A Nuclear Factor Required for Specific Translation of Cyclin B May Control the Timing of First Meiotic Cleavage in Starfish Oocytes

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In most animals, the rate of cyclin B synthesis increases after nuclear envelope breakdown during the first meiotic cell cycle. We have found that cyclin B-cdc2 kinase activity drops earlier in emetine-treated than in control starfish oocytes, although the protein synthesis inhibitor does not activate the cyclin degradation pathway prematurely. Moreover, protein synthesis is required to prevent meiotic cleavage to occur prematurely, sometimes before chromosomes have segregated on the metaphase plate. In normal conditions, increased synthesis of cyclin B after germinal vesicle breakdown (GVBD) balances cyclin degradation and increases the time required for cyclin B-cdc2 kinase to drop below the level that inhibits cleavage. Taken together, these results point to cyclin B as a possible candidate that could explain the need for increased protein synthesis during meiosis I. Although direct experimental evidence was not provided in the present work, cyclin B synthesis after GVBD may be important for correct segregation of homologous chromosomes at the end of first meiotic metaphase, as shown by a variety of cytological disorders that accompany premature cleavage. Although the overall stimulation of protein synthesis because of cdc2 kinase activation is still observed in oocytes from which the germinal vesicle has been removed before hormonal stimulation, the main increase of cyclin B synthesis normally observed after germinal vesicle breakdown is suppressed. The nuclear factor required for specific translation of cyclin B after GVBD is not cyclin B mRNA, as shown by using a highly sensitive reverse transcription followed by polymerase chain reaction procedure that failed to detect any cyclin B mRNA in isolated germinal vesicles.

INTRODUCTION

During the process of oogenesis, oocytes of most animal species undergo meiotic arrest before the completion of chromosomal reduction followed by tremendous growth. The length of time that oocytes remain in this arrested state and the nature of the stimulus that reinitiates meiosis are species-dependent (see Masui and Clarke, 1979 for review). However, action of this variety of stimuli is indirect in reinitiating meiosis. They first activate a nonspecies specific cytoplasmic factor called maturation-promoting factor (MPF), which was extensively purified from starfish and *Xenopus* oocytes and identified to cyclin B-cdc2 kinase, the master regulator in the mitotic control network (see Dorée 1990; Nurse, 1990 for reviews).

During the mitotic cell cycle, exit from mitosis requires cyclin degradation, which is mediated by an ubiquitin-dependent process (Glutzer *et al.*, 1991). Cyclin degradation is followed by dephosphorylation of the cdc2 subunit on threonine 161 (Lorca *et al.*, 1992a), and both events bring about MPF inactivation. Then chromosomes segregate, and a nuclear envelope reforms around the decondensing chromosomes. Although MPF activity also drops to some extent between the two meiotic divisions (Dorée *et al.*, 1983; Gerhart *et al.*, 1984), as does the overall state of protein phosphorylation (Dorée *et al.*, 1983), chromosomes stay condensed, and a nuclear envelope does not reassemble. This may suggest that a control mechanism specific for meiotic maturation limits depletion of cyclin B because of its proteolysis at ana-

phase of the first meiotic cell cycle. Alternatively, the interval of time between first and second meiotic metaphase may simply be too short for chromosome decondensation and nuclear envelope assembly to proceed.

In most animals, the overall rate of protein synthesis increases around the time of MPF activation during the first meiotic cell cycle (Smith and Ecker, 1971; Baltus *et al.*, 1973; Guerrier and Dorée, 1975; Blankstein and Kiefer, 1977; Schultz *et al.*, 1978). This overall stimulation may depend on phosphorylation of the S6 and S1 ribosomal proteins, which was reported to be strongly increased as a consequence of MPF activation in hormone-stimulated oocytes (Hanocq-Quertier and Baltus, 1981; Nielsen *et al.*, 1982; Peaucellier *et al.*, 1988) or on maturation-specific polyadenylation of mRNA (Rosenthal *et al.*, 1982; Mc Grew *et al.*, 1989; Paris and Richter, 1990). One of the major proteins whose synthesis increases when oocytes are released from arrest at the G2-prophase boundary is cyclin B (Standart *et al.*, 1987; Kobayaishi *et al.*, 1991; Van Loon *et al.*, 1991).

However, new synthesis of cyclin B does not appear to be required, at least not for oocytes to complete the first meiotic cell cycle (Gerhart *et al.*, 1984; Picard *et al.*, 1985; Neant and Guerrier, 1988; McGrew *et al.*, 1989, 1990; Westendorf *et al.*, 1989; Dubé and Dufresne, 1990; Minshull *et al.*, 1991; Hunt *et al.*, 1992) with the possible exception of mammals, which were reported not to form a functional meiotic spindle in the absence of protein synthesis (Hashimoto and Kishimoto, 1988). It was even extrapolated from biochemical data that complete meiotic maturation of *Xenopus* oocytes would occur in the absence of any cyclin synthesis (Minshull *et al.*, 1991), although neither formation of meiotic spindles nor meiotic cleavages were analyzed in this study.

