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Quantification of Cyclin B1 and p34^{cdc2} in Bovine Cumulus-Oocyte Complexes and Expression Mapping of Genes Involved in the Cell Cycle by Complementary DNA Macroarrays[1]

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ABSTRACT

Although high amounts of cyclin B1 mRNA are present in bovine oocytes arrested at the germinal vesicle (GV) stage, the protein is not detectable. Furthermore, there is a depletion of the stored cyclin B1 mRNA in the oocyte as follicular growth progresses. To assess the effect of follicular growth on the accumulation of M-phase promoting factor (MPF) components, mRNA and protein levels of cyclin B1 and p34^{cdc2} were measured in GV oocytes collected from diverse follicle size groups (<2 mm, 3–5 mm, and >6 mm). Because oocytes collected from very small follicles have high levels of cyclin B1 mRNA, the onset of its accumulation in the oocytes was evaluated by in situ hybridization of fetal ovaries. Also, a comparative expression map of cell cycle-related genes expressed in the oocyte and cumulus cells was established using nylon-based cDNA arrays, which allowed the detection of 35 different genes transcribed mostly in oocytes. Both components of the pre-MPF complex were expressed at the mRNA level in GV oocytes, whereas p34^{cdc2} was the only pre-MPF protein detected at that stage, thus indicating that meiosis resumption in bovine oocytes is differentially regulated as compared with other mammals, and meiosis resumption seems to be regulated by the translation of cyclin B1 mRNA.

cumulus cells, gametogenesis, gene regulation, oocyte development, ovum

INTRODUCTION

In mammals, a finite number of oocytes arrested at the prophase stage of meiosis (late G_2 of the cell cycle) are stored in the ovaries. These oocytes can remain dormant in some species for many years until maturation commences due to follicular recruitment triggered by an unknown signal. Following recruitment, oocyte growth occurs concomitantly with follicular growth; during this phase, the oocyte acquires meiotic competence [1, 2]. This meiotic competence allows the oocyte, either ovulated or physically extracted from the follicle, to resume meiosis, which triggers a cascade of morphological events that bring the oocyte to the second metaphase (MII) stage, where it awaits fertilization (for review see [3]). The first observable event characterizing the meiotic resumption of an oocyte is germinal vesicle (GV) breakdown (GVBD), which occurs in nearly all full-size oocytes [4]. However, GVBD is not dependent on meiotic competence; bovine oocytes <100 μm in diameter from very small follicles (<1.8 mm) can undergo GVBD, but most cannot reach the MII stage [2, 4]. Furthermore, oocyte growth and expansion is correlated with follicular growth until the follicle reaches 3 mm, at which point the oocyte diameter reaches a plateau [2]. Thus, small bovine oocytes (<100 μm) collected from small follicles (<2 mm) are impaired in their capacity to reach the MII phase.

An important aspect of oocyte growth is the accumulation of RNA in the cytoplasm and the nucleolus [5], which stops when the oocyte reaches a diameter of 110 μm [2]. These pools of stored RNA are essential to ensure proper protein synthesis during transcriptional silencing of the bovine embryonic genome that occurs during meiotic maturation, fertilization, and the first embryonic cell divisions. Transcription in the early bovine embryo is mostly inactive until the 8- to 16-cell stage, at which point the maternal to zygotic transition (MZT) occurs [6]. Although minor transcriptional activity is detected as early as the two-cell stage [7], it is not necessary for further embryonic development because most embryos treated with an RNA polymerase II transcription inhibitor (α-amanitin) during the first cell divisions reach the 8- to 16-cell stages [8, 9]. Therefore, it is most probable that the proteins required to undergo these first cell cycles are stored as maternal RNAs during early oocyte growth. One of our objectives was to establish an inventory of the stored cell cycle-related maternal RNAs, which was accomplished by the use of cDNA macroarrays.

In addition to mRNA, the proteins accumulated during oocyte growth are also important for the resumption of meiosis. Once the oocyte is extracted from the follicular environment, resumption of meiosis is followed by a latent period during which the M-phase promoting factor (MPF), a protein complex composed of two subunits, cyclin B and p34^{cdc2} [10], is activated [11]. The MPF activity in oocytes has been well characterized by fusion experiments between meiotically incompetent and competent oocytes, demonstrating that active MPF from a competent oocyte can induce condensation of chromatin in the immature oocyte nucleus [12]. Therefore, the ability of the oocyte to resume meiosis is associated with MPF activity, yet species-specific control of MPF activation exists (reviewed in [13]). In the mouse, the acquisition of meiotic competence is partly associated with the synthesis of p34^{cdc2} [14–17], suggesting that a lack of one of the MPF components keeps the oocyte in meiotic arrest. Similarly, although both cyclin B1 mRNA and protein have been detected in meiotically competent

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TABLE 1. Primer sequences used to amplify the studied genes.

Gene	Strand	Sequence	Temperature of fluorescence acquisition (°C)
Cyclin B1	Upper	5′-TTGTGACTGACAACACCTACACC-3′	83
	Lower	5′-AGAATTCAGCTGTGCTAGAGTGC-3′	
$p34^{cdc2}$	Upper	5′-AGGAAGGGGTTCCTAGTACTG-3′	82
	Lower	5′-GAAAAAGTGGTTTCTTCGTTGC-3′	
β-Actin	Upper	5′-CGTGACATTAAGGAGAAGCTGTGC-3′	88
	Lower	5′-CTCAGGAGGAGCAATGATCTTGAT-3′	
Ubiquitin	Upper	5′-TTTTCGTGAAGACCCTGACCG-3′	86
	Lower	5′-TAAATGGCTAGAGTGCAGAAGG-3′	
Globin	Upper	5′-GCAGCCCACGGTGGCGAGTAT-3′	90
	Lower	5′-GTGGGACAGGAGCTTGAAAT-3′	

