The Role of Cyclin B in Meiosis I

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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 GO 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), 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
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 promi-
nently 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 (Boober and Beach, 1987, 1988; Solomon et al., 1988; Goebl and Byers, 1988; Reed et al., 1988). Unfortunately, a direct immunological 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; Pines and Hunt, 1987; Standart et al., 1987). These considerations suggest the existence of a related protein, possibly cyclin B, that is stored in 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; Pines and Hunt, 1987; Standart et al., 1987). These considerations suggest the existence of a related protein, possibly cyclin B, that is stored in meiosis I.

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Hybrid Select Translation

DNA from potential cyclin B cDNA-containing plaques was isolated (Davis et al., 1989; Helms et al., 1985) and bound to nitrocellulose filters (Kafatos et al., 1973). DNA from potential cyclin B cDNA-containing plaques was isolated (Davis et al., 1989; Helms et al., 1985) and bound to nitrocellulose filters (Kafatos et al., 1973). DNA from potential cyclin B cDNA-containing plaques was isolated (Davis et al., 1989; Helms et al., 1985) and bound to nitrocellulose filters (Kafatos et al., 1973).
hybridization was carried out for 2 h at 37°C. hybridization was carried out with 12 μg poly[A] 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, JW101 (2,383-bp insert) and JW102 (2,773-bp insert), were subcloned into M13mp8 or Bluescribe M13- (Shuttle) 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 deletions subclones, were cloned into PATH (Koerner, T. J., personal communication) or Bluescribe vectors. Two other cDNAs, JW103 (=1,500-bp insert) and JW104 (=2,300-bp insert), were subcloned into Bluescribe M13- to confirm 5′ cDNA terminal sequences. 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 JW101 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 pW401 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 at 30°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 (Pel-Freeze Biologicals), 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-Freeze Biologicals), 1 mM MgCl2, and 191.5 mM KCl, 116 mM Tris base, pH 7.0 at time of use). Cells were fixed with 0.1 μCi/ml 125I-labeled donkey anti-rabbit antibodies (Amersham Corp.) and incubating the suspension at room temperature for 30 min. Reaction products were washed twice with cold kinase buffer, T 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 (2 μg preimmune antibodies 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 SP6(+) (Krieg and Melton, 1984).

**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 I, and mitosis II. 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 cell equivalents) were run on a 10% 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 (Chemicon Laboratories, Rogers, AR), and 0.1% Tween-20 followed by 0.1 μg/ml 125I-labeled donkey anti-rabbit antibodies (Amersham Corp., Arlington Heights, ILs).

**Cell Fractionation**

100-μl 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 taursine, 40 mM NaCl, 10 mM EGTA, 2.5 mM MgCl2, 0.1 M Heps) at pH 6.8 or 7.2, and resuspended in the appropriate buffer. Cells were lysed with 0.25 M sucrose. A few 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-dimethylaminopropyl]carbodiimide [Sigma Chemical Co.], 0.875 ml H2O, 30 ml 25 mM NaHPO4, 20 mM Na2HPO4, 2 mM MgCl2, 10 mM EGTA, 191.5 mM KCl, 116 mM Tris base, pH 7.0 at time of use). Cells were fixed with 0.1 μg/ml Hoechst 33342. 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.
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 immunomic (0.25% NaDeoxycholate, 0.5% NP-40, 0.5% SDS, and 1 x 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 x immunomic, 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 phosphatase 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 λgt10 was constructed as described in Materials and Methods. Replica filters of the library were screened with probe 1 [32P-cDNAs made against the original template RNA, which contained high levels of cyclin B mRNA] or probe 2 (a mix of 32P-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 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 ~50%. At the end of meiosis II, cyclin B levels drop by ~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 λJW101 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 125I-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 min), the chromosomes remain condensed and proceed directly through meiosis II, exiting into the interphase of the first mitotic cell cycle at 48 min. 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 (λJW102) 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 (λJW101), which extends from nt 378 through the polyA tail, was also sequenced; the individual nucleotide differences in it are indicated above the sequence of λJW102 (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 λJW101 is shorter than that of λJW102 by 11 nt and contains a single polyA addition recognition sequence, whereas λJW102 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...
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 HI sites-KTPxK, ATPK, KSPK and KTPK—which are phosphorylated in vitro during mitosis and phosphorylated in vitro by partially purified growth-associ-ated 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 (Hanks et al., 1988; Brenner, 1988).

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 could provide the six ligands typical of calcium-binding regions (Tufty and Kretsinger, 1975; Vyas et al., 1987). This sequence is potentially significant in light of numerous reports of intra-cellular calcium transients occurring at key points in the cell cycle such as sperm–egg contact, nuclear envelope break-down, 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., 1985, 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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Figure 4. Comparisons of the amino acid sequences of cyclin B, cyclin A, and sea urchin cyclin I. The deduced amino acid sequences (single letter code) for cyclin B, cyclin A (Swenson et al., 1986), and sea urchin cyclin I (Pines and Hunt, 1987) were aligned by eye to emphasize the common features.

Both urchin cyclin 1 (Fig. 4) and the fission yeast cyclin (not shown) encoded by cdcl3 (Booher and Beach, 1988; Solomon et al., 1988; Goebl and Byers, 1988) are more like cyclin B than cyclin A. These identities are most concen-
tive with the corresponding region of cyclin A and 65% identity with the cyclin A site (at position 209-218). Both ur-
chin cyclin 1, like cyclin B, contains two potential zinc binding sites, one near the 
H2o terminus and the other aligned with one of the two cyclin B sites (at position 209-218). Both ur-
chin and yeast cyclin contain a cAMP-dependent protein ki-
nase phosphorylation site aligned with the site in cyclin B. Neither yeast cdcl3 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).

Most interestingly, oocytes entered meiosis more rapidly in response to the mixture of the two cyclin mRNAs than to each mRNA alone (Fig. 5). Oocytes injected with a combination of 0.05 ng/mL cyclin A plus B mRNAs entered meiosis considerably ahead of those injected with either 1 ng/mL 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 12,000-g pellet, a 124,000-g supernatant, and 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 fertilization, most of these aggregates had dispersed. Interestingly, after fertilization a small amount of localized staining at the nuclei was seen.

Earlier work from this lab has shown that clam oocytes lack a pool of cyclin A protein, cyclin A begins to be synthe-
sized within minutes of fertilization, and cyclin A is an ex-
cellent 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 the 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 μCi [35S]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 [35S]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 thecdc2 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 [35S]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 35S-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 35S 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-32P]ATP and the phosphorylated reaction products were analyzed by gel electrophoresis, several 32P-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; suprt 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 μm.
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 these 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 (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 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-
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.
Oocyte with stockpiles of cyclin B protein across interphase. Cyclin B synthesis leads to formation of active MPF at end of interphase.

Embryo enters meiotic M phase

Proteolytic destruction of Cyclin B at end of meiosis inactivates MPF.

Embryo enters Interphase

Cyclin A and B protein across interphase leads to formation of active MPF at end of interphase.

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 protein 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 (Cyect 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.

We thank Cord Dorhmann and Michael Greene for their technical assistance in some of these experiments and Stephen Johnston for his help with sequencing primers.

This work was supported by National Institutes of Health grant HD23696 to J. V. Ruderman.

Received for publication 30 November 1988 and in revised form 11 January 1989.

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 (Lohtia et al., 1988), are not shown.

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