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Expression of Mos Proto-Oncoprotein in Bovine Oocytes during Maturation In Vitro[1]

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ABSTRACT

The c-*mos* proto-oncogene product Mos is believed to be an active component of the cytostatic factor that stabilizes and sustains the activity of maturation-promoting factor. Mos has been found to be responsible for the metaphase arrest of oocytes at the second meiotic division in both *Xenopus* and the mouse. In this study, we have demonstrated, by Western blot and immunoprecipitation analysis, that an approximately 39-kDa protein, identified as Mos, was present in in vitro-matured (metaphase II stage) bovine oocytes but disappeared in parthenogenetically activated oocytes. The oocytes actively synthesized $p39^{mos}$ at the metaphase II stage (between 22 and 26 h of in vitro maturation [IVM]), whereas little $p39^{mos}$ synthesis was detected during the first 4 h of IVM and it was nondetectable during aging at 44–48 h of IVM, when oocytes lose the capability of normal development after fertilization. Ethanol activation of mature oocytes led to the disappearance of $p39^{mos}$. β-Tubulin, but not $p34^{cdc2}$, was co-precipitated with Mos when extracts of metaphase II-stage bovine oocytes were incubated with Mos antiserum. These results demonstrated that Mos is present and actively synthesized in mature bovine oocytes and that oocytes aged beyond the optimal time for fertilization seem to lose the ability to synthesize the Mos protein. β-Tubulin was found to be associated with Mos, which suggests a possible role for the cytoskeletal protein in maintaining the meiotic arrest in mature bovine oocytes.

INTRODUCTION

Oocyte maturation is a complex phenomenon involving both the nucleus and the cytoplasm. Its regulation is multifactorial and is normally dependent on the follicular environment in which oocyte maturation takes place. In most mammalian species, fully grown oocytes are naturally arrested at the prophase of the first meiotic division, or prophase I. Resumption of meiosis in mammalian oocytes occurs in response to the preovulatory surge of LH after the animal reaches puberty. The meiotic resumption is characterized by germinal vesicle breakdown, chromosomal condensation, progression to metaphase of the first meiosis, release of the first polar body, and then arrest at the metaphase of the second meiosis (MII) [1, 2]. Most mammalian oocytes are ovulated at MII and remain arrested at this stage until activated by a fertilizing spermatozoon (fertilization) or by artificial stimuli (parthenogenetic activation) [3, 4]. This meiotic arrest (MII arrest) is maintained by the persistently high activity of maturation-(or M-phase-) promoting factor (MPF) [5]. MPF, which was later found to be a universal cell cycle regulator of both mitosis and meiosis, is a complex of B cyclins and a cyclin-dependent kinase, cdk1 or $p34^{cdc2}$ [6–8]. Active MPF is responsible for the onset of M-phase in all eukaryotic cells including oocytes [9, 10]. Fertilization or parthenogenetic activation of oocytes releases the metaphase arrest through inactivation of MPF by degradation of the cyclin component [8, 10]. It was discovered that cytoplasm from ovulated oocytes contained a cytostatic factor (CSF) that could block cell division at metaphase after injection of the oocyte cytoplasm into the blastomere [2]. It is believed that CSF promotes meiotic arrest by either directly or indirectly stabilizing MPF activity during MII [8, 11] and hence prevents oocytes from progressing beyond metaphase. The product of the c-*mos* proto-oncogene, Mos, has been identified as an active component of CSF and, in cooperation with cyclin-dependent kinase 2 (cdk2), has been found to be necessary for the metaphase II arrest of mature oocytes in mice [11–13].

