0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(8):3351–3361

doi: 10.1210/en.2005-0305

Cloning and Characterization of a Functional Type II Gonadotropin-Releasing Hormone Receptor with a Lengthy Carboxy-Terminal Tail from an Ancestral Vertebrate, the Sea Lamprey

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A full-length transcript encoding a functional type II GnRH receptor was cloned from the pituitary of the sea lamprey, *Petromyzon marinus*. The current study is the first to identify a pituitary GnRH receptor transcript in an agnathan, which is the oldest vertebrate lineage. The cloned receptor retains the conserved structural features and amino acid motifs of other known GnRH receptors and notably includes a C-terminal intracellular tail of approximately 120 amino acids, the longest C-terminal tail of any vertebrate GnRH receptor identified to date. The lamprey GnRH receptor was shown to activate the inositol phosphate (IP) signaling system; stimulation with either lamprey GnRH-I or lamprey GnRH-III led to dose-dependent responses in transiently transfected COS7 cells. Furthermore, analyses of serially truncated lamprey GnRH receptor mutants indicate perturbations of the C-terminal tail disrupts IP accumulation, however, the tailless lamprey GnRH receptor was not only functional but was also capable of stimulating IP levels equal to wild type. Expression of the receptor transcript was demonstrated in the pituitary and testes using RT-PCR, whereas *in situ* hybridization showed expression and localization of the transcript in the proximal pars distalis of the pituitary. The phylogenetic placement and structural and functional features of this GnRH receptor suggest that it is representative of an ancestral GnRH receptor. In addition to having an important role in lamprey reproductive processes, the extensive C-terminal tail of this lamprey GnRH receptor may have great significance for understanding the evolutionary change of this vital structural feature within the GnRH receptor family. (*Endocrinology* 146: 3351–3361, 2005)

GnRH ACTION IS MEDIATED through a class A rhodopsin-like 7-transmembrane G protein-coupled receptor (GPCR) (1, 2). Known GnRH receptors share a number of unique features that distinguish them from other class A GPCRs, including variations of the conserved transmembrane domain motifs, and most distinctly the evolutionary loss of the carboxy-terminal (C-terminal) tail in certain mammalian GnRH receptors (2). Since the first successful cloning of a GnRH receptor transcript from the mouse (3), a total of 38 GnRH receptor cDNAs has been cloned: 11 type I (tailless; lacking a C-terminal tail) forms and 27 type II (tailed; containing a C-terminal tail) forms (1, 5). Since the description of the catfish GnRH receptor 1, which was the first identified GnRH receptor to retain the evolutionarily conserved intracellular C-terminal tail, it has become evident that the main structural difference within the GnRH receptor family is the presence or absence of the intracellular C-terminal tail. This tail has been shown to affect not only effective GnRH binding and activation of signal transduction but also desensitization and internalization pathways as well (6–15). Three GnRH receptor subtypes, IA, IB, and II, have been suggested based on phylogenetic and sequence analysis of extracellular loop 3 (16). These authors proposed that these subtypes arose from an ancestral gene that underwent duplication and gave rise to the two distinct types, with the type I receptor undergoing another gene duplication to produce the type IA and IB receptor subtypes (16).

Multiple GnRH receptors have been characterized in several species of vertebrates, suggesting that most organisms likely contain two or more functional GnRH receptors in the pituitary and brain (17–23). Investigations in these organisms have demonstrated differential tissue distribution of GnRH receptor subtypes as well as changes in receptor transcript expression based on reproductive stage (18, 23). Although the representation of GnRH receptors across the vertebrate lineage extends from mammals to *Osteichthyes*, there have not yet been any GnRH receptors isolated and cloned in earlier evolved vertebrates from *Chondrichthyes* or *Agnatha*.

Lampreys, along with hagfish, are the only living representatives of the agnathans, the most ancient class of vertebrates, whose lineage dates back over 550 million years (24). Lampreys, which express two forms of GnRH, lamprey GnRH-I and lamprey GnRH-III (4, 25–27), are important to our understanding of the reproductive success of the first vertebrates and are likely to have retained key characteristics of the ancestral GnRH and GnRH receptor from which modern GnRH isoforms and GnRH receptors arose. The main objective of this study was to isolate and characterize a GnRH receptor from the sea lamprey pituitary. From this study, a GnRH receptor has been cloned from pituitary cDNA, ex-

First Published Online May 5, 2005

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Abbreviations: aa, Amino acids; GPCR, G protein-coupled receptor; IP, inositol phosphate; RACE, rapid amplification of cDNA ends; TM, transmembrane.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

pressed, and characterized. The isolated transcript encodes a pituitary receptor that shares the characteristic transmembrane region motifs and hydropathic profile of GnRH receptors as well as a C-terminal tail of approximately 120 amino acids, considerably longer than any previously identified vertebrate GnRH receptor. The cloned receptor was shown to encode a functional product as determined by inositol phosphate (IP) assays, in which stimulation of transiently transfected COS7 cells with either lamprey GnRH-I or lamprey GnRH-III led to dose-dependent responses. Furthermore, analyses of serial truncations of the lamprey GnRH receptor indicate that the C-terminal tail has a significant effect on signal propagation and internalization. RT-PCR expression analysis has shown detectable levels of this transcript in pituitary and testes but not in the brain. The putative amino acid sequence and the expression pattern of this GnRH receptor transcript provide evidence that it is representative of an ancestral GnRH receptor and that it likely plays a key role in regulation of reproduction in the sea lamprey.

Materials and Methods

Tissues

A total of 1200 male and female adult sea lampreys, *Petromyzon marinus*, were used in this study. These lamprey were collected at the Cocheco River fish ladder in Dover, NH. These fish were maintained at the University of New Hampshire (UNH) Anadromous Fish and Aquatic Invertebrate Research Laboratory in accordance with UNH animal care guidelines. The animals were decapitated, immediately after which pituitary, brain, heart, liver, muscle, kidney, eye, and ovary/testes were dissected and frozen in liquid nitrogen. Pituitary was used for RNA isolation and cDNA synthesis, liver was used for genomic DNA isolation, and all tissues were used for RT-PCR expression studies.

Twenty parasitic phase sea lampreys were obtained from the Hammond Bay Biological Station (Hammond Bay, MI). These fish were sent to UNH and maintained at the Anadromous Fish and Aquatic Invertebrate Research Laboratory in accordance with UNH animal care guidelines. Fish were decapitated and their heads were dissected to expose the pituitary and brain for horizontal cryomicrotomy in preparation for *in situ* hybridization.