and incompetent goat oocytes [18], only $p34^{cdc2}$ mRNA is present in meiotically incompetent oocytes [19]. In contrast, both components of MPF are present in the pig oocytes, suggesting that meiotic arrest in the pig is likely controlled by another pathway that involves protein phosphate transfer (kinase and/or phosphatase) [20]. In the cow, previous studies from our laboratory have shown that cyclin B1 is not present at the protein level in oocytes at the GV stage [21]. However, cyclin B1 mRNA is present in bovine GV stage oocytes, and it appears to be depleted during follicular growth [22]. This depletion could be associated with mRNA degradation or translation into cyclin B1 proteins. Although translation into protein is plausible, it is not supported by previous studies in which cyclin B1 protein has not been detected in GV stage oocytes [21]. In an attempt to clarify whether translation to protein occurs, we analyzed the transcript and protein levels of the pre-MPF components in bovine GV oocytes collected from groups of follicles of different sizes. We demonstrated how stored pre-MPF components may control meiosis resumption in the bovine oocyte and identified the cell cycle-related gene transcripts stored in the maternal RNA pool.

MATERIALS AND METHODS

Oocyte and Cumulus Cell Collection

Bovine ovaries were collected from a commercial slaughterhouse. The cumulus-oocyte complexes (COCs) from 3- to 5-mm follicles were collected by aspiration, and the COCs from <2-mm and >6-mm follicles were collected by dissection with the aid of a stereomicroscope. The cumulus cells were mechanically separated from the oocytes, recovered, pelleted by centrifugation, and frozen immediately at −80°C until RNA extraction. The oocytes were washed three times in PBS buffer to prevent cumulus cell contamination of the samples and were frozen at −80°C in a minimum volume until RNA extraction.

Quantitative Real-Time Polymerse Chain Reaction

Six pools of 20 oocytes were used for each follicle size group. The RNA was extracted using Microprep SpinColumn (Stratagene, La Jolla, CA), and a DNase I treatment was performed during the extraction. To account for RNA loss during the extraction procedures, 0.1 pg of rabbit globin (Life Technologies, Burlington, ON, Canada) was added to the samples prior to the RNA extraction. The total RNA was eluted from the columns in a volume of 50 μl. The RNA was coprecipitated by adding 10 μg of glycogen (10 mg/ml; Boeringer-Manheim, Laval, PQ, Canada) to 5 μl of 3 M sodium acetate (pH 5.2) and 55 μl of isopropanol. The pelleted RNA was washed in 70% ethanol and dried prior to reverse transcription. First-strand cDNA was synthesized using a SuperScript Preamplification System for First Strand cDNA Synthesis kit (Life Technologies) and 100 ng of $oligo(dT)_{12\text{-}18}$ primer in a total reaction volume of 20 μl. A cDNA aliquot of 2 μl was subjected to quantitative polymerase chain reaction (PCR) amplification using a Light Cycler apparatus (Roche Diagnostics, Laval, PQ, Canada). Products were detected with SYBR Green (Roche Diagnostics). The sequences of the primers used for each amplification are listed in Table 1. Prior to quantification, optimization procedures were performed by running PCRs with or without the purified template to identify the melting temperatures of the primer dimers and the specific product. To measure the level of mRNA in the samples, the fluorescence values were taken at a temperature associated with the beginning of the peak for the specific product (Table 1). The standard curve was generated using the DNA from a PCR product purified by agarose electrophoresis and band extraction using Qiaquick columns (Qiagen, Mississauga, ON, Canada) and five serial dilutions ranging from 100 pg to 1 fg. The amplification program was as follows: preincubation for FastStart polymerase activation at 95°C for 10 min followed by 40 amplification cycles of denaturation at 95°C for 0 sec (20°C/sec), annealing for every gene at 57°C for 5 sec (20°C/sec), elongation at 72°C for 16 sec (2°C/sec), and acquisition of fluorescence (see Table 1 for temperature) for 5 sec (20°C/sec). After the end of the last cycle, the melting curve was obtained by starting the fluorescence acquisition at 72°C and taking measurements every 0.1°C until 95°C was reached.

In Situ Hybridization

Eight fetal ovaries were collected from the slaughterhouse. The approximate age of the fetus was estimated based on the tail-head length. The ovaries were fixed at 4°C in 4% buffered paraformaldehyde (pH 7.2) for 48 h, dehydrated, embedded in paraffin, and sliced into 6-μm-thick sections. A subset of four sections was mounted on slides, and the paraffin was removed by several washes in xylene. The tissue was then rehydrated and treated with 0.2 M HCl, 1 μg/ml proteinase K, 0.1 M triethanolamine (pH 8), and 0.25% anhydrous acetic acid for 5 min at room temperature. The cyclin B1 probe was cloned as previously described [19] and labeled with ^{35}S-dATP (Amersham-Pharmacia, Baie D'Urfé, PQ, Canada) using the Nick-translation kit (Boeringher Manheim). Prehybridization and hybridization were performed at 42°C in a humidified chamber with optimized buffers as described previously [23]. The specific activity of the probe was 2×10^8 cpm/μg, and 2×10^5 cpm were used per hybridization. Posthybridization washes in 0.1× standard saline citrate (SSC) were performed three times at 42°C for 60 min each time. The tissue was dehydrated, air dried, covered with NTB_2 emulsion, and exposed in the dark at 4°C. After 4 wk of exposure, the slides were developed in D19 buffer (Eastman-Kodak, Rochester, NY), fixed, and stained with Giemsa.

