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F. Rentier-Delrue, D. Swennen, Patrick Prunet, M. Lion, J.A. Martial. Tilapia prolactin: Molecular cloning of two cDNAs and expression in *Escherichia coli*. *DNA Barcodes*, 1989, 8 (4), pp.261-270. 10.1089/dna.1.1989.8.261 . hal-02728004

HAL Id: hal-02728004

<https://hal.inrae.fr/hal-02728004>

Submitted on 2 Jun 2020

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Tilapia Prolactin: Molecular Cloning of Two cDNAs and Expression in *Escherichia coli*

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ABSTRACT

We have isolated cDNA clones encoding tilapia prolactin (tiPRL) from a cDNA library prepared from tilapia (*Oreochromis niloticus*) anterior pituitary glands. A trout PRL cDNA fragment was used as hybridization probe to select the recombinant plasmids carrying the tiPRL coding sequence. Two types of PRL cDNA were isolated and their complete nucleotide sequence determined. The larger cDNA (tiPRL-I) codes for a polypeptide of 212 amino acids, including a putative signal sequence of 24 amino acids, and contains a 3' untranslated region of 787 bp. The second prolactin cDNA (tiPRL-II) encodes a polypeptide of 200 amino acids, including a presumptive signal peptide of 23 amino acids, and contains a noncoding region of 512 bp. tiPRL-I and tiPRL-II cDNA sequences are 81% similar, whereas the encoded proteins share 69% amino acid identity at optimal alignment. Mature tiPRL-I was efficiently expressed in *Escherichia coli* carrying a plasmid in which the tiPRL-I cDNA was under the control of the $\Phi 10$ promoter of T7 bacteriophage. The new recombinant protein representing about 45% of the total cellular proteins was found in inclusion bodies and cross-reacted with salmon PRL antiserum.

INTRODUCTION

AS IN OTHER VERTEBRATES, prolactin (PRL) in fish has a pleiotropic spectrum of biological activities that are involved in a variety of physiological mechanisms such as osmoregulation, reproduction, metabolism, behavior, and mucus production. Most studies devoted to this hormone in fish deal with its osmoregulatory activity. PRL's role in supporting freshwater adaptation has thus become increasingly established (for review, see Clarke and Bern, 1980; Loretz and Bern, 1982; Hirano, 1986).

Tilapia is generally considered as a euryhaline fish capable of adapting to high saltwater salinity. Recent development of tilapia aquaculture in brackish water or in seawater has emphasized salt tolerance problems and has raised new interest in the physiology of osmoregulation in this species (Prunet and Bornancin, 1989). PRL is certainly among the most important hormones involved in the control of hydromineral balance in tilapia (Dharmamba, 1970; Dharmamba and Maetz, 1972, 1976; Clarke, 1973; Foskett *et al.*, 1982).

Recently, two tilapia PRLs have been isolated from culture medium of pituitaries from the tilapia species *Oreochromis mossambicus* (Specker *et al.*, 1985a; Yamagushi *et*

al., 1988). Biochemical analyses indicated that these two forms are distinct proteins of different sizes, but studies on the osmoregulatory activities of both PRLs did not indicate any clear differences (Specker *et al.*, 1985a; Young *et al.*, 1988).

A more complete characterization of biological activities of both PRLs in tilapia would be of interest. However, this would require large amounts of pure hormone. To achieve such hormonal production and to gain more insight into gene regulation requirements underlying biological activities of both PRLs, we constructed a tilapia (*Oreochromis niloticus*) pituitary cDNA library. In this report, we describe the molecular cloning and characterization of two PRL cDNAs differing in size, and the expression of the larger of these cDNAs in *Escherichia coli*.