Nucleotide isolation

Genomic DNA was isolated from 100 mg of lamprey liver using the prescribed protocol from Sambrook and Russell (section 6.7) (28). Total RNA was isolated from 1000 lamprey pituitaries (approximately 1 g) using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) per the manufacturers guidelines.

PCR from genomic DNA template

Lamprey genomic DNA was used as template for PCR with degenerate primers designed to GnRH receptor transmembrane (TM) regions 6 and 7. The sequences for these primers were provided to us by Troskie *et al.* (29): JH5s, 5′-CTCGAATTCGGNATHTGGTAYTGGT-3′; and JH6α_2, 5′-ACACTCGAGCCRTADATNTRNGGRTC-3′.

These oligos were obtained from operon.com. Reactions were mixed to total volume of 50 μl [1× Amplitaq Gold PCR buffer, 1 mM deoxynucleotide triphosphates, 1.25 U Amplitaq DNA polymerase (all from PE Biosystems, Foster City, CA), 2 μM each primer, 1 μl dimethylsulfoxide, and 4.35 μg genomic DNA]. These reactions were cycled on an Eppendorf PCR gradient thermocycler under the following conditions: 94 C for 9 min, 35 cycles of 93 C for 1 min, 53 C for 2 min, 72 C for 3 min, and 72 C for 5 min.

First-strand cDNA construction

First-strand cDNA was constructed using the first-strand cDNA synthesis kit (Amersham Pharmacia, Buckinghamshire, UK) from pituitary total RNA with the *Not*I-dT$_{18}$ primer. This first-strand cDNA was then used as template for PCR with combinations of gene-specific primers (see Table 1), the degenerate JH5s and JH6α_2 primers, and the *Not*I and poly-dT reverse primers.

3′- and 5′-Rapid amplification of cDNA ends (RACE)

Total RNA from lamprey pituitary was used to construct double-stranded cDNA using the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA). 3′- and 5′-RACE was performed with this system using various gene-specific primers (Integrated DNA Technologies, Coralville, IA) (Table 1). RACE reactions were performed with the following parameters: 95 C for 1 min, five cycles of 94 C for 10 sec and 74 C for 5 min, five cycles of 94 C for 10 sec and 72 C for 5 min, 10 cycles of 94 C for 10 sec and 70 C for 5 min, and 15 cycles of 94 C for 10 sec and 68 C for 5 min.

Experimental 3′- and 5′-RACE reactions were performed using various combinations of the above gene-specific primers and the Marathon adaptor primers. These combinations were used to amplify overlapping portions of the GnRH receptor cDNA. Clones were prepared for sequencing by standard TA-cloning with the pGEM T-easy vector system (Promega, Madison, WI), and inserts were sequenced at the Huntsman Cancer Institute DNA Sequencing Facility at the University of Utah. Sequences were analyzed using the Lasergene DNAStar suite of analysis programs. The full-length lamprey GnRH receptor cDNA was deposited in GenBank under accession no. AF439802.

Construct development, cell culture, and transfection

The coding region of the wild-type lamprey GnRH receptor was amplified via PCR and inserted into the pcDNA3.1 HisTOPO mammalian expression vector (Invitrogen, Carlsbad, CA). Mutant lamprey GnRH receptor constructs were generated via PCR using primers containing 3′-stop codons to produce serial truncations of the intracellular C-terminal tail [80, 40, and 0 (tailless) amino acids (aa)], which were inserted into the pcDNA3.1 HisTOPO vector. All constructs were verified by sequence analysis. COS7 cells were cultured in 10% fetal bovine serum in DMEM (Invitrogen), and were maintained at 37 C in 5% CO_2.

TABLE 1. Lamprey GnRH receptor gene-specific primers

Primer name	Nucleotide sequence (5′–3′)
lGnRHrGSP1	CGGAGCATTGTGTCACGCAAGGTCG
lGnRHrGSP2	CGACCTTGCGTGACACAATGCTCCG
lGnRHrGSP3	ATCCGTGTGATGGAGATTTGTGCCA
lGnRHrGSP4	TGGCACAAATCTCCATCACACGGAT
lGnRHrGSP5	CCGAACGCCAGCCACACAGGC
lGnRHrGSP6	GCCTGTGTGGCTGGCGTTCGG
lGnRHrGSP7	GCGGGCGAGTTCGTCTGCCG
lGnRHrGSP8	CGGCAGACGAACTCGCCCGC
lGnRHrGSP9	GGCTCGGCTCAAAGTGAATCCGCTG
lGnRHrGSP10	CAGCGGATTCACTTTGAGCCGAGCC
lGnRHrGSP11	AGCGTTCTGCTGGCGGTCCC
lGnRHrGSP12	TCACCCACGGCAACTTCGTCGAGCAG
lGnRHrGSP13	GCCCCTCCGAACGCCAGCCACACA
lGnRHrGSP14	GCCCCTGGACGCCGTGTGGCA
lGnRHrGSP15	CGGTCCCGCAGCTCTTTCTGTTCC
lGnRHrGSP16	CAAAAACTTCGTTCAGTGCGTCACCCACG
lGnRHrGSP17	TGGTATTGGTTCGACCGGAGCATTGTGTCACGC
lGnRHrGSP18	AACTTGACCGTGTTGTGCACCATCT
lGnRHrGSP19	GATCTCCAAGAGGATGCGAGAAGGA
lGnRHrGSP20	CAAAAACTTCGTTCAGTGCGTCACC
lGnRHrGSP21	TCTCCAAGAGGATGCGAGAAGGAAG
lGnRHrGSP22	CAAGAGGATGCGAGAAGGAAGCATT
lGnRHrGSP23	CCACCACTGGCATCACAGAACG
lGnRHrGSP24	GAGGCCGAGTAGCGAAAGGAGC
lGnRHrGSP25	GCCGCTGCTGCCGCTGGC
lGnRHrGSP26	CGTCACGGGCTCCTTTCGCTACTC
lGnRHrGSP27	CACGGGCTCCTTTCGCTACTCGGC
lGnRHrGSP28	CGCAGTGTCTGCGTTATTCTCA

GSP = 2, 4, 6, 8, 10, 23, and 24 are 3′→5′ (antisense) primers, and all others are 5′→3′ (sense) primers.

The day before transfection, 5×10^5 cells were seeded in 60-mm culture plates. Transfection was performed using 5 μg of vector and 15 μl of lipofectamine (Invitrogen) in 2.4 ml total volume in Opti-MEM-I (Invitrogen) and an incubation time of 5 h at 37 C in 5% CO_2, after which, 2.5 ml of 20% fetal bovine serum in DMEM was added and cultures were grown overnight.

