Presence of LH receptor mRNA in granulosa cells as a potential marker of oocyte developmental competence and characterization of the bovine splicing isoforms

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As the expression of the LH receptor (LH-R) in granulosa cells is thought to be associated with later stages of folliculogenesis, this study was undertaken to evaluate the presence of LH-R mRNA as a suitable marker for developmental competence of oocytes. Granulosa cells and cumulus-oocyte complexes (COCs) were recovered from cows that had received ovarian stimulation. The COCs were subjected to embryo production procedures *in vitro* to assess the embryonic potential of the oocyte, and the corresponding granulosa cells were used to evaluate the presence of LH-R mRNA by RT-PCR. The presence of LH-R transcripts in granulosa cells is not a key characteristic of a follicle bearing a competent oocyte, although a higher proportion of oocytes reach the blastocyst stage when LH-R mRNA is detected in the granulosa cells. Different LH-R isoforms were cloned and sequence discrepancies among

six of the isoforms enabled the design of specific oligonucleotides to study the presence of the isoforms in different follicular cells. All LH-R transcripts studied and the 80 kDa protein product corresponding to the full length receptor were found in granulosa cells of small (< 4 mm) and large (> 5 mm) follicles. When the granulosa cells were cultured, the transcripts were downregulated by the culture conditions; downregulation was more acute in granulosa cells from small follicles. The addition of LH to the culture media enhanced LH-R mRNA downregulation. The presence of several LH-R transcript isoforms was tissue specific and in the theca cells LH-R mRNA was restricted mainly to cells from larger follicles. This finding indicates that the expression and the splicing of LH-R mRNA are regulated in a cell-specific and follicular size-specific manner.

Introduction

The physiological events involved in follicular recruitment, growth, selection, dominance and ovulation are not yet fully understood. LH plays an important role in the regulation of ovarian function, as it is responsible for ovulation and transformation of follicular cells into luteal cells (Smith *et al.*, 1994). The action of LH is mediated by the binding to its receptor (LH-R) which is anchored on the cell membrane (Davis, 1994). However, the events that trigger the production of functional receptors that are located on the surface of the granulosa cells appear to be regulated in a complex manner and it is still unclear whether the presence of LH-R is associated with follicular dominance. Peng *et al.* (1991) reported that the temporal expression of LH-R is different between follicular cells, as the theca

cells produce LH-R early in the follicular growth phase, whereas the mural granulosa cells acquire LH-R near the preovulatory LH surge. In support of these results, LH-R is expressed only in the granulosa cells of one follicle of ≥ 8 mm in diameter per cow (Bao *et al.*, 1997; Manikkam *et al.*, 2001). This finding implies that there is a relationship between follicular dominance and LH-R production in granulosa cells. On the contrary, Evans and Fortune (1997) reported that the selection of the dominant follicle occurs without observed differences regarding the LH-R mRNA. Even though patterns of LH-R expression in the follicular cells have not yet been clearly demonstrated, it is generally accepted that the receptor appears on the surface of granulosa cells in the late stages of folliculogenesis and that probably only in the dominant follicle does it have the capacity to ovulate.

The competence of oocyte development is also partly associated with follicular growth, as oocytes recovered from follicles of < 2 mm in diameter are meiotically

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impaired and display poor capacities to reach the blastocyst stage, whereas most of the oocytes collected from follicles that are 3–8 mm in diameter are meiotically competent and 30% reach the blastocyst stage when subjected to *in vitro* maturation, fertilization and development (Motlik and Fulka, 1986; Pavlok *et al.*, 1992; Lonergan *et al.*, 1994; Fair *et al.*, 1995). The mechanisms by which the oocyte acquires the capacity to reach the blastocyst stage during its follicular maturation is unknown. However, it is most probable that all the different types of cell that compose the follicle are in close association with each other and that the status of oocyte maturation is reflected in the surrounding granulosa cells. Direct evidence that two types of follicular cell influence each other is the LH-R expression, as the mRNA is not detected in the cumulus cells surrounding the oocyte, whereas it is present in the mural granulosa cells (Peng *et al.*, 1991; van Tol *et al.*, 1996), indicating that the physical distance between the oocyte and the granulosa cells influences LH-R mRNA expression. These observations are supported by Eppig *et al.* (1997) who showed that the mouse oocyte represses the expression of the LH-R in cultured granulosa cells. Considering these reports, it was proposed that the expression of the LH-R in granulosa cells could be associated with the presence of a mature and, hence, developmentally competent oocyte, as the repressive control of LH-R expression could be removed once the oocyte acquires the capacity for embryonic development, thus ensuring the ovulation of a competent oocyte.

