

The Role of Cyclin B in Meiosis I

Joanne M. Westendorf,* Katherine I. Swenson,† and Joan V. Ruderman*§

*Program in Cell and Developmental Biology and †Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115; and §Department of Zoology, Duke University, Durham, North Carolina 27706

Abstract. In clams, fertilization is followed by the prominent synthesis of two cyclins, A and B. During the mitotic cell cycles, the two cyclins are accumulated and then destroyed near the end of each metaphase. Newly synthesized cyclin B is complexed with a small set of other proteins, including a kinase that phosphorylates cyclin B *in vitro*. While both cyclins can act as general inducers of entry into M phase, the two are clearly distinguished by their amino acid sequences (70% nonidentity) and by their different modes of expression in oocytes and during meiosis. In contrast to cyclin A, which is stored solely as maternal mRNA, oocytes contain a stockpile of cyclin B

protein, which is stored in large, rapidly sedimenting aggregates. Fertilization results in the release of cyclin B to a more disperse, soluble form. Since the first meiotic division in clams can proceed even when new protein synthesis is blocked, these results strongly suggest it is the fertilization-triggered unmasking of cyclin B protein that drives cells into meiosis I. We propose that the unmasking of maternal cyclin B protein allows it to interact with cdc2 protein kinase, which is also stored in oocytes, and that the formation of this cyclin B/cdc2 complex generates active M phase-promoting factor.

PROGRESS through the cell cycle in eukaryotes is regulated both by external signals (such as growth factors, mating factors, and fertilization) and the synthesis or modification of internal regulators. In mammalian somatic tissue culture cells, for example, the major regulatory events controlling cell proliferation occur during G1. Exposure of quiescent G0 or early G1 cells to tissue-specific growth factors promotes the transcription of certain genes whose products probably control the transcription of other genes; this leads to the ability to traverse G1 and enter S phase (for reviews see Baserga, 1986; Rollins and Stiles, 1989). The next well-studied regulatory step occurs late in G2 with the appearance of a cytoplasmic activity called MPF (M phase-promoting factor),¹ which is recognized by its ability upon microinjection or addition to cell-free systems to drive nuclei into meiosis or mitosis. As cells exit from M phase into the next interphase, MPF activity disappears (Smith and Ecker, 1971; Masui and Markert, 1971; Inglis et al., 1976; Wasserman and Smith, 1978; Sunkara et al., 1979; Loidl and Grobner, 1982; Weintraub et al., 1982; Adlahka et al., 1983; Tachibana et al., 1987).

Some, but not all, of these mechanisms operate during the specialized cell cycles of oocytes and early embryos. Like somatic cells, the first regulatory events involve external signals: in many cases hormones trigger the meiotic divisions

at the end of oogenesis and fertilization activates the onset of the mitotic cell cycles (Masui and Clarke, 1979). However, in contrast to somatic cells, most embryonic cells have abbreviated cell cycles consisting of rapid, alternating periods of DNA synthesis (interphase) and mitosis (M phase). The G1 and G2 periods are absent or very short in these "cleavage divisions," presumably because most of the necessary materials have been laid down previously during oogenesis. There is no evidence that other external signals, like growth factors, play any role in controlling the very early cell cycles. Instead, during the cleavage divisions, the cells seem to rely entirely on internal regulation. The key regulatory events control the rise in MPF activity across the cycle, which drives cells into M phase, and the loss of MPF activity at the end of mitosis, which allows cells to exit into the next interphase (Smith and Ecker, 1971; Masui and Markert, 1971; Wasserman and Smith, 1978; Newport and Kirschner, 1984; Gerhart et al., 1984; Miake-Lye and Kirschner, 1985; Lohka and Maller, 1985).

During the mitotic cell cycles, both protein synthesis and protein phosphorylation are needed for the activation of MPF and entry into M phase. Both are needed for meiosis II as well, but in some cases meiosis I can proceed without ongoing protein synthesis. If either protein synthesis or phosphorylation is blocked during the mitotic cell cycles, cells will arrest at the interphase/mitosis border (Neant and Guerrier, 1988; Labbé et al., 1988; for review see Swenson et al., 1989). Where cells remain in M phase for an extended time (as in colchicine arrest or during naturally occurring meiotic metaphase arrest), protein synthesis and phosphorylation are

1. *Abbreviations used in this paper:* buffer T, 0.3 M glycine, 0.12 M K gluconate, 0.1 M taurine, 40 mM NaCl, 10 mM EGTA, 2.5 mM MgCl₂, 0.1 M HEPES; MPF, M phase-promoting factor; MSW, Millipore-filtered sea water; nt, nucleotide.

required to maintain that M phase arrest (Ziegler and Masui, 1976; Clarke and Masui, 1983; Neant and Guerrier, 1988; Hashimoto and Kishimoto, 1988; Hunt, T. and J. V. Ruderman, manuscript in preparation). Finally, proteolysis is needed for loss of MPF activity and exit from M phase into the next interphase (Picard et al., 1985, 1987; Shoji-Kasai et al., 1988; Schollmeyer, 1988).

Among the proteins encoded by stored mRNAs and prominently synthesized during the rapid meiotic and mitotic cleavage divisions of marine invertebrate embryos are the cyclins (Rosenthal et al., 1980, 1982, 1983; Evans et al., 1983; Swenson et al., 1986; Pines and Hunt, 1987; Standart et al., 1987). Cyclins are synthesized and accumulated across each cell cycle and then destroyed at the end of each mitosis, immediately preceding the metaphase-anaphase transition. This unusual behavior has suggested a role for the cyclins in the cell cycle, possibly as activators or integral components of MPF. While a complete molecular description of MPF is not yet available, one of its components in frogs has been identified as the homolog of the yeast *cdc2/CDC28* protein kinase (Gautier et al., 1988; Dunphy et al., 1988), a protein thought to interact with the yeast homolog of the invertebrate cyclins (Booher and Beach, 1987, 1988; Solomon et al., 1988; Goebel and Byers, 1988; Reed et al., 1988). Unfortunately, a direct immunochemical test of the idea that cyclins are components of highly purified, active MPF is precluded at the present time by technical constraints: so far, purified MPF has been obtained from only one organism, the frog *Xenopus* (Lohka et al., 1988), and the only available cyclin antibodies do not cross-react with frog cyclins. However, a role for cyclins as integral components of active MPF is strongly supported by our recent demonstration that, in clam embryos, newly synthesized cyclins join preexisting *cdc2* protein kinase to form 220-kD complexes that resemble MPF in several important ways (Draetta et al., 1989).

In clams, there are two prominent cyclins, A and B. Both rise and fall in parallel with the cell cycle, but their destruction periods are slightly offset. We have previously shown that when pure cyclin A is introduced into frog oocytes, which are physiologically arrested at the G2/M border of meiosis I and contain a pool of *cdc2* protein kinase, it causes entry into M phase and the resumption of meiosis (Swenson et al., 1986). Thus cyclin A, which begins to be made before meiosis I in both frogs and clams, can act as a general inducer of M phase. Paradoxically, however, clam cyclin A itself cannot be responsible for driving meiosis I in clams: when protein synthesis is blocked, fertilized clam oocytes proceed perfectly well through meiosis I in the absence of any maternally stockpiled or newly synthesized cyclin A (Luca et al., 1987). These considerations suggest the existence of a related protein, possibly cyclin B, that is stored in the oocyte and can be mobilized during meiosis I.

Here we show that unfertilized clam oocytes do indeed contain a stockpile of cyclin B protein and that cyclin B is a potent inducer of meiosis I. In oocytes, cyclin B protein is stored as large cytoplasmic aggregates. Within minutes of fertilization, this material disperses, probably in response to the rise in intracellular pH. Thus cyclin B has all the properties required of the M phase inducer for meiosis I. We suggest that the unmasking of maternal cyclin B protein after fertilization allows a productive interaction with *cdc2* protein kinase, which is also stored in oocytes, and that this interaction leads to the formation of active MPF.

Materials and Methods

Preparation of Embryos

Adult clams (*Spisula solidissima*) were collected by the Marine Resources staff of the Marine Biological Laboratory, Woods Hole, MA, and maintained in sea water at 14–18°C for up to several months. Oocytes were collected by dissection (Rosenthal et al., 1980), settled 3–5 times at room temperature in Millipore-filtered (0.45- μ m pore size) sea water (MSW) and resuspended in MSW plus 50 μ g/ml gentamycin sulfate (Sigma Chemical Co., St. Louis, MO) at 20,000–40,000 oocytes/ml (\sim 0.25 ml packed oocytes/100 ml MSW). Cultures were kept suspended by stirring with a 60-rpm paddle motor and maintained at 18°C using a refrigerated water bath. When oocytes were kept for several days, they were settled and resuspended each day in fresh MSW plus antibiotic.

50 μ l sperm (Rosenthal et al., 1980) was diluted into 5 ml MSW and kept at 18°C for up to 2 h. For fertilization, 1 vol sperm stock was added to 1,000 vol oocyte suspension. Only cultures showing >98% fertilization and synchronous progress into meiosis (<5 min differences among all individual embryos in the population) were used.

In some experiments, oocytes were parthenogenetically activated by adding an extra 40 mM KCl to the cell suspension. Upon completion of nuclear envelope breakdown and entry into meiosis I at 10–12 min, cells were pelleted and resuspended in an equal volume of MSW.

Progress through the meiotic and mitotic cell cycles was monitored routinely using two methods (Hunt, T., and J. V. Ruderman, manuscript in preparation). 1 μ g/ml Hoechst 33342 (Calbiochem-Behring Corp., San Diego, CA) was added to cultures and 10- μ l aliquots were monitored during the experiment by fluorescence microscopy. For quantitative analysis, 100- μ l aliquots were pipetted into 1.5-ml microfuge tubes containing 1 ml 75% EtOH, 25% glacial acetic acid (vol/vol) and held for up to 12 h. Tubes were spun briefly in a microfuge, supernatant was drained off, 100 μ l lacto-orcein stain (1 gm orcein [Sigma Chemical Co.; O-1127], 20 ml glacial acetic acid, 80 ml 85% lactic acid [Fisher Scientific Co., Pittsburgh, PA; A162]) was added, and cells were stained for 20–30 min. 1 ml 40% acetic acid was added, cells were settled, the supernatant was removed, and 200 μ l 40% acetic acid was added. 8.5 μ l of the cell suspension was mounted under an 18-mm cover slip and cells were viewed with brightfield optics.

