Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1423–1427, February 1994
Neurobiology

A second gene for gonadotropin-releasing hormone: cDNA and expression pattern in the brain

(gonadoliberin/preprohormone/peptide/reproduction/teleost)

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Communicated by Theodore H. Bullock, November 8, 1993

ABSTRACT **In vertebrates, the gonadotropin-releasing hormone (GnRH) decapeptide is secreted from hypothalamic nerve terminals to regulate reproduction via control of synthesis and release of pituitary gonadotropins. Only one GnRH peptide has been found in mammals, with one exception, although numerous other vertebrate species express more than one of the eight known decapeptide forms as shown by immunocytochemical labeling of distinct cell groups in the brain. However, neither the functional nor the evolutionary relationships among these GnRH forms are clear, because only one preprohormone gene sequence from any species has been reported. The most ubiquitous alternative form of GnRH is [His^5,Trp^7,Tyr^8]GnRH (also referred to as chicken-II), which differs from the mammalian sequence at amino acids 5, 7, and 8. This peptide has been shown to have the most potent releasing-hormone activity, although immunocytochemical staining has suggested it is synthesized only in the mesencephalon. Here we report the cloning and expression pattern of the gene for the precursor of this form from the teleost fish *Haplochromis burtoni*. This is the second GnRH-encoding gene to be characterized in this species. The newly discovered preprohormone gene differs from that previously reported in two ways. First, whereas the original gene predicts only a single associated peptide, this one predicts two associated peptides, both of which appear to be unique. Second, the gene for [His^5,Trp^7,Tyr^8]GnRH is expressed in only one cell group in the mesencephalon. In contrast, the previously reported gene is expressed only in the terminal nerve. The striking differences between the preprohormone structure and localization suggest that the genes coding for the two known GnRH forms in *H. burtoni* did not arise from a recent duplication event. Interestingly, neither of the two genes found to date in this species is expressed in cells which project from the hypothalamus to the pituitary, suggesting that yet a third gene coding for GnRH may exist.**

The amino acid sequence of the gonadotropin-releasing hormone (GnRH) family of decapeptides has been remarkably conserved during 500 million years of vertebrate evolution. During this time, eight decapeptide forms have evolved, and immunocytochemistry has shown them to be expressed in vertebrate brains (1–6). The most ubiquitous alternative form of GnRH is [His^5,Trp^7,Tyr^8]GnRH (also referred to as chicken-II), which differs from the mammalian sequence at the fifth, seventh, and eighth amino acids. This peptide has been shown to have the most potent releasing-hormone activity (7), although immunocytochemical staining has localized it only in cell bodies of the mesencephalon (8). The GnRH motif may have served much longer, since yeast α mating factor has been reported to have 80% amino acid sequence homology (9) and demonstrated releasing-factor properties when applied to mammalian pituitary tissue (9). In the eight GnRH forms sequenced to date at the peptide level (refs. 10 and 11; see Fig. 1 for sequence comparisons and nomenclature conventions), 5 of the 10 amino acids are invariant, including the cyclized amino terminus and amidated carboxyl terminus. [His^5,Trp^7,Tyr^8]GnRH is one of the most widespread and ancient forms, represented in all nonmammalian vertebrates analyzed to date. Thus, with immunocytochemistry, certain species of cartilaginous fish, bony fish, reptiles, and birds have been shown to contain [His^5,Trp^7,Tyr^8]GnRH in addition to their unique GnRH isoforms (reviewed in ref. 10). Among these species, the [His^5,Trp^7,Tyr^8]GnRH peptide elicits the greatest release of gonadotropins from dispersed pituitary cells *in vitro* (23–26) and is the most effective form in stimulating ovulation when administered *in vivo* (7). This potency is surprising, since [His^5,Trp^7,Tyr^8]GnRH is not the predominant GnRH isoform expressed in cells that project to the pituitary but, rather, is typically found in midbrain neuronal populations (27–34). Moreover, neither expression levels (35) nor inhibition by specific antibodies of [His^5,Trp^7,Tyr^8]GnRH correlates with the reproductive cycle (36). Characterization of the cDNA encoding this enigmatic GnRH form could provide insight about the evolutionary origins of GnRH and may serve as a tool in the search for its expression in mammalian species.

