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Dynamics of Maturation-Promoting Factor and Its Constituent Proteins during In Vitro Maturation of Bovine Oocytes[1]

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ABSTRACT

Maturation-promoting factor (MPF) is known to be a key regulator of both mitotic and meiotic cell cycles. MPF is a complex of a B cyclin and the cyclin-dependent kinase cdk1 ($p34^{cdc2}$). Oocyte maturation and its arrest at metaphase of meiosis II (MII) are regulated by changes in MPF activity. In this study, experiments were conducted to examine the dynamics of MPF activity and its constituent proteins during in vitro maturation of bovine oocytes. Bovine oocytes displayed relatively low levels of MPF (histone H1 kinase) activity at the germinal vesicle stage during the first 8 h of maturation. MPF activity increased gradually thereafter, and its first peak of activity occurred at 12–14 h of maturation (presumptive metaphase I), which was followed by an abrupt reduction in activity at 16–18 h, during presumptive anaphase and telophase. MPF activity then increased, reaching a plateau at 20–24 h of maturation (MII stage). This high level of MPF activity was maintained for several hours but decreased gradually after 30 h of maturation and became barely detectable by 48 h of in vitro maturation (IVM) culture. At each time point, there was a significant variation among individual oocytes in histone H1 kinase activity, which was probably due to asynchronous maturation. Abundance of cdk1 increased gradually during the first 8 h and then remained relatively constant except for an apparent reduction at 18–22 h of IVM. The level of cyclin B_2 increased quickly during the initial 2 h of culture, and this high level was maintained until 16 h, after which a significant reduction was observed between 18 and 22 h of IVM. The de novo synthesis of cyclin B_2, however, exhibited a biphasic oscillation during maturation, with peaks before the onset of MI and of MII. These results have defined the profiles of MPF activity and its individual components during bovine oocyte maturation in vitro. We conclude that active MPF regulates bovine oocyte maturation and that de novo synthesis of cyclin B_2 occurs during the process of maturation.

INTRODUCTION

In most mammalian species, oocytes are formed during fetal life and are arrested at the prophase stage of the first meiotic division until around the time of ovulation. Resumption of meiosis in vivo requires hormonal stimulation, which leads to germinal vesicle (GV) breakdown (GVBD) and chromosomal condensation, followed by progression through metaphase of the first meiosis (MI), release of the first polar body, and then arrest at metaphase of the second meiosis (MII) [1]. Early investigations using amphibian oocytes and cytoplasmic transfer demonstrated that mature oocytes contain a powerful maturation-promoting factor (MPF) in their cytoplasm and that the meiotic arrest of these oocytes is maintained by a persistently high level of MPF activity [2, 3]. MPF was later found to be a universal cell cycle regulator of both mitosis and meiosis. It is a serine/threonine protein kinase composed of the catalytic subunit cyclin-dependent kinase 1 (cdk1, also known as $p34^{cdc2}$) and the regulatory subunit, cyclin B [4–6]. Active MPF induces chromosomal condensation, nuclear envelope breakdown, and cytoplasmic reorganization with entry into M-phase of either mitotic or meiotic cell cycles [7–12]. MPF activity during oocyte maturation is controlled by the association of cdk1 with cyclin B_2, forming pre-MPF, which is subsequently activated by specific dephosphorylation of the cdk1 by the homolog of the yeast phosphatase cdc25 [13, 14]. Mechanisms involved in the cell cycle regulation of the meiotic resumption and of the meiotic arrest have been studied extensively with *Xenopus* oocytes and more recently in limited studies in some mammalian oocytes, particularly mice [12, 15, 16].

MPF activity may be determined by measuring its kinase activity using exogenous histone H1 as a substrate [10]. Arion et al. [17] showed that H1 kinase and MPF activity are one and the same, and that histone H1 is an authentic member of the array of natural substrates of MPF. Fluctuations in MPF activity during oocyte maturation have been demonstrated in amphibian and marine invertebrate oocytes [17, 18]. Without exception, the results in all species studied so far showed that histone H1 kinase activity increases sharply at the G_2 to M-phase transition, decreases at the anaphase and telophase stages, and then increases at the metaphase of the second meiotic division [19]. These measurements have recently been extended to include changes in histone H1 kinase activity during maturation of mouse [20–22], pig [23–26], and rabbit [27] oocytes. To our knowledge, there have been no reports on the dynamics of MPF activity and its component proteins during oocyte maturation in cattle, although H1 kinase levels have been determined in matured bovine oocytes [28] and after activation of matured oocytes [29].

Cyclins were initially described as cell cycle-regulated proteins synthesized periodically during early embryonic development of marine invertebrates [30]. Molecular cloning of cyclin genes from a variety of eukaryotes revealed at least eight distinct cyclin genes in the mammalian genome [31]. Cyclins A, B_1, and B_2, and the G_2 cyclins are normally synthesized and then destroyed during meiotic division [32, 33]. Functional cyclin B protein is required for entry into M-phase [34, 35], while cdk1 is the catalytic subunit of MPF, and its protein kinase activity is responsible for governing entry into M-phase in all eukaryotes.

In the present study, the objectives were to examine MPF activity and the relative levels of its key components,

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cdk1 and cyclin B_2, during maturation in vitro of bovine oocytes. We report in this paper that MPF activity fluctuates during bovine oocyte maturation despite the relatively constant levels of cyclin B_2 and cdk1 that exist throughout maturation, and that the biphasic synthesis of cyclin B_2 is found to precede the two elevations of MPF activity.

