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Gonadotropin-Releasing Hormone (GnRH) in Ancient Teleosts, the Bonytongue Fishes: Putative Origin of Salmon GnRH

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The molecular forms of gonadotropin-releasing hormone (GnRH) were examined in the bonytongue fishes (Osteoglossomorpha), one of the most ancient living teleost groups. These fish represent a phylogenetic link between the early ray-finned fishes and the modern teleosts. Five representative species from four of six bonytongue families were examined for GnRH using high-performance liquid chromatography and radioimmunoassay techniques with antisera raised against salmon (s), chicken-II (c-II), and mammalian (m) forms of GnRH. Salmon GnRH and cGnRH-II were identified in four of the species (arawana, elephantnose, false featherfin, Asiatic featherfin) whereas in the butterfly fish, mGnRH and cGnRH-II were identified. Our data suggest that teleosts such as eels and butterfly fish, which have mGnRH like that of even earlier ray-finned fishes, may have evolved before fish with sGnRH. We also suggest that sGnRH first appeared in the Osteoglossomorpha. The phylogenetic relationship of the eels (Anguillidae), butterfly fish (Pantodontidae), and bonytongue fish among other teleosts needs to be reexamined using additional characteristics.

The first gonadotropin-releasing hormone (GnRH) to be identified was isolated from pig (Matsuo *et al*., 1971) and sheep (Burgus *et al*., 1972). To date, 12 distinct forms of GnRH have been isolated and the peptide sequence has been determined. Ten forms have been identified in vertebrates from jawless fish to humans (Jimenez-Linan *et al*., 1997; Sherwood *et al*., 1994, 1997) and an additional two forms have been isolated and sequenced from a protochordate (Powell *et al*., 1996).

The pattern of GnRH evolution has been elucidated through the screening of the molecular forms of the neuropeptide in many representative species from fishes, amphibians, reptiles, birds, and mammals (King and Millar, 1995; Sherwood *et al*., 1994, 1997). These phylogenetic studies have shown that most species have two or three forms of GnRH in the brain. The 10 forms of GnRH identified to date in the vertebrates are widely distributed, but show a clear pattern in evolution. Five of the 10 forms have been identified and sequenced from bony fish (Osteichthyes): two of these forms also occur in other vertebrates whereas three forms are specific to bony fish. The shared forms, which are named after the animals from which the GnRH form was identified first, are mammalian GnRH (mGnRH) and chicken GnRH-II (cGnRH-II). The specific forms are salmon GnRH (sGnRH), catfish GnRH (cfGnRH), and seabream GnRH (sbGnRH).

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The pattern of GnRH distribution in bony fish is quite simple. All bony fish (ray-finned and lobe-finned groups) tested to date have cGnRH-II present in the brain. This characteristic is shared with cartilaginous fish, amphibians, reptiles, birds, and mammals (King and Millar, 1995; Sherwood *et al.*, 1994, 1997). The second form of GnRH in the brain of some bony fish is mGnRH. This form is expressed in lobe-finned fish (e.g., lungfish, Joss *et al.*, 1994) and the most ancient of the living ray-finned fish, (Crim *et al.*, 1985; Lescheid *et al.*, 1995; Sherwood *et al.*, 1991). The presence of mGnRH is a characteristic shared with amphibians and mammals (Conlon *et al.*, 1993; Lescheid *et al.*, 1995). The ancient bony fishes with mGnRH include the four major groups whose ancestors evolved before those of the teleosts and also include one of the earliest teleosts to evolve, the eels (King *et al.*, 1990). The teleosts that evolved later, the herring group (Clupemorpha, Sherwood, 1986) and euteleosts (Sherwood *et al.*, 1997) do not have the mGnRH form, but have sGnRH instead. The other two specialized forms of GnRH, cfGnRH and sbGnRH, appeared in more recent euteleosts. Thus, GnRH is tightly conserved in vertebrates as might be expected for a molecule that controls reproduction. Therefore, a change in the molecular form of GnRH in vertebrates is a useful characteristic or marker in studies of evolution.

An important group of fish that has not been examined for the presence of GnRH is the bonytongue fishes (Osteoglossomorpha). One of the oldest living groups of the teleosts, the bonytongues emerged during the Jurassic Period (Lauder and Liem, 1983a), possibly on the supercontinent Pangaea. Bonytongues form a link between the ancient bony fish (ray-finned line) and the modern teleosts. The ancient origin of the Osteoglossomorpha has made it difficult to determine the relationship of the bonytongued families to each other and to the teleosts (Lauder and Liem, 1983a; Li and Wilson, 1996; Patterson and Rosen, 1977).

The objective of this study was to determine whether GnRH forms changed with the emergence of these early teleosts. Phylogenetically, the species that occur directly before the teleosts are the ''Chondrostei'' (reedfish and sturgeon), Ginglymodi (gars), and Halecomorphi (*Amia*) (Grande and Bemis, 1996; Nelson, 1994). In the first three species, mGnRH and cGnRH-II were identified (Lescheid *et al.*, 1995; Sherwood *et al.*, 1991); in *Amia* mGnRH was identified, but cGnRH-II was not studied (Crim *et al.*, 1985). However, there are no reports of GnRH in the osteoglossomorphs, yet these are a phylogenetically relevant group of teleosts. In the present study we examine five species from four of six families (Nelson, 1994) for GnRH molecular forms using high-performance liquid chromatography and radioimmunoassay methods. The implications of these findings on the evolution of both GnRH and the ancient teleosts are discussed.

