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Zebrafish Gonadotropins and Their Receptors: II. Cloning and Characterization of Zebrafish Follicle-Stimulating Hormone and Luteinizing Hormone Subunits—Their Spatial-Temporal Expression Patterns and Receptor Specificity[1]

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ABSTRACT

Gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH) play critical roles in vertebrate reproduction. In the present study, we cloned and characterized zebrafish FSHβ (*fshb*), LHβ (*lhb*), and GTHα (*cga*) subunits. Compared with the molecules of other teleosts, the cysteine residues and potential glycosylation sites are fully conserved in zebrafish Lhb and Cga but not in Fshb, whose cysteines exhibit unique distribution. Interestingly, in addition to the pituitary, *fshβ, lhβ,* and *cga* were also expressed in some extrapituitary tissues, particularly the gonads and brain. In situ hybridization showed that zebrafish *fshβ* and *lhβ* were expressed in two distinct populations of gonadotrophs in the pituitary. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that all the three subunits increased expression before ovulation (0100–0400) when the germinal vesicles in the full-grown follicles were migrating toward the periphery, but the levels dropped at 0700, when ovulation occurred. Recombinant zebrafish FSH (zfFSH) and LH (zfLH) were produced in the Chinese hamster ovary (CHO) cells and their effects on the cognate receptors (zebrafish Fshr and Lhr) tested. Interestingly, zfFSH specifically activated zebrafish Fshr expressed together with a cAMP-responsive reporter gene in the CHO cells, whereas zfLH could stimulate both Fshr and Lhr. In conclusion, the present study systematically investigated gonadotropins in the zebrafish in terms of their structure, spatial-temporal expression patterns, and receptor specificity. These results, together with the availability of recombinant zfFSH and zfLH, provide a solid foundation for further studies on the physiological relevance of FSH and LH in the zebrafish, one of the top biological models in vertebrates.

FSH, Fshr, LH, Lhr, pituitary, zebrafish

INTRODUCTION

Gonadotropins (GTHs), namely, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are the key regulators of vertebrate reproduction, and they are synthesized and secreted from the gonadotrophs in the anterior pituitary. Together with thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG), FSH and LH belong to the glycoprotein hormone family whose members bear carbohydrate moieties that influence their biosynthesis, secretion, half-life, and biological potency [1, 2]. All members of the hormone family are heterodimers consisting of noncovalently linked α and β subunits. The α subunit is shared by all members of the family, while the β subunit is structurally distinct and hormone specific, determining the specificity of the hormones [1]. Until the mid-1980s, all the gonadotrophic functions of the pituitary in teleosts were attributed to a single gonadotropin called maturational gonadotropin. However, the successful purification of two chemically distinct gonadotropins from chum salmon (*Oncorhynchus keta*) pituitary in 1988 [3–5] led to the proposal that similar to the situation in tetrapods, there also existed two gonadotropins in teleosts. This concept has since been confirmed in a large number of fish species by either purification or cloning [6, 7].

In the female, FSH and LH exert their effects on ovarian functions through membrane receptors (GTHR) on the granulosa and theca cells. In fish, the presence of their specific binding sites in the ovary has been demonstrated in the amago salmon (*Oncorhynchus rhodurus*) [8] and murrel (*Channa punctatus*) [9]. Different from mammalian FSH and LH receptors that are highly specific for their cognate hormones with less than 0.1% cross activation [10–12], the functional duality and receptor specificity of FSH and LH seem to be less apparent in teleosts. In salmonid ovaries, LH (previously called GTH-II) purified from coho salmon (*Oncorhynchus kisutch*) pituitary recognized both types of GTHRs, the type I and type II receptors, whereas FSH (GTH-I) bound only to the type I receptor (now termed FSHR) with limited interaction with the type II receptor (LHR) [13]. In contrast, purified chum salmon FSH stimulated cAMP production by COS cells expressing amago salmon FSHR or LHR, whereas chum salmon LH activated only amago salmon LHR [14, 15]. In the African catfish (*Clarias gariepinus*), both purified and recombinant LH and FSH could elevate intracellular cAMP level in FSHR-expressing HEK-T 293 cells; however, the cells expressing LHR could be activated only by LH [16–18]. In an Indian carp (*Labeo rohita*), the situation is even more complicated. The two gonadotropin receptors purified from the ovarian follicles recognized both salmon FSH and LH in receptor binding assays, albeit with preference for their cognate ligands [19]. However, this result should be interpreted cautiously because the hormones used in the study were from different species.

Despite the numerous reports on FSH and LH in teleosts in the past 15 yr, we are still far from understanding the functions of these gonadotropins in controlling fish reproduction. In salmonids, it has been proposed that FSH may be responsible mainly for ovarian growth, whereas LH is important in inducing final oocyte maturation and ovulation

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TABLE 1. Primers for cloning zebrafish gonadotropin subunits.

Primer	Gene		Primer sequence
1	*lhb*	Sense:	5′-CAAGAGCCCATTTTCCAC-3′
2		Antisense:	5′-AGGCTGCAGTC(GA)CAGCT-3′
3	*cga*	Sense:	5′-T(TG)GG(AC)TGTGAGGA(GA)TGC-3′
4		Antisense:	5′-AGCATGT(AG)GCTTC(TA)GA(TG)GTG-3′
5	*fshb*	Sense:	5′-CATTGATTCCCAGATGAGGA-3′
6		Antisense:	5′-TTG(CA)T(GA)CA(TAC)TC(AG)CAGCT-3′
7	*fshb*	Antisense:	5′-CAGAGCCACGGGGTACACAAAGACTG-3′
8	*lhb*	Antisense:	5′-CCGGGTATGTGATCTGCGGGTCCAC-3′
9	*cga*	Antisense:	5′-CTTGGACCTCAGGGGTGTGGGGTAAGC-3′
10	*fshb*	Sense:	5′-CGGGGGTCTCCAGCGAAACTCC-3′
11	*lhb*	Sense:	5′-AGCCTGCTGAGCAACCGCAACG-3′
12	*cga*	Sense:	5′-GACACTCATCACGCTCCGCCGG-3′

[20–23]. However, whether this is also the situation in other groups of teleosts such as cyprinids remains to be elucidated, especially when we consider the fact that the specificity of FSH and LH for their receptors varies significantly among different species. Although fish FSH and LH are homologs of mammalian counterparts [6], lines of evidence from different fish models show beyond doubt that they do not work in exactly the same way as those in tetrapods. To gain insight into the functionality of the two gonadotropins in fish, it is imperative to carry out systematic studies in representative model species, and the success of such studies would depend on the availability of 1) recombinant FSH and LH with proven biological activities, 2) information about the receptor systems involved in FSH and LH signaling, 3) a sound assay system to assess and analyze the roles of the two hormones, and 4) bioinformatics information of the species.

In the past two decades, especially with the recent development of genomics, zebrafish (*Danio rerio*), a member of cyprinids, has been quickly rising as a top vertebrate model, first in developmental biology but now in a variety of fields including physiology. Using zebrafish ovary as the model, our laboratory has been working on the local ovarian regulatory network of growth factors and its regulation by gonadotropins [24–35]. Although all the studies we reported so far used human chorionic gonadotropin (hCG), these, together with the work from others [36–38], have made zebrafish ovary a well-validated and sound platform for further analysis of the functions of endogenous FSH and LH and their mechanisms of actions. As the first step toward this, the present study was undertaken to clone the subunits of zebrafish FSH (zfFSH) and LH (zfLH) and characterize their spatial expression patterns in the pituitary and extrapituitary tissues as well as their temporal expression profiles in the pituitary during the daily ovulatory cycle. To analyze the receptor specificity of the two zebrafish gonadotropins, we further established two stable Chinese hamster ovary (CHO) cell lines that express recombinant zfFSH and zfLH followed by testing their activities on the stable cell lines that express zebrafish gonadotropin receptors Fshr and Lhr. This represents one of the few studies in teleosts and the first in cyprinids on the receptor specificity of the two gonadotropins.

MATERIALS AND METHODS

Animals and Chemicals

Zebrafish were purchased from local pet stores and maintained in flow-through aquaria (36 L) at 26°C on a 14L:10D photoperiod with the light on at 0800 and off at 2200. Under this photoperiod condition, the fish normally spawn within 1 h after the light was on. The fish were fed twice per day with commercial tropical fish food. All experiments were performed under license from the government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

All chemicals were obtained from Sigma (St. Louis, MO) or Amersham Biosciences (Piscataway, NJ), and all enzymes used for cloning were from Promega (Madison, WI) unless otherwise stated. The culture medium and fetal bovine serum (FBS) were purchased from Gibco Invitrogen (Carlsbad, CA).

Genomic DNA Isolation

The caudal fins were excised from five zebrafish and digested at 50°C for 14 h with proteinase K (100 μg/ml; Gibco) in digestion buffer (100 mM NaCl, 100 mM Tris-HCl, 25 mM EDTA, 0.5% SDS). The digest was then extracted with phenol/chloroform, and the genomic DNA was precipitated with ammonium acetate and ethanol. The residual RNA was removed from the preparation by treatment with RNase A (50 μg/ml) followed by phenol/chloroform extraction. The genomic DNA was then precipitated again and finally dissolved in 20 μl TE buffer.

Total RNA Isolation and Reverse Transcription

Total RNA was extracted from pooled zebrafish pituitaries using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. Briefly, the pituitaries were isolated from the zebrafish of mixed sexes under the dissecting microscope and homogenized in 2 ml Tri-Reagent. Chloroform (500 μl) was then added and the mixture vortexed on the Thermomixer Comfort (Eppendorf, Hamburg, Germany) for 2 min at 1300 rpm. After centrifugation, the aqueous layer (500 μl) was transferred to a new tube and mixed well with 500 μl isopropanol to precipitate the RNA. The same protocol was also used to extract RNA from other tissues and cultured CHO cells. To synthesize cDNA from the total RNA, reverse transcription was performed at 42°C for 2 h in a reaction of 10 μl containing 3 μg RNA, 1× M-MLV buffer, 0.5 mM each dNTP, 0.5 μg oligo-dT, and 80 U M-MLV reverse transcriptase.

Cloning of Zebrafish fshb *(FSHβ),* lhb *(LHβ), and* cga *(GTHα) Fragments*

Degenerate primers for LHβ (Table 1, Primers 1 and 2) and GTHα (Primers 3 and 4) were designed based on the conserved regions of the molecules from several teleosts. The primers do not flank any potential intron sites. PCR was performed on the genomic DNA on the Thermal Cycler 9600 (Eppendorf) in a volume of 30 μl consisting of 1 μg of genomic DNA, 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 μM each primer, and 0.75 U *Taq* polymerase. Thirty-five cycles of amplification were carried out with the reaction profile of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. The amplified PCR fragments were resolved on agarose gel, isolated, cloned into pBluescript II KS (+) (Stratagene, La Jolla, CA) through T/A cloning, and sequenced.

The approach used for amplifying zebrafish *lhb* and *cga* using degenerate primers was not successful for amplifying *fshb*. Considering that both zebrafish and goldfish (*Carassius auratus*) are cyprinids, we then tried a sense primer specific for goldfish FSHβ [39] (Table 1, Primer 5) to amplify the 3′ region of zebrafish *fshb* cDNA using the SMART-RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). This was followed by a nested PCR amplification with Primer 5 and a degenerate primer (Table

TABLE 2. Primers for real-time PCR amplification.