RNA and In Vitro Translation

PolyA⁺ RNA was prepared as described by Rosenthal et al. (1983) and translated in reticulocyte lysates, made as described by Pelham and Jackson (1976), containing 500 μ Ci/ml [³⁵S]methionine (New England Nuclear, Boston, MA; NEG-009T, 1,142 Ci/mmol). 10- μ l translation reactions contained 3–6 μ g of total RNA, or smaller amounts of hybrid-selected RNA. 50 μ l SDS gel sample buffer (Laemmli, 1970) was added and a portion, usually 10 μ l, was analyzed on SDS-polyacrylamide gels as described in Anderson et al. (1973).

cDNA Cloning

10 μ g of polyA⁺ RNA isolated from KCl-activated oocytes was the template for the synthesis of double-stranded cDNA as described by Gubler and Hoffman (1983) and as modified by Bruck et al. (1986). Eco RI linkers (New England BioLabs, Beverly, MA; No. 1020) were added and the DNA was ligated into λ gt10 DNA as described by Bruck et al. (1986). The DNA was packaged into phage particles and plated with host cells C600r⁺m⁺Hfl. About 10,000 recombinant plaques in the library were screened separately with two radioactive probes (Maniatis et al., 1982). Probe 1 contained single-stranded cDNA complementary to the same polyA⁺ RNA used for library construction. 5 μ g polyA⁺ RNA was denatured in 10 mM methylmercurichydroxide for 10 min at room temperature and neutralized with 100 mM 2-mercaptoethanol. cDNA was synthesized in a 33- μ l reaction containing 60 U reverse transcriptase, 47 mM Tris-HCl, pH 8.3, 10 mM MgCl₂, 300 ng oligo dT, 10 mM DTT, 33 mM KCl, 4.5 mM NaPyrophosphate (Sigma Chemical Co.), 1.35 mM dGTP, 1.35 mM dTTP, 0.01 mM dCTP, 0.01 mM dATP, 50 μ Ci [α -³²P]dCTP, 50 μ Ci [α -³²P]dATP (600 Ci/mmol; New England Nuclear), and 10 U RNasin (Promega Biotec, Madison, WI).

Hybrid Select Translation

DNA from potential cyclin B cDNA-containing plaques was isolated (Davis et al., 1980; Helms et al., 1985) and bound to nitrocellulose filters (Kafatos et al., 1979). Filters were used for hybrid select translation as described by Alexandraki and Ruderman (1981) with the following modifications. Pre-

hybridization was carried out for 2 h at 37°C, hybridization was carried out with 12 µg polyA⁺ RNA for 8 h at 37°C, and posthybridization washes were carried out at 60°C. The mRNAs selected by each of the cloned cDNAs were precipitated with ethanol and translated in reticulocyte lysate.

DNA Sequencing

Two overlapping cDNA clones, λJW101 (2,383-bp insert) and λJW102 (2,772-bp insert), were subcloned into M13mp8 or Bluescribe M13 (Stratagene) vectors in both orientations. Deletions of various sizes were made at the end nearest the sequencing primer site according to the methods of Hong (1982) or Henikoff (1984). Restriction fragments, representing cDNA sequences that were not close to the primer site in any of the deletion subclones, were cloned into pATH (Koerner, T. J., personal communication) or Bluescribe vectors. Two other cDNAs, λJW103 (≈1,500-bp insert) and λJW104 (≈2,300-bp insert), were subcloned into Bluescribe M13- to confirm 5' cDNA sequence. All clones were sequenced by the dideoxy chain termination method (Sanger et al., 1977) as shown in Fig. 4. DNA and protein sequences were analyzed using IntelliGenetics programs or others available through the Molecular Biology Computer Research Resource, Dana-Farber Cancer Institute, Boston.

Preparation of Anti-Cyclin B Antibodies

The λJW101 cDNA insert (2,383 bp; position 378 [amino acid 96] to the end of the polyA tail; Fig. 4) was excised with Eco RI and cloned into the Eco RI site of bacterial expression vector pATH 1 and transfected into the host bacteria RRI. As described in the text, the resulting plasmid pJW401 was shown to encode a 75-kD fusion protein consisting of 323 NH₂-terminal amino acids of TrpE, followed by 7 amino acids specified by the linker and the 332 COOH-terminal amino acids of cyclin B. Several milligrams of the TrpE-cyclin B fusion protein were prepared as follows. Each of ten flasks containing 100 ml M9 salts, 0.5% casamino acids, 1 mM MgSO₄, 0.1 mM CaCl₂, 0.2% glucose, 20 µg/ml thiamine was inoculated with a 10-ml overnight culture and grown for 1.5 h with vigorous shaking. 5 µg/ml idoleacrylic acid (Sigma Chemical Co.) was added, and cells were grown for an additional 5 h. Cells were pelleted and lysed in 1% SDS, 10 mM NaPO₄, pH 7.2, 6 M urea, 1% β-mercaptoethanol. Lysates were separated on 15% SDS-polyacrylamide gels (Anderson et al., 1973). Fusion protein was visualized by staining with 4 M sodium acetate (Higgins and Dahmus, 1979), excised, electroluted from the gel (McDonald et al., 1986), and dialyzed against PBS. White New Zealand female rabbits were injected intramuscularly, intradermally, or subcutaneously with 250 µg TrpE-cyclin B fusion protein emulsified with Freund's complete adjuvant. Rabbits were boosted with 100 µg fusion protein emulsified with Freund's incomplete adjuvant at 4-wk intervals. Sera were collected 4–5 d after boosting. For affinity purification of antibodies (Lazarides, 1982), TrpE-cyclin B was attached to CNBr-activated Sepharose CL4B (Sigma Chemical Co.); antibodies specific for the fusion protein were bound to the matrix, eluted with 0.2 M glycine-HCl, pH 2.5, and immediately neutralized with 0.1 vol 1.5 M Tris-HCl, pH 8.8. The concentration of affinity-purified antibodies was determined by measuring the A₂₈₀ of the eluate (Layne, 1957).

Analysis of Cyclin B Levels during Meiosis and Mitosis

1-ml aliquots of an embryo culture (20,000/ml) were taken at 4-min intervals from fertilization across meiosis I, meiosis II, mitosis 1, and mitosis 2. These were precipitated with 12.5% TCA, washed twice with acetone, and dissolved in 100 µl SDS-urea gel sample buffer. 20-µl samples (4,000 embryos/sample) were run on a 15% polyacrylamide gel containing SDS and blotted onto nitrocellulose (Swenson et al., 1986). The blot was incubated with 0.5 µg/ml affinity-purified antibody in PBS plus 3% BSA, 10% donkey serum (Pel-Freez Biologicals, Rogers, AR) and 0.1% Tween-20 followed by 0.1 µCi/ml ¹²⁵I-labeled donkey anti-rabbit antibodies (Amersham Corp., Arlington Heights, IL).

Cell Fractionation

100-ml aliquots of oocytes and activated embryos taken at 7 and 20 min postactivation, all at 50,000 cells/ml, were dechorionated, washed twice in buffer T (0.3 M glycine, 0.12 M K gluconate, 0.1 M taurine, 40 mM NaCl, 10 mM EGTA, 2.5 mM MgCl₂, 0.1 M Hepes) at pH 6.8 or 7.2, and resuspended in the appropriate buffer. Cells were lysed and 0.25 M sucrose was added. 5 ml of each lysate was spun at 1,000 g for 10 min. The supernatant was spun at 13,000 g for 10 min, and the resulting supernatant was spun at 124,000 g for 1 h. Each of the pellets were resuspended in 1 ml buffer T plus 0.25 M sucrose. Aliquots of each fraction were dissolved in SDS-

urea sample buffer and samples containing equal cell equivalents were electrophoresed and blotted. Blots were reacted with cyclin B antibodies as above.

Immunofluorescence

Oocytes were fertilized and stained *in vivo* with 1 µg/ml Hoechst 33342. 50-ml aliquots were taken at 0, 6, and 15 min, spun, and resuspended in fixative (0.5 g 1-ethyl-3-[3-dimethylamino-propyl]carbodiimide [Sigma Chemical Co.], 0.875 ml 20% formaldehyde added 4 min before use to 50 ml 25 mM Na₂HPO₄, 25 mM NaH₂PO₄, 2 mM MgCl₂, 10 mM EGTA, 191.5 mM KCl, 116 mM Tris base, pH 7.0 at time of use). Cells were fixed for 30 min, washed three times in PBS and once in 0.3 M glycine, 1 mM MgCl₂, and stored overnight at 4°C. Cells were incubated with 20 µg/ml affinity-purified cyclin B antibodies or preimmune immunoglobulins in PBS, 0.2% Tween-20, 20% goat serum (Pel-Freez Biologicals), 1 mM MgCl₂ for 4 h at 37°C, washed three times in PBS plus 1 mM MgCl₂, and incubated with 25 µg/ml affinity-purified fluorescein-conjugated goat anti-rabbit antibodies (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) in PBS, 0.1% Tween-20, 10% goat serum, 1 mM MgCl₂ for 4 h at 37°C. Final washes were in PBS, 1 mM MgCl₂, 0.1 µg/ml Hoechst 33342. Cells were placed on slides, flattened with pieces of agarose 0.15 mm thick (Fukui et al., 1987), and mounted. For photography, each cell was exposed to the light beam for the same amount of time before and during each picture.

Microinjection of Cyclin mRNAs into Frog Oocytes

Xenopus females (Nasco Biologicals, Fort Atkinson, WI) were anesthetized by immersion in 0.15% ethyl-*n*-aminobenzoate. Ovarian lobes were surgically removed into calcium-free modified Barth's saline (Colman, 1984) made up with distilled deionized water. The lobes were manually dissected into clumps of ~100 oocytes and digested with 20 mg/ml collagenase for 20 min. Oocytes were freed of the surrounding layer of follicle cells by dissection and kept at 19°C in modified Barth's saline. Oocytes were microinjected near the centers of the vegetal hemispheres. Capped cyclin A mRNA was transcribed *in vitro* with SP6 polymerase using pAXH(+) that had been linearized with Hind III (Swenson et al., 1986). Capped cyclin B mRNA was transcribed *in vitro* using Eco RI-linearized DNA pCD102 which was constructed by inserting the cyclin B fragment 88–1,504 into the Bgl II site of SP64T (Krieg and Melton, 1984).

