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Expression cloning of a cDNA encoding a fish prolactin receptor

(tilapia/kidney/osmoregulation/conserved receptor domains/mammalian cells)

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ABSTRACT **By using an expression cloning strategy, we isolated a single positive clone encoding a tilapia prolactin (PRL) receptor. Tilapia PRL_{188} was used to screen a freshwater tilapia kidney expression library transfected in COS cells. The tilapia PRL receptor is a mature protein of 606 amino acids. The extracellular domain is devoid of the tandem repeat units present in birds and has two pairs of cysteine residues, a Trp-Ser-Xaa-Trp-Ser motif, and two potential N-glycosylation sites. The cytoplasmic domain contains 372 amino acids, including box 1, a sequence previously shown to be important for signal transduction in mammalian species. Thus, the general structure is similar to the long form of mammalian PRL receptors; however, amino acid comparisons reveal a rather low identity (≈37%). Northern blot analysis shows the existence of a single transcript in osmoregulatory tissues and reproductive organs. This localization is in agreement with known functions of PRL in teleosts.**

Prolactin (PRL) is a pituitary polypeptide hormone that is implicated in many physiological actions in vertebrates including fish (1). Since the pioneering work by Pickford and Phillips in 1959 (2) demonstrating that hypophysectomized *Fundulus heteroclitus* require PRL for survival in freshwater, numerous studies have confirmed that PRL is one of the major hormones regulating the maintenance of water and electrolyte homeostasis on osmoregulatory surfaces (3–5). In tilapia, two distinct PRL forms have been well characterized ($tiPRL_{188}$ or $tiPRL_I$ and $tiPRL_{177}$ or $tiPRL_{II}$), which share only 69% identity (6–8). These two tilapia PRLs (tiPRLs) were shown to be differentially regulated during adaptation to a hyperosmotic environment (9–11) and to exhibit both common and distinct biological effects and potencies (6, 10, 12). These effects are mediated by a specific cell membrane receptor. In tilapia, initial studies using ovine PRL (oPRL) as a ligand revealed the presence of PRL receptors in various tissues (13–15). Using bioactive recombinant tiPRL forms (16), high specific binding of PRL (up to 45% with $tiPRL_{188}$) has recently been shown in gill and kidney, involving only one class of receptors, which binds $tiPRL_{188}$ with higher affinity than $tiPRL_{177}$ (17).

PRL receptor cDNAs have been cloned from several mammalian species and sources (18–20) and two avian species (21, 22). These receptors belong to a superfamily including the receptors for growth hormone (GH), cytokines, and erythropoietin (18). This superfamily has several common structural features (23) including two pairs of conserved extracellular cysteine residues, a single transmembrane domain, and an intracellular proline-rich region (24). In lower vertebrates, however, no PRL receptor cDNA has been identified.

We report in this paper the isolation of a tiPRL receptor cDNA¶ by an expression cloning approach (25). The characterized tiPRL receptor is a mature protein of 606 amino acids with a single extracellular unit and a long cytoplasmic domain. Moreover, tissue distribution studies indicate the presence of a single tiPRL receptor mRNA in various targets.

MATERIALS AND METHODS

Hormones and Preparation of Radiolabeled Ligands. Recombinant tiPRLs were kindly provided by F. Rentier-Delrue (Université de Lièges, Belgium). Recombinant tilapia GH (tiGH) was a gift from J. Smal (Eurogentec, Lièges, Belgium). oPRL (NIDDK-oPRL-19) was a gift from the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program (Bethesda, MD). Recombinant human growth hormone (hGH) was provided by Serono (Geneva). hGH was labeled according to the chloramine-T method (26), and $tiPRL_{188}$ was labeled according to Aupérin *et al.* (10). The specific activity was 80–140 μCi/μg for ^{125}I-labeled hGH (^{125}I-hGH) and 30–40 μCi/μg for ^{125}I-labeled $tiPRL_{188}$ (^{125}I-$tiPRL_{188}$; 1 Ci = 37 GBq).

Expression Cloning. An expression library was constructed in pcDNA1 by Invitrogen with polyadenylylated RNA (27) prepared from kidneys removed from freshwater-adapted adult tilapia (*Oreochromis niloticus*). The yield was 1.2×10^6 primary recombinants.