MATERIALS AND METHODS

Oocyte Collection and In Vitro Maturation

Ovaries were obtained from cows and heifers slaughtered at a local slaughterhouse and were transported to the laboratory in Dulbecco's PBS (DPBS) at 25–30°C. Oocytes were collected from antral follicles (2–8 mm in diameter) by aspiration with an 18-gauge needle within 5 h after slaughter, as described by Yang et al. [36]. Oocytes with at least 4 layers of cumulus cells were selected for in vitro maturation (IVM). The medium for oocyte maturation was M199 (Earle's salts), supplemented with 25 mM Hepes, 7.5% fetal calf serum, and the hormones ovine (o) FSH (0.5 μg/ml), oLH (5.0 μg/ml), and estradiol (1.0 μg/ml). Ovine FSH and LH were obtained from the NHPP, NIDDKD, NICHHD, and the USDA. Maturation took place in 100-μl drops of medium covered by paraffin oil, under 5% CO_2 and 95% humidified air at 39°C. Culture time varied from 0 to 48 h as required for specific experiments.

Histone H1 Kinase Assay

Histone H1 kinase activity was measured in lysates of single cumulus-free oocytes, prepared by twice freezing and thawing in 10 μl histone kinase buffer: 15 mM 3-(*n*-morpholino)propanesulfonic acid (MOPS), pH 7.2, containing 80 mM β-glycerophosphate, 10 mM EGTA, 15 mM $MgCl_2$, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml cAMP-dependent protein kinase inhibitor peptide. Kinase reactions were initiated by the addition of 10 μl of substrate buffer containing 2 mg/ml histone H1 (Sigma Type III-S; Sigma Chemical Company, St. Louis, MO), 2 mM dithiothreitol (DTT), and 5 μCi [γ-^{32}P]ATP; and the reactions were carried out for 45 min at 35°C. The reaction mixture of each individual oocyte was immediately diluted with an equal volume of double-strength SDS sample buffer containing β-mercaptoethanol and boiled for 3 min. Kinase reaction products were subjected to SDS-PAGE [37] and autoradiography to demonstrate histone H1 phosphorylation. To determine the relative activity of MPF in each oocyte, the autoradiographic image of the histone H1 bands at each meiotic stage was scanned by laser densitometry (LKB 2222–010 UltraScan XL; LKB, Bromma, Sweden), and an average value was calculated for each observation. The average value at the GV stage was used as the baseline against which all other single oocytes on each gel were compared. Values of < 2, 2–3, or > 3 times the base value within the same experiment were considered to represent low, medium, or high histone H1 kinase activity, respectively. Three independent replicates were conducted, and at least three oocytes were analyzed at each time point in each replicate. Oocytes at each stage were classified with respect to the three ranges of histone H1 kinase activity, and their proportions were determined.

To quantitatively determine histone H1 kinase activity, lysates of single oocytes were aliquoted into two equal portions (5 μl each). Kinase reactions were carried out under the same conditions by adding 5 μl complete substrate buffer or substrate buffer without histone H1 (control). Upon completion, the reaction mixture was spotted onto Whatman P81 (Clifton, NJ) phosphocellulose paper discs, which were washed 5 times in 1% phosphoric acid and twice in methanol, and air-dried. Total ^{32}P incorporation was determined by liquid scintillation spectrometry. Histone H1 kinase activity was measured by subtracting control values from those of complete reactions. Twenty individual oocytes per replicate were used at each time point of IVM for the quantitative analysis of H1 kinase activity, and the experiment was repeated three times.

Immunoblotting Analysis of Cyclin B_2 and cdk1 Proteins

Levels of cdk1 and cyclin B2 were determined by Western blot analysis with enhanced chemiluminescence (ECL) to maximize sensitivity. In experiment 1, 50 oocytes/group were harvested at 0, 4, 8, 12, 16, 20, 24, and 48 h of IVM; and in experiment 2, 50 oocytes/group were collected at 0, 2, 4, 8, 10, 14, 18, and 22 h of IVM. Cumulus-free oocytes were lysed in 50 μl of lysis buffer containing 100 mM Tris-HCl (pH 6.8), 1% (w:v) SDS, 10% (w:v) sucrose, and 5% (v:v) β-mercaptoethanol. Solubilized proteins were resolved by SDS-PAGE and transferred to polyvinyl difluoride (PVDF; Immobilon P; Millipore Corp., Bedford, MA) using standard conditions [38]. Membranes were blocked with 3% gelatin in PBS and then probed with the appropriate primary and secondary antibodies. Anti-cdc2 monoclonal antibody was purchased from Transduction Laboratories (cat. # C12720; Lexington, KY), and anti-cyclin B_2 antiserum was kindly provided by Steve Reed (Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA). Immunoreactive proteins were detected by ECL according to the manufacturer's protocol (Amersham, Buckinghamshire, England, UK). The relative amounts of specific proteins were determined by scanning densitometry (LKB 2222–010 UltraScan XL). Each experiment was repeated three times.