IP assay

The IP stimulation and extraction protocol used was adapted from previous studies (21, 30, 31). Briefly, 24 h after transfection, cells were trypsinized and seeded in 12-well plates at approximately 1.5×10^5 cells/well. At h 72 cells were washed in PBS and incubated in 1 ml of 2% dialyzed fetal bovine serum and 2 μCi/ml myo[2-^{3}H]inositol (Amersham) in medium 199 (Invitrogen). At h 96 cells were washed two times with IP buffer (20 mM HEPES, 20 mM LiCL in 1× Hank's balanced salt solution) and were preincubated in IP buffer for 15 min at 37 C, followed by stimulation with either lamprey GnRH-I or lamprey GnRH-III in IP buffer (concentrations ranging from 10^{-6} to 10^{-11} M for dose-response analysis or 10^{-6} M for the C-terminal tail functional analysis) for 1 h at 37 C with gentle shaking. The reactions were stopped with the addition of 0.2 ml of prechilled 20% perchloric acid, and the plates were placed on ice for 30 min. The wells were scraped and the extracts were transferred to sterile 1.5-ml tubes and neutralized with 5 M KOH, followed by a 1-h incubation at 4 C. Tubes were centrifuged at 5000 rpm at 4 C for 15 min, and 1.2 ml of supernatant was transferred to a new sterile 1.5 ml tube. IPs (IP, IP_2, and IP_3) were isolated by ion exchange chromatography using AG1×8–200 resin (Bio-Rad Laboratories, Hercules, CA) in formate form. IPs were eluted with 1 M ammonium formate/0.1 M formic acid, samples were counted by liquid scintillation, and data were analyzed using Prism (GraphPad, San Diego, CA). Treatments were performed in triplicate in three independent experiments, and cells transfected with blank vector and nontransfected cells were used as negative controls.

Phylogenetic analysis

Positive sequences were aligned by amino acid-coding sequence using the ClustalW method with MegAlign (Lasergene, DNAStar, Madison, WI) to create a consensus sequence representing the lamprey GnRH receptor protein-coding sequence. This sequence was aligned, using ClustalV, with 36 other known GnRH receptor amino acid sequences and analyzed using Phylogenetic Analysis Using Parsimony 4.0 beta10 (32). Trees were constructed using a neighbor joining analysis with 1000 bootstrap replicates; only branches with 50% frequency or better were retained.

RT-PCR

Total RNA from adult male and female lamprey was isolated from the pars intermedia, proximal pars distalis, rostral pars distalis, brain, heart, liver, muscle, eye, testes, ovary, and kidney using Tri-reagent (Molecular Research Center). These RNA stocks were then treated (1 μg/reaction) with RQ1 RNase-free DNase (Promega), and 4 μl of each reaction were then used with the AccessQuick RT-PCR system (Promega). LGnRHr GSP5 and lGnRHr GSP 2 were used (2.5 pmol/reaction) with the following cycling parameters: 48 C for 45 min, 95 C for 2 min, 30 cycles of 95 C for 15 sec, 68 C for 1 min, 72 C for 1 min, and 72 C for 5 min. Negative controls were performed by adding 1 μl RNase (10 mg/ml) and incubating at 37 C for 1 h before adding AccessQuick reagents and cycling.

In situ hybridization

In situ hybridization was performed as described previously (33) with modifications as described by Root *et al.* (34). Brain and pituitary were dissected from two parasitic lampreys and were oriented for horizontal sectioning. GnRH-R probes were produced by reverse transcription of two PCR-amplified (GSP13 *vs.* GSP8 and GSP19 *vs.* GSP24) portions of the GnRH receptor transcript using the RiboProbe synthesis system (Promega). These represent a 500-nucleotide sequence that includes the coding sequence from the extracellular end of transmembrane region 1 through the C-terminal end of extracellular loop 1 and a 336-nucleotide sequence corresponding to the coding region of intracellular loop 3 through the middle of the C-terminal tail. A mixture of these two digoxigenin-labeled probes was used (10.0 ng/μl of each).

Results

Lamprey GnRH receptor cDNA isolation and sequencing

Using PCR from genomic template with degenerate primers and subsequent 5′ and 3′-RACE from pituitary cDNA, a 1838-base full-length cDNA was identified and confirmed with at least three separate clones to each portion of the sequence. This transcript contains a 55-base 5′-untranslated region, a 1380-base coding region, and a 458-base 3′-untranslated region. Translation of the coding region demonstrated that the identified transcript encoded a GnRH receptor sequence of 460 amino acids. Within this open reading frame, all of the regions of a 7-TM GPCR were predicted (Fig. 1).

Lamprey GnRH receptor cDNA sequence analysis

The amino acid sequence encoded by the lamprey GnRH receptor transcript has high identity to numerous GnRH receptors previously identified. The receptor amino acid sequence was compared with all previously identified GnRH receptors using MegAlign (Lasergene). It has highest identity with those of the aquatic caecilian *Typhlonectes natans* (61.2%) (GenBank no. AF174481), the amphibian *Rana catesbeiana* (receptor 1: 60.7% and receptor 2: 59.8%) (35), and the striped sea bass *Morone saxitilis* (59.6%) (36). The chicken GnRH receptor (37) and the human GnRH receptor (38, 39) were also used in this analysis and were found to have 43 and 40% identity, respectively (Fig. 2).

The lamprey GnRH receptor amino acid sequence was examined for conservation of the characteristic motifs of class A GPCRs and GnRH receptors (Fig. 3). The lamprey GnRH receptor maintains all of the conserved motifs of class A GPCRs with the exceptions characteristic of GnRH receptors. Based on this comparison, the lamprey GnRH receptor appears more closely related to type II GnRH receptors than type I GnRH receptors.

Lamprey GnRH receptor functional analysis

Both lamprey GnRH-I and lamprey GnRH-III stimulated a significant response in IP accumulation, in a dose-dependent manner, in COS7 cells that were transiently transfected with the lamprey GnRH receptor (Fig. 4). The $\log EC_{50}$ (represented as mean ± SEM; n = 3) of lamprey GnRH-III (-9.37 ± 0.243) was significantly ($P < 0.0002$) lower then the $\log EC_{50}$ of lamprey GnRH-I (-8.10 ± 0.150). This significant difference in IP activation suggests the presently cloned lamprey GnRH receptor is lamprey GnRH-III selective. Cells transfected with blank pcDNA3.1 vector showed no response in IP accumulation after treatment with either lamprey GnRH-I or lamprey GnRH-III (data not shown).

