

Characterization of a Chicken Luteinizing Hormone Receptor (cLH-R) Complementary Deoxyribonucleic Acid, and Expression of cLH-R Messenger Ribonucleic Acid in the Ovary¹

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ABSTRACT

Studies were conducted to characterize the chicken ovarian LH receptor (cLH-R) cDNA and to evaluate expression of cLH-R mRNA in follicles at different stages of development. A total of 1.89 kb of nucleic acid sequence corresponding to the cLH-R (1.79 kb of the predicted coding region) was isolated by a combination of reverse transcription-polymerase chain reaction and cDNA library screening techniques. Also of interest was the finding that two of three positive clones isolated from the hen ovarian cDNA library contained an 86-bp insert located in the extracellular domain within 69 bp of the putative transmembrane domain. This insert contains an inframe TGA stop codon, suggesting that an alternatively spliced transcript results in translation of a truncated protein corresponding to the extracellular domain of the cLH-R.

Considering all protein domains thus far characterized, the deduced amino acid sequence of the cLH-R shares 73.2% and 74.2% identity with the rat and porcine LH-R sequences, respectively, with highest homology occurring within the seven transmembrane spanning regions (86–88% identity vs. mammalian sequences). Northern blot analysis determined that cLH-R mRNA levels in the theca layer tend to increase through follicle development to the second largest (F2) preovulatory follicle ($p = 0.084$), and to decrease in the largest preovulatory (F1) follicle ($p < 0.02$ vs. F2). By comparison, cLH-R mRNA levels are nondetectable (by Northern blot analysis) in granulosa cells from prehierarchal (3–8-mm diameter) follicles. Constitutive expression of cLH-R mRNA in granulosa cells is first detectable at the 9–12-mm diameter stage of follicle development, and levels are further increased in cells from large preovulatory (F1, F2, and F3) follicles ($p < 0.01$ vs. 9–12-mm stage). Collectively, these results are consistent with previous observations that granulosa cells from prehierarchal follicles fail to produce cAMP or steroids in response to short-term incubation with ovine LH, *in vitro*, and that granulosa cells acquire LH responsiveness only subsequent to follicle selection into the rapid growth phase.

INTRODUCTION

While much information has been published concerning gonadotropin-induced steroid production in granulosa and theca tissue from hierarchal (for review, see [1]) and non-hierarchal [2–4] follicles of the hen, little is known about the gonadotropins and gonadotropin receptors that mediate this response. For instance, while the cDNA sequences for the avian LH α and β subunits have been characterized [5–7], there is still no information available concerning the chicken FSH β subunit.

With respect to biological actions of gonadotropins within the hen ovary, FSH binding to granulosa and theca cell membranes has been reported to decrease as the stage of follicle development approaches the time of ovulation [8, 9]. The marked reduction in FSH binding to granulosa cells of the largest preovulatory (F1) follicle is consistent with the inability of FSH to induce measurable progesterone production from these cells *in vitro* [10]. Nevertheless, neither the FSH receptor cDNA sequence nor the protein structure has yet been reported. There is even less information regarding the LH receptor (LH-R) within the avian ovary, and reports have been essentially limited to the immunocytochemical detection and localization of the receptor protein on chicken granulosa and theca cells [11, 12], the internalization of LH complexes in interstitial tissue from embryo ovaries [13], and a preliminary evaluation of LH-R binding in chicken granulosa cells by conventional radioreceptor assay [14]. As in the mammalian receptor, there is considerable evidence that after interaction of LH with the LH-R, the second messenger signaling mechanisms in the hen ovary include activation of the adenylyl cyclase/protein kinase A and phospholipase C/phosphatidylinositol systems [15, 16]. The primary objectives of the present studies were to isolate and characterize a partial cDNA for the chicken (c) LH-R, and subsequently to evaluate the expression and localization of cLH-R mRNA in granulosa and theca tissue during follicle development.

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 exposed to a photoperiod of 15L:9D, with lights-on at midnight. Individual laying cycles were monitored by the daily timing of oviposition. Hens were killed approximately 16–18 h prior to a mid-sequence ovulation by cervical dislocation. 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 and 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.