The c-*mos* proto-oncogene was found to be expressed at high levels in testes and ovaries, and specifically, in male and female germ cells [14]. In the mouse and *Xenopus*, Mos disappears rapidly upon fertilization of oocytes, paralleling the pattern of expression typical for mammalian maternal messages [15, 16]. Microinjection of Mos-specific antisense oligonucleotides into mouse or *Xenopus* oocytes blocked oocyte maturation, whereas injection of Mos sense RNA into the oocytes promoted maturation of these oocytes [11, 17–19]. Introduction of Mos antibody into immature mouse oocytes blocks the formation of a polar body [20], and when injected into mouse zygotes, Mos antibody prevents the first cleavage by arresting zygotes at the pronuclear stage [21]. These results indicated that Mos is required both for the activation of MPF during meiosis I and II and for the meiotic arrest of oocytes at the metaphase of meiosis II [22, 23]. Although the study of c-*mos* proto-oncogene function from different species has shown strong correlation among Mos activity (a serine-threonine kinase), transforming efficiency, oocyte maturation-inducing activity, and CSF activity [24], notable interspecies differences do exist among different vertebrates. Oocytes of female mice which lacked the c-*mos* proto-oncogene could mature but failed to arrest at MII, and the fertility of the c-*mos*-deficient female mice was severely impaired [25, 26]. Aberrations of these female mice included spontaneous parthenogenetic activation of their oocytes and the development of ovarian cysts and teratomas [27]. These findings suggest that the expression and function of Mos appear to play a lesser role in murine meiotic maturation than in *Xenopus* [27].

The objectives of this study were 1) to detect the expression of c-*mos* proto-oncogene in bovine oocytes matured in vitro and after oocyte activation, 2) to determine the timing of Mos synthesis during oocyte maturation, and 3) to examine the possible association of Mos with other cell cycle proteins, such as $p34^{cdc2}$ and β-tubulin.

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MATERIALS AND METHODS

Oocyte Collection and In Vitro Maturation (IVM)

Ovaries were obtained from cows and heifers at a local slaughterhouse. The ovaries were transported in Dulbecco's PBS (DPBS) at 25–30°C to the laboratory within 5 h after slaughter. Oocytes were aspirated from antral follicles (2–8 mm in diameter) with an 18-gauge needle and placed in DPBS containing 0.1% polyvinyl alcohol (PVA), as described previously [28]. Oocytes with at least 4 layers of cumulus cells were selected for this study. The medium used for oocyte maturation was Medium 199 with Earle's salts, 25 mM Hepes, and 7.5% fetal calf serum (FCS), with added hormones (ovine (o) FSH, 0.5 μg/ml; oLH, 5.0 μg/ml; and estradiol, 1.0 μg/ml). Maturation was accomplished with cumulus-oocyte complexes in 100-μl drops of medium, covered with paraffin oil in Falcon culture dishes (Falcon 1008; Fisher, Bridgeport, NJ). The culture environment was 5% CO_2:95% humidified air at 39°C for various times, as required for the specific experiments. These procedures have been routinely used in our laboratory and normally result in 90–95% maturation, 85–90% fertilization, and 40–45% blastocyst development [28].

Oocyte Activation

Activation of bovine oocytes was carried out by a combined ethanol and cycloheximide treatment [29]. After 22 h of IVM, cumulus cells were removed by placing the matured oocytes in 0.2% hyaluronidase in DPBS-PVA for 1–2 min followed by vortexing. The nude oocytes were assigned randomly to treatment and control groups. The treatment group oocytes were activated by bathing them for 5 min in 7% ethanol in DPBS, followed by washing with DPBS-PVA and M199+FCS. The oocytes were then incubated in M199+FCS containing 10 μg/ml cycloheximide for 12 h. The combined activation treatment was needed to effectively activate the newly matured bovine oocytes [29].

Antibody Sources

Because the nucleotide and corresponding amino acid sequences for the *c-mos* proto-oncogene from large animals, including cattle, are not available, those from divergent species were compared to determine highly conserved sequences that could be expected to result in an antiserum with broad applicability. Accordingly, an antiserum was generated in rabbits after immunization with a mixture of peptides (Ile/Val-Leu/Gln-Tyr-Ala-Val-Val-Ala-Tyr-Asp/Asn-Cys-Arg-Asp-Gly-Gly-Cys) based on the homologous nucleotide sequence of human, *Xenopus*, and mouse Mos in the laboratory of G.F. Vande Woude (National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD) [17, 30, 31]. The antiserum thus generated, along with the peptide antigen, was used throughout this study where appropriate. Mouse monoclonal anti-rat β-tubulin antibody, anti-cdc2, and enzyme-conjugated secondary antibodies were obtained, respectively, from Maxim Biotech, Inc. (S. San Francisco, CA), Transduction Laboratories (Lexington, KY), and Sigma Chemical Company (St. Louis, MO).