MATERIALS AND METHODS

Plasmid pUC13 and cDNA synthesis kit were purchased from Amersham Laboratories. The sequencing kit was from the United States Biochemical Corporation. Enzymes were purchased from Amersham Laboratories and Boehr-

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inger Mannheim. The synthetic oligonucleotides were provided by Eurogentec S.A. (Liege, Belgium). They were purified by electrophoresis on 16% polyacrylamide gels containing 7 M urea (Narang *et al.*, 1979).

cDNA library construction

Fresh pituitaries from tilapia (*O. niloticus*) raised in freshwater were collected at the CERER (Warm Water Pisciculture Center, Tihange, Belgium). To enrich the preparation in PRL mRNA-producing cells, the anterior part of the pituitary gland was isolated. Total RNA was purified by the guanidium isothiocyanate procedure (Ulrich *et al.*, 1977; Chirgwin *et al.*, 1979) and mRNA was purified by chromatography on oligo(dT)-cellulose. Oligo(dT)-primed cDNA was synthesized according to the Gubler and Hoffman (1983) procedure. The double-stranded cDNA was size-fractionated by chromatography on Sepharose 4B (Pharmacia), and blunt-ended molecules larger than 700 nucleotides were inserted into the pUC13 plasmid (Viera and Messing, 1982), previously cut by *Sma* I, and dephosphorylated. The ligated product was introduced into *E. coli* RR1 Δ M15 (lacZ, M15, F', lacI^q, ZM15, proA) (ATCC collection), according to Mandel and Higa (1970), and grown on Luria broth (L broth) plates containing 100 μ g/ml of ampicillin, 0.5 mM isopropyl- β -D-thiogalactoside (IPTG), and 0.01% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal).

cDNA screening

White colonies containing plasmid with cDNA insert were transferred to nitrocellulose filters and grown overnight. They were lysed, neutralized, and fixed (Grunstein and Hogness, 1975). Prehybridization was carried out in 6 $\times$ SSC (1 $\times$ SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1 $\times$ Denhardt's solution (0.02% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin), and denatured salmon sperm DNA (100 μ g/ml). Hybridization was performed overnight at 45°C, in the same buffer, with ³²P-labeled probe. Filters were washed in 6 $\times$ SSC/1 $\times$ Denhardt for 30 min at room temperature, then washed for 30 min at temperatures up to 54°C.

DNA sequencing

DNA sequence analysis was carried out by the dideoxynucleotide chain-termination method according to Sanger *et al.* (1977) after cloning of the entire insert in the bacteriophage M13 (mp18 and mp19 vectors) (Yanish-Perron *et al.*, 1985). Use was made of synthetic oligonucleotide primers in the sequencing strategy.

Construction of tilapia PRL-I expression vector

Vector pAR3040 (Rosenberg *et al.*, 1987) is a derivative of pBR322 that contains the Φ 10 promoter and the natural T Φ terminator for T7 RNA polymerase. This plasmid was modified in our laboratory to facilitate cloning by (i) re-

FIG. 1. Partial restriction map and sequencing strategy of tiPRL-I (A) and tiPRL-II (B) inserts. From left to right, the diagram shows: the 5' untranslated region (thick line), the region coding for the peptide signal (hatched box), the mature tiPRL (open box), and the 3' untranslated region including the poly(A) tail (thick line). Thin line represents the region of the pUC13 plasmid multiple cloning sites. Restriction enzyme cleavage sites are indicated as follows: Ba, *Bam* HI; Bc, *Bcl* I; Bg, *Bgl* II; Ec, *Eco* RI; Hi, *Hind* III; Ps, *Pst* I; Pv, *Pvu* II; Ta, *Taq* I. Arrows indicate direction and extent of sequencing.

BL21 (DE3), a λ lysogen of BL21 (r_B^- m_B^- rif^s) in which the prophage carries a copy of the gene for T7 RNA polymerase under the control of the *lac* UV5 promoter, was grown from inoculum, overnight, in 5 ml of Luria broth, in the presence of 100 μ g/ml of ampicillin. Of this culture, 0.4 ml was used to inoculate 10 ml of the same medium with ampicillin. Synthesis of T7 RNA polymerase was induced by adding 0.4 mM IPTG when the culture reached $OD_{600} = 0.8$. Three hours later, cells were collected, dissolved in Laemmli sample buffer (1970), and analyzed by NaDodSO₄ polyacrylamide gel electrophoresis with Coomassie blue staining.

