

On the Synthesis and Destruction of A- and B-type Cyclins during Oogenesis and Meiotic Maturation in *Xenopus laevis*

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Abstract. We have measured the levels of cyclin mRNAs and polypeptides during oogenesis, progesterone-induced oocyte maturation, and immediately after egg activation in the frog, *Xenopus laevis*. The mRNA for each cyclin is present at a constant level of $\sim 5 \times 10^7$ molecules per oocyte from the earliest stages of oogenesis until after fertilization. The levels of polypeptides show more complex patterns of accumulation. The B-type cyclins are first detectable in stage IV and V oocytes. Cyclin B2 polypeptide is present at $\sim 2 \times 10^9$ molecules (150 pg) per oocyte by stage VI. The amount increases after progesterone treatment, but returns to its previous level after GVBD and undergoes no further change until it is destroyed at fertilization. Cyclin B1 is present at 4×10^8 molecules per oocyte in stage VI oocytes,

and rises steadily during maturation, ultimately reaching similar levels to cyclin B2 in unfertilized eggs. Unlike the B-type cyclins, cyclin A is barely detectable in stage VI oocytes, and only starts to be made in significant amounts after oocytes are exposed to progesterone. A portion of all the cyclins are destroyed after germinal vesicle breakdown (GVBD), and cyclins B1 and B2 also experience posttranslational modifications during oocyte maturation. Progesterone strongly stimulates both cyclin and p34^{cdc2} synthesis in these oocytes, but whereas cyclin synthesis continues in eggs and after fertilization, synthesis of p34^{cdc2} declines strongly after GVBD. The significance of these results is discussed in terms of the activation and inactivation of maturation-promoting factor.

UNFERTILIZED amphibian eggs are stably arrested in metaphase of meiosis II and contain an activity called maturation-promoting factor (MPF),¹ which is the cytoplasmic agent responsible for promoting the G2→M transition of the eukaryotic cell cycle (Maller, 1990; Masui and Markert, 1971; Newport and Kirschner, 1984; Nurse, 1990; Smith and Ecker, 1971). MPF can be assayed by microinjection into oocytes of *Xenopus laevis*, which respond by undergoing meiotic maturation, comprising a complex series of events including the appearance of a white spot in the animal pole of the oocyte. This is a consequence of changes inside the oocyte that include the breakdown of the oocyte nucleus, known as germinal vesicle breakdown (GVBD). In the course of maturation, the oocytes complete the first meiotic division, expel the first polar body, and enter second meiosis. As originally shown for *Xenopus* by Gerhart et al. (1984), and in starfish by Kishimoto and Kanatani (1976) (reviewed by Meijer and Guerrier, 1984), MPF activity appears at the time of GVBD, disappears between the

two meiotic divisions, and reappears at second meiotic metaphase.

Purified MPF is a complex containing p34^{cdc2}, the *Xenopus* homologue of the yeast cdc2/CDC28 cell cycle control gene, and a B-type cyclin (reviewed by Nurse, 1990, and by Pines and Hunter, 1990). On the basis of studies of the rapid cell cycles that comprise cleavage, the simple two-state cell cycle originally defined by Newport and Kirschner (1984), is now regarded as a cyclical turning on and off of cdc2-kinase activity (Murray and Kirschner, 1989a,b). The accumulation of cyclin(s) permits alterations in the phosphorylation state of p34^{cdc2} and turns on its protein kinase activity (Gould and Nurse, 1989; Solomon et al., 1990). As well as promoting entry into M-phase, cdc2-kinase also triggers the proteolysis of cyclin, which occurs after a certain lag and leads to rapid loss of cdc2-kinase activity and return to interphase (Félix et al., 1990).

One of the most striking properties of MPF, which was discovered before its molecular components had been identified, is its apparent ability to activate itself (Wu and Gerhart, 1980). Although in nature the hormone progesterone triggers *Xenopus* maturation, and protein synthesis is needed for the first appearance of MPF activity, when maturation is triggered by injection of a small amount of MPF, MPF activity is "amplified" in a reaction that does not require protein

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1. *Abbreviations used in this paper:* CSF, cytosolic factor; GVBD, germinal vesicle breakdown; MPF, maturation promoting factor.

synthesis. Immature oocytes must therefore contain an inactive precursor of MPF that MPF itself can somehow activate. This hypothetical precursor is known as pre-MPF (Cyert and Kirschner, 1988). It is of considerable interest and importance to determine the components of pre-MPF and to discover how it is activated.

Although MPF activity drops between the two meiotic divisions (Gerhart et al., 1984), paradoxically, cells do not re-enter interphase. This is true of clams and starfish, whose transparent eggs permit a clear view of the behaviour of chromosomes and nuclei during the process (Dorée et al., 1983; Meijer and Guerrier, 1984; Westendorf et al., 1989). After meiosis I and expulsion of the first polar body, the chromosomes stay condensed, the nuclear envelope does not reassemble, and perhaps most importantly for the success of the whole procedure, there is no DNA replication. Why not? In mitotic cycles it is now usually assumed that high MPF equals mitosis and low MPF equals interphase. What special mechanisms apply during meiosis to abrogate this rule? In starfish, cyclin B is destroyed between the two meiotic divisions (Standart et al., 1987; M. Dorée and A. Picard, personal communication), whereas cyclin B is not proteolyzed until the end of meiosis II in clams (Westendorf et al., 1989). We wished to determine what happened to cyclins during the maturation of *Xenopus* oocytes. The opacity of frog oocytes makes it more difficult to follow the cytology of oocyte maturation in this organism, but there is no reason to doubt that the essentials of the process are the same.

We recently cloned and expressed all three *Xenopus* cyclins in bacteria, and raised specific antibodies against them. As a prerequisite for understanding the pre-MPF → MPF activation and the state of the cell between meiosis I and II, we investigated the levels of cyclins and cdc2 before, during, and after meiosis in *Xenopus*. We find that stage VI oocytes contain a considerable maternal endowment of B-type cyclin polypeptides and p34^{cdc2}. We further show that cyclin A is synthesized during progesterone-induced maturation and that some portion of all the cyclins are destroyed during meiotic maturation. The behavior of cyclin B1, which seems largely to avoid destruction at first meiosis and of B2, which is progressively phosphorylated during maturation, may provide clues to understanding the paradox of how cells stay in M-phase while MPF levels fall.

Materials and Methods

Preparation of Oocytes

Ovaries were excised from non-primed *Xenopus laevis* females. Oocytes were treated with 500 µg/ml collagenase (Boehringer Mannheim UK Ltd., UK) for one hour to dislodge follicle cells and cultured in modified Barth medium (Gurdon and Wickens, 1983). Full-grown stage VI oocytes were chosen according to the description of Dumont (1972). To induce maturation, 5 µg/ml progesterone was added to the medium and the oocytes were cultured at 22°C. In some experiments, oocytes were labeled in medium containing 1 mCi/ml ³⁵S-methionine (SJ1515; Amersham International, Amersham, UK). After labeling, the oocytes were washed several times with fresh media and then collected or cultured further. Germinal vesicle breakdown was monitored by observing white spot formation in a separate group of oocytes. The uptake of ³⁵S-methionine into oocytes during a 2-h "pulse" label declined to ~20% of its initial value after GVBD. To enable the rates of protein synthesis to be corrected for the resulting lower specific activity of the precursor pool, we measured the total radioactivity in a small sample of the homogenate. The results shown in Fig. 9 C were normalized using these data.

Typically, 10 oocytes were homogenized by pipetting in an appropriate extraction buffer as described in each section and centrifuged at 10,000 rpm for 1 min to remove the yolk. The supernatants were used for further analysis.

