughout the nucleus in cells that enter S-phase 15 late (see arrow in Figure 9). We emphasize that BrdU incorporation is also found in the late replicating heterochromatin in double mutant embryos. This observation indicates that DNA replication during S-phase 15 is complete in double mutant embryos. We conclude, therefore, that cell cycle progression in the absence of zygotic expression of cyclins A and B is arrested in the G_2 phase before mitosis 15.

Discussion

The analysis of cell proliferation in embryos homozygous for the cyclin B deficiencies (*Df(2R)59AB* and *Df(2R)59AD*) yielded an unexpected result. Proliferation was found to continue in mutant embryos that cannot express cyclin B

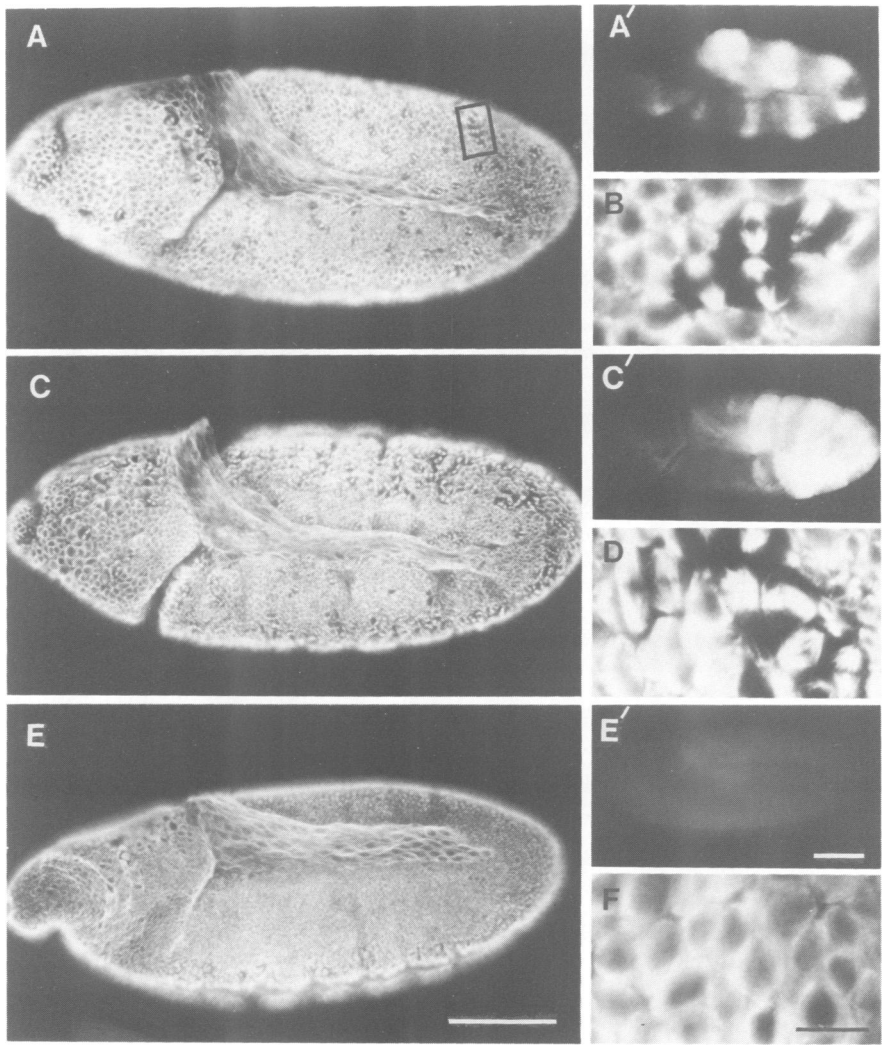

Fig. 8. Synergistic action of cyclin A and B during mitosis 15. Embryos derived from parents heterozygous for *cycA*^{5} and *Df(2R)59AB* were fixed at the time of mitosis 15 and double labeled with antibodies against β-tubulin (A−F) and β-galactosidase (A′, C′ and E′). The anti-β-galactosidase labeling allowed the identification of embryos (see Materials and methods) that were homozygous for either *cycA*^{5} (A, A′ and B) or *Df(2R)59AB* (C, C′ and D) or both *cycA*^{5} and *Df(2R)59AB* (E, E′ and F). The anti-β-tubulin labeling allowed the identification of cells in mitosis. Mitotic spindles are clearly visible in the high magnification views (B and D) and can also be detected in the low magnification views (A and C; see for instance the boxed region in A showing the same region as B) throughout the epidermis of embryos deficient for either cyclin A or cyclin B. In contrast, mitotic spindles are not observed in double mutant embryos (E and F). Bars in E, E′ and F = 100, 100 and 10 μm, respectively.

