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 analysis. 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 glyconeogenesis [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.

Received: April 26, 1999
Accepted: May 14, 1999
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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

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 μl AMV Reverse Transcriptase XL (Takara), 4 μl 5 x reaction buffer, 8 μl dNTP mixture, 2 μl random primer mixture (Takara) and 0.5 μl ribonuclease inhibitor (Takara) to 1 μl 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 μl of cDNA, 0.125 μl EX taq polymerase (Takara), 2.5 μl 10 × EX taq buffer (Takara), 2 μl dNTP mixture (Takara) and 20 μM 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 (ø 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 remainding 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 μg/ml estradiol-17β (Sigma) for 21 hours at 39°C under 5% CO_2 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 remainding COCs were fertilized with Percoll-washed bovine spermatozoa [9] in modified defined medium containing 1 μg/ml heparin for 18 hours at 39°C under 5% CO_2 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% CO_2, 5% O_2 and 90% N_2 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

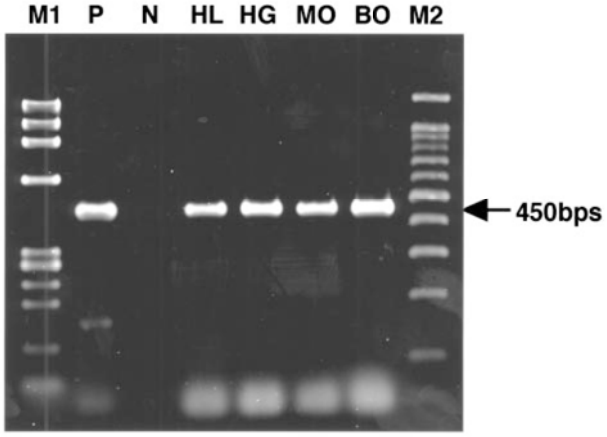

Fig. 1. Detection of G3PDH mRNA in bovine ovarian tissue. M1, molecular marker (øX174-Hae III digest); P, positive control (control cDNA); N, negative control (distilled water); HL, human lymphocytes; HG, human granulosa cells; MO, mouse ovarian tissue; BO, bovine ovarian tissue; M2, molecular marker (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

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                          10         20         30         40         50
bovine G3PDH     1  accacagtcc atgccatcac tgccacccag aagactgtgg 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

                          60         70         80         90        100
bovine G3PDH    51  cgggaagctg tggcgtgacg gccgaggggc tgcccagaat atcatccctg 100
human  G3PDH    51  cgggaaactg tggcgtgatg gccgcggggc tctccagaac atcatccctg 100
mouse  G3PDH    51  tggaaagctg tggcgtgatg gccgtggggc tgcccagaac atcatccctg 100
rat    G3PDH    51  tggaaagctg tggcgtgatg gccgtggggc agcccagaac atcatccctg 100

                         110        120        130        140        150
bovine G3PDH   101  cttctactgg cgctgccaag gccgtgggca aggtcatccc tgagctcaac 150
human  G3PDH   101  cctctactgg cgctgccaag gctgtgggca aggtcatccc tgagctagac 150
mouse  G3PDH   101  catccactgg tgctgccaag gctgtgggca aggtcatccc agagctgaac 150
rat    G3PDH   101  catccactgg tgctgccaag gctgtgggca aggtcatccc agagctgaac 150

                         160        170        180        190        200
bovine G3PDH   151  gggaagctca ctggcatggc cttccgcgtc cccactccca acgtgtctgt 200
human  G3PDH   151  gggaagctca ctggcatggc cttccgtgtc cccactgcca acgtgtcagt 200
mouse  G3PDH   151  gggaagctca ctggcatggc cttccgtgtt cctaccccca atgtgtccgt 200
rat    G3PDH   151  gggaagctca ctggcatggc cttccgtgtt cctaccccca atgtatccgt 200