FIG. 3. Hydropathy profile of tilapia PRL-I (A) and PRL-II (B). Hydropathy score for each residue was obtained by averaging the hydropathy indices of 7 amino acids at a time.

RESULTS

Isolation of two prolactin cDNA clones

Poly(A)⁺RNA purified from tilapia pituitaries was used as a template for DNA synthesis. Approximately 4,200 recombinant clones were obtained. These clones were hybridized with a ³²P-labeled 700-bp *Eco* RI fragment of trout prolactin (Mercier *et al.*, 1989) corresponding to the coding region of the molecule. The washing temperature was raised up to 54°C. At this temperature, 156 clones hybridized selectively. Among these, 22 full-length clones were analyzed by restriction mapping. They could be divided into two groups based on the restriction map of the inserted cDNA (Fig. 1, A and B). ptiPRL-I contained one *Bgl* II, one *Hind* III, and one *Taq* I site as well as two *Bcl* I and *Pst* I sites. Its full length was about 1,600 bp. ptiPRL-II contained single *Eco* RI and *Pvu* II sites, two *Pst* I and two *Hind* III sites, and three *Taq* I sites. Its full length was about 1,300 bp. Out of 22 recombinant plasmids, 16 had the type I pattern and 6 had the type II pattern.

Characterization of tiPRL-I and tiPRL-II

To confirm that tiPRL-I and tiPRL-II represent tiPRL clones, the nucleotide sequences were determined. Figure

1, A and B, depict the overall structure and sequencing strategy of both prolactin cDNAs, and the complete nucleotide sequences are shown in Fig. 2. The tiPRL-I insert contains a large open reading frame of 636 nucleotides. The 3' untranslated region is 787 bp long and contains the hexanucleotide AATAAA which precedes the polyadenylation tail of many eukaryotic mRNAs. The encoded polypeptide has 212 amino acids and a calculated molecular weight of 23,534 daltons. The amino-terminal end of the molecule includes a large number of hydrophobic amino acid residues, consistent with the putative role of this segment as a signal peptide that is cleaved off in the secretion process. The mature protein sequence starts at valine residue number 1. This interpretation is based on the known amino acid sequence (Specker *et al.*, 1985a; Yamagushi *et al.*, 1988). The mature protein is thus 188 amino acids long and has a molecular weight of 20,891 daltons.

The tiPRL-II sequence includes a 38-bp 5' noncoding sequence, an open reading frame of 600 bp, and a 3' untranslated region of 512 bp which also contains a polyadenylation signal. The coding region of tiPRL-II encodes a polypeptide of 200 amino acids and a mature protein of 177 amino acids. The molecular weights of the pre- and mature proteins are 22,129 daltons and 19,591 daltons, respectively. The first 23 amino acids constitute the signal peptide.

Comparison of tiPRL-I and tiPRL-II cDNA

The nucleotide sequence of tiPRL-I cDNA is much longer than that of tiPRL-II cDNA, due to a longer coding region (36 additional nucleotides) and a longer 3' untranslated region (270 additional nucleotides) (Fig. 2). Codon usage is uneven for both proteins with a preference for guanosine and cytosine in the third position (data not shown). Comparison of tilapia prolactins deduced from the nucleotide sequence reveals that tiPRL-II preprotein is 12 amino acids shorter than tiPRL-I. Furthermore, 50 amino acid substitutions are located at random. In the signal peptide, 1 amino acid is missing in tiPRL-II and 4 amino acids are substituted. The hydropobicity profiles (Kyte and Doolittle, 1982) of the proteins encoded by tiPRL-I and tiPRL-II cDNA are shown in Fig. 3. For both proteins, a strongly hydrophobic region is found at the amino terminus which corresponds to the signal peptide of the molecule.