In the present work, we investigated how changes in the rate of cyclin B synthesis and degradation regulate the size of the cyclin B pool during the first meiotic cell cycle in starfish oocytes. We found that although protein synthesis does not appear to be required for completion of the first meiotic cell cycle, it is required to limit growth of the meiotic spindle and prevent premature meiotic cleavage and emission of the first polar body. Cyclin B-cdc2 kinase activity drops earlier in emetine-treated than in control oocytes. Nonetheless, the protein synthesis inhibitor does not advance cyclin degradation at the end of first meiotic cell cycle. In addition we show that an unidentified component, sequestered before hormonal stimulation in the nucleus of G2-arrested oocytes, is required for specific stimulation of cyclin B translation after nuclear envelope breakdown in maturing oocytes. Therefore our results suggest that a specific mechanism enhances synthesis of cyclin after nuclear envelope breakdown and introduces a delay in the timing of subsequent meiotic events during this crucial cell cycle which allows segregation of appaired chromosomes. Cyclin B synthesis was however not blocked specifically by injection of anti-sens oligonucleotides in the present

work, thus cyclin B is only one possible candidate that could explain the need for increased protein synthesis during meiosis I.

MATERIAL AND METHODS

Handling Starfish Oocytes

Females of the starfish *Astropecten aranciatus* and *Asterias rubens* were collected near Banyuls and Roscoff, respectively, during their breeding season and kept in running natural sea water before use. Prophase-arrested oocytes were prepared free of follicle cells by washing them several times in artificial Ca^{2+} -free sea water (Dorée and Guerrier, 1975) and finally were transferred to natural seawater. Meiotic maturation was induced by adding 1 μM 1-MeAde (Sigma, St. Louis, MO). The procedure used to enucleate oocytes has been described previously (Picard *et al.*, 1988a).

Immunochemical Procedures

The polyclonal antibodies against starfish cyclin B and the immunoblotting or immunoprecipitation procedures have been described previously (Picard *et al.*, 1991), as well as the procedures used for cytological observation of microtubule networks and chromosomes (Picard *et al.*, 1988a).

Labeling Oocytes with [^{35}S]-Methionine

[^{35}S]-methionine (Amersham, Arlington Heights, IL) was added to sea water containing oocytes to a final concentration of 300 $\mu\text{Ci}/\text{ml}$. Incorporation of radioactivity into proteins was stopped by transferring oocytes either in sea water containing 100 mM methionine or both 100 mM methionine and 0.1 mM emetine. At the end of the pulse-labeling period, oocytes were fixed in Laemmli buffer, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were dried, and the pattern of protein synthesis was analyzed by autoradiography.

Assay of H1 Kinase Activities

Oocytes were taken in 2 μl of a buffer containing 8 mM Na- β glycerophosphate, 1.5 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mM MgCl_2 , and 0.5 mM dithiothreitol (DTT) at pH 7.4 and frozen in liquid nitrogen. After thawing the samples, 8 μl of a reaction mixture containing 20 mM Hepes pH 7.4, 10 mM MgCl_2 , 1 mM DTT, 1 mg/ml H1 histone (Boehringer Mannheim, Indianapolis, IN), and 0.1 mM ATP (300 cpm/pmol) was added. Reaction was allowed to proceed for 10 min at room temperature and stopped by adding Laemmli buffer. Phosphorylation of H1 histone was quantified by SDS-PAGE followed by autoradiography and counting the radioactive area by liquid scintillation counting.

Cyclin B Oligonucleotides and Proteins

The sequences of the two cyclin B oligonucleotides primers are: 5'-GCT CTG CAG CGA GTT TGT AA-3' and 5'-ATA TTG CAC TCC ATG GAA CG-3'. They hybridize to the central coding region of *Marthasterias glacialis* cyclin B cDNA (Labbé *et al.*, 1989) and yield a 372-base pair (bp) product. The two c-mos oligonucleotides primers are: 5'-AGA CCA GTG CCG AGA G-3' and 5'-TGT TCA GTC ACA CGG AGG AAG-3'. These two oligonucleotides primers hybridize to the 3' terminal coding region of *Xenopus* c-mos cDNA (Freeman *et al.*, 1989) and yield a 193-bp product. Taq polymerase was obtained from Promega (Madison, WI) and avian myeloblastosis virus (AMV) reverse transcriptase (RT) and RNase A from GIBCO (Grand Island, NY). The oligonucleotides were synthesized on an Applied Biosystem 380 B DNA synthesizer (Foster City, CA) then purified on a G25 column (Pharmacia, Piscataway, NJ). Goldfish GST-cyclin B, prepared as previously described (Hirai *et al.*, 1992), was a generous gift of Dr. Yamashita, Okazaki, Japan.

RNA Preparation

Thirty germinal vesicles were sucked up from fully grown oocytes into a sterile micropipet containing ~2 ng of in vitro translated *Xenopus* c-mos mRNA (control). The content of the micropipet was immediately mixed with 40 μ l of lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 100 mM DTT, 0.5% sodium lauroyl sarcosinate, and 1 μ g *Escherichia coli* 5S RNA). The corresponding 30 enucleated oocytes were also taken in 40 μ l of lysis buffer. Total RNAs were extracted according to Belyavsky *et al.* (1989) and subsequently resuspended in 5 μ l diethyl pyrocarbonate-treated water.