Despite this difference in $\log EC_{50}$, both lamprey GnRH-I and lamprey GnRH-III were shown to stimulate a statistically indistinguishable maximum level of IP accumulation when the wild-type or mutant lamprey GnRH receptors were treated with a high dose (10^{-6} M) (Fig. 5). Serial truncation of the lamprey GnRH receptor resulted in an initial decrease in magnitude of IP signaling. The 80-aa C-terminal tail re-

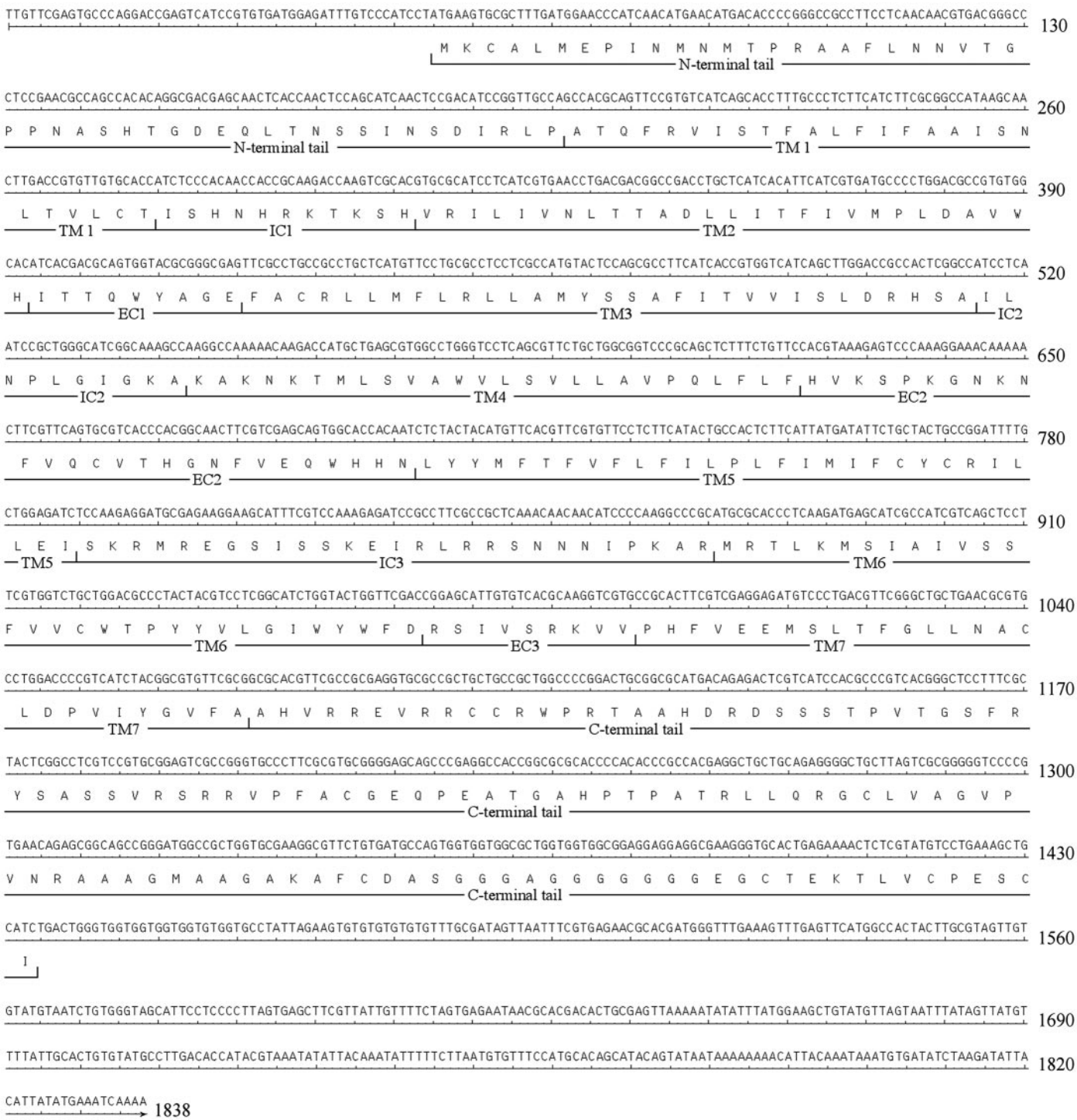

FIG. 1. Sequence map. The lamprey GnRH receptor transcript is shown with the amino acid coding sequence of the predicted protein. N-term, Amino-terminal tail; IC, intracellular loop; TM, transmembrane domain; EC, extracellular loop; C-term, carboxyl-terminal tail.

sulted in an approximately 43% reduction in signal relative to wild type. Further truncation of the C-terminal tail to 40 aa also showed a decrease in signal relative to wild type (28% reduction) but to a lesser extent, compared with the 80-aa C-terminal tail. Finally, the magnitude of signaling of the tailless lamprey GnRH receptor mutant was shown to fully recover, producing IP levels comparable with wild type.

Phylogenetic analysis of GnRH receptors

Phylogenetic analysis was performed using an alignment (MegAlign, ClustalW) of the predicted lamprey GnRH receptor protein sequence with 36 previously identified GnRH receptor protein sequences (Fig. 6). The GnRH receptors grouped into three phylogenetic clades, with the lamprey receptor grouping with

