

Quantitative Analysis of Messenger RNA Abundance for Ribosomal Protein L-15, Cyclophilin-A, Phosphoglycerokinase, β -Glucuronidase, Glyceraldehyde 3-Phosphate Dehydrogenase, β -Actin, and Histone H2A During Bovine Oocyte Maturation and Early Embryogenesis In Vitro

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ABSTRACT Real-time reverse transcription PCR has greatly improved the ease and sensitivity of quantitative gene expression studies. However, measurement of gene expression generally requires selection of a valid reference (housekeeping gene) for data normalization to compensate for inherent variations. Given the dynamic nature of early embryonic development, application of this technology to studies of oocyte and early embryonic development is further complicated due to limited amounts of starting material and a paucity of information on constitutively expressed genes for data normalization. We have validated quantitative procedures for real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of mRNA abundance during bovine meiotic maturation and early embryogenesis and utilized this technology to determine temporal changes in mRNA abundance for ribosomal protein L-15, cyclophilin-A, phosphoglycerokinase, β -glucuronidase, glyceraldehyde-3-phosphate dehydrogenase, β -actin, and histone H2A. Quantification of amounts of specific exogenous RNAs added to samples revealed acceptable rates of RNA recovery and efficiency of reverse transcription with minimal variation. Progression of bovine oocytes to metaphase II resulted in reduced abundance of polyadenylated, but not total transcripts for majority of above genes; however phosphoglycerokinase exhibited a significant decline in both RNA populations. Abundance of mRNAs for above genes in early embryos generally remained low until the blastocyst stage, but abundance of ribosomal protein L-15 mRNA was increased at the morula stage and histone H2A mRNA showed dynamic changes prior to embryonic genome activation. Results demonstrate

a valid approach for quantitative analysis of mRNA abundance in oocytes and embryos, but do not support constitutive expression of above genes during early embryonic development. *Mol. Reprod. Dev.* 73: 267–278, 2006. © 2005 Wiley-Liss, Inc.

Key Words: real-time RT-PCR; gene expression; polyadenylation and deadenylation

INTRODUCTION

The translational control of oocyte mRNAs during meiotic maturation and early embryogenesis is dependent on evolutionarily conserved processes termed cytoplasmic polyadenylation and deadenylation, which involve addition or deletion of adenosine residues on the tail of mRNAs (Richter, 1999; Eichenlaub-Ritter and Peschke, 2002). The early developmental cell fate decisions of an embryo are regulated through translational activation of maternally stored mRNAs prior to embryonic genome activation, also termed the maternal zygotic

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transition (Eichenlaub-Ritter and Peschke, 2002). Embryonic genome activation (at 2- to 8–16-cell stages, depending on the species) will further influence embryonic development via innate synthesis of new transcripts (Schultz, 1993; Telford et al., 1990). Gaining a clear insight into the molecular events controlling mammalian oocyte maturation and early embryonic development is crucial to advancements in developmental biology and improvements in assisted reproductive technologies. Technical limitations and dearth of starting material have restricted accurate, widespread quantitative analysis of mRNA abundance for genes of interest in mammalian oocytes and early embryos using traditional molecular approaches. However in recent years, real-time reverse transcription polymerase chain reaction (RT-PCR) procedures have been applied for quantitative analysis of mRNA abundance in oocytes and embryos (Robert et al., 2002; Lonergan et al., 2003a,c; McGraw et al., 2003; Fair et al., 2004b; Gutierrez-Adan et al., 2004; Lequarre et al., 2004; Leyens et al., 2004; Rizos et al., 2004; Vigneault et al., 2004). Although the approach is highly sensitive, inherent technical limitations associated with limited amounts of starting material (variation in RNA recovery, efficiency of reverse transcription) may influence results generated. The most common way to account for inherent variation in real-time RT-PCR assays of mRNA abundance is to use amounts of mRNA detected for a constitutively expressed (housekeeping) gene for data normalization. However, the biology of oocytes and early embryos is unique, since maternal pools of mRNA are gradually depleted during meiotic maturation and early development until embryonic genome activation.

Therefore, despite utilization of select housekeeping genes for data normalization in previously published studies of this nature, the identity of an appropriate control gene for data normalization in real-time RT-PCR assays that is constitutively expressed throughout early embryogenesis and (or) in response to various treatments has remained elusive. An alternative approach would be to use exogenous control RNAs added during sample preparation as sensitive indicators to account for potential variation during RNA extraction, reverse transcription procedures, and for normalization of results (Wrenzycki et al., 1999; Yaseen et al., 2001).

The objective of this study was to develop and validate appropriate procedures for quantitative real-time RT-PCR analysis of mRNA abundance in bovine oocytes and early embryos to account for potential variation in efficiency of RNA extraction and reverse transcription. In addition, temporal changes in mRNA abundance for seven known housekeeping genes [ribosomal protein L-15 (RPL-15), cyclophilin-A (CYC-A), phosphoglycerokinase (PGK), β -glucuronidase (GUSB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β -actin, and histone H2A (H2A)] during bovine oocyte maturation and early embryogenesis were determined in an attempt to identify a suitable housekeeping gene for normalization of real-time RT-PCR data for oocytes and early embryos.

MATERIALS AND METHODS

Materials

All materials were obtained from Sigma Aldrich (St. Louis, MO) unless stated otherwise.

Oocyte Recovery and In Vitro Maturation

Briefly, ovaries obtained at a local abattoir were transported to the laboratory in sterile saline solution. Upon return to the laboratory, ovaries were washed in sterile saline and cumulus oocyte complexes (COCs) were aspirated from 2–7 mm visible follicles using an 18-gauge needle and 50–60 mm Hg of negative pressure. After sedimentation, the COCs were individually selected and washed 3–4 times in HEPES-buffered hamster embryo culture medium (HECM) [114 mM NaCl, 3.2 mM KCl, 2 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 $\mu\text{l/ml}$ MEM nonessential ($10\times$) amino acids, 17 mM sodium lactate, 0.1 mM sodium pyruvate, 2 mM NaHCO_3 , 1 mM HEPES, 0.183 mM penicillin-G, 3 mg/ml BSA; pH 7.3–7.4; 275 ± 10 mOsm/kg] under a stereomicroscope. COCs with more than four compact layers of cumulus cells and homogeneous cytoplasm were matured in TCM 199 [supplemented with 0.2 mM sodium pyruvate, 5 mg/ml gentamicin sulfate, 6.5 mM L-glutamine, 156 nM bovine LH (Sioux Biochemical, Sioux Center, IA), 15.6 nM bovine FSH (Sioux Biochemical), 3.67 nM 17β -estradiol, and 10% v/v defined FBS (Hyclone, Logan, UT)] for 24 hr in groups of 50 in 4-well dishes containing 400 μl of maturation medium at 38.5°C , 5% CO_2 in air with maximum humidity. For germinal vesicle (GV) stage oocyte RNA samples, cumulus cells were completely removed by hyaluronidase (0.1%) digestion and repeated pipetting and denuded oocytes in groups of 10 were snap frozen in 100 μl lysis solution (RNAqueous Micro Kit, Ambion, Inc., Austin, TX) and stored at -80°C until RNA isolation. For MII oocyte RNA samples, cumulus cells were removed after maturation (as described above) and mature oocytes in groups of 10 were snap frozen in 100 μl lysis solution and stored at -80°C until RNA isolation. Metaphase II oocytes were selected based on the presence of a single polar body.

