Detection of Messenger Ribonucleic Acid Coding for Glyceraldehyde-3-Phosphate Dehydrogenase in Single Bovine Oocytes and Early Embryos

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Abstract: The reverse transcription-polymerase chain reaction (RT-PCR) method is widely used for studying mRNA expression in cells and tissues. In this study, we examined whether the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene could be used as an endogenous control for gene expression in single bovine oocytes and early embryos. First, sequencing of partial bovine G3PDH cDNA from ovarian tissue was performed. The sequence analysis of bovine G3PDH cDNA after subcloning indicated a high homology with human, mouse and rat G3PDH cDNA. Next, we examined whether G3PDH could be detected in single bovine oocytes and early embryos by using the RT-PCR method. Signals for G3PDH mRNA were detected in single immature and mature oocytes and single embryos at the one-cell to blastocyst stages. Thus, G3PDH is suitable as an endogenous control for examining mRNA expression even with single bovine oocytes or early embryos by using the RT-PCR method.

Key words: Bovine, mRNA, Glyceraldehyde-3-phosphate dehydrogenase, Oocytes, Early embryos.

The reverse transcription-polymerase chain reaction (RT-PCR) is a powerful tool for detecting the mRNA expression of genes. It is commonly used for detecting expression in mammalian gametes and early embryos, since they contain much smaller numbers of cells than other tissues or organs. Several house-keeping genes are often used as endogenous controls for RT-PCR analy-

sis. The mRNA for β -actin has been widely used as a positive control [1], but its expression varies among different tissues [2]. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) plays a central role in glycolysis and glycconeogenesis [3]. Expression of the G3PDH gene has been detected in various tissues in humans [4], mice [5], rats [6], pigs [2] and chickens [7], but there are few reports on the gene coding of bovine G3PDH (GenBank accession nos. AJ000039 and U85042). In the present study, we isolate and identify cDNA clones containing part of the coding sequence for bovine G3PDH, and examine whether mRNA expression of the gene can be used as an endogenous control for RT-PCR analysis of gene expression in bovine oocytes and early embryos.

Materials and Methods

Cloning of partial bovine G3PDH cDNA

Bovine ovaries were collected from a local slaughterhouse and stored at -80°C until RNA extraction. A piece of the tissue was then homogenized and total RNA was isolated with Trizol (Gibco BRL Life Technologies, Rockville, MD, USA). Briefly, the tissue was homogenized with 5 ml Trizol and 2 ml chloroform, then centrifuged to separate the organic and aqueous phases. RNA was precipitated by adding an identical volume of isopropanol to the aqueous phase. The RNA pellet obtained after centrifugation was washed with 75% ethanol, then dried before being dissolved in 300 μl DEPC-treated water. Residual DNA in the extracts was removed by digestion with 70 unit/ μl Dnase I (Takara Biomedicals, Tokyo, Japan). Reverse transcription was then performed

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at 30°C for 10 min, 42°C for 60 min, 99.9°C for 6 min, 5°C for 5 min and 4°C for 5 min after the addition of 2 μ l AMV Reverse Transcriptase XL (Takara), 4 μ l 5 x reaction buffer, 8 μ l dNTP mixture, 2 μ l random primer mixture (Takara) and 0.5 μ l ribonuclease inhibitor (Takara) to 1 μ l of the RNA extract. After the RT reaction, Rnase H (Takara) treatment was performed to remove the RNA. The cDNA was then amplified with human G3PDH specific primers, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. PCR amplification was performed with a mixture of 1 μ l of cDNA, 0.125 μ l EX taq polymerase (Takara), 2.5 μ l 10 $\times$ EX taq buffer (Takara), 2 μ l dNTP mixture (Takara) and 20 μ M of each primer. After an initial phase of 5 min at 94°C, 40 cycles of amplification were carried out at 94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds. A final extension step of 10 min at 72°C ended the reaction. MessengerRNA from human lymphocytes and cumulus-granulosa cells, and mouse ovarian tissues was used as a positive control. The resulting amplification products were separated by 2% agarose gel electrophoresis and then cloned into the pGEM-T Easy vector (Promega, Madison WI, USA). After analysing 6 plasmid clones with the ALF express DNA sequencer (Pharmacia Biotech AB, Uppsala, Sweden), we determined the sequence of partial bovine G3PDH cDNA. Its nucleotide and predicted amino acid sequences were compared and aligned to human (GenBank accession no. M17851), mouse (GenBank accession no. M32599), rat (GenBank accession no. M17701) and previously reported bovine (GenBank accession nos. AJ000039 and U85042) G3PDH cDNA.