                         210        220        230        240        250
bovine G3PDH   201  tgtggatctg acctgccgcc tggagaaacc tgccaagtat gatgagatca 250
human  G3PDH   201  ggtggacctg acctgccgtc tagaaaaacc tgccaaatat gatgacatca 250
mouse  G3PDH   201  cgtggatctg acgtgccgcc tggagaaacc tgccaagtat gatgacatca 250
rat    G3PDH   201  tgtggatctg acatgccgcc tggagaaacc tgccaagtat gatgacatca 250

                         260        270        280        290        300
bovine G3PDH   251  agaaggtggt gaagcaggcg tcagagggcc ctctcaaggg cattctaggc 300
human  G3PDH   251  agaaggtggt gaagcaggcg tcggagggcc ccctcaaagg catcctgggc 300
mouse  G3PDH   251  agaaggtggt gaagcaggca tctgagggcc cactgaaggg catcttgggc 300
rat    G3PDH   251  agaaggtggt gaagcaggcg gccgagggcc cactaaaggg catcctgggc 300

                         310        320        330        340        350
bovine G3PDH   301  tacactgagg accaggttgt ctcctgcgac ttcaacagcg acactcactc 350
human  G3PDH   301  tacactgagc accaggtggt ctcctctgac ttcaacagcg acacccactc 350
mouse  G3PDH   301  tacactgagg accaggttgt ctcctgcgac ttcaacagca actcccactc 350
rat    G3PDH   301  tacactgagg accaggttgt ctcctgtgac ttcaacagca actcccattc 350

                         360        370        380        390        400
bovine G3PDH   351  ttctaccttc gatgctgggg ctggcattgc cctcaacgac cactttgtca 400
human  G3PDH   351  ctccaccttt gacgctgggg ctggcattgc cctcaacgac cactttgtca 400
mouse  G3PDH   351  ttccaccttc gatgccgggg ctggcattgc tctcaatgac aactttgtca 400
rat    G3PDH   351  ctccaccttt gatgctgggg ctggcattgc tctcaatgac aactttgtga 400

                         410        420        430        440        450
bovine G3PDH   401  agctcatttc ctggtacgac aatgaatttg gctacagcaa cagggtggtg 450
human  G3PDH   401  agctcatttc ctggtatgac aacgaatttg gctacagcaa cagggtggtg 450
mouse  G3PDH   401  agctcatttc ctggtatgac aatgaatacg gctacagcaa cagggtggtg 450
rat    G3PDH   401  agctcatttc ctggtatgac aatgaatatg gctacagcaa cagggtggtg 450

                         460        470        480        490        500
bovine G3PDH   451  ga........ .......... .......... .......... .......... 500
human  G3PDH   451  ga........ .......... .......... .......... .......... 500
mouse  G3PDH   451  ga........ .......... .......... .......... .......... 500
rat    G3PDH   451  ga........ .......... .......... .......... .......... 500
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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%.

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                                  10          20          30          40          50
bovine G3PDH    1   TTVHAITATQ  KTVDGPSGKL  WRDGRGAAQN  IIPASTGAAK  AVGKVIPELN   50
human  G3PDH    1   TTVHAITATQ  KTVDGPSGKL  WRDGRGALQN  IIPASTGAAK  AVGKVIPELD   50
mouse  G3PDH    1   TTVHAITATQ  KTVDGPSGKL  WRDGRGAAQN  IIPASTGAAK  AVGKVIPELN   50
rat    G3PDH    1   TTVHAITATQ  KTVDGPSGKL  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
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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%.

