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Quantification of Housekeeping Transcript Levels During the Development of Bovine Preimplantation Embryos[1]

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ABSTRACT

In mammals, the study of gene expression in the preimplantation embryo has been difficult because the standard procedures used to quantify mRNA generally require large amounts of starting material. The development of protocols using different quantitative strategies generally involving the polymerase chain reaction (PCR) has provided new tools for exploration of gene expression in preimplantation embryos. However, the use of an internal standard, often referred as a housekeeping gene, is essential to normalize the mRNA levels. RNA levels of eight housekeeping genes were quantified using real time PCR throughout the preimplantation period of the bovine embryo to find the most suitable gene to be used as standard. Histone H2a was the best internal standard because the transcript levels were constant across the preimplantation period. Linear amplification of antisense RNA using the T7 promotor for in vitro transcription of the entire RNA pool was evaluated as a suitable way to preamplify the starting material prior to quantification and was effective in providing accurate RNA abundance profiles throughout the preimplantation period. However, the amplification appears to be template dependent because the amplification factors were higher for some genes.

developmental biology, early development, embryo, oocyte development, ovum

INTRODUCTION

Quantitative measurements of mRNA levels can be achieved using several approaches; however, some of these approaches are not suitable when working with mammalian oocytes or preimplantation embryos because of the difficulty in gathering the large amount of starting material needed to obtain enough RNA to perform these techniques. The Northern blot analysis has not been widely used to study gene expression during preimplantation development because about 300 oocytes or 54 blastocysts are needed to analyze the expression of a single gene [1, 2]. The RNase protection assay approach has been reported to be sensitive enough to detect specific messengers in only 0.1 blastocyst equivalent per reaction [3], but the technique is tedious and requires much sample manipulation, rendering it more prone to technical errors. The use of the polymerase chain reaction (PCR) has overcome the problems associated with tissue scarcity and has enabled efficient detection of specific transcripts. However, the amplification plateau phase renders the approach more qualitative than quantitative. To date, the numerous studies using qualitative PCR to detect the presence of gene transcripts throughout the preimplantation period generally involved 5–15 oocytes or embryos per reaction [4–10]. Nested PCR approaches, which involve a second round of PCR amplification, have also been used to detect specific gene transcripts in bovine oocytes and human embryos [11, 12]. However, the need for more quantitative measurements has prompted the development of semiquantitative methods involving the amplification of an endogenous standard, often referred as a housekeeping gene, for which the mRNA level is considered to be constant [13] and/or the amplification of an exogenously added mRNA (rabbit globin) to account for technical imprecision due to the manipulation of the samples [14–19]. Other methods involving the blotting of the PCR products and hybridization with a specific radiolabeled probe allowing quantification similar to that with the Southern blot technique have also been proposed [20–22]. The plateau phase of the PCR amplification, which generally occurs in the later cycles, keeps the PCR method from being truly quantitative. However, a simple way to prevent overamplification is to restrict the number of PCR cycles by previously evaluating the optimal number of cycles during the optimization steps [23]. The competitive PCR procedure is a truly quantitative method that uses serial dilutions of a known amount of an exogenous cDNA template (which is generally similar to the targeted genes but of slightly different size) in combination with a serial dilution of the sample. The added quantified cDNA template will compete with the endogenous sequence for the primers and the nucleotides during the PCR, and according to normal probabilities, the more abundant template will produce more amplified product. By comparing the relative intensities of the bands for the two amplified products after gel electrophoresis, it is possible to precisely measure the amount of mRNA in the sample. This technique has been successfully used to measure gene expression in preimplantation embryos [24–27]. However, the procedure is time consuming because of the need to design the standardized cDNA template and often requires optimization before a systematic measurement of the mRNA levels can be performed in several samples. The development of

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TABLE 1. Information on the primers used for amplification.

Gene	GenBank accession number	Species	Sequence	Location	Product size (bp)	Temperature of fluorescence acquisition (°C)
β-Actin	BC008633	Mouse	5′-CGTGACATTAAGGAGAAGCTGTGC-3′	609–632	374	87
			5′-CTCAGGAGGAGCAATGATCTTGAT-3′	960–983		
GAPDH	U85042	Cow	5′-GAAGACTGTGGATGGCCCCTCC-3′	519–540	357	89
			5′-GTTGAGGGCAATGCCAGCCCC-3′	856–876		
Ubiquitin	Z18245	Cow	5′-TTTTCGTGAAGACCCTGACCG-3′	780–798	284	86
			5′-TAAATGGCTAGAGTGCAGAAGG-3′	1043–1064		
Lamin B	XM_003777	Human	5′-ACTGACCTCATCTGGAAGAACC-3′	1173–1194	393	84
			5′-CAAAACTTTCAGTGTCAAAAAGG-3′	1544–1566		
Tubulin	BC008659	Human	5′-CTCTGCTGAGAAAGCCTACCA-3′	898–918	400	87
			5′-CCACGTACCAGTGAACAAAGG-3′	1278–1298		
Histone H2a	U62674	Mouse	5′-GTCGTGGCAAGCAAGGAG-3′	1014–1031	182	88
			5′-GATCTCGGCCGTTAGGTACTC-3′	1178–1195		
U2snRNA	U57614	Human	5′-CTCGGCCTTTTGGCTAAGAT-3′	4889–4908	172	83
			5′-CGTTCCTGGAGGTACTGCAA-3′	5042–5061		
18S rRNA	AF176811	Cow	5′-GAGAAACGGCTACCACATCC-3′	55–74	337	87
			5′-GGACACTCAGCTAAGAGCATCG-3′	371–392		