Gene		Primer sequence	Expected size (bp)	GenBank accession no.
fshb	Sense:	5′-CAGATGAGGATGCGTGTGC-3′	281	AY424303
	Antisense:	5′-ACCCCTGCAGGACAGCC-3′		
lhb	Sense:	5′-ATGTTATTGGCTGGAAATGG-3′	423	AY424304/AY424305
	Antisense:	5′-CTAGTATGCGGGGAAATCC-3′		
cga	Sense:	5′-GACACTCATCACGCTCCG-3′	383	AY424306
	Antisense:	5′-TAGTAACAGGTGCTGCAGTGG-3′		
gapd	Sense:	5′-AATGAAGGGAATTCTGGGA-3′	398	AW826687
	Antisense:	5′-AACAACTACAGCAATGCCTG-3′		

1, Primer 6) designed for a conserved region of FSHβ. The PCR reaction profile was the same as that for *lhb* and *cga.*

Cloning of Full-Length cDNAs for fshb, lhb, *and* cga

Antisense primers (Table 1, Primers 7–9) were designed based on the sequences of the cloned cDNA fragments and used for 5′-RACE (rapid amplification of cDNA ends). The 5′-RACE products were cloned and sequenced for designing new primers for 3′-RACE. The 3′-RACE primers (Table 1, Primers 10–12) were designed based on the sequences near the 5′ ends of the 5′-RACE products to amplify the full-length cDNAs. RACE PCR was performed with a profile consisting of 5 cycles at 94°C for 5 sec and 72°C for 3 min; 5 cycles at 94°C for 5 sec, 70°C for 10 sec, and 72°C for 3 min; followed by 31 cycles at 94°C for 5 sec, 68°C for 10 sec, and 72°C for 3 min. The PCR products of desired length were excised from agarose gel and cloned into pBluescript II KS (+) through T/A cloning. Both strands of the 3′-RACE products or full-length cDNAs were sequenced with the BigDye Terminator Cycle Sequencing Kit v3.1 and analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

In Situ Hybridization

Sexually mature female zebrafish were sacrificed by decapitation, and their heads were fixed in Bouin solution and processed for paraffin sectioning. The cross sections at 7-μm thickness were mounted on the slides coated with poly-(L)-lysine. For in situ hybridization, the consecutive sections were hybridized with antisense cRNA probes specific for *fshb*, *lhb*, and *cga* according to a reported protocol [40] with modifications. The probes were prepared by in vitro transcription, and *lhb* sense probe was used as the negative control. Briefly, the sections were rehydrated, fixed with 4% paraformaldehyde, and washed in TBS buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). After treatment with 0.2 N HCl for 10 min, the sections were heated at 70°C for 15 min in 2× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) to denature RNA. After rinsing with TBS and fixing with 4% paraformaldehyde again, the sections were acetylated with 0.5% acetic anhydride and permeabilized at 37°C for 10 min with proteinase K (50 μg/ml) in TBS with 2 mM $CaCl_2$. The sections were then dehydrated, treated with the prehybridization solution (2× SSC, 50% formamide, 0.02% SDS, and 0.01% salmon sperm DNA) at 58°C for 30 min and hybridized at 58°C overnight with 300 ng/ml DIG-labeled antisense or sense probes in the hybridization buffer (2× SSC, 50% formamide, 0.02% SDS, 0.01% salmon sperm DNA, and 10% dextran sulfate). The hybridization was carried out in the Hybri-well press-seal hybridization chamber (Sigma). After a series of washing with 2× SSC, 50% 2× SSC/50% formamide (58°C,) and 1× SSC, the sections were treated for 15 min with the blocking solution containing 10% sheep serum and 1% Blocking Reagent (Roche, Mannheim, Germany), and the DIG-labeled probes were detected with anti-DIG-alkaline phosphatase (Roche) according to the manufacturer's protocol. Color development was stopped by 20-min washing in distilled water with gentle shaking. The sections were finally mounted in gelatin glycerol (Sigma) and viewed with Nikon microscope Microphot-FX (Nikon, Tokyo, Japan). All images were captured with a Nikon DXM 1200 digital camera (Nikon) and analyzed with the software Nikon ACT-1 version 2.12 (Nikon).

Real-Time RT-PCR Quantitation of fshb, lhb, *and* cga *Expression During the Ovulatory Cycle*

To examine the expression of all the subunits during daily ovulatory cycle, about 50 sexually mature spawning female zebrafish and 50 males of similar body size were chosen and randomly divided into six groups for sampling at different times (1900, 2200, 0100, 0400, 0700, and 1200) of the ovulatory cycle. At each time point, the females were killed by decapitation and the pituitary glands isolated. The total RNA from each pituitary was extracted with 100 μl Tri-Reagent and reverse transcribed as described previously.

The expression levels of zebrafish gonadotropin subunits were analyzed by real-time RT-PCR. The standard for each subunit and *gapd* (glyceraldehyde-3-phosphate dehydrogenase) was prepared by PCR amplification of cDNA fragment with specific primers (Table 2), the amplicon was purified and quantitated by electrophoresis along with the Mass Ruler DNA Marker (MBI Fermentas, Hanover, MD), and the copy number of DNA molecule was calculated. The amplified DNA molecule of each gene was used to construct standard curve in all real-time PCR assays.

The real-time PCR was performed on the iCycler iQ Real-time PCR Detection System (Bio-Rad) in a volume of 30 μl containing 0.33 μl RT reaction product, 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 μM each primer, 0.75 U *Taq* polymerase, SYBR Green I (1:35 000; Molecular Probes, Leiden, The Netherlands), and 20 nM fluorescein (Bio-Rad). The reaction profile consisted of 40 cycles of 94°C for 30 sec, 60°C (58°C for *gapd*) for 30 sec, 72°C for 1 min, and 82°C for 7 sec for signal detection. To assess the specificity of the PCR amplification, a melt curve analysis was performed at the end of the reaction consisting of 175 cycles of 7 sec with temperature increased at a rate 0.2°C/cycle. In addition, the specificity of PCR amplification was also confirmed by agarose gel electrophoresis at the end of real-time assay, and the identities of the amplified products for all genes were further confirmed by cloning the PCR products into pBluescript II KS (+) and sequencing.

Construction of Expression Constructs

Gene-specific primers flanking the open reading frame (ORF) of the three subunits were used to amplify the ORF of each gene directly from

TABLE 3. Primers for PCR amplification of open reading frames.

Gene		Primer sequence[a]	Expected size (bp)
fshb	Sense:	5′-CCC**AAGCTT**GGGCCACCATGAGGATGCGTGTGCTTG-3′	422
	Antisense:	5′-CCG**CTCGAG**CGGCTAGTGTATGCTGCAGCTGG-3′	
lhb	Sense:	5′-CCC**AAGCTT**GGGCCACCATGTTATTGGCTGGAAATGG-3′	452
	Antisense:	5′-CCG**CTCGAG**CGGCTAGTATGCGGGGAAATCC-3′	
cga	Sense:	5′-CCC**AAGCTT**GGGCCACCATGTTTTGGACAAGATACG-3′	383
	Antisense:	5′-CCG**CTCGAG**CGGTTAAGACTTATGATAGTAAC-3′	

[a] The restriction enzyme sites added are bolded and the modified sequences before ATG are underlined to conform to the Kozak consensus sequence (GCCGCC[A/G]CCATGG). All the primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

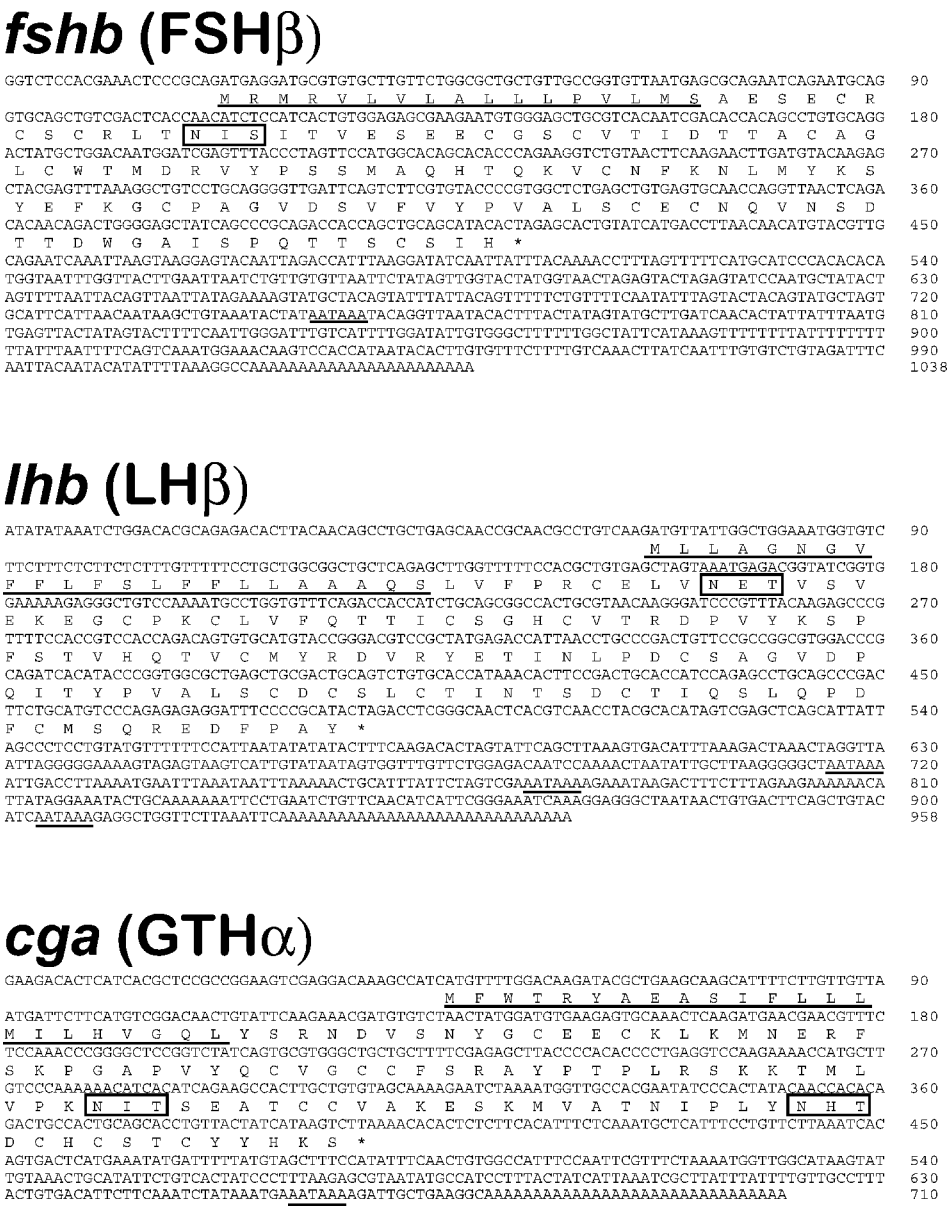


FIG. 1. DNA and deduced amino acid sequences of zebrafish Fshb (FSHβ) (AY424303), Lhb1 (LHβ1) (AY424304 for Lhb1 and AY424305 for Lhb2), and Cga (GTHα) (AY424306). The putative polyadenylation signals (AATAAA) are underlined in the 3′-untranslated regions, and the predicted signal peptides are underlined at the N-terminal. The potential N-linked glycosylation sites are marked by boxes.

the pituitary. Restriction sites for *Hind*III and *Xho*I were added at the 5′-end of the sense and antisense primers, respectively, for subsequent cloning. The Kozak sequence was included in all sense primers before the start codon ATG to enhance translation efficiency [41] (Table 3). PCR was carried out for 30 cycles in a volume of 25 μl containing 0.5 μl RT reaction product, 1× PCR buffer, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 0.2 μM each primer, and 1.5 U *Pfu* polymerase with the profile of 30 sec at 94°C, 30 sec at 60°C, and 1.5 min at 72°C. The PCR products were double digested with *Hind*III and *Xho*I and cloned into pcDNA5/FRT vector (Invitrogen, Carlsbad, CA) at *Hind*III and *Xho*I sites downstream of the CMV promoter to generate three constructs: pcDNA5/FRT/*fshb*, pcDNA5/FRT/*lhb*, and pcDNA5/FRT/*cga*. To construct the expression plasmids that coexpress α and β subunits, pcDNA5/FRT/*cga* was double digested with *Pvu*II and *Bgl*II to release the insert and promoter and blunt ended with Klenow fragment. The plasmids pcDNA5/FRT/*fshb* and pcDNA5/FRT/*lhb* were cut open at *Bgl*II site and blunt ended. The *Pvu*II/*Bgl*II fragment containing *cga* ORF and CMV promoter was then inserted into the blunt-ended *Bgl*II site on pcDNA5/FRT/*fshb* and pcDNA5/FRT/*lhb* to generate the expression constructs for zfFSH and zfLH, respectively. Since the inserts were amplified directly from the zebrafish pituitary, they were independent of the cloned cDNAs. All the expression constructs were sequenced to confirm sequence fidelity (the clone obtained for *lhb* was confirmed to be *lhb*2).

