

# Cloning, Characterization, and Tissue Distribution of Prolactin Receptor in the Sea Bream (*Sparus aurata*)

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The prolactin receptor (PRLR) was cloned and its tissue distribution characterized in adults of the protandrous hermaphrodite marine teleost, the sea bream (*Sparus aurata*). An homologous cDNA probe for sea bream PRLR (sbPRLR) was obtained by RT-PCR using gill mRNA. This probe was used to screen intestine and kidney cDNA libraries from which two overlapping clones (1100 and 2425 bp, respectively) were obtained. These clones had 100% sequence identity in the overlapping region (893 bp) and were used to deduce the complete amino acid sequence of sbPRLR. The receptor spans 2640 bp and encodes a protein of 537 amino acids. Features characteristic of PRLR, two pairs of cysteines, WS box, hydrophobic transmembrane domain, box 1, and box 2, were identified and showed a high degree of sequence identity to PRLRs from other vertebrate species. SbPRLR is 29 and 32% identical to tilapia (*Oreochromis niloticus*) and goldfish (*Carassius auratus*) PRLRs, respectively. In the sea bream two PRLR transcripts of 2.8 and 3.2 kb were detected in the intestine, kidney, and gills and a single transcript of 2.8 kb was detected in skin and pituitary by Northern blot. Spermiating gonads (more than 95% male tissue; gonado-somatic index of 0.6) contained, in addition to the 2.8-kb transcript, three more transcripts of 1.9, 1.3, and 1.1 kb. RT-PCR, which is a far more sensitive method than Northern blot, detected PRLR mRNA in gills, intestine, brain, pituitary, kidney, liver, gonads, spleen, head-kidney, heart, muscle, and bone. Immuno-

histochemistry using specific polyclonal antibodies raised against an oligopeptide from the extracellular domain of sbPRLR detected PRLR in several epithelial tissues of juvenile sea bream, including the anterior gut, renal tubule, choroid membrane of the third ventricle, saccus vasculosus, branchial chloride cells, and branchial cartilage. © 2001 Academic Press

**Key Words:** prolactin receptor; marine teleost; osmoregulation; reproduction.

## INTRODUCTION

Prolactin receptors (PRLRs) belong to an extensive family of hormone and cytokine receptors (class 1 cytokine receptor superfamily), which includes growth hormone (GH) and several interleukin receptors (Finidori and Kelly, 1995). These receptors share several structural features in common: an extracellular domain (ECD) with two pairs of cysteines and a conserved motif (WS box) composed of a conserved amino acid sequence, Trp-Ser-X-Trp-Ser, where X can be any amino acid, a single-transmembrane domain composed largely of hydrophobic amino acids, and an intracellular domain (ICD). The latter domain contains a proline-rich region (box 1), lying immediately adjacent to the membrane, which is composed largely of negatively charged residues and is followed by an-

other consensus region (box 2), comprising a series of hydrophobic residues followed by negatively charged residues (Bole-Feysot *et al.*, 1998; Kelly *et al.*, 1993, 1991b). These two highly conserved motifs are essential for receptor activity (Goupille *et al.*, 1997).

A PRLR cDNA was first cloned from rat liver (Boutin *et al.*, 1988) and shown to encode a protein of 291 amino acid residues; subsequently, a longer form (591 aa) of the receptor was identified in rat liver and ovary. PRLR cDNAs have now been cloned from species in all vertebrate groups except reptiles (for review see Bole-Feysot *et al.*, 1998). Short and long forms of the PRLR have been identified in rat, mouse, and sheep (Davis and Linzer, 1989; Moore and Oka, 1993; Bignon *et al.*, 1997). These two receptors differ only in the ICD, which is 357 amino acids in the long form and 57 amino acids in the short form which lacks box 2 (Shirota *et al.*, 1990; Bole-Feysot *et al.*, 1998). A third form of PRLR has also been identified in the rat PRL-dependent immune cell line (Nb2); this form contains a partial deletion of the cytoplasmic domain (Ali *et al.*, 1991). Short and long forms of PRLRs originate from alternative splicing of the mRNA transcribed from a single gene (Ormandy *et al.*, 1998) and only long forms are capable of inducing the transcription of milk proteins, whereas short forms are capable of inducing cell proliferation (Das and Vonderhaar, 1995). A single form of PRLR has been identified in birds, which most closely resembles the long form found in mammals, with the exception of a tandem repeat within the ECD (Chen and Horseman, 1994; Zhou *et al.*, 1996). In tetrapods, PRLR has a widespread distribution and has been identified in brain and pituitary, liver, kidney, lungs, skin, gonads and accessory glands, skeletal muscle, and tissues of the immune system (for review Bole-Feysot *et al.*, 1998); it has also recently been detected in bone (Clement-Lacroix *et al.*, 1999).

PRLRs have also been cloned in an amphibian (Yamamoto *et al.*, 1998), in a euryhaline teleost, Nile tilapia (*Oreochromis niloticus*) (tiPRLR) (Sandra *et al.*, 1995), and in a fresh water teleost, the goldfish (*Carassius auratus*) (gf PRLR) (Tse *et al.*, 2000). In tilapia there is a single transcript of 3.2 kb which shares greatest similarity with the long forms of mammalian PRLRs, although the overall homology is low (37%) (Sandra *et al.*, 1995, 2000). In the goldfish two transcripts, of 3.5 and 4.6 kb, were identified. The 4.6-kb transcript encodes a 600-amino acid protein 48.3%

identical to tiPRLR (Tse *et al.*, 2000). The expression of PRLR was analyzed in tilapia by Northern blot and the results obtained demonstrated that the osmoregulatory organs, gills, kidney, and intestine expressed PRLR at higher levels. The ovary, testis, and head-kidney (which has hematopoietic function in fish) also expressed PRLR but at much lower levels (Sandra *et al.*, 1995, 2000). A similar distribution of PRLR was also observed in the goldfish (Tse *et al.*, 2000). These results confirmed the results obtained with homologous ligand binding studies in the gills and kidney of tilapia (Auperin *et al.*, 1994) and identified alternative target tissues for PRL in fish. The high levels of PRLR expression in osmoregulatory tissues of fish corroborate the well-documented action of PRL in the maintenance of hydromineral balance in euryhaline teleosts (Bern, 1975).

The function of PRL in marine teleosts is unclear, particularly since in euryhaline fish its principal role appears to be in freshwater adaptation and it inhibits seawater adaptation. High levels of expression of PRL have been reported in the sea bream pituitary gland (Santos *et al.*, 1999) but relatively little is known about its physiological role in marine fish. In the present study the cloning of PRLR cDNA from a marine teleost, the sea bream (*Sparus aurata*), is described and its expression and tissue localization in adult fish is characterized. In addition, part of the sbPRLR gene was cloned and compared with the gene for mouse PRLR.

## MATERIALS AND METHODS

### *Animals and Tissues*

Adult sea bream (~350 g) maintained in through-flow seawater tanks at  $17 \pm 2^\circ$  under natural photoperiod for winter in the Algarve, Portugal were killed by stunning and decapitation. Liver, kidney, intestine, brain, pituitary, skin, gills, skeletal muscle, heart, bone, spleen, head-kidney, and mature gonadal tissue (more than 95% male tissue; gonado-somatic index of 0.6) were frozen in liquid nitrogen and stored at  $-70^\circ$ .

### *Total RNA and mRNA Purification*

Total RNA was extracted from the tissues mentioned above using "TRI reagent" (Sigma, St. Louis,

MO). The poly(A)<sup>+</sup> RNA fraction was obtained from total RNA by chromatography on columns of oligo(dT) cellulose (Aviv and Leder, 1972).

