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Five Different Types of Putative GnRH Receptor Gene are Expressed in the Brain of Masu Salmon (*Oncorhynchus masou*)

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ABSTRACT—Recent studies have shown that there are multiple genes encoding gonadotropin-releasing hormone receptor (GnRH-R) in single species. In salmonids, however, only a single gene has been identified in the rainbow trout. We therefore isolated partial cDNAs from the brain and the pituitary of masu salmon *Oncorhynchus masou* by reverse transcription-polymerase chain reaction and 5'-rapid amplification of cDNA ends, using primers corresponding to conserved transmembrane domains (TMs). Five different partial cDNAs were isolated from an individual and termed as msGnRH-R1, R2, R3, R4 and R5. They are divided into two groups, msGnRH-R1, R2, R3 and msGnRH-R4, R5. Two groups share 59-71% nucleotide sequence identities. Phylogenetic analysis showed that the former group is closely related to the goldfish GnRH-R GfA, and the latter to GfB. All five msGnRH-R genes were expressed in the brain and msGnRH-R1, R3 and R5 were expressed in the pituitary. In addition, we found mRNA for msGnRH-R1 in the kidney and ovary, and R2 in the ovary, whereas msGnRH-R5 gene was widely expressed in the muscle, heart, kidney and testis. Differences in the expression of msGnRH-R genes between maturing and spawning fish were observed in the brain and pituitary, except for the constantly expressed msGnRH-R5. A splicing variant of msGnRH-R1 mRNA that is capable of generating a truncated GnRH-R that consists of 5TMs was also expressed in the brain, pituitary and kidney. These results indicate that five different types of putative GnRH-R gene are present and expressed in the brain of masu salmon.

Key words: GnRH receptor, salmon, brain, pituitary, gene expression

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) plays a central role in the reproductive system of vertebrates. At least two forms of GnRH coexist in the brain of most vertebrate species. One GnRH form, hypothalamic GnRH, stimulates gonadotropin (GTH) synthesis and release from the pituitary and regulates gonadal maturation. Second GnRH, chicken GnRH-II (cGnRH-II), is highly conserved among all classes of vertebrates and shown to influence sexual behavior in some vertebrate species (Millar, 2003). In most teleosts, three molecular forms of GnRH have been identified; species-specific hypophysiotropic GnRH in the ventral telencephalon and preoptic area, cGnRH-II in the midbrain, and neuromodulatory salmon GnRH (sGnRH) in the olfactory neurons and terminal nerve (Dubois *et al*., 2002). In salmonids, only two forms of GnRH, sGnRH and cGnRH-II, have been identified in the brain, except for a primitive salmonid, lake whitefish, in which three distinct GnRH molecules were recently identified (Adams *et al*., 2002). It is well established in salmonids that sGnRH regulates gonadal maturation through stimulation of GTH synthesis and release, whereas cGnRH-II does not participate in gonadal maturation and is thought to function as a neuromodulator in the brain (Amano *et al*., 1997).

The presence of multiple forms of GnRH implies the existence of multiple cognate receptor types. Indeed, two types of GnRH receptor (GnRH-R) have been characterized in the goldfish (Illing *et al*., 1999), medaka (Okubo *et al*., 2001) and African catfish (Tensen *et al*., 1997; Bogerd *et al*., 2002) and three types of GnRH-R in the bullfrog (Wang *et al*., 2001a). Moreover, a second gene encoding mammalian GnRH-R was identified from Primates (Millar *et al*., 2001). This type II receptor is highly selective for cGnRH-II, while formerly identified type I receptor shows high affinity for

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Note: The nucleotide sequences of msGnRH-R1, R2, R3, R4 and R5 reported in this paper have been deposited in DDBJ database with the respective accession numbers AB107910, AB107911, AB107912, AB107913 and AB107914.

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mammalian GnRH (mGnRH). Although the two types of mammalian GnRH-R have distinct ligand selectivity, multiple GnRH-Rs of lower vertebrates show the same order of affinities for the natural forms of GnRH (cGnRH-II > hypothalamic GnRH and/or sGnRH). It is therefore difficult to discriminate the multiple types of nonmammalian GnRH-R in terms of ligand at present.

The multiple types of GnRH-R are expressed in differential spatiotemporal patterns. In the bullfrog, mRNA of one type of three GnRH-Rs was restricted to the pituitary, and those of the other two GnRH-Rs were detected only in the forebrain and hindbrain. Also, differences in the temporal expression for the three GnRH-R mRNAs were observed (Wang *et al*., 2001a). In teleosts, only one type of two catfish GnRH-Rs (cfGnRH-Rs) is expressed in the ovary (Bogerd *et al*., 2002), and two goldfish GnRH-Rs show different distributions in the brain and ovary (Illing *et al*., 1999). Differences in tissue distribution for the multiple types of GnRH-R suggest their different roles in GnRH action, although precise functions of multiple types of GnRH-R remain to be elucidated.

In salmonids, only a single type of GnRH-R has been identified from the brain of rainbow trout (rtGnRH-R) (Madigou *et al*., 2000). Recently, a second mRNA isoform (mRNA-2) generated by alternative promoter usage and splicing was characterized (Madigou *et al*., 2002). These two GnRH-R isoforms show distinct tissue distributions, and were differently distributed in the testis and ovary during gametogenesis. However, it is still unknown whether multiple types of GnRH-R genes exist in salmonids. In the present study, we therefore isolated putative cDNAs encoding GnRH-R in the brain and the pituitary of masu salmon (*Oncorhynchus masou*) by reverse transcription-polymerase chain reaction (RT-PCR) and 5'-rapid amplification of cDNA ends (5'-RACE), using degenerate primers designed from conserved transmembrane domains (TMs). Here, we demonstrate that five distinct putative GnRH-R genes are present in masu salmon and they were expressed in different spatiotemporal patterns.

