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Three forms of gonadotropin-releasing hormone characterized from brains of one species

(neuropeptides/teleost/reproduction/evolution)

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ABSTRACT **Most vertebrate species have more than one form of gonadotropin-releasing hormone (GnRH) in their brains, but it is not clear whether each form has a distinct function. We report that sea bream (*Sparus aurata*) brains have three forms of GnRH, one of which is described herein and is called sea bream GnRH (sbGnRH). The primary structures of two forms were determined by Edman degradation and mass spectral analysis. The amino acid sequence of sbGnRH is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2. The second peptide is identical to a form originally isolated from chicken brains (cGnRH-II): pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2. cGnRH-II is the most ancient form of GnRH identified to date in jawed fish and the most prevalent form throughout the vertebrates. The third form of GnRH has previously been identified as salmon GnRH by cDNA studies and is confirmed here by chromatographic and immunological studies. Phylogenetic distribution of GnRH peptides suggests sbGnRH arose in the perch-like fish as a gene duplication of the existing cGnRH-II or salmon GnRH genes. All three identified GnRH peptides were synthesized and shown to release gonadotropin *in vivo* in the sea bream. The dominant form of GnRH stored in the pituitary was sbGnRH. Not only was the content of sbGnRH 500-fold greater than that of salmon GnRH but also cGnRH-II was not detected in the pituitary. The latter evidence suggests that sbGnRH is the endogenous releaser of gonadotropin II.**

Gonadotropin-releasing hormone (GnRH) is both a releaser of gonadotropins and a neuromodulator (1, 2), but the relationship between structure and function remains unclear. Most vertebrate species have more than one form of GnRH in the brain (see ref. 3). The placental mammals appear to have a single form of GnRH, but even within this group there is an exception, the primitive musk shrew, *Suncus murinus* (4). Two forms of GnRH within a species are characteristic of nonplacental mammals, birds, reptiles, amphibians, and most fishes.

Distinct locations of the two forms of GnRH within the brain of a given species suggest that each form has a different function. In African catfish, *Clarias gariepinus*, evidence from *in situ* hybridization shows that the cell bodies containing one form of GnRH (chicken GnRH-II, cGnRH-II) are in the midbrain, whereas cell bodies with the second type of GnRH (catfish GnRH, cfGnRH) are in the forebrain and infundibular stalk (5). Further, immunohistochemical or chromatographic studies support the conclusion that there is a discrete localization of cell bodies with different GnRH forms in several fish species (5–9).

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Teleost fish make a good model for determining if one or more of the GnRH peptides is the gonadotropin releaser. Known to lack a hypothalamo-pituitary portal system, teleosts have GnRH axons that grow into the pituitary and release GnRH near the gonadotrophs. Hence, the presence of a specific form of GnRH in the pituitary is a good indication of releaser function. In contrast, relative potency of the GnRH forms does not identify the gonadotropin releaser because all endogenous forms of GnRH release gonadotropin if tested in the species of origin (see refs. 10 and 11).

Localization studies to date show that the number of GnRH forms in the pituitary varies with the species. The single form of salmon GnRH (sGnRH) in the salmon pituitary (7, 12) and both sGnRH and cGnRH-II in the goldfish pituitary (9) are potent releasers of pituitary gonadotropins either *in vivo* or *in vitro* (11, 13). When two forms of GnRH are detected in the pituitary, it is not clear if the forms have different functions such as separate release of the follicle-stimulating hormone-like (GTH-I) or luteinizing hormone-like (GTH-II) hormones. An additional question is whether GnRH peptides in axons that terminate outside of the pituitary (2, 14) have more than one neuromodulatory role.

Three forms of GnRH are present in a number of modern teleosts based on HPLC and immunological studies. Examples of fish for which indirect evidence shows three GnRHs in the brain are the winter flounder, *Pseudopleuronectes americanis* (15), and snook, *Centropomus undecimalis* (16).

The hypothesis that three forms of GnRH are present in one species and have discrete locations and functions is testable, but only if the primary structure of each of the three forms is identified and synthesized. We provide evidence that the three forms of GnRH present in the sea bream (*Sparus aurata*) are distinct. To our knowledge, the primary structure of one of these forms has not been previously described for any species. In addition, we show that only one form of GnRH is likely to be the gonadotropin releaser and report that all three native GnRH peptides release gonadotropin in sexually mature sea bream.

