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Poly(A) RNA Is Reduced by Half During Bovine Oocyte Maturation but Increases when Meiotic Arrest Is Maintained with CDK Inhibitors[1]

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ABSTRACT

Variations in the amount of different RNA species were investigated during in vitro maturation of bovine oocytes. Total RNA content was estimated to be 2 ng before meiosis, and after meiosis resumption, no decrease was observed. Ribosomal RNA did not appear to be degraded either, whereas poly(A) RNA was reduced by half after meiosis resumption, from 53 pg to 25 pg per oocyte. Real-time polymerase chain reaction was performed on growth and differentiation factor-9 (*GDF-9*), on cyclin B1, and on two genes implicated in the resistance to oxidative stress, glucose-6-phosphate-dehydrogenase (*G6PD*) and peroxiredoxin-6 (*PRDX6*). When these transcripts were reverse-transcribed with hexamers, the amplification results were not different before or after in vitro maturation. But when reverse transcription was performed with oligo(dT), amplification was dramatically reduced after maturation, except for cyclin B1 mRNA, implying deadenylation without degradation of three transcripts. Although calf oocytes have a lower developmental competence, their poly(A) RNA contents were not different from that of cow oocytes, nor were they differently affected during maturation. When bovine oocytes were maintained in vitro under meiotic arrest with CDK inhibitors, their poly(A) RNA amount increased, but this rise did not change the poly(A) RNA level once maturation was achieved. The increase could not be observed under transcription inhibition and, when impeding transcription and adenylation, the poly(A) RNA decreased to a level normally observed after maturation, in spite of the maintenance of meiotic arrest. These results demonstrate the importance of adenylation and deadenylation processes during in vitro maturation of bovine oocytes.

gamete biology, gene regulation, kinases, meiosis, oocyte development

INTRODUCTION

During oocyte growth, when the follicle develops from the primordial stage to the antral stage, transcription and translation are intense and result in the production of RNAs and protein, both for immediate use and for storage [1]. Messenger RNAs can be stored for long periods, with half-lives of up to 28 days in mouse oocytes [2]. Stable storage of mRNAs implies variable length of their 3′ poly(A) tail, but also different association with specific RNA binding proteins, especially the masking proteins [3, 4]. Once a mouse oocyte has reached its full size (diameter 75–80 μm), it contains about 0.6 ng of total RNA [5], with 8% being poly(A) RNA [6], whereas the poly(A) RNA represents only 1% of the total RNA in somatic cells. During meiotic resumption of a mouse oocyte, the amount of polyadenylated RNA is reduced by more than half [7], and concomitantly, important changes occur in protein synthesis, probably due to differential recruitment of stored mRNAs [8]. Such a poly(A) RNA decrease is also observed during maturation of *Xenopus* oocytes [9], but studies in other species are scarce.

In cattle, once a follicle reaches 3 mm in diameter, the oocyte has achieved maximum size, and the transcription essentially ceases [10, 11]. The competence for completing meiosis up to metaphase II is already acquired [12], but the developmental competence will be further enhanced through the subsequent steps of folliculogenesis [13]. The molecular events occurring during that period are mostly unknown. When a bovine oocyte is aspirated from an antral follicle with a diameter >3 mm, spontaneous meiotic resumption occurs, but it requires a short burst of transcription in the cumulus-oocyte-complex during the initial hours of maturation [14]. Before chromatin condensation, some transcription can be detected in the germinal vesicle (GV) of the oocyte [15, 16], but it is no longer detectable after GV breakdown (GVBD), whereas polyadenylation appears intense at least up to metaphase I [16].

The goal of this study was to analyze variations in the amount of different RNA species during in vitro maturation of the bovine oocyte, because other studies had focused on the growth period of the oocyte [17] or during preimplantation embryonic development [18]. Therefore, we measured the total RNA content and estimated the proportion of ribosomal RNA before and after in vitro maturation. Previously, poly(A) RNA quantification required large numbers of oocytes [19], but a more sensitive assay was developed (i.e., the poly(A) RNA detection system, by Promega, Madison, WI), which allows quantification with fewer oocytes. Poly(A) RNA was quantified before and after in vitro maturation not only in cow oocytes, but also in calf oocytes. The lower developmental potential of calf oocytes has been largely documented [20–22]. Calf oocytes show a reduced relative protein expression [23, 24] and delayed ooplasmic maturation [21, 25] with biochemical failures [26]. These cytoplasmic aspects could be related to a lower amount or to a different processing of poly(A) RNA during meiosis resumption.

