Three distinct types of GnRH receptor characterized in the bullfrog

Li Wang*, Jan Bogerd†, Hueng S. Choi*, Jae Y. Seong*, Jae M. Soh*, Sang Y. Chun*, Marion Blomenröhr†, Brigitte E. Troskie‡, Robert P. Millar§, Wen H. Yu¶, Samuel M. McCann¶, and Hyuk B. Kwon*‖

*Hormone Research Center and Department of Biology, Chonnam National University, Kwangju 500-757, Republic of Korea; †Research Group for Comparative Endocrinology, Department of Experimental Zoology, Utrecht University, Utrecht 3584, The Netherlands; Research Unit for Molecular Reproductive Endocrinology, Department of Chemical Pathology, University of Cape Town, South Africa 7925; §MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh, EH3 9ET, Scotland; and ¶Pennington Biomedical Research Center, Louisiana State University, 6400 Perkins Road, Baton Rouge, LA 70808-4124

Contributed by Samuel M. McCann, October 25, 2000

It has been proposed recently that two types of GnRH receptors (GnRHR) exist in a particular species. Here we present data demonstrating that at least three types of GnRHR are expressed in a single diploid species, the bullfrog. Three different cDNAs, encoding distinct types of bullfrog GnRHR (bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3), were isolated from pituitary and hindbrain of the bullfrog. BfGnRHR-1 mRNA was expressed predominantly in pituitary, whereas bfGnRHR-2 and -3 mRNAs were expressed in brain. The bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 proteins have an amino acid identity of ≈30% to ≈35% with mammalian GnRHRs and ≈40% to ≈50% with nonmammalian GnRHRs. Interestingly, bfGnRHR-2 has an 85% amino acid homology with *Xenopus* GnRHR. Less than 53% amino acid identity was observed among the three bfGnRHRs. All isolated cDNAs encode functional receptors because their transient expression in COS-7 cells resulted in a ligand-dependent increase in inositol phosphate production. Notably, all three receptors exhibited a differential ligand selectivity. For all receptors, cGnRH-II has a higher potency than mGnRH. In addition, salmon GnRH also has a strikingly high potency to stimulate all three receptors. In conclusion, we demonstrated the presence of three GnRHRs in the bullfrog. Their expression in pituitary and brain suggests that bfGnRHRs play an important role in the regulation of reproductive functions in the bullfrog.

Gonadotropin-releasing hormone (GnRH) plays a key role in controlling gonadotropin secretion and reproductive functions in vertebrates (1). To date, 11 structural variants of GnRH have been identified in vertebrates (2–4), and 2 variants of GnRH have been identified in an invertebrate, the tunicate (5). At least two forms of GnRH are expressed in a particular vertebrate species (2–4), and one of these GnRHs invariably is chicken GnRH-II (cGnRH-II). The events leading to GnRH-stimulated gonadotropin synthesis and release from the anterior pituitary depend on the presence of GnRH receptors (GnRHRs). Knowledge of the GnRHR structure, its interaction with GnRH and GnRH analogues, and its physiological regulation is vital to our understanding of the functioning of the hypothalamic-pituitary-gonadal axis. The first functional GnRHR cDNA was cloned from mouse (6, 7). Subsequently, GnRHR cDNAs were cloned from other mammals (8–11). More recently, GnRHR cDNAs were also identified in nonmammalian vertebrates, such as African catfish, goldfish, and *Xenopus* (12–14). The availability of the GnRHR cDNAs facilitated studies on the mechanisms of GnRH action. The presence of at least two GnRHs in a particular species led to a concept of the existence of more than two types of GnRHR (15). The cloning of two subtypes (type IA and type IB) of GnRHR cDNAs from goldfish confirmed that, indeed, both subtype genes are expressed in a single species (13). However, no full-length cDNAs encoding a second or a third type of GnRHR have been identified so far. The research reported here demonstrated that there are three distinct GnRHRs in bullfrog pituitary (bfGnRHR-1) and brain (bfGnRHR-2 and bfGnRHR-3).

Materials and Methods

GnRHs. Mammalian GnRH (mGnRH), salmon GnRH (sGnRH), and chicken GnRH-I (cGnRH-I) were purchased from Sigma, whereas cGnRH-II was obtained from American Peptide, Sunnyvale, CA. Seabream GnRH was obtained from Bachem, and lamprey GnRH-III (lGnRH-III) was synthesized in the Louisiana State University protein facility in Baton Rouge, LA.

Animals and Tissue Preparation. Bullfrogs were purchased from the local market in Kwangju, Korea, and housed in flow-through tanks under simulated natural conditions. Frogs were killed by decapitation. The tissues of interest were quickly dissected, immediately frozen in liquid nitrogen, and stored at −80°C until use.

RNA and DNA Isolation. Total RNA was extracted by using Trireagent (Sigma) according to the manufacturer's instructions. Poly(A)$^+$ RNA was purified from total RNA by using QIAGEN Oligotex mRNA kit (Qiagen, Chatsworth, CA). Genomic DNA was isolated from liver by using a genomic DNA isolation kit (Qiagen).

Amplification of Partial cDNAs by Reverse Transcription–PCR. Pituitary or hindbrain poly(A)$^+$ RNA (50 ng) was reverse transcribed by using an oligo(dT) primer and SuperScript II RNase H$^-$ reverse transcriptase (Life Technologies, Rockville, MD). These cDNAs served as templates for subsequent PCR amplification by using two degenerate deoxyoligonucleotide primers, DG-F and DG-R (see Table 1), corresponding to DNA sequences encoding conserved amino acid sequences in the transmembrane domains III and VI of other GnRHRs. PCR conditions were denaturation at 95°C for 5 min, followed by 35 cycles at 94°C for 15 sec, at 55°C for 10 sec, and at 72°C for 30 sec. PCR products of the expected size (≈390 bp) were excised and purified by using a Geneclean II kit (Bio 101). Next, the PCR products were subcloned into pGEM-T (Promega). Positive clones were isolated and purified by using a QIAGEN Plasmid Miniprep Kit (Qiagen). Plasmids containing an insert of the expected size were subjected to DNA sequence analysis by the dideoxy chain-termination method, according to the manufacturer's instruction (Sequenase; United States Biochemical).