RT-Polymerase Chain Reaction (PCR)

The first strand was synthesized as follows. One-half of total nuclear RNAs and the entire amount of RNA extracted from enucleated oocytes were specifically reverse transcribed with cyclin B reverse-primer oligonucleotide in 25 μ l of a buffer containing 50 mM tris(hydroxymethyl)aminomethane(Tris)-HCl pH 8.3, 40 mM KCl, 1 mM DTT, 6 mM MgCl₂, 0.4 mM of each dNTP, 40 pmol of each primer oligonucleotide and 2.5 units AMV-RT. Synthesis was allowed to proceed for 120 min at 37°C. As a control, the other half of total nuclear RNAs was reverse transcribed according to the same procedure with c-mos reverse primers.

The PCR amplification step was conducted in a total volume of 50 μ l containing 25 μ l of the previous RT reaction mixtures supplemented with 50 pmol of each primer oligonucleotide, 50 μ M of each dNTP, 50 mM KCl, 10 mM Tris HCl pH 8.8, 1.5 mM MgCl₂, 0.1% Triton X-100, and 1 unit Taq polymerase. Each sample was overlaid with 40 μ l mineral oil and incubated in a Biometra Trio Thermoblock DNA thermal cycler according to the following sequence: 5 min at 80°C followed by 3 min at 81°C (when Taq polymerase is added), then 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 2 min, and finally 10 min at 72°C.

RESULTS

Newly Synthesized Cyclin B Is Destroyed at First Meiotic Anaphase in Hormone-stimulated Starfish Oocytes

In starfish oocytes, maturation is controlled by a gonadostimulin originating from radial nerves (Kanatani, 1964), which itself induces the release from follicle cells of 1-methyladenine (1 MeAde), the natural effector of meiotic maturation (Kanatani *et al.*, 1969). Although starfish oocytes arrested at first meiotic prophase already contain a preformed and inactive cyclin B-cdc2 complex (Strausfeld *et al.*, 1991), readily enter first meiotic cycle, form a meiotic spindle, and emit first polar body in the absence of protein synthesis (Houk and Epel, 1974; Guerrier and Dorée, 1975; Dorée, 1982; Picard *et al.*, 1985), preliminary results showed that cyclin B is one of the most efficiently synthesized proteins during first meiotic cell cycle.

Why do oocytes produce cyclin B in excess if it is not required for the first meiotic cell cycle? Both cdc2 kinase and the overall state of protein phosphorylation rise very rapidly after their drop at first meiotic anaphase in starfish oocytes (Dorée *et al.*, 1983; Picard *et al.*, 1985). Moreover, the second meiotic cell cycle is shorter than the first one, as also observed in most animals that like starfish do not arrest at second meiotic metaphase (see

Masui and Clarke, 1979 for review). Therefore, one possibility could be that excess cyclin is synthesized during the first meiotic cell cycle to speed up the next one, although protein synthesis is still required for maturing oocytes to enter second meiotic metaphase (Picard *et al.*, 1985). To investigate this possibility, oocytes of the starfish *A. aranciatus* were induced to mature with 1-MeAde, and newly synthesized proteins, including cyclin B, were pulse-labeled for 10 min with ³⁵S-methionine just after germinal vesicle breakdown (GVBD). Then samples were taken at various times of meiotic maturation and analyzed for stability of newly synthesized cyclin. As shown in Figure 1, >95% of cyclin B molecules that were synthesized during the pulse had disappeared just after first polar body emission. Thus, the burst of cyclin B synthesis after GVBD is not aimed to speed up second meiotic cell cycle.

Protein Synthesis Is Required to Prevent Premature Exit from First Meiotic Cell Cycle in Maturing Starfish Oocytes

As shown in Table 1, first polar body was emitted 4–6 min earlier in oocytes of the starfish *A. aranciatus* whose protein synthesis was suppressed by adding 0.1 mM

Figure 1. Autoradiograms showing that cyclin B is one of the major neosynthesized proteins during first meiotic cell cycle. (a) Oocytes of the starfish *A. aranciatus* were pulse-labeled for 10 min in the presence of 300 μ Ci/ml ³⁵S-methionine either before hormonal stimulation (lane 1) or 35 min after hormonal stimulation (5 min after GVBD, lanes 2 and 3). Oocytes were fixed either just at the end of the labeling period (lanes 1 and 2) or 30 min later, at the time of first polar body emission (lane 3). In all instances, the pulse-labeling period was terminated by transferring oocytes in a large excess sea water containing 100 mM methionine. The same amount of solubilized oocytes was loaded in lanes 1–3. The arrowhead points to cyclin B, the major newly synthesized protein after GVBD that disappears at the end of first meiotic cell cycle. (b) Same experiment as in a, but cyclin B was immunoprecipitated with a polyclonal antibody raised against starfish cyclin B before running the gel. (c) Quantification of data in b by scanning the autoradiogram (arbitrary units).

