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Molecular Cloning, Functional Characterization, and Gene Expression of a Follicle-Stimulating Hormone Receptor in the Testis of Newt *Cynops pyrrhogaster*

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We previously demonstrated *in vitro* that mammalian follicle-stimulating hormone (FSH) stimulates the proliferation of newt secondary spermatogonia and their differentiation into primary spermatocytes. In the current study, we isolated a cDNA from newt testis that encodes a FSH receptor (FSH-R). The total sequence homology in the deduced protein of the newt was approximately 70% with mammalian FSH-Rs. Mammalian cells, transiently transfected with the cloned newt *FSH-R* cDNA, displayed specific binding to [^{125}I] human FSH and cAMP accumulation, indicating that the cloned cDNA encodes a functional newt FSH-R protein. Northern blot analysis revealed a single transcript of approximately 3.0 kb length that was synthesized in testicular somatic cells (mainly Sertoli cells) from spermatogonial to spermatid stages with the highest level expressed during the primary spermatocytes stage. These results demonstrate that FSH stimulates newt spermatogenesis through the FSH-R. This study, as far as we know, reports for the first time the cloning of an amphibian *FSH-R* cDNA. © 2000 Academic Press

Key Words: **follicle-stimulating hormone; receptor; cloning; cDNA; functional expression; spermatogenesis; newt; amphibian.**

Gonadotropins (GTH) are pituitary hormones regulating folliculogenesis, ovulation (1) and spermatogenesis (2–5) in vertebrates. The GTHs are glycoproteins and include follicle-stimulating hormone (FSH) and luteinizing hormone (LH) that have been identified in mammals, avians, amphibians and some teleost fishes (6). FSH and LH bind to specific receptors on the surface of target cells in the gonads. FSH is essential for initiating and maintaining spermatogenesis (4, 7). The biochemical effects of FSH in mammals are mediated through its interaction with its receptor that is specifically expressed in Sertoli cells (8, 9).

We previously showed in organ cultures of newt testes that mammalian FSH stimulates the proliferation of secondary spermatogonia and their differentiation into primary spermatocytes (10, 11). Furthermore, experiments involving spermatogonia cultured alone or with testicular somatic cells revealed that this stimulation of germ cells by FSH is mediated through Sertoli cells (12). These results indicated that FSH is a major regulator of amphibian spermatogenesis as it is in other vertebrates.

The FSH receptor (FSH-R) belongs to a superfamily of receptors that act through interactions with G-proteins, as do the receptors for LH/CG (chorionic gonadotropin) and thyroid stimulating hormone (TSH) (13). These receptors in the superfamily contain seven transmembrane domains and a large glycosylated extracellular domain required for interaction with the complex heterodimeric structure formed by the α and β subunits of these hormones. Binding of the hormones to the receptor activates a G-protein, which then stimulates the membrane-bound adenylyl cyclase, resulting in the elevation of the intracellular cAMP concentration (13).

FSH-R cDNAs have been cloned from several species of mammals (14–20) and chicken (21, 22). In the case of quail and reptiles the extracellular domains of the FSH-Rs were cloned (23, 24). Recently, the cDNA structure of amago salmon GTH receptors (sGTH-Rs) was determined (25, 26), but no studies have been reported on amphibian FSH-Rs.

Our long-range goal is to determine the molecular mechanism of FSH's action. To this end, in the current study, we cloned a cDNA for a FSH-R in newt, charac-

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terized its properties by expressing the receptor proteins in cells transfected with the cDNA, and examined its expression in various tissues by the reverse transcription-polymerase chain reaction (RT-PCR), as well as its expression in testes by Northern blot and RT-PCR analysis.

MATERIALS AND METHODS

Animal and reagents. Adult male newts (*Cynops pyrrhogaster*), collected during winter and early spring, were purchased from a dealer (Hamamatsu Seibutsu Kyozai Ltd. Hamamatsu, Japan). All chemicals were obtained from Nacalai, Kyoto, Japan, unless otherwise stated.