MATERIALS AND METHODS

Collection of Brains and Extraction of Peptides. A total of 1.8 kg of intact brains (10,000) and dissected hypothalami (2500) was collected from mature male and female gilthead sea bream during the spawning season (January) at the Israel National Center for Mariculture. The tissues were immedi-

Abbreviations: GnRH, gonadotropin-releasing hormone; sGnRH, salmon GnRH; sbGnRH, sea bream GnRH; cGnRH-II, chicken GnRH-II; irGnRH, immunoreactive GnRH; GTH-II, gonadotropin II; TFA, trifluoroacetic acid; cfGnRH, catfish GnRH; mGnRH, mammalian GnRH; MALDI, matrix-assisted laser desorption/ionization; LSI, liquid secondary ionization.

ately frozen in liquid nitrogen, shipped on dry ice to the University of Victoria, B.C., Canada, and stored at −90°C. Brains were divided into four groups; lots A, B, and C were predominantly brains and some hypothalami, whereas lot D was predominantly hypothalami.

For each lot, the brain tissue was frozen with liquid nitrogen and powdered in a Waring Blendor. Extraction of peptides with acetone and HCl and subsequent removal of substances soluble in petroleum ether were done as described (17). The final aqueous phase for each lot was evaporated in a vacuum centrifuge to a volume of <500 ml. Each extract was pumped onto separate columns of 10 Sep-Pak cartridges in series.

Each Sep-Pak column was connected to a Beckman model 166 HPLC chromatograph. Solvent A was 0.05% trifluoroacetic acid (TFA) in water; solvent B was 80% acetonitrile/19.95% water/0.05% TFA. A gradient was applied at an increasing rate of 1.4% solvent B per min and a flow rate of 1 ml/min. Eluant was collected in 1-ml fractions for 60 min. Aliquots of 10 μl from each fraction were used for radioimmunoassay (RIA) to determine immunoreactive GnRH (ir-GnRH; see below for antisera). Fractions that contained irGnRH in each lot were pooled and reduced in volume in a vacuum centrifuge.

HPLC Analysis. After Sep-Pak HPLC, each lot of extract was applied to a C_{18} column [solution A, 0.1 M heptafluorobutyric acid (HFBA), pH 2.5; solution B, 0.1 M HFBA in 75% acetonitrile (ACN); 5–65% B in 60 min]. Early-eluting irGnRH fractions from all lots were pooled from this step and designated sea bream GnRH-I (sbGnRH-I). Further purification of sbGnRH-I involved application in sequence to a C_{18} column [solution A, 0.13 triethylammonium formate (TEAF), pH 2.5; solution B, ACN; 5–60% B in 55 min], another C_{18} column [solution A, 0.1 M triethylammonium phosphate (TEAP), pH 6.5; solution B, ACN; 5–20% B in 10 min, 20–40% B in 40 min], and a phenyl column (solution A, 0.05% TFA, pH 2.0; solution B, 0.05% TFA in 80% ACN; 5–20% B in 10 min, 20–40% B in 40 min).

Late-eluting irGnRH fractions for all lots from the HFBA step above were combined, reduced in volume, and applied to a C_{18} column (solution A, 0.25 TEAF; solution B, ACN; 17–24% B in 7 min, 24% B for 50 min). The isocratic portion of this program separates sGnRH from other irGnRH forms (16). The early- and late-eluting irGnRH fractions from this step were designated sbGnRH-II and sbGnRH-III, respectively. Further purification of sbGnRH-II and -III followed the protocol used for the last two steps of sbGnRH-I purification.

RIA Measurement. Aliquots of 10 μl from fractions collected at each successive step in the purification were assayed for irGnRH by methods previously described (18). Heterologous assays were used with mammalian (mGnRH) as the labeled hormone and standards. Antiserum GF-4 was used in a final dilution of 1:5000 resulting in 26–37% binding of ^{125}I-labeled mGnRH. Antiserum GF-4 recognizes six of the eight known forms of GnRH (17, 19, 20). Lamprey GnRH-I is not recognized by GF-4, whereas lamprey GnRH-III has not been tested. Limits of detection (B/B_0 = 80%) averaged 7.2 pg. Serial dilutions were done if fractions of 10 μl had values of irGnRH that exceeded B/B_0 = 20%. Antiserum R-42 does not recognize forms of GnRH that are altered at the NH_2 and COOH termini (19, 21). R-42 was, therefore, used after the last step in the purification to verify that the peptide was intact. A dilution of 1:100,000 of R-42 had a binding of 31% and a limit of detection of 2.8 pg.