The expression cloning strategy was performed as described in Mathews and Vale (25). DNA minipreps (28) were prepared from pools of clones and transiently transfected in COS-7 cells grown on chamber slides (Nunc). Cells were incubated with ≈6×10^5 cpm of ^{125}I-$tiPRL_{188}$ (≈800 pM per chamber) in 0.6 ml of DMEM/20 mM Hepes/0.1% bovine serum albumin/0.1% glucose, pH 7.4, for 4 hr at room temperature. The same procedure was applied to a miniprep of the rat PRL receptor cDNA (26) using binding of ^{125}I-hGH as a control. Slides were processed as described (25).

The isolated cDNA clone was sequenced by the dideoxy chain-termination method (29) using modified T7 DNA polymerase (Sequenase, United States Biochemical). Comparisons of sequences were performed using the BISANCE program (30).

Expression of the tiPRL Receptor cDNA. COS-7 cells were transfected using the same protocol (see above) with 5 μg of full-length cDNA per 100-mm culture dish. Cell membranes were prepared according to Boutin *et al.* (26).

Ten micrograms of the membrane preparation, in 0.1% bovine serum albumin/25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$, was incubated for 15 hr at 20°C, in the presence of

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Abbreviations: PRL, prolactin; tiPRL, tilapia PRL; oPRL, ovine PRL; GH, growth hormone; hGH, recombinant human GH; tiGH, tilapia GH.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. L34783).

21,000 cpm of ^{125}I-tiPRL$_{188}$ (33 pM per tube) and various concentrations of unlabeled hormone (final volume: 0.4 ml). The reactions were stopped by the addition of 2 ml of chilled binding buffer. Bound hormone was separated from unbound hormone by centrifugation at 3000 × *g* for 30 min. The pellets were counted using a LKB Pharmacia γ spectrometer. Binding parameters were determined using the LIGAND program (31).

tiPRL Receptor Northern Blots. Polyadenylylated RNA was purified from tissues removed from pools of adult or juvenile tilapias reared in freshwater or brackish water. Northern blot analysis and membrane hybridizations were conducted as described (10, 28), with a 0.9-kb probe corresponding to the extracellular domain of tiPRL receptor and a rainbow trout β-actin cDNA (32) as control. In fish, the level of actin gene expression between tissues is variable but is relatively constant within the same tissue. Both probes were labeled using [α-^{32}P]dCTP and the Megaprime DNA labeling system (Amersham). Quantitation of signals was performed by scanning densitometry using a PhosphorImager (Molecular Dynamics). The relative expression of tiPRL receptor mRNA was determined by calculating the ratio of the intensity of tiPRL receptor mRNA signal to the intensity of β-actin mRNA. These data were analyzed statistically using Duncan's multiple range test.

RESULTS

Expression Cloning. From 40 pools of 2000–2500 clones tested by transient transfection, we found 1 pool with four positive cells, corresponding to 1 out of ≈10^5 recombinant clones tested. This pool was subdivided into 14 subpools containing about 200–300 colonies. Two subpools were positive, and one was split into 15 subpools (30–40 colonies) of which 2 were positive. One pool was plated according to the columns–rows method (33) and found to contain two identical positive clones with an insert of ≈3 kb. Fig. 1 shows the density of labeled cells after transient transfection with the cloned cDNA.

Analysis of Nucleotide and Amino Acid Sequences. The cDNA insert of ≈3000 bp consists of 206 bp in the 5′-untranslated region and an open reading frame of 1890 bp (Fig. 2). The 3′-untranslated region is ≈750 bp with a putative polyadenylylation consensus signal (AATAAA) and a poly(A) tail.

No clear Kozak consensus sequence (34) was found surrounding the initial ATG. This codon is followed by bases encoding 24 residues that have all the characteristics of a signal peptide. The mature protein would thus consist of 606 aa with a theoretical molecular mass of 68.2 kDa and an isoelectric point of 5.53. This receptor can be divided into three parts: (*i*) an extracellular domain of 210 aa with five cysteine residues and a Trp-Ser-Xaa-Trp-Ser motif sequence, which have been shown to be extremely conserved among the PRL receptors of vertebrates (18), and two consensus sequences (Asn-Xaa-Ser/Thr) for potential N-linked glycosylation sites; (*ii*) a single transmembrane domain of 24 hydrophobic residues; and (*iii*) a cytoplasmic domain of 372 residues, which is slightly longer than the mammalian long form of the PRL receptor. The membrane-proximal region contains a proline-rich region (PPVPGP) termed box 1, which has been shown to be highly conserved among the GH/PRL/cytokine receptor family (23). Box 2, a less conserved region (35), is also present and contains two tyrosine residues. No other known signal transduction motif was found in the intracellular domain of this receptor.