MATERIALS AND METHODS

Amplification of partial cDNAs by RT-PCR

Total RNA was extracted from an individual pituitary of maturing female masu salmon (body weight 146 g, fork length 24.8 cm, gonadosomatic index 0.82%) by the guanidium thiocyanate hot phenol-chloroform method (Chirgwin *et al*., 1979). A reverse transcription was performed on 5 μg of total RNA using an oligo(dT) primer (Amersham Biosciences Corp., NJ, USA) and SuperScript II RNase H^- reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. These cDNAs were served as templates for PCR amplification with degenerate primers, GnRf1, 5'-ATGACCTTYRTGGTGATGCC-3' and GnRr1, 5'-AGGTAGTAGGGCGTCCAGCA-3', corresponding to conserved sequences in the TM2 and TM6 of teleost GnRH-Rs (see Fig. 1). A PCR mixture (10 μl) contained 1x Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, primers (1 μM each) and 0.53 units Expand High Fidelity Enzyme (Roche Applied Science, Mannheim, Germany). PCR condition was: denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 15 sec, 55°C for 30 sec, and 72°C for 1 min, and finally by additional 7 min at 72°C. PCR fragments of expected size (approximately 570 bp) were amplified and purified by a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and cloned into a pCR-Script Amp SK(+) cloning vector (STRATAGENE, CA, USA). Nucleotide sequence was determined by the dideoxy chain-termination method (Sanger *et al*., 1977), using a SQ-5500 DNA sequencer (Hitachi, Tokyo, Japan). Multiple cDNA clones containing an insert of the expected size were subjected to DNA sequence analysis.

5'-rapid amplification of cDNA ends (5'-RACE)

Total RNA was extracted from the brain containing the telencephalon and hypothalamus of the same individual used in the RT-PCR. The total RNA was reverse transcribed and 5'-RACE was performed using SMART™ RACE cDNA Amplification Kit (CLONTECH Laboratories, Inc., CA, USA) according to the manufacturer's instruction. A gene-specific primer, 5'-GnR1, 5'-CTGACCACCACCAGGATGAAGGC-3', was designed on the basis of four different partial sequences obtained by RT-PCR (Fig. 1). 5'-RACE condition was: denaturation at 94°C for 2 min, followed by 5 cycles of 94°C for 5 sec and 72°C for 3 min, 5 cycles of 94°C for 5 sec, 70°C for 10 sec and 72°C for 3 min, and 27 cycles of 94°C for 5 sec, 68°C for 10 sec and 72°C for 3 min, and finally by additional 7 min at 72°C. In addition, to obtain the required number of cDNA clones for determination of the sequences of msGnRH-R4 and R5, we designed the specific primer, 5'-GnR2, 5'-GGTCTGCAGAGGC-

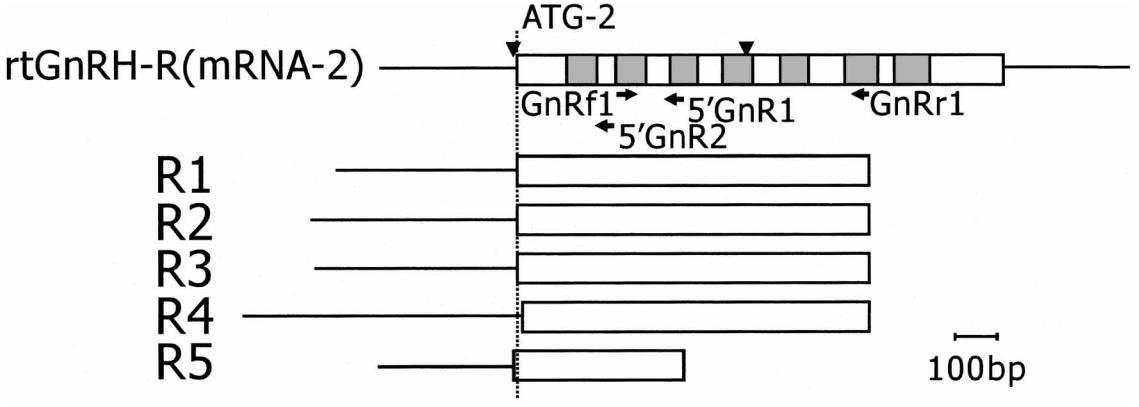


Fig. 1. Schematic representation of msGnRH-R cDNAs compared with rtGnRH-R (mRNA-2). The primers used for RT-PCR and 5'-RACE are indicated by arrows. Filled triangles (▼) indicate positions of introns in the rtGnRH-R. The transmembrane domains are indicated by gray boxes.

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CAGGCTGGCA-3', on the basis of their partial sequences obtained by the first 5'-RACE (Fig. 1). The second RACE-PCR, cloning and sequencing were performed as described above.

Phylogenetic analysis of msGnRH-Rs

The partial amino acid sequences of msGnRH-Rs, spanning from the extracellular N terminal domain to TM3, were aligned with the corresponding sequences of vertebrate GnRH-Rs by GENETYX (Software Development Co., Ltd, Japan). A phylogenetic tree was generated by PHYLIP software (Felsenstein, 1989) using the neighbor-joining method (Saitou and Nei, 1987). The *Drosophila* GnRH-R homolog was used as an outgroup.