PCR modules capable of reading fluorescence during the PCR (real time PCR) has overcome most of these problems and allows precise and efficient measurement of the mRNA level before the reaction reaches the plateau phase (for review, see [28]). The successful use of such a system has been reported for quantifying mitochondrial DNA, β-actin, and hypoxanthine phosphoribosyltransferase in very small samples such as single oocytes, polar bodies, and subcellular components [29–31].

The study of gene expression during the preimplantation period also requires an endogenous standard to normalize the RNA levels to justify the variations observed. With tissue samples or somatic cell cultures, it is common to standardize the measured mRNA levels to housekeeping genes such as β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and 18S rRNA. To date, most of the preimplantation work has involved these common housekeeping genes for normalization. However, the expression profiles of these genes during the preimplantation period have not been evaluated. The amount of mRNA present throughout the preimplantation period probably is not constant for most genes because most mRNAs are degraded after fertilization. This variability introduces considerable bias when the values of the candidate gene are corrected to the values of an inconsistent housekeeping gene. Thus, the objective of the present study was to use real-time PCR to measure the mRNA levels of several so-called housekeeping genes in a developmental series covering the bovine preimplantation period to identify the genes that are most suitable to be used as endogenous standards in gene expression studies.

MATERIALS AND METHODS

Oocyte and Embryo Collection

Bovine ovaries were collected at a slaughterhouse. The cumulus-oocyte complexes (COCs) from follicles 3–5 mm in diameter were collected by aspiration, and the follicles <2 mm in diameter were dissected with the aid of a stereomicroscope. The COCs where morphologically classified according to Blondin and Sirard [32]. Each developmental set was composed of germinal vesicule (GV) and mature oocytes, two-cell and eight-cell embryos, and blastocysts. Oocytes were in vitro matured in synthetic oviductal fluid (SOF) medium [33] with 1 μg/ml estradiol-17β, 0.5 μg/ml FSH, and 5 μg/ml LH for 22–24 h. Oocytes were fertilized and cultured in SOF medium supplemented with 0.6 mM of cystein at 38.5°C under an atmosphere of 5% CO_2, 20% O_2 up to the eight-cell stage. For further development, the embryos were transferred at the same temperature under an atmosphere of 7% O_2 until they reached the blastocyst stage. The efficiency rates of the in vitro production SOF system were as described previously [34]. The GV and matured oocytes were mechanically separated from the cumulus cells by vortexing, washed three times in PBS buffer, and stored at −80°C until RNA extraction. Three replicates of 20 oocytes or embryos were used for each developmental stage.

Total RNA Extraction

Total RNA was extracted and DNase I treatment was performed simultaneously using StrataPrep columns (Stratagene, La Jolla, CA). The RNA was coprecipitated using 1 μg of glycogen (Roche Molecular Biochemicals, Laval, PQ, Canada) as carrier by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol (Sigma, St. Louis, MO). The RNA was pelleted by centrifugation and washed using 400 μl of 70% ethanol. The RNA/glycogen pellet was dried quickly at 65°C using a thermal cycler (MJ Research, Watertown, MA) and then resuspended in 10 μl of nuclease-free water.

Reverse Transcription and Linear Antisense RNA Amplification

Of the three replicates, two were used for T7 linear RNA amplification. The other replicate was directly reverse transcribed using a modified version of the standard protocol described above. In addition to the oligo$(dT)_{12\text{-}18}$ primer, 200 ng of random hexamers (Roche) was added to the RNA and allowed to anneal by denaturing the secondary structure at 70°C for 2 min and then rapidly quenching the mix on ice. After the reverse transcription (RT) reaction, the final volume was increased to 30 μl with nuclease-free water.