Preparation of *Xenopus* Egg Extracts and Cycling Egg Extracts

Extracts were prepared as described by Murray and Kirschner (1989a) and Murray (1991).

Microinjection of Cyclin mRNA into *Xenopus* Oocytes

Stage VI oocytes were injected with 20 nl of cyclin A or B1 (850 ng/µl) mRNA and cultured at 21°C in modified Barth medium containing 1 mCi/ml ³⁵S-methionine. Samples of three oocytes were taken at different times, washed once, and homogenized in 50 µl of TE (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). The resultant supernatants were mixed with SDS sample buffer and run on the SDS polyacrylamide gel. Control oocytes were injected with water. The mRNAs for cyclin A and B1 were transcribed with T7 RNA polymerase from cDNA clones of cyclin A or B1 in pGEM1 linearized with Sall (cyclin A) or BamHI (cyclin B1).

Immunoblotting

Ten oocytes were homogenized with 60 µl of a buffer containing 80 mM Tris-Cl (pH 6.8), 200 mM sucrose, 100 mM NaCl, 5 mM EDTA, 3 mM PMSF, and 5 mM NEM, followed by the addition of 2 µl of ³⁵S-labeled Δ133-truncated cyclin A polypeptide synthesized in the reticulocyte lysate to control for protein degradation during sample preparation. Yolk was removed by centrifugation at 10,000 rpm for 1 min, the supernatant was mixed with 15 µl of 4 × SDS sample buffer, boiled, and then applied onto a 15% SDS polyacrylamide gel (Anderson et al., 1973). Immunoblotting was as previously described (Gautier et al., 1990). Blots were probed with affinity-purified polyclonal anticyclin antibodies: anticyclin A antibody K3, anticyclin B1 antibody L6, and anticyclin B2 antibody N5. ³⁵S-protein A (SJ444, 820 Ci/mmol; Amersham International) at 0.1 µCi/ml was used as the secondary probe and immunoreactive bands were detected by autoradiography on Hyperfilm β-max (Amersham International). Protein concentrations were determined by scanning densitometry and comparison with standards synthesized in a reticulocyte lysate.

The absolute concentrations of cyclin protein standards were determined in two different ways. We used ³⁵S-methionine diluted to a known specific activity in the reticulocyte lysate to synthesize cyclins in a parallel incubation (since we were using ³⁵S-protein A as the detection system, the actual standards were synthesized with no added label). We precipitated the labeled protein with TCA, determined its concentration by liquid scintillation counting, and compared its concentration with that of the unlabeled control on an immunoblot using the alkaline phosphatase secondary detection system as previously described (Gautier et al., 1990). As a second check, we diluted bacterially produced cyclins to appropriate concentrations in virgin reticulocyte lysate and analyzed them alongside the radiolabeled and unlabeled reticulocyte standards. We estimate that our concentration determinations, which ultimately depend on this standardization, are reliable to within a factor of about two.

Affinity Chromatography with p13^{Suc1} Sepharose Beads

Affinity chromatography on p13^{Suc1} beads was performed by a modification of a method previously described (Minshull et al., 1990). Ten oocytes labeled with ³⁵S-methionine were homogenized in 500 µl of histone H1 kinase buffer (80 mM Na β-glycerophosphate, 20 mM EGTA, 15 mM MgCl₂, 1 mM DTT) and centrifuged at 10,000 rpm for 1 min. The supernatants were incubated for 1 h at 4°C with 20 µl equivalent of p13-beads pre-equilibrated with H1 kinase buffer. The beads were collected by centrifugation and washed three times with bead buffer (50 mM Tris-Cl, pH 7.4, 5 mM NaF, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP-40) containing 5 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml trypsin inhibitor, and 100 µM benzamidine. The beads were transferred to a new tube at the second wash, and finally resuspended in SDS sample buffer. The bound materials were analyzed by SDS-PAGE and autoradiography on Hyperfilm β-max (Amersham International).

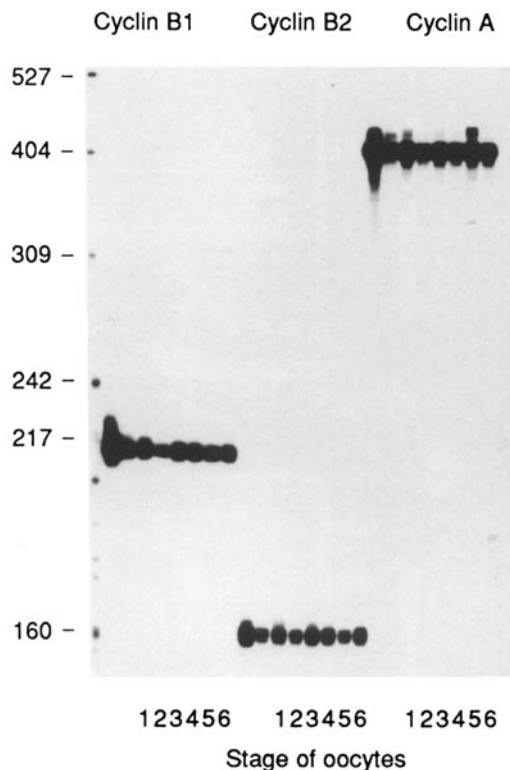


Figure 1. The mRNA levels of cyclin A, B1 and B2 during *Xenopus* oogenesis. One oocyte equivalent of total RNA prepared from pools of five staged oocytes (stages I to VI) was analyzed by RNase protection as previously described (Minshull et al., 1989). Pure cyclin mRNA made in vitro was used as a standard to estimate the mRNA concentrations. The first two lanes of each series contained 100 and 25 pg of the appropriate pure cyclin mRNA, while the lanes labeled 1-6 represent the stages of oocytes, with the particular cyclin indicated at the top of each series. The numbers in the left column indicate the sizes of MspII-digested pBR322 fragments used as markers.

Immunoprecipitation of Individual Cyclins

10-20 oocytes labeled with ³⁵S-methionine were homogenized in 1.5 ml of modified RIPA buffer (80 mM Na-β-glycerophosphate, 50 mM NaF, 10 mM Na₂PO₄, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, pH 7.2), containing 1 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 15 μg/ml benzamidine and then mixed with 50 μl of affinity-purified anticyclin antibody. Immunoprecipitation was then performed as previously described (Minshull et al., 1990).

Results

Xenopus eggs contain the mRNA for three cyclins, called A, B1, and B2 (Minshull et al., 1989a; Minshull et al., 1990), although, as will be seen below, we cannot be absolutely certain that this is a complete roster. To determine at what stage of oogenesis the mRNA for these cyclins are made, and to measure their relative levels, RNA was extracted from oocytes of increasing maturity from a single female, and the level of each cyclin mRNA measured by RNase protection mapping as previously described by Minshull et al. (1989a). Fig. 1 shows that the mRNAs for cyclins A, B1, and B2 were already present in the smallest oocytes at $\sim 5 \times 10^7$ molecules per oocyte each, and that the total amount of mRNA did not increase during oogenesis. These data do not distin-

guish between the possibilities that cyclin mRNA is synthesized in a burst and stored for months in a completely stable form, or that the constant level is maintained by continuous synthesis balanced by turnover. The early accumulation of maternal mRNA is characteristic of maternal mRNA in *Xenopus* (Golden et al., 1980; Sagata et al., 1989a), however, and means that the synthesis and accumulation of cyclin polypeptides could in principle occur at any time before, during or after meiotic maturation.