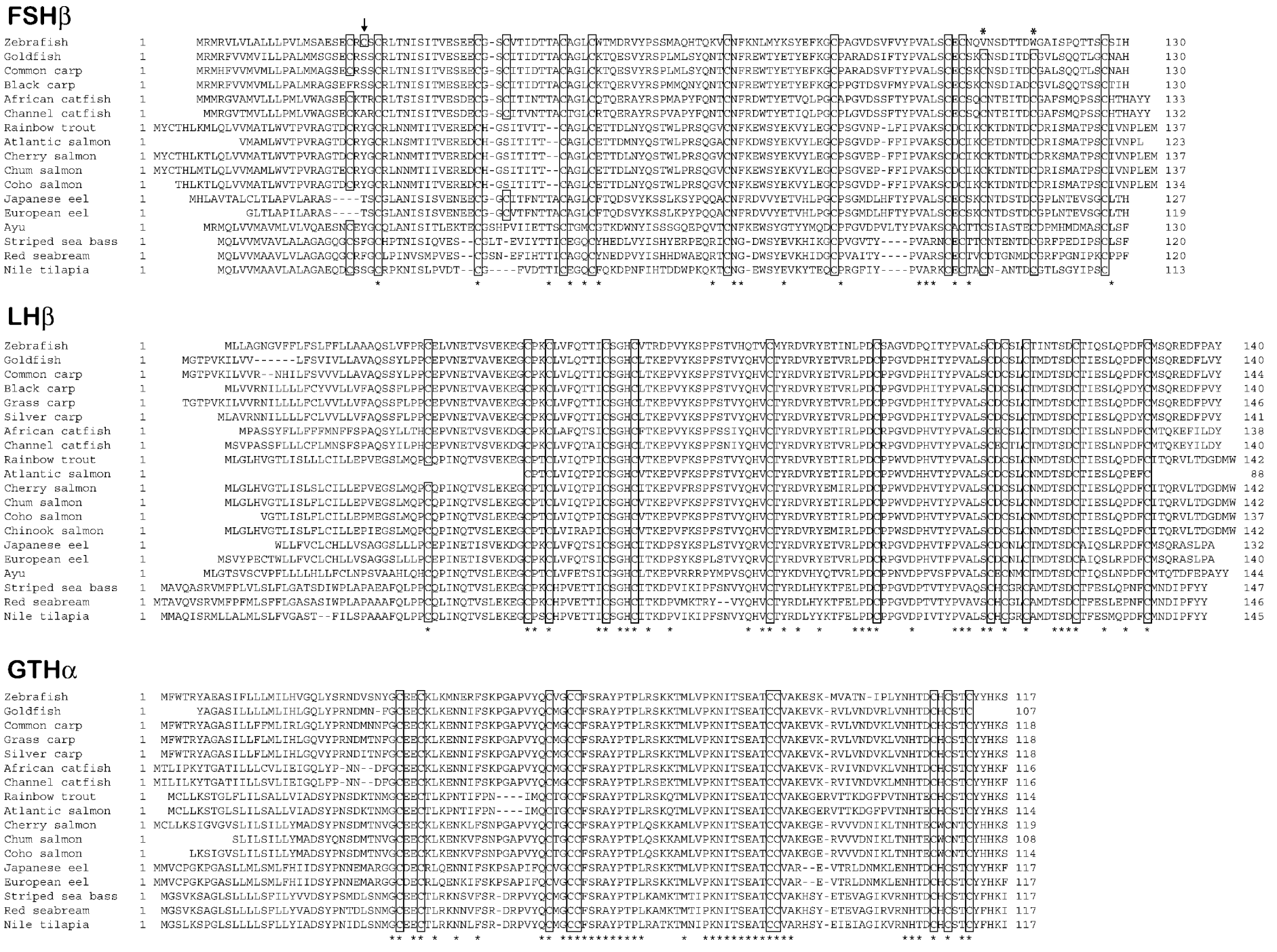
Cell Culture and Transfection of Flp-In CHO Cells

Flp-In CHO cells (Invitrogen) were cultured in Ham F-12 medium containing antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml) and 10% FBS at 37°C with 5% CO_2. The cells were subcultured in 10-cm culture dishes (Falcon, Franklin Lakes, NJ) and allowed to grow to 25% confluence before transfection. The expression construct for zfFSH or zfLH (2 μg) was cotransfected into the CHO cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol together with 18 μg of plasmid pOG44 (Invitrogen) that encodes a recombinase to facilitate homologous recombination at the specific FRT site.

Recombinant Production of zfFSH and zfLH

The transfected CHO cells were selected by hygromycin B (Invitrogen) at 500 μg/ml and cloned by limited dilution in 96-well plates. The wells containing single cells or colonies were marked, and some were expanded when the cells had grown to half confluence for RT-PCR characterization and functional test. The hormone activities were assayed by adding the conditioned medium to the CHO-K1 cells that stably express zebrafish gonadotropin receptors Fshr or Lhr and the cAMP-responsive reporter SEAP (secreted human placental alkaline phosphatase) (Kwok et al., published separately).

The clones that exhibited activities of FSH or LH were isolated and



expanded for further RT-PCR and Northern blot characterization. The confirmed clones were then used for the production of recombinant proteins according to the protocol described by Schatz et al. [42]. Briefly, the cells (1×10^6) were subcultured into a 750-ml flask (Falcon) in 50 ml Ham F-12 medium with 10% FBS and allowed to grow to about 90% confluence at 37°C for 4 days. The FBS-containing medium was then replaced with 50 ml FBS-free medium and the culture temperature reduced to 28°C. After further incubation for 5 days at the reduced temperature, the medium was harvested and concentrated by 200 folds with Amplicon Ultra 10 000 MWCO (Millipore, Bedford, MA), and the cells were collected for RNA extraction for further characterization by RT-PCR and Northern blot hybridization.

Northern Blot Hybridization

Northern blot hybridization was performed on both zebrafish pituitary and cloned CHO cells according to our previous report [43] to analyze the expression of the three gonadotropin subunits. About 130 zebrafish pituitaries were used to extract total RNA. The RNA from zebrafish pituitaries (13 μg) or CHO cells (10 μg) was resolved on 1% denaturing agarose gel containing 2.2 M formaldehyde and transferred to positively charged nylon membrane (Roche). The blots were UV cross-linked using the GS Gene Linker (Bio-Rad) and hybridized with DIG-labeled antisense RNA probes. The signals were detected with the Chemiluminescent Detection Kit according to the manufacturer's instruction and analyzed on the Lumi-Imager F1 Workstation (Roche).

SEAP Reporter Gene Assay

The assay for the biological activity of recombinant zfFSH and zfLH was based on the cAMP-induced expression of SEAP by the CHO cells that express either zebrafish *fshr* or *lhr*. Briefly, the CHO-Fshr and CHO-Lhr cells were plated in 48-well plates (Falcon) at the density of 2.5×10^4 cells/well in Ham F-12 medium supplemented with 10% FBS. After 24-h incubation, the concentrated conditioned media from zfFSH or zfLH cell lines were added at different concentrations. After 18-h treatment at 37°C, the medium was collected from each well and the level of SEAP activity quantified with the Chemiluminescent SEAP Reporter Gene Assay Kit (Roche) according to the instruction of the manufacturer and our recent report [44]. The chemiluminescent signal was visualized, quantified, and analyzed with the Lumi-Imager and the software LumiAnalyst 3.1 (Roche).

Data Analysis

The sequence analysis was performed with MacVector 7.2.2 (Accelrys, San Diego, CA). The signal peptides were predicted with the software SignalP 3.0 at the ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics. For reporter gene assay, the dose response of SEAP activity was analyzed by nonlinear regression using Prism 4.0b on Macintosh OS X (GraphPad Software, San Diego, CA). The mRNA levels of *fshb*, *lhb*, and *cga* in each pituitary were first calculated as the ratio to that of *gapd*, which was measured as the internal control, and then expressed as the percentage of those at 1900. The experiment was repeated three times using different batches of fish, and the data were combined for analysis with one-way ANOVA followed by Dunnett's test.

RESULTS

Cloning and Sequence Analysis of Zebrafish fshb, lhb, *and* cga

In the present study, the full-length cDNAs encoding zebrafish *fshb*, *lhb*, and *cga* subunits were cloned by RACE and sequenced. The cloned *fshb* cDNA consists of 1038 bp

FIG. 2. Comparison of deduced amino acid sequences of zebrafish Fshb, Lhb, and Cga with those of representative teleost species. The conserved cysteines are boxed. The asterisks at the bottom of each alignment indicate the amino acids that are fully conserved among the species listed. For the alignment of FSHβ, the arrow on the top marks the additional unique cysteine in the zebrafish only, and the two missing cysteines at the C-terminal are marked by asterisks on the top.

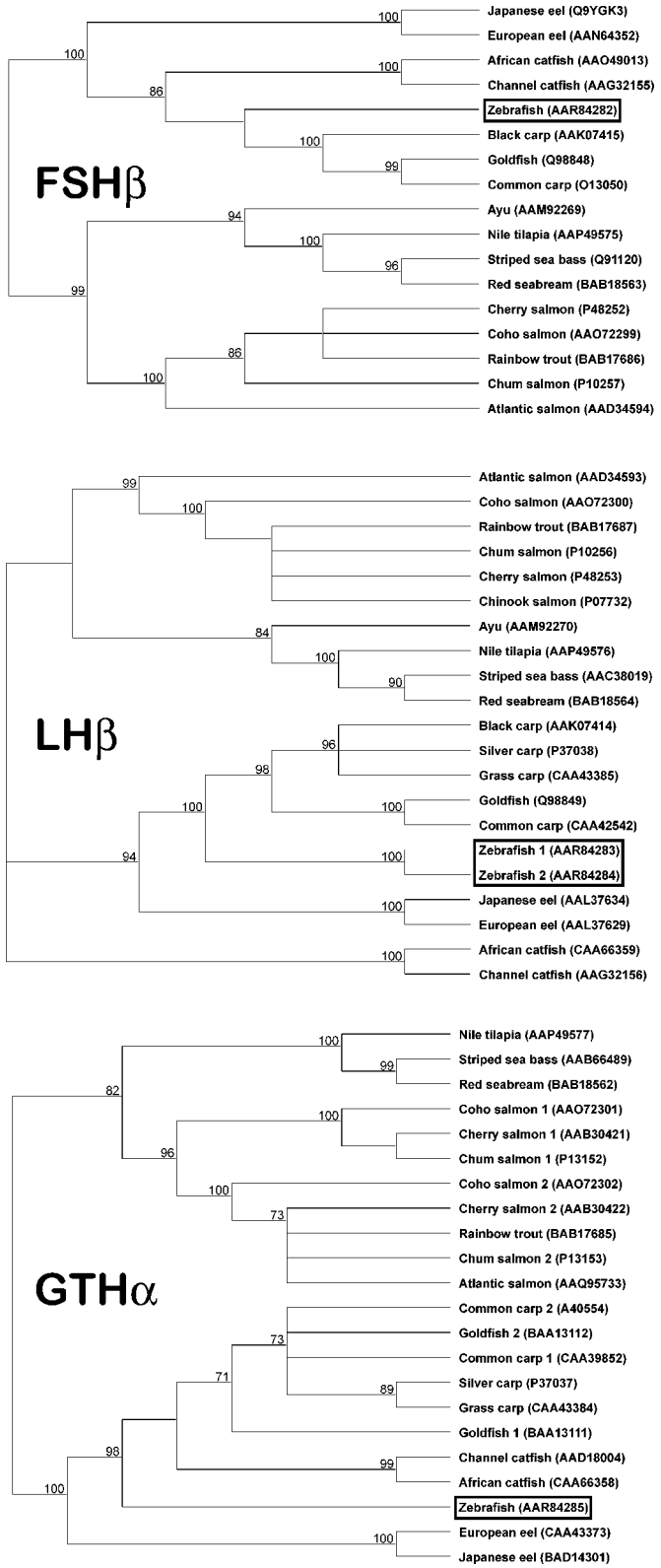


FIG. 3. Phylogenetic relationship of zebrafish Fshb, Lhb, and Cga with their counterparts from other representative teleosts as demonstrated by bootstrapping analysis using neighbor joining method. The numbers at the forks are the bootstrap proportions.

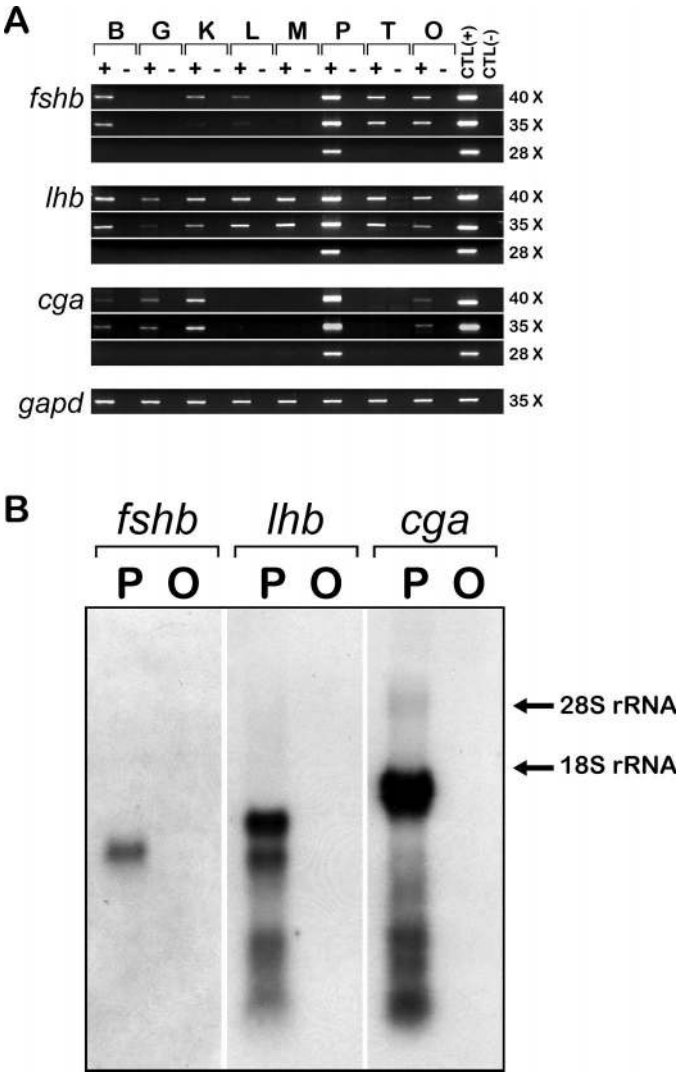


FIG. 4. Tissue distribution of *fshb, lhb,* and *cga* expression as analyzed by reverse transcription-polymerase chain reaction for different cycle numbers (28×, 35×, and 40×) (1 μg RNA for the pituitary and 3 μg for other tissues) (**A**). The abundant expression of these subunits in the pituitary was confirmed by Northern blot hybridization with the ovary as the control (**B**). B, brain; G, gill; K, kidney; L, liver; M, muscle; P, pituitary; T, testis; O, ovary. CTL(+), positive control on cloned cDNA; CTL(−), negative control without templates.

with an ORF of 393 bp (including the stop codon). A putative polyadenylation signal AATAAA is located about 260 bp upstream of the poly (A) tail. The putative protein contains 130 amino acids, including a signal sequence of 17 amino acids (Fig. 1). At the amino acid level, zebrafish Fshb shares 64–67% homology with its counterparts in cyprinids and only 30–57% with those of other teleosts (Figs. 2 and 3). As expected, the mature peptide of Fshb contains a single putative N-linked glycosylation site (NIS) and 12 cysteine residues. Interestingly, zebrafish Fshb exhibits unique cysteine distribution as compared to the molecules from other teleosts (Fig. 2). Among the 13 cysteines conserved in other cyprinids, two cysteines located at the C-terminal are absent in zebrafish Fshb (the 10th and 11th according to the numbering in tetrapods [7]); however, zebrafish Fshb has an additional cysteine at the N-terminal before the first cysteine in tetrapods. The amino acid sequence of zebrafish Fshb was confirmed by cloning and sequencing of five independent clones.

