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The A- and B-type cyclins of *Drosophila* are accumulated and destroyed in temporally distinct events that define separable phases of the G2-M transition

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We show that the sequence of *Drosophila* cyclin B has greater identity with B-type cyclins from other animal phyla than with *Drosophila* cyclin A, suggesting that the two cyclins have distinct roles that have been maintained in evolution. Cyclin A is not detectable in unfertilized eggs and is present at low levels prior to cellularization of the syncytial embryo. In contrast, the levels of cyclin B remain uniformly high throughout these developmental stages. In cells within cellularized embryos and the larval brain, cyclin A accumulates to peak levels in prophase and is degraded throughout the period in which chromosomes are becoming aligned on the metaphase plate. The degradation of cyclin B, on the other hand, does not occur until the metaphase-anaphase transition. In cells arrested at c-metaphase by treating with microtubule destabilizing drugs to prevent spindle formation, cyclin A has been degraded in the arrested cells, whereas cyclin B is maintained at high levels. These observations suggest that cyclin A has a role in the G2-M transition that is independent of spindle formation, and that entry into anaphase is a key requirement for the degradation of cyclin B.

Key words: cell cycle/cyclin/*Drosophila*/mitosis

Introduction

The cyclins are a family of proteins that accumulate throughout the cell cycle to be degraded around the time of the metaphase-anaphase transition. Although originally discovered in the eggs of marine invertebrates (Evans *et al.*, 1983; Swenson *et al.*, 1986; Standart *et al.*, 1987), they have since been found in insects (Lehner and O'Farrell, 1989; Whitfield *et al.*, 1989), amphibians (Minshull *et al.*, 1989 and personal communication), and mammals (Pines and Hunter, 1989) and thus appear to play a role in mitosis in all eukaryotes. This idea was strengthened by the finding that the fission yeast cell cycle gene, *cdc13*, encodes a protein homologous to cyclin B (Booher and Beach, 1988; Goebl and Byers, 1988; Hagan *et al.*, 1988; Solomon *et al.*, 1988).

Genetic evidence indicates that the *cdc13* gene is required for the G2-M transition and furthermore that it interacts with *cdc2* (Booher and Beach, 1987). *cdc2* encodes a protein kinase, $p34^{cdc2}$, that is functionally interchangable with homologues from *Saccharomyces cerevisiae* (Beach *et al.*, 1982) and from human cells (Lee and Nurse, 1987). The protein has also been found in highly purified preparations of M-phase promoting factor (MPF) (Gautier *et al.*, 1988), a factor first described as being required for frog oocytes to enter into their meiotic divisions (Masui and Markert, 1971; Reynhout and Smith, 1974; Gerhart *et al.*, 1984). The *cdc2* protein is also a component of histone H1 kinase of starfish, the equivalent of MPF (Arion *et al.*, 1988; Labbé *et al.*, 1988). The activation of cdc2 kinase protein is controlled both by its association with cyclins and by its dephosphorylation at a specific tyrosine residue (Dunphy and Newport, 1989; Gautier *et al.*, 1989; Gould and Nurse, 1989; Labbé *et al.*, 1989a,b; Morla *et al.*, 1989).

Very few studies have focused upon the different roles of cyclins A and B. Murray and Kirschner (1989) have shown that mitotic cycles can be reinitiated in *Xenopus* egg extracts that have been denuded of all endogenous mRNAs, solely by the addition of cyclin B mRNA. These results suggest that cyclin B might be sufficient for the mitotic cycle, although it is possible that there are other cyclical activities that occur *in vivo* which require cyclin A and which are not easily assayed in this *in vitro* system. This is supported by the finding that mutations in the *Drosophila* cyclin A gene abolish progression through the cell cycle (Lehner and O'Farrell, 1989), indicating that in this organism, cyclin B cannot substitute for cyclin A. A role for both cyclins in the meiotic divisions of *Xenopus* is implied by the observations that the injection of synthetic clam cyclin A mRNA or sea urchin cyclin B mRNA induces the maturation of G2 arrested *Xenopus* oocytes (Swenson *et al.*, 1986; Pines and Hunt, 1987). Indeed, Draetta and coworkers (1989) concluded that both cyclins form separate complexes with cdc2 in clams, as antibodies against either cyclin would immunoprecipitate H1 histone kinase activity. A number of observations in clams suggest that the two cyclins have different roles in both meiotic and mitotic divisions. Clam oocytes have a substantial maternal provision of cyclin B protein which is not degraded until after the second meiotic division (Westendorf *et al.*, 1989). However, as they have no maternal cyclin A protein, the maternal cyclin B protein appears sufficient for the first meiotic division which will take place even if protein synthesis is inhibited. Under these conditions, the second meiotic division does not take place, however, presumably reflecting a requirement for cyclin A. A difference in the behaviour of the two cyclins is also seen in the subsequent mitotic cycles in the fertilized egg, in which cyclin A is degraded before cyclin B.

We have previously shown that maternally derived transcripts encoding the *Drosophila* cyclins A and B differ in their localization in embryos, cyclin B but not cyclin A mRNAs being preferentially associated with pole cells, precursors of the germ-line (Whitfield *et al.*, 1989). In this paper, we report the sequence of the *Drosophila* cyclin B, and show, using antibodies raised against the cyclin A and B proteins expressed in *E.coli*, that the two proteins exhibit different kinetics of synthesis and degradation. Arrest of the

cell cycle with microtubule destabilizing drugs has enabled us to separate the phases of the cell cycle at which degradation of the two cyclins occurs.

Results

Sequence analysis of the Drosophila cyclins

We have previously described the isolation of *Drosophila* cyclin A and B cDNAs from an adult female *Drosophila melanogaster* library using degenerate oligonucleotide probes (Whitfield *et al.*, 1989). A restriction endonuclease cleavage map of these two cDNAs is shown in Figure 1. We have also sequenced these cDNAs and found that one encodes cyclin A whose sequence was previously reported by Lehner and O'Farrell (1989). The nucleotide sequence of the longest cyclin B cDNA has revealed an open reading frame encoding a polypeptide of 530 amino acids (Figure 1). The first methionine codon in the open reading frame is flanked by the consensus sequence for translational start sites in *Drosophila* (Cavener, 1987). The 3′ non-translated portion of the mRNA corresponds to 776 nucleotides excluding the poly(A) tail which is preceded by a typical polyadenylation signal. It is not yet clear whether the cDNA represents all of the 5′ non-translated portion of the gene.

Figure 2A shows a comparison of the amino acid sequences of *Drosophila* cyclins A and B both with each other and with cyclins A and B from the surf clam, *Spisula solidissima* (Swenson *et al.*, 1986; Westendorf *et al.*, 1989) using DIAGON plots (Staden, 1982). It is clear that although *Drosophila* cyclins A and B share an obvious similarity, they both show similarity over a considerably longer region when compared with their clam homologues. The linear alignments in Figure 2B indicate the considerable degree of amino acid sequence identity between the cyclins. Within a central region spanning 206 amino acid residues (Figure 2B, underlined), the *Drosophila* cyclins A and B share 35% identity whereas clam cyclin A and *Drosophila* cyclin A share 53% identity as do clam cyclin B and *Drosophila* cyclin B. In particular, the *Drosophila* cyclin B sequence contains a consensus cAMP-dependent protein kinase site, a feature diagnostic of all those cyclin sequences published to date (Minshull *et al.*, 1989).