For linear RNA amplification, the general procedure is called the Eberwine technique [35]. The principle behind the linear amplification is to add a T7 RNA polymerase promotor sequence during cDNA synthesis by using an oligo(dT) primer bearing a T7 promotor sequence upstream. The second step is to produce double-stranded DNA from the newly synthesized cDNA. Finally, the T7 promoting sequence is used to transcribe in vitro the entire double-stranded DNA pool corresponding to the entire mRNA pool, thus linearly amplifying the entire pool while maintaining the relative abundance of the messengers [35]. The T7 amplification protocol followed for the present study was described by Luo and collaborators [36], except that random hexamers were also added during the first RT reaction to reverse transcribe the RNA without a poly(A) tail. The RT reactions were done in 20 μl using an oligo$(dT)_{30}$ with the T7 promotor sequence in 5′ random hexamers (Roche) and Superscript II (Life Technologies, Burlington, ON, Canada). The second strand was processed using *Escherichia coli* ligase (Life Technologies), *E. coli* polymerase 1 (Life Technologies), and RNase H (Life Technologies). The double-stranded DNA was blunted using T4 DNA polymerase (Life Technologies). The DNA was purified using Qiaquick PCR purification columns (Qiagen, Mississauga, ON, Canada), coprecipitated with 1 μg of glycogene (Roche), and washed with 70% ethanol. The RNA amplification was per-

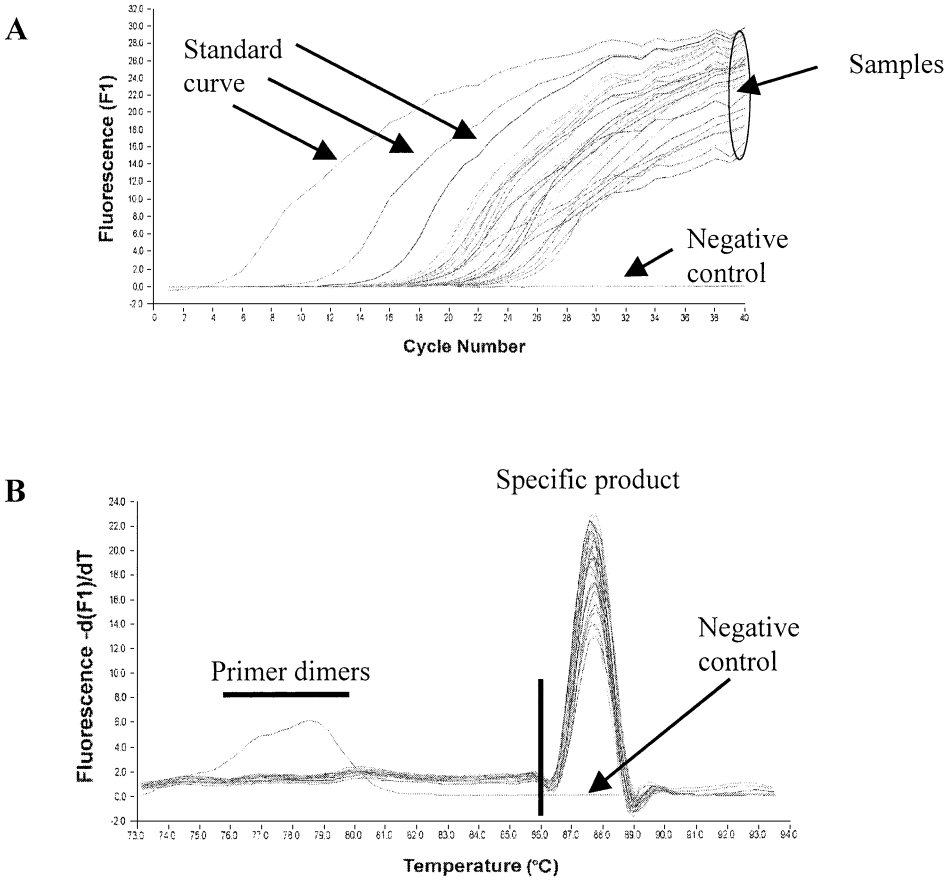

FIG. 1. **A**) Typical amplification profile of ubiquitin. **B**) Melting curve profile indicating the specific product and primer dimers formed in the negative control. This curve was used during optimization procedures to determine the temperature at which the fluorescence reading should be taken. In this case, the readings were taken at 86°C as indicated by the vertical line. In both panels, all the samples are represented along with the standard curve and the negative control.

formed using the AmpliScribe T7 in vitro transcription system (Epicentre, Madison, WI). The resulting antisense RNA (aRNA) was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol (Sigma), and the pellet was washed with 70% ethanol. The aRNA was reverse transcribed using the standard protocol described above in a 20-μl volume without the oligo(dT)$_{12-18}$, which was replaced with 200 ng of random hexamers (Roche) to prime the reaction. The final volume of the samples amplified using the T7 system was adjusted to 50 μl with nuclease-free water.

