Reproduction (2001) **121**, 455–461

Research

Expression of mRNA for the LH and FSH receptors in mouse oocytes and preimplantation embryos

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The gonadotrophins LH and FSH are known to regulate gonadal growth, and differentiation, endocrine function and gametogenesis. The LH receptor is expressed in ovarian theca, granulosa and luteal cells, and in testicular Leydig cells. The FSH receptor is expressed only in ovarian granulosa cells and in testicular Sertoli cells. The expression of the FSH and LH receptors was analysed by RT–PCR to study the role of these receptors in early mouse development. After reverse transcription, strategically designed nested primers were used for amplification from cDNA. Transcripts for the receptors were present in mouse oocytes and preimplantation embryos. The presence of mRNA for FSH and LH receptors in oocytes, zygotes and preimplantation embryos indicates a potential role for the gonadotrophins in the modulation of meiotic resumption and completion of oocyte maturation, as well as a beneficial effect on early embryonic development in mice.

Introduction

The pituitary gonadotrophin hormones, LH and FSH, are structurally related glycoproteins that are required for the normal development and function of the gonads (Gharib *et al.*, 1990). The cDNAs encoding LH and FSH receptors have been cloned from several species and there is considerable similarity in the amino acid sequences and the structural organization of the receptors (Minegishi *et al.*, 1990; Sprengel *et al.*, 1990; Gudermann *et al.*, 1992). The gonadotrophin receptors belong to the large family of G protein-coupled receptors, which traverse the plasma membrane with seven helical domains and have an extracellular amino terminus and an intracellular carboxyl terminus (McFarland *et al.*, 1989). In most mammalian species, the gene encoding the mRNA for the LH receptor consists of 11 exons, and that encoding the mRNA for the FSH receptor consists of ten exons. In both cases, the last exon encodes the entire transmembrane and intracellular domain of the receptor (Kelton *et al.*, 1992; Gromoll *et al.*, 1996).

LH and FSH receptors are responsible for the transduction of the biological actions of LH and FSH to their target cells, using cAMP as the main, although not the only, intracellular second messenger (Segaloff and Ascoli, 1993). It has been reported that the block in *in vitro* embryo development caused by several nucleotides, such as hypoxanthine, can be reversed by the presence of compounds, such as LH and FSH, which lead to an increase in intracellular cAMP (Loutradis *et al.*, 1987, 1994; Fissore *et al.*, 1992). It is well established that LH elicits ovarian actions by increasing the concentration of cAMP. cAMP is a key molecule in the regulation of oocyte maturation and there is evidence for its inhibitory and stimulatory actions (Tsafriri *et al.,* 1972). In the preovulatory follicle, cAMP concentrations are increased in response to LH, which affects the cumulus cells and interrupts communication in the cumulus–oocyte complex. As a result of this change, the flow of cAMP to the oocyte decreases, inhibition is relieved and meiosis is resumed (Dekel, 1988). Spontaneous maturation of cultured oocytes is inhibited by cAMP, FSH and other agents (Schultz *et al.*, 1983). These agents increase the concentration of cAMP in cumulus cells and in the oocyte. *In vivo* maturation of oocytes is initiated by the LH surge, which results in an increase in the concentration of cAMP in the cytoplasm of follicular cells, thus stimulating production of steroid hormones (Suzuki *et al.*, 1988).

In the present study, the expression of mRNA for the FSH and LH receptors was examined in mouse oocytes and preimplantation embryos at various stages of development using RT–PCR to investigate the role of these receptors in early development.

Materials and Methods

Collection of mouse oocytes and preimplantation embryos

Mouse oocytes and preimplantation embryos were obtained from 6–10-week-old female mice (New Zealand Black (NZB) × New Zealand White (NZW), Pasteur Institute, Athens) that had been induced to superovulate with i.p. injections of 5 iu equine chorionic gonadotrophin

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1470-1626/2001

(eCG, Sigma, St Louis, MO) followed by administration of 5 iu hCG (Sigma) 48 h later.