Characterization of the Primary Structure. An aliquot (10% by volume) of the peptides purified by HPLC on a phenyl column was subjected to a narrow-bore C_{18} column employing 0.05% TFA and acetonitrile for elution. Fractions were collected and analyzed with a Bruker reflex matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometer. When accurate mass measurements were required, these were carried out with a Jeol HX100 mass spectrometer operating in a liquid secondary ionization (LSI) mode using an accelerated voltage/electric field scan as described elsewhere (17). The residual 90% of each sample was evaporated to dryness and subjected to pyroglutamate aminopeptidase treatment as described (22). This was followed by HPLC separation and sequence analysis on an ABI protein sequencer (model 470A).

Peptide Synthesis. Solid-phase synthesis of sbGnRH was carried out on a methylbenzhydrylamine resin (Boc strategy) using previously established methods (23) and the following protecting groups: pyro-Glu(carbobenzoxy), Boc-His(tosyl), Boc-Ser(benzyl), and Boc-Tyr(2-bromocarbobenzoxy). sbGnRH was deprotected and cleaved from the solid support with hydrofluoric acid. After purification with reverse-phase HPLC (>97% pure) in two solvent systems (24), the structure was confirmed by mass spectral and amino acid composition analyses.

HPLC Analysis of Pituitary Extract. Sea bream pituitaries (100, 0.75 g total) were collected during the spawning season from 2-year-old females with preovulatory oocytes (500–600 μm o.d.). The pituitaries were extracted for peptides as described above. The extract was applied to a separate C_{18} HPLC column and eluted using an isocratic method (TEAF, as above). Reference peptides of sbGnRH, cGnRH-II, and sGnRH (200 ng each) were applied to the HPLC column after the pituitary extract and the procedure was repeated. Fractions (1 ml) were collected separately for each HPLC application and assayed for irGnRH. A blank (800 μl of solvent A injected) HPLC was done prior to that of the pituitary extract. irGnRH was not detected in 500 μl of each blank fraction.

RESULTS

Initial HPLC Analysis. Assay of HPLC fractions from the four lots that eluted from the Sep-Pak columns showed irGnRH activity in fractions 21–41 (Fig. 1*A*). Fractions that contained <15 ng per fraction of irGnRH activity were not used in subsequent steps.

sbGnRH-I Purification. Each HPLC run in the HFBA step showed an early-eluting peak at 46 min (Fig. 1*B*). These irGnRH fractions (called sbGnRH-I) from the four lots were pooled and shown to contain a total of 153 ng of irGnRH. A large proportion (110 ng) of the combined irGnRH was derived from lot D containing predominantly hypothalamic tissue.

In the TEAF step of the purification, 297 ng of irGnRH was detected in fractions 33 and 34, with <10 ng of activity in the next 4 fractions. Fractions 33 and 34 were combined for the TEAP step of the procedure. Three fractions (28–30) were found to have a total of 220 ng of irGnRH and were further purified. At the last step of the purification, fractions 23 and 24 contained 232 ng of irGnRH. Fraction 23 (179 ng of irGnRH) was selected for digestion with pyroglutamyl aminopeptidase and protein sequencing. RIA using antiserum R-42 confirmed the presence of intact irGnRH in these fractions but detected only 82 ng in fraction 23 and 120 ng in combined fractions 23 and 24.

Edman degradation of the pyroglutamyl aminopeptidase-treated fraction of sbGnRH-I yielded the sequence: His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly. The molecule ion mass of the nontreated sbGnRH-I fraction was m/z 1113.6 (LSI). The calculated monoisotopic mass (MH^+) for pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2 (elemental composition $C_{52}H_{69}N_{14}O_{14}$) is 1113.51 Da.

sbGnRH-II and -III Purification. Late-eluting fractions (>50 min) from the HFBA step of the procedure were