In Vitro Fertilization and Embryo Culture

For in vitro fertilization, spermatozoa from a frozen-thawed semen straw were selected through a Percoll density gradient. The semen was layered on a Percoll gradient consisting of 0.5 ml each of 30%, 60%, and 90% Percoll in HEPES buffered Tyrode's lactate (TL) sperm medium (100 mM NaCl, 25 mM NaHCO_3 , 10 mM HEPES, 0.29 mM NaH_2PO_4 , 0.035 mM sodium lactate, 1.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2.61 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ supplemented with 0.2 mM sodium pyruvate, 6 mg/ml BSA, and 50 $\mu\text{g/ml}$ gentamicin) and centrifuged at 2,000 rpm for 10 min. Then, the pellet was resuspended in 4 ml of TL sperm medium and centrifuged at 800 rpm for 10 min. Finally, the pellet was resuspended in 100 μl of TL sperm medium and concentration determined

in a hemocytometer. Matured oocytes and sperm (10^6 sperm/ml) were co-incubated for 20 hr in groups of 50 in 4-well dishes containing 400 μ l of fertilization medium (114 mM NaCl, 25 mM NaHCO₃, 3.2 mM KCl, 0.34 mM NaH₂PO₄, 0.183 mM penicillin-G, 16.6 mM sodium lactate, 0.5 mM MgCl₂·6H₂O, 2.7 mM CaCl₂·2H₂O, 0.2 mM sodium pyruvate, 6 mg/ml BSA, and 1.5 U of heparin) at 38.5°C, 5% CO₂ in air with maximum humidity. Semen from the same bull was used for all experiments. To separate cumulus cells, the presumptive zygotes were vortexed for 2 min and washed three times in HEPES buffered-HECM. Embryo culture was then performed in groups of 50 presumptive zygotes in 4-well dishes containing 400 μ l of KSOM medium (Specialty Media, Phillipsburg, NJ) supplemented with 3 mg/ml BSA under mineral oil. Culture was carried out at 38.5°C, 5% CO₂ in air with high humidity. Embryos at the 8–16-cell stage were separated 72 hr after fertilization and cultured in fresh KSOM medium supplemented with 3 mg/ml BSA and 10% FBS until day 7. The 2-cell embryos were collected 33 hr following fertilization, 4-cell embryos 44 hr later, 8-cell embryos 52 hr later, 16-cell embryos 72 hr later, morula 5 days later, and blastocyst-stage embryos 7 days later. All the embryos were snap frozen in 100 μ l lysis solution and stored at –80°C until RNA isolation. As a control for IVF procedure, a pool of embryos in each IVF run were cultured until the blastocyst stage to assess the developmental competence of the fertilized eggs. Only embryos collected from controlled experiments with rates of development to blastocyst stage of >25% (day 7) were used in the analysis. For each of the oocyte and embryo stages, five pools of samples were collected from a total of 12 different IVF runs.

In Vitro Transcription and RNA Quantification

For synthesis of green fluorescent protein (GFP) RNA, linear DNA templates having a SP6 promoter sequence at the 5'-end and poly(T₁₈) tail on the 3'-end were generated by polymerase chain reaction (PCR) from plasmid vector pCX-EGFP (generously provided by Jeffrey B. Kopp, NIH). The following primers 5'-TCATTTAGGTGACACTATAGAATTCGCCACCATGGTGAGCAAGG-3' (forward) and 5'-TTTTTTTTTTTTTTTTTTTACTCCAGCAGGACCATGTGATCGCGC-3' (reverse) were used to PCR amplify a 719 bp segment from above GFP plasmid. For PCR, 500 ng of pCX-EGFP plasmid DNA was added to 100 μ l PCR mixture [10 μ l of 10 \times PCR buffer, 3 μ l of 50 mM MgCl₂, 5 μ l of 100% DMSO, 5 μ l of 10 mM dNTP mix, 5 μ l each of 10 μ M forward and reverse primers, and 5 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA)]. PCR conditions used were as follows: initial denaturation, 15 min at 94°C; amplification 40 cycles of 30 sec at 94°C, 30 sec at 55°C, and 45 sec at 72°C. PCR amplified product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA). GFP RNA was synthesized by in vitro transcription from PCR amplified GFP DNA template with SP6 RNA polymerase

using the RiboProbe-SP6 system (Promega, Madison, WI). After in vitro transcription, template DNA was removed with RNase-free DNase and reactions subjected to phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Precipitated cRNA was re-suspended in RNA storage solution (Ambion) and stored at –80°C until use. Concentration and integrity of cRNA was quantified on the Agilent Bioanalyzer 2100 using RNA 6000 Nano chip (Agilent Technologies, Palo Alto, CA).

Generation of far-red fluorescent protein (HcRed1) cRNA was conducted basically as described above for GFP RNA, but with different primers. The 738 bp linear DNA templates for in vitro transcription were generated by PCR from plasmid vector pHc-Red1-Nuc (BD Biosciences, San Jose, CA) using the following primers, 5'-TCATTTAGGTGACACTATAGCCATGGTGAGCGG-CCTGCTGAA-3' (forward) and 5'-TTTTTTTTTTTTTTTTTTTGTATCTGAGTCCGGAGTTGGCCTT-3' (reverse) with a poly (T₁₈) tail fused to HcRed1 sequence.

RNA Extraction and Reverse Transcription

Total RNA was extracted from each pool of oocytes/embryos (n = 5 pools of 10 oocytes/embryos per time point), and residual genomic DNA removed by DNase I digestion, using the RNAqueous micro kit (Ambion) according to manufacturer's instructions, but with slight modifications. Before RNA extraction, each sample was spiked with 250 fg of GFP synthetic RNA as an exogenous control and 50 μ g of tRNA as a carrier. RNA was eluted twice from the silica-based microfilter cartridge using 10 μ l volume of pre-warmed (75°C) elution solution according to manufacturer's instructions. The RNA from each pool of oocytes and embryos was divided into two samples so that the RNA equivalent of five oocytes (10 μ l) was primed with 1 μ l (450 ng) of oligo dT₍₁₅₎ and the other half of the RNA was primed with 1 μ l of 20 μ M random hexamers. Before reverse transcription (RT), 250 fg (1 μ l) of HcRed1 synthetic RNA was added to each reaction. RNA/primer mixture was incubated at 70°C for 10 min and rapidly cooled to 4°C prior to RT. RT was performed at 42°C for 1 hr in a final volume of 20 μ l containing 8 μ l RT mix [4 μ l of 5 \times RT buffer, 2 μ l of 0.1 M dithiothreitol, 1 μ l of 10 mM dNTP mix, and 1 μ l (200 U) of Superscript II reverse transcriptase (Invitrogen, Life Technologies)] followed by incubation at 70°C for 10 min to terminate the reaction. Each RT reaction was then diluted with nuclease free water (Ambion) to a final volume of 100 μ l (one oocyte or embryo equivalent = 20 μ l of cDNA).