In the African cichlid fish *Haplochromis burtoni*, reproductive capacity is regulated through social interactions (37, 38), and GnRH-containing neurons in the hypothalamic preoptic area are implicated in this process (39, 40). HPLC analysis and differential immunochemistry have identified three distinct GnRH decapeptides in *H. burtoni* (N. Sherwood and R.D.F., unpublished data). The primary sequence for one of these forms, [Trp^7,Leu^8]-GnRH (previously referred to as salmon GnRH), has been deduced from the cloned cDNA (4). Based on its HPLC profile, the second GnRH form in *H. burtoni* has properties similar to those of [His^5,Trp^7,Tyr^8]GnRH whereas the third form appears to be unique. The existence of three peptide forms, as well as the puzzling nonhypothalamic expression of [Trp^7,Leu^8]GnRH (see below), makes *H. burtoni* an ideal model for studying the physiological functions and evolutionary relationships of this potent peptide family. As a first step, we chose to search for the gene sequence‡ and expression site of [His^5,Trp^7,Tyr^8]-GnRH.

MATERIALS AND METHODS

cDNA Construction and Reverse Transcription (RT)–PCR. To obtain nucleotide sequences encoding a [His^5,Trp^7,-Tyr^8]GnRH-like peptide from *H. burtoni*, we employed a

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Abbreviation: GnRH, gonadotropin-releasing hormone.

‡The sequence reported in this paper has been deposited in the GenBank database (accession no. L27435).

A

GnRH form	Source	Amino acid sequence										Citations	
		1	2	3	4	5	6	7	8	9	10	Peptide	Nucleotide
GnRH	Mammal	pE	H	W	S	Y	G	L	R	P	GNH2	12, 13	1-3
[Gln^8]-GnRH	Chicken (I)	pE	H	W	S	Y	G	L	Q	P	GNH2	14-17	
[Trp^7 Leu^8]-GnRH	Salmon	pE	H	W	S	Y	G	W	L	P	GNH2	18	4-6
[His^5 Asn^8]-GnRH	Catfish	pE	H	W	S	H	G	L	N	P	GNH2	19	
[His^5 Trp^7 Tyr^8]-GnRH	Chicken (II)	pE	H	W	S	H	G	W	Y	P	GNH2	20	
[His^5 Trp^7 Leu^8]-GnRH	Dogfish	pE	H	W	S	H	G	W	L	P	GNH2	21	
[Tyr^3 Leu^{5}Glu^{6}Trp^7 Lys^8]-GnRH	Lamprey (I)	pE	H	Y	S	L	G	W	K	P	GNH2	22	
[Trp^3 His^5 Asp^{6}Trp^7 Lys^8]-GnRH	Lamprey (III)	pE	H	W	S	H	D	W	K	P	GNH2	11	
	yeast α mating factor	W	H	W		L	Q	L	K	P	G	9	

B

PCR 5' Chains

[Trp^7 Leu^8]-GnRH		Q	H	W	S	**Y**	G	W	**L**	P	GNH2
[His^5 Trp^7 Tyr^8]-GnRH		Q	H	W	S	**H**	G	W	**Y**	P	GNH2
	round 1:	5'-CAA/G	CAT/C	TGG	TCX AGT/C	CA-3'					
	round 2:					5'-CAT/C	GGX	TGG	TAT/C	CC-3'	

FIG. 1. Comparison of known GnRH amino acid sequences. All decapeptide sequences are given with reference to the mammalian GnRH sequence (12, 13). Residues which differ from those in the mammalian form are identified by both position and residue; thus, salmon GnRH (4) is referred to as [Trp^7,Leu^8]GnRH because, in salmon, the leucine found in position 7 in mammalian GnRH is tryptophan, and the arginine in position 8 is leucine. (*A*) The primary structures of the eight known GnRH forms and of the first nine amino-terminal residues from yeast α mating factor (9–11). In GnRH, positions 1, 2, 4, 9, and 10 are invariant, and positions 3 and 7 show only conservative changes. (*B*) The predicted amino acid sequence of the preprohormone for *Haplochromis burtoni* [Trp^7,Leu^8]GnRH is compared with that of [His^5,Trp^7,Tyr^8]GnRH. The two residues at which these forms differ are indicated in bold print. Degenerate 5′ oligonucleotides used for PCR are shown below the amino acid sequences.