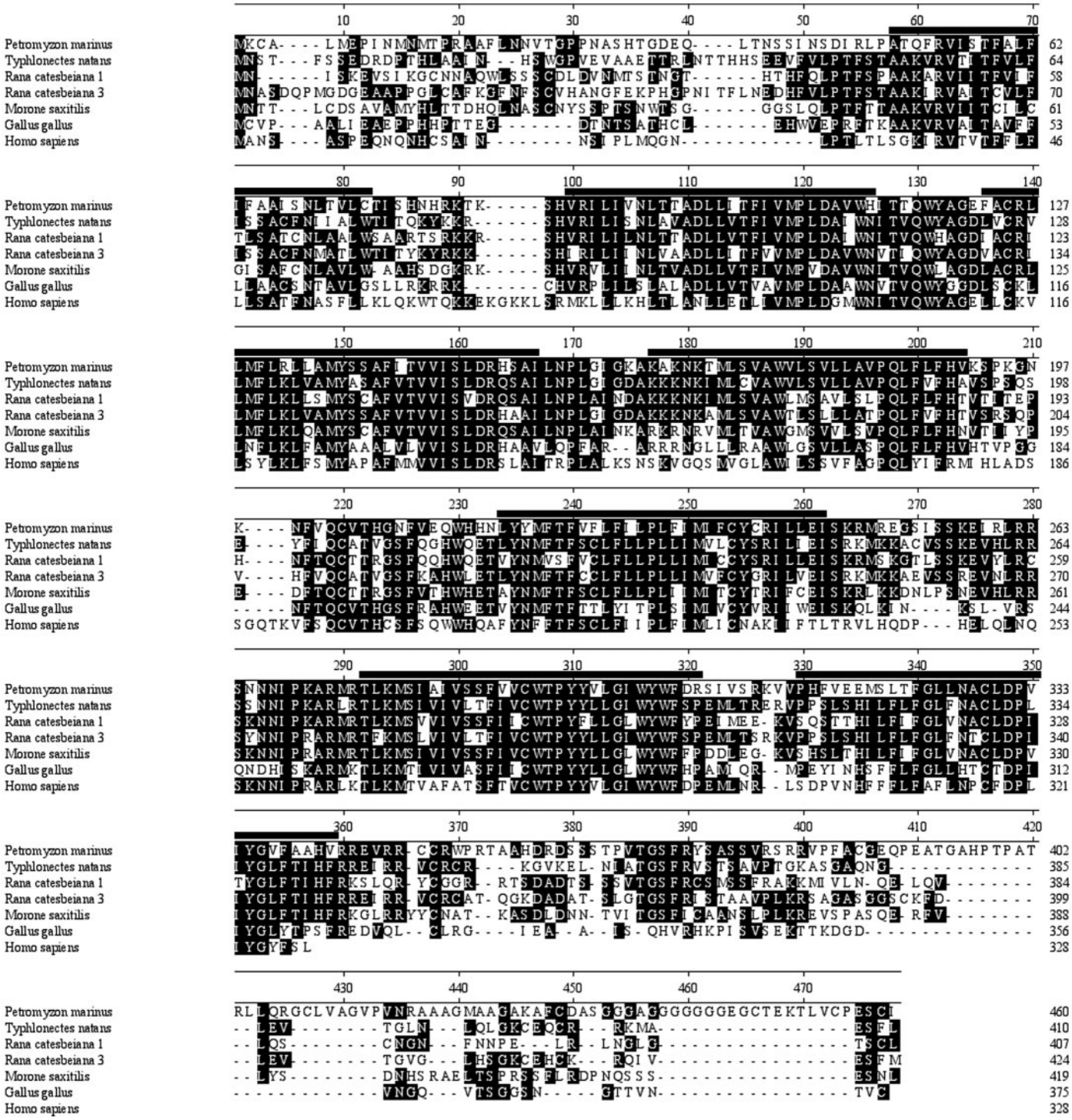

FIG. 2. Amino acid alignment of lamprey GnRH receptor with representative GnRH-Rs. *Shaded* amino acids are shared with the consensus formed from this alignment (consensus not shown). *Black bars* above the sequence indicate transmembrane domains.

type II GnRH receptors from amphibians and mammals as well as with three fish receptors. The GnRH-like receptors identified from the tunicate form outgroups.

Sequences used were: mouse (3, 40, 41), human (38, 39), rat (42–44), sheep (45), cow (46), pig (47), bonnet macaque (48), rhesus monkey (20), dog (49), horse (GenBank no. AF018072), guinea pig (50), wallaby (51), gecko (31), tunicate (52), amberjack (GenBank no. AJ130876), Rio Cauca caecilian (GenBank no. AF174481), African green monkey (20), marmoset (19), chicken (37), bullfrog (35), brown frog (22), African clawed frog (29), catfish (17, 53), goldfish (18), striped sea bass (36), rainbow trout (54), Japanese eel (55), and Japanese medaka (21).

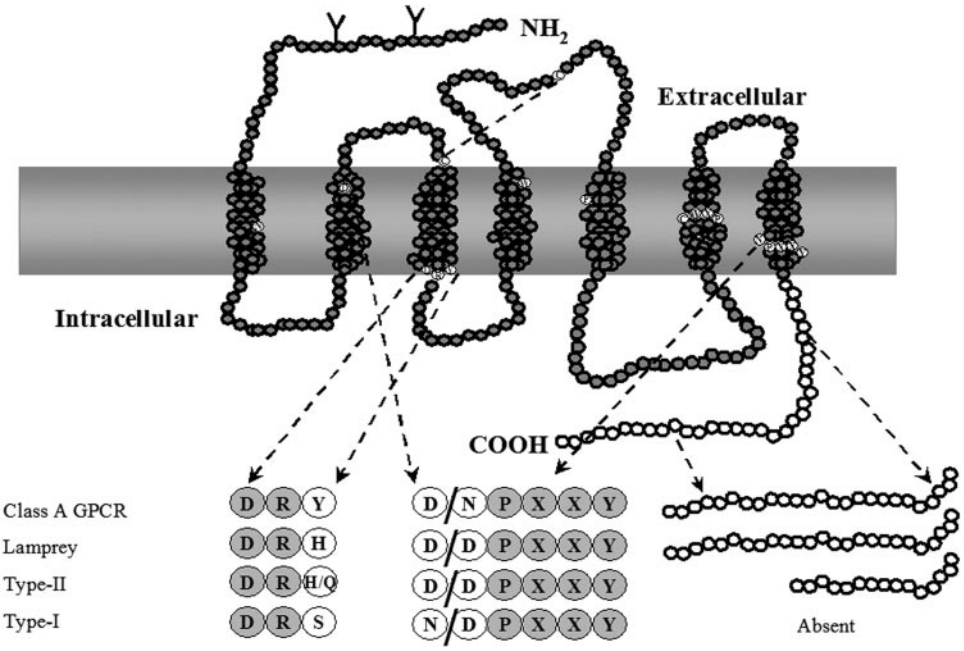

FIG. 3. Pattern of motif change through GnRH receptors. The conserved amino acid motifs of class A GPCRs are shown in *white* with the conserved residue indicated. Three main motifs have changed significantly through GnRH receptor evolution. The tyrosine in TM3 has become variable, the aspartate/asparagine motif in TM2/7 has reversed, and the C-terminal tail has shortened to the point of nonexistence in mammalian type I receptors.

