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Characterization of the Chicken Follicle-Stimulating Hormone Receptor (cFSH-R) Complementary Deoxyribonucleic Acid, and Expression of cFSH-R Messenger Ribonucleic Acid in the Ovary[1]

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ABSTRACT

Studies were conducted to characterize the chicken (c) FSH receptor (R) cDNA, and to evaluate expression of cFSH-R mRNA in the hen ovary at known stages during follicle development. A total of 2.5 kb of nucleic acid sequence including the complete cFSH-R coding region was isolated by a combination of the reverse-transcription polymerase chain reaction and 5′- and 3′-rapid amplification of cDNA ends techniques. Overall, the nucleic acid sequence homology of the cFSH-R cDNA coding region is 71.8% and 72.2% compared to the rat and bovine FSH-R, respectively, while the deduced amino acid sequence identity for the receptor protein (693 amino acids) is 71.9% and 72.4%, respectively. By comparison, the cFSH-R nucleic acid and amino acid sequences are 60.1% and 49.4% identical to the respective cLH-R sequences. Northern blot analysis detected a single 4.3-kb cFSH-R mRNA transcript, which was selectively expressed in ovarian (granulosa, theca, and stromal) tissues, but not the oviduct, adrenal, liver, muscle, or brain. As the follicle developed from the prehierarchical (6- to 8-mm diameter) to the largest preovulatory (F1 follicle) stage, cFSH-R mRNA levels progressively declined within both the granulosa and theca layers ($p < 0.05$). Moreover, cFSH-R mRNA levels were lower in whole atretic than in morphologically normal 3- to 5-mm follicles ($p = 0.0015$). The pattern of cFSH-R mRNA expression within the granulosa layer during follicle development was notably different from that of the recently reported cLH-R, in that cLH-R mRNA levels increase to become readily detectable coincident with dramatically increased steroidogenic capacity during the last few days before ovulation of the follicle. On the other hand, highest levels of cFSH-R mRNA in 6- to 8-mm (prehierarchical) follicles were consistent with a role for the cFSH-R in maintaining the viability of prehierarchical follicles and in initiating granulosa cell differentiation at the time when follicles are selected into the preovulatory hierarchy.

INTRODUCTION

The gonadotropins FSH and LH are heterodimeric glycoproteins produced within the adenohypophysis that, in the female, act primarily at the level of the ovarian follicle. While the complementary DNA sequences of the chicken (c) gonadotropin α and LHβ subunits have recently been fully characterized [1–3], there is as yet no information available concerning the nucleic acid sequence for the avian FSHβ subunit. In addition, despite the fact that the cFSH protein has been purified to apparent homogeneity [4], there remains considerable ambiguity regarding the potential biological activity of FSH within the largest (F1) through sixth largest (F6) preovulatory follicles. For instance, while LH is generally thought to be the more active gonadotropin in promoting progesterone production from preovulatory follicle granulosa cells, FSH has been reported to demonstrate limited [4–6] to no [7, 8] biological activity when steroid production is evaluated as a physiological endpoint.

By contrast, results from in vitro studies indicate that recombinant human (rh) FSH (a preparation devoid of detectable LH bioactivity [8]) acts within prehierarchical (6- to 8-mm diameter) follicle granulosa cells to increase levels of cytochrome P450 cholesterol side-chain cleavage ($P450_{scc}$) and P450 17α-hydroxylase ($P450_{17\alpha OH}$) mRNA, initiate $P450_{scc}$ and $P450_{17\alpha OH}$ enzyme activity, and promote progesterone and androgen synthesis. These actions occur, at least in part, via the adenylyl cyclase/protein kinase A second messenger signaling pathway [9, 10] and are proposed to occur during the final stages of granulosa cell differentiation following selection of a follicle into the preovulatory hierarchy. There is also evidence that FSH can induce modest, but significant, progesterone, androgen, and estrogen production from the prehierarchical follicle theca layer in vivo [11] and prevent granulosa cells from undergoing apoptosis in vitro [12]. Moreover, relatively low levels of FSH binding have been detected within ovarian stroma, the theca layer, and granulosa tissue, and such binding generally decreases during follicle development [13, 14]. Recently, a partial chicken (c) LH receptor (R) cDNA has been characterized, and the cLH-R mRNA transcript in granulosa cells is found to be expressed only within preovulatory follicles [15]. By comparison, there is essentially no change in LH-R mRNA levels during development in the theca, and taken together these data are consistent with previously determined biological effects of exogenously administered LH on granulosa and theca tissue steroidogenesis in vitro. To better understand the relationship between LH and FSH actions with regard to the process of hen follicle selection and the initiation of granulosa cell differentiation, we conducted the present studies to 1) characterize the cFSH-R cDNA and 2) evaluate changes in FSH-R mRNA levels in granulosa and theca tissues during growth and differentiation of ovarian follicles.

MATERIALS AND METHODS

Animals and Reagents

Single-comb white Leghorn hens (H&H Poultry, Portland, IN), 25–40 wk of age and laying regular sequences of at least 5–6 eggs, were used in all studies described. Birds were housed individually in laying batteries, provided with free access to feed (Purina Layena Mash; Purina Mills, St. Louis, MO) and water, and were exposed to a photo-

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TABLE 1. Nucleic acid and deduced cFSH-R amino acid sequences compared to the rat [22] and bovine [23] FSH-R, and chicken LH-R [15] sequences, considering the extracellular (EC), transmembrane (TM) and intracellular (IC) domains.[a]

Sequence	Nucleic acid	Amino acid	Amino acid by receptor domains		
			EC	TM	IC
Rat FSH-R	71.8	71.9 (87.2)	64.8 (83.1)	83.0 (93.6)	66.7 (84.1)
Bovine FSH-R	72.2	72.4 (87.4)	65.0 (83.6)	83.7 (93.6)	68.3 (84.1)
Chicken LH-R	60.1[b]	49.4 (70.7)[b]	35.1 (56.7)[b]	74.6 (94.3)	33.3 (65.1)

[a] Numbers are percentage of rat, bovine, and chicken vs. cFSH-R. Numbers outside parentheses represent the percentage of exact amino acid matches, whereas numbers within parentheses consider conservative amino acid substitutions.
[b] Represents incomplete sequence from the extracellular domain of the cLH-R; see [15].