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Isolation and Characterization of a Partial cLH-R cDNA

Given that F1 follicle granulosa cells are highly responsive to LH (see [1]), it was reasoned that this cell type should express relatively high levels of cLH-R mRNA. Thus, total RNA was isolated from F1 granulosa cells and reverse-transcribed into first-strand cDNA by use of oligo(deoxythymidine)-primer and recombinant M-MLV reverse transcriptase in the presence of deoxynucleotides (Perkin-Elmer, Norwalk, CT). Sequences for oligonucleotide primer pairs were based on the recently published quail partial LH-R cDNA sequence [17] (site of nucleotides corresponding to published quail sequence in parentheses): forward primer, ATCTCTTGACATTGAGGACGG (bp 23–44); reverse primer, TAGGTCAGAACAGCTTCCAGCAGG (bp 518–495).

First-strand cDNA was subjected to 35 cycles of PCR amplification using GeneAmp core reagents (Perkin-Elmer; 2-min denaturation at 94°C for the first cycle and 1 min per cycle thereafter, 1-min annealing at 55°C, and 1-min extension at 72°C for the first 34 cycles, 7-min extension on the final cycle). The amplified 495-bp PCR product was resolved through a 1.2% agarose gel, isolated, purified (GeneClean; Bio 101, La Jolla, CA), and subcloned into the pCRII plasmid vector (Invitrogen, San Diego, CA) for large-scale plasmid preparation and nucleic acid sequence analysis.

Subsequently, a complete chicken ovarian cDNA library (prepared in λ gt11 vector by Stratagene, La Jolla, CA) was screened by using the cLH-R PCR product, random-prime labeled with [α -³²P]dCTP (Megaprime DNA Labelling System, Amersham Corp., Arlington Hts., IL), as a cDNA probe. The labeled probe was purified by use of Centri-Sep columns (Princeton Separations, Inc., Adelphia, NJ). Three different positive clones were identified after primary, secondary and tertiary screens from an initial estimated 5×10^5 plaques. Complementary DNA inserts from each of the three plaques were amplified by PCR with primers derived from the λ gt11 sequence adjacent to the site of insertion. All three PCR products were subsequently subcloned into pCRII plasmid vector, amplified by plasmid preparations, and then sequenced by the dideoxychain termination method using ³⁵S-dATP (Amersham Corp.) and the Sequenase version 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH). Sequence data were assembled and analyzed by means of 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 (9–12-mm follicles), and prehierarchal (6–8-mm and 3–5-mm) follicles as previously described [2]. In addition, morphologically normal and atretic follicles (3–5-mm diameter) were collected and processed without separating granulosa and theca layers. Atretic follicles were identified on the basis of the presence of follicle haemorrhagia, collapsed morphology, and opaque appearance. Total cellular RNA from tissue at each stage of development was isolated by use of Trizol Reagent (Gibco-BRL, Richmond, CA), 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 electrophoresed in 1% agarose gels in the presence of 6% formaldehyde, then transferred

to nitrocellulose Nitro ME membranes (MSI, Westboro, MA) by capillary action overnight.

Antisense RNA probe was synthesized by in vitro transcription from the linearized (*Bam*HI) cLH-R PCR product plasmid template using T7 RNA polymerase, [α -³²P]CTP (3000 Ci/mmol; Amersham), and the Gemini II Riboprobe Core System (Promega, Madison, WI). Membranes were prehybridized at 65°C for 4 h in a buffer containing 50% formamide, 5-strength Denhardt's solution, 0.5 mg/ml yeast tRNA, 1 mM EDTA, 50 mM NaPO₄, and 0.8 M NaCl. Hybridizations were conducted overnight in an identical buffer containing the cRNA probe (specific activity approximately 10⁹ cpm/ μ g; 2×10^6 cpm/ml buffer), and membranes were subsequently washed under highly stringent conditions (double-strength sodium chloride-sodium citrate solution [SSC; single-strength SSC = 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0] containing 0.1% sodium dodecyl sulfate [SDS] for 10 min followed by 0.1-strength SSC plus 0.1% SDS for 15–30 min) at 65°C. Membranes were exposed to autoradiographic film at –70°C for 1–5 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 evaluation of ethidium bromide-stained 28S and 18S bands of ribosomal RNA. The rationale for this analysis is that β -actin mRNA, a common housekeeping gene, is not expressed at consistent levels during hen follicle development (unpublished observations). 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 versus the appropriate control) are presented as the mean \pm SEM of scanning data from replicate experiments. Data were analyzed by a one-way analysis of variance, and significant interactions were determined by use of Student's *t*-test or the Newman-Keuls multiple range test.