Cloning of newt FSH-R cDNA. Degenerate primers were synthesized that correspond to the peptide sequences conserved in the extracellular region of mammalian FSH-Rs. Two regions (HEIRIEKA and FNPCEPIM) of the extracellular region of mammalian FSH-Rs were selected. The sequence of the set of primers for PCR were 5′-CAYGARATHMGNATHGARAARGC-3′ and 5′-CATDATRTCYTCRCANGGRTTRAA-3′. Using oligo(dT)-primed cDNAs prepared from testes poly(A)$^+$-RNA as a template, PCR amplification was performed for 40 cycles. A PCR cycle consisted of 1 min at 94°C, 90 sec at 45°C, and 1 min at 72°C. The PCR products were analyzed on 2% agarose gel and subcloned in a pT7Blue T-vector (Novagen, Madison, WI, USA). To obtain a cDNA clone containing the newt *FSH-R* cDNA, we performed Southern hybridization using the degenerate oligonucleotides [5′-SATDGARGCHRRCYTYACMTAYCCHAGCCAYTGCTGTGCHTTYRC-3′] that correspond to the peptide sequence (residues 264–278) of human FSH-R (14). Recombinant plasmid was loaded on agarose gel and blotted onto Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, England) and then hybridized with [^{32}P] labeled degenerate oligonucleotide. Hybridization to and washing of the membrane were performed according to the method of Benedum *et al.* (27). Positive clones contained 800 bp of insert cDNA, and their sequences were determined by BigDye terminator cycle sequencing ready reaction (PE Biosystems, Foster, CA) using a DNA sequencer (Model 310, PE Biosystems, Foster, CA).

As the nucleotide sequence of the 800 bp fragment revealed a high homology to those of mammalian FSH-R, this fragment was used as a probe for screening a newt testis cDNA library. A positive clone was isolated, and its insert cDNA was excised with *Eco*RI and *Xho*I and subcloned in pBluescript II SK(−). The cloned cDNA was sequenced as described above. All sequence data were obtained for both strands and deposited into the DDBJ data bank with the Accession No. AB005587.

Construction of expression vectors. For expression studies, a cDNA containing the entire coding sequence for the newt FSH-R was amplified from the cDNA library clone using a set of primers (5′-ACTCGAGATGTCTCTGGCCATCCTTTGC-3′) containing *Xho*I cleavage site and 5′-CGGATCCTTAGTTCAAGTTATTCAGAGG-3′ containing *Bam*HI site). Thirty thermal cyclings for PCR amplification were performed with *Pfu* DNA polymerase (1 min at 94°C, 1 min at 52°C, and 2.5 min at 72°C). An amplified cDNA (2.1 kb) was cleaved with *Xho*I and *Bam*HI and inserted into pcDNA 3.1(−) (Invitrogen, Carlsbad, CA). Authenticity of this cDNA was confirmed by sequencing.

Ligand-binding assay. COS-7 cells, a mammalian cell line, were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% FCS. 2×10^6 cells were seeded in 75 cm^2 tissue-culture flasks 1 day before transfection and transfected with the plasmid (15 μg) using DEAE-dextran methods as described by Gonzalez and Joly (28). Three days after transfection, cell membranes were prepared according to Yarney *et al.* (17). The membrane preparation (100 μg) in 25 mM Tris–HCl (pH 7.2) containing 10 mM $MgCl_2$ and 0.1% BSA was incubated overnight at 22°C with 30,000 cpm of [^{125}I] human FSH (NEN, Boston, MA) and various concentration of unlabeled human FSH or LH (Calbiochem, La Jolla, CA) in a total volume of 300 μl. The reaction was stopped by the addition of ice-cold 25 mM Tris–HCl (pH 7.2, 1 ml) containing 10 mM $MgCl_2$ and 0.1% BSA. Bound hormone was separated from the unbound by centrifugation at 10,000*g* for 10 min. The radioactivity of the pellet was counted using an automatic gamma counter (1480 Wizard, Wallac OY, Turku, Finland). Total and nonspecific binding (>10% of the total binding) were determined by adding [^{125}I] human FSH in the absence or presence of excess unlabeled human FSH. In all the assessments of [^{125}I]FSH binding, only vector-transfected cells were incubated as negative control.