### Generation of a Homologous sbPRLR cDNA Probe by RT-PCR

PCR primers were designed within the most highly conserved regions of PRLRs identified after multiple sequence alignments of all PRLR sequences available (GenBank). A forward primer based on a highly conserved region of the ECD domain of PRLR (TFTCWW), 5' ACA TTC ACC TGC TGG TGG3' (Pharmacia Biotech, Uppsala, Sweden) (primer PF1), was synthesized and the reverse primer, in which inosine (I) was introduced to minimize degeneracy at the 3' end 5' GAT CTT TGG CAC IGG IGG3' (primer PR1), was located within the proline-rich motif (PPVPGPKI).

cDNA was synthesized from 1 µg of gill RNA poly(A)<sup>+</sup> in a 30-µl reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol (DTT), 1 mM dNTP, 15 pmol oligo(dT) primer, RNase inhibitor (3.2 U; Pharmacia Biotech), and M-MLV reverse transcriptase (20 U; Gibco BRL, Barcelona), for 2 h at 37°. PCR was carried out in a 50-µl reaction containing 5 µl of gill cDNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl<sub>2</sub>, 1 mM dNTP, 40 pmol of forward primer, 200 pmol of reverse primer, and *Taq* DNA polymerase (1.25 U; Promega, Madison, WI); sterile water substituted for cDNA in control reactions.

The reaction was initially denatured for 5 min and then submitted to 45 cycles of denaturing (94°, 1 min), annealing (52°, 2 min), and extension (72°, 1 min) in a thermocycler (Robocycler; Stratagene, La Jolla, CA). The amplified products were analyzed on a 1.5% agarose gel containing ethidium bromide (0.5 µg/µl), excised from the gel, and purified using a column containing a silica-gel membrane (Quiagen).

The purified DNAs were ligated into pGEM-T vector (Promega) in a 10-µl reaction containing purified DNA (7 µl), 1 µl of T4 DNA ligase (3 U/ml), 1 µl of pGEM-T (50 ng), and 1 µl of 10× buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl<sub>2</sub>, 100 mM DTT, and 10 mM ATP), incubated overnight at 4°, and used to transform *Escherichia coli* (XL1B MRF' strain). DNA was isolated from clones and analyzed in 1.5% agarose

gels after restriction digest. Sequencing was carried out using a modification of the dideoxy chain termination method (Sanger *et al.*, 1977; T7 sequencing Kit; Pharmacia Biotech).

### Construction of Sea Bream Intestine and Kidney cDNA Libraries

cDNA libraries were constructed from 5 µg of sea bream intestine and kidney poly(A)<sup>+</sup> RNA using the Lambda ZAP cDNA and UNI-ZAP XR cDNA cloning kits (Stratagene), respectively. The double-stranded cDNA was ligated into the corresponding vectors and packaged into Gigapack Gold III packaging extracts (Stratagene).

### Screening of cDNA Libraries

The sea bream intestine library was screened with a [ $\alpha$ -<sup>32</sup>P]dCTP-labeled sbPRLR probe (Rediprime, random labeling kit; Amersham, Little Chalfont, UK) generated by RT-PCR. Filters were hybridized overnight at 65° and washed at 65° in 0.1× SSC/0.1% SDS. Two positive plaques were obtained from 400,000 recombinants, isolated, automatically excised into pBluescript (Stratagene), and sequenced using an adaptation of the dideoxy chain termination method (Sanger *et al.*, 1977) with the universal primers within the flanking regions of the polylinker of pBluescript. This clone, named I3a2.2, was then used to screen the kidney cDNA library, and one positive plaque was isolated from 400,000 recombinants. This clone, named KA13.1, was also sequenced from the 3' and 5' ends using internal walking primers designed from the newly achieved sequence (Fig. 1).

### Northern Blot Analysis

Two micrograms of mRNA from sea bream liver, kidney, intestine, brain, pituitary, skin, gill, head-kidney, spleen, and skeletal muscle and 10 µg of gonad mRNA were fractionated on a 5.5% formaldehyde/1.5% agarose gel, transferred to a nylon filter (Hybond-N; Amersham) with 10× SSC, and cross-linked at 80° for 2 h. Prior to hybridization the filter was washed at 60° for 20 min in 1× SSC, 0.1% SDS and prehybridized in 50% formamide, 50 mM NaPO<sub>4</sub>, 5× Denharts, 0.1% SDS, 5× SSC, 50 µg/ml DNA from calf

thymus for 4 h at 42°. Hybridization was allowed to proceed overnight at 42° in fresh prehybridization solution containing sbPRLR clone I3A2.2 labeled with [ $\alpha$ -<sup>32</sup>P]dCTP. Filters were then washed for 30 min at 42° in prehybridization solution and stringency washes carried out at 55° for 15 min in 1× SSC, 0.1% SDS and then at 60° for 30 min. The membrane was exposed to Biomax MS film (Kodak, Rochester, NY) with intensifying screens at -70° for 5 days. To evaluate the relative amounts of mRNA for each tissue, the blot was stripped with a boiling solution of 0.1% SDS and hybridized with a sea bream  $\beta$ -actin probe (Santos *et al.*, 1997) following the same protocol as before but reducing exposure time to 1 h.

### *Tissue Distribution by RT-PCR*

The tissue expression of sbPRLR was also analyzed with the more sensitive method of RT-PCR. Specific primers were designed using the Primer Premier computer program (Version 4.04; Premier Biosoft International) based on the sbPRLR sequence. Forward primer (PF2) (5'AGTCCGGCTGGGTCACCATTA) and reverse primer (PR2) (5'GGTGGCGACCAAGATCCAAAAC3') were synthesized by MWG-Biotech GmbH (Germany) and were predicted to amplify a fragment of 249 bp.

cDNA was synthesized in a 30- $\mu$ l reaction as previously described from total RNA (0.5  $\mu$ g) from the following tissue samples: liver, kidney, intestine, brain, pituitary, skin, gills, heart, skeletal muscle, bone, ovary, and testis. As a negative control, a PCR which contained all the reagents, but in which cDNA was omitted, was run simultaneously. RT-PCR was carried out with this cDNA (5  $\mu$ l) in a 50- $\mu$ l reaction as previously described, with the exception that a lower concentration of MgCl<sub>2</sub> (1.5 mM), 50 pmol of each primer, and *Taq* DNA polymerase (1.25 U; Promega) were used. The thermocycling protocol (Robocycler; Stratagene) was as follows: initial denaturing step (94°, 2 min) followed by 35 cycles of denaturation (94°, 1 min), annealing (56°, 1 min), and extension (72°, 30 s).

### *Southern Blot Analysis*

PCR products were separated on a TBE/agarose gel (1.5%). The gel was soaked in denaturing solution (0.5

M NaOH, 1.5 M NaCl) for 15 min with gentle agitation, washed twice for 2 min in sterile water, and soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 30 min. Transfer to a nitrocellulose membrane (Hybond C; Amersham) was carried out using 10× SSC as transfer buffer. The membrane was baked for 2 h at 80° and hybridized using sbPRLR clone I3A2.2 as the probe and the same procedure described for cDNA library screening. The blot was exposed for 5 min and 2 h at room temperature (24°).

### *Partial Cloning of sbPRLR Gene*

Genomic DNA was extracted using TRI reagent (Sigma). The PCR protocol already described above for analyzing sbPRLR expression in adult tissue was applied to approximately 10 ng of sea bream genomic DNA using primers PF2 and PR2. The product obtained was cloned into pGEM-T vector using the protocol already described for cloning the sbPRLR probe and its sequence was determined.

### *Tissue Distribution by Immunohistochemistry (IHC)*

The methodology used for antibody production is detailed in Nevalainen *et al.* (1996). Briefly, an oligopeptide from the extracellular domain of sbPRLR was synthesized by Sheffield University Krebs Institute Molecular Synthesis Service using Applied Biosystems Model 476A. The chosen sequence, 35–49 (RLYY-ERERLEGVHEC) from the extracellular domain of the sbPRLR, was conjugated to bovine thyroglobulin (Sigma T-1001) using carbodiimide. The oligopeptide was chosen on the basis of amino acid composition and because a similar region of rat prolactin receptor has been found to induce high-quality antisera used for detecting PRLR in many mammalian tissues. The conjugate was dissolved in normal saline, emulsified in Freund's Complete Adjuvant, and injected intramuscularly into rabbits. A second injection of antigen, emulsified in Freund's Incomplete Adjuvant, was given 2 months later, and after an initial test bleed the serum was collected 14 days after the second injection.