Cyclins B1 and B2 Are Present in Stage VI Oocytes, while Cyclin A Does Not Appear Until Just Before First Meiotic Metaphase

Extracts of activated *Xenopus* eggs (Murray and Kirschner, 1989) actively synthesize cyclin A, B1, and B2 (Minshull et al., 1989a). Highly purified preparations of MPF prepared from mature eggs contain both cyclin B1 and B2 (Gautier et al., 1990). The latter observation indicates that the B-type cyclin polypeptides are present in unfertilized eggs. To test which cyclin polypeptides accumulate at what stages during oogenesis, we measured the amount of each cyclin polypeptide by immunoblotting, using affinity-purified anticyclin antibodies that were highly specific for each cyclin (Minshull et al., 1990). The levels of each cyclin were quantitated by comparison with known amounts of cyclin protein that had been synthesized under the direction of synthetic mRNA in a reticulocyte cell-free system (see Materials and Methods).

Fig. 2 shows that cyclin B1 polypeptide was first detectable in stage IV oocytes, but did not increase greatly in amount

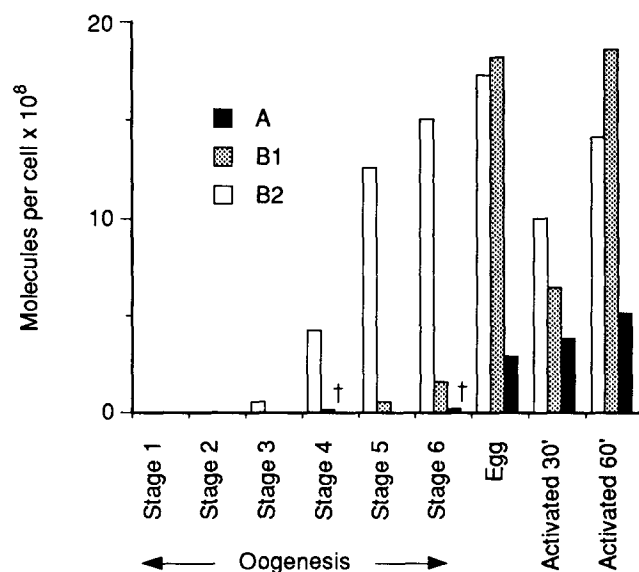


Figure 2. Accumulation of cyclin A, B1, and B2 during oogenesis and after egg activation. The levels of cyclins A, B1, and B2 were quantified as described in Materials and Methods (and see Fig. 3), and plotted as a bar graph with respect to developmental time during oogenesis or in eggs before and after electrical activation. Note that 2×10^9 molecules per cell corresponds to a concentration of ~ 0.15 μg/ml or ~ 3 nM. The precise amounts may be underestimates, because we found that about half the added labeled marker cyclin Δ133 was trapped in the bulky yolk pellet. The '+' mark means that a band was detectable by eye, but less than 1 pg was present.

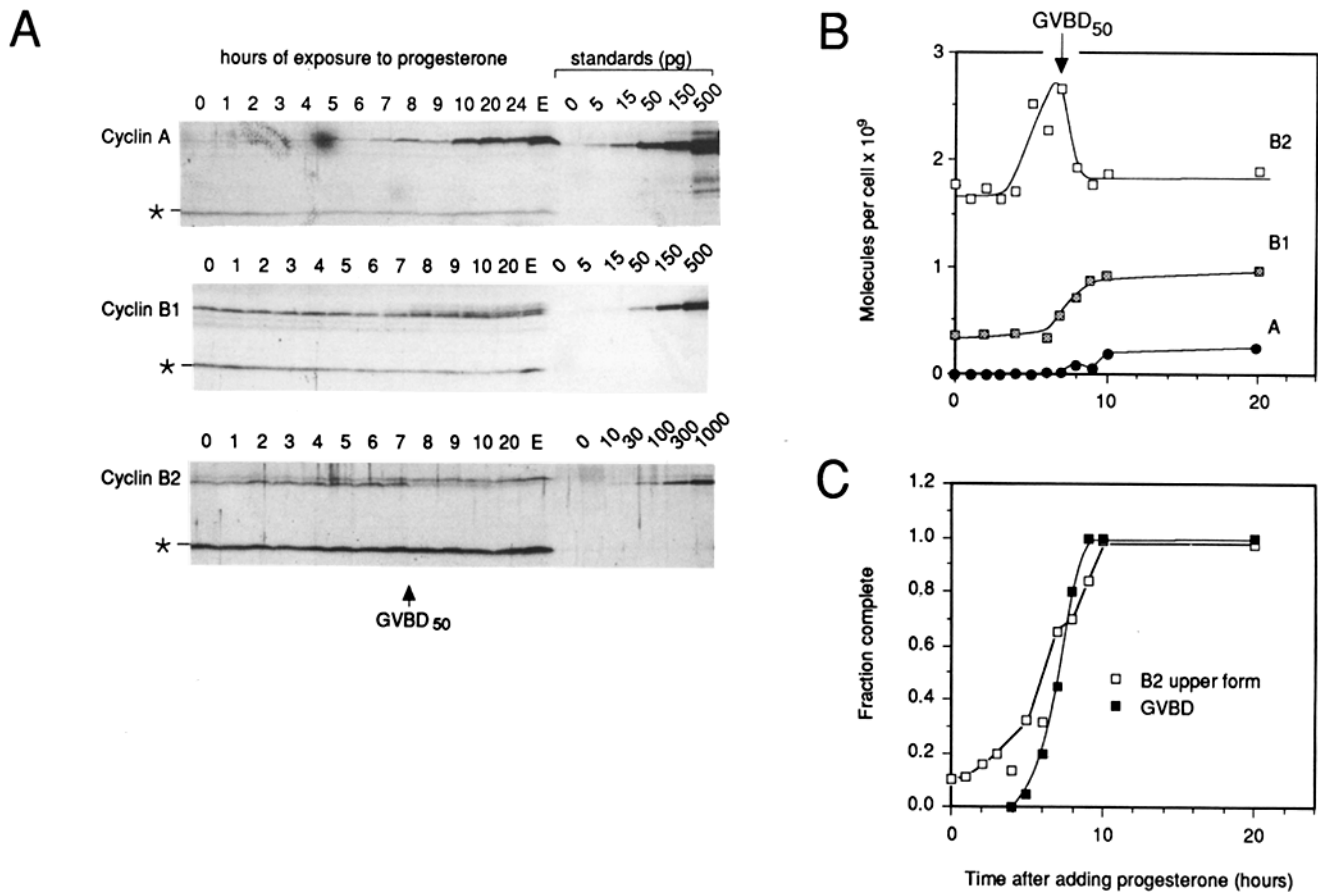


Figure 3. Accumulation of cyclin A, B1, and B2 during *Xenopus* oocyte maturation. (A) Stage VI oocytes were incubated in 5 $\mu\text{g/ml}$ progesterone and 10 oocytes were sampled at hourly intervals for analysis by immunoblotting. Each lane contains the equivalent of two oocytes. (A) Autoradiograms of immunoblots detected with ^{35}S -protein A for each cyclin as indicated. The asterisk (*) marks the internal control band of ^{35}S -labeled $\Delta 133$ cyclin A polypeptide, which was added to oocytes at the time of lysis (the intensity of this band is different in each case owing to different exposure times of the three autoradiograms). The time of sampling is indicated above each lane. (lane E) Unfertilized eggs (from a different female). Standards of each cyclin were run in the same gel as indicated above each lane. This allowed the quantitation shown in B. (B) Quantitation of cyclin levels during oocyte maturation obtained by densitometry of the films shown in A. The time of GVBD_{50} is shown at top. (C) Correlation of the fraction of cyclin B2 in the upper band during oocyte maturation (*open squares*), with the appearance of the white spot signifying GVBD (*filled squares*).

until oocyte maturation, when its level rose from ~ 30 pg (4×10^8 molecules) per cell in stage VI oocytes to ~ 60 – 80 pg ($\sim 1 \times 10^9$ molecules) per cell in metaphase II arrested eggs. Cyclin B2 was synthesized even earlier in oogenesis; stage III oocytes gave a detectable signal on the immunoblots, and the level increased to between 100–200 pg (1 – 2×10^9 molecules) per cell in stage VI oocytes and eggs. The level of cyclin B2 in unfertilized eggs is very similar to that of stage VI oocytes. Thus, the major cyclin component in stage VI oocytes is cyclin B2, whereas in unfertilized eggs, it is a roughly equal mixture of cyclins B1 and B2.