Cloning the Housekeeping Genes

Primers were designed according to the sequences found in GenBank. The reported bovine sequences were preferentially used; otherwise, an alignment was done with the sequences of different species, and conserved regions were utilized. The specificity of the primers was tested by submitting them to a BLAST analysis. The sequences and information on the source of the primers are listed in Table 1. For optimization and confirmation of the specificity, the housekeeping genes were amplified and cloned using liver and kidney RT reactions, which were performed using a standard procedure on 5 μg of total RNA using 200 ng of oligo(dT)$_{12-18}$ and Superscript II (Life Technologies). The oligo(dT)$_{12-18}$ was first added to the RNA samples and allowed to anneal by denaturing the secondary structures at 70°C for 2 min and then quenching the reaction rapidly on ice. The RT buffer, dithithreitol, dNTP, and 1 μl of Superscript II (200 U/μl) were added, and the reaction was performed at 42°C for 1 h, followed by a denaturing step at 70°C for 10 min.

A cDNA aliquot of 2 μl was subjected to PCR amplification using a PTC-100 Programmable Thermal Controller (MJ Research) using the following program: preincubation with *Taq* Gold polymerase (Applied Biosystems, Streetsville, ON, Canada), activation at 95°C for 10 min, and then 35 amplification cycles of denaturation at 95°C for 1 min, annealing (for every housekeeping gene) at 57°C for 1 min, and elongation at 72°C for 1 min. The last of the 35 cycles was followed by a final elongation step at 72°C for 5 min. Aliquots of the PCRs were electrophoresed to confirm the amplification, and the amplicons from successful reactions were cloned by TA cloning (Invitrogen, Burlington, ON, Canada). The true positives were manually sequenced to confirm their identity with the T7 sequencing kit (Amersham-Pharmacia, Baie D'Urfé, PQ, Canada).

Real-Time PCR

The standard curve was prepared as follows. A standard PCR was performed using the verified plasmids as template for each housekeeping gene as described above. The products were electrophoresed, and the specific band for each gene studied was extracted from the gel using Qiaquick gel extraction columns (Qiagen). The purified PCR products were quantified by running an aliquot along with a DNA mass ladder (Life Technologies) and diluted for the standard curves.

The PCRs were conducted in a Light Cycler apparatus (Roche), and products were detected with SYBR Green, which is included in the FastStart Master SYBR Green I mix (Roche). Prior to the 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). For each quantification, a 2-μl aliquot of the RT reaction was used. The standard curve was performed using the DNA prepared as described above, and five serial dilutions were used ranging from 100 pg to 1 fg. For each housekeeping gene, all the samples were quantified simultaneously during the same run. 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 housekeeping 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 genreated by starting the fluorescence acquisition at 72°C and taking measurements every 0.1°C until 95°C was reached.

After the completion of the quantitative PCR analysis, 10% of the PCR products was electrophoresed on a standard 1% agarose gel stained with ethidium bromide and visualized by exposure to ultraviolet light. The images were obtained using a gel doc apparatus (Bio Rad, Mississauga, ON, Canada).

RESULTS

Figure 1A shows the amplification profile of ubiquitin in all the samples studied. As expected, the logarithmic