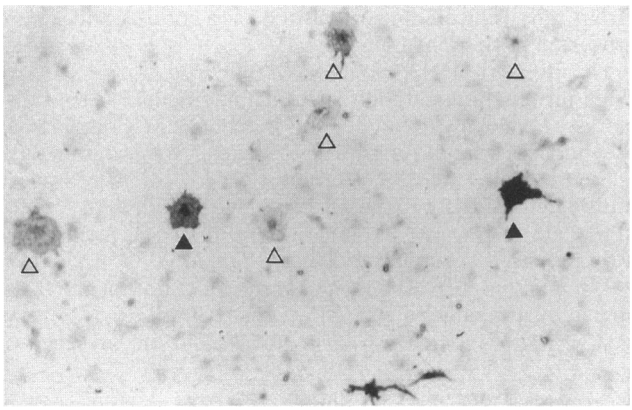

FIG. 1. Bright-field photomicrograph of COS cells transfected with the tiPRL receptor cDNA and labeled with ^{125}I-tiPRL$_{188}$. (×35.) Cells expressing high and moderate levels of radioactivity are indicated by filled and open arrowheads, respectively.

As shown in Table 1, the overall amino acid identity is 34.8–38.4% with PRL receptors and 26–28.1% with GH receptors from other species. This higher similarity with PRL receptors is explained by an increased sequence identity in the extracellular domains (49.0–56.7%) of PRL receptors vs. 28.9–35.8% for GH receptors. Interestingly, the sequence identity between the cytoplasmic domain of the tiPRL receptor and avian or mammalian PRL receptors is low (26.6–30.6%), in the same range as that found for intracellular domains of various GH receptors (21.8–23.9%).

Competition and Scatchard Analysis. Membranes were prepared from COS-7 cells transiently transfected with the entire tiPRL receptor clone and used for binding experiments (Fig. 3). The association constant (K_a) of the expressed receptor was 1.7 × 10^9 M^{-1} using ^{125}I-tiPRL$_{188}$ as a tracer, which is about 10-fold lower than the association constant (K_a = 2.3 × 10^{10} M^{-1}) previously reported in tilapia kidney (17). Unlabeled fish hormones and oPRL were used for competition curves. As expected, tiGH failed to compete with ^{125}I-tiPRL$_{188}$, even at the highest concentrations. Both tiPRL$_{177}$ and oPRL were able to compete with the ligand (relative potency: tiPRL$_{188}$ > oPRL > tiPRL$_{177}$) but with differing potency than previously observed in tilapia kidney microsomes, in which oPRL was less potent than tiPRL$_{177}$ (17).

Northern Blot Analysis of tiPRL Receptor mRNA. By using a cDNA probe corresponding to the extracellular domain of the receptor, the tissue distribution of tiPRL receptor was carried out in various tissues collected from freshwater-adapted tilapia. As shown in Fig. 4*A*, a single transcript of ≈3.2 kb was observed in all tissues displaying a signal: kidney, gill, and gut. A clear but weak signal was seen in testis and liver at 5 days of exposure (data not shown). Under these conditions, no transcript was seen in skin and muscle. The effect of salinity changes on PRL receptor transcripts in gill was also examined. In tilapia transferred for 6 days to brackish water, no additional signal appeared compared to freshwater-adapted fish, while the relative tiPRL receptor mRNA level normalized to β-actin mRNA decreased significantly ($P < 0.05$; Fig. 4*B*).

DISCUSSION

A fish PRL receptor cDNA has been isolated by screening a tilapia kidney expression library transfected in COS cells with ^{125}I-tiPRL$_{188}$. This study provides, to our knowledge, the first sequence of PRL receptor in lower vertebrates and shows that expression cloning in mammalian cells is a powerful strategy for isolating lower vertebrate receptor cDNA. The high level of specific binding seen with radioactive tiPRL$_{188}$ (17) is probably important to the success of this technique.