Assay of Protein Kinase Activity in Cyclin B Immunoprecipitates

Oocytes (50,000/ml) were fertilized or activated with 40 mM KCl. 10 µCi/ml [³⁵S]methionine ("Translabel," 1,027 Ci/mmol, ICN) were added to 100 ml cell suspension at the end of meiosis II (55–60 min), and cells were cultured until the beginning of mitosis 1 (70–75 min), as marked by nuclear envelope breakdown. Cells were spun for 15 s in a clinical centrifuge and the pellets were lysed by vortexing in 8 ml cold buffer T, pH 7.2, plus 0.2% Tween-20 and 1 mM PMSF. The lysate was spun at 13,000 g for 10 min and 0.2-ml aliquots of the supernatant (~125,000 cells) were mixed with 2 µg affinity-purified antibodies (or 2 µg preimmune antibodies that had been affinity purified on protein A-Sepharose CL4B; Sigma Chemical Co.), and incubated overnight at 4°C. Each aliquot received 50 µl of a 1:1 slurry of protein A-Sepharose CL4B in buffer T plus 0.2% Tween-20 and was incubated for 2–4 h at 4°C. Antibody-containing pellets were recovered by spinning in a microfuge for 10 s, and washed three times in buffer T plus 0.2% Tween-20 and twice in kinase buffer (7.5 mM MgCl₂, 20 mM Tris-HCl, pH 7.2, 1 mM NaF).

In vitro kinase reactions were carried out by resuspending washed pellets in 10 µl kinase buffer containing 10 µCi [³²P]ATP (3,000 Ci/mmol, Amersham Corp.) and incubating the suspension at room temperature for 30 min. Reaction products were washed twice with cold kinase buffer, suspended in 20 µl SDS gel sample buffer minus thiol reducing agent, and boiled for 2 min. The labeled proteins in 10 µl of the reaction were separated by electrophoresis on a 15% polyacrylamide gel. Dried gels were autoradiographed at –80°C using Kodak XAR5 film and a DuPont Lightning-plus screen.

When exogenous proteins were tested for phosphoacceptor activity, 2 µg of the test protein (which had been heated at 56°C for 10 min to inactivate contaminating kinases) was included in the kinase reaction. 20 µl SDS gel sample buffer minus reducing agent was added directly to the sample at the end of the incubation. The following substrates were tested: histones H1, histone H2A, histone H2B, histone H3, protamine sulfate, and casein (all from Sigma Chemical Co.).

Reprecipitation of ^{32}P -phosphoprotein products in the immune precipitate kinase assay was carried out as follows. The reaction products were washed and suspended in 20 μl SDS gel sample buffer as described above, 1 μl β -mercaptoethanol was added, the sample was boiled for 2 min and brought to 0.8 ml of immunomix (0.25% NaDeoxycholate, 0.5% NP-40, 0.5% SDS, and 1 \times PBS). Any remaining antibody that could be bound by protein A was removed by adding 25 μl protein A-Sepharose 6MB (Pharmacia Fine Chemicals, Piscataway, NJ) and spinning for 10 s; the supernatant was divided into 0.2-ml aliquots, incubated overnight at 4°C with 2 μg of affinity-purified anti-cyclin B antibody, precipitated with 25 μl protein A-Sepharose CL4B, washed three times with 1 \times immunomix, twice with PBS, and eluted with 20 μl SDS gel sample buffer minus reducing agent.

Some immune precipitates were dephosphorylated before the *in vitro* kinase reaction. Precipitates were incubated for 10 min at 30°C in 40 mM Pipes, pH 6.0, 1 mM DTT, 1 mM PMSF with or without 170 mU potato acid phosphatase (Sigma Chemical Co.; P6760) (Cooper and King, 1986). The reaction products were washed three times with buffer T containing 0.2% Tween-20 and twice with kinase buffer. The phosphatased proteins were then taken through the kinase reaction as described above.

Results

Cloning and Identification of Cyclin B cDNA

Cyclin B mRNA is one of the three most abundant polyA⁺ maternal mRNAs found in early clam embryos, but was not represented in an earlier cDNA library (Rosenthal et al., 1980, 1983; Rosenthal and Ruderman, 1987). A new cDNA library in $\lambda\text{gt}10$ was constructed as described in Materials and Methods. Replica filters of the library were screened with probe 1 (^{32}P -cDNAs made against the original template RNA, which contained high levels of cyclin B mRNA) or probe 2 (a mix of ^{32}P -labeled cloned cDNAs encoding the two other abundant maternal mRNAs stored in the oocyte, cyclin A, and ribonucleotide reductase [Rosenthal et al., 1983; Standart et al., 1985; Swenson et al., 1986]). Plaques hybridizing with the first probe, but not with the second, were selected as potential cyclin B clones. DNA was isolated from several candidate clones and tested by an mRNA hybridization selection translation assay. The radioactive translation products encoded by RNAs selected by individual clones were examined on autoradiographs of SDS-polyacrylamide gels. Of the first five clones tested, three selected mRNAs that translated to give a protein of the same electrophoretic mobility as cyclin B (Fig. 1, lanes 3–5).

To further test the identity of these clones, we asked if the protein encoded by one of these clones showed the characteristic behavior of cyclins; i.e., accumulation across the cell cycle followed by abrupt disappearance at the metaphase-anaphase transition. To do this, we produced a rabbit polyclonal antiserum against a bacterial fusion protein containing 37 kD of NH₂-terminal bacterial TrpE and 38 kD of the potential cyclin B sequence carried by λJW101 . Samples from a population of synchronously dividing embryos were taken at 4-min intervals across meiosis I, meiosis II, mitosis 1, and mitosis 2, separated on a gel, blotted to nitrocellulose, and reacted with affinity-purified antibody. This antibody recognized a protein that comigrated with marker cyclin B protein (data not shown), increased across the cell cycle, and disappeared at the metaphase-anaphase transition of each mitosis, as expected (Fig. 2). The antibody did not show detectable binding to the other cyclin in these embryos, cyclin A, which migrates on these gels at 60 kD. We conclude that λJW101 contains cyclin B coding sequences.

During the mitotic cycles, cyclin B levels peaked in early

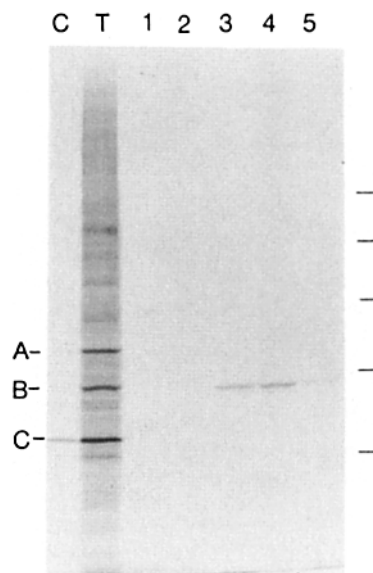


Figure 1. Identification of cyclin B cDNA clones by an mRNA hybrid selection–translation assay. mRNAs hybridizing to five potential cyclin B cDNA clones (selected as described in the text) and to the cDNA clone λJWC (encoding the small subunit of ribonucleotide reductase) were translated in reticulocyte lysate. The [^{35}S]methionine-labeled translation products were analyzed by SDS-PAGE followed by autoradiography. A, B, and C indicate the electrophoretic mobilities of cyclin A, cyclin B, and ribonucleotide reductase, which is referred to as protein C in earlier publications. Translation products programmed by (lane C) RNA selected by λJWC cDNA; (lane T) total embryonic RNA; and (lanes 1–5) RNA selected by potential cyclin B cDNA clones; lane 3 shows the product of RNA selected by clone λJW101 . The positions of molecular mass marker proteins are indicated by dashes on the right, from top to bottom: 116, 94, 68, 56, and 41 kD.

M phase (Fig. 2). The rapid loss of cyclin B began several minutes before the end of mitosis, defined here as the time when 50% of the embryos have passed through the metaphase-anaphase transition, and was completed in <10 min. During the destruction period, up to 90% of cyclin B disappeared from the population as a whole. However, because of the slight asynchrony among individual embryos in the population, it is impossible to distinguish between (a) incomplete disappearance in all cells and (b) complete disappearance in all individual cells that is obscured by the asynchrony of the population. The blot also shows that the disappearance of the 56-kD cyclin B band was not accompanied by the appearance of any higher or lower molecular mass bands. This result suggests cyclin B is proteolytically destroyed, rather than reversibly modified, at the end of each mitosis.

Unlike cyclin A, which is undetectable in oocytes and requires protein synthesis for its appearance after fertilization, there is a pool of cyclin B protein present in the unfertilized oocyte (Fig. 2). Also in contrast to cyclin A, which almost completely disappears at the metaphase-anaphase transition of meiosis I, cyclin B is not completely destroyed at the end of meiosis I; instead, cyclin B drops by $\sim 50\%$. At the end of meiosis II, cyclin B levels drop by $\sim 90\%$ and then proceed to show the characteristic oscillations across each mitotic cell cycle.

