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Cloning and Expression of a cDNA Encoding a Prolactin Receptor of the Japanese Red-Bellied Newt, *Cynops pyrrhogaster*

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ABSTRACT—Primary structure of a newt prolactin receptor (nPRL-R) was deduced from a cDNA clone isolated from a kidney cDNA library as well as from a polymerase chain reaction (PCR) product. The predicted nPRL-R protein was composed of 626 amino acids (aa), and contained a signal sequence and a transmembrane region. The extracellular domain had two pairs of cysteine residues and a WSXWS motif. The cytoplasmic domain comprised 368 residues and contained both box 1 and box 2 sequences which are considered to be required for the signal transduction of the cytokine/growth hormone (GH)/PRL-R family in mammals. The nPRL-R shares 50–52% protein sequence identity with mammalian PRL-Rs. When nPRL-R was expressed in COS-7 cells, specific binding of [¹²⁵I] rat prolactin (PRL) was observed. Northern blot analysis revealed the existence of a single transcript, more than 10 kb in length, which was expressed in the kidney and brain. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed the expression of PRL-R mRNA in the testis, ovary, liver and bladder of the newt. This is, as far as we know, the first report of cloning on amphibian PRL-R.

INTRODUCTION

Prolactin (PRL) is a pituitary polypeptide hormone that has diverse functions in vertebrates (Nicoll and Bern, 1972). In amphibians, PRL is involved in development (Kikuyama *et al.*, 1980), metamorphosis (Kikuyama *et al.*, 1993), osmoregulation (Nicoll, 1982) and reproduction (Mazzi *et al.*, 1967). Amphibian PRLs have been purified and characterized in the bull frog (Yasuda *et al.*, 1991), the Japanese toad (Yamamoto *et al.*, 1986), *Xenopus* (Yamashita *et al.*, 1993) and the newt (Matsuda *et al.*, 1990).

The physiological effects of PRL are mediated by the specific cell surface receptor. Prolactin receptor (PRL-R) cDNAs have been cloned in several mammals (Boutin *et al.*, 1988, 1989; Davis and Linzer, 1989; Edery *et al.*, 1989; Shirota *et al.*, 1990), avians (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996) and fish (Sandra *et al.*, 1995). The PRL-Rs belong to the cytokine/ growth hormone (GH)/PRL receptor superfamily and have common structural features such as two pairs of cysteine residues (Bazan, 1989), a WSXWS motif (Baumgartner *et al.*, 1994) in the extracellular domain and a proline-rich sequence, box 1 (Murakami *et*

al., 1991), in the cytoplasmic domain. Binding analysis using ovine PRL as a ligand showed that kidney and bladder are major target organs of PRL in amphibians (White, 1981; Tarpey and Nicoll, 1987). However, no PRL-R cDNA has been cloned in amphibians. Thus, the molecular mechanism of PRL action in amphibians is still unclarified.

In this study, as a first step to investigate the function of PRL in amphibian tissues, we isolated a cDNA for newt PRL-R (nPRL-R) and demonstrated that nPRL-R expressed in COS-7 cells bound specifically to [¹²⁵I] rat PRL. Moreover, we examined the tissue distribution of the mRNA by Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analysis.

MATERIALS AND METHODS

Animals and reagents

Adult newts (*Cynops pyrrhogaster*) collected during winter and early spring, were purchased from Hamamatsu Seibutsu Kyozaï Ltd, Hamamatsu, Japan. All chemicals were obtained from Nacalai Tesque, Inc., Kyoto, Japan, unless otherwise stated.

Degenerate polymerase chain reaction

Degenerate primer 1 (5'-ATGGATCCTTYWSNTGYTGTTG-3') containing *Bam*HI site at the 5' end and primer 2 (5'-ATGAATTC-GNRTYTCCCAATC-3') containing *Eco*RI at the 5' end were synthe-

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sized corresponding to peptide sequences (FT/SCWW and EWET/I) conserved among mammalian PRL-Rs. Total RNA was prepared from newt kidney by acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)⁺-RNA was fractionated from the total RNA by chromatography on oligo(dT)-cellulose (type 7, Pharmacia Biotech, Piscataway, NJ, USA). Double-strand cDNA was synthesized from newt kidney poly(A)⁺-RNA by oligo(dT) priming with a cDNA synthesis kit (Amersham, Arlington Heights, IL, USA) and amplified with Taq DNA polymerase (Takara Shuzo Co. Ltd, Kyoto, Japan) by 40 cycles of PCR, using primer 1 and primer 2. PCR cycle consisted of 30 sec of denaturing at 94°C, 30 sec of annealing at 55°C, and 3 min of extension at 72°C. PCR product was analyzed on 2% agarose gel. The PCR product was then extracted from the agarose gel, treated with both *Bam*HI and *Eco*RI, and then subcloned into pBluescript II SK (-) (Stratagene, La Jolla, CA, USA).