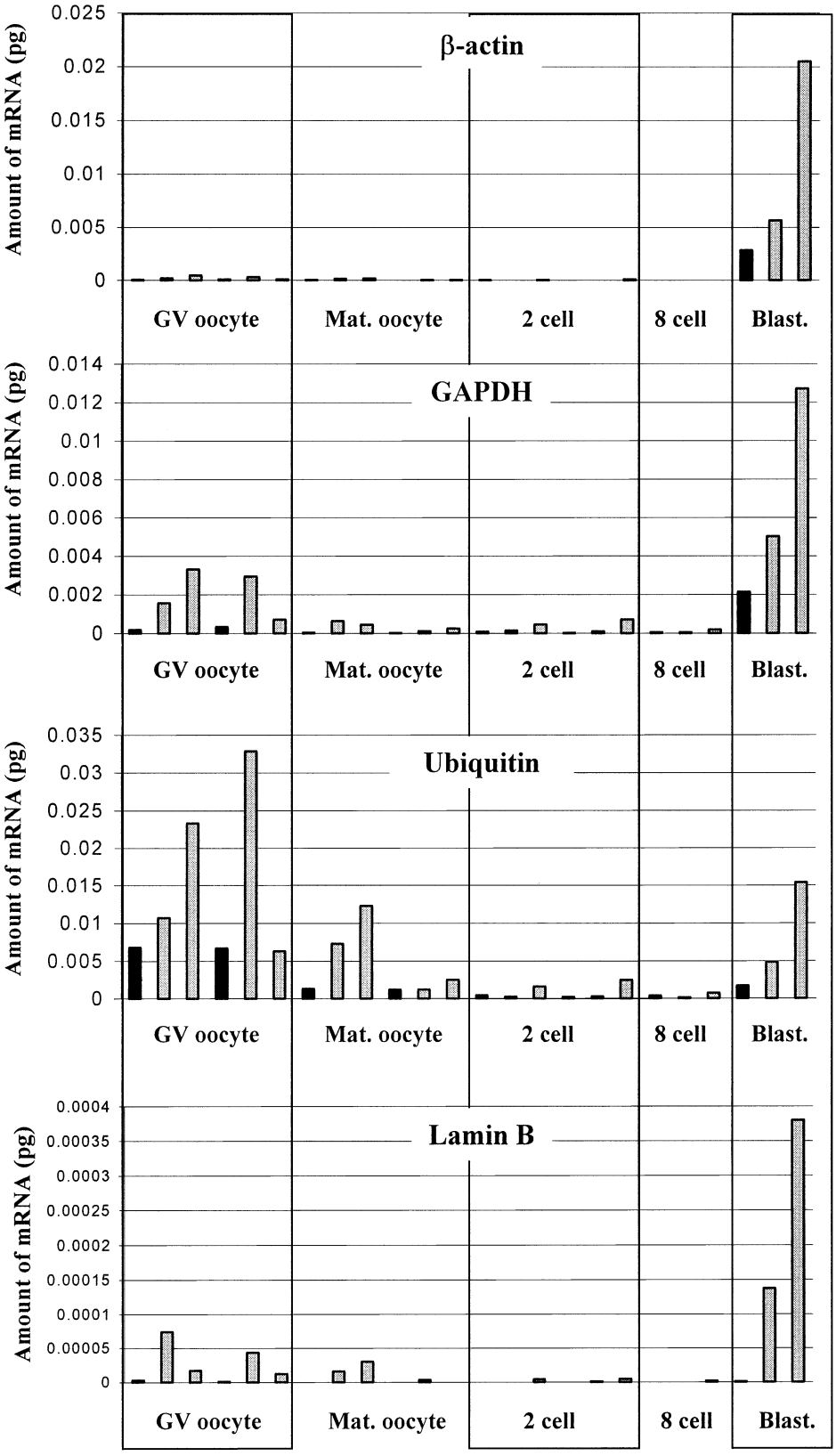

FIG. 2. Quantification of the mRNA levels of housekeeping genes during the bovine preimplantation period. For each developmental stage, some samples were directly evaluated and others were submitted to linear T7 RNA amplification prior to quantification by real-time PCR. For GV oocytes, matured oocytes, and two-cell embryos, the first three samples represented were collected from follicles <2 mm in diameter and the other three samples were collected from follicles 3–5 mm. For the eight-cell embryos and blastocysts, all the oocytes were collected from 3- to 5-mm follicles.

segment of the amplification occurs sooner when the sample is more concentrated in the targeted template. For all genes studied, the standard curve was composed of five samples of a serial dilution of a purified PCR product ranging from 100 pg to 1 fg. Even at the lowest concentration, some samples were not included in the range of the standard curve, and the amount of the targeted gene transcript was calculated by linear extrapolation. The real-time PCR is a novel technique. In addition to the quantification analysis of the amplification curves produced by the augmentation of fluorescence as the number of amplicons increases, there is a second important step that differs from regular