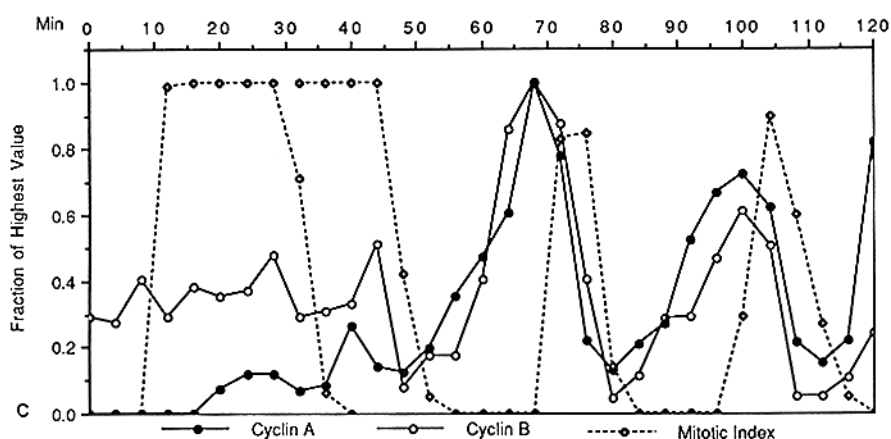
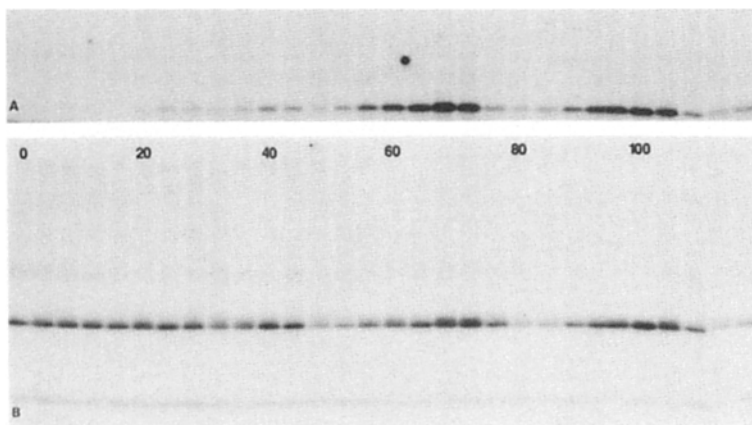


Figure 2. Antibodies directed against a fusion protein containing sequences from the potential cyclin B cDNA clone λ JW101 recognize on immunoblots a protein with the characteristics of cyclin B. (A and B) Immunoblot analysis of cyclin A and cyclin B, respectively, across meiosis and the first two mitotic cell cycles of the clam embryo. Duplicate samples of oocytes and embryos were taken at 4-min intervals from 0–120 min after fertilization. One set was electrophoresed on an SDS–polyacrylamide gel, blotted onto nitrocellulose, reacted with affinity-purified rabbit antibodies against TrpE–cyclin A or –cyclin B fusion proteins, and then incubated with 125 I-labeled donkey anti-rabbit antibodies. Autoradiograms of the blots are shown. The reacting bands in section B comigrated with authentic marker cyclin B loaded on an adjacent lane (not shown). (C) The second set of samples was analyzed by lacto-orcein staining of the chromosomes to monitor progress across the mitotic cell cycles. The rise in mitotic index represents the point at which cells enter M phase, as indicated by the onset of chromosome condensation. The fall in mitotic index represents the point at which cells exit M phase, as indicated by the metaphase–anaphase transition. After anaphase of meiosis I (36') the chromosomes remain condensed and proceed directly through meiosis II, exiting into the interphase of the first mitotic cell cycle at 48'. Cyclin levels were determined by quantitative densitometry of the autoradiograms shown in A and B.

The Nucleotide and Derived Amino Acid Sequence of Cyclin B

The complete DNA sequence of the longest cloned cyclin B cDNA (λ JW102) is presented in Fig. 3. The 2,772-bp sequence represents most of the mRNA length, which was estimated at 2,800 nucleotides (nt) by RNA gel blots (not shown). The longest open reading frame begins with two adjacent ATG codons (at nt 88 and 91) and terminates at nt 1,374. The two upstream ATGs do not function as initiators, since their removal does not affect the size of the translated protein (see below). Kozak's (1986) rules argue that translation is initiated at the fourth ATG (preceded by A at nt 88) rather than the third ATG (preceded by T at nt 85). The predicted size of the protein is 48 kD (428 amino acids), compared to the 56-kD mobility of cyclin B on SDS–polyacrylamide gels. We do not consider this discrepancy very significant since the electrophoretic mobilities of the cyclins on various gel systems show considerable variability. The coding region is followed by a 1,398 nt 3' noncoding sequence which contains no open reading frames encoding more than 60 amino acids; it shows two polyA addition recognition sequences, AATAAA (Proudfoot and Brownlee,

1976), one starting at nt 2,733 and the other at nt 2,748, followed by a short stretch of polyA beginning at nt 2,751.

Most of a second cyclin B cDNA clone (λ JW101), which extends from nt 378 through the polyA tail, was also sequenced; the individual nucleotide differences in it are indicated above the sequence of λ JW102 (Fig. 3). Within the presumptive coding regions, there is only one difference and it does not change the predicted amino acid sequence. The 3' end of λ JW101 is shorter than that of λ JW102 by 11 nt and contains a single polyA addition recognition sequence, whereas λ JW102 has two.

The Amino Acid Sequence of Cyclin B

When the deduced amino acid sequence of cyclin B was compared with other available sequences, including those present in the National Biomedical Research Foundation Protein Sequence Database, the only extensive similarities seen were with the related protein clam cyclin A (Swenson et al., 1986), sea urchin cyclin (Pines and Hunt, 1987), and yeast cyclin (Booher and Beach, 1988), as discussed later. While cyclin B contains short stretches of amino acids related to those found in some kinases (Fig. 4), the homologies are much

AAGAAAAACCCCAACTAGTCTAGTGAATATGTTGAAITGGCACCITTTGTTTACTGTTGTTTCAATGATATTTGTTGTTTGTGAATGATGACTACA 99
 M T T 3
 AGAGCCGGCTTCGGGAAATCTTGCTAATGCAAGAATGATGCATATATGGAGGAAGCTGGCCACATGGCTAACTCAAAGGTAGACACACACGCTCACAT 198
 R A A S A N L A N A R M M H N H E E A G H M A N L K G R P H T S H 36
 GCTCCACAGAAACCTTTGGGGATTTGGCAATCAAGTGGCCATCACAATATCAGATGTCACAGGAAAGGATCCCATCAAGAAAGAAATA 297
 A S Q R N T L G D I G N Q V S A I T I S D V P R K D P I I K K E I 69
 GTTCATTTATCTAGTCAACCAAGATACCAAGAGGCAAGCTACACATCAATAAAGTTTAGCAGAAAGGATCATATACCTAAGAAACAA 396
 V H L S S H Q H K I L T K S K A T T S L X S L A E E S H I P K K Q 102
 GAAGCATTACTTTTTTGAACCTGTTGCTGCCAAGCCAAACCAAGTGGCTACTGCAACAGTACTTCCACAACCAAGTCCCGGTTCTCTATG 495
 E A F T F L E P V A A M P K P T T V P T A T V L P Q P T V P V P M 135
 GATATAAGTGAATGTTCCAGAAATGTTCTCCAGAGTTTGGCTCAATGATGACAAAATATAGATGCAAAATGCAAAAGAAACCCCAACTAGTCAAGTAA 594
 D I S E N V P E S F S R V L L N V Q N I D A N D K E N P Q L V S E 168
 YATGCAATGACATCTAGTATACAGAGATTTAGAGGAAATATCCATACGACATAATATTTAGAAATCAAGAAATCAGAGAAAGATGGGT 693
 Y V N D I Y D Y M R D L E G K Y P I R H N Y L E N Q E I T G K M R 201
 GCAATTTAATAGATGGTATGTCAGGTCATCATGATTCACACTGTTTACAGAGACACTTTACCTCAGTACCCATTATAGACAGATTTGTTACAG 792
 A I L I D W L C Q V H R R F H L L Q E T T L Y L T V A I I D R L L Q 234
 GAAAGCCAGCTGCTAGAAATAACACTCAATAGTCCGAGTTACTTCTATGTTAATAGCTTCCAAATATGAAGAAATGATGACACCTGAAGTTGACAG 891
 E S P V P R N K L Q L V G V T S M L I A S K Y E E M Y A P E V A D 267
 TTTGTATATAACTGCAATGCTTATACGAAAAAAGAAATCTTAGAAATGGAACAACATATACTTAAAAAATAAACTTCAGTTTGGTCCGACACTT 990
 F V Y I T D N A Y T K K E I L E M E Q H I L K K L N F S F G R P L 300
 TGTCTGATTTCTTACGAGAGATTCAAAAGCTGGCCAGGTTGATGCCAACAACACAGTTAGCAAGTATCTTATGGAGCTGACTATCAGAGAT 1089
 C L H F L R R D S K A G Q V D A N K H T L A K Y L H E L T I T E Y 333
 GATATGTCATATCTACTTCAAAAATAGCTGCTGAGCCCTGTCTTCTTCAAACTTCTGTATAGTACTCATTGGACGGAGACATTAACACAC 1188
 D M V Q Y L P S K I A A A L C L S M K L L D S T H W T E T L T H 366
 TACAGTTCATATGTAAGAGGATTTGGTGTCAACATGCGAAGACTTCCAGTCTAGTCAATTAAGCAGAGAACGCAAACTCCTGCTTCATACA 1287
 Y S S Y C E K D L V S T M Q K L A S L V I K A E N S K L T A V H T 399
 AAGTACTCAAGCTTAAATTTAAGAGTCAAGTAAATAGCTGCCTTGAAATCTCCATTTGGTTAAAGAAATGGCAGCTGCTCAGTATGATCTGTGATCT 1387
 K Y S S S K F M K I S K L A A L K S P L V K E L A A A S V 428
 TGTAAATATAACACAAAGCTAACCGGGATATAGTCAAAATAGTCCGGATCTATTGTATGATGATAGGACTTGTCTCTCAITTCATCTGTTATTT 1487
 TATTAGTGTGGTTGTCGGAGTTTATTTATTTTGTGAATTTGATGACTAAGTATGGGGCAATATGAGTTATGATAGGTGGTGGATGTTCTTCTG 1587
 TATTGTAGTGAACAATAGTTGGAAGGTTGCAAGAAAAAACAATGCACTTGTGCAATTTATTTGATTTATATATATATAGTATGATCAACTCGTTGACAAA 1687
 ATCAAAAAAATGCAAAACTGATTTGGCAAGCATACATGTTGTTAAATGTTGTTTAACTTTTAACTTCAAAAAGTTTACCATAACCATGTTCC 1787
 GTCAAAATTTTAAAAATTTCCGACATAAATCATGTATGCTTGTGATGATCAAAAAGCAATCTATGCTGGTGGTTTAACTTATTTTAAAGATCT 1887
 CGAGTGTGCTGATTTGGGAATAGATGGGTGCTAGTGTCTTGTCTTCTAAAATTTGAGGGCTTAAATATATGATGATGATGATGATGATGATGATG 1987
 ATAGATTTGTTGTTGCTTTGTTGTCATTAACCTTTCCGAGAGAGACTTATGCAATCTTTGGCTTAACTAAAATATATAAAAAAATCAT 2087
 CATAGATTTATAGATATATTTTCACTATTTAGATTTAATAAAAAAACCACCTTGAACAACAAAGCCATAGGTTATTCATTTATTTGACATAAA 2187
 TGATAAATAACCATGAATGATCATTTATT GTATTTTTTTTAGGAATGTAAGCATATTTGCTTCTTTAAAACATGTAACCTTTATAACT 2287
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 TTGACCGAGCTGCAACTTTAGGATCTGCCAAAGTCTATGTTGTTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 2487
 AATATGAT 2587
 TCAATGACAGGCACTAAGAGTGGATTAATGCTTTTCACTGATAGATAGCATTTAGTGGTATAGTATGATGATGATGATGATGATGATGATGATGAT 2687
 ACTATTTTATGCTGTTGATACATAATTTTTTATACATAAATTTTGTATATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAAAATAAAAA 2772

Figure 3. The sequence of cyclin B cDNA. The complete sequence of λ JW102 is shown. Most of another cDNA, λ JW101 which starts at nt 378, was also sequenced; the single nucleotide differences, insertions, and deletions (–) are indicated above the λ JW102 sequence. None of these differences changed the predicted cyclin B amino acid sequence which is given in the single letter code. The first four start codons (ATG) at nt 29, 36, 88, and 91 and the two polyA addition sequences (AATAAA) beginning at nt 2,726 and 2,741 are underlined.

weaker than those between known kinases (for reviews see Hanks et al., 1988; Brenner, 1988).