polymerase chain reaction (PCR) strategy (Fig. 1*B*) using brain regions known to contain GnRH neuronal populations (see Fig. 4 for schematic view of *H. burtoni* brain). Animals were sacrificed and whole brains were removed as described (41). The dorsal portion of the telencephalon and the entire optic tectum and cerebellum from each brain were removed. Total RNA was isolated from the remaining ventral portions by guanidine thiocyanate/acid/phenol extraction and converted into cDNA by reverse transcriptase (Superscript, BRL). cDNA synthesis was primed with 0.5 μg of a bipartite oligonucleotide consisting of a homopolymer of nine thymidine residues at the 3′ end. The 5′ domain comprised a sequence of 14 nucleotides which included a restriction endonuclease recognition site [5′-GCAGAAGCTTCAGC(T_9)-3′].

The resulting cDNA was used as substrate for nested PCR. In both rounds of amplification, the downstream primer was equivalent to the 5′ domain of the bipartite oligonucleotide, described above. In the first PCR, the upstream primer was a pool of 24 tetradecamers representing all possible coding sequences for the first five amino acids of [His^5,Trp^7,-Tyr^8]GnRH; 12.5 pmol of upstream primer was used. After 40 cycles of 94°C/30 s, 55°C/30 s, 72°C/15 s, 0.05 μl of the product of this reaction was aliquoted to a second reaction using 12.5 pmol of a new upstream primer together with the same downstream primer under the same cycling conditions. The new primer pool of 24 hexadecamers consisted of all possible coding combinations for residues 5–9 of the decapeptide. Reactions were performed with *Taq* DNA polymerase (synthesized courtesy of R. Moses, Vollum Institute, Oregon Health Science University, Portland) on a Perkin–Elmer 9600 thermocycler. Oligodeoxynucleotides were synthesized as needed (Applied Biosystems model 391).

PCR products were separated by electrophoresis in a 1.5% GTG agarose gel (FMC) and products >300 bases were electroeluted and subcloned into M13 phage vector for sequence analysis (4). Nucleotide sequences were analyzed for the presence of the second upstream primer (ending in the codon for amino acid 9; see Fig. 1), followed by a codon for the final conserved amino acid of the decapeptide, Gly^{10}, and sequences for the canonical GnRH amidation and peptide processing site, Gly^{11}-Lys^{12}-Arg^{13} (42).

Construction of cDNA Library and Sequence Determination. To obtain full-length coding sequences, polyadenylylated RNA was isolated from *H. burtoni* ventral brain regions (see above) and used to construct a cDNA library in λgt10. Two hundred fifty thousand primary recombinants were screened (Genescreen; DuPont/NEN) with a radiolabeled oligonucleotide 29 bases in length (5′-GGGAATGCAGC-TACCTGAGACCCCAGAGG-3′) derived from a putative [His^5,Trp^7,Tyr^8]GnRH PCR product. Positively hybridizing phage were purified by successive rescreening at reduced density. The cDNA inserts were subcloned into M13 phage and the nucleotide sequences were determined by the conventional dideoxy chain-termination method. A cDNA containing the full-length coding sequence was subcloned into a transcription vector (Pselect, United States Biochemical) for generation of sense and antisense RNA probes.

Northern Analysis of [His^5,Trp^7,Tyr^8]GnRH Precursor. Northern blots of total RNA were prepared (4) and probed with antisense RNA probes synthesized with SP6 RNA polymerase, incorporating [α-^{32}P]UTP to a specific activity of 3×10^8 dpm/μg. In addition, poly(A)$^+$ RNA was isolated from whole brains (RiboSep, Collaborative Biomedical Products, Bedford, MA), prepared as a Northern blot, and used for both high- and low-stringency hybridizations. Hybridization mixtures contained ≈10^7 dpm per ml. High-stringency conditions were obtained in 50% formamide at 65°C with washes in 0.1× standard saline citrate (SSC) at 70°C. Low-stringency hybridizations were with 50% formamide at 37°C and washes in 0.2× SSC at 52°C.