Metabolic Labeling and Mos Immunoprecipitation Assay

In order to determine the extent and timing of Mos synthesis, cumulus-oocyte complexes at different IVM stages were labeled in the presence of [^{35}S]methionine for 4 h (Amersham Corp., Buckinghamshire, England, UK). During the labeling period, oocytes were cultured in methionine-free B_2 medium (Sigma) in 4-well culture dishes overlaid with paraffin oil. The labelled oocytes were then freed of cumulus cells and lysed in RIPA buffer on ice [31] containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.05% SDS, 0.5% sodium deoxycholate, and 1% NP-40 (Sigma) supplemented with protease inhibitors PMSF (1 mM), leupeptin (20 μM), pepstatin (1 μM), benzamidine (1 mM), and antipain (2 μM). Mos antiserum or antiserum that had been preabsorbed with excess peptide antigen was added to the lysates. After 1-h incubation on ice, Mos immune complexes were collected by absorption (30 min at 0°C) to insoluble protein A by adding one-half volume of the suspension (10% w:v) of washed, formalin-fixed *Staphylococcus aureus* Cowan strain I (SAC; Sigma). The SAC-Mos immune complexes were recovered by centrifugation, washed extensively in RIPA buffer, and solubilized by boiling in SDS sample buffer.

SDS-PAGE, Immunoblotting, and Fluorography

For Western blot analysis, groups of oocytes were lysed in 50 μl SDS sample buffer at 30°C containing 5% β-mercaptoethanol. Lysed oocytes and solubilized immune complexes were resolved by SDS-PAGE (12% total acrylamide, 2.6% cross-linked acrylamide; National Diagnostics, Atlanta, GA) under reducing conditions [32]. Standard electroblotting procedures [33] were used to transfer proteins to polyvinyl difluoride (PVDF; Immobilon P; Millipore Corp., Bedford, MA) for immune detection. After incubation with primary antibody and washes, appropriate secondary antibody conjugates were introduced, and enhanced chemiluminescence (ECL) detection of immunoreactive proteins was performed according to the manufacturer's guidelines (Amersham). Where indicated, membranes, or individual lanes cut therefrom, were stripped of bound antibodies by incubation in 50 mM Tris-HCl (pH 5.0), 2% SDS, and 5% β-mercaptoethanol at 55–60°C. After stripping, the membranes were washed three times and reblocked before additional antibody probes were performed.

After immunoprecipitation and SDS-PAGE, gels containing radiolabeled proteins were fixed, stained to visualize molecular weight markers, and processed for fluorography by impregnation with 2,5-diphenyloxazole [34]. Dried gels were used to expose pre-flashed x-ray films for 8 days at −80°C [35].

RESULTS

Identification of Mos in Bovine Oocytes

The expression of *c-mos* proto-oncogene protein in matured (22 h IVM) bovine oocytes was verified using duplicate samples (1000 oocytes per lane) probed with Mos antiserum or the corresponding preimmune serum. ECL Western blots revealed an approximately 39-kDa presumptive Mos proto-oncoprotein (Mos) after incubation with antiserum (Fig. 1, lane 3), while the protein was not detected when incubated with preimmune serum (Fig. 1, lane 1). The Mos protein identified in the bovine oocytes is indistinguishable from the Mos protein observed in NIH 3T3 cells [19] in terms of mobility on the same SDS-PAGE gel (data not shown). When the same membrane in lane 3 was stripped, reblocked, and reprobed with antiserum preabsorbed with Mos peptide, immune recognition of Mos was abolished (Fig. 1, lane 4). Likewise when the same membrane in lane 1 was reincubated with Mos antiserum (with-