To confirm the modifications of poly(A) tails at the 3′ end of mRNAs during maturation, the efficiency of amplification of several transcripts was tested before and after in

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vitro maturation, using reverse transcription (RT) either with oligo(dT) or with hexamers. Two of the genes analyzed were implicated in the maturation process: first, cyclin B1, as a member of the M-phase promoting factor (MPF), whose activity is absolutely required to resume meiosis [27] (in the mouse oocyte, the expression level of cyclin B1 protein depends mostly on post-transcriptional modifications [28]); and second, growth and differentiation factor-9 (*GDF-9*), an oocyte-specific transcript involved in folliculogenesis [29] and in cumulus expansion, and in the induction of hyaluronic acid during maturation [30]. Two genes code for antioxidant enzymes: glucose-6-phosphate dehydrogenase (*G6PD*), which leads to the generation of NADPH [31]; and peroxiredoxin-6 (*PRDX6*), whose product reduces hydrogen peroxide and alkyl hydroperoxide [32]. Both enzymes are involved in maintaining the redox state of the cell. A good protection against oxidative stress is an important factor for successful in vitro embryo production, moreover, G6PD expression can also be modulated by post-transcriptional modifications [31]. Two reporter genes were also amplified; the histone, H2a, as an endogenous standard reported to be constant through the maturation process [33], and the rabbit globin mRNA, which was exogenously added to each sample to account for the variations caused by the different manipulations.

Although most oocytes extruded from 3- to 6-mm antral follicles are able to resume meiosis in vitro, they have a lower developmental ability compared to in vivo-matured oocytes [34]. In order to enhance their developmental capacity, these oocytes could be maintained in vitro at the GV stage using inhibitors of cyclin-dependant kinases (CDKs) without compromising subsequent developmental competence. This was demonstrated for butyrolactone-1 (BL-I) [35, 36] or for roscovitine [37], and for a combination of reduced concentrations of both inhibitors [38]. This step would give time to mimic some of the subsequent folliculogenesis steps in culture (i.e., by adding different growth factors [39]). However, CDK inhibitors may interfere with gene expression [40, 41]. When bovine oocytes were treated with one or the other inhibitor, a convolution of the nuclear membrane and aberrant structures within the nucleoplasm were observed [42]. To know whether chemically delayed maturation may influence the transcription or the stability of stored maternal mRNAs, the poly(A) RNA content within oocytes maintained under meiotic arrest was investigated and tested in the presence of inhibitors of transcription or polyadenylation.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise indicated.

Collection of Oocytes

Oocytes were collected by puncturing follicles from ovaries of slaughtered cows or slaughtered calves (6–9 mo old). Only intact cumulus oocyte complexes (COCs) with three or more layers of cumulus cells were considered. COCs were directly analyzed or matured during 24 h in tissue culture medium 199 (TCM-199) supplemented with 10 ng/ml epidermal growth factor (EGF) and 0.4 mM pyruvate at 39°C and under 5% CO_2 in humidified air. Under these conditions, more than 85% of the oocytes reached metaphase II [43].

RNA Extraction

Immature and mature oocytes were carefully denuded by repeated pipetting, and washed three to four times in TCM-199. For total RNA quantification and Northern blot analysis, pools of 500 immature and matured oocytes were collected, whereas for poly(A) RNA quantification, pools of 50 oocytes were used. Each pool was stored at −80°C in a minimum volume of medium. Total RNA from each pool was extracted with 100 μl of Tripure Isolation Reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Twenty micrograms of glycogen (Roche Applied Science) was used as a carrier during extraction for the pools containing 50 oocytes. After precipitation with isopropanol, the RNA was centrifuged at 12 000 × *g*, and the pellet was washed twice with 70% ethanol, dried, and resuspended in RNase-free water.

Experiment 1: Total RNA Quantification

Total RNA extracted from two pools of 500 immature oocytes, and two pools of 500 matured oocytes were resuspended in 100 μl of RNase-free water, and the optical density of the solution was read both at 260 nm and 280 nm using a spectrophotometer (Ultraspec 3000; Pharmacia-Biotech, Amersham Biosciences, Little Chalfont, UK).