zygotically, even after the maternally derived cyclin B appeared exhausted according to immunofluorescent labeling. By quantitative immunoblotting, we found that the level of cyclin B present in mutant embryos before mitosis 16 is <5% of the wildtype level. After mitosis 16, cyclin B expression and cell proliferation are restricted in wildtype embryos to a few cells predominantly in the developing nervous system. This circumstance in conjunction with detection limits precluded a quantitative analysis at later developmental stages. However, assuming that the half life of the maternal cyclin B contribution is constant, we estimate that there must be >1000-fold less cyclin B in mutants than in wildtype embryos during the latest stages that we have analyzed. The fact that mitotic divisions still occur in mutants at these late stages indicates that cyclin B is either not essential for mitosis or required only at very low concentrations.

In contrast to these observations, cyclin B function has been found to be clearly required for mitosis in a variety of systems. Mutations in cdc13 encoding a *Schizosaccharomyces pombe* B-type cyclin and disruption of B-type cyclin genes in *Saccharomyces cerevisiae* block entry into mitosis (Booher and Beach, 1988; Hagan *et al.*, 1988; Ghiara *et al.*, 1991; Surana *et al.*, 1991). Ablation of cyclin B mRNA in *Xenopus* egg extracts also prevents entry into mitosis (Minshull *et al.*, 1989). Interestingly, however, cyclin B mRNA ablation in *Xenopus* embryos results in a transient arrest only, and cell cycle progression resumes in the absence of cyclin B mRNA (Weeks *et al.*, 1991).

Analyses of *S.cerevisiae* and *Xenopus* egg extracts have also revealed multiple B-type cyclins with overlapping functions (Minshull *et al.*, 1989; Ghiara *et al.*, 1991; Surana *et al.*, 1991). Multiple B-type cyclins have also been identified in humans (Pines and Hunter, 1989; S.Reed, personal communication). If there were multiple B-type cyclins in *Drosophila*, this might explain the continued divisions seen in cyclin B deficient embryos. Although we cannot exclude this possibility, we emphasize that we have failed to detect a closely related B-type cyclin by low stringency hybridizations and PCR approaches (Lehner and