cDNA cloning

Double-strand cDNA was synthesized from newt kidney poly(A)⁺-RNA by using oligo(dT) and random hexanucleotide priming with a cDNA synthesis kit (Amersham) as described above and cloned into λ gt11 vector using λ gt11 cDNA cloning kit (Amersham). A nPRL-R cDNA was obtained by screening the λ gt11 cDNA library with a 420 bp fragment of nPRL-R cDNA labeled with [α -³²P]dCTP by the Megaprime DNA labeling system (Amersham). The insert cDNA was cleaved from the λ gt11 and subcloned into *Eco*RI site of pBluescript II SK(-).

DNA sequencing

Cloned DNA was sequenced by ABI PRISM dye primer cycle sequencing ready reaction kit (Perkin Elmer, Norwalk, CT, USA) using DNA sequencer (model 373S, Applied Biosystems, Foster, CA, USA). All sequence data were obtained for both strands.

Amplification of 3' region of nPRL-R cDNA

3' RACE (rapid amplification of cDNA ends) method was performed according to the procedure of Frohman *et al.* (1988). First strand cDNA was synthesized from poly(A)⁺-RNA from kidney by using oligo(dT)-adaptor primer (5'-CTACGAATTCGATCGGATCCTTTT-TTTTTTTTTT-3') with a cDNA synthesis kit (Amersham). The cDNA was amplified by 20 cycles of PCR with *Pfu* DNA polymerase (Stratagene) using nPRL-R specific-primers 3 (5'-GACTGTGCAG-ATCTTCTGGT-3') and adaptor primer (5'-CTACGAATTCGATCGGATCC-3'). A PCR cycle consisted of 1 min of denaturing at 94°C, 1 min of annealing at 55°C, and 3 min of extension at 72°C. Second round of amplification was then performed under the same conditions described above using a second nested nPRL-R-specific primer 4 (5'-CTCGAGGTAGATGACAGTA-3') and adaptor primer. PCR products were subcloned into pT7blue T-vector (Novagen, Madison, WI, USA). Three independent clones were sequenced to determine whether PCR-induced errors had occurred in the cDNA sequences.

Expression of nPRL-R cDNA and binding experiments

A nPRL-R cDNA containing the entire coding region was amplified from newt kidney cDNA using primer 5 (5'-TCTAGAATGCG-GCAAACCTGAAATA-3') containing *Xba*I cleavage site and primer 6 (5'-GAGCTCGTGGCTTTACCCGAACAAGTA-3') containing *Sac*I cleavage site. Forty cycles of PCR amplification was performed with *Pfu* DNA polymerase and the cycle consisted of 30 sec of denaturing at 94°C, 30 sec of annealing at 55°C, and 5 min of extension at 72°C. The 1.9 kb amplified cDNA was subcloned into pT7blue T-vector. Authenticity of this cDNA was confirmed by sequencing three independent clones. The 1.9 kb cDNA was cleaved with *Sac*I and *Xba*I, and was inserted into the pSVL expression vector (Pharmacia Biotech, Piscataway, NJ, USA). The resultant recombinant, pnPRL-R, was purified by an alkaline method, followed by CsCl ultracentrifugation to obtain a supercoiled circular recombinant (Maniatis *et al.*, 1989). pnPRL-R or pSVL vector was transfected into COS-7 cells by the