Immunoprecipitation Assay for Cyclin B_2 and cdk1 Synthesis

In order to determine the synthesis of cdk1 and cyclin B_2 during bovine oocyte maturation, metabolic labeling and immunoprecipitation analyses as described by Harlow and Lane [39] were conducted. A total of 40 oocytes per group were harvested at various maturation times. Before labeling, cumulus cells were removed, and denuded oocytes were washed 3 times in methionine-free B2 medium (Sigma, Arlington Heights, IL) and then incubated for 3 or 4 h in the same B2 medium supplemented with 1 mCi/ml [^{35}S]methionine (in vivo cell-labeling grade, specific activity $>$ 1000 Ci/mmol; Amersham). After labeling, oocytes were washed 3 times in PBS containing 3 g/L of polyvinylpyrrolidone (PVP) and then were lysed and stored frozen (−80°C) in RIPA buffer [39] supplemented with protease inhibitors: PMSF (1 mM), leupeptin (20 μm), pepstatin (1 μM), benzamidine (1 mM), and antipain (2 μM). Lysates were then precleared by incubation on ice for 2 h in the presence of an equal volume of preloaded (nonimmune rabbit serum), 10% Omnisorb Cells (Calbiochem, La Jolla, CA). Insoluble materials were removed by centrifugation at 10 000 $\times$ *g* for 15 min at 4°C. Protein synthesis in each sample was determined by [^{35}S]methionine incorporation after trichloroacetic acid (TCA) precipitation and liquid scintillation spectroscopy. Equivalent amounts of radiolabeled proteins in each sample were treated with antibodies (either cyclin B_2 or anti-cdc2); after 1-h incubation

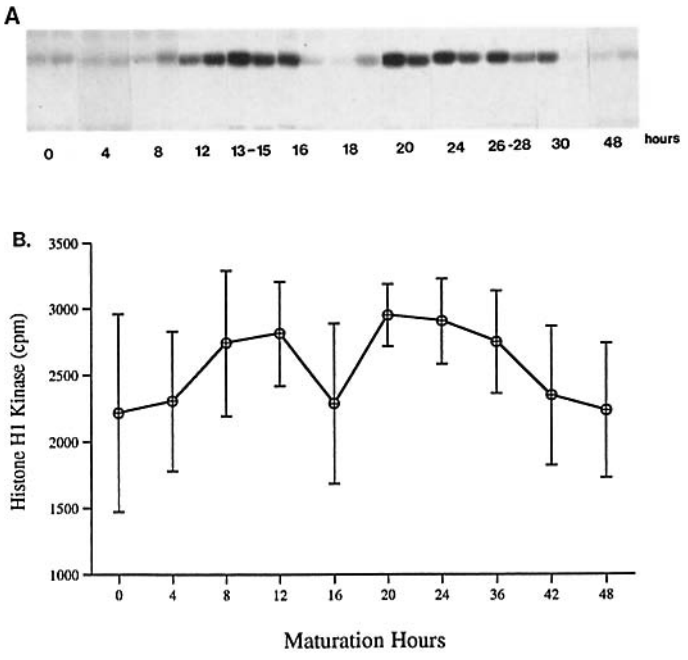

FIG. 1. Histone H1 activity during bovine oocyte maturation in vitro. **A**) Pattern of histone H1 kinase activity demonstrated by SDS-PAGE and autoradiography. A minimum of three individual oocytes were used at each time point, and the experiment was repeated three times. Shown here is a summarized pattern of the three replicates. **B**) Quantitative histone H1 kinase activity was determined by liquid scintillation spectrometry after capture on phosphocellulose. Data represent means (± SD) of incorporation for 20 individual oocytes at each time of IVM conducted in three replicates. The nuclear progression during IVM corresponds to GV (0–4 h), GVBD (8 h), MI (12–15 h), anaphase and telophase (16–19 h), and MII (20 h and onward).

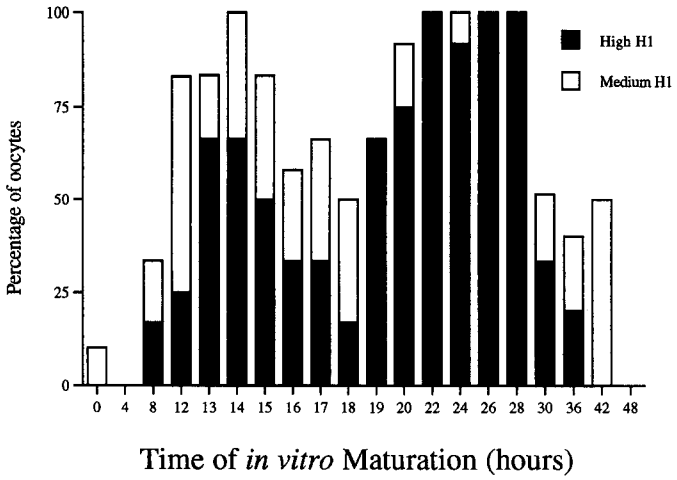

FIG. 2. Proportions of oocytes with active MPF (medium or high histone H1 kinase activity) at various maturation stages. Individual oocytes were collected at times indicated and assayed for histone H1 kinase activity after the removal of cumulus cells. The reaction products were resolved by SDS-PAGE and visualized by autoradiography, as in Figure 1A. The relative intensity of phosphorylated histone H1 bands at each meiotic stage was determined by scanning densitometry. The value of 2–3 or > 3 times of base value within the same experiment was considered to be medium or high H1 kinase activity, respectively. The nuclear progression during IVM corresponds to GV (0–4 h), GVBD (8 h), MI (12–15 h), anaphase and telophase (16–19 h), and MII (20 h and onward).

at 0°C, immune complexes were collected by adding 1/2 volume of washed 10% Omnisorb Cells followed by a further 30-min incubation with occasional mixing. Immune complexes were collected by centrifugation at 10 000 × *g*, washed extensively with RIPA buffer, and then solubilized by boiling for 5 min in SDS sample buffer containing β-mercaptoethanol. Each sample was concentrated to about 50 μl, resolved by SDS-PAGE, and visualized by fluorography [40, 41].