Table 1. Blocking protein synthesis advances the time of first polar body emission in oocytes of the starfish *Astropecten aranciatus*

Experiment N°	Time of first polar body emission ^a	
	No emetine (control)	0.1 mM emetine
I	75 ± 3 min.	69 ± 4 min.
II	78 ± 4 min.	72 ± 5 min.
III	74 ± 3 min.	70 ± 3 min.
IV a	78 ± 7 min.	72 ± 9 min.
IV b	∞	∞

^a The kinetics of first polar body emission were monitored as a function of time elapsed from 1-MeAde addition in four separate experiments (four different females). Emetine was added 10 min after 1-MeAde. Values refer to the times when 50% of the oocytes (200 oocytes counted in experiments I–III, 20 oocytes counted in experiment IV) had completely emitted the first polar body. In experiment IV 20 oocytes were injected before hormonal stimulation with either 0.1 mg/ml GST-cyclin B (IVb) or the same volume (400 pl) of distilled water (IVa).

emetine 15 min after 1-MeAde addition, thus before the increased synthesis of cyclin B. In contrast cleavage was not advanced when protein synthesis was suppressed later. This suggested that premature cleavage could be because of suppression of cyclin B synthesis after GVBD. Cyclin B was thus quantified from immunoblots along first meiotic cell cycle in control and emetine-treated oocytes. As shown in Figure 2, premature cleavage was indeed associated with both a premature drop in the amount of cyclin B and premature cyclin B-cdc2 kinase inactivation in emetine-treated oocytes.

The above results were open to two different interpretations. Emetine could speed up cyclin degradation during first meiotic cell cycle (for example by suppressing synthesis of an inhibitor of cyclin degradation). Alternatively, increased synthesis of cyclin B after GVBD could be required to balance cyclin degradation during first meiotic cell cycle and to slow down disappearance of cdc2 kinase activity. According to this view, the time of activation of the cyclin degradation pathway should be the same in the presence or absence of emetine.

To discriminate between both possibilities, oocytes of the starfish *A. aranciatus* were released from G2/prophase arrest by 1-MeAde and ³⁵S-methionine added 30 min later, thus 5 min after nuclear envelope breakdown. Oocytes were incubated for 10 min in the presence of ³⁵S-methionine, then diluted in 1000 vol of sea water containing either 100 mM unlabeled methionine or both 100 mM methionine and 0.1 mM emetine to stop incorporation of ³⁵S-methionine into proteins. Groups of 10 oocytes were then solubilized in Laemmli buffer at various times from isotopic dilution to first meiotic cleavage to monitor degradation of ³⁵S-methionine-labeled cyclin B by SDS-PAGE and autoradiography. As expected, first meiotic cleavage occurred ~5 min earlier

in emetine-treated oocytes. Nonetheless, neither premature nor amplified degradation of ³⁵S-labeled cyclin B was detected in such oocytes (Figures 3 and 4a). The same result was obtained when oocytes were preloaded overnight with ³⁵S-methionine before hormonal stimulation (Figure 4b). We conclude that treatment of starfish oocytes with emetine, which blocks protein synthesis and advances first meiotic cleavage, results in a premature and more extensive drop of cyclin B concentration at the end of the first meiotic cell cycle without advancing the onset of cyclin degradation.

Thus, the difference of cyclin B content between control and oocytes treated at the time of hormonal stimulation with emetine is at any time an estimate of the amount of cyclin B that was newly synthesized during meiotic maturation in control oocytes (Figure 2b). Significant degradation of cyclin B already occurs shortly after GVBD. From GVBD to first meiotic cleavage, the

Figure 2. The amount of cyclin B and the H1 kinase activity of cyclin B-cdc2 drop prematurely at the end of first meiotic cell cycle in emetine-treated oocytes of the starfish *A. aranciatus*. (a) Immunoblot showing variations in the amount of cyclin B during the course of the first meiotic cell cycle in control and emetine-treated oocytes (all samples were run on the same gel). Zero corresponds to the time of hormone addition. Arrows point to the times when 50% of the oocytes reached metaphase (M) or completed first polar body (p.b.) emission, respectively. (b) Quantification of Figure 2a by scanning the immunoblots (arbitrary units). The dashed line (synthesis) shows the difference between the amount of cyclin B in control and emetine-treated oocytes at a given time. (c) Changes of cyclin B-cdc2 kinase activity along first meiotic cell cycle in control and emetine-treated oocytes. At the indicated times, homogenates were prepared from 10 oocytes, and H1 histone kinase activity (expressed as femtomoles of phosphate transferred to H1 histone/min/oocyte) was assayed in anti-cyclin B immunoprecipitates.

Figure 3. Emetine does not prematurely open the window for cyclin degradation during first meiotic cell cycle. Thirty minutes after hormonal stimulation, oocytes of the starfish *A. aranciacus* were pulse-labeled for 10 min with 300 $\mu\text{Ci}/\text{ml}$ ^{35}S -methionine. The 10-min labeling period was terminated by transferring aliquots of the oocyte suspension in 1000 vol of sea water containing either 100 mM unlabelled methionine alone (top) or both 100 mM methionine and 0.1 mM emetine (bottom). Aliquots were taken at the indicated times and run on SDS-gels; changes in the pattern of newly synthesized proteins were analyzed by autoradiography. Arrowheads point to cyclin B. The same batch of oocytes was used for experiments depicted in Figures 2 and 3.

pool of "old" cyclin B is progressively replaced by newly synthesized cyclin B. Earlier investigators failed to detect degradation of cyclin B starting shortly after the time of GVBD in starfish oocytes because they monitored newly synthesized, not the whole pool, cyclin B (Standard *et al.*, 1987). Another interesting result is that the pool of cyclin B at second meiotic metaphase is only about one-third of that found at prometaphase of the first meiotic cell cycle (not shown). This may be related to the shorter duration of the second meiotic cell cycle as compared to the first one.