***In Situ* Hybridization.** The site of [His^5,Trp^7,Tyr^8]GnRH mRNA production in *H. burtoni* brain was assessed by *in situ* hybridization. Animals (n = 8) were sacrificed, brains were removed, and tissue was fixed as described (41). Cryostat sections (40 μm) were mounted on poly(L-lysine)-coated slides for hybridization to the digoxygenin-UTP (Boehringer Mannheim)-labeled [His^5,Trp^7,Tyr^8]GnRH RNA probe. Hybridizations were carried out at high stringency (55°C in 50% forma-

mide) according to Boehringer Mannheim's instructions with the following modifications: 150 μl of hybridization solution was applied to slides containing four to five brain sections. After hybridization, three washes were done in 2× SSC at 55°C for 1 hr each, followed by 1 wash in 1× SSC for 30 min at room temperature. To prepare the RNA probe, subcloned prepro-[His^5,Trp^7,Tyr^8]GnRH plasmid was linearized with *Eco*RI (GIBCO/BRL), the recessed 3′ termini were filled in by the Klenow fragment of DNA polymerase I, and the DNA was treated with proteinase K. The template DNA was purified (Magic DNA clean-up system; Promega) and 300 ng was used for transcription of an RNA probe with SP6 RNA polymerase (GIBCO/BRL) in the presence of digoxygenin-UTP. Final concentrations in a 100-μl reaction volume were as follows: 1× SP6 reaction buffer, 1× NTP labeling mixture (Boehringer Mannheim), 4 mM dithiothreitol, 80 units of RNasin (Promega), 13.2 μg of bovine serum albumin, and 120 units of SP6 polymerase. Solutions for both Northern analysis and *in situ* hybridizations were prepared with water treated with 0.075% diethyl pyrocarbonate.

RESULTS

Isolation and Comparison of Prepro-[His^5,Trp^7,Tyr^8]GnRH. Six positively hybridizing clones were identified from the *H. burtoni* ventral brain cDNA library and the nucleotide sequences of the inserts were determined. The longest open reading frame containing the [His^5,Trp^7,Tyr^8]GnRH coding sequence begins with a methionine initiator codon and predicts a 90-amino acid protein (Fig. 2). The first 23 residues are largely hydrophobic and most likely constitute a signal peptide as is found in many polyprotein neuroendocrine precursors (42). The signal sequence is in direct linkage with [His^5,Trp^7,Tyr^8]GnRH, which is followed by Gly^{11}-Lys^{12}-Arg^{13}. These residues follow the decapeptide sequence in all of the other cloned GnRH preprohormones and serve as substrates for posttranslational processing (42). The remainder of the precursor may code for two additional peptides also generated by proteolytic processing, neither of which shows any homology to sequences in protein databases. One peptide, 28 residues in length, is followed by dibasic residues (Arg-Arg; see Fig. 2 legend), and the remaining 19 amino acids could comprise a second processing product. There is ≈10% amino acid identity between the two *H. burtoni* GnRH preprohormones when the GnRH motif is excluded.

Size and Distribution of [His^5,Trp^7,Tyr^8]GnRH mRNA in the Brain. Northern blot analysis of total RNA extracted from dorsal, ventral, and whole *H. burtoni* brain revealed a single transcript of ≈530 bases expressed in ventral and whole brain but absent from dorsal structures (Fig. 3). Radiolabeled antisense RNA probes for both the [His^5,Trp^7,Tyr^8]GnRH and [Trp^7,Leu^8]GnRH preprohormones were prepared and

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                    -20          -10          +1          10
[Trp7, Leu8]-GnRH:        MEAGSRVIMQVLLLALVVQVTLS QHWSYGWLPG GKR
[His5, Trp7, Tyr8]-GnRH:  MCVSRLALLLGLLLCVGAQLSFA QHWSHGWYPG GKR

  20          30          40          50          60
SVGELEATIRMMGTGGVVSLPDEANAQIQERLRPYNIINDDSSHFDRKKRFPNN*
ELDSFGTSEISEEIKLCEAGECSYLRPQRRSILRNILLDALARELQKRK*
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FIG. 2. Predicted amino acid sequence of *H. burtoni* prepro-[His^5,Trp^7,Tyr^8]GnRH. Amino acids are numbered beginning at the first residue in the GnRH decapeptide. The functional domains of prepro-[His^5,Trp^7,Tyr^8]GnRH are illustrated and compared with *H. burtoni* prepro-[Trp^7,Leu^8]GnRH (4). In both, hydrophobic signal sequences (negative numbers) are directly followed by the GnRH decapeptide region (bold) and residues involved with posttranslational processing. A second processing site (underlined) indicates the beginning of a coding sequence for the second of two novel peptides.

