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Expression of Two Gonadotropin-Releasing Hormone (GnRH) Precursor Genes in Various Tissues of the Japanese Eel and Evolution of GnRH

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ABSTRACT—We isolated and characterized two distinct cDNAs for mammalian gonadotropin-releasing hormone (mGnRH) and chicken GnRH-II (cGnRH-II) precursors from the Japanese eel by rapid amplification of cDNA ends. Each GnRH precursors were composed of a signal peptide, a GnRH decapeptide, a processing site and a GnRH-associated peptide. Northern blot and reverse transcription-polymerase chain reaction analysis revealed that the mGnRH precursor gene is expressed in all tissues tested including the brain, pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and peripheral blood leukocyte. In contrast, the cGnRH-II precursor gene expression was detected only in the brain, pituitary, olfactory epithelium, ovary and testis. These findings suggest unknown physiological function(s) for mGnRH besides the well-documented role in the pituitary gonadotropin synthesis and release. The eel mGnRH and cGnRH-II precursors have high amino acid homologies with seabream GnRH (sbGnRH) precursors of the Perciforms and cGnRH-II precursors of other teleosts, respectively. Phylogenetic analysis showed the existence of three distinct evolutionary arms of GnRHs; multiple GnRH forms (mGnRH, guinea pig GnRH, chicken GnRH-I, sbGnRH and catfish GnRH (cfGnRH)) on the first, cGnRH-II on the second, and salmon GnRH (sGnRH) on the third arm. This analysis suggests that mGnRH progenitor has undergone sequence divergence to give rise to sbGnRH and cfGnRH, whereas sGnRH represents a separate evolutionary line.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) was originally isolated from mammalian hypothalamus as the physiologic regulator of gonadotropin (GTH) release from the pituitary gland (Matsuo *et al*., 1971; Amoss *et al*., 1971). To date, ten distinct GnRH molecular forms have been characterized from vertebrates and two from a protochordate (see Sherwood *et al*., 1997; Jimenez-Linan *et al*., 1997). It is generally accepted that two or three GnRH molecular forms are present in the brain of a single vertebrate species. The coexistence of multiple GnRH forms in a single species raises questions concerning their respective distribution, regulation and physiological function. One form of GnRH known as chicken GnRH-II (cGnRH-II) has been found in the brains of representatives of all six vertebrate classes from cartilaginous fish to mammals, whereas other GnRH forms display marked diversity among vertebrates. Although multiple GnRH forms have been found in the brain of more than twenty teleost species, the primary structures of only four GnRH forms, salmon GnRH (sGnRH), cGnRH-II, seabream GnRH (sbGnRH) and catfish GnRH (cfGnRH), have been confirmed by amino acid sequencing and/or cDNA analysis (see Amano *et al*., 1997; see Yu *et al*., 1997). Mammalian GnRH (mGnRH) has not yet been isolated or sequenced from teleost species. To date, therefore, there is no direct evidence that mGnRH occurs in teleosts. In addition to the hypothalamus, GnRH precursor gene expression has also been found recently in several reproductive tissues including the placenta, mammary gland, ovary and testis, indicating peripheral paracrine or autocrine role(s) for GnRH (see Sherwood *et al*., 1997; Suetake and Aida, 1997).

The Japanese eel, *Anguilla japonica*, is one of the most primitive living teleost species and, therefore, provides a good animal model to study the functions of GnRH and the structure and expression of its gene in an evolutionary point of view. In the brain of the Japanese eel, mGnRH has been detected by immunocytochemistry (Nozaki *et al*., 1985). In the brain of the European eel, *Anguilla anguilla*, mGnRH and cGnRH-II have been detected by combination of high performance liquid chromatography and radioimmunoassay (King *et al*., 1990).

In the present study,we isolated and characterized two

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eel GnRH precursor cDNAs by rapid amplification of cDNA ends (RACE). We also examined whether the two GnRH precursor genes are expressed in various tissues by Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis. In addition, we discussed the evolution of GnRH precursors in vertebrates.

MATERIALS AND METHODS

PCR and oligonucleotide primers

Each following PCR amplification was performed in a final volume of 20 μl containing 1×PCR buffer (Takara, Otsu, Shiga, Japan), 200 μM of dNTPs (Takara), 0.5 U *Taq* DNA polymerase (Takara), 0.5 μM of each primer and 1 μl cDNA template, using a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA) .