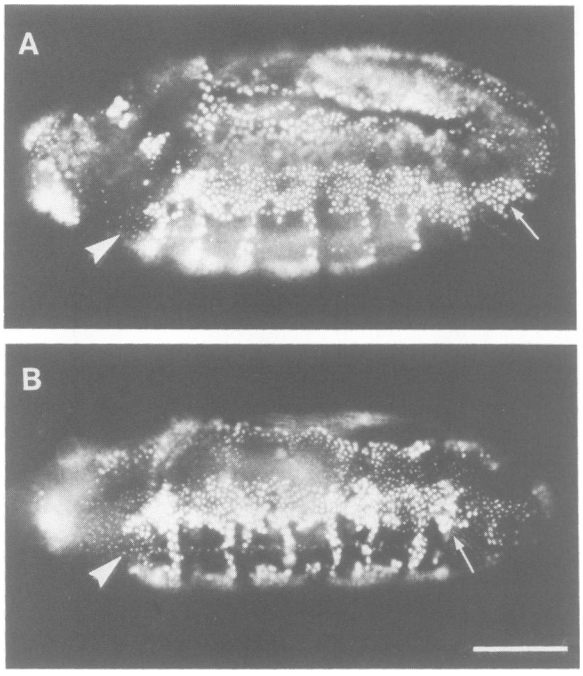

Fig. 9. DNA replication during S-phase 15 in embryos deficient for cyclins A and B. Embryos derived from parents heterozygous for *cycA*^{5} and the cyclin B deficiency *Df(2R)59AB* were pulse labeled with BrdU during S-phase 15, fixed and double-labeled with antibodies against BrdU (A and B) and β-galactosidase (not shown). The β-galactosidase labeling allowed the identification of embryos homozygous deficient for both *cycA*^{5} and *Df(2R)59AB* (see Materials and methods). BrdU incorporation occurred in these double mutant embryos (B), and indistinguishably in the control embryos (A), in the early replicating euchromatin (arrows) and in the late replicating heterochromatin (arrowheads). Bar in B = 100 μm.

O'Farrell, 1990; J.A.Knoblich, unpublished observations).

In addition to B-type cyclins, higher eukaryotes express cyclin A, which has not been found in yeast so far. Cyclin A is known to associate with the same p34^{cdc2} kinase that is also bound by cyclin B. Therefore, cyclins A and B might have overlapping functions. Our experiments demonstrate that cyclin A and cyclin B act synergistically *in vivo*. While high levels of either cyclin in combination with low levels of the other cyclin are sufficient for mitosis 15, this division does not occur when both cyclins are only present at low levels.

The notion that cyclin A has a mitotic function has been suggested before based on analyses in *Xenopus* egg extracts and in cyclin A deficient *Drosophila* embryos (Minshull *et al.*, 1990; Lehner *et al.*, 1991; Roy *et al.*, 1991). In these mutant embryos, cell cycle progression in the epidermis was found to be blocked before mitosis 16, and pulse labeling experiments with BrdU demonstrated that the block occurred after entry into S-phase 16. However, the possibility that chromosomes might not be completely replicated during S-phase 16 in cyclin A deficient embryos was not excluded in these analyses. Incomplete replication is expected to result in a block of entry into mitosis (checkpoint arrest; see Hartwell and Weinert, 1989). The interpretation that the block of mitosis 16 in cyclin A deficient embryos may represent a checkpoint arrest is fostered by the recent, direct demonstrations that cyclin A has a role in the control of S-phase in mammalian tissue culture cells (Girard *et al.*, 1991; Pagano *et al.*, 1992; Zindy *et al.*, 1992). We have also not directly excluded a checkpoint arrest in the case of the mitosis 15 block in double mutant embryos. However, we discount this possibility based on the following arguments. First, BrdU incorporation in double mutant embryos is also found in the late replicating chromocenter indicating that S-phase 15 is complete. Second, the previous analysis of cyclin A deficient embryos has demonstrated that the maternal cyclin A contribution is sufficient (if required at all) for at least substantial DNA replication during S-phase of the following cycle 16 (Lehner *et al.*, 1991). We conclude, therefore, that cell cycle progression is arrested in the G_2-phase of cycle 15 in double mutant embryos. This result demonstrates that cyclin A has a mitotic function. Based on results of microinjection of antibodies against cyclin A in human tissue culture cells, Pagano *et al.* (1992) have reached the same conclusion very recently.

The finding that cyclins A and B have overlapping functions in mitosis might explain the observation that mitotic divisions proceed in the virtual absence of cyclin B. However, these mitotic divisions are clearly abnormal indicating that the functions of cyclins A and B are not identical. Recent analyses in *Xenopus* and clam egg extracts have also revealed functional differences between cyclins A and B. In contrast to cyclin B/p34^{cdc2} kinase, cyclin A/p34^{cdc2} kinase was found to escape inhibition by phosphorylation on Tyr15 to a large extent in these extracts (Clarke *et al.*, 1992). Moreover, in contrast to cyclin B, cyclin A was also found to be incapable of triggering cyclin degradation (Luca *et al.*, 1991; Lorca *et al.*, 1992a). We emphasize that mitosis in cyclin B deficient embryos was still regulated by *string* (which encodes the cdc25 phosphatase that reverses the inhibitory phosphorylation of Tyr15) and was still accompanied by cyclin A degradation. *In vitro*, cyclin A and cyclin B associated kinase activities also have different effects on microtubule dynamics and microtubule nucleation from centrosomes (Buendia *et al.*, 1992). Cyclin A specifically stimulates microtubule nucleation and cyclin B specifically leads to microtubule shrinkage. The spindle abnormalities observed in cyclin B deficient embryos are entirely consistent with these *in vitro* results. Microtubules in metaphase and early anaphase cells appeared less organized in the mutants than in wildtype cells, where straight microtubules connect the spindle poles and chromosomes. Typically, astral microtubules appeared more abundant in the mutant metaphase cells. These observations are therefore consistent with the idea that a cyclin B dependent increase in microtubule dynamics facilitates *in vivo* the reorganization of prophase into metaphase asters. Reduced microtubule dynamics might also be responsible for the mitotic delay observed in cyclin B deficient embryos.