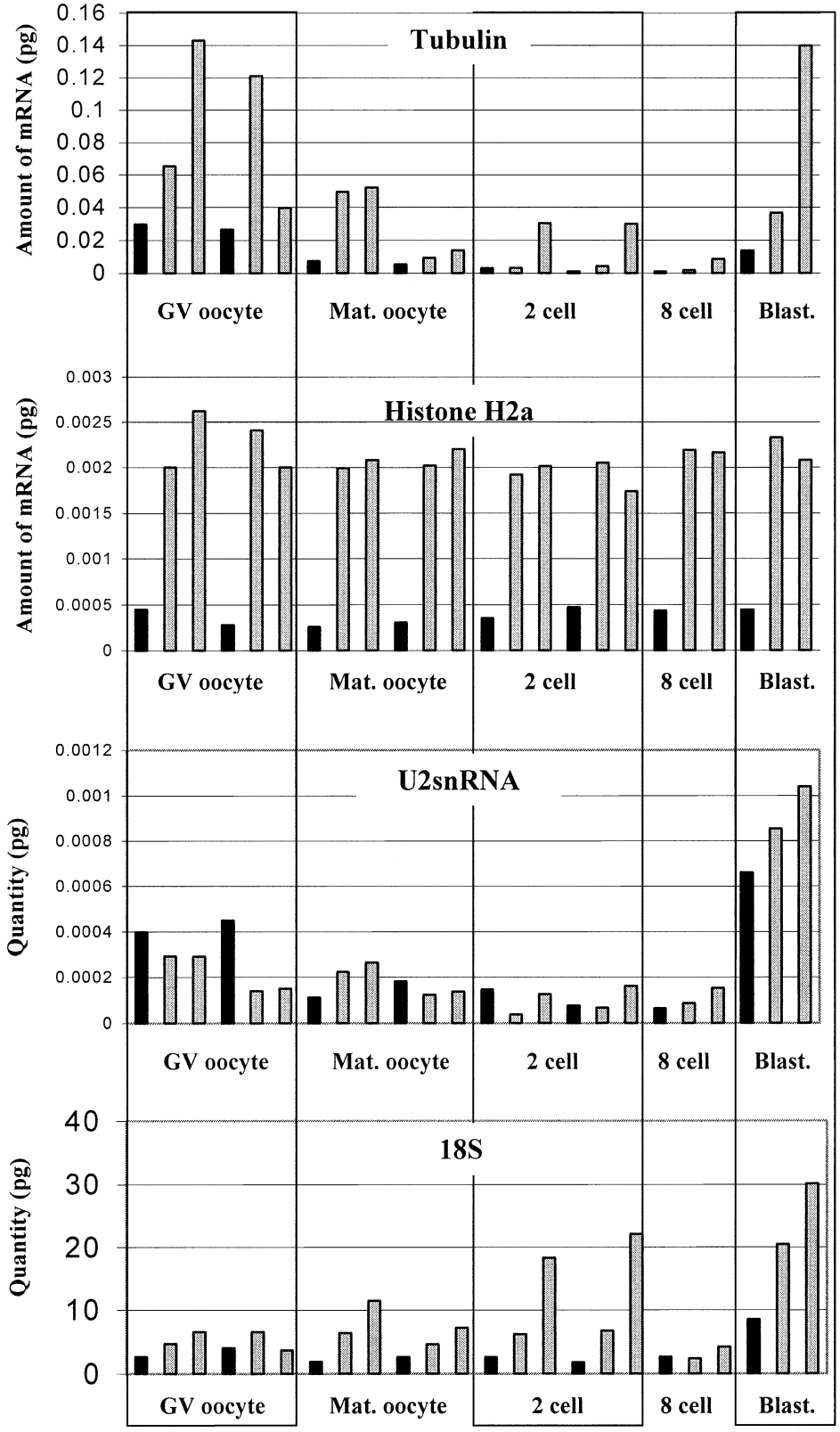

FIG. 2. Continued.

PCR, i.e., the melting curve (Fig. 1B). Because the melting temperature of the amplicon is sequence specific, a melting curve with only one peak demonstrates the specificity of the amplification. A lack of specificity would result in the presence of several peaks different from that of the confirmed sequence samples used for the standard curves. When no target template is present in the sample, the primers often dimerize, as indicated in the negative control. Two types of detection are available for real-time PCR: specific detection involves fluorescent probes that will bind the amplified product, and nonspecific detection, as we used, involves SYBR Green, which binds to all double-stranded DNAs. For this nonspecific detection, it is important to measure the fluorescence precisely before the predeter-

TABLE 2. Estimation of the amplification factor resulting from the linear amplification procedures.

Gene	Amplification factor[a]
β-Actin	20
GAPDH	20
Ubiquitin	10
Lamin B	200
Tubulin	10
Histone H2a	20
U2snRNA	5
18S rRNA	5

[a] The amplification factor was estimated by comparing the values of the unamplified sample to the values obtained after the T7 linear RNA amplification.

mined melting temperature of the specific product to avoid the additional fluorescence of the contaminating double-stranded DNA produced by primer dimers. The optimized temperature of fluorescence acquisition for all the genes studied are listed in Table 1.

The quantification of transcripts for the selected housekeeping genes revealed that there are relatively large amounts of most of these transcripts in the GV oocyte, and the levels decrease regularly as the embryo develops until it reaches the eight-cell stage. This decrease is followed by a sharp increase at the blastocyst stage. As indicated in Figure 2, β-actin, GAPDH, ubiquitin, lamin B, and tubulin mRNA levels followed this pattern of maternal RNA degradation. By contrast, U2 small nuclear RNA (U2snRNA) and 18S rRNA levels were mostly constant until the eight-cell stage, followed by an increase at the blastocyst stage. The mRNA level of histone H2a was very stable across the entire preimplantation period. For both oocyte stages and the two-cell embryos, two follicle size groups were considered because the oocytes collected from <2-mm and 3- to 5-mm follicles were processed by the in vitro procedures separately. Although no statistical analyses were performed, no clear differences between the two follicle size groups were observed, indicating that the RNA levels of these genes does not seem to be influenced by follicle size group from which the oocytes were collected. The effect of the mRNA preamplification using the T7 promotor followed by in vitro transcription prior to the quantification indicates that the profiles are conserved for all the genes studied (Fig. 2). By comparing the levels of RNA detected between the preamplified and the nonamplified samples and by adjusting this value to the differences in RT reaction final volumes, amplification factors were estimated (Table 2). The different values among the genes suggest that the T7 linear amplification is template sensitive. The U2snRNA and the 18S rRNA are not polyadenylated and therefore cannot be reverse transcribed using the oligo(dT) coupled with the T7 promoter sequence, thus impairing the linear amplification of these RNAs.