The primers used in the present study are shown in Fig. 1. For RACE for the mGnRH precursor cDNA, we used the following primers: MF-1, 5'-CA(A/G)CA(C/T)TGGTC(A/C/G/T)TA(C/T)GG-3'; MF-2, 5'-CA(C/T)TGGTC(A/C/G/T)TA(C/T)GG(A/C/G/T)(C/T)T-3'; MR-1, 5'-CGTCCAGTTTCTCTGTCAGCCAG-3'; MR-2, 5'-GATTTGCCAGTATTTCCTTCAG-3'; MR-3, 5'-CTTCAGGCTGGAAAGTGTAAT-3'; RTG, 5'-AACTGGAAGAATTCGCGGCCG-3'; RTG-nested, 5'-TGGAAGAATTCGCGGCCGCAG-3'; AAP, 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3'; AUAP, 5'-CACGCGTCGACTAGTACGGG-3'. MF-1 and MF-2 were designed for 3'-RACE, based on the mGnRH amino acids 1–6 and 2–7, respectively. After determining the nucleotide sequences from the mGnRH decapeptide encoding regions to the 3'-end, new specific primers, MR-1, MR-2 and MR-3, were synthesized for 5'-RACE, based on the nucleotide sequences of the GnRH-associated peptide (GAP) region. RTG, RTG-nested, AAP and AUAP were used as adaptor primers. For RACE for cGnRH-II precursor cDNA, we used the following primers: CF-1, 5'-CA(A/G)CA(C/T)TGGTCICA(C/T)GGITGGTA-3'; CF-2, 5'-TGGTCICA(C/T)GGITGGTA(C/T)CCIGG-3'; CR-1, 5'-TCCATTTGGTCCCAGTCGTTCC-3'; CR-2, 5'-ACTGACTTGTCCATCTGGATAC-3'; CR-3, 5'-GGATACAGTTGCTATGTGAAG-3'; dT-adaptor, 5'-GACTCGAGTCGACATCGA(T)17-3'; T-adaptor, 5'-GACTCGAGTCGACATCGAT-3'. CF-1 and CF-2 were designed for 3'-RACE, based on the cGnRH-II amino acids 1–8 and 3–10, respectively. After determining the nucleotide sequences from the cGnRH-II decapeptide encoding regions to the 3'-end, new specific primers, CR-1, CR-2 and CR-3, were synthesized for 5'-RACE, based on the nucleotide sequences of the 3'-untranslated region. RTG, RTG-nested, dT-adaptor and T-adaptor were used as adaptor primers. In order to generate cDNA probes for Northern blot analysis, we used the following primers: MF-3, 5'-GGCAAGAGAGGAGCAGATAGT-3'; MR-4, 5'-GTCATGTTTTATTGAAGGAATGC-3'; CF-3, 5'-AGAATGCAGCTACTTGAGACC-3'; CR-4, 5'-TAGAGCGAGTCATAGGAGAC-3'. MF-3/MR-4 and CF-3/CR-4 were used to generate the mGnRH and cGnRH-II precursor cDNA probes, respectively. For RT-PCR analysis, we designed the following primer: CF-4, 5'-CTTTAGCTGTGATGGTGAAC-3'. MF-3/MR-4 and CF-4/CR-4 were used to amplify the mGnRH and cGnRH-II precursor cDNAs, respectively.

RACE for the eel mGnRH precursor cDNA

Animals and RNA preparation. Immature eels were purchased from a commercial dealer. Four fish (One female and three males), body weight 444–461g and gonadosomatic index (GSI) 0.23–1.67%, were anesthetized in 0.06% 2-phenoxyethanol and sacrificed by decapitation. Dissected brain tissue was frozen in liquid nitrogen and stored at –80°C until total RNA preparation. Total RNA was extracted from the whole brain using RNA extraction solution (ISOGEN; Nippongene, Toyama, Japan), according to the manufacturer's instructions.

cDNA synthesis for 3'-RACE. First-strand cDNA was synthcted from 5 μg of total RNA with *Not*I dT primer (5'-AACTGGAAGAATTCGCGGCCGCAGGAA$(T)_{18}$-3') using Ready-To-Go™ T-primed first-strand cDNA synthesis kit (Pharmacia Biotech, USA) according to the manufacturer's instructions.

3'-RACE. The first-strand reaction mixture was amplified with MF-1 and RTG primers. Forty cycles of PCR was carried out under a cycle profile of 94°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec. Then a 100-fold-diluted first PCR reaction mixture was used as template in a nested PCR for 35 cycles with MF-2 and RTG-nested primers. The nested PCR was conducted under the same cycle profile as that used for the first PCR but annealing temperature was increased to 55°C. The final elongation step was carried out at 72°C for 10 min.

cDNA synthesis for 5'-RACE. First-strand cDNA was synthesized

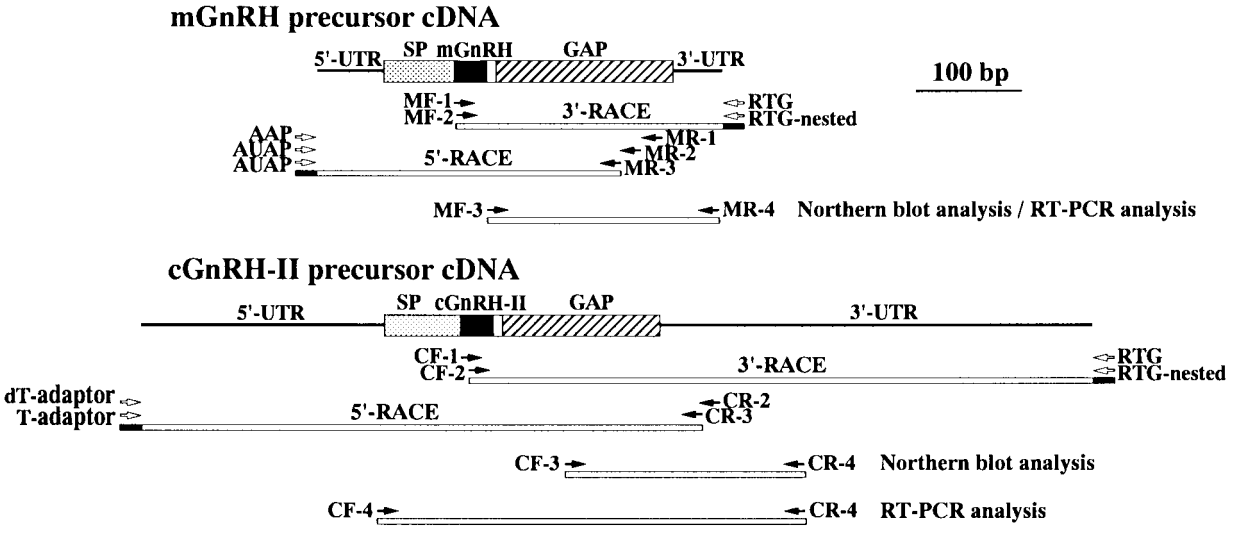

Fig. 1. Location of the primers used for 3'-RACE, 5'-RACE, generation of probes for Northern blot analysis, and RT-PCR analysis. Location of forward (1{), reverse (1|) and adaptor (1qand 1r) primers are shown and correspond to oligonucleotides listed in *MATERIALS AND METHODS*. 5'-UTR, 5'-untranslated region; SP, signal peptide; GAP, GnRH-associated peptide; 3'-UTR, 3'-untranslated region.