Abbreviations: bf, bullfrog; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; RACE, rapid amplification of cDNA ends; GSP, gene-specific primer; bfGnRH, bullfrog GnRH; mGnRH, mammalian GnRH; sGnRH, salmon GnRH; cGnRH, chicken GnRH; lGnRH, lamprey GnRH.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF153913, AF144062, and AF144063).

‖To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.011508498. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.011508498

Table 1. Nucleotide sequence of primers

Primer	Sequence
DG-F	5′-GCMGCWTTMRTKCTRGTRGTKRTBAGC-3′
DG-R	5′-GGTCATYTTYAGSGTYYTYAKHCKDGC-3′
3′-GSP(I)	5′-GTGTACAACGAGGGGCAGCTTCCAGCGAGCA-3′
3′-NGSP(I)	5′-CCAAGCGCATGAGCAAAGGAACGCTTTC-3′
3′-GSP(II)	5′-TGTGCAACACATGGGAGCTTTGCCCAAC-3′
3′-NGSP(II)	5′-GCACCCTATTTGTCACTCCGCTAGGCGTCA-3′
3′-GSP(III)	5′-TGTTTGTGTTCCACACGGTGAGCCGGTC-3′
3′-NGSP(III)	5′-CCGCTCCTCATCATGGTCTTCTGCTACGGA-3′
5′-GSP(I)	5′-TCTCCTGCCAGTGCAGCTGGAAGCTGC-3′
5′-NGSP(I)	5′-GCTGTGGGAGTGAGAGCACTGCGCTCATT-3′
5′-GSP(II)	5′-CCAGGGACCGTCCGCAAACGGAACA-3′
5′-NGSP(II)	5′-TCCACAGCATGATACGGTTGCGTTGTCCA-3′
5′-GSP(III)	5′-CCATGATGAGGAGCGGGAGAAGGAAGAGGC-3′
5′-NGSP(III)	5′-GTGACCGGCTCACCGTGTGGAACACAAA-3′
bfGnRHR-1A	5′-CGCGAATTC*GCCACC*ATGAATATCTCAAAGGAAGTTAGCAT-3′
bfGnRHR-1B	5′-CGCCTCTAGATTCATATTATACACATCTGTAATTGACT-3′
bfGnRHR-2A	5′-CGCGAATTC*GCCACC*ATGGCCATGCAGCTAGCTATCGTAAAT-3′
bfGnRHR-2B	5′-CGCCTCTAGATTCAAAAGACAGACTGTACTGTGGTGGCC-3′
bfGnRHR-3A	5′-CGCGAATTC*GCCACC*ATGAACGCTAGTGACCAACCTATGG-3′
bfGnRHR-3B	5′-CGCCTCTAGATTCACATAAAGCTCTCTACGATTTGAC-3′

For the DG-F and DG-R primers: B = T or C or G, D = A or T or G, H = A or T or C, K = G or T, M = A or C, R = A or G, S = G or C, W = A or T, Y = T or C. The bfGnRHR-1A, bfGnRHR-2A, and bfGnRHR-3A primers each contain an *Eco*RI restriction enzyme site (underlined) and a Kozak consensus sequence (italics), and the bfGnRHR-1B, bfGnRHR-2B, and bfGnRHR-3B primers each contain a, *Xba*I restriction enzyme site (underlined), engineered in their 5′-ends.

Cloning of GnRHR cDNAs by Rapid Amplification of cDNA Ends (RACE). Two sets of gene-specific primers (GSPs) were designed on the basis of each of the three different partial cDNA sequences obtained by reverse transcription–PCR. For 3′-RACE, 3′-GSP [I, II or III] and 3′-NGSP [I, II or III] were used; for 5′-RACE, 5′-GSP [I, II or III] and 5′- NGSP [I, II or III] primers (Table 1) were used. Poly(A)$^+$ RNA purified from pituitary or hindbrain was used to synthesize adapter-ligated double-stranded cDNA by using the Marathon cDNA Amplification Kit (CLONTECH). Next, 3′- and 5′-RACE were performed by using the GSPs in combination with the adapter primers AP1 and AP2, respectively. RACE products were cloned in pGEM-T (Promega). On the basis of the three different partially overlapping DNA sequences of the 5′- and 3′-RACE products, bfGnRHR-1A and bfGnRHR-1B, bfGnRHR-2A and bfGnRHR-2B, and bfGnRHR-3A and bfGnRHR-3B primers (Table 1) were designed to specifically amplify the coding regions of three bullfrog GnRHR cDNAs.

Cell Culture, Transfection, and Inositol Phosphate Assay. The coding regions of the bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 cDNAs were subcloned into the mammalian expression vector pcDNA3 (Invitrogen) and named pc-bfGnRHR-1, pc-bfGnRHR-2, and pc-GnRHR-3, respectively. Before transfection (24 h), COS-7 cells (1×10^5 per well) were plated out in a 12-well plate. The next day the cells were transfected by using SuperFect (Qiagen). After transfection, the cells were incubated in inositol-free DMEM (Life Technologies) containing 2% (vol/vol) dialyzed FCS and labeled with 1 μCi (1 Ci = 37 GBq)[^{3}H]myo-inositol per well (Amersham Pharmacia) for 48 h. Medium was then removed, and cells were washed with 0.5 ml buffer A (140 mM NaCl/20 mM Hepes/4 mM KCl/8 mM D-glucose/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mg/ml fatty acid-free BSA). Then cells were preincubated with buffer A containing 10 mM LiCl for 15 min, followed by addition of the GnRHs at various concentrations at 37°C for 45 min. The reaction was stopped by removing the incubation medium and adding 0.5 ml of ice-cold 10 mM formic acid. After 30 min at 4°C, the formic acid extracts were transferred to columns containing Dowex anion-exchange resin. Total inositol phosphates were then eluted with 1 ml of 1 M ammonium formate/0.1 M formic acid, and radioactivity was determined. The GnRH concentrations inducing half-maximal stimulation (EC_{50}) were calculated by using GraphPad PRISM2 software package.