Immunohistochemistry was carried out using a modification of Sternberger (1974). Briefly, tissues were fixed in sublimated Bouin-Hollande (Kraicer *et al.*, 1967). Sections were cut at 4  $\mu$ m and mounted on APES-coated slides. For IHC, sections were dewaxed

in xylene and rehydrated in graded alcohols, and heavy metal ions were removed by immersion in 1% iodine in 70% ethanol and reduced in 5% sodium thiosulphate. For the IHC procedure, sections were immersed first in 1% hydrogen peroxide in phosphate buffer (100 mM, pH 7.4) containing 20% methanol to destroy endogenous peroxidase; then nonspecific binding sites were blocked using 4% swine serum in 1% bovine serum albumin (BSA) in phosphate buffer. Sections were then covered in the primary rabbit antiserum to sea bream PRLRs diluted in phosphate-buffered saline (PBS)/BSA (1/200) and incubated at 4° overnight; control slides received normal rabbit serum instead of specific primary antiserum. After washing, the sections were incubated at room temperature (ca. 20°) in swine anti-rabbit serum diluted 1/50 (Dako, Denmark) and then in peroxidase/anti-peroxidase reagent (Dako). Color was generated by immersion in diaminobenzidine in Tris-HCl buffer, pH 7.6. Sections were dehydrated, cleared, and mounted in Depex. Cross-reaction to bovine thyroglobulin was checked by absorption of diluted antiserum with 500 µg/ml of bovine thyroglobulin and testing on sections of larvae containing thyroid follicles.

## RESULTS

### *sbPRLR Probe*

A cDNA fragment spanning 429 bp was obtained by RT-PCR using primers PF1 and PR1. The sequence of this fragment was compared to sequences available in the EMBL databases (NCBI, BLASTX) and was found to have the highest homology with tiPRLR (43% identity). The sea bream RT-PCR product was found to align with tiPRLR in the region predicted from primer localization and consisted of

part of the ECD and part of the ICD. The PCR product was of a smaller size than predicted as the forward primer annealed downstream to the region for which it was designed. In addition to the overall sequence similarity to tiPRLR, other structural features characteristic of PRLR were also present in this fragment, such as the WS box (WSEWT) and a 24-amino acid transmembrane domain with a high content of hydrophobic amino acids.

### *cDNA Library Screening*

The RT-PCR product was used as a probe to screen a sea bream intestine cDNA library containing 450,000 primary recombinants. Two clones were isolated from 400,000 recombinants; the clone inserts were excised and sized by agarose gel electrophoresis, where they were observed to be of a similar size (1100 bp). Sequencing revealed that one insert was 23 bp longer at the 5' end (clone I3A2.2). Both inserts shared 100% identity in the overlap. The deduced amino acid sequence showed that they encoded the complete ECD, the transmembrane domain, and part of the cytoplasmic domain (Fig. 1). The longer clone (I3A2.2) was then used to screen the sea bream kidney cDNA library ( $2 \times 10^6$  primary recombinants) from which another clone, 2425-bp long, was isolated from 400,000 recombinants. This clone was incomplete at the 5' end. Alignment of the sequences of the clones obtained from the intestine and kidney libraries showed that both were 100% identical in the overlap (893 bp) and, in combination, permitted elucidation of the full-length sequence of sbPRLR.

SbPRLR spans 2.6 kb and contains an open reading frame (ORF) encoding a protein of 537 amino acids (Fig. 1). This ORF consists of a signal peptide of 20 amino acids followed by a mature protein of 517 amino acids. The 5' untranslated region (UTR) com-

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FIG. 1. Nucleotide sequence and deduced amino acid sequence of the sea bream PRLR. The sequence underlined comprises the overlapping region between clones I3A2.2. and KA13.1. The cysteine residues are circled; the four potential N-glycosylation sites are enclosed by pentagons. The WS motif is boxed and the transmembrane domain is in italics. Within the intracellular domain, box 1 (next to the transmembrane domain) and box 2 are boxed. Single tyrosine residues are enclosed by a star. In the 3' untranslated region three potential polyadenylation signals are printed in boldface and underlined. Nucleotide sequences printed in boldface correspond to the primers used to analyze tissue distribution by RT-PCR. Walking primers used to elucidate the complete nucleotide sequence of sbPRLR are enclosed by an arrow.