Bovine oocyte collection and embryo production

Bovine *in vitro* maturation, fertilization and culture were carried out as previously described [8]. Briefly, cumulus-oocytes complexes (COCs) were collected from the small antral follicles (ϕ 5 to 10 mm) of bovine slaughterhouse ovaries. Some of the COCs were vortexed to remove the cumulus and corona cells surrounding the oocytes, and the denuded oocytes, which were regarded as being immature oocytes, were collected and stored at -80°C until RNA extraction. The remaining COCs were matured in TCM-199 (with Earle's salts and 25 mM HEPES-buffer, Gibco) supplemented with 1 mg/ml polyvinyl alcohol (cold water soluble, Sigma Chemical Co., St. Louis, MO, USA), 0.5 mM sodium pyruvate, 0.02 AU/ml follicle stimulating hormone (Antrin, Denka Seiyaku, Tokyo, Japan) and 1 μ g/ml estradiol-17 β (Sigma) for 21 hours at 39°C under 5% CO₂ in air with high humidity. Some of the matured COCs were then

vortexed to remove the cumulus and corona cells surrounding the oocytes, and the denuded oocytes with the first polar body, which were regarded as being mature oocytes, were collected and stored at -80°C until RNA extraction. The remaining COCs were fertilized with Percoll-washed bovine spermatozoa [9] in modified defined medium containing 1 μ g/ml heparin for 18 hours at 39°C under 5% CO₂ in air with high humidity [8]. Following fertilization, cumulus cells and attached spermatozoa were removed from the embryos by vortex agitation. The embryos were cultured to the blastocyst stage in modified synthetic oviduct fluid medium [10] for 168 hours at 39°C under 5% CO₂, 5% O₂ and 90% N₂ with high humidity [11]. Single embryos were collected at 24, 48, 72, 96, 120, 144 and 168 hours post insemination (hpi), and then stored at -80°C until RNA extraction. The developmental stages of embryos at each hpi were 1-cell, 2-cell, 8-cell, 8- to 16-cell, 16- to 32-cell, morula and blastocyst stages, respectively.

RT-PCR analysis in bovine oocytes/embryos

Messenger RNA extracted from bovine oocytes and embryos was reverse-transcribed and then the cDNA was amplified by means of a Superscript One-step PCR (Gibco) according to the manufacturer's protocol. PCR for bovine G3PDH was performed with the same primers as described previously. To confirm that the RNA extract did not contain residual genomic DNA, the PCR of the extracts was done without the RT reaction. Both RT-PCR and PCR

Fig. 1. Detection of G3PDH mRNA in bovine ovarian tissue. M1, molecular marker (ϕ X174-Hae III digest); P, positive control (control cDNA); N, negative control (distilled water); HL, human lymphocytes; HG, human granulosa cells; MO, mouse ovarian tissue; BO, bovine ovarian tissue; M2, molecular marker (100 bp DNA ladder).

amplification products were separated by 2% agarose gel electrophoresis. Experiments were repeated three times.