RESULTS

Histone H1 Kinase Activity

Histone H1 kinase (MPF) activity during bovine oocyte maturation was measured qualitatively by gel electrophoresis and autoradiography, and quantitatively by liquid scintillation counting. Oocytes were harvested every 1–4 h during maturation; assay results are shown in Figures 1 and 2. Bovine oocytes displayed a low level of histone H1 kinase activity at the GV stage during the first 8 h of IVM (Fig. 1A). An initial peak of histone H1 kinase activity occurred at about 12–14 h of maturation (onset of metaphase I) and was followed by an abrupt reduction in activity at 16–18 h, which corresponded to the anaphase and telophase stages of the first meiotic division. Thereafter, histone H1 kinase activity again increased, reaching a plateau at 20 h of IVM, corresponding to the time when these oocytes reach metaphase II. More quantitative liquid scintillation counting revealed that the H1 kinase activity increased gradually between GV and GVBD stages and peaked at MI (Fig. 1B). The H1 kinase activity dropped at 16 h (anaphase I and telophase I) but peaked again at 20 h (metaphase II). MPF measurements with more sampling intervals demonstrated that the majority of oocytes had high H1 kinase activity between 13 and 14 h, and low H1 kinase activity between 16 and 18 h of IVM (Fig. 2). Over 70% of oocytes contained high H1 kinase activity at 19 to 20 h of IVM and almost all oocytes (90–100%) had high H1 kinase activity between 22 and 28 h. This activity decreased gradually after 30 h and reached basal levels by 48 h of IVM.

Cyclin-Dependent Kinase 1 ($p34^{cdc2}$ or cdk1)

Cyclin-dependent kinase 1 is the catalytic component of MPF, and as such it plays a pivotal role in controlling cell cycle progression. To detect and quantify cdk1 protein during maturation, oocytes were collected every 4 h between 0 and 24 h of IVM, and at 48 h for aged oocytes. Western blot analysis indicated that the amount of cdk1 in oocytes appeared to increase gradually during the first 8 h of maturation and then remained relatively constant between 8 and 16 h of IVM (Fig. 3). However, a relatively low level of cdk1 was consistently observed at 20 h of IVM in all three replicates of the experiment. To further confirm this observation, oocytes were again collected at 0, 2, 4, 8, 10, 14, 18, and 22 h of IVM and subjected to the same Western blot analysis. The results showed that cdk1 again displayed relatively low levels at 18 and 22 h of IVM. Therefore, cdk1 seemed to increase gradually during the first 8 h of IVM, to maintain this high level until 16 h, and then to decline between 18 and 22 h of IVM. A slight increase of cdk1 was observed at 24 h of IVM and later.

Immunoprecipitation assays revealed that cdk1 was synthesized almost entirely between 0 and 13 h of IVM, and almost no synthesis was detected after 16 h of IVM (data not shown).

Cyclin B_2

Cyclin B is periodically synthesized and degraded during cell cycles and thus is a major determinant of cell cycle

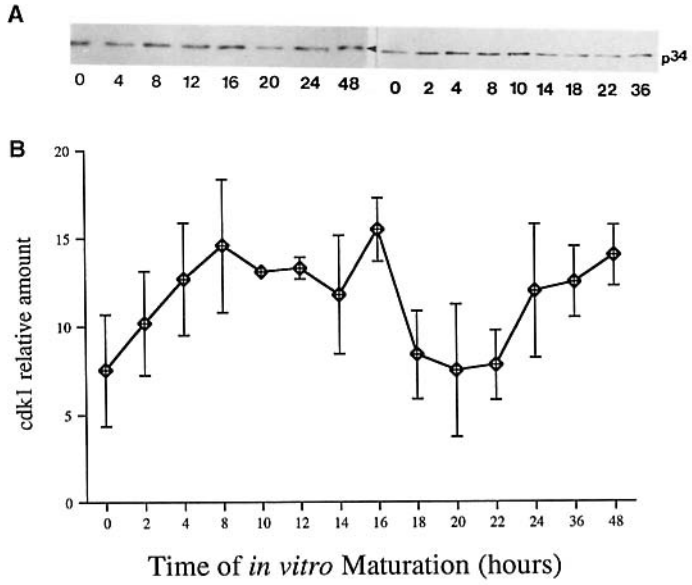

FIG. 3. Appearance of cdk1 during bovine oocyte maturation. Fifty oocytes were harvested at each time point and subjected to SDS-PAGE and Western blot analysis. **A**) Left and right sides indicate the two different experiments. Each experiment was repeated three times; representative results are shown. **B**) Relative amounts of cdk1 (means ± SD) were determined by scanning densitometry of blots. The nuclear progression during IVM corresponds to GV (0–4 h), GVBD (8 h), MI (12–15 h), anaphase and telophase (16–19 h), and MII (20 h and onward).