The functional structure of the receptor is interesting as several splicing isoforms have been observed in many species (Sokka *et al.*, 1992; VuHai-Luu Thi *et al.*, 1992; Bacich *et al.*, 1994; Koo *et al.*, 1994; Mamluk *et al.*, 1998) and to our knowledge, no physiological function has been assigned to any of the splice forms. The LH-R is composed of a large extracellular LH-CG binding domain followed by a transmembrane domain spanning seven times the cytoplasmic membrane that anchors the receptor at the cell surface and a relatively short intracellular domain responsible for signal transduction (Dufau *et al.*, 1995).

The present study characterizes the presence of LH-R mRNA in granulosa cells of superstimulated cows to evaluate the expression of LH-R in granulosa cells as a marker of oocyte developmental competence. The study was extended by characterizing the presence of LH-R isoforms in bovine follicular cells of various sources using semi-quantitative RT-PCR. The association of LH-R expression with follicular size was studied at the mRNA level using specific primers segregating the isoforms and at the protein level in granulosa cells from different follicular size groups. Finally, RT-PCR was used to evaluate, in a time-dependent manner, the effect of addition of LH on the presence of LH-R mRNA in cultured bovine granulosa cells.

Materials and Methods

Bovine ovarian stimulation protocols

Fourteen cyclic Holstein heifers were used. Two days before the start of the superstimulation procedures, the ovaries of heifers with palpable or functional corpora lutea (days 3–15) were aspirated transvaginally to remove all large follicles. The ovaries were then stimulated for 2 days with FSH (Folltropin® V, CDMV, St-Hyacinthe, QC) which was administered in four doses of 3 ml (50 mg). As described by Blondin *et al.* (2002), at 33 or 48 h after the last injection, follicles from each ovary were counted by ultrasonography and classified according to diameter: small (< 4 mm) or medium to large (> 5 mm). Follicle collection was performed by transvaginal aspiration of small groups of three to five follicles. The recovered cumulus–oocyte complexes (COCs) were cultured *in vitro* to assess their developmental capability and the remaining granulosa cells were snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction to study the presence of LH-R mRNA.

Embryo production in vitro

The COCs were kept in separate groups and were matured in 50 µl droplets of maturation medium for 24 h. The maturation medium included TCM-199 supplemented with Earle's salts (Gibco BRL Life Technologies, Burlington, ON), bicarbonate (Sigma, St Louis, MO) and 20% (v/v) heat-treated fetal calf serum (FCS), 0.5 µg FSH-P ml^{–1} (NIDDK), 5.0 µg LH I^{–1} (NIDDK), 1.0 µg oestradiol ml^{–1} (Sigma), 0.2 mmol pyruvic acid I^{–1} and 50.0 µg gentamicin ml^{–1} (Sigma). The droplets were covered with mineral oil (Aldrich Chemical, Milwaukee, WI) and preincubated in a humidified atmosphere containing 5% CO₂ and 95% O₂ at 38.5°C for a minimum of 2 h before the addition of the COCs.

After maturation, the COCs were washed twice in Hepes-buffered Tyrode's medium (TLH) containing 0.3% (w/v) fatty acid-free BSA (Sigma), 0.2 mmol pyruvic acid I^{–1} (Sigma), 2.0 µg heparin ml^{–1} (Sigma) and 50.0 µg gentamicin ml^{–1} (Sigma). The COCs were washed and transferred into 48 µl fertilization medium to which 2 µl of 2 mmol penicillamine I^{–1}, 1 mmol hypotaurine I^{–1}, 250 µmol adrenaline I^{–1} (PHE, all from Sigma) was added followed by bovine spermatozoa. The fertilization procedure lasted 15–18 h. The fertilization medium contained Tyrode's lactate medium, 0.6% (w/v) fatty acid-free BSA (Sigma), 0.2 mmol pyruvic acid I^{–1} (Sigma), 2.0 µg heparin ml^{–1} (Sigma) and 50.0 µg gentamicin ml^{–1} (Sigma). The bull spermatozoa used was kindly provided by Le Centre d'Insémination Artificielle du Québec (CIAQ) and were supplied in frozen straws that were thawed in a 35°C water bath for 1 min and then separated using the swim-up technique as described by Parrish and Foote (1986). An aliquot of 2 µl sperm suspension was added to each of the fertilization

droplets to a final concentration of 1×10^6 spermatozoa ml^{-1} .