Experiment 2: Northern Blot Analysis of Ribosomal RNA

Total RNA extracted from 500 immature oocytes and from 500 matured oocytes was size-separated by electrophoresis through a denaturing formaldehyde-agarose gel (1%). After migration, the RNA was transferred to a nylon membrane (hybond-N; Amersham Biosciences) by overnight capillary blotting in a 20× saline-sodium-citrate (SSC) solution. After UV exposure for RNA cross-linking, the nylon membrane was prehybridized for 2 h at 42°C in 6× SSC, 5× Denhardt reagent, 0.5% SDS, 50% formamide, and 100 μg/ml denaturated salmon sperm DNA. The membrane was hybridized overnight with a 18S ribosomal probe added to the same solution but without Denhardt reagent. The 18S probe was a 307-base pair fragment amplified by polymerase chain reaction (PCR) from the cDNA of bovine granulosa cells with two primers: 5′-CTGAGAAACGGCTACCACA-3′ (forward) and 5′-AGAGCAAGGGGCGGGGACG-3′ (reverse). The PCR product was purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and labeled with the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA) as specified by the manufacturer's instructions, with 50 μCi of (α-^{32}P) dCTP (Amersham Biosciences; 3000 Ci/mmol). After three washes, the last one being in 0.1× SSC, the membrane was exposed overnight to an autoradiographic film (Kodak X-Omat; Eastman Kodak, Rochester, NY). The intensity of the 18S band was quantified by densitometric scanning with a computer-assisted image analysis (NIH image). This experiment was repeated twice.

Experiment 3: Poly(A) RNA Quantification

Poly(A) RNA was quantified with the Poly(A) RNA Detection System (K4040; Promega, Madison, WI) as described by the manufacturer's instructions (technical bulletin TB282). Briefly, total RNA from each pool of 50 oocytes was hybridized with an excess of 18-mer oligo(dT) containing a single deoxyguanosine, deoxycytidine, or deoxyadenosine at the 3′ end in order to anchor the oligonucleotide to the very start of the poly(A) tail. Pyrophosphorylation of anchored oligo(dT) produced free dNTPs proportionally to the amount of hybridized anchored oligo(dT). With the nucleoside diphosphate kinase (NDPK) enzyme, the terminal phosphate from the free dNTPs was transferred to ADP to form ATP. The net result of these two reactions was the production of an amount of ATP proportional to the number of poly(A) tails present in the sample. The ATP was measured in a third reaction with a very sensitive luciferase/luciferin reagent producing light that was detected with a luminometer (Fluoroskan Ascent FL Labsystems, Thermo Electron, Waltham, MA). A standard curve was established with serial concentrations of a 1.2-kilobase polyadenylated synthetic kanamycin transcript (supplied by the manufacturer). The detection limit of the assay was 40 pg/μl of standard mRNA. Each measure was performed in triplicate. Hybridization reactions without RNA were prepared to check the absence of ATP contaminant in the mix, and the RNA sample without NDPK was also measured to demonstrate the lack of contaminants that could contribute to the signal.

Experiment 3a: poly(A) RNA amount in cow and calf oocytes before and after maturation. Poly(A) RNA was measured in 10 pools of 50 immature cow oocytes and in 9 pools of 50 matured cow oocytes. For calf oocytes, nine pools of immature oocytes and seven pools of matured oocytes were analyzed.

Experiment 3b: poly(A) RNA amount in cow oocytes after 24 h of in vitro meiotic arrest using a combination of inhibitors of cyclin-dependant kinases. In each experiment, 8 pools of 50 oocytes were used. Two pools of 50 immature oocytes were directly analyzed (immature). Four pools of 50 oocytes were cultured during 24 h in TCM-199 with 6.25 μM BL-I

TABLE 1. Information on primers used for real-time PCR.

Gene	GenBank accession number	Primer sequence	Primer position (bp)	Product size (bp)	Probe sequence	Probe position (bp)	Slope standard curve
globin	V00879	GGGCAACGTGAATGTGGAAG	101	100	CAGGCTGCTGGTTGTCTACCCATGGA	143	−3.48
		ACAGGTCCCCCAAAGGACTCG	201				
PRDX6	AF090194	GGCAAGAAATACCTCCGCTAC	682	66	AGCCATAGGCT0GCCAT	710	−3.35
		GGCAGCTCCAGAACCATCTC	728				
G6PD	XMD49337	GAGGCCGTCACCAAGAACAT	1031	150	ATGGTGCTGAGATTTGCCAACAGGAT	1108	−3.41
		GGTAGTGGTCGATGCGGTAGA	1181				
GDF-9	AP307092	TCTTAGCGCCCTCACTGCTT	810	120	AACGACACAAGTGCTCAGGCTTTTCA	839	−3.31
		GACAGCCCTCTCTTCTGGTCA	930				
Cyclin B	L26548	AACAGCTCTTGGAGACATCGGT	249	73	CAAAGTCAGTGAACAACCGCAGGCC	272	−3.22
		TTGCTTCCTTTTTCAGAGGCA	322				
Histone H2a	U57614	AGAAGACGCGCATCATCCC	267	78	CATCCGCAACGACGAGGAGCTCA	304	−3.38
		ACTTTGCCCAGCAGCTTGTT	345				