Western Blots

Pools of 200 GV oocytes were used for each follicle size group. Except for the Hela cell proteins, which were already extracted and included as a positive control with the cyclin B1 antibody, the proteins from the other samples were extracted using a modified RIPA medium: 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM sodium fluoride, and 1 μg/ml of other protease inhibitors (aprotinin, leupeptin, and pepstatin). The protein extracts were loaded on a 12% SDS-polyacrylamide gel. The proteins were transferred by electrophoresis on Hybond-enhanced chemiluminescence membranes (Amersham-Pharmacia) using a Trans-blot apparatus (Bio-Rad, Mississauga, ON, Canada). The membranes were blocked with freshly prepared PBS containing 3% nonfat dry milk. The primary antibodies for cyclin B1 (no. 05-158) and $p34^{cdc2}$/CDK1 PSTAIR (no. 06-923) were purchased from Upstate Biotechnologies (Lake Placid, NY). The hybridizations were performed using a 1:1000 dilution of cyclin B1 or a 1:2000 dilution of $p34^{cdc2}$ antibodies during an overnight incubation at 4°C with agitation. The hybridized mem-

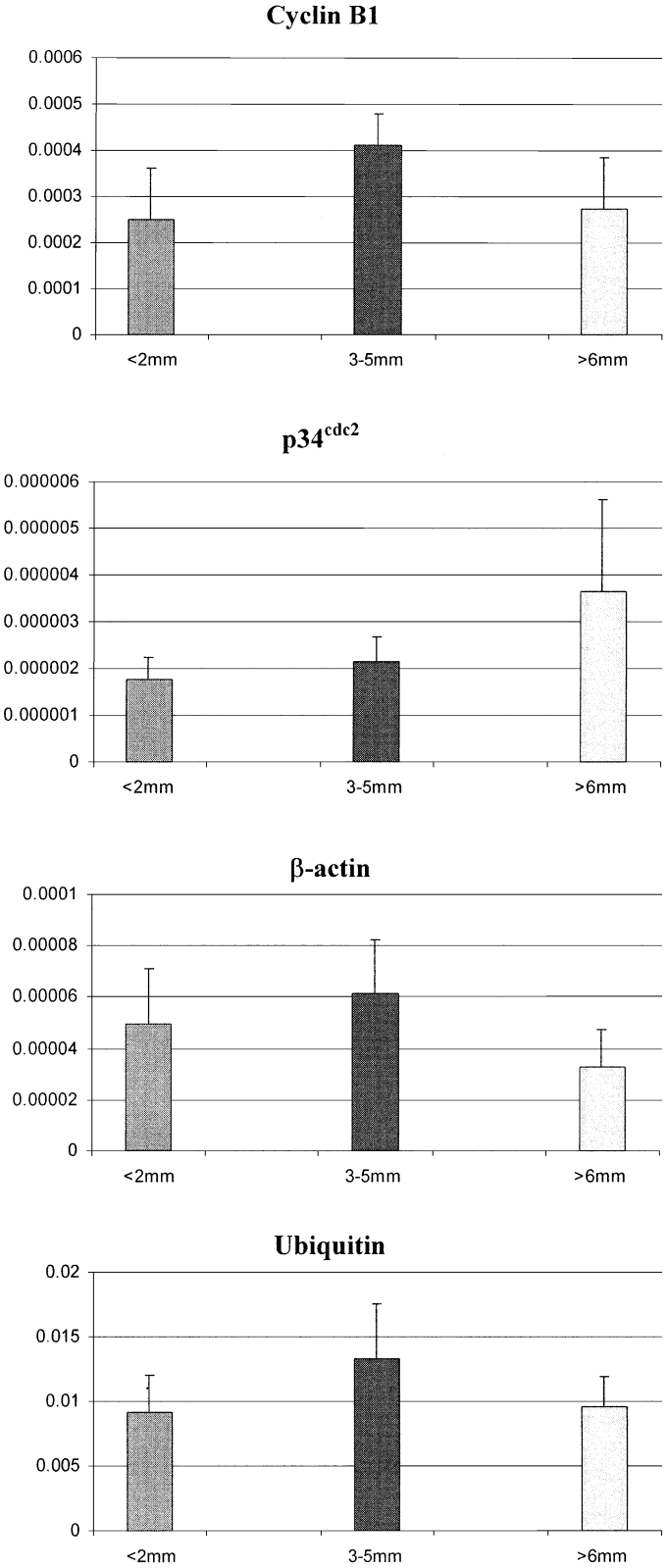

FIG. 1. Messenger RNA level of cyclin B1, p34^{cdc2}, β-actin, and ubiquitin, relative to the exogenous rabbit globin, was evaluated in bovine GV oocytes from different follicle size groups using real time PCR. The follicle size groups are indicated under each graph. Vertical bars represent SD of the mean (n = 6).

branes were washed with water and hybridized with the second antibody coupled with horseradish peroxidase; for cyclin B1 an anti-mouse IgG (Upstate Biotechnologies) was used, and for p34^{cdc2} an anti-rabbit IgG (Upstate Biotechnologies) was used. The second hybridization was performed at room temperature for 1 h. The detection was done by enhanced chemiluminescence using the ECL kit (Amersham-Pharmacia) and autoradiographic films (Eastman-Kodak).

Complementary DNA Macroarray

Two nylon-based human cDNA macroarrays were purchased from Clontech Laboratories (La Jolla, CA). For both macroarrays, all the cDNAs were spotted in duplicate. The first array was the Atlas Human Array Trial Kit containing 82 human cDNAs and nine housekeeping control cDNAs. It was hybridized with a probe prepared from a pool of 1200 GV oocytes collected from 3- to 5-mm follicles. The second macroarray was the Atlas Human Apoptosis Array containing 205 human cDNAs and nine housekeeping control cDNAs. The apoptosis array was hybridized with a probe prepared from a pool of 1700 GV oocytes from the same follicle size group (3–5 mm). Two identical membranes are provided with each Atlas array kit, and for both macroarrays the second membrane was hybridized with probes prepared from the corresponding cumulus cells. The total RNA was extracted using Trizol reagent (Life Technologies). To eliminate DNA contamination, the samples were treated with DNase I and further purified by phenol/chloroform (Sigma, St. Louis, MO) extraction. The probe preparation and hybridization procedures were performed as described by the manufacturer. The total RNA was reverse transcribed using an oligonucleotides cocktail specific to the targeted genes displayed on the array. The probes were radiolabeled by the incorporation of [^{32}P]dATP (Amersham-Pharmacia) during the reverse transcription reaction. The probes were added to the prewarmed ExpressHyb solution (Clontech Laboratories), and the hybridizations were performed at 68°C overnight in an air incubator. The membranes were washed thoroughly in 2× SSC and 1% SDS three times for 30 min each at 68°C. Another wash was performed in 0.1× SSC and 0.1% SDS for 30 min also at 68°C. A final wash was performed in 2× SSC for 5 min at room temperature. The membranes were revealed using a PhosphorImager apparatus (Amersham-Pharmacia), and for the second array the membranes were also exposed to autoradiographic film (Eastman-Kodak).