The embryos were washed twice in development medium and then transferred to 50 μl of preconditioned development droplets. The embryos were cocultured with bovine oviductal cells prepared as described by Blondin and Sirard (1995).

Culture of granulosa cells

The granulosa cells were collected from ovaries from an abattoir; the ovaries were kept on ice during transportation to the laboratory and follicular dissection. Cells were cultured as described by Rouiller *et al.* (1996) in a 24-well polystyrene dish at 1×10^6 viable cells (as determined by trypan blue exclusion) per well in Ham's F-12 medium supplemented with human apo-transferin (10 mg l^{-1}) (Sigma), ascorbic acid (17.6 mg l^{-1}) (Sigma) and pig FSH ($50 \text{ } \mu\text{g l}^{-1}$) (National Institute of Diabetes, Digestive and Kidney Diseases; NIDDK, Bethesda, MD) in a humidified atmosphere containing 5% CO_2 and 95% O_2 at 38.5°C . LH (100 ng ml^{-1}) (NIDDK) was added to the media to study the effect of the presence of LH on the receptor mRNA. Once the cells were collected, they were snap-frozen in liquid nitrogen and stored at -80°C .

Tissue collection and RNA extraction

Total ovarian tissue samples were collected from ovaries obtained from an abattoir and stored on ice during transportation. The total ovary sample included entire ovaries with corpora lutea. The granulosa and theca cells were collected by dissecting the follicles on ice under a stereomicroscope. The cumulus cells of COCs collected by aspiration of follicles of 3–7 mm in diameter were dissociated mechanically. All of the different types of cell were washed in PBS, collected by centrifugation at 900 *g* for 2 min and frozen at -80°C . Tissue samples from liver, kidney and muscle were snap-frozen and stored at -80°C until RNA extraction. Total RNA was isolated from the tissues using Trizol reagent (Gibco BRL Life Technologies) and quantified by measuring its absorbancy at 260 nm. An aliquot of each sample was subjected to electrophoresis to verify the integrity of RNA. The RNA samples were treated by DNase I (RNase free) (Ambion, Austin TX) and re-extracted by phenol–chloroform before the RT–PCR reactions. Genomic DNA contamination in the RNA samples was evaluated by performing PCR reactions directly on an aliquot of the RNA samples without the reverse transcription step.

Amplification and cloning of different isoforms of the LH-R mRNA

Primers were designed according to the bovine sequence found in Genbank (U20504) to amplify 713 bp

of the extracellular LH binding domain of the LH-R cDNA. Another primer was designed (LH-R G to H low) in a region located downstream and was used in combination with the upstream primer LH-R A to B up to find other different LH-R isoforms. First-strand cDNA was synthesized from total RNA (5 μg) using a 'SuperScriptTM Preamplification System for First Strand cDNA Synthesis' kit (Gibco BRL Life Technologies) and 200 ng oligo(dT)_{12–18} primer in a total reaction volume of 20 μl . An aliquot of 2 μl cDNA was subjected to PCR amplification using a Programmable Thermal Controller (PTC-100TM, MJ Research Inc., Watertown, MA). The PCR reaction mixture contained 30 pmol of each primer, $1 \times$ PCR buffer (PE Applied Biosystems, Foster City, CA), 0.35 mmol dNTPs l^{-1} and water to 50 μl . The reaction was heated to 94°C for 10 min (hot start) after the addition of Taq Gold polymerase (PE Applied Biosystems). The amplification profile comprised 35 cycles: 94°C for 1 min (dissociation), 58°C for 1 min (annealing) and 72°C for 1 min (extension). The final cycle included a further 5 min at 72°C to complete strand extension. An aliquot of the PCR reaction was subjected to electrophoresis on a 1% (w/v) agarose gel.