In summary, our results demonstrate that cyclin A has a mitotic role and acts synergistically with cyclin B which facilitates the organization of the mitotic spindle and allows a rapid progression through mitosis.

Materials and methods

Drosophila stocks

The *Drosophila* stock *A2 2nd 27* containing a P-element insertion marked with w^{+} in the chromosomal region 59A was isolated in an enhancer trap screen in the laboratory of Y.N.Jan, UCSF, San Francisco (Jongens *et al.*, 1992).

Deficiencies that delete cyclin A [*CycA*^{3}, synonym *l(3)183* (Hoogwerf *et al.*, 1988; Lehner *et al.*,1991)] or cyclin B [*Df(2R)59AB* and *Df(2R)59AD*,

see below] were balanced with balancer chromosomes (*CyO,P[w+,ftz-lacZ]* and *TM3,P[w+,Ubx−lacZ]*) carrying transgenes that direct the expression of β-galactosidase in characteristic spatial patterns. Homozygous deficient progeny from these stocks could therefore be identified based on the absence of anti-β-galactosidase labeling. In some experiments, $CycA^5$ [synonym *l(3)neo114* (Lehner and O'Farrell, 1989)] was used instead of $CycA^3$.

The fly stock with the *hs-stg3* transgene which allows expression of a *string* cDNA under control of a heat shock promoter has been described previously (Edgar and O'Farrell, 1990) and was kindly provided by B.Edgar, UCSF, San Francisco.

Fly stocks with transgenes (*hs-cycA* and *hs-cycB*) allowing the expression of either a cyclin A cDNA or a cyclin B cDNA under the control of a heat shock promoter were obtained by P-element transformation (Spradling, 1986) with appropriate constructs in the vector pCaSpeR-hs (Pirotta, 1988).

Isolation of deficiencies

According to *in situ* hybridization on polytene chromosomes and Southern blot experiments (T.Jongens, Y.N.Jan and C.F.Lehner, unpublished observations), the P-element insertion *A2 2nd 27* carrying w^+ was found to be 4 kb downstream of the cyclin B gene (see Figure 1A). In order to isolate deficiencies deleting the cyclin B gene, we screened for w^- revertants after X-ray mutagenesis. 5000 males (*A2 2nd 27*/SM1) were irradiated (4500 rad) and crossed with 5000 virgin females (*A2 2nd 27*/SM1). Among 50 000 progeny (*A2 2nd 27*/SM1), 16 w^- revertants were identified, but only two of the lines that were established proved to be deficiencies according to genetic, cytological and molecular analyses. The breakpoints of the first deficiency, *Df(2R)59AB*, were determined cytologically as 59A1-3;59B1-2, and in the case of the second deficiency, *Df(2R)59AD*, as 59A1-3;59D1-4.

Antibodies, BrdU labeling and immunofluorescence experiments

Mouse monoclonal antibodies against cyclin B were produced by the monoclonal antibody core facility at UCSF, San Francisco using a bacterially expressed cyclin B fusion protein as immunogen (Lehner and O'Farrell, 1990). Hybridoma supernatant with the mouse monoclonal antibody F2 against cyclin B was used at a dilution of 1:3 in immunofluorescence experiments, and of 1:40 in immunoblotting experiments. The affinity-purified, rabbit polyclonal antibody against cyclin A, and the mouse monoclonal anti-PSTAIR antibody have been described previously (Lehner and O'Farrell, 1990; Yamashita *et al.*, 1991). Mouse monoclonal antibodies against bromodeoxyuridine (BrdU, Becton-Dickinson) and β-tubulin (Amersham), and rabbit antibodies against β-galactosidase (Cappel), as well as all secondary antibodies (Dianova) were obtained commercially. Immunofluorescence experiments were done as described previously (Lehner and O'Farrell, 1989; Lehner *et al.*, 1991). Pulse labeling with BrdU, followed by immunofluorescence analysis was done as described previously (Edgar and O'Farrell, 1990).