Female mice were killed by cervical dislocation, and ovaries were removed and washed in Dulbecco's PBS (Gibco BRL, Gaithersburg). Oocytes were collected by ovarian puncture in Dulbecco's PBS containing 4 mg BSA ml^{-1} (Sigma) 15–17 h after hCG administration. Cumulus cells were removed by gentle pipeting after 2–3 min of incubation with 60 U hyaluronidase ml^{-1} in Dulbecco's PBS with BSA. Oocytes were examined under a dissecting microscope to ensure complete removal of cumulus cells. Cumulus-free oocytes were washed twice with fresh medium to remove hyaluronidase and were transferred to culture dishes.

Female mice were mated with male mice of the same strain and age. A single male was placed with two females overnight. Mating was detected by the presence of a vaginal plug and mice were killed by cervical dislocation 15–17 h after hCG administration. Oviducts were removed and washed in Dulbecco's PBS. Zygotes were isolated from the oviducts by puncture. Two-cell embryos were flushed from the oviducts 34–40 h after hCG administration. Four-cell embryos were obtained after culture of zygotes for 42–46 h in modified Ham's culture medium without hypoxanthine (Loutradis *et al.,* 1994) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Morulae and blastocysts were obtained after culture of zygotes for 70 and 96 h, respectively. All embryos were rinsed several times in Dulbecco's PBS with BSA before mRNA was isolated.

Preparation of RNA from mouse tissue

Ovaries were removed from female mice, and total RNA was prepared using TRIzol (Gibco, BRL). The total RNA was used as a positive control for specific FSH receptor and LH receptor primer pairs. RNA was also isolated from cumulus cells surrounding oocytes, zygotes, two-cell embryos and from oviduct flushings after removal of zygotes. RNA (0.2 μg) was added to the reverse transcription reaction to synthesize cDNA.

RT–PCR analysis

Groups of oocytes and embryos at the same stage of development were examined by RT followed by two rounds of nested PCR for LH receptor and FSH receptor mRNAs, and by one round of PCR for β-actin (internal standard) mRNA. Sequences of cDNA clones for the mRNAs have been published (LH receptor: O'Shaughnessy *et al.,* 1994; FSH receptor: Gudermann *et al.*, 1992). The primer sequences used for the first PCR of LH and FSH receptors were described by O'Shaughnessy *et al.* (1997). Nested primer pairs for increased sensitivity were also designed. Inner pairs for the second PCR were constructed with the help of the Primer 3 program (Rozen and Skaletsy, 1996, 1997).

Oligonucleotide primers were designed to cross intron and exon boundaries for discrimination of products from genomic DNA and cDNA. The approximate positions of the primer pairs used for both receptors are shown (Fig. 1). The oligonucleotide primers used in the present study for amplification of β-actin mRNA were described by Roelen *et al.* (1997). All primers were produced to order by MWG Biotech (High Point, NC). As a negative control for specific primers, a defined volume of culture medium in which the embryos were cultured was processed in parallel and was subjected to the same RT–PCR reaction. The primer cDNA sequences and the sizes of the amplified products are listed (Table 1).

The expression of cytochrome P450 aromatase was examined by RT–PCR to exclude contamination of oocytes, zygotes and embryos by cumulus cells or other cell fragments. In mice, cytochrome P450 aromatoase is expressed in cumulus cells, but not in oocytes or embryos (Hickey *et al.*, 1988; Ishimura *et al.*, 1989; Whitelaw *et al.*, 1992). The forward and reverse specific primers used for amplification of P450 aromatase were 5′-TCAATACCAGGTCCTGGCTA-3′ and 5′-GTATGCACTGATTCACGTTC-3′ (Terashima *et al.*, 1991; Greco and Payne, 1994). P450 aromatase RT–PCR was performed as described by Greco and Payne (1994); the specific primers give rise to a fragment of 760 bp. In addition,