period of 15L:9D, with lights-on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed by cervical dislocation approximately 12–18 h before a mid-sequence ovulation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Oligonucleotide primers (for polymerase chain reaction [PCR] amplification, 5′- and 3′ rapid amplification of cDNA ends [RACE], and nucleic acid sequencing) were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) or the University of Notre Dame Biotechnology Core Facility. Additional reagents were acquired from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

Isolation and Characterization of a Partial cFSH-R cDNA

Total RNA was isolated from small white ovarian follicles (≤ 5-mm diameter) and reverse-transcribed into first-strand cDNA using oligo(deoxythymidine) primer and recombinant M-MLV reverse transcriptase in the presence of deoxynucleotides (Perkin-Elmer, Norwalk, CT). Sequences for degenerate oligonucleotide primer pairs were based on the published human FSH-R cDNA sequence ([16]; location of amino acids corresponding to published human sequence in parentheses): forward primer (amino acids 397–404) CCN MGN TTY CTG ATG TGY AAY CT; reverse primer (amino acids 590–584) AAG SWG ATK GGR GCC ATR CA; where M = A, C; Y = T, C; S = G, C; W = A, T; K = G,T; R = G, A; N = A, T, G, C.

First-strand cDNA was subjected to 40 cycles of PCR amplification using GeneAmp core reagents (Perkin-Elmer; 30-sec denaturation at 95°C, 30-sec annealing at 55°C, and 30-sec extension at 72°C). The amplified 440-bp PCR product was resolved through a 1.2% agarose gel, isolated, purified (GeneClean; Bio 101, La Jolla, CA), and subcloned into the pBluescript SK(+) vector (Stratagene, La Jolla, CA) for large-scale plasmid preparation and nucleic acid sequence analysis.

Subsequently, the RACE technique was utilized to further characterize the FSH-R cDNA sequence in the 5′ and 3′ directions, and was accomplished essentially as described by the manufacturer (Clontech Laboratories, Inc., Palo Alto, CA). Briefly, poly(A)$^+$-enriched RNA from granulosa tissue of the second largest (F2) preovulatory plus prehierarchical (6- to 8-mm) follicles was isolated using the Micro-Fast Track Kit (Invitrogen, San Diego, CA). Double-stranded cDNA was synthesized, and Marathon cDNA Amplification adaptors (Clontech) were ligated. Nested gene-specific primers for the 5′ direction (GSP1: 5′-TGG GAA GTG CCA CCG TGA AAG-3′ [bp 1513–1490 from cFSH-R sequence] and GSP2: 5′-TTG CGG TTG AGT TGC ATG GCA TAG G-3′ [bp 1439–1415]) and 3′ direction (GSP3: 5′-AAC TCA ACC GCA AGG TTC GAC-3′ [bp 1427–1447] and GSP4: 5′-GCA TCT ACT TTA CTG TGA GAA ACC CC-3′ [bp 1652–1677]) were designed from the transmembrane cFSH-R PCR product described above.

An initial amplification of the 5′ region by PCR was performed using GSP1 and Adaptor Primer 1 (Clontech) under the following conditions: 1 min, 94°C; 0.5 min, 94°C; and 4 min, 72°C for five cycles; 0.5 min, 94°C, and 4 min, 70°C, for five cycles; 0.5 min, 94°C, and 4 min, 68°C, for 25 cycles. A second amplification was conducted using one-tenth the volume of the first reaction as template, with the GSP2 and Adaptor Primer 2 as internal primers and amplification conditions as described above. Three PCR products (ranging from 1.1 to 1.5 kb in length) were gel-purified and ligated into the PCRII vector (TA Cloning Kit, Invitrogen), then further amplified and purified from plasmid preparations. All three products were determined to be homologous to mammalian FSH-R, and the sequence of one of these extended beyond the 5′-end of the predicted start codon. These cloned products were fully sequenced in both directions by the dideoxychain termination method using ^{35}S-dATP (Amersham Corp., Arlington Heights, IL) and the Sequenase version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH).

Initial and secondary amplifications of the 3′ region were conducted using GSP3 and Adaptor Primer 1, and GSP4 and Adaptor Primer 2, using the same amplification conditions for each combination as described above, with the exception that during the final 25 cycles annealing was at 65°C for 0.5 min. Two different PCR products (each approximately 1.5 kb in length) were gel-purified and ligated into the PCRII vector, and then amplified and purified from plasmid preparations. Sequence analysis of each subsequently determined that both products were derived from the cFSH-R and extended through the end of the proposed coding region. Sequence data were assembled from multiple products over the entire coding region and analyzed by the MacVector and AssemblyLIGN programs (IBI, New Haven, CT).

RNA Isolation and Northern Blot Analysis

Total cellular RNA was collected from granulosa and theca tissue from hierarchal (F1, F2, F3) follicles, follicles recently selected into the follicle hierarchy (9- to 12-mm follicles), and prehierarchical (6- to 8-mm and 3- to 5-mm) follicles as previously described [17]. To evaluate selective tissue expression of the cFSH-R transcript, total cellular

RNA was prepared from liver, cerebrum, cerebellum, adrenal, kidney, oviduct, and ovarian stromal (interstitial tissue plus follicles < 1 mm in diameter [18]) and 3- to 5-mm follicle granulosa tissue. In addition, morphologically normal and atretic follicles (3- to 5-mm diameter) were collected and processed for RNA without separating granulosa and theca layers. Atretic follicles were identified on the basis of the presence of follicle haemorrhagia, collapsed morphology, and opaque appearance [12]. Total cellular RNA from all tissues was isolated using Trizol Reagent (Gibco-BRL, Grand Island, NY), and the purity and quality were assessed by measuring the optical density of each sample at 260 and 280 nm. Fifteen micrograms of total cellular RNA was resolved in 1% agarose gels in the presence of 6% formaldehyde, then transferred to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by capillary action overnight.