Quantitative Real-Time RT-PCR

The quantification of all gene transcripts was done by real-time quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Absolute quantification using this method is described elsewhere (Li and Wang, 2000; Whelan et al., 2003). Primers were designed using Primer Express program (Applied Biosystems) and derived from bovine

TABLE 1. Details of Primers Used in Real-Time PCR

Gene	GenBank accession number	Species	Sequence
GAPDH	BG691477	Cow	F: 5'-GCCATCAATGACCCCTTCAT-3' R: 5'-TGCCGTGGGTGGAATCA-3'
β -Actin	BG689033	Cow	F: 5'-GGATGAGGCTCAGAGCAAGAGA-3' R: 5'-TCGTCCCAGTTGGTGACGAT-3'
RPL-15	AY786141	Cow	F: 5'-TGGAGAGTATTGCGCCTTCTC-3' R: 5'-CACAAGTTCCACCACACTATTGG-3'
Cyclophilin-A	BF230516	Cow	F: 5'-GCCATGGAGCGCTTTGG-3' R: 5'-CCACAGTCAGCAATGGTGATCT-3'
Phosphoglycerokinase	BG688981	Cow	F: 5'-GTTGCAGAACATCTCAGCTCATCT-3' R: 5'-CGGTTTAGTCTCTAAGAAATTCAAATGG-3'
β -Glucuronidase	BE751040	Cow	F: 5'-TGTCATCGCACACAGAGCAA-3' R: 5'-CACAAAATCCAGGTGAGAAGCTT-3'
Histone H2A	BF076713	Cow	F: 5'-GAGGAGCTGAACAAGCTGTG-3' R: 5'-TTGTGGTGGCTCTCAGTCTTC-3'

sequences found in GenBank (see Table 1) and the amplicon size for each of the genes studied ranged from 80–150 bp. A primer matrix was performed for each gene tested to determine optimal primer concentrations. Each reaction mixture consisted of 2 μ l of cDNA (corresponding to 1/10 of an oocyte/embryo), 1.5 μ l each of forward (5 μ M) and reverse primers (5 μ M), 7.5 μ l of nuclease free water, and 12.5 μ l of SYBR Green PCR Master Mix in a total reaction volume of 25 μ l (96-well plates). Reactions were performed in duplicate for each sample in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The thermal cycler program consisted of 40 cycles of 95°C for 15 sec and 60°C for 1 min. Standard curves for each gene and controls were constructed using tenfold serial dilutions of corresponding plasmids and run on same plate as samples. Copies of GFP and HcRed1 RNA in each pool were determined using standard curves constructed from the plasmid pCX-EGFP and pHc-Red1-Nuc, respectively. Real-time primer sequences for GFP and HcRed1 were as follows: GFP forward 5'-CAACAGCCACAACGTCTATATCATG-3'; GFP reverse 5'-ATGTTGTGGCGGATCTTGAAG-3'; HcRed1 forward 5'-GCCCGGCTTCCACTTCA-3'; and HcRed1 reverse 5'-GGCCTCGTACAGCTCGAAGTA-3'. Partial cDNAs for RPL-15 and H2A were amplified from bovine GV oocytes, cloned into pCR2.1 Topo vector (Invitrogen Life Technologies), and subjected to fluorescent dye primer sequencing to confirm identity. Resulting plasmids, along with plasmids containing cDNA inserts for CYC-A, PGK, GUSB, GAPDH, and β -actin [vector pCMV Sport6 (Invitrogen Life Technologies)] obtained from the MSU Center for Animal Functional Genomics NBFGC (National Bovine Functional Genomics Consortium) library were used to construct standard curves. Representative R^2 for GFP, HcRed1, GAPDH, β -actin, RPL-15, CYC-A, PGK, GUSB, and H2A standard curves were all >0.98. For each measurement, threshold lines were adjusted to intersect amplification lines in exponential portion of amplification curve and cycles to threshold (Ct) recorded. For each sample, amounts of mRNA (copies) for GFP, HcRed1, and housekeeping genes analyzed were determined from their respective standard curves.

Statistical Analysis

The copy numbers of GFP and HcRed1 RNA added to samples were calculated according to molecular weight of the RNA (base pair size of the RNA product \times 330 Da) and then converted into copy numbers based upon Avogadro's number (1 mol = 6.022×10^{23} molecules). Percent RNA recovery was determined by calculation of copies of GFP RNA detected versus number of copies added prior to RNA extraction and efficiency of reverse transcription by comparison of copies of HcRed1 measured versus number of copies added to RNA samples immediately prior to reverse transcription. Copies of RNA for each individual gene of interest within each sample were normalized relative to copies of GFP RNA measured in each sample, and differences in normalized RNA data across developmental stages were determined by one-way analysis of variance using the GLM procedure of SAS. Individual mean comparisons were performed using Tukey's test. Differences of $P < 0.05$ were considered significant.

RESULTS

Validation of Real-Time RT-PCR Procedures

To assess potential variation in RNA recovery and efficiency of reverse transcription, samples were spiked with 250 fg of GFP and HcRed1 cRNAs prior to RNA isolation or cDNA synthesis, respectively. Representative electropherogram for in vitro synthesized GFP RNA is shown in Figure 1A. Results of real-time RT-PCR assay of MII and GV oocyte cDNA generated with oligo dT primers (used for quantification of amounts of polyadenylated transcripts) indicate an acceptable rate of GFP RNA recovery (~50%) in both sample types that was not significantly different ($P > 0.05$), and with minimal variation within samples (Fig. 1B). Furthermore, percent recovery of GFP RNA (~95%) was greater in GV and MII cDNA samples primed with random hexamers (used for quantification of total transcripts), but did not differ between samples (Fig. 1C). The copies of GFP RNA detected in oligo dT primed cDNA generated from early embryos at the pronuclear, 2-, 4-, 8-, 16-cell, morula, and blastocyst stages also did not differ (Fig. 1D). In