Two potential phosphorylation sites were seen in cyclin B. At position 416 we found the sequence KSPxxK which resembles four histone H1 sites—KTPxK, ATPK, KSPK and KTPK—which are phosphorylated *in vivo* during mitosis and phosphorylated *in vitro* by partially purified growth-associated histone kinase (Langan et al., 1980; Langan, 1982). At position 306 we found the sequence RRxS, the consensus sequence for phosphorylation by cAMP-dependent protein kinase (for review see Edelman et al., 1987). These sites are possibly significant since (a) highly purified preparations of MPF and cdc2/cyclin complexes have considerable histone H1 kinase activity, as discussed below, and (b) injection of the R subunit of cAMP-dependent protein kinase can trigger meiotic maturation in frog oocytes (Maller and Krebs, 1977).

The cyclin B sequence contains two possible zinc-chelating sites: HLSSHQH (aa 71–77) and CQVHHRFH (aa 209–216). These resemble known zinc-chelating sites in which two ligands, most frequently histidine (H) and cysteine (C), are optimally separated by three amino acids, or one turn of an α helix (Sulkowski, 1985; Miller et al., 1985). As discussed below, there is some experimental evidence that these sites may be functional.

Cyclin B also contains a sequence, NIDANDKENPQLVSE (aa 154–168), which has nine potential calcium-chelating amino acids (glutamate [E], aspartate [D], glutamine [Q], asparagine [N], and serine [S]) interspersed in a way that they could provide the six ligands typical of calcium-binding regions (Tufty and Kretsinger, 1975; Vyas et al., 1987). This is potentially significant in light of numerous reports of intracellular calcium transients occurring at key points in the cell cycle such as sperm-egg contact, nuclear envelope breakdown, and the metaphase-anaphase transition (Izant, 1983; Poenie et al., 1985, 1986; Hepler, 1985; Twigg et al., 1988). However, this sequence is embedded in a longer polyanionic region (aa 136–195 = 22% aspartate and glutamate) that could easily have some other function.

Relationships among the Cyclins

Earlier work identified a single major cyclin in sea urchin embryos, referred to here as urchin cyclin 1, whereas there are two prominent cyclins in clam embryos; cyclins A and B (Rosenthal et al., 1980, 1983; Evans et al., 1983; Swenson et al., 1986; Pines and Hunt, 1987). The amino acid sequence of clam cyclin B is different from that of cyclin A, indicating clearly that the two clam cyclins are different pro-

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CYCLIN B----MTTRASANLANANRMMHNEEAGHMANLKRPHSTHASQRNTLGDIGNQVSAITI--SDVPRKDPKIIK
CYCLIN A-----MSQFALLHGDENQMRGKQNTSRNGLSGOKRAALGVI TNQVQVRIQPSRAAKPKSS
SU CYCLIN----MALGTNNMNLHGESKHTFNENVSARLGGKSLAVQKPAQRAALGNISNVVRTAQAQS---KRVVKKD
CYCLIN B----MTTRASANLANANRMMHNEEAGHMANLKRPHSTHASQRNTLGDIGNQVSAITI--SDVPRKDPKIIK

      80              100              120              140
KEIVLSSHQKILTKSKATTSLKSLAESHIPKQEAFTFLEPVAAMPKPTTVPATVLPQVTPV--PMDI--SENVPES
EFNIQDENAFKQKARTFQQQPSQSFVFDPTFAAFVQKAPTSVHTDIPAAITLQRV--PLTEVPGSPDITSLDSHES
TRQAMKTKKATSSLHVVGL-----PVEDLPTMSTSPDVL-----AMEVDQAEAFS
KEIVLSSHQKILTKSKATTSLKSLAESHIPKQEAFTFLEPVAAMPKPTTVPATVLPQVTPV--PMDI--SENVPES

      160              180              200              220
FSRVLLVQNTDANKENPQLVSEVNDIYDMRDELGKYPFRHNYLENQE--ITGKMRALLDNLCOVHRHFLLQETL
PMLLDLPEEEKFLDREAVILTVPEYEDLYNYLQAEKRNRAKPYMKRQTD--ITSMRCILVQWLVSEEDKLRHRETL
QQLIALQVDEIDIDGDNQFCLSEYADITLRLRLEVEEMAPNYLDRETDITGRMRLIADVMLVQVHLRFLLQETL
FSRVLLVQNTDANKENPQLVSEVNDIYDMRDELGKYPFRHNYLENQE--ITGKMRALLDNLCOVHRHFLLQETL

      240              260              280              300
YLTVAIDRLQFSPVPRNQLQVGVTSMLIASKYEEMVPEVADFVYITDNAVTKKEILEMEOHILKKNLSPGREFLCL
FLGVNYIDRFLSKISVLRGKLVGAASMFIAKYEEIYVPOVKEFAYITDQVYTSQVLRMELHLLKVLTFDVAPEVTN
FLTVQLIDRFLEHSHVSKGKLVGVGTAMFLASKYEEMVPEVADFVYITDNAVTKKEILEMEOHILKKNLSPGREFLCL
YLTVAIDRLQFSPVPRNQLQVGVTSMLIASKYEEMVPEVADFVYITDNAVTKKEILEMEOHILKKNLSPGREFLCL

      320              340              360
HFLRRDSKAGQVDANKHTLAKYIMELTITEYDMWQ--YLEPSIAAALCLSNKLLDS--TH--WTETLTHYSYCEKDLVST
WFCEDFLKSQDAADKLSLTMPLTELTLIDMDAYKLEPSTAAALCLLARYSLGIEP---WPNQLVKKTYGIEGHFVDC
HFLRRDSKAGQVDANKHTLAKYIMELTITEYDMWQ--YLEPSIAAALCLSNKLLDS--TH--WTETLTHYSYCEKDLVST
HFLRRDSKAGQVDANKHTLAKYIMELTITEYDMWQ--YLEPSIAAALCLSNKLLDS--TH--WTETLTHYSYCEKDLVST

      381              400              420
MOKLASLVKAE----NSKLTAVHTKYSSKFMKSKLAALKSPVVKELAAASV
LKDLKHTSLGAE----SHQQQAVQEKYKQKRYHQVSDFSKMFVPHNLALLAL
VQR--IVQILLRDDSASQKSAVRYKYGSSKFMKSKLAALKSPVVKELAAASV
MOKLASLVKAE----NSKLTAVHTKYSSKFMKSKLAALKSPVVKELAAASV

Protein Kinase Consensus Sequences XXXXX
Growth-Associated Histone Kinase-Like Phosphorylation Site XXXXX
cAMP-Dependent Protein Kinase phosphorylation Site XXXXX
Zinc-Chelating Sites XXXXX

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Figure 4. Comparisons of the amino acid sequences of cyclin B, cyclin A, and sea urchin cyclin 1. The deduced amino acid sequences (single letter code) for cyclin B, cyclin A (Swenson et al., 1986), and urchin cyclin 1 (Pines and Hunt, 1987) were aligned by eye to emphasize the common features.

teins. There is, however, considerable (31%) overall identity between the two clam cyclin proteins (Fig. 4).

Both urchin cyclin 1 (Fig. 4) and the fission yeast cyclin (not shown) encoded by *cdc13* (Booher and Beach, 1988; Solomon et al., 1988; Goebel and Byers, 1988) are more like cyclin B than cyclin A. These identities are most concentrated in the middle regions of the proteins. For example, the sequence spanning aa 166–294 in cyclin B shows 50% identity with the corresponding region of cyclin A and 65% identity with that of urchin cyclin 1. It is also this region that is most similar to the yeast cyclin. We note that urchin cyclin 1, like clam cyclin B, contains two potential zinc binding sites, one near the NH₂ terminus and the other aligned with one of the two cyclin B sites (at position 209–218). Both urchin and yeast cyclin contain a cAMP-dependent protein kinase phosphorylation site aligned with the site in cyclin B. Neither yeast *cdc13* cyclin, urchin cyclin 1, nor clam cyclin A show any regions resembling kinase consensus sequences.

Cyclin B Is an M Phase Inducer

To ask if cyclin B can act as a general inducer of M phase, we introduced cyclin B into frog oocytes, which are naturally

arrested at the G2/M border of meiosis I. Pure cyclin B mRNA was transcribed in vitro from plasmid pCD102 and injected into *Xenopus* oocytes. It translated to give cyclin B protein and the recipient oocytes entered meiosis (Fig. 5). Various control RNAs (globin, gap junction protein, and cyclin antisense) not did induce meiosis. Thus cyclin B, like cyclin A, can induce entry into M phase and the resumption of meiosis.

Most interestingly, oocytes entered meiosis more rapidly in response to a mix of the two cyclin mRNAs than to each mRNA alone (Fig. 5). Oocytes injected with a combination of 0.05 ng/nl cyclin A plus B mRNAs entered meiosis considerably ahead of those injected with either 1 ng/nl of cyclin A or B mRNA alone. This result argues that there is a strong degree of cooperativity between cyclin A and cyclin B.