Intracellular cAMP assay. The expression vector was transfected into COS-7 cells as described in the ligand-binding assay. Sixteen to 18 h after transfection, the cells were replated (5×10^4 cells/well, 24-well tissue culture plates). At 69–72 h posttransfection, the cells were washed with DMEM (1 ml) and then incubated in DMEM (250 μl) containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Chemical Co., St. Louis, MO) for 15 min at 37°C. Increasing concentrations of ovine FSH or LH (Sigma) were then added and the cells were incubated for 30 min at 37°C. The assay was terminated by aspiration of the medium. Cell lysis and measurement of intracellular cAMP concentration were carried out using BIOTRAK cAMP enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. In all the assessments of cyclic AMP production, only vector-transfected cells were incubated as negative control. All measurements were performed in triplicate and mean and standard errors were calculated.

Northern blot analysis. Total RNA was prepared from testis fragments by the guanidinium thiocyanate-phenol–chloroform method (29). Twenty micrograms of total RNA were electrophoresed in a 1% formaldehyde agarose gel and blotted to a nylon membrane, Hybond-N$^+$ (Amersham Pharmacia Biotech). Digoxygenin-labeled antisense newt *FSH-R* cRNA probes were prepared by *in vitro* transcription (Boehringer-Mannheim, Mannheim, Germany) of a subclone of cDNA, corresponding to nucleotides 715–1308 (DDBJ Accession No. AB005587). Hybridization was carried out at 68°C overnight in the hybridization solution {0.2 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 10% SDS, 0.5% blocking reagent (Boehringer-Mannheim)}. After hybridization, the membranes were washed with 2× standard sodium citrate (SSC) containing 0.1% SDS. A final wash was performed with 0.1× SSC containing 0.1% SDS at 68°C. The immunological detection of the signal was carried out according to the manufacturer's instruction (Boehringer-Mannheim).

RT-PCR and Southern blot analysis. Fractionation of somatic cells (mostly Sertoli cells, 98% somatic cells and 2% germ cells) and germ cells (98% germ cells and 2% somatic cells) from testicular fragments was carried out as described previously (12). Total RNA was prepared from the isolated testicular cells and several other tissues as described in Northern blot analysis. One microgram of total RNA from several tissues, testicular somatic cells or germ cells that had been treated with DNase I (Gibco-BRL, Tokyo, Japan) were reverse-transcribed using an oligo(dT) primer with the Thermoscript RT-PCR system (Gibco BRL, Tokyo, Japan). PCR was carried out with KOD DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). The primers used for newt *FSH-R* and *β-tubulin* genes were: *FSH-R* F, 5′-CTGTGGGTTCCAGTTTCGGATGCCA-3′; *FSH-R* R, 5′-GATTAACTGGTATTTGAGGCTCGGAAG-3′; *β-tubulin* F, 5′-GGGAAGTAATCAGCGATGAG-3′, *β-tubulin* R, 5′-AGGAGTGGGTCAGCTGGAAT-3′. The annealing temperatures were 61°C for *FSH-R* and 55°C for *β-tubulin*, and 30 cycles (*FSH-R*) and 25 cycles (*β-tubulin*) were performed. The PCR products were separated on agarose gels, transferred to Hybond-N$^+$ nylon membranes (Amersham Pharmacia Biotech) and detected by Southern hybridization. Labeling of the cDNA probe as well as hybridization and detection of signals were carried out using the AlkPhos direct system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

cDNA Cloning and Structure of Newt FSH-R

To isolate a cDNA for a newt FSH-R, we performed PCR with degenerate primers containing the sequences present in the conserved extracellular domain of mammalian FSH-Rs. Using cDNAs from newt testis as templates, we obtained a faint signal with 800 bp of PCR products that we subcloned in plasmid vectors. Forty recombinant plasmids were screened with a synthetic degenerate 45-mer oligonucleotide derived from the reported extracellular region of human FSH-R. One positive clone was isolated and the nucleotide sequence was determined. The deduced amino acid sequence is 61% homologous to human FSH-R. With the PCR product as a hybridization probe, we isolated a cDNA clone containing the entire coding region of a newt FSH-R from a testis cDNA library. This clone is 3075 bp in length and encodes a 696 amino acid residue containing a signal peptide of 17 amino acids at the N-terminal region. The mature protein is predicted to be 679 amino acids with a molecular mass of 77 kDa.