Statistical Analysis

The effects of follicular growth on the cyclin B1 and p34^{cdc2} mRNA levels were analyzed. Each mRNA value was corrected to account for RNA loss during procedures, using the quantified values of the rabbit globin that was added to each sample prior to RNA extraction as an exogenous standard. The means from six replicates for each follicle size group were compared by ANOVA. The variance between the replicates was used as the error term, and a confidence level of 95% ($P < 0.05$) was used to compare differences between means.

RESULTS

To quantify relative amounts of cyclin B1 and p34^{cdc2} mRNA, two housekeeping genes were considered because the β-actin mRNA was too variable among oocyte pools, with particularly low levels in the >6-mm follicle group. Therefore, the ubiquitin transcript levels were also measured and were more stable between the different follicle size groups.

At the mRNA level, the transcripts for both components of MPF were detected in the oocyte samples using material equivalent to two oocytes. Even with such small sample sizes, the cyclin B1 transcript was measured in relatively high amounts in all samples; the relative abundance of the cyclin B1 mRNA was about 10-fold higher than that of β-actin mRNA (Fig. 1). However, p34^{cdc2} mRNA was almost undetectable; even after PCR amplification of 50 cycles, the levels of p34^{cdc2} remained about 100-fold lower than the cyclin B1 mRNA levels (Fig. 1). p34^{cdc2} mRNA was undetectable in some oocyte samples; even when the transcript was faintly detected with the real time PCR system, no bands for the PCR products were seen on agarose gels (data not shown). There was no relationship ($P > 0.05$)

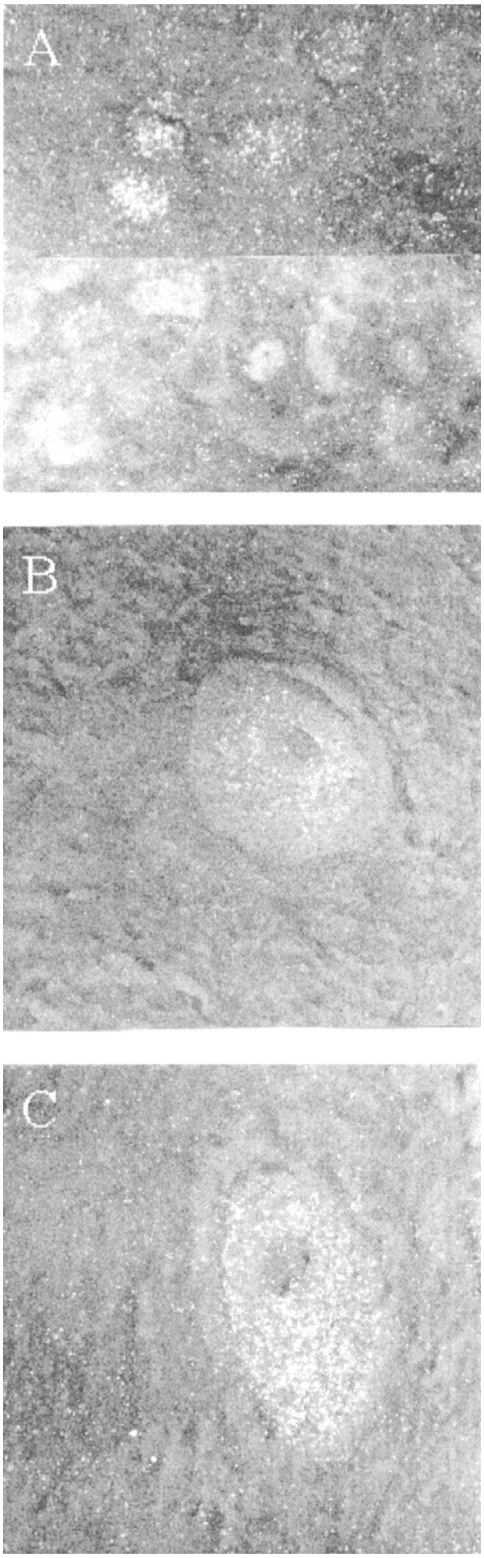

FIG. 2. In situ hybridization for cyclin B1 mRNA in fetal bovine ovaries at 135 dpc (**A**), 245 dpc (**B**), and 265 dpc (**C**). Most of the grains are distributed over the cytoplasm of the oocytes (**A**–**C**) or oogonia (**A**) but are absent from the GVs (**B** and **C**) and surrounding somatic cells (**A**–**C**). ×32.

between the relative abundance of cyclin B1 and $p34^{cdc2}$ mRNA, and follicle size (Fig. 1).