Figure 3 shows the intensity of the band patterns of the genes following the real-time PCR amplification. For β-actin, GAPDH, lamin B, tubulin, and histone H2a, the band intensity profiles throughout the preimplantation period are similar to the quantitative measurement profiles (Fig. 2). However, for ubiquitin the progressive degradation of the transcript until the eight-cell stage detected by real-time PCR was not supported by the band intensity profile (Fig. 3). As for U2snRNA, the band intensity profile suggests that the RNA level is constant throughout the preimplantation period and does not increase markedly at the blastocyst stage, as indicated in Figure 2. The levels of β-actin are very low during most of the developmental stages studied as opposed to higher levels at the blastocyst stage (Fig. 2). The β-actin transcript was detected by real-time PCR at every developmental stage studied (Fig. 3), and the levels of β-actin rose dramatically at the blastocyst stage.

DISCUSSION

The measurement of the mRNA levels of the different housekeeping genes by real-time PCR revealed profiles similar to the maternal RNA degradation pattern seen in bovine embryos. The bovine embryonic genome is transcriptionally inactive during the first cell divisions until the embryo reaches the 8- to 16-cell stage, when the major resumption of transcription occurs [37]. Therefore, maternal RNA accumulated during oocyte growth ensures protein synthesis during genomic silencing [38–40] and is gradually degraded until the resumption of embryonic transcription, explaining the sharp increase in RNA level at the blastocyst stage. Most of the mRNA profiles determined with real-time PCR were confirmed by comparing the band intensities of the electrophoresed PCR products. Thus, although the previous studies using semiquantitative reverse RT-PCR procedures [14–19] were not considered quantitative per se, the profiles observed were probably accurate. Among the housekeeping genes evaluated, only U2snRNA mRNA levels have been studied during the bovine preimplantation period [1], and those authors concluded that the transcript level was constant until the 8- to 16-cell stage, followed by an increase in RNA level at the blastocyst stage, which corroborates our results (Fig. 2). Of all the candidate genes studied, only histone H2a mRNA levels were constant across the entire preimplantation period. A suitable endogenous standard is critical to the quantification process and a definition of such standard during the preimplantation development is needed. In Northern blot analysis, the amount of mRNA of the candidate gene is normalized to the amount of a mRNA of a housekeeping gene (generally one of the major rRNA subunits) to account for differences in the amount of RNA loaded per lane. However, during the bovine preimplantation period when the total RNA content is higher in the oocyte than in the eight-cell embryo [39], this approach would result in normalizing values to those of a gene following the normal maternal RNA degradation pattern to compensate for the RNA lost. Normalization to such a gene will bias the result toward the conclusion that there is more mRNA of a candidate gene in the eight-cell embryo than in the GV oocyte if the candidate mRNA is not degraded as quickly as is that of the housekeeping gene. Would the results make more sense if they where expressed per oocyte or per embryo? If so, normalization to histone H2a mRNA levels would be appropriate; otherwise, normalization to another housekeeping gene should be considered.

The linear amplification of RNA did not result in the expected 2000-fold yield reported by Phillips and Eberwine [35]. The low amplification factors obtained for U2snRNA and 18S rRNA can be easily explained by the lack of a poly(A) tail on these RNAs, preventing the addition of the T7 promoter sequence coupled to the oligo$(dT)_{12-18}$ and thus preventing amplification during the in vitro transcription step. To reverse transcribe these RNAs, random hexamers were used to prime the reaction. We expected the poly(A) tail of the mRNA to be available for annealing with the oligo$(dT)_{12-18}$ even in the presence of the random hexamers. However, the higher quantity of hexamers outcompeted the oligo$(dT)_{12-18}$, resulting in poor aRNA amplifi-