Cyclin B Is Sequestered in Oocytes and Released after Fertilization

Despite possessing a full complement of the M phase inducer cyclin B, clam oocytes remain arrested at the G2/M border of meiosis I indefinitely unless they are fertilized or activated by artificial agents such as calcium ionophore or KCl. This suggests that the oocyte stockpile of cyclin B might be masked in some way, making it unavailable until fertilization leads to some change in its status. We tested this idea using two different approaches. Oocytes, or embryos taken shortly after KCl activation, were lysed in a solution buffered at pH 6.8, the prefertilization pH (Finkel and Wolf, 1978), and separated by centrifugation into four fractions: a 1,000-g pellet, a 13,000-g pellet, a 124,000-g pellet, and a 124,000-g supernatant. Aliquots of each fraction were separated by gel electrophoresis, blotted, and reacted with cyclin B antibodies (Fig. 6 A). More than half of cyclin B in oocytes was found in the 1,000-g pellet. By 7 min after activation much of this had been released from the 1,000-g pellet and was recovered primarily in the 124,000-g supernatant. (For clarity of presentation, the small changes in the other two fractions are not shown.) This shift was even more marked by 20 min. Preliminary attempts to visualize this redistribution by immunofluorescence confirmed that much of the maternal stockpile of cyclin B protein is sequestered in the oocyte and released after fertilization (Fig. 6 C). In unfertilized oocytes, cyclin B appeared as discrete cytoplasmic aggregates. By 6 min after fertilization, most of these aggregates had dispersed. Interestingly, after fertilization a small amount of localized staining at the nucleolus was seen.

Earlier work from this lab has shown that clam oocytes lack a pool of cyclin A protein, cyclin A begins to be synthesized within minutes of fertilization, and cyclin A is an excellent inducer of M phase in frog oocytes (Swenson et al., 1986). However, cyclin A itself cannot be the natural inducer of meiosis I in clams since blocking the appearance of cyclin A and all other newly synthesized proteins with emetine does not block meiosis I (Hunt, T., and J. V. Ruderman, manuscript in preparation). A resolution of this puzzle is now provided by our findings that (a) oocytes contain a pool of cyclin B protein, (b) cyclin B has M phase-inducing activity, and (c) after fertilization, sequestered cyclin B appears to be released to a more soluble and presumably more available form. Thus cyclin B has all of the properties required of the M phase inducer used for meiosis I.

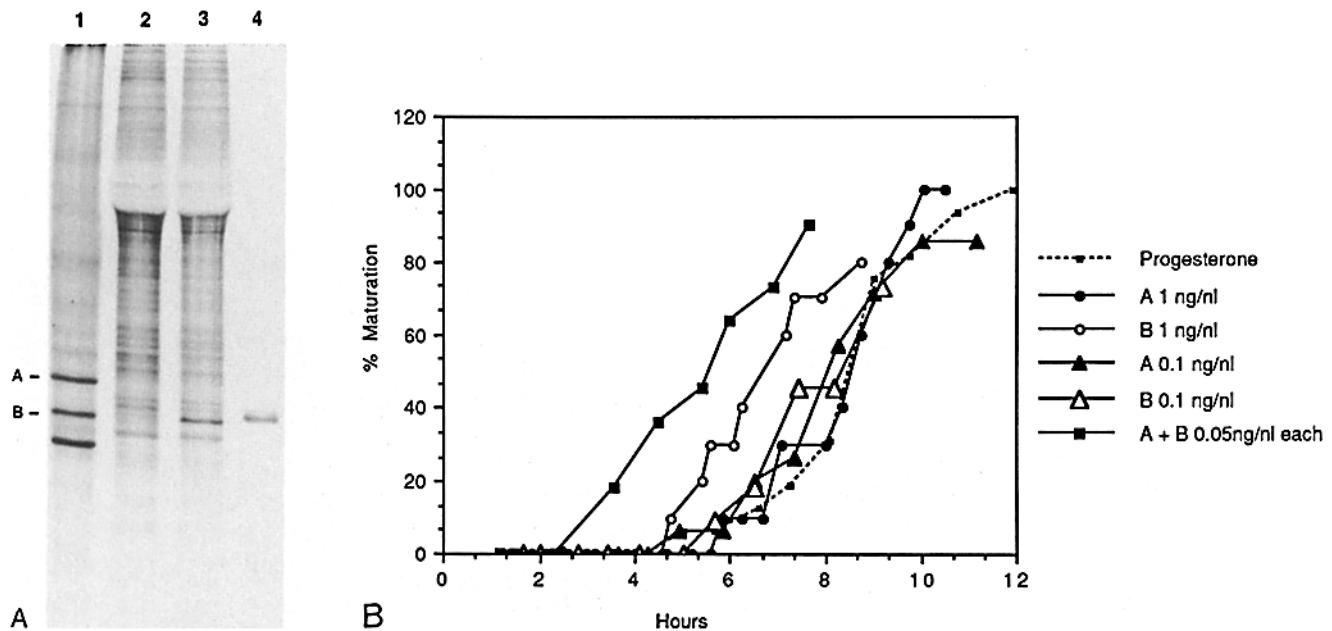


Figure 5. The response of *Xenopus* oocytes to injected cyclin A and cyclin B mRNAs. (A) Frog oocytes were injected with 1 μ Ci [35 S]methionine dissolved in either 40 nl water or 40 nl cyclin B mRNA and then incubated for 6 h at 18°C. Cyclin B antibodies were used to prepare an immune precipitate from one of the oocytes injected with cyclin B mRNA. The autoradiogram shows the [35 S]methionine-labeled proteins synthesized by (lane 1) clam embryos in first mitosis; (lane 2) frog oocytes injected with water; (lane 3) frog oocytes injected with cyclin B mRNA; and (lane 4) frog oocytes injected with cyclin B mRNA, anti-cyclin B immune precipitate. Control immune precipitations carried out using oocytes injected with water did not contain any radioactive proteins (not shown). The slight difference in the electrophoretic mobilities of cyclin B in total cell cytoplasm (lane 3) and the immune precipitate (lane 4) appears to be an artifact of the large difference in the total protein content of the two samples (not shown). (B) Frog oocytes were injected with 40 nl of cyclin A, cyclin B, or cyclin A and B mRNA at the concentrations indicated. Entry into meiosis (% maturation) was scored by the appearance of the "maturation white spot" at the pigmented animal pole cortex.

In many species, fertilization triggers a rapid series of ionic changes that result in a significant rise in the intracellular pH. In clams, the internal pH rises from 6.8 to 7.2 (Finkel and Wolf, 1978). When oocytes were lysed and fractionated at the prefertilization pH of 6.8, more than half of the cyclin B was found in the 1,000-g pellet. When oocytes were lysed at the postfertilization pH of 7.2, much of the cyclin B was lost from the 1,000-g pellet and recovered in more soluble fractions (Fig. 6 B). This result suggests that the postfertilization change in intracellular pH contributes to the release of cyclin B from a sequestered to a more soluble, possibly more available form, and that this unmasking of cyclin B drives the cells into meiosis I.

Immunoprecipitates of Cyclin B Have Protein Kinase Activity

We have recently shown that clam oocytes contain a pool of a 33-kD protein homologous to the *cdc2* protein kinase (Draetta et al., 1989) that is a component of MPF and a key regulator of mitosis in organisms ranging from yeast to humans (Lee and Nurse, 1987; Gautier et al., 1988; Dunphy et al., 1988; Draetta and Beach, 1988). After fertilization of clam oocytes, newly synthesized cyclins join the maternally inherited *cdc2* protein kinase to form complexes that can be precipitated by *cdc2* antibodies or by Sepharose beads cross-linked to the fission yeast 13-kD protein *sucl* (Draetta et al.,

1989), another protein that interacts with the *cdc2* protein kinase (Hindley et al., 1986; Brizuela et al., 1987) and MPF (Dunphy et al., 1988). To investigate some of the properties of the cyclin B/*cdc2* complex, we prepared immune precipitates containing cyclin B, took them through an *in vitro* kinase assay, and examined the phosphorylated reaction products. This approach is essentially identical to that used to demonstrate, in yeast cell lysates, that the CDC28 kinase is complexed with a 40-kD substrate protein (Reed et al., 1985; Mendenhall et al., 1987). Embryos were labeled with [35 S]methionine during the first mitotic cell cycle, an M phase lysate was prepared (Fig. 7, lane a), and cyclin B immune precipitate complexes were recovered. Cyclin B was the only prominent 35 S-labeled protein present in the immune complex (Fig. 7, lane c), even on very long exposures (not shown). Since there is little or no synthesis of the *cdc2* protein kinase in the early clam embryo (Draetta et al., 1989), the absence of 35 S incorporation in the 33-kD region of the gel is to be expected. When the immune complex was subsequently incubated in kinase reaction buffer with [γ - 32 P]ATP and the phosphorylated reaction products were analyzed by gel electrophoresis, several 32 P labeled endogenous reaction products were detected. These included proteins migrating at 78, 56, and 45 kD, a doublet at 33 kD, and some heavily labeled material near the top of the resolving gel.