RESULTS

Identification and Characterization of cLH-R cDNA

A 495-bp product was generated from an F1 granulosa cDNA template by PCR. After sequence analysis, the chicken PCR product (excluding primer sequence) was determined to have 98% amino acid identity (100% homology considering conservative substitutions; [18]) compared to the recently published N-terminal region of the quail LH-R sequence (Table 1). When this PCR product was used as a cDNA probe to screen a complete chicken ovarian cDNA library, three positive clones were identified from approximately 5×10^5 plaques. Collectively, the three clones (cLH-R#3, cLH-R#4, cLH-R#8) plus the PCR product were determined to span over 75% of the extracellular domain corresponding to the mammalian LH-R, plus the entire transmembrane spanning and intracellular domains (Fig. 1).

The deduced amino acid sequence for the cLH-R contained four potential *N*-linked glycosylation sites within the extracellular domain and two potential protein kinase C consensus phosphorylation sites within the intracellular domain (Fig. 2). A single area of sequence (at bp 651) proved

TABLE 1. Identity of the deduced cLH-R amino acid sequence compared to rat, porcine, and quail LH-R sequences, considering the extracellular (EC), transmembrane (TM), and intracellular (IC) domains.

Sequence	% vs. cLH-R ^a			Overall
	EC	TM	IC	
Rat LH-R	64.8 (81.0)	86.4 (95.8)	53.9 (72.4)	73.2 (86.4)
Porcine LH-R	67.4 (83.7)	87.9 (96.2)	48.1 (64.9)	74.2 (86.9)
Quail LH-R	97.6 (100)	NA ^b	NA ^b	—

^a Numbers outside parentheses represent the percentage of exact matches; numbers in parentheses consider conservative substitutions.

^b NA, quail cDNA sequence not available.

difficult to resolve, even after the substitution of dITP for dGTP; therefore, the glycine residue corresponding to amino acid 217 remains speculative. Considering all domains thus far characterized, the cLH-R showed 73.2% and 74.2% identity (86.4% and 86.9% homology considering conserved amino acid substitutions) to the corresponding regions of the rat [19] and porcine [20] LH-R, respectively (Table 1).

Two of the three positive cDNA clones isolated from the chicken ovarian library (cLH-R#4 and cLH-R#8) contained an insert (the full insert determined from clone cLH-R#4 is 86 bp in length) not previously described in LH-R mRNA from mammalian species (Fig. 2). The insert sequence, located within the extracellular domain 23 AA upstream from the predicted transmembrane domain, encoded for 17 AA followed by an inframe TGA stop codon. Presumably, this alternatively spliced transcript results in the translation of a truncated cLH-R protein corresponding to all but the terminal 23 AA of the extracellular receptor domain. The 51-bp coding region within the insert had no detectable homology to sequence from any other LH-R or G-protein-coupled receptor cDNA available from GenBank. With the exception of this insert, the remaining sequence among all clones and the PCR product was identical, with the exception of six single base substitutions, none of which are related to the insert.