Results

Sequence of partial bovine G3PDH cDNA

Agarose gel electrophoresis of RT-PCR products from

bovine ovarian tissue total RNA displayed positive signals of similar size (approximately 450 bp) to those of human lymphocytes and cumulus-granulosa cells, and mouse ovarian tissues (Fig. 1). As shown in Figs. 2 and 3, the partial cDNA was 452 bp and encoded 150 amino acids. The 452 bp of the non-primer sequence were 83.2% identical and the corresponding 150 amino

bovine G3PDH	1	accacagtcc	10	atgccatcac	20	tgccacccag	30	aagactgtgg	40	atggcccctc	50
human G3PDH	1	accacagtcc		atgccatcac		tgccacccag		aagactgtgg		atggcccctc	50
mouse G3PDH	1	accacagtcc		atgccatcac		tgccacccag		aagactgtgg		atggcccctc	50
rat G3PDH	1	accacagtcc		atgccatcac		tgccactcag		aagactgtgg		atggcccctc	50
bovine G3PDH	51	cgggaaagctg	60	tgccgtgacg	70	gccgaggggc	80	tgcccagaat	90	atcatccctg	100
human G3PDH	51	cgggaaagctg		tgccgtgacg		gccgaggggc		tgcccagaat		atcatccctg	100
mouse G3PDH	51	tggaagagctg		tgccgtgacg		gccgtggggc		tgcccagaac		atcatccctg	100
rat G3PDH	51	tggaagagctg		tgccgtgacg		gccgtggggc		agcccagaac		atcatccctg	100
bovine G3PDH	101	cttctactgg	110	cgctgccaaag	120	gccgtggggc	130	aggtcatccc	140	tgagctcaac	150
human G3PDH	101	cctctactgg		cgctgccaaag		gctgtggggc		aggtcatccc		tgagctagac	150
mouse G3PDH	101	catccactgg		tgctgccaaag		gctgtggggc		aggtcatccc		agagctgaac	150
rat G3PDH	101	catccactgg		tgctgccaaag		gctgtggggc		aggtcatccc		agagctgaac	150
bovine G3PDH	151	gggaagctca	160	ctggcatggc	170	cttccgcgtc	180	cccactccca	190	acgtgtctgt	200
human G3PDH	151	gggaagctca		ctggcatggc		cttccgcgtc		cccactccca		acgtgtcagt	200
mouse G3PDH	151	gggaagctca		ctggcatggc		cttccgcgtt		cctaccacca		atgtgtccgt	200
rat G3PDH	151	gggaagctca		ctggcatggc		cttccgcgtt		cctaccacca		atgtatccgt	200
bovine G3PDH	201	tgtggatctg	210	acctgccgcc	220	tgagagaaacc	230	tgccaagtat	240	gatgagatca	250
human G3PDH	201	ggtggacctg		acctgccgct		tagaaaaacc		tgccaaatat		gatgacatca	250
mouse G3PDH	201	cgtggatctg		acgtgccgcc		tgagagaaacc		tgccaagtat		gatgacatca	250
rat G3PDH	201	tgtggatctg		acatgccgcc		tgagagaaacc		tgccaagtat		gatgacatca	250
bovine G3PDH	251	agaaggtggt	260	gaagcaggcg	270	tcagagggcc	280	ctctcaaggg	290	cattctaggg	300
human G3PDH	251	agaaggtggt		gaagcaggcg		tcggagggcc		ccctcaagg		catcctgggc	300
mouse G3PDH	251	agaaggtggt		gaagcaggcg		tctgagggcc		cactgaaggg		catcttgggc	300
rat G3PDH	251	agaaggtggt		gaagcaggcg		gccgagggcc		cactaaaggg		catcctgggc	300
bovine G3PDH	301	tacactgagg	310	accaggttgt	320	ctcctgcgac	330	ttcaacagcg	340	acactcactc	350
human G3PDH	301	tacactgagg		accaggttgt		ctcctctgac		ttcaacagcg		acaccactc	350
mouse G3PDH	301	tacactgagg		accaggttgt		ctcctgcgac		ttcaacagca		actcccactc	350
rat G3PDH	301	tacactgagg		accaggttgt		ctcctgtgac		ttcaacagca		actcccattc	350
bovine G3PDH	351	ttctaccttc	360	gatgctgggg	370	ctggcattgc	380	cctcaacgac	390	cactttgtca	400
human G3PDH	351	ctccaccttc		gacgctgggg		ctggcattgc		cctcaacgac		cactttgtca	400
mouse G3PDH	351	ttccaccttc		gatgccgggg		ctggcattgc		tctcaatgac		aactttgtca	400
rat G3PDH	351	ctccaccttc		gatgctgggg		ctggcattgc		tctcaatgac		aactttgtga	400
bovine G3PDH	401	agctcatttc	410	ctggtacgac	420	aatgaatttg	430	gctacagcaa	440	cagggtggtg	450
human G3PDH	401	agctcatttc		ctggtatgac		aacgaatttg		gctacagcaa		cagggtggtg	450
mouse G3PDH	401	agctcatttc		ctggtatgac		aatgaataacg		gctacagcaa		cagggtggtg	450
rat G3PDH	401	agctcatttc		ctggtatgac		aatgaatatg		gctacagcaa		cagggtggtg	450
bovine G3PDH	451	ga.....	460		470		480		490		500
human G3PDH	451	ga.....									500
mouse G3PDH	451	ga.....									500
rat G3PDH	451	ga.....									500