MATERIALS AND METHODS

Collection of Brains and Pituitaries

Butterfly fish (*Pantodon buchholzi* $n = 11$), false featherfin (*Xenomystus nigri* $n = 11$), elephantnose (*Gnathonemus petersii* $n = 10$), Asiatic featherfin (*Chitala chitala* $n = 11$), and arawana (*Osteoglossum bicirrosum*) were purchased from pet shops. Whole brains (0.4 g) from butterfly fish, separated brains (1.2 g) and pituitaries (0.01 g) from false featherfin, and brains combined with pituitaries from elephantnose (1.91 g) and from Asiatic featherfin (1.04 g) were frozen on dry ice. Arawana brains ($n = 11$) and pituitaries ($n = 2$) with a total weight of 1.27 g were removed at the University of Toronto, flash frozen in liquid nitrogen, and shipped on dry ice to the University of Victoria. All samples were stored at −80°C. The age of the fishes was not known. Butterfly fishes and false featherfins were immature, but the reproductive status of the remaining fishes was not determined.

Peptide Extraction

All samples were extracted in the same manner. Butterfly fish, elephantnose, Asiatic featherfin, and arawana brains were extracted with pituitaries. False featherfin brains and pituitaries were extracted separately.

Frozen tissues were powdered in liquid nitrogen with a mortar and pestle precooled on dry ice. Pow-

dered material was extracted in 10 ml of cold acetone/0.1 N HCl mixture (100/3 v/v). The mixture was stirred for 3 h and then vacuum filtered through Whatman No. 1 filter paper. Remaining solids were reextracted in 4–5 ml acetone:0.01 N HCl (4:1 v/v), stirred for 3 min, and filtered again. The two filtrates were combined and lipids were removed by five successive petroleum ether treatments (4:1 v/v, filtrate: ether). The extract was concentrated to 1–3 ml on a speed vacuum concentrator (Savant, Farmingdale, NY) and filtered through a 0.45-μm, nonpyrogenic, low protein binding filter (Costar, Cambridge, MA).

High-Performance Liquid Chromatography

Extracts were fractionated on a Supelco Supelcosil LC-18 high-performance liquid chromatography (HPLC) column (4.6 mm × 25 cm × 5 μm particle diameter) with guard columns. The column was linked to a Beckman solvent module 166 and detector module 125. The column was prewashed with increasing and decreasing gradients of solvents A and B (see below). A blank sample consisting of 800 μl of Milli Q water was injected onto the column before each extract and eluted using the program as for extracts. Sixty fractions were collected and 100–500 μl of each was assayed as described below. The extract in aliquots of 600 μl was injected onto the column in a solvent environment of 83% triethylammonium formate (0.25 M, pH 6.5, solvent A) and 17% acetonitrile (solvent B) in 2-min intervals. After 10 min following the first injection, the solvent mixture was changed to 76% A and 24% B over a 7-min period and held for 43 min. Fractions were collected for 60 min, one fraction per minute at a flow rate of 1 ml/min. Samples of 25–200 μl were taken from each fraction, vacuum dried, and stored at 4°C for radioimmunoassay.

Standards

Following HPLC fractionation of each extract, columns were washed as above. Synthetic GnRH standards were injected on the column and eluted in the same solvent program as the extracts. Synthetic mGnRH, cGnRH-II, and sGnRH (200 ng each) were mixed and injected after elution of the butterfly and false featherfin extracts. Synthetic mGnRH, cGnRH-II, sGnRH, and dogfish GnRH (dfGnRH) (4000 ng each) were injected following elution of the elephantnose and Asiatic featherfin extracts. Synthetic mGnRH, sGnRH, and dfGnRH standards (160 ng each) and synthetic cGnRH-II standard (360 ng) were injected following elution of the arawana extract. Fractions of the standard runs were collected in the same manner as the extracts, and 10-μl aliquots were removed and dried for radioimmunoassay (RIA).

Radioimmunoassay

A competitive radioimmunoassay as described in Sherwood *et al.* (1983) was employed for detecting GnRH immunoreactivity in fractionated extracts. Polyclonal antisera raised in three different rabbits were used to screen extracts for GnRH immunoreactivity. Butterfly fish and false featherfin extracts were screened with antisera GF-4 and B-6 (antisera letter designations refer to the rabbit, tailing number indicates bleed number). Elephantnose and Asiatic featherfin extracts were screened with GF-6 and 7CR-10. Arawana extract was screened with GF-6 and B-7. Mammalian GnRH standard and mGnRH^{125}I trace were used with GF-4, GF-6, B-6, and B-7 at final dilutions of 1:5000 (GF-4, GF-6), 1:1000 (B-6), and 1:2000 (B-7). Chicken GnRH-II standard and cGnRH-II^{125}I trace were used with 7CR-10 at a final dilution of 1:7500. Antisera cross-reactivities are reported by Lescheid *et al.* (1997) and Quanbeck *et al.* (1997). Antisera GF-4 and GF-6 were raised against sGnRH and cross-react with most known forms of GnRH. B-6 and B-7 were raised against mGnRH. B-6 was tested extensively and found to cross-react exclusively with mGnRH. B-7 was the bleed following B-6 and is assumed to have similar cross-reactivity characteristics to those of B-6. Antiserum 7CR-10 was raised against dfGnRH and has a high cross-reactivity for dfGnRH, cGnRH-II, and sGnRH. Previously, cross-reactivity studies with 7CR-10 used a lamprey GnRH^{125}I trace and cGnRH-II standard (Lescheid *et al.*, 1997). In the present study 7CR-10 had an average binding of 11.6% and limit of detection (B/B_0 = 80%) of 5.8 pg when used with the cGnRH-II^{125}I trace and cGnRH-II standard. Average binding activities and detection limits of the remaining antisera were as follows: 22.9% and 6.1 pg (GF-4), 31% and 10.7 pg (GF-6), 76.3% and 3.0 pg (B-6), and 22.9% and 6.1 pg (B-7).

