The Requirements for Protein Synthesis and Degradation, and the Control of Destruction of Cyclins A and B in the Meiotic and Mitotic Cell Cycles of the Clam Embryo

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Abstract. Fertilization of clam oocytes initiates a series of cell divisions, of which the first three—meiosis I, meiosis II, and the first mitotic division—are highly synchronous. After fertilization, protein synthesis is required for the successful completion of every division except meiosis I. When protein synthesis is inhibited, entry into meiosis I and the maintenance of M phase for the normal duration of meiosis occur normally, but the chromosomes fail to interact correctly with the spindle in meiosis II metaphase. By contrast, inhibition of protein synthesis immediately after completion of meiosis or mitosis stops cells entering the next mitosis. We describe the behavior of cyclins A and B in relation to these "points of no return." The cyclins are synthesized continuously and are rapidly destroyed shortly before the metaphase–anaphase transition of the mitotic cell cycles, with cyclin A being degraded in advance of cyclin B. Cyclin destruction normally occurs during a 5-min window in mitosis, but in the monopolar mitosis that occurs after parthenogenetic activation of clam oocytes, or when colchicine is added to fertilized eggs about to enter first mitosis, the destruction of cyclin B is strongly delayed, whereas proteolysis of cyclin A is maintained in an activated state for the duration of metaphase arrest. Under either of these abnormal conditions, inhibition of protein synthesis causes a premature return to interphase that correlates with the time when cyclin B disappears.

It has been known for many years that inhibiting protein synthesis blocks entry into mitosis and cleavage in marine invertebrate embryos (Hultin, 1961; Kishimoto and Lieberman, 1964; Timourian and Uno, 1967; Wilt et al., 1967; Wagenaar and Mazia, 1978; Picard et al., 1985; Dubé, 1988). Inhibition of protein synthesis in these cells causes a specific cell cycle arrest at the G2-M transition, and does not inhibit progression through mitosis or S phase. Until recently, the reason for the protein synthesis requirement for this transition was not well understood. In the past three years, however, it has become clear that the G2-M transition requires an active protein kinase, generally known as maturation promoting factor (MPF)[1] (for reviews see Murray and Kirschner, 1989*b*; Nurse, 1990; and Pines and Hunter, 1990). This entity, which is also known as cdc2-kinase (although this is an ambiguous term in view of the existence of protein kinases that use $p34^{cdc2}$ as a substrate), contains two components: a catalytic kinase subunit encoded by the homologue of the *S. pombe cdc2* gene, and a companion cyclin subunit (Dunphy et al., 1988; Gautier et al., 1988; Draetta et al., 1989; Labbé et al., 1989; Gautier et al., 1990). The activity of cdc2-kinase appears to depend absolutely on cyclin (Minshull et al., 1989*a*; Murray et al., 1989), but it is also regulated by the pattern of phosphorylation on certain key threonine and tyrosine residues in $p34^{cdc2}$ (Draetta and Beach, 1988; Morla et al., 1989; Dunphy and Newport, 1989; Gould and Nurse, 1989; Solomon et al., 1990; Krek and Nigg, 1991; Kumagai and Dunphy, 1991; Meijer et al., 1991). The cyclins have the unusual property of periodic cell cycle–regulated destruction (Evans et al., 1983; Standart et al., 1985; Swenson et al., 1986; Meijer et al., 1989; Minshull et al., 1989*b*; Westendorf et al., 1989), and there is considerable evidence that their disappearance is what turns off cdc2-kinase, allowing cells to complete mitosis and enter the next cell cycle (Draetta et al., 1989; Murray et al., 1989; Glotzer et al., 1991; Luca et al., 1991). Transition through mitosis is thus regulated by both translational and posttranslational mechanisms, which involve protein phosphatases as well as protein kinases and proteases (Doonan and Morris, 1989; Luca and Ruderman, 1989; Ohkura et al., 1989; Cyert and Thorner, 1989; Félix et al., 1990; Glotzer et al., 1991; Lee et al., 1991; Lorca et al., 1991).

In this paper, we briefly describe the meiotic and mitotic cycles of fertilized oocytes of the clam *Spisula solidissima*, and show the consequences of inhibiting protein synthesis with emetine at various times after fertilization. We find that

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1. *Abbreviation used in this paper*: MPF, maturation promoting factor.

protein synthesis is not required for germinal vesicle breakdown, completion of meiosis I, or progression into meiosis II (which follows meiosis I after only 15 min). Whereas inhibition of protein synthesis in the mitotic cell cycle leads to inhibition of the G2-M transition, however, inhibition of protein synthesis after fertilization or parthenogenetic activation leads to a failure of proper chromosome–spindle interactions at second meiotic metaphase.

The accumulation of cyclins during the mitotic cycles probably accounts for at least part of the protein synthesis requirement for entry into M-phase. We show that cyclin is a stable protein during interphase, and is only destroyed during a narrow window of <5 min duration, which opens 2 min earlier for cyclin A than for cyclin B. The disappearance of cyclin B precedes the metaphase–anaphase transition by ~1 min. The proteolysis of cyclin is normally turned off as soon as cells reenter interphase, but in cells with monopolar spindles or colchicine-disrupted microtubules, the proteolytic machinery is maintained in an unusual state for several hours. The destruction of cyclin A is kept on under these conditions, whereas the proteolysis of cyclin B only occurs after a lag of ~50 min. The resulting high levels of cyclin B probably account for the mitotic block caused by microtubule inhibitors. In support of this idea, the mitotic arrest produced by colchicine or parthenogenetic activation is shortened considerably by inhibition of protein synthesis. This inhibition leads to a premature loss of cyclin B and concomitant return to interphase, presumably when cyclin B levels fall below the threshold required to maintain the activity of $p34^{cdc2}$-associated protein kinase.

Materials and Methods

Animals and Gametes

Spisula solidissima were obtained from the Department of Marine Resources (Marine Biological Laboratory, Woods Hole, MA) and kept in running sea water at 12–15°C. The gonads were cut open with scissors to yield sperm, which was stored as a concentrated exudate at 4°C, or oocytes, which were washed in 0.45 μm (Millipore) filtered sea water by repeated settling. The oocytes were stored as a gently stirred suspension containing not more than 50,000 oocytes/ml for up to 3 d at 15–18°C with daily changes of water. Gentamycin was added at a final concentration of 50 μg/ml to inhibit bacterial growth. This suspension was diluted to ~20,000 cells/ml for experiments. Glass beakers were used for volumes of 20–60 ml, and 20-ml glass scintillation vials for incubations of 1–10 ml. Cells were kept in suspension by swirling just before sampling, which was performed at intervals of 3–5 min in the experiments described in this paper. Incubation was at 18°C unless otherwise indicated.

Fertilization was achieved by diluting concentrated sperm 100-fold in sea water, and adding 1/200th vol of this dilute suspension to the egg suspension. This routinely gave 100% fertilization with no polyspermy. Parthenogenetic activation was achieved by addition of 1/11th vol of 0.5 M KCl to the suspension of oocytes. After germinal vesicle breakdown, ~10 min after activation, the cells were pelleted and resuspended in normal sea water. We found it was important to remove the excess KCl, for development in its continuing presence proceeded variably and abnormally after meiosis II, particularly when emetine was added.

Orcein Staining to Visualize Chromosomes

The method described by Kuriyama et al. (1986) was adapted slightly to permit rapid repeated sampling. Samples of cell suspensions (typically 100 μl) were added to 1 ml of 75% vol/vol 100% ethanol, 25% glacial acetic acid (Carnoys fixative) and allowed to sit for several hours to overnight (though the samples can be processed immediately with indistinguishable results). The cells and precipitated salt were harvested by a 1-2-s spin on an Eppendorf microcentrifuge, and the supernatant was decanted or removed with a drawn-out pasteur pipette. The cells were gently suspended in lacto-orcein stain (1% wt/vol orcein in 50% vol/vol acetic acid, 50% vol/vol lactic acid [85%, L1250; Sigma Chemical Co., St. Louis, MO]). To prepare lacto-orcein stain, orcein (Sigma Chemical Co.) was dissolved in boiling acetic acid, diluted with the lactic acid, and then filtered. The cells were left in stain for up to 60 min before staining was terminated by addition of 1 ml of 40% vol/vol acetic acid. The cells were harvested by brief centrifugation and washed once with 40% vol/vol acetic acid. The cell suspension (8.5 μl) was placed on the slide and covered with an 18-mm coverslip, which was secured and sealed with nail varnish. These preparations were good for a few days, though they ultimately dried out. The mitotic chromosomes of *Spisula* are easy to see when stained in this way. It is a relatively straightforward matter to score cells for condensed chromosomes, and the metaphase–anaphase transition is very easy to score with certainty.

Immunofluorescent Staining of the Meiotic Spindle

To visualize meiotic spindles and their associated chromosomes, cells undergoing meiosis were incubated in 1 μg/ml HOECHST 33342 (Calbiochem-Behring Corp., San Diego, CA), which allows chromosomes in living cells to be seen by epifluorescent shortwave UV illumination, permitting very precise staging. At this concentration, the dye does not delay or impair development (although the UV-irradiated observed cells are quickly killed). At the desired moment, ~200,000 cells were pelleted and gently resuspended in 5 ml of lysis buffer, modified after Vallee and Bloom (1983) (100 mM Pipes, 1 mM EGTA, 1 mM $MgSO_4$, 0.025% vol/vol NP-40, pH 6.6). Using a wide-bore pipetter, ~300 μl of this suspension was layered on coverslips coated with 10 mg/ml poly-L-lysine. The excess buffer was removed by blotting, and the cells were fixed by immersing the coverslips in 90% methanol containing 50 mM EGTA, pH 6.5 at −20°C for 6 min (Harris et al., 1980). The samples were rinsed in PBS, incubated in 10% sheep serum for 30 min at 37°C followed by mouse monoclonal anti α-tubulin antibody (N. 356; Amersham Corp., Arlington Heights, IL) for 1.5 h at 37°C, washed four times for 4 min in PBS, and incubated with the fluorescein-conjugated second antibody (affinity-purified sheep anti-mouse IgG, 16110084; Cappel Laboratories, Malvern, PA) for a further 1.5 h, followed by four washes in PBS containing 1 μg/ml Hoechst 33342 and a final rinse in water before mounting in Elvanol.

Analysis of Patterns of Protein Synthesis and Destruction

The rates and patterns of protein synthesis were followed by labeling with [^{35}S]methionine (Amersham Corp.) at a final concentration of 50 μCi/ml, the label usually being added at 45–55 min, around the time of second polar body formation. Before this point, the cells take up label very slowly. To analyze the pattern of radioactive polypeptides, 100-μl samples of the egg suspension (~2,000 cells) were added to 200 μl of cold 25% wt/vol TCA. The cells were harvested by centrifugation and the supernatant was carefully removed with a drawn-out pasteur pipette (the cells form a loose pellet). Distilled water (50 μl) was added to enable the cells to be resuspended by vortex mixing. This step significantly improved the procedure. 1 ml of acetone was then added, the cells were pelleted, and the supernatant was removed and replaced with fresh acetone, which was in turn removed. The residue was dried briefly under water suction, and taken up in SDS gel sample buffer containing 5% vol/vol mercaptoethanol. After repeated vortex mixing, and freezing and thawing, the samples were heated at 90°C for 30 s to obtain as complete solubilization of all proteins as possible. (It is salutory that the fertilization envelopes of sea urchin eggs remained completely intact after this entire procedure as seen under the microscope; so one should perhaps beware of assuming that even such apparently catholic sampling procedures necessarily displays all proteins.) The best, most highly resolved gels were obtained when the samples were worked up promptly; the longer samples were allowed to remain in TCA at room temperature, the less well resolved were the autoradiograms. Total acid-insoluble radioactivity in the samples was determined by TCA precipitation of the gel samples in the presence of 100 μg of carrier albumin, collection on glass-fiber filters (GF/C; Whatman Laboratory Products, Inc., Clifton, NJ), and scintillation counting.

Inhibitors

Emetine (Boehringer Mannheim Biochemicals, Indianapolis, IN) was prepared as a 20-mM stock solution in water and stored at −20°C. Aphidicolin and taxol were gifts of Dr. Matthew Suffness (National Cancer Institute, Bethesda, MD). They were made up at 1 mg/ml in DMSO and used at a

final dilution of 1:1,000. Colchicine was purchased from Sigma Chemical Co., and used at 20 μg/ml final concentration from an aqueous stock solution.

Results

The Meiotic and First Mitotic Cell Cycles of Clam Oocytes Are Highly Synchronous

Clam oocytes are arrested in prophase of meiosis I at the G2-M border. Fertilization or parthenogenetic activation releases the cells from this arrest, and the oocytes enter M phase 10 min later (Allen, 1953; Kuriyama et al., 1986). The oocytes complete meiosis I at ~35 min and meiosis II at 50 min, and the first mitotic division occurs at 80 min. To monitor these cell cycles, cells were fixed in ethanol/acetic acid and stained with orcein as described in Materials and Methods. The nuclear envelopes were poorly preserved by this procedure and, accordingly, the events of the cell cycle were followed mainly in terms of the behavior of the chromosomes. Fig. 1 shows a series of micrographs of the salient stages of orcein-stained clam embryos, starting with an unfertilized oocyte and ending at second mitotic anaphase. Fig. 2 *A* plots the timing of these stages.