Two controls tested the possibility that the observed phos-

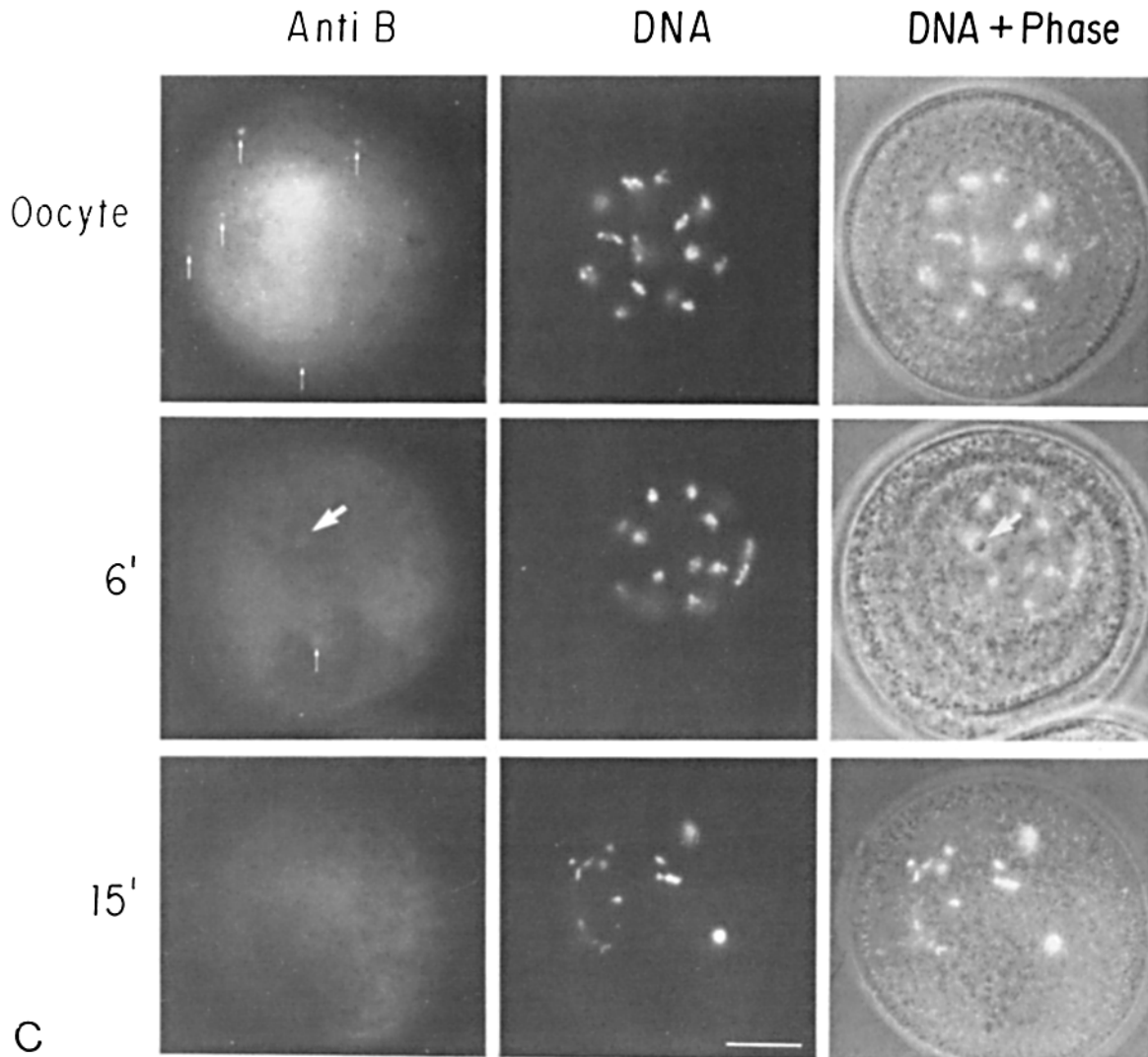
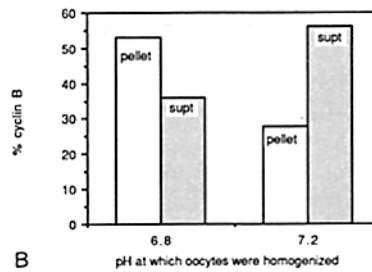
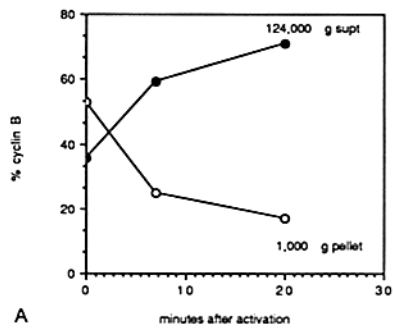


Figure 6. Fertilization is accompanied by a redistribution of cyclin B. (A) Clam oocytes or embryos taken at 7 and 20 min postfertilization were homogenized in buffer T, pH 6.8, and fractionated into four compartments by centrifugation as described in the text. Each fraction was electrophoresed onto a polyacrylamide gel, blotted, and reacted with cyclin B antibodies. The amount of cyclin B in each fraction was quantitated by scanning autoradiograms of the immunoblot. Only the values for the 1,000-g pellet and the 124,000-g supernatant showed significant changes. (B) Oocytes or embryos were homogenized in buffer T, pH 6.8 or 7.2, and fractionated as above. *pellet* indicates the 1,000-g pellet; *supt* indicates the 124,000-g supernatant. (C) Oocytes or embryos taken at 6 and 15 min postfertilization were fixed, permeabilized, reacted with cyclin B antibodies, and stained with fluorescein-conjugated second antibody. Chromosomes were visualized by vital staining with the DNA-binding dye Hoechst 33342. Small arrows mark some of the aggregates of cyclin B; large arrows mark the nucleolus. Bar, 10 μ m.

phorylation could be due to a nonspecifically precipitating kinase. First, kinase reactions were carried out using precipitates made with preimmune serum. None of the phosphoproteins were seen when preimmune serum was used (Fig. 7, lane *d*). Second, immune precipitates with anti-cyclin B antibodies were made with embryos lysates lacking cyclin B. To make these lysates, we took advantage of earlier work showing that the addition of emetine to embryos in M phase blocks the synthesis of new cyclin proteins but does not interfere with the destruction of the cyclins (Evans et al., 1983; Hunt, T., and J. V. Ruderman, manuscript in preparation). Immune complexes made from lysates lacking cyclin B had no detectable kinase activity (Fig. 7, lane *k*).

These results indicate that newly synthesized cyclin B is closely associated with a small number of other proteins which coprecipitate with cyclin B by virtue of this association, one or more of which has protein kinase activity. Because these other proteins are not among those labeled with [³⁵S]methionine, it seems likely that they represent preexisting proteins made during oogenesis. It is, of course, also possible that some or all of those proteins are deficient in methionine or present in substoichiometric amounts. While we strongly suspect that the 33-kD phosphoprotein that coprecipitates with cyclin B represents the maternal cdc2 protein kinase (see below), this particular experiment does not establish this point directly.

To test if the 56-kD phosphoprotein was cyclin B, the reaction products of the *in vitro* kinase assay were reduced, denatured, diluted, and taken through a second round of immunoprecipitation with anti-cyclin B antibody. Cyclin B antibodies (Fig. 7, lane *h*), but not preimmune serum or cyclin A antibodies (not shown), precipitated a phosphoprotein that comigrated with cyclin B. This result establishes one of the *in vitro* phosphoprotein products as cyclin B itself and suggests that cyclin B can be phosphorylated *in vivo* by cdc2.

We also tested the ability of cyclin B immune precipitates to phosphorylate exogenous substrates. Of those tested (individual histone fractions, casein, and protamine sulfate) histone H1 was by far the best substrate (data not shown).

The identification of potential zinc binding sites in cyclin B and the enhancement by zinc of the kinase activities of two other cell cycle regulators, the CDC28 kinase of budding yeast (Reed et al., 1985; Mendenhall et al., 1987) and cyclin A (Swenson, K. I., and J. V. Ruderman, manuscript submitted for publication), prompted us to test the effect of 1 mM zinc on cyclin B kinase activity *in vitro*. In the presence of zinc, phosphorylation of the 33-kD protein was depressed and that of the 45-kD substrate enhanced (Fig. 8, lane *d*). This result suggests that the zinc binding sites of cyclin B are functional and can influence the activity of the complex, at least *in vitro*.

Discussion

The work presented here shows that there are two different and distinctive cyclins, A and B. The two are distinguished by their amino acid sequences, their differential expression in full-grown oocytes, and their behavior during meiosis. Despite these differences, however, cyclins A and B share the important ability to act as general, nonspecies-specific inducers of M phase. This is judged by the revealing, although relatively crude, bioassay of injecting cyclin mRNAs into

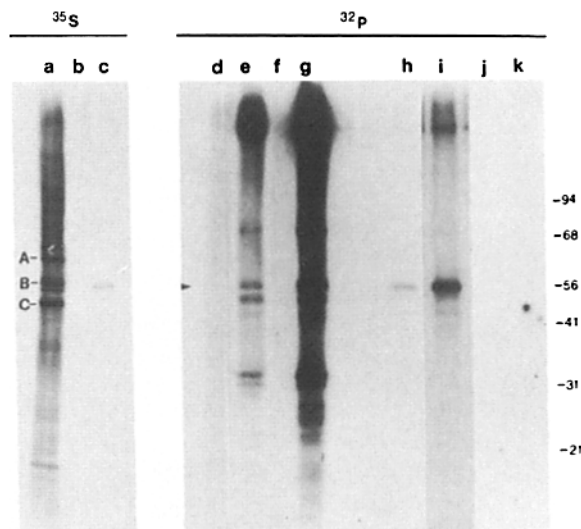


Figure 7. Immune precipitate complexes containing cyclin B have kinase activity. Clam oocytes were activated and labeled with [³⁵S]methionine during the first mitotic cell cycle. Cells in M phase were lysed in buffer containing 0.2% Tween and a 13,000-g supernatant was prepared. Affinity-purified anti-cyclin B antibody was added to aliquots of the lysate, and immune precipitates were prepared. An autoradiogram of the reaction products is shown. The exposure time for all lanes was 2 h, except lane *i* which was 16 h. (Lanes *a-c*) [³⁵S]Methionine label. (Lane *a*) [³⁵S]Methionine-labeled proteins in the lysate; (lane *b*) immune precipitate using preimmune antibodies; and (lane *c*) immune precipitate using anti-cyclin B antibodies. (Lanes *d-k*) Immune precipitate complexes were subsequently incubated with [³²P]ATP to assay for endogenous protein kinase activity. (Lane *d*) Immune precipitate using preimmune serum; (lane *e*) immune precipitate using anti-cyclin B antibodies; (lane *f*) immune precipitate using preimmune serum, treated with phosphatase before kinase assay; (lane *g*) immune precipitate using anti-cyclin B antibodies treated with phosphatase before kinase assay; and (lane *h*) immune precipitate using anti-cyclin B antibodies was treated with phosphatase and incubated with [³²P]ATP. The ³²P-labeled reaction products were denatured by boiling in SDS gel sample buffer containing β -mercaptoethanol and diluted with immunoprecipitation buffer. The solution was cleared of dissociated antibodies by reacting with protein A-Sepharose, and fresh anti-cyclin B antibodies were added to reprecipitate denatured cyclin B. The efficiency of reprecipitation is low; parallel samples taken through the kinase assay reveal that essentially all of the radioactive signal seen in lane *h* is due to ³²P (not shown); (lane *i*) same as lane *h*, 16-h exposure; (lanes *j* and *k*) embryos in first mitosis were treated with emetine to block protein synthesis. The embryos subsequently arrested in interphase with no detectable cyclin A or B (not shown). A lysate was prepared and tested for *in vitro* kinase activity as above. (Lane *j*) immune precipitate using preimmune serum; and (lane *k*) immune precipitate using anti-cyclin B antibodies.

frog oocytes and observing that they can drive the recipients into meiosis.