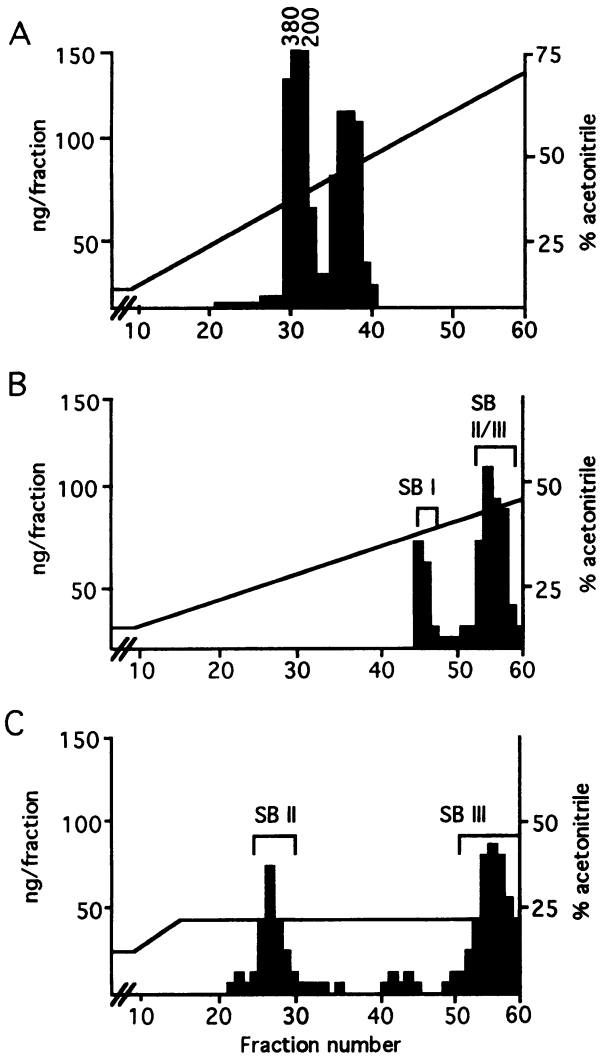

FIG. 1. Purification of GnRH from sea bream brains. Percent acetonitrile is shown by a solid line. (*A*) irGnRH was eluted from Sep-Pak HPLC using TFA in the mobile phase from lot B brains. (*B*) irGnRH from lot B brains was eluted from a C_{18} column using HFBA in the mobile phase. (*C*) Purification of SB-II and SB-III using a C_{18} column with the isocratic TEAF HPLC method. Samples were from late-eluting fractions in all lots of brains after the HFBA step.

assumed to contain two forms of irGnRH as indicated by our preliminary studies in which extracts were compared to synthetic GnRH standards. Likewise, in the present study, irGnRH eluted as two areas in the TEAF step (Fig. 1*C*). The earlier-eluting peak was designated sbGnRH-II and was applied to the remaining steps of purification. The later-eluting peak was designated sbGnRH-III and was separately applied to the remaining steps of purification.

Identification of sbGnRH-II. Purification of sbGnRH-II and subsequent protein sequencing and mass spectrometry showed that sbGnRH-II is identical in sequence and mass with cGnRH-II. The amino acid sequence of the pyroglutamyl aminopeptidase-treated sbGnRH-II was determined to be His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly. The molecule ion mass of this peptide was m/z 1236.6 (MALDI). The calculated monoisotopic mass (MH^+) for pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (elemental composition $C_{60}H_{70}N_{17}O_{13}$) is 1236.53 Da. These data confirm that sbGnRH-II is identical in structure to the previously described cGnRH-II (25).

Identification of sbGnRH-III. sbGnRH-III shares its elution position with synthetic sGnRH (16). The late elution position of sbGnRH-III in the TEAF step (Fig. 1*C*), is consistent with sGnRH in other species (16). Likewise, sbGnRH-III maintained a hydrophobic elution position throughout purification but was not present in sufficient quantity to sequence. During the identification of GnRH forms in the pituitary, irGnRH eluted in the same position with the sGnRH standard (see below). In addition, the cDNA for sGnRH from sea bream has been determined (Y.Z., unpublished data).

Identification of Pituitary GnRH. The dominant form of GnRH eluted at the same time as the sbGnRH standard (Fig. 2). A total of 166 ng of irGnRH (antiserum GF-4) was detected in fractions 23–24. Only 0.33 ng of irGnRH was detected in fractions 46–47 corresponding to the elution position of sGnRH. irGnRH eluting with the cGnRH-II standard was not detected, although antiserum cross-reactivity of GF-4 with cGnRH-II is 4%.