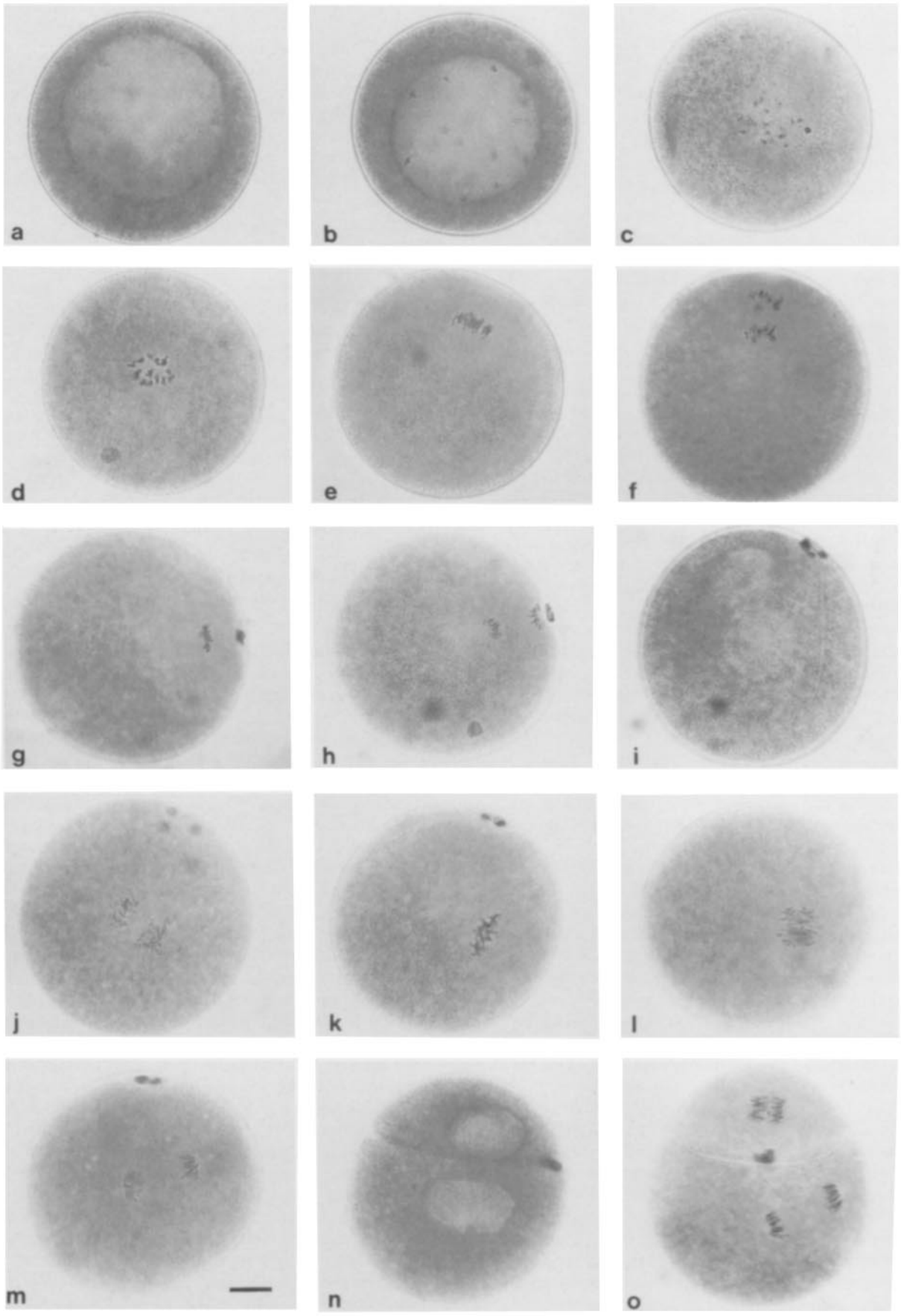
Oocytes have a large nucleus, the germinal vesicle, which contains highly condensed chromosome bivalents as revealed by staining with Hoechst 33342 (not shown), although they do not stain with orcein (Fig. 1 *a*). The chromosomes become strongly orcein-positive ~8 min after fertilization (Fig. 1 *b*) and the nuclear envelope breaks down at ~10 min. A large spindle forms and the condensed chromosomes move from their perinuclear positions to form the first meiotic metaphase plate (Fig. 1, *c–e*). The spindle moves toward the edge of the oocyte, lining up perpendicular to the surface so that when the first anaphase occurs at ~35 min (Fig. 1 *f*), one set of chromosomes is expelled from the oocyte, forming the first polar body (Fig. 1 *g*). The chromosomes that remain within the oocyte form a second meiotic plate after a short delay and after second anaphase (Fig. 1 *h*), the second polar body forms. It is noteworthy that the chromosomes do not decondense between meiosis I and II, nor does the nuclear envelope reassemble during this interval (Fig. 1, *c–h*). The sperm pronucleus remains partially condensed throughout meiosis, seen as an orcein-positive disc with no discrete chromosomes visible (e.g., Fig. 1, *d* and *h*).

A few minutes after the meiosis II metaphase–anaphase transition, both male and female sets of chromosomes decondense, and form separate nuclei (Fig. 1 *i*). About 24 min later, after DNA replication is complete, the chromosomes become visible as fine orcein-positive threads (Fig. 1 *j*). The nuclear envelopes break down and the separate male and female sets of chromosomes align on the metaphase plate (Fig. 1 *k*). Anaphase occurs at ~80 min, 10 min after the chromosomes first become visible (Fig. 1, *l* and *m*). This transition from metaphase to anaphase was easily quantified, and we used it as a timing landmark (Fig. 1, compare *k* with *l* and *m*). Cytokinesis follows ~5 min after anaphase, and the chromosomes decondense while cytokinesis is in progress. The cycle is then repeated at ~30-min intervals (depending on the temperature) with cells spending roughly equal time in interphase and mitosis as judged by when chromosomes are orcein positive. In many of the figures in this paper the first appearance of orcein-positive chromosomes is marked as the start of M phase and anaphase onset as the end of M phase, although the chromosomes remain condensed for a short time after the onset of anaphase.

Fig. 2 *A* plots these cell cycle transitions from fertilization until the end of the third mitotic division. Populations of developing embryos develop highly synchronously. Typically, >95% of the cells in a culture undergo anaphase within a 5-min window. The first mitotic division yields two cells of unequal size, however, and is followed by further asymmetric cleavage divisions (Fig. 1, *n* and *o*). At each of these divisions, the larger cells divide earlier than the smaller cells. Therefore, even though the population of embryos continues to develop in step, synchrony within the embryo itself is progressively lost after the second mitotic division. Accordingly, most of the experiments here concern only the first three cell cycles of the embryo: meiosis I, meiosis II, and mitosis 1. Fig. 2 *b* shows the effect of adding 1 μM emetine to the culture, which inhibited protein synthesis by ~40%. This partial inhibition of protein synthesis prolonged interphase and reduced the synchrony of development without significantly affecting the length of meiotic or mitotic M phase. A similar result was obtained in cleaving sea urchin eggs by Dubé (1988). It implies that newly made proteins are required to trigger entry into M phase, but not for the process of mitosis itself.

Protein Synthesis Is Required for the Completion of Every Cell Cycle after Fertilization Except Meiosis I

To define the protein synthesis requirements for the meiotic and mitotic cell cycles, the protein synthesis inhibitor emetine was added to oocytes and embryos at various times after fertilization, and its effect on the ability of the embryo to proceed through meiosis and mitosis was scored. Inhibition of protein synthesis was complete and prompt when 100 μM emetine was added (data not shown). Emetine did not prevent fertilization, entry into, or completion of first meiosis. Chromosome condensation, nuclear envelope breakdown, metaphase I, anaphase I, and the expulsion of the first polar body all occurred with normal morphology and kinetics (Fig. 3, *a–c*). However, second meiosis was seriously deranged when protein synthesis was inhibited. The chromosomes failed to form a second meiotic metaphase plate (Fig. 3 *c*). Instead of lining up between the two poles, the chromosomes tended to float about in disorganized clusters that often separated into two groups containing unequal numbers of chromosomes. In some cases sister chromatids could be seen traveling together rather than separating (Fig. 3 *d*). Thus, emetine appeared to block correct formation or function of the meiosis II spindle. To check this, normal and emetine-treated cells were stained for microtubules. Control cells displayed displayed a typical asymmetric spindle aligned perpendicular to the cell surface at the time of meiosis II metaphase (Fig. 4 *A*). By contrast, the emetine-treated cells contained abnormal symmetrical bipolar structures that resembled two closely spaced asters with variable orientation (Fig. 4, *C* and *E*). At no time did these asters develop into normal-looking second meiotic spindles, nor did the chromosomes align between them. In addition, as Figs. 4, *C* and *E* show, numerous short microtubules were present in a disorganized meshwork throughout the cytoplasm in the emetine-treated embryos, whereas in uninhibited cells, all the microtubules were associated with the spindle and asters.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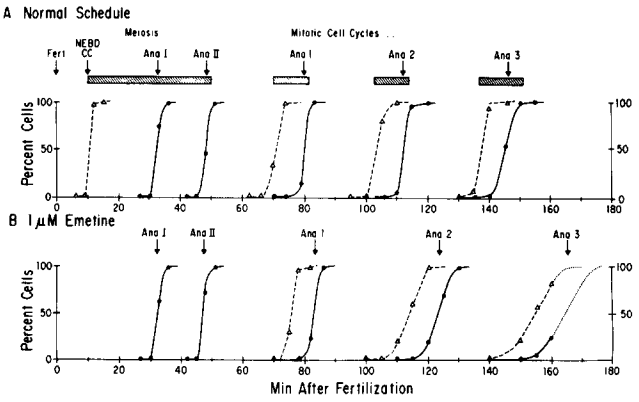


Figure 2. The time course of cell cycle transitions in the meiotic and first three mitotic cell cycles of fertilized clam oocytes. (*A*) Normal schedule. Dashed lines with triangles show when chromosomes first become visible; solid lines show the fraction of cells that had undergone the metaphase–anaphase transition. For clarity, the period of M phase is denoted by shaded bars, but it should be recalled that chromosomes remain condensed for a few minutes after the onset of anaphase (see Figure 1, *m* and *o*). (*B*) Cell cycle events in the presence of 1 μM emetine, which inhibited protein synthesis by ~40% in this experiment. Interphase is prolonged and the synchrony is impaired, but the duration of M phase is essentially unchanged.

At the end of second meiosis, when control embryos formed second polar bodies, the emetine-treated cells underwent a transient contraction (Fig. 3 *e*), but as described by Longo et al., 1991, this did not lead to formation of a second polar body. The more interior clusters of female chromosomes and all the male chromosomes then decondensed and formed nuclei on schedule (Fig. 3 *f*), while the chromosomes close to the cortex condensed even further, often giving the deceptive appearance of a second polar body (Fig. 3 *g*). In other cases the arrested embryos contained multiple nuclei that separately enclosed the male pronucleus and various clusters of female chromosomes that had drifted apart (e.g., Fig. 3 *g*). Thus de novo protein synthesis is not needed for the resumption and completion of first meiosis, but is essential for the proper execution of meiosis II. One interpretation (almost a description) of these observations is that the kinetochores fail to function in meiosis II when protein synthesis is inhibited, for microtubules connecting the chromosomes to the poles appear to be missing.

These data indicate that a newly made protein or proteins are at least needed for the correct alignment or attachment of the chromosomes to the second meiotic spindle, if not the actual formation of the spindle, and for subsequent splitting of sister chromatids. Surprisingly, however, even when protein synthesis was completely inhibited, the chromosomes remained condensed for the appropriate duration of meiosis I and II: they decondensed and formed nuclei on the normal schedule, suggesting that there are at least two different controls, one for proper chromosome alignment and splitting, and another for chromosome decondensation. The first requires new proteins, the second does not.

Identification of the Commitment Points for Meiosis II, Mitosis 1, and Mitosis 2

To define the time at which enough of the newly made proteins had accumulated to allow completion of meiosis II, we carried out experiments modeled after those of Wilt et al., (1967) and Wagenaar (Wagenaar and Mazia, 1978; Wagenaar, 1983). A batch of embryos was fertilized and samples were removed at 3-min intervals into 100 μM emetine to inhibit further protein synthesis. Aliquots of each emetine-inhibited sample were fixed and stained with orcein at regular intervals, and scored for the fraction of embryos that were able to complete meiosis II and the first two mitotic divisions. In the example shown in Fig. 5, inhibition of protein synthesis at 20 min prevented any of the embryos carrying out meiosis II. By 23 min, however, ~5% had accumulated enough newly synthesized protein to complete meiosis II, as indicated by the hatched bars. When protein synthesis was blocked at 26 min, 48% were capable of traversing meiosis and by 29 min, well before anaphase of meiosis I, emetine no longer affected meiosis II. We define the 'commitment point' for each cell cycle transition as the time when 50% of the embryos become capable of executing a morphologically normal meiosis II or of entering mitotic M phase.

In the experiments shown here (Fig. 5), the commitment point for meiosis II occurred at ~26 min after fertilization, well in advance of anaphase of first meiosis. The commitment point for first mitosis occurred at ~58 min. This is ~10 min after second meiotic anaphase (48 min) and >10 min before control cells first showed condensed chromosomes. Similarly, the commitment point for mitosis 2 was at ~88 min, ~10 min after anaphase of mitosis 1 and well before the cells entered M phase of mitosis 2. The cells in which protein synthesis was blocked always arrested in interphase, with an intact nuclear envelope and decondensed chromosomes (Fig. 3, *h* and *i*).