Tissue-specific expression

Tissue-specific expression was analyzed by RT-PCR using RNA isolated from 20 adult male and female lamprey. Tissues examined included the proximal pars distalis, rostral pars distalis, brain, ovary, testes, heart, muscle, liver, kidney, and eye. Amplified products were analyzed by gel electrophoresis in the presence of ethidium bromide and were visualized using a UV transilluminator. The target sequence of approximately 840 bases was visualized in the pars intermedia, proximal pars distalis, rostral pars distalis, and testes (Fig. 7).

In situ hybridization

Expression was visualized by *in situ* hybridization in the proximal pars distalis of the lamprey pituitary (n = 2) (Fig. 8). This expression is seen in the same portion of the lamprey pituitary in which GnRH binding sites are known to be concentrated (56). GnRH receptor transcript expression was not detected in the parasitic phase lamprey brain.

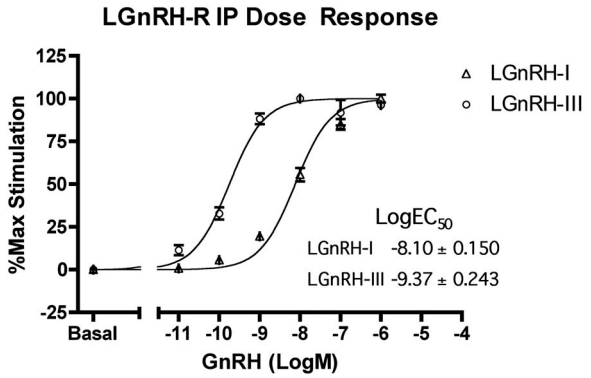

FIG. 4. Lamprey GnRH receptor dose-response curve. The lamprey GnRH receptor was shown to activate the IP signaling system, in a dose dependent manner, in transiently transfected COS7 cells. Lamprey GnRH-III stimulated IP accumulation at a significantly lower $\log EC_{50}$ when compared with lamprey GnRH-I ($P < 0.002$). $\log EC_{50}$ shown as mean ± SEM; n = 3 independent experiments.

Discussion

A 1838-base full-length cDNA encoding a GnRH receptor has been identified from the sea lamprey pituitary. This transcript sequence includes a 55-base 5′-untranslated region; a 1380-base reading frame, based on translation starting at the first AUG methionine codon, encoding a full 7-TM receptor protein of 460 aa; and a 403-base 3′-untranslated region. Analysis of the encoded amino acid sequence showed maintenance of the characteristic motifs of GnRH receptors and high overall similarity to previously identified GnRH receptors. The lamprey GnRH receptor was shown to be functional through stimulation of IPs in transiently transfected COS7 cells. Lamprey GnRH receptor C-terminal tail

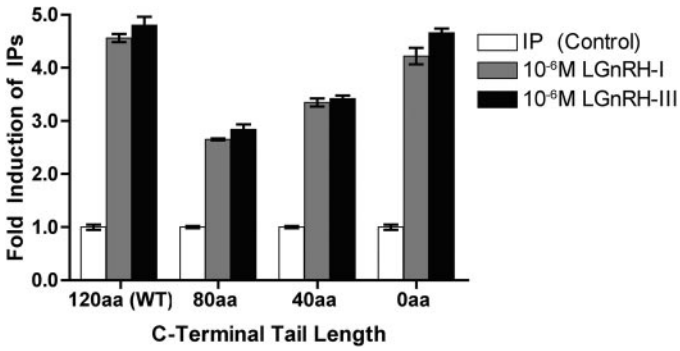

FIG. 5. Functional analysis of the lamprey GnRH receptor intracellular C-terminal tail. The wild-type lamprey GnRH receptor and mutants containing intracellular C-terminal tail truncations were expressed in COS7 cells and treated with a maximum dose (10^{-6} M) of lamprey GnRH-I, lamprey GnRH-III, or control (IP buffer). Functional analysis was performed based on the fold induction of IP stimulation relative to control within each group. Truncation of the lamprey GnRH receptor results in a decrease in IP production, which is fully recovered in the tailless mutant form.

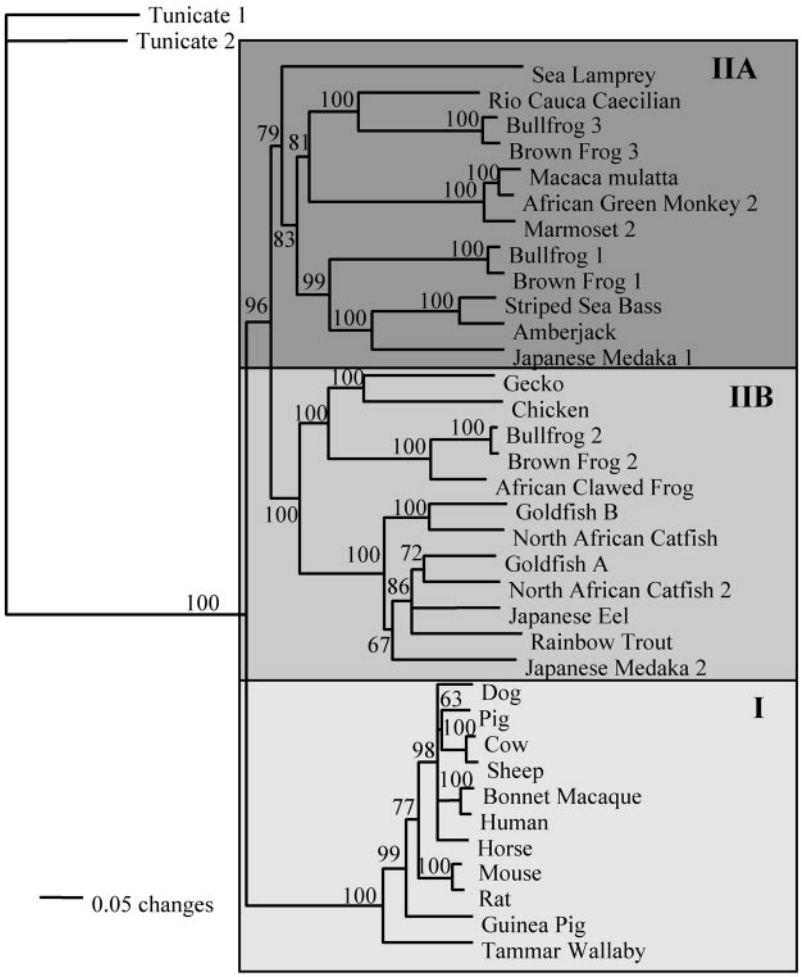

FIG. 6. GnRH receptor phylogenetic tree. This phylogenetic analysis of known GnRH receptor amino acid sequences was performed using the neighbor joining method with 1000 bootstrap replicates. *Dark gray*, Type IIA receptors; *gray*, type IIB receptors; *light gray*, type I receptors. *Numbers* indicate the percentage of bootstrap replicates in which the labeled branch was reproduced.

truncations resulted in an initial decrease in IP production relative to wild type, which was fully recovered by the tailless mutant. Expression of the receptor transcript was demonstrated by RT-PCR in the proximal pars distalis and rostral pars distalis of the pituitary as well as the testes, whereas expression was also visualized in the proximal pars distalis of the juvenile lamprey pituitary by *in situ* hybridization with digoxigenin-labeled riboprobes.