Because cyclin B1 mRNA could be detected in oocytes from small follicles (<2 mm), the time of acquisition of cyclin B1 mRNA was evaluated by in situ hybridization of

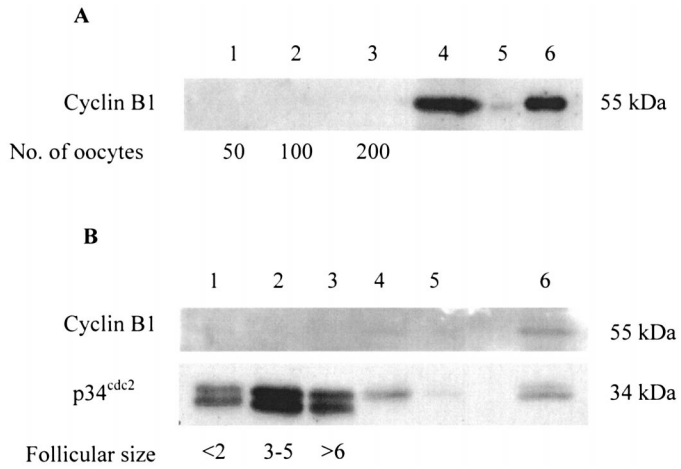

FIG. 3. Western blot analysis of the presence of cyclin B1 and $p34^{cdc2}$ in bovine oocytes, cumulus cells, and granulosa cells. **A**) Various numbers of GV oocytes from 3- to 5-mm oocytes were used to detect the presence of cyclin B1 protein. The number of oocytes used is indicated under lanes 1, 2, and 3. Lanes 4, 5, and 6 are protein extracts from granulosa, cumulus, and Hela cells, respectively. **B**) Pools of 200 GV oocytes were used in lanes 1, 2, and 3. The follicle size group (mm) from which they were collected is indicated below each lane. Lanes 4, 5, and 6 are protein extracts from granulosa, cumulus, and Hela cells, respectively.

fetal bovine ovaries. Cyclin B1 mRNA was already visible in fetal ovaries 135 days postcoitum (dpc) and could be detected up to 265 dpc (Fig. 2). This mRNA was clearly excluded from the GV of the oocytes at 245 and 265 dpc and from the surrounding somatic cells. At 135 dpc, cyclin B1 mRNA was detected as two to four patches of grains, likely over young oocytes surrounded by a single layer of flattened somatic cells, as inside fetal primordial follicles.

No cyclin B1 protein was detected by Western blot analysis in any of the oocyte pools used (Fig. 3, A and B); a slight amount of protein was detected in the cumulus cells, and strong signals were obtained in the mural granulosa and human Hela cells, used here as positive controls (Fig. 3A). However, when the same blot was hybridized to detect the presence of $p34^{cdc2}$, a strong signal was obtained in all of the oocyte samples, with the highest signal detected in the 3- to 5-mm follicles (Fig. 3B). Moreover, two bands were detected in the oocyte samples, indicating that $p34^{cdc2}$ is either present in two phosphorylated states in the GV stage oocytes or that the anti-PSTAIR antibody recognizes $p33^{cdk2}$ in addition to $p34^{cdc2}$. Because cyclin B1 and $p34^{cdc2}$ are the two components of pre-MPF, this analysis shows that the immature (GV) bovine oocyte lacks the cyclin B1 component to form the pre-MPF complex. In summary, the protein and transcript expression patterns of these two pre-MPF components are opposite in bovine oocytes at the GV stage: cyclin B1 is stored as cytoplasmic mRNA with undetectable levels of the protein, whereas high quantities of $p34^{cdc2}$ protein are stored in the oocyte with very low levels of the corresponding mRNA.

To evaluate the presence of cell cycle-associated mRNAs in bovine oocyte and cumulus cells, cDNA macroarrays were evaluated to establish an expression map (Figs. 4 and 5). Confirming the results obtained by real time PCR, both components of the MPF complex were found at the mRNA level, and the cyclin B1 mRNA levels were higher than those for $p34^{cdc2}$ (Fig. 5). The same observation was made for ubiquitin and β-actin mRNAs (Figs. 4 and 5); ubiquitin mRNA levels were higher than those for β-actin. Overall, numerous transcripts for cell cycle-related genes were found

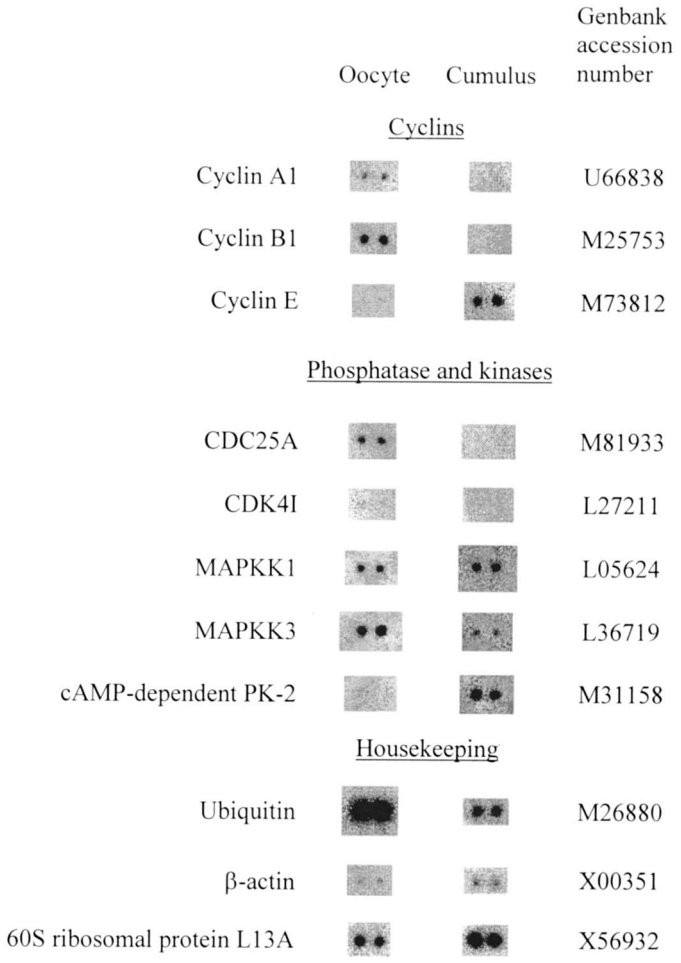

FIG. 4. Comparative expression mapping of genes involved in the cell cycle based on information from a human cDNA nylon-based array. The hybridizations were performed using probes prepared from total RNA extracted from 1200 denuded GV oocytes from 3- to 5-mm follicles or the corresponding cumulus cells.

in the oocyte, whereas the cumulus cells showed very weak signals for the same genes (Figs. 4 and 5). These hybridizations showed that among the gene transcripts found in the GV oocytes, several cyclins, numerous protein kinases, and members of the mitogen-activated protein kinase (MAPK) pathway were present (Figs. 4 and 5). Proliferating cell nuclear antigen (PCNA) mRNA was detected in the GV oocyte but was absent in the cumulus cells. Cyclin B1, MAPKK1, and the housekeeping cDNAs were present on both arrays and produced similar signals, indicating that the lack of signal in the cumulus cells is not the result of poor probe preparation. Only two mRNAs, cAMP-dependent PK-2 and cyclin E, were highly expressed in cumulus cells but almost undetectable in the oocyte (Fig. 4).