Abundance of cyclins during embryogenesis

We have recloned the cDNAs for both cyclins A and B into vectors that permit their expression in *E.coli*. This has enabled us to use bacterially synthesized protein in order to raise rabbit antisera specific for the two proteins. Affinity purification and absorption of these antisera against various full length or deleted polypeptides of cyclins A and B (synthesized in *E.coli*), demonstrated that the antibodies were specific for their respective cyclins (see Materials and methods). Neither antiserum showed any cross-reactivity with its partner cyclin on Western blots. However, the anti-cyclin B serum was able to recognise synthetic cyclin B2 from *Xenopus* (data not shown), suggesting that it contains antibodies against epitopes of cyclin B that are conserved between species.

In extracts of soluble protein from *Drosophila* embryos, anti-cyclin A recognizes a polypeptide doublet with an apparent molecular weight of 60 kd, whereas anti-cyclin B recognizes a single polypeptide of ~65 kd. We have used the specific antisera to follow the accumulation and relative

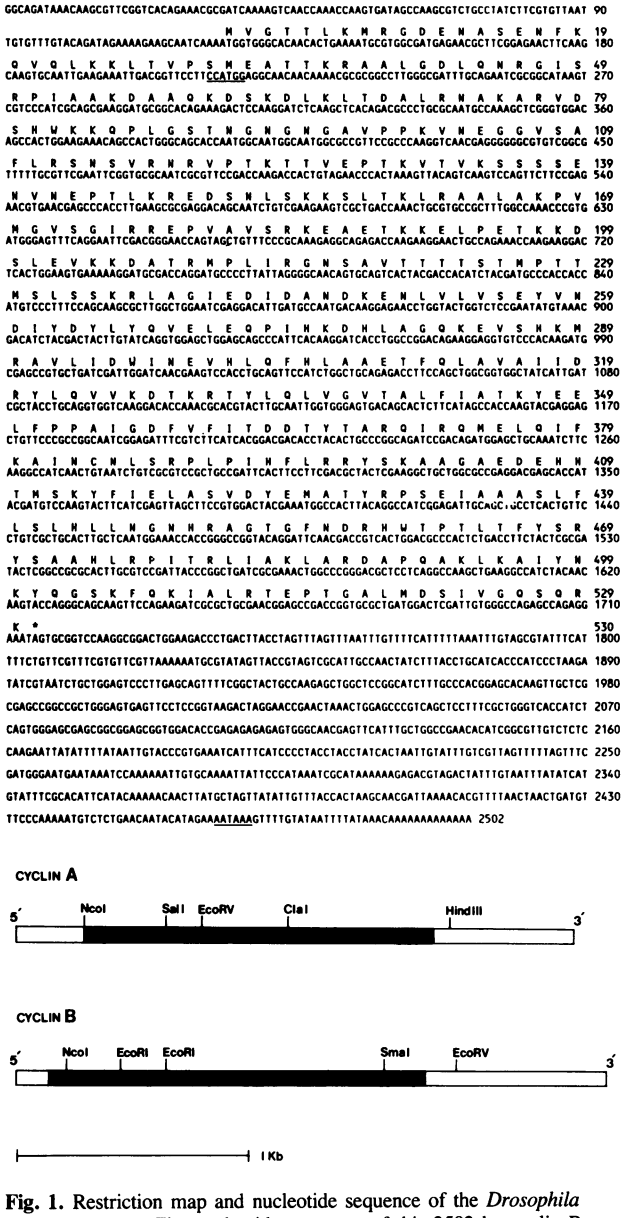
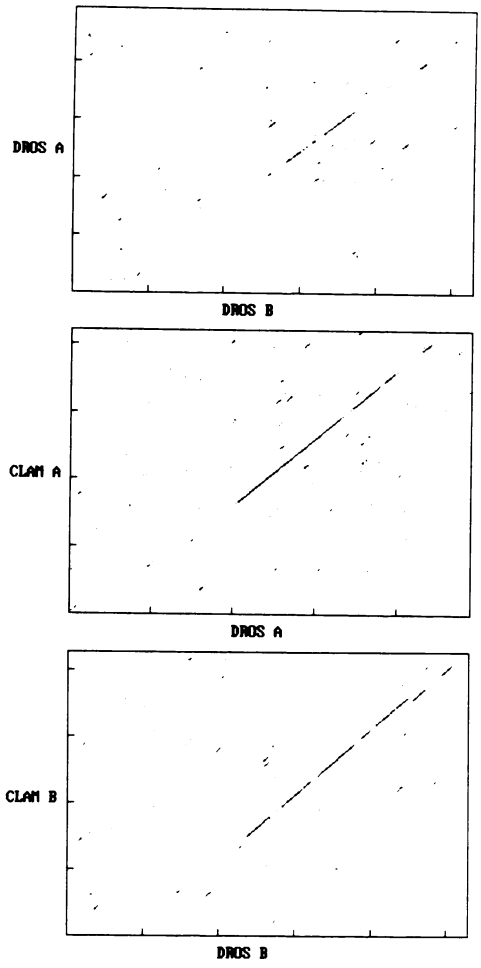


Fig. 1. Restriction map and nucleotide sequence of the *Drosophila* cyclin B cDNA. The nucleotide sequence of this 2502 bp cyclin B cDNA has an open reading frame encoding a polypeptide of 530 amino acid residues. The derived amino acid sequence is shown in single letter code, beginning at an ATG codon with flanking bases that match the consensus sequences for translational start sites in *Drosophila* (Cavener, 1987). The *Nco*I site, coinciding with an in-frame ATG codon, and a putative polyadenylation signal are indicated by underlining.

abundance of both cyclins during embryonic development (Figure 3). As previously demonstrated by Lehner and O'Farrell (1989), cyclin A is just detectable in 0−1 h embryos. There appears to be a significant increase in levels of cyclin A in 3−4 h embryos. This level is maintained until around 8 h of development, after which cyclin A levels fall (Figure 3A). In contrast, cyclin B is present at a relatively high level in 0−1 h embryos and is maintained at this level for up to 10 h of development, thereafter declining gradually throughout the remainder of embryogenesis. We have also examined the levels of the two cyclins in the unfertilized eggs laid by virgin females. As in the unfertilized eggs of the clam (Westendorf *et al.*, 1989), cyclin B is present at