Table 1. Oligonucleotide primers used for RT–PCR assays

mRNA	PCR primer pair	Primers 5′–3′	Sequence of oligonucleotide	Annealing temperature (°C)	Product size (bp)	Restriction enzyme and product size (bp)
FSH receptor	Outer pair	5′-end	GGGCTGGAGTCCATTCAGACG	55		
		3′-end	CAGTTTATAACGACTGGTCAG	55	318	
	Inner pair	5′-end	AATTCACCAGCCTACTGGTTG	55		
		3′-end	CAGTTTATAACGACTGGTCAG	55	175	HincIII-126/49
LH receptor	Outer pair	5′-end	AATACACAACTGTGCATTCAAC	55		
		3′-end	ATTTGGATGAAGTTCAGAGGTT	55	451	
	Inner pair	5′-end	TTTGGAAGAATTGCCTGATGAT	55		
		3′-end	CATGACAAACTTGTCTAGACTA	55	313	DdeI-185/128
β-actin		5′-end	TGAACCCTAAGGCCAACCGTC	56		
		3′-end	GCTCATAGCTCTTCTCCAGGG	56	396	

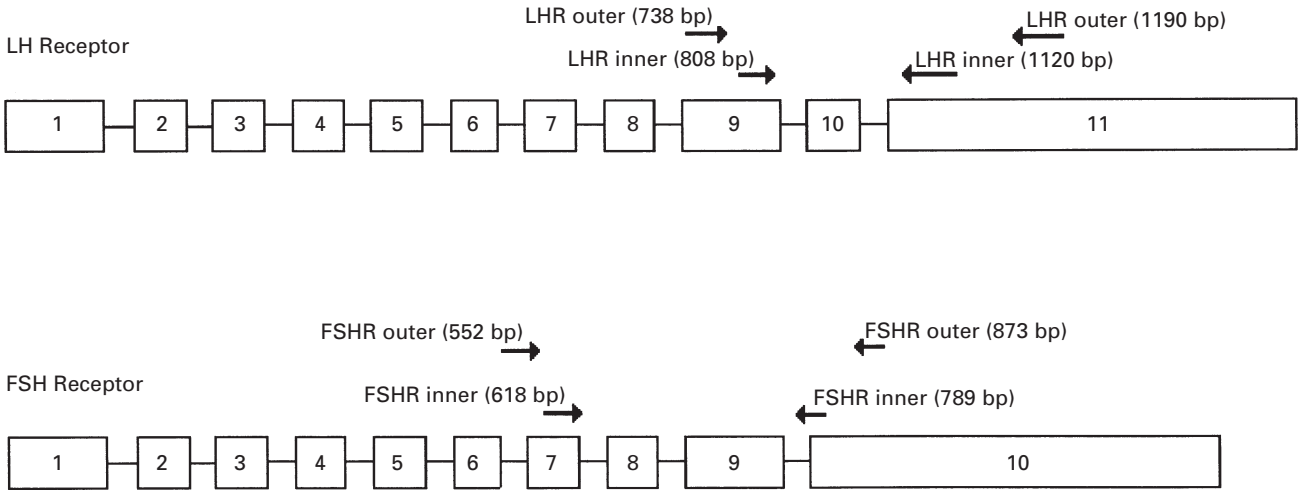

Fig. 1. Gene structure of LH receptor (LHR) and FSH receptor (FSHR) showing the approximate position of PCR primers. The exons are indicated in numbered boxes.

RT–PCR analysis with specific LH and FSH receptor primers was performed on mRNA isolated from oviductal fluids containing cumulus cells and various cell fragments, after collection of zygotes.