-120 cgttcacgacaatggtgtggccgtaggaggtctctgttcgcaacatggagttcagtgactcagtcggtgtaggtcgacact -41  
 -21 M W R D L G L A V L -12  
 -40 cgtcctcagctggacacactgcgggacagtggcgccagg ATG TGG AGA GAT CTT GGA TTG GCA GTT CTT 30  
 -11 V L L S A A V E S N S T S P P G K P V L 9  
 31 GTG CTG CTG TCT GCA GCT GTA GAG TCC AAC AGC ACG TCT CCA CCA GGG AAA CCG GTC CTG 90  
 10 L S C R S P E K E T F T C W W E P G S D 29  
 91 CTC AGC TGC AGG TCC CCT GAG AAG GAG ACG TTT ACC TGC TGG TGG GAC CCA GGC TCT GAT 150  
 30 G G L P T T Y R L Y Y E R E R L E G V H 49  
 151 GGA GGG CTT CCC ACC ACG TAC CGA CTC TAT TAT GAG AGA GAA AGA TTG GAG GGA GTG CAC 210  
 50 E C P D Y W S A G S N S C F F N K T H T 69  
 211 GAG TGT CCG GAC TAT TGG TCA GCA GGT AGC AAC TCC TGT TTC TTC AAC AAG ACC CAC ACC 270  
 70 L I W V D Y Y L T V V A S N A L G N A T 89  
 271 TTA ATC TGG GTG GAC TAC TAC CTG ACC GTG GTG GCC TCC AAT GCC CTC GGG AAT/GCC ACC 330  
 90 S D I F K M D V M E I M K P N V P E N V 109  
 331 TCA GAC ATC TTT AAA ATG GAC GTG ATG GAA ATC ATG AAG CCC AAT GTT CCT GAA AAT/GTA 390  
 110 T L L V V E T E D S P Y L H I R W E H P 129  
 391 ACG CTG CTG GTG GTG GAG ACG GAG GAC AGT CCA TAT CTC CAT ATC AGA TGG GAA CAT CCC 450  
 130 R N T D T E S G W V T I K Y E L R I K Q 149  
 451 CGT AAC ACA GAC ACC GAG TCC GGC TGG GTC ACC ATT AAA TAT GAA CTG AAG ATC AAA CAA 510  
 150 E K N N K W K E Y M S G T Q S H F S L Y 169  
 511 GAA AAA AAC AAC AAG TGG AAG GAG TAC ATG TCA GGT ACA CAA AGC CAT TTC AGC CTG TAC 570  
 170 S I A P G G V Y T V Q V R C K L D H G S 189  
 571 AGC ATC GCT CPT GGA GGG GTT TAC ACA GTT CAG GTG CGC TGT AAA CTG GAC CAC GGA TCC 630  
 190 W S E W T N S T N V K V P N Y V Q N E R 209  
 631 TGG AGT GAG TGG ACC AAC AAT ACC AAC GTG AAG GTT CCA AAC TAT GTT CAA AAT GAG AGA 690  
 210 S F T W I L V A T L S A I P F M A A M C T 229  
 691 TCG TTT TGG ATC TTG GTC GCC ACC CTG TCT CAC ATT CCA TTT ATG GCA GCA ATG TGC ACC 750  
 230 L I V K R E N V K Q C V L P P V P G P K 249  
 751 TTG ATC GTG AAG AGG GAA AAT GTG AAG CAG TGT GTT CTG CCT CCT GTT CCT GGT CCA AAG 810  
 250 I R G V D F H L L M S G Q S E D S T N A 269  
 811 ATC AGA GGA GTT GAT TTT CAC CTC CTC ATG AGT GGA CAA TCT GAA GAC AGC ACC AAT GCC 870  
 270 L I I N Q N F P L I V G W K D Q M E E 289  
 871 CTG ATC ATC AAC CAG AAC TTT CCT CTC ATT GTG GGC TGG AAA GAC CAG ATG GAG GAG TAC 930  
 290 L I V T E N D I G P P S D A S H C Q K R 309  
 931 CTC ATC GTG ACA GAG AAT GAC ATC GGG CCT CCG TCG GAT GCC TCC CAT TGT CAA AAA AGG 990  
 310 K K S L I I P A G G F C S D W E I Q C K S 329  
 991 AAA AAG AGC TTG ATT ATT CCG GCT GGC TTC TGC TCA GAC TGG GAA ATC CAA TGC AAG TCA 1050  
 330 T H I Q N D C A G G T K N E T G N F P K 349  
 1051 ACC CAC ATT CAG AAT GAC TGT GCA GGG GGA ACA AAG AAT GAA ACG GGT AAC TTT CCC AAG 1110  
 350 N N K S L S G E S L S M E P S K L Q K 369  
 1111 AAC AAT AAG TCT CTC TCA GGG GAG AGT TTA TCT TAC ATG GAG CCA TCC AAG CTG CAG AAA 1170  
 370 Q Q C L G Q N F V N T E A T D P S P L N 389  
 1171 CAG CAG TGT TTA GGT CAA AAC TTT GTG AAC ACA GAA GCA ACA GAT CCC AGT CCA TTG AAC 1230  
 390 D N A V K P F T N S G V D I R K H V 409  
 1231 TAC GAC AAT GCT GTC AAA CCC TTC ACA AAC AGC GGC TAT GTG GAT ATT CGG AAA CAT GTG 1290  
 410 E N T Q E V N V K Q V D S R V E D V N 429  
 1291 GAG AAC ACG CAG GAG GTG AAT GTG AAA CAG GTG GAC TAC AGC AGA GTG GAA GAT GTG AAT 1350  
 430 G D N V F I L N N K N L A F N S S G M 449  
 1351 GGT GAC AAT GTG TTC ATC CTA AAC AAC AAA AAT CTT GCA TTT AAC AGC TCT GGC TAC ATG 1410  
 450 D F Q R Q E E K L S E D S R V K E V G 469  
 1411 GAT TTT CAG AGR CAA GAG GAG AAA CTA TCA GAG GAT TAC AGC AGG VTG AAA GAG VTG GGC 1470  
 470 S N N V V V L Q I Q D M S A D T K N P H 489  
 1471 AGT AAC AAT GTG GTG GTC CTG CAG ATA CAA GAC ATG TCA GCT GAC ACT AAA AAT CCT CAT 1530  
 490 A T G A H K M G V C T E L I N S E I D 509  
 1531 GCG ACT GGA GCC CAC AAA ATG GGG GTG TGC ACA GAA CTC ATC AAT AGC GAA TAT ATA GAT 1590  
 510 T I P A P P L M \* 518  
 1591 ACC ATC CCT GCA CCA CCT TTA ATG TAA tgtttagtttaacatgcaatgtccaaatacaaatggctgatgtc 1661  
 1662 ccoctatgatttactgttcagagtttaataaaaaggctgcaggttattccatagttttctcaataataactctgaggagtttcc 1741  
 1742 atgacatttggcaagagacatttcgtggtcaccagaagataaaacttaccgacttcagtgataccctgaacttccctacag 1821  
 1822 caccatgaagttgcccgttttgggttcagagtgaaatattcttgacacttctgaatttattgtgtgaaatttgctacatat 1901  
 1902 attcaggggtccccagagataaaacctaaagatcttgggtgacctgactctccatcgagccccataatcatgccaaagttt 1981  
 1982 caatcgctccaataactttggtaaaataaaatataaccagacttactgtatataacagtagtttgaattgtaatacattaca 2061  
 2062 taataggatgtaacttttttggctcaggctcaggtaactttttacaactctgaaaagaaaaaactctgccactcctt 2141  
 2142 ttggatccttaccactactgggacacctgatgtattataacaacatattgatgtactgaagcacaggtcatgtctggtgtg 2221  
 2222 aagtaaaaccatgtgataagatgattactctcagacagggatatttactccttggtaactatggattccttactctga 2301  
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 2382 ttgtagaggaagtactgtatttgaacttcggtaaaatagcaataactacagttgtaaaaatccatgtacacgttaattgtcct 2461  
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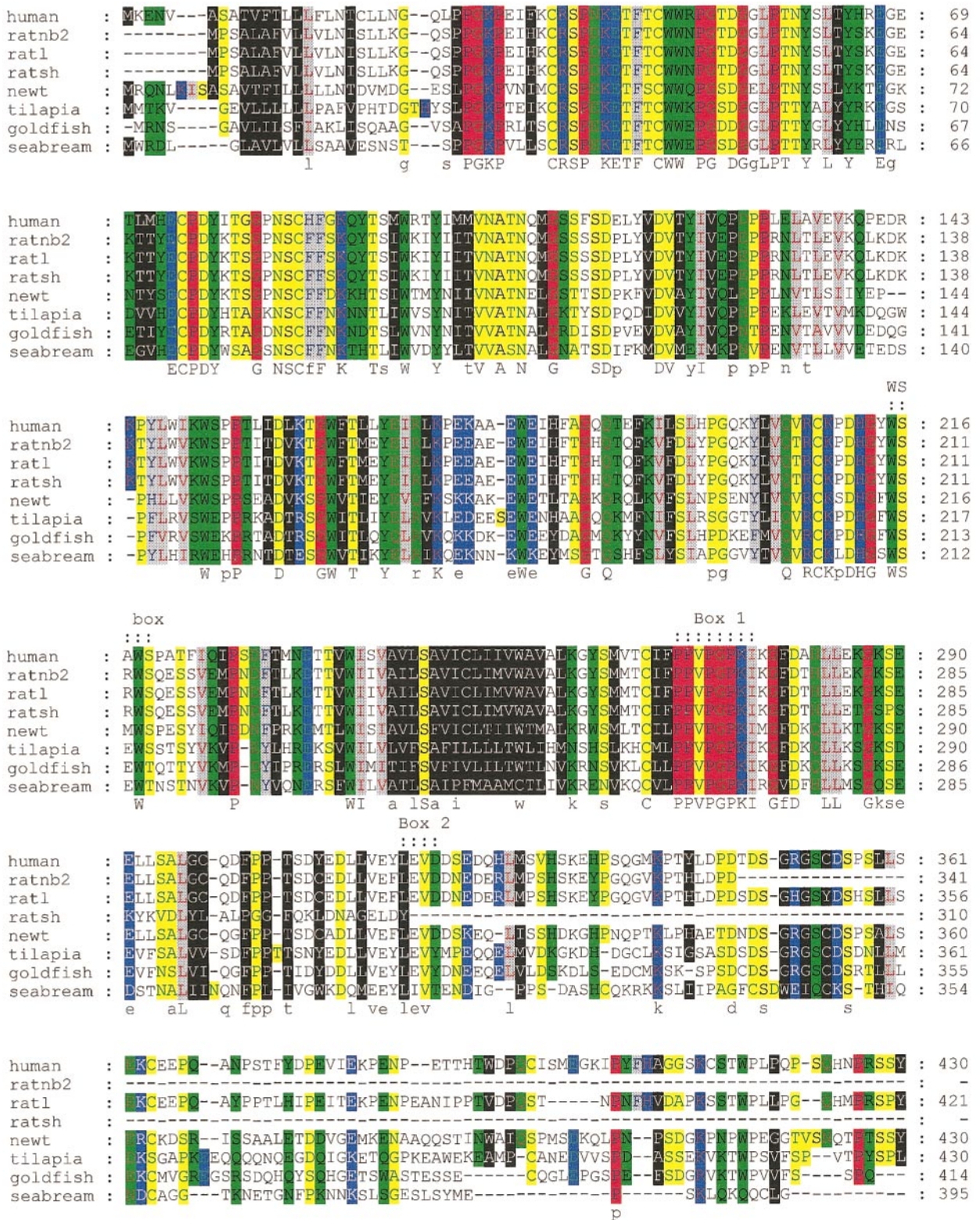


FIG. 2. Multiple sequence alignment of sea bream PRLR (sbPRLR) with PRLR from several other vertebrates. Colored blocks refer to the physicochemical properties of the amino acids according to the key. Short form of rat PRLR, ratsh; intermediate form of rat PRLR, ratnb2; long form of rat PRLR, ratl; newt PRLR, newt; tilapia PRLR, tilapia; goldfish PRLR, goldfish. On the last line of each block, amino acids identical in all species are indicated by capital letters; small letters represent the most common amino acid in that position. WS box, box 1, and box 2 are indicated.