Statistical Analysis. All data are presented as the mean ± SEM of three independent experiments. Statistical analysis was performed by using one-way analysis of variance, and where $P < 0.05$, it was followed by the Bonferroni test. A $P < 0.05$ was considered significant.

Northern and Southern Blot Analyses. For Northern blot analysis, total RNA from pituitary, forebrain (including olfactory lobe and telencephalon), and hindbrain (including hypothalamus, cerebellum, and spinal cord) was isolated from different seasons (January, hibernation season; June, breeding season; September, after-breeding season). Ten micrograms of total RNA was electrophoresed in a 1% formaldehyde agarose gel and transferred by capillary action to a Zeta-Probe GT membrane (Bio-Rad). For Southern blot analysis, 20 μg of genomic DNA, digested with different restriction endonucleases (*Eco*RI, *Xba*I, *Pst*I, and *Bam*HI [Kosco, Seoul]), was electrophoresed in a 0.8% agarose gel and transferred by capillary action to a Zeta-Probe GT membrane. Random-primed bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 cDNA fragments were labeled (Roche Molecular Biochemicals) with [α-^{32}P]dCTP (Amersham Pharmacia) and used as probes for Northern and Southern blot analyses. Hybridization was performed according to the instruction manual of Zeta-Probe GT membrane. The membranes were washed twice with 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and 0.1% SDS at room temperature for 5 min each, and then once with 0.1 × SSC and 0.1% SDS at 65°C for 50 min. The membranes were then exposed to x-ray films (X-Omat; Kodak) at −70°C for 1 or 2 days.

Results

Cloning and Identification of Three Different Bullfrog GnRHR cDNAs. To isolate bullfrog GnRHR cDNAs, degenerate DG-F and DG-R primers were used for PCR amplification with pituitary

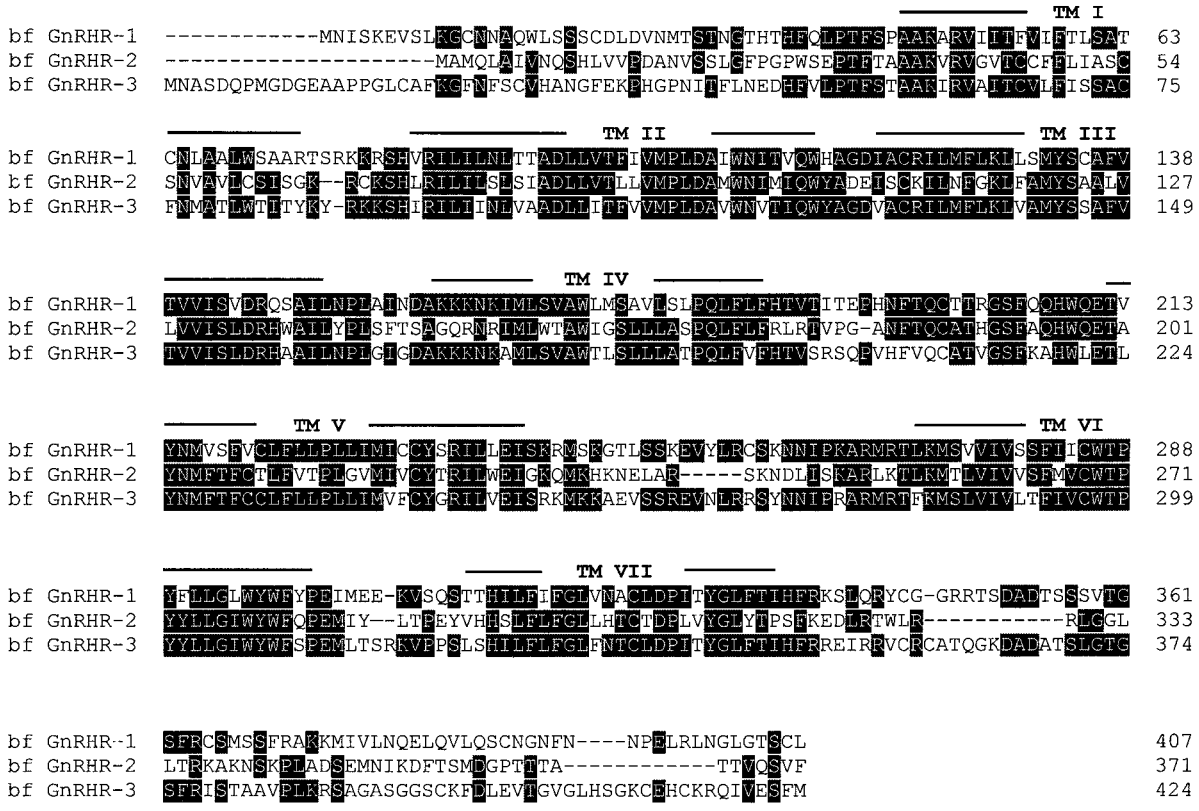

Fig. 1. Comparison of the deduced amino acid sequences of the three types of bfGnRHRs. *Heavily shaded* residues are identical in two or three of the receptors. Amino acid numbers are shown (*Right*). Gaps introduced for optimal alignment are indicated as *dashes*. The putative transmembrane domains are indicated (*Top*). BfGnRHR-1 shares 40% amino acid identity with bfGnRHR-2 and 53% with bfGnRHR-3, whereas bfGnRHR-2 shares 40% amino acid identity with bfGnRHR-3.