Analysis of the lamprey GnRH receptor amino acid sequence revealed that it contains four potential methionine start codons within the first 13 codons of the reading frame (Fig. 1). It is unknown which one of these start codons is used, but multiple start codons are not unusual in GnRH receptor transcripts (45, 54, 57). The regional comparison of this transcript to other GnRH receptors demonstrates maintenance of the high sequence conservation of the transmembrane helices and intracellular loop 2. The relatively low conservation of extracellular loop 3, the most crucial region of the receptor for ligand specificity (16), was also maintained. The variations in the conserved GPCR motifs were also similar to other type II GnRH receptors identified to date, as was the hydropathy plot of the predicted protein (data not shown).

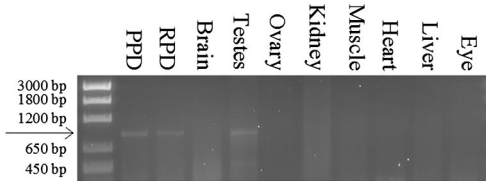

FIG. 7. Tissue-specific expression of the lamprey GnRH receptor transcript. The target product of approximately 850 bases was produced in the proximal pars distalis, rostral pars distalis, and testes. PPD, Proximal pars distalis; RPD, rostral pars distalis; PI, pars intermedia.

Activation of GnRH receptors is known to primarily stimulate the IP_3 second messenger system (2, 31, 50), which in turn leads to several downstream responses including the synthesis and secretion of the gonadotropins. The cloned lamprey GnRH receptor was shown to be functional as it responded in a dose-dependent manner to both lamprey GnRH-I and lamprey GnRH-III via accumulation of IP in transiently transfected COS7 cells. Lamprey GnRH-III was shown to be the most potent of the two forms (see Fig. 4), with a $\log EC_{50}$ approximately 20-fold lower than the $\log EC_{50}$ of lamprey GnRH-I, and therefore may be the native ligand for this cloned receptor. This differential activation correlates well with the previous identification of two high-affinity GnRH binding sites in the lamprey pituitary, further supporting the hypothesis that the lamprey express at least two GnRH receptors (56).

The GnRH receptor family is unique among GPCRs be-

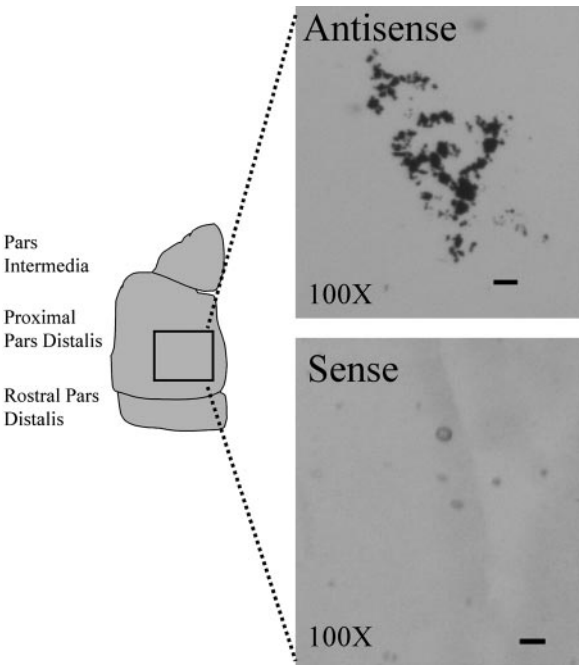

FIG. 8. *In situ* hybridization in the pituitary of a parasitic lamprey. Antisense probe showed expression of the lamprey GnRH receptor transcript in the proximal pars distalis of a parasitic lamprey pituitary. Sense probe in the same pituitary showed no staining. *Bar*, 100 μm. This photomicrograph is representative of two parasitic lampreys.

cause a number of its members lack an intracellular C-terminal tail. All of these tailless receptors have been identified from mammalian species, and because the first six GnRH receptors identified were tailless, it was originally thought that all GnRH receptors lacked a C-terminal tail. In 1997 the first GnRH receptor with a C-terminal tail was identified in the African catfish (53). The implications of this variation in presence or absence of the C-terminal tail remains an important subject of investigation. Various studies have shown that the C-terminal tail of GPCRs has an important role in controlling expression, membrane cycling, desensitization, and G protein activation (2, 58). Progressive truncation of the C-terminal tail of the catfish receptor 1 was shown to drastically and progressively reduce surface expression of the receptor (6, 17), and addition of the catfish GnRH receptor 1 C-terminal tail to the rat GnRH receptor greatly increased the surface expression of the receptor construct (10). The known tailed GnRH receptors contain intracellular tails of varying sizes from 40 to 80 aa in length. The lamprey GnRH receptor includes a C-terminal tail of 120 aa; this is the longest C-terminal tail identified to date in a GnRH receptor. An initial analysis of the functional significance of the lamprey GnRH receptor's lengthy C-terminal tail was performed based on the magnitude of IP signaling using mutants of the lamprey GnRH receptor containing serial truncations of the intracellular C-terminal tail [120 aa (wild type), 80 aa, 40 aa, and 0 aa (tailless)], which were stimulated with a maximal dose (10^{-6} M) of either lamprey GnRH-I or lamprey GnRH-III. Partial truncations of the C-terminal tail resulted in a decrease in the magnitude of IP accumulation, compared with wild type; however, signaling was fully recovered by the tailless mutant of the lamprey GnRH receptor, as shown in Fig. 5. Interpretation of these data can be difficult; however, in conjunction with previous studies of the function of other GnRH receptor C-terminal tails (6, 10, 13, 59), we suggest these findings indicate that truncation of the lamprey GnRH receptor C-terminal tail may cause a structural alteration, which decreases ligand binding affinity and therefore decreases IP signaling. Alternatively, this structural alteration could lead to a disturbance in G protein binding to the activated receptor; however, it may likely be a combination of both (6, 68). Interestingly, the magnitude of IP signaling of the tailless lamprey GnRH receptor is equivalent to the wild type, which we speculate to be due to a compensation resulting from a reduced level of ligand-dependent internalization, which is typically seen in tailless type I GnRH receptors and some tailless type II GnRH mutants (6, 10, 59). Additionally, the long C-terminal tail of the lamprey GnRH receptor may function, in part, to produce the high levels of surface expression demonstrated in our previous GnRH binding studies (56, 60). This analysis indicates the lamprey GnRH receptor possesses several of the conserved structural and functional attributes of both type I and type II GnRH receptors and is therefore likely a representative of the ancestral form. Further analysis of the function of the lamprey GnRH receptor's lengthy C-terminal tail on the level of cell surface expression, ligand binding affinity, and internalization is the subject of ongoing research in our laboratory, which will be necessary to fully describe this unique system.