One striking consequence of inhibiting protein synthesis at the time of, or just after, the commitment point for mitosis 1 was that such cells as divided under these conditions formed blastomeres of equal size (not shown) instead of the normal asymmetric daughters (Fig. 3 *i*). We did not further investigate this point.

Figure 1. Oocytes and embryos stained with orcein from meiosis I until mitosis 2. Oocytes and embryos taken at various times after fertilization or KCl activation were stained and examined as described in Materials and Methods. (*a*) Unfertilized oocyte, (*b* and *c*) KCl-activated oocytes, and (*d–o*) fertilized cells: (*b*) 8 min after activation, (*c*) early meiosis I pro-metaphase, ~15 min after activation, (*d*) late meiosis I prometaphase, ~25 min after fertilization, (*e*) meiosis I metaphase, (*f*) meiosis I anaphase, (*g*) meiosis II metaphase, (*h*) meiosis II anaphase, (*i*) first interphase (two pronuclei faintly visible), (*j*) early M phase of mitosis 1 (note the separate groups of condensing chromosomes), (*k*) mitosis I metaphase, (*l*) mitosis 1 anaphase, (*m*) mitosis 1 anaphase/telophase, (*n*) two-cell embryo interphase, and (*o*) mitosis 2 anaphase. The sperm nucleus can be seen particularly clearly in *d* and *h* as a compact stained body without distinct chromosomes (compare with *j*). Bar, 10 μm.

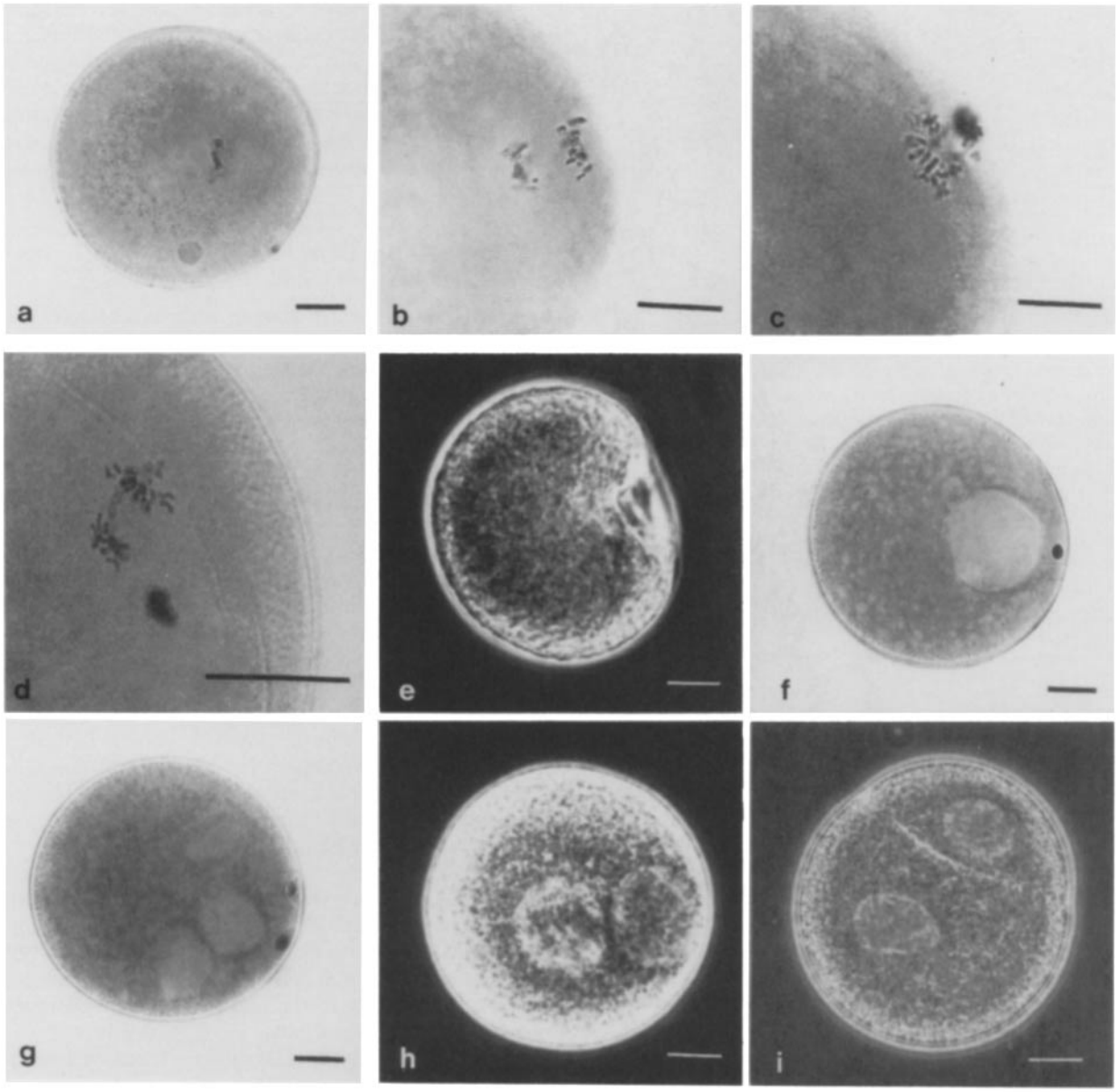
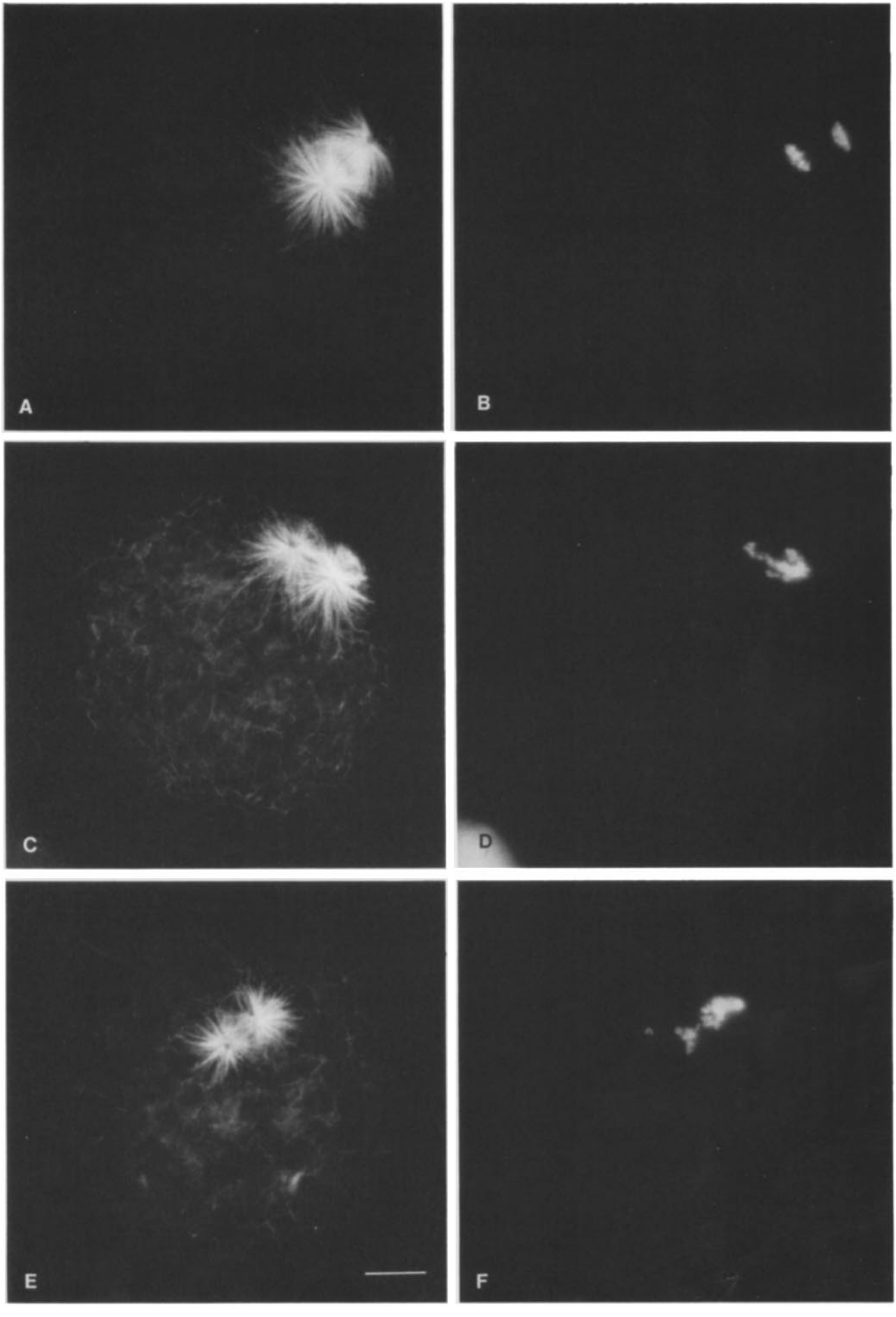


Figure 3. When protein synthesis is blocked, the chromosomal events of meiosis I occur normally but those of meiosis II do not. Oocytes were fertilized (*a* and *i*) or activated with KCl (*b–h*) and cultured in the presence of 100 μM emetine. Samples were taken at various times during the first hour of development and analyzed by orcein staining (*a–d*, *f*, and *g*) or by phase-contrast microscopy (*e*, *h*, and *i*). (*a*) Meiosis I metaphase, (*b*) meiosis I anaphase, (*c*) misaligned chromosomes during meiosis II metaphase, (*d*) aberrant dispersal of chromosomes into two clusters during meiosis II anaphase, (*e*) transient contraction seen at the end of meiosis II anaphase, and (*f–h*) cells arrested in postmeiosis II interphase, showing variable numbers of nuclei. In *i*, the addition of emetine was delayed until 70 min in a culture of fertilized embryos. These cells proceeded through first mitosis and arrested at the two-cell stage in interphase, with relatively normal morphology. Bars, 10 μm.

The Time to Reach Mitosis Depends on the Amount of Newly Made Protein but the Time Spent in Mitosis Does Not

Cells become competent to carry out mitosis when they have passed the commitment point, but the amount of protein synthesis that has been allowed beyond the commitment point strongly affects the time taken to enter mitosis. In the experiment shown in Fig. 6, emetine was added at regular 3-min intervals from 59 to 74 min after fertilization. Samples of in-

Figure 4. Emetine allows the formation of two asters at meiosis II, but the chromosomes are not properly connected with it. Oocytes were activated with KCl and allowed to develop with or without 100 μM emetine until second meiotic metaphase, at which time samples were fixed and stained for tubulin (*A*, *C*, and *E*) and examined under shortwave UV to show chromosomes (*B*, *D*, and *F*) as described in Materials and Methods. (*A* and *B*) No added emetine, 53 min, normal second meiotic anaphase. (*C–F*) Two examples of emetine-treated activated oocytes sampled at the same time. (These images can be compared with those in Fig. 3, *a–d*, which show normal and emetine-deranged metaphase II and anaphase II as seen with orcein staining.) Bar, 10 μm.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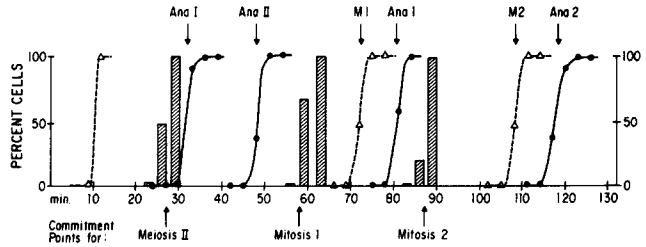


Figure 5. The first three "points of no return." A large culture of fertilized oocytes was set up, and subcultures were removed at 3-min intervals into vials containing 100 μM emetine (final concentration). Parallel samples were taken for orcein staining. At the end of the experiment, the emetine-containing samples were scored for their arrest point. Shaded bars indicate the fraction of cells that successfully completed the following cell cycle transition when emetine was added. For example, addition of emetine at 30 min allowed normal completion of meiosis II, but failure to enter mitosis 1. When emetine was added to a culture at 60 min, however, more than half the embryos completed mitosis 1 and then arrested at the two cell stage, unable to enter mitosis 2. Dashed lines and triangles denote condensed chromosomes, solid lines and filled circles the metaphase–anaphase transitions of the uninhibited master culture.