The predicted protein of the newt FSH-R contains a large extracellular domain and seven transmembrane domains, structures also present in the G-protein-coupled receptor family (Fig. 1). The extracellular domain in the newt FSH-R contains ten cysteine residues (Cys-18, -24, -31, -187, -274, -275, -291, -341, -349, -359), considered necessary for folding the protein (30), and five potential N-linked glycosylation sites (Asn-46, -190, -198, -267, -292). In mammalian FSH-Rs two of the three conserved N-linked glycosylation sites in the extracellular domain are required for proper folding of the protein (31). As 10 cysteine residues and two glycosylation sites (Asn-190, -292) are conserved in the newt FSH-R at the same positions as in mammalian FSH-Rs, it is likely that these motifs are also required for proper folding of the newt protein. There are two additional N-linked glycosylation sites (Asn-46, -267) in the newt FSH-R that are not present in mammalian FSH-Rs. Although the biological significance of these additional glycosylation sites is unknown, one site (Asn-46) is conserved in amago salmon's GTH-RI (sGTH-RI) and the chicken's FSH-R, and another site (Asn-267) is conserved in amago salmon's sGTH-R.

The transmembrane domain in newt FSH-R contains two cysteine residues in the first and second extracellular loops that are predicted to form an intramolecular disulfide bridge (32). The acidic-Arg(R)-aromatic motif (ERW, position 469–471), considered important in the interaction between the receptor and G-proteins in mammals (33), is also present in newt FSH-R. This motif is conserved in receptors from newt to mammals but not in amago sGTH-RI. These results suggest that the FSH-Rs in tetrapods of mammals, chickens and newts have a common signal transduction pathway mediated by an acidic-Arg(R)-aromatic motif. In addition, there are consensus sites (Thr-558 and Ser-599) for protein kinase C phosphorylation in the third intracellular loop and the sixth transmembrane domain. The cytoplasmic domains contain three cysteine residues and a potential protein kinase C phosphorylation site (Thr-635); one of the cysteine residues is conserved from amago sGTH-RI to mammalian FSH-Rs, but the phosphorylation site is conserved only from amago sGTH-RI to chicken FSH-R.

Comparison of Amino Acid Sequence

The amino acid sequence of the newt FSH-R was compared with those of some vertebrates (Fig. 1). The complete amino acid sequence of newt FSH-R was 69–70% and 72% homologous with mammals and chicken, respectively, but only 48% with amago sGTH-RI. However, the amino acid sequence of the transmembrane domain of newt FSH-R shared a high sequence identity with mammals, chicken and amago salmon (82–83, 86, and 68%, respectively). But the extracellular domain of newt FSH-R shared a low amino acid sequence identity with the mammalian and chicken FSH-Rs and amago sGTH-RI (60–62, 63, and 35%, respectively) and the least homology with mammalian LH receptors (less than 52%).

Phylogenetic Analysis

To determine if the newt FSH-R was phylogenetically related to other glycoprotein hormone receptors, we compared its amino acid sequence in the transmembrane domain with that in sea anemone, *Drosophila*, amago, newt, chicken, rat, and human (Fig. 2). A phylogenetic tree was constructed by the neighbor-joining method (34), using the Phylogeny Inference Package (PHYLIP) version 3.573c (35). The distance matrices were calculated with the Dayhoff PAM matrix of the PROTDIST program in the PHYLIP. The robustness of the tree was assessed by 100 bootstrap resamplings. The analysis revealed that newt FSH-R, mammalian and chicken FSH-Rs formed a group with the bootstrap value of 100%, but sGTH-RI and tetrapod FSH-Rs, though clustered, were not related to newt FSH-R with high statistical confidence (58%).