The cFSH-R cDNA probe was produced by the random-primer labeling method using the Megaprime DNA labeling System (Amersham) and [α^{32}P]-dCTP (3000 Ci/mmol; Amersham). The labeled probe was subsequently purified from unincorporated nucleotide using Centri-Sep columns (Princeton Separations, Adelphia, NJ) according to the manufacturer's recommendations. Northern blots were prehybridized in a solution containing 10-strength Denhardt's solution, 4-strength sodium chloride-sodium citrate solution (SSC; single-strength SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0), and 100 μg/ml herring sperm DNA (Promega, Madison, WI), plus 0.5% SDS for 30 min at 60°C. Hybridizations were conducted at 60°C overnight with a solution of 10-strength Denhardt's solution, 4-strength SSC, 0.1% SDS, 10 mM Tris-HCl (pH 7.4), and 100 μg/ml herring sperm DNA, plus the heat-denatured cDNA probe (2×10^6 cpm/ml). Membranes were subsequently washed twice (for 5 min each) with double-strength SSC at room temperature, followed by twice at 60°C for 15 min in double-strength SSC containing 0.1% SDS, and finally with 2–3 washes in 0.1-strength SSC plus 0.1% SDS (for 15 min each wash) at 60°C. Membranes were exposed to autoradiographic film at −70°C for 1 to 5 days.

Data Analysis

Northern blot analyses were repeated a minimum total of three times. The relative extent of hybridization was evaluated by densitometry (UltraScan XL laser densitometer; Pharmacia LKB, Piscataway, NJ), and equal loading of RNA onto gels was verified after the evaluation of ethidium bromide-stained 28S and 18S bands of ribosomal RNA. The rationale for this analysis is that β-actin mRNA, a common house-keeping gene, is not expressed at consistent levels during hen follicle development (unpublished results). Moreover, it is reasoned that potential bias due to uneven loading, incomplete transfer, and/or unequal hybridization efficiency within a blot is virtually eliminated by combining data from several independent, replicate analyses. Messenger RNA levels (fold increase vs. the appropriate control) are presented as the mean ± SEM of scanning data from replicate experiments. Data were analyzed by regression analysis, paired *t*-test, or one-way analysis of variance, with significant interactions determined by Scheffe's F-test.

RESULTS

Identification and Characterization of cFSH-R cDNA

The nucleotide and deduced amino acid sequences of the cFSH-R are presented in Figure 1. The nucleic acid sequence of the cloned cDNA has an open reading frame of 2079 bp, and includes 51 bp encoding a putative signal peptide (including the 5′ ATG start codon [19]), 1047 bp corresponding to the extracellular domain, 792 bp from the predicted transmembrane region, and 189 bp from the intracellular domain [20]. The deduced translated sequence for cFSH-R encodes a total of 693 amino acids, the first 17 amino acids of which represent the proposed signal peptide. Accordingly, the mature protein consists of 676 amino acids, corresponding to an estimated molecular mass of 78 767 daltons.

The extracellular domain region consists of 349 amino acids and has three potential *N*-linked glycosylation sites and a total of 11 cysteine residues. The transmembrane domain is represented by 264 amino acids arranged as seven transmembrane-spanning segments typical of a G protein-related receptor [21]. The intracellular loops between transmembrane segments (TM) I-II, TM III-IV, and TM V-VI contain multiple tyrosine, serine, and threonine residues that may represent potential phosphorylation sites. Two sites within the TM domain (Thr^{555} and Ser^{596}) may represent consensus sites for protein kinase C phosphorylation. A total of nine cysteine residues and one potential *N*-linked glycosylation site (Asn^{380}) are located within this domain.

The intracellular domain consists of 63 amino acids, including potential sites for phosphorylation by protein kinase C (Thr^{632}) and protein kinase A (Thr^{658}). Nucleic and amino acid (identity and conservative substitutions [22]) homologies of the cFSH-R aligned with the rat [23] and bovine [24] FSH-R sequences, as well as the recently characterized cLH-R [15] sequences, are compared in Table 1 and Figure 2.

Selective Tissue Expression of cFSH-R mRNA

Northern blot analysis of the cFSH-R mRNA detected a single 4.3-kb transcript from total cellular RNA prepared from ovarian stromal tissue, and there was no evidence of expression in any nonovarian tissues including the oviduct (Fig. 3). When compared on the basis of total cellular RNA, FSH-R mRNA levels were determined to be 6.3 ± 1.1-fold higher in the granulosa, compared to the theca, layer within the F1 follicle ($p < 0.05$; n = 4).

Complementary FSH-R mRNA during Follicle Development and Atresia

Complementary FSH-R mRNA levels progressively declined within both the theca ($p = 0.038$, by regression analysis; Fig. 4) and granulosa ($p < 0.001$, by regression analysis; Fig. 5) layers as the follicle developed from the prehierarchical (3- to 8-mm diameter) to the largest preovulatory (F1) follicle stage ($p < 0.05$). Highest levels of cFSH-R mRNA were repeatably detected within granulosa cells from 6- to 8-mm follicles ($p < 0.05$ vs. F1 and F2 follicles). Finally, Northern analysis demonstrated that c-FSH-R mRNA levels were decreased in atretic vs. morphologically normal follicles (mean decline, 54.8 ± 4.9%, range 43–53%; $p = 0.0015$; Fig. 6).

DISCUSSION

A cDNA corresponding to the cFSH-R has been cloned, and the overall sequence was determined to be considerably more homologous to the rat and bovine FSH-R than the recently characterized cLH-R ([15]; Table 1, Fig. 2). The mature cFSH-R protein sequence (693 amino acids) is sim-

A

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-23      AGGAGACAAATGTGAAGACAGAG ATG TCC TTG GAT CTC ACC TGC TTG CTG   27
                                 Met Ser Leu Asp Leu Thr Cys Leu Leu    9

 28  ATT CTC CTG GCC AGC TGC TCT GGC TGC CAG CAC CAC ACG TGC CTC TGT   75
 10  Ile Leu Leu Ala Ser Cys Ser Gly Cys Gln His His Thr Cys Leu Cys   25

 76  GAA GGC AGG ATA TTC ATC TGC CAG GAG ATC AAG GTG GTC CAG CTG CCC  123
 26  Glu Gly Arg Ile Phe Ile Cys Gln Glu Ile Lys Val Val Gln Leu Pro   41
                             *
124  CGG GAC ATT CCC ACC AAT GCC ACA GAA CTG AGA TTT GTC CTC ACC AAG  171
 42  Arg Asp Ile Pro Thr Asn Ala Thr Glu Leu Arg Phe Val Leu Thr Lys   57

172  ATG AGA GTC ATT CCG AAG GGA GCT TTC ACA GGA CTT CAT GAC CTA GAG  219
 58  Met Arg Val Ile Pro Lys Gly Ala Phe Thr Gly Leu His Asp Leu Glu   73