Analysis of tissue distribution of msGnRH-R mRNAs by RT-PCR

Total RNAs were extracted from the brain and the pituitary of maturing (April, n=8) and spawning (September, n=3) female masu salmon, and other peripheral tissues including the muscle, ventricle of the heart, gill, liver and kidney and ovary of maturing females, and the testis of maturing males. The total RNAs (200 or 500 ng) were reverse transcribed and used for RT-PCR. Gene-specific primer pairs were designed: msGnRH-R1, 5'-primer, 5'-AAATGGATCATCCGAAATGG-3', 3'-primer, 5'-GGCACACACTGACCAATAAT-3'; msGnRH-R2, 5'-primer, 5'-AGATGGATCAACCGAAACGCTG-3', 3'-primer, 5'-ATCTTACACATGGCGTCCCCG-3'; msGnRH-R3, 5'-primer, same as msGnRH-R2, 3'-primer, 5'-GCATCTTACACATGGCATCT-3'; msGnRH-R4, 5'-primer, 5'-TTGGTCTTTGTCGCAGTGC-3', 3'-primer, 5'-CACTACTGGGAACATTGGAG-3'; msGnRH-R5, 5'-primer, 5'-TGGGAAACTCGACTCTTTGG-3', 3'-primer, 5'-TGCCACGCTAATCAGCACG-3'. PCR mixture (10 μl) for msGnRH-R1, R4 and R5 contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, primers (1 μM each) and 0.25 units TaKaRa Taq™ (TAKARA SHUZO CO., LTD., Shiga, Japan), and that for msGnRH-R2 and R3 contained 1x Expand High Fidelity buffer, 1.25 mM $MgCl_2$ (R2) or 1.5 mM $MgCl_2$ (R3), 0.2 mM dNTP, primers (1 μM each) and 0.53 units Expand High Fidelity Enzyme (Roche). PCR condition was: denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec, 54°C (R1), 69.5°C (R2), 60°C (R3), 63°C (R4) or 56°C (R5) for 30 sec and 72°C for 1 min 15 sec, and finally by additional 7 min at 72°C. Specific amplification of each msGnRH-R mRNA was confirmed by PCR using heterologous combinations of primer sets and template msGnRH-R cDNAs. Amplification of β-actin mRNA was conducted as an internal control using the following primers, 5'-primer, 5'-TGTAACATGGGCAGTATCCTT-3' and 3'-primer, 5'-GATGTCGGAACACATGTGCACT-3'. A PCR mixture (10 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, primers (1 μM each) and 0.25 units TaKaRa Taq™ (TAKARA). PCR condition was: denaturation at 94°C for 1 min, 40 cycles of 94°C for 30 sec, 65°C for 30 sec and 72°C for 2 min, and finally by additional 10 min at 72°C. The PCR products were electrophoresed on 1.5% (msGnRH-R1, R2, R3 and R5), 2.0% (msGnRH-R4) and 2.5% (β-actin) agarose gels, detected by staining with ethidium bromide and visualized by illumination with UV light.

RESULTS

Identification of partial GnRH-R cDNAs

In a preliminary RT-PCR using pooled pituitaries of maturing fish as a source of total RNA, 15 kinds of partial cDNA encoding GnRH-R were obtained. They consist of highly similar 14 cDNA sequences and one with low similarity with others. Some of the differences in the 14 cDNA sequences seemed to be due to individual difference. Therefore, to check its possibility, we performed PCR with genomic DNAs from four individuals as templates. Several genomic sequences were present in each fish, but three major sequences were commonly distributed. We thus decided to obtain GnRH-R cDNAs corresponding to the three genomic sequences from an individual pituitary of maturing female masu salmon. In the result of RT-PCR, three different GnRH-R cDNAs were identified. Subsequently, the 5' cDNA ends of these three and the one obtained from the preliminary RT-PCR and an additional new GnRH-R mRNAs were determined by 5'-RACE from the brain of the same fish.

The five partial cDNAs consisted of 1270, 1332, 1319, 1542 and 727 bp, and were designated as msGnRH-R1, R2, R3, R4 and R5, respectively. The msGnRH-R1, R2, R3 and R4 contain the 5' untranslated regions (5'UTRs) and partial open reading frames (ORFs) encoding the extracellular N terminal domain and TMs1-6. The msGnRH-R5 contains the 5'UTR and a partial ORF encoding the extracellular N terminal domain and TMs1-3 (Fig. 1). Five msGnRH-Rs are divided into two groups, one group includes msGnRH-R1, R2 and R3 and the other includes msGnRH-R4 and R5. The nucleotide sequences of msGnRH-R1, R2 and R3 share 96-99% identities, while those of msGnRH-R4 and R5 have 81% identity (Table 1). The nucleotide sequence identities between the two groups are 59-71%. The former group has the higher sequence identities (96-97%) to the mRNA-2 variant of rtGnRH-R than does the latter group (62-74%).

Table 1. Nucleotide sequence identities (%) among msGnRH-Rs and rtGnRH-R (mRNA-2)

	R1	R2	R3	R4	R5
R2	96				
R3	96	99			
R4	71	70	70		
R5	59	61	61	81	
rtGnRH-R	97	96	96	74	62

The deduced amino acid sequences of msGnRH-Rs

The alignment of the deduced amino acid sequences of five msGnRH-Rs shows that there are many differences in the extracellular N terminal domain between the two groups, although a number of important amino acids for receptor functions are commonly conserved (Fig. 2). Potential glycosylation sites in the extracellular N terminal domain are present in msGnRH-R1, R2 and R3 (Asn^6 and Asn^{27}), msGnRH-R4 (Asn^{23} and Asn^{27}), and msGnRH-R5 (Asn^9, Asn^{23}, and Asn^{27}). Two cysteines in the first and the second extracellular loops, which are necessary for correct folding of the receptor, are conserved in the corresponding position of msGnRH-R1, R2, R3 and R4 (Cys^{121} and Cys^{198}). Likewise, putative ligand contact sites in the cfGnRH-R1 (Asn^{105} and Lys^{124}) (Blomenröhr *et al.*, 2001) are found at the homologous positions (Asn^{109} and Lys^{128}). The residues that affect receptor function and are highly conserved