Groups of 10–15 oocytes or embryos at the same stage in 10 μl Dulbecco's PBS were placed in a 0.5 ml thin walled PCR tube. Oocytes and embryos were lysed by addition of 10 μl lysis buffer (0.5% (v/v) NP40, 10 mmol Tris l^{-1} (pH 8.0), 10 mmol NaCl l^{-1} and 3 mmol $MgCl_2$ l^{-1}). Medium (10 μl) from each embryo collection was processed in parallel as a negative control. The tubes were centrifuged at 12 000 ***g*** for 1–2 min and 5 μl of the supernatant containing crude RNA was used for cDNA synthesis in the presence (RT+) and absence (RT–) of reverse transcriptase. RNA samples were denatured at 70°C for 5 min and cooled to 30°C. DNA was degraded by incubating the samples with 1 U DNase I (Gibco, BRL) for 30 min at 37°C. The reaction mixture was made up to a total volume of 20 μl, containing 5 μl crude RNA, 5 μl of 5 × reverse transcriptase buffer, 10 mmol dithiothreitol l^{-1}, 0.5 μg oligo $(dt)_{12-18}$ primer, 1 U RNase inhibitor, 1 mmol of each dNTP l^{-1}, with (RT+) or without (RT–), and 100 U M-MLV reverse transcriptase (Gibco, BRL). The mixture was incubated at 37°C for 1 h and the reaction was terminated by heating at 95°C for 5 min and quenching at 4°C. Products were stored at –20°C until the subsequent PCR.

For the first PCR, 3 μl reverse transcribed cDNA was added to the first PCR mastermix to a total volume of 50 μl containing 10 μl of 10 × PCR buffer, 1.5 mmol $MgCl_2$ l^{-1}, 0.2 μmol of each specific 3′ and 5′ outer primer, 0.2 mmol of each dNTP l^{-1} and 1.5 U Taq DNA polymerase (Gibco BRL). Each reaction was overlaid with light white oil and heated to 94°C for 5 min to denature all proteins and DNA. PCR cycling conditions were 94°C denaturation, temperature of annealing specific for primers (Table 1), 72°C extension, each step 1 min. PCR was carried out for 30 cycles. The reaction was terminated at 72°C for 10 min. First round PCR products were quenched at 4°C and stored at –20°C until the second round of PCR.

For the second round of PCR, 5 μl of the primary product was added to 45 μl freshly prepared PCR mastermix as described above, containing 0.2 μmol l^{-1} of each specific 3′ and 5′ inner primer. All reactions were overlaid with light white oil and PCR was carried out for 30 cycles with inner primer pairs using the same programme. Samples were stored at –20°C until electrophoresis was performed.

The amplified products were subjected to electrophoresis on 2% (w/v) agarose gels and stained with ethidium bromide. Ten microlitres of each PCR product and dye buffer was analysed in parallel with a 100 bp DNA ladder (Gibco BRL) as a standard. After gel electrophoresis was completed, gels were visualized under ultraviolet light and photographed with a Polaroid camera.

Verification of RT–PCR products

The identities of RT–PCR products (LH and FSH receptors) were verified by restriction enzyme analysis and sequencing. Verification of RT–PCR products was performed using 10 μl of the secondary PCR product with the appropriate enzyme in a final reaction volume of 20 μl with the appropriate restriction enzymes at 37°C for 1 h (Table 1). Digestion with DdeI (Gibco BRL) would be expected to yield fragments of 185 and 128 bp from the RT–PCR product for the LH receptor, whereas digestion with Hinc III (Gibco BRL) would be expected to yield fragments of 126 and 49 bp from the RT–PCR product for the FSH receptor. The size of the digested products was analysed by electrophoresis as described above. For sequence analysis, the secondary PCR products for LH and FSH receptors were purified using a kit (Wizard PCR Preps DNA Purification System, Promega, Madison). DNA was sequenced after PCR with dye-labelled

dideoxy terminators in a VGI automated sequencer (Visible Genetics Inc., Toronto)

Results

Expression of mRNA transcripts of LH and FSH receptors in mouse oocytes and embryos at different stages of development

Electrophoresis of RT–PCR products for the LH receptor produced bands corresponding to fragment sizes (313 bp) produced by the specific primers used in the second round PCR. LH receptor mRNA transcripts were present in unfertilized oocytes, in zygotes and in embryos at the two-cell, four-cell, morula and blastocyst stages (Fig. 2b).