(Calbiochem, EMD Biosciences, San Diego, CA) and 12.5 μM roscovitine (kindly provided by Dr. L. Meijer, Centre National de la Recherche Scientifique, Station Biologique de Roscoff, France): two were directly analyzed (premature), whereas the remaining two were further cultured for 24 h in a classic maturation medium (premature and mature). The two last pools of 50 oocytes were matured for 24 h (mature). This experiment was repeated four times.

Experiment 3c: poly(A) RNA amount in oocytes maintained under meiotic arrest and in the presence of inhibitors of transcription or adenylation. In each experiment, five pools of 50 oocytes were used. One pool of immature oocytes was directly analyzed. Three pools were cultured for 24 h in TCM-199 with 6.25 μM BL-I and 12.5 μM roscovitine: 1) alone, or 2) with 500 μg/ml of 3′-deoxyadenosine (cordycepin) an adenosine analogue that terminates poly(A) tail elongation and inhibits transcription, or 3) with 500 μg/ml of 3′-deoxyguanosine that inhibits transcription but not polyadenylation. These three pools were directly analyzed after 24 h of meiotic arrest. The last pool matured only after 24 h. This experiment was repeated four times.

Experiment 4: Real-Time PCR After RT with Hexamers or oligo(dT)

Total RNA was extracted from two pools of 80 immature oocytes, and two pools of 80 in vitro-matured bovine oocytes as described above but with 5 pg of polyadenylated rabbit globin mRNA (Invitrogen) added to each pool. The RNA from each pool was divided into eight samples so that the RNA equivalent of 10 oocytes was reverse transcribed either with 250 ng of hexamers (Roche Applied Science) or with 200 ng of oligo(dT) (Amersham Biosciences). The reproducibility of the results obtained with each method was assessed by four equally treated replicates. All samples were denatured at 65°C, flash-cooled to 4°C, then reverse-transcribed for 1 h at 42°C in a final volume of 15 μl containing 10 mM dithiothreitol, 1 mM dNTPs, 25 units of Expand RT (all from Roche Applied Science), and 10 units of RNA-guard (Amersham Biosciences). After RT, the volume of each sample was extended to 65 μl.

Six genes were quantified in each sample using real-time PCR and a specific molecular beacon for each gene (hybridization probe with a quencher [TAMRA] at the 5′ end and a fluorescent dye [FAM] at the 3′ end). The four genes of interest were two antioxidant enzymes, *PRDX6* and *G6PD*, one transcript specific of the oocyte, *GDF-9*, and one member of the MPF complex, cyclin B1. Two reporter genes were amplified; the exogenous rabbit globin gene was spiked to account for the variations caused by manipulation of the samples (differences in pipetting or in the efficiency of reverse transcription between tubes), and the endogenous histone, H2a, to normalize different RNA amounts between pools. According to a study on the quantification of several housekeeping genes during maturation and early development in the bovine [33], only histone H2a mRNA levels appeared constant across the entire preimplantation period. The sequences of the different primers used are presented in Table 1.

PCRs were performed on an ABI Prism 7700 (Applied Biosystems, Foster City, CA). The amplification reaction used 5 μl of the cDNA and the Platinum Quantitative PCR Super Mix-UDG (2×) (from Invitrogen Life Technologies). This mix contains each dNTP, but dUTP instead of dTTP, 40 U/ml uracil-DNA-glycosylase (UDG), 60 U/ml Platinum *Taq* polymerase, and ROX as a passive fluorescent dye for well normalization; 400 nM of each primer (forward and reverse), and 200 nM of the Taqman probe were added to the reaction. The PCR protocol included a first step at 50°C (2 min) for the activity of UDG, preventing ''carryover'' contamination from previous PCR products, then 10 min at 95°C to activate the *Taq* polymerase, followed by 40 cycles for 15 sec at 95°C, and 1 min at 60°C. For each gene, a standard curve of amplification was established using five serial dilutions (in triplicate) of a reference cDNA. The same stock of cDNA was used in all experiments (cDNA from granulosa cells, except for *GDF-9*, for which we used a cDNA derived from a pool of oocytes).