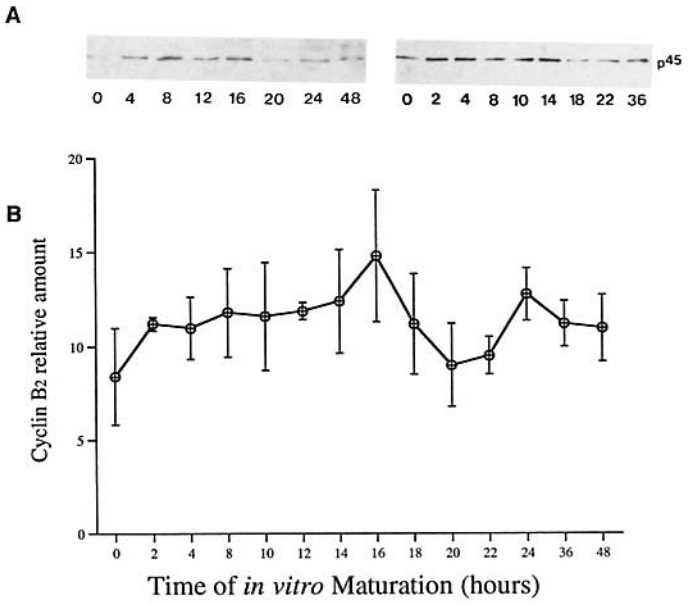

FIG. 4. Cyclin B_2 protein levels during bovine oocyte maturation. The content of cyclin B_2 protein was examined during maturation by Western blot analysis using 50 oocytes per time point. Relative quantities of cyclin B_2 were determined by scanning densitometry of blots. **A**) Left and right sides indicate two different experiments. Each experiment was repeated three times; representative results are shown. **B**) Relative amounts of cyclin B_2 (means ± SD) during oocyte in vitro maturation. The nuclear progression during IVM corresponds to GV (0–4 h), GVBD (8 h), MI (12–15 h), anaphase and telophase (16–19 h), and MII (20 h and onward).

progression. Relative levels of cyclin B_2 in bovine oocytes during IVM were examined by Western blot analysis, as above, but using anti-recombinant human cyclin B_2 antibodies. Cyclin B_2 was detected at low levels in GV-stage (0 h) oocytes, and the level of cyclin B_2 increased rapidly during the first 4 h of maturation (Fig. 4). Thereafter, the elevated level of cyclin B_2 was maintained until at least 16 h of IVM, but fell by about 70% by 20 h and then increased again by 24 h of IVM and thereafter (Fig. 4).

Cyclin B_2 synthesis was measured by metabolic labeling and immunoprecipitation. The pattern of cyclin B_2 synthesis manifested fluctuations that displayed two peaks of synthesis before the first and the second meiosis, respectively (Fig. 5). Little, if any, synthesis of cyclin B_2 synthesis was detected during MI (10–16 h of IVM) and MII (21–31 h of IVM).

DISCUSSION

In this study, we report that bovine oocytes, like other mammalian oocytes, displayed two obvious metaphases (MI and MII) correlated with the biphasic appearances of high MPF activity in their cytoplasm during meiotic maturation. The fluctuation of MPF activity during oocyte maturation, which matches well with oocyte nuclear progression reported previously [42], reflects the fact that MPF induces GVBD and chromosome condensation before the onset of MI, while the reduction of its activity triggers the metaphase-anaphase transition. A resurgence in MPF activity after release of the first polar body brings about MII, and the sustained high levels of MPF account for the characteristic metaphase arrest that occurs in the absence of fertilization or parthenogenetic activation. Our observations showed that bovine oocytes incubated in M199 containing hormones exhibited an initial peak of MPF activity at 12–14 h of IVM, by which time 70% of the oocytes had reached meiosis I ([42] and our observations). Fully grown pig oocytes also exhibit low levels of histone H1 kinase activity during the early stages of maturation [25]. Most bovine oocytes reach MI after 12 h of IVM, with the anaphase-telophase transition occurring between 15 and 18 h of IVM, though variation exists among oocytes [42]. The mean level of H1 kinase at 16 h of IVM was about half of that measured at 12 h of IVM. Accordingly, approximately 40% of the oocytes studied here were judged to be at the anaphase-telophase stage by 16 h of IVM. After 20 h of IVM, approximately 80% of oocytes have reached metaphase of the second meiosis [43]. This corresponds to the second peak of MPF activity observed in the present study. The fluctuations in mean levels of MPF activity observed here closely matched the nuclear events.

Oocytes collected from a given batch of ovaries from slaughtered cattle include oocytes from a heterogenous follicular population. Even though all these oocytes are arrested at the GV stage, they proceed through meiosis at different rates. Our results clearly showed that individual bovine oocytes exhibit varying MPF activity at any time point observed. This variation, brought about by asynchro-

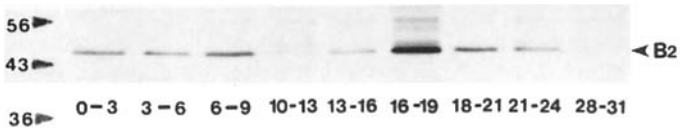

FIG. 5. Cyclin B_2 synthesis during oocyte maturation in vitro. Oocytes were labeled with [^{35}S]methionine for 3 h at different times of oocyte maturation culture (0–31 h), then lysed and subjected to immunoprecipitation with anti-cyclin B_2 antibody. A 45-kDa cyclin B_2 protein was specifically precipitated and detected by fluorography after SDS-PAGE. The nuclear progression during IVM corresponds to GV (0–4 h), GVBD (8 h), MI (12–15 h), anaphase and telophase (16–19 h), and MII (20 h and onward). Molecular weight standards are shown at left (× 10 $^{-3}$).

nous maturation, was particularly evident at the onset of MI (ranging from 12 h to 15 h of IVM) and during the anaphase/telophase-to-metaphase II transition (ranging from 15 h to 19 h of IVM) (Fig. 2). Noticeably, bovine oocytes began to exhibit a decline in MPF activity at 30 h of IVM, and most oocytes have minimal MPF activity after 40 h of IVM, which may help to explain why spontaneous activation occurs more readily in these aging oocytes [44–46]. This may also account for the increased efficiency of nuclear transfer using aging oocytes after 30 h of IVM [46]. Additionally, when bovine oocytes at 25–26 h of IVM were treated for 4 h with 6-dimethylaminopurine, an inhibitor of protein phosphorylation that induces a decline in cdk1 kinase activity [47], their activation rate was about 48%; longer treatment times did not increase the oocyte activation rate, possibly because of the already-reduced MPF activity in the aged oocytes (unpublished result).