Chicken LH-R mRNA during Follicle Development

Northern blot analysis of cLH-R mRNA in theca tissue detected a predominant 3.0-kb transcript throughout follicle development; levels tended to increase through development to the F2 stage ($p = 0.084$), then decrease in the F1 follicle ($p < 0.02$ vs. F2 theca, with use of a post hoc paired *t*-test; Fig. 3). The cLH-R mRNA transcript was not de-

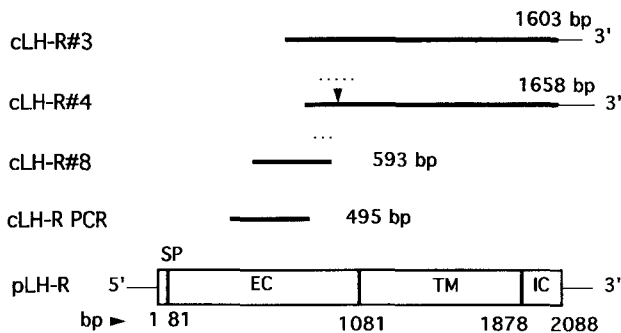


FIG. 1. Schematic diagram of cLH-R PCR product plus chicken cDNA library clones aligned to porcine (p) LH-R cDNA. EC, extracellular domain; TM, transmembrane spanning domain; IC, intracellular domain, SP, secretory peptide. Dotted lines represent the insert that encodes the TGA stop codon (represented by arrowhead).