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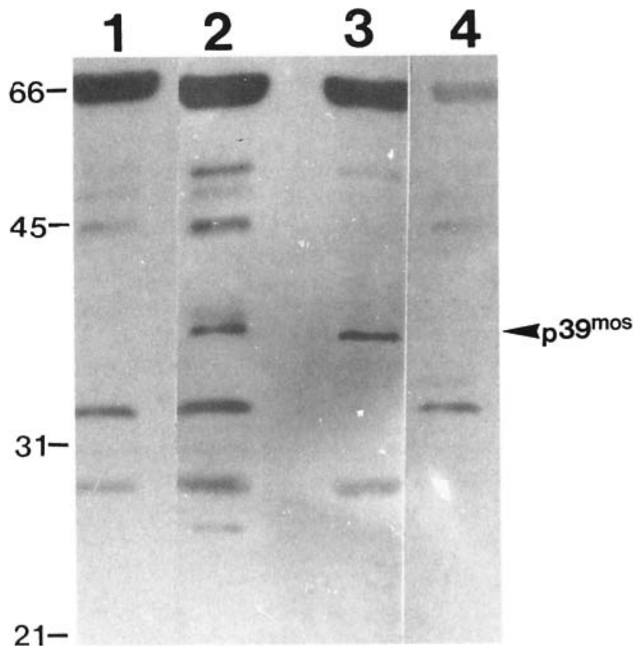

FIG. 1. Western blot identification of Mos proto-oncoprotein in matured (22 h) bovine oocytes. Duplicate samples of 1000 matured oocytes in each lane were resolved by SDS-PAGE, transferred, and incubated with either the preimmune serum (lane 1) or Mos antiserum (lane 3). Immunoreactive proteins were visualized by ECL after the addition of appropriate secondary antibody. The transfer membrane segment represented by Lane 1 was then incubated with Mos antiserum (lane 2), while lane 3 was stripped of bound antibodies and reprobed with Mos antiserum preabsorbed with the Mos peptide antigen (lane 4). p39^{mos} is present in lanes 2 and 3 (Mos antiserum), but not in lanes 1 and 4 (preimmune and peptide-absorbed antisera, respectively). Molecular weight markers ($\times 10^{-3}$) are shown at left.

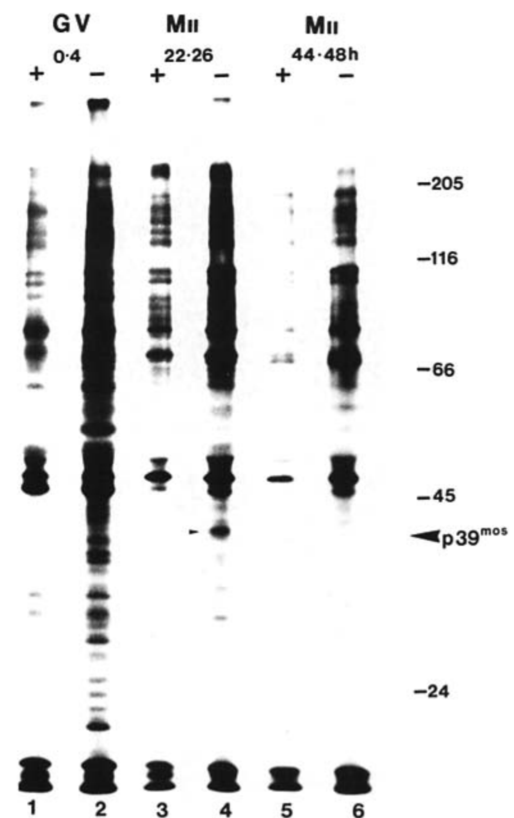

FIG. 2. Fluorographic detection of Mos synthesis in bovine oocytes during IVM. Oocytes (600/group) were labeled in methionine-free medium supplemented with 1 mCi/ml [^{35}S]methionine at times and stages as indicated. Lysates were prepared from each group, divided in half, and subjected to immunoprecipitation using *Mos* antiserum (–, lanes 2, 4, 6) or peptide-absorbed antiserum (+, lanes 1, 3, 5). Immune complexes were isolated by absorption to insoluble protein A, solubilized, resolved by SDS-PAGE, and detected by fluorography. p39^{mos} synthesized during the 22–26-h labeling period (lane 4) is indicated by the arrow. Molecular weight markers ($\times 10^{-3}$) are shown at right. GV, germinal vesicle stage.

out prior stripping), p39^{mos} was detected as expected (Fig. 1, lane 2).