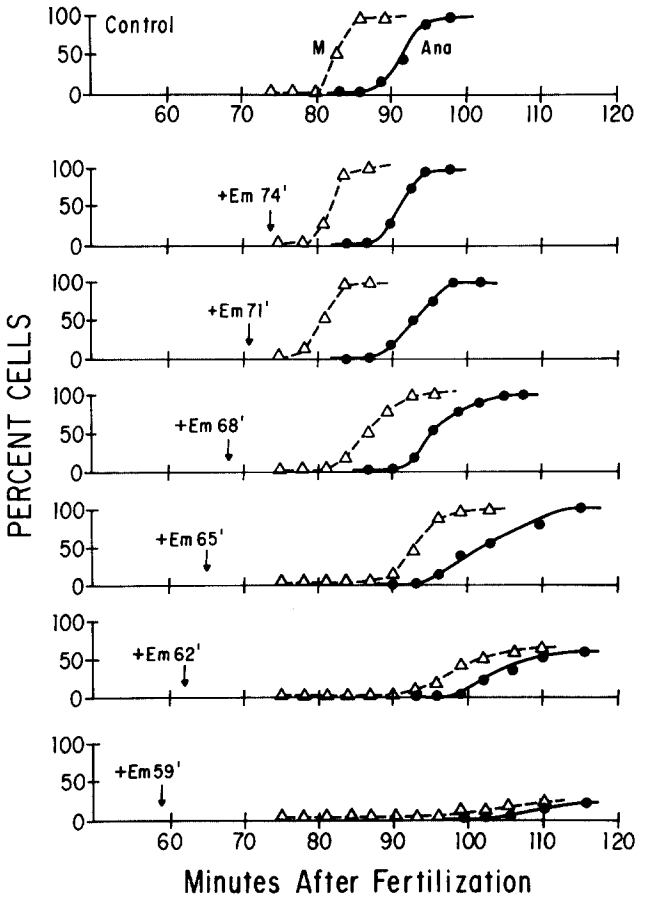


Figure 6. Emetine can delay entry into mitosis even when it does not block it. Emetine (100 μM final) was added to cultures of fertilized oocytes at the times indicated in each panel, and samples were taken at intervals for staining with orcein. Open triangles denote the first appearance of orcein-positive chromosomes, closed circles the metaphase–anaphase transition.

hibited embryos were fixed, stained, and scored for entry and exit from M phase. The culture temperature was lower in this experiment to allow finer resolution of the cell cycle. Control cells entered M phase at 83 min and left M phase at 92 min. When emetine was added close to the commitment point for mitosis 1 (~61 min), entry into mitosis eventually occurred, but was very delayed. For example, when emetine was added at 62 min, ~60% of the cells had passed the commitment point, but those cells did not enter M phase until ~100 min, 18 min later than controls. When emetine was added 3 min later, at 65 min, all of the cells had passed the commitment point and those cells entered M phase with a lag of only 14 min compared with controls. As more protein synthesis was allowed beyond the commitment point, the time to enter mitosis was further reduced until by 71 min, inhibition of protein synthesis no longer delayed entry into mitosis.

These results suggest that cells require newly synthesized proteins for more than one cell cycle–related function. First, the commitment point defines the time at which sufficient amounts of certain proteins required for mitosis have passed a threshold level. Second, the inverse relationship between the amount of protein synthesis allowed beyond the commitment point and the length of time taken to reach the onset of M phase suggests the existence of a catalytic reaction, whose rate determines when cells enter M phase. The less enzyme present, the slower the reaction it catalyzes and the longer it takes to enter mitosis. The proper spatial positioning of the first mitotic cleavage plane apparently also requires newly synthesized proteins, which may or may not be the same as those needed for entering mitosis.

Regardless of the length of time spent in interphase, Figs. 2 *B* and 6 show that the total time spent in mitosis (defined as the time from when chromosomes first stain as orcein-positive threads until the metaphase–anaphase transition) is essentially the same. This argues that the newly made proteins needed for progress past the commitment point are mainly concerned with setting the conditions for allowing entry into mitosis rather than providing material for the execution of mitosis itself.

The Accumulation and Destruction of Cyclins A and B Occur in Phase with the Mitotic Cell Cycles

As outlined in the introduction, the behavior of cyclins A and B strongly suggested an explanation for at least part of the protein synthesis requirement for mitosis. To define the kinetics of cyclin synthesis and destruction more closely, we performed experiments in which fertilized oocytes were labeled with [^{35}S]methionine and parallel samples were taken for inspection of chromosome morphology and analysis on SDS gels. Fig. 7 shows one of several such experiments covering the first two mitotic divisions. The period during which chromosomes were orcein positive is indicated by shading, and the metaphase–anaphase transition shown as a dotted line. The important results, which were highly reproducible, were twofold. Cyclin A was degraded ~2 min before cyclin B, and cyclin B was destroyed ~60 s before the metaphase–anaphase transition (see also Fig. 9). Both cyclins began to accumulate again soon after mitosis was complete, which suggested that destruction ended as suddenly as it began, an interpretation which we confirmed as described

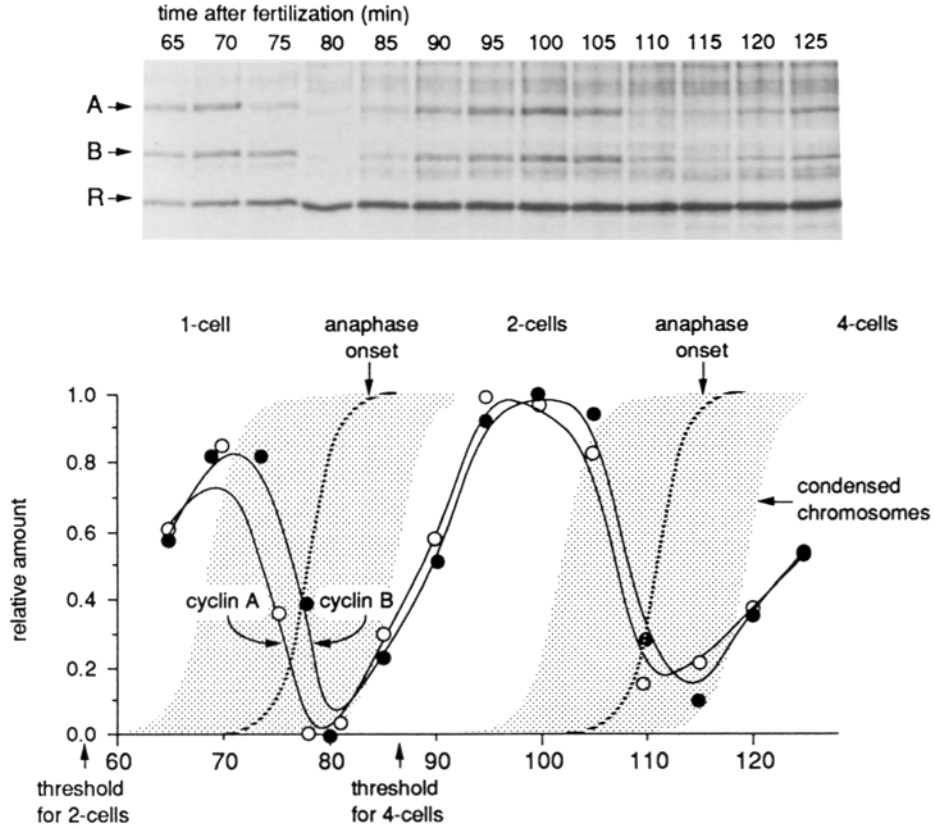


Figure 7. Cyclin oscillations in relation to events of the mitotic cell cycle. Duplicate samples were removed at 5-min intervals (indicated above each lane) from a culture of fertilized oocytes to which [^{35}S]methionine had been added at 50 min. One sample was worked up for analysis by SDS-PAGE. (*A*) Cyclin A; (*B*) cyclin B; (*R*) small subunit of ribonucleotide reductase. The other sample was stained with orcein and scored for the presence of condensed chromosomes (*shaded areas*) and the fraction in anaphase (*dotted line*); these data are plotted together with the densitometer scans from the autoradiograph in the lower panel. (○) Cyclin A; (●) cyclin B.

in the next section. When oocytes were pulse labeled for 5 min throughout the mitotic cell cycle, synthesis of both cyclins was essentially constant (data not shown). This suggests that newly synthesized cyclins are exempt from destruction, even if they are synthesized during the period of rapid cyclin proteolysis (see below). It also means that in the cell cycles of early clam embryos there is little or no reduction in the rate of protein synthesis during mitosis.

The Periods of Cyclin Instability Are Extremely Brief

To define the period of cyclin stability and instability more

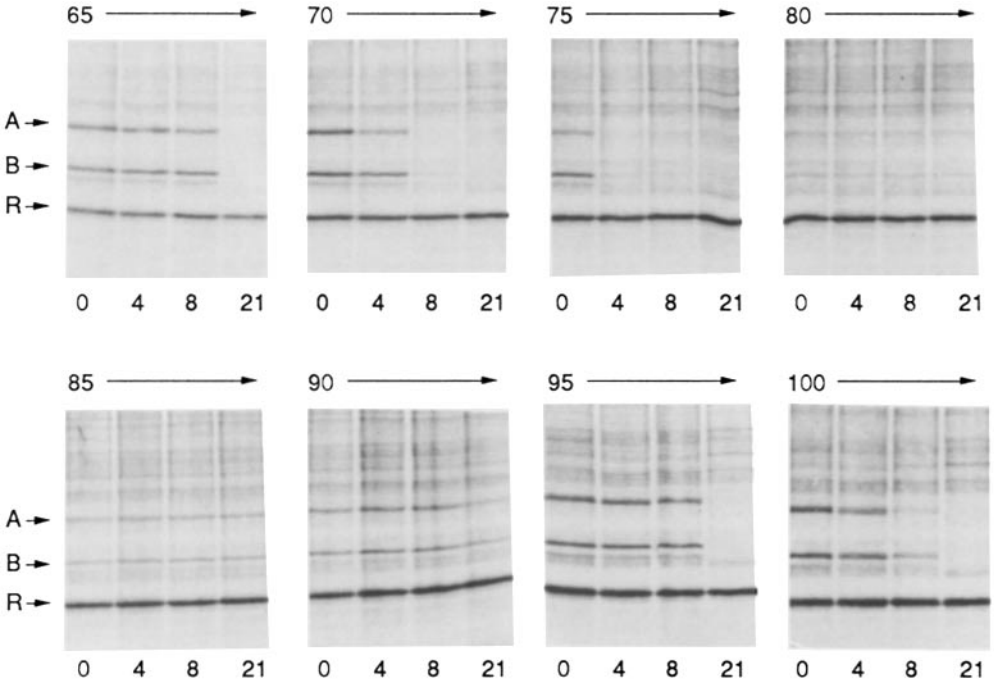
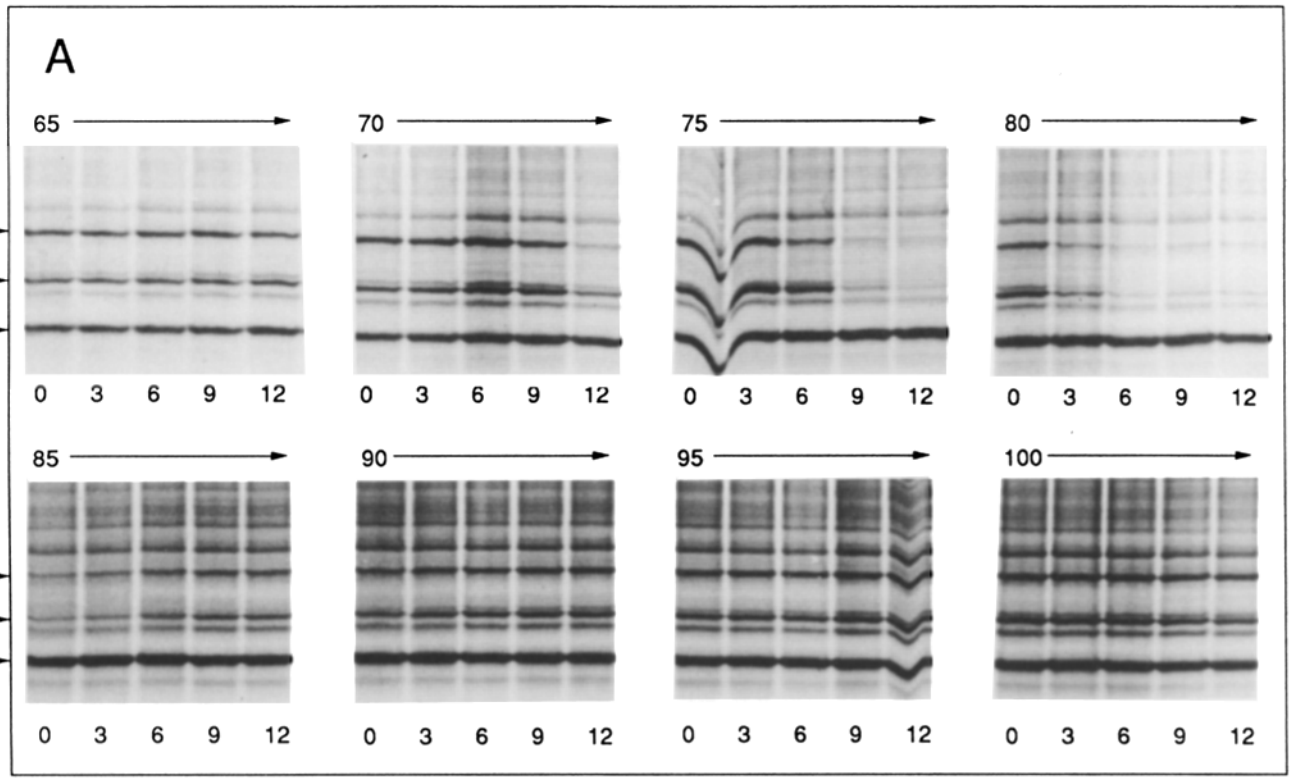