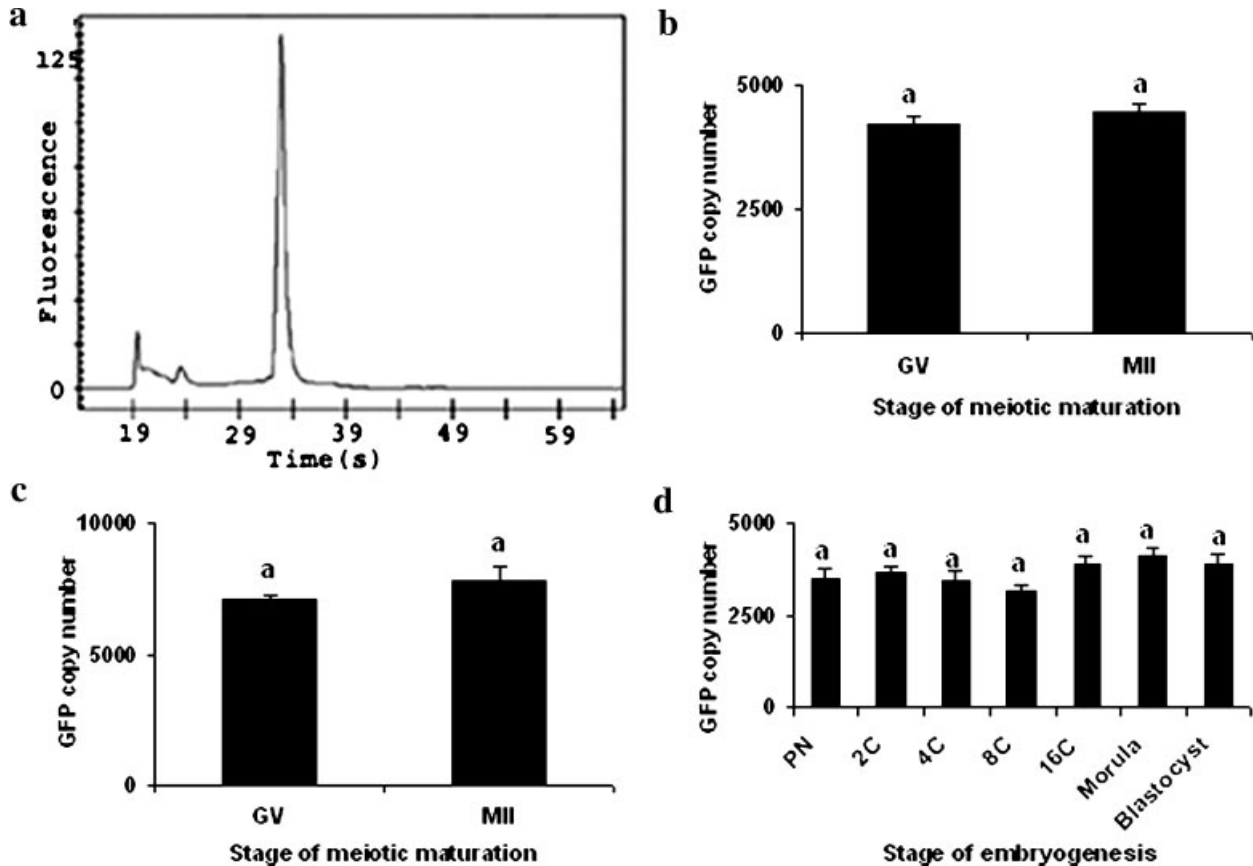


Fig. 1. Quantitative real-time RT-PCR analysis of amounts of green fluorescent protein (GFP) RNA in oocyte and embryo samples spiked prior to RNA isolation. (a) A representative electropherogram generated by Agilent Bioanalyzer nanochip for in vitro synthesized GFP RNA. Quantification of amounts of exogenous GFP RNA in samples of germinal vesicle (GV) and in vitro matured metaphase (MII) stage oocytes, (b) reverse transcribed with oligo dT₍₁₅₎, or (c) random

hexamers ($n = 5$ each). (d) Quantification of amounts of exogenous GFP RNA in samples of in vitro derived embryos collected at pronucleus (PN), 2-cell (2-C), 4-cell (4-C), 8-cell (8-C), 16-cell (16-C), morula and blastocyst stages ($n = 5$ each) reverse transcribed with oligo dT₍₁₅₎. Data are shown as mean \pm SEM. Means with common superscripts are not different.

addition, copies of GFP RNA recovered from early embryos primed with random hexamers were greater than oligo dT but did not differ significantly (data not shown). Similar results as reported in Figure 1 were obtained when copies of HcRed1 RNA in above described samples were assayed to specifically assess variation in efficiency of reverse transcription reactions ($>85\%$; data not shown).

Quantification of Abundance of GAPDH, β -Actin, RPL-15, CYC-A, PGK, and GUSB mRNAs During Oocyte Maturation

Amounts of polyadenylated GAPDH, β -actin, RPL-15, CYC-A, PGK, and GUSB mRNAs (normalized relative to amounts of GFP RNA in each sample) decreased ($P < 0.05$) 1.5- to 5-fold during meiotic maturation (Fig. 2). However, amounts of total transcripts (polyadenylated + nonadenylated, determined by real-time RT-PCR using RT reaction primed with random hexamers) for GAPDH, β -actin, RPL-15, CYC-A, and GUSB genes, were not decreased in MII versus GV oocytes (Fig. 3). In contrast, total transcripts for the PGK gene were significantly decreased in MII oocytes ($P < 0.05$; Fig. 3).

Temporal Regulation of mRNA for GAPDH, β -Actin, RPL-15, CYC-A, PGK, and GUSB During Early Embryonic Development

Quantification of polyadenylated transcripts for CYC-A, PGK, GUSB, GAPDH, and β -actin genes revealed a similar temporal expression pattern, where mRNA abundance for each gene remained low through embryonic genome activation until the blastocyst stage when a significant increase in mRNA abundance was detected (Fig. 4). A similar temporal expression pattern for RPL-15 mRNA was observed, except that a significant increase in transcript abundance was observed at the morula stage and further increased at the blastocyst stage (Fig. 4).

Dynamic Regulation of Histone H2A mRNA During Bovine Oocyte Maturation and Early Embryogenesis

A distinct overall temporal expression pattern for H2A mRNA was observed. Like observed for other genes analyzed, the abundance of polyadenylated H2A mRNA decreased during meiotic maturation (Fig. 5A), but