Expression of tiPRL-I in *E. coli*

Figure 4 shows the construction of the expression vector based on the T7 RNA polymerase (Studier and Moffatt, 1986) for the production of an unfused protein. The PARAE vector, a derivative of pAR3040, was digested by *Nde* I and *Bam* HI. Cutting the ptiPRL-I DNA with *Taq* I and *Bgl* II released a 877-bp fragment containing the complete region coding for the tiPRL-I mature protein, except for its first 20 nucleotides. These nucleotides were synthe-

FIG. 4. Schematic representation of the construction of plasmid pT7-tiPRL-I. Plasmid pAR3040 was modified as described in the Materials and Methods section (*) and called pARAE. Plasmid pT7-tiPRL-I was constructed from pARAE and ptiPRL-I using synthetic oligonucleotides as described in the text. Stippled box and hatched box represent the $\Phi 10$ promoter and T Φ terminator for T7 RNA polymerase, respectively. Arrow indicates orientation of the $\Phi 10$ promoter. Heavily hatched box in ptiPRL-I represents the entire tilapia PRL-I cDNA. Ap^R, Gene coding for the ampicillin resistance.

FIG. 5. Analysis of tiPRL-I synthesis by NaDodSO₄ polyacrylamide gel electrophoresis. *E. coli* BL21(DE3) harboring plasmid pT7tiPRL-I was grown at 37°C in L broth containing 100 µg/ml ampicillin. At OD₆₀₀ = 0.8, synthesis was induced or not with IPTG and culture was grown for 3 hr at 37°C. Total cell lysate was prepared by centrifuging 1 ml of cells. The pellet was dissolved in 100 µl of Laemmli sample buffer and 10 µl were heated at 90°C for 5 min and loaded onto a 12.5% polyacrylamide gel. A. Total cellular proteins of uninduced (lane 1) and induced (lane 2) cells. Lane 3, Total cellular proteins of BL21(DE3) containing the pARAE plasmid without insert. B. Western blot analysis of tiPRL-I. Total proteins were transferred to nitrocellulose (Towbin, 1979) and tiPRL was detected using a salmon PRL antiserum. The immune complexes were revealed by peroxidase-conjugated antibodies.

sized chemically with *Nde* I and *Taq* I protruding ends to ensure the correct cloning and orientation behind the T7 promoter in plasmid pARAE. The ligation products were cloned in *E. coli* strain HB101. The correct recombinant plasmid was identified by standard techniques and called pT7tiPRL-I. To direct the expression *in vivo*, it was transferred into *E. coli* strain BL21 (DE3).

Expression of tiPRL-I was directed by T7 RNA polymerase. In the presence of IPTG, T7 RNA polymerase is switched on, which allows the expression of genes under the control of the T7 promoter. Total extracts from induced and noninduced cells were analyzed by NaDodSO₄ polyacrylamide gel. In the presence of IPTG, a new protein of about 24 kD appeared, which was absent from extracts of bacteria containing the vector plasmid only (Fig. 5A). The amount of tiPRL was estimated to represent 45% of the total cellular proteins. In addition, inclusion bodies were observed in bacterial cells carrying the recombinant expression vector. These particles were easily purified by

sonication and centrifugation. To confirm the identity of the tiPRL in the induced pT7tiPRL-harboring BL21(DE3) cells, a Western blot analysis was performed on proteins extracted from both induced and noninduced cells, using a salmon PRL antiserum. The results showed that the extra protein whose expression is induced upon addition of IPTG, cross-reacts with the salmon antiserum and should correspond to the tiPRL-I (Fig. 5B).