As for *lhb*, two cDNA clones coding for distinct amino acid sequences were isolated and designated *lhb*1 (958 bp) and *lhb*2 (956 bp), respectively. Both clones have a coding region of 423 bp and contain three potential polyadenylation signals in the 3′-untranslated region (Fig. 1). A signal peptide of 23 amino acids is predicted at the N-terminal. The two cDNAs have 14 nucleotide differences, and five nucleotide deletions are present in *lhb*2 (data not shown). The two putative proteins have high homology at the amino acid level, with only one amino acid difference at position 55. The location of the N-linked glycosylation site (NET) and the 12 cysteine residues are fully conserved compared with the sequences from other teleosts (Fig. 2). The amino acid sequence of zebrafish Lhb shows about 72–75% homology with those of cyprinids and 47–64% with the molecules of other fish species (Fig. 2 and 3). Zebrafish Fshb and Lhb share very low overall homology (40%) between themselves; however, the number and location of cysteine residues are much more conserved with 10 out of 12 cysteines identical in the two molecules (Fig. 2).

The cloned cDNA of *cga* consists of 710 bp with a polyadenylation signal at 14 bp upstream of the poly (A) tail. The coding region encodes a peptide of 117 amino acids with a putative signal peptide of 23 amino acids at the N-terminal (Fig. 1). The number and location of the two potential N-linked glycosylation sites (NIT, NHT) and 10 cysteine residues are fully conserved compared with the molecules from other teleosts (Fig. 2). The amino acid sequence of zebrafish Cga shows high homology (70–80%) with its counterparts in cyprinids but lower homology with those of other teleosts (55–70%) (Figs. 2 and 3).

Tissue Distribution of fshb, lhb, *and* cga *Expression*

Gonadotropins are well known to be expressed by the pituitary. However, conventional RT-PCR analysis in the zebrafish using the primers listed in Table 2 revealed that in addition to the pituitary, which exhibited the highest level of expression for all subunits, each subunit of FSH and LH also showed novel extrapituitary expression in other tissues, particularly the gonads (Fig. 4A). The expression of *fshb* could be consistently detected in the ovary, testis, brain, kidney, and liver, whereas *lhb* had obviously wider tissue distribution with the signal detectable in all the tissues examined, including the ovary, testis, brain, gill, kidney, liver, and muscle. The expression of *cga* could also be detected in the brain, gill, and kidney; however, in the gonads, only a weak signal was observed in the ovary but not in the testis. It should be noted that the extrapituitary expression of all three subunits could be detected only with high cycle numbers of PCR amplification (35× and 40×), and the pituitary was the only tissue that generated signals with low amplification cycle number (28×). However, despite that the expression levels were generally low in the extrapituitary tissues, the expression patterns of the three subunits were highly reproducible with different individuals.

The abundant expression of *fshb*, *lhb*, and *cga* in the pituitary was further demonstrated by Northern blot hybridization on the total RNA isolated from 130 pituitary glands (Fig. 4B). The mRNA transcripts were easily detected for all three subunits with *fshb* showing the weakest signal and *cga* the strongest. The transcript of *cga* was the biggest of the three subunits. For *lhb* and *cga*, in addition to the major transcripts, several minor bands were also detected, which could be due partly to RNA degradation be-

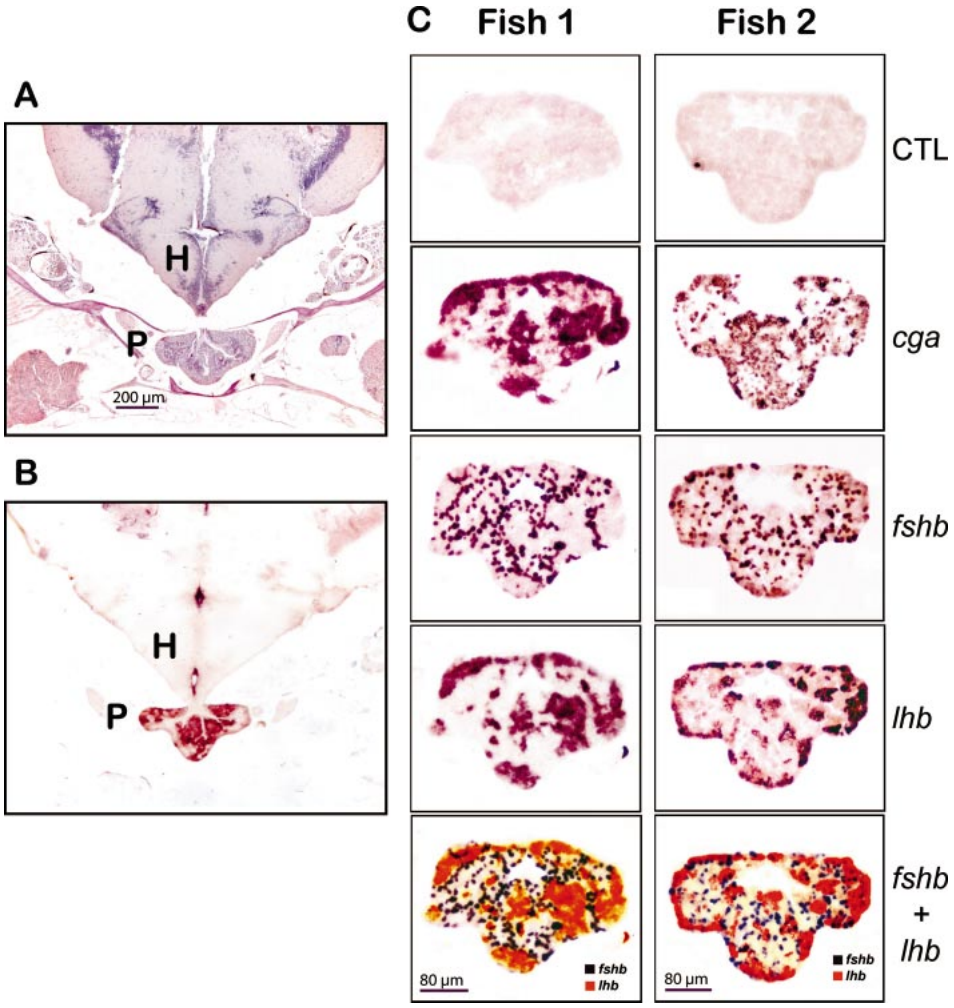


FIG. 5. Spatial expression patterns of *fshb, lhb,* and *cga* in the pituitary. **A**) Cross section of zebrafish head stained with hematoxylin and eosin. **B**) In situ hybridization with *fshb* probe showing specific staining in the pituitary only. **C**) Two series of consecutive sections from two fishes stained with the antisense *fshb, lhb,* and *cga* probes. The control (CTL) sections were stained with the sense *lhb* probe. The images shown at the bottom of each series are the superimposed pictures of *fshb* and *lhb* cells generated with Photoshop CS. H, hypothalamus; P, pituitary.

cause the pituitary samples were collected and stored over a certain time before they were pooled for RNA extraction. However, we cannot rule out the possibility that there might be multiple transcripts for these subunits. As a negative control, no signal was detected in the zebrafish ovary for any subunit, although all three subunits showed expression in the ovary when assayed with more sensitive RT-PCR (Fig. 4).

Localization of fshb, lhb, *and* cga *Expression in the Pituitary*

Before in situ hybridization was performed, we first examined the general histological morphology of the zebrafish pituitary using hematoxylin-eosin (H.E.) staining. As shown in Figure 5A, the pituitary gland was located in a bony hollow right beneath the hypothalamus of the brain. The nerve fibers from the hypothalamus extended downward and ramified into the pituitary. To localize the expression of *fshb*, *lhb*, and *cga*, we performed in situ hybridization on consecutive cross sections of the pituitary. The detection was specific for all three subunits with the signals detectable only in the pituitary (Fig. 5B with *fshb* probe).

The mRNAs of *fshb*, *lhb*, and *cga* could be easily and clearly detected in the pituitary, whereas no signal was detected in the control sections using *lhb* sense probe (Fig. 5C). Interestingly, the *fshb*-positive and *lhb*-positive cells showed distinct patterns of distribution in the zebrafish pituitary. The *fshb*-positive cells were well separate and scattered as single cells or small cell clusters, whereas the *lhb*-positive cells were generally arranged as large clusters in the same area. The numbers of the two gonadotrophic cells seemed to be similar; however, it was difficult to quantitate accurately. When the two images for *fshb* and *lhb* staining on consecutive sections were superimposed using photo editing software Photoshop CS (Adobe Systems, San Jose, CA), the resulted combined image clearly illustrated that *fshb* and *lhb* mRNAs were expressed in two distinct populations of cells with only limited overlappings, and this could be due to the fact that the staining for the two subunits was not performed on the same section. Hybridization with *cga*-specific probe revealed extensive signals that apparently covered all the areas where *fshb*- and *lhb*-positive cells were present.

Temporal Expression Profiles of fshb, lhb, *and* cga *During Daily Ovulatory Cycle*

In contrast to the pituitary of other fish models, zebrafish pituitary is a tiny organ that can be isolated only under the microscope. This makes RNA extraction and quantitation of gene expression a challenging task. To test the feasibility of performing real-time RT-PCR on a single zebrafish pituitary, we first performed conventional RT-PCR using gene-specific primers for *fshb*, *lhb*, *cga*, and *gapd* (Table 2). As shown in Figure 6A, the expression could be easily detected at the pituitary level for all genes investigated,