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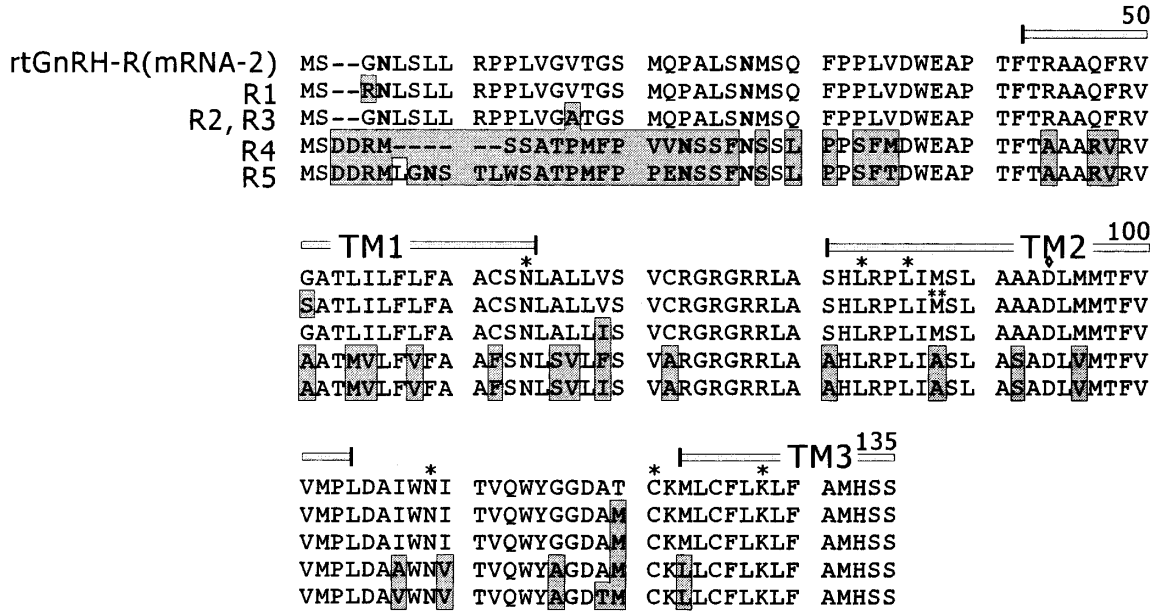


Fig. 2. Alignment of deduced amino acid sequences of msGnRH-Rs and rtGnRH-R. Different residues between msGnRH-Rs and rtGnRH-R are shaded. Lines at the top of the sequences represent putative transmembrane domains. Putative glycosylation site are indicated by bold type. The important amino acids for receptor functions are indicated by single asterisks, and the amino acid conserved in nonmammalian GnRH-R and GPCRs but not in mammalian GnRH-R by an open diamond (◇). Double asterisks indicate the putative translation initiation site of a splicing variant of msGnRH-R1 (R1-v). Numbers of amino acids above the sequence refer to msGnRH-R5.

among G protein-coupled receptors (GPCRs) (Asn^{64}, Leu^{83} and Leu^{86}) are also conserved in msGnRH-Rs. Similar to other nonmammalian GnRH-Rs, an aspartic acid residue in TM2 (Asp^{94}) is conserved in msGnRH-Rs.

Phylogenetic analysis of GnRH-Rs

The phylogenetic tree constructed using the *Drosophila* GnRH-R homolog as an outgroup showed that msGnRH-R1, R2 and R3 are closely related to the goldfish GnRH-R, GfA, and cfGnRH-R2, while msGnRH-R4 and R5 are related to GfB and cfGnRH-R1 (Fig. 3). The tree indicated that vertebrate GnRH-Rs fell into three lineage groups. Mammalian type I receptors solely formed the first lineage group (Group 1). Second lineage included the mammalian type II receptors and GnRH-Rs of *Typhlonectes natans*, bullfrog (GnRHR-1 and -3), *Xenopus* (GnRH-R II), medaka (GnRH-R1), yellow tail, striped bass and European seabass (Group 2). Third lineage included the msGnRH-Rs in addition to lower vertebrate GnRH-Rs of chicken, *Xenopus* (GnRH-R I) and bullfrog (GnRHR-2) (Group 3). Therefore, the fish GnRH-Rs fell into three lineage subgroups (Group 2, 3a and 3b) (Fig. 3).

Truncated mRNA variants

In the 5'-RACE, we identified variant forms of msGnRH-R1 (R1-v) and R4 (R4-v). The msGnRH-R1-v lacks 183 bp between nucleotides 430 and 612 of msGnRH-R1, which corresponding to a region from the 5'UTR to TM1 (Fig. 4). Since the nucleotide 430 corresponds to the first nucleotide of the exon 2 of rtGnRH-R (Madigou *et al.*, 2002), and the nucleotides 611 and 612 are AG, that is, splicing acceptor site, its deletion most probably occurs by alternative splicing of intron I of the msGnRH-R1 gene. The putative translation initiation site of msGnRH-R1-v corresponds to the Met^{88} of msGnRH-R1. Hydrophobicity analysis of the deduced amino acid sequence shows that the msGnRH-R1-v encodes a truncated protein consisting of 5TMs from TM3 to TM7 (Fig. 4). On the other hand, the msGnRH-R4-v lacks 358 bp between 356 and 713 of msGnRH-R4 corresponding to the 5'UTR, therefore the deduced amino acid sequence of its product is identical to that of msGnRH-R4.