The LH-R isoforms were cloned as follows: the PCR products were purified using Qiaquick columns (QIAGEN Inc., Argentia Road, ON) and subcloned into a TA cloning system (Invitrogen Corporation, Faraday Ave, CA). One microgram of ligated DNA was transformed by heat shock in 100 μl of competent DH5 α *Escherichia coli* cells. Colonies were grown on Luria-Bertoni (LB) medium containing ampicillin for 16 h at 37°C . Plasmid DNA of selected colonies was extracted using the plasmid miniprep system (QIAGEN Inc.). The preps were digested with EcoRI, PST I and Hind III restriction enzymes to identify eight plasmids with different LH-R isoforms. The authenticity of the cloned cDNAs was verified by sequencing using T7 SequencingTM kit (Pharmacia Biotech, Baie D'Urfé, QC). Further sequencing was performed using an automated sequencing service.

RT–PCR to evaluate the presence of each of the cloned LH-R isoforms

The comparative sequence analysis of the different cloned LH-R isoforms revealed deleted and inserted sequences allowing the design of primers intended to discriminate three pairs of the cloned LH-R isoforms (Table 1). The RT–PCR procedure was as described above. The primers used to discriminate clones A and B were LH-R A to B up and LH-R A to B low. The upstream primer used to discriminate clones C and D was LH-R C to D up and the downstream primer was LH-R low. The upstream primer used to discriminate clones E and F was LH-R up and the downstream primer was LH-R E to F low.

Table 1. Details of the primers used to amplify different isoforms of the bovine LH receptor mRNA and the β -actin used as internal standard

Name	Primer sequence	Position on Genbank sequence U20504
LH-R up	5'-TCCCTCGGTAAAATACCTAAGC-3'	397–421
LH-R low	5'-GTAGCCCATAATGTCTTCACAGG-3'	1088–1111
LH-R A–B up	5'-AACCACCATACCAAGAAATGC-3'	517–537
LH-R A–B low	5'-ATCCCAGCCACTCAGTTCAC-3'	1005–1024
LH-R C–D up	5'-GCCAGTTGAATCTGTTTCTGC-3'	N/A
LH-R E–F low	5'-GCACACAGGTTTGATCCCTT-3'	N/A
LH-R G–H low	5'-CAACAGAAAGAAATCCCTTTGG-3'	1924–1945
β -actin up	5'-CGTGACATTAAGGAGAAGCTGTGC-3'	N/A
β -actin low	5'-CTCAGGAGGAGCAATGATCTTGAT-3'	N/A

Western blot analysis

The antibody used is specific to the first 11 amino acids of rat LH-R extracellular domain. The antibody was a kind gift of P. C. Roche (Mayo Clinic, Rochester, MN). Granulosa and theca cells were collected from bovine ovaries from an abattoir as described above. For a positive control of the antibody, a protein extraction of rat ovary was included. The tissue samples were homogenized in a modified radioimmunoprecipitation buffer containing 50 mmol Tris-HCl l^{-1} (pH 7.4), 1% (w/v) NP-40, 0.25% (w/v) sodium deoxycholate, 150 mmol NaCl l^{-1} , 1 mmol EDTA l^{-1} , 1 mmol phenylmethylsulfonyl (PMSF) l^{-1} , 1 μg aprotinin ml^{-1} , 1 μg leupeptin ml^{-1} , 1 μg pepstatin ml^{-1} , 1 mmol sodium orthovanadate (Na_3VO_4) l^{-1} and 1 mmol sodium fluoride l^{-1} (all from Sigma). Fifty micrograms of protein and a coloured molecular size marker (Bio-Rad, Mississauga, ON) were separated by 10% SDS-PAGE under reducing conditions. The proteins were electro-transferred on to a membrane (CBS Scientific Co. Inc., Delmar, CA) and the hybridization was performed as described by Han *et al.* (1997). In brief, non-specific binding sites were blocked with 5% (w/v) non-fat dry milk and the blot was incubated for 2 h at room temperature with 1:1500 dilution of LH-R antibody. The blot was washed and reincubated for 1 h at room temperature with 1:3000 dilution of horseradish peroxidase-labelled anti-rabbit IgG (Bio-Rad). The blot was washed and revealed by chemiluminescence (Pharmacia Biotech).