Normally, amino acid identity between PRL receptors from various species averages ≈70% within mammals and ≈52% between birds and mammals, which in both cases is higher than the value of ≈37% found between fish and higher vertebrates. This represents about the same level of amino acid identity between fish and mammalian PRL hormones (≈31%), which

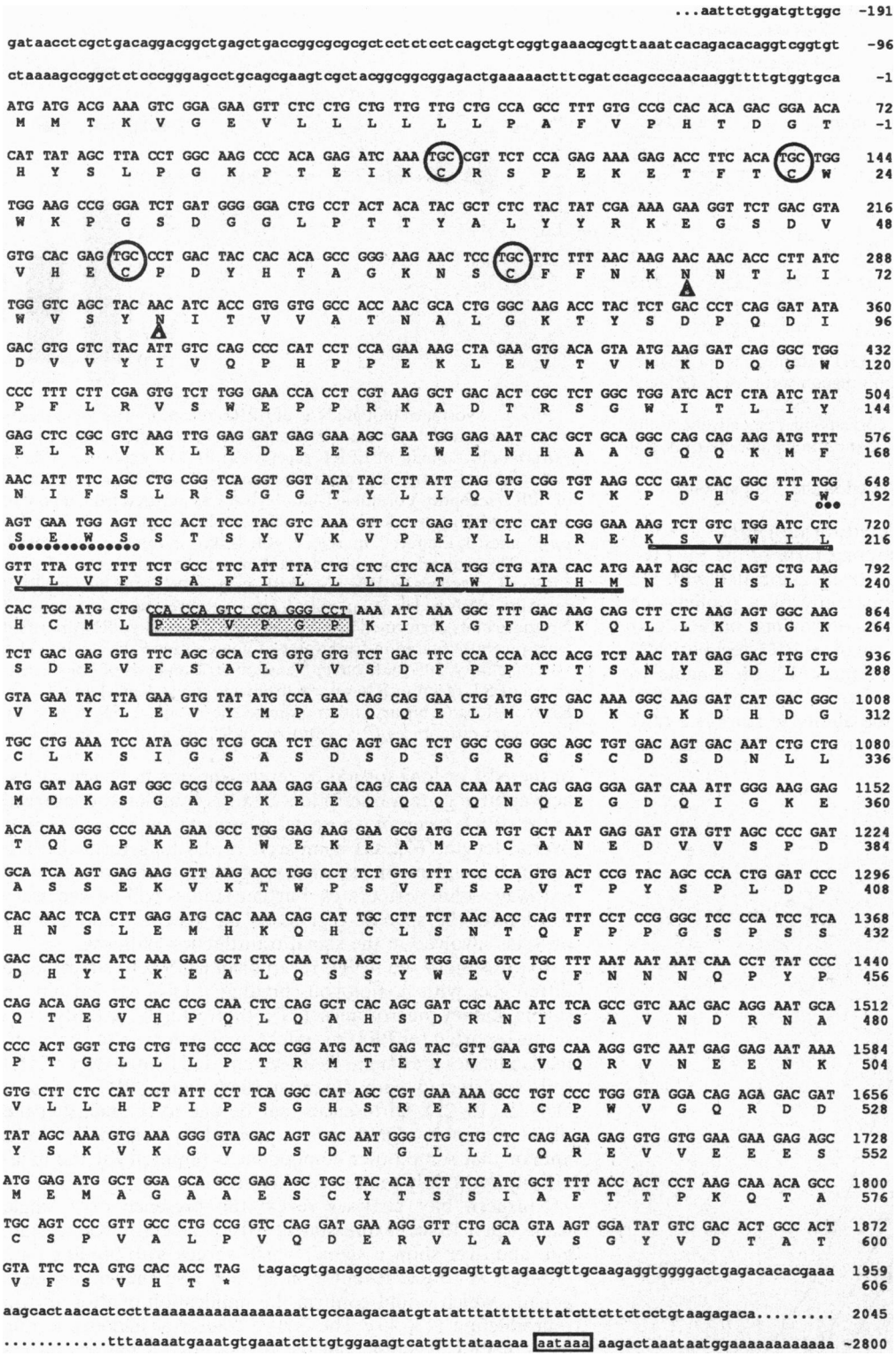

FIG. 2. Nucleotide and deduced amino acid sequences of the tiPRL receptor clone. Nucleotides are positively numbered from the first base of the codon for the initiation methionine. Amino acids are positively numbered from the first amino acid after the potential cleavage site of the signal peptide. The two pairs of cysteine residues of the extracellular domain are circled, and the two potential N-linked glycosylation sites are marked by filled triangles. The Trp-Ser-Xaa-Trp-Ser motif is underlined with black dots. The putative transmembrane is underlined with a solid line. Box 1 is surrounded with a stippled box. The asterisk denotes the stop codon. The potential polyadenylylation signal is boxed.

could suggest a coevolution of hormones and receptors. Moreover, evaluation of gene structure confirms that GH, PRL, and placental lactogens arise from a common ancestral gene (36). Cloning of PRL and GH receptors in lower vertebrates (fish and amphibians) would be of great interest in order to confirm the hypothesis of a common ancestral gene for the PRL/GH receptor family.