The macroarrays used displayed human cDNA, whereas the probes were prepared from bovine RNA. The hybridization effectiveness using the macroarray technology for cross-species gene expression analysis showed that for the 82 cDNAs on the trial membrane, 37 and 30 targets were detected with the oocyte and cumulus cell probes, respectively, whereas with the apoptosis macroarray bearing 205 cDNAs, 82 and 51 targets were detected using the oocyte and cumulus cell probes, respectively. Both membranes had the same nine housekeeping genes, eight of which were detected in both membranes with both probe types.

DISCUSSION

Several follicle size groups were used to analyze the influence of follicular maturation on mRNA and protein levels of the MPF components. Follicle size is indirectly associated with the capacity of the enclosed oocyte to resume meiosis [2, 4]; in the early stages of follicular development an increase in follicle size is strongly correlated with oocyte growth until the oocyte reaches its maximum size when the follicle reaches 3 mm [2]. On average, the bovine oocyte acquires the capacity to resume meiosis to the MII stage after reaching a diameter of 110 μm in antral follicles of 2 mm [2, 24]. The transcriptional silencing of the bovine genome occurs when the oocyte is 110 μm in diameter [2, 24]. Therefore, oocytes collected from follicles of <2 mm are smaller in size and are meiotically less competent than those collected from larger follicles. Taken together, we would not expect to observe much difference between oocytes from the 3- to 5-mm and >6-mm size groups with regard to MPF components because at these follicle sizes, the oocytes are transcriptionally inactive, at maximum size, and able to resume meiosis. In a previous study using a suppressive subtraction hybridization (SSH) approach to identify maternal transcripts accumulated during oocyte growth [22], cyclin B1 mRNA levels were higher in the oocytes collected from <2-mm follicles than in those collected from the 3- to 5-mm follicles [22]. However, the results presented here indicate that follicle size does not influence the transcript levels of both components of the MPF. The discrepancies between both reports can be explained technically. In our previous study [22], cyclin B1 was identified as more abundant in oocytes collected from smaller follicles (<2 mm) than in oocytes collected from 3- to 5-mm follicles. The SSH approach required a PCR amplification step prior to the subtraction to obtain sufficient amount of starting material. During that global amplification step, a visual evaluation of the amplification is made to ensure that an equal amount of total cDNA of both cell types is added in the subtractive reaction. Because the total RNA content is lower in the oocytes collected from <2-mm follicles [2, 4] and the amount of cyclin B1 is similar between both size groups, amplification of both samples to obtain the same amount of total cDNA would result in a higher amount of cyclin B1 in the <2-mm sample, which was effectively detected by the SSH.

As reported previously [25], when using an oligo dT to prime the reverse transcription reaction, the amount of PCR product detected can be affected by the polyadenylated state of the mRNAs because the lack of a poly(A) tail on the stored maternal mRNAs can impair the reverse transcription step. Reports showing that stored maternal mRNAs have a shorter poly(A) tail are numerous [5, 26, 27]; however, they do not indicate that the tail is totally removed. It has been clearly reported that the poly(A) tails are short when the maternal mRNAs are stored and are elongated when they are recruited for translation [28, 29]. Given the complex nature of mRNA storage and recruitment, the length of the poly(A) tail remaining after the deadenylation process is more or less specific to each template [30]. Because we do not have sufficient information concerning the length of the poly(A) tail of the stored mRNAs studied in the present paper, we do not have sufficient evidence to conclude that the length of the poly(A) tail could be different in the oocytes of different follicle size groups.