1	AT CTC TTG CAC ATT GAG GAC GGA GCA TTC AGA AAC CTT CCA AGG CTA AAA TAT TTG AGC	59
1	L L H I E D G A F R N L P R L K Y L S	19
60	ATC TGT AAC ACC GGA ATA ATA GAA TTC CCA GAC TTG ACG CAG ATC TTC TCC TCA GAA GCT	119
20	I C N T G I I E F P D L T Q I L K S E A	39
120	CAT TTT ATT TTG GAG CTG TGT GAC AAC TTG CGT ATG ACA ACC ATA CCT CAA AAT GCC TTC	179
40	H F I L E L C D N L R M T T I P Q N A F	59
180	CAG GGC ATG AGC AAC GAA TCG CTG ACA CTC AKA CTT TAT AAA AAT GGA TTT GAA GAT ATC	239
60	Q G M S N E S L T L R L L T A T T A A T G A D I	79
240	CAC AGC CAT GCC TTC AAT GGG ACC AAG CTA AAT CAA TTG ATT CTG AAG GAC AAC AAA AAC	299
80	H S H A F M G T K L N Q L I L K D N K N	99
300	CTC AGG CCG ATA CAC AAC GAT GCC CTG AGA GGG GCC ACC GGG CCA GAC CTC CTG GAT ATT	359
100	L R R I H N D A L R G A T G P D V L D I	119
360	TCT TCA ACG GCA CTG GAG TCC CTG CCF AGC TAT GGG CTG GAG GCC ATC CAG GTC CTT AAT	419
120	S S T A L E S L P S Y G L E A I Q V L N	139
420	GCA ATG TCA TCG TAC TCA TTG AAG AGG CTG CCG CCT GTG AAT AAA TTC AGC AGC CTG CTG	479
140	A M S S Y S L K R L P P L D K F S S L L	159
480	GAA GCC GTT CTG ACC TAC CCC AGC CAC TGC TGT GCT TTT CAA AAG GGC AGC GAA AAG	539
160	E A V L T Y E S H C C A E Q N L R T E K	179
540	CAA AAT TCC TTG CTT TCC ATT TCT GAC AAC TTC TCC AAA CAG TGT GAA AGC ACA ATG AGG	599
180	Q N S L L S I S D N F S K A Q C E S T M R	199
600	AAA CCA ACT AGT GAA GTA TTT TAC AGA GAT GCT TCT TCC AAC ACA TCG CTG GGC CAG CAG	659
200	K P T S E V F Y R D A S S N T S L G Q Q	219
660	AAA AAC ACA TGT ACC CAC TTG AAA CAG C C C C A G A T A C T A C T C C A G A A C C A	719
220	K N T H L K Q P K I L T C T P E	239
720	GAC GCC TTT AAT CCG TGT GAA GAC ATC CTA GGA TAC AAG TTT CTC AGG GTC CTG ATC TGG	779
240	D A F C C E D I L G Y G V D A Q T S G C Q Y Y	259
780	TTG ATA AAC ATC CTT GCC CTT GCT GGC AAC TTC ATT CTG CTC CTC GTC CTC ATA ACC ACC	839
260	F I N I L A L A G N F I V L L V L I T S	279
840	CAC TAC AAG CTC ACG GTT CCT CGC TTG CTC ATG TGC AAC CTC TCC TTT GCT GAT TTT TGC	899
280	H Y K L T V P R F L M C N L S F A D F C	299
900	ATG GGG CTT TAC TTG CTG CTC ATT GCT TCG GTG GAT CAG ACA ACG GGG CAG TAC TAC	959
300	M G C L Y L L L I A S V D A Q T S G C Q Y Y	319
960	AAC CAC GCC ATA GAC TGG CAA ACA GCG AGC GGC TGC ACC ACT GCT GGC TTT TTC ACC GTC	1019
320	N H A I D W Q T G S G C S T A G F F T V	339
1020	TTT GCA AGC GAG CTC TCC GTC TAC ACA CTA ACA GTC ATT ACC ATA GAA AGG TGG CAT ACC	1079
340	F A S E L S V Y T L T V I T I E R W H T	359
1080	ATC ACC TAC GCC ATG CAG CTG GAC CGA AAG CTA CCG CTG GGC CAC GGC GTC CCC ATC ATG	1139
360	I T Y A M Q L D R K L R L R H A V P I M	379
1140	CTT GGT GGC TGG CTG TTC TCC ATC CTG ATA GCG GTG CTG CCG CTA CTG GGG GTC AGC AGC	1199
380	L G G W V F S I L I A V L L L G V S S	399
1200	TAC ATG AAG GTC AGC ATC TGT TTG CCC ATG GAT ATT GAA ACG GGG CTT TCC CAA GCC TAG	1259
400	Y H K V S I C L P M D I E T G L Q A Y	419
1260	ATA TTG CTG ATC TTA ATG CTC AAC GTS APA GCC TTC CTT GTC APT TGT GCT TGT TAC ATC	1319
420	I L L I L M L N V I A P F L V I C A C Y I	439
1320	AAG ATA TAC GTC GCC CTG CAG AAC CCT GAG CTG GTA GCC ACC AAT AAA GAC ACC AAG ATT	1379
440	K I Y V A V Q N P E L V A C A N K D T K I	459
1380	GCC AAG AGA ATG GGC ATA CTG ATC TTC ACG GAT TTT ACC TGC ATG GCC GCC ATC TCC TTG	1439
460	A K R M A I L I F T D F T C M A P I S F	479
1440	TTT GGC ATA TCT GCT GCC ATT AAG GTA CCT CTC ATC ACA GTG ACG AAC TCC AAG ATT CTG	1500
480	F A I S A A I K V P L I T V T N S K I L	499
1500	CTG GTT TTA TTC TAC CCA GTC AAC TCC TGC GCA AAC CCA TTT CTG TAT GCC ATT TTC ACT	1560
500	L V L F Y P V N S C A N P F L I T T	519
1560	AAA GCA TTC CAA AGA GAT TTC TTC CTG ATG AGC AAA CTA GGT TGC TGT AAG AGC CGA	1620
520	K A F Q R R D F F L L M S K L G C C K S R	539
1620	GCA GAG CTC TAT CGA CTG AAC TAC TTC TCT TCT TAC ACC CCC AAC TGC AAA AAT GCC AGC	1680
540	A E L Y R V N Y F S A Y T P N C K N G S	559
1680	TCA GCC CCA GGC CCC AGT AAA GCC TCA CAG GCA TTG CTG CTT TTG TCA GGT TCA GAG AAG	1740
560	S A P G P S K A S Q A L L S A S E K	579
1740	CTG TGC AAA ACA AGA CGC TCC ACA AAG AAA AGC CAG CCA GAG TGC CAG TAG GTGCTGTCC	1800
580	L C E T E E E T K K S Q P E C Q ***	595
1800	TCTATGGTGC CAGCTGCAGT TATTCTACC AAGTAGGAT TGTTCGTAT CTTGACATC TCACCCTAAA	1870
1870	TAAATCATC AGTCACTCGT GCCG	1894

INSERT (between bases 687-688)
 1 GTG AAG AAG CCT TCC CTT ACA GCT ACT CAA CTA TTT TTT ATG AGG ATG AAA TGA CCG GCT 60
 1 V K K F S L T A T Q L F F M R H R *** 17
 61 TTG ACT TTG AGT ATG ACT TCT GTC AG 86