Expression of Newt FSH-R Protein

To determine whether the newt *FSH-R* cDNA expresses a functional protein, we subcloned its entire coding region in an expression vector. COS-7 cells were then transfected with these vectors and competitive radioligand receptor assays were performed. Increasing concentrations of unlabeled human FSH competed with [^{125}I] human FSH (hFSH) for binding to the membranes (Fig. 3A). Likewise, human LH (hLH) competed

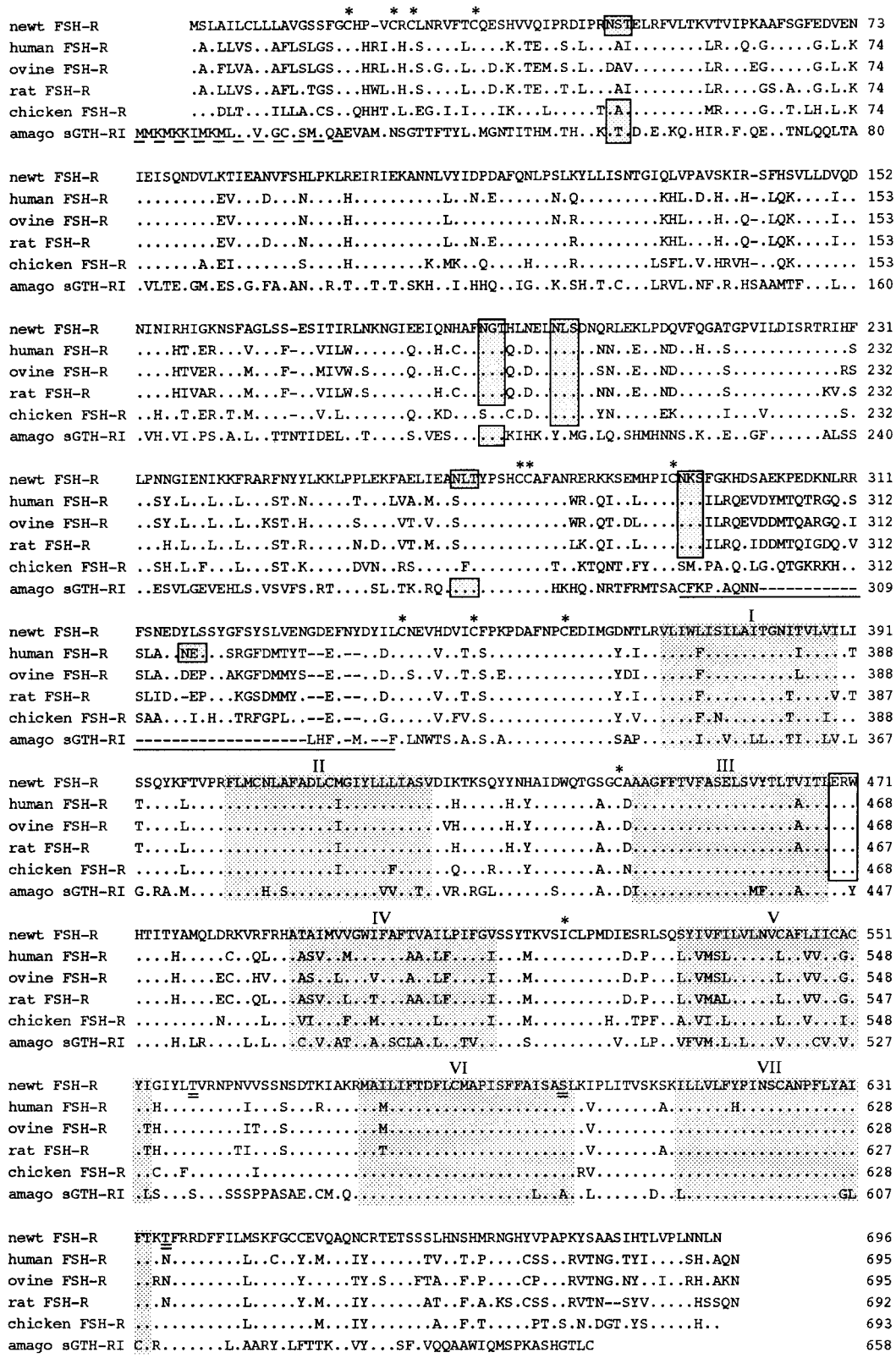

FIG. 1. Alignment of the deduced amino acid sequences of the newt, human (14), ovine (17), rat (15), and chicken (22) FSH-Rs, and amago salmon GTH-RI (26). The signal peptide is underlined with dashed lines. Asterisks indicate cysteine residues. A highly heterologous region is underlined with a solid line. Seven transmembrane domains are lightly shaded (numbered above with roman numerals). Potential N-glycosylation sites are surrounded by stippled boxes. Acidic-Arg(R)-aromatic motif (ERW) is indicated by an open box. Potential protein kinase C phosphorylation sites are double underlined.

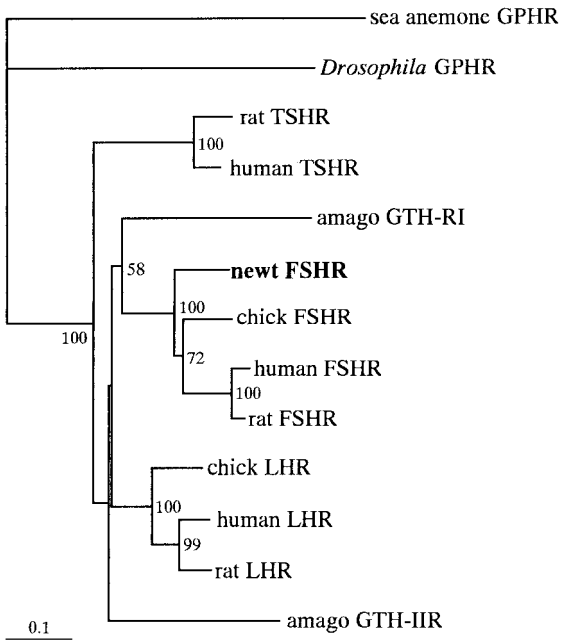

FIG. 2. Phylogenetic tree of the amino acid sequences of the transmembrane domain of the glycoprotein hormone receptor family, constructed with the neighbor-joining method. Lengths of horizontal lines indicate the genetic distance. The numbers indicate bootstrap values from 100 replicates.