and hindbrain cDNAs as templates. PCR products of ≈390 base pairs were subcloned and sequenced. Surprisingly, three different types of putative GnRHR cDNA sequences were identified. To rule out potential PCR artifacts, the experiment was repeated by using new pituitary and hindbrain cDNAs. To obtain additional 5′- and 3′-cDNA sequences for three receptors, several gene-specific primers were designed and used in 5′- and 3′-RACE experiments. The first cDNA consisted of 1,839 nucleotides encoding a protein of 407 amino acids, designated bfGnRHR-1. The second cDNA consisted of 2,086 nucleotides encoding a protein of 371 amino acids, designated bfGnRHR-2. The third cDNA consisted of 1,772 nucleotides encoding a protein of 424 amino acids, designated bfGnRHR-3 (Fig. 1). Hydropathy analysis of bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 identified seven stretches of hydrophobic amino acids in each receptor protein, characteristic for members of the G protein-coupled receptor family (16). Several potential sites for posttranslational modification, including N-linked glycosylation sites and phosphorylation sites are present in the three receptors. bfGnRHR-1 shares 40% and 53% amino acid identity with bfGnRHR-2 and bfGnRHR-3, respectively, whereas bfGnRHR-2 shares 40% amino acid identity with bfGnRHR-3 (Table 2). bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 are only 30–50% homologous to other known GnRHRs. Interestingly, bfGnRHR-2 shows an 85% homology with that of *Xenopus*. In contrast to the mammalian GnRHRs, but similar to nonmammalian GnRHRs, bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 contain C-terminal cytoplasmic tails of 74, 57, and 79 amino acids, respectively.

Functional Characterization of the Bullfrog GnRHRs. To examine whether bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 are functional, we transiently transfected cDNA constructs harboring the bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 into COS-7 cells. Two amphibian forms of GnRH, mGnRH, and cGnRH-II (17,18), and other GnRHs (sGnRH, cGnRH-I, lGnRH-III, and seabream GnRH) stimulated inositol phosphate formation in a dose-dependent manner, indicating that all three bullfrog receptors couple to G_q/G_{11} in COS-7 cells (Fig. 2 and Table 3). All three receptors have a higher potency for cGnRH-II than for mGnRH (Table 3). However, for bfGnRHR-2, cGnRH-II is about 100 times more potent than mGnRH, whereas for bfGnRHR-1 and bfGnRHR-3, cGnRH-II is only 10 to 20 times more potent than mGnRH. In addition, sGnRH also has a strikingly high potency to stimulate all three receptors (Fig. 2). It is also of interest that bfGnRHR-1 exhibits relatively high responsiveness for lGnRH-III, bfGnRHR-3 exhibits relatively high responsiveness for

Table 2. Amino acid identity between various GnRH receptors

	bfGnRHR-1	bfGnRHR-2	bfGnRHR-3
human GnRHR	32%	36%	32%
rat GnRHR	34%	36%	33%
ovine GnRHR	32%	36%	32%
catfish GnRHR	40%	51%	42%
goldfish GnRHR-A	39%	51%	41%
goldfish GnRHR-B	40%	50%	41%
Xenopus GnRHR	40%	85%	40%
bfGnRHR-1	100%	40%	53%
bfGnRHR-2	40%	100%	40%
bfGnRHR-3	53%	40%	100%

Amino acid identity was determined using the CLUSTAL W(1.4) alignment program.

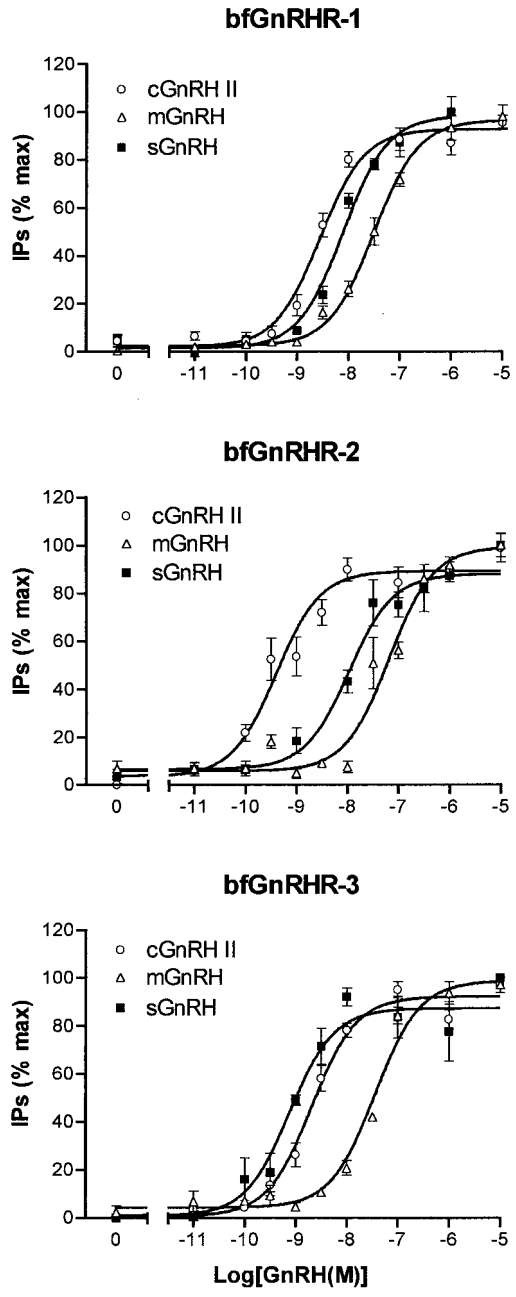

Fig. 2. Inositol phosphate production in COS-7 cells transiently transfected with the bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 constructs, after stimulation for 45 min with mGnRH (△), cGnRH-II (○), and sGnRH (■). Data are represented as means ± SEM of triplicate determinations from a representative experiment of three independent experiments.

cGnRH-I, whereas bfGnRHR-2 shows a similar responsiveness for lGnRH-III, cGnRH-I, and seabream GnRH (Table 3).