Statistical analysis

ANOVA was carried out to determine the effect of the follicular size and the presence of LH-R mRNA in granulosa cells on the percentage of blastocysts obtained, to evaluate whether the presence of LH-R transcripts are associated with oocyte developmental competence. All percentage data were ARCSIN transformed before analysis.

Table 2. Blastocyst production in relation to follicular size and presence of LH receptor (LH-R) mRNA in bovine granulosa cells detected by RT-PCR

Size of follicle (mm)	Presence of LH-R*	<i>n</i>	Oocyte	Blastocyst (%)
< 4	+	4	13	8 (61.5%) ^a
	–	6	21	8 (38.1%) ^b
> 5	+	8	53	31 (58.5%) ^a
	–	3	29	12 (41.4%) ^b

*The primers used can detect isoforms A to F.

n represents the number of oocyte pools used.

^{ab}Percentages with different superscripts are significantly different ($P < 0.05$).

Results

Qualitative RT-PCR was carried out to detect the presence of LH-R mRNA in granulosa cells collected during oocyte recovery, to evaluate the receptor transcript as a potential marker of oocyte developmental competence. The primers were designed to amplify 713 bp of the large extracellular domain of the LH-R cDNA spanning exon 4 to the beginning of the transmembrane domain (exon 11). The LH-R mRNA was not detected in all of the granulosa cell pools, whereas β -actin, used as positive control, was detected in all samples. Higher blastocyst rates were observed ($P < 0.05$; Table 2) when LH-R mRNA was detected in the corresponding granulosa cells indicating a beneficial effect.

Several bands were detected after PCR amplification, indicating the presence of multiple transcripts (Fig. 1). The LH-R transcripts were detected in granulosa cells collected from small (< 4 mm) and large (> 5 mm) follicles. With this first set of primers, six different cDNAs were cloned (Clones A to F; Fig. 2) and to find other splicing isoforms a second set of primers was designed to amplify a region located further downstream into exon 11. From this second set of amplifications, two more isoforms were isolated (Clones G and H; Fig. 2). Sequence analysis and comparisons between clones indicate that clone A is identical to the targeted Genbank

Fig. 1. Agarose gel electrophoresis of RT-PCR amplifications of the bovine LH receptor (LH-R) showing several bands, and of β -actin. The RT-PCR amplifications were performed on total RNA extracted from bovine granulosa cells and ovary. The sample of the ovary used as a positive control represents total RNA extracted from an ovarian cortex with a corpus luteum. The follicular size group (< 4 mm and > 5 mm) is indicated above each lane.

LH-R sequence (U20504), whereas clone B is missing 82 bp. Clone A and clone B are identical to Genbank sequences U41414 and U41413, respectively. When the rat LH-R gene structure was used as a model to identify the size of the exons and the splicing sites, the 82 bp gap corresponded to the entire exon 10 which is absent in clones B, D, F and H.

Clones C to D and E to F differ by the insertion of a supplementary 323 bp and 174 bp sequence, respectively (Fig. 2). The additional portions of these clones are not homologous with any LH-R Genbank sequences. Furthermore, when they were submitted to a BLAST analysis, the supplementary fragment of clones C to D did not find any match, whereas a portion of the additional 174 bp of clones E to F showed 91% identity with the short interspersed repetitive element (SINE) sequences and the second exon of bovine adenylate kinase isozyme 2 (Genbank D90066) also showed a 92% identity over 65 nucleotides, whereas the bovine phosphoprotein called Bucentaur (BCNT) (Genbank AB005652) showed a 84% identity over 102 nucleotides. The sequence discrepancy associated to clone G was a deletion of 265 bp at the beginning of exon 11, whereas clone H combined the deletion of exon 10 to the same deleted portion of exon 11.

Computerized open reading frame (ORF) analysis revealed that only clones A and B have an ORF over their entire sequences, whereas the insertion in clones C to D leads to a stop codon early in the extra sequence inserted between exons 7 and 8 (Fig. 2). The sequence discrepancies in the other clones (E to H) produce a stop codon near the transmembrane domain (Fig. 2).