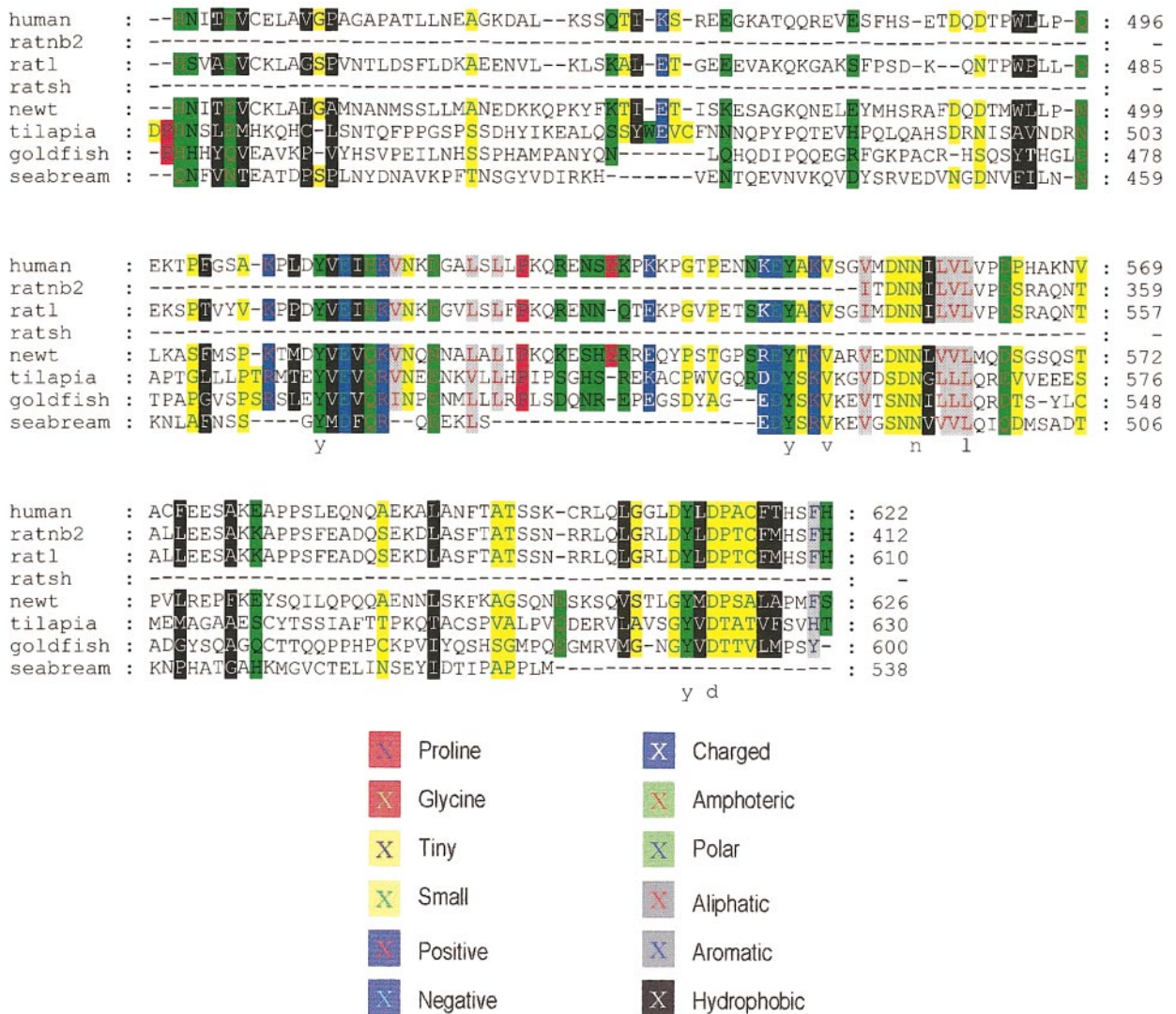


FIG. 2—Continued

prises 120 nucleotides and the 3'UTR comprises 906 nucleotides. Three clear polyadenylation signals are present at positions 1687, 1919, and 2004 and precede the poly(A) tail.

All the characteristic structural features of PRLRs are present (Fig. 1): two pairs of cysteine residues in the ECD at positions 12, 22, 51, and 62, a WS box in which the last serine was conservatively substituted by threonine, the transmembrane domain comprising 24 hydrophobic amino acids and box 1, and a proline-rich region (PPVPGPKI) in the transmembrane proximal to ICD at position 243. Four potential N-linked glycosylation sites were identified in

the ECD. Within the ICD tyrosine residues were found at positions 289, 361, 390, 402, 422, 448, 462, and 507 (Fig. 1).

Multiple sequence alignment of sbPRLR with other previously isolated PRLRs (Fig. 2) shows that the overall identity of sbPRLR with tilapia and goldfish PRLR is 29 and 32%, respectively, and that with mammalian and amphibian counterparts is 23–27% (Table 1). However, if the physicochemical properties of the amino acids are taken into consideration, receptor sequence conservation is remarkable, not only between fish but also among all vertebrates (Fig. 2).



**TABLE 1**  
Amino Acid Identities (%) in PRLRs from Several Species

	Human	Ratnb2	Rat1	Ratsh	Newt	Tilapia	Goldfish	Seabream
Human	—	49	66	33	48	35	33	26
Ratnb2	—	—	67	68	35	28	28	25
Rat1	—	—	—	46	46	34	33	26
Ratsh	—	—	—	—	25	22	22	23
Newt	—	—	—	—	—	34	36	27
Tilapia	—	—	—	—	—	—	46	29
Goldfish	—	—	—	—	—	—	—	32

*Note.* Short form of rat PRLR, ratsh; intermediate form of rat PRLR, ratnb2; long form of rat PRLR, rat1; newt PRLR, newt; tilapia PRLR, tilapia; goldfish PRLR, goldfish.

### Partial Genomic Sequence of *sbPRLR*

PCR of sea bream genomic DNA using primers PR1 and PR2 yielded a 1000-bp fragment. This fragment contains three exons separated by two introns of 540 and 38 bp (Fig. 3). The intron–exon boundaries follow the gt/ag rule (Mount, 1982) and the organization appears to be conserved compared with mouse PRLR gene (Ormandy *et al.*, 1998).

### Expression Analysis of *sbPRLR* by Northern Blot

Northern blot analysis using 2  $\mu$ g of mRNA (Figs. 4A and 4B) demonstrated that PRLR is encoded by two transcripts of 2.8 and 3.2 kb in the intestine, kidney, and gills. In the pituitary and skin a single transcript of 2.8 kb was observed. When 10  $\mu$ g of mRNA extracted from spermiating gonads, which contained more than 95% male tissue (Fig. 4C), was