Quantitative analysis of cyclin protein levels in mutant embryos

In order to determine the level of the residual, maternally derived cyclin protein present in embryos homozygous for deficiencies deleting either the cyclin A or the cyclin B gene, we developed a PCR assay which allowed the identification of such embryos. Eggs from either the $CycA^3$/*TM3* or the *Df(2R)59AB*/*CyO* stock were collected for 1 h on apple juice agar plates and aged for 6 h at 25°C. Individual embryos were homogenized at 4°C in 4 μl PCR buffer (50 mM KCl, 0.01% gelatin, 10 mM Tris−HCl, pH 8.3) containing 0.5% Nonidet P-40 and 0.5% Tween 20. 3 μl of the homogenate were combined with 3 μl 3×SDS gel sample buffer, heated at 95°C for 5 minutes, and immediately stored at −70°C for subsequent analysis by immunoblotting. 1 μl of the homogenate was added to 4 μl PCR buffer with 50 μg/ml proteinase K, covered with mineral oil, and incubated for 1 h at 50°C. Subsequently, proteinase K was inactivated by a 10 min incubation at 94°C. After cooling to 4°C, 15 μl of a PCR mix (PCR buffer with 3 mM $MgCl_2$, 1.5 μM of each primer, 250 μM dGTP, dCTP, dATP and dTTP, 0.5 U *Taq* polymerase) were added.

For the identification of cyclin A deficient embryos, we used primer 1 [GCA GGATCC ATG GG(CGT) GCC AT(TC) (TC)T(CGT) AT(TC) GA(TC) TGG] and primer 2 [GCA GTCGAC GG(GT) GG(AG) TA(GTC) ATC TCC TC(AG) TA (TC) TT]. These two primers allow the efficient amplification of a 260 bp fragment from the cyclin A gene and the somewhat less efficient amplification of a 220 bp fragment from the cyclin B gene.

For the identification of cyclin B deficient embryos, we used primer 2 in combination with primer 3 (ATG CGA GCC GTG CTG ATC GAT TGG) and primer 4 (ATG CGC TCC ATC CTT ATT GAT TGG). These three primers allowed an efficient amplification of a 250 bp fragment from the cyclin A gene and an equally efficient amplification of a 210 bp fragment from the cyclin B gene.

After enzymatic amplification (1 min at 94°C, 2 min at 55°C, 1 min at 72°C, 35 cycles), reaction products were analyzed on agarose gels.

The residual homogenates of embryos scored as homozygous deficient were pooled. For controls, we pooled the residual homogenates of embryos that did not score as homozygous deficient. Serial dilutions of the control homogenates next to the homogenate from homozygous deficient embryos were resolved by SDS−PAGE, transferred to nitrocellulose and probed sequentially with the affinity purified rabbit anti-cyclin A antibodies, the mouse monoclonal anti-cyclin B antibody (F2) and mouse monoclonal anti-PSTAIR antibody. Bound antibodies were visualized using an ECL kit (Amersham).