with [^{125}I] hFSH, but only at high concentrations. The newt receptor had a 50-fold higher affinity for human FSH than for human LH. Binding of labeled FSH to cells transfected with the vector alone was less than 1% of that bound by cells transfected with the vector containing the cDNA for newt FSH-R (data not shown). These results indicate that the cloned cDNA encodes a functional newt FSH-R.

According to Dattatreyamurty and Reichert (36), the amino acid residues 9–30 in the extracellular domain of rat FSH-R comprise a specific FSH binding domain. The corresponding sequence in newt FSH-R is highly homologous with those in mammals, chicken and reptiles. Thus, our current results and those of others suggest that in general this sequence is important for FSH binding in vertebrates. However, in rats there is an additional site (residues 300–315) for interaction with FSH (37), but this sequence is highly heterologous between mammals and newt, suggesting that this region contributes little, if any, to hormone-receptor binding in newt.

COS-7 cells containing the newt FSH-R increased significantly their intracellular cAMP concentration following stimulation by ovine FSH (oFSH) but did not respond to ovine LH (oLH) (Fig. 3B). As the cells transfected with the vector alone did not respond to these hormones (data not shown), we conclude that the newt FSH-R expressed in COS-7 cells can transduce the FSH signal in the cytoplasm to elevate cAMP concentration as well as mammalian FSH-Rs, suggesting that vertebrate FSH-Rs have a common signal transduction pathway.