Both increases in MPF activity during oocyte maturation require active protein synthesis and phosphorylations [23, 48–50]. Most studies have shown that cyclin B synthesis is necessary for the activation of MPF [35, 51] and that cdk1 levels change little during the cell cycle. To investigate the changes in these MPF components during bovine oocyte maturation, we examined their relative levels and degree of synthesis at various times of maturation. As previously reported in the pig [19], basal levels of cdk1 changed little during IVM, at least up to 16 h, except for a slight increase in the first few hours. While cdk1 kinase activity, the equivalent of histone H1 kinase activity, is known to be responsible for governing M-phase entry in all eukaryote cells [14], our results indicated that MPF activity in bovine oocytes seemed to depend on cdk1 activation, rather than on its abundance. Although lower levels of cdk1 protein occurred at 20 h of IVM, histone H1 kinase activity was increasing at this time point. The dephosphorylation of $p34^{cdc2}$ coincides with an increase in histone H1 kinase activity, again suggesting that activation of pre-MPF is the locus of control [50, 52].

Since cdk1 is constitutively present in dividing cells, the implication is that the synthesis and degradation of cyclin B regulates the activity of MPF. For the cyclin B family, only cyclin B_2 levels increase sharply before GVBD and decline thereafter during the anaphase and telophase transition in *Xenopus laevis* oocytes [32]. Cyclin B activates MPF [30, 51], and it is the only protein whose de novo synthesis is necessary for the induction of MPF activity and cell cycle progression in *Xenopus* oocytes [53, 54]. Similarly, in maturing mouse oocytes, the synthesis of cyclin B is required to drive the continued increase in histone H1 kinase activity seen after GVBD [47, 55]. We show here that cyclin B_2 increased during the first several hours of IVM and was then maintained at a relatively steady level until a slight reduction was observed between 18 and 22 h of IVM. Bovine oocytes synthesized cyclin B_2 before MI and before MII, thus displaying a biphasic pattern of expression. The fact that peak cyclin B_2 synthesis occurred before the rise in MPF activity suggests that cyclin B_2 synthesis is needed for, but does not directly govern, the onset of MPF activation. When MPF activity reached its first peak (MI), cyclin B_2 synthesis had already occurred, and this newly synthesized cyclin B_2 seemed to be necessary to form pre-MPF, which then becomes activated during MI. Despite the lack of interphase between MI and MII, bovine oocytes displayed a second round of cyclin B_2 synthesis before MII at 16–20 h of IVM, which correlated with an increase in histone H1 kinase activity during this time, suggesting that the entry to meiosis II also depends on the newly synthesized cyclin B_2. It appears that active MPF requires a critical period of cyclin synthesis shortly before each metaphase in bovine oocytes, similar to that reported for maturing mouse oocytes, in which cyclin B synthesis drives the increase in MPF after GVBD [55].

In domestic mammals, unlike rodents, the synthesis of cyclins and associated proteins does not occur during follicular development before the LH surge [16]. Low levels of cyclin B/cdk1 complexes present in immature oocytes are maintained in the inactive form (see Fig. 2). Proteins synthesized during the first few hours of IVM may induce GVBD in bovine oocytes and lead to the autocatalytic amplification of MPF [56]. Should additional cyclin B be provided, it may complex with cdk1 faster than it can be inactivated, providing a pre-MPF pool, the substrate for the cdc25 phosphatase that generates MPF [14, 16, 35]. Once a threshold level of MPF activity has been reached, autocatalytic MPF activation follows, resulting in oocyte maturation. Although growing porcine oocytes have sufficient key components for the G2 to M transition, they remain incapable of converting these components to active MPF until growth is virtually completed [25]. The accumulation of cyclins permits alterations in the phosphorylation state of cdk1 and turns on its protein kinase activity [57, 58]. Microinjection of cyclins into immature oocytes also causes active MPF formation [35]. Our results indicated that the rapid increase of cyclin B_2 during early maturation is necessary to activate pre-MPF even though bovine oocytes contain some cyclin B_2 before initiating metaphase. Curiously, cyclin B_2 levels were reduced at 20–22 h of IVM, similar to the pattern of cdk1 expression, suggesting that maintenance of high MPF activity and metaphase arrest is not dependent on the high levels of cyclin B_2 and cdk1. After MPF becomes fully active, it may be maintained by other cytoplasmic factors, e.g., cytostatic factor (CSF or Mos) [59, 60], even in the face of declining amounts of MPF constituent subunits. The other interesting fact is that, despite the reduced MPF activity at 40–44 h of IVM, the MII configuration in the aging oocyte remains unchanged (Figs. 1 and 2). This suggests that the fact that the chromatin arresting in MII configuration may be due to factors other than just MPF as suggested previously [61]. Matured bovine oocytes have been shown to contain Mos protooncoprotein ([62, 63] and the companion paper [65]), which is also synthesized in a cell cycle-dependent manner. In the absence of Mos, the cyclin B component of MPF is degraded, leading to the release of MII arrest [64].