Synthesis of Mos during Bovine Oocyte Maturation

Metabolic labeling and immunoprecipitation assays were used to demonstrate the synthesis of Mos in bovine oocytes (300 per group) during the course of IVM. The complexity of labeled proteins solubilized from Mos immune complexes (due to nonspecific antiserum effects and/or trapping within recovered SAC-complex pellets) required that comparisons be made between immunoprecipitations performed in the presence and absence of competing Mos peptide. Nonetheless, fluorography following immunoprecipitation and SDS-PAGE revealed that bovine oocytes actively synthesize p39^{mos} in the newly matured oocytes between 22 and 26 h of IVM (Fig. 2, lane 4). There was little synthesis during the first 4 h of IVM culture and no synthesis in aging MII-stage oocytes at 44–48 h of IVM (Fig. 2, lanes 2 and 6). Several additional immunoprecipitation assays were performed with essentially the same results.

Mos Protein Disappearance in Activated Bovine Oocytes

In order to examine the changes in Mos protein levels after parthenogenetic activation, matured MII oocytes (22 h of IVM) and oocytes at 12 h after activation were collected and lysed in SDS sample buffer. Figure 3, lane A, shows that p39^{mos} was detected only in the matured bovine oocytes, whereas the protein was not detected in the acti-

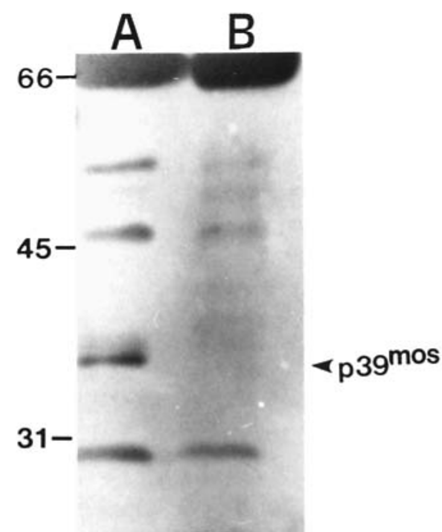

FIG. 3. The disappearance of Mos protein in activated bovine oocytes. Five hundred oocytes per group were used to detect the presence of Mos in matured bovine oocytes (lane A) and in activated oocytes (lane B) with Western blot analysis using the methods described for Figure 1. An approximately p39-kDa Mos protein appeared in lane A, but not in lane B, as indicated by the arrow. Molecular weight markers ($\times 10^{-3}$) are shown at left.

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vated oocytes (Fig. 3, lane B), indicating that the activation events resulted in the loss of Mos from bovine oocytes.

Analysis of Mos Immune Complex Co-Precipitants

Mos possesses an intrinsic serine-threonine kinase activity that is required for meiotic maturation of oocytes [22, 36]. Studies of Mos expression in human neuroblastoma cells [37] and Mos activity in *Xenopus* oocytes have shown that tubulin can form stable associations with Mos and serves as a substrate for its kinase activity [38]. Mos was also found to associate with $p34^{cdc2}$ in Mos-transformed NIH 3T3 cells [39], while the cyclin B-$p34^{cdc2}$ complex (MPF) has been shown to play a role in microtubule dynamics in starfish oocytes [40].

Mos immune complexes prepared from metaphase-arrested bovine oocytes, as described above, were resolved by SDS-PAGE and analyzed for the presence of tubulin and $p34^{cdc2}$. While $p34^{cdc2}$ could be detected in unfractionated lysates of oocytes (Fig. 4, lane O), none was observed to co-precipitate with Mos (lane P). $p34^{cdc2}$ remained in the assay supernatant (lane S) at levels comparable to that observed in the unfractionated lysate. β-Tubulin, on the other hand, was partitioned between the supernatant fraction and the Mos immune complex (lane P). Thus β-tubulin, but not $p34^{cdc2}$, was co-precipitated by Mos antiserum.