The sequence discrepancies enabled the design of primers to segregate the first six clones and semi-quantitative RT-PCR was used to study the presence of these LH-R isoforms in several tissues (Fig. 3). As expected, all LH-R transcripts were detected in the ovary and corpus luteum samples, whereas they were not detected in liver and kidney samples. Weak signals for isoforms A and E were observed in the muscle sample. The cumulus cell sample showed only a very weak band for clone E (data not shown), whereas all the transcript isoforms were expressed in granulosa cells collected from follicles of < 4 mm or > 5 mm in diameter. By contrast, the isoform regulation in theca cells appears to be related to follicular size, as the presence of the LH-R isoforms was largely absent from theca cells obtained from follicles of < 4 mm in diameter except for the detection of a very faint band for clone E (data not shown). The theca cells collected from follicles of > 5 mm in diameter did not show a signal for clones C to D and only a weak band was found for clone A.

The same approach was used to analyse the effect of culture and the presence of LH on the presence of the first six LH-R clones (A to F) by culturing bovine granulosa cells collected from follicles with a diameter of < 4 mm or $\geq$ 5 mm with or without LH supplementation. A fraction of the cells were harvested after a time course to assess the influence of culture over time (Fig. 4). Follicular size had a clear impact *in vitro* on the presence of LH-R mRNA, whereas the duration of culture resulted in downregulation of the LH-R transcripts. Nearly all LH-R transcripts were undetectable in granulosa cells from follicles of < 4 mm in diameter after 2 h in culture, whereas for the follicles of > 5 mm in diameter, the downregulation effect was observed after 4 h of culture. The addition of LH to the culture media further enhanced the downregulation of LH-R transcripts as shown (Fig. 4).

Western blot analyses revealed that the reported 80 kDa full length LH-R is translated in granulosa cells from follicles of < 4 mm and > 5 mm in diameter. A doublet was also found at 45 kDa in the granulosa cells of both size groups and the corpus luteum, but was absent in the theca cells of follicles of < 4 mm in diameter (Fig. 5).

Discussion

In mice, the oocyte suppresses the expression of LH-R in the granulosa cells (Eppig *et al.*, 1997) and in cows, LH-R mRNA is not detected in the cumulus cells but is

Fig. 2. Illustration of the alignment for the different LH receptor (LH-R) clones. The bovine LH-R illustration is based on Genbank sequence U20504. The limits of the exons were determined according to the splicing sites found in the rat LH-R gene structure (Dufau *et al.*, 1995) (Genbank number AH004953). Specific cDNA and protein domains are indicated by 5' untranslated region (5'UTR), extracellular, transmembrane (TM), intracellular (IC) and 3'UTR. On the different LH-R clones, boxes 1 and 2 represent missing portions, whereas for clones C, D, E and F the extra portion size and the insertion site are represented by solid black boxes. The arrows indicate the priming sites used to discriminate the isoforms and the stop codon is indicated by (*). The size of the fragment amplified using primers LH-R up and low (Table 1) is indicated for each isoform.

present in the mural granulosa cells (Peng *et al.*, 1991; van Tol *et al.*, 1996). Taking these reports into consideration together with the generally accepted contention that only the dominant follicle expresses LH-R in the granulosa cells, it was proposed that the oocyte would allow the expression of LH-R in granulosa cells once it has completed its cytoplasmic maturation and awaits the preovulatory LH surge. If this proposal is correct, the presence of LH-R mRNA in granulosa cells could be a valuable tool for oocyte management in an *in vitro* production programme because it would be a good marker of oocyte developmental competence and the technique does not destroy the gamete.

In the present study, the granulosa cells were collected *in vivo* from ovaries that were stimulated using a protocol that enhances the recovery of developmentally competent oocytes (Sirard, 2001; Blondin *et al.*, 2002).

This ovarian stimulation regimen induces acquisition of developmental competence of oocytes in as many as 80% of the recovered oocytes (Blondin *et al.*, 2002). When LH-R mRNA was detected in these granulosa cells, a higher proportion of the associated oocytes reached the blastocyst stage but, unfortunately, development was normal in some groups of oocytes in which LH-R mRNA was not detectable in the granulosa cells. Therefore, the presence of LH-R mRNA in the granulosa cells is not by itself a reliable marker of oocyte developmental competence. Other unidentified factors may have an important impact on oocyte developmental competence and may act with LH-R in a synergistic manner. For instance, it has been shown that the more competent oocytes are recovered from follicles in the early stage of atresia (Blondin and Sirard, 1995). During the ovarian stimulation protocol used in the present