Tissue Expression of Newt FSH-R mRNA

We examined by RT-PCR the mRNA expression of FSH-R in several newt tissues (Fig. 4). A high level of RNA expression was observed in testis, a low level in ovary, and none in kidney, brain, spleen, and liver.

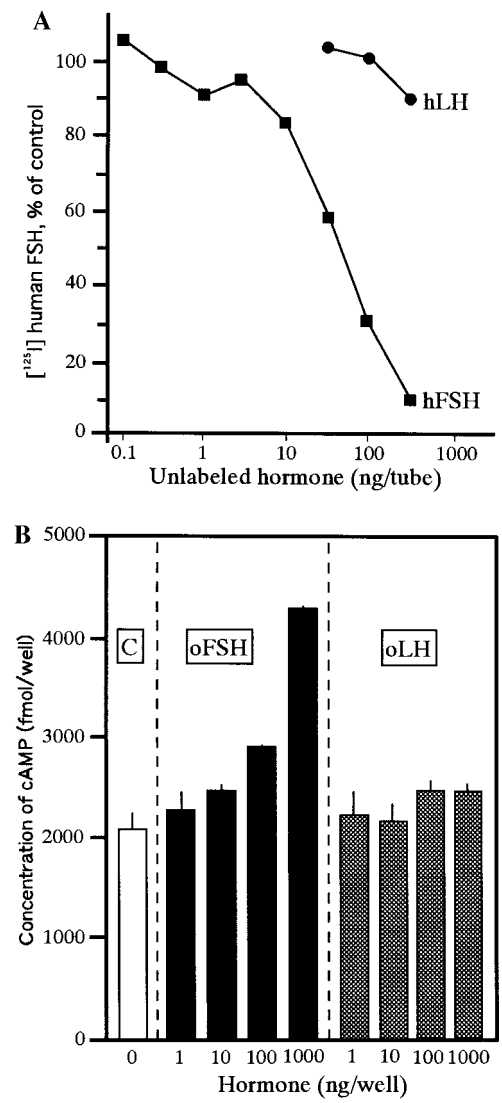

FIG. 3. (A) Competitive binding of [^{125}I] human FSH to the membranes from COS-7 cells transfected with pcDNA-*FSH-R* cDNA. The results are expressed as the percentage of the maximal specific binding observed in the absence of competitor. Nonspecific binding was assessed with 1000-fold excess of unlabeled hormone. Three independent transfection experiments gave similar results. (B) Cyclic AMP accumulation in COS-7 cells transiently transfected with newt *FSH-R* cDNA. Intracellular cAMP concentration was measured as a function of hormone concentration. Each point is the mean of triplicate determinations. C, without hormone.

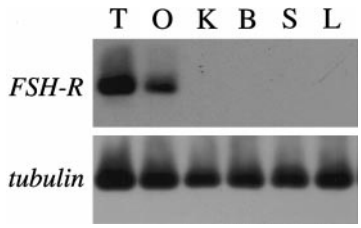

FIG. 4. Tissue expression of *FSH-R* mRNA. One microgram of total RNA from testis (T), ovary (O), kidney (K), brain (B), spleen (S), and liver (L) was reverse-transcribed and amplified by PCR using primers *FSH-R* F and *FSH-R* R. The PCR products were loaded on agarose gel and analyzed by Southern blot analysis. β-Tubulin was used for control.

This expression pattern correlates well with that found in mammals, chicken and amago salmon where FSH-Rs and sGTH-RI were expressed only in reproductive organs. Although Kubokawa and Ishii (38) reported gonadotropin-binding sites in amphibian liver, we did not detect mRNA expression for FSH-R in newt liver. It is possible that the cycles of PCR that we performed to amplify the *FSH-R* mRNA were too few and/or our experimental conditions were not appropriate for detecting low levels of *FSH-R* expression in the liver.