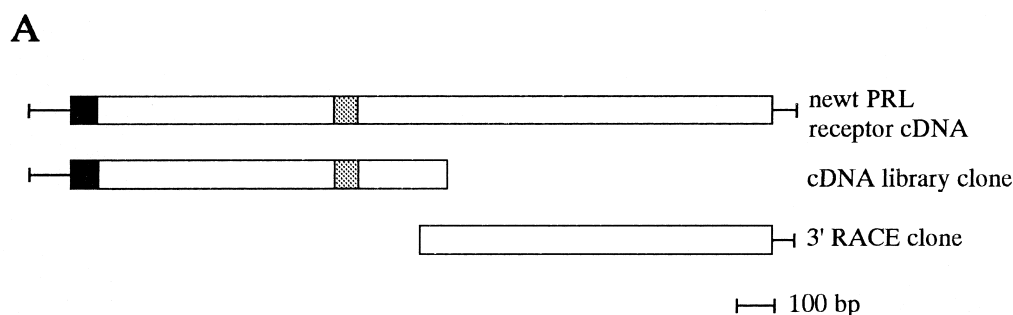
DEAE-dextran method as described by Ausubel *et al.* (1987). Cells (5×10^5) were plated on a 10-cm culture dish and transfected with 10 μ g of each plasmid. Three days after transfection, cell membranes were prepared according to Boutin *et al.* (1988). Cells were scraped with 25 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂, and lysed by 2 freeze-thaw cycles. Membranes were collected by centrifugation for 5 min and the pellet was resuspended in 25 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂ and 1 mM PMSF. Binding experiment was performed according to Sandra *et al.* (1995). One hundred microgram of the membrane preparation in 25 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂ and 0.1% BSA was incubated for 12 hr at 20°C in the presence of 30,000 cpm of [¹²⁵I] rat PRL (NEN, Boston, MA, USA) and various concentrations of unlabeled rat PRL (Biogenesis, Poole, England), ovine PRL (Sigma Chemical Co.), rat GH (Biogenesis, Poole, England) or ovine GH (Biogenesis, Poole, England). The reactions were stopped by the addition of 2 ml of ice-cold 25 mM Tris-HCl (pH 7.5) containing 10 mM MgCl₂. Bound hormone was separated from unbound hormone by centrifugation at 10,000 \times g for 10 min. The radioactivity of the pellets were counted using an automatic gamma counter.

Northern blot analysis

Poly(A)⁺-RNA was prepared from various tissues of adult newts as described above. Northern blot analysis was done as described previously (Yamamoto *et al.*, 1996). Five microgram of poly(A)⁺-RNA was electrophoresed in a 1% formaldehyde agarose gel and blotted to a nylon-membrane, Hybond(N)+ (Amersham). Membranes were prehybridized at 42°C for 2 hr in the hybridization buffer [5 \times standard saline phosphate ethylenediaminetetra-acetate (SSPE), 50% formamide, 0.5% sodium dodecyl sulfate (SDS), 5 \times Denhart's and 20 μ g/ml salmon sperm DNA]. The 1.1 kb of nPRL-R cDNA isolated from cDNA library was labeled with [α -³²P]dCTP by the Megaprime DNA labeling system (Amersham), added to the hybridization buffer and hybridized at 42°C for 16 hr. After hybridization, membranes were washed successively in 2 \times SSPE containing 0.1% SDS at 65°C for 30 min, 1 \times SSPE containing 0.1% SDS at 65°C for 30 min and 0.1 \times SSPE containing 0.1% SDS at 65°C for 30 min.

Reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis

Two microgram of total RNA from various tissues which had been treated with DNase I (Gibco-BRL, Tokyo, Japan) was reverse-transcribed using a random hexanucleotide primer with a cDNA synthesis kit (Amersham). The cDNAs were amplified by 40 cycles of PCR using nPRL-R cDNA-specific primer 7 (5'-CAGAGGGGAAAAAC-ACTTACTC-3') and 8 (5'-GATGTGGAGGCTCATAGATTA-3'). A PCR cycle consisted of 30 sec of denaturing at 94°C, 30 sec of annealing at 55°C, and 2 min of extension at 72°C. The amplified products were separated on agarose gel and transferred to Hybond(N)+. The 185 bp of nPRL-R cDNA, which does not contain the regions of primer 6 and 7, was amplified with *Pfu* DNA polymerase using nPRL-R cDNA-specific primer 9 (5'-TCCAGACTACAAAACCTCT-3') and primer 10 (5'-AGTCACGTTCAACGGGGGA-3'), and subcloned into pT7blue T-vector. Authenticity of this cDNA was confirmed by sequencing. The 185 bp of cDNA labeled with [γ -³²P]ATP by the MEGALABEL (Takara) was used as hybridization probe. Hybridization was performed as described in Northern blot analysis. To control for potential contamination of the PCR reactions, reactions were performed with RNA that had not been reverse-transcribed or with samples lacking RNA in the reverse transcription. Each of these control reactions yielded negative results.