Fig. 3. RT-PCR discriminating the different bovine LH receptor (LH-R) transcripts cloned in diverse tissue samples of β-actin. For the negative control, water was added instead of template during reverse transcription. The total ovarian sample was extracted from several ovaries with corpora lutea. The granulosa (G) and theca (T) cells were collected by dissecting bovine ovaries obtained from an abattoir. The size of the follicles is indicated above the lanes. The cumulus cells were dissociated mechanically from the cumulus-oocyte complexes collected by aspiration of follicles of 3–7 mm in diameter from bovine ovaries obtained from an abattoir.

Fig. 4. RT-PCR discriminating the different LH receptor (LH-R) transcripts in cultured bovine granulosa cells and β-actin. The duration of culture of the granulosa cells is indicated. The size of the follicles from which the granulosa cells were collected is indicated (< 4 mm or > 5 mm in diameter). The culture media supplemented with LH is indicated with + (with LH) or - (without LH). Before the cells were cultured, a portion of the cells was immediately frozen as indicated by n.c. (not cultured).

Fig. 5. Western blot analysis of the presence of LH receptor (LH-R) in granulosa and theca cells and corpora lutea (CL). All the cells are from bovine ovaries obtained from an abattoir except for the last lane which is the antibody positive control. The reported molecular mass of the full length LH-R is 80 kDa for humans (Han *et al.*, 1997) and rats (Al-Hader *et al.*, 1997).

study, this early stage of follicular atresia is purposely induced by the 'coasting' period after the last FSH injection and it significantly increases the developmental competence of the oocytes (Barnes and Sirard, 2000; Sirard, 2001; Blondin *et al.*, 2002). However, follicular atresia decreases the abundance of LH-R transcripts (Tilly *et al.*, 1992), therefore diminishing the efficiency of LH-R as a marker of oocyte developmental competence.

The RT-PCR detected the presence of several splicing isoforms of LH-R mRNA in the granulosa cells, theca cells and corpus luteum. Similar findings have been reported in bovine corpora lutea (Mamluk *et al.*, 1998) and in ovarian cells of other species, such as pigs (Sokka *et al.*, 1992; VuHai-Luu Thi *et al.*, 1992), sheep (Bacich *et al.*, 1994) and rats (Wang *et al.*, 1991; Koo *et al.*, 1994). In the present study, eight different LH-R transcripts were cloned, the sequences of clones A and B of which have already been reported by van Tol *et al.* (1996). Furthermore, the deletion of the entire exon 10 and partial deletion of exon 11 have also been reported in sheep (Abdennebi *et al.*, 2002). By contrast, clones C to F are novel. Structure analysis demonstrated that clones C to D and E to F have an extra portion, whereas clones G to H have a deleted region. Similar sequence discrepancies have also been found in turkeys as two LH-R sequences were isolated: in one sequence a portion was deleted, whereas the other sequence had an insertion; both were near the transmembrane domain (You *et al.*, 2000). The inserted sequences of clones C to D and E to F are not associated with known LH-R sequences and as the genomic organization of the bovine LH-R gene is unknown, the possibility that these isoforms contain an unspliced intron cannot be ruled out. In the present study, sample contamination by genomic DNA is unlikely because the RNA samples were treated with DNase I before reverse transcription. Moreover, PCR reactions were performed directly on RNA without the reverse transcription step and no amplification was detectable. Thus, the isolated clones must result from alternate splicing and must have a polyA tail to be reverse

transcribed using an oligo dT. The genomic organization of the rat LH-R gene reveals that all the introns are evenly distributed in the extracellular domain and the region covered by clones A to F should include five introns. If these clones were heterogeneous nuclear RNA being processed, other isoforms containing the other introns should have been found. By contrast, the deleted sequences of clones G to H indicate that these isoforms have been 'over spliced' relative to clone A. It is also interesting to note that the isoforms can be paired, so that one isoform in each group differs from the others by the deletion of exon 10. From this finding, it is possible that the bovine genome has two copies of the LH-R gene, one copy of which does not contain exon 10. The number of genomic copies of LH-R in cows is still unknown. However, species differences have been reported: the rat genome has a single copy of the LH-R gene and the human genome has at least two copies (Tsai-Morris *et al.*, 1998).