Analysis of the amino acid sequence indicates that the structure of the tiPRL receptor is similar to that of the long form of the mammalian PRL receptor. The extracellular region, which does not contain the tandem repeat present in avian PRL receptors (21, 22), is the most conserved region (≈53%). This domain contains two pairs of cysteine residues and a Trp-Ser-Xaa-Trp-Ser motif reported to be important for high-affinity PRL binding (37, 38). Only two potential N-glycosylation sites (Asn-67 and Asn-76) are present, in the same relative position as those of the distal unit of the avian extracellular domain. The third site found between the second and the third extracellular cysteine residues, present in every mammalian PRL receptor, is absent in the fish. Thus, asparagine-linked N-glycosylation sites in the PRL receptor appear to have undergone some evolutionary changes.

Table 1. Comparison of amino acid identities of the tiPRL receptor with PRL and GH receptors of various vertebrates

	Percent amino acid identity of the tiPRL receptor					
	Extracellular domain		Cytoplasmic domain		Overall identity	
Receptor	PRL	GH	PRL	GH	PRL	GH
Bovine	49.0	28.9	27.7	22.6	34.8	26.7
Human	51.4	30.5	30.6	22.6	38.1	27.0
Mouse	52.4	32.9	27.1	21.8	35.7	26.0
Rabbit	56.2	32.1	28.8	23.4	38.1	27.7
Rat	53.8	32.8	26.6	22.6	35.7	27.3
Chicken	49.0/56.7*	35.8	29.3	23.9	38.4†	28.1
Pigeon	47.6/55.2*	—	27.4	—	37.0†	—

Amino acid sequences of PRL and GH receptors from bovine, human, mouse, rabbit, rat, chicken, and pigeon were used. Original references can be found in refs. 18–22.

*The first value and the second value correspond, respectively, to the identity with the membrane-distal unit and the membrane-proximal unit.

†Membrane-distal units were not included in the comparison.

Recently, both the internal deleted form of PRL receptor found in the Nb2 cell line and the long form of the rat PRL receptor were shown to be constitutively associated with the tyrosine kinase Jak2 (39–41), apparently by a proline-rich motif, box 1, located in the membrane-proximal intracellular region (42). Moreover, the short form of rat PRL receptor (43) and the truncated rabbit PRL receptor (42) are unable to transduce a lactogenic message, whereas the long and Nb2 forms of rat PRL receptor have the ability to stimulate milk protein gene transcription (44). Therefore, the C-terminal part of the Nb2 or long form of receptor appears to be critical for activation of gene transcription. Box 1 is completely conserved in the tiPRL receptor whose cytoplasmic domain has a similar overall length (372 aa) compared to the long form of PRL receptor found in other species, suggesting a common signaling pathway within vertebrates. Further studies will be necessary to identify the specific regions of the receptor and cellular proteins involved in the signal transduction pathway.