In view of previous reports that inhibition of cyclin degradation or microinjection of undegradable cyclins

suppresses cleavage (Picard *et al.*, 1985; Murray *et al.*, 1989; Ghiara *et al.*, 1991; Gallant and Nigg, 1992), the above results suggest that emetine caused premature cleavage by suppressing cyclin B synthesis, thereby allowing cyclin B-cdc2 kinase to drop prematurely below the level that prevents meiotic cleavage to occur. To provide a more direct evidence in support of this view, we microinjected GST-cyclin B in emetine-treated oocytes and monitored the time of first meiotic cleavage in cyclin B- or mock-injected oocytes. Mock-injected oocytes readily emitted first polar body, and as expected earlier, in the presence of emetine. In contrast, meiotic cleavage did not occur in oocytes injected with the undegradable cyclin, even in the presence of emetine, although they readily underwent GVBD (Table 1, experiment IV). In this experiment the injected cyclin B may have caused M phase arrest by inhibiting destruction of endogenous cyclins. Conversely, this experiment supports the view that emetine advances cleavage by suppressing synthesis of cyclin B not that of another protein.

Blocking Protein Synthesis Impairs First Meiotic Cell Cycle in Starfish Oocytes

Emetine-induced premature cdc2 kinase inactivation resulted in cytological disorders as shown by microtubules and DNA staining. Although no obvious difference with control was detected until 50 min after hormonal stimulation, first polar body was bigger and emitted prematurely in emetine-treated oocytes. After cdc2 kinase had dropped prematurely, chromosomes did not usually align correctly onto a metaphase plate; spindle-enlarged and microtubule-driven chromosomes did not properly segregate (Figure 5). The extent of cytological disorders was quite variable among oocytes. In some cases metaphase occurred quite normally, although spindle rotation and first polar body were advanced. In others cases spindle did not form at all, thus first polar body was not emitted. In some starfish species (for example *A. rubens*) blocking protein synthesis at the time of hormonal stimulation resulted in the formation of an enormous polar body (Figure 6), and first meiotic cleavage was even more dramatically advanced, occurring in some females as much as 25 min earlier than in control oocytes. This suggested that advanced cleavage was not simply because of shortening of metaphase. In agreement with this view, anomalous segregation of chromosomes was observed at the normal time of prometaphase in emetine-treated oocytes. Whereas in control oocytes appaired chromosomes segregated with separating asters shortly after GVBD before moving back at metaphase in the equatorial plane of the meiotic spindle, furrowing already occurred at that time in emetine-treated oocytes, resulting in aberrant distribution of chromosomes (Figure 7).

The above results strongly suggested that enhanced translation of cyclin B after GVBD is essential for cy-

Figure 4. Timing of ³⁵S-labeled cyclin B degradation during first meiotic cell cycle. (a) Quantification of data in Figure 3. Autoradiograms were scanned and quantified for ³⁵S-cyclin B (arbitrary units). (b) Oocytes were preloaded for 18 h with ³⁵S-methionine, washed, and induced to mature in the presence or absence of emetine. Samples were taken at the indicated times, and their ³⁵S-cyclin B content was determined after immunoprecipitation.

tological events of the first meiotic cell cycle to proceed normally. To investigate the possibility that some component of the germinal vesicle, released in cytoplasm after breakdown of its envelope, could increase at that time the rate of cyclin B translation, the content of a germinal vesicle was sucked up into a micropipet from an immature donor oocyte and microinjected into an

immature recipient oocyte. The injected oocyte was then induced to mature with 1-MeAde together with control noninjected oocytes, and 5 min after GVBD they were pulse-labeled for 10 min with ³⁵S-methionine then solubilized in Laemmli buffer. Proteins were separated by SDS-PAGE, and the amount of labeled cyclin was estimated by autoradiography. As shown in Figure 8 (lanes

Figure 5. Emetine impairs events of first meiotic cell cycle in oocytes of the starfish *A. aranciatus*. The top panels show antitubulin immunofluorescence, and the lower panels show the corresponding Hoechst fluorescence, revealing the distribution of chromosomes. (a and b) Control maturing oocytes at early metaphase (a) and late metaphase after spindle rotation (b). (c) Maturing oocytes treated with 0.1 mM emetine 10 min after hormonal stimulation with 10^{-6} M 1-MeAde and fixed at the time of "metaphase".

Figure 6. Blocking protein synthesis results in the formation of an enormous first polar body in oocytes of the starfish *A. rubens*. (a) Micrograph taken 70 min after hormonal stimulation of a control oocyte (after first polar body emission, arrow). (b) Micrograph taken 60 min after hormonal stimulation of an oocyte treated 10 min before hormonal stimulation with 0.1 mM emetine. Bar, 50 μ m.

1 and 2), synthesis of cyclin B was higher in the microinjected oocyte. The same experiment was repeated, but this time the contents of two germinal vesicles were microinjected. As shown in Figure 8 (lane 3), the extent of cyclin B synthesis increased with the amount of microinjected nuclear material.