By contrast, cyclin A protein was essentially undetectable in oocytes of any stage. Stage VI oocytes contain < 1 pg (10^7 molecules), whereas unfertilized eggs contain 20–30 pg ($\sim 2 \times 10^8$ molecules) per cell.

As expected, the level of B-type cyclins rapidly declined and then built up again after egg activation. The overall level of cyclin A stayed relatively constant at this time, and increased steadily up to 75 pg/cell equivalent at the time of first mitosis (data not shown).

Cyclin Protein Levels Vary in a Complex Pattern during Oocyte Maturation

Fig. 2 shows the steady-state levels of cyclin polypeptides at relatively stable stages of development. To examine the details of cyclin synthesis and destruction during maturation, when oocytes complete the first meiotic division and arrest at metaphase of meiosis II in the span of a few hours, the amounts of cyclin A, B1 and B2 proteins were measured in stage VI oocytes that were incubated with progesterone (Fig. 3).

Cyclin A, which is essentially absent from stage VI oocytes, first appeared in easily detectable amounts (5–10 pg/cell) at about the time of GVBD. Its level decreased slightly when GVBD was completed and then increased again, suggesting that a round of cyclin A destruction had occurred between the two meiotic divisions. The existence of this brief period of destruction was confirmed by following the fate of radiolabeled cyclin A, as will be described in a later section (Fig. 7).

The levels of cyclin B1 in oocytes increased steadily after progesterone treatment, and reached a plateau shortly after GVBD. An immunoreactive polypeptide with slightly retarded gel mobility appeared after GVBD. It is not clear whether this represents a posttranslationally modified form of cyclin B1, or the de novo translation of mRNA for a closely related polypeptide. In contrast to the pattern seen for cyclin B1, cyclin B2 protein levels increased after progesterone treatment up to the point of GVBD, at which time they rapidly fell back to the initial level. This decline took place at about the same time that cyclin A levels dipped, suggestive of a round of partial proteolysis during meiosis I. In no case, however, did the amount of cyclin B2 drop below $\sim 10^9$ molecules per cell, in contrast to what happens at fertilization or at anaphase of mitotic divisions, when essentially all the cyclin B2 is destroyed (Minshull et al., 1990a).

As already noted by Gautier and Maller (1991), two immunoreactive bands of cyclin B2 were detectable during maturation, of which the upper band is a phosphorylated form (Gautier and Maller, 1991; R. Golsteyn, unpublished observations). In oocytes up to stage VI, most of the cyclin B2 was present as the lower (unphosphorylated) form. During maturation, a smooth transition to the phosphorylated form occurred, until the dephosphorylated form was essentially undetectable in fully mature oocytes. Fig. 3 C shows that the shift to the upper form started ~ 1 h before germinal vesicle breakdown, and went almost to completion in unfertilized eggs, but the time course of conversion to the upper form proceeded more slowly than the time course of GVBD. We never observed any conversion of the upper band to the lower one between the two meiotic divisions.

Thus, each cyclin shows an individual and surprisingly complex picture of differentially timed synthesis, which gives rise to an even more complex pattern of accumulation because of posttranslational modifications that include partial proteolysis during meiosis I.

Partial Destruction of B-type Cyclins Occurs after GVBD

The immunoblotting data in Fig. 3 suggested that the small amount of accumulated cyclin A was destroyed shortly after GVBD, and at least a portion of the pool of cyclin B2 likewise seemed to undergo proteolysis at about the same time. To examine the destruction of cyclins during meiotic maturation in more detail, oocytes were incubated in medium containing ^{35}S -methionine, starting 30 min after addition of progesterone. After 3 h, the cells were transferred to unlabeled medium (still with progesterone). Labeled cyclins were detected either by immunoprecipitation with each specific antibody or by affinity chromatography using p13^{suc1} Sepharose, an affinity matrix for p34^{cdc2} (Brizuela et al., 1987; Dunphy et al., 1988).

Fig. 4 shows that ~ 2 h after GVBD₅₀, the radioactivity in p13-bound B-type cyclins (solid line, open circles) dropped to $\sim 30\%$ of its initial value, consistent with the idea that the majority of the B-type cyclins were destroyed at this time. At the same time that label was lost from cyclin, the labeling of other proteins increased. In particular, there was increased incorporation into bands at ~ 34 kD, which, as shown below, correspond to p34^{cdc2} and related polypeptides. In such "pulse-chase" experiments, the labeled cyclins

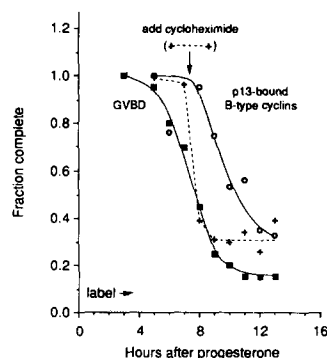


Figure 4. Kinetics of destruction of B-type cyclins during oocyte maturation. Stage VI oocytes were incubated with ^{35}S -methionine from 0.5 to 3.5 h after exposure to progesterone. The labeled oocytes were then divided into two groups. The first group was incubated in unlabeled medium (still with progesterone) and samples of 10 oocytes were taken at 1-h intervals thereafter. Homogenates of

these samples were chromatographed on p13-Sepharose and analyzed by gel electrophoresis, autoradiography, and scanning densitometry as described in Materials and Methods (○—○). The other group of labeled oocytes had cycloheximide (20 $\mu\text{g}/\text{ml}$) added 7 h after adding progesterone (approximately GVBD₅₀). These were analyzed in the same way as the first group (+-----+). We did not attempt to resolve the different forms of B-type cyclins in this experiment. Filled squares (■—■) indicate the proportion of cell that had not undergone GVBD.

that survived destruction displayed characteristic phosphorylation-associated mobility shifts (see Fig. 6).

To completely block protein synthesis and thereby eliminate reincorporation of label into newly synthesized cyclins, 100 μM cycloheximide was added to a sample of the same batch of labeled oocytes 7 h after progesterone, at about the time of GVBD₅₀. Fig. 4 (dashed line, crosses) shows that the presence of cycloheximide accelerated the destruction of the B-type cyclins. Loss of label occurred ~ 2 h earlier than that in the absence of the drug, and the destruction of the cyclins occurred more rapidly. Moreover, cycloheximide inhibited the conversion of the surviving cyclins to the phosphorylated form (data not shown). The fraction of newly synthesized B-type cyclins that were destroyed in the presence of the cycloheximide was again $\sim 70\%$. In the following paper, we will show a similar experiment using immunoblotting that gave essentially the same results (Minshull et al., 1991). At the same time as the cyclins were being destroyed, levels of the labeled 34-kD protein that bound to p13-beads were unchanged (see Fig. 6). This provided an internal control for recovery, and also illustrates the specificity of cyclin destruction. Their accelerated disappearance is not due to generalized toxicity of protein synthesis inhibitors.