Nevertheless, the PCR analysis revealed that cyclin B1

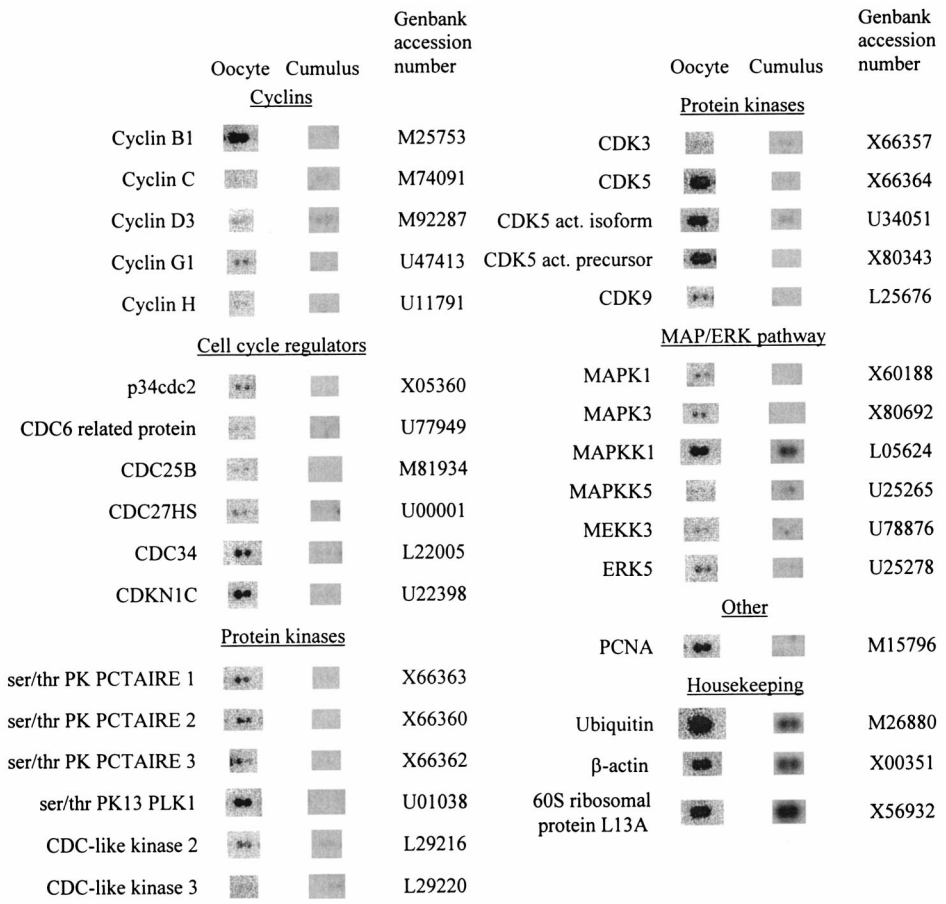

FIG. 5. Comparative expression mapping of genes involved in the cell cycle based on information from a human cDNA nylon-based array. The hybridizations were performed using probes prepared from total RNA extracted from 1700 denuded GV oocytes or the corresponding cumulus cells. The hybridization spots for the cumulus cells were obtained from an image produced by a prolonged exposure of the membrane to autoradiographic film, whereas the image for the oocytes was obtained with a PhorphorImager apparatus.

mRNA is present in very small antral follicles, indicating that this transcript is accumulated very early during follicular development. In situ hybridization revealed cyclin B1 mRNA in fetal ovaries from 135 to 265 dpc, showing that the accumulation of the transcript occurs extremely early in ovarian development. However, it was unclear whether this transcript is exclusively present in oocytes or whether it is also present in some oogonias. Because meiosis begins in cattle as early as 75–80 dpc but oogonial mitosis goes on discontinuously until 150–170 dpc [31, 32], prophase-engaged oocytes (42%), dividing oogonia (30%), and degenerating (28%) oogonia coexist at 135 dpc in fetal ovaries [25], leading to an uncertainty at that stage. In older ovaries, however, the result is clear: the cyclin B1 gene is highly expressed in fetal GV oocytes, with almost no transcript inside the GV or in the surrounding somatic cells.

At the protein level, the highest level of the other component of the MPF, $p34^{cdc2}$, was found in oocytes collected from 3- to 5-mm follicles. The smallest amounts of $p34^{cdc2}$ found in the <2-mm follicles could suggest that this protein is accumulated during oocyte growth, whereas larger amounts of the protein in the larger follicles (>6 mm) could be associated with larger quantities of degenerating oocytes found in this follicle size group. The present data did not take into account the relative differences in oocyte size and were not corrected for oocyte volume [19] or total protein content [13] or absolute amount [16] because the MPF should be associated with the number of nuclei that reinitiate meiosis rather than the cytoplasmic volume or total protein content. That is, the capacity to resume meiosis is most probably associated with a threshold amount of active MPF independently of the cytoplasmic volume rather than a concentration level.

The activation mechanisms of the MPF have been studied in several species of mammalian oocyte, and several species-specific differences have been identified. The mouse oocyte is dependent on the synthesis of $p34^{cdc2}$ [13, 14, 16] to resume meiosis, whereas in the pig the mechanism must be controlled at a different level because both MPF components are present [17, 33]. In goat oocytes, cyclin B1 and $p34^{cdc2}$ were detected in both meiotically competent and incompetent oocytes [19, 20], suggesting that additional protein synthesis and/or protein modifications may be necessary to allow MPF formation and activation. Our results indicate that the meiotically competent bovine oocyte is unique when compared with other mammalian oocytes studied because cyclin B1 protein was not detected in any follicle size group. These findings support the results of Lévesque and Sirard [21], who showed that bovine oocytes cannot resume meiosis in the presence of cycloheximide, an inhibitor of protein synthesis, but the microinjection of exogenous cyclin B1 protein can override its inhibitory effect. Altogether, these results demonstrate that the lack of cyclin B1 protein results in arrest of the fully grown bovine oocyte at the G2/M transition. However, if exogenous cyclin B1 triggers meiosis resumption while the translational mechanisms are inhibited, the other MPF component ($p34^{cdc2}$) must be present at the protein level. This finding is in agreement with the results from the present study, which demonstrated that $p34^{cdc2}$ protein is present at relatively high levels.

The mechanism of meiotic arrest inhibition is still under

investigation in large mammals, but we suspect this inhibition is due to an active factor secreted by the theca cells [34]. To better understand the mechanism by which the follicular environment inhibits the ability of the oocyte to resume meiosis, it would be interesting to evaluate whether the repressive effect of the factor secreted by the theca cells acts directly and specifically on the control of the cyclin B1 mRNA translation in the bovine oocyte.

The cyclin B family is composed of two members, cyclin B1 and cyclin B2, and both types are reported to be part of the MPF in the *Xenopus* oocyte [12]. The temporal fluctuation of cyclin B2 has been studied during in vitro maturation of bovine oocytes; it is present at the GV stage at a low level [35]. However, it is unknown whether the cyclin B2 present in the bovine oocyte can induce meiosis resumption. In knockout models, the cyclin B2-null mice are perfectly viable, whereas the cyclin B1-null mice die at a very early stage in utero, suggesting that cyclin B1, which is both bound to the intracellular membranes and free in the cytoplasm, is capable of compensating for the absence of the membrane-associated cyclin B2 [36]. In humans, cyclin B1 is associated with the microtubules, whereas cyclin B2 is linked to the Golgi apparatus [37]. Unlike cyclin B1, cyclin B2 does not relocate into the nucleus at the prophase stage of the cell cycle [37]. Therefore, both cyclins could play very different roles in meiotic resumption.