We showed previously that microinjection of the content of a germinal vesicle triggers cdc2 kinase acti-

vation in recipient starfish oocytes (Picard and Dorée, 1984; Picard *et al.*, 1988b). Therefore the above experiments did not, at first sight, discriminate between a direct stimulation of cyclin synthesis, because of microinjecting nuclear sap in cytoplasm, and an indirect stimulation, merely because of activation of cdc2 kinase by microinjected nuclear material. Nonetheless we found that the extent of cdc2 kinase activation in recip-

Figure 7. Blocking protein synthesis induces premature furrowing and severely impairs chromosome distribution at prometaphase in oocytes of the starfish *A. rubens*. The top panels show antitubulin immunofluorescence, and the lower panels show the corresponding Hoechst fluorescence. (a) Control oocyte fixed at prometaphase 35 min after hormonal stimulation. Well-formed asters are separating, and the metaphase spindle is not yet formed. Note that chromosomes are exclusively distributed in the immediate vicinity of the meiotic asters. (b) Emetine-treated oocyte fixed 35 min after hormonal stimulation (simultaneously with the control oocyte shown in a). The top panel shows that furrowing already occurred (arrow). One of the meiotic asters is anchored in the cortex; the second one is enormous and its center somewhat difficult to localize (arrowhead). The bottom panel shows that part of the chromosomes segregated (although imperfectly) with the giant aster, but the others failed to segregate with the cortex-anchored aster. Bar, 30 μ m.

Figure 8. A component of the germinal vesicle increases the extent of cyclin B translation in *A. aranciacus* oocytes. Lane 1: 5 min after GVBD, a maturing oocyte was incubated for 10 min with 300 $\mu\text{Ci/ml}$ ^{35}S -methionine, solubilized in Laemmli buffer, and analyzed by SDS-PAGE and autoradiography for cyclin B synthesis (arrowhead). Lane 2: same experiment as in 1, but the content of a germinal vesicle (taken from an unlabelled oocyte) was microinjected before hormonal stimulation. Lane 3: same experiment as in 1 or 2, but the contents of two germinal vesicles were microinjected (the same experiment was repeated 5 times with similar results).

ient oocytes (from <2 to ~ 340 femtomol of P transferred to H1 histones/min/oocyte) was independent of the number of microinjected nuclei, in contrast to stimulation of cyclin synthesis, that increased in proportion of this number. This suggested that stimulation of cyclin synthesis was at least partially a direct dose-dependent effect of the microinjected nuclear material rather than simply a consequence of cdc2 kinase activation.

In the next experiment the germinal vesicle was removed, and enucleated oocytes were put simultaneously with nucleated immature oocytes (control) into sea water containing 10^{-6}M 1-MeAde. Then groups of 10 oocytes, enucleated or not (control), were transferred every 10 min, starting from hormonal stimulation, in sea water containing 1 mCi/ml ^{35}S -methionine in the continuous presence of 10^{-6}M 1-MeAde. After 10 min pulse-labeling, homogenates were prepared from each group of 10 oocytes and used for quantification of ^{35}S -methionine-labeled cyclin B. Striking differences were observed in the pattern of cyclin B synthesis (Figure 9). In control oocytes, a moderate stimulation of cyclin B synthesis was detected simultaneously with the overall stimulation of protein synthesis around the time when the first cytological signs of nuclear envelope disorganization (germinal vesicle "fading") became obvious. Then a major increase of cyclin B synthesis occurred ~ 10 min after nuclear envelope breakdown. This increase was highly specific for cyclin B. No significant increase in the synthesis of any other protein was detected at that time with exception of a doublet of low molecular weight proteins (presumably histones). This large and specific stimulation of cyclin B synthesis was not observed in enucleated oocytes, although the moderate and nonspecific stimulation that follows cdc2 kinase activation was readily detected. Taken together, the above results demonstrate that a nuclear factor sequestered in the germinal vesicle and released in cytoplasm after nuclear envelope breakdown is required

for a specific burst of cyclin B translation to occur during the first meiotic cell cycle of starfish oocytes.

We next considered the possibility that this nuclear factor could be cyclin B mRNA (Figure 10). Total RNA was extracted from either 15 manually isolated germinal vesicles (lane 2) or the 15 corresponding enucleated oocytes (lane 4), then both extracts were monitored for the presence of cyclin B mRNA using the RT approach followed by a PCR step. As a control, a small amount of *in vitro*-transcribed *Xenopus* c-mos mRNA was introduced in the tip of the sterile micropipet used to suck up the germinal vesicles and mixed with the nuclear sap released from them in the micropipet before RNA extraction (lane 3). The expected length of the amplified products were 372 and 193 bp for cyclin B and c-mos, respectively (see MATERIAL AND METHODS). As shown in Figure 9, no cyclin B mRNA was detected in nuclei, whereas a strong and specific amplification was observed in enucleated oocytes. Specific amplification of cyclin B was readily detectable even using a single

Figure 9. Overall stimulation of protein synthesis occurs, but specific stimulation of cyclin B synthesis is suppressed in hormone-stimulated oocytes from which the germinal vesicle has been removed. Top: groups of 10 oocytes were taken at the indicated times after 1-MeAde addition and pulse-labeled with 1 mCi/ml ^{35}S -methionine. After 10 min the 10 oocytes were taken and fixed with Laemmli buffer. Newly synthesized proteins were analyzed by SDS-PAGE and autoradiography. Arrowhead points to cyclin B. Bottom: same experiment as in top, but oocytes were first enucleated by sucking up the germinal vesicle with a micropipet before adding 1-MeAde.