220  AAA ATA GAG ATC TCA CAG AAT GAT GCC TTG GAG ATC ATA GAA GCA AAT  267
 74  Lys Ile Glu Ile Ser Gln Asn Asp Ala Leu Glu Ile Ile Glu Ala Asn   89

268  GTG TTT TCC AGC CTT CCC AAA CTA CAT GAA ATA AGA ATT GAG AAG GCC  315
 90  Val Phe Ser Ser Leu Pro Lys Leu His Glu Ile Arg Ile Glu Lys Ala  105

316  AAC AAA CTC ATG AAG ATT GAT CAA GAC GCC TTC CAG CAC CTT CCA AGC  363
106  Asn Lys Leu Met Lys Ile Asp Gln Asp Ala Phe Gln His Leu Pro Ser  121

364  CTC AGA TAT TTG TTA ATA TCA AAC ACA GGC CTT AGC TTT TTA CCC GTC  411
122  Leu Arg Tyr Leu Leu Ile Ser Asn Thr Gly Leu Ser Phe Leu Pro Val  137

412  GTC CAT AGG GTG CAC TCC TTC CAG AAA GTT TTG CTA GAT GTT CAA GAC  459
138  Val His Arg Val His Ser Phe Gln Lys Val Leu Leu Asp Val Gln Asp  153

460  AAT ATC CAT ATA CGT ACA ATT GAA AGG AAC ACG TTC ATG GGC CTG AGT  507
154  Asn Ile His Ile Arg Thr Ile Glu Arg Asn Thr Phe Met Gly Leu Ser  169

508  TCT GAA AGT GTG ACT CTA CGG CTA AAT AAA AAT GGG ATT CAG GAA ATC  555
170  Ser Glu Ser Val Thr Leu Arg Leu Asn Lys Asn Gly Ile Gln Glu Ile  185
                                                    *
556  AAG GAT CAT GCA TTT AGT GGA ACC TGC CTG GAT GAG CTA AAT CTA AGT  603
186  Lys Asp His Ala Phe Ser Gly Thr Cys Leu Asp Glu Leu Asn Leu Ser  201

604  GAC AAT TAC AAC TTA GAA AAA TTG CCG GAG AAA GTC TTC CAA GGA GCC  651
202  Asp Asn Tyr Asn Leu Glu Lys Leu Pro Glu Lys Val Phe Gln Gly Ala  217

652  ATC GGG CCT GTT GTT TTG GAT ATT TCA AGG ACA AGA ATC AGC TTC CTG  699
218  Ile Gly Pro Val Val Leu Asp Ile Ser Arg Thr Arg Ile Ser Phe Leu  233

670  CCA AGT CAT GGA TTA GAA TTC ATT AAG AAG CTA AGA GCG AGG TCT ACA  747
234  Pro Ser His Gly Leu Glu Phe Ile Lys Lys Leu Arg Ala Arg Ser Thr  249

748  TAC AAG TTA AAA AAA CTT CCT GAT GTA AAC AAA TTT AGA TCT TTG ATT  795
250  Tyr Lys Leu Lys Lys Leu Pro Asp Val Asn Lys Phe Arg Ser Leu Ile  265
          *
796  GAG GCA AAC TTC ACC TAT CCT AGC CAT TGC TGT GCA TTT ACA AAT CGG  843
266  Glu Ala Asn Phe Thr Tyr Pro Ser His Cys Cys Ala Phe Thr Asn Arg  281

844  AAA ACA CAA AAC ACA GAA TTT TAC CCA ATA TGT AGC ATG TCT CCG GCA  891
282  Lys Thr Gln Asn Thr Glu Phe Tyr Pro Ile Cys Ser Met Ser Pro Ala  297

892  AAG CAA GAC CTT GGT GAG CAG ACT GGC AAA AGG AAA CAC AGA CGA TCT  939
298  Lys Gln Asp Leu Gly Glu Gln Thr Gly Lys Arg Lys His Arg Arg Ser  313

940  GCA GCT GAA GAT TAT ATT TCC CAT TAT GGC ACG CGT TTT GGC CCG TTG  987
314  Ala Ala Glu Asp Tyr Ile Ser His Tyr Gly Thr Arg Phe Gly Pro Leu  329

988  GAG AAC GAA TTT GAC TAT GGC TTG TGC AAC GAA GTC GTT GAT TTT GTT 1035
330  Glu Asn Glu Phe Asp Tyr Gly Leu Cys Asn Glu Val Val Asp Phe Val  345

1036 TGC TCA CCC AAA CCT GAT GCC TTC AAT CCG TGT GAA GAT ATC ATG GGA 1083
346  Cys Ser Pro Lys Pro Asp Ala Phe Asn Pro Cys Glu Asp Ile Met Gly  361
                                          I
1084 TAC AAC GTG CTG AGA GTT CTG ATA TGG TTT ATC AAC ATT TTA GCT ATC 1131
362  Tyr Asn Val Leu Arg Val Leu Ile Trp Phe Ile Asn Ile Leu Ala Ile  377

1132 ACT GGG AAC ACC ACC GTC CTC ATT ATT TTA ATA AGC AGT CAA TAC AAA 1179
378  Thr Gly Asn Thr Thr Val Leu Ile Ile Leu Ile Ser Ser Gln Tyr Lys  393
                                          II
1180 CTC ACT GTA CCT CGT TTT CTA ATG TGC AAT CTT GCA TTT GCA GAT CTC 1227
394  Leu Thr Val Pro Arg Phe Leu Met Cys Asn Leu Ala Phe Ala Asp Leu  409

1228 TGT ATA GGT ATC TAT CTG TTG TTT ATT GCA TCA GTA GAT ATC CAG ACC 1275
410  Cys Ile Gly Ile Tyr Leu Leu Phe Ile Ala Ser Val Asp Ile Gln Thr  425

1276 AAA AGC CGG TAT TAC AAC TAT GCC ATA GAC TGG CAA ACC GGG GCA GGA 1323
426  Lys Ser Arg Tyr Tyr Asn Tyr Ala Ile Asp Trp Gln Thr Gly Ala Gly  441
                                       III
1324 TGC AAT GCT GCA GGA TTT TTT ACT GTT TTT GCA AGT GAA CTC TCA GTC 1371
442  Cys Asn Ala Ala Gly Phe Phe Thr Val Phe Ala Ser Glu Leu Ser Val  457