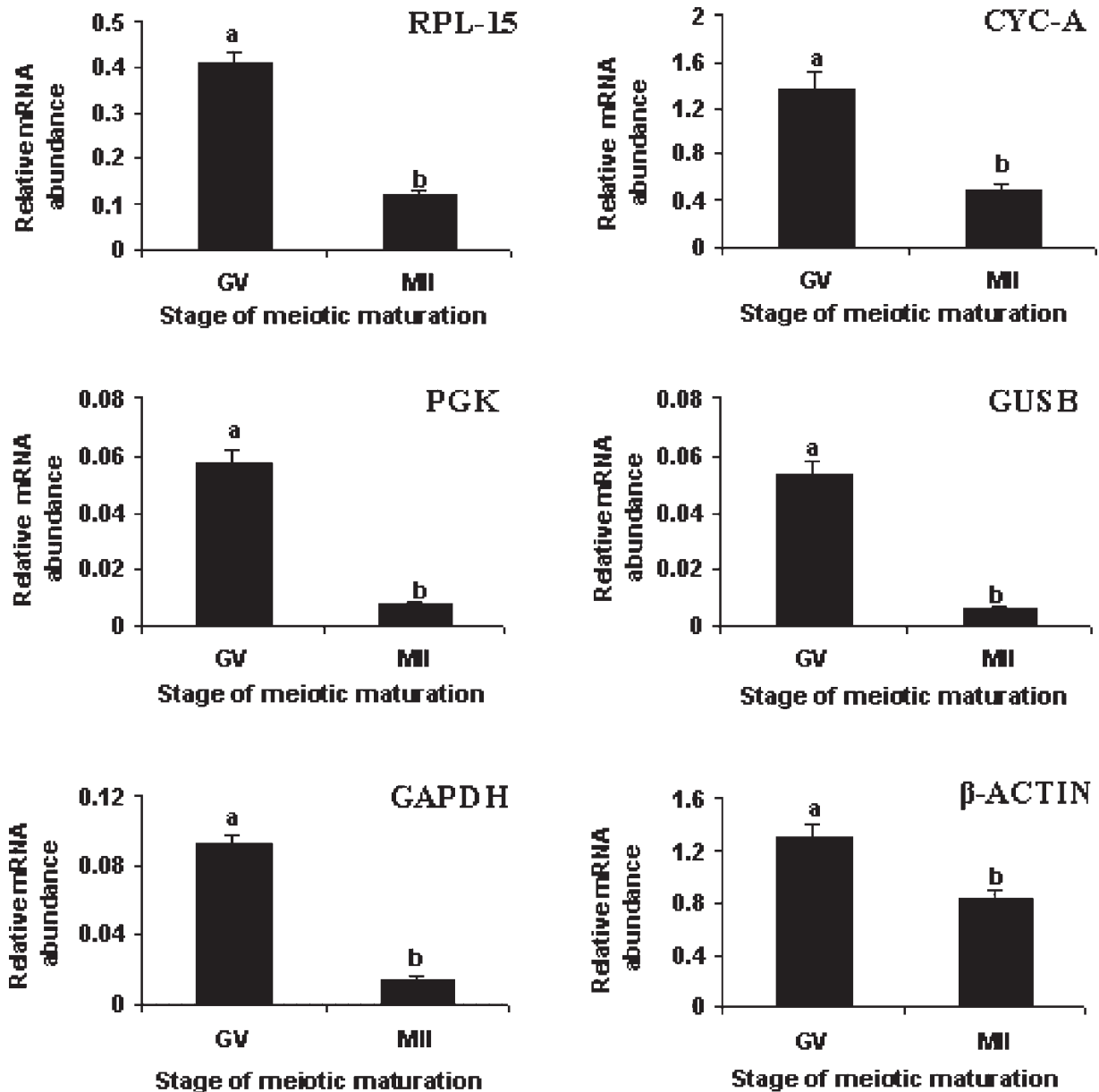


Fig. 2. Quantitative real-time RT-PCR analysis of polyadenylated RPL-15, CYC-A, PGK, GUSB, GAPDH, and β -actin transcripts in samples of germinal vesicle (GV) and metaphase (MII) stage bovine oocytes ($n = 5$ each). Data are normalized relative to abundance of exogenous control (GFP) RNA and shown as mean \pm SEM. Time points without a common superscript are significantly different, $P < 0.005$.

there was no difference noted in total number of transcripts detected at the GV versus MII stages (Fig. 5B). However, the decrease in polyadenylated H2A mRNA at the MII stage was transient, as amounts of RNA recovered to prematuration levels following fertilization (at the pronuclear stage) and were gradually depleted through the 16-cell stage (Fig. 5A). A similar depletion of total H2A transcripts was observed from the pronuclear through 16-cell stages (Fig. 5B). In contrast to other housekeeping genes examined, abundance of H2A transcripts (polyadenylated and total) remained low in morula and blastocyst stage embryos (Fig. 5C,D).

DISCUSSION

Technical constraints, including limited amounts of starting material and a paucity of information on constitutively expressed genes required for data normalization, have restricted widespread quantitative analysis of mRNA abundance for genes of interest in mammalian oocytes and early embryos using quantitative RT-PCR procedures. In the present studies, procedures for quantitative analysis of mRNA abundance in oocytes and early embryos (using quantification of exogenous RNAs added to samples to account for variation

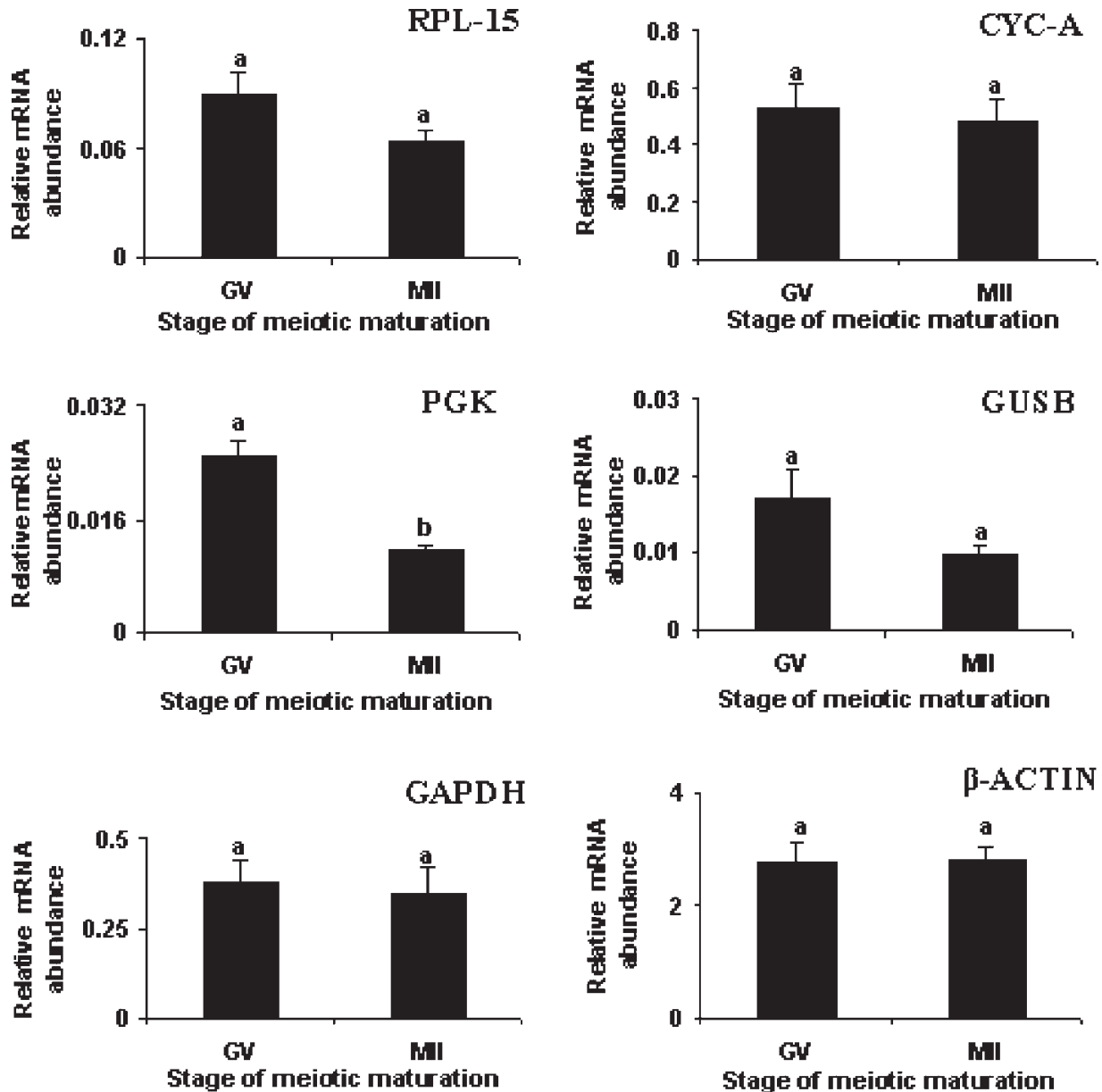


Fig. 3. Quantitative real-time RT-PCR analysis of total RPL-15, CYC-A, PGK, GUSB, GAPDH, and β -actin transcripts in samples of germinal vesicle (GV) and metaphase (MII) stage bovine oocytes ($n = 5$ each). Data are normalized relative to abundance of exogenous control (GFP) RNA and shown as mean \pm SEM. Time points without a common superscript are significantly different, $P < 0.0001$.

