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Rainbow Trout Prolactin cDNA Cloning in *Escherichia coli*

L. MERCIER,* F. RENTIER-DELRUE,§ D. SWENNEN,§ M. LION,§ P. LE GOFF,†
P. PRUNET,‡ and J.A. MARTIAL§

ABSTRACT

We describe the isolation and characterization of a cDNA for trout prolactin (tPrl). An extensive analysis of tPrl recombinant clones by restriction analysis and sequencing revealed the presence of only one form of tPrl mRNA. The deduced protein sequence consists of 210 amino acids, including a signal peptide of 23 amino acids. The amino acid sequence of the mature protein is compared among teleosts and mammals, showing two domains of strong similarity that may be involved in biological activity.

INTRODUCTION

PROLACTIN (Prl) and growth hormone (GH) are polypeptide hormones of pituitary origin. Including mammalian placental lactogen and proliferin (Linzer and Nathans, 1984), they belong to a protein family with related structure and function.

Evaluation of the biological and physiological roles of these hormones in Salmonids is based mostly on experiments in which hormones from other species were used (Donaldson *et al.*, 1979; Clarke and Bern, 1980). Recently, homologous salmon GH has been used in Salmonids and its effect on growth has been well characterized (Furuya, 1985; Agellon and Chen, 1986; Sekine *et al.*, 1986). However, there is still little information concerning the role of Prl and GH in fish adaptation to new osmotic environments (Loretz and Bern, 1982; Hirano, 1986).

The development of seawater stock farming experiments has raised considerable interest in factors involved in growth and adaptation to changes in water salinity (see Harache, 1986, for a review). According to Nicoll (1980), the primary role of prolactin in fish is to regulate water and electrolytes homeostasis. Likewise, Prunet *et al.* (1985) reported increased plasma Prl when immature rainbow trout were transferred from seawater to freshwater. Moreover, Hirano (1986) suggests that Prl inhibits, while GH favors, seawater adaptation in Salmonids and reports distinct effects of the proteins on plasma ion levels in most assays.

To study the structural requirements for biological activities of Prl and GH in rainbow trout, we constructed a trout pituitary cDNA library and isolated a cDNA encoding trout Prl. Trout GH cloning and expression in *Escherichia coli* are described in the accompanying paper (Rentier-Delrue *et al.*, 1989).

MATERIALS AND METHODS

cDNA library construction

Pituitaries collected from rainbow trout (*Salmo gairdneri*), raised in seawater at the SODAB experimental farm (Tredarzec, France), were immediately frozen in liquid nitrogen and stored at −75°C.

Poly(A)$^+$RNA isolation, cDNA synthesis, and library construction were performed as described in the accompanying paper (Rentier-Delrue *et al.*, 1989).

mRNA translation and immunoprecipitation

The poly(A)$^+$RNA was translated *in vitro* with a rabbit reticulocyte cell-free translation kit in the presence of [^{35}S]-

*Université d'Angers, INSERM U298, CHR, 49033 Angers Cédex, France.
†Laboratoire de Biologie Moléculaire, Campus de Beaulieu, 35042 Rennes Cédex, France.
‡Laboratoire de Physiologie des Poissons, INRA, Campus de Beaulieu, 35042 Rennes Cédex, France.
§Laboratoire Central de Génie Génétique, Université de Liège, 4000 Sart Tilman, Belgium.

methionine (Amersham Laboratories, 50 Tbq/mmole). Trout prePrl synthesized in the cell-free system was immunoprecipitated with rabbit antiserum to salmon Prl (Prunet *et al.*, 1985). Control immunoprecipitation was carried out using a normal rabbit serum. Immune complexes were precipitated with protein A of the Cowan strain of *Staphylococcus aureus*, as described (Martial *et al.*, 1977). ^{35}S-labeled proteins were analyzed by electrophoresis on $NaDodSO_4$-polyacrylamide slab gels and autoradiography with the Kodak X-omat S film.

Screening and sequencing

Recombinant colonies immobilized on nitrocellulose filters were screened (Wooks, 1984) with the mixed synthetic 24-mer oligonucleotide probes described in Fig. 1. These probes were kindly provided by Dr. P. Valenzuela from Chiron Corporation (Emeryville, CA). The nucleotide sequence was predicted from the amino acid sequence of salmon Prl (*Oncorhynchus keta*) (Yasuda *et al.*, 1986) and the codon usage in salmon GH mRNA (Sekine *et al.*, 1985). It was expected to be complementary to nucleotides coding for amino acids 160–167 of tPrl.