DISCUSSION

We have identified two PRL cDNAs in a tilapia (*O. niloticus*) pituitary cDNA library. Identification was done by hybridization under low stringency with a trout PRL cDNA probe. Compared at a preprotein level, these two tilapia PRLs share 151 out of 212 amino acids. The mature proteins have 132 out of 188 amino acids in common. The larger protein, tiPRL-I, is 11 amino acids longer than the smaller one, tiPRL-II.

A "large" (tiPRL₁₈₈) and a "small" (tiPRL₁₇₇) tilapia PRL have already been purified from cultured pituitaries of tilapia *O. mossambicus*, and their complete amino acid sequence determined (Yamaguchi *et al.*, 1988). Our PRLs have the same amino acid content as the ones sequenced by these authors with the exception of two amino acid substitutions: Phe₇ and Thr₁₀₀ in *O. niloticus* PRL-I are replaced by Leu₇ and Ser₁₀₀ in the corresponding PRL of *O. mossambicus*. These minor changes are probably species specific.

Ninety-one nucleotide substitutions and 36 deletions distinguish the coding region of tiPRL-I and II cDNAs. This corresponds to 81% similarity. However, the two proteins share only 69% amino acid identity at optimal alignment. The discrepancy in the degree of similarity between the nucleotide and protein sequences is a consequence of the high number of nucleotide mutations that cause amino acid replacements. Indeed, only 21 substitutions are located in the third or "wobble" position of their respective codons. It should be noted that, when correctly aligned, two regions of the 3' noncoding sequences are highly conserved (Fig. 2): the first, close to the stop codon, is 64 bp long and 81% similar. The second, 292 bp long and located near the poly(A) tail, is 66% similar. The rest of the 3' noncoding sequences is completely dissimilar.

A comparison of the nucleotide and the polypeptide sequences clearly shows that the two forms of PRL are products of distinct genes. Two forms of PRL have also been described in salmon (*Oncorhynchus keta*) (Yasuda *et al.*, 1986). However, in the latter case, both PRLs are highly similar with only 4 amino acid replacements. In trout and salmon, the majority of pituitary hormones are always isolated in pairs with little variation in the amino acid content (Kawauchi, 1983). These genetic variant forms are explained by the tetraploid origin of the Salmonidae family (Ohno *et al.*, 1968). However, it appears that, except for a few cases of gene duplications by tetraploidization among certain so-called "primitive" teleosts such as Salmonidae and Cyprinidae, gene duplication of most teleost fish oc-

FIG. 6. Amino acid sequences comparison of seven mammalian and five fish mature PRLs. Gaps were made to maximize homology. Arrows indicates identical or nearly identical (one mismatch allowed) among all PRLs. Identical residues among all mammalian PRLs are represented by dots and those among all fish PRLs are represented by stars.

followed by divergent evolution. It is noteworthy that only one growth hormone has been found so far in two species of tilapia, at the protein level for *O. mossambicus* (Farmer *et al.*, 1976; Specker *et al.*, 1985b) and at the cDNA level for *O. niloticus* (Rentier-Delrue *et al.*, 1989; accompanying

FIG. 7. Superimposed hydropathy profiles of tiPRL-I and tiPRL-II. tiPRL-II profile has been interrupted at two locations, corresponding to the deleted amino acids and indicated by gaps in the upper frame, to match the corresponding profile of tiPRL-I. Vertical lines and horizontal lines represent profiles of tiPRL-I and tiPRL-II, respectively. Black areas represent overlapping profiles.

paper). In the eel family, two growth hormones and one PRL have been isolated from the species *Anguilla japonica* (Kishida *et al.*, 1987).