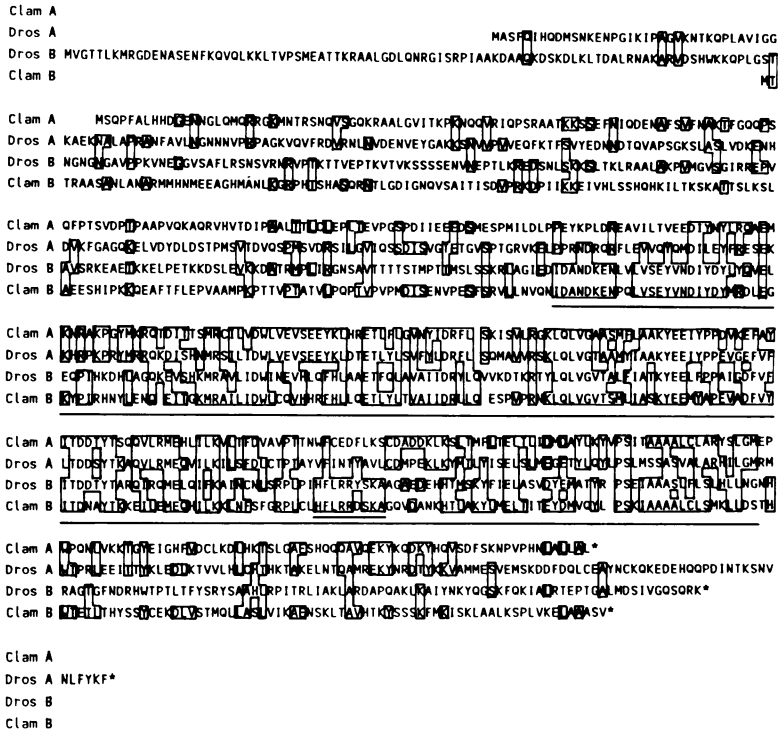


Fig. 2. Comparisons of the amino acid sequence of cyclins A and B from *Drosophila melanogaster* and the surf clam *Spisula solidissima*. (**A**) Comparisons were carried out between the predicted amino acid sequences of *Drosophila* cyclin A (Lehner and O'Farrell, 1989) and cyclin B (this work); *Drosophila* cyclin A and *Spisula* cyclin A (Swenson *et al.*, 1986); and *Drosophila* cyclin B and clam cyclin B (Westendorf *et al.*, 1989), using the DIAGON program of Staden (1982). A score of 132 for a window of 11 was used. (**B**) Manual alignment of the predicted amino acid sequences of the *Drosophila* and *Spisula* cyclins A and B. Regions of identity are boxed. The central region of the cyclins showing greatest similarity is underlined and the conserved region of the B-type cyclins containing a consensus cAMP-dependent protein kinase site RRxSx (Feramisco *et al.*, 1980) is double underlined.

high levels prior to fertilization whereas cyclin A is undetectable.

Kinetics of cyclin accumulation and degradation in embryonic cell cycles

We have also used the antisera in indirect immunofluorescent microscopy to follow the distribution of the two proteins in embryonic cell cycles. The *Drosophila* embryo is a syncytium for the first 13 rounds of mitosis which occur at ~10 min intervals (Foe and Alberts, 1983). During the 14th cycle, individual nuclei undergo cellularization and the first round of cell divisions occur within clusters or 'domains' of cells following a temporally controlled pattern (Foe, 1989). Staining syncytial embryos with either anti-cyclin A or anti-cyclin B antisera reveals no obvious pattern of cyclical variation in concentration with the mitotic cycle (data not shown). Following cellularization, however, both cyclin A and B begin to undergo clear patterns of cycling. Figure 4 shows mitotic domains from cellularized embryos stained with antisera against either cyclin A (left) or B (right). Cyclin A accumulates in cells prior to entry into M-phase. It appears to reach peak levels in prophase, and is degraded as the chromosomes are becoming aligned on the metaphase plate, so that few metaphase cells show any staining. Cyclin A then remains at low levels during anaphase and telophase. Cyclin B also accumulates during G2 and, while prophase cells often show a more uniform distribution of the protein over the whole cell, degradation of the protein does not occur until the metaphase–anaphase transition. Thus we regularly see metaphase cells which have high levels (Figure 4, cell **m1**) or low levels (Figure 4, cell **m2**) of cyclin B. As with cyclin A, levels remain low during anaphase and telophase.

Cyclin behaviour in dividing cells of the larval nervous system

We wished to see whether the two cyclins followed the same pattern of behaviour in mitotic cycles at different developmental stages. We chose to examine the larval central nervous system (CNS), a tissue highly suited to the study of cell division. The ventral ganglion of the CNS has a set of large stem cells, thought to be persistent embryonic neuroblasts, which continue to undergo asymmetric divisions throughout development to produce sets of smaller ganglion mother cells (GMCs). The GMCs then divide symmetrically

to produce a pair of neurons (Truman and Bate, 1988). Using indirect immunofluorescence, we have followed the synthesis and degradation of cyclins A and B in the mitotically dividing neuroblasts and GMCs in the ventral ganglia of third instar larvae. We have found, once again, that the levels of cyclin A peak around prophase and then fall, whereas cyclin B expression is steadily maintained at its maximum from prophase until metaphase. These observations are illustrated in Figure 5. Cells at different stages of their cycle, stained to reveal DNA and cyclin A, are presented in panels A,E and B,F respectively. The cell in prophase (p) shows the highest levels of immunostaining, whereas the one in metaphase (m) shows very little. The cell in anaphase (a) shows no staining at all. Cells stained to reveal DNA and cyclin B are illustrated in panels C,G and D,H respectively. In this case, the cell in prophase (p) shows a high level of staining which is maintained, or perhaps slightly increased, by metaphase (m). Cyclin B levels dramatically drop at the onset of anaphase (a). Although the cyclin B levels are reduced in this anaphase neuroblast, there is some residual staining that appears associated with the spindle, being strongest in the mid-body region. The degree of spindle-like staining is highly variable within these preparations. The two cyclins thus appear to undergo a differing course of accumulation and destruction with respect to mitotic events, independent of either the cell type or the developmental stage. The accumulation and degradation of the two cyclins is similar in a variety of cell types in the larval CNS to the cycles observed within the mitotic domains of embryos.

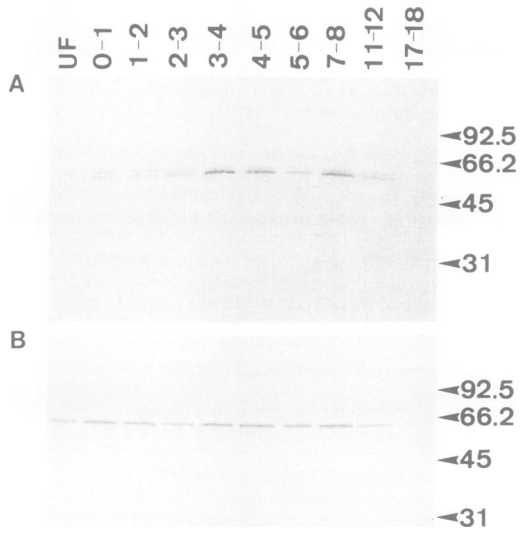


Fig. 3. Comparison of the abundance of cyclin A and B during embryonic development. Total protein extracts from *Drosophila* embryos, collected and aged at 24°C, were separated on 10% SDS−PAGE, transferred to nitrocellulose and immunostained with polyclonal antisera to either cyclin A (**panel a**) or cyclin B (**panel b**), followed by a peroxidase conjugated second antibody. The colour reaction was developed over 5 min for both panels. Developmental stages of the embryos are indicated in hours above the tracks and the mobility of molecular weight markers is shown at the right hand side in kd. The faint bands of lower mobility than cyclin B in panel b are not cyclin related peptides, since they are not recognized when affinity purified antisera are used (data not shown).