The probes were 5′-labeled with [γ-^{32}P]dATP (Amersham Laboratories, 185 TBq/mmole) with T4 polynucleotide kinase (Bethesda Research Laboratories).

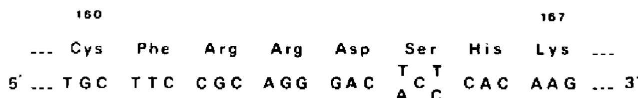

FIG. 1. Synthetic mixed oligonucleotide probes corresponding to amino acids 160–167 of chum salmon Prl sequence (Yasuda *et al.*, 1986). The oligonucleotide sequence was deduced on the basis of codon usage for chum salmon GH mRNA (Sekine *et al.*, 1985).

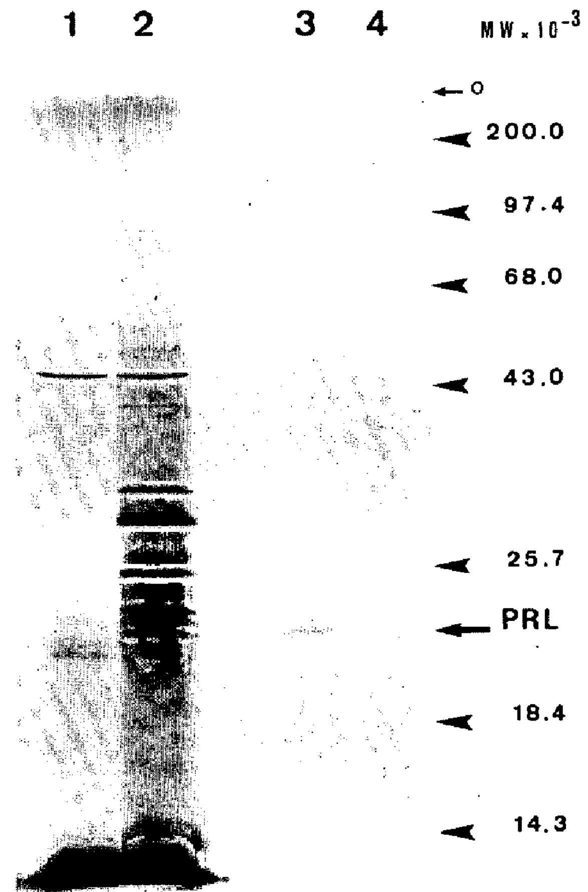

FIG. 2. Translation of pituitary mRNA in a cell-free rabbit reticulocyte system. Lane 2 contains proteins translated from poly(A)$^+$mRNA. The background, with no RNA added, is shown on lane 1. Lane 4, Immunoprecipitate of the translation products, using a rabbit antiserum to salmon Prl; lane 3, immunoprecipitation control using normal rabbit serum. Position of the prestained molecular weight markers (Bethesda Research Laboratories, 14,300–200,000 daltons), are indicated by arrows.

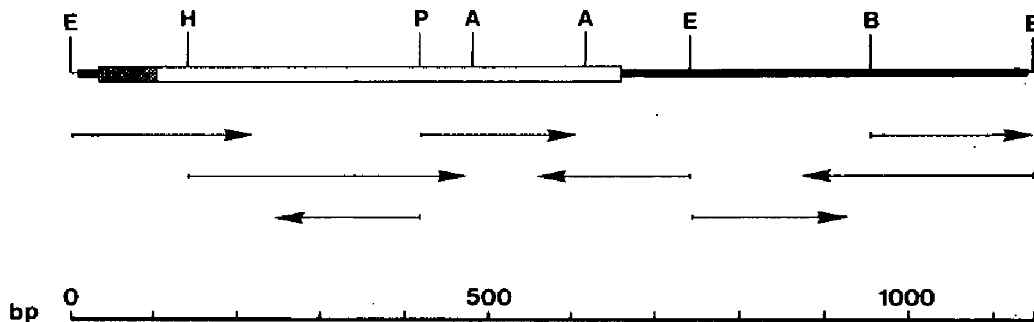

FIG. 3. Schematic representation of the cDNA corresponding to tPrl mRNA. The hatched box and open box represent the regions coding for the peptide signal and the mature protein respectively. Thick line represents the 5′ and 3′ untranslated region of the mRNA. The sequencing strategy is indicated by the arrows showing the direction and the extent of sequencing. The main restriction sites are: A, *Ava* II; B, *Bam* HI; E, *Eco* RI; H, *Hind* III; P, *Pst* I. Sequencing was done by subcloning restriction fragments in bacteriophage M13 and using the universal primer, except in one case where we