Northern Blot Analysis. To determine the spatiotemporal expression of bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 mRNAs, we performed Northern blot analysis by using pituitary, forebrain, and hindbrain RNA samples isolated during hibernation season, breeding season, and after-breeding season (Fig. 3). For each bfGnRHR mRNA, a different size was detected. There were two major bands of ≈2.7 kb and ≈0.5 kb for bfGnRHR-1, a single band of ≈8.0 kb for bfGnRHR-2, and a single band of ≈4.0 kb

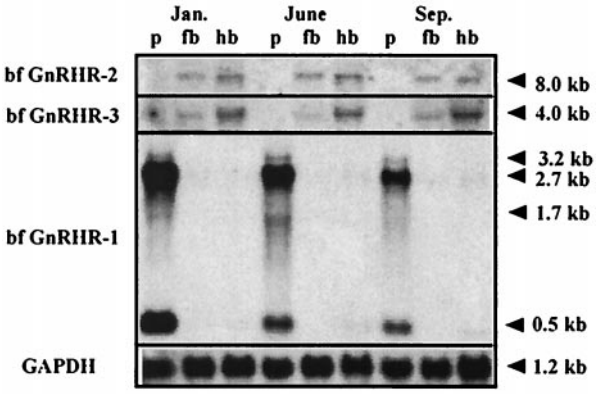

Fig. 3. Northern blot analysis of bfGnRHRs expression by using ^{32}P-labeled bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 cDNAs: p, pituitary; fb, forebrain; hb, hindbrain. Total RNA (10 μg) prepared from each brain region in different season (January, hibernation; June, breeding; and September, after-breeding) was subjected to Northern blot analysis under high-stringency conditions. The different transcripts and the sizes for each receptor are indicated (*Left*). GAPDH was used as an internal control indicating equal loading. Exposure times were 2.5 days for Northern blots of bfGnRHR-2 and bfGnRHR-3 and 12 h for Northern blots of bfGnRHR-1 and GAPDH.

for bfGnRHR-3 (Fig. 3). Longer exposure of the Northern blots revealed weak hybridizing bands of ≈3.2 kb and ≈1.7 kb for bfGnRHR-1 in both forebrain and hindbrain (data not shown). In addition, differences in spatial expression patterns were observed for each of the three bfGnRHR mRNAs. The bfGnRHR-1 mRNA is expressed in pituitary, whereas bfGnRHR-2 and bfGnRHR-3 mRNAs are expressed in forebrain and hindbrain. Also, differences in the temporal expression for each of the three bfGnRHR mRNAs were observed. The expression of the bfGnRHR-1 mRNAs in pituitary of 2.7 and 0.5 kb is highest in hibernation season, lower in the breeding season, and lowest in the after-breeding season (Fig. 3). The bfGnRHR-3 mRNA expression is highest in September, both in forebrain and hindbrain, whereas bfGnRHR-2 mRNA expression is lowest in September, both in forebrain and hindbrain (Fig. 3).

Genomic Southern Blot Analysis. Southern blot analysis was carried out by using the bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 cDNAs as probes. Two hybridizing bands were observed in the lanes containing bullfrog genomic-DNA cut with *Eco*RI, *Xba*I, *Pst*I, and *Bam*HI, respectively, by using the bfGnRHR-2 probe, although none of these enzymes cut within the cDNA sequence (Fig. 4). Likewise, two bands were observed in lanes containing bullfrog genomic-DNA cut with *Eco*RI and *Xba*I, respectively, although

Table 3. EC_{50} values of GnRHs for inositol phosphate production in COS-7 cells transiently transfected with bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 constructs

	EC_{50} (logM)		
Ligand	bfGnRHR-1	bfGnRHR-2	bfGnRHR-3
cGnRH-II	-8.64 ± 0.08^{a}	-9.41 ± 0.12^{a}	-8.85 ± 0.13^{a}
mGnRH	-7.51 ± 0.11^{bc}	-7.36 ± 0.14^{b}	-7.53 ± 0.01^{b}
sGnRH	-7.97 ± 0.15^{ab}	-8.01 ± 0.07^{b}	-9.12 ± 0.13^{a}
cGnRH-I	-6.25 ± 0.09^{d}	-7.36 ± 0.19^{b}	-8.51 ± 0.17^{a}
lGnRH-III	-6.92 ± 0.21^{cd}	-7.47 ± 0.13^{b}	-6.60 ± 0.22^{c}
sbGnRH	-6.45 ± 0.10^{d}	-7.89 ± 0.13^{b}	-7.75 ± 0.12^{b}

Data are mean ± S.E.M. of three independent experiments. Means in the same column that are significantly different are indicated by different superscript letters.