DISCUSSION

Sea bream brains contain three distinct forms of GnRH, one of which has a previously unidentified structure, whereas the other two are known forms, cGnRH-II and sGnRH. To our knowledge, three forms of GnRH have not previously been established for one species. Two forms are identified here by primary structure and the third form is identified by chromatographic evidence that supports the deduced structure obtained from cDNA (Y.Z., unpublished data). The early-eluting form (Fig. 1*B*) is an amidated decapeptide with the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH_2, hereafter referred to as sbGnRH. The primary structure of the second form of GnRH (Fig. 1*C*) in seabream brain is identical to cGnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2). The third, late-eluting form of GnRH is chromatographically characterized as sGnRH by elution position throughout the purification process (Fig. 1*C*), by comparison with the synthetic standard (Fig. 2) and by identification of the sGnRH cDNA in other studies. Proof of the structural identity of sbGnRH and cGnRH-II included Edman degradation to determine the sequence of the GnRH-(2–10) fragment. The NH_2-terminal pyroglutamic acid was identified by specific cleavage with pyroglutamyl aminopeptidase for all purified peptides. The presence of an amidated COOH terminus was established by mass spectrometry for sbGnRH and cGnRH-II.

The identification of the primary structure of sbGnRH increases the known structures in the GnRH family to nine forms (Fig. 3). All of these forms are decapeptides that have conserved amino acids in positions 1, 2, 4, 9, and 10. The

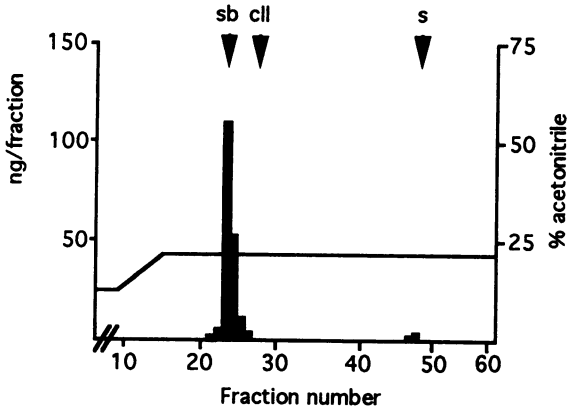

FIG. 2. HPLC analysis of GnRH from sea bream pituitaries. Percent acetonitrile is shown by the solid line. irGnRH was eluted from a C_{18} column using an isocratic method with TEAF and acetonitrile as mobile phase. The elution positions of synthetic standards are indicated by arrowheads (sb = sbGnRH, cII = cGnRH-II, s = sGnRH).

	1	2	3	4	5	6	7	8	9	10
SEA BREAM	pGLU	HIS	TRP	SER	TYR	GLY	LEU	SER	PRO	GLY-NH2
MAMMAL	pGLU	HIS	TRP	SER	TYR	GLY	LEU	ARG	PRO	GLY-NH2
CHICKEN-I	pGLU	HIS	TRP	SER	TYR	GLY	LEU	GLN	PRO	GLY-NH2
CATFISH	pGLU	HIS	TRP	SER	HIS	GLY	LEU	ASN	PRO	GLY-NH2
SALMON	pGLU	HIS	TRP	SER	TYR	GLY	TRP	LEU	PRO	GLY-NH2
CHICKEN-II	pGLU	HIS	TRP	SER	HIS	GLY	TRP	TYR	PRO	GLY-NH2
DOGFISH	pGLU	HIS	TRP	SER	HIS	GLY	TRP	LEU	PRO	GLY-NH2
LAMPREY-III	pGLU	HIS	TRP	SER	HIS	ASP	TRP	LYS	PRO	GLY-NH2
LAMPREY-I	pGLU	HIS	TYR	SER	LEU	GLU	TRP	LYS	PRO	GLY-NH2

FIG. 3. Amino acid sequence of the identified GnRH peptides. Boxes indicate amino acids that differ from sbGnRH identity.

highest variability is observed at position 8; seven different amino acids are present in position 8. Other positions of variability (3 and 5–7) show fewer amino acid substitutions. Presumably, multiple forms of GnRH arise from nucleotide substitutions in existing GnRH genes. The presence of a serine in position 8 of sbGnRH represents only a single amino acid substitution compared with mGnRH or cGnRH-I.