In samples in which tracer binding dropped to 20% and below, serial dilutions at 1:2 were assayed and the concentration of immunoreactive GnRH closest to 50% tracer binding is reported.

RESULTS

Butterfly Fish and False Featherfin

Antibody GF-4 detected immunoreactive material in fractions 19–21 of the butterfly fish extract at a total concentration of 1.4 ng/brain. The elution position corresponds with that of synthetic mGnRH standard, which eluted in fractions 21–22. Immunoreactivity was also detected in fraction 26 (0.03 ng/brain) which corresponds with the elution position of the synthetic cGnRH-II standard (Fig. 1). Antibody B-6, which cross-reacts with only mGnRH, confirmed the presence of mGnRH in fractions 19–21 (0.37 ng/brain) of the butterfly fish brain.

In the false featherfin brain extract, GF-4 revealed immunoreactive material in fractions 25–26 (0.14 ng/brain) and fractions 43–48 (1.08 ng/brain) which eluted in a similar position to that of synthetic cGnRH-II (fractions 26–27) and sGnRH (fraction 47), respectively (Fig. 2). In the false featherfin pituitary extract, immunoreactive material was detected in fraction 26 (0.01 ng/brain) and fractions 44–47 (0.05 ng/brain) by GF-4 (Fig. 2). Shoulder-immunoreactive peaks about posi-

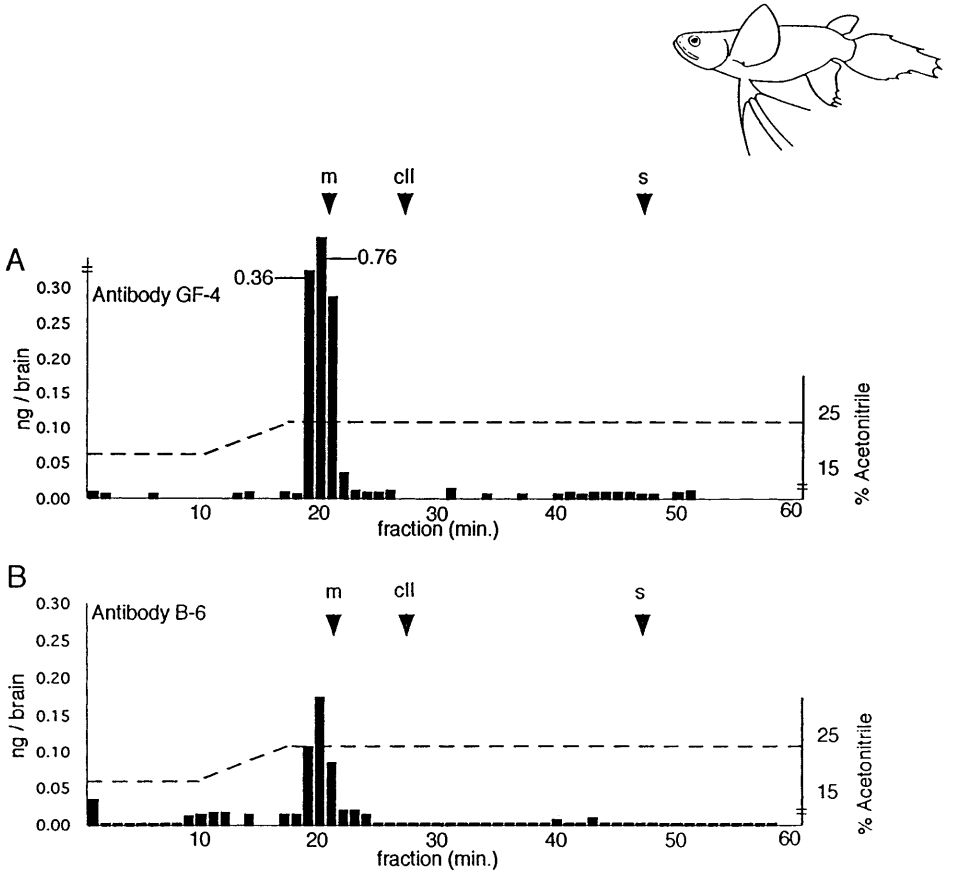

FIG. 1. Immunoreactive GnRH in brain and pituitary extract of butterfly fish (*Pantodon buchholzi*). Chromatograms illustrate HPLC elution position (minutes) and the concentration of immunoreactive GnRH material (ng/brain) detected by specific antisera. Dashed line indicates percentage of solvent B with time. Arrows at the top of the chromatogram indicate the elution position of synthetic GnRH standards. (A) Immunoreactive GnRH detected by antiserum GF-4. (B) Immunoreactive GnRH detected by antiserum B-6 (see text for cross-reactivity characteristics of specific antisera with different forms of GnRH). m, mGnRH; cII, cGnRH-II; s, sGnRH.