Fig. 2. The nucleotide sequence of partial G3PDH cDNA from bovine ovarian tissue. The nucleotide sequence of RT-PCR products from bovine ovarian tissue was compared with nucleotide sequences of humans, mice and rats by using DNASIS software. The homology was 83.2%.

			10	20	30	40	50	
bovine G3PDH	1	TTVHAITATQ	KTVDGPGSKL	WRDGRGAAQN	IIPASTGAAK	AVGKVIPELN	50	
human G3PDH	1	TTVHAITATQ	KTVDGPGSKL	WRDGRGALQN	IIPASTGAAK	AVGKVIPELD	50	
mouse G3PDH	1	TTVHAITATQ	KTVDGPGSKL	WRDGRGAAQN	IIPASTGAAK	AVGKVIPELN	50	
rat G3PDH	1	TTVHAITATQ	KTVDGPGSKL	WRDGRGAAQN	IIPASTGAAK	AVGKVIPELN	50	
			60	70	80	90	100	
bovine G3PDH	51	GKLTGMAFRV	PTPNVSVVDL	TCRLEKPAKY	DEIKKVVKQA	SEGPLKGILG	100	
human G3PDH	51	GKLTGMAFRV	PTANVSVVDL	TCRLEKPAKY	DDIKKVVKQA	SEGPLKGILG	100	
mouse G3PDH	51	GKLTGMAFRV	PTPNVSVVDL	TCRLEKPAKY	DDIKKVVKQA	SEGPLKGILG	100	
rat G3PDH	51	GKLTGMAFRV	PTPNVSVVDL	TCRLEKPAKY	DDIKKVVKQA	AEGPLKGILG	100	
			110	120	130	140	150	
bovine G3PDH	101	YTEDQVVSCD	FNSDTHSSTF	DAGAGIALND	HFVKLISWYD	NEFGYSNRVV	150	
human G3PDH	101	YTEHQVVSSD	FNSDTHSSTF	DAGAGIALND	HFVKLISWYD	NEFGYSNRVV	150	
mouse G3PDH	101	YTEDQVVSCD	FNSNSHSSTF	DAGAGIALND	NFVKLISWYD	NEYGYSNRVV	150	
rat G3PDH	101	YTEDQVVSCD	FNSNSHSSTF	DAGAGIALND	NFVKLISWYD	NEYGYSNRVV	150	

Fig. 3. Predicted amino acid sequence of partial G3PDH cDNA from bovine ovarian tissue. The predicted amino acid sequence of RT-PCR products from bovine ovarian tissue was compared with amino acid sequences of humans, mice and rats by using DNASIS software. The homology was 94.4%.