Reproduction in sea bream is of interest not only because individual fish spawn daily over a period of several months but also because this species undergoes sexual inversion. In captivity, female sea bream mature sexually but do not undergo final ovarian maturation, ovulation, and spawning; this is thought to be due to low GTH-II release. Exogenous GnRH causes these fish to ovulate (26). To address the question of which of the three forms of GnRH releases the gonadotropins, pituitaries were examined for irGnRH content. Clearly, sbGnRH was the dominant form (500-fold) of GnRH in the axons terminating in the pituitary. This provides strong evidence that this form of GnRH is the releaser of GTH-II during the peri-ovulatory period. The similarity between mammals and fish is striking as only a single form of GnRH apparently releases the gonadotropin necessary for ovulation. This explains the earlier observation (27) that sGnRH antiserum in sea bream did not cross-react with cell bodies in the preoptic nucleus, the origin of the axons that terminate in the pituitary. Rather, the antiserum against mGnRH (only one amino acid different from sbGnRH) cross-reacted with the preoptic cells.

The identification of three forms of GnRH within the brain of one species raises the possibility that each form has a distinct function and is related to a specific phase of the reproductive cycle. Injection of each of the native GnRH peptides (5 or 25 μg/kg of body weight) into mature female sea bream resulted in elevated plasma GTH-II levels. However, the order of potency for the peptides at 1.5 and 4 hr after injection was cGnRH-II > sGnRH > sbGnRH. The ratio of potency at both doses at 1.5 hr was 7:2:1 (Y.Z. and A.E., data not shown). These data clearly demonstrate that each form of GnRH from sea bream brains elicits a significant increase in the release of GTH-II from the pituitary gland. However, this does not explain why sbGnRH, the dominant form of GnRH in the pituitaries of mature sea bream, is less potent than sGnRH or cGnRH-II in inducing GTH-II release. This may indicate that sbGnRH is degraded faster than the two other native GnRHs by specific peptidases present in the pituitary, kidney, and liver of the female sea bream (28, 29). Another possibility is that sbGnRH, the most recently evolved form, may use one of the existing receptors that have higher affinities for sGnRH and cGnRH-II, the established forms of GnRH in the more primitive teleosts. Similarly, in the catfish, *Clarias gariepinus*, the more recently evolved catfish GnRH is less potent than cGnRH-II for induction of GTH-II secretion and has a lesser binding affinity to the pituitary GnRH receptor (30).

Evidence presented in this paper shows that the newly described sbGnRH form is present in perciform fishes, a group of fish that originated ≈100 million years ago. However, a review of the immunological and chromatographic data shows that a third form of GnRH, which may be identical with sbGnRH, is also present in other perciform fishes, including snook (16), mullet, *Mugil cephalus* (ref. 31; Fig. 4), and cichlid, *Haplochromis burtoni* (unpublished data). In all cases, a hydrophilic irGnRH elutes in a similar position to that of the purified sbGnRH. A third form of GnRH was also indirectly identified in orders that are closely related to perciformes: pleuronectiformes (flounder; ref. 15) and gasterosteiformes (stickleback; ref. 32). It is probable that the third hydrophilic form in these fish is sbGnRH.

An additional three orders of fish that evolved earlier than the perch-like fish also have three forms of GnRH that have different elution profiles from the HPLC (Fig. 4). The fish in these orders are gadiformes (tomcod, *Microgadus proximus*;