with MR-1 primer from 1 μg of the same total RNA as that used for the 3'-RACE. Subsequently, a dC-tail was added to the 3'-end of the synthesized cDNA using terminal transferease included in the 5'-RACE PCR kit (5'-RACE System for Rapid Amplification of cDNA Ends. Ver 2.0; GIBCO/BRL, Gaitherburg, MD, USA).

5'-RACE. 5'-RACE was carried out by three-stage PCR amplifications for 35 cycles each: the first stage PCR was performed with MR-1 and AAP primers under a cycle profile of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. For the second stage, a 100-fold-diluted first stage PCR reaction mixture was subjected to a nested PCR with MR-2 and AUAP primers. Then a 100-fold-diluted second stage PCR reaction mixture was subject to the third stage PCR with MR-3 and AUAP primers. The following cycle profiles was used for the second and third stage PCRs: 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The final elongation step was carried out at 72°C for 10 min after the third PCR amplification.

RACE for the eel cGnRH-II precursor cDNA

3'-RACE. Thirty-five cycles of PCR was carried out with CF-1 and RTG primers, using the same first strand cDNA as that used in 3'-RACE for mGnRH precursor cDNA. Then a 100-fold-diluted first PCR reaction mixture was subjected to a nested PCR for 35 cycles with CF-2 and RTG-nested primers. A cycle profile of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec was used for these PCRs. The final elongation step was carried out at 72°C for 10 min.

cDNA synthesis for 5'-RACE. First-strand cDNA was synthesized with CR-1 primer from 1 μg of the same total RNA as that used for the 3'-RACE. Then a dA-tail was added to the 3'-end of the synthesized cDNA as described above.

5'-RACE. Thirty-five cycles of first PCR was performed with CR-2 and dT-adaptor primers under a cycle profile of 94°C for 30 sec, 45°C for 30 sec and 72°C for 30 sec. A 100-fold-diluted first PCR reaction mixture was subjected to a nested PCR for 35 cycles with CR-3 and T-adaptor primers under a cycle profile of 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec. The final elongation step was carried out at 72°C for 10 min.

Subcloning and DNA sequencing

The cDNA fragments amplified by RACE described above were ligated into pBluescript II SK(-) vector (Stratagene, La Jolla, CA, USA). The plasmid DNA was purified by an alkaline lysis method. Both strands of the plasmid DNA were sequenced with T3 or T7 primer, using a Model 373A DNA sequencer with dye terminator cycle sequencing kits (Applied Biosystems, Foster City, CA, USA).

Northern blot analysis

Total RNA of the brain, liver, ovary, and testis were isolated from 20 immature eels of both sexes, body weight 312–429g and GSI 0.02-0.97%, as described above. Poly(A)$^+$RNA was purified from the total RNA of each tissue with Oligotex™-dT 30 (Takara) according to the manufacturer's instructions. Ten micrograms of poly(A)$^+$RNA from each tissue was denatured at 65°C for 15 min in 50% formamide and subjected to electrophoresis on 0.9% agarose gel. After the electrophoresis, the RNA was transferred to nitrocellulose membranes (Hybond-N$^+$, Amersham, UK) and UV cross-linked using Spectrolinker™ XL-1500 (Spectronics Corporation, Westburg, NY, USA) for 30 sec. The cDNA probes for the mGnRH (217 bp) and cGnRH-II precursors (225 bp) were generated and labeled with [α-^{32}P] dCTPs by PCR using MF-3/MR-4 and CF-3/CR-4 primer pairs, respectively (Fig. 1). The membranes were prehybridized in 6×SSC containing 50% formamide, 1×Denhardt's solution, 0.5% SDS and calf thymus DNA (100 ng/ml) at 42°C for 3 hr. Then the membranes were hybridized with either a cDNA probe of mGnRH or cGnRH-II precursor (1×10^9cpm/ml each) in the prehybridization buffer at 42°C for 18 hr. The membranes were washed in 2×SSC containing 0.1% SDS at room temperature for 10 min, and then washed twice in 0.2×SSC containing 0.1% SDS at 65°C for 10 min. The membranes hybridized with the mGnRH and cGnRH-II precursor cDNA probes were exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan) at –80°C for 2 and 6 days, respectively.

RT-PCR analysis

The brain, pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin and fin were dissected from four immature eels of both sexes, body weight 273–805g and GSI 0.07–0.73%. Peripheral blood was collected in heparinized syringes from the caudal vessel and diluted at 2 fold with PBS. The diluted blood was then applied to a discontinuous Percoll (Research Organics, Cleveland, Ohio, USA) density gradient of 1.032 and 1.100 g/ml and centrifuged at 1600g for 30 min at 4°C. Cells at the interface densities of 1.032 and 1.100 g/ml were collected and washed twice with PBS. These cells consisted of peripheral blood leukocyte (PBL). Total RNA of each tissues was isolated as described above. Five micrograms of RNA treated with DNase (Promega, Tokyo, Japan) was reverse transcribed as described in cDNA synthesis for 3'-RACE. MF-3/MR-4 and CF-4/CR-4 primer pairs were used to amplify the mGnRH and cGnRH-II precursor cDNAs (Fig. 1). Each PCR for 40 cycles was conducted under a cycle profile of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. One half of each PCR reaction mixture was analyzed on 2% agarose gels and stained with ethidium bromide. For control, PCRs were also conducted with the genomic DNA template and absence of cDNA template to examine if PCR products result from the amplification of genomic DNA and of cross-contamination of sample, respectively. Genomic DNA was taken from the peripheral blood erythrocyte.