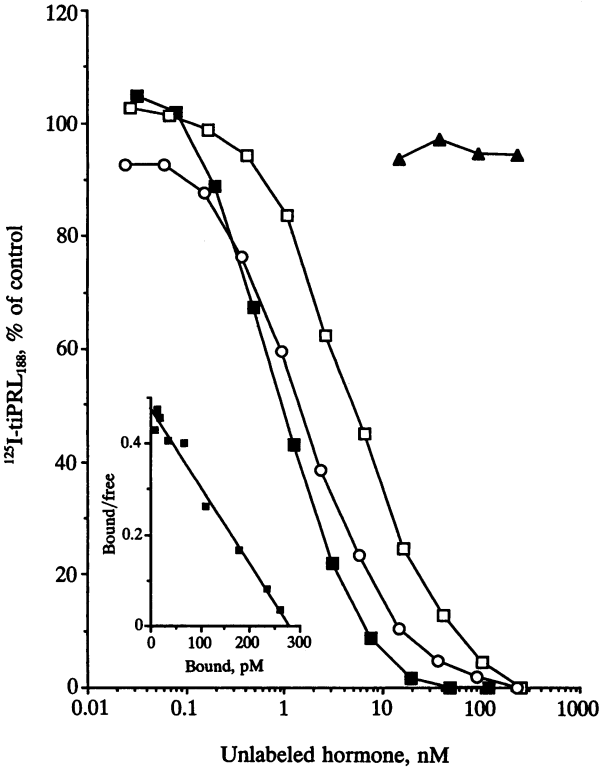

FIG. 3. Competition binding of ^{125}I-tiPRL$_{188}$ to membranes from COS cells transfected with the tiPRL receptor cDNA. Membranes were incubated in the presence of ligand and various concentrations of unlabeled tiPRL$_{188}$ (■), tiPRL$_{177}$ (□), oPRL (○), and tiGH (▲). The results are expressed as percentage of the maximal specific binding observed in the absence of competitor. (*Inset*) Scatchard plot of the competition assay with unlabeled tiPRL$_{188}$; K_a = 1.7 ± 0.2 nM^{-1} and B_{max} = 10.6 ± 0.7 pmol/mg of protein (n = 3).

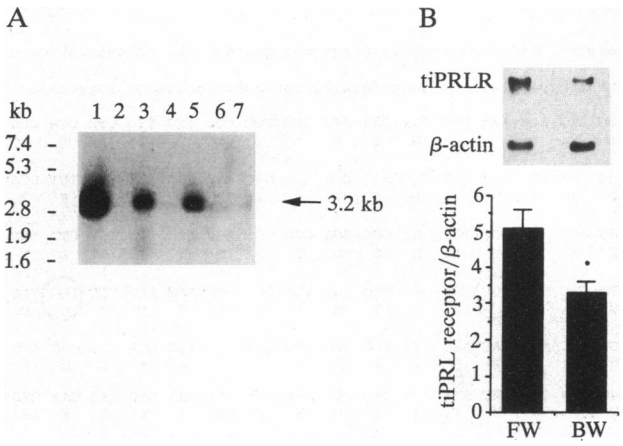

FIG. 4. Northern blot analysis of tiPRL receptor. The membranes were successively hybridized with the probe corresponding to the extracellular domain of tiPRL receptor (tiPRLR; exposure: 3 days) and rainbow trout β-actin probe (not shown). (*A*) Tissue distribution of tiPRL receptor. Polyadenylylated RNA was purified from a pool of adults, and 5 μg was loaded for each sample except for the liver (15 μg). Lanes: 1, kidney; 2, muscle; 3, gill; 4, skin; 5, intestine; 6, liver; 7, testis. The sizes of molecular markers are indicated on the left. (*B*) Effect of a freshwater (FW)-brackish water (BW) transfer on tiPRL receptor mRNA levels in gill. (*Upper*) Representative results of Northern blots performed with 5 μg of polyadenylylated RNA purified from juveniles reared in freshwater or adapted for 6 days in brackish water (salinity: 20‰). (*Lower*) Densitometric analysis of polyadenylylated tiPRL receptor RNA normalized with polyadenylylated β-actin RNA. Values (arbitrary units) indicate the means ± SEM (n = 4). Significant differences ($P < 0.05$) are indicated with an asterisk (*).

Expression of the tiPRL receptor in COS cells shows some differences with homologous binding studies carried out on tilapia kidney microsomes (17). Interestingly, affinity constants reported for PRL receptors in pigeon crop sac or mammalian tissues are similar to those reported from COS or CHO cells transfected with avian or mammalian PRL receptor cDNAs (18, 22). Differences may be due to the fact that the tiPRL receptor cDNA was expressed in mammalian cells and/or that some other component is required for the high-affinity form only seen in fish tissues.

Northern blot analyses reveal the presence of a single transcript in tissues expressing the tiPRL receptor. Kidney, gill, gut, and liver show a signal, which agrees with binding data (13–15). A tiPRL receptor mRNA is also present in male gonads, which would confirm the implication of PRL in fish reproduction (13, 45). The 3.2-kb transcript encodes a long form of PRL receptor, but the tilapia mRNA pattern differs from patterns reported in mammalian species, where several transcripts encode a long form of PRL receptor (18, 46). Interestingly, a single transcript argues rather for the existence of a single high-affinity PRL binding site as described (17).

In spite of the structural relatedness of PRL and GH (36), PRL is well established as the hormone responsible for freshwater adaptation in euryhaline teleosts, whereas GH has an important role in the seawater adaptation, particularly in salmonids (47, 48). Transfer of tilapia for 6 days to brackish water resulted in a low but significant decrease of the tiPRL receptor transcript level in gill. Binding studies have also shown various modifications of PRL receptor levels during seawater

adaptation (15, 49). In addition to the use of this cDNA for cloning other fish PRL receptors, it will also provide an important tool for better understanding the regulation of the tiPRL receptor and the specific biological actions of each form of tiPRL.

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