Together, these results have defined the profiles of MPF activity and its key components during bovine oocyte maturation in vitro. It is concluded that active MPF regulates bovine oocyte maturation, while the activation of MPF seems to depend on synthesis of cyclin B_2 during the process of maturation.

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REFERENCES

1. Smith LD. The induction of oocyte maturation: transmembrane signaling events and regulation of the cell cycle. Development 1989; 107: 685–699.
2. Masui Y, Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool 1971; 177:129–146.
3. Smith LD, Ecker RE. Regulatory processes in the maturation and early cleavage of amphibian eggs. Curr Top Dev Biol 1971; 5:1–38.
4. Lohka MJ, Hayes MK, Maller JL. Purification of maturation-promoting factor, an intracellular regulator of early mitotic events. Proc Natl Acad Sci USA 1988; 85:3009–3013.
5. Gautier J, Minshull J, Lohka M, Giotzer M, Hunt T, Maller JL. Cyclin is a component of maturation-promoting factor from *Xenopus*. Cell 1990; 60:487–494.
6. Murray AW. Creative blocks: cell-cycle checkpoints and feedback controls. Nature 1992; 359:599–604.
7. Kishimoto T, Kanatani H. Cytoplasmic factor responsible for germinal vesicle breakdown and meiotic maturation in starfish oocytes. Nature 1976; 221:273–274.
8. Murray AW, Kirschner MW. Domineos and clocks: the union of two views of the cell cycle. Science 1989; 246:614–621.
9. Murray AW, Solomon MJ, Kirschner MW. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature 1989; 339:280–286.
10. Nurse P. Universal control mechanism regulating onset of M-phase. Nature 1990; 344:503–507.
11. Motlik J, Kubelka M. Cell-cycle aspects of growth and maturation of mammalian oocytes. Mol Reprod Dev 1990; 27:366–375.
12. Parrish JJ, Kim CI, Bae IH. Current concepts of cell-cycle regulation and its relationship to oocyte maturation, fertilization and embryo development. Theriogenology 1992; 38:277–296.
13. Lewin B. Driving the cell cycle: M phase kinase, its partners and substrates. Cell 1990; 61:743–752.
14. Norbury C, Nurse P. Animal cell cycle and their control. Annu Rev Biochem 1992; 61:441–470.
15. Yew N, Strobel M, Vande Woude GF. *Mos* and cell cycle: the molecular basis of the transformed phenotype. Curr Opin Genet Dev 1993; 3:19–25.
16. Homa ST. Calcium and meiotic maturation of the mammalian oocyte. Mol Reprod Dev 1995; 40:122–134.
17. Arion D, Meijer L, Brizuela L, Beach D. *cdc*2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell 1988; 55:371–378.
18. Draetta G, Luca F, Westendorf J, Brizuela L, Ruderman J, Beach D. *cdc*2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell 1989; 56:829–838.
19. Moor R, Hutchings A, Hawkins C, Jung T. Towards a molecular understanding of meiosis in domestic animals. In: Lauria A, Gandolfi F (eds.), Embryonic Development and Manipulation in Animal Production Trends in Research and Applications. London: Portland Press; 1992: 1–15.
20. Hashimoto N, Kishimoto T. Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. Dev Biol 1988; 126:242–252.
21. Fulka J Jr, Flechon JE, Motlik J, Fulka J. Does autocatalytic amplification of maturating-promoting factor (MPF) exist in mammalian oocytes? Gamete Res 1988; 21:185–192.
22. Fulka J Jr, Jung T, Moor RM. The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. Mol Reprod Dev 1992; 32:378–382.
23. Mattioli M, Galeati G, Bacci ML, Barboni B. Change in maturation-promoting activity in the cytoplasm of pig oocytes throughout maturation. Mol Reprod Dev 1991; 30:119–125.
24. Naito K, Toyoda Y. Fluctuation of histone H1 kinase activity during meiotic maturation in porcine oocytes. J Reprod Fertil 1991; 93:467–473.
25. Christmann L, Jung T, Moor RM. MPF components and meiotic competence in growing pig oocytes. Mol Reprod Dev 1994; 38:85–90.
26. Kikuchi K, Naito K, Daen FP, Izaike Y, Toyoda Y. Histone H1 kinase activity during in vitro fertilization of pig follicular oocytes matured in vitro. Theriogenology 1995; 43:523–532.
27. Jelinkova L, Kubelka M, Motlik J, Guerrier P. Chromatin condensation and histone H1 kinase activity during growth and maturation of rabbit oocytes. Mol Reprod Dev 1994; 37:210–215.
28. Campbell KHS, Ritchie WA, Wilmut I. Nuclear-cytoplasmic interactions during the first cell cycle of nuclear transfer reconstructed bovine embryos: implications for deoxyribonucleic acid replication and development. Biol Reprod 1993; 49:933–942.
29. Collas P, Sullivan EJ, Barnes FL. Histone H1 activity in bovine oocytes following calcium stimulation. Mol Reprod Dev 1993; 34:224–231.
30. Evans T, Rosenthal ET, Youngblom J, Distel D, Hunt T. Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell 1983; 33:389–396.
31. Hunter T. Oncogenes and cell proliferation. Curr Opin Genet Dev 1993; 3:1–4.
32. Kobayashi H, Minshull J, Ford C, Golsteyn R, Poon R, Hunt T. On the synthesis and destruction of A- and B-type cyclins during oogenesis and meiotic maturation in *Xenopus* laevis. J Cell Biol 1991; 114: 755–765.
33. Chapman DL, Wolgemuth DJ. Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis. Development 1993; 118:229–240.
34. Westendorf JM, Swenson KI, Ruderman JV. The role of cyclin B in meiosis I. J Cell Biol 1989; 108:1431–1444.
35. Minshull J. Cyclin synthesis: who needs it? Bioessays 1993; 15:149–155.
36. Yang X, Jiang S, Foote RH. Bovine oocyte development following different oocyte maturation and sperm capacitation procedures. Mol Reprod Dev 1993; 34:94–100.
37. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 277:680–685.
38. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76:4350–4354.
39. Harlow E, Lane D. Immunoprecipitation. In: Antibody, A Laboratory Manual. New York: Cold Spring Harbor Laboratory; 1988: 421–469.
40. Bonner WM, Laskey RA. A film detection method for tritium labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 1974; 46:83–88.
41. Laskey RA, Mills AD. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 1975; 56:335–341.
42. Sirard MA, Florman HM, Leibfried-Rutledge ML, Barnes FL, Sims ML, First NL. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol Reprod 1989; 40:1257–1263.
43. Shamsuddin M, Larsson B, Rodriguez-Martinez H. Maturation-related changes in bovine oocytes under different culture conditions. Anim Reprod Sci 1993; 31:49–60.
44. Nagai T. Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. Gamete Res 1987; 16:243–249.
45. Ware CB, Barnes FL, Maiki-Laurila M, First NL. Age dependence of bovine oocyte activation. Gamete Res 1989; 22:265–275.
46. Yang X, Jiang S, Kovacs A, Foote RH. Age dependent activation, enucleation and nuclear transfer of bovine oocytes matured in vitro and in vivo. Biol Reprod 1991; 44(suppl 1):141.
47. Hampl A, Eppig JJ. Translation regulation of the gradual increase in histone H1 kinase activity in maturating mouse oocytes. Mol Reprod Dev 1995; 40:9–15.
48. Kubelka M, Rimkevicava Z, Guerrier P, Motlik J. Inhibition of protein synthesis affects histone H1 kinase, but not chromosome condensation activity, during the first meiotic division of pig oocytes. Mol Reprod Dev 1995; 41:63–69.
49. Levesque JT, Sirard MA. Effects of different kinases and phosphatases on nuclear and cytoplasmic maturation of bovine oocytes. Mol Reprod Dev 1995; 42:114–121.
50. Tatemoto H, Terada T. Activation of p34*cdc*2 kinase around the meiotic resumption in bovine oocytes cultured in vitro. Theriogenology 1996; 45:427–437.
51. Levesque JT, Sirard MA. Meiotic resumption is mediated by the accumulation of cyclin B in bovine immature oocytes. Theriogenology 1996; 45:188.
52. Choi T, Aoki F, Mori M, Yamashita M, Nagahama Y, Kohmoto K. Activation of p34^{cdc2} protein kinase activity in meiotic and mitotic cell cycle in mouse oocytes and embryos. Development 1991; 113: 789–795.
53. Minshull J, Blow JJ, Hunt T. Translation of cyclin mRNA is necessary for extracts of activated *Xenopus* eggs to enter mitosis. Cell 1989; 56: 947–956.
54. Murray AW. The cell cycle as a *cdc*2 cycle. Nature 1989; 342:14–15.