1372 TAC ACA CTG ACT GTG ATA ACT CTG GAA AGG TGG CAT ACC ATT ACC TAT 1419
458  Tyr Thr Leu Thr Val Ile Thr Leu Glu Arg Trp His Thr Ile Thr Tyr  473
                                                          IV
1420 GCC ATG CAA CTC AAC CGC AAG GTT CGA CTT CGG CAT GCT GTG ATC ATA 1467
474  Ala Met Gln Leu Asn Arg Lys Val Arg Leu Arg His Ala Val Ile Ile  489

1468 ATG GTT TTT GGC TGG ATG TTT GCT TTC ACG GTG GCA CTT CTT CCC ATA 1515
490  Met Val Phe Gly Trp Met Phe Ala Phe Thr Val Ala Leu Leu Pro Ile  505

1516 TTT GGC ATC AGC AGC TAC ATG AAG GTC AGC ATC TGT TTG CCC ATG CAC 1563
506  Phe Gly Ile Ser Ser Tyr Met Lys Val Ser Ile Cys Leu Pro Met His  521
                                                    V
1564 ATA GAA ACA CCG TTT TCT CAG GCT TAT GTT ATA TTT CTT TTA GTG TTG 1611
522  Ile Glu Thr Pro Phe Ser Gln Ala Tyr Val Ile Phe Leu Leu Val Leu  537

1612 AAT GTA CTT GCC TTT GTG ATC ATC TGC ATC TGC TAC ATC TGC ATC TAC 1659
538  Asn Val Leu Ala Phe Val Ile Ile Cys Ile Cys Tyr Ile Cys Ile Tyr  553

1660 TTT ACT GTG AGA AAC CCC AAT GTT ATC TCT TCA AAC AGC GAC ACC AAA 1707
554  Phe Thr Val Arg Asn Pro Asn Val Ile Ser Ser Asn Ser Asp Thr Lys  569
                                                VI
1708 ATT GCC AAG CGC ATG GCT ATA TTG ATC TTC ACA GAC TTC CTC TGC ATG 1755
570  Ile Ala Lys Arg Met Ala Ile Leu Ile Phe Thr Asp Phe Leu Cys Met  585

1756 GCA CCA ATA TCT TTT TTT GCA ATA TCA GCT TCT CTC AGG GTT CCT CTC 1803
586  Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala Ser Leu Arg Val Pro Leu  601
                                                    VII
1804 ATC ACA GTC TCC AAA TCC AAG ATC CTT CTG GTT TTG TTT TAC CCT ATT 1851
602  Ile Thr Val Ser Lys Ser Lys Ile Leu Leu Val Leu Phe Tyr Pro Ile  617

1852 AAT TCC TGT GCT AAC CCT TTC CTC TAT GCC ATT TTC ACA AAG ACT TTT 1899
618  Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala Ile Phe Thr Lys Thr Phe  633

1900 CGC AGG GAT TTC TTC ATT CTG TTG AGC AAG TTT GGT TGC TGT GAA ATG 1947
634  Arg Arg Asp Phe Phe Ile Leu Leu Ser Lys Phe Gly Cys Cys Glu Met  649

1948 CAA GCC CAG ATT TAC AGA ACA GAG ACT TCC TCA TCT GCT CAT AAT TTC 1995
650  Gln Ala Gln Ile Tyr Arg Thr Glu Thr Ser Ser Ser Ala His Asn Phe  665

1996 CAC ACA AGA AAT GGC CAT TAC CCT ACT GCA TCA AAA AAC AGT GAT GGG 2043
666  His Thr Arg Asn Gly His Tyr Pro Thr Ala Ser Lys Asn Ser Asp Gly  681

2044 ACT ATT TAT TCA CTG GTT CCT CTG AAT CAC TTG AAC TGA AATGCTTGCAT 2093
682  Thr Ile Tyr Ser Leu Val Pro Leu Asn His Leu Asn ***               693

2094 GAATTTGTGTCTGAGGCAGTGTGATAATCACTTTCAACTATTTGAATAATGACTTCAACATAG 2156

2157 CATGCAAACTTATTTTTTATGAGAAAACATTTCTATTTCCACTTTGCTTTTTTGCTCATCCTC 2219

2220 CGAGATGGCACATTATCTTCATTCGTTCTTGAAGGACAAAGAATGAAACTGCAGTATAGGTCA 2282

2283 AAGTGTCCTTGTAGAATTCTGCCCAGAAATAAACGAGCTTATAAATGCATATCACCTGCTATA 2345

2346 CAGGCAGAGATTCCCCTCAGTCTAGGAAGACGCTTATGAGAACGAAGTAATGGTTATATGTGG 2408

2409 TCAAAAAAGGGAGGGAAAAAAAATATATATATTTTTG                          2445
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B

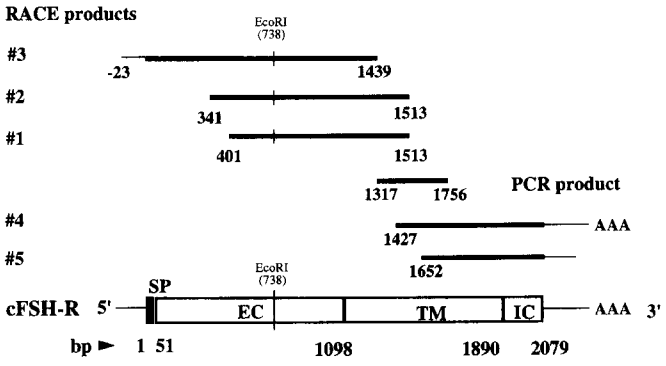

FIG. 1. **A**) Nucleic acid and deduced amino acid sequences for the partial cFSH-R cDNA. Nucleotides and amino acids are numbered to the left and right. Shown are 23 bases of the untranslated 5′ region and 5′ ATG start codon (nucleic acid +1), followed by 2079 bases of open reading frame. The seven predicted transmembrane-spanning domains are single-underlined (numbered above with roman numerals); a 17-amino acid putative leading signal peptide is double-underlined. Putative *N*-linked glycosylation sites are marked above with an asterisk. **B**) Schematic diagram of the composite cFSH-R cDNA, 5′- and 3′-RACE products, and original PCR product derived from degenerate oligonucleotide primers.