FSH receptor transcripts (Fig. 2c) of the predicted size (175 bp) were observed at all stages examined. FSH receptor mRNA transcripts were present in unfertilized oocytes, in zygotes and in embryos at the two-cell, four-cell, morula and blastocyst stages. All of the embryos at each developmental stage expressed β-actin mRNA (Fig. 2a), confirming the integrity of the RNA and the RT–PCR. In control samples of medium alone in which two-cell, four-cell, morula and blastocyst stage embryos were cultured and subjected to the same RT–PCR reaction, no amplification of the LH receptor and FSH receptor mRNAs was observed (Fig. 3). RT–PCR analysis with LH and FSH receptor primers and mRNA isolated from oviductal fluids containing cumulus cells and various cell fragments after collection of zygotes gave rise to the expected fragments for LH and FSH receptors, respectively (Fig. 3).

The experiments for RT–PCR of LH and FSH receptors were repeated at least three times with oocytes and embryos at all stages of development using different lysates, and transcripts were detected consistently.

Digestion of the two second round PCR products with restriction enzymes yielded fragments of the expected sizes.

Expression of mRNA transcripts of P450 aromatase in mouse cumulus cells, oocytes and embryos at different stages of development

Oocytes and embryos at all stages of development were examined by RT–PCR with P450 aromatase specific primers, and no amplification was observed. Samples of cumulus cells surrounding oocytes, zygotes and two-cell embryos were subjected to the same RT–PCR reaction and produced bands of 760 bp (Fig. 4).

Sequencing of RT–PCR products

Sequencing of second round PCR products showed that the 313 bp RT–PCR product for the LH receptor (nucleotides 808–1120, GenBank accession number M81318) and the 175 bp RT–PCR product for the FSH receptor (nucleotides 618–789, GenBank accession number AF095642), respectively, matched published GenBank sequences.

Discussion

Recent studies have increased understanding of the role of FSH and LH in reproduction, and especially in folliculogenesis and steroidogenesis (Tena-Sempere and Hoger, 1999). Gonadotrophins are required for a normal rate of follicular development, and the period from the primary to the mid- to late secondary stages is associated with significant expression of both gonadotrophin receptors (O'Shaughessy *et al.*, 1997).

Gonadotrophins cause an increase in ovarian cAMP (Marsh *et al.*, 1972). However, high cAMP concentrations inhibit oocyte maturation (Dekel and Beers, 1978). The cAMP concentration decreases in mouse oocytes before the resumption of meiosis (Schultz *et al.*, 1983a) and it has been suggested that in human oocytes, the decrease in cAMP concentration triggers maturation (Tornell and Hillesjo, 1993). FSH initially increases cAMP concentrations in cumulus–oocyte complexes and in the oocyte, possibly by cAMP transfer via gap junctions, whereas FSH decreases cAMP concentrations at a later stage (Salustri *et al.*, 1985). LH increases the cAMP concentrations in follicles, but decreases the concentration of cAMP in maturing oocytes (Schultz *et al.,* 1983b), possibly via a decrease in the transport of cAMP from the cumulus cells into the oocyte as a result of a loss of the gap junctions (Dekel *et al.*, 1981; Dekel, 1988). The initial FSH-induced increase in cAMP in the oocyte induces an increase in cAMP degradation, and the resulting decrease in cAMP induces oocyte maturation (Salustri *et al.*, 1985). In oocytes treated with a combination of FSH and LH, LH may inhibit the initial FSH-induced increase in cAMP, resulting in a failure to increase the rate of cAMP degradation (Jinno *et al.,* 1989).