By adding cycloheximide at intervals after the end of the 3-h labeling period, we were able to test the stability of cyclins at various stages during maturation. As previously noted, addition of cycloheximide early after progesterone blocks maturation, but if the inhibitor is added after a certain critical time, the oocytes undergo GVBD (Gerhart et al., 1984; Wasserman and Masui, 1975). At intermediate times, batches of oocytes display a heterogeneous response; some oocytes mature while others do not. Fig. 5 shows that in the presence of cycloheximide, cyclins B1 and B2 were only destroyed (or unstable) in oocytes that underwent maturation (Fig. 5, lane 3), and not in the oocytes in which cycloheximide inhibited germinal vesicle breakdown (Fig. 5, lanes 2 and 4). As in the previous experiments, scanning densitometry showed that $\sim 70\%$ of the newly made cyclins were destroyed in the oocytes that matured. This result shows that

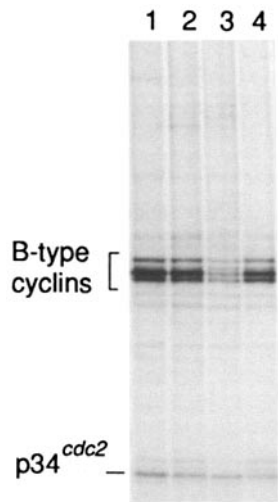


Figure 5. B-type cyclins are degraded in the oocytes that undergo maturation in the presence of cycloheximide, and are not degraded in the oocytes that do not mature. Stage VI oocytes were labeled with ^{35}S -methionine as described in Fig. 4. Cycloheximide ($20\ \mu\text{g}/\text{ml}$) was added at the onset of white spot appearance (5 h after adding progesterone in this experiment). At 10 h, when GVBD was complete in a parallel batch of uninhibited oocytes, the cycloheximide-treated oocytes were divided into two groups: those that had undergone maturation in the presence of cycloheximide and those in which cycloheximide had inhibited maturation. Each

sample was analyzed by p13-binding assay. (lane 1) The oocytes at 5 h after adding progesterone; (lane 2) the nonmatured oocytes at 10 h; (lane 3) the matured oocytes at 10 h (lane 4) the nonmatured oocytes at 13 h. About 20% of the cycloheximide-treated oocytes underwent maturation compared with 85% of the untreated oocytes in this experiment.

the destruction of B-type cyclins is closely related to meiotic maturation, and cyclins are only unstable if the cells enter meiosis I. Cyclin is a relatively stable protein in interphase or in mature eggs. This is an important result, because it rules out the idea that oocyte maturation in response to *c-mos* mRNA could be ascribed to the stabilization of a rapidly turning-over cyclin pool (Freeman et al., 1990; Sagata et al., 1989a,b), which would thereby promote the accumulation of cyclin past a critical threshold for *cdc2* kinase activation.

The Destruction of Cyclin B1 at First Meiosis Is Slower and Less Complete than that of Cyclin B2

To follow the behavior of the individual B-type cyclins more precisely, oocytes were again placed in medium containing ^{35}S -methionine and progesterone for 3 h. The oocytes were then washed and incubated in nonradioactive medium containing progesterone, and samples taken for immunoprecipitation with anticyclin B1 and B2 antibodies. Fig. 6 shows the results, which confirm several previous data. In the case of cyclin B2, three strongly labeled polypeptides, a doublet at $\sim 55\ \text{kD}$ and a band at $34\ \text{kD}$, were selectively retained in the immunoprecipitates. They correspond to the two forms of cyclin B2 and to $\text{p}34^{\text{cdc}2}$, as will be shown below in Fig. 8. About 80% of the initial radioactive cyclin B2 was degraded 2 h after GVBD_{50} , whereas there was no loss of radioactivity from the 34-kD band. After GVBD, the upper band of cyclin B2 predominated and there were signs that the 34-kD band split in two.

The pattern of bands in the anticyclin B1 immunoprecipitates was more complex. In addition to the expected cyclin and $\text{p}34^{\text{cdc}2}$ bands, radioactivity was detected in two additional polypeptides, labeled X1 and X2 (Fig. 6, lower panel). These are probably cross-reacting polypeptides, since they were not seen on the B2 immunoprecipitates or in the p13-bound material. The behavior of these unidentified polypep-

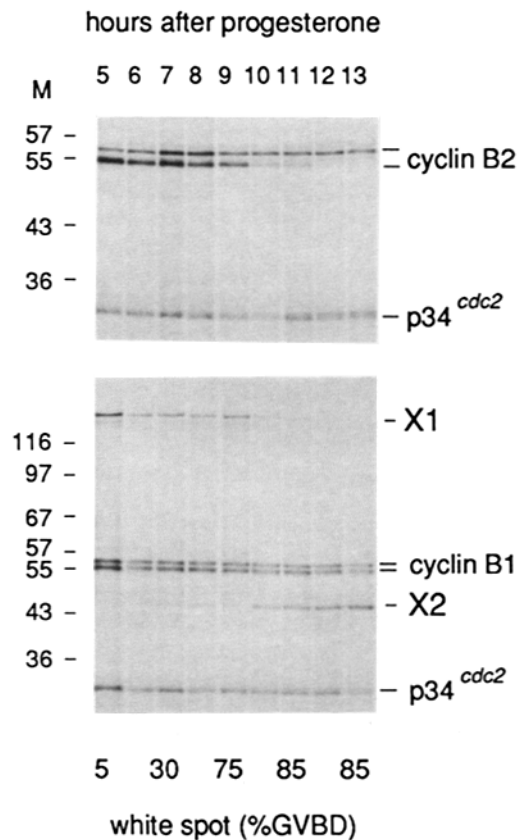


Figure 6. Destruction of cyclin B2 and B1 during oocyte maturation. Portions of the noncycloheximide-treated homogenates from the experiment described in the legend of Fig. 4 were immunoprecipitated with anticyclin B2 antibody (upper panel) or anticyclin B1 antibody (lower panel) and analyzed by gel electrophoresis and autoradiography (see Materials and Methods). The time of sampling in hours after adding progesterone is shown at the top, and the percent of oocytes showing white spot (GVBD) is shown underneath. GVBD_{50} occurred at $\sim 7.5\ \text{h}$ in this experiment (see the GVBD curve in Fig. 4).

ptides is interesting, however, with X1 disappearing and X2 appearing in a reciprocal relationship. We do not know their identity. Label in cyclin B1 started to be lost from the immunoprecipitates $\sim 2\ \text{h}$ after GVBD_{50} , but the rate of cyclin degradation thereafter was slower than that of cyclin B2. Indeed, even by 6 h after GVBD_{50} , less than half the starting label in cyclin B1 had been lost. This behavior is consistent with the immunoblotting results of Fig. 3, which showed a steady accumulation of cyclin B1 during meiotic maturation, with no sign of sudden destruction at GVBD, in contrast to the levels of cyclin B2, which rose and then fell.