Microtubule destabilization can be used to separate phases of the G2−M transition at which the two cyclins accumulate

As it seemed that the accumulation and degradation of the two cyclins were following different kinetics in the progression from prophase to anaphase, we sought to study the effect of arresting the cell cycle during this period. To this end, we treated whole mount preparations of larval brains with microtubule destabilizing drugs, and followed the patterns of cyclin expression. In the absence of microtubules, most of the features of the G2−M transition take place, except spindle formation. Consequently, chromosomes neither undergo prophase movements, nor alignment on the

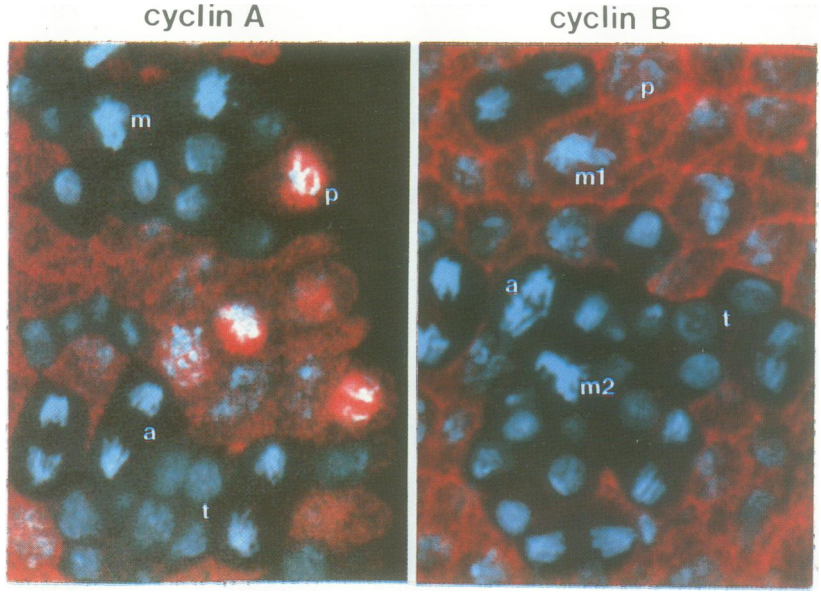
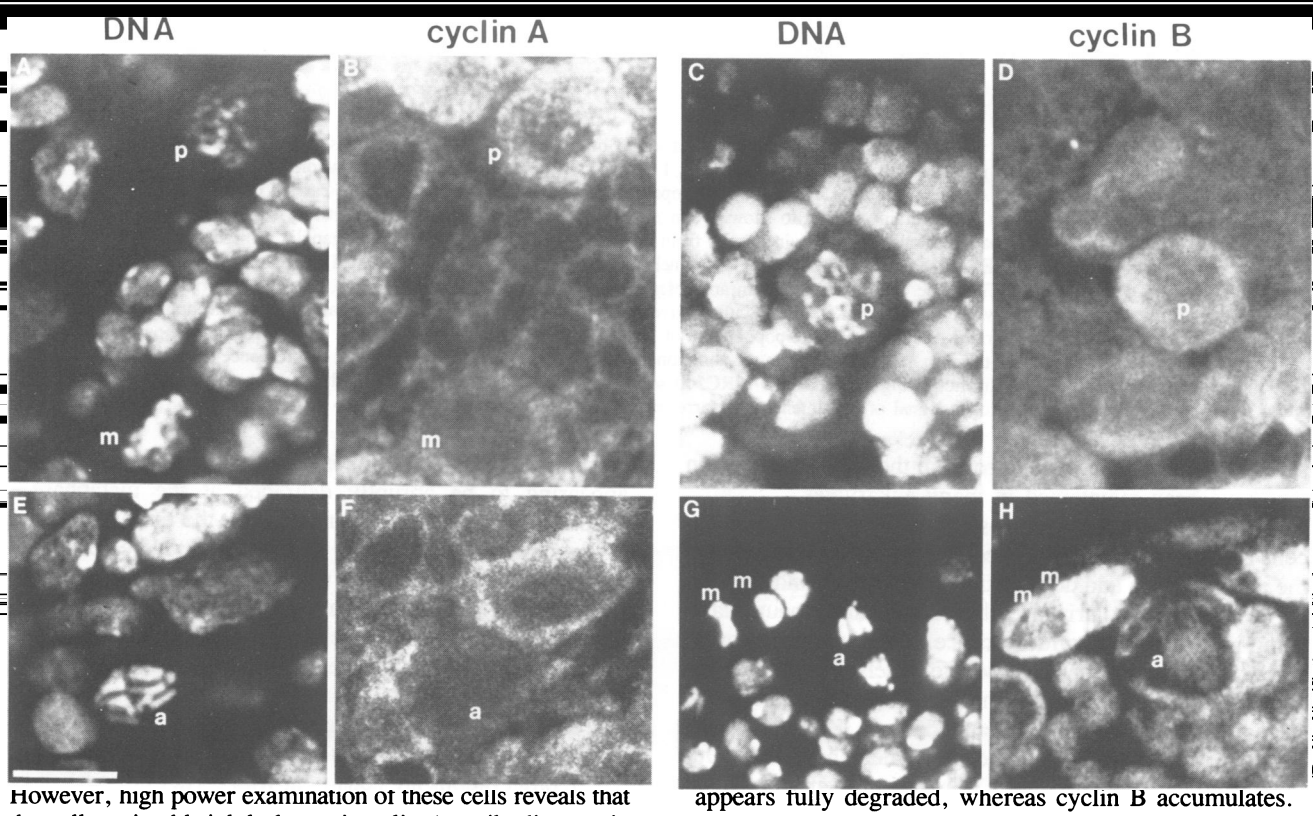


Fig. 4. Distribution of *Drosophila* cyclins A and B in mitotic domains of cellularized embryos. Embryos immunostained with antisera against cyclin A (**left panel**) or cyclin B (**right panel**) and examined by confocal microscopy as described in Materials and methods. The antibody staining is in red, whereas the counterstaining of DNA with propidium iodide is shown in blue. Individual cells within these mitotic domains have been labelled as follows, according to the phase they have reached in the mitotic cycle: **p**, prophase; **m**, metaphase; **a**, anaphase; and **t**, telophase. The scale bar indicates 10 μm.

metaphase plate, nor anaphase movements, but continue condensing to produce what have been called c-metaphases. To rule out the possibility of the drugs having side effects unrelated to microtubule destabilization, we examined the effects of three different reagents, colchicine, nocodazole and MTC (Fitzgerald, 1976). We present only the results of colchicine treatment since each drug gave similar effects upon cell cycle arrest and the expression of the two cyclins. Figure 6 shows whole mount preparations of larval brains that have been incubated in colchicine for 1 h and then immunostained with antibodies against cyclins A and B. This



However, high power examination of these cells reveals that the cells stained brightly by anti-cyclin A antibodies are in prophase (Figure 6, panels E and F; cell **p**), whereas the cells stained brightly by the anti-cyclin B antibodies are arrested in metaphase (Figure 6, panels G and H; cells **m**). Metaphase arrested cells are not stained by the anti-cyclin A antibodies (Figure 6, panels E and F; cell **m**). This can be seen even more dramatically if the time of incubation in colchicine is increased. Figure 7 (panels A and B) cells incubated in colchicine for 2 h before immunostaining. The neuroblasts in metaphase are stained for cyclin B (Figure 7, panel B; cell **m**), but not cyclin A (Figure 7, panel A; cell **m**). In contrast, the anti-cyclin A antibodies stain the two prophase GMCs very strongly (Figure 7, panel A; cell **p**). Increasing the length of incubation in colchicine to 4 h increases the number of cells in metaphase arrest (Figure 7, panels C and D—these fields show several metaphase cells only one of which is labelled **m** in each field). The field of cells stained with anti-cyclin A serum is from the brain lobes. None of these metaphase cells shows cyclin A, illustrating that the effect of colchicine arrest is seen in all brain regions. A similar increase in the proportion of

appears fully degraded, whereas cyclin B accumulates.