Figure 8. Emetine "chase" experiment to time the onset of cyclin destruction and restabilization. The experiment was similar to that shown in Fig. 7, except that at 5-min intervals, at the times indicated above each panel, samples were transferred to fresh vials containing emetine (100 μM final). These "chase" vials were sampled at 0, 4, 8, and 21 min after mixing (the times are shown below each lane). Cleavage occurred at 85 and 120 min in this experiment.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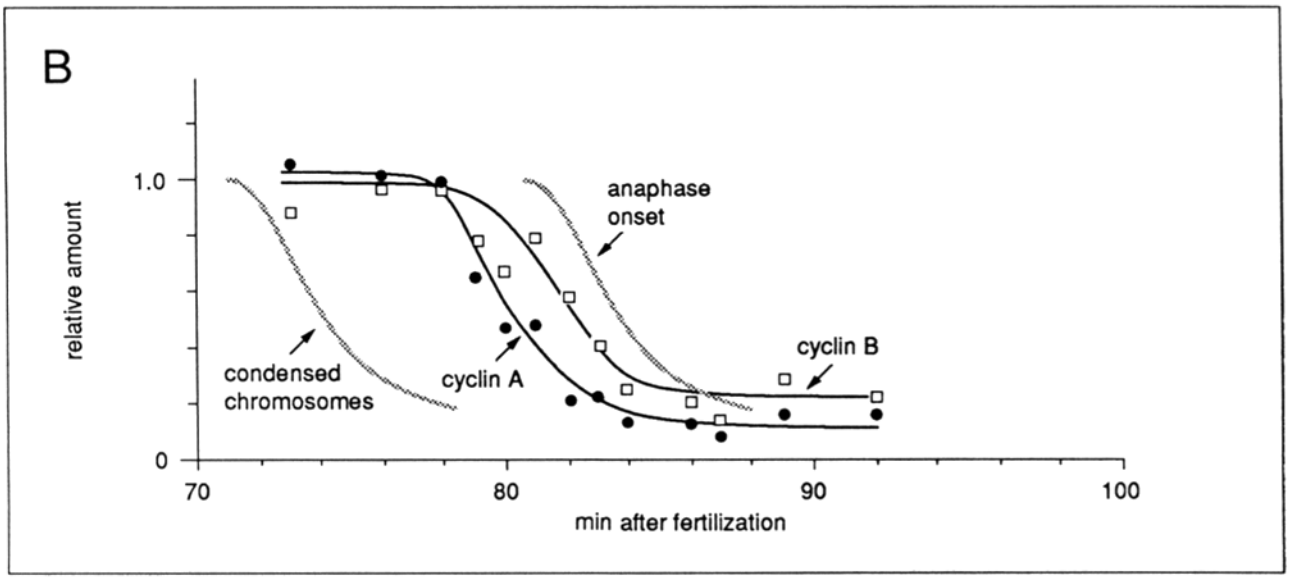
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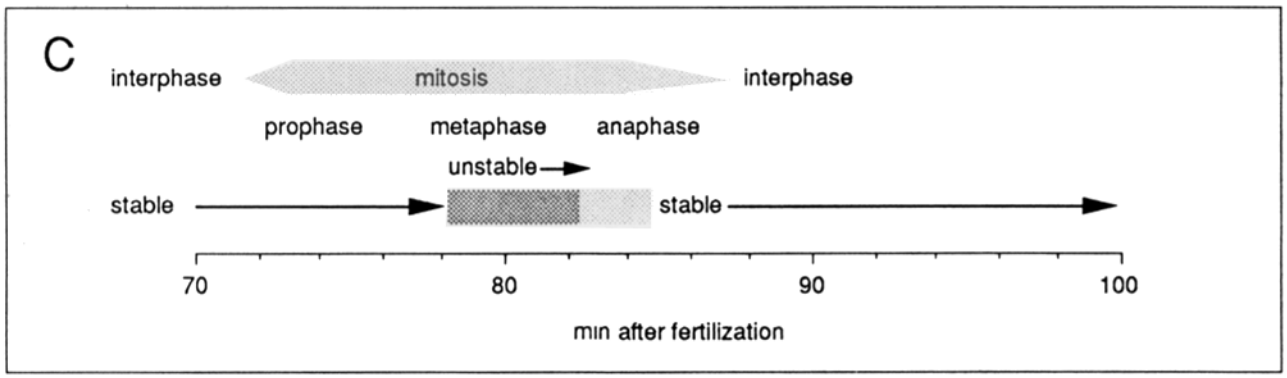
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closely, and to correlate these periods with the cell cycle, we performed two different kinds of "chase" experiments. In the first, fertilized embryos were allowed to develop for 50 min, at which time [^{35}S]methionine was added. 1-ml samples were removed at 5-min intervals into vials containing emetine (to give 100 μM final concentration). Samples were removed from these emetine chase samples at 20 s, 4 min, 8 min, and 21 min. The disappearance of the cyclins was especially obvious when resynthesis was prevented, the strong and persistent ribonucleotide reductase polypeptide (indicated by an *R*) serving as an internal control (Fig. 8). In this experiment, the destruction of cyclin A started at 74 min and was complete by 78 min, while cyclin B followed slightly later. The cyclins that were present at 80 min (the time of cytokinesis) up to 100 min were completely stable until the next period of destruction, which started at 108 min, just before the metaphase–anaphase transition of the second cleavage division. These results indicate that the cyclins are essentially stable during interphase and early M phase, but become highly unstable for a period of ~5 min near the end of metaphase. Sometime in telophase, the cell switches back to "cyclin stability mode" and newly made cyclins can accumulate again. Since the cyclin destruction curves run parallel to the cell cycle synchrony curves, the minimum length of the destruction phase cannot be defined for an individual cell. It is possible that "destruction mode" occupies a shorter period than the 5-min outer limit set by the degree of synchrony of these cultures.

However, as can be seen in Fig. 6, inhibition of protein synthesis around the time of the commitment point delays both entry and exit from mitosis. To circumvent this problem, we used a "chase" of unlabeled methionine that reduced the incorporation of [^{35}S]methionine 10,000-fold, but did not inhibit protein synthesis. Fig. 9 shows the results of an experiment that allowed a very detailed view of the kinetics of cyclin destruction in relation to the appearance of condensed chromosomes and the onset of anaphase, as plotted in Fig. 9 *B* and shown schematically in Fig. 9 *C*. In this experiment, destruction of cyclin A started at 79 min and was complete ~3 min later. Cyclin B destruction followed cyclin A by ~2 min, and by 85 min both cyclins were completely stabilized. The destruction of cyclin B preceded anaphase onset by ~1 min (Fig. 9 *B*). We were unable to detect any obvious cellular change associated with the disappearance of cyclin A. It has recently been found that indestructible mutants of cyclin A arrest cells in mitosis (Luca et al., 1991).

Cyclin B Levels Stay High in M phase–arrested Cells

Colchicine strongly delayed and slowed the destruction of cyclin B in sea urchins eggs (Evans et al., 1983). At the time those experiments were done, cyclin A had not been detected in those cells (although it is almost certainly present, albeit at low levels). To investigate the effects of colchicine on cyclins A and B in clam embryos, the inhibitor was added to

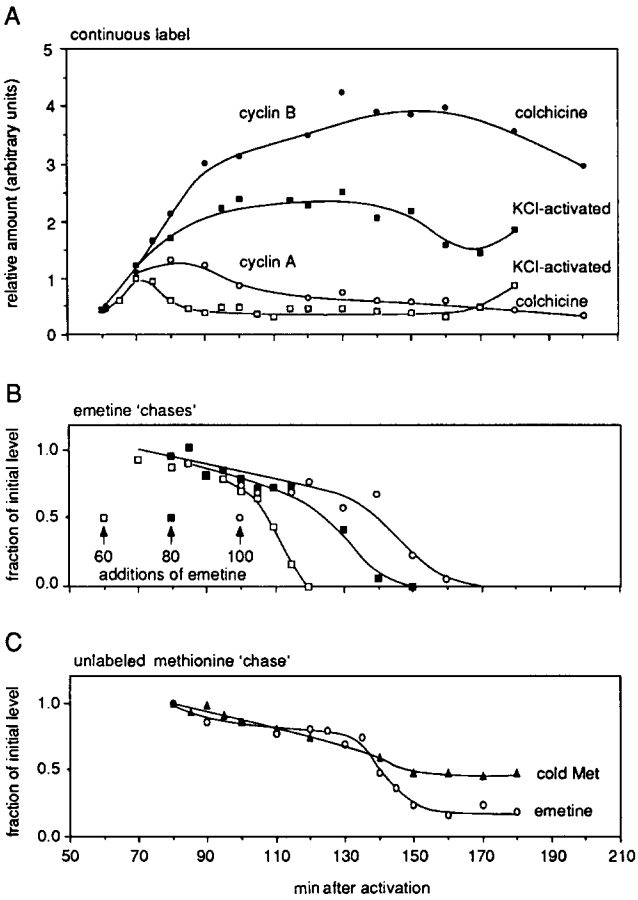

Figure 10. Cyclin destruction is impaired and prolonged in parthenogenetically activated and colchicine-inhibited embryos. (*A*) Colchicine (20 μg/ml) and [^{35}S]methionine were added to a culture of fertilized oocytes at 55 min (after second polar body emission), sampled at intervals, and analyzed by SDS-PAGE and densitometry of the cyclin bands. (*B*) A culture of parthenogenetically activated clam oocytes was labeled with [^{35}S]methionine starting 55 min after addition of KCl. At 80 and 100 min, emetine was added to portions of the culture and samples were removed for analysis by SDS-PAGE and cytological examination. The figure plots the radioactivity in cyclin B determined by densitometry of gel bands (see Minshull et al., 1989*b* for an example of autoradiographs from a similar experiment). (*C*) [^{35}S]Methionine was added to parthenogenetically activated oocytes at 50 min. At 80 min, samples were transferred to fresh vials with either 0.1 mM emetine (*open circles*) or 0.2 mM L-methionine (*closed triangles*).

[^{35}S]methionine-labeled cells at 50 min, the time of second polar body formation. In the presence of the drug, the cells entered first mitotic metaphase on schedule at 75 min, but failed to assemble a mitotic spindle or enter anaphase. Cells were arrested in M phase for several hours, the chromosomes becoming progressively more condensed and tightly clustered as time passed. Eventually, starting at 4–5 h after

Figure 9. Unlabeled methionine "chases" define the time of cyclin restabilization. This experiment was similar in design to the one shown in Fig. 8, except that 1 mM L-methionine was substituted for 100 μM emetine. The unlabeled methionine was added at the times indicated above each panel, and samples from these aliquots were removed into TCA at the times indicated below each lane. The autoradiograms were scanned and the data plotted together with the cytological statistics in *B*. *C* shows a schematic diagram to illustrate the periods of cyclin stability and instability in relation to the events of mitosis.

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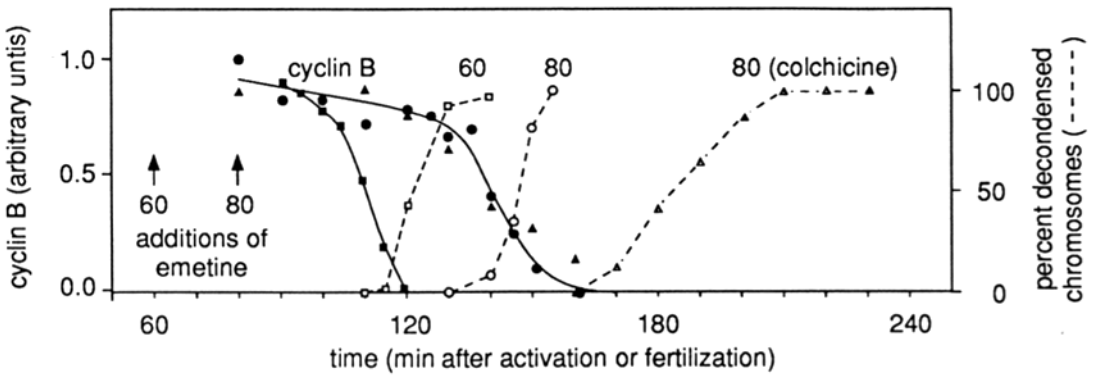