The Western blot analysis revealed two bands for $p34^{cdc2}$ that may be associated with the phosphorylation state of the protein. In the *Xenopus* oocyte, the phosphorylation of $p34^{cdc2}$ on Thr 14 by Myt1 and on Thr 15 by wee1 inactivates the complex, whereas dephosphorylation of Thr 14 and Thr 15 by the phosphatase cdc25 activates MPF [15, 16, 38, 39]. The macroarray analysis presented here indicated the presence of cdc25A and cdc25B at the transcript level. However, among the multiple variants of cdc25, cdc25A is more involved in the G1/S transition, whereas cdc25C has been associated with the G2/M border [40]. There is a very low level of cdc25C protein in porcine GV oocytes, but this protein is actively synthesized until the MII stage [40], suggesting that the oocyte stores cdc25C mRNA during its growth before the transcriptional silencing that occurs during meiotic maturation. In the present study, the presence of cdc25C mRNA was not investigated but cdc25A and cdc25B mRNAs were detected in the bovine GV oocyte.

Another explanation for the presence of a second band in the oocyte samples is the accumulation of $p33^{cdk2}$, because the antibody used to detect $p34^{cdc2}$ was raised against the PSTAIR region of the protein, which is also shared with $p33^{cdk2}$. The physiological functions of $p33^{cdk2}$ are associated with DNA synthesis during the G1/S transition when it forms a complex with cyclin E [41]. The role of $p33^{cdk2}$ during meiosis is unclear; in *Xenopus* oocytes the $p33^{cdk2}$/cyclin E complex was involved in the MII arrest [42], whereas the specific inhibition of $p33^{cdk2}$ did not alter the capacity of the *Xenopus* oocyte to pursue normal meiosis [43].

As mentioned previously, the bovine genome is mainly trancriptionally inactive during oocyte meiotic resumption, fertilization, and the first zygotic cell cycles. Therefore, all of the transcripts necessary to synthesize the proteins involved in the first zygotic cell divisions and those involved later in the maternal control of zygotic gene expression during MZT should be found in all stored maternal mRNAs [44–46]. In this context, the use of macroarrays is an efficient way to establish a quick overview of the expression map and to evaluate the presence of specific transcripts, as shown here for the GV oocyte. This approach may also become a valuable tool for simultaneously following the fluctuations of multiple transcripts at specific embryonic stages until the MZT to better understand the use of the maternal RNA pools. In the present study, we were able to use cross-species hybridization under stringent conditions to generate a large amount of information, because the genes involved in meiosis resumption are often conserved between species. However, when no signal is detected for a particular cDNA, it is not possible to determine whether the lack of hybridization is due to the absence of the messenger or to the lack of homology between the species. By using a second tissue as a control, a portion of the cDNA not detected can be attributed to the absence of the transcript in the sample. In the present study, the use of cumulus cells as a control revealed that cyclin E and cAMP-dependent PK-2 mRNAs (Fig. 4) are present at a very low level in the bovine oocyte. The use of proliferating cells would have been a more appropriate control and would certainly have resulted in stronger signals. Cumulus cells showed weak hybridization for cell cycle genes because they are not involved in a mitotic process, as indicated by the lack of hybridization for PCNA, which is a marker for cell growth and proliferation. Even though the GV oocyte itself is not a dividing cell but shows high hybridization for PCNA, it is reasonable to suppose that PCNA is stored in the maternal RNA stock to be used during the first cell divisions of the early embryo. Strong staining for PCNA protein has previously been reported in the bovine oocyte [47], but because large amounts of stored mRNA and proteins are not mutually exclusive, both may be present.

The quantitative measurements of transcript levels revealed that cyclin B1 mRNA is more abundant than $p34^{cdc2}$ mRNA and that β-actin mRNA is less abundant than the ubiquitin transcript. By comparing the intensity of the macroarray hybridizations, the same conclusion can be drawn, thus highlighting the quantitative potential of the macroarray screening process. Nylon-based array hybridization involves a high probe volume that requires a large number of oocytes, thereby limiting the use of specific classes of oocytes. Alternatively, the real time PCR technology offers a solution to tissue scarcity by increasing the sensitivity of the quantification; two oocyte equivalents were used per PCR as compared with 1200 or 1700 oocytes per macroarray evaluation. However, real time PCR is not a high-throughput technology suitable for overall pictures of gene expression patterns. The development of glass slide microarrays should result in the reduction of the amount of starting material required, and both technologies should be complementary.

The array analysis revealed several cyclins and cell division cycle proteins at the mRNA level and cyclin-dependent kinases (CDK) and MAPKs. The ERK1 (MAPK1) and ERK2 (MAPK2) genes are involved in GVBD in pig oocytes [48, 49] and were also present and active in the bovine oocytes [50, 51]. By contrast, the involvement of MAPK in meiosis resumption occurred after GVBD in the goat oocyte [52]. The macroarray analysis indicated the presence of MAPK1 mRNA (Fig. 5), in agreement with the previous reports and further extending the list of messengers for kinases phosphorylating the MAPKs (MAPKK) and several CDKs that are also present in GV bovine oocytes. The specific inhibition of CDK by butyrolactone I inhibits meiosis resumption [51], highlighting the importance of these genes in meiotic arrest.

The control of meiosis resumption is different in bovine oocyte than in the other mammalian oocytes studied so far; cyclin B1 protein is the missing component forming the pre-MPF complex. A large amount of cyclin B1 mRNA is present in meiotically competent and incompetent GV oocytes, and the transcript is readily detectable in primordial oocytes of 130-day-old fetuses. Nylon-based cDNA macroarrays can be used with heterologous probes to establish a large expression map in tissues and oocytes, with reasonable quantitative results.

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