The fragments of 217 and 406 bp obtained with MF-3/MR-4 and CF-4/CR-4 primer pairs, respectively, from the brain, pituitary, ovary and testis were subcloned and sequenced as described above to confirm their authenticity as the mGnRH and cGnRH-II cDNA amplicons, respectively.

Phylogenetic analysis

The amino acid sequences of the full-length GnRH precursors of the eel and those of several other vertebrates reported to date were aligned to each other by CLUSTAL W (Thompson *et al*., 1994) with default setting. After the alignment, a phylogenetic tree was generated by PHYLIP (Felsenstein, 1989) using neighbor-joining method (Saito and Nei, 1987).

Full species names and GenBank accession numbers are as follows: Human (*Homo sapiens*) mGnRH: X01059, cGnRH-II: AF036329; Tree shrew (*Tupaia glis belangeri*) mGnRH: U63326, cGnRH-II: U63327; Rat (*Rattus norvegicus*) mGnRH: M15527; Guinea pig (*Cavia porcellus*) guinea pig GnRH (gpGnRH): AF033346; Chicken (*Gallus gallus*) chicken GnRH-I (cGnRH-I): X69491; Frog (*Xenopus laevis*) mGnRH: L28040; Gilthead seabream (*Sparus aurata*) sbGnRH: U30320, sGnRH: U30311, cGnRH-II: U30325; African cichlid (*Haplochromis burtoni*) sbGnRH: U31865, sGnRH: S63657, cGnRH-II: L27435; African catfish (*Clarias gariepinus*) cfGnRH 1: X78049, cfGnRH 2: X78048, cGnRH-II: X78047; Goldfish (*Carassius auratus*) sGnRH 1:AB017271, sGnRH 2: AB017272, cGnRH-II 1: U30386, cGnRH-II 2: U40567; Sockeye salmon (*Oncorhynchus nerka*) sGnRH 1: D31868, sGnRH 2: D31869.

RESULTS

Isolation and characterization of the eel GnRH precursor cDNAs

From the sequence analysis of the 3'- and 5'-RACE products, the full-length cDNAs for the mGnRH and cGnRH-II of the Japanese eel were identified. The mGnRH precursor cDNA consists of 379 bp, comprising a short 5'-untranslated region (61 bp), an open reading frame (273 bp), a stop codon (TAG),

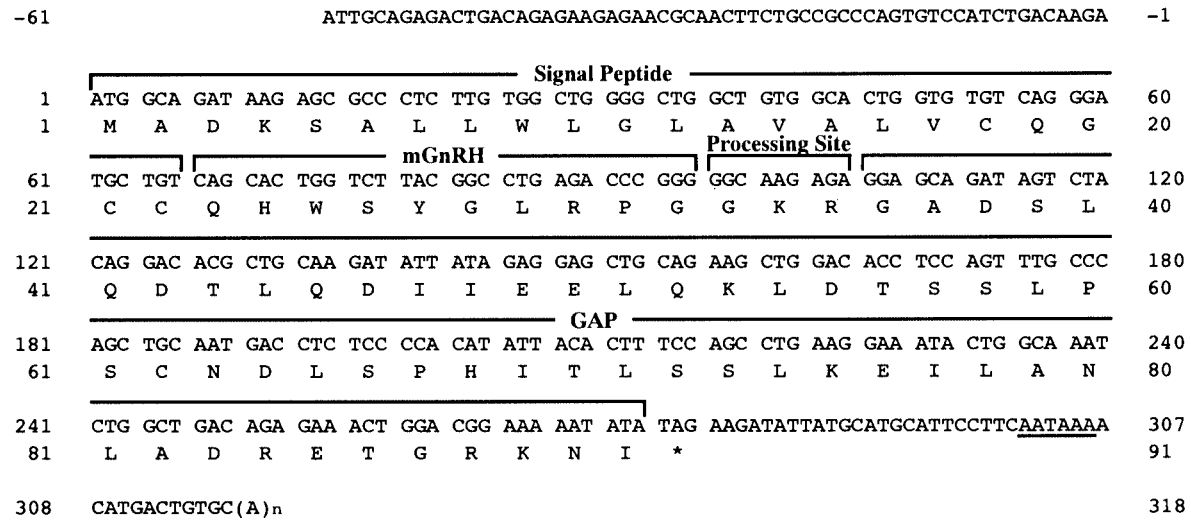

Fig. 2. Nucleotide and deduced amino acid sequences of the cDNA for the mGnRH precursor in the Japanese eel. Nucleotides are numbered 5' to 3', beginning with the first ATG codon in the open reading frame (ORF). Amino acid residues are numbered beginning with the first residue (Met) in the ORF. The polyadenylation signal (AATAAA) is underlined. The asterisk indicates the stop codon. GAP, GnRH-associated peptide. These sequences have been deposited in GenBank under accession number AB026989.