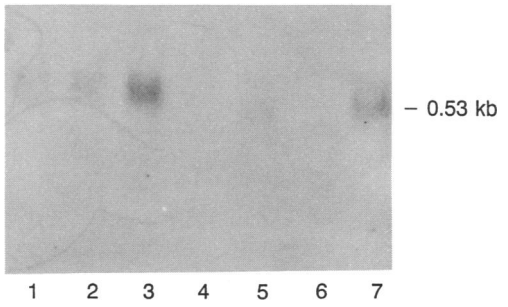

FIG. 3. Characterization of prepro-[His^5,Trp^7,Tyr^8]GnRH mRNA in *H. burtoni*. High-stringency Northern blot analysis was performed with total RNA from ventral, dorsal, or whole brain and a ^{32}P-labeled prepro-[His^5,Trp^7,Tyr^8]GnRH RNA probe. Lanes 1–3, total RNA (5, 10, and 20 μg, respectively) extracted from a pool of three brains; lanes 4–7, total RNA samples (10 μg), each from half of a single brain; lanes 4 and 6, RNA from an adult ventral brain part; lanes 5 and 7, RNA from the corresponding dorsal brain halves. One band of ≈530 bases is seen in lanes containing ventral or total brain RNA. No signal was detected in total RNA extracted from dorsal brain halves, localizing the expression of this transcript exclusively to more ventral brain regions. Methylene blue staining of ribosomal RNA bands confirmed that equal amounts of total RNA were loaded onto lanes 4–7.

used to probe Northern blots of total brain poly(A)$^+$ RNA at both high and low stringencies. The results (not shown) indicate that the probes for the two preprohormones do not cross-hybridize at either stringency with the alternative transcript.

[His^5,Trp^7,Tyr^8]GnRH mRNA, localized by *in situ* hybridization, was found only in a cluster of neurons found in the mesencephalon (Fig. 4 *Upper*) previously shown to contain GnRH by immunocytochemistry (39). Interestingly, [His^5,Trp^7,Tyr^8]GnRH mRNA was not expressed in the hypothalamic preoptic nucleus (Fig. 4 *Lower Left*), which projects to the pituitary and hence probably does not directly influence gonadotropin activation. The probes for the two species of GnRH found in *H. burtoni* do not crossreact either when applied *in situ* (see Fig. 4) or in Northern blots (see above). Thus, different GnRH-encoding genes are expressed in distinct brain regions.

DISCUSSION

In *H. burtoni*, as in other vertebrates, three neuronal populations have been shown to contain GnRH by immunocytochemistry: the terminal nerve, the hypothalamic/preoptic area, and the mesencephalon (refs. 39 and 41; see Fig. 4 *Upper*). Here we have shown that [His^5,Trp^7,Tyr^8]GnRH mRNA is expressed only in the mesencephalic population (Fig. 4 *Lower Left*). In contrast, [Trp^7,Leu^8]GnRH mRNA is localized only in the terminal nerve nucleus of the telencephalon (Fig. 4 *Lower Right*). Further, the lack of *in situ* hybridization within the GnRH-immunoreactive cells of the hypothalamic/preoptic area (Fig. 4) reveals that the GnRH-encoding gene expressed there is sufficiently different to elude detection by either probe. Thus, despite its potent releasing-factor activity (7), [His^5,Trp^7,Tyr^8]GnRH is unlikely to influence pituitary function directly. Moreover, this suggests that yet a third gene coding for GnRH may be responsible for regulating reproduction.