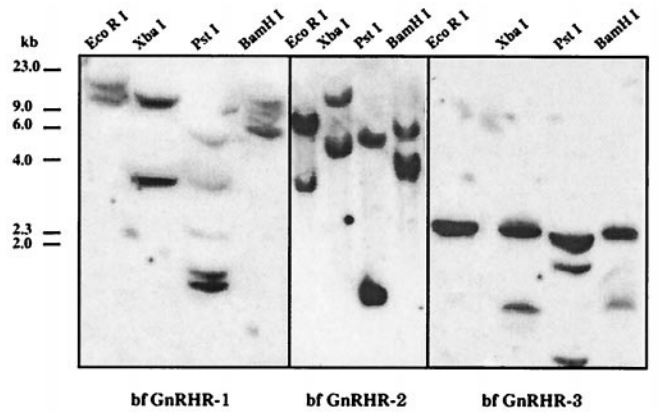

Fig. 4. Southern blot analysis of bullfrog genomic DNA. Ten micrograms of high molecular weight genomic DNA were cut with *Eco*RI, *Xba*I, *Pst*I, and *Bam*HI. Southern blot analysis was performed under high-stringency conditions by using ^{32}P-labeled bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 full-length cDNAs.

recognition sites for these enzymes are absent in the bfGnRHR-1 cDNA sequence. On the other hand, *Bam*HI, which has one cleavage site in the bfGnRHR-1 cDNA, generated one strong and three weak hybridizing bands, whereas *Pst*I, which has two cleavage sites in the bfGnRHR-1 cDNA, generated two strong and three very weak hybridizing bands (Fig. 4). Three bands hybridized with the *Pst*I-digested genomic DNA by using the bfGnRHR-3 probe, which is consistent with the bfGnRHR-3 cDNA containing two *Pst*I sites (Fig. 4). In the other lanes, the genomic DNA (cut with *Eco*RI) and *Bam*HI (having no cleavage sites within the bfGnRHR-3 cDNA sequence) yielded single hybridizing bands. Although *Xba*I has no cleavage sites in the bfGnRHR-3 cDNA sequence, two hybridizing bands were observed (Fig. 4).

Discussion

We cloned and functionally characterized three distinct bullfrog cDNAs, each encoding a different type of GnRHR. Each GnRHR mRNA displayed a distinct spatiotemporal expression pattern. In addition, each bfGnRHR exhibited a differential ligand selectivity.

In vertebrates, 11 naturally occurring structural GnRH variants have been identified (2–4). At least two forms of GnRH are expressed in a particular species (4). The mammalian type GnRH (GnRH1) appears in the hypothalamus, the cGnRH-II type GnRH (GnRH2) appears in the midbrain, and the third, salmon type GnRH (GnRH3), is found in the terminal nerve and olfactory system of the forebrain in some fish (ref. 19; GnRH nomenclature according to ref. 20). These findings lead to the suggestion that probably more than three GnRH gene families exist (21). We have demonstrated the presence of mGnRH and cGnRH-II in the bullfrog (L.W., M. S. Yoo, H. M. Kang, I. B. Im, H.S.C., J.B., and H.B.K., unpublished data). We cannot, however, exclude the possible existence of a third form of GnRH, such as sGnRH or lGnRH-III.

All three bfGnRHRs are functional receptors, because both native ligands, mGnRH and cGnRH-II, were able to stimulate inositol phosphate production in COS-7 cells in a dose-dependent manner. Similar to the situations for catfish (12) and goldfish (13) GnRHRs, cGnRH-II is the most potent native GnRH triggering inositol phosphate production. Interestingly, however, the relative potency of cGnRH-II to various receptors is different. For bfGnRHR-1, cGnRH-II is only about 10-fold more potent than mGnRH, whereas it is about 100-fold more potent for bfGnRHR-2 (Fig. 2, Table 3). It is highly probable that the native ligand for bfGnRHR-1, which is expressed in pituitary, is mGnRH, and that the native ligand for bfGnRHR-2, which is expressed in brain, is cGnRH-II. The high potency of sGnRH for bfGnRHR-3 suggests the presence of a third form of GnRH (GnRH3), such as salmon-like GnRH in bullfrog brain. Recently, we succeeded in cloning three different types of GnRHR cDNAs from *Rana dybowskii* (rdGnRHRs). Each type of rdGnRHR cDNA has an amino acid identity of >95% with the corresponding bfGnRHRs and has a similar distribution, expression, and pharmacological function (L.W., J.B., H.S.C., J.Y.S., S.Y.C., J.M.S., and H.B.K., unpublished data).

On the basis of differences in the amino acid sequence of extracellular loop 3, Troskie *et al.* (15) have proposed distinguishing two types of GnRHR: type I and II GnRHRs. GnRHRs from a wide range of species, including all mammalian and nonmammalian GnRHRs, seem to belong to type I GnRHR. According to their classification, bfGnRHR-2 and bfGnRHR-3 that are expressed in the brain seem to belong to type I and II GnRHRs, respectively. However, bfGnRHR-1 that is mainly expressed in pituitary does not belong to either type I or type II GnRHRs. Thus, their GnRHR classification, which is based solely on extracellular loop 3 analysis, may not be useful in amphibians.

Mammalian and nonmammalian GnRHRs share several common features that are also observed in bfGnRHRs. Three potential glycosylation sites in the N-terminal domain, which are known to be important for mGnRHR expression (22), are present in catfish and goldfish GnRHRs. Also, the bfGnRHR-1 protein (at positions 2, 27, and 32), the bfGnRHR-2 protein (at positions 9 and 19), and the bfGnRHR-3 protein (at positions 2, 25, and 41) contain such sites. In addition, bfGnRHR-1 and -2 also contain potential glycosylation sites in their extracellular loop II (positions 195 and 183, respectively). Likewise, putative ligand-binding sites in human GnRHR (Asp^{98}, Asn^{102}, and Lys^{121}; ref. 3), which are conserved in both goldfish GnRHRs, are also conserved in three bfGnRHRs (Asp^{105}, Asn^{109}, and Lys^{128} for bfGnRHR-1; Asp^{94}, Asn^{98}, and Lys^{117} for bfGnRHR-2; Asp^{116}, Asn^{120}, and Lys^{139} for bfGnRHR-3). This fact suggests that a similar ligand-binding site is present in both mammalian and nonmammalian GnRHRs. Glu^{301}, which was shown to be important for interacting with Arg^{8} of mGnRH in the mammalian GnRHRs (23), was predicted to be absent in nonmammalian GnRHRs. Interestingly, such a glutamate residue, which is conserved in catfish and goldfish GnRHRs, is also conserved at the homologous position in bfGnRHR-2 (Glu^{291}). However, the amino acid at the homologous position of Glu^{301} is replaced by Gln^{311} and Pro^{321} in the bfGnRHR-1 and bfGnRHR-3 proteins, respectively. In addition, several aromatic amino acids, which are supposed to interact with the most critical part of the GnRH molecule (His^{2}-Trp^{3}; ref. 24), are conserved in the bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 proteins. Aromatic amino acid residues Phe^{219}, Phe^{275}, Trp^{279}, and Tyr^{282} that are found in the rat GnRH receptor are also positioned at the same loci of the bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 proteins.