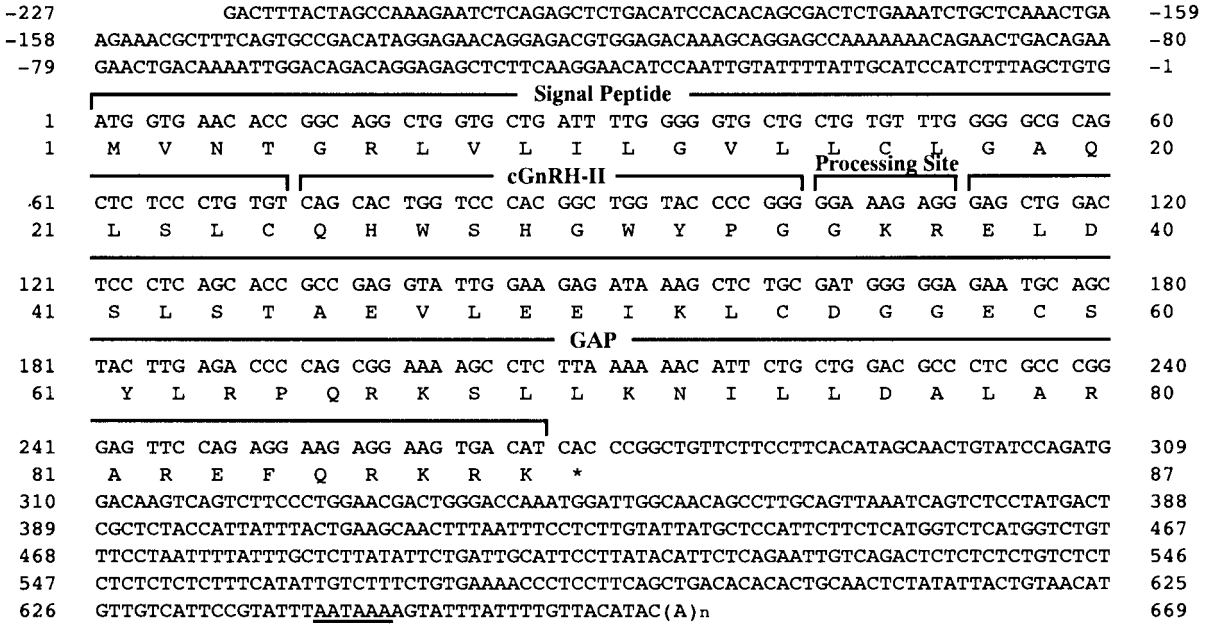

Fig. 3. Nucleotide and deduced amino acid sequences of the cDNA for the cGnRH-II precursor in the Japanese eel. For details, see legend to Fig. 2. These sequences have been deposited in GenBank under accession number AB026990.

and a short 3'-untranslated region (42 bp) (Fig. 2). The cGnRH-II precursor cDNA consists of 896 bp, comprising a long 5'-untranslated region (227 bp), an open reading frame (261 bp), a stop codon (TGA) and a long 3'-untranslated region (405 bp) (Fig. 3). In both mGnRH and cGnRH-II precursor cDNAs, the open reading frame starts at the first ATG codon, and a polyadenylation signal (AATAAA) is present 18 and 27 bp upstream from a poly (A) tail, respectively. The deduced amino acid sequences of both GnRH precursors have the same molecular architecture as those of other vertebrates reported to date: each composed of a signal peptide (22 and 24 amino acids for the mGnRH and cGnRH-II precursor, respectively), the GnRH decapeptide, a processing site (Gly-Lys-Arg), and a GAP (56 and 50 amino acids, respectively). The nucleotide sequence was determined by at least four independent amplifications to avoid PCR errors. An additional confirmation was achieved by performing PCR to generate the full-length cDNA from the independent brains of four eels.

Northern blot analysis

The result of Northern blot analysis is shown in Fig. 4. A single band of approximately 400 and 900 bases was observed in the brain by hybridization with the mGnRH and cGnRH-II precursor cDNA probes, respectively. No hybridization signal was detected in the liver, ovary, and testis with both the two probes.

RT-PCR analysis

After 40 cycles of PCR using MF-3/MR-4 primer pair, a single cDNA fragment of the expected size (217 bp) was obtained from all tissues tested; the brain, pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and PBL (Fig. 5A). Amplification of the same cDNA samples with CF-4/CR-4 primer pair produced a single cDNA fragment of the expected size (406 bp) from the brain, pituitary, olfactory epithelium, ovary and testis but not from the eye, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and PBL (Fig. 5B). Sequencing of these two bands revealed that both of them are indeed the respective GnRH precursor cDNA amplicons. The sizes of the PCR products from the genomic DNA were longer than those from tissue samples. No product was detected from the negative controls.

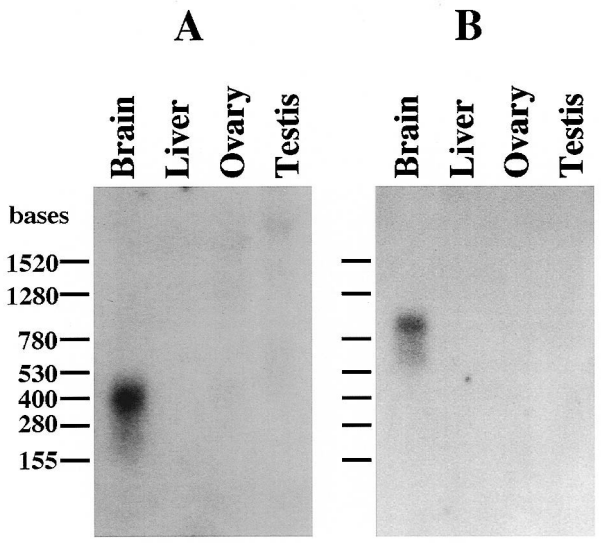

Fig. 4. Northern blot analysis for the mGnRH and cGnRH-II precursor mRNAs. It was performed on poly $(A)^+$ RNA (10 μg) obtained from the brain, ovary, testis and liver. RNA size makers are shown on the left. A, Hybridization with the mGnRH precursor cDNA probe. B, Hybridization with the cGnRH-II precursor cDNA probe.