RT-PCR indicated expression of the lamprey GnRH receptor transcript in both the pituitary and testes. The receptor transcript was strongly detected in the proximal pars distalis of the pituitary and in the testes and was also weakly detected in the rostral pars distalis of the pituitary. These data are consistent with previous GnRH binding studies in which high-affinity binding has been detected in the pituitary and gonad of the adult male and female sea lamprey (56, 60, 61). Pituitary and gonadal expression of GnRH receptor transcripts has also been demonstrated in other models, including mammals and teleost fish (29, 39, 54, 55, 62, 63). *In situ* hybridization with a specific riboprobe to the lamprey GnRH receptor showed moderate levels of expression in the proximal pars distalis of the pituitary (Fig. 8). This distribution of GnRH receptor expression is similar to the distribution of GnRH binding sites characterized in the sea lamprey pituitary by quantitative *in vitro* autoradiography (56, 60). Characterization of GnRH binding sites within the lamprey pituitary at various stages of development has shown a dramatic increase in GnRH receptor expression levels during gonadal maturation (60). These data showed that the levels of GnRH receptor in the pituitary of sexually mature lamprey are high, reaching a maximal binding capacity of 1.0×10^{-11} (60), compared with the range reported in goldfish, 10^{-9} (64). A corresponding increase has been shown in lamprey GnRH concentrations as lampreys mature, with a particularly dramatic increase in the level of lamprey GnRH-III preceding ovulation (65, 66). Pituitary expression and differential activation of the present GnRH receptor strongly indicate that this newly identified GnRH receptor is a key player in the reproductive development and function of the sea lamprey.

The similarity of the lamprey GnRH receptor amino acid

sequence to that of other type II GnRH receptors and its close phylogenetic grouping with the recently identified type II mammalian GnRH receptors suggest that this receptor represents an ancestral form of the type II GnRH receptor (19, 20). The visualization of the lamprey receptor transcript in multiple tissues implies that, like other type II receptors (19, 20, 29, 54, 63, 67), this receptor may have functions outside the pituitary in addition to binding to GnRH in the pituitary.

The phylogenetic relationship of the GnRH receptor family has been described in numerous studies. One classification of GnRH receptors is based on a scheme presented by Troskie *et al.* (16) using the amino acid motifs in extracellular loop 3 to group the GnRH receptors using phylogenetic analysis into two main groups, type I and type II. Millar *et al.* (1) recently provided a new classification of the GnRH receptors (types I, II, and III) using phylogenetic analysis based on the amino acid structures of the full-length cloned receptors. Okubo *et al.* (21) presented an additional phylogenetic analysis of a larger groups of GnRH receptors and used the entire receptor sequence. They identified three main groups as well and suggested grouping of the receptors based on the presence or absence of the C-terminal tail and the intron structure of the GnRH receptor genes (21). Based on our data, we provide a modification in the classification scheme of GnRH receptors. Our phylogenetic analysis, using the full-length GnRH receptor sequences and using virtually all known GnRH receptor sequences, shows three main groupings. These groups agree well with the groups identified by Troskie *et al.* and Okubo *et al.* in the relative members of each group; however, our analysis, like that of Okubo *et al.*, suggests that the two clades that are not exclusively made up of mammalian tailless receptors are more closely related to one another than either is to the mammalian tailless clade. In light of these data, we show that, rather than a subdivision among the type I receptors, the subdivision is more appropriately assigned within the type II receptors.

In conclusion, a full-length cDNA for a GnRH receptor was identified from the sea lamprey pituitary. The coded amino acid sequence of this transcript was similar to that of known GnRH receptors and maintained the conserved properties of these receptors. Stimulation of the cloned lamprey GnRH receptor led to a dose-dependent response in accumulation of IP when treated with either lamprey GnRH-I or lamprey GnRH-III. Functional analysis of the lamprey GnRH receptor C-terminal tail indicates its significant role in IP signaling and internalization. Expression analysis of this transcript by RT-PCR showed that it is detectable in both the pituitary and testes of adult lampreys. Expression mapping of this transcript using *in situ* hybridization in the brain and pituitary of the sea lamprey demonstrated that it is expressed primarily in the proximal pars distalis of the lamprey pituitary, in concurrence with GnRH *in vitro* autoradiography studies in the lamprey pituitary. The current study is the first to identify a pituitary GnRH receptor transcript in an agnathan, the oldest lineage of vertebrates. The phylogenetic placement, structural and functional features of this GnRH receptor suggest that it is representative of an ancestral GnRH receptor. In addition to having an important role in lamprey reproductive processes, the extensive C-terminal tail of this lamprey GnRH receptor has great significance for understanding the evolutionary change of this vital structural feature within the GnRH receptor family.

Acknowledgments

We thank Drs. Beverly Rubin, Stuart Tobet, Karen Carleton, Charles Warren, and Andrew Laudano, whose assistance and expertise were vital to this research. We also thank Drs. Brigitte Troskie, Hiroshi Kawauchi, Masumi Nozaki, Shunsuke Moriyama, Akiyoshi Takahashi, and Kara Lee for their input and assistance. We thank Cari Bourn, Adele Marone, and Brian Sullivan for their technical assistance and Drs. Thomas Foxall, James Haney, and Charles Walker for the use of their microscopy facilities.

Received March 16, 2005. Accepted April 27, 2005.

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This work was supported by National Science Foundation Grants 0090852 and 0421923 (to S.A.S.). This is Scientific Contribution 2193 from the New Hampshire Agricultural Experiment Station.

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