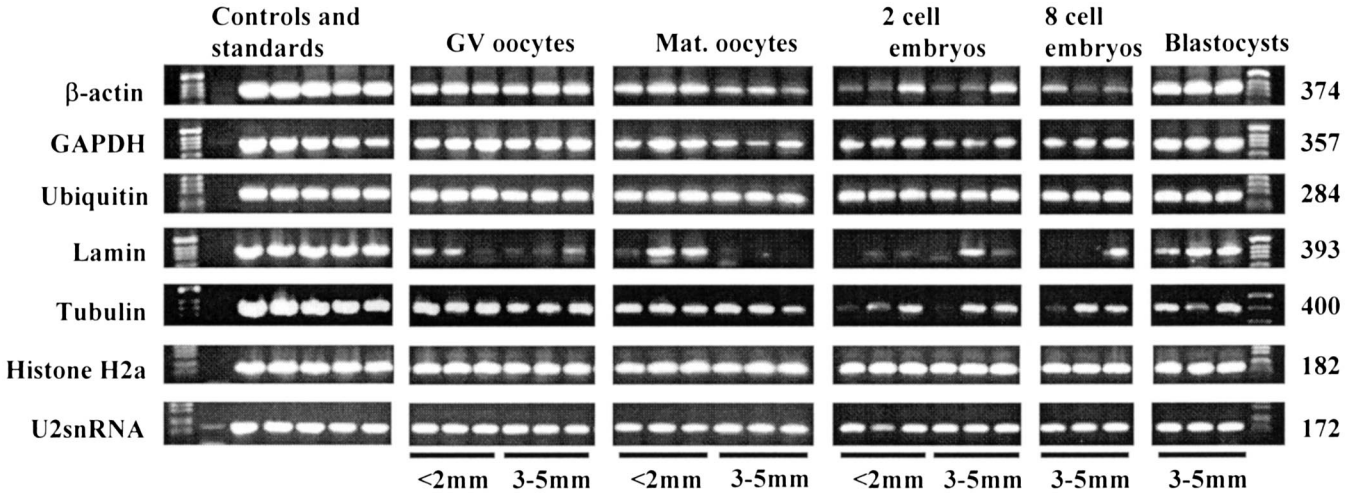

FIG. 3. Representative PCR amplification profiles of candidate housekeeping genes. The oocytes were classified according to the follicule size group (<2 mm or 3–5 mm). Three pools of 20 oocytes or embryos were used for each developmental stage. The first lane of each triplicate represents standard RT-PCR amplification, whereas the other lanes represent PCRs for amplified aRNA using the T7 linear amplification procedures.

cation. However, the results indicate that the limited amplification achieved was enough to maintain the relative abundances of the transcripts among the samples. The mRNA level profiles were the same with or without preamplification, suggesting that this approach could be used to amplify the mRNA pool to obtain a better signal with a smaller amount of starting material or to measure the expression level of more genes per sample. Thus, if comparisons are to be made across the preimplantation period for a specific gene, the use of the T7 amplification system should be reliable, whereas the differences between the amplification factors demonstrate that the relative abundance between the different genes was biased. Therefore, if comparisons of transcript levels between different genes are to be made, the T7 linear amplification process should be avoided. However, because the addition of the random hexamers seemed to negatively affect the reaction, a more extensive evaluation of the technique is needed. The maternal mRNAs stored within the oocyte cytoplasm may be deadenylated [38, 41–43]. It is not clear whether the poly(A) tail is totally removed or whether a small portion remains. In the case of complete removal of the poly A tail, these RNAs would not be reverse transcribed using an oligo(dT) primer, thus the need to use random hexamers.

To study gene expression in very small samples and ultimately to measure several candidates in a single oocyte or embryo, a preamplification step should be used to amplify the signal to reach the dynamic range of detection of the apparatus. When measurements are taken in the lower portion of the dynamic range, variations between replicates are often observed. Moreover, because there is certainly some inherent biological variation among samples, discriminating among these sources of variations adds to the complexity of finding gene expression differences associated with a phenotypic impact. Optimization of the preamplification system is therefore important.

Exogenous control mRNA (rabbit globin) added prior to the extraction step has been used to account for the variations caused by manipulation of the samples [14–19]. However, the rabbit globin mRNA commercially sold is extracted from reticulocytes and, according to the manufacturer, is not totally pure, possibly containing contaminating mRNAs other than those of the two globin chains. Therefore, the presence of the mRNA of the gene of interest in the exogenous standard should be confirmed before the standard is added to the samples. A perfect exogenous standard would be a quantified synthetic mRNA containing a poly(A) tail produced by transcription in vitro, as reported by Lequarre and collaborators [13]. However, this kind of standard would not account for the differences in the amount of starting material or RNA degradation as an endogenous control would. Although one of the major benefits of working during the preimplantation period over somatic cell work is the possibility to precisely control the amount of oocytes or embryos added to each reaction, normalization of the measured mRNA level to that of a constant housekeeping is still necessary to validate the observed differences among gene samples. In the present study, the addition of an exogenous synthetic mRNA to the samples prior to extraction would have been useful to evaluate whether the variations observed among replicates were caused by differences in the RNA extraction procedure or in the effectiveness of the amplification reactions. Because histone H2a levels were stable across the entire set of samples, these variations could be associated with intrinsic mRNA level differences.

For evaluation of candidate genes during the preimplantation period, a suitable endogenous control should be used in combination with an exogenous mRNA standard. Real-time PCR is a very efficient and precise method for evaluating the transcript level of multiple genes within small samples composed of a restricted amount of oocytes or preimplantation embryos.

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