Discussion

We conclude from a comparison of the amino acid sequences of the A- and B-type cyclins of *Drosophila* with those of the clam that the separate identity of these two types of cyclin has been maintained throughout evolution. Thus A-type cyclins show greater sequence identity across species barriers than when compared with B-type cyclins of the same species. Such sequence conservaton of these two cyclin types points

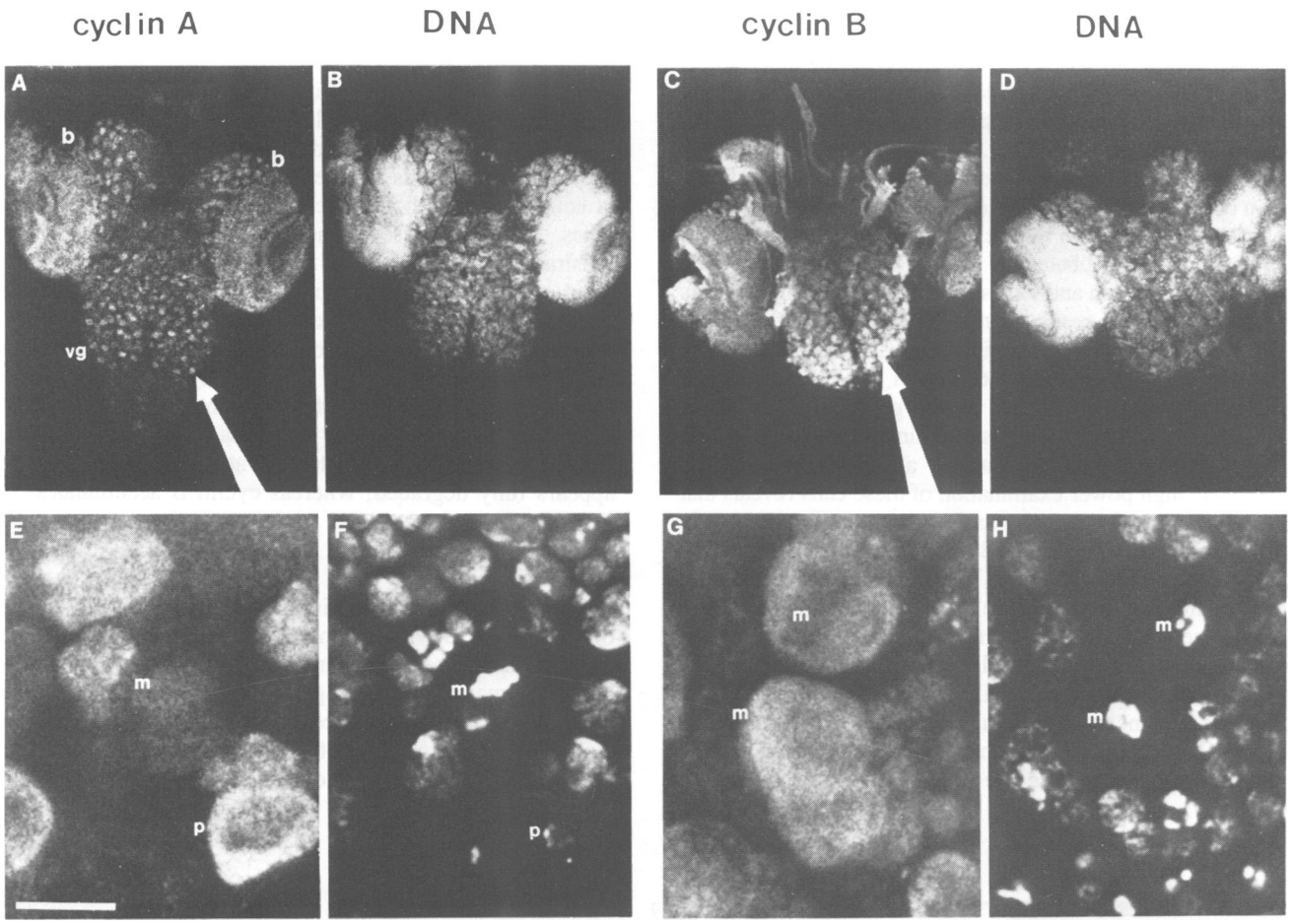


Fig. 6. Immunostaining of colchicine treated whole mount preparations of larval brains with antibodies to cyclins A and B. Brains dissected from third instar larvae, incubated for 1 h in tissue culture medium containing 1×10^{-5} M colchicine and fixed as whole mount preparations. Immunostaining with antisera against either cyclin A or cyclin B, and preparation for confocal microscopy were carried out as described in Materials and methods. The upper panels show whole brains stained to show cyclin A and DNA (panels **A and B**, respectively), or cyclin B and DNA (panels **C and D**, respectively). The lower panels show brightly stained cells from the indicated regions of the ventral ganglia at higher magnification stained to show cyclin A and DNA (panels **E and F**) or cyclin B and DNA (panels **G and H**). Individual cells labelled **p** and **m** are at prophase and c-metaphase (see text) respectively. The scale bar indicates 10 μm and refers to the lower series of micrographs. The upper series are at a magnification of 95×, showing the whole larval brain. It is difficult to give meaningful quantification of such data from whole brains kept in short-term culture since the tissue is comprised of many complex populations of cells, each undergoing quite different mitotic regimes. However, if we consider the giant neuroblasts of the thoracic region of the ventral ganglion, then the cells that show very bright fluorescence with anti-cyclin A (in the 90th percentile, following normalization using the BIORAD-MRC500 software), are all in prophase. Similarly, in preparations stained with anti-cyclin B, all cells showing a fluorescence signal of this intensity are arrested in c-metaphase.

towards them having distinct roles in the G2–M transition that have been maintained in many eukaryotes.