Statistical Analysis

Total and poly(A) RNA content in immature and mature cow and calf oocytes were analyzed by two-way analysis of variance with the maturation status and the origin of the oocytes (cow or calf) as fixed factors. In experiments 3b and 3c, the poly(A) RNA content was analyzed with one-way analysis of variance. For analysis of mRNA expression assayed by quantitative RT-PCR, one-way repeated measures analysis of variance were used. Differences of $P < 0.05$ were considered significant.

RESULTS

Total RNA Quantification Before and After In Vitro Maturation

Total RNA within one oocyte was about 1.9 ± 0.2 ng (mean ± SEM) before maturation and 2.0 ± 0.15 ng after maturation, with a ratio OD_{260}:OD_{280} always >1.85. No significant change in total RNA occurred during maturation.

Ribosomal RNA Before and After In Vitro Maturation

The intensity of the 18S ribosomal band hybridized to Northern blots did not significantly change after maturation. No ribosomal degradation appeared during meiotic resumption of bovine oocyte (Fig. 1).

Poly(A) RNA Quantification

Experiment 3a: immature and matured cow and calf oocytes. The amount of poly(A) RNA significantly decreased during maturation ($P < 0.0001$), but there was no effect of the origin of the oocyte (cow or calf). Poly(A) RNA in a cow oocyte was 53 ± 4 pg (mean ± SEM) before maturation and declined to 24.5 ± 2.5 pg at the end of 24 h of maturation. In calf oocytes, the poly(A) RNA amount was quite similar, but the variability appeared more pronounced. The average amount was 57 ± 8 pg before maturation and declined significantly to 32 ± 6.5 pg after maturation.

Experiment 3b: oocytes maintained 24 h under meiotic arrest with CDK inhibitors. After 24 h of in vitro meiotic arrest with BL-I and roscovitine, the poly(A) RNA amount in bovine oocytes significantly increased to reach 91 ± 12

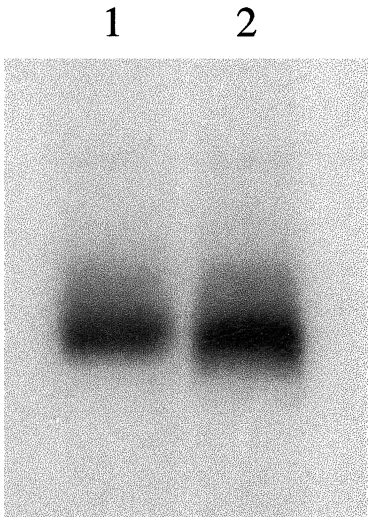

FIG. 1. Comparison of the amount of ribosomal RNA in bovine immature and mature oocytes by hybridization with an 18S rRNA probe to Northern blot. This experiment was repeated twice. Lane 1: RNA from 500 immature oocytes; lane 2: RNA from 500 matured oocytes.

pg. Once these oocytes were allowed to mature after 24 h, the poly(A) RNA significantly decreased to reach a value that was not different from that measured in simply matured oocytes (Fig. 2).

Experiment 3c: oocytes 24 h under meiotic arrest with CDK inhibitors and in the presence of inhibitors of transcription or adenylation. Under maintenance of meiotic arrest with a combination of BL-I and roscovitine, the poly(A) RNA amount again significantly increased (88.5 ± 10.5 pg; $P < 0.05$). If we simultaneously added deoxyguanosine, there was no significant increase. On the other hand, in the presence of both deoxyadenosine and CDK inhibitors, the level of poly(A) RNA sharply dropped to a value as low as that observed in matured oocytes (14.3 ± 7 pg/oocyte) (Fig. 3).

Real-Time PCR

For relative quantification, the efficiency of amplification of the target (the four genes of interest) and of the reference genes (globin or histone H2a) must be similar. This efficiency is given by the slope obtained with standard curves and was included between −3.2 (100% efficiency) and −3.5 for all studied genes (Table 1). According to the globin standard curve, the mean quantity of rabbit globin found in each replicate was 0.042 ± 0.003 pg, as expected. When the PCR results of endogenous genes were normalized with the globin value, the variability between the replicates did not exceed 10%. If RT was performed with hexamers, normalization with globin or histone H2a values gave similar results, the intrinsic RNA quality of pools of 80 oocytes was apparently not quite different.