ilar to the size previously reported for the FSH-R sequences of the rat (675 amino acids [23]) and bovine (678 amino acids [24]) species. Not unexpectedly, the predicted transmembrane region is the most highly conserved domain when the two chicken gonadotropin receptors are compared, as well as when the cFSH-R sequence is compared to those from mammalian species. Ten of 11 cysteine residues (including 5 of 5 cysteines located near the junction between the putative extracellular and transmembrane domains) and two cysteine residues (Cys^{442} and Cys^{517}) that reportedly form a disulfide bridge between the first and second extracellular loops are conserved in the cFSH-R compared to the rat and bovine FSH-R cDNA [20]. Cysteine residues are generally thought to be required for maintain-

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c FSH-R     MSLDLTCLLI LLASCSGCQH HTCLCEGRIF ICQEIKVVQL PRDIPTNATE LRFVLTKMRV   60
r FSH-R      A L VS  A F GTG   H  WL H SN V  L  DS  TEI  T L R  I
b FSH-R      A L VA  A F SLG   H  RL H SNGV  L   S  TEI  S L RD V


c FSH-R     IPKGAFTGLH DLEKIEISQN DALEIIEANV FSSLPKLHEI RIEKANKLMK IDQDAFQHLP  120
r FSH-R        S A FG             V  V   D    N              N LY  NPE   N
b FSH-R          S FG             V  V        N              N LY   P    N
c LH-R                    I     S  S  R   SA  D   A S   L LNTKN LH  EDG  RN


c FSH-R     SLRYLLISNT GLSFLPVVHR VHSFQKVLLD VQDNIHIRTI ERNTFMGLSS ESVTLRLNKN  180
r FSH-R                 IKH  A  K IQ L       I    N HIV A  S     F    I W S
b FSH-R     N           IKH  A  K IQ L       I    N H V   S     F   M VW S
c LH-R      R K  S C    IIEF DLTQ IF SAHFI E LC  LRMT   PQ A Q M N   L  K Y


c FSH-R     GIQEIKDHAF SGTCLDELNL SDNYNLEKLP EKVFQGAIGP VVLDISRTRI SFLPSHGLEF  240
r FSH-R       E  HNC   N  Q          N   E   ND     S    I        KV HS  N    N
b FSH-R          HNC   N  Q          S   E   ND     S    I           RS   Y   N
c LH-R       FED HS    N  K NQ I  K  K  RRIH NDALR  T   D     S AL ES   Y   A


c FSH-R     IKKLRARSTY KLKKLPDVNK FRSLIEANFT YPSHCCAFTN RKTQNTEFYP ICSMSPAKQD  300
r FSH-R     L          R     NLD   VT M  SL          A  L R IS LH    NK ILR
b FSH-R     L     K    R     SLE   VT V  SL          A  WRR TSDLH    NK ILR E
c LH-R       QV N M S  S  R  PLD   S  L  VL          Q  LR E-K-QNS --LL-SISDN


c FSH-R     LGEQTGKRKH RRSAAEDYIS HYGTRFGPLE NEFDYGLCNE VVDFVCSPKP DAFNPCEDIM  360
r FSH-R     IDDM QIGDQ  V LID -EP S  KGSDMMY      D        VT
b FSH-R     VDDM QA GQ  V L   DEP S AKG DVMY S    D        VT   E
c LH-R      FSK CEST-M  KPTS VFYR DASS-NTSLV G-QQKNT TH LKILT T E           L


c FSH-R     GYNVLRVLIW FINILAITGN TTVLIILISS QYKLTVPRFL MCNLAFADLC IGIYLLFIAS  420
r FSH          I         S            VV TT                              L
b FSH-R      DDI         S         IL  V   T                              L
c LH-R        SF             LA   FI  LV  T  H             S   F  M L   L


c FSH-R     VDIQTKSRYY NYAIDWQTGA GCNAAGFFTV FASELSVYTL TVITLERWHT ITYAMQLNRK  480
r FSH          H   Q H              D                    A          H    EC
b FSH-R       VH  TE H              D                    A          H    EC
c LH-R        A  SGQ    H       S   ST                      I            D


c FSH-R     VRLRHAVIIM VFGWMFAFTV ALLPIFGISS YMKVSICLPM HIETPFSQAY VIFLLVLNVL  540
r FSH        Q    ASV   L  T   AA   F                   D DS L  L   MA
b FSH-R      Q    AS   LV  I   A    F                   D DS L  L   MS
c LH-R      L      P   LG  V SILI  V  LL V              D   GL     ILLI M   I


c FSH-R     AFVIICICYI CIYFTVRNPN VISSNSDTKI AKRMAILIFT DFLCMAPISF FAISASLRVP  600
r FSH          V  G  T H  L     T IV  S          T                        K
b FSH-R        V  G  T H  L       IT  S          M                        K
c LH-R        LV  A    K  VA Q  E LVAA K                T              AIK


c FSH-R     LITVSKSKIL LVLFYPINSC ANPFLYAIFT KTFRRDFFIL LSKFGCCEMQ AQIYRTETSS  660
r FSH            A                            N              Y
b FSH-R                                       N              Y V    T  S
c LH-R         TN          V                  A Q    L  M  L   KSR  EL  V-NYF


c FSH-R     SAHNFHTRNG HYPTASKNSD GTIYSLVPLN HLN*                              693
r FSH       AT    A KS  CSS PRVTN --S V       SS
b FSH-R     T     P     C P PRVTN  SN T I  R   A
c LH-R          YTPNCK SSAPGPSKAS QALLL SASE K C
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FIG. 2. Comparison of the deduced c-FSH-R amino acid sequence with the rat and bovine FSH-R, and cLH-R, sequences. Blank spaces within aligned sequences represent residues similar to the cFSH-R sequence; hyphen (-) indicates corresponding residues deleted from respective sequence. Three additional residues (QPK) exist within the chicken LH-R sequence between amino acids 352 and 353 from the chicken FSH-R sequence.

ing the appropriate tertiary structure required for biological function. Thus much of the extracellular binding region would be predicted to be similar in conformation among species.