This is supported by our observations of cyclin levels in cells at different stages of the cell cycle. We have examined the accumulation and degradation of cyclins A and B in the cell cycles of cellularized embryos and larval neuroblasts using indirect immunofluorescence. The two cyclins cycle in a comparable manner at these two widely separated stages of develoment. Cyclin A accumulates throughout interphase to peak during prophase. It then undergoes degradation and is no longer present in the majority of metaphase cells. Our observations of cyclin A levels in embryos are similar to those made by Lehner and O'Farrell (1989). However, these authors showed an example of one cell at metaphase in which cyclin A degradation has not occurred. We also observe such cells, and it is our impression that the cyclin A degradation is somewhat variable with respect to metaphase both in cellularized embryos and larval neuroblasts. Cyclin B also accumulates throughout interphase, but persists for longer, appearing to be abruptly degraded at the metaphase–anaphase transition. In order to clarify the timing of cyclin A degradation relative to metaphase, we treated brain preparations with colchicine which destabilizes microtubules and thus physically prevents progression into anaphase. Under these conditions, cyclin A first accumulates to high levels at prophase and is degraded as cells come to arrest at metaphase. In contrast, cyclin B is maintained at high levels, thus clearly separating two stages of the mitotic cycle at which these proteins reach their maximum concentrations. These observations are consistent with earlier pulse labelling studies of cyclin synthesis in clam embryos showing that cyclin A is degraded 2–3 min before cyclin B, and that in the presence of colchicine, cyclin A is degraded whilst cyclin B accumulates (Minshull *et al.*, 1989). However our study of fixed whole mount preparations of either embryos or larval brains from *Drosophila* permits the first direct comparisons

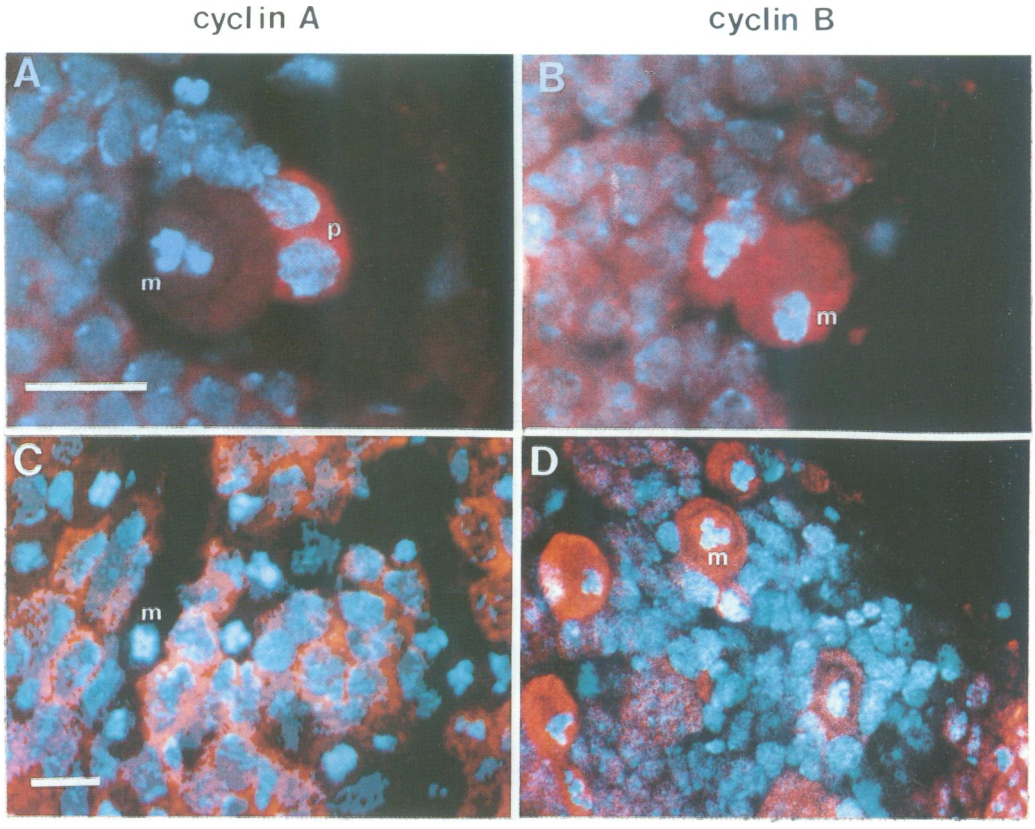


Fig. 7. The time dependent accumulation of metaphase figures with low levels of cyclin A and high levels of cyclin B in the presence of colchicine. Brains dissected from third instar larvae, incubated in tissue culture medium containing 1×10^{-5} M colchicine for either 1 h (panels **A and B**) or 4 h (panels **C and D**) and fixed as whole mount preparations. These have been stained with antisera against either cyclin A (panels **A and C**) or cyclin B (panels **B and D**) for confocal microscopy as described in Materials and methods. Antibody staining is in red and DNA staining in blue. The cells in panels A, B and D are from a region of the ventral ganglion comparable to those illustrated in Figure 6. Panel C shows cells from one of the brain lobes. Individual cells labelled **p** and **m** are at prophase and c-metaphase (see text) respectively. The mitotic index (proportion of cells in c-metaphase) increases with the length of time in colchicine, and only one of several cells with highly condensed chromosomes at c-metaphase is labelled both in panel C and D. The scale bar for panels A and B and for panels C and D indicates 10 μm.

of the levels of both cyclins in individual cells at different cell cycle stages.

In terms of cyclin behaviour, neuroblasts arrested at metaphase by colchicine treatment may not differ fundamentally from the clam oocyte arrested at metaphase in its first meiotic division. These oocytes have no cyclin A protein, but do have a stockpile of maternal cyclin B protein that is released following fertilization. This maternal cyclin B is the only cyclin required for the completion of the first meiotic division (Westendorf *et al.*, 1989), although experiments with protein synthesis inhibitors suggest that cyclin A is needed for subsequent divisions in the clam. Our finding of high levels of cyclin B protein and barely detectable levels of cyclin A protein in unfertilized *Drosophila* eggs is reminiscent of these similar findings by Westendorf *et al.* (1989) in unfertilized clam eggs. We do not know, however, whether there are any requirements for cyclin A during meiosis in *Drosophila*, since unlike clam eggs, unfertilized *Drosophila* eggs are able to undergo both meiotic divisions, the completion of which is thought to be triggered by the act of oviposition. The low levels of cyclin A which we observe in unfertilized *Drosophila* eggs might indicate either that it was degraded in the second meiotic divison or that it was never required.

Both A- and B-type cyclins have been shown to be able to associate with the cdc2 kinase in clams and in frogs suggesting that such associations might be universal (Draetta *et al.*, 1989; Minshull,J., Golsteyn,R., Hill,C. and Hunt,T., submitted). The activation of cdc2 kinase is dependent upon its dephosphorylation and its association with the cyclins. It is possible that the different temporal profiles of accumulation and degradation of the two cyclins reflect a need to direct the cdc2 kinase to particular substrates or activating phosphatases at different stages of the cycle. Our observations on the fluctuations in cyclin levels are consistent with recent data from Hunt and colleagues, who have shown that the cyclin A–cdc2 complex peaks in its kinase activity before the cyclin B–cdc2 complex. Furthermore, they find that the two complexes have different preferences for the substrates that they phosphorylate (Minshull,J., Golsteyn,R., Hill,C. and Hunt,T., submitted). Together with our observations, this suggests that cyclin A is required earlier in the cycle concomitant with chromosome condensation and the reorganization of both the nuclear envelope and the whole of the cytoskeleton at prophase. This is perhaps the true G2–M transition. Cyclin B, on the other hand, might be required to establish and maintain the metaphase state until chromosomes become correctly aligned on the equatorial plane of the spindle ready for anaphase.