Tissue distribution of msGnRH-R mRNAs

Tissue distribution of msGnRH-R mRNAs was examined by RT-PCR with specific primer sets (Fig. 5). For a preliminary estimation of reproductive stage-dependent variation in expression of msGnRH-R genes, the brain and the pituitary total RNAs from maturing (April) and spawning (September) female masu salmon were used as templates in the RT-PCR. All types of msGnRH-R mRNAs were detected in the brain, and msGnRH-R1, R3 and R5 mRNAs were detected in the pituitary. Differences in the temporal expression of msGnRH-R genes between maturing and spawning fish were observed, except for the constantly expressed msGnRH-R5. The msGnRH-R2, R3 and R4 genes were expressed exclusively in the brain of maturing fish. The msGnRH-R1 and R3 mRNAs were detected only in the pituitary of maturing fish. Only msGnRH-R5 mRNA was found in the pituitary of spawning fish. In other peripheral tissues, the msGnRH-R1 mRNA was found in the kidney and ovary, and the msGnRH-R2 mRNA in the ovary, whereas the msGnRH-R5 mRNA was widely distributed in

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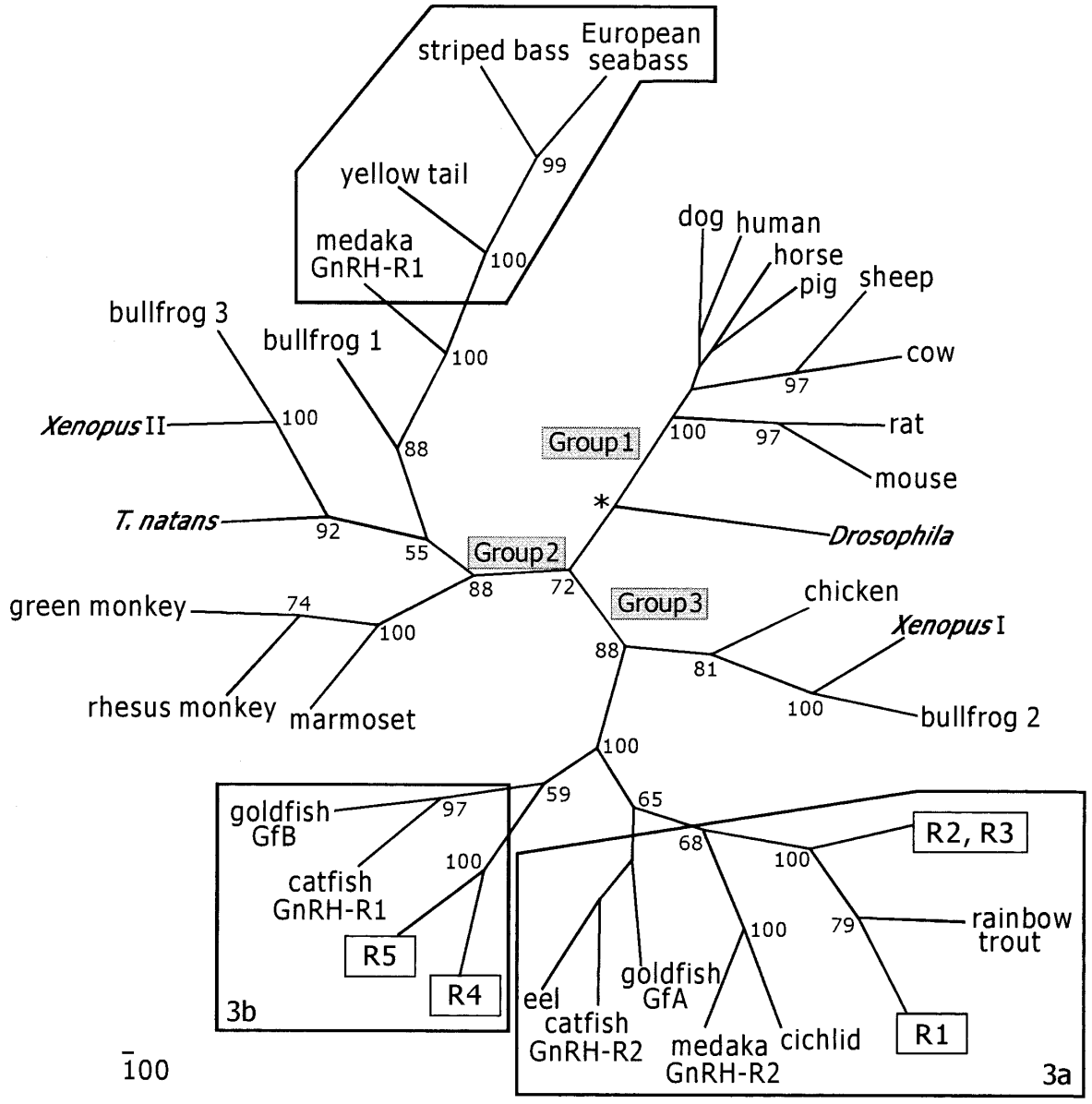