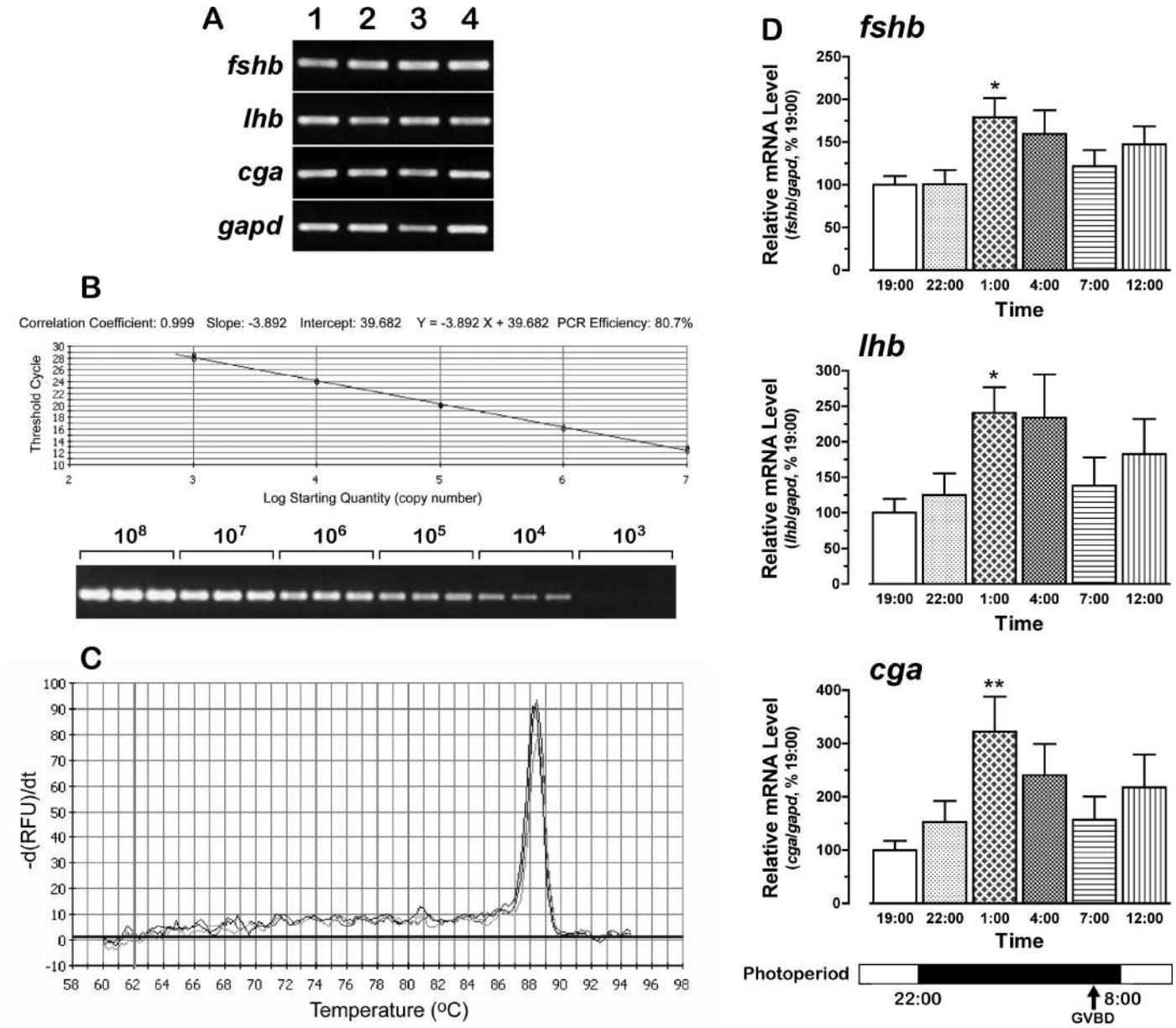


FIG. 6. Temporal expression profiles of *fshb, lhb,* and *cga* during the daily ovulatory cycle. All three subunits and housekeeping gene *gapd* could be easily amplified by reverse transcription-polymerase chain reaction at the single pituitary level, and the results were reproducible on four individuals (**A**). A typical standard curve for real-time PCR measurement of *fshb* expression is shown in (**B**) with the agarose gel image at the bottom to demonstrate specific PCR reaction. The specificity of the amplification was further confirmed by melt curve analysis (**C**). The expression levels of *fshb, lhb,* and *cga* at different time points are presented in (**D**) with the photoperiod shown at the bottom. The data were combined from three independent experiments with each data point representing the mean ± SEM of 19–25 samples. * $P < 0.05$; ** $P < 0.001$ vs. 1900. GVBD, germinal vesicle breakdown.

demonstrating the feasibility of RT-PCR quantitation at the single pituitary level.

Real-time RT-PCR assays were then developed and validated for all target genes including *gapd* as the control. A typical standard curve for *fshb* is shown in Figure 6B with the melt curve showing the single amplicon in the reaction (Fig. 6C). We then used the real-time RT-PCR assays to investigate the temporal expression profiles of zebrafish gonadotropin subunits during the daily ovulatory cycle, with emphasis on the periovulatory period. Sexually mature spawning females were sampled at several time points (1900, 2200, 0100, 0400, 0700, and 1200) and individual pituitary glands isolated for real-time RT-PCR analysis. At 2200, there was either a slight increment in expression (*lhb* and *cga*) or no change (*fshb*) compared with the levels at 1900. However, at 0100, when the germinal vesicle had started its migration under our aquarium conditions, the mRNA levels of all three subunits elevated significantly, particularly *cga* expression, which showed more than 3-fold increase, whereas the increases in *fshb* and *lhb* expression were about 1.8- and 2.2-fold, respectively. After reaching their maximal levels at 0100, a decreasing trend was noticed afterward for all the three subunits. The expression levels at 0400, when the germinal vesicle had migrated to the periphery, were lower but still seemed to be comparable to those at 0100. At 0700, when the final oocyte maturation (germinal vesicle breakdown, GVBD) and ovulation occurred, the expression of the three subunits had decreased

to the levels comparable to those at 1900. A slight increase in the expression of the subunits was observed at 1200, but it was not statistically significant.

Recombinant Production of zfFSH and zfLH

To study the biological activities and physiological relevance of the two gonadotropins, the availability of homologous hormones is essential. With the cDNAs of zebrafish gonadotropin subunits and the receptor-based bioassays for the two hormones available, we went on to produce recombinant zfFSH and zfLH using CHO cells as the bioreactor. After transfection and selection with hygromycin B, three CHO-zfFSH (F1, F2, and F3) and two CHO-zfLH (L1 and L2) clones were isolated by limited dilution. RT-PCR analysis showed that both L1 and L2 expressed *lhb* and *cga*; however, only one CHO-zfFSH clone (F2) expressed both *fshb* and *cga* subunits. The clones F2 and L1 were expanded, and their expression of *cga* and *fshb* or *lhb* was further confirmed by Northern blot hybridization. Both *fshb* and *lhb* were expressed as a single transcript at high levels in the two cell lines. A single transcript could also be detected for *cga,* but its expression level seemed to be lower than that of the β subunit in each cell line (Fig. 7).

To increase the yield of recombinant production, we adopted a novel approach of cell culture as reported [42] that involved two phases: a growth phase at 37°C in FBS-containing medium followed by a production phase at 28°C in serum-free medium. This approach would also ease the subsequent purification process because the serum-free medium was used in the production phase [45]. Our results also showed an increase in the biological activities detected for zfFSH and zfLH using this culture scheme (data not shown).

Functional Analysis of Recombinant zfFSH and zfLH

The functional identity of the three subunits cloned and the receptor specificity of the recombinant zfFSH and zfLH were investigated using the two stable CHO cell lines that express zebrafish gonadotropin receptors *fshr* or *lhr* and a reporter gene SEAP whose expression is driven by a cAMP-responsive promoter. Recombinant zfFSH significantly stimulated SEAP expression in CHO-Fshr cells in a dose-response manner, but it had no effect on CHO-Lhr cells at any concentrations tested. To our surprise, recombinant zfLH significantly stimulated both zebrafish Fshr and Lhr with a slight preference for the latter (Fig. 8). The medium from a control CHO cell line that was transfected with the vector and selected with hygromycin B showed no effects on either receptors.

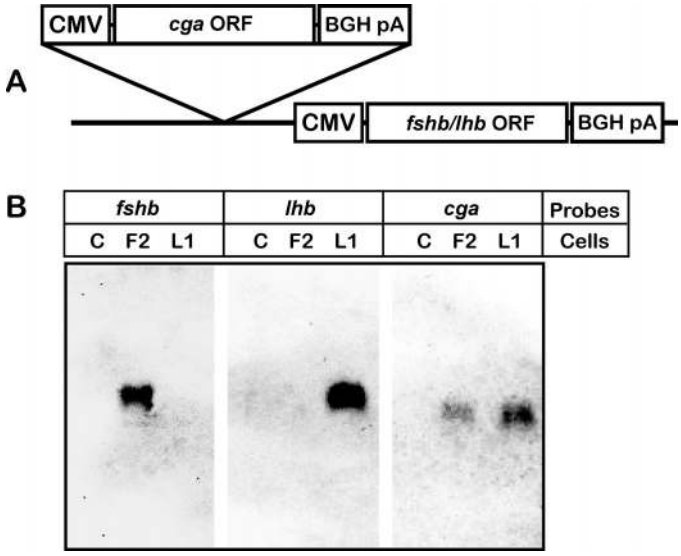


FIG. 7. Establishment of stable CHO cell lines that express recombinant zfFSH and zfLH. **A**) Schematic representation of the expression constructs for zfFSH and zfLH. **B**) Northern blot analysis for the expression of *fshb, lhb,* and *cga* in the two established cell lines (F2 and L1). CMV, cytomegalovirus promoter; BGH pA, bovine growth hormone polyadenylation sequence; C, control CHO cells.

DISCUSSION

The present study cloned and sequenced gonadotropin subunits *fshb, lhb,* and the common *cga* from the zebrafish pituitary, providing a clear proof for the duality of gonadotropins in this model organism. Compared with the similar work in other species, this part of work has been particularly challenging because of the extremely small size of the pituitary gland in this species.

Among the three subunits cloned, *fshb* shows the greatest structural variation from that of other fish species. The β subunits of glycoprotein hormones are characterized by 12 cysteines [1, 6, 7]. However, the FSHβ in the goldfish, common carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), and African catfish has 13 cysteines [39, 46–48]. In the zebrafish, although the number of cysteine residues agrees well with that in tetrapods and most teleosts, they displayed unique distribution with a cysteine located at the N-terminal before the conserved first cysteine in tetrapods and two cysteines missing at the C-terminal (the 10th and 11th according to the numbering in tetrapods [7]). Other cysteine residues are well conserved. The third cysteine unique for cyprinids, catfish, and mammals is also conserved in zebrafish Fshb. Consistent with that in other cyprinids, zebrafish Fshb possesses only one potential N-linked glycosylation site.

For zebrafish Lhb, the number and location of all cysteines and *N*-linked glycosylation site are fully conserved. Two isoforms of *lhb* cDNA were isolated in the present study, which is consistent with the reports on LHβ polymorphism in teleosts and tetrapods, including the common carp [49] and human [50]. In many teleost species, including the goldfish [51], common carp [46], chum salmon [52], and coho salmon [53], two GTHα variants have been reported. The existence of isoforms of gonadotropin subunits is likely due to the tetraploidity of the fish species studied. With the zebrafish genome project close to completion, the issue of gene copy number in the genome will soon be resolved. Since the two isoforms of zebrafish Lhb exhibit only one amino acid change, the possibility that they are different in function is small.

The amino acid sequences of zebrafish Fshb and Lhb showed very low homology (40%) between themselves. However, the positions of cysteines seem to be more conserved. Some short sequences, including PVA and GVD, which have been suggested to be essential for the association between LHβ and GTHα [47], are identical in zebrafish Fshb and Lhb, suggesting that these sequences may also be responsible for the interaction of FSHβ with GTHα in this species. In tetrapods, LHβ showed more rapid divergence than FSHβ at the amino acid level [54]. In contrast, teleost LHβ is more conserved than FSHβ in structure [39, 55–58]. The significantly lower conservation in fish

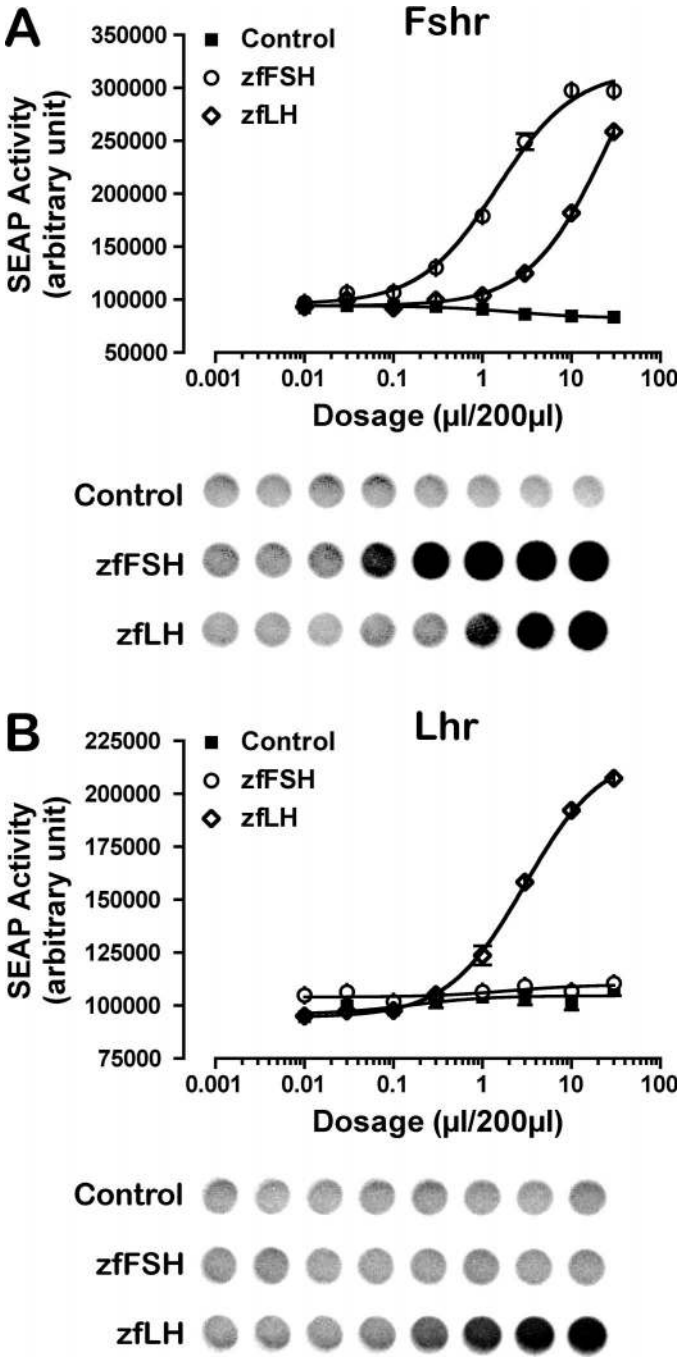


FIG. 8. Dose response of zebrafish Fshr- and Lhr-expressing cell lines to recombinant zfFSH and zfLH. Both cell lines also coexpress the cAMP-responsive reporter gene SEAP. The zebrafish Fshr could be significantly stimulated by both zfFSH and zfLH (**A**), whereas its Lhr responded only to zfLH without cross reaction with zfFSH (**B**). The medium from control CHO cells transfected with the vector alone showed no effect on either receptors. Each data point represents mean ± SEM of three replicates. The typical chemiluminescent images are shown at the bottom of each graph.