B

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ACCCAGGTCTTCTGCTCGTGTAAAGACAAGTTTGGACCAACCTCGGCACAGATGACTATCACAGTGAGCAAGTGATCCTGCGTTTCCAC 90
TCGGAGCAGAAATAAGCCATCAACATGCGGCAAAACCTGAAATATCTGCCTCAGCCGTCGCATTATTTTATTGCTTTTACTAAACACA 180
      M R Q N L K I S A S A V A F I L L L L N T
GATGTGATGGACGGAGAATCACTTCCTGGAAAGCCTGTTAACATCATGTGCGCTCTCCAGAAAAAGAACCTTTCTGCTGGTGGCAA 270
D V M D G E S L P G K P V N I M (C) R S P E K E T F S (C) W W Q
CCAGGTTCTGATGGGGACTTCCAATACTACAGCCTGTTATACAAAACAGAGGGAAAAACACTTACTCTGAATGTCCAGACTACAAA 360
P G S D G G L P T N Y S L L Y K T E G K N T Y S E (C) P D Y K
ACCTCTGGTCCCACTCATGTTTTTTTGTATAAAAAGCACAGCTCAATCTGGACGATGTATAATATCATGTGAATGCAACCAACGAGCTG 450
T S G P N S (C) F F D K K H T S I W T M Y N I I V N A T N E L
GGGAGTACTACGTCTGATCCCAATTTGTGGATGTAGCGTATATAGTTTTCAGCTTCGTCCTCCCGTTGAACGTGACTCTTTCTATAATCTAT 540
G S T T S D P K F V D V A Y I V Q L R P P L N V T L S I I Y
GAGCCTCCACATCTTCTGGTGAATGGTCACCAACCGTCAGAGGCGGATGTCAAGTCTGGTTGGGTAACCATGAATACGAGGTACAATTC 630
E P P H L L V K W S P P S E A D V K S G W V T I E Y E V Q F
AAATCTAAGAAAGCAAGGAATGGGAGACACTCACTGCTGTTAAACAACGACAGCTCAAGGTGTTTGTATTTAAATCCAGTGAGAACTAC 720
K S K K A K E W E T L T A G K Q R Q L K V F S L N P S E N Y
ATTGTACAAGTGCCTGTAAATCTGACCATGGGTTTTGGAGCATGTGGAGTCCAGAAAGCTACATCCAGATTCCAGACAACTTCCCAAGA 810
I V Q V R C K S D H G F W S M W S P E S Y I Q I P D N F P R
AAAGACATGACATTATGATATCGATCGCAGTGTATCATTTGTCTTTTGAACAATCATTTGGACAATGGCTCTTAAACGCTGGAGC 900
K D M T L W I S I A V L S F V I C L T I I W T M A L K R W S
ATGTTGACTTGCATCCTGCCCTGTTCCAGGGCCCAAAATATGGGCTTTGATAAGCAGTTACTTAAAGACAGGAAATCAGAAGAGCTG 990
M L T C I L P P V P G P K I M G F D K Q L L K T G K S E E L
TTAAGTGCCCTGGGTGTCAAGGTTTTCCACCAACATCAGACTGTGCAGATCTTCTGGTGAATTTCTCGAGGTAGATGACAGTAAAGAG 1080
L S A L G C Q G F P P T S D C A D L L V E F L E V D D S K E
CAGCTGATCTTAGCCATGACAAAGGGCACCCAAACCAACCAAGTACCCATGCTGAAACAGACAATGATTACAGGAGAGGGAGC 1170
Q L I S S H D K G H P N Q P T K L P H A E T D N D S G R G S
TGTGACAGTCTCTCGGCACTCTCGGAGAGATGCAAAAGACTCCAGAATCTTTCGGCAGCTCTTGAATCCGATGATGTTGGTGAAATGAAA 1260
C D S P S A L S E R C K D S R I S S A A L E S D D V G E M K
GAAATGCTGCACAACAAAGTACCGTAAATTTGGGCAATTCAAAGTCCAATGTCTGACAAACAGTTGCCTAACCAGTATGGGAAACCA 1350
E N A A Q Q S T V N W A I Q S P M S D K Q L P N P S D G K P
AACCCTTGGCCTGAAGGAGGAACAGTCAGTAATCAGACTCCTACATCCTCCTACCACAACATTACTGAAGTTTGTAAATTTGGCCCTTGGT 1440
N P W P E G G T V S N Q T P T S S Y H N I T E V C K L A L G
GCCATGAATGCAAAATATGTATCACTTCTAATGGCCAAATGAGGACAAGAAGCAGCCAAAATCACTTTAAACAGATTGAGACGATTAGCGAG 1530
A M N A N S S L L M A N E D K K Q P K Y F K T I E T I S E
GAGAGCGCAGGGAAGCAGACGATTGGAGTACATGCACCTCCAGAGCTTTTGACGAGACACAATGTGGCTTTTGCCCAACCTGAAGGCA 1620
E S A G K Q N E L E Y M H S R A F D Q D T M W L L P N L K A
TCTTTCATGTCTCCAAGACAATGGATTATGTGGAGGTTTCAAGAGTTAAACCAAAACATGCACTTGCCTGATACCAAAACAGAAGGAA 1710
S F M S P K T M D Y V E V Q K V N Q N N A L A L I P K Q K E
AGCCATGGGAGAAGGAAACAGTACCCTTCTACGGTCCGAGCAGGGAATATGCCAAGTGGCAGGAGTGGAGGATAAATATGTCTAGTA 1800
S H G R R E Q Y P S T G P S R E Y A K V A R V E D N N V L V
CTAATGCAGGACTCAGGATCTCAGAGCACCCAGTCTCCGAGAGCCATTAAAGAGTATTTCGAGATCCTCAACCAACAGGCTGAG 1890
L M Q D S G S Q S T P V L R E P F K E Y S Q I L Q P Q Q A E
AATAATTTGAGTAAATTTAACGCAGGGAGTCAAAATGAGAGCAAGAGCCAGGTGAGCAGACTGGGTTACATGGATCCAAGTGCCTGGCT 1980
N N L S K F N A G S Q N E S K S Q V S T L G M D P S A L A
CCCATGTTTGTAAATGAAAGTTTGATCATTATGAGTACTTGTTCGGGTAAAGCCACAGACTAAAAA 2058
P M F S *
  