Fig. 3. A phylogenetic tree generated by the neighbor-joining method. One thousand bootstrap replicates were performed and expressed as percentages for the nodes on the tree. The *Drosophila* GnRH-R homolog is used as an outgroup and an asterisk indicates the root of this tree. The scale bar corresponds to estimated evolutionary distance units. Full species names and GenBank accession numbers are as follows: rainbow trout *Oncorhynchus mykiss*, AJ272116; cichlid *Astatotilapia burtoni*, AY028476; medaka *Oryzias latipes* GnRH-R1, AB057675, GnRH-R2, AB057674; goldfish *Carassius auratus* GfA, AF121845, GfB, AF121846; catfish *Clarias gariepinus* GnRH-R1, X97497, GnRH-R2, AF329894; eel *Auguilla japonica*, AB041327; European seabass *Dicentrarchus labrax*, DLA419594; yellow tail *Seriola dumerilii*, AJ130876; bullfrog *Rana catesbeiana* GnRHR-1, AF144063, GnRHR-2, AF153913, GnRHR-3, AF144062; *Xenopus laevis* type I, AF172330, type II, AF257320; *Typhlonectes natans*, AF174481; green monkey *Cercopithecus aethiops*, AF353988; rhesus monkey *Macaca mulatta*, AF353987; marmoset *Callithrix jacchus*, AF368286; chicken *Gallus gallus*, AJ304414; dog *Canis familiaris*, AF206513; human *Homo sapiens*, S60587; horse *Equus caballus*, AF018072; pig *Sus scrofa*, AH009128; sheep *Ovis aries*, X72088; cow *Bos taurus*, U00934; rat *Rattus sp.*, S59525; mouse *Mus musculus*, L01119; *Drosophila melanogaster*, AF077299.

the muscle, heart, kidney and testis. Moreover, the msGnRH-R1-v gene was expressed weakly in the brain and pituitary, strongly in the kidney of maturing fish. The msGnRH-R4-v mRNA was also found in the brain and ovary of maturing fish. In the gill and liver, any types of msGnRH-R mRNA were not detected.

DISCUSSION

We have demonstrated that five different putative GnRH-R mRNAs are present in masu salmon, and they are distributed in the brain and other different tissues. Furthermore, differences in the expression of msGnRH-R genes between maturing and spawning fish were observed in the

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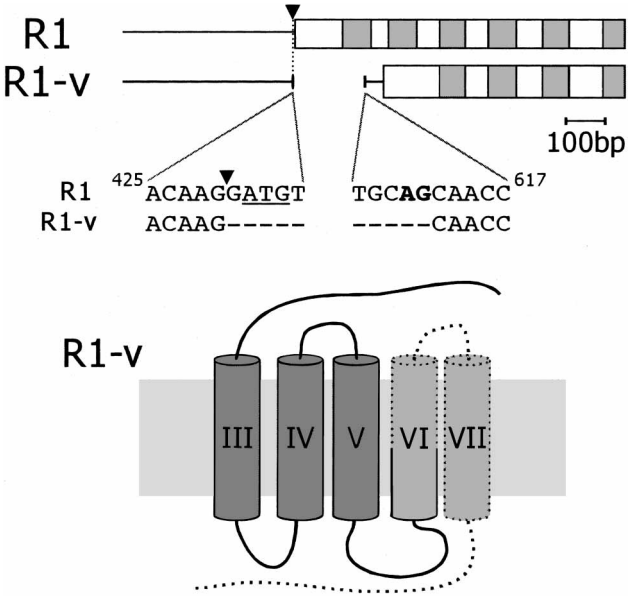


Fig. 4. Schematic representation of msGnRH-R1 and R1-v (upper). The transmembrane domains (TMs) are indicated by gray boxes, and the putative position of first intron of msGnRH-R1 by filled triangle (▼) and dotted line. msGnRH-R1-v lacks a part of 5'UTR and ORF encoding the N terminal domain and TM1. Dashes indicate sequence gaps introduced to maximize sequence homology. Translation initiation site of msGnRH-R1 is underlined and splicing acceptor site is denoted by bold type. Numbers of nucleotides above the sequence refer to msGnRH-R1. Putative two-dimensional model of msGnRH-R1-v (lower). msGnRH-R1-v may consist of 5TMs from TM3 to TM7. The putative region is indicated by dotted line. The extracellular N terminal domains of msGnRH-R1 and R1-v are quite different.

brain and the pituitary. Also noted is that there are variants of msGnRH-R1 and R4 mRNAs, and the former is capable of generating a truncated GnRH-R that consists of 5TMs. This study is the first to show that more than three GnRH-R genes are differentially expressed in a single species.

Five different msGnRH-Rs are grouped into two types, msGnRH-R1, R2, R3 and msGnRH-R4, R5. Two groups share 59-71% identities in their nucleotide sequences. The phylogenetic analysis indicated that they are closely related to the two types of goldfish and catfish GnRH-Rs, which also share 60-70% nucleotide sequence identities. msGnRH-R1, R2 and R3 are related to the GfA and cfGnRH-R2, and msGnRH-R4 and R5 are related to the GfB and cfGnRH-R1 (Fig. 3). Since these three fish species are ancient tetraploid teleosts (Ohno *et al*., 1968; Allendorf and Thorgaard 1984), the two groups may arise from a genomic duplication. This notion is supported by the evidence that only GnRH-Rs of the tetraploid teleosts belong to the latter group (Group 3b in Fig. 3). The former group includes msGnRH-R1, R2 and R3, which are closely related to the rtGnRH-R. Although these msGnRH-Rs share quite high sequence identities with each other (96-99%), they are not derived from individual or allelic difference but from three distinct genes, because that all three msGnRH-R genes were isolated from four individuals in the preliminary experiment. Genomic Southern blot analysis using specific oligonucleotides (msGnRH-R1, 5'-CAACCTCGGAGACGGACAAGGATTGG-3'; msGnRH-R2, 5'-AGTGGTATGGCGGGGACGCCATGTGT-3'; msGnRH-R3, 5'-CAGTGGTATGGCGGAGATGCCATGTG-3') as probes suggested that these are single copy genes (data not shown). Moreover, their different expression patterns (Fig. 5) support that the three msGnRH-R mRNAs are encoded by three distinct genes. The high sequence similarities among them suggest that they arose from gene duplications at evolutionarily late stages. Taken together, the present results indicate that five distinct GnRH-R genes are present in masu salmon.