Comparison of both tilapia PRLs with PRLs of trout (*Salmo gairdneri*) (Mercier *et al.*, 1989), chum salmon (*Oncorhynchus keta*) (Yasuda *et al.*, 1986), and carp (*Cyprinus carpio*) (Yasuda *et al.*, 1987) reveals that tiPRL-I is more similar to all fish PRL than tiPRL-II. tiPRL-I is 68% similar to trout or chum salmon PRL (which differ by only 1 amino acid) and 64% similar to carp PRL, while tiPRL-II is only 53% and 50% similar to trout or chum salmon and carp PRL, respectively. Seven mammalian and five teleost PRLs were compared (Fig. 6). The most striking structural difference between fish and mammalian PRL is the absence, in the fish hormones, of the small disulfide loop at the amino terminus, as occurs in all GH molecules. However, it has been shown that this Cys₄₋₁₁ disulfide loop in PRL is not required to maintain the biological activity of PRL in vertebrates (Doneen *et al.*, 1979). In all PRLs, four regions of highly conserved amino acids are found (18–32, 58–72, 80–98, and 176–193 in this alignment). The divergence between mammalian and teleost PRLs in the nonconserved regions is obvious and probably reflects the physiological differences that exist between mammals and teleost fish. When fish PRLs are compared among themselves, 84 amino acids out of 188 are identical. However, when tiPRL-II is excluded from the comparison, the number of identical residues climbs to 103. This tends to put tiPRL-II aside from the other PRLs and leads us to believe that tiPRL-II evolved under less stringent constraints and might have distinct biological properties, except for its osmoregulatory functions, which appear to be maintained. Indeed, tiPRL-I and tiPRL-II act on freshwater osmoregulation in a similar fashion. Both PRLs restore normal plasma ion levels and transepithelial potential (TEP) in hypophysectomized tilapia transferred to freshwater (Specker *et al.*, 1985a; Young *et al.*, 1988). Moreover, neither PRL restores gill Na⁺-K⁺ ATPase activity in hypophysectomized tilapia, suggesting that, in freshwater,

both hormones may act on epithelial permeability to Na⁺ (Young *et al.*, 1988). Studies on the neuroendocrine control of tiPRL-I and tiPRL-II production indicate that somatostatin (SRIF) and urotensin II inhibit similarly the *in vitro* release of both tiPRLs (Rivas *et al.*, 1986). The only difference between tiPRL-I and tiPRL-II in the osmoregulatory aspects studied so far is found in the dose-dependency of the effects on integumental TEP in salamander (Young *et al.*, 1988).

Superimposition of hydropathy profiles of both tilapia PRLs, taking into account the deleted regions of tiPRL-II, reveals that the first missing domain is hydrophilic and the second is very hydrophobic and therefore believed to be located inside the molecule (Fig. 7). If these deletions induce a change in the conformation of the molecule, they have a very weak effect on the osmoregulatory functions of tiPRLs. Growth-promoting activity seems to differ more markedly in the two proteins, since tiPRL-I displays this type of activity in juveniles of the tilapia *O. mossambicus* while tiPRL-II does not (Specker *et al.*, 1985b).

To characterize the tilapia PRLs better, we have expressed tiPRL-I in *E. coli*. The expression system based on the T7 RNA polymerase was used because it offers stringent control and good level of expression (Studier and Moffatt, 1986). Using this system, we constructed a recombinant expression vector containing the cDNA coding for the tiPRL-I. The expression plasmid directed the synthesis of tiPRL-I very efficiently, since the level of expression achieved was approximately 45% of the total cellular protein. The newly synthesized protein was found as insoluble granules. A similar strategy currently is being used to express the smaller prolactin (tiPRL-II).

ACKNOWLEDGMENTS

This work was supported by grant C320 from the Région Wallonne, Ministère des Technologies Nouvelles, Belgium. We are grateful to Dr. A. Renard (Eurogentec, Liège, Belgium) for helpful discussions. We also thank Dr. J.C. Philippart (Laboratoire de Démographie des Poissons et de Pisciculture, Université de Liège, Belgium) and the members of the CERER-Pisciculture (Tihange, Belgium) for providing tilapias and for their help in collecting the pituitaries.

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Received for publication January 12, 1989.