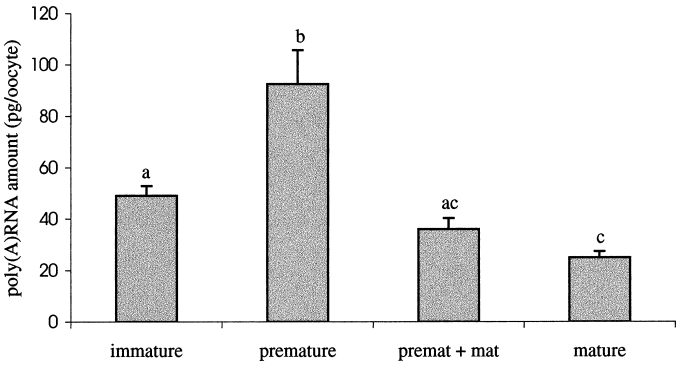

FIG. 2. Poly(A)RNA amount per oocyte (in picograms) before and after maturation, prematuration, or both. Immature: oocytes directly analyzed after follicle extrusion; premature: oocytes analyzed after 24 h of meiotic arrest with a combination of two CDK inhibitors (BL-1 and roscovitine). Premature + mat: oocytes maintained 24 h under meiotic arrest with CDK inhibitors followed by 24 h maturation. Mature: oocytes matured for 24 h. Columns with different superscripts are significantly different, $P < 0.05$ (one-way ANOVA).

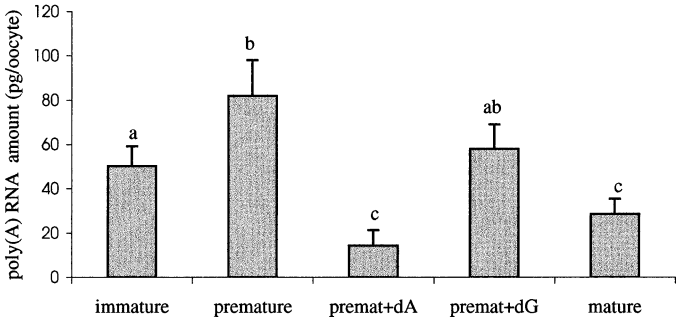

FIG. 3. Poly(A)RNA amount per oocyte (in picograms) in the presence of CDK inhibitors and inhibitors of transcription and polyadenylation. Immature: oocytes directly analyzed after follicle extrusion; premature: oocytes analyzed after 24 h of meiotic arrest with a combination of two CDK inhibitors (BL-1 and roscovitine); premat + dA: oocytes incubated for 24 h with CDK inhibitors and deoxyadenosine; premat + dG: oocytes incubated for 24 h with CDK inhibitors and deoxyguanosine; mature: oocytes matured for 24 h. Columns with different superscripts are significantly different, $P < 0.05$ (one-way ANOVA).

RT with Hexamers

As reported [43], the number of histone H2a transcripts was not affected during in vitro maturation (data not shown), thus relative quantification could be performed with *H2a* used as the reference gene according to the standard curve method (ABI Prism 7700 Sequence Detection System; User Bulletin #2, Applied Biosystems). The relative quantity of the four genes of interest obtained after maturation is shown in Figure 4. The relative number of *G6PD* and *GDF-9* transcripts was not affected during maturation, whereas there was an increase for *PRDX6* and cyclin B1 messengers.

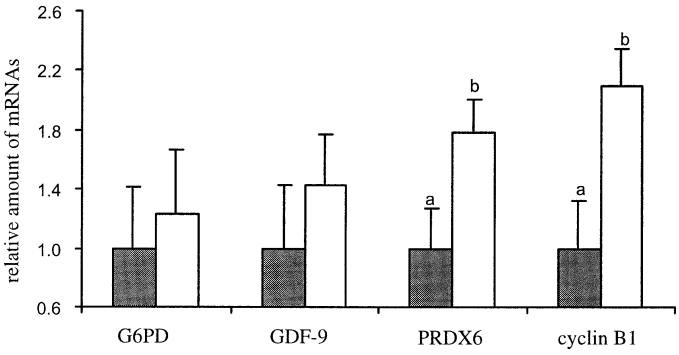

FIG. 4. Relative quantification (arbitrary units) of the expression of four transcripts reverse-transcribed with hexamers and normalized with *H2a* used as the reference gene. Gray bars: before maturation; open bars: after maturation. a≠b, $P < 0.05$.