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References

Booher,R. and Beach,D. (1988) *EMBO J.*, **7**, 2321−2327.
Buendia,B., Clarke,P.R., Felix,M.A., Karsenti,E., Leiss,D. and Verde,F. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 523−532.
Buendia,B., Draetta,G. and Karsenti,E. (1992) *J. Cell Biol.*, **116**, 1431−1442.
Clarke,P.R., Leiss,D., Pagano,M. and Karsenti,E. (1992) *EMBO J.*, **11**, 1751−1761.
Devoto,S.H., Mudryj,M., Pines,J., Hunter,T. and Nevins,J.R. (1992) *Cell*, **68**, 167−176.
Ducommun,B., Brambilla,P., Félix,M.-A., Franza,B.R.,Jr, Karsenti,E. and Draetta,G. (1991) *EMBO J.*, **10**, 3311−3319.
Dunphy,W.G. and Kumagai,A. (1991) *Cell*, **67**, 189−196.
Edgar,B.A. and O'Farrell,P.H. (1990) *Cell*, **62**, 469−480.
Fang,F. and Newport,J.W. (1991) *Cell*, **66**, 731−742.
Featherstone,C. and Russell,P. (1991) *Nature*, **349**, 808−810.
Gautier,J., Solomon,M.J., Booher,R.N., Bazan,J.F. and Kirschner,M.W. (1991) *Cell*, **67**, 197−211.
Ghiara,J.B., Richardson,H.E., Sugimoto,K., Henze,M., Lew,D.J., Wittenberg,C. and Reed,S.I. (1991) *Cell*, **65**, 163−174.
Girard,F., Strausfeld,U., Fernandez,A. and Lamb,N.J.C. (1991) *Cell*, **67**, 1169−1179.
Glotzer,M., Murray,A.W. and Kirschner,M.W. (1991) *Nature*, **349**, 132−138.
Gould,K.L. and Nurse,P. (1989) *Nature*, **342**, 39−45.
Gould,K.L., Moreno,S., Owen,D.J., Sazer,S. and Nurse,P. (1991) *EMBO J.*, **10**, 3297−3309.
Hagan,I., Hayles,J. and Nurse, P (1988) *J. Cell Sci.*, **91**, 587−595.
Hartwell,L.H. and Weinert,T.A. (1989) *Science*, **246**, 629−634.
Hoogwerf,A.M., Akam,M. and Roberts,D. (1988) *Genetics*, **118**, 665−670.
Jongens,T.A., Hay,B., Jan,L.Y. and Jan,Y.N. (1992) *Cell*, **70**, 569−584.
Koff,A., Cross,F., Fisher,A., Schumacher,J., Leguellec,K., Philippe,M. and Roberts,J.M. (1991) *Cell*, **66**, 1217−1228.
Krek,W. and Nigg,E.A. (1991a) *EMBO J.*, **10**, 305−316.
Krek,W. and Nigg,E.A. (1991b) *EMBO J.*, **10**, 3331−3341.
Kumagai,A. and Dunphy,W.G. (1992) *Cell*, **70**, 139−151.
Lahue,E.E., Smith,A.V. and Orr-Weaver,T.L. (1991) *Genes Dev.*, **5**, 2166−2175.
Lehner,C.F. and O'Farrell,P.H. (1989) *Cell*, **56**, 957−968.
Lehner,C.F. and O'Farrell,P.H. (1990) *Cell*, **61**, 535−547.
Lehner,C.F., Yakubovich,N. and O'Farrell,P.H. (1991) *Cold Spring Harbor Symp. Quant. Biol.*, **56**, 465−475.
Léopold,P. and O'Farrell,P.H. (1991) *Cell*, **66**, 1207−1216.
Leptin,M. and Grunewald,B. (1990) *Development*, **110**, 73−84.
Lew,D.J. and Reed,S.I. (1992) *Trends Cell Biol.*, **2**, 77−80
Lew,D.J., Dulic,V. and Reed,S.I. (1991) *Cell*, **66**, 1197−1206.
Lorca,T., Labbé,J.-C., Devault,A., Fesquet,D., Strausfeld,U., Nilsson,J., Nygren,P.-A., Uhlen,M., Cavadore,J.-C. and Dorée,M. (1992a) *J. Cell Sci.*, **102**, 55−62.
Lorca,T., Labbé,J.-C., Devault,A., Fesquet,D., Capony,J.-P., Cavadore,J.-C., Le Bouffant,F. and Dorée,M. (1992b) *EMBO J.*, **11**, 2381−2390.
Luca,F.C., Shibuya,E.K., Dohrmann,C.E. and Ruderman,J.V. (1991) *EMBO J.*, **10**, 4311−4320.
Lundgren,K., Walworth,N., Booher,R., Dembski,M., Kirschner,M. and Beach,D. (1991) *Cell*, **64**, 1111−1122.