FIG. 2. Nucleic acid and deduced amino acid sequences for partial cLH-R cDNA. The location of the insert (between bases 687 and 688) is noted. Single underlines indicate the seven predicted transmembrane-spanning domains. Double underlines indicate the putative N-linked glycosylation sites, and single lines indicate two overlapping protein kinase C consensus phosphorylation sites. At the bottom is nucleic acid sequence for 86-bp insert together with deduced amino acid sequence and stop codon.

etectable in tissue from the shell gland (data not shown). By comparison, there were marked changes in granulosa cell cLH-R mRNA levels during follicle development (Fig. 4). The transcript was nondetectable in granulosa collected from 3-5-mm and 6-8-mm follicles as determined by Northern blot analysis, and was first evident during devel-

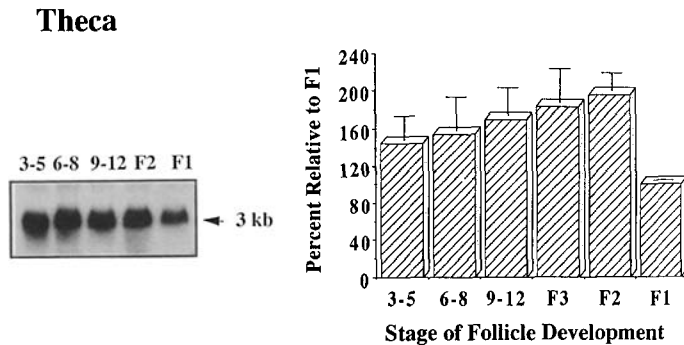


FIG. 3. Representative Northern blot of cLH-R mRNA from theca tissue during follicle development (excluding RNA from F3 follicle; **left panel**) and summary of scanning data (**right panel**; mean densitometric units expressed as percentage of F1 \pm SEM; data from 4–6 replicate autoradiographs). F1, F2, and F3 indicate the largest, most mature; the second largest; and the third largest preovulatory follicles, respectively; 3–5, 6–8, and 9–12 refer to diameter of follicles (mm).

opment in granulosa cells collected from 9–12-mm follicles. By comparison, total cellular RNA from 3–5-mm- and 6–8-mm-follicle granulosa cells subjected to amplification by reverse-transcriptase (RT) PCR produced variable results. In one experiment, no products corresponding to cLH-R were detected, while in the replicate experiment a small amount of amplified cLH-R product was detected at both the 3–5-mm and 6–8-mm stage of development. In each experiment, the appropriate negative controls were established, and the integrity of the cDNA template was subsequently verified by the successful amplification of unrelated products (data not shown). The most pronounced expression was evident in hierarchal (F1 to F3) follicles ($p < 0.01$ vs. 9–12-mm follicle granulosa). Finally, Northern analysis determined that cLH-R mRNA levels were decreased in atretic versus morphologically normal follicles (mean decline, $85 \pm 9\%$, range 40–97%; $p < 0.05$; Fig. 5).

DISCUSSION

Multiple LH-R mRNA transcripts have been detected in mammalian species, and the size and relative abundance is both tissue- and species-specific. For example, there are six discernible LH-R transcripts in rat luteal tissue (a predominant 1.2-kb species with lesser abundant 1.6-, 1.9-, 2.6-, 4.3-, and 7.7-kb transcripts), whereas in rat whole ovary the predominant mRNA transcript is 6.7 kb in length with less abundant 1.2-, 2.6-, and 4.3-kb species [21]. By comparison, both the porcine ovary and porcine testis express LH-R mRNA species of 1.4, 2.6, 4.0, 4.7, 5.8, 6.7 kb in length, but in different relative proportions [20]. While the physiological significance of most transcripts is currently not understood, it has been demonstrated that the 1.2-kb species of the rat LH-R mRNA encodes a truncated form of the receptor [22].