The patterns of cyclin expression in colchicine treated brains not only accentuate differences in the normal profiles of the levels of the two cyclins, they also offer a clue to

the logic of cyclin degradation during mitosis. It is clear from our results that cyclin A accumulation and degradation is independent of spindle function, i.e. that cyclin A accumulates and is degraded in the same manner in both colchicine treated cells and untreated control cells. However, this is quite clearly not the case for cyclin B. Whereas its accumulation seems to be independent of spindle function, its degradation is inhibited by colchicine treatment. Indeed, the most likely explanation for this is that the degradation of cyclin B is dependent upon the correct functioning of the spindle. In fact, the sudden drop of cyclin B levels at anaphase in untreated neuroblasts points towards anaphase movements as being the key control step that triggers the degradation of this cyclin.

Are there other hints as to what the different roles of the two cyclins might be in any of these events? The work of Murray and Kirschner (1989) suggested that the translation of cyclin B alone may be sufficient to drive multiple mitotic cycles in a *Xenopus* cell free system. However, while this system can be used to monitor cycles of chromosome condensation–decondensation, nuclear envelope breakdown–reformation and cdc2 kinase activity, there are many other mitotic events that do not take place in this system: spindle formation rarely occurs and the complex reorganization of the cytoskeleton that occurs *in vivo* is impossible. Thus cyclin A might activate a cdc2 kinase with a role that cannot be assayed by this system and which may not be required for the continuing cycling of events dependent upon cyclin B. That different mitotic events can cycle independently of each other is well documented in rapidly dividing embryonic systems. Thus, when frog eggs are enucleated, for example, cyclical contractions of the cortical cytoplasm continue to take place in the absence of the nuclear mitotic cycles (Hara *et al.*, 1980). The syncytial *Drosophila* embryo could be similar to the *Xenopus* cell free system in having a greater requirement for cyclin B than cyclin A. This might reflect the comparatively simple cytoskeletal changes that take place in the syncytium in the absence of cytokinesis. We presume, however, that there must be some requirement for cyclin A in the syncytial *Drosophila* embryo as we are able to detect low amounts of the protein. Neither cyclins A or B show obvious cycling in the syncytial stages, however. This could indicate either that the two cyclins are not required to cycle in these very rapid mitoses, or that only a small proportion of the protein undergoes cycling and the changes in cyclin levels are not detectable by immunofluorescence. We show that the amounts of cyclin A protein increase substantially around 3–4 h, whereas cyclin B stays at roughly the same level during this time period. This corresponds to the time in development when the transition occurs between the rapid mitotic divisions in the syncytial embryo and the true cell divisions of the cellularized embryo. This we confirmed by experiments in which, rather than follow the temporal progression of cyclin A levels in a population of embryos, we hand-picked sufficient syncytial embryos and newly cellularized embryos to compare cyclin A levels by Western blot analysis (data not shown). This could support the thesis that cyclin A is specifically required to regulate the complex pattern of cell cycle dependent cytoskeletal rearrangements and other cellular events required of cells that must ultimately undergo cytokinesis.

Alternatively, the appearance of high levels of cyclin A upon cellularization may relate either directly or indirectly to the transition between the non-regulated syncytial cycles and the developmentally regulated cell divisions in the 14th division cycle. Until this point in *Drosophila* development, the mitotic cycles are ~10 min long and consist of rapidly alternating S and M phases with no G1 or G2. It is possible to disrupt aspects of these 'abbreviated' cycles using either drugs or mutations and still permit other events to continue cycling (Freeman *et al.*, 1986; Raff and Glover, 1988). The 14th division cycle which occurs after cellularization is the first to have a G2 phase, the length of which is independently regulated in different 'domains' of the embryo (Foe, 1989). The entry of these domains into mitosis is controlled by the zygotic expression of the gene *string* whose transcription is regulated both spatially and temporally (Edgar and O'Farrell, 1989; O'Farrell *et al.*, 1989). *string* is the *Drosophila* homologue of *cdc25*, a fission yeast gene known to be required for cdc2 activation. As cdc25 must act in concert with at least one or other of the cyclins and a cdc2 phosphatase to activate cdc2, the increase in level of cyclin A protein following cellularization could be a critical requirement of this first regulated G2–M transition. A detailed examination of cdc2 activation in the 14th mitotic cycle of *Drosophila* may help illuminate the roles played by the two cyclins in cell cycle regulation.

Materials and methods

cDNA cloning and DNA sequencing

cDNA clones of both cyclin A and cyclin B were isolated from a *Drosophila* adult female cDNA library using redundant oligonucleotide probes (Whitfield *et al.*, 1989). Inserts from these cDNAs and from longer homologous cDNAs isolated from a 0–4 h embryo library (kindly provided by N.Brown, Harvard), were subcloned into pKS Bluescript (Stratagene). Random fragments, of mean size 0.5 kb, were generated from the subclones by sonication and gel purification and subcloned into the *Sma*I site of M13 tg131. All clones were sequenced using a Sequenase kit (United States Biochemical Corporation), according to the manufacturer's instructions.

Antibodies

Drosophila cyclin A and cyclin B cDNAs have *Nco*I restriction sites (CCATGG) in which the ATG codon is in-frame with the largest open reading frame. For cyclin A, the *Nco*I ATG corresponds to the putative initiation codon of the cDNA, whereas the *Nco*I ATG of cyclin B corresponds to the third in-frame methionine codon, 31 amino acid residues downstream of the initiation codon (Figure 1). In order to produce full length unfused cyclin A protein, we modified the T7 expression vector pAR3038 (Studier and Moffatt, 1986) by replacing its unique *Nde*I site with an *Nco*I site. This modified vector (pWW1) allowed the *Nco*I–*Hin*dIII fragment encoding the entire open reading frame of cyclin A to be transferred directly into *Nco*I/*Hin*dIII cut pWW1. The *Nco*I–*Eco*RI fragment from cyclin B (encoding all but the first 31 amino acid residues of the protein), was also subcloned into pWW1. The modified vector was prepared by first cutting with *Bam*HI, end filling, then cutting with *Nco*I. Both the cyclin A construct (pWW1cycA) and the cyclin B construct (pWW1cycB), were expressed in the host BL21 (DE3) as described in Studier and Moffatt (1986). Cells from induced cultures were pelleted and lysed by incubation in 50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$ containing 200 μg/ml lysozyme, for 30 min at 0°C. Lysis was completed by two cycles of freeze–thaw before adding DNase I to 10 μg/ml and incubating for a further 10 min at 0°C. The lysate was then vigorously homogenized in a hand held Dounce and centrifuged at 10 000 r.p.m. for 15 min in a Sorvall HB-4 rotor. The pellet was resuspended by homogenization in ice-cold 0.5 M NaCl, 0.5% Triton X-100, 1 mM PMSF, and recentrifuged as before. For the pWW1cycA construct, the pellet was dissolved in 50 mM Tris–HCl, pH 8.0, 8 M urea, 1 mM PMSF; analysis of this soluble extract by SDS–PAGE indicated that it consisted of >90% intact cyclin A protein, with the majority of the contaminants being of lower molecular weight (probably degradation products of cyclin A). The truncated cyclin B protein was not soluble in 50 mM Tris–HCl, pH 8.0, 8 M urea; the suspension was therefore centrifuged as before and the pellet raised in 50 mM Tris–HCl pH 8.0, 9.5 M urea, 0.1% SDS, 1 mM PMSF. This soluble fraction proved to be >95% intact