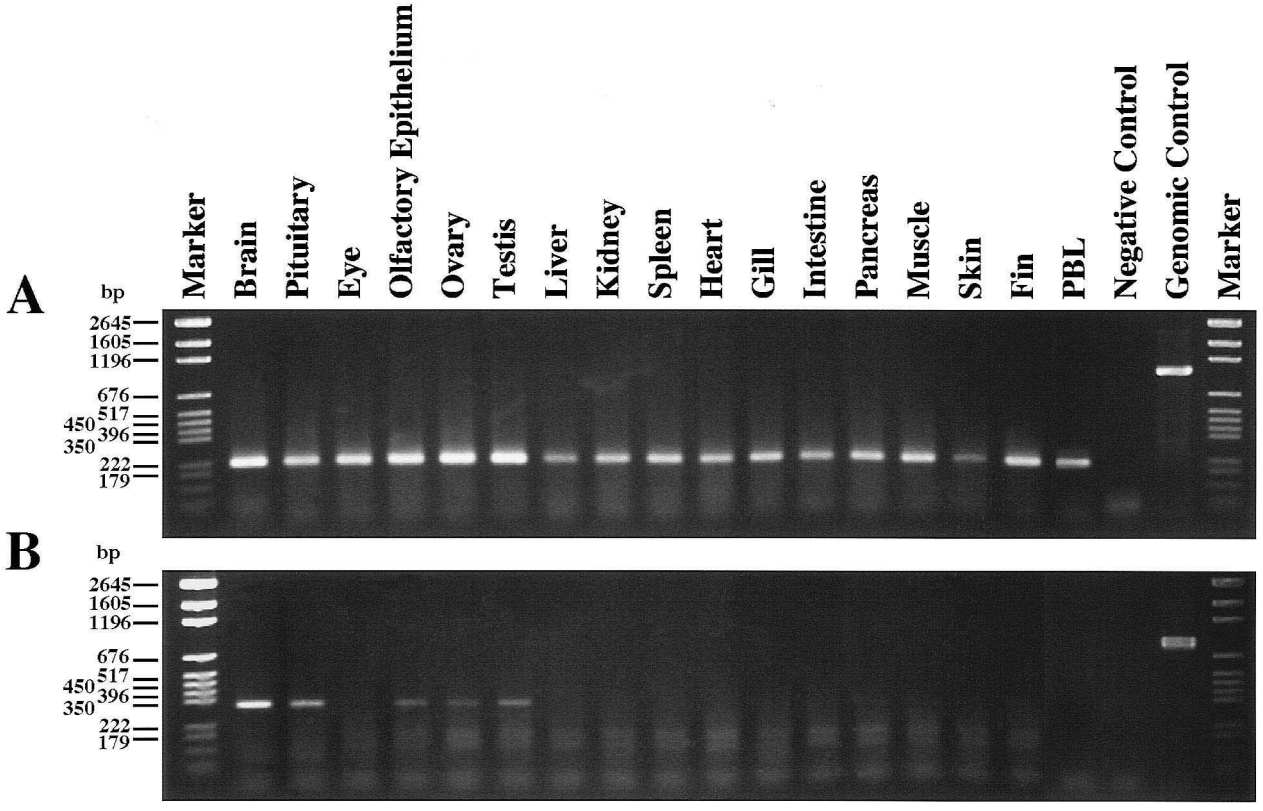

Fig. 5. RT-PCR analysis of the mGnRH and cGnRH-II precursors in various tissues. Total RNAs from various tissues were reverse transcribed, amplified by PCR, fractionated on a agarose gel, and stained with ethidium bromide. A, RT-PCR amplification with the mGnRH precursor cDNA-specific primer pair. B, RT-PCR amplification with the cGnRH-II precursor cDNA-specific primer pair. PBL, peripheral blood leucocyte.