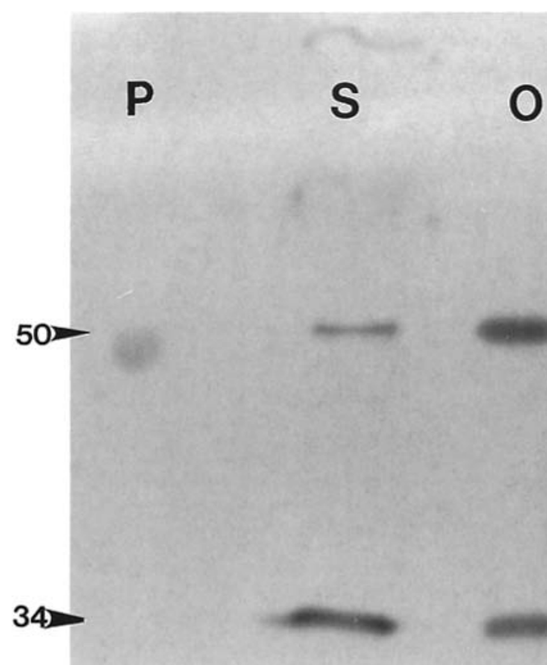

FIG. 4. Co-immunoprecipitation of Mos and β-tubulin. MII-stage oocytes (1000 oocytes) were lysed in RIPA buffer and immunoprecipitated using Mos antiserum. Both the immune complex pellet (P) and the supernatant fraction (S) were resolved by SDS-PAGE and transferred to PVDF membrane. Lane O represents a similarly resolved and blotted whole oocyte lysate (1000 oocytes). The membrane was sequentially probed using anti-β-tubulin and anti-cdc2 antibodies and was processed for ECL detection. Both cdc2 (34-kDa band) and β-tubulin (50-kDa band) were detected in the unfractionated lysate. The Mos immune complex (P) contains β-tubulin but not $p34^{cdc2}$. Molecular weight markers ($\times 10^{-3}$) are shown at left.

DISCUSSION

In the past decade, studies on the expression of c-*mos* proto-oncogene have been conducted extensively at either the mRNA or protein level in amphibia and mammals [20, 41–43]. While the v-*mos* proto-oncogene encodes a 37.8-kDa primary gene product having an intrinsic serine/threonine protein kinase activity [44], a 43-kDa Mos protein was demonstrated in mouse testes [45] and rat skeletal muscle [46] with antibodies distinct from those used here. In addition, Mos candidates of 37 kDa (major form) and both 40 and 35 kDa (minor forms) were detected in a human neuroblastoma cell line [37]. In *Xenopus* and mouse oocytes, a 39-kDa protein identified as Mos was shown to be a key component of CSF and an initiator of MPF activity as demonstrated by several early studies [19, 47–49]. These findings suggest that the size of Mos may vary among different species and/or tissues. We used a "universal" Mos antibody raised against highly conserved sequences in *Xenopus*, mice, and humans and detected an approximately 39-kDa Mos protein in bovine oocytes. Additional Mos detection experiments using large numbers of bovine oocytes suggested that the molecular mass of the Mos protein varied between 37.5 and 39 kDa, which seemed to be similar to the size of the v-*mos* oncogene primary gene product of 37.8 kDa [45]. When bovine oocyte samples were compared with NIH 3T3 cells transformed by c-mos^{xe}, Mos from these two sources was indistinguishable in terms of mobility on the same SDS-PAGE (data not shown). Accordingly we designated the protein detected in bovine oocytes as $p39^{mos}$. It remains possible that there may be less sequence conservation between cattle and other species [12, 19]. Recently, $p39^{mos}$ expression was also detected in bovine oocytes with another Mos antibody [50]. In our experiments, although Mos could be detected in approximately 200 bovine oocytes by Western blot analysis, its weak signal along with nonspecific binding by the antiserum necessitated the use of a large number (about 1000) of bovine oocytes in most experiments. This also implied that bovine oocytes contained very small amounts of Mos. The analysis with preimmune serum and that which had been neutralized with Mos peptide as control substantiated the protein's identity as $p39^{mos}$. These results indicated that bovine oocytes expressed the c-*mos* proto-oncogene during maturation via de novo protein synthesis. The fact that the Mos protein disappeared in activated oocytes confirmed early findings in other species that Mos plays a role in maintaining meiotic arrest in oocytes [11, 15, 19].