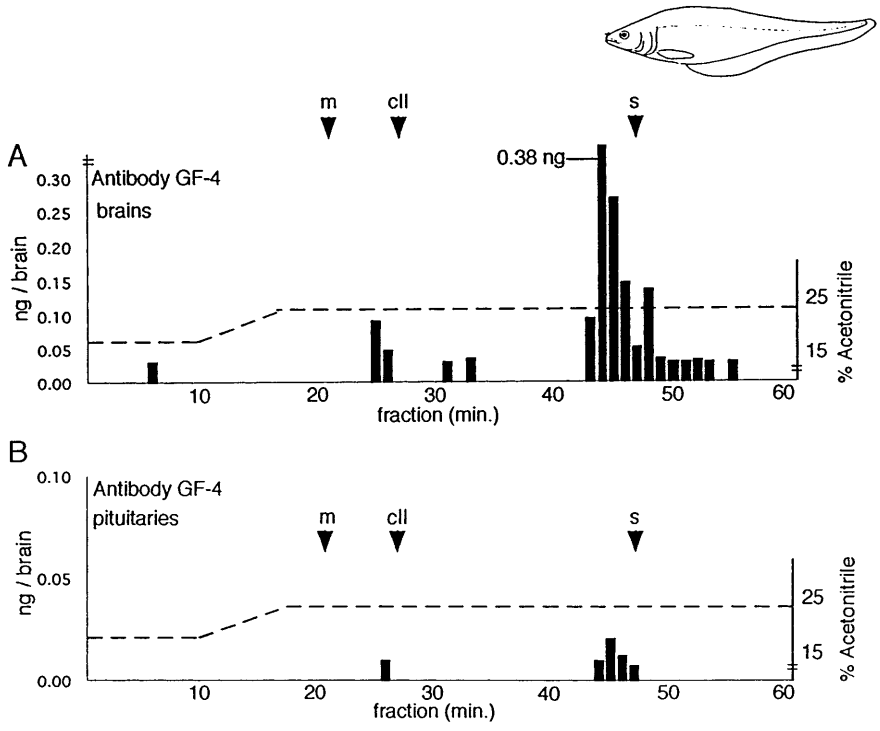

FIG. 2. Immunoreactive GnRH in brain and pituitary extracts of the false featherfin (*Xenomystus nigri*). Chromatograms illustrate HPLC elution position (minutes) and the concentration of immunoreactive GnRH material (ng/brain) detected by antiserum GF-4. Dashed line indicates percentage of solvent B with time. Arrows at the top of the chromatogram indicate the elution position of synthetic GnRH standards. (A) Immunoreactive GnRH detected in brain extract. (B) Immunoreactive GnRH detected in pituitary extract. m, mGnRH; cII, cGnRH-II; s, sGnRH.

tion 47 of the brain extract are probably sGnRH which tends to elute as a broad peak over several fractions. A similar tailing pattern is seen in the pituitary extract. Mammalian GnRH was detected neither by GF-4 in brains and pituitaries of the false featherfin nor by B-6 in the brain. The lack of detection of mGnRH is supported by the fact that mGnRH cross-reacts with GF-4 more than any other known form of GnRH.

Antibody 7CR-10, raised against dogfish GnRH, cross-reacts almost exclusively with dfGnRH (25%), cGnRH-II (100%), and sGnRH (85%) when cross-reactivities are referenced against synthetic cGnRH-II standard. This antibody was not available at the time the butterfly fish and false featherfin extracts were assayed. As a result, the presence of cGnRH-II, which has a low cross-reactivity (4%) with antiserum GF-4 requires further confirmation in these two species.

Elephantnose and Asiatic Featherfin

Immunoreactive material detected by GF-6 in fractions 26–27 (0.03 ng/brain) and fractions 47–49 (0.15 ng/brain) of the elephantnose brain-pituitary extract eluted in the same position as the synthetic cGnRH-II (fraction 26) and sGnRH (fractions 47–48) standards (Fig. 3). The presence of cGnRH-II (0.07 ng/brain) and sGnRH (0.17 ng/brain) was confirmed by antibody 7CR-10. A similar pattern of immunoreactive material was seen in the brain-pituitary extract of the Asiatic featherfin (Fig. 4). Chicken GnRH-II was detected at a concentration of 0.06 ng/brain (fractions 26–27) by 7CR-10. Salmon GnRH was detected at concentrations of 0.44 by GF-6 and 0.06 ng/brain (fractions 47–49) by 7CR-10. GnRH-like immunoreactivity detected by GF-6 in fractions 38–39 of the Asiatic featherfin extract probably indicate sGnRH, because a GnRH-like peptide of the same primary structure and mass of sGnRH