cyclin B protein. Both protein preparations were dialysed extensively against 10 mM Tris−HCl, pH 8.0, 2 mM DTT and stored in aliquots at −70°C at a concentration of ~5 mg/ml. Rabbits were immunized by subcutaneous injection of ~200 μg of cyclin proteins in 1.0 ml of an emulsion of PBS and complete Freund's adjuvant (1:1 v/v). Booster injections containing 100 μg of protein in incomplete Freund's adjuvant were administered at monthly intervals. Blood was removed from the marginal vein of the ear 7 days after each booster injection and the serum stored at −70°C until use.

Affinity purification of antibodies

Antibodies were affinity purified against pWW1cycA and B proteins bound to Sepharose. The proteins were coupled to CNBr-activated Sepharose CL4B (Pharmacia) according to the manufacturer's instructions. Antisera were recycled three times through 2 ml columns of affinity matrix and the columns washed with 50 volumes of 50 mM Tris−HCl, pH 8.3, 0.5 M NaCl, 0.1% NP40, before eluting the bound antibodies with 20 mM triethylamine in 10% glycerol. The eluate was immediately neutralized by the addition of 0.2 volumes of 1 M Tris−HCl pH 7.5, and fetal calf serum added to a final concentration of 30%. Affinity purified antibodies were stored in small aliquots at −70°C. Antisera were used at a dilution of ×1000 on Western blots, and affinity purified antisera at ×500.

Electrophoresis and Western blotting

Drosophila embryos were collected over a 1 h period on yeasted grape juice/agar plates from wild-type population cages. Two 1 h precollections were taken in order to ensure that retained embryos had been laid prior to the sample collection. Embryos were harvested from the plate, washed in water and divided into groups of 50. Embryos were aged at 24°C, groups of 50 being frozen on solid CO_2 at 1 h intervals. Unfertilized eggs were collected from virgin females (Oregon R) over a period of 4 h before freezing. Samples of total protein from staged embryos of *Drosophila* were prepared by homogenization of 50 embryos in 25 μl of ice-cold gel sample buffer (2% SDS, 0.125 M Tris−HCl, pH 6.9, 5% 2-mercaptoethanol, 2 mM Na_2EDTA, 10% glycerol). Immediately after homogenization, samples were heated in a boiling water bath for 5 min and then centrifuged in a microfuge for 1 min to pellet insoluble material. Samples were either used immediately or stored frozen at −20°C. Protein samples were analysed on 10% SDS−PAGE (Laemmli, 1970). Gels were either fixed and stained in 25% isopropanol, 10% acetic acid, 0.2% Coomassie Blue R250, or electrophoretically transferred to nitrocellulose filters in a BIORAD Transblot apparatus according to the method of Towbin *et al.* (1979), except that methanol was not added to the transfer buffer. Filters were incubated overnight at 4°C in 5% powdered skimmed milk (PSM) in PBS, rinsed in PBS and then incubated for 2 h at room temperature in sealed polythene bags containing the first antibody diluted in 5% PSM−PBS. After washing the filters for 3 × 10 min in PBS containing 0.2% Tween 20, they were then resealed in polythene bags containing peroxidase conjugated second antibody diluted 1 to 1000 in 5% PSM−PBS and incubated at room temperature for 2 h. The washing cycle was repeated, including an extra wash in PBS before developing the staining in a filtered solution of 0.05% 4-chloro-1-napthol (Aldrich Chemical Company Ltd) in PBS to which 0.0002 volumes of H_2O_2 (30% w/v) had been added.

Fixation and immunostaining of embryos

Embryos were fixed and prepared for staining using the method of Dequin *et al.* (1984), which is based on the technique of Mitchison and Sedat (1983). Embryos were dechorionated in 60% hypochlorite bleach in 0.7% NaCl, 0.1% Triton-X and then rinsed several times in NaCl−Triton. Embryos were then transferred to a glass vial containing 1 volume of 4% paraformaldehyde in PBS, to 3 volumes of heptane and were left to fix for 1 h with gentle agitation. The aqueous layer was then removed, an equal volume of methanol added and the tube was shaken vigorously for 1 min. Embryos that had lost their vitelline membrane sank to the bottom of the tube and were rinsed twice in methanol. The embryos were then rehydrated in PBS−0.1% Triton and blocked in 10% fetal calf serum for at least 1 h at 22°C. The embryos were incubated overnight at 4°C in the same buffer containing the appropriate antibody and RNase A at 2 mg/ml. After washing (3 times for 15 min), embryos were incubated with a fluorescein conjugated goat anti-rabbit (Jackson Immunoresearch Inc.) secondary antibody for 2 h at 22°C. Excess secondary antibodies were washed off in PBS + 0.1% Triton. DNA was stained using propidium iodide (1 μg/ml) followed by a final rinse in PBS before mounting in 85% glycerol containing 2.5% propylgallate. The preparations were viewed using an MRC-500 Confocal Imaging system in conjunction with a Nikon Optiphot compound microscope. Colour prints were obtained using a Sony Color Video Printer UP5000-P.

Immunostaining whole mount brain preparations

Third instar larvae were washed and dissected in 0.7% NaCl. In those experiments in which colchicine treatment was carried out, the brains were transferred to tissue culture medium as described by Truman and Bate (1988), containing 1×10^{-5} M colchicine, and incubated for the times indicated in the results. Brains were fixed by transferring them to a drop of 0.7% NaCl containing 3.7% formaldehyde and left for 30 min. They were then incubated in 0.7% NaCl, 0.3% Triton X-100, 10% fetal calf serum, 2 mg/ml RNase A, for 1 h before incubating overnight with the working dilution of antibodies to either cyclin A or B in the same buffer. The brains were washed in PBS and then incubated with fluorescein conjugated goat anti-rabbit (Jackson Immuno Research Inc.) secondary antibody for 2 h at 22°C. Excess secondary antibodies were washed off using PBS + 0.1% Triton. DNA was stained using propidium iodide (1 μg/ml) which was rinsed off with PBS before mounting in 85% glycerol containing 2.5% propylgallate. The preparations were viewed as above. Each of the immunostaining experiments was repeated 5 times with 5−10 brains depending upon the final quality of the preparations. The levels of cyclin A and B staining in all of the giant neuroblasts of the thoracic region of the ventral ganglia were examined, and found to be highly reproducible between preparations.

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Note added in proof

Since this paper was submitted, a paper has been published by C.Lehner and P.O'Farrell (1990) (*Cell*, **61**, 535–547) which also presents the cyclin B sequence and confirms our previous findings [Whitfield *et al.*, (1989)].