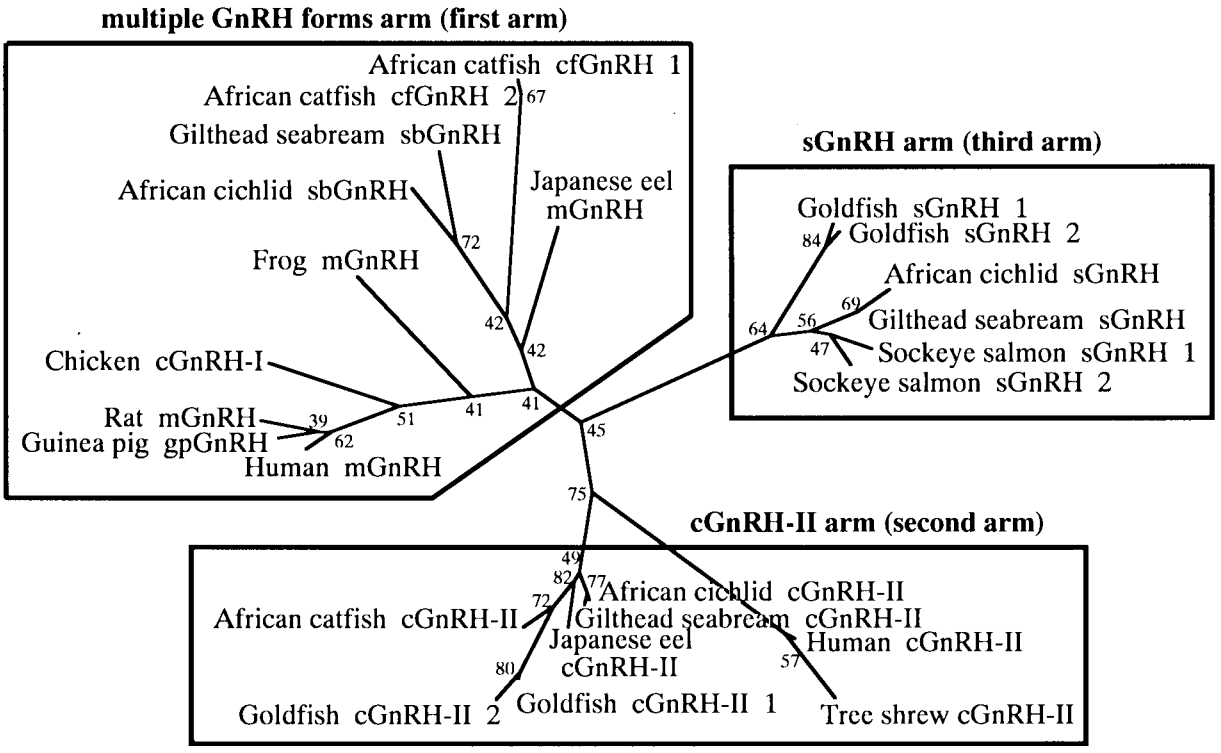

Fig. 6. A phylogenetic tree of GnRH precursor in vertebrates. This unrooted tree was constructed by neighbor-joining method (Saito and Nei, 1987) using PHYLIP software (Felsenstein, 1989), based on the alignments of the amino acid sequences of whole GnRH precursors using CLUSTAL W (Thompson *et al.*, 1994). The values at the nodes are bootstrap probabilities (%) estimated by 100 times replications. Evolutionary distances are represented by the length of the branches. An unrooted tree is shown because of the lack of an obvious outgroup. For accession numbers and species names, see MATERIALS AND METHODS.

Sequence similarity and phylogenetic analysis

The percentage similarity for the deduced amino acid sequence was determined between the two GnRH precursors of the eel and those of other vertebrates. The mGnRH precursor of the eel has high similarity with those of tetrapods (49–57%), cGnRH-I precursor of the chicken (55%) and in particular sbGnRH precursors of the Perciforms (61–66%). On the other hand, the cGnRH-II precursor of the eel has very high similarity (76–85%) with those of other teleosts.

Phylogenetic analysis revealed that known GnRH precursors including mGnRH and cGnRH-II precursors of the eel are classified into 3 groups as shown in Fig. 6. Mammalian GnRH, gpGnRH, cGnRH-I, sbGnRH and cfGnRH precursors are localized on the first arm, cGnRH-II precursors on the second arm, and sGnRH precursors on the third arm.

DISCUSSION

Characterization of the eel GnRH precursors

We isolated and characterized the mGnRH and cGnRH-II precursor cDNAs of the Japanese eel, and provided direct evidence for the existence of mRNAs for these two GnRH forms in the eel. This is the first report that mGnRH precursor cDNA is isolated and characterized from teleosts. The signal peptides of the two GnRH precursors typically include high proportions of hydrophobic amino acids: 13 out of 22 residues for the mGnRH precursor, and 16 out of 24 residues for the cGnRH-II precursor. In addition to the Gly-Lys-Arg processing site, the GAPs of the mGnRH and cGnRH-II precursors contain one and two Arg-Lys site(s), respectively, which typically represent enzymatic cleavage recognition site, similar to several other neuropeptide precursors (Douglass *et al.*, 1984).

Expression of the eel GnRH precursor genes

Northern blot analysis revealed the presence of a single mGnRH precursor mRNA of approximately 400 bases and a single cGnRH-II precursor mRNA of approximately 900 bases in the brain (Fig. 4). The sizes of these two mRNAs correspond to those of the isolated cDNAs. No hybridization signals for the two GnRH precursor mRNAs were detected in the liver, ovary and testis. However, low abundance of the mRNAs, but not absence of the mRNAs, may account for the absence of signals in these tissues. Thus, we next carried out RT-PCR analysis in several tissues.

RT-PCR analysis revealed that the mGnRH precursor mRNA is present not only in the brain, but also in all tissues tested: the pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and PBL (Fig. 5A), and that the cGnRH-II precursor

mRNA is present not only in the brain, but also in the pituitary, olfactory epithelium, ovary and testis (Fig. 5B). Several lines of evidence support the validity of our observations in RT-PCR analysis. First, sequencing of the PCR fragments revealed that those are indeed the fragments of the GnRH precursor mRNAs. Second, the possibility of the genomic DNA contamination is ruled out, since the sizes of the PCR products from the genomic DNA template differed from those of the cDNA samples. Third, the possibility of cross-contamination seems highly unlikely, since there were no products for both GnRH precursor cDNAs from negative control and for the cGnRH-II precursor cDNA from several tissues. Finally, the same results were obtained from four individuals including both sexes (A representative data is shown in Fig. 5).

The present study demonstrates the widespread expressions of the GnRH precursor genes. Notably, the mGnRH precursor gene is expressed in all tissues examined. Compared with the GnRH precursor gene expression in the reproductive tissues, that in the non-reproductive tissues has been investigated only in a few studies. In mammals, the mGnRH precursor gene expression has been reported in several types of different lymphocytes (Azad *et al*., 1991; Azad *et al*., 1993a; Wilson *et al*., 1995), liver, heart, skeletal muscle and kidney (Kakar and Jennes, 1995). In teleosts, there has been only one study that demonstrates the GnRH precursor gene expression in non-reproductive tissues. White and Fernald (1998) demonstrated that the sbGnRH precursor gene is expressed in all tissues tested (heart, liver, spleen, and kidney) of the African cichlid, but the cGnRH-II and sGnRH precursor genes are not observed to be expressed in non-reproductive tissues. Our present result, in combination with these previous studies, demonstrates the same tissue-specific expression patterns of the eel mGnRH, mammalian mGnRH and cichlid sbGnRH precursor genes, but not the cichlid sGnRH precursor gene. This suggests that mGnRH and sbGnRH, but not sGnRH, are evolutionary and functionary homologous.