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      S   G   W   V   T   I   K   Y   E   L   R   I   K   Q   E   K
AG TCC GGC TGG GTC ACC ATT AAA TAT GAA CTA AGA ATC AAA CAA GAA AAA
      N   N   K   W   K
AAC AAC AAG TGG AAG gtg agt ttt aat gag ttt taa tca aca gaa tct
cca aca gaa gga tcc gct agt gct aaa gcc gtc act cgg ata ggt cat
ttt acg aca ggg gtc ggt act aga caa tag tta act ccg tgt tat aaa
tgt tta tga gtc tgt tac agg ttt att tta gtg ctg aaa caa tca att
gac tca gca aca act cac aat tta aac aag gga ctg tct ggt aac aaa
ctg tat gct ata gat gta att tac ttc agt att gga gta aaa taa cta
gtc ttt aaa aac tga aaa gac taa aga agt gcc taa cga att tca agg
tga ggt cat agt agt gtg atc caa aga tga ggg gtt tca tag gaa ctt
ttg aaa gta aat gtt caa gtt gtt act tag cca gcc agt aga ggg ctg
cct cca gaa ctt tca gca gtc aag ttg atc caa ttc cat act tta ccc
ttt tta taa aca ccc aca cac att tga ccc aca tat cat tta acc tgc
      E   Y   M   S   G   T   Q
gta caa tgt tca tta ttt gtt ctt cag GAG TAC ATG TCA GGT ACA CAA
      S   H   F   S   L   Y   S   I   A   P   G   G   V   Y   T   V
AGC CAT TTC AGC CTG TAC AGC ATC GCT CCT GGA GGG GTT TAC ACA GTT
      Q   V   R   C   K   L   D   H   G   S   W   S   E   W   T   N
CAG GTG CGC TGT AAA CTG GAC CAC GGA TCC TGG AGT GAG TGG ACC AAC
      S   T   N   V   K   V   P   N
AGT ACC AAT GTG AAG GTT CCC AAC tgt gag tgt caa aaa gtc ttt cct
cca tct ttg act cac cag tga ggt ggc cac tat tat taa ctt gaa aat
      Y   V   Q   N   E   R   S   F   W   I   L
cac ctt ttg ttt tag TAT GTT CAA AAT GAG AGA TCG TTT TGG ATC TTG
      V   A   T
GTC GCC ACC A

```

**FIG. 3.** Nucleotide sequence and deduced amino acid sequence of the PCR-amplified genomic sequence of sea bream PRLR. The primers used are indicated by boldface/italic. The nucleotides corresponding to coding regions are in capitals and those within the noncoding regions are in lowercase letters.

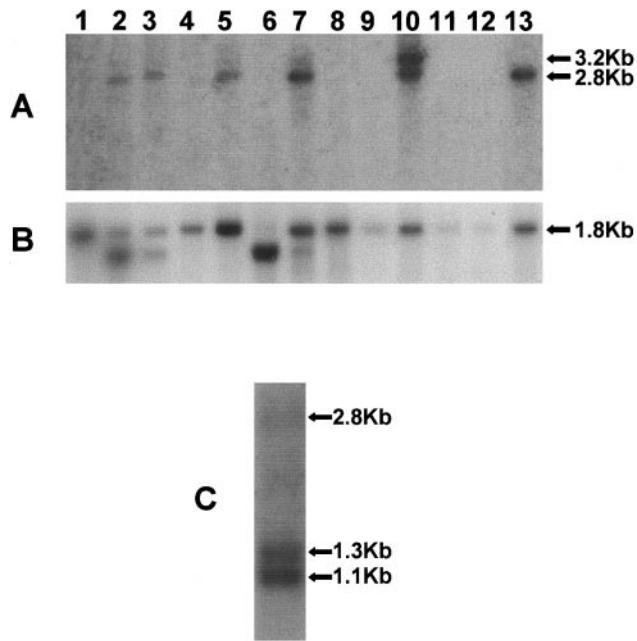


FIG. 4. (A) Northern blot analysis of approximately equivalent quantities of mRNA ( $2 \mu\text{g}$ ) from several sea bream tissues. With the exception of sample 2, all are from adult fish: 1, head-kidney; 2, larvae; 3, skin; 4, brain; 5, kidney; 6, muscle; 7, gill; 8, spleen; 9, liver; 10, intestine; 11, testis; 12, ovary; 13, pituitary. Hybridization was carried out with sbPRLR (clone I3A2). (B) Results obtained from the hybridization of the same filter with sea bream  $\beta$ -actin. (C) Northern blot analysis of mRNA ( $10 \mu\text{g}$ ) extracted from mature (spermiating) male gonads. Hybridization was carried out with sbPRLR (clone I3A2). The arrows on the right side of the filter indicate transcript size.

analyzed, the major transcripts identified were 1.3 and 1.1 kb. The 2.8-kb transcript, which was the principle transcript in larvae, skin, kidney, gill, and pituitary, was present, but at low levels, in gonads and a further transcript of 1.9 kb was observed in gonads. Intestine, gill, kidney, and pituitary had the highest levels of PRLR expression and no signal was detected in the other tissues analyzed using the same amount of mRNA (Fig. 4A).

### Tissue Distribution by RT-PCR

RT-PCR with sea bream receptor-specific primers of approximately equivalent quantities of cDNA from kidney, skin, gill, intestine, brain, testis, ovary, liver, skeletal muscle, heart, and bone resulted in detection of transcripts in gill and intestine (Figs. 5A

and 5B). The Southern blot of these reactions also permitted detection of PRLR transcripts in skin, kidney, brain, testis, ovary, and liver (Fig. 5C). Although no signal was visible in skeletal muscle, heart, and bone after the blot had been exposed for 5 min (Fig. 5C), longer exposure time (2 h) permitted detection of PRLR transcripts in these tissues (Fig. 5D).

### Immunohistochemistry of Sea Bream Tissues

Only tissues from juvenile sea bream were examined for PRLR distribution by specific immunohistochemistry using antiserum to an oligopeptide of the external domain of the receptor; some of these are illustrated in Fig. 7. The antiserum used for these studies was prepared by conjugation of a specific oligopeptide to bovine thyroglobulin. When tested for the presence of antibodies to thyroglobulin, the antiserum produced only a weak reaction with colloid of thyroid follicles in sea bream. This cross-reaction could be eliminated by incubation with bovine thyroglobulin without significant effect on specific reaction with PRLR in other tissues; this effect is illustrated in Figs. 6A–6D. Figure 6A shows

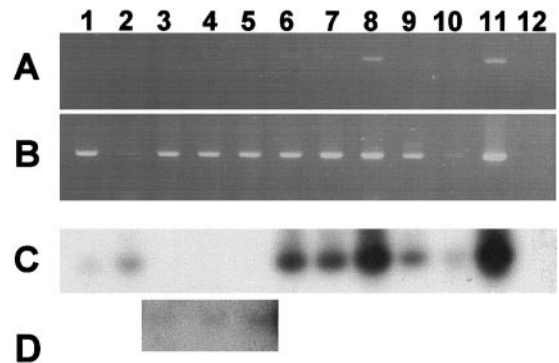
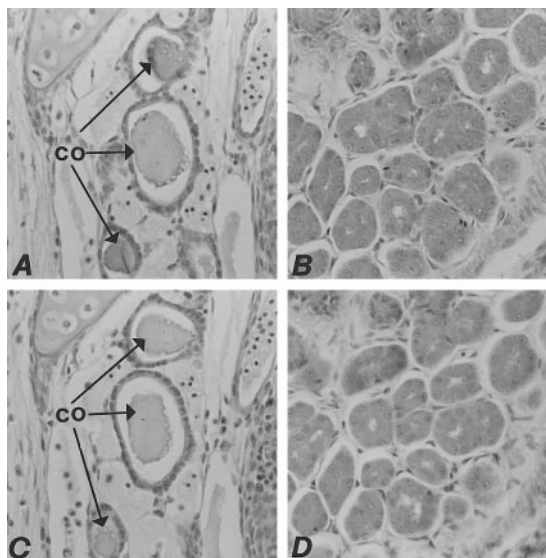


FIG. 5. (A) Distribution of PRLR in adult tissues by RT-PCR: 1, brain; 2, testis; 3, heart; 4, muscle; 5, bone; 6, kidney; 7, skin; 8, gills; 9, ovary; 10, liver; 11, intestine; 12, PCR negative control. (B) The cDNA used to determine PRLR tissue distribution were also amplified with sea bream  $\beta$ -actin primers to evaluate the relative amount of cDNA used in each PCR. (C) Southern blot of the reactions shown in A (5 min exposure). (D) Heart, muscle, and bone samples after 2 h exposure. The sensitivity of RT-PCR coupled with Southern blotting permitted detection of PRLR transcripts in brain, testis, kidney, skin, gills, ovary, liver, and intestine after 5 min exposure and in heart, muscle, and bone after 2 h exposure.



**FIG. 6.** Immunohistochemistry of thyroid follicles of sea bream (A and C) and proventricular gland cells (B and D). (A and B) show immunoreaction with unabsorbed antiserum to PRLR peptide antibody conjugated to bovine thyroglobulin, and (C and D) are adjacent sections reacted with the same antiserum absorbed with bovine thyroglobulin. Immunoreaction has been abrogated only in thyroid follicle colloid (co) by thyroglobulin absorption (cf. A and C).

reaction with unabsorbed antiserum with colloid in thyroid follicles, and in Fig. 6B there is strong reaction with the proventricular gland cells on the same section. Figures 6C and 6D show the same areas of tissue as those from the adjacent section but reacted with antiserum absorbed with bovine thyroglobulin. The weak reaction in thyroid colloid was absorbed by thyroglobulin but the reaction in the proventricular cells was not reduced.

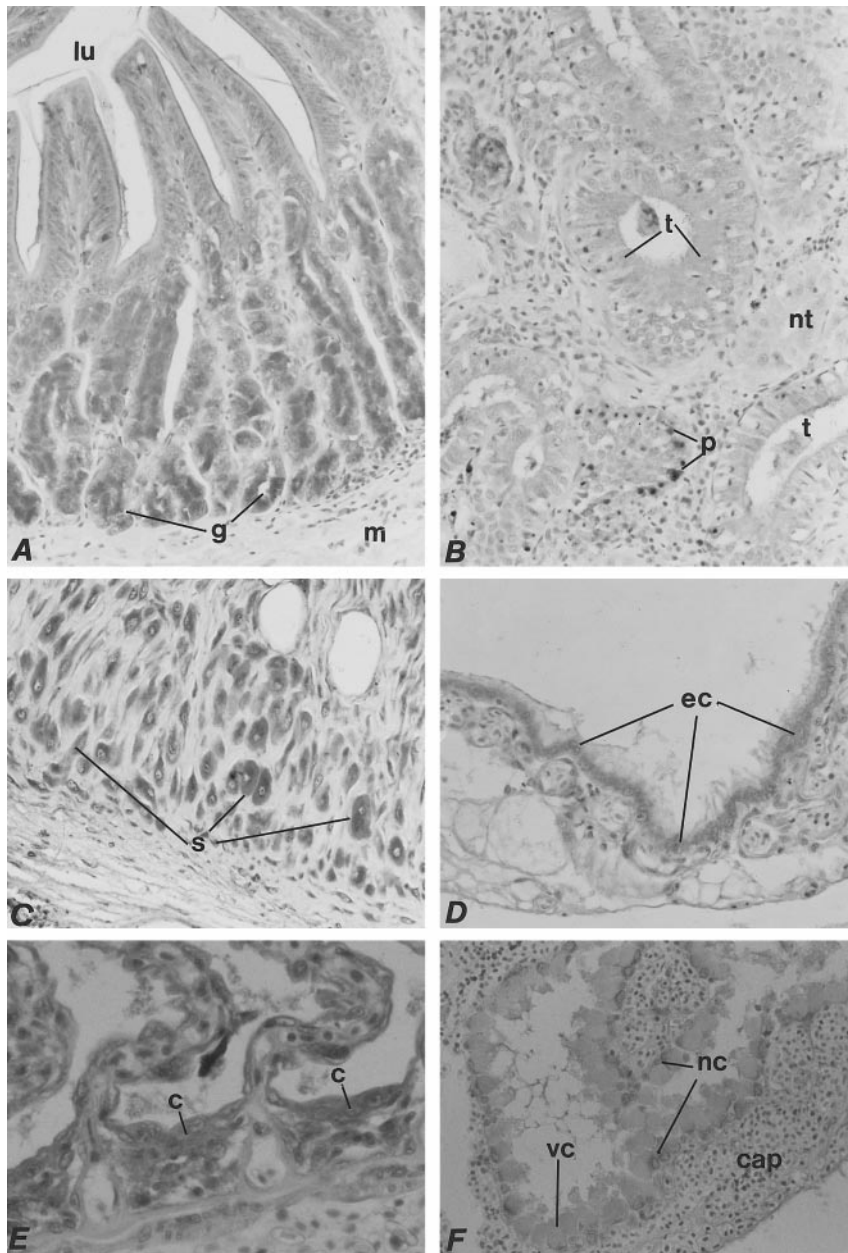
Figure 7 shows immunoreactions in renal tubules (Fig. 7B) and gut epithelium, with the most intense reaction in the basal glandular region of the proventriculus (Fig. 7A); epithelia of other regions of the gut contained only low levels of receptor protein. In the gill, the chloride cells of the filament epithelium reacted strongly (Fig. 7E) but stromal cells of the fibrous cartilage of the gill bars appeared to contain abundant receptor protein (Fig. 7C). The single layer of epithelial cells of the choroid plexus was rich in receptor protein (Fig. 7D) and it is interesting that the epithelial cells of the saccus vasculosus also contained prolactin receptors (Fig. 7F).