In the present study, the expression of LH and FSH receptors was examined in preimplantation mouse embryos. The results demonstrate that the receptors are present in oocytes, zygotes, two- and four-cell embryos, morulae and blastocysts. Contamination of oocytes and preimplantation embryos with cumulus cells was excluded by examining mRNA preparations by RT–PCR for P450 aromatase. The presence of this cumulus cell-expressed gene was not observed in isolated oocytes or preimplantation embryos. The expression of LH and FSH receptors in preimplantation mouse embryos indicates that FSH and LH have a direct effect on the reversal of the hypoxanthine-induced two-cell block by increasing the concentration of cAMP.

Expression of LH and FSH receptors at the zygote and two-cell embryo stages probably represents product from the maternal genome, as in mice major activation of embryonic gene transcription begins at the two-cell stage (Flach *et al.*, 1982). The presence of transcripts for LH and FSH receptors at later stages of development indicates activation of the embryonic genome at these stages. However, without quantitative PCR or the detection of polymorphisms, it is not possible to determine whether transcripts for LH and FSH receptors are of maternal or embryonic origin. Therefore it is not possible to draw

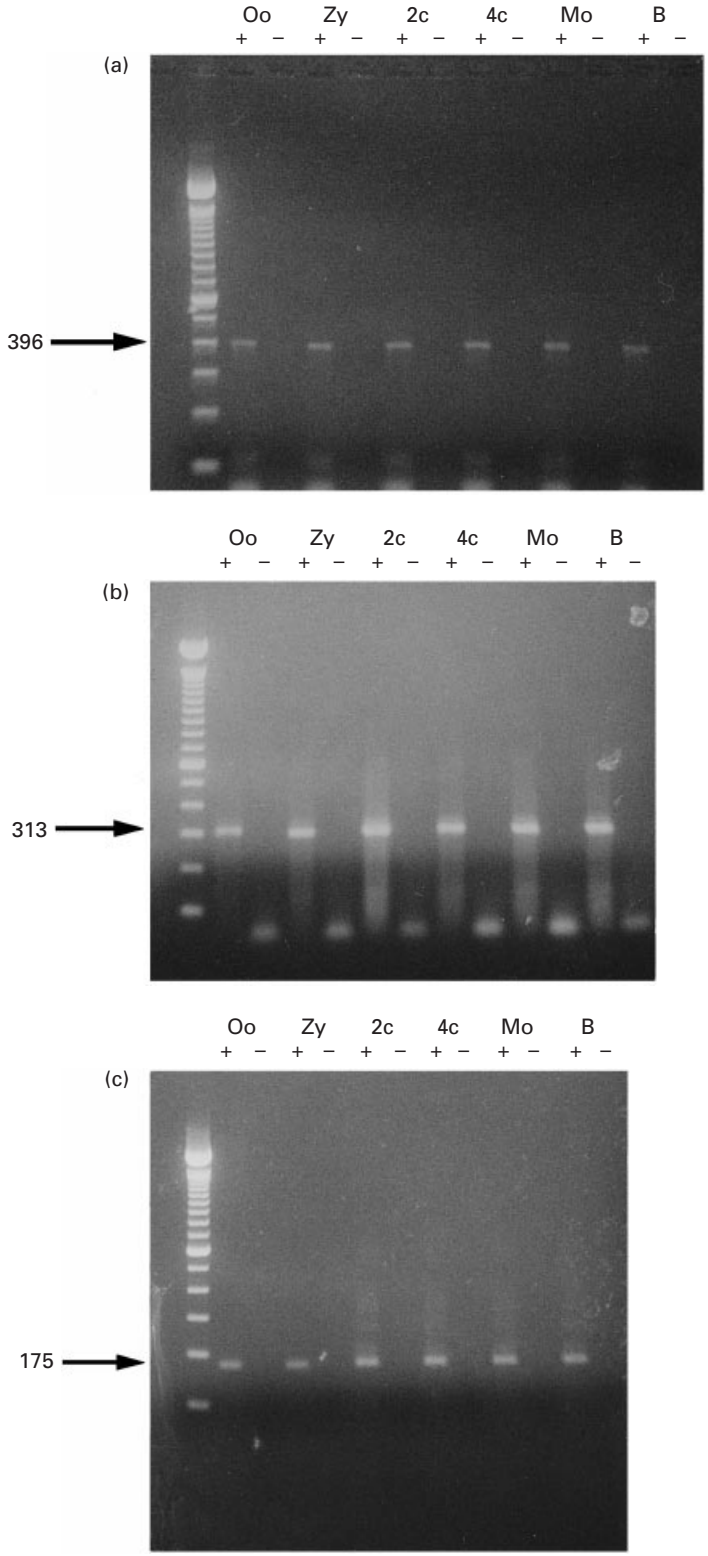

Fig. 2. Gel electrophoresis of RT–PCR products for (a) β-actin control, (b) LH receptor mRNA and (c) FSH receptor mRNA in mouse oocytes and embryos. Oocytes (Oo) with and without reverse transcriptase (RT+/RT–); zygotes (Zy) (RT+/RT–); two-cell (2c) embryos (RT+/RT–); four-cell (4c) embryos (RT+/RT–); morulae (Mo) (RT+/RT–); blastocysts (B) (RT+/RT–). Detection of β-actin mRNA ensured correct RNA extraction and cDNA synthesis. After amplification, samples were subjected to electrophoresis on 2% agarose gels and visualized under ultraviolet light. Arrows show RT–PCR products. A 100 bp molecular size marker was used.

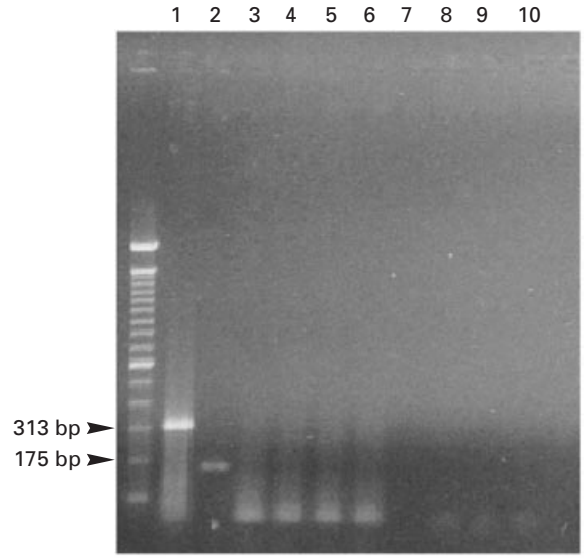

Fig. 3. Gel electrophoresis of RT–PCR products for mouse LH and FSH receptors with mRNA from oviductal fluids remaining after zygote collection and with mRNA from culture medium. LH receptor (lane 1) and FSH receptor (lane 2) RT–PCR analysis with mRNA from oviductal fluids after zygote collection; LH receptor RT–PCR analysis with culture medium of two-cell embryos (lane 3), four-cell embryos (lane 4), morulae (lane 5) and blastocysts (lane 6); FSH receptor RT–PCR analysis with culture medium of two-cell embryos (lane 7), four-cell embryos (lane 8), morulae (lane 9) and blastocysts (lane 10). A 100 bp molecular size marker was used.