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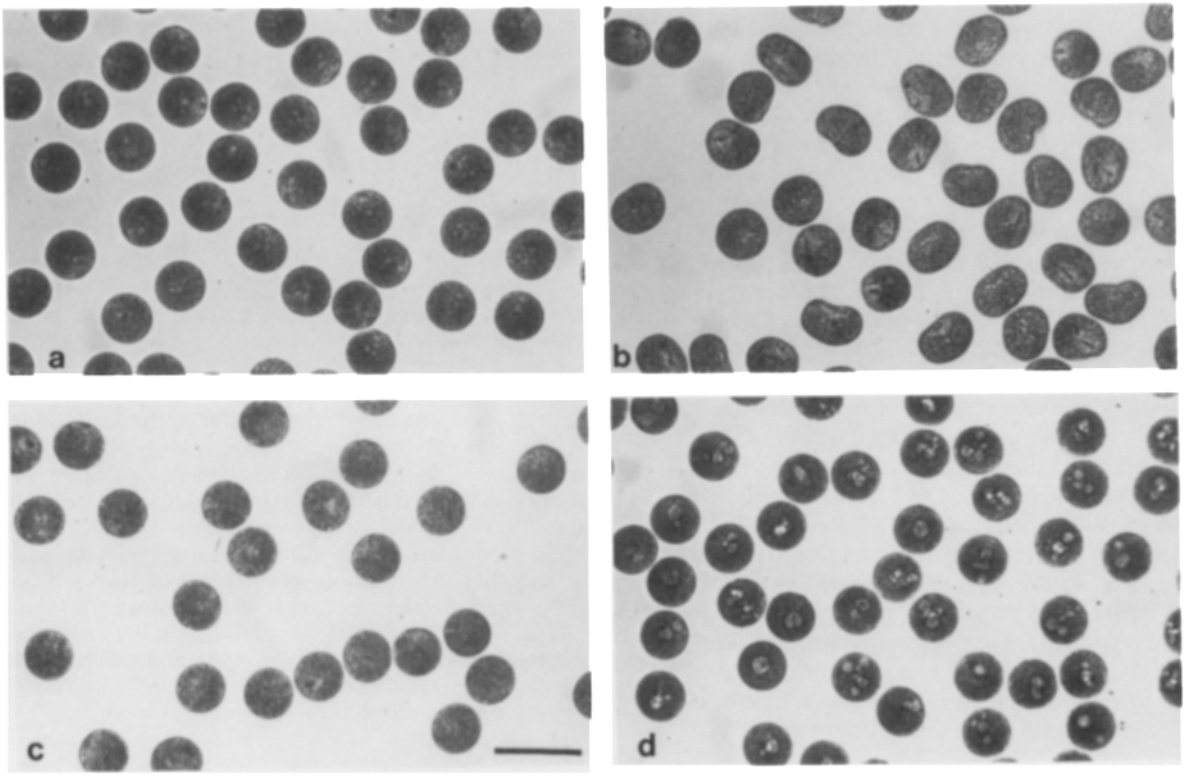


Figure 11. Embryos are arrested in metaphase by high levels of cyclin B, and can be returned to interphase by addition of emetine. Colchicine and [^{35}S]methionine were added to a culture of fertilized embryos after second polar body formation. Emetine was added at 70 min (after nuclear envelope breakdown) and samples were removed for cytological inspection and analysis by SDS-PAGE. The intensity of the cyclin B band and the fraction of cells with decondensed chromosomes are plotted as a function of time. A parallel culture without emetine remained in M phase (i.e., contained tightly condensed chromosomes) for the entire duration of the time frame shown here. *B* shows the microscopic appearance of the oocytes as they passed from mitosis (*a*) back to interphase (*d*; note the multiple nuclei) in the presence of colchicine and emetine. *b* shows the typical transient "lima bean" appearance that occurs during this transition, which would herald cytokinesis were it not for the presence of the colchicine. The cells relax (*c*) again before the nuclei re-form. Bar, 100 μm.

colchicine addition, occasional cells left M phase and began to reassemble nuclei (not shown). The level of cyclin A remained high until ~90–100 min after fertilization (considerably longer than usual) and then dropped to a new, low steady-state level that was maintained for the next 90 min or so (Fig. 10). In contrast, cyclin B levels rose to higher than normal levels in the presence of colchicine and maintained this level for 2–4 h past the normal time of cleavage.

The behavior of cyclins in the presence of colchicine was obviously distorted by the presence of the drug, and it was

impossible to be sure that the effect was not directly on the destruction system, rather than indirect via microtubule dissociation. There is an alternative way in clam embryos to maintain cells in mitotic M phase, however, which does not require addition of any inhibitor. Parthenogenetic activation of clam oocytes by 45 mM (total) KCl in sea water allows meiosis I and II to occur normally (Allen, 1953), followed by entry into first mitosis on schedule. Things go wrong after this point. The cells form a monopolar spindle with condensed chromosomes and remain like this for 2–4 h, with considerable variation among individual embryos (Kuriyama et al., 1986). Just as with colchicine, individual cells escape metaphase asynchronously, reassemble nuclei, and replicate their chromosomes. The cells then enter another long period of M phase arrest. They do not undergo cytokinesis. These slow cell cycles, in which metaphase is enormously prolonged with respect to the other events of the cell cycle, continue for at least 12 h, eventually giving rise to single cells packed with chromosomes in one enormous nucleus (not shown). Cyclin A fell to a low level approximately on schedule in these embryos, but just as in the colchicine-arrested embryos, cyclin B rose to much higher levels than normal and then stayed there, with destruction balanced by new synthesis (Fig. 10). Addition of emetine at 60, 80, and 100 min revealed that cyclin A was turning over rapidly at all times after the start of destruction mode at 75 min (data not shown), but cyclin B showed very different behavior (Fig. 10 *B*). After emetine was added, the level of cyclin B stayed relatively constant for ~50 min (considerably shorter than with colchicine, but at least 10 times longer than in uninhibited fertilized embryos) and then dropped abruptly. Cyclin B thus behaved as though it had a long, but finite lifetime of ~50 min under these circumstances.

When unlabeled methionine chases were used to follow the behavior of prelabeled cyclin B in parthenogenetically activated cells that were in metaphase arrest, the kinetics of loss of labeled cyclin B appeared somewhat different. Whereas emetine-chased cells showed a sudden loss of cyclin B ~50 min after inhibition of protein synthesis, methionine-chased samples showed a gradual, more or less linear loss of cyclin B after cessation of [^{35}S]methionine incorporation (Fig. 10 *C*). This is consistent with individual cyclin B molecules having a finite life expectancy. If no new cyclin is made, all labeled molecules are of similar age when they are degraded together. If on the other hand new unlabeled cyclin continues to be made, it apparently can compete for destruction with the labeled molecules. Once a molecule is fated to be destroyed, however, it has a finite life expectancy. It is unclear how cyclin is tagged for destruction, which would seem to be a requirement of this model. There is one other difference between the "emetine chase" and the unlabeled methionine chase experiments, which may be significant. The level of cyclin A should be significantly higher in the methionine chase experiments, and this may influence the timing of proteolysis of cyclin B.

In any case, the state of microtubules and the form of the spindle clearly determine the life expectancy of the cyclins. When the spindle is absent or improperly formed, cyclin A stays in "destruction mode" for long periods at a time, while cyclin B shifts to a state in which it displays a finite life expectancy. It may well be pertinent that the *cdc13*-117 strain in *S. pombe*, in which the yeast B-type cyclin is mutated, showed hypersensitivity to microtubule inhibitors (Booher and Beach, 1988). And it has also been shown that in both HeLa cells and yeast, disruption of microtubules leads to mitotic arrest associated with exaggerated levels of histone H1 kinase activity associated with p34^{cdc2}/cyclin complexes (Draetta and Beach, 1988; Moreno et al., 1989).

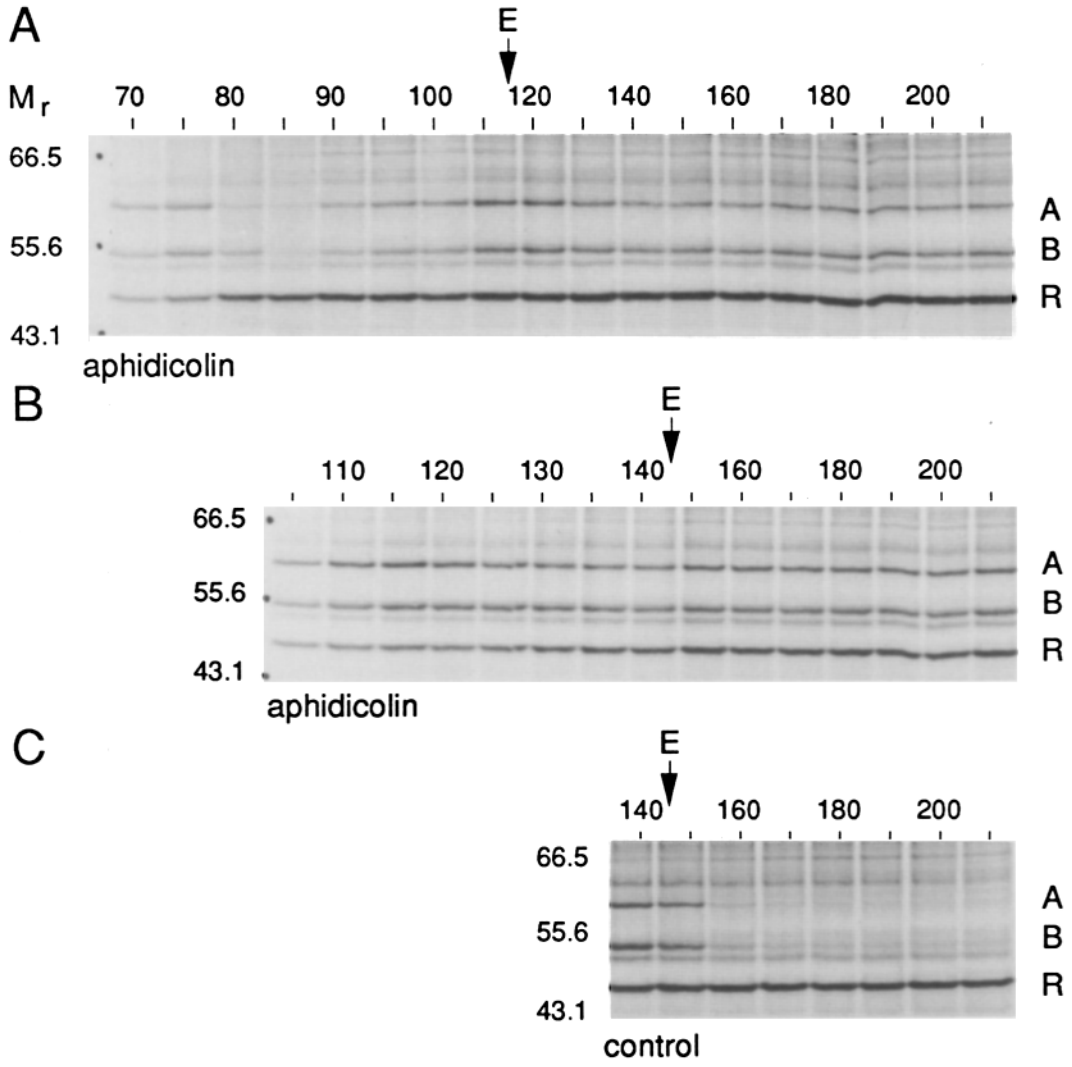
Inhibiting Protein Synthesis during Mitotic Arrest Advances Entry into the Next Cell Cycle

The preceding experiments show that when proper duplex mitotic spindles fail to form, either because of the presence of colchicine or because only one spindle pole is present, the destruction of cyclin B is delayed and the cells remain in M phase with highly condensed chromosomes. It seemed possible that the continuing presence of high levels of cyclin B might be responsible for keeping the cells in M phase. As shown in the previous section, the addition of emetine eventually leads to the loss of cyclin B, and we might expect the cells to exit mitosis prematurely when this occurred. This proved to be true both for KCl-activated and colchicine-inhibited cells arrested in M phase (Fig. 11 *A*). A transient surface contraction reminiscent of what normally preceded cytokinesis occurred at about the time of disappearance of cyclin B, but the cells did not divide. Interphase nuclei formed shortly thereafter (Fig. 11 *B*). The surface contraction was transient and impossible to preserve by fixation, so the response could not be placed on a good statistical footing. However, our strong impression was that the disappearance of cyclin B preceded or coincided with the surface contractions that mark the onset of anaphase in normal cells, and that this was the earliest visible manifestation of entry into the next cycle. It may represent an abortive attempt at cleavage.

The Effects of Other Cell Cycle Inhibitors on Cyclin Behavior

We tested the effects of several other inhibitors that interfere with the cell cycle. For example, cytochalasin B, which inhibits actin-based processes including cytokinesis did not inhibit nuclear division cycles and had very little effect on the kinetics or extent of the destruction of cyclin (data not shown). Taxol, which stabilizes microtubules, had no effect on the kinetics of cyclin synthesis or destruction, though it seriously impaired chromosome segregation and cytokinesis.