By contrast, there appears to be a single 3.0-kb cLH-R transcript expressed in chicken ovarian tissues; this transcript is essentially the same size as the apparent single LH-R mRNA species previously reported from quail testes [17]. Nevertheless, the identification of an alternatively spliced mRNA transcript containing an inframe stop codon suggests that a truncated product corresponding to the extracellular region of the cLH-R protein may be translated. At the present time, we have no evidence that the alternatively spliced transcript is translated within either granulosa or

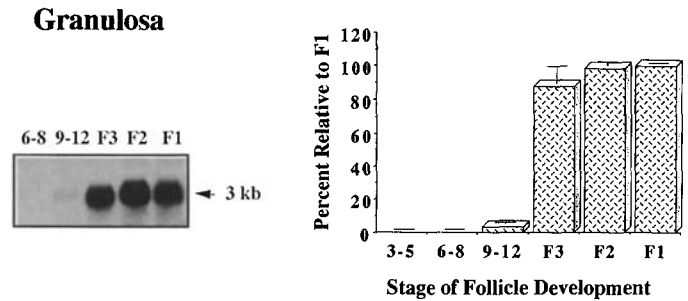


FIG. 4. Representative Northern blot of cLH-R mRNA from granulosa tissue during follicle development (excluding RNA from 3–5-mm follicles; **left panel**) and summary of scanning data (**right panel**; mean densitometric units expressed as a percentage of F1 \pm SEM; data from 3–5 replicate autoradiographs). Abbreviations as in Figure 2.

theca tissue, nor, if it is, whether the protein is physiologically functional. Interestingly, transient transfection of the rat luteal LH-R extracellular domain in human kidney 293 cells results in the expression of a protein with high binding affinity for the hCG ligand. However, as the majority of truncated receptor was found to remain sequestered within 293 cells (i.e., is not secreted), the physiological relevance of this protein remains in question [22]. The cLH-R mRNA transcript can be differentiated from cFSH receptor (cFSH-R) mRNA on the basis of both size of transcript (an approximate 4.3-kb transcript for cFSH-R mRNA) and pattern of expression during follicle development (unpublished data).

All 19 cysteine residues and three of the four potential sites for *N*-linked glycosylation encoded within the cLH-R cDNA are conserved compared to the porcine LH-R amino acid sequence [20]. Moreover, a highly conserved sequence of amino acids (YPSHCCAF) located at the carboxy terminal of exon 9 from the extracellular domain of mammalian LH-R, FSH-R, and thyroid-stimulating hormone receptors is entirely conserved within the cLH-R sequence (corresponds to AA 165 to 172, Fig. 2); this region has been proposed to form a pocket for glycoprotein hormones to bind to its specific receptor [23].

The predicted second transmembrane spanning region of cLH-R (AA 288 to 310, Fig. 2) is identical to the rat, porcine, and human amino acid sequences, including the location of the aspartic acid residue corresponding to Asp³⁸³ from the rat LH-R sequence. Moreover, the location of this Asp residue within the second transmembrane spanning region is conserved in virtually all mammalian G-protein coupled receptors; this amino acid has been specifically implicated in preserving high-affinity ligand binding and

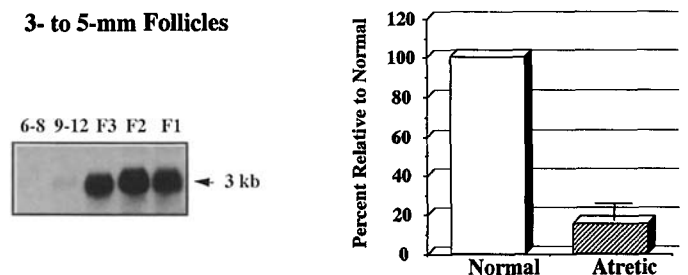


FIG. 5. Representative Northern blot of cLH-R mRNA from 3–5-mm morphologically normal and atretic follicles (**left panel**) and summary of scanning data (**right panel**; mean densitometric units expressed as a percentage of normal follicles \pm SEM; $n = 6$).

cAMP production in response to interaction with the ligand [24]. The entire transmembrane domain shares greater than 86% identity to the rat and porcine sequences, and in general, there is a high degree of homology within the seven transmembrane spanning regions of the G-protein coupled receptor family [25].