in RNA recovery and reverse transcription) were validated and used in studies to elucidate temporal changes in mRNA abundance for seven presumptive house-keeping genes (RPL-15, CYC-A, PGK, GUSB, GAPDH, β -actin, and H2A) during bovine oocyte maturation and early embryogenesis. The present studies demonstrate validity of above approach for quantitative studies of mRNA abundance in oocytes and early embryos. Furthermore, results of present studies demonstrated a decrease in abundance of polyadenylated, but not total mRNA transcripts for above genes (excluding PGK) during meiotic maturation. During early embryogenesis, temporal changes in mRNA abundance for above

genes (excluding H2A) were not observed until the morula or blastocyst stages. But in contrast, dynamic temporal changes in H2A mRNA abundance were observed in early embryos prior to embryonic genome activation (16-cell stage). Collectively, results of present studies demonstrate distinct temporal regulation of mRNA abundance for above genes during oocyte maturation and early embryogenesis and thus support normalization of quantitative PCR data from oocytes and early embryos relative to measured amounts of exogenous RNAs added to samples (as described), rather than relative to amounts of mRNA for above house-keeping genes.

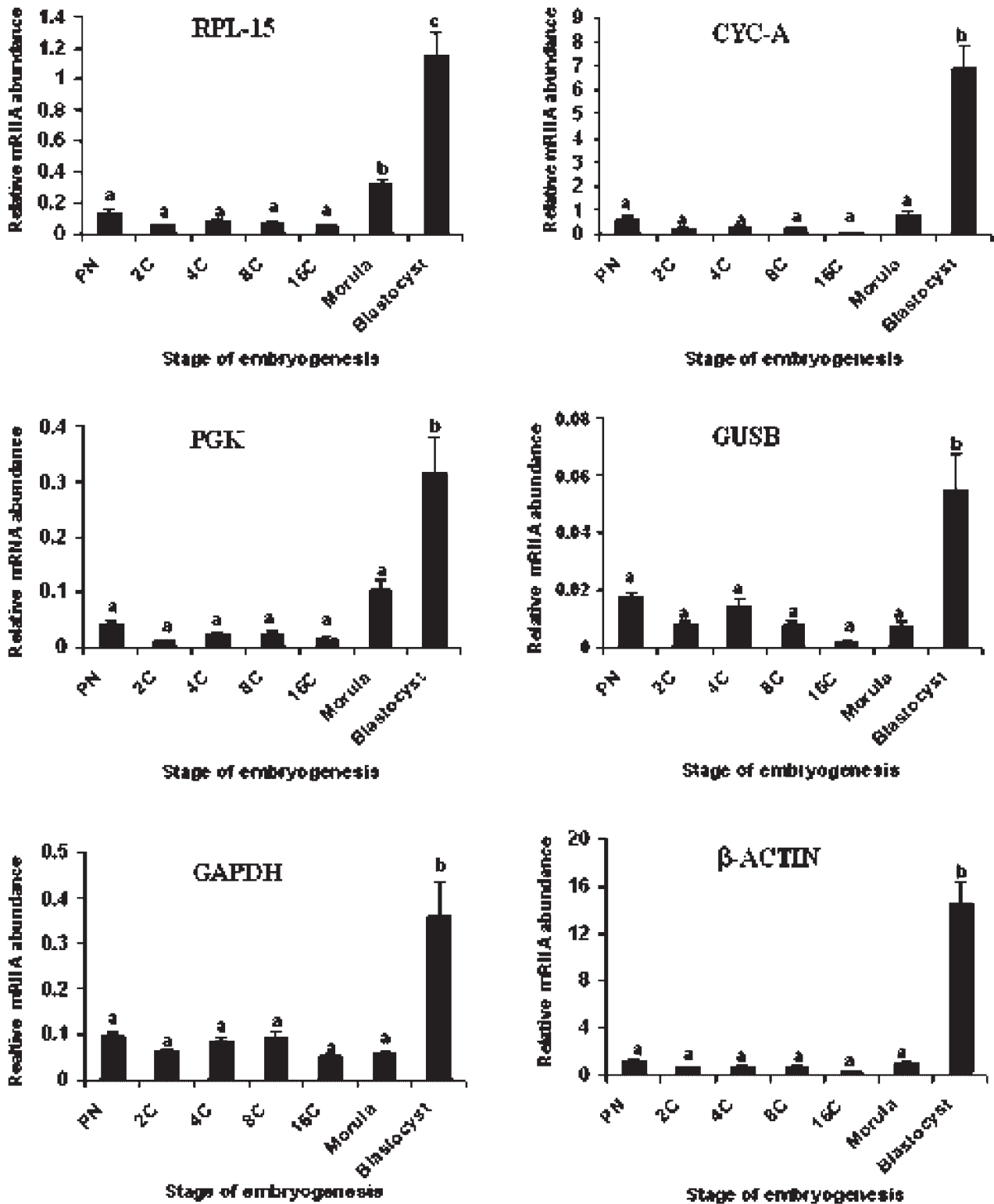


Fig. 4. Quantitative real-time RT-PCR analysis of polyadenylated RPL-15, CYC-A, PGK, GUSB, GAPDH, and β -actin transcripts in samples of in vitro derived bovine embryos collected at pronucleus (PN), 2-cell (2-C), 4-cell (4-C), 8-cell (8-C), 16-cell (16-C), morula, and blastocyst stages (n = 5 each). Data are normalized relative to abundance of exogenous control (GFP) RNA and shown as mean \pm SEM. Time points without a common superscript are significantly different, $P < 0.05$.