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Fig. 1. (A) Schematic representation of the full length nPRL-R, and cDNA library and 3' RACE clones. The boxes indicate sequences of the coding region. The black and dotted areas are the putative signal peptides and the predicted transmembrane regions, respectively. Untranslated regions are given as lines. (B) Nucleotide and the deduced amino acid sequence of nPRL-R. The transmembrane region is underlined with a solid line. Two pairs of cysteine residues in the extracellular domain are circled. Potential N-linked glycosylation sites are underlined with dashed lines. A WSMWS sequence is surrounded by a stippled box. Box 1 is indicated by an open box and box 2 is double-underlined. A C-terminus tyrosine residue is surrounded by a slashed box. An asterisk indicates the termination codon. The nucleotide sequences here will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB005045.

RESULTS

cDNA cloning and structure of nPRL-R

A cDNA fragment of 420 bp was amplified from newt kidney cDNA using RT-PCR with degenerate primers, based on the sequences in the conserved extracellular domain of mammalian PRL-Rs. The amplified DNA showed 54% identity with human PRL-R and was used as a probe to screen a newt kidney cDNA library. Consequently, one positive clone was isolated by screening 5×10^5 phage clones. Sequence analysis revealed that this clone contained 1108 bp cDNA insert which, however, did not cover the entire coding sequence of nPRL-R cDNA. The missing 3' region of nPRL-R cDNA was amplified by RACE-PCR method (Fig. 1A).

The nucleotide sequences of nPRL-R cDNA and the deduced amino acid (aa) sequences are shown in Fig. 1B. The cDNA is 2058 bp in length and contains an open reading frame of 1878 bp. The open reading frame encodes 626-aa residues; there is a putative 26-aa signal peptide at the N-terminal region and a single transmembrane domain between residues 235 and 258. Thus, the mature protein is predicted to be a 600-aa protein with a 208-aa extracellular domain, a 24-aa transmembrane domain, and a 368-aa cytoplasmic domain. The predicted molecular mass is 67.1 kDa with an isoelectric point of 5.41. The extracellular domain of nPRL-R contains a WSXWS motif and two pairs of cysteine residues which are conserved in cytokine/GH/PRL receptor family. In addition, three potential N-glycosylation sites (Asn-62, Asn-107 and Asn-135) are present in the extracellular domain. The cytoplasmic domain of nPRL-R contains both a box 1 and a box 2 sequences, which are conserved in cytokine/GH/PRL receptor family (Murakami *et al.*, 1991). In addition, the C-terminal region of nPRL-R contains a tyrosine residue which is required for signal transduction of mammalian PRL-R (Lebrum *et al.*, 1995). These structural features of extracellular and cytoplasmic domains indicate that nPRL-R is similar to the mammalian long form of PRL-Rs.