TABLE 2. Ratio of PCR signals obtained after RT with oligo(dT) versus hexamers.

Oocyte status	Rabbit globin	Histone H2a	*G6PD*	*GDF-9*	*Cyclin B1*	*PRDX6*
Immature	9.85 ± 1.05	0.08 ± 0.01	2.95 ± 0.63	5.48 ± 1.08[a]	2.45 ± 0.45	3.7 ± 0.5[a]
Matured	11.05 ± 1.55	0.03 ± 0.0	0.51 ± 0.11	0.69 ± 0.13[b]	1.65 ± 0.05	0.7 ± 0.1[b]

[a,b] a ≠ b, $P < 0.05$.

RT with oligo(dT)

The amplification results were quite different if RT was performed with oligo(dT) or with hexamers (Table 2). The amplification of the histone H2a transcript, normally not adenylated, was very low when using oligo(dT), and could no longer be used as a reference gene. For the fully polyadenylated rabbit globin, amplification was much higher using oligo(dT) instead of hexamers. For the other genes, the efficiency of amplification after RT with oligo(dT) changed, depending on the maturation status of the oocyte. In these conditions, relative quantification was difficult to realize. However, for each gene, we compared the ratio of RT-PCR results with oligo(dT) on RT-PCR results with hexamers for immature and matured oocytes. For *GDF-9* and *PRDX6*, this ratio dropped significantly during maturation. For *G6PD* and for *H2a*, the decrease was close to significance ($P = 0.06$ and $P = 0.09$, respectively), whereas for the exogenously added rabbit globin, the ratio was not affected by in vitro maturation.

DISCUSSION

For the first time, variations in the amount of different RNA species were analyzed during in vitro maturation of bovine oocytes. Olszanska et al. [44], using ethidium bromide-induced fluorescence, estimated the total RNA content of a bovine oocyte before meiosis resumption to be close to 1 ng. Bilodeau-Goeseels and Schultz [18], using Northern blot techniques, estimated the total RNA content of a matured bovine oocyte to be around 2.4 ng. We found no report of total RNA content before and after meiosis resumption with the same detection technique. Here, with a classic RNA detection system, we quantified total RNA content to be close to 2 ng before and after maturation, between the two values previously reported; more specifically, we demonstrated that total RNA content does not decrease during in vitro meiotic resumption. In the mouse, a 20% decrease of bulk RNA was observed during maturation, including a 23% decrease in rRNA [45, 46]. Our Northern blot analysis showed that in bovine oocyte, ribosomal RNA was apparently not degraded during in vitro maturation. Thus, rRNA appeared stable up to the 4-cell stage in a bovine embryo [18]. The amount of poly(A) RNA in a bovine oocyte was estimated to be around 55 pg before meiosis resumption, consequently, it would represent 5% of total RNA, slightly lower than the 8% reported in a mouse oocyte [6]. This amount of poly(A) RNA was dramatically affected during meiosis resumption, being reduced by half at the end of the process as described for mouse [7] and *Xenopus* oocytes [9].

In bovine oocytes, a shortening of the poly(A) tail was reported for 6 out of 10 transcripts analyzed before and after maturation [47, 48]. Our results confirmed the importance of deadenylation during meiosis resumption. This process could not be detected during the first 4 h of maturation, but was completed after 18 h (data not shown). So, according to the kinetics of meiosis resumption in the bovine [49], deadenylation apparently occurs after GVBD as reported in *Xenopus* [50]. Besides the deadenylation wave, several transcripts can also be specifically polyadenylated depending on the sequence of their 3′ untranslated region [51]. In the bovine oocyte, polyadenylation was described for several transcripts as well [47, 48], and polyadenylation activity was detected between 6 and 10 h after the beginning of meiosis resumption [16].