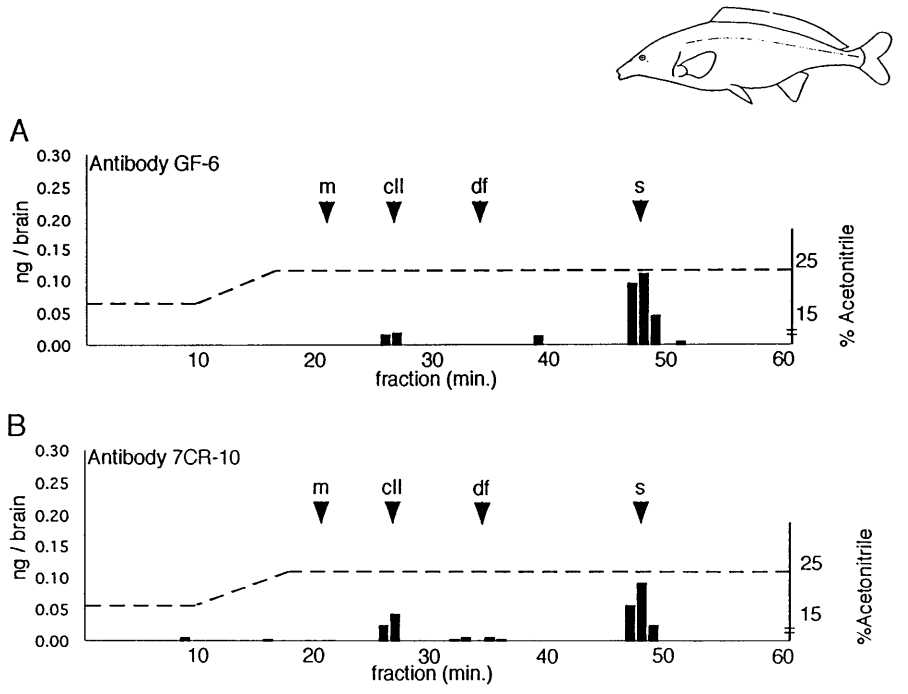

FIG. 3. Immunoreactive GnRH in brain and pituitary extract of the elephantnose (*Gnathonemus petersii*). Chromatograms illustrate HPLC elution position (minutes) and the concentration of immunoreactive GnRH material (ng/brain) detected by specific antisera. Dashed line indicates percentage of solvent B with time. Arrows at the top of the chromatogram indicate the elution position of synthetic GnRH standards. (A) Immunoreactive GnRH detected by antiserum GF-6. (B) Immunoreactive GnRH detected by antiserum 7CR-10 (see text for cross-reactivity characteristics of specific antisera with different forms of GnRH). m, mGnRH; cII, cGnRH-II; s, sGnRH.

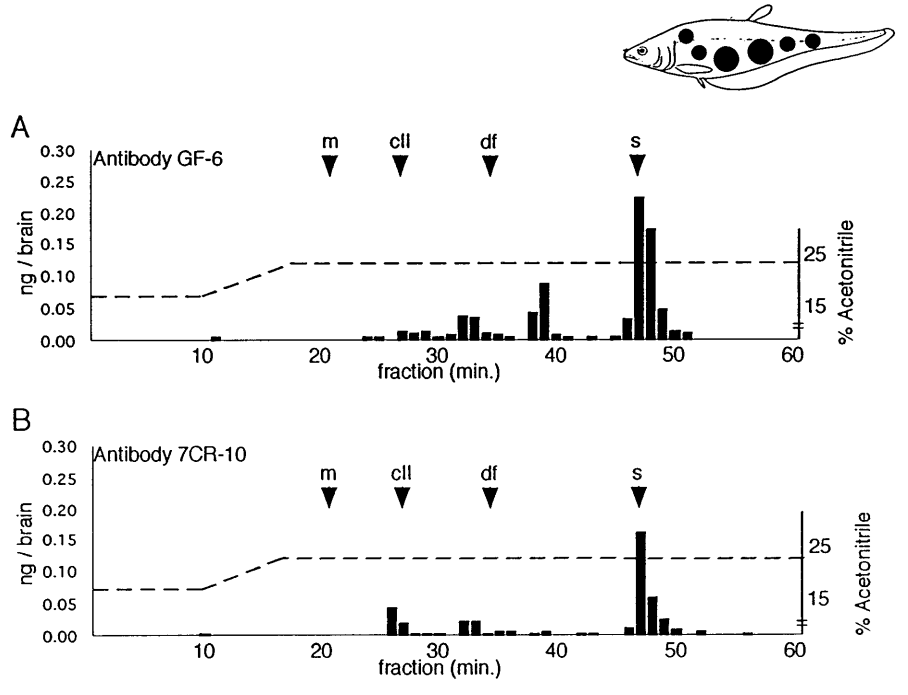

FIG. 4. Immunoreactive GnRH in brain and pituitary extract of the Asiatic featherfin (*Chitala chitala*). Chromatograms illustrate HPLC elution position (minutes) and the concentration of immunoreactive GnRH material (ng/brain) detected by specific antisera. Dashed line indicates percentage of solvent B with time. Arrows at the top of the chromatogram indicate the elution position of synthetic GnRH standards. (A) Immunoreactive GnRH detected by antiserum GF-6. (B) Immunoreactive GnRH detected by antiserum 7CR-10 (see text for cross-reactivity characteristics of specific antisera with different forms of GnRH). m, mGnRH; cII, cGnRH-II; s, sGnRH.

occasionally elutes in this position for unknown reasons (Powell, unpublished data).