Newly Synthesized Cyclin A Translated from Injected mRNA Is Destroyed after GVBD

Cyclin A synthesis was undetectable during the labeling period in experiments like those shown in Figs. 4–6, because as Figs. 3 and 9 B show, cyclin A synthesis starts later than that of the B-type cyclins. As a result, this approach did not give a clear result as regards cyclin A destruction at meiosis I. To circumvent this problem, we analyzed cyclin A synthesis and destruction in oocytes that had been induced to mature by microinjected synthetic cyclin A mRNA (Swenson et

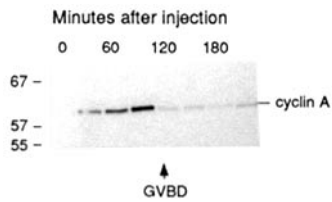


Figure 7. Cyclin A mRNA-induced maturation is accompanied by the destruction of cyclin. Cyclin A mRNA (17 ng) was injected into oocytes, which were labeled with ^{35}S -methionine. Samples were taken every 30 min and analyzed by immunoprecipitation with anticyclin A antibody. An arrow indicates the time of GVBD₅₀.

lyzed by immunoprecipitation with anticyclin A antibody. An arrow indicates the time of GVBD₅₀.

al., 1986). The injected oocytes were incubated in ^{35}S -methionine, and labeled cyclin A was immunoprecipitated from the homogenates. Fig. 7 shows that the newly synthesized cyclin A was easily detectable in the cells that had been injected with exogenous mRNA, and that it was destroyed abruptly at GVBD. Similar results were obtained for B-type cyclins (data not shown; but see Pines and Hunt, 1987).

In summary, it appears that cyclins A and B2 undergo relatively sharp proteolysis at first meiosis of *Xenopus* oocyte maturation, which leads to loss of 70–80% of the polypeptides accumulated up to that time. By contrast, the destruction of cyclin B1 is slower and less complete, unless cycloheximide is added. As a result, the levels of cyclin B1 normally increase fairly smoothly during meiotic maturation. We do not understand the reason for the different behaviors of cyclin B1 and B2, nor why inhibiting protein synthesis makes such a big difference.

The Relative Rates of Cyclin and p34^{cdc2} Synthesis during Oocyte Maturation

In Figs. 5 and 6, we drew attention to the synthesis of polypeptides with apparent molecular masses in the 34-kD range which we strongly suspected to correspond to p34^{cdc2}. Two such polypeptides were selectively retained by p13-Sepharose, but only the larger of the two was found in the anti-B-type cyclin immunoprecipitates. The labeled bands both gave positive reactions with a monoclonal anti-PSTAIR antibody (data not shown) and as Fig. 8 shows, they comigrated with marker polypeptides corresponding to *Xenopus* p34^{cdc2} and p32^{Eg1}. Although p32^{Eg1} was bound by p13-Sepharose, it was not detectable in the anticyclin immunoprecipitates, consistent with its previously noted absence from anticyclin immunoprecipitates of extracts prepared from activated eggs (Minshull et al., 1990; Solomon et al., 1990).

To assess the relative rates of synthesis of the cyclins and p34^{cdc2} during oocyte maturation, stage VI oocytes were incubated with ^{35}S -methionine for 2 h “pulse-labeling” periods before, during, and after GVBD. Homogenates were prepared and analyzed by affinity chromatography using either p13^{suc1}-Sepharose (Fig. 9 A) or anticyclin A antibodies (Fig. 9 B). Fig. 9 A shows that synthesis of B-type cyclins was detectable before stage VI oocytes were incubated with progesterone and increased three- to fourfold during maturation (Fig. 9 C). We were unable to detect cyclin A synthesis in stage VI oocytes, but label in cyclin A could be detected at the time of GVBD (Fig. 9 B).

The synthesis of p34^{cdc2} was also easily detectable in stage VI oocytes, and increased about twofold in response to progesterone. After GVBD, however, the labeling of p34^{cdc2} declined markedly. In fact, by 20 h after addition of progesterone it was barely detectable, which is in agreement

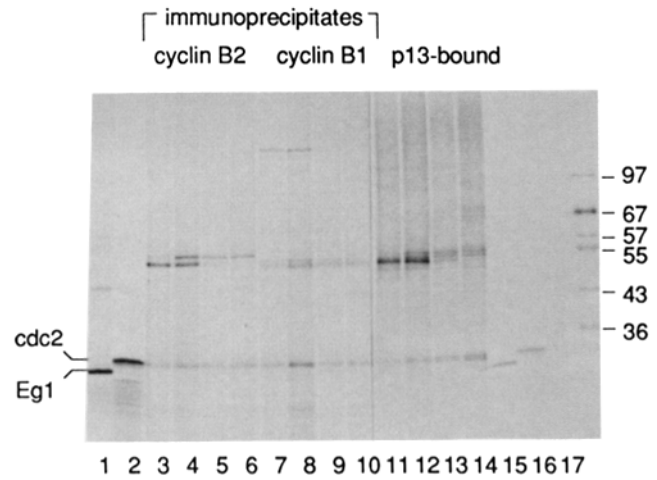


Figure 8. The identification of p34^{cdc2}-related proteins in oocytes. The same samples as were analyzed in Figs. 4 and 6 were rerun on an SDS-polyacrylamide gel with markers provided by cell-free translation of synthetic mRNA made from cloned p32^{Eg1} and p34^{cdc2}. (lanes 1 and 15) p32^{Eg1}; (lanes 2 and 16) p34^{cdc2}; (lanes 3–6) cyclin B2 immunoprecipitates; (lanes 7–10) cyclin B1 immunoprecipitates; (lanes 11–14) p13^{suc1}-Sepharose-retained material. In each series, samples were taken 3, 7, 10, and 13 h after addition of progesterone. Lane 17 shows the molecular weight markers.

with our previous failure to detect p34^{cdc2} synthesis in extracts of activated eggs (Minshull et al., 1990; and see Fig. 10). The data shown in Fig. 9 C are normalized to take account of the gradually decreasing permeability of the maturing oocytes to ^{35}S -methionine as described in Materials and Methods.

We estimate that stage VI oocytes contain $\sim 0.1 \mu\text{M}$ p34^{cdc2} (6×10^{10} molecules per oocyte) using anti-PSTAIR antibodies and immunoblotting. This is roughly 10 times the amount of total cyclins present in oocytes (data not shown).

Cyclin A Does Not Bind to p13 Beads until after Egg Activation

Cyclin A was conspicuously absent from the p13-bound material in these experiments, which we found surprising because the immunoblotting data of Fig. 3 and the immunoprecipitation data of Fig. 9 B led us to expect to be able to detect its synthesis at about the time of GVBD. Fig. 10 shows that cyclin A could be detected in the p13-retained material from activated eggs (Fig. 10, lanes 4 and 5; see also Minshull et al., 1990). The failure of cyclin A from oocytes and unfertilized eggs to bind to p13-Sepharose does not appear to be due simply to its low abundance, because as shown in Fig. 2, its level increased relatively little immediately after egg activation, yet it was now retained. This finding probably explains why we could never detect histone kinase activity in anticyclin A immunoprecipitates prepared from unactivated eggs, whereas strong activity appeared soon after activation (J. Minshull, data not shown). These are provocative observations, since they suggest that endogenous cyclin A may not normally associate with p34^{cdc2} until after fertilization. Further experiments are required to confirm this. It is difficult to understand how or why cyclin A mRNA causes oocyte maturation if cyclin A protein does not normally interact with p34^{cdc2} until after fertilization.