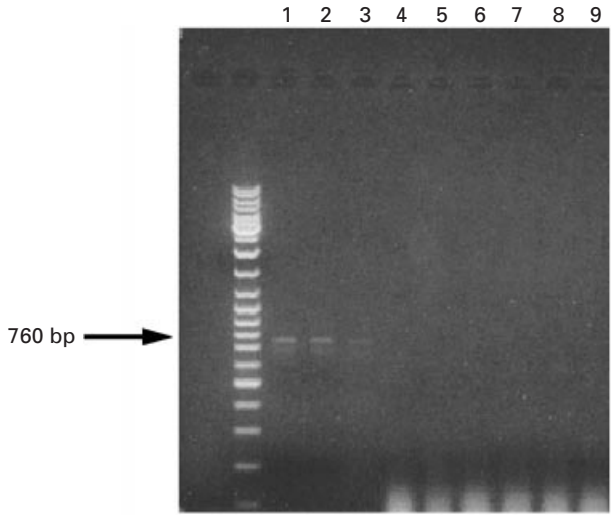

Fig. 4. RT–PCR amplification of P450 aromatase in mouse cumulus cells, oocytes and embryos at different stages of development. Cumulus cells surrounding oocytes (lane 1); cumulus cells surrounding zygotes (lane 2); cumulus cells surrounding two-cell embryos (lane 3); oocytes (lane 4); zygotes (lane 5); two-cell embryos (lane 6); four-cell embryos (lane 7); morulae (lane 8); blastocysts (lane 9). A 100 bp molecular size marker was used.

conclusions on the timing of the switch from maternal to embryonic transcripts for the two genes.

Loutradis *et al.* (1994) studied the effect of LH and FSH in reversing the hypoxanthine-induced block in the development of mouse embryos. In some strains of mouse, hypoxanthine arrests the development of the embryo at the two-cell stage (Loutradis *et al.*, 1987; Nureddin *et al.*, 1990). The mechanism underlying this block is poorly understood, although it is known that hypoxanthine does not inhibit development by blocking transcription or translation (Fissore *et al.*, 1992). Hypoxanthine inhibits resumption of meiosis in mouse oocytes and it has been suggested that this is a result of the inhibition of phosphodiesterase, which leads to an increase in the concentration of cAMP (Eppig *et al.*, 1985; Downs *et al.*, 1989). However, hypoxanthine does not block embryo cleavage via phosphodiesterase inhibition (Nureddin *et al.*, 1990). Compounds that increase the concentration of cAMP reverse the hypoxanthine-induced block (Nureddin *et al.*, 1990; Fissore *et al.*, 1992). The combination of FSH and LH reverses the block, whereas FSH alone does not have this positive effect. hCG, which has the same action as LH, reverses the two-cell block and supports the development of embryos to the blastocyst stage.

The presence of the LH and FSH mRNA transcripts in oocytes and cleavage stage mouse embryos indicates a possible mechanism for the involvement of gonadotrophins in the reversal of the two-cell block through the presence of receptors. The receptors may be present from the early stages of preimplantation development enabling the embryo to respond to gonadotrophins, which increase cAMP concentrations, thus reversing the two-cell block.

Furthermore, *in vitro* maturation is used widely for primary oocytes at the germinal vesicle stage recovered from ovaries that have had no prior stimulation with exogenous gonadotrophins. Human menopausal gonadotrophin (hMG) and hCG are used most commonly as the main components of the culture system for oocyte maturation (Barnes *et al.*, 1996). Increased fertilization rates have been reported after the addition of hMG for 6–8 h in the culture medium of human oocytes recovered from small follicles (Zhang *et al.*, 1993). It has been suggested that gap junction-mediated transmission of follicular cell cAMP to the oocyte inhibits oocyte maturation, whereas gonadotrophin stimulation terminates cumulus–oocyte communication and initiates resumption of meiosis, thus interrupting the direct transfer of cAMP to the oocyte (Dekel 1980; Loutradis *et al*, 1994). However, the expression of LH and FSH receptors in denuded oocytes, zygotes and preimplantation embryos, as determined in the present study, indicates a new mechanism for the maturation of denuded oocytes (free of cumulus cells) mediated by gonadotrophins directly.

Further studies are required to elucidate these mechanisms completely. The presence of alternate transcripts for the two receptors is currently being investigated as these have been reported in the neonate mouse ovary (O'Shaughessy *et al.*, 1997).

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Received 12 July 2000.
Accepted 1 November 2000.