Expression of FSH-R mRNA during Newt Spermatogenesis-Stage and Cell Type

To examine whether the expression of *FSH-R* mRNA is stage-dependent, Northern blot analysis was performed on total RNA from newt testis fragments derived from different stages of spermatogenesis, and a single transcript of approximately 3.0 kb was detected (Fig. 5A). In contrast to newt testes, several species of *FSH-R* mRNAs (0.5–6.7 kb) were observed in chicken (22) and mammalian testes (16–18, 39). These different species of transcripts are thought to originate from different polyadenylation sites and/or alternative splicing (39–41). Thus, our current study indicates that the regulation of spermatogenesis by FSH in newt testis is simpler than that in avians and mammals, as we found only a single species of mRNA for the FSH-R.

The synthesis and amount of newt *FSH-R* mRNA during spermatogenesis was stage-dependent (Fig. 5A). The transcript was most abundant in the primary spermatocyte-stage and least in the spermatid-stage. Likewise, stage-dependent expression of *FSH-R* mRNA was observed in rat testis with a high level synthesized at stages I, XIII, and XIV (41, 42). Differences in testicular structure between newt and rat preclude a direct comparison of stages; however, stage-specific synthesis of *FSH-R* mRNA appears to be required for both newt and mammalian spermatogenesis.

To determine which cell type expresses *FSH-R* mRNA in newt testes, we performed RT-PCR using oligo(dT)-primed cDNAs as a template prepared from total RNA; the RNA was derived from highly purified fractions of germ or somatic cells (mostly Sertoli cells) (Fig. 5B). As expected, we detected expression of *FSH-R* mRNA in somatic cells but barely in germ cells, consistent with our previous findings that mammalian FSHs bind to membrane fractions of somatic cells (mostly Sertoli cells) (43). Thus, FSH acts in newt testis via Sertoli cells as it does in other vertebrates.

The effect of FSH on mammalian spermatogenesis is still complex. Recently, mice lacking the FSH-R were shown to be fertile males but had testes reduced in size and weight (44, 45). Furthermore, the testes of such knockout mice (FORKO) contained a significant increase in the percentage of spermatogonia but a decrease in the percentage of elongated spermatids (46), suggesting that FSH, though important for normal spermatogenesis, is not essential for initiating spermatogenesis. As mammalian FSH alone can stimulate newt spermatogenesis and its FSH signaling pathway, the newt and mammalian pathways must be quite similar. This relationship should permit us to investigate further the mechanism of FSH action in newt testes—a simpler model with the advantage of a refined *in vitro* organ culture (11) and a cell coculture consisting of spermatogonia and Sertoli cells (47).

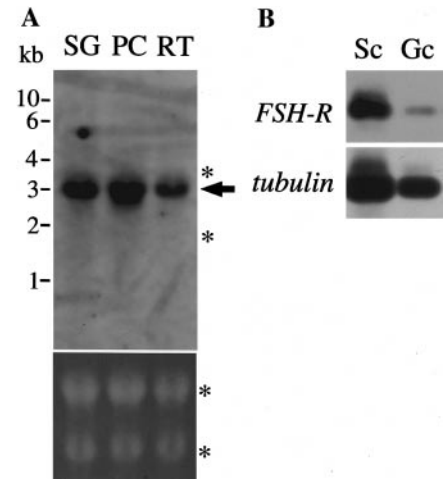

FIG. 5. Expression of *FSH-R* mRNA in newt testis. (A) Northern blot analysis. Total RNA (20 μg) from testes fragments rich in spermatogonia (SG), primary spermatocytes (PC), and round spermatids (RT) were loaded on 1% agarose-formaldehyde gel. The gel was stained with ethidium bromide (lower panel) and used for detecting *FSH-R* mRNA (upper panel). The arrow indicates the position of the *FSH-R* mRNA. Asterisks show the positions of ribosomal RNA (28S and 18S rRNA). (B) RT-PCR analysis. Total RNA (1 μg) from purified fractions of somatic (Sc, mostly Sertoli cells) and germ (Gc) cells was reverse-transcribed and amplified by PCR using primers *FSH-R* F and *FSH-R* R. The PCR products were loaded on agarose gel and analyzed by Southern blot analysis. β-Tubulin was used for control.

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