Inhibition of DNA synthesis with 10 μg/ml aphidicolin permitted one mitotic cell division and one round of cyclin destruction (Fig. 12), but no further divisions occurred and both cyclins were strongly stabilized thereafter. The aphidicolin-treated cells did not display properly condensed chromosomes or anything resembling metaphase plates or anaphase figures. Instead, two orcein-positive smudges corresponding to the two pronuclei could be seen shortly before the cells began to divide (Fig. 12 *D*). Two cells of equal size were formed by this cleavage and, in many cases, the cleavage furrow did not pass completely across the cell. Shortly after cytokinesis, the faintly staining nucleoid bodies lost their stain, but this was later regained and kept for > 1 h (not shown). No clear nuclei formed in such cells, which did not resemble any normally occurring stage; because of the aberrant form of their chromosomes it was impossible to tell whether they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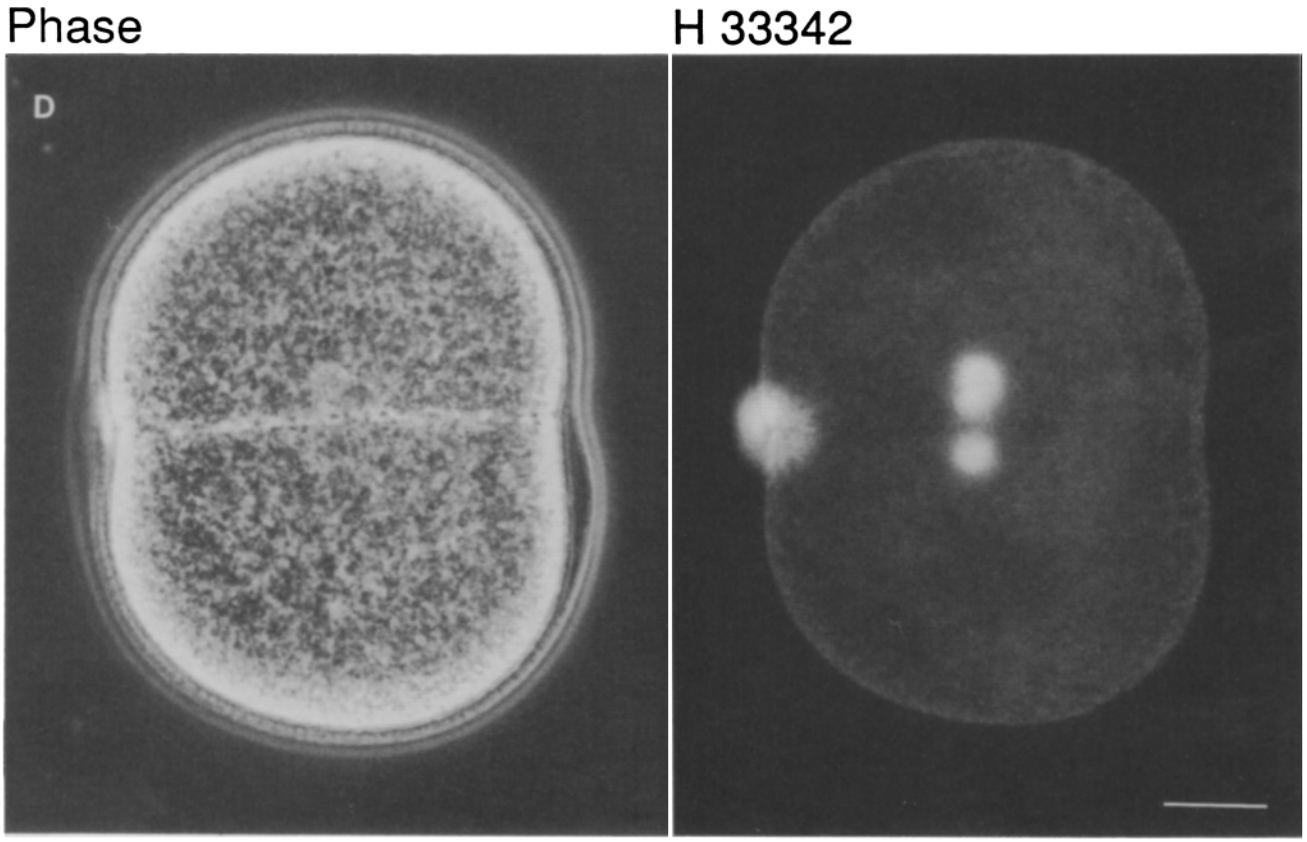
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were in mitosis, interphase, or some bizarre unnatural state. Further investigation would be necessary to interpret this interesting "phenotype." Clearly, however, some sort of coupling between the completion of DNA replication and cell cycle progression exists in clam embryos, unlike the situation in very early frog embryos, which do not arrest the cell cycle in response to inhibitors of DNA synthesis (Dasso and Newport, 1990; Newport and Kirschner, 1984). This is probably because the volume of cytoplasm relative to the nucleus is ~500 times greater in the frog compared with the clam oocyte.

Discussion

The initial object of these studies, which were carried out in 1984–5, before cyclin had been shown to act as an M phase inducer (Swenson et al., 1986; Minshull et al., 1989*a*; Murray and Kirschner, 1989), was to delineate the meiotic and mitotic cell cycles of fertilized clam oocytes after fertilization in relation to the protein synthesis requirement for these rapid early cell cycles. In particular, we wished to explore the relationships between these requirements and the behavior of cyclins A and B. Failure to synthesize cyclins during interphase could account, at least in part, for why protein synthesis inhibitors arrest the cell cycle at the G2-M transition of mitosis. The requirement for cyclin synthesis for cell cycle progression has been well documented by experiments in *Xenopus* cell-free systems (Murray and Kirschner, 1989*a*; Murray et al., 1989) and the observations that neither *S. pombe cdc13* nor *Drosophila* cyclin A mutants can perform the G2-M transition (Booher and Beach, 1988; Lehner and O'Farrell, 1990). It is worth pointing out that neither the lack of cyclins caused by these mutations nor inhibition of protein synthesis in early frog and sea urchin embryos seems to block DNA replication (Miake-Lye et al., 1983; Wagenaar, 1983), although this point has not been checked in the clam embryo.

Conversely, destruction of cyclins appears to be necessary to leave M phase; we found a striking correlation between the timing of the disappearance of cyclin B and reentry to interphase in cells arrested in metaphase by colchicine or monopolar spindles. The discovery that truncated cyclins lacking the NH_2-terminal "destruction box" are stable and keep cells arrested in mitosis (Murray et al., 1989; Belmont et al., 1990; Glotzer et al., 1991; Luca et al., 1991; Ghiara et al., 1991) provides a direct demonstration that cyclin destruction is required for cells to leave M phase and enter interphase. It is a moot point whether destruction of cyclin A is required solely to get it out of the way, so to speak, or whether its destruction is causally connected with a cell cycle transition that occurs before the onset of anaphase. We were never able to see any obvious correlation between the timing of cyclin A proteolysis and changes in cellular structure that might have provided clues as to the separate functions of cyclins A and B.

The Timing of Cyclin Degradation and the Metaphase–Anaphase Transition

This paper also describes the precise timing of the destruction of cyclins A and B in relation to anaphase onset at first mitosis in dividing clam embryos. Cyclin A is degraded before cyclin B, and cyclin B destruction precedes anaphase by ~60 s. Normally, the duration of "destruction phase" in mitotic cycles is extremely brief, as though the destruction pathway for cyclin was rapidly turned off at the end of M phase (it is difficult to say whether the destruction of cyclin A is normally turned off before the destruction of cyclin B, but the results with microtubule inhibitors and parthenogenetically activated embryos shown in Figs. 10 and 11 suggest that cyclin A proteolysis stays switched on for as long as the levels of cyclin B – and also presumably of cdc2-kinase – stay high). This behavior is also found in cell-free systems in which added NH_2-terminally truncated (and hence indestructible) cyclin of one type leads to constitutive destruction of both types (Luca et al., 1991). The transience of the proteolytic activity was previously only implied, by the rapid accumulation of cyclins after the end of mitosis. By testing the stability of cyclins at different stages of the cell cycle we demonstrate unequivocally that the oscillations in cyclin level occur as a result of constant synthesis and an alternation between stability during interphase and rapid destruction at the end of metaphase. The window during which cyclins are unstable is normally ~5 min or less. When mitotic spindles are altered, however, this window of cyclin destruction is lengthened and altered in very interesting ways.

Complete dissociation of microtubules by colchicine has a severe effect on cyclin turnover. Once cells enter M phase in the presence of this drug, cyclin A destruction is kept on for the entire duration of metaphase arrest, and cyclin A levels are maintained at low levels. At the same time, the destruction of cyclin B is impaired and delayed, so that its levels rise abnormally high. The same effect is seen in only slightly less marked form in parthenogenetically activated cells containing monopolar spindles. This suggests that cyclin B destruction does not simply depend on intact microtubules, but requires a correctly assembled bipolar spindle. Indeed, the drug taxol, which stabilized microtubules and caused extraordinary hypertrophy of the two mitotic asters in these cells (data not shown), did not affect cyclin destruction. The B-type cyclin mutant of *S. pombe*, *cdc13*-117 is hypersensitive to thiabendazole, an antimicrotubule drug related to nocodazole that is effective against yeast microtubules (Booher and Beach, 1988). Several authors have reported finding cyclin and cdc2 localized at spindle poles (Bailly et al., 1989; Alfa et al., 1990; Pines and Hunter, 1991), and Kellogg et al. (1991) found by affinity chromatography that both cyclins A and B were associated with microtubules. One explanation for the delayed kinetics of cyclin B destruction in metaphase-arrested cells is that cyclin/cdc2 complexes need to be transported to a particular subcellular location where

Figure 12. Aphidicolin stabilizes cyclins A and B after the first mitotic cell cycle. Aphidicolin was added at 10 min to a culture of fertilized oocytes, and [^{35}S]methionine was added at 50 min (*A*). Portions of the culture were removed into separate vials with emetine at 70 (*B*) or 100 min (*C*), and sampled for SDS-PAGE. *D* shows a micrograph of a typical embryo that underwent equal cleavage in the presence of aphidicolin, on the left viewed under phase-contrast illumination, at right seen under UV illumination (Hoechst 33342 was present at 1 μg/ml). Bar, 10 μm.

the destruction machinery resides, and that this is greatly slowed when the spindle is dissociated or improperly formed. But this does not easily explain why monopolar spindles should display altered kinetics of cyclin destruction, and an alternative explanation for the effect is that misplaced chromosomes may transmit a signal that delays cyclin destruction. There is considerable evidence from micromanipulation experiments that unattached chromosomes are capable of delaying the onset of anaphase by a "checkpoint" mechanism (Nicklas and Koch, 1969; Sluder and Begg, 1983; Nicklas and Kubai, 1985; Hartwell and Weinert, 1989; for review see Murray and Szostak, 1985). Experiments in cell-free systems will probably be required to explore the mechanism of this effect.

Are Cyclins the Only Cell Cycle Regulators Synthesized after Fertilization?

How many of the noncyclin mRNAs that are newly recruited by ribosomes after fertilization encode cell cycle regulators? Apart from the cyclins, which clearly belong in this category, the only other mRNAs in clam oocytes to be identified so far encode the small subunit of ribonucleotide reductase and histone H3 (Standart et al., 1985; Swenson et al., 1987). Although the proteins encoded by these mRNAs are required for cell cycle progression, it is unlikely that they have a direct regulatory role. Moreover, the very different effects of inhibition by aphidicolin, a DNA synthesis inhibitor, from those caused by emetine make it unlikely that failure to provide ribonucleotide reductase or histones could account for the emetine-associated lesions either in meiosis II or even first mitosis. Nevertheless, the idea that the defect in second meiotic metaphase might be caused by lack of newly synthesized A- or B-type cyclins is probably incorrect. Clam oocytes contain a store of cyclin B, but not of cyclin A. The maternal stock of cyclin B largely evades destruction between the two meiotic divisions, so there should be plenty of cyclin B even in the complete absence of protein synthesis (Westendorf et al., 1989; Shibuya, E., and J. V. Ruderman, manuscript in preparation). So, could lack of cyclin A account for the meiosis II metaphase defect? At first sight, this too seems unlikely, because the protein synthesis requirement for second meiosis is complete by ~25 min, and what little new cyclin A that has accumulated by this time is destroyed shortly after this (Westendorf et al., 1989). Thus, if cyclin A were required for second meiosis, it would have to act before anaphase of first meiosis. This is not impossible, but it is not a very attractive idea. It seems more probable that other components, which are required to correctly form the second meiotic spindle, are synthesized after fertilization. A good candidate would be the molluscan equivalent of c-*mos*, which plays an important part in vertebrate meiosis (Daar et al., 1991; Sagata et al., 1988, 1989), especially in the progression of meiosis I to meiosis II (Kanki and Donoghue, 1991). There may well be others, however, which remain to be identified.

Why Do Oocytes Not Reenter Interphase between Meiosis I and Meiosis II?

One of the most interesting questions raised by these studies, albeit indirectly, is: What happens in the interval between the two meiotic divisions? We are somewhat limited in our discussion by lack of data concerning MPF levels in clam oocytes; given the variability of oocyte maturation, it is probably hazardous to extrapolate from animals as far distant phylogenetically as starfish, frogs, and clams. Nevertheless, it is likely that MPF levels drop between the two meiotic divisions in clams, because the histone kinase activity of $p34^{cdc2}$ transiently declines in this interval (Shibuya, E., and J. V. Ruderman, manuscript in preparation). Yet as can be clearly seen, the nucleus does not reassemble, the chromosomes do not decondense, and there is no S phase. (There must not be, or an important objective of meiosis fails!) It is by no means clear in clams how much cyclin B is lost at meiosis I, another interesting point which is under careful investigation (see Westendorf et al., 1989). We want to emphasize here, however, that loss of MPF and a drop in $p34^{cdc2}$ kinase activity does not inevitably lead to exit from M phase and return to interphase. We imagine that other factors maintain the phosphorylation state of the correct set of proteins so as to keep the chromosomes condensed and the nuclear envelope disassembled. Perhaps a special meiotic protein kinase is responsible, or perhaps the phosphatases which normally undo M phase are temporarily shut off; our data do not tell. But understanding the molecular basis of this seeming paradox (really, only a paradox because of previous simple interpretations of how $p34^{cdc2}$/cyclin kinases control the cell cycle) will undoubtedly deepen our comprehension of the M phase/interphase transition. It is also important to recall that at least three events—namely, the splitting of the first meiotic spindle pole to form the two poles of the second meiotic spindle, the functional duplication of the first meiotic kinetochores, and cytokinesis—must occur in this interval. These processes may well depend on, and in some sense be coordinated by, the transient drop in $p34^{cdc2}$-associated kinase that marks the passage from meiosis I to II.