Arawana

In the arawana brain-pituitary extract, GnRH-specific immunoreactivity in fractions 26 (0.01 ng/brain) and 46–47 (0.02 ng/brain) were detected by GF-6 (Fig. 5). The peaks had the same elution positions as the synthetic cGnRH-II and sGnRH standards. Antibody B-6 did not detect mGnRH in the arawana fractions.

A Novel Peak

Small immunoreactive peaks were consistently detected in fractions 30–33 by GF-4, GF-6, and 7CR-10 in all species studied. Synthetic dfGnRH standard eluted in fraction 34. This immunoreactivity, however, is unlikely to be dfGnRH because most of this immunoreactive material eluted before, and not in, fraction 34.

DISCUSSION

From evidence based on high-performance liquid chromatography and radioimmunoassay, we report the presence of sGnRH in representative species of three families of bonytongue fishes and mGnRH in the only species of a fourth family. The bonytongue fishes are one of the most ancient extant groups of teleosts and the earliest phylogenetic group of fishes in which sGnRH has been reported.

The presence of mGnRH in the butterfly fish, based on comparison of HPLC elution position to that of a synthetic mGnRH standard, was confirmed by the antiserum B-6. B-6 is a polyclonal antiserum raised

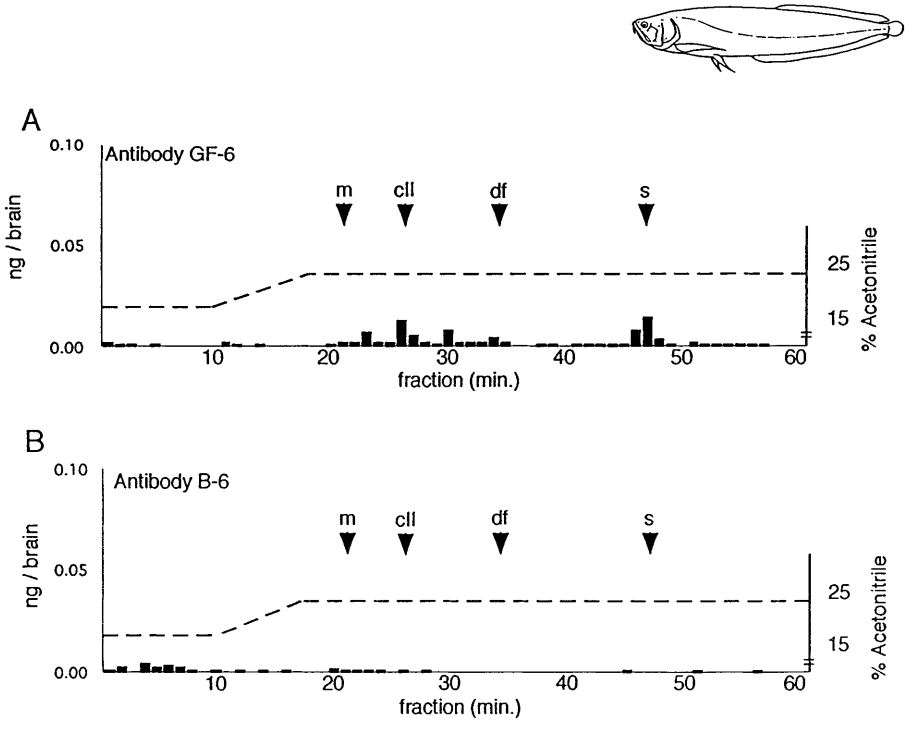

FIG. 5. Immunoreactive GnRH in brain and pituitary extract of arawana (*Osteoglossum bicirrosum*). Chromatograms illustrate HPLC elution position (minutes) and the concentration of immunoreactive GnRH material (ng/brain) detected by specific antisera. Dashed line indicates percentage of solvent B with time. Arrows at the top of the chromatogram indicate the elution position of synthetic GnRH standards. (A) Immunoreactive GnRH detected by antiserum GF-6. (B) Mammalian GnRH was not detected by antiserum B-6. m, mGnRH; cII, cGnRH-II; s, sGnRH.