## DISCUSSION

The present study describes the cloning and characterization of a PRLR in a marine teleost. Conservation of all the structural and functional features characteristic of PRLRs, the physicochemical properties, both in the ECD and in the ICD, and gene organization, substantiate the identification of this protein as a PRLR and make it improbable that it is a receptor for another member of the growth hormone/PRL gene family.

The tissue distribution of the receptor was determined in adult fish both by Northern blotting, which permitted the identification of size and number of transcripts, and by the more sensitive method of RT-PCR, which allowed detection of PRLR in tissue with low transcript number. Cellular localization of PRLR protein was determined by IHC with a sea bream-specific receptor antiserum. In addition, part of the genomic organization of sbPRLR was elucidated and compared to the PRLR gene of mammals.

PRLR in the sea bream exhibits all the characteristic features of long forms of PRLRs. In common with other species sbPRLR contains a single transmembrane spanning domain and an ECD and ICD. The ECD comprises 208 amino acids and contains two pairs of cysteines which were identified at positions topologically equivalent to those of the mammalian, amphibian, and other fish PRLR, representing areas of high homology. In mammals these two pairs of cysteines form ligation pockets specific for each ligand and mutational studies demonstrated that they are important for receptor structure and function (Roza-kis-Adcock and Kelly, 1991, 1992). The similar constitution and organization in sbPRLR, as well as in the other fish, suggest a common function. In the WS box of the ECD, there has been a conservative substitution of the last serine by threonine; an identical substitution is present in goldfish (Tse *et al.*, 2000) and in mouse (Davis and Linzer, 1989) serine has been replaced by glycine. The WS box can be found in all the members of the class 1 superfamily of cytokine receptors with the exception of the growth hormone receptor (GHR), in which conservative substitutions have occurred (Kelly *et al.*, 1991a). The high degree of conservation of the WS box suggests that it has an important role in receptor function, and mutations in this



**FIG. 7.** Immunohistochemistry of some tissues from juvenile sea bream using rabbit antiserum to an oligopeptide in the external domain of the prolactin receptor. (A) The proventricular region of the gut showed the strongest reaction of all the gut epithelia; moreover, the basal glandular cells (g) of this region appear to have greater content of receptor protein. There is also immunostaining in the epithelial cells lining the lumen of the gut (lu), but no reaction in the smooth muscle (m). (B) A section through the renal kidney showing immunostaining in epithelial cells of some tubules (t), but no reaction in smaller proximal tubules (nt). The reaction in most epithelial cells is spread throughout the cytoplasm but in others it is concentrated in the basal region of the cell as punctate bodies (p). (C) Fibrous cartilage of the gill bars showing strong reaction in the stromal cells (s). (D) There is a strong prolactin receptor immunoreaction in the single layer of epithelial cells of the choroid (ec) of the midbrain. (E) Section of a gill filament showing prolactin receptors in the chloride cells (c). (F) Epithelial cells of the saccus vasculosus showed two patterns of immunostaining similar to the kidney tubule cells in B. In some cells prolactin receptors appeared to be spread throughout the cytoplasm (vc) but in others there was also a concentration around the nucleus (nc) in the basal portion of the cell adjacent to the capillary (cap).

region are detrimental to receptor affinity (Rozakis-Adcock *et al.*, 1991), suggesting a role in ligand binding. It seems that the WS motif is required for maintenance of the correct folding of the molecule (Goffin *et al.*, 1998). In common with mammalian PRLR, the ECD of sbPRLR contains four potential N-linked glycosylation sites, although their relative positions are not conserved. In tiPRLR only two N-linked glycosylation sites were found. The importance of glycosylation sites for adoption of an active conformation of PRLR has been demonstrated in rat (Buteau *et al.*, 1998) but is unknown for other species, including fish. Unlike in avian species, tandem repeats (Chen and Horseman, 1994; Zhou *et al.*, 1996) were not identified within the ECD of sbPRLR. The transmembrane domain of sbPRLR comprises 24 amino acids and shares the hydrophobic properties described for previously characterized PRLRs. The ICD of sbPRLR comprises 284 amino acids and contains the proline-rich motif (box 1), which shares 100% identity with all PRLRs known so far. This is by far the most highly conserved region within the PRLRs ICD among cytokine receptors and it plays an important role in the signal transduction pathway (Dinerstein *et al.*, 1995; Pezet *et al.*, 1997). The ICD of different PRLR forms varies in size and several isoforms of PRLR which differ from each other in length and composition have been found in the same species (Boutin *et al.*, 1989, 1988; Shirota *et al.*, 1990). Additional important structural and functional features are also present in sbPRLR ICD and include box 2 and several tyrosine residues. In general sbPRLR resembles the long form of PRLR, although six deletions scattered throughout the ICD, which lacks 6–21 amino acids, make it 62–92 residues shorter than all the other long forms of PRLR so far identified. Most of these deletions are also present in gfPRLR but do not occur in tiPRLR.

Despite the presence of all the characteristic features of PRLRs, the amino acid sequence of sbPRLR shares a relatively low level of identity with tiPRLR (Sandra *et al.*, 1995) and gfPRLR (Tse *et al.*, 2000). However, sequence identity is higher (46%) in tilapia and goldfish PRLR. Whether the sequence differences observed in fish PRLR are important for specific functions of the PRLR and related to differences in the environment that they inhabit remains to be examined; for example, tilapia is euryhaline and goldfish is a freshwater stenohaline species.