Figure 10. The germinal vesicle does not sequester cyclin B mRNA in starfish oocytes. Total RNA extracted either from manually isolated germinal vesicles or from the corresponding enucleated oocytes was assayed for the presence of cyclin B mRNA by RT-PCR (see MATERIALS AND METHODS). (1) Standard oligonucleotides (From top to bottom: 1358, 1078, 872, 603, 310, 234, 194, and 118 bp). (2) Total RNA was extracted from germinal vesicles. (3) Control: same experiment as in lane 2 but *Xenopus* c-mos mRNA (2 ng) was added in the micropipet used to collect the germinal vesicles. (4) RNA was extracted from 15 enucleated oocytes. (5) RNA was extracted from a single enucleated oocyte. (6) Same experiment as in lane 5 but the sample was treated with 1 μ g RNase before the RT-PCR step.

oocyte (lane 5). Absence of amplified cyclin B products in the nuclear material was not because of mRNA degradation because the c-mos control mRNA was readily amplified.

The above results rule out the possibility that cyclin B mRNA might be sequestered to a significant extent in the germinal vesicle of immature oocytes. The nuclear factor required for the dramatic and specific stimulation of cyclin B translation after GVBD has not yet been identified.

DISCUSSION

The eukaryotic cell cycle is driven by changes in the activity of complexes between cdc2 (or cdc2-like proteins) and cyclins. Cyclins have been classified into G1 and G2-M cyclins, depending on the function they are supposed to play in regulating cell cycle. Mechanisms that control expression of G1 cyclins are the object of intense investigation (see McKinney and Heintz, 1991; Reed, 1991; Sherr, 1993 for reviews), but paradoxically little is known about regulation of G2/M cyclins expression although they were first discovered (Evans *et al.*, 1983). Yet, expression of G2/M cyclins has been reported to change along cell cycle. For example, both transcription and translation of cyclin A seem to start coincidentally with entry into S-phase in mammalian cell lines (Pines and Hunter, 1990; Girard *et al.*, 1991; Pagano *et al.*, 1992) as well as in the regenerating liver (Lu *et al.*, 1992; Zindy *et al.*, 1992). Oocytes, which were

excellent models for biochemical investigations leading to identification of MPF and of mechanisms that control its activity, may also prove to be suitable tools to investigate expression of G2/M cyclins and its regulation.

Recently, it was shown that the amount of cyclin B1 increases as much as 3–10-fold during progesterone-induced meiotic maturation of *Xenopus* oocytes, but the mechanism responsible for this increase was not further analyzed (Kobayashi *et al.*, 1991). In goldfish oocytes 17 α -20 β -dihydroxy-4-pregnen-3-one, the maturation-inducing steroid in this species, was also reported to trigger cyclin B synthesis. Such oocytes, which do not appear to contain a preformed cyclin B-cdc2 complex before hormonal stimulation, look promising to investigate the hormonal control of G2/M cyclins expression (Hirai *et al.*, 1992).

In starfish oocytes, which contain a preformed cyclin B-cdc2 complex, cyclin B synthesis has been reported to increase after hormonal stimulation (Standart *et al.*, 1987). This increased synthesis was suggested to be because of an increase in the extent of cyclin B mRNA polyadenylation that indeed occurs at that time. Because the poly-A tail of many mRNAs increases in length after hormonal stimulation of oocytes (Jeffery, 1977; Mc Grew *et al.*, 1989; Mc Grew and Richter, 1990; Paris and Richter, 1990; Wickens *et al.*, 1990), the possibility was not previously considered that some specific mechanism could be involved in the dramatic increase of cyclin B synthesis. In the present report we monitored changes in the amount of cyclin B during the first meiotic cell cycle of starfish oocytes, as well as its kinetics of synthesis and degradation. At variance with *Xenopus* oocytes, no increase in the amount of cyclin B was detected during meiotic maturation, although a dramatic increase in cyclin B synthesis occurred after GVBD. Although enucleation did not suppress the overall stimulation of protein synthesis observed after cdc2 kinase activation in hormone-stimulated oocytes, the main increase of cyclin B synthesis normally observed 5–10 min after germinal vesicle breakdown did not occur in oocytes from which the germinal vesicle had been removed before hormonal stimulation. In contrast, microinjection of the content of supernumerary germinal vesicles in nucleated oocytes increased in a dose-dependent fashion cyclin B translation, without having such an effect on translation of other proteins. These results strongly suggested that a nuclear factor, released after nuclear envelope breakdown in cytoplasm of hormone-stimulated oocytes, strongly and specifically stimulated cyclin B translation.

The possibility was considered that this factor could be cyclin B mRNA. According to this view, translation of cyclin B would be limited in hormone-stimulated enucleated oocytes by the amount of cytoplasmic mRNA. This possibility was ruled out by using a highly sensitive RT-PCR procedure, which failed to detect any cyclin B mRNA in isolated germinal vesicles. Therefore

the nuclear factor essential for enhanced cyclin B translation after nuclear envelope breakdown is not cyclin B mRNA but rather a factor that specifically recognizes a motif in cyclin B mRNA, perhaps related to the unidentified maternal factor responsible for specific localization of cyclin B transcripts in syncytial *Drosophila* embryos (Raff *et al.*, 1990). At the present time of our investigations, we do not know what it is, although it certainly plays an important role in timing and regulating events of the first meiotic cell cycle.