Consistent with virtually all G protein-coupled receptors thus far characterized, the aspartic acid residue within TM II (Asp^{408}), the asparagine residue within TM VII (Asn^{618}), and proline residues within TM IV, TM VI, and TM VII ($Pro^{504, 587, 616, 623}$) are conserved within the cFSH-R compared to the rat FSH-R [23]. Also indicative of this family of protein receptors as a whole is the highly conserved acidic-Arg-aromatic triplet motif (ERW, amino acids 466–468; Fig. 1) located in the second intracellular loop, which is proposed to be the site of interaction with G proteins [25].

Northern blot analysis of tissues from the hen ovary detected a single transcript corresponding to approximately 4.3 kb for the cFSH-R; this size is larger than that recently determined for the cLH-R mRNA transcript (3.0 kb [15]). On the basis of published results from the rat, selective expression of the cFSH-R mRNA within only ovarian tissues is not unexpected for this gonadotropin receptor [26]. On the other hand, the finding of a single cFSH-R transcript is in contrast to the expression of at least two major tran-

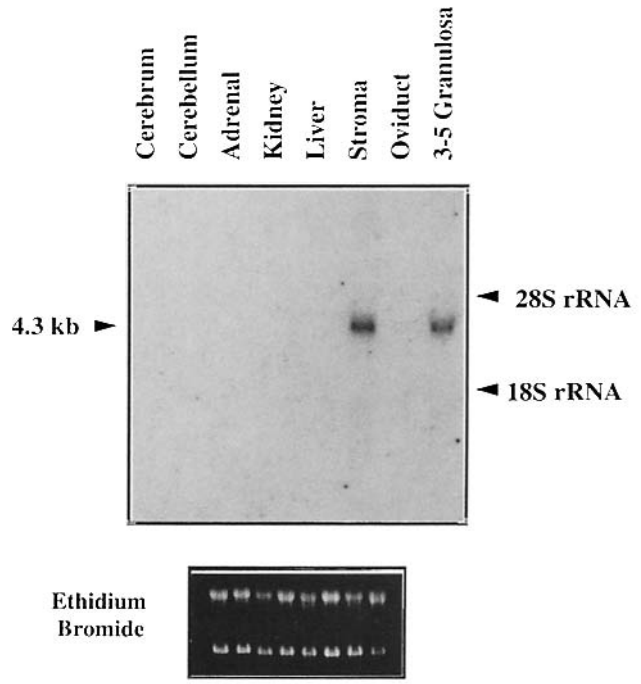

FIG. 3. Northern blot of representative tissues showing selective expression of the 4.3-kb FSH-R mRNA transcript in ovarian (stromal and 3- to 5-mm follicle granulosa) samples. The ethidium bromide-stained gel, depicting 28S and 18S rRNA, is pictured below. This experiment was repeated once, with similar results.

scripts (approximately 2.5 and 7.0 kb) and a variable number of minor transcripts found in mRNA extracts from the mammalian ovary (e.g., 1.6, 3.3 and 3.8 kb for bovine [24]; 1.8 and 4.2 kb for rat [26]). Studies in which the mammalian FSH-R protein has been expressed in vitro have determined that the 2.5-kb band codes for a complete and functional receptor [23, 27]; thus it is concluded that the 4.3-kb cFSH-R transcript is in excess of a size required to encode the functional receptor. While we have recently suggested that cLH-R mRNA may be expressed as both a full-length as well as an alternatively spliced, truncated form of protein corresponding to the extracellular domain [15], there is as yet no evidence for the expression of an alternatively spliced form of the cFSH-R gene. Similarly, isoforms of the FSH-R representing truncated forms of the

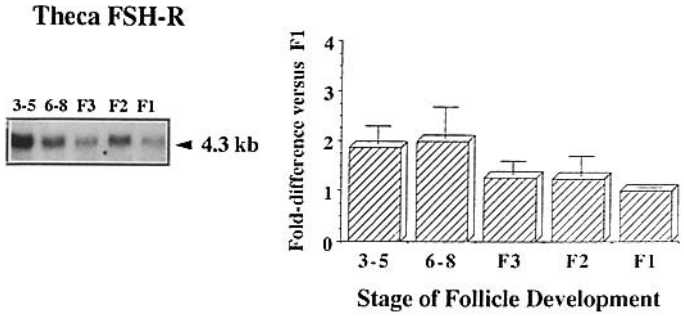

FIG. 4. Representative Northern blot of cFSH-R mRNA from theca tissue during follicle development (left) and summary of scanning data (right; mean densitometric units expressed as fold difference vs. F1 ± SEM; data from 4 replicate autoradiographs). F1, F2, and F3 indicate the largest, most mature; the second largest; and the third largest preovulatory follicles, respectively; 6–8 and 3–5 refer to the diameters of follicles, in millimeters.

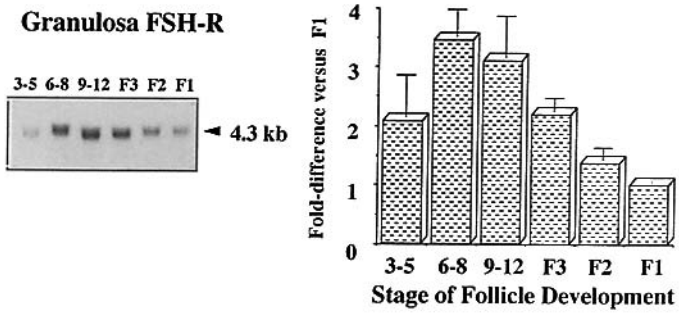

FIG. 5. Representative Northern blot of cFSH-R mRNA from granulosa tissue during follicle development (left) and summary of scanning data (right; mean densitometric units expressed as fold difference vs. F1 ± SEM; data from 4–6 replicate autoradiographs). Hyphenated numbers refer to diameters of follicles, in millimeters.

receptor have been identified in ovine and monkey testes [28, 29], though none have yet been found to be expressed in mammalian granulosa cells [24].

Binding of FSH to hen theca tissue has been identified by radioreceptor assay [13] and immunohistochemical [30] methods, and levels of FSH binding are reported to slightly decrease during follicle maturation. Both methods indicate a relatively limited capacity of theca to bind mammalian FSH. The comparatively lower levels of cFSH-R mRNA in theca vs. granulosa and progressively declining levels of transcript in the theca layer throughout follicle development are consistent with these previous reports (Fig. 4).