The striking differences in the expression of cyclins A and B during oogenesis and meiosis I suggest that the two cyclins have different functions but, under certain circumstances, can substitute for each other. This idea is further supported by the finding that introducing cyclins A and B individually into frog oocytes is far less effective than introducing the two cyclins together. The frog oocyte injection assay, while help-

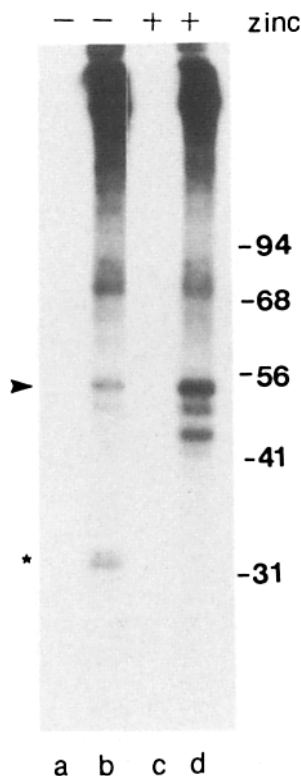


Figure 8. The effect of 1 mM zinc on cyclin B-associated kinase activity. Immune precipitate complexes containing cyclin B were prepared from lysates of fertilized embryos that had been cultured to mid-M phase of first mitosis. In vitro kinase assays were carried out as described in Fig. 7 in the presence or absence of 1 mM $ZnCl_2$. The ^{32}P -labeled reaction products were analyzed by SDS-PAGE followed by autoradiography. (Lane a) Preimmune serum; (lane b) anti-cyclin B; (lane c) preimmune serum, plus zinc; and (lane d) anti-cyclin B, plus zinc.

ful in the original identification of the abilities of the cyclins to induce M phase, is not well suited for certain approaches that might tell us more about functional differences between the cyclins. Needed next are investigations using cell-free systems (Lohka and Masui, 1984; Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Newport and Spann, 1988) that allow detailed biochemical and morphological analyses of the multiple events that occur when cells enter meiosis and mitosis.

What are the roles of the two cyclins during the normal meiotic and mitotic cell cycles of naturally developing clam embryos? Oocytes contain abundant amounts of cyclin A and B mRNAs, both of which are completely inactive in the full-grown oocyte and are loaded onto polysomes soon after fertilization (Rosenthal et al., 1980, 1983; Swenson et al., 1986). Oocytes lack any detectable cyclin A protein, but do contain cyclin B protein which must have been made earlier in oogenesis. Much of the maternal stockpile of cyclin B protein appears to be sequestered as large cytoplasmic aggregates in oocytes. Within minutes of fertilization it is released to a more disperse, soluble form, possibly in response to the postfertilization increase in cytoplasmic pH. We presume it is this unmasking of maternal cyclin B protein that drives cells into meiosis I, rather than unmasking of maternal cyclin mRNAs, since when the postfertilization appearance of new cyclin is blocked by emetine, the cells can still proceed through meiosis I with completely normal kinetics and morphology (Luca et al., 1987).

There is now considerable experimental support for the idea that cyclins join with other components to form active MPF. First, there is strong genetic evidence for a requisite interaction between cyclin and *cdc2* for entry into M phase

in yeast (Booher and Beach, 1987, 1988; Reed et al., 1988). Second, the biochemical experiments of Gautier et al. (1988) and Dunphy et al. (1988) show that the frog oocyte *cdc2* homolog is a component of active MPF. Finally, we have shown that clam oocytes contain a pool of *cdc2* protein kinase and, after fertilization, *cdc2* is joined by newly synthesized cyclins A and B to form *cdc2*/cyclin A and *cdc2*/cyclin B complexes ~220 kD in size (Draetta et al., 1989).

A close association between cyclins, *cdc2*, and MPF is further reflected in the observations that purified MPF preparations (Lohka et al., 1988), *cdc2*-containing complexes (Gautier et al., 1988), and cyclin B-containing complexes (this paper; Draetta et al., 1989) all show high levels of histone H1 kinase activity in vitro. Mitosis-specific histone kinase appears to exist in two interconvertible forms (Mitchelson et al., 1978; Zeilig and Langan, 1980; Meijer et al., 1987), and conversion to the active form probably requires phosphorylation (Pelech et al., 1987). Starfish oocytes store inactive histone kinase; during development histone kinase activity rises and falls across the meiotic and mitotic cycles with kinetics remarkably similar to those of MPF and the cyclins (Meijer et al., 1987; Meijer and Pondaven, 1988). Also, in starfish and sea urchins, MPF and histone kinase activities show identical requirements for new protein synthesis, namely that activation in meiosis I does not depend on new protein synthesis, while activation in meiosis II and mitosis does. An additional linkage between the cyclins, MPF and histone kinase is suggested by the observations that both histone kinase (Pelech et al., 1987) and cyclin B-associated kinase activities (this paper) are affected by the presence of zinc. Furthermore, during extended times in metaphase arrest, MPF activity (for review see Masui and Shibuya, 1987), histone kinase activity (Lake and Salzman, 1972; Meijer and Pondaven, 1988), and cyclin B levels (Hunt, T., and J. V. Ruderman, manuscript in preparation) stay high. Significantly, mitosis-specific histone kinase that has been purified on the basis of its ability to use histone H1 as a preferred substrate contains *cdc2* and exhibits considerable MPF activity (Bradbury, 1974; Inglis et al., 1976; Labbé et al., 1988; Arion et al., 1988).

Taken together, these results suggest that (a) the sequestered form of the maternal cyclin B protein stored in oocytes cannot interact, or cannot interact properly, with the *cdc2* protein kinase that is also stored in oocytes; (b) fertilization leads to the unmasking and subsequent availability of cyclin B; and (c) the recruitment of cyclin B protein into complexes with the *cdc2* protein kinase is an essential step in generating active MPF for the onset of meiotic M phase. The proteolytic destruction of the cyclins at the metaphase-anaphase transition would inactivate MPF by removing an essential component. Newly synthesized cyclins would then be required to generate active MPF in the next cell cycle and in each subsequent one. This idea is summarized by the model presented in Fig. 9.

This simple idea does not, however, account for the apparently conflicting observations that the cyclins are synthesized and accumulated steadily across interphase of each cell cycle, yet MPF activity appears abruptly and only at the end of interphase. This apparent discrepancy could be resolved if newly synthesized cyclins are masked during interphase of each mitotic cycle as well. Alternatively, the cyclins could be activated late in interphase by a posttranslational modification.

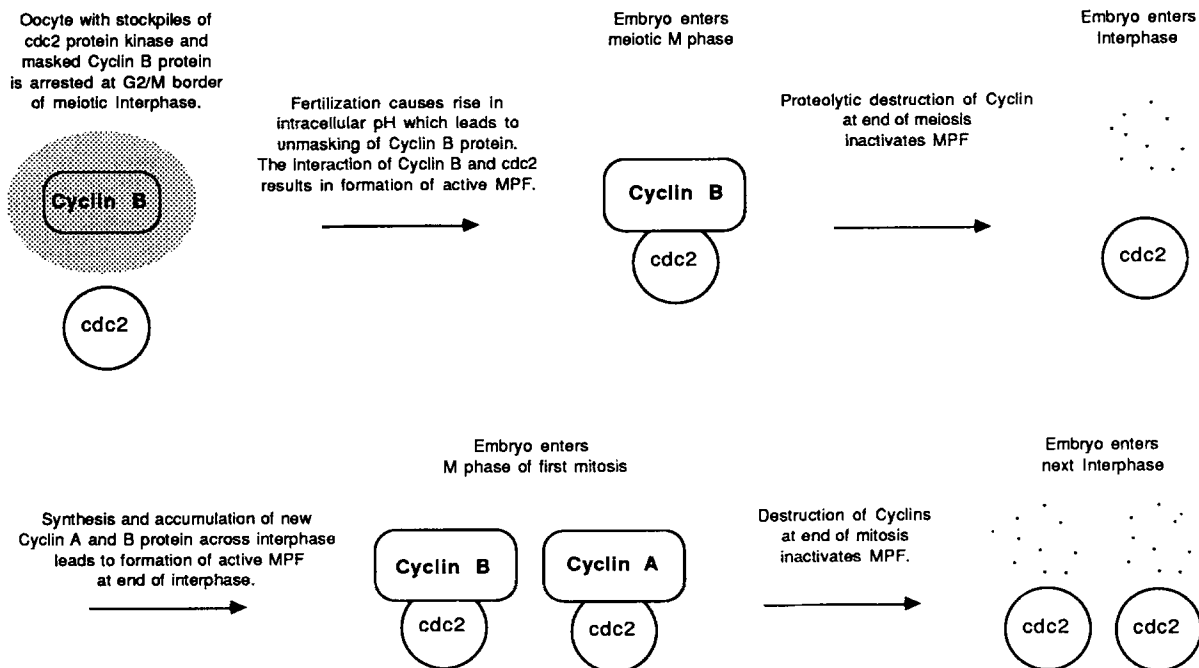


Figure 9. The model outlines the proposed unmasking of maternal cyclin B protein, its subsequent interaction with cdc2 protein kinase which generates active MPF, and the destruction of cyclin at the end of each meiotic and mitotic division (marked by the metaphase-anaphase transition) which results in the loss of MPF activity. Two other components thought to interact with the complex, namely the 13-kD *sucl* gene product (Hindley et al., 1987; Brizuela et al., 1987; Dunphy et al., 1988) and a 45-kD phosphoprotein that copurifies with active MPF (Lohka et al., 1988), are not shown.

We strongly suspect that frog oocytes, like those of clams, contain a stockpile of cyclin B protein that is prevented from interacting productively with the oocyte cdc2 kinase until an appropriate signal leads to changes in the cytoplasmic milieu. This idea is supported by the finding that frog oocytes have stores of latent MPF and latent histone H1 kinase which, under certain circumstances, can be converted to their active forms *in vitro* in the absence of protein synthesis (Cyert and Kirschner, 1988; Labbé et al., 1988). While protein synthesis is normally required for hormonally stimulated frog oocytes to proceed through meiosis I, this could be explained by the need for a newly synthesized noncyclin protein that somehow unmask the stored cyclin or promotes interaction between cyclin and cdc2. One good candidate is the kinase *c-mos*, whose synthesis after hormone stimulation is essential for meiotic maturation in frogs (Sagata et al., 1988). This step could be bypassed when clam or urchin cyclin mRNAs are microinjected into the frog oocyte if they direct the synthesis of cyclin proteins that are not properly masked, either because the frog's masking system is saturated by the production of excess cyclins or does not efficiently deal with foreign cyclins.

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