Increased cyclin B synthesis is observed after GVBD in both invertebrate and vertebrate (Dubé and Dufresne, 1990; Kobayaishi *et al.*, 1991; Van Loon *et al.*, 1991), and it may be a general feature in animal kingdom. It may be required for arrest at first meiotic metaphase, which is frequently observed in invertebrate (see Masui and Clarke, 1979, for review). In agreement with this view, *Patella* oocytes exit from metaphase I when microinjected with cyclin antisense oligonucleotides (Van Loon *et al.*, 1991) as does a variety of molluscan eggs that escape metaphase I arrest when treated with protein synthesis inhibitors (Neant and Guerrier, 1988; Dubé and Dufresne, 1990). In species like the annelid *Sabellaria*, arrest at metaphase I appears to be facultative, as if it was playing a regulatory rather than an essential role in meiotic maturation (Peaucellier, 1977). Perhaps the specific mechanism for enhanced translation of cyclin B after GVBD is set at a very high level of activity in species that arrest at metaphase I.

Although in the absence of experiments showing that blocking cyclin B synthesis by injection of antisense oligonucleotides cyclin B is only one possible candidate that could explain the need for increased protein synthesis during meiosis I, our results support the view that enhanced translation of cyclin B after GVBD may be required to prevent scattering of meiotic chromosomes in the prematurely cleaving oocyte and to ensure their correct alignment on the metaphase plate. Indeed cyclin B-cdc2 kinase activity was found to drop earlier when the burst of enhanced cyclin B synthesis was suppressed by emetine, although the inhibitor did not prematurely turn on the cyclin degradation pathway, in contrast to previous assumptions (Kobayaishi *et al.*, 1991; Van Loon *et al.*, 1991). As a consequence, meiotic metaphase was bypassed, and first polar body was prematurely emitted. This confirms that inactivation of cdc2 kinase is a rate-limiting event for cleavage, as suggested by previous reports that expression of nondegradable mitotic cyclins arrests cells before cleavage (Murray *et al.*, 1989; Ghiara *et al.*, 1991; Luca *et al.*, 1991; Gallant and Nigg, 1992). It also fits the proposal that phosphorylation of myosin light chain by cyclin B-cdc2 kinase inhibits interaction with actin and prevents cytokinesis to occur until the kinase has been inactivated (Satterwhite and Pollard, 1992). Finally, our result that the size of the meiotic spindle increases considerably in emetine-

treated oocytes is also in agreement with a previous report that cyclin B-cdc2 kinase activity reduces the apparent growth rate of centrosome-nucleated microtubules (Verde *et al.*, 1990).

The cyclin degradation pathway is believed to be activated at the metaphase to anaphase transition of the cell cycle, with degradation of cyclin A starting somewhat earlier than that of cyclin B (Luca and Ruderman, 1989; Minshull *et al.*, 1990; Whitfield *et al.*, 1990; Hunt *et al.*, 1992). Our results show that this may not apply to the first meiotic cell cycle, at least in starfish oocytes, where degradation of cyclin B is already detected shortly after the time of GVBD. Cyclin B-cdc2 kinase has been shown to be the trigger of cyclin degradation during cell cycle (Felix *et al.*, 1990) and cyclin A-cdc2 kinase to delay this process to some extent, at least in vitro (Luca *et al.*, 1991; Lorca *et al.*, 1992b). During the first cycle of meiotic maturation, starfish oocytes do not appear to contain cyclin A-cdc2 kinase (Picard, unpublished data), as also observed in other animals (Luca *et al.*, 1990; Kobayaishi *et al.*, 1991). Moreover cyclin B-cdc2 kinase is rapidly activated after hormonal stimulation, at least partially because of accumulation of a preformed inactive complex in immature oocytes. Yet oocytes will enter metaphase only after a relatively long-lasting prometaphase: in the starfish *A. aranciacus*, for example, cyclin B-cdc2 kinase undergoes activation 6–8 min after hormonal activation, GVBD occurs about 20 min later, yet chromosomes will not align on the metaphase plate until 60 min after hormonal stimulation, thus prometaphase lasts as much as 40 min, about one-half of the first meiotic cell cycle. The early activation of cyclin B-cdc2 kinase, combined with the absence of cyclin A-cdc2 kinase to antagonize its effect, may explain the early activation of the cyclin degradation pathway during first meiotic cell cycle.

The different kinds of cells in an embryo are produced in a regular spatial pattern. This is a consequence of the initial polarity of the egg, which itself depends on the correct distribution of maternal mRNA during oogenesis and meiotic maturation. To limit loss of specific mRNAs and ensure their correct distribution during the course of meiotic maturation, oocytes restrict the area where disjunction of homologous chromosomes will occur. This is achieved without any exception in animal kingdom by restricting growth of the meiotic spindle to a very eccentric area. If the potentially catastrophic consequences for embryo development of impairing meiotic cleavage are considered, it is not surprising that oocytes may have developed specific strategies to limit growth of the meiotic spindle and to delay cleavage until they have checked that alignment of homologous chromosomes on the metaphase plate is correct. Possibly enhanced translation of cyclin B after is one of these strategies, which may have been widely used throughout evolution.

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