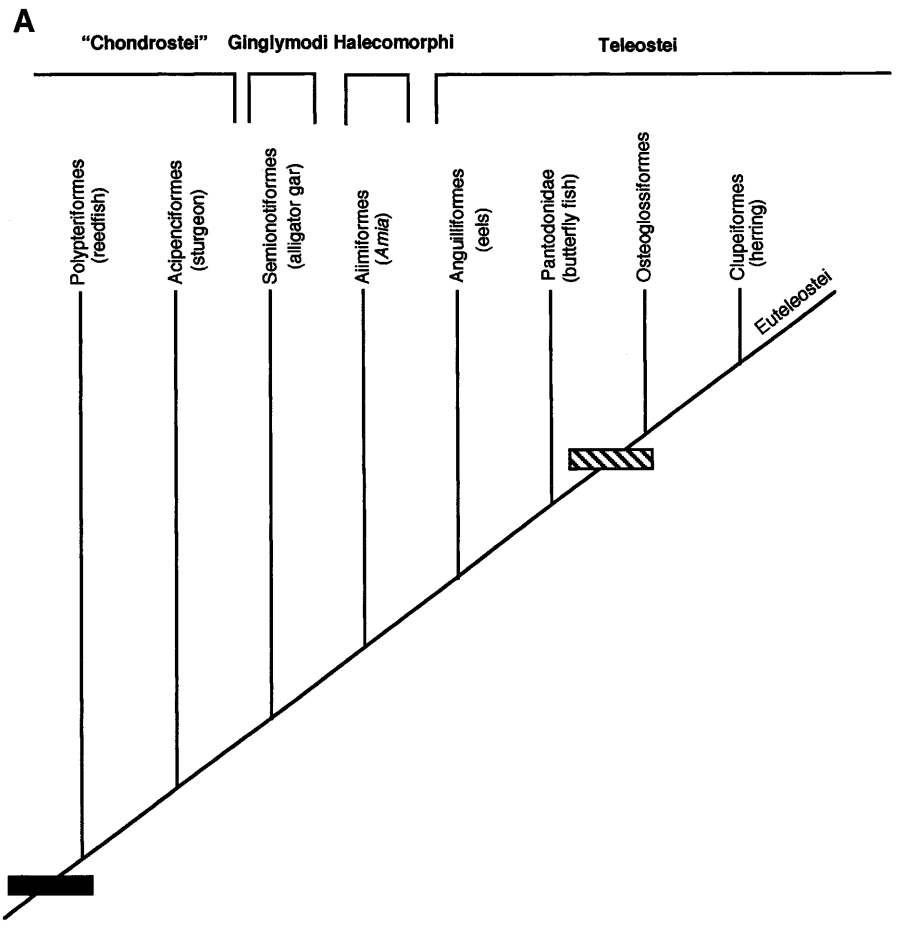

FIG. 6. Hypothetical cladograms depicting the evolution of mammalian and salmon GnRH in bony fish (ray-finned). The black box indicates the proposed emergence of mGnRH, whereas the hatched box indicates the replacement of mGnRH by sGnRH. (A) Cladogram of all bony fish (ray-finned). Mammalian GnRH has been detected in two species of "Chondrostei" (reedfish and sturgeon), one species of Ginglymodi (gar) and one species of Halecomorphi (*Amia*). In addition, two species of basal teleost (eel and butterfly fish) have mGnRH present. In osteoglossomorphs and teleosts, that evolved thereafter, mGnRH is not detected, rather is replaced by sGnRH or later specialized teleost forms of GnRH. Cladogram is based on De Pinna (1996) and Lauder and Liem (1983a), but is modified for eels and butterfly fish to reflect the GnRH data presented here. Use of "Chondrostei" is as described in Grande and Bemis (1996) and Lauder and Liem (1983a). (B) A detailed portion of the cladogram in A showing Osteoglossiformes and Anguilliformes families. Mammalian GnRH was found in the Anguillidae (eels) and Pantodonidae (butterfly fish), but is replaced by sGnRH in the Osteoglossidae (arawana), Notopteridae (featherfins), and Mormyridae (elephantnose) families. GnRH was not studied in the Hiodontidae and Gymnarchidae families as indicated by an asterisk.

against mGnRH and one that cross-reacts with only mGnRH (Lescheid *et al.*, 1997). The presence of cGnRH-II was determined by HPLC elution position. With the HPLC methods described herein, none of the other known forms of GnRH elute at positions 25–27. All immunoreactive peaks in these positions that we have sequenced have been consistently identical to cGnRH-II (Lovejoy *et al.*, 1991, 1992; Ngamvongchon *et al.*, 1992; Powell *et al.*, 1994, 1997; Weber *et al.*, 1997). Salmon GnRH is the most hydrophobic of all known GnRHs and elutes at a position more distant than all other synthetic forms of GnRH.

The presence of sGnRH in the majority of the species examined here indicates that sGnRH likely first appeared in the osteoglossomorphs. This conclusion is based on evidence of the distribution of mGnRH or sGnRH in vertebrates. Figure 6A shows that mGnRH is thought to have first appeared in evolution in early-evolving bony fish (Osteichthyes) based on evidence from two species of "Chondrostei" (reedfish and sturgeon), one species of Ginglymodi (gar), and one species of Halecomorphi (*Amia*) (Crim *et al.*, 1985; Lescheid *et al.*, 1995; Sherwood *et al.*, 1991). In contrast, the salmon form of GnRH, first isolated and sequenced