Nevertheless, it appears that PRLR are more variable (29–46% sequence identity) among fishes than among mammals, birds, and amphibia, which share 71–83% sequence identity. However, since there are many more species of teleost fish than all the rest of the vertebrates, it is too early to make general comparisons. It is important to note, however, that the motifs known to be essential for the mechanisms of signal transduction are conserved, suggesting that similar mechanisms operate throughout the vertebrates. If the physicochemical properties of the amino acids are taken into consideration, conservation is remarkable, principally in the ECD but also within the ICD. Even though the amino acid composition of the receptors varies, substitutions, which occurred during evolution, appear to have preserved the overall physicochemical properties of the receptor. Analysis of a partial sequence of genomic DNA for sbPRLR shows that, within the region analyzed, gene organization also appears to have been conserved and resembles that of the mouse (Ormandy *et al.*, 1998). In particular, the sea bream WS box and transmembrane domain, in common with the situation reported in mouse, are coded by different exons.

Northern blot analysis of several sea bream tissues revealed the presence of two differently sized transcripts, which had differential levels of expression in the diverse tissues analyzed. In gills, intestine, and kidney, both transcripts could be observed, though the 2.8-kb transcript was predominant in gills and kidney, whereas the 3.2-kb transcript appears to be more abundant in the intestine. In the pituitary and skin, only the smaller transcript was observed. In addition, the gonads contained three further transcripts of 1.9, 1.3, and 1.1 kb, suggesting that more than one form of PRLR is transcribed in the sea bream gonad. It is possible that these three transcripts encode a short form of PRLR. The expression of PRLR in sea bream differs from that reported in tilapia, in which a single transcript of 3.2 kb was identified, and although more than one transcript of gfPRLR has been identified, they are much larger, 4.6 and 3.5 kb. The existence of alternative forms of PRLR in the sea bream remains to be clarified but expression may be more like that of mouse, in which several transcripts encode the long and short forms of PRLR in a tissue-specific

pattern (Buck *et al.*, 1992) as a consequence of alternative splicing of the same gene (Ormandy *et al.*, 1998). Moreover, in sea bream, Northern blotting demonstrated that the intestine, gill, kidney, and pituitary are the tissues expressing sbPRLR at the highest levels. PRL has long been recognized as a freshwater-adapting hormone in fish. In fish adapted to hyperosmotic environments, PRL is known to modulate the absorption of NaCl in the gastrointestinal tract (Morley *et al.*, 1981) and in hyposmotic environments, PRL reduces branchial sodium efflux and water permeability of gills, renal tubules, intestine, and urinary bladder; some of these actions are due to a reduction to Na<sup>+</sup>K<sup>+</sup> AT-Pase activity (Flick *et al.*, 1994; Shepherd *et al.*, 1997). Adult sea bream spend their life in a marine environment without a severe hydromineral challenge; nevertheless, the high level of expression of receptor in the osmoregulatory organs suggests that these are important targets for PRL and raises intriguing questions about the function of PRL at these sites in this marine teleost. In tilapia, somewhat paradoxically, there was an increase in PRLR numbers in fish transferred from fresh to brackish water (Auperin *et al.*, 1995), suggesting that they are prepared for unexpected changes in external ionic or osmotic concentrations. The expression of PRLR in skin may suggest a function at the level of hydromineral balance or at the level of fish immunoprotection. Fish skin is a major barrier against external aggressions and pathogens and PRL enhances the production of mucus, which further protects the animal. This mechanism of protection has already been described in other species of fish (Sage, 1970). The presence of PRLR transcripts in the gonads suggests a likely involvement of PRL in the reproductive cycle of this species. Sea bream is a protandrous hermaphrodite (Hape and Zohar, 1988). The mechanisms underlying its sex reversal are still poorly understood, although it appears that estrogens induce feminization of the gonads, whereas androgens induce masculinization (Badura and Friedman, 1988; Hape and Zohar, 1988; Condeça and Canario, 1999). The involvement of PRL in the production of steroid hormones and the onset of gonadal development has already been reported in cichlid species (Rubin and Specker, 1992; Tan *et al.*, 1988), suggesting that PRL may be involved in the process of sex reversal

through control of steroid production and may also be involved in control of gonad maturation.

The analysis of receptor tissue distribution carried out by RT-PCR supports the results obtained by Northern blotting and IHC, and due to its higher sensitivity, PRLR expression was detected in other tissues, such as the liver, muscle, bone, and brain, suggesting that PRL may also be involved in growth and behavior, other putative functions already described in cichlid fish (Blum and Fiedler, 1965; Lam and Hoar, 1967; Shepherd *et al.*, 1997).

Immunohistochemistry of PRLR in tissues of sea bream using the antibody to an oligopeptide of the external domain of the receptor showed specific reaction in several tissues. Reaction with thyroglobulin, the carrier protein of the peptide immunogen, was shown to be weak and occurred only with the thyroglobulin-rich colloid within the thyroid follicles.

Distribution of specific PRLR was noted in tissues concerned with control of water and ion balance, including gills, intestine, and renal tubule epithelium. Although PRL is associated with survival of euryhaline fish in water of low osmotic potential, principally by inhibition of sodium loss, PRL receptors have also been shown to increase in gills of *Oreochromis niloticus* when transferred from fresh water to brackish water (Auperin *et al.*, 1995). The presence of PRLR in these epithelial cells may suggest roles for PRL other than ion and water regulation since PRL influences the cell cycle, through inhibition of apoptosis and differentiation (Clarke and Bern, 1980; Hirano, 1986). The presence of PRLR in the saccus vasculosus and in the choroid plexus may be related to the function of these epithelia in monitoring the composition of cerebrospinal fluid in relation to plasma composition. PRLR have been described in the choroid plexus epithelia of other vertebrates (Posner *et al.*, 1983; Buntin and Walsh, 1988; Muccioli *et al.*, 1988) and their presence in fish suggests that they are an earlier evolutionary development. PRLR also occur in mesodermally derived connective tissues, having been described in mammalian chondrocytes of developing cartilage and mesenchymal precartilage (Freemark *et al.*, 1997), and their recent detection in osteoblasts (Clement-Lacroix *et al.*, 1999) shows that they do have a role(s) in skeletal tissues. Only further physiological studies will determine the functions of PRL through receptor binding in these various tissues.

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