Five putative msGnRH-R genes were expressed in different tissues. In the rainbow trout, mRNA-2 isoform of the rtGnRH-R mRNA was detected in the brain, pituitary, retina, ovary, and testis, but not in the liver (Madigou *et al*., 2002). The msGnRH-R1, which is the most related to the rtGnRH-R, showed similar expression pattern: its mRNA was detected in the brain, pituitary and ovary, but not in the testis and liver. In the testis of rainbow trout, the level of rtGnRH-R mRNA-1 was low at the early spermatogenic stages and increased with spermatogenesis, whereas that of mRNA-2 appeared constant throughout spermatogenesis (Madigou *et al*., 2002). Since the primers used in the RT-PCR are specific to msGnRH-R1 mRNA corresponding to the mRNA-2, the expression of msGnRH-R1 gene was expected in the testis. However, only msGnRH-R5 mRNA was detected in the testis in the present study. This might be due to species difference in the testicular expression of the two mRNA isoforms of msGnRH-R1 gene. Nevertheless, we are not sure of the presence of its mRNA-1 isoform in masu salmon. We could not obtain any cDNAs corresponding to the mRNA-1 in the 5'-RACE, suggesting that mRNA-1 is minor or null species of msGnRH-R1 mRNA in the masu salmon. It should be of interest and importance to determine if the two mRNA isoforms of msGnRH-R1 gene exist and their testicular expression in masu salmon.

The tissue distribution of msGnRH-R mRNAs was significantly different from each other. In particular, msGnRH-R5 gene was widely expressed in the peripheral tissues such as muscle, heart, kidney and testis, whereas others were expressed only in the kidney (msGnRH-R1 and R1-v) and ovary (msGnRH-R1, R2 and R4-v). Similar differential expression of two types of GnRH-R has been reported in the goldfish and catfish. In the goldfish, both GfA and GfB were expressed in the brain and pituitary, but only GfA was expressed in the ventral telencephalon, ovary and liver (Illing *et al*., 1999). In the catfish, cfGnRH-R2 mRNA was most abundantly expressed in the brain, whereas cfGnRH-R1 mRNA was predominantly expressed in the pituitary (Bogerd *et al*., 2002). Only cfGnRH-R2 was expressed in the ovary. Therefore, two features common between GfA and cfGnRH-R2, which are related to msGnRH-R1, R2 and R3, are specific expression in the ovary and relatively wide tissue distribution. In masu salmon, however, both two groups of msGnRH-R are expressed in the ovary. Moreover,

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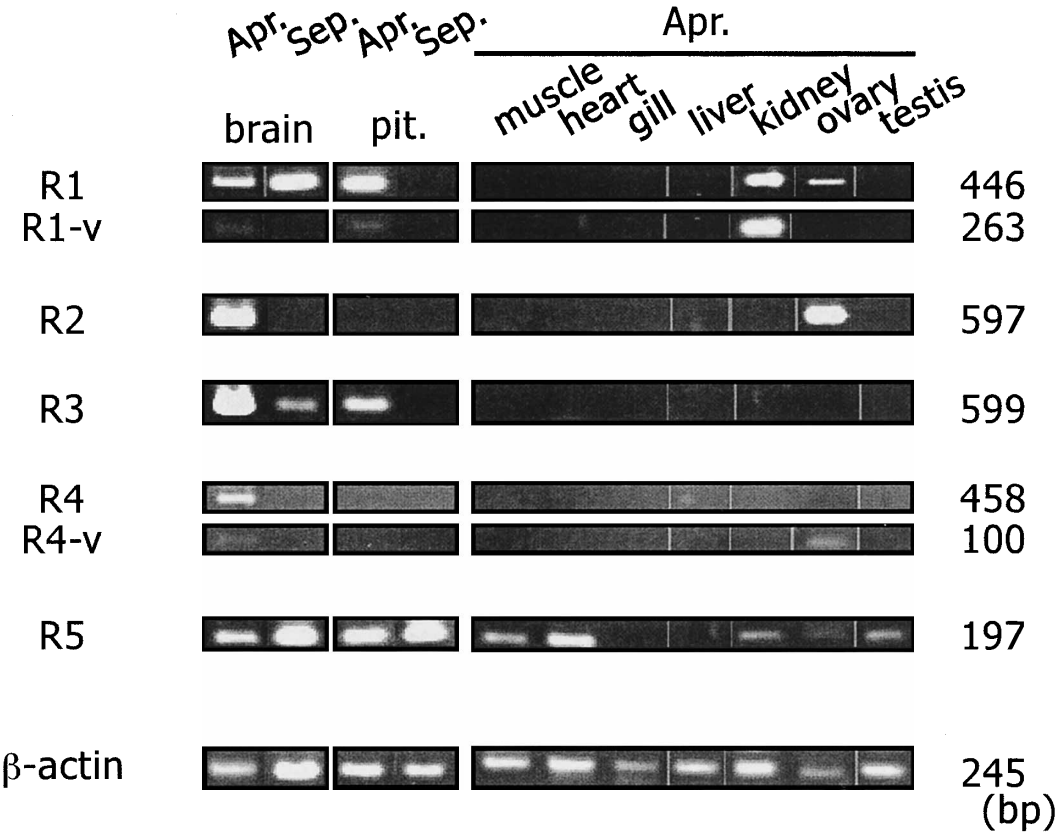


Fig. 5. Analysis of gene expression of msGnRH-Rs by RT-PCR in various tissues of masu salmon. Total RNAs were prepared from the brain, pituitary and other peripheral tissues in different seasons in April (maturing fish) and September (spawning fish). β-actin was used as an internal control.

msGnRH-R5 corresponding to the other group of goldfish and catfish GnRH-Rs showed wide distribution as described above. It is therefore considered that specific tissue distribution of multiple GnRH-Rs is not solely related to the phylogenetic relationship. Considering that even the highly conserved msGnRH-R2 and R3 showed different expression patterns, regulatory regions involved in the tissue specific expression of GnRH-R genes may diversify more rapidly than their coding regions do.