Although all bullfrog GnRHRs share low homology with their nonmammalian counterparts, they display some of the typical structural characteristics observed only in nonmammalian GnRHRs. First, bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 contain C-terminal intracellular tails of 74, 57, and 79 amino acids, respectively. It is well established that this cytoplasmic domain, which is absent in the mammalian GnRHRs, is functionally important for agonist-dependent phosphorylation, desensitization, and internalization of other G protein-coupled receptors as well as nonmammalian GnRHRs (2, 25–27). Second, mammalian GnRHRs are characterized by a reciprocal exchange of Asp^{87} in transmembrane domain II and Asn^{318} in transmembrane domain VII, as compared with other G protein-coupled receptors (28, 29). Nonmammalian receptors, however, contain only aspartic acid at these positions (12, 13). Likewise, bfGnRHR-1 has Asp^{94}/Asp^{326}, bfGnRHR-2 has Asp^{83}/Asp^{308}, and bfGnRHR-3 has Asp^{105}/Asp^{338} at these positions. Third, the conserved DRY motif of the proximal second intracellular domain in other G protein-coupled receptors (2) is

modified to DRS in all mammalian GnRHRs. This motif is changed to DRH in all nonmammalian GnRHRs, including bfGnRHR-2 and bfGnRHR-3. However, bfGnRHR-1 contains the sequence DRQ in this region.

Although mGnRH has a similar EC_{50} for the three receptor types, bfGnRHR-3 has a Glu at the position homologous to Glu^{301} in mammalian GnRHRs, whereas bfGnRHR-1 and bfGnRHR-2 have no homologous Glu residue. This Glu^{301} has been shown to interact with Arg^{8} of mGnRH (23). However, we were not able to determine receptor affinities in binding studies. It might be that K_i values of the three receptor types differ for mGnRH, but this difference is compensated for by a difference in coupling efficiency to the inositol phosphate-signaling pathway.

Differences in particular structural motifs among three receptors may be responsible for this selectivity. It is of great interest to note that the results presented here might be indicative of the presence of homologous second and/or third types of GnRHR in other nonmammalian as well as mammalian species. Such additional GnRHRs are also likely to display a differential ligand selectivity for the specific GnRHs present in that species.

Bullfrogs adjust their body temperature to the outside environment, so they enter a dormant stage during the winter season, and that adjustment is accompanied by a decreased metabolism. To investigate differences in GnRHR gene expression accompanying this physiological phenomenon, we performed a seasonal Northern blot analysis. This analysis revealed a spatiotemporal difference in the expression of bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 transcripts. bfGnRHR-2 and bfGnRHR-3 mRNA expression were detected only in forebrain and hindbrain, whereas bfGnRHR-1 mRNA expression was restricted to the pituitary. The bfGnRHR-2 and bfGnRHR-3 mRNA levels are similar during the hibernation season. The bfGnRHR-3 mRNA levels decrease in the forebrain during the breeding season and increase during the after-breeding season in the hindbrain, whereas bfGnRHR-2 mRNA levels decrease only during the after-breeding season, both in forebrain and hindbrain. These results suggest an important role for GnRHs in reproductive behavior in the bullfrog as in mammals (30). In addition, Northern blot analysis revealed a clear reduction of bfGnRHR-1 mRNA levels from breeding to after-breeding season in the pituitary. However, the highest bfGnRHR-1 mRNA levels were detected in the hibernation season, suggesting an important role for bfGnRHR-1 during hibernation, apart from its role in reproductive functions. These results suggest an important role for GnRHs in controlling gonadotropin secretion in the bullfrog. This role, both in the pituitary and in the brain, may be played by mGnRH. On the other hand, cGnRH-II may play an important role in the brain. It is not yet identified whether a third form of GnRH (GnRH3), such as sGnRH or lGnRH-III, exists in the bullfrog. However, if a GnRH3 form is present in the bullfrog brain, it may also play a role in the reproductive behavior in the bullfrog.

Southern blot analysis indicated that bfGnRHR-1 and bfGnRHR-3 are encoded by separate, single-copy genes. For bfGnRHR-2, two bands were detected in each lane in the Southern blot analysis. However, we favor the opinion that bfGnRHR-2 is also encoded by a single-copy gene, because only a single transcript was detected in the tissues tested. Possibly, a nonexpressed (pseudo) gene, homologous to the bfGnRHR-2 gene, exists in the bullfrog.

In summary, three different bfGnRHR cDNAs have been cloned and functionally expressed. bfGnRHR-2 and bfGnRHR-3 are expressed only in the brain. This report on the cloning of these types of GnRHRs in vertebrates demonstrates that three different types of GnRHR are expressed in a single species. Their differential spatiotemporal expression pattern suggests that the differential regulation of bfGnRHR-1, bfGnRHR-2, and bfGnRHR-3 gene expression might play an important role in the regulation of reproductive functions. Our finding in the bullfrog supports the possibility that multiple GnRHRs are expressed in other species, including mammals, because several forms of GnRH ligand are present in mammals. In addition, the identification of three distinct types of GnRHR in the bullfrog may provide important clues for a better understanding of the phylogenetic origin of the GnRHR family.