Millar,J.B.A., McGowan,C.H., Lenaers,G., Jones,R. and Russell,P. (1991) *EMBO J.*, **10**, 4301–4309.
Minshull,J., Blow,J.J. and Hunt,T. (1989) Cell, **56**, 947–956.
Minshull,J., Golsteyn,R., Hill,C.S. and Hunt,T. (1990) *EMBO J.*, **9**, 2865–2875.
Motokura,T., Bloom,T., Kim,H.G., Jüppner,H., Ruderman,J.V., Kronenberg,H.M. and Arnold,A. (1991) *Nature*, **350**, 512–515.
Norbury,C., Blow,J. and Nurse,P. (1991) *EMBO J.*, **10**, 3321–3329.
Pagano,M., Pepperkok,R., Verde,F., Ansorge,W. and Draetta,G. (1992) *EMBO J.*, **11**, 961–971.
Parker,L.L., Atherton-Fessler,S., Lee,M.S., Ogg,S., Falk,J.L., Swenson,K.I. and Piwnica-Worms,H. (1991) *EMBO J.*, **10**, 1255–1263.
Pines,J. and Hunter,T. (1989) *Cell*, **58**, 833–846.
Pines,J. and Hunter,T. (1990) *Nature*, **346**, 760–763.
Pines,J. and Hunter,T. (1991) *J. Cell Biol.*, **115**, 1–17.
Pirrotta,V. (1988) In R.L. Rodriguez and D.T. Denhardt (eds), *Vectors. A Survey of Molecular Cloning Vectors and Their Uses*. Butterworth, Boston, pp.437–456.
Rosenblatt,J., Guy, Y. and Morgan,D.O. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 2824–2828.
Roy,L.M., Swenson,K.I., Walker,D.H., Gabrielli,B.G., Li,R.S., Piwnica-Worms,H. and Maller,J.L. (1991) *J. Cell Biol.*, **113**, 507–514.
Russell,P. and Nurse,P. (1987) *Cell*, **49**, 559–567.
Shirodkar,S., Ewen,M., DeCaprio,J.A., Morgan,J., Livingston,D.M. and Chittenden,T. (1992) *Cell*, **68**, 157–166.
Solomon,M.J., Glotzer,M., Lee,T.H., Philippe,M. and Kirschner,M.W. (1990) *Cell*, **63**, 1013–1024.
Solomon,M.J., Lee,T. and Kirschner,M.W. (1992) *Mol. Biol. Cell*, **3**, 13–27.
Spradling,A. (1986) In Roberts,D.B. (ed.), *Drosophila—A Practical Approach*. IRL Press, Oxford, pp. 175–198.
Strausfeld,U., Labbé,J.C., Fesquet,D., Cavadore,J.C., Picard,A., Sadhu,K., Russell,P. and Dorée,M. (1991) *Nature*, **351**, 242–245.
Surana,U., Robitsch,H., Price,C., Schuster,T., Fitch,I., Futcher,A.B. and Nasmyth,K. (1991) *Cell*, **65**, 145–161.
Thomas,L., Clarke,P.R., Pagano,M. and Gruenberg,J. (1992) *J. Biol. Chem.*, **267**, 6183–6187.
Tsai,L.-H., Harlow,E. and Meyerson,M. (1991) *Nature*, **353**, 174–177.
Weeks,D.L., Walder,J.A. and Dagle,J.M. (1991) *Development*, **111**, 1173–1178.
Whitfield,W.G.F., González,C., Sánchez-Herrero,E. and Glover,D.M. (1989) *Nature*, **338**, 337–340.
Xiong,Y., Connolly,T., Futcher,B. and Beach,D. (1991) *Cell*, **65**, 691–699.
Yamashita,M. Yoshikuni,M., Hirai,T., Fukada,S. and Nagahama,Y. (1991) *Dev. Growth Diff.*, **33**, 617–624.
Zindy,F., Lamas,E., Chenivesse,X., Sobczak,J., Wang,J., Fesquet,D., Henglein,B. and Bréchot,C. (1992) *Biochem. Biophys. Res. Commun.*, **182**, 1144–1154.

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