The msGnRH-R genes were expressed with different temporal patterns in the brain and/or the pituitary of maturing and spawning fish, except for the widely expressed msGnRH-R5. The msGnRH-R2, R3 and R4 genes were expressed exclusively in the brain of maturing fish, and the msGnRH-R1 and R3 mRNAs were detected only in the pituitary of maturing fish. Accordingly, the msGnRH-R5 mRNA was the only species of msGnRH-R mRNA expressed in the pituitary of the spawning fish. Relatively low expression of msGnRH-R genes in the spawning fish suggest that GnRH is less or restrictively functional after completion of final sexual maturation. Although further studies are necessary to depict their spatial and temporal expression in detail, the present results indicate that multiple msGnRH-R genes are expressed in different spatiotemporal patterns during sexual maturation, suggesting that they exert different roles in GnRH action. Moreover, these results are consistent with the multiple functions of GnRH in the brain and pituitary of pre-spawning salmonids, that is, stimulation of reproductive behavior in the brain and stimulation of gonadotropes in the pituitary (Urano *et al*., 1999). The significant differences in temporal expression of the msGnRH-R genes suggest that GnRH plays different roles in maturing and spawning masu salmon.

A crucial point in addressing the function of msGnRH-Rs is ligand-receptor relationships. It is becoming more obvious that any types of GnRH-R of lower vertebrates show high affinity for cGnRH-II together with similar or lower affinities for other native GnRHs. However, relatively different selectivity is shown by multiple types of GnRH-R in a single species. For example, the GfA showed a greater preference of cGnRH-II than GfB (Illing *et al*., 1999). In the catfish, cfGnRH-R1 had a higher affinity than cfGnRH-R2 for cGnRH-II and cfGnRH, but no difference was observed with respect to inositol phosphate and cAMP signaling properties (Bogerd *et al*., 2002). In the masu salmon, physiological ligand in the pituitary is thought to be sGnRH, because cGnRH-II was not detected in the pituitary (Amano *et al*., 1997). It is thus conceivable that msGnRH-R1, R3 and R5, which were expressed in the pituitary, show high affinity for sGnRH. Furthermore, all of msGnRH-Rs are expected to

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bind cGnRH-II like other nonmammalian GnRH-Rs. Although their structural properties need to be determined, it is possible that they bind both sGnRH and cGnRH-II, and their physiological ligand in the brain may depend on spatio-temporal distribution of the two ligands and five receptors.

We previously showed that expression of sGnRH genes was significantly changed in the brain of masu salmon during their growth and sexual maturation, and the levels of sGnRH mRNAs were already high in the prepubertal stages. The levels declined in the pre-spawning stages, followed by an increase in the spawning period (Ando *et al*., 2001). These changes correspond well with that of sGnRH level in the brain (Amano *et al*., 1992, 1993). In contrast, the sGnRH level in the pituitary gradually increased with sexual maturation and reached a maximum in the spawning season. On the other hand, the concentrations of cGnRH-II in the brain were much lower than those of sGnRH (approximately one tenth of sGnRH), and did not show any significant changes in relation to maturation (Amano *et al*., 1992, 1993). Therefore, msGnRH-Rs expressed in the brain of maturing and spawning fish may respond to relatively high levels of sGnRH. However, physiological significance of msGnRH-R gene expression in the pituitary of maturing fish with relatively low levels of sGnRH is not unknown at present.

The putative splicing variant of msGnRH-R1 can generate distinct extracellular N terminal domain and 5TMs. Similar truncated 5TM GnRH-R of human type II receptor has been proposed (Neill, 2002). Moreover, chemokine receptors with only 5TMs from TM3 to TM7, due to a genetic deletion, appear to act as functional receptors in the aspects of receptor expression, signaling, internalization, and desensitization (Ling *et al*., 1999). The msGnRH-R1-v was expressed weakly in the brain and pituitary, strongly in the kidney of maturing fish, suggesting that the msGnRH-R1-v is a functional receptor. Since it was co-expressed with the intact receptor in these tissues, it may interact with them as reported in the truncated GnRH-Rs corresponding to the N terminus and TM1 to TM5 in the bullfrog (Wang *et al*., 2001b) and human (Grosse *et al*., 1997).

Phylogenetic analysis clustered fish GnRH-Rs into three subtypes of lineage. The first type group (Group 2) contains the GnRH-Rs of medaka (GnRH-R1), striped bass, yellow tail and European seabass. The second type (Group 3a) includes the msGnRH-R1, R2, R3, rtGnRH-R, goldfish GfA, cfGnRH-R2, medaka GnRH-R2, eel GnRH-R and cichlid GnRH-R. The third type (Group 3b) contains the msGnRH-R4, R5, goldfish GfB and cfGnRH-R1. The Group 3b may correspond to GnRH-Rs of only tetraploid fish. Accordingly, the Groups 2 and 3a seem to be two major types of fish GnRH-Rs. It is of considerable interest to determine if the Group 2 type of GnRH-R exists in salmonids, like medaka.

In conclusion, the present study demonstrates that five different putative GnRH-R genes are present in masu salmon and they are differentially expressed in the brain, pituitary and other peripheral tissues. We propose that they have different functions in the brain and the pituitary during sexual maturation. The five partial msGnRH-R mRNA sequences obtained in the present study enable us to further examine their specific spatial expression by *in situ* hybridization and also temporal expression by quantitative real-time PCR. The information on their different expression and also structural properties will provide us a new insight into physiological significance of multiple GnRH-Rs in vertebrates.

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