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References

Alfa, C. E., J. S. Hyams, B. Ducommun, and D. Beach. 1990. Distinct nuclear and spindle pole body populations of cyclin-cdc2 in fission yeast. *Nature (Lond.).* 347:680–682.

Allen, R. D. 1953. Fertilization and artificial activation in the egg of the surf-clam, *Spisula solidissima. Biol. Bull. (Woods Hole)* 105:213–239.

Bailly, E., M. Dorée, P. Nurse, and M. Bornens. 1989. $p34^{cdc2}$ is located in both nucleus and cytoplasm; part is centrosomally associated at G_2/M and enters vesicles at anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3985–3995.

Belmont, L. D., A. A. Hyman, K. E. Sawin, and T. J. Mitchison. 1990. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell.* 62:579–589.

Booher, R., and D. Beach. 1988. Involvement of cdc13$^+$ in mitotic control in *Schizosaccharomyces pombe*: possible interaction of the gene product with microtubules. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2321–2327.

Cyert, M. S., and J. Thorner. 1989. Putting it on and taking it off: phosphoprotein phosphatase involvement in cell cycle regulation. *Cell.* 57:891–893.

Daar, I., R. S. Paules, and G. F. Vande Woude. 1991. A characterization of

cytostatic factor activity from *Xenopus* eggs and c-*mos*-transformed cells. *J. Cell Biol.* 114:329–335.

Dasso, M., and J. W. Newport. 1990. Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. *Cell.* 61:811–823.

Doonan, J. H., and N. R. Morris. 1989. The *bimG* gene of Aspergillus nidulans, required for completion of anaphase, encodes a homologue of mammalian phosphoprotein phosphatase 1. *Cell.* 57:987–996.

Draetta, G., and D. Beach. 1988. Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell.* 54:17–26.

Draetta, G., F. Luca, J. Westendorf, L. Brizuela, J. Ruderman, and D. Beach. 1989. cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell.* 56:829–838.

Dubé, F. 1988. Effect of reduced protein synthesis on the cell cycle in sea urchin embryos. *J. Cell. Physiol.* 137:545–552.

Dunphy, W. G., and J. W. Newport. 1989. Fission yeast p13 blocks mitotic activation and tyrosine phosphorylation of the Xenopus *cdc2* protein kinase. *Cell.* 58:181–191.

Dunphy, W. G., L. Brizuela, D. Beach, and J. Newport. 1988. The *Xenopus* cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell.* 54:423–431.

Evans, T., E. T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt. 1983. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell.* 33:389–396.

Félix, M.-A., P. Cohen, and E. Karsenti. 1990. Cdc2 H1 kinase is negatively regulated by a type 2A phosphatase in the *Xenopus* early embryonic cell cycle: evidence from the effects of okadaic acid. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:675–683.

Gautier, J., C Norbury, M. Lohka, P. Nurse, and J. Maller. 1988. Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene cdc^{2+}. *Cell.* 54:433–439.

Gautier, J., J. Minshull, M. Lohka, M. Glotzer, T. Hunt, and J. L. Maller. 1990. Cyclin is a component of MPF from Xenopus. *Cell.* 60:487–494.

Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I Reed. 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation by cyclin prevents exit from mitosis. *Cell.* 65:163–188.

Glotzer, M., A. W. Murray, and M. W. Kirschner. 1991. Cyclin is degraded by the ubiquitin pathway. *Nature (Lond.).* 349:132–138.

Gould, K. L., and P. Nurse. 1989. Tyrosine phosphorylation of the fission yeast $cdc2^{+}$ protein kinase regulates entry into mitosis. *Nature (Lond.).* 342: 39–45.

Harris, P., M. Osborn, and K. Weber. 1980. Distribution of tubulin containing structures in the egg of the sea urchin *Strongylocentrotus purpuratus* from fertilization through first cleavage. *J. Cell Biol.* 84:668–679.

Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science (Wash. DC).* 246:629–634.

Hultin, T. 1961. The effect of puromycin on protein metabolism and cell division in fertilised sea urchin eggs. *Experientia (Basel).* 17:410–411.

Kanki, J. P., and D. J. Donoghue. 1991. Progression from meiosis I to meiosis II in *Xenopus* oocytes requires *de novo* translation of the mos^{xe} protooncogene. *Proc. Natl. Acad. Sci. USA.* 88:5794–5798.

Kellogg, D. R., K. Oegama, J. W. Raff, W. Sullivan, W. Theurkauf, and B. Alberts. 1991. Studies on the centrosome of the early *Drosophila* embryo. *Cold Spring Harbor Symp. Quant. Biol.* In press.

Kishimoto, S., and I. Lieberman. 1964. Synthesis of RNA and protein required for the mitosis of mammalian cells. *Exp. Cell Res.* 36:92–103.

Krek, W., and E. A. Nigg. 1991. Differential phosphorylation of vertebrate $p34^{cdc2}$ kinase at the G1/S and G2/M transitions of the cell cycle: identification of major phosphorylation sites. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:305–316.

Kumagai, A., and W. G. Dunphy. 1991. The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. *Cell.* 64:903–914.

Kuriyama, R., G. G. Borisy, and Y. Masui. 1986. Microtubule cycles in oocytes of the surf clam, *Spisula solidissima*: an immunofluorescence study. *Dev. Biol.* 114:151–160.

Labbé, J.-C., J.-P. Capony, D. Caput, J.-C. Cavadore, J. Derancourt, M. Kaghdad, J.-M. Lelias, A. Picard, and M. Dorée. 1989. MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3053–3058.

Lee, T. H., M. J. Solomon, M. C. Mumby, and M. W. Kirschner. 1991. INH, a negative regulator of MPF, is a form of protein phosphatase 2A. *Cell.* 64:415–423.

Lehner, C. F., and P. H. O'Farrell. 1990. The roles of Drosophila cyclins A and B in mitotic control. *Cell.* 61:535–547.

Longo, F. J., S. Cook, L. Mathews, and S. J. Wright. 1991. Nascent protein requirement for completion of meiotic maturation and pronuclear development: examination of fertilized and A-23187-activated surf clam (*Spisula solidissima*) eggs. *Dev. Biol.* 148:75–86.

Lorca, T., D. Fesquet, F. Zindy, F. Le Bouffant, M. Cerruti, C. Brechot, G. Bevauchelle, and M. Dorée. 1991. An okadaic acid-sensitive phosphatase negatively controls the cyclin degradation pathway in amphibian eggs. *Mol. Cell. Biol.* 11:1171–1175.

Luca, F. C., and J. V. Ruderman. 1989. Control of programmed cyclin destruction in a cell-free system. *J. Cell Biol.* 109:1895–1909.

Luca, F. C., E. K. Shibuya, C. E. Dohrmann, and J. V. Ruderman. 1991. Both cyclin AΔ60 and BΔ97 are stable and arrest cells in M-phase, but only BΔ97 turns on cyclin destruction. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:4311–4320.

Luca, F. C., E. K. Shibuya, C. E. Dohrmann, and J. V. Ruderman. 1991. Truncated cyclin A is stable and blocks the completion of mitosis. *EMBO (Eur. Mol. Biol. Organ.) J.* In press.

Meijer, L., D. Arion, R. Golsteyn, J. Pines, L. Brizuela, T. Hunt, and D. Beach. 1989. Cyclin is a component of the sea urchin egg M-phase specific histone H1 kinase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:2275–2282.

Meijer, L., L. Azzi, and J. Y. J. Wong. 1991. Cyclin B targets $p34^{cdc2}$ for tyrosine phosphorylation. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1545–1554.

Miake-Lye, R., J. Newport, and M. Kirschner. 1983. Maturation-promoting factor induces nuclear envelope breakdown in cycloheximide-arrested embryos of *Xenopus laevis*. *J. Cell Biol.* 97:81–91.

Minshull, J., J. J. Blow, and T. Hunt. 1989*a*. Translation of cyclin mRNA is necessary for extracts of activated Xenopus eggs to enter mitosis. *Cell.* 56:947–956.

Minshull, J., J. Pines, R. Golsteyn, N. Standart, S. Mackie, A. Colman, J. Blow, J. V. Ruderman, M. Wu, and T. Hunt. 1989*b*. The role of cyclin synthesis, modification and destruction in the control of cell division. *J. Cell Sci. Suppl.* 12:77–97.

Moreno, S., J. Hayles, and P. Nurse. 1989. Regulation of $p34^{cdc2}$ protein kinase during mitosis. *Cell.* 58:361–372.

Morla, A., G. Draetta, D. Beach, and J. Y. J. Wang. 1989. Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entrance into mitosis. *Cell.* 58:193–203.

Murray, A. W., and M. W. Kirschner. 1989*a*. Cyclin synthesis drives the early embryonic cell cycle. *Nature (Lond.).* 339:275–280.

Murray, A. W., and M. W. Kirschner. 1989*b*. Dominoes and clocks: the union of two views of the cell cycle. *Science (Wash. DC).* 246:614–621.

Murray, A. W., and J. W. Szostak. 1985. Chromosome segregation in mitosis and meiosis. *Annu. Rev. Cell Biol.* 1:289–315.

Murray, A. W., M. J. Solomon, and M. W. Kirschner. 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. *Nature (Lond.).* 339:280–286.

Newport, J. W., and M. W. Kirschner. 1984. Regulation of the cell cycle during early Xenopus development. *Cell.* 37:731–742.

Nicklas, R. B., and C. A. Koch. 1969. Chromosome manipulation. III. Spindle fiber tension and the reorientation of mal-oriented chromosomes. *J. Cell Biol.* 43:40–56.

Nicklas, R. B., and D. F. Kubai. 1985. Microtubules, chromosome movement, and reorientation after chromosomes are detached from the spindle by micromanipulation. *Chromosoma.* 92:313–324.

Nurse, P. 1990. Universal control mechanism regulating cell cycle timing of M-phase. *Nature (Lond.).* 344:503–508.

Ohkura, H., K. Noriyuki, S. Miyatani, T. Toda, and M. Yanagida. 1989. The fission yeast $dis2^{+}$ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell.* 57:997–1007.

Picard, A., G. Peaucellier, B. F. Le Bouffant, C. Le Peuch, and M. Dorée. 1985. Role of protein synthesis and proteases in production and inactivation of maturation-promoting activity during meiotic maturation of starfish oocytes. *Dev. Biol.* 109:311–320.

Pines, J., and T. Hunter. 1990. $p34^{cdc2}$. The S and M kinase? *New Biol.* 2:389–401.

Pines, J., and T. Hunter. 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. *J. Cell Biol.* 115:1–17.

Sagata, N., M. Oskarsson, T. Copeland, J. Brumbaugh, and G. F. Vande Woude. 1988. Function of c-mos proto-oncogene product in meiotic maturation in Xenopus oocytes. *Nature (Lond.).* 335:519–526.

Sagata, N., I. Daar, M. Oskarsson, S. D. Showalter, and G. F. Vande Woude. 1989. The product of the mos proto-oncogene as a candidate "initiator" for oocyte maturation. *Science (Wash. DC).* 245:643–646.

Sluder, G., and D. A. Begg. 1983. Control mechanisms of the cell cycle: role of the spatial arrangement of spindle components in the timing of mitotic events. *J. Cell Biol.* 97:877–886.

Solomon, M. J., M. Glotzer, T. H. Lee, M. Philippe, and M. W. Kirschner. 1990. Cyclin activation of $p34^{cdc2}$. *Cell.* 63:1013–1024.

Standart, N. M., S. J. Bray, E. L. George, T. Hunt, and J. Ruderman. 1985. The small subunit of ribonucleotide reductase is encoded by one of the most abundant translationally regulated maternal RNAs in clam and sea urchin eggs. *J. Cell Biol.* 100:1968–1976.

Swenson, K. I., K. M. Farrell, and J. V. Ruderman. 1986. The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in *Xenopus* oocytes. *Cell.* 47:861–870.

Swenson, K. I., N. Borgese, G. Pietrini, and J. V. Ruderman. 1987. Three translationally regulated mRNAs are stored in the cytoplasm of clam oocytes. *Dev. Biol.* 123:6–10.

Timourian, H., and J. Uno. 1967. Protein synthesis requirements for the first two cleavages in sea urchin eggs. *Exp. Cell Res.* 48:173–176.

Vallee, R. B., and G. S. Bloom. 1983. Isolation of sea urchin egg microtubules

with taxol and identification of mitotic spindle microtubule-associated proteins with monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80:6259–6263.

Wagenaar, E. B. 1983. The timing of synthesis of proteins required for mitosis in the cell cycle of the sea urchin embryo. *Exp. Cell Res.* 144:393–403.

Wagenaar, E., and D. Mazia. 1978. The effect of emetine on first cleavage division in the sea urchin *Strongylocentrotus purpuratus*. *In* Cell Reproduction: In Honor of Daniel Mazia. E. Dirksen, D. Prescott, and C. Fox, editors. Academic Press, New York. 539–545.

Westendorf, J. M., K. I. Swenson, and J. V. Ruderman. 1989. The role of cyclin B in meiosis. *J. Cell Biol.* 108:1431–1444.

Wilt, F. H., H. Sakai, and D. Mazia. 1967. Old and new protein in the formation of the mitotic apparatus in cleaving sea urchin eggs. *J. Mol. Biol.* 27:1–7.