The structure of the [His^5,Trp^7,Tyr^8]GnRH cDNA predicts two novel peptides in addition to it, in contrast to the single associated peptide predicted by [Trp^7,Leu^8]GnRH cDNA. The lack of homology between the two known GnRH preprohormones in *H. burtoni* suggests that these genes have either evolved independently or diverged from a common ancestor well before the appearance of teleost fish.

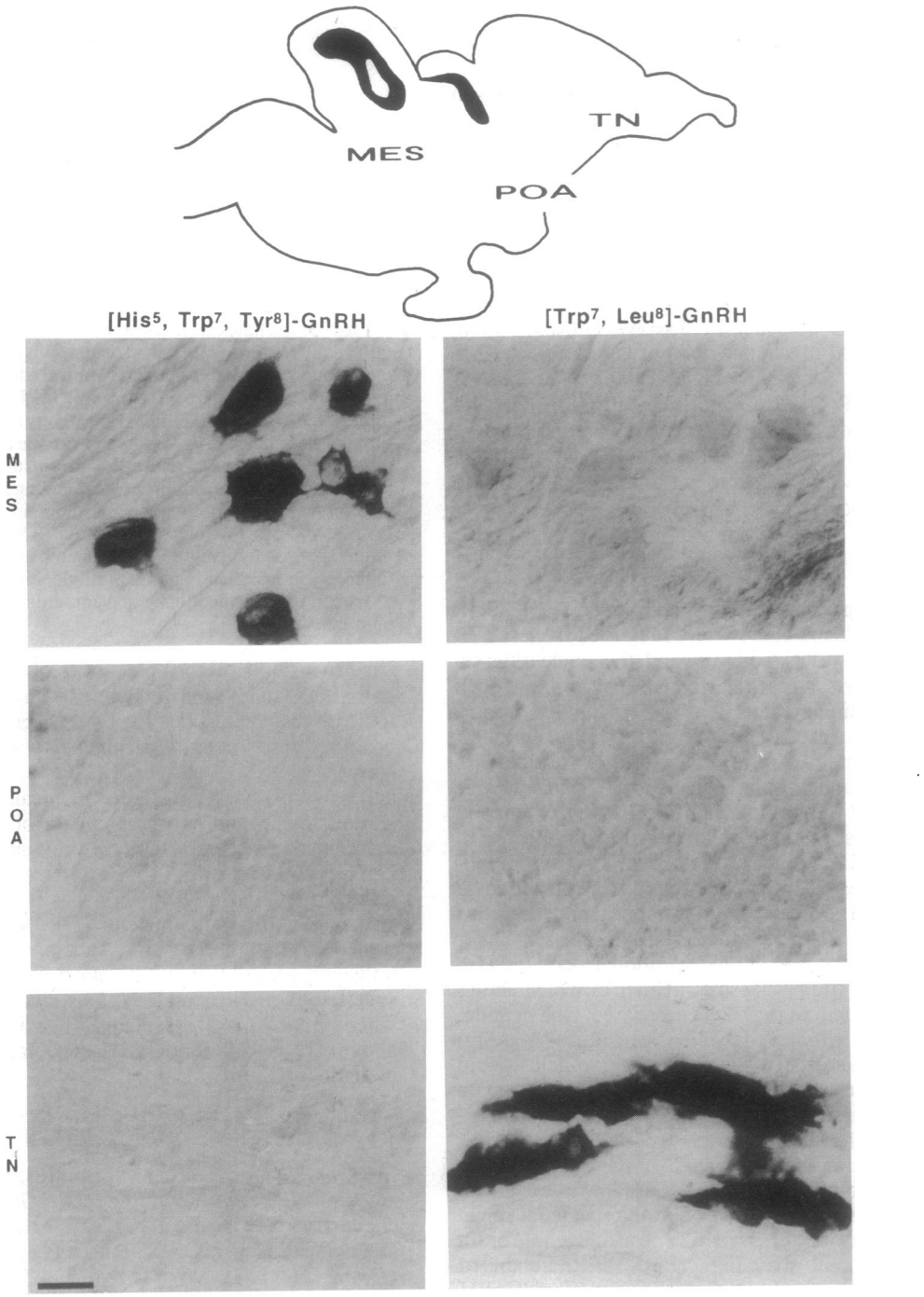

FIG. 4. Localization of prepro-[His^5,Trp^7,Tyr^8]GnRH mRNA in *H. burtoni* brain. (*Upper*) Schematic view of a midsagittal section from *H. burtoni* brain showing the three populations of neurons immunoreactive for GnRH (39, 41). For simplicity, the clusters are represented within the same plane although the mesencephalic (MES) population lies lateral to both the terminal nerve (TN) and hypothalamic preoptic area (POA) populations. (*Lower*) The six panels show the labeling with each of the two GnRH gene probes in the three brain regions with cells known to contain GnRH from immunocytochemistry (39). (*Left*) *In situ* hybridization with a prepro-[His^5,Trp^7,Tyr^8]GnRH-specific, digoxygenin-UTP-labeled RNA probe. Only the mesencephalic population was labeled. (*Right*) *In situ* hybridization with a prepro-[Trp^7,Leu^8]GnRH-specific, digoxygenin-UTP-labeled RNA probe. Only the terminal nerve population was labeled. Note that neither probe labeled the hypothalamic preoptic group of cells. (Bar = 100 μm.)

The preprohormone structure and extrahypothalamic location of the *H. burtoni* [His^5,Trp^7,Tyr^8]GnRH precursor may indicate a unique function for this GnRH form and/or its associated peptides. While hypothalamic GnRH clearly functions as a releasing hormone, neuromodulatory and neurotransmitter roles have been postulated for GnRHs in other brain regions (reviewed in ref. 10). The localization of the [His^5,Trp^7,Tyr^8]GnRH mRNA to the mesencephalon supports previous immunological studies in which [His^5,Trp^7,-Tyr^8]GnRH-like peptides were found concentrated in caudal brain areas (27–34). Such mesencephalic GnRH-containing neurons may play a role in the central organization of reproductive behavior as suggested for poecilid fish, where a similar neuronal population has been described (43). These cells contain GnRH and project to a spinal neurosecretory group which, in turn, has been postulated to play a role in gonadal duct contractility.