FSHβ implies a functional divergence of FSH, and it immediately raises an interesting question about the roles and physiological relevance of FSH in different groups of teleosts. The availability of recombinant FSH and LH in the zebrafish will provide a powerful tool to address this issue in the future.

Zebrafish Cga is a well-conserved molecule, with its putative N-linked glycosylation sites and cysteine residues fully conserved. The higher sequence conservation of Cga is likely due to the fact that this subunit is shared by all pituitary glycoprotein hormones, including FSH, LH, and TSH, and therefore has been under higher selective pressure during vertebrate evolution. Recently, the sequence of *cga* has also been reported by another laboratory (AY522553) [59]. However, sequence comparison shows that although the two *cga* cloned have identical amino acid sequence and there are only two nucleotide differences in the coding region, the 3′-untranslated regions of the two sequences exhibit great variation (data not shown).

Although the pituitary gland has been well accepted to be the site where FSH and LH are produced and released, RT-PCR analysis in the zebrafish clearly demonstrated that the subunits of gonadotropins were also widely expressed in a variety of extrapituitary tissues, including the gonads, brain, kidney, and liver, although the expression levels were obviously lower than those in the pituitary. A similar phenomenon of extrapituitary/placental expression of gonadotropins (FSH, LH, and/or hCG) in tissues such as gonads, brain, kidney, and liver has also been reported in mammals, including humans [60–64]. In teleosts, the expression of FSHβ and LHβ has been demonstrated in the brain of tilapia (*Oreochromis niloticus*) by both RT-PCR and immunocytochemical staining [65]. A recent study in the gilthead seabream (*Sparus aurata*) using Northern hybridization and/or RT-PCR demonstrated that all the subunits of FSH and LH were expressed in the ovary, and their expression was localized to the oocyte by in situ hybridization and/or immunocytochemistry. The expression seemed to be stage dependent and was subject to the regulation by gonadotropin-releasing hormone (GnRH) [66]. These results, together with the evidence in the zebrafish, suggest that the extrapituitary expression of gonadotropins may be a universal phenomenon in vertebrates. However, the physiological significance of the nonpituitary gonadotropins remains unknown and will be an interesting issue to address in the future. In the zebrafish, it is interesting to note that the extrapituitary expression of *fshb* was limited to only a few tissues, including the gonads, brain, kidney, and liver; however, *lhb* exhibited much wider tissue distribution. What is also interesting is that in some tissues, such as the testis, liver, and muscle, the expression of *fshb* and/or *lhb* was not accompanied by that of *cga*, suggesting that in some tissues *fshb* and *lhb* may be expressed as monomers. In mammals, the free LHβ subunit has been demonstrated to bind to LHR in the ovary without increasing cAMP level, and its presence significantly suppresses the binding of hCG to the ovarian LHR [67].

Although the duality of gonadotropins is well documented in all teleosts studied and numerous studies have demonstrated that the gonadotrophs are located in the proximal pars distalis [68–74] and the periphery of pars intermedia as well [40, 75–85], it is still ambiguous whether the two gonadotropins are synthesized in the same cells. The duality of gonadotrophs has been established by immunocytochemical staining in species like the killifish (*Fundulus heteroclitus*) [74] and Mediterranean yellowtail (*Seriola dumerilii*) [85] using antisera against the homologous gonadotropins or their β subunits and in the bluefin tuna (*Thunnus thynnus*) [84], pejerrey (*Odontesthes bonariensis*) [73], gilthead seabream [86], rainbow trout (*Oncorhynchus mykiss*), and Atlantic salmon (*Salmon salar*) [87] using heterologous antisera; however, the same antisera failed to reveal two types of gonadotrophs in other teleosts [76, 81, 88, 89].

Because of the structural similarity between FSH and

LH, the production of specific antisera for FSH or LH has been difficult, and, to date, no specific antiserum has been obtained for FSH in cyprinids, which makes it impossible to distinguish the two hormone-producing cells by immunostaining in this family of teleosts. To address this issue, we investigated the spatial localization of *fshb*, *lhb*, and *cga* expression in the pituitary by in situ hybridization. The results clearly demonstrated that *fshb* and *lhb* were expressed in two separate populations of gonadotrophs that exhibited distinct patterns of distribution in the zebrafish pituitary. As the common subunit shared by FSH, LH, and TSH, the mRNA of *cga* was detected throughout the areas where *fshb* and *lhb* expression was localized. These results are consistent with the reports using in situ hybridization in other fish species, including the rainbow trout [72], African catfish [48], tilapia [65, 90], and Atlantic halibut (*Hippoglossus hippoglossus*) [40].

We also investigated the temporal expression profiles of *fshb*, *lhb*, and *cga* in the pituitary during the daily ovulatory cycle using real-time RT-PCR. According to the observations in our laboratory, the germinal vesicle in the full-grown oocyte start to migrate from the center to the periphery at around 0100 under our aquarium conditions [35]. Interestingly, this also seemed to be the time when the expression of all GTH subunits reached their peak levels. The expression levels appeared to remain high until 0400, when the germinal vesicle had already reached the periphery. This pattern of expression is understandable for *lhb* because LH has been well known to be involved in inducing final oocyte maturation and ovulation. A preovulatory LH surge has been reported in the rainbow trout [20], spotted seatrout (*Cynoscion nebulosus*) [91], gilthead seabream [92], and goldfish [93]. What is interesting was the obvious concurrent elevation of *fshb* expression at 0100 and 0400 before GVBD at 0700, suggesting a functional role for zfFSH in the final stage of follicle development. However, according to the information available, FSH in teleosts has generally been considered to be the gonadotropin involved in promoting follicle growth or vitellogenesis, and it is less likely to play significant roles in oocyte maturation or ovulation. FSH is less potent than LH in stimulating maturation-inducing hormone (MIH) production from the postvitellogenic follicles in the amago salmon [3], and it is incapable of inducing maturational competence development and oocyte maturation in the red seabream (*Pagrus major*) in vitro [94]. Although the present study provides no clues to the physiological significance of zfFSH in the final stage of oocyte development, the preovulatory increase of *fshb* expression implies that FSH may not simply be involved in promoting vitellogenesis in the growing follicles, and it may have direct actions on the postvitellogenic follicles as well. This speculation is further supported by our recent evidence that high expression level of *fshr* could be detected in the full-grown follicles, although the level was slightly lower than that in the midvitellogenic stage (Kwok et al., published separately). Recently, a significant elevation of FSHR mRNA level was reported in the postvitellogenic follicles of rainbow trout during the acquisition of maturational competence [95]. Whether FSH plays any direct role in oocyte maturation and/or ovulation will be an interesting issue to address in the future, particularly with the recombinant zfFSH available from the present study. One possibility is that FSH may augment LH action by up-regulating LH receptor expression as it does in mammals [96–98].

To confirm the functional identity of the cDNAs cloned and develop a source of zebrafish FSH and LH for future studies, we expressed recombinant zfFSH and zfLH in the CHO cells and tested their effects on the homologous gonadotropin receptors (Fshr and Lhr). The production of recombinant gonadotropins from the CHO cell system has been reported in mammals [99–101], and their bioactivities resembled those of natural hormones purified from the pituitary [101, 102]. Recently, our laboratory has cloned both zebrafish *fshr* and *lhr* from the ovary and established two stable CHO cell lines expressing Fshr or Lhr and the cAMP-responsive reporter gene SEAP. These two cell lines were adopted in the present study to investigate the functionality and receptor specificity of the recombinant zfFSH and zfLH. Compared with the conventional bioassays of gonadotropins, bioassays based on cell lines expressing cloned receptors are easier and safer to perform with high specificity and sensitivity [103, 104].

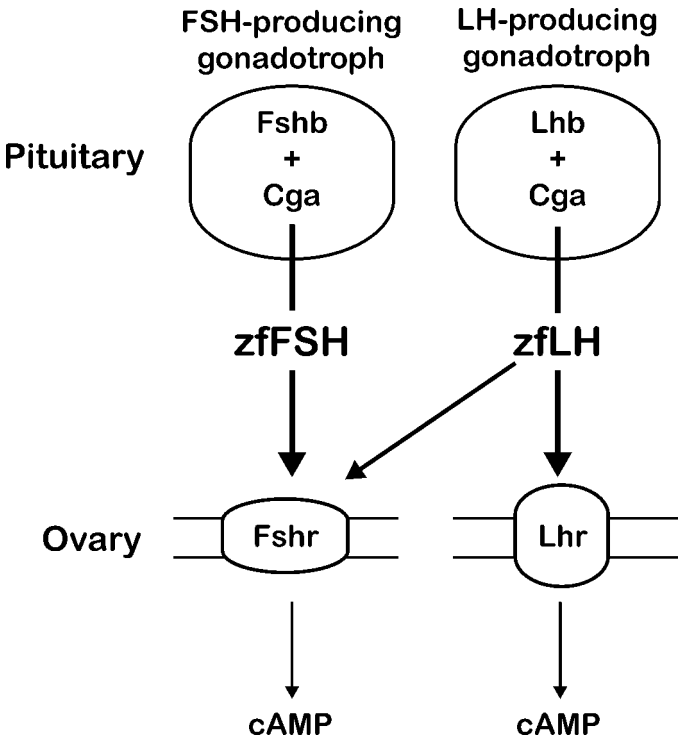


FIG. 9. Schematic representation of the duality of gonadotrophs, gonadotropins (FSH and LH), and their receptors (Fshr and Lhr) as well as ligand-receptor interaction in the zebrafish.

In contrast to the high specificity of mammalian gonadotropins, the specificity of gonadotropins to their receptors has been shown to be less apparent in teleosts according to the limited studies in salmonids and catfish [13–18]. The only report on receptor specificity in cyprinids was conducted in an Indian carp using purified GTH receptors [19]. However, this study used heterologous salmon FSH and LH, and therefore its results should be interpreted with caution because of the high plasticity of gonadotropin-receptor interaction in teleosts. Using recombinant gonadotropins and their native receptors in the zebrafish, the present study, for the first time in cyprinids, provides information about the ligand-receptor relationship. Recombinant zfFSH was highly specific for zebrafish Fshr, and it showed no activity on Lhr in a wide range of concentrations. However, zfLH could stimulate both Lhr and Fshr, although it seemed to have higher preference for the former. This finding is surprisingly similar to the reports in the African catfish and

coho salmon, whose LH or GTH-II also recognizes both receptors, whereas FSH binds only to FSHR (type I receptor) with little interaction with LHR (type II receptor) [13, 16–18]. Opposite to the situation in the zebrafish, African catfish, and coho salmon, purified chum salmon FSH stimulates cAMP production by the COS cells expressing amago salmon FSHR or LHR, whereas chum salmon LH activates only amago salmon LHR [14, 15]. This result should again be treated with caution because of the use of heterologous hormones from a different species. In a companion study, we have also tested the ligand-receptor relationship using bovine FSH and LH. Interestingly, bovine FSH and LH worked in a way opposite to that of zfFSH and zfLH. Bovine LH was highly specific to zebrafish Lhr, whereas bovine FSH could stimulate both Fshr and Lhr to the similar extent at the dosage tested (Kwok et al., published separately). This result supports the argument that despite the structural conservation of gonadotropin subunits across vertebrates, phylogenetically related gonadotropins can exert unpredictable receptor specificity when applied in different species. Chicken LH acts like FSH, and it is three times more potent than rat FSH in terms of binding to rat ovarian FSHR [105]. Pregnant mare serum gonadotropin (PMSG), which is supposed to act like LH, has both LH- and FSH-like effects when applied in rats [106]. Together with the evidence from other fish models, the present study suggests that although exogenous mammalian gonadotropins may elicit biological activities by binding to fish gonadotropin receptors, the effects may not reflect those of endogenous hormones because of their difference in receptor binding specificity and affinity. It is therefore desirable to use homologous fish gonadotropins, especially for studies on the physiological relevance of FSH and LH and their signaling mechanisms.