Oocytes from a wide variety of organisms accumulate mRNA during their growth phase [51]. In mouse oocytes, Mos mRNA transcripts increased as the oocytes reached full size [41, 52], and human oocytes also contain abundant Mos transcripts [43]. Polyadenylation of Mos mRNA is also a pivotal regulatory step in *Xenopus* meiotic maturation [53]. In order to observe the onset and extent of Mos synthesis, we examined three groups of oocytes at different stages. Mos synthesis was observed in oocytes as they reached the MII stage but not in aged MII oocytes. These findings suggest that 1) MII arrest probably requires Mos synthesis, 2) Mos may play a critical role in stabilizing MPF activity to maintain MII arrest, and 3) spontaneous activation, which occurs more readily in aging oocytes, may be due to the reduced synthesis of Mos. Spontaneous activation of bovine oocytes after 30 h of IVM may also result from gradual Mos degradation and the subsequent decline in MPF activity [54]. Related observations of MPF activity, as measured by histone H1 kinase levels in single bovine oocytes, indicated that most bovine oocytes lose MPF activity after 30 h of IVM [55]. The levels of Mos

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and its synthesis at additional times of maturation in bovine oocytes remain to be investigated.

Tubulin was shown to be co-precipitated with Mos in the Mos immune complexes (Fig. 4). Several previous studies suggested that Mos can associate with phosphorylated tubulin in *Xenopus* oocytes, in Mos-transformed fibroblasts [38], and in a human neuroblastoma cell line [37]. v-Mos protein and the mouse c-Mos protein were capable of copolymerizing with tubulin [56]. The serine-threonine Mos kinase was believed to be involved in microtubule reorganization leading to formation of the spindle and spindle pole [1, 20, 27, 57, 58]. Thus tubulin not only functions as the microtubular cytoskeleton in cells; it also forms the meiotic spindle in the maturing oocytes, the dynamics of which may be regulated by Mos [5, 44, 59]. Microtubule-associated protein (MAP) is also a potential substrate for Mos kinase activity [38]. In our experiments, while only a small proportion of tubulin was co-precipitated with Mos antibody, the fact that Mos associates with at least some β-tubulin during bovine oocyte meiotic arrest suggests that Mos may be responsible, either directly or indirectly, for modifications of tubulin, thus providing for a further modulation of MPF activity.

We were not able to demonstrate an association or the formation of a stable complex between Mos and $p34^{cdc2}$ in mature, metaphase-arrested bovine oocytes. Other studies in both interphase and metaphase cells indicated that Mos may associate with $p34^{cdc2}$, but this complex lacks H1 kinase activity [39, 46, 48]. Oocytes matured in vitro for 22 h are known to be arrested in MII [28] and to contain a high level of H1 kinase activity [55, 60–62]. The fact that $p39^{mos}$ did not form a complex with $p34^{cdc2}$ in MII-stage oocytes may be responsible for, or may be a consequence of, the consistently high level of histone H1 kinase activity at this time. Consequently, a Mos-$p34^{cdc2}$ association may be found in bovine oocytes at times other than the metaphase arrest. It is also possible that Mos stabilizes MPF through other pathways such as binding to tubulin or B-type cyclins [39, 56] since tubulins and cyclin B_2 are known to be the primary substrates for Mos kinase activity during metaphase arrest [13, 16, 63].

In conclusion, the data presented here demonstrate that Mos is present and actively synthesized in mature bovine oocytes and that Mos disappears after parthenogenetic activation of the oocytes. β-Tubulin was found to be associated with Mos, which suggests a possible role for the cytoskeletal protein in contributing to the meiotic arrest in mature bovine oocytes. Therefore, we conclude that the presence of Mos may play an important regulatory role in the arrest at meiosis MII in bovine oocytes.

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