Comparison of aa sequence

The aa sequence of nPRL-R is compared with those of various vertebrates (Table 1). The extracellular domain of nPRL-R shows 52-62% sequence identities with those of mammals, chicken and fish PRL receptors. However, the cytoplasmic domain of nPRL-R shows lower sequence identity with other PRL receptors although the box 1 and box 2 are conserved; there is 30-48% identity between nPRL-R and other PRL-Rs. Therefore, overall identity between nPRL-R and mammalian or chicken PRL-Rs is 50-52%. This value is the same level of identity as that between mammalian and chicken PRL-Rs. On the other hand, overall identity between newt and fish PRL-R is significantly low (38%), the same level as that found between mammalian PRL-Rs and fish PRL-R (35-38%). In addition, overall identity between nPRL-R and other vertebrate growth hormone receptors (GH-Rs) is less than 25%.

COS-7 cell expression and binding experiment

To express the nPRL-R in COS-7 cells, we cloned by RT-PCR a 1.9 kb of nPRL-R cDNA containing the entire coding region of nPRL-R and transfected into COS-7 cells. Membranes were prepared from COS-7 cells transfected with pnPRL-R (containing the 1.9 kb cDNA) and used for binding experiments. The various concentrations of unlabeled rat PRL competed with [125 I] rat PRL bound to the membranes (Fig. 2). In addition, ovine PRL competed with [125 I] rat PRL in the same range of concentrations as unlabeled rat PRL. A slight displacement of [125 I] rat PRL was observed with rat GH or ovine GH. From these results it is indicated that the cloned cDNA encodes functional nPRL-R. As a negative control, using the membranes from COS-7 cells transfected with pSVL (no insert), no specific binding was observed (data not shown).

Expression of PRL-R in newt tissues

Expression of nPRL-R in various tissues was analyzed by Northern blot analysis. A single mRNA species of more than 10 kb was identified in the kidney and brain (Fig. 3A). In the liver, testis and ovary, however, no transcripts were de-

Table 1. Comparison of aa identity of the nPRL-R with PRL-Rs of various vertebrates

Receptor	% aa sequence identity of the nPRL-R					
	Extracellular domain		Cytoplasmic domain		Overall identity	
	PRL-R	GH-R	PRL-R	GH-R	PRL-R	GH-R
Human	55	28	48	19	51	22
Rat	60	30	42	21	50	24
Rabbit	62	31	46	22	52	25
Chicken	52/62 ^a	29	43	20	50 ^b	22
Fish	55	—	30	—	38	—

^a The first and the second values correspond to the identity with the membrane-distal and -proximal units, respectively.

^b Membrane-distal unit was excluded in the comparison.

tected. Then, we performed RT-PCR analysis using nPRL-R-specific primers (Fig. 3B). A strong signal was detected in the bladder in addition to the kidney and brain. In the testis, ovary and liver, weak but significant signals were detected.

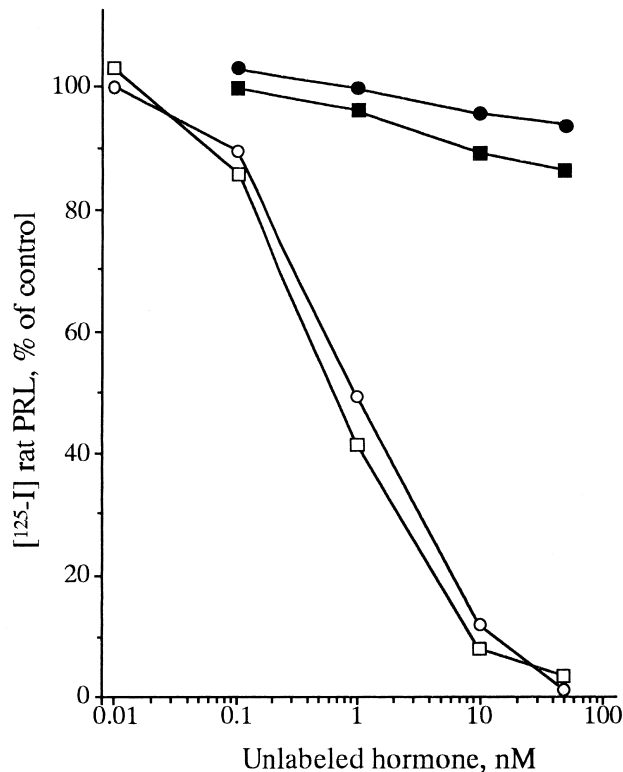


Fig. 2. Competitive binding of [125 I] rat PRL to membranes from COS-7 cells transfected with the nPRL-R cDNA. Membranes were incubated in the presence of ligand and various concentrations of unlabeled rat PRL (○), ovine PRL (□), rat GH (●) or ovine GH (■). The results are expressed as the percentage of the maximal specific binding observed in the absence of competitor.