We are grateful to Dr. K. S. Lee, Dr. H. M. Kang, and Ms. Alison McGregor for helpful advice and technical assistance. This work was partly supported by grants awarded to H.B.K. from the Ministry of Education of Korea (BSRI-98–4425) and the Korea Science and Engineering Foundation (KOSEF) through the Hormone Research Center (HRC-98–0101).

1. Sealfon, S. C. & Millar, R. P. (1995) *Cell. Mol. Neurobiol.* **15,** 25–42.
2. Sealfon, S. C., Weinstein, H. & Millar, R. P. (1997) *Endocr. Rev.* **18,** 180–205.
3. Jimenez-Linan, M., Rubin, B. S. & King, J. C. (1997) *Endocrinology* **138,** 4123–4130.
4. Carolsfeld, J., Powell, J. F. F., Park, M., Fischer, W. H., Craig, G., Chang, J. P., Rivier, J. E. & Sherwood, N. M. (2000) *Endocrinology* **141,** 505–512.
5. Powell, J. F., Reska-Skinner, S. M., Prakash, M. O., Fisher, W. H., Park, M., Rivier, J. E., Craig, A. G., Mackie, G. O. & Sherwood, N. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10461–10464.
6. Tsutsumi, M., Zhou, W., Millar, R. P., Mellon, P. L., Roberts, J. L., Flanagan, C. A., Dong, K., Gillo, B. & Sealfon, S. C. (1992) *Mol. Endocrinol.* **6,** 1163–1169.
7. Reinhart, J. M., Mertz, L. M. & Catt, K. J. (1992) *J. Biol. Chem.* **267,** 21281–21284.
8. Kakar, S. S., Musgrove, L. C., Devor, D. C., Sellers, J. C. & Neill, J. D. (1992) *Biochem. Biophys. Res. Commun.* **189,** 289–295.
9. Kaiser, U. B., Zhao, D., Cardona, G. R. & Chin, W. W. (1992) *Biochem. Biophys. Res. Commun.* **189,** 1645–1652.
10. Illing, N., Jacobs, G. F., Becker, I. I., Flanagan, C. A., Davidson, J. S., Eales, A., Zhou, W., Sealfon, S. C. & Millar, R. P. (1993) *Biochem. Biophys. Res. Commun.* **196,** 745–751.
11. Weesner, G. D. & Matteri, R. L. (1994) *J. Anim. Sci.* **72,** 1911.
12. Tensen, C., Okuzawa, K., Blomenröhr, M., Rebers, F., Leurs, R., Bogerd, J. Schulz, R. & Goos, H. (1997) *Eur. J. Biochem.* **243,** 134–140.
13. Illing, N., Troskie, B. E., Nahornlak, C. S., Hapgood, J. P., Peter, R. E. & Millar, R. P. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 2526–2531.
14. Troskie, B. E., Hapgood, J. P., Millar, R. P. & Illing, N. (2000) *Endocrinology* **141,** 1764–1771.
15. Troskie, B., Illing, N., Rumbak, E., Sun, Y.-M., Hapgood, J., Sealfon, S., Conklin, D. & Millar, R. P. (1998) *Gen. Comp. Endocrinol.* **112,** 296–302.
16. Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J. & Sealfon, S. C. (1992) *DNA Cell Biol.* **11,** 1–20.
17. Sherwood, N. M., Zoeller, R. T. & Moore, F. L. (1986) *Gen. Comp. Endocrinol.* **61,** 313–322.
18. Conlon, J. M., Collin, F., Chiang, Y. C., Sower, S. A. & Vaudry, H. (1993) *Endocrinology* **132,** 2117–2123.
19. King, J. A. & Millar, R. P. (1995) *Cell. Mol. Neurobiol.* **15,** 5–23.
20. Fernald, R. D. & White, R. B. (1999) *Front. Neuroendocrinol.* **20,** 224–240.
21. Kasten, T. L., White, S. A., Norton, T. T., Bond, C. T., Adelman, J. P. & Fernald, R. D. (1996) *Gen. Comp. Endocrinol.* **104,** 7–19.
22. Davidson, J. S., Flanagan, C. A., Zhou, W., Becker, I. I., Elario, R., Emeran, W., Sealfon, C. S. & Millar, R. P. (1995) *Mol. Cell. Endocrinol.* **107,** 241–245.
23. Flanagan, C. A., Becker, I. I., Davidson, J. S., Wakefield, I. K., Zhou, W., Sealfon, S. C. & Millar, R. P. (1994) *J. Biol. Chem.* **269,** 22636–22641.
24. Davidson, J. S., Flanagan, C. A., Becker, I. I., Illing, N., Sealfon, S. C. & Millar, R. P. (1994) *Mol. Cell. Endocrinol.* **100,** 9–14.
25. Willars, G. B., Heding, A., Vrecl, M., Sellar, R., Blomenröhr, M., Nahorski, S. R. & Eidne, K. A. (1999) *J. Biol. Chem.* **274,** 30146–30153.
26. Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P. L. & Eidne, K. A. (1998) *J. Biol. Chem.* **273,** 11472–11477.
27. Blomenröhr, M., Heding, A., Sellar, R., Eidne, K. A., Leurs, R., Bogerd, J. & Willars, G. B. (1999) *Mol. Pharmacol.* **56,** 1229–1237.
28. Blomenröhr, M., Bogerd, J., Leurs, R., Schulz, R. W., Tensen, C. P., Zandbergen, M. A. & Goos, H. J. (1997) *Biochem. Biophys. Res. Commun.* **238,** 517–522.
29. Zhou, W., Flanagan, C., Ballesteros, J. A., Konvicka, K., Davidson, J. S., Weinstein, H., Millar, R. P. & Sealfon, S. C. (1999) *Mol. Pharmacol.* **45,** 165–170.
30. Mani, S. K., Allen, J. M., Rettori, V., McCann, S. M., O'Malley, B. W. & Clark, J. H. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6468–6472.