In summary, the full-length cDNAs encoding *fshb*, *lhb*, and *cga* were cloned from zebrafish pituitary in the present study. In addition to the pituitary, *fshb*, *lhb*, and *cga* also appeared to be expressed in a variety of extrapituitary tissues. In the zebrafish pituitary, the two gonadotropins were obviously expressed by two distinct populations of gonadotrophic cells. During the daily ovulatory cycle, all three gonadotropin subunits showed a preovulatory surge in expression, suggesting roles for both zfFSH and zfLH in the final stage of follicle development. Using CHO cells as the bioreactor, the present study also established two stable cell lines that produce bioactive recombinant zfFSH and zfLH. Besides the information about receptor specificity, the availability of zfFSH and zfLH will allow for more in-depth studies on the physiological functions of FSH and LH in zebrafish reproduction and their signal transduction mechanisms. The dualities of gonadotropins, gonadotrophic cells, and the receptors as well as the ligand-receptor interaction in the zebrafish are schematically summarized in Figure 9.

REFERENCES

1. Pierce JG, Parsons TF. Glycoprotein hormones: structure and function. Annu Rev Biochem 1981; 50:465–495.
2. Ulloa-Aguirre A, Timossi C, Damian-Matsumura P, Dias JA. Role of glycosylation in function of follicle-stimulating hormone. Endocrine 1999; 11:205–215.
3. Suzuki K, Nagahama Y, Kawauchi H. Steroidogenic activities of two distinct salmon gonadotropins. Gen Comp Endocrinol 1988; 71:452–458.
4. Suzuki K, Kawauchi H, Nagahama Y. Isolation and characterization of subunits from two distinct salmon gonadotropins. Gen Comp Endocrinol 1988; 71:302–306.
5. Suzuki K, Kawauchi H, Nagahama Y. Isolation and characterization of two distinct gonadotropins from chum salmon pituitary glands. Gen Comp Endocrinol 1988; 71:292–301.
6. Li MD, Ford JJ. A comprehensive evolutionary analysis based on nucleotide and amino acid sequences of the α- and β-subunits of glycoprotein hormone gene family. J Endocrinol 1998; 156:529–542.
7. Swanson P, Dickey JT, Campbell B. Biochemistry and physiology of fish gonadotropins. Fish Physiol Biochem 2003; 28:53–59.
8. Kanamori A, Kagawa H, Nagahama Y. Gonadotropin receptors in the postovulatory ovary of amago salmon (*Oncorhynchus rhodurus*). Gen Comp Endocrinol 1987; 66:210–217.
9. Jamal Uddin M, Bhattacharya S. In-vitro binding of gonadotrophin to fish ovary. J Endocrinol 1986; 111:407–413.
10. Tilly JL, Aihara T, Nishimori K, Jia XC, Billig H, Kowalski KI, Perlas EA, Hsueh AJ. Expression of recombinant human follicle-stimulating hormone receptor: species-specific ligand binding, signal transduction, and identification of multiple ovarian messenger ribonucleic acid transcripts. Endocrinology 1992; 131:799–806.
11. Braun T, Schofield PR, Sprengel R. Amino-terminal leucine-rich repeats in gonadotropin receptors determine hormone selectivity. EMBO J 1991; 10:1885–1890.
12. Moyle WR, Campbell RK, Myers RV, Bernard MP, Han Y, Wang X. Co-evolution of ligand-receptor pairs. Nature 1994; 368:251–255.
13. Yan L, Swanson P, Dickhoff WW. A two-receptor model for salmon gonadotropins (GTH I and GTH II). Biol Reprod 1992; 47:418–427.
14. Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H, Nagahama Y. The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochem Biophys Res Commun 1999; 265:366–371.
15. Oba Y, Hirai T, Yoshiura Y, Yoshikuni M, Kawauchi H, Nagahama Y. Cloning, functional characterization, and expression of a gonadotropin receptor cDNA in the ovary and testis of amago salmon (*Oncorhynchus rhodurus*). Biochem Biophys Res Commun 1999; 263: 584–590.
16. Bogerd J, Blomenrohr M, Andersson E, van der Putten HH, Tensen CP, Vischer HF, Granneman JC, Janssen-Dommerholt C, Goos HJ, Schulz RW. Discrepancy between molecular structure and ligand selectivity of a testicular follicle-stimulating hormone receptor of the African catfish (*Clarias gariepinus*). Biol Reprod 2001; 64:1633–1643.
17. Vischer HF, Granneman JC, Linskens MH, Schulz RW, Bogerd J. Both recombinant African catfish LH and FSH are able to activate the African catfish FSH receptor. J Mol Endocrinol 2003; 31:133–140.
18. Vischer HF, Bogerd J. Cloning and functional characterization of a gonadal luteinizing hormone receptor complementary DNA from the African catfish (*Clarias gariepinus*). Biol Reprod 2003; 68:262–271.
19. Basu D, Bhattacharya S. Purification of two types of gonadotropin receptors from carp ovarian follicles: overlapping recognition by two different ligands. Gen Comp Endocrinol 2002; 129:152–162.
20. Breton B, Govoroun M, Mikolajczyk T. GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. Gen Comp Endocrinol 1998; 111:38–50.
21. Swanson P. Salmon gonadotropins: reconciling old and new ideas. In: Scott AP, Sumpter JP, Kime DE, Rolfe MS (eds.), Reproductive Physiology of Fish. Sheffield, UK: FishSymp91; 1991:2–7.
22. Prat F, Sumpter JP, Tyler CR. Validation of radioimmunoassays for two salmon gonadotropins (GTH I and GTH II) and their plasma concentrations throughout the reproductive cycle in male and female rainbow trout (*Oncorhynchus mykiss*). Biol Reprod 1996; 54:1375–1382.
23. Weil C, Bougoussa-Houadec M, Gallais C, Itoh S, Sekine S, Valotaire Y. Preliminary evidence suggesting variations of GtH 1 and GtH 2 mRNA levels at different stages of gonadal development in rainbow trout, *Oncorhynchus mykiss*. Gen Comp Endocrinol 1995; 100:327–333.
24. Pang Y, Ge W. Activin stimulation of zebrafish oocyte maturation in vitro and its potential role in mediating gonadotropin-induced oocyte maturation. Biol Reprod 1999; 61:987–992.
25. Wang Y, Ge W. Cloning of zebrafish ovarian carbonyl reductase-like 20β-hydroxysteroid dehydrogenase and characterization of its spatial and temporal expression. Gen Comp Endocrinol 2002; 127:209–216.
26. Pang Y, Ge W. Gonadotropin regulation of activin βA and activin type IIA receptor expression in the ovarian follicle cells of the zebrafish, *Danio rerio*. Mol Cell Endocrinol 2002; 188:195–205.
27. Pang Y, Ge W. Gonadotropin and activin enhance maturational com-

petence of oocytes in the zebrafish (*Danio rerio*). Biol Reprod 2002; 66:259–265.
28. Pang Y, Ge W. Epidermal growth factor and TGFα promote zebrafish oocyte maturation in vitro: potential role of the ovarian activin regulatory system. Endocrinology 2002; 143:47–54.
29. Wang Y, Ge W. Gonadotropin regulation of follistatin expression in the cultured ovarian follicle cells of zebrafish, *Danio rerio*. Gen Comp Endocrinol 2003; 134:308–315.
30. Wang Y, Wong AO, Ge W. Cloning, regulation of messenger ribonucleic acid expression, and function of a new isoform of pituitary adenylate cyclase-activating polypeptide in the zebrafish ovary. Endocrinology 2003; 144:4799–4810.
31. Wang Y, Ge W. Spatial expression patterns of activin and its signaling system in the zebrafish ovarian follicle: evidence for paracrine action of activin on the oocytes. Biol Reprod 2003; 69:1998–2006.
32. Wang Y, Ge W. Involvement of cyclic adenosine 3′,5′-monophosphate in the differential regulation of activin βA and βB expression by gonadotropin in the zebrafish ovarian follicle cells. Endocrinology 2003; 144:491–499.
33. Wang Y, Ge W. Cloning of zebrafish ovarian P450c17 (CYP17, 17α-hydroxylase/17, 20-lyase) and characterization of its expression in gonadal and extra-gonadal tissues. Gen Comp Endocrinol 2004; 135: 241–249.
34. Wang Y, Ge W. Cloning of epidermal growth factor (EGF) and EGF receptor from the zebrafish ovary: evidence for EGF as a potential paracrine factor from the oocyte to regulate activin/follistatin system in the follicle cells. Biol Reprod 2004; 71:749–760.
35. Wang Y, Ge W. Developmental profiles of activin βA, βB, and follistatin expression in the zebrafish ovary: evidence for their differential roles during sexual maturation and ovulatory cycle. Biol Reprod 2004; 71:2056–2064.
36. Kohli G, Hu S, Clelland E, Di Muccio T, Rothenstein J, Peng C. Cloning of transforming growth factor-β1 (TGF-β1) and its type II receptor from zebrafish ovary and role of TGF-β1 in oocyte maturation. Endocrinology 2003; 144:1931–1941.
37. Garg RR, Bally-Cuif L, Lee SE, Gong Z, Ni X, Hew CL, Peng C. Cloning of zebrafish activin type IIB receptor (ActRIIB) cDNA and mRNA expression of ActRIIB in embryos and adult tissues. Mol Cell Endocrinol 1999; 153:169–181.
38. Wu T, Patel H, Mukai S, Melino C, Garg R, Ni X, Chang J, Peng C. Activin, inhibin, and follistatin in zebrafish ovary: expression and role in oocyte maturation. Biol Reprod 2000; 62:1585–1592.
39. Yoshiura Y, Kobayashi M, Kato Y, Aida K. Molecular cloning of the cDNAs encoding two gonadotropin β subunits (GTH-Iβ and -IIβ) from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 1997; 105:379–389.
40. Weltzien FA, Norberg B, Helvik JV, Andersen O, Swanson P, Andersson E. Identification and localization of eight distinct hormone-producing cell types in the pituitary of male Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp Biochem Physiol A Mol Integr Physiol 2003; 134:315–327.
41. Kozak M. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res 1984; 12:857–872.
42. Schatz SM, Kerschbaumer RJ, Gerstenbauer G, Kral M, Dorner F, Scheiflinger F. Higher expression of Fab antibody fragments in a CHO cell line at reduced temperature. Biotechnol Bioeng 2003; 84:433–438.
43. Yam KM, Yu KL, Ge W. Cloning and characterization of goldfish activin βA subunit. Mol Cell Endocrinol 1999; 154:45–54.
44. Lau MT, Ge W. Cloning of Smad2, Smad3, Smad4 and Smad7 from the goldfish pituitary and evidence for their involvement in activin regulation of goldfish FSHβ promoter activity. Gen Comp Endocrinol; 2005; 141:22–28.
45. Furukawa K, Ohsuye K. Effect of culture temperature on a recombinant CHO cell line producing a C-terminal α-amidating enzyme. Cytotechnology 1998; 26:153–164.
46. Chang YS, Huang CJ, Huang FL, Lo TB. Primary structures of carp gonadotropin subunits deduced from cDNA nucleotide sequences. Int J Pept Protein Res 1988; 32:556–564.
47. Liu Z, Kim S, Karsi A. Channel catfish follicle-stimulating hormone and luteinizing hormone: complementary DNA cloning and expression during ovulation. Mar Biotechnol (NY) 2001; 3:590–599.
48. Vischer HF, Teves AC, Ackermans JC, van Dijk W, Schulz RW, Bogerd J. Cloning and spatiotemporal expression of the follicle-stimulating hormone β subunit complementary DNA in the African catfish (*Clarias gariepinus*). Biol Reprod 2003; 68:1324–1332.
49. Chang YS, Huang FL, Lo TB. Isolation and sequence analysis of carp gonadotropin β-subunit gene. Mol Mar Biol Biotechnol 1992; 1:97–105.
50. Haavisto AM, Pettersson K, Bergendahl M, Virkamaki A, Huhtaniemi I. Occurrence and biological properties of a common genetic variant of luteinizing hormone. J Clin Endocrinol Metab 1995; 80:1257–1263.
51. Kobayashi M, Kato Y, Yoshiura Y, Aida K. Molecular cloning of cDNA encoding two types of pituitary gonadotropin α subunit from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 1997; 105: 372–378.
52. Kitahara N, Nishizawa T, Gatanaga T, Okazaki H, Andoh T, Soma G. Primary structure of two mRNAs encoding putative salmon α-subunits of pituitary glycoprotein hormone. Comp Biochem Physiol B Biochem Mol Biol 1988; 91:551–556.
53. Swanson P, Suzuki K, Kawauchi H, Dickhoff WW. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol Reprod 1991; 44:29–38.
54. Wallis M. Episodic evolution of protein hormones in mammals. J Mol Evol 2001; 53:10–18.
55. Kato Y, Gen K, Maruyama O, Tomizawa K, Kato T. Molecular cloning of cDNAs encoding two gonadotrophin β subunits (GTH-Iβ and -IIβ) from the masu salmon, *Oncorhynchus masou*: rapid divergence of the GTH-Iβ gene. J Mol Endocrinol 1993; 11:275–282.
56. Gen K, Okuzawa K, Senthilkumaran B, Tanaka H, Moriyama S, Kagawa H. Unique expression of gonadotropin-I and -II subunit genes in male and female red seabream (*Pagrus major*) during sexual maturation. Biol Reprod 2000; 63:308–319.
57. Elizur A, Zmora N, Rosenfeld H, Meiri I, Hassin S, Gordin H, Zohar Y. Gonadotropins β-GtHI and β-GtHII from the gilthead seabream, *Sparus aurata*. Gen Comp Endocrinol 1996; 102:39–46.
58. Mateos J, Mananos E, Martinez-Rodriguez G, Carrillo M, Querat B, Zanuy S. Molecular characterization of sea bass gonadotropin subunits (α, FSHβ, and LHβ) and their expression during the reproductive cycle. Gen Comp Endocrinol 2003; 133:216–232.
59. Nica G, Herzog W, Sonntag C, Hammerschmidt M. Zebrafish *pit1* mutants lack three pituitary cell types and develop severe dwarfism. Mol Endocrinol 2004; 18:1196–1209.
60. Goldsmith PC, McGregor WG, Raymoure WJ, Kuhn RW, Jaffe RB. Cellular localization of chorionic gonadotropin in human fetal kidney and liver. J Clin Endocrinol Metab 1983; 57:654–661.
61. Zhang FP, Markkula M, Toppari J, Huhtaniemi I. Novel expression of luteinizing hormone subunit genes in the rat testis. Endocrinology 1995; 136:2904–2912.
62. Markkula M, Hamalainen T, Loune E, Huhtaniemi I. The follicle-stimulating hormone (FSH) β- and common α-subunits are expressed in mouse testis, as determined in wild-type mice and those transgenic for the FSH β-subunit/herpes simplex virus thymidine kinase fusion gene. Endocrinology 1995; 136:4769–4775.
63. Markkula M, Kananen K, Klemi P, Huhtaniemi I. Pituitary and ovarian expression of the endogenous follicle-stimulating hormone (FSH) subunit genes and an FSH β-subunit promoter-driven herpes simplex virus thymidine kinase gene in transgenic mice; specific partial ablation of FSH-producing cells by antiherpes treatment. J Endocrinol 1996; 150:265–273.
64. Emanuele N, Connick E, Howell T, Anderson J, Hojvat S, Baker G, Souchek J, Kirsteins L, Lawrence AM. Hypothalamic luteinizing hormone (LH): characteristics and response to hypophysectomy. Biol Reprod 1981; 25:321–326.
65. Parhar IS, Soga T, Ogawa S, Sakuma Y. FSH and LH-β subunits in the preoptic nucleus: ontogenic expression in teleost. Gen Comp Endocrinol 2003; 132:369–378.
66. Wong TT, Zohar Y. Novel expression of gonadotropin subunit genes in oocytes of the gilthead seabream (*Sparus aurata*). Endocrinology 2004; 145:5210–5220.
67. Muralidhar K, Moudgal NR. Studies on rat ovarian receptors for lutropin (luteinizing hormone). Interaction with β-subunit of sheep lutropin. Biochem J 1976; 160:615–619.
68. Dubourg P, Burzawa-Gerard E, Chambolle P, Kah O. Light and electron microscopic identification of gonadotrophic cells in the pituitary gland of the goldfish by means of immunocytochemistry. Gen Comp Endocrinol 1985; 59:472–481.
69. Kaneko T, Kobayashi M, Aida K, Hanyu I. Ultrastructural immunocytochemistry of gonadotrophs in the goldfish pituitary gland. Cell Tissue Res 1985; 239:337–342.
70. Cambre ML, Verdonck W, Ollevier F, Vandesande F, Batten TF, Kuhn ER. Immunocytochemical identification and localization of the differ-