Northern blot analysis of cLH-R mRNA levels shows no dramatic changes within theca tissue during development from prehierarchical follicles through the second largest (F2) preovulatory follicle, and this is generally consistent with the ability of LH to promote steroid production from whole follicles both throughout development (including prehierarchical follicles) and in ovarian stromal tissue [26–28]. On the other hand, there is a significant decrease in cLH-R mRNA levels in F1, compared to F2, follicle theca cells (by approximately 42%; Fig. 3). This decline in cLH-R mRNA corresponds to a period of decreased steroidogenic responsiveness to LH during the hours immediately preceding ovulation [29]. While this decline in steroidogenesis has previously been attributed to post-receptor events, including the loss of $C_{17,20}$ -lyase activity, the present data suggest that there may also be a concomitant decrease in LH receptor expression.

LH-induced cAMP accumulation and steroid production from granulosa cells of hierarchical (F1 to F3) follicles is well characterized [30–32], and the pronounced LH responsiveness of these cells correlates well with the high levels of cLH-R mRNA detected at this stage of development (Fig. 4). By contrast, granulosa cells from follicles less than 9 mm in diameter are steroidogenically incompetent, and this has been attributed primarily to the lack of cytochrome P450 cholesterol side-chain cleavage ($P450_{\text{SCC}}$) activity; moreover, it has been determined that granulosa cells from nonhierarchical follicles are responsive to FSH as demonstrated by gonadotropin-induced cAMP accumulation [2, 33]. The working hypothesis has previously been presented that steroidogenic enzyme ($P450_{\text{SCC}}$ and $P450_{17\alpha}$ -hydroxylase) activity in granulosa cells is induced at or around the time of follicle selection in response to the stimulatory actions of FSH and possibly vasoactive intestinal peptide [3, 4, 34]. Under physiological conditions, in vivo, FSH-induced steroid production is first detectable at the 9–12-mm stage of development [10].

While prehierarchical follicle granulosa cells are responsive to FSH, there is a lack of increased cAMP accumulation or steroid production following a challenge with LH [2, 10]. These published data are consistent with those presented herein, as cLH-R mRNA in granulosa cells is not detectable by Northern blot analysis until the follicle has entered the rapid growth phase (9–12-mm stage); however, because LH-induced progesterone production from granulosa cells is not evident until sometime subsequent to the 9–12-mm-follicle stage [10], it is possible that either significant levels of receptor protein have yet to be translated or that the receptor protein has yet to become coupled to appropriate second messenger pathways. The ability to amplify low levels of cLH-R cDNA by RT-PCR and Southern blot analysis in one of two replicate experiments using 3–8-mm-follicle granulosa cells indicates a very low-level expression of the message.

It was also not unexpected that cLH-R mRNA levels decrease in atretic versus normal follicles (Fig. 5), as similar results have been reported from the rat ovary [35]. Follicle atresia in the hen occurs almost exclusively in prehierarchical follicles [36], and accordingly, follicles selected for this experiment were \leq 5-mm diameter. In light of the

virtual absence of cLH-R mRNA in prehierarchical follicle granulosa cells, the observed decrease in cLH-R mRNA levels during atresia occurs exclusively from within the theca layer. We have previously reported that hen atretic follicles, particularly within granulosa cells, exhibit extensive oligonucleosome formation, characteristic of apoptosis [37, 38]. It should be noted that considerable variation was found in the extent of decline (ranging from 40 to 97%) in cLH-R mRNA levels for atretic follicles. 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 cLH-R mRNA is a cause or simply an effect of apoptosis and atresia.

In summary, cloning of the cLH-R cDNA has provided additional information regarding the conservation of G-protein coupled receptors among animal classes (e.g., aves vs. mammalia), as well as an important new tool for investigating the physiology of gonadotropin actions within the hen ovary. The results described herein are of particular significance given the previous difficulties in evaluating the presence of the ovarian cLH-R by conventional radioreceptor assays. Additional studies are currently in progress to evaluate hormonal control of cLH-R mRNA expression in granulosa and theca tissue and to evaluate levels of the receptor protein.

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