DISCUSSION

In this study, we cloned nPRL-R cDNA from newt kidney and confirmed that the cDNA encodes nPRL-R by binding experiment. As far as we know, this is the first report on cloning of amphibian PRL-R cDNA.

Comparison of aa sequences shows that nPRL-R has about 50% identity to mammalian and chicken PRL-Rs, and 38% identity to fish PRL-R. On the other hand, mammalian PRL-Rs show about 70% identity with each other, about 52% identity to avian PRL-Rs and 35-38% identity to fish PRL-R. These relations of identity among vertebrate PRL-Rs are also found in those of identity among vertebrate PRLs. The mammalian PRLs show 60-70% identity with each other, 68% identity to chicken PRL and about 30% identity to fish PRLs. The bullfrog (*Rana catesbeiana*) PRL shows 70% identity to chicken PRL, 60 and 50% sequence identity to human and rat PRL, respectively, and about 30% sequence identity to fish PRLs (Yasuda *et al.*, 1991). These findings may suggest a coevolution of PRLs and PRL-Rs in vertebrates.

The predicted structure of nPRL-R is similar to that of mammalian long form of PRL-Rs. The nPRL-R contains a single unit of extracellular domain and no homologous repeats as observed in avian PRL-Rs (Tanaka *et al.*, 1992; Chen and Horseman, 1994; Zhou *et al.*, 1996). In the extracellular domain of nPRL-R, conserved are two pairs of cysteine residues and a WSXWS motif both of which are required for ligand-receptor interactions in mammalian PRL-Rs (Rozakis-Adcock and Kelly, 1991; Baumgartner *et al.*, 1994). This suggests that these motifs play crucial roles in ligand-receptor interaction in amphibian PRL-Rs as well as in mammalian PRL-Rs. Three potential N-glycosylation sites of nPRL-R are present in the same relative position as those of the mammalian PRL-Rs. Since the N-glycosylation site between the second and the

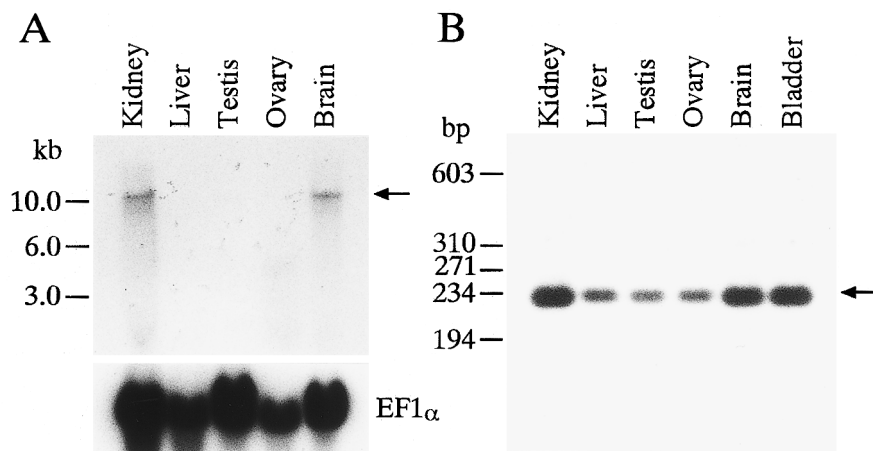


Fig. 3. Expression of PRL-R mRNA in various newt tissues. (A) Northern blot analysis. Five μ g of poly(A)⁺-RNA was loaded on denaturing agarose gel, blotted, and hybridized with 1.1 kb of 32 P-labeled nPRL-R cDNA. The arrow indicates the position of the nPRL-R mRNA. Same filter was reprobbed with 1 kb of 32 P-labeled newt EF1 α cDNA (The nucleotide sequence of newt EF1 α has been submitted to the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number AB005588). (B) RT-PCR analysis. Two μ g of total RNA was reverse-transcribed and amplified by 40 cycles of PCR using primers 5 and 6. The amplified products were loaded on agarose gel and analyzed by Southern blot analysis. The arrow indicates 234 bp of PCR amplified products.