ent cell types in the pituitary of the seabass (*Dicentrarchus labrax*). Gen Comp Endocrinol 1986; 61:368–375.
71. Sanchez Cala F, Portillo A, Martin del Rio MP, Mancera JM. Immunocytochemical characterization of adenohypophyseal cells in the greater weever fish (*Trachinus draco*). Tissue Cell 2003; 35:169–178.
72. Naito N, Hyodo S, Okumoto N, Urano A, Nakai Y. Differential production and regulation of gonadotropins (GTH I and GTH II) in the pituitary gland of rainbow trout, *Oncorhynchus mykiss*, during ovarian development. Cell Tissue Res 1991; 266:457–467.
73. Miranda LA, Strussmann CA, Somoza GM. Immunocytochemical identification of GtH1 and GtH2 cells during the temperature-sensitive period for sex determination in pejerrey, *Odontesthes bonariensis*. Gen Comp Endocrinol 2001; 124:45–52.
74. Calman B, Lin YW, Wallace RA. Preparation and use of specific antibodies to the β-I and β-II subunits of gonadotropic hormone from *Fundulus heteroclitus* pituitary. Gen Comp Endocrinol 2001; 123: 203–209.
75. Fantodji A, Follenius E, N'Diaye AS. Immunocytochemistry of gonadotropic cells in the pituitary of *Pomadasys jubelini* (teleost fish). Gen Comp Endocrinol 1990; 79:439–445.
76. Yan HY, Thomas P. Histochemical and immunocytochemical identification of the pituitary cell types in three sciaenid fishes: Atlantic croaker (*Micropogonias undulatus*), spotted seatrout (*Cynoscion nebulosus*), and red drum (*Sciaenops ocellatus*). Gen Comp Endocrinol 1991; 84:389–400.
77. Toubeau G, Poilve A, Baras E, Nonclercq D, De Moor S, Beckers JF, Dessy-Doize C, Heuson-Stiennon JA. Immunocytochemical study of cell type distribution in the pituitary of *Barbus barbus* (Teleostei, Cyprinidae). Gen Comp Endocrinol 1991; 83:35–47.
78. Vissio PG, Paz DA, Maggese C. The adenohypophysis of the swamp eel, *Synbranchus marmoratus*, an immunocytochemical analysis. Biocell 1996; 20:155–161.
79. Rendon C, Rodriguez-Gomez FJ, Munoz-Cueto JA, Pinuela C, Sarasquete C. An immunocytochemical study of pituitary cells of the Senegalese sole, *Solea senegalensis* (Kaup 1858). Histochem J 1997; 29:813–822.
80. Ekengren B, Peute J, Fridberg G. Gonadotropic cells in the Atlantic salmon, *Salmo salar*. An experimental immunocytological, electron microscopical study. Cell Tissue Res 1978; 191:187–203.
81. Saga T, Yamaki K, Doi Y, Yoshizuka M. Chronological study of the appearance of adenohypophysial cells in the ayu (*Plecoglossus altivelis*). Anat Embryol (Berl) 1999; 200:469–475.
82. Quesada J, Lozano MT, Ortega A, Agulleiro B. Immunocytochemical and ultrastructural characterization of the cell types in the adenohypophysis of *Sparus aurata* L. (teleost). Gen Comp Endocrinol 1988; 72:209–225.
83. Segura-Noguera MM, Laiz-Carrion R, del Rio MP, Mancera JM. An immunocytochemical study of the pituitary gland of the white seabream (*Diplodus sargus*). Histochem J 2000; 32:733–742.
84. Kagawa H, Kawazoe I, Tanaka H, Okuzawa K. Immunocytochemical identification of two distinct gonadotropic cells (GTH I and GTH II) in the pituitary of bluefin tuna, *Thunnus thynnus*. Gen Comp Endocrinol 1998; 110:11–18.
85. Garcia-Hernandez MP, Garcia-Ayala A, Zandbergen MA, Agulleiro B. Investigation into the duality of gonadotropic cells of Mediterranean yellowtail (*Seriola dumerilii*, Risso 1810): immunocytochemical and ultrastructural studies. Gen Comp Endocrinol 2002; 128:25–35.
86. Garcia Ayala A, Villaplana M, Garcia Hernandez MP, Chaves Pozo E, Agulleiro B. FSH-, LH-, and TSH-expressing cells during development of *Sparus aurata* L. (Teleostei). An immunocytochemical study. Gen Comp Endocrinol 2003; 134:72–79.
87. Nozaki M, Naito N, Swanson P, Miyata K, Nakai Y, Oota Y, Suzuki K, Kawauchi H. Salmonid pituitary gonadotrophs. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. Gen Comp Endocrinol 1990; 77:348–357.
88. Magliulo-Cepriano L, Schreibman MP, Blum V. Distribution of variant forms of immunoreactive gonadotropin-releasing hormone and β-gonadotropins I and II in the platyfish, *Xiphophorus maculatus*, from birth to sexual maturity. Gen Comp Endocrinol 1994; 94:135–150.
89. Laiz-Carrion R, del Mar Segura-Noguera M, del Pilar Martin del Rio M, Mancera JM. Ontogeny of adenohypophyseal cells in the pituitary of the American shad (*Alosa sapidissima*). Gen Comp Endocrinol 2003; 132:454–464.
90. Melamed P, Rosenfeld H, Elizur A, Yaron Z. Endocrine regulation of gonadotropin and growth hormone gene transcription in fish. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1998; 119:325–338.
91. Thomas P, Pinter J, Das S. Upregulation of the maturation-inducing steroid membrane receptor in spotted seatrout ovaries by gonadotropin during oocyte maturation and its physiological significance. Biol Reprod 2001; 64:21–29.
92. Gothilf Y, Meiri I, Elizur A, Zohar Y. Preovulatory changes in the levels of three gonadotropin-releasing hormone-encoding messenger ribonucleic acids (mRNAs), gonadotropin β-subunit mRNAs, plasma gonadotropin, and steroids in the female gilthead seabream, *Sparus aurata*. Biol Reprod 1997; 57:1145–1154.
93. Stacey NE, Cook AF, Peter RE. Ovulatory surge of gonadotropin in the goldfish, *Carassius auratus*. Gen Comp Endocrinol 1979; 37:246–249.
94. Kagawa H, Tanaka H, Okuzawa K, Kobayashi M. GTH II but not GTH I induces final maturation and the development of maturational competence of oocytes of red seabream in vitro. Gen Comp Endocrinol 1998; 112:80–88.
95. Bobe J, Maugars G, Nguyen T, Rime H, Jalabert B. Rainbow trout follicular maturational competence acquisition is associated with an increased expression of follicle stimulating hormone receptor and insulin-like growth factor 2 messenger RNAs. Mol Reprod Dev 2003; 66:46–53.
96. Segaloff DL, Wang HY, Richards JS. Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization. Mol Endocrinol 1990; 4:1856–1865.
97. Piquette GN, LaPolt PS, Oikawa M, Hsueh AJ. Regulation of luteinizing hormone receptor messenger ribonucleic acid levels by gonadotropins, growth factors, and gonadotropin-releasing hormone in cultured rat granulosa cells. Endocrinology 1991; 128:2449–2456.
98. Zeleznik AJ, Midgley AR Jr, Reichert LE Jr. Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. Endocrinology 1974; 95:818–825.
99. Schmidt A, Gromoll J, Weinbauer GF, Galla HJ, Chappel S, Simoni M. Cloning and expression of cynomolgus monkey (*Macaca fascicularis*) gonadotropins luteinizing hormone and follicle-stimulating hormone and identification of two polymorphic sites in the luteinizing hormone β subunit. Mol Cell Endocrinol 1999; 156:73–83.
100. Hakola K, Boogaart PV, Mulders J, de Leeuw R, Schoonen W, Heyst JV, Swolfs A, Casteren JV, Huhtaniemi I, Kloosterboer H. Recombinant rat luteinizing hormone; production by Chinese hamster ovary cells, purification and functional characterization. Mol Cell Endocrinol 1997; 128:47–56.
101. Hakola K, Van der Boogaart P, Mulders J, de Leeuw R, Schoonen W, Van Heyst J, Swolfs A, Van Casteren J, Huhtaniemi I, Kloosterboer H. Recombinant rat follicle-stimulating hormone; production by Chinese hamster ovary cells, purification and functional characterization. Mol Cell Endocrinol 1997; 127:59–69.
102. Mannaerts B, de Leeuw R, Geelen J, Van Ravestein A, Van Wezenbeek P, Schuurs A, Kloosterboer H. Comparative in vitro and in vivo studies on the biological characteristics of recombinant human follicle-stimulating hormone. Endocrinology 1991; 129:2623–2630.
103. Christin-Maitre S, Vasseur C, Fauser B, Bouchard P. Bioassays of gonadotropins. Methods 2000; 21:51–57.
104. Christin-Maitre S, Bouchard P. Bioassays of gonadotropins based on cloned receptors. Mol Cell Endocrinol 1996; 125:151–159.
105. Iwasawa A, Hattori M, Fukuhara Y, Kawashima M, Wakabayashi K, Kamiyoshi M. Chicken lutropin acts like follitropin in rat ovarian follitropin receptor: an isoelectric focusing study. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 1998; 120:83–89.
106. Licht P, Gallo AB, Aggarwal BB, Farmer SW, Castelino JB, Papkoff H. Biological and binding activities of equine pituitary gonadotrophins and pregnant mare serum gonadotrophin. J Endocrinol 1979; 83:311–322.