Kowalski et al. [11] reported that relatively high doses of cFSH (100–200 ng/ml) and rhFSH (1–2 IU/ml) stimulated progesterone, androgen, and estrogen production from theca cells prepared from 6- to 8-mm follicles, while Robinson et al. [7] concluded that preparations of bovine, turkey, or chicken FSH had little effect on steroidogenesis in whole prehierarchical follicles or theca cells from hierarchal follicles. On the other hand, various preparations of ovine, bovine, turkey, and chicken LH all have a pronounced stimulatory effect on steroid production in whole prehierarchical follicles or in stromal tissue and theca cells at all stages of follicle development [7, 11, 18]. Thus, while FSH may play a supportive role in making steroid precursors available for steroid biosynthesis or maintaining expression of steroidogenic enzymes, the physiological significance of FSH in directly stimulating steroid production within the theca layer remains in question.

Gilbert et al. [31] detected FSH labeling in granulosa cells from prehierarchical and hierarchal follicles by immunohistochemical methods, and Ritzhaupt and Bahr [14]

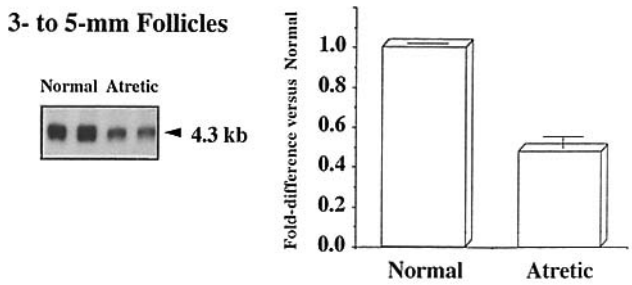

FIG. 6. Representative Northern blot of cFSH-R mRNA from 3- to 5-mm morphologically normal and atretic follicles (left) and summary of scanning data (right; mean densitometric units expressed as fold difference vs. normal follicles ± SEM; n = 4).

reported specific binding of porcine FSH within the F1 follicle granulosa layer by radioreceptor assay. Whereas measurable levels of FSH-R mRNA are expressed within the granulosa layer throughout follicular development (Fig. 5), it has yet to be unequivocally established to what extent the FSH-R is biologically active in granulosa cells from hierarchal (9- to 12-mm through F1) follicles. For instance, while rhFSH stimulates progesterone secretion from 9- to 12-mm follicles [8], highly purified preparations of bovine, chicken, and turkey FSH fail to initiate detectable progesterone production in granulosa cells from the three largest (F3 to F1) preovulatory follicles [7, 8]. On the other hand, Krishnan et al. [4] concluded that cFSH stimulates progesterone production from F1 follicle granulosa cells, in vitro, although at relatively minor levels compared to the effects of ovine LH.

Taken together, some of the discrepancies in the relative amount of FSH bioactivity (limited vs. none) in preovulatory follicle granulosa cells may be attributed to species differences within the FSH protein (e.g., ability to bind to the cFSH-R, as discussed above), variability in the purity of the FSH preparation (i.e., amount of contamination with LH [7]), and/or the stage of follicle development investigated [32]. For instance, mammalian species are generally proposed to contain three (e.g., bovine [24], rat [23], ovine [33]), or four (human [16, 27], *Macaca* [28], equine [34]) glycosylation sites (important in peptide ligand recognition) within the extracellular binding domain of the FSH-R; yet only one of three glycosylation sites predicted within the cFSH-R amino acid sequence are conserved compared to the rat, ovine and bovine sequences. It is also conceivable that while the FSH-R protein may in fact be present within the membranes of preovulatory follicle granulosa cells, albeit at lower levels than in prehierarchical follicles ([14]; Fig. 5), such receptors may not be fully coupled to an active adenylyl cyclase enzyme or they may be functionally coupled to alternative second messenger pathways [31, 35]. Nevertheless, it is clear that LH is the more important gonadotropin in promoting granulosa cell steroidogenesis in hierarchal follicles.

By contrast, the effects of rhFSH on granulosa cells from 6- to 8-mm follicles have been well-documented. For instance, rhFSH induces the expression and activity of cytochrome $P450_{scc}$ and $P450_{17\alpha OH}$ enzymes in prehierarchical follicle granulosa cells at a time when cFSH-R mRNA levels are highest and cLH-R mRNA is virtually undetectable [9, 10, 15]. Furthermore, FSH treatment attenuates the onset of apoptotic cell death in prehierarchical follicle granulosa cells incubated in serum-free medium [12], and induces fibronectin production [36]. These effects are presumably mediated via activation of the adenylyl cyclase/protein kinase A intracellular signaling pathway [12, 36]. The positive effects of FSH on granulosa cell viability are consistent with a recent report, which concluded that FSH is a major survival factor for maintaining early antral follicles of the rat [37].

Finally, it is not unexpected to find that cFSH-R mRNA levels decrease in atretic compared to normal follicles (Fig. 6), as similar results have been reported from the rat ovary [38]. Follicle atresia occurs almost exclusively in prehierarchical follicles [39], and accordingly, follicles selected for this experiment were from 3- to 5-mm in diameter. It has previously been reported that hen atretic follicles exhibit extensive oligonucleosome formation characteristic of apoptosis, and this occurs almost exclusively within the granulosa layer [12, 40]. Thus, we would predict that, in similarity to the decrease in cLH-R transcript recently reported for atretic compared to healthy follicles [15], much of the decrease in cFSH-R transcript levels is specific to granulosa cells. The decrease in gonadotropin receptor mRNA levels may account for the loss of gonadotropin-induced steroidogenesis in atretic follicles, despite the previous observation that both 3β- and 17β-hydroxysteroid dehydrogenase activities persist until the latter stages of follicle resorption [41]. Given that all atretic follicles in these experiments were selected on the basis of morphology, and thus were undoubtedly collected at various stages of the atretic process, it is not possible to speculate on whether the loss of cFSH-R mRNA is a cause or simply an effect of apoptosis and atresia.

In summary, expression profiles of the cFSH-R mRNA transcript are consistent with a role for FSH and the FSH-R in initiating, or at least facilitating, final differentiation of this follicle cell layer [9, 10], as well as in preventing prehierarchical follicle granulosa cells from succumbing to programmed cell death [12]. This shift from an FSH-dominant to an LH-dominant environment in granulosa cells during hen follicle development is not unlike that which occurs in mammalian granulosa cells during the transition from the preantral and antral stages to the preovulatory stage of differentiation [35].

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