Only clones A and B have an ORF over the entire cloned sequence, indicating that they are the only isoforms to produce functional LH-R. As the extracellular domain of the receptor near the transmembrane domain is important for signal transduction and translocation of the receptor to the cell surface (Alvarez *et al.*, 1999), clone B may not be able to activate signal transduction appropriately or may not be present on the cytoplasmic membrane. If the other LH-R isoforms are translated, the presence of an early stop codon indicates that they produce truncated receptors. In pigs and sheep, respectively, three and four truncated LH-R mRNA lacking the transmembrane domain have been found (Loosfelt *et al.*, 1989; Bacich *et al.*, 1994), and it was suggested that these mRNAs produce secreted soluble receptors (Bacich *et al.*, 1994). For a complete understanding of the role of these isoforms, it is necessary to localize the product of these isoforms to evaluate whether they are located on the cell surface or contained within the cytoplasm. In sheep, the isoforms G and H are translated *in vivo* and are located in the cytosol (Bacich *et al.*,

1999). Furthermore, radioimmunoassay analysis would be useful to verify whether some of LH-R isoforms are secreted in the follicular fluid. The role of these secreted isoforms may be to capture LH to prevent it from binding to the cell surface receptors and in turn to prevent premature cellular differentiation and ovulation before the LH surge. A similar hormone capture mechanism has been demonstrated for the insulin-like growth factors (IGF) as the IGF-binding proteins secreted in the follicular fluid act to keep the IGF concentrations under a certain threshold (Schams *et al.*, 1999). Full length and shorter LH-R isoforms were detected by western blot analysis as a band of 80 kDa corresponding to the full length LH-R detected in humans (Han *et al.*, 1997) and rats (Al-Hader *et al.*, 1997), whereas the other bands observed at 45 kDa could be associated with the truncated receptors produced by clones E to H. In the event that these isoforms are not secreted or even translated, these isoforms could result from voluntary splicing modification controlling the production of full length LH-R. Such a mechanism involving a splicing switch associated with a specific physiological state has been proposed in ewes, in which clones G to H are abundant during anoestrus, but are almost absent during the breeding season (Abdennebi *et al.*, 2002).

The results of the present study indicate that there are tissue-specific and follicular size-specific LH-R isoforms. This finding may be related to follicular growth and differentiation, as changes in LH-R splicing during cellular differentiation have been reported in rat testis (Tena-Sempere *et al.*, 1994). The downregulation of LH-R mRNA by the presence of the ligand has been reported by LaPolt *et al.* (1991) and Peegle *et al.* (1994), but to our knowledge, the downregulation associated with follicular size observed in the present study is a novel finding.

The results of the present study demonstrate that LH-R mRNA is expressed in granulosa cells collected from all follicular size groups. This finding is in contrast to other studies, as it has been reported that the expression of LH-R in granulosa cells is restricted to one follicle of ≥ 8 mm in diameter per cow (Bao *et al.*, 1997; Mannikam *et al.*, 2001). However, this result is also a controversial issue as Evans and Fortune (1997) reported that the selection of the dominant follicle occurs without concomitant LH-R mRNA differences because they did not observe any LH-R mRNA hybridization in granulosa cells. Furthermore, Peegle *et al.* (1994) did not detect any LH-R mRNA hybridization in rat granulosa cells at 6 h after LH administration. This absence of detectable LH-R hybridization may be due to technical differences because all these studies used *in situ* hybridization, which is less sensitive than RT-PCR.

The presence of LH-R mRNA in granulosa cells is not a specific marker of oocyte developmental competence, but it may prove to be useful in combination with other gene markers. The identification of such markers can be

achieved using a more global approach that compares total gene expression of cells collected from follicles from which the developmental competence of the oocyte is known (Robert *et al.*, 2001). Overall, these results indicate that little is known about the regulation of temporal expression of LH-R in the different follicular cells. Although the results of the present study highlight complex regulatory mechanisms associated with follicular size, cell specificity and culture conditions, the role of these isoforms is unknown; to understand their physiological impact, extensive characterization of the transcript and protein contents in granulosa and theca cells collected from precise follicular environment are needed. At present, the complex nature of LH-R expression is reflected in the literature by the inconsistencies of published reports.

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