The wide spread expressions of the GnRH precursor genes suggest that GnRH may have physiological function(s) in an autocrine or paracrine manner, besides the well-documented role on the pituitary GTH synthesis and release. This hypothesis raises a question whether the function(s) are tissue specific or common among all tissues. Further studies, including the identification of GnRH peptide and its receptor in peripheral tissues, are necessary to clarify the physiological function(s) of GnRH in these tissues.

In contrast to the gene expression of the mGnRH precursor, that of the cGnRH-II precursor is limited to several tissues. This finding suggests that the mGnRH and cGnRH-II precursor genes are differentially regulated and have distinct functions. Although the function of cGnRH-II is unknown, the expression of the cGnRH-II precursor gene in the olfactory epithelium suggests that cGnRH-II is involved in the pathway through which environmental and behavioral inputs influence the neuroendocrine systems, and supports the speculation that cGnRH-II plays a role in control of sexual behavior (Muske, 1993; King and Millar, 1995). It is possible that cGnRH-II in the olfactory epithelium has an important role for the reproductive migration of the eel.

Evolution of GnRH precursors

The eel is a key vertebrate in the GnRH evolution because it has mGnRH. Mammalian GnRH occurs in primitive teleosts, amphibians and mammals, but not in reptiles and birds. This unique phylogenetic distribution of mGnRH raises a question as to the evolution of GnRHs. The analysis on the eel mGnRH precursor cDNA provides an important information to examine the evolution of GnRHs. The mGnRH precursor of the eel has much higher similarity with sbGnRH precursors than with sGnRH precursors of other teleosts. Different from a lot of speculations to date, this result suggests mGnRH progenitor has undergone sequence divergence to give rise to sbGnRH, but not to sGnRH. This idea is supported by the phylogenetic tree (Fig. 6) and the same tissue-specific expressions between the mGnRH and sbGnRH precursor genes, but not the sGnRH precursor gene as described above.

Here we suggest the following evolutionary scheme of GnRHs. Duplication of an ancestral GnRH gene gave rise to the distinct evolutionary arms, multiple GnRH forms (mGnRH, gpGnRH, cGnRH-I, sbGnRH, and cfGnRH) on the first, cGnRH-II on the second, and sGnRH on the third arm. Although the point of this gene duplication is not known, the separation of these three GnRH arms clearly occurred prior to the emergence of cartilaginous fish which has three GnRH forms; dogfish GnRH (dfGnRH), cGnRH-II, and a novel GnRH form (see King and Millar, 1997). The amino acid sequence of dfGnRH differs from sGnRH at only fifth residue. This suggests that sGnRH originated from dfGnRH by one amino acid residue change, and the novel GnRH form in cartilaginous fish is ancestral to multiple GnRH forms on the first evolutionary arm. On this arm, mGnRH gene of primitive teleost has undergone sequence divergence during evolution, to give rise to cfGnRH and sbGnRH gene. Catfish GnRH or sbGnRH is present as an alternative variant to mGnRH in late teleost species. Chicken GnRH-I and gpGnRH were also derived from mGnRH. Chicken GnRH-II represents a separate evolutionary arm (the second arm). Chicken GnRH-II has been highly conserved during the evolution and has been found in the brains of representatives of all six vertebrate classes from cartilaginous fish to mammals. Salmon GnRH also represents a separate evolutionary arm (the third arm). This evolutionary scheme is in part similar to that described by Gothilf *et al*. (1995) and White *et al*. (1998). Our present scheme, however, differs from theirs in that duplications of an ancestral GnRH gene to give rise to three evolutionary arms occurred prior to the emergence of cartilaginous fish, and that cfGnRH was derived from mGnRH progenitor by point mutation.

Several lines of evidence support our evolutionary scheme. First, in addition to the African cichlid, gilthead seabream, and cartilaginous fish, there are several species in which three GnRH forms were detected, and each of the three forms is within each distinct evolutionary arm. Most species of amphibians and one species of eutherian mammals were

also reported to have three GnRH forms: mGnRH, cGnRH-II, and a novel form with properties similar to sGnRH (see King and Millar, 1997; Montaner *et al.*, 1998). Although sGnRH has been isolated and sequenced only from several teleost species so far, sGnRH-like form may not yet be found in other vertebrates. It could be universal that there are three distinct GnRH forms in a single species through vertebrates. Second, the branching order within each arm matches the evolutionary branching order of the species. Third, the GnRH forms on the distinct evolutionary arms show distinct localization, multiple GnRH forms on the first arm are expressed in the hypothalamus, cGnRH-II in the midbrain, and sGnRH in the telencephalon.

In conclusion, two distinct cDNAs for the mGnRH and cGnRH-II precursors of the eel were isolated and characterized. We provided the direct evidence for the presence of these two GnRH precursor mRNAs in the eel. The eel mGnRH precursor gene is expressed in all tissues examined in the present study, suggesting unknown physiological role(s) for mGnRH in addition to the well-documented role on the pituitary GTH synthesis and release. We also suggested the existence of three GnRH evolutionary arms; multiple GnRH forms (mGnRH, gpGnRH, cGnRH-I, sbGnRH, and cfGnRH) on the first, cGnRH-II on the second, and sGnRH on the third arm. This indicates that mGnRH progenitor has undergone sequence divergence to give rise to sbGnRH and cfGnRH, but not to sGnRH.

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