Fig. 4. Detection of G3PDH mRNA in single bovine oocytes. M1, molecular marker (ϕ X174); P, positive control (bovine ovarian tissue); N, negative control (distilled water); PCRw/oRT- 1, 40, PCR products made without the RT reaction from single and 40 oocytes, respectively; RT-PCR- 1, 40, RT-PCR products from single and 40 oocytes, respectively; M2, molecular marker (100 bp DNA ladder). G3PDH mRNA was detected in both single and 40 oocytes.

acids were 94.4% identical with human (GenBank accession no. M17851), mouse (GenBank accession no. M32599) and rat (GenBank accession no. M17701) sequences. When the nucleotide sequence was compared to the other bovine sequences for G3PDH (GenBank accession nos. AJ000039 and U85042), a novel 7 nucleotide sequence was found. Therefore, based on the evidence, the cDNA fragments isolated in this study are partial cDNA coding bovine G3PDH.

Expression of G3PDH mRNA in bovine oocytes and early embryos

RT-PCR analysis with mRNA from bovine oocytes was performed to confirm G3PDH gene expression in single oocytes (Fig. 4). This indicated that it is possible to detect the gene expression of G3PDH in a single cell.

In the next experiments, expression of the G3PDH gene was evaluated in single bovine cells during oocyte maturation and embryo development. G3PDH messages were expressed in all stages of oocytes and embryos (Fig. 5). The G3PDH expression was, however, substantially weaker in embryos at 72 and 96 hpi, although it is difficult to precisely quantify the amount of RNA by RT-PCR. This observation suggests that transcriptional activity decreases between 72 and 96 hpi in early bovine embryogenesis.

Discussion

RT-PCR analysis is commonly used for detection of gene expression in mammalian oocytes or early embryos. When using the RT-PCR method the abundance of mRNA and its expression at all stages of development of oocytes/embryos is an important criterion in the selection of an endogenous control for gene expression.

In the first experiment we isolated and identified G3PDH cDNA clones from bovine ovarian tissue. Our results also indicated that the cDNA fragment is highly conserved in cattle, mice, rats and humans (Figs. 2 to 3). The high homology of the sequences may be due to the nature of G3PDH. In glycolysis, G3PDH reversibly catalyzes the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate [7]. G3PDH is a tetrameric enzyme composed of chemically

Fig. 5. Detection of G3PDH mRNA in single bovine oocytes and early embryos. M1, molecular marker (ϕ X174); P, positive control (bovine ovarian tissue); N, negative control (distilled water); IO, an immature oocyte; MO, a mature oocyte; Hours post insemination- 24 to 168, an embryo at 24, 48, 72, 96, 120, 144 and 168 hpi, respectively; M2, molecular marker (100 bp DNA ladder). G3PDH mRNA was detected in all single oocytes and embryos.

identical subunits and the amino acid sequence of the subunit has been determined in many kinds of animals and bacteria [3].

Many studies have been done on gene expression in oocytes and/or early embryos with RT-PCR [12–15], but usually several tens or hundreds of oocytes and/or embryos are used for extraction of total RNA. Recently it has been reported that RNA can be extracted from single bovine oocytes or embryos [16]. Our results indicate that mRNA including the G3PDH gene can be extracted from a single bovine oocyte (Fig. 4). This may be because our RNA extraction method is highly efficient and G3PDH mRNA is abundant in bovine oocytes. It is clear that there was no contamination of genomic DNA coding G3PDH in the mRNA extracts from oocytes in this study because no PCR product signals were detected when the reaction was done without the RT reaction (Fig. 4).

G3PDH mRNA was detected in immature and mature bovine oocytes and early embryos at the one-cell to blastocyst stages by RT-PCR analysis in this study (Fig. 5), but relatively weak signals were detected with embryos at 72 and 96 hpi. During the 8- to 16-cell stage in bovine embryos there is a major onset of embryonic gene transcription [18]; the main metabolic pathway after the 8- to

16-cell stage is glycolysis [17]. Because the embryo samples in this study at 72 and 96 hpi were at the 8- to 16-cell developmental stage, the weak signals may be due to the relatively low transcription activity of the G3PDH gene during this developmental stage.

In conclusion, G3PDH is suitable as an endogenous control for examining gene expression even with single bovine oocytes or early embryos by RT-PCR analysis.

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