Variable RT-PCR results using oligo(dT) versus hexamers may indicate deadenylation [52]. Our data point out, for three out of four mRNAs analyzed, the removal of poly(A) tails during meiosis resumption, whereas cyclin B1 mRNA was not deadenylated. Cyclin B1 protein heterodimerizes with a cyclin-dependant kinase (CDK1) to form MPF, a complex essential to meiosis resumption [27]. During mouse oocyte maturation, the level of cyclin B1 protein rises steadily [53] in correlation with a lengthening of the poly(A) tail of the transcript [28]. We showed here that the number of polyadenylated cyclin B1 transcripts was stable during bovine oocyte maturation. It has been recently reported that cyclin B1 mRNA would undergo cytoplasmic polyadenylation before the beginning of in vitro maturation, which had already occurred during the time when the ovaries were transported from the slaughterhouse to the laboratory [54].

Although they were deadenylated, none of the transcripts we analyzed was degraded during maturation. Recently, transcriptome analysis of bovine oocytes using cDNA arrays revealed that the relative abundance of most messengers was effectively stable during maturation [55], with 10% of them showing a decrease, and 10% of them showing an increase, as we observed here for cyclin B1 and *PRDX6* transcripts. This increase could be the result of the low transcriptional activity detected in the GV at the beginning of maturation [15, 16]. A higher level of cyclin B1 protein during bovine oocyte maturation may depend on both an increase in transcription and translation.

The different amplification results obtained with one or the other RT method clearly underline the importance of adenylation for the regulation of expression during maturation in bovine oocytes. Studies on purified poly(A) RNA [56, 57] or on cDNA reverse transcribed only with oligo(dT) [58] do not take into account transcripts without a poly(A) tail or with a too-short poly(A) tail. So the differences detected in those studies may reflect changes in the number of transcripts during maturation, but also the loss or addition of a poly(A) tail to transcripts.

Histone transcripts are unique because they usually lack a poly(A) tail, ending instead in a conserved stem-loop structure. However, some of the stored histone mRNAs in amphibian oocytes have short oligo(A) tails added to the stem-loop structure [59]. These oligo(A) tails are removed at oocyte maturation [60]. Similarly, the low PCR signal obtained for histone H2a reverse transcribed with oligo(dT) could come from a small proportion of polyadenylated transcripts present in immature bovine oocyte. This proportion also appeared to decrease during maturation.

The lack of cytoplasmic competence of calf oocytes was neither reflected by a reduced content of poly(A) RNA, nor

by a different processing during maturation. However, the amount of poly(A) RNA was apparently more subject to variations between pools of calf oocytes.

Unexpectedly, the poly(A) content of oocytes under meiotic arrest with a combination of BL-I and roscovitine increased. Because there is still some transcription in the GV [15, 16], this could be due to neotranscription, or to the addition of poly(A) tails to transcripts not yet adenylated, or both. To solve this, we used deoxyadenosine (cordycepin) or deoxyguanosine in combination with CDK inhibitors; the first one impedes transcription and adenylation, whereas the second one interacts only with transcription. As the rise in poly(A) content was no longer detectable in the presence of deoxyguanosine, it probably resulted from transcription. When polyadenylation was hampered, the poly(A) RNA dropped to a very low level. Perhaps factors that prevent deadenylation during meiotic arrest would themselves depend on polyadenylation to be functional. Poly(A) tails could also be the result of a dynamic process [61] and could not be further elongated. Interactions of the inhibitors with the different factors responsible for translational control should also be considered. Under meiotic arrest of bovine oocytes with BL-I, the phosphorylation of the cap-binding factor eIF4E, linked to the mRNA recruitment to polyribosomes, is blocked [62], whereas cordycepin prevents the dissociation of maskin from eIF4E [63]. Finally, as the rise of poly(A) RNA obtained in the presence of CDK inhibitors was no longer detectable after 24 h of maturation, the maintenance of meiotic arrest in vitro could not improve the quantity of poly(A) RNA, but the specific pattern of the transcripts affected is not known.

In conclusion, total RNA amount as well as ribosomal RNA did not decrease during bovine oocyte maturation, but half of the poly(A) RNA disappeared. Real-time PCR data indicated that several mRNAs were deadenylated but not degraded, whereas cyclin B1 transcript was not deadenylated. The poly(A) RNA content within a calf or a cow oocyte was not different. The poly(A) RNA content in oocytes maintained under meiotic arrest with CDK inhibitors increased. This increase, apparently due to transcription, did not allow obtaining a higher poly(A) RNA amount once the oocyte has finally achieved meiosis resumption. If polyadenylation was hampered during the maintenance of meiotic arrest, the poly(A) RNA amount decreased. These results clearly underline the importance of addition and removal of poly(A) tails during meiosis resumption and meiotic arrest in bovine oocyte.

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