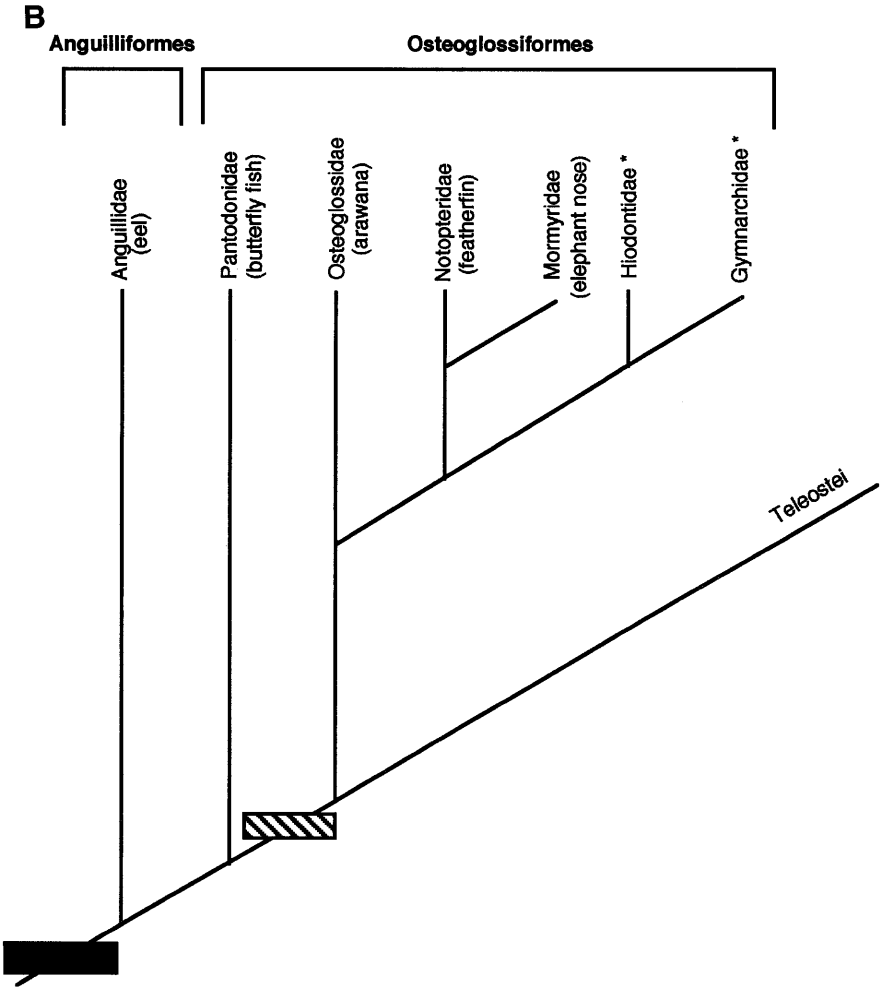

FIG. 6—*Continued*

from chum salmon (Sherwood *et al.*, 1983), was subsequently shown to be distributed in almost all groups of teleosts that evolved after the eels and now in some of the bonytongue fishes. Sherwood *et al.* (1983) hypothesized that sGnRH was derived from mGnRH and indicated that, although the frequency of amino acid substitutions replacing Leu^7-Arg^8 in mGnRH to Trp^7-Leu^8 in sGnRH is very low, such substitutions would require only one base change in each of the two codons.

Until the present study, eels were the only teleosts reported to express mammalian GnRH (King *et al.*, 1990). The eels are a highly specialized group of fish and cladistically have been positioned both after (Lauder and Liem *et al.*, 1983b; Nelson, 1994) and before (DePinna, 1996) the bonytongue fishes. If the eels evolved after the bonytongue fish, the evidence found in the present study suggests one of two things: (1) that sGnRH independently evolved twice from mGnRH in teleosts, first with the earliest teleost fishes (e.g., Osteoglossomorpha) and second with the emergence of the herring (Clupeomorpha) (Sherwood *et al.*, 1994); or (2) a loss of expression of mGnRH following a gene duplication event, but a silent retention of the gene within the genome. The possibility of sGnRH independently emerging twice in evolution from the same mammalian form of GnRH is intriguing, but the probability is low. Such a case in GnRH evolution has never been reported. The second possibility in which the mGnRH gene is not expressed cannot be

confirmed without identifying both the mGnRH and the sGnRH genes. To date, neither the peptide nor cDNA encoding mGnRH have been identified in any teleost classified as evolving after bonytongue fish or eels.

If the eels evolved before the bonytongues fishes, and the butterfly fish was the first bonytongue to appear, then a third and more parsimonious explanation is that substitutions occurred in the mGnRH gene resulting in the sGnRH gene, the event occurring after the eels evolved. Our data support this option (Fig. 6A).

The presence of mGnRH in the butterfly fish also supports the idea that sGnRH first appeared in bonytongue fishes that evolved after the butterfly fish (Fig. 6B). These data also imply that the pantodon family is distinct from the other osteoglossomorph families. The butterfly fish has been classified as the single member of a single genus in the family Pantodonidae (Nelson, 1994). Analysis of anatomical characteristics (Li and Wilson, 1996) and spermatozoal morphology (Jamieson, 1991) has placed the butterfly fish as an outlying member of the bonytongue fishes. An immunohistochemical study on protein hormones in gastroentero–pancreatic tissue confirms that the butterfly fish has a number of distinct characteristics compared to other osteoglossomorphs (Al-Mahrouki and Youson, 1998). The presence of mGnRH in the butterfly fish is consistent with a unique status.

Molecular techniques have been used to analyze interrelationships of teleosts, but produced results inconsistent with anatomical data (see De Pinna, 1996). Kocher and Stepien (1997) also review the use of molecular systematics to show that some techniques have inherent problems, although they can be effective in some cases. Maley and Marshall (1998) argue that the use of proteins in phylogenetic studies has some distinct advantages compared with RNA and DNA. Here we present data in several species concerning a small, highly conserved peptide that controls reproduction. The results suggest that the phylogenetic classification of Anguillidae (eels) and Pantodonidae (butterfly fish) needs to be reexamined using more characteristics. Meanwhile, it is clear that sGnRH likely first appears in evolution in the Osteoglossomorpha, an ancient teleost group.

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