55. Hampl A, Eppig JJ. Analysis of the mechanism(s) of metaphase I arrest in maturing mouse oocytes. Development 1995; 121:925–933.
56. Tatemoto H, Horiuchi T. Requirement for protein synthesis during the onset of meiosis in bovine oocytes and its involvement in the autocatalytic amplification of maturation-promoting factor. Mol Reprod Dev 1995; 41:47–53.
57. Gould KL, Nurse P. Tyrosine phosphorylation of the fission yeast *cdc*2 protein kinase regulates entry into mitosis. Nature 1988; 342:39–45.
58. Solomon MJ, Glotzer M, Lee TH, Philippe M, Kirschner MW. Cyclin activation of $p34^{cdc2}$. Cell 1990; 63:1013–1024.
59. Sagata N, Watanabe N, Vande Woude GF, Ikawa Y. The products of the *mos* proto-oncogene product is a cytostatic factor responsible for meiosis arrest in vertebrate eggs. Nature 1989; 342:512–518.
60. Sagata N. Meiotic metaphase arrest in animal oocytes: its mechanisms and biological significance. Trends Cell Biol 1996; 6:22–28.
61. Dominko T, First NL. P34*cdc*2 and P33*CDK*2 in regulation of metaphase II arrest in bovine oocytes. Theriogenology 1996; 45:157.
62. Wu B, Ignotz GG, Currie BW, Yang X. Identification of c-*mos* proto-oncoprotein and its association with β-tubulin in mature bovine oocytes. Theriogenology 1995; 43:356.
63. Tatemoto H, Terada T. On the c-*mos* proto-oncogene product during meiotic maturation in bovine oocytes culture in vitro. J Exp Zool 1995; 272:159–162.
64. Watanabe N, Vande Woude GF, Ikawa Y, Sagata N. Specific proteolysis of the c-*mos* proto-oncogene product by calpain on fertilization of *Xenopus* eggs. Nature 1989; 342:505–511.
65. Wu B, Ignotz G, Currie WB, Yang X. Expression of Mos proto-oncoprotein in bovine oocytes during maturation in vitro. Biol Reprod 1997; 56:260–265.