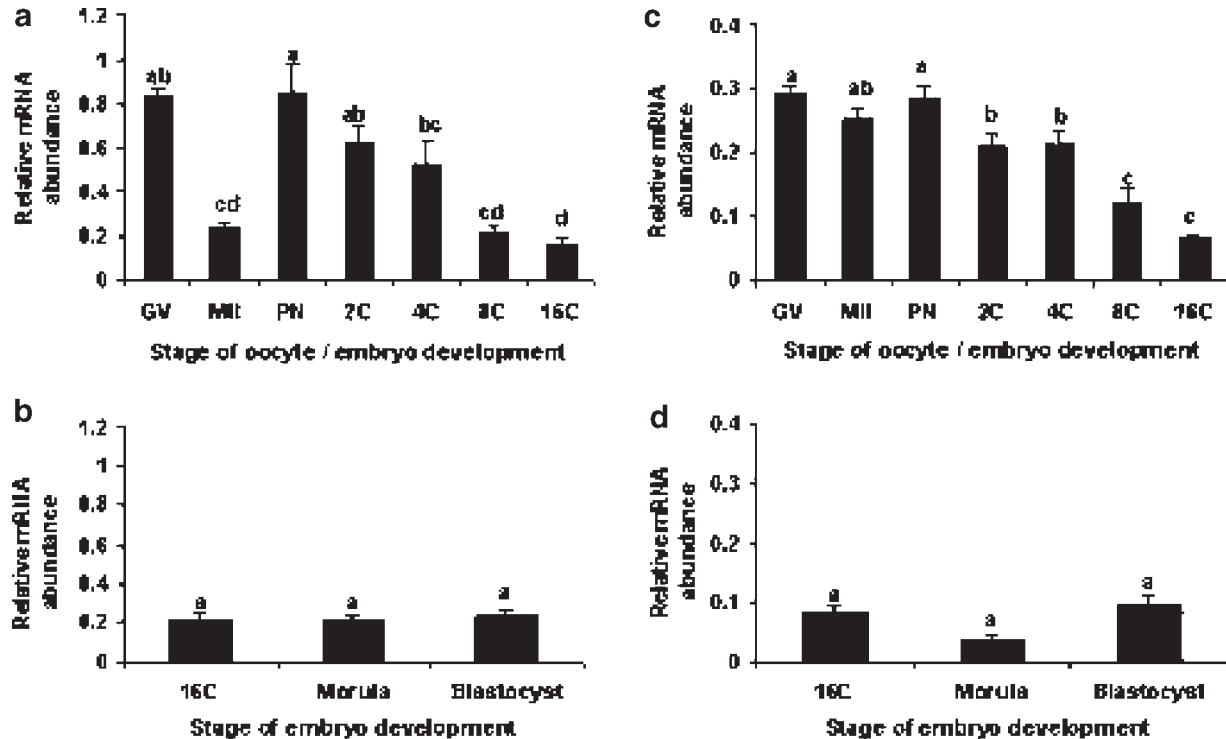


Fig. 5. Quantitative real-time RT-PCR analysis of H2A mRNA in oocytes and in vitro derived embryos. (a) Relative abundance of polyadenylated H2A mRNA transcripts in samples collected during meiotic maturation and early embryogenesis through embryonic genome activation [n=5 each of bovine germinal vesicle (GV) and in vitro matured metaphase (MII) stage oocytes, pronucleus (PN), 2-cell (2-C), 4-cell (4-C), 8-cell (8-C), and 16-cell (16-C) stage embryos]. (b) Relative abundance of total H2A transcripts in same samples (n = 5 each) as depicted in (a). (c) Relative abundance of polyadenylated H2A

transcripts in samples collected throughout subsequent stages of early embryonic development (16-C, morula and blastocyst stages; n=5 each). (d) Relative abundance of total H2A transcripts in samples collected throughout subsequent stages of early embryonic development (16-C, morula and blastocyst stages; n=5 each). Data are normalized relative to abundance of exogenous control (GFP) RNA and shown as mean ± SEM. Time points without a common superscript are significantly different, $P < 0.05$.

Use of exogenous RNAs as a control in real-time RT-PCR analysis of RNA abundance in oocytes and early embryos has been reported previously, but with distinct differences in approach and (or) limited details of assay validation provided (Lequarre et al., 2004; Vigneault et al., 2004). For example, in one previous study, the highest GFP value obtained was used as a reference sample and relative amounts of GFP RNA in remaining samples corrected relative to amount of GFP RNA detected in the reference sample (Vigneault et al., 2004). However, no indication of variation in recovery of RNA was reported, and precipitation of RNA prior to RT was required in this protocol, which may increase variation in RNA recovery. In another study, polyadenylated rabbit globin mRNA was added to oocyte samples before RNA extraction, but only two pools of samples were used for analysis (Lequarre et al., 2004). In our extraction procedure, which does not require precipitation of RNA prior to RT, we were able to specifically adjust for and quantify variation in RNA recovery and efficiency of RT, which did not differ between sample sets analyzed.

Abundance of mRNA for specific housekeeping genes has also been utilized for normalization of quantitative RT-PCR data when comparing effects of treatments on oocytes or embryos at a single time point in develop-

ment, but validity of such results is also dependent on demonstration that endogenous mRNA levels for selected housekeeping gene are not affected by treatment. CYCA, PGK, GUSB, β -actin, and H2A have previously been utilized as internal controls for real-time RT-PCR assays in somatic cells/tissues (Overbergh et al., 1999; Specht et al., 2001; Birot et al., 2003; Bock et al., 2004) and oocytes and early embryos (Fair et al., 2004a; Gutierrez-Adan et al., 2004; Lequarre et al., 2004; Leyens et al., 2004; Rizos et al., 2004). In the present studies, GAPDH RNA transcripts showed the least variation in copy number from pronuclear through the morula stage (day 5), and could potentially be used as an internal control for data normalization for embryos during early cleavage divisions (prior to the blastocyst stage). However, in vitro culture conditions during oocyte maturation and early embryonic development have been shown to affect abundance of specific transcripts and global gene expression profiles, respectively (Wrenzycki et al., 1999, 2001; Rief et al., 2002; Rizos et al., 2002, 2003; Lonergan et al., 2003b,c; Gutierrez-Adan et al., 2004; Rinaudo and Schultz, 2004). Thus, quantification of amounts of exogenous control RNA added prior to RNA extraction should serve as the preferred method for data normalization in real-time

RT-PCR assays of mRNA abundance in oocytes and early embryos.

Irrespective of described technical applications of the results of present studies, results also shed new biologically relevant insight into temporal changes in mRNA abundance for seven presumptive housekeeping genes during bovine oocyte maturation and early embryonic development. The relative abundance of polyadenylated RPL-15, CYC-A, GUSB, GAPDH, and β -actin mRNA transcripts significantly decreased during meiotic maturation, whereas abundance of total transcripts for above genes was not affected. Measurement of total transcripts for GAPDH, H2A, and β -actin in bovine oocytes and embryos has been reported previously (Robert et al., 2002). However, results of previous studies are difficult to interpret because a reference standard to account for variation in RNA recovery or RT efficiency was not utilized, and data were not subjected to statistical analysis (Robert et al., 2002). The observed decrease in number of polyadenylated transcripts for above genes during meiotic maturation in our studies could be due to transcript deadenylation. A number of maternal mRNAs are stored in the mammalian oocyte in an inactive form (Eichenlaub-Ritter and Peschke, 2002; Schultz, 2002), and their translation is ultimately controlled in a precise temporal and spatial pattern during oocyte maturation and early embryogenesis (Oh et al., 2000). These include mRNAs encoding for essential factors required for cell cycle progression (Tay et al., 2000). Changes in length of the poly(A) tail and *cis* elements in the 3'-UTR are involved in post-transcriptional regulation and translational control during early development in *Xenopus* and mammalian embryos (Richter, 1999; Oh et al., 2000). During meiotic maturation, some mRNAs undergo a significant reduction in poly (A) tail length resulting in translational repression, which has been reported for actin and alpha tubulin in mouse oocytes (Bachvarova et al., 1985). Furthermore, a reduction in poly (A) tail length occurs for connexin-43, heat shock protein-70, Oct-4, plakophilin, pyruvate dehydrogenase, and RNA poly (A) polymerase (PAP) transcripts in bovine oocytes (Brevini-Gandolfi et al., 1999). However, previous studies indicate that β -actin mRNAs do not undergo any changes in the length of their poly (A) tails during meiotic maturation in bovine oocytes (Brevini-Gandolfi et al., 1999). Further studies will be required to confirm extent of deadenylation of RPL-15, CYC-A, GUSB, and GAPDH transcripts during bovine meiotic maturation.