third extracellular cysteine residues is absent in fish PRL-R, it seems that N-glycosylation sites in PRL-Rs are conserved from amphibians to mammals and some evolutionary changes occurred between fishes and amphibians. In the cytoplasmic domain of nPRL-R, box 1 and box 2 sequences, which are involved in signal transduction of cytokine/GH/PRL receptor family (Murakami *et al.*, 1991) in mammals, are conserved. In addition, conserved also is a C-terminal tyrosine residue which is responsible for activation of lactogenic genes in mammals (Lebrum *et al.*, 1995a). These structural features in the cytoplasmic domain are also observed in avians and fish PRL-Rs. From these findings it is suggested that vertebrate PRL-Rs have a common signal transduction pathway mediated by box 1 and box 2 sequences and a C-terminal tyrosine residue. Recently, Lebrum *et al.* (1995b) showed that the association of Jak 2 with box 1 is required for signal transduction of PRL-R. In amphibians, PCR fragment of a Jak family tyrosine kinase was cloned from newt testis (Takamune *et al.*, unpublished results). It is possible that Jak family kinase is also involved in the signal transduction of amphibian PRL-Rs.

Binding experiments showed specific binding of rat PRL to COS-7 cell membranes transfected with nPRL-R. In previous PRL-binding studies for amphibian tissues, ovine prolactin was widely used and served as a ligand for the endogenous amphibian PRL-Rs (White, 1981; Tarpey and Nicoll, 1987). Thus, we used ovine PRL as a competitor in our binding experiments and specific competition was observed. It is reported that ovine PRL has GH-like activity in amphibians (Nicoll, 1982) and binds specifically to eel GH-R (Hirano, 1991). Therefore, the cDNA isolated in the present study could encode newt GH-R which binds to mammalian PRL. However, this possibility is excluded by the result that both rat and ovine GHs scarcely competed with [¹²⁵I] rat PRL bound to the membranes. A slight displacement of [¹²⁵I] rat PRL by rat and ovine GHs may be due to the contamination of PRL in the rat and ovine GHs. Hence, we concluded that cDNA isolated in the present study encodes the nPRL-R.

Northern blot analysis revealed a single transcript of more than 10 kb in the newt kidney and brain. This result is consistent with previous studies that PRL binds strongly to the microsomal fraction of kidney (White, 1981; Tarpey and Nicoll, 1987) and brain (Lüthy *et al.*, 1985; Muccioli *et al.*, 1990) in amphibians, suggesting that PRL directly regulates hydro-mineral balance and its own secretion in the respective organs in the newt. The newt mRNA expression pattern, where a single transcript encodes a PRL-R, is similar to the pattern reported in fish (Sandra *et al.*, 1995). In contrast to newt and fish, several transcripts for PRL-R are expressed in mammals (Buck *et al.*, 1992). This result suggests that multiple transcripts derived from varied sites of polyadenylation and/or alternative splicing do not exist in the newt and fish PRL-Rs.

RT-PCR analysis showed that nPRL-R was expressed strongly in the bladder and weakly in liver. The expression in the bladder agrees with the binding data (White, 1981) and supports the idea that PRL regulates the hydromineral balance. Compared to mammals, the level of PRL-R expression

in the liver was lower in the newt. This result is consistent with the previous studies that PRL binds weakly to the microsomal fraction of amphibian livers (White, 1981), suggesting that the liver is not a major target organ of PRL in amphibians and PRL does not play a major role in the regulation of liver enzymes, hepatic water contents or energy substrates in the amphibian liver. In the reproductive organs of newt, low level of PRL-R expressions was observed. Mazzi *et al.* (1967) showed that PRL induces the degeneration of spermatogonia, suggesting that PRL represses the FSH production in the hypothalamus. We demonstrated that low temperature caused elevation of prolactin concentration in the newt blood, which induced cell death of spermatogonia just before meiosis (Yazawa *et al.*, submitted). Although the function of PRL in reproductive organs are still unclear, our results indicate that PRL acts directly to those organs.

In amphibians, PRL exerts a versatile physiological action: It induces larval growth, inhibition of metamorphosis and regulation of osmolarity (Ensor, 1978; Kikuyama *et al.*, 1993). PRL also plays important roles in reproduction especially in urodeles (Mazzi and Vellano, 1987; Polzonetti-Magni *et al.*, 1995). The availability of a nPRL-R cDNA will contribute to a better understanding of the molecular mechanism of PRL actions in various tissues of amphibians.

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