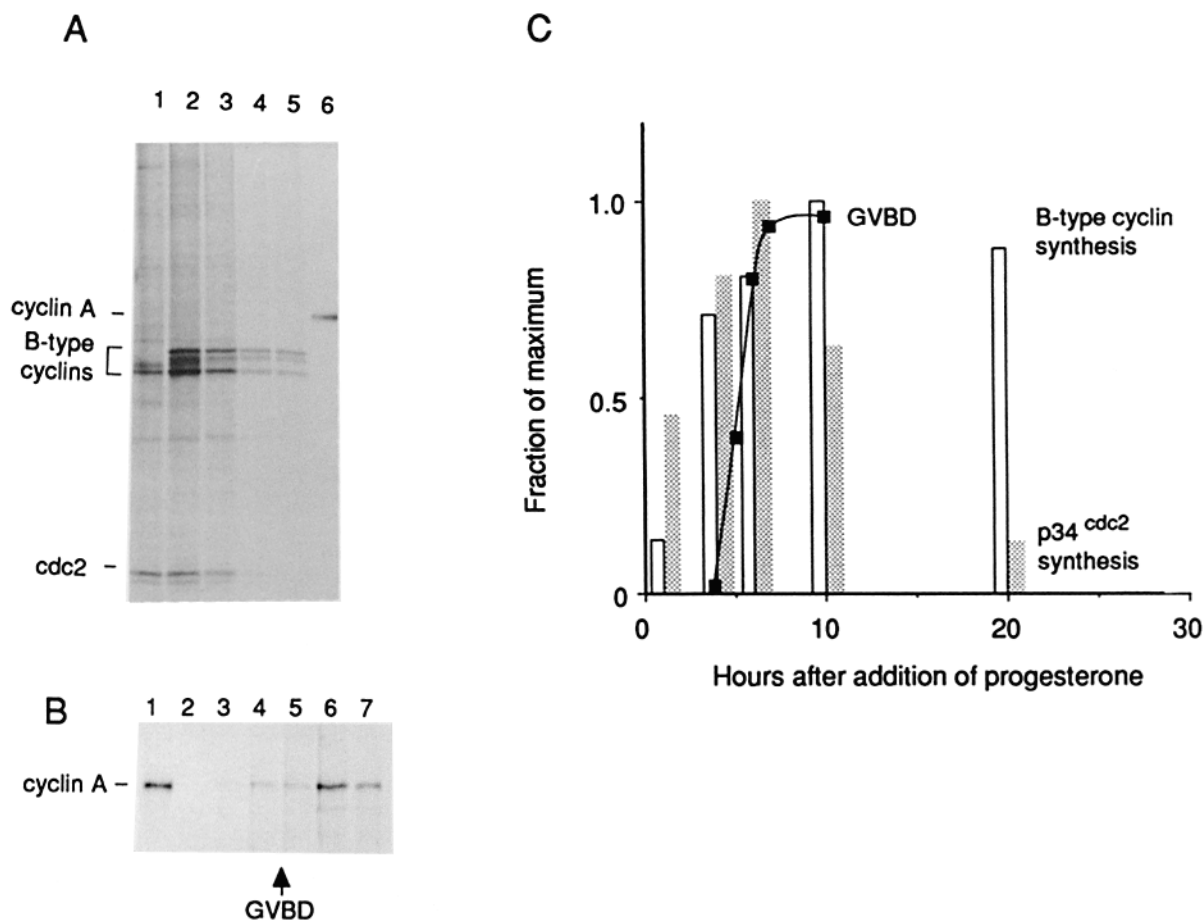


Figure 9. Comparison of the synthesis of *cdc2* and cyclins during oocyte maturation. (A) Stage VI oocytes were pulse labeled with ³⁵S-methionine for 2 h at different times after adding progesterone and the homogenates analyzed by chromatography on p13-Sepharose. (lane 1) 0–2 h; (lane 2) 3–5 h; (lane 3) 5–7 h; (lane 4) 8–10 h; (lane 5) 18–20 h; (lane 6) ³⁵S-cyclin A marker. Equal amounts of protein were analyzed in this experiment. (B) Immunoprecipitation with anticyclin A antibodies was used in place of p13-Sepharose. (lane 1) ³⁵S-cyclin A marker; (lane 2) no antibody; (lanes 3–7) immunoprecipitates using purified anticyclin A antiserum. (lane 3) 0–2 h; (lane 4) 3–5 h; (lane 5) 5–7 h; (lane 6) 8–10 h; (lane 7) 10–12 h. The uptake of ³⁵S-methionine decreased by ~50% at GVBD and by 80% by 10–12 h, and correspondingly larger samples were analyzed at later times to correct for this. The time of GVBD₅₀ (5.5 h in this experiment) is indicated below. (C) Relative rates of synthesis of p34^{cdc2} and B-type cyclins during oocyte maturation. The bands in A were scanned with a densitometer and corrected for ³⁵S-methionine uptake as described in Materials and Methods.

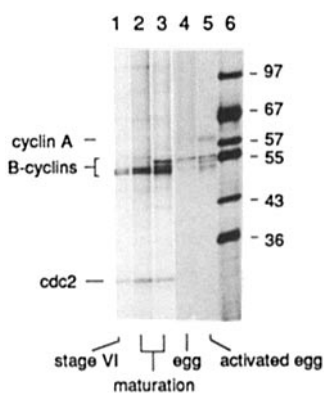


Figure 10. Cyclin A does not bind to p13-Sepharose in oocytes or mature eggs; cyclin B does, even in stage VI oocytes. Stage VI oocytes from a single female were incubated in the absence of hormone (lane 1) or with progesterone for 2 h (lane 2) or 7 h (lane 3) with ³⁵S-methionine present for the last 2 h. Homogenates were analyzed by chromatography on p13-Sepharose followed by SDS-PAGE and autoradiography. Lanes 4 and 5 show a similar analysis of ³⁵S-methionine-labeled mature eggs and electrically activated eggs (from a different female). Lanes 4–6 were from older samples and were exposed four times longer to compensate for the low residual ³⁵S-methionine content. The positions of the cyclin and *cdc2* bands are indicated at the side.

Figure 10 also shows that detectable amounts of B-type cyclins are being synthesized in stage VI oocytes, and that they are retained by p13-Sepharose. This means that they are probably already associated with p34^{cdc2} in the unactivated oocytes. If this interpretation is correct, the activation of pre-MPF requires only posttranslational changes to a preformed cyclin-cdc2 complex, and the protein synthesis requirement for oocyte maturation does not represent synthesis of any of the components of MPF. The companion paper (Minshull et al., 1991) further explores this point.

Discussion

The aim of the present work was to describe the time course of cyclin synthesis, destruction, and posttranslational modification during *Xenopus* oogenesis and oocyte maturation. This is a prerequisite to understanding the mechanism of activation of MPF by progesterone and the transition from meiosis I to meiosis II. The salient observations in this paper can

be summarized as follows. (a) Oocytes contain a store of ~ 5 nM B-type cyclin. Cyclin A is present at less than 1/100th this amount, and can be considered as effectively absent compared to the levels it achieves during the rapid cycles of early embryonic cell cycles. (b) The stored B-type cyclin bind to p13-Sepharose, and hence probably is associated with (inactive) p34^{cdc2} in stage VI oocytes. (c) Progesterone treatment leads to an increase in synthesis of the B-type cyclins, whose concentration rises accordingly. There is no evidence for rapid turnover of cyclins until after GVBD. (d) Cyclin B2 starts to get phosphorylated before GVBD. (e) Cyclin A starts to accumulate concomitantly with GVBD. (f) Shortly (1–2 h) after GVBD, up to 80% of cyclins A and B2 are destroyed; much less cyclin B1 is destroyed at this time, unless protein synthesis is blocked with cycloheximide. (g) After the destruction phase that marks the intermeiotic period, all three cyclins accumulate and are stable until fertilization. (h) Cyclin B2 is almost exclusively present as its phosphorylated form in matured oocytes. Lower mobility forms of cyclin B1 also appears after GVBD, which either correspond to phosphorylated molecules or the synthesis of a closely related B-type cyclin. Cyclin A does not undergo detectable mobility shifts. (i) The B-type cyclins in extracts of oocytes at all stages of maturity bind to p13-Sepharose, whereas cyclin A is not retained by this matrix until after fertilization.