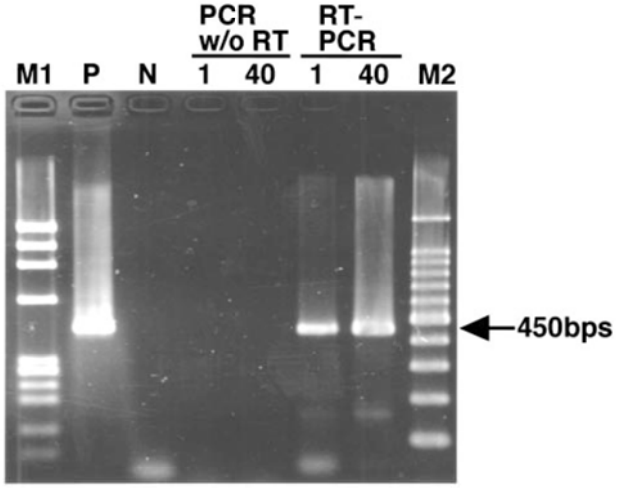

Fig. 4. Detection of G3PDH mRNA in single bovine oocytes. M1, molecular marker (øX174); P, positive control (bovine ovarian tissue); N, negative control (distilled water); PCRw/oRT- 1, 40, PCR products made without the RT reaction from single and 40 oocytes, respectively; RT-PCR- 1, 40, RT-PCR products 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

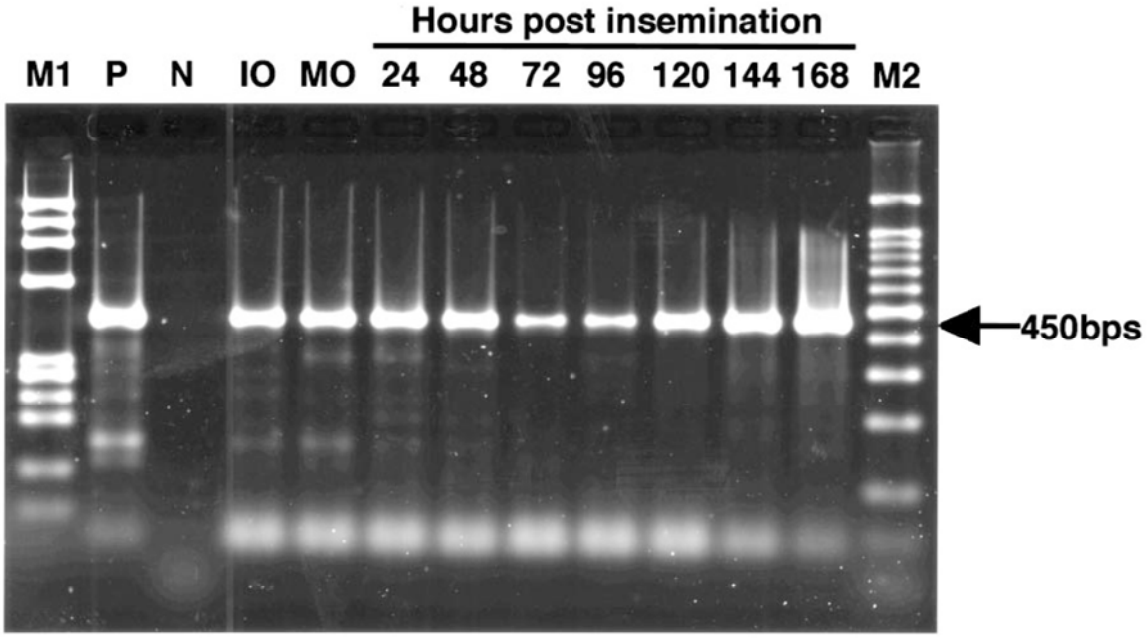

Fig. 5. Detection of G3PDH mRNA in single bovine oocytes and early embryos. M1, molecular marker (øX174); P, positive control (bovine ovarian tissue); N, negative control (distilled water); IO, an immature oocyte; MO, a mature oocyte; Hours post insemination- 24 to 168, an embryo at 24, 48, 72, 96, 120, 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.

Acknowledgments

We thank the staff of the Meat Inspection Office in Osaka City for providing the ovaries in this study. This study was supported in part by Molecular Bioengineering of Food Animal Protein Resources, Research for the Future Program, Japan Society for the Promotion of Science and by a Grant-in-Aid for Scientific Research (No. 10298937) from Japan's Ministry of Educatuion, Science and Culture.

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