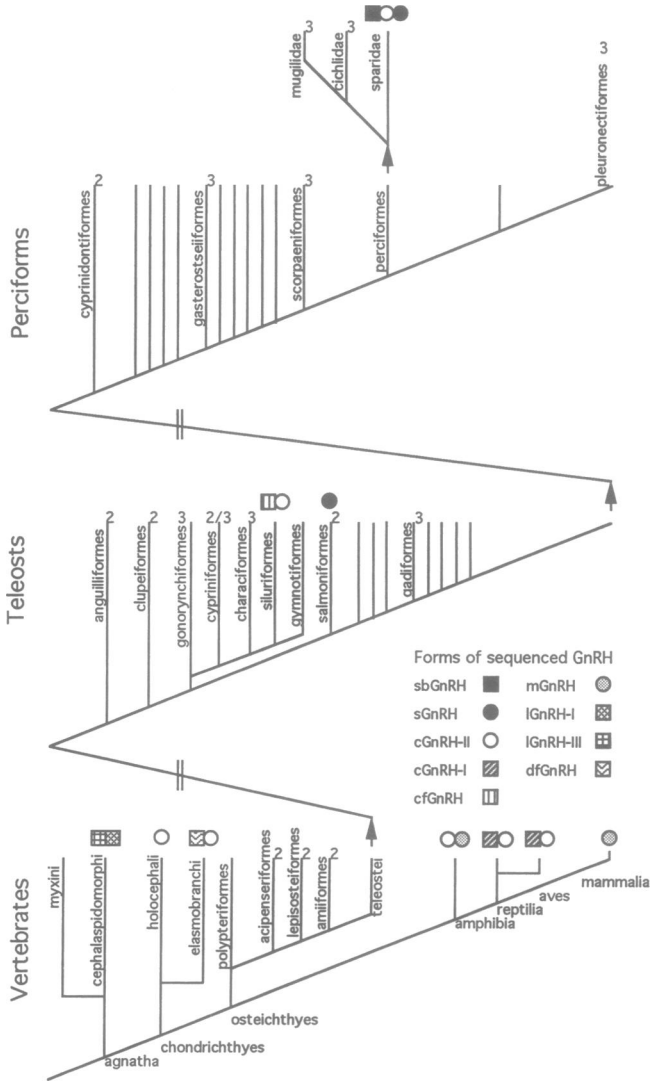

FIG. 4. Phylogenetic distribution of the forms of GnRH among the vertebrates. Number of GnRH forms in representatives of a given order is shown by a number. These data are based on chromatographic and immunological data. Where a sequence of the peptide has been determined, a symbol represents the form(s) present. In orders of fishes where forms of GnRH have not been determined, the vertical bar is not identified. dfGnRH, dogfish GnRH; l-GnRH, lamprey GnRH.

ref. 33), characiformes (sabalo, *Prochilodus lineatus*; ref. 38), and gonorynchiformes (milkfish, *Chanos chanos*; ref. 31). In all of the fishes listed above, the three forms of GnRH in the brain follow a similar pattern to that of the sea bream. Indirect evidence shows that the first form is a hydrophilic form of irGnRH that elutes in a similar position compared to sbGnRH. The second form is cGnRH-II, and the third form is sGnRH. Although the first form of GnRH in fish listed above is similar to sbGnRH based on chromatographic and immunological evidence, it is unresolved whether all fish with three forms of GnRH have sbGnRH. Only the sequence of the peptide or cDNA in each species will establish the identity of the first form. It is certainly possible that these forms have an identity distinct from sbGnRH, especially in the more phylogenetically ancient fish.

Multiple forms of a peptide could have arisen from multiple copies of genes as a result of genome duplication, unequal sister chromatid exchange, duplication resulting in a redundant DNA sequence, or homologue crossover during meiosis. After each of these events, it is thought that one copy of the gene is conserved, whereas the other copy that is not required for normal function diverges. Eventually, two distinct gene products may be produced.

Genome duplication has occurred in some fish species such as the salmon and sturgeon (see ref. 34). The haploid number of chromosomes, however, is 24 for sea bream (Y.Z., unpublished data). This makes it unlikely that sbGnRH resulted from a genome duplication in sea bream.

Unlike genome duplication, exon duplication would result in a tandem organization of two GnRH coding regions within one gene. The cDNA for sbGnRH has not been reported, but two distinct forms of GnRH are not encoded in one precursor based on the cDNAs determined to date: sGnRH in sea bream, mGnRH (35), sGnRH (36), cfGnRH, and cGnRH-II (5) and chicken GnRH-I (37) in other species. The lack of evidence for polyploidy or exon duplication in the GnRH gene of the sea bream opens the possibility that unequal crossover or unequal transfer of genetic material may have occurred.

The distribution of GnRH among the teleosteans (Fig. 4) suggests that sbGnRH must have arisen from sGnRH or cGnRH-II. cGnRH-II is present in all teleosts examined (3). In contrast, sGnRH appears to have arisen from mGnRH in species that evolved near the origin of the teleosts; the sGnRH continued to be expressed in all teleosts that subsequently evolved with the exception of some catfish, in which cfGnRH replaces sGnRH.

It is more likely that sbGnRH arose from a modification of sGnRH rather than cGnRH-II. Evidence to support this hypothesis is that sbGnRH differs from sGnRH by two amino acid substitutions but differs from cGnRH-II by three. Also, the minimum number of nucleotide substitutions that would be required for the change from sGnRH to sbGnRH is two, whereas the number of substitutions from cGnRH-II to sbGnRH is three.

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