These findings readily account for the “autocatalytic” amplification of MPF activity, which can occur even in the presence of cycloheximide. We suppose that the preexisting cyclin B–p34^{cdc2} complexes (predominantly cyclin B2–p34^{cdc2}) correspond to the “pre-MPF” of Cyert and Kirschner (1988), and that microinjection of a “starter” dose of MPF leads to activation of the cdc2-kinase by dephosphorylation of key tyrosine (and possibly threonine) phosphates (Dunphy and Newport, 1989; Gould and Nurse, 1989). At the end of first meiotic metaphase, destruction of some fraction of the cyclins (but not of p34^{cdc2}) can be detected. Normally, a considerable pool of both B-type cyclins appears to survive this period of proteolysis, and as shown in the accompanying paper, the surviving cyclins appear to be sufficient to enable a second round of MPF activation. By contrast, when protein synthesis is inhibited, the destruction of the preformed cyclins during first meiosis is accelerated and increased, and it is not possible to obtain a second round of autocatalytic amplification in such oocytes (Miake-Lye et al., 1983). It is not clear why cyclin destruction occurs earlier and more rapidly when protein synthesis is inhibited with cycloheximide, but this agrees very well with the observations of Gerhart et al. (1984), who observed premature loss of MPF activity under these conditions. We interpret this result to mean that at least one other protein apart from the cyclins themselves participates in oocyte maturation by stabilizing cyclins; *c-mos* is obviously a strong candidate for such a role (Sagata et al., 1989a,b). This does raise a difficult point, however. Why, if it is true that *c-mos* corresponds to CSF and can prevent cyclin destruction, are cyclins not stable in meiosis I, and why do oocytes not arrest at meiosis I metaphase? Two simple kinds of explanation might resolve this difficulty; perhaps there is insufficient *c-mos* at the time of meiosis I, or other components are required besides *c-mos* to form active CSF.

The behavior of cyclin B2 is interesting in the light of the question of what happens in the short space between the two

meiotic divisions. The phosphorylated fraction of this protein rises steadily during oocyte maturation, and shows no tendency to revert to its nonphosphorylated form despite the loss of MPF kinase activity that normally occurs in this interval. This observation suggests that either some other kinase remains active during the intermeiotic interval and thereby helps keep M-phase substrates in their phosphorylated form, or that the phosphatases that would normally return cells to interphase after the disappearance of MPF are temporarily turned off. This is a point worthy of further investigation.

Compared with the relatively large amounts of stockpiled B-type cyclins in prophase arrested oocytes, the level of cyclin A is very low (in effect, it is undetectable) at the outset of oocyte maturation. The accumulation of cyclin A closely follows the curve for GVBD (Fig. 3), and labeled methionine can first be detected in cyclin A just before GVBD (Fig. 9B). This pattern of accumulation strongly implies that cyclin A is not required for entry into first meiosis. This conclusion is supported by the data in the following paper, which show that progesterone-induced maturation can occur in the complete absence of translatable cyclin A mRNA. Yet cyclin A is clearly required for mitotic cell cycles, since a *Drosophila* mutant deficient for cyclin A stops dividing when the maternal stores of protein and mRNA run out (Lehner and O'Farrell, 1990). The evidence thus suggests that cyclin A is not involved in first meiosis, or that if it is, it must have acted long before the G2 → M transition of this cell cycle will take place. Our data do not permit any conclusion as to the possible role of cyclin A in second meiosis, but since translation of the stored cyclin A mRNA starts at GVBD and the protein accumulates before the second meiotic division, it may play some part in the later proceedings.

If cyclin A does not normally participate in meiosis I, why is cyclin A mRNA so effective at promoting maturation? This is another paradox whose resolution is really not clear. One possible explanation, however, is as follows. The G2 → M transition for meiosis I evidently uses an unusual and perhaps unique mechanism. This is suggested by the unusually long period of latency (weeks, if not months) between accumulation of cyclin and the activation of MPF, and by the unusual progesterone-mediated triggering mechanism. After fertilization, however, the rapid cleavage cycles almost certainly depend on cyclin A. This probably means that the normal mitotic trigger mechanism that involves cyclin A is present in oocytes, but is not normally used in these cells. We think that inappropriately expressed cyclin A may cause the activation of preformed cyclin B–p34^{cdc2} complexes by activating this latent mechanism. It is worth pointing out that oocytes that are induced to mature by injection of cyclin mRNA do not end up as quietly arrested “eggs” with a nice white spot. They generally pass quickly through this stage and start what may be abortive cleavages, often showing necrotic changes leading to the so-called “white puffball” phenotype after several hours of incubation (data not shown).

There is a considerable difference between the levels of cyclin and p34^{cdc2}. Whereas the cyclins are present in the range of $1\text{--}5 \times 10^9$ molecules per oocyte, there are about 10–100 times more molecules of p34^{cdc2} $\sim 10^{11}$ molecules (5 ng) per oocyte. Since B-type cyclins are associated with p34^{cdc2} in equimolar amounts (Erikson and Maller, 1989;

Labbé et al., 1989), the majority of cdc2 molecules in oocytes are not bound to cyclin molecules. Since the cyclin-p34^{cdc2} complex is the active form of MPF, it is likely that it is the number of cyclin molecules, rather than the amount of p34^{cdc2}, that places a limit on the total amount of MPF that can form in meiosis I. In agreement with these estimates, Solomon et al. (1990) and J. Maller and his colleagues (J. Maller, personal communication) found that addition of cyclin protein to oocytes or extracts of oocyte led to at least five times more cdc2-kinase activity than is normally found.

Xenopus oocytes contain more than one cdc2-related molecule that is recognized by anti-PSTAIR antibodies. The major form corresponds to p34^{cdc2}, the homologue of the yeast p34^{cdc2} (Gould, K.L., J. Newport, unpublished observations). A slightly lower molecular weight form, p34^{Eg1} (Paris and Philippe, 1990; Paris et al., 1991) is also present, although as previously noted, it does not appear to be complexed with any of the cyclins we yet know (Minshull et al., 1990; Solomon et al., 1990). When oocyte extracts were passed over p13-Sepharose, B-type cyclins and cdc2-related proteins were retained both before and after progesterone treatment. These results suggest that B-type cyclins are already complexed with p34^{cdc2} in stage VI oocytes. Presumably, these represent pre-MPF. It remains to be seen precisely how progesterone leads to the activation of these cyclin-cdc2 complexes. It is worth noting that, whereas stage VI oocytes contain predominantly cyclin B2, mature eggs contain equal amounts of cyclins B1 and B2; hence, the composition of the MPF that appears before meiosis I is somewhat different from that present in cytostatic factor-arrested eggs. It is possible that this difference has functional consequences, and it also means that the classical MPF from *Xenopus* oocytes contains at least two slightly different kinases.

Despite the clear evidence for cyclin synthesis and accumulation we show in this paper, we present evidence in the following paper that oocyte maturation can occur in the complete absence of cyclin synthesis, which means that the maternal stockpile of cyclin is sufficient to activate cdc2 kinase. Despite the impressive progress of recent years, there is still much to be learned about the control of meiosis in *Xenopus* oocytes.

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