Until recently, only one GnRH peptide (previously called mammalian GnRH) had been reported in mammals, that which regulates the pituitary gonadotropes. This form can be

detected in some bony fish but is absent in *H. burtoni*, as well as in reptiles and birds (see ref. 10 for review). In contrast, [His^5,Trp^7,Tyr^8]GnRH has widespread expression among vertebrates with the notable exception of eutherian mammals. Since nonmammalian animals express more than one GnRH peptide, it is enigmatic that no alternative forms, particularly [His^5,Trp^7,Tyr^8]GnRH, have been found in mammals with one exception (ref. 44 and cited in ref. 10). These forms may be truly absent, their functions obsolete or appointed to the hypothalamic form, or possibly they have been overlooked due to structural modifications which prevent their detection by GnRH-specific antibodies.

As puzzling as the apparently limited distribution of [His^5,Trp^7,Tyr^8]GnRH in mammals is the lack of a detectable hypothalamic GnRH precursor in *H. burtoni*. Reproductive capacity in this fish is controlled by social interactions (37, 38) and mediated via the GnRH-containing neurons in the preoptic area (39–41). It seems likely that a third GnRH form in *H. burtoni* is produced in these neurons and represents the teleost counterpart to mammalian GnRH responsible for controlling reproductive circuitry. Thus, in *H. burtoni*, the three GnRH-containing neuronal populations might each contain unique decapeptide and precursor forms serving reproductively related but distinct functions. The peptides associated with the two nonhypothalamic forms of GnRH are likely to play important physiological roles which may have contributed to their distinct spatial expression during the course of evolution.

The isolation of the cDNA encoding the [His^5,Trp^7,Tyr^8]GnRH preprohormone will allow homologous genes in other species to be identified, offering a window onto the functional and evolutionary relationships among the forms of GnRH.

We thank R. Yamawaki for technical assistance and T. Bushnik-Harris, B. Evans, S. McConnell, M. Nadal-Vicens, J. Wine, and two reviewers for useful comments on earlier drafts of the manuscript. This work was supported by National Institutes of Health grants to S.A.W. (MH09986), R.D.F. (HD23799), and J.P.A. (NS28504).

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