In contrast to RPL-15, CYC-A, PGK, GUSB, GAPDH, and β -actin mRNA, abundance of both total and polyadenylated transcripts for PGK was significantly decreased during meiotic maturation. Although we are unable to prove conclusively from present studies, such results are likely due to depletion of PGK mRNA during translation and (or) transcript degradation. Several oocyte-derived mRNAs have been demonstrated to undergo translational activation during meiotic maturation, including transcripts for the SPIN gene, β -

catenin, E-cadherin, tissue-type plasminogen activator, and c-mos (Gebauer et al., 1994; Stutz et al., 1998; Oh et al., 2000).

An extensive change in pattern of protein synthesis is observed following fertilization/egg activation and prior to genome activation in mammalian embryos (Latham et al., 1991; Xu et al., 1997). Maternally recruited mRNAs undergo polyadenylation and are translationally activated following fertilization (Aoki et al., 2003). A similar temporal pattern of mRNA abundance for CYC-A, PGK, GUSB, GAPDH, and β -actin during early embryogenesis was observed, where abundance remained low through the 16-cell and morula stage, followed by a sharp increase at the blastocyst stage. The expression pattern of GAPDH and β -actin in early bovine embryos was reported previously with a sharp increase observed at the blastocyst stage, but studies did not analyze pronuclear, 4-, 16-cell, and morula stages (Robert et al., 2002). To our knowledge, temporal changes in RPL-15 mRNA abundance during early development have not been reported previously. A similar temporal expression pattern was denoted for RPL-15 mRNA as for other housekeeping genes listed above, with the exception that RPL-15 mRNA abundance was significantly increased at the morula stage and further increased at the blastocyst stage. In a recent study, abundance of mitochondrial Mn-superoxide dismutase transcripts was significantly increased at the morula stage (day 5) in bovine embryos with a further increase at the blastocyst stage (Lonergan et al., 2003c). Blastocyst formation, which is a prerequisite for implantation and establishment of pregnancy, is driven by the expression of a specific set of critical gene products, which direct the acquisition of cell polarity, trophoblast differentiation, and establishment of inner cell mass (Watson and Barcroft, 2001). The observed upregulation of mRNA transcript abundance for above genes at the blastocyst stage could be associated with transcription coupled developmental processes including cell proliferation, compaction, and blastocoel formation, which would potentially require increased expression of key metabolic and housekeeping genes. However, we cannot discount the potential relative contribution of increased cell numbers to the elevated abundance of housekeeping gene transcripts at the blastocyst stage because data were not normalized relative to numbers of nuclei at a given stage of development.

A distinct temporal pattern for H2A mRNA abundance was observed in the present studies. In somatic tissues of metazoan species, the mRNAs encoding for all five classes of histone proteins are unique because they are the only known mRNAs that lack poly (A) tails and instead end in a conserved stem-loop sequence, which is highly conserved in evolution (Marzluff, 1992; Dominski and Marzluff, 1999; Marzluff and Duronio, 2002). In contrast, histone mRNAs in GV stage *Xenopus* oocytes have a short poly (A) tail attached to the stem-loop sequence, which plays an active role in translation repression (Ballantine and Woodland, 1985; Sanchez and Marzluff, 2004) and deadenylation causes transla-

tional activation (Sanchez and Marzluff, 2004). However, to our knowledge, data in mammalian oocytes/embryos regarding the relationship between polyadenylation and translational activation/repression of H2A mRNA are not available. In the present studies, cDNAs were prepared from pools of bovine oocyte/embryo RNA primed with oligo dt, and differences were observed versus results obtained with random primers (total transcripts). Therefore, our results indirectly suggest that H2A mRNA in bovine oocytes and early embryos is polyadenylated, in contrast to what is reported in somatic tissues (Marzluff, 1992; Dominski and Marzluff, 1999; Marzluff and Duronio, 2002). Abundance of polyadenylated H2A mRNA in bovine oocytes was decreased during meiotic maturation (at MII vs. the GV stage), but no difference in total number of transcripts was detected, similar to results observed for RPL-15, CYC-A, GUSB, GAPDH, and β -actin. However, abundance of polyadenylated H2A transcripts was higher at the pronuclear stage than observed in MII oocytes, suggestive of potential transcript readenylation during fertilization. Similar phenomena, where the length of the poly (A) tail is decreased during meiotic maturation and then begins to elongate in fertilized embryos, have been observed for PAP, HSP-70, and Glut-1 transcripts in cattle (Brevini et al., 2002). During early embryogenesis, total and polyadenylated H2A mRNA was maximal at the pronuclear stage, progressively decreased through the 8-cell stage and remained low from the 16-cell through the blastocyst stage, in contrast to the temporal mRNA expression pattern during early embryogenesis observed for other housekeeping genes examined in the present studies.

The biological significance of the distinct regulation of H2A mRNA observed during meiotic maturation and early embryogenesis is not clear and will require further investigation. Following fertilization, protamines are removed from sperm chromatin, and the decondensing haploid sperm nucleus is simultaneously associated with histones mobilized from the mammalian oocyte cytoplasm (Perreault, 1992). From the present experiments, we are unable to conclude whether H2A mRNA underwent translational activation during meiotic progression, due to loss of poly (A) tail as in *Xenopus* oocytes, or whether translational activation accompanied the increase in polyadenylated transcripts during fertilization. In previous studies, H2A mRNA has been widely utilized as an internal standard to normalize measurements of mRNA abundance in bovine oocytes and embryos at similar stages of development and across stages of development (Lonergan et al., 2003c; Fair et al., 2004a; Gutierrez-Adan et al., 2004; Lequarre et al., 2004; Leyens et al., 2004; Rizos et al., 2004). Our results clearly demonstrate that H2A mRNA is not constitutively expressed, but rather is dynamically regulated during early development. Thus, results suggest that use of H2A mRNA abundance for data normalization could potentially influence results and further illustrate the importance of use of exogenous control RNAs to normalize data and account for inherent variation in

real-time RT-PCR assays of mRNA abundance in oocytes and early embryos.

In summary, we have validated procedures for quantitative analysis of changes in mRNA abundance in oocytes and early embryos using real-time RT-PCR and addition of exogenous control RNAs to normalize data for differences in RNA recovery and efficiency of reverse transcription. We have also analyzed temporal changes in mRNA abundance for RPL-15, CYC-A, PGK, GUSB, GAPDH, β -actin, and H2A during bovine oocyte maturation and early embryonic development. Results provide new information on dynamic regulation of RNA transcripts for such genes during bovine oocyte maturation and early embryonic development and further illustrate the potential importance of validation of constitutive expression of internal control (housekeeping genes) used for data normalization across development time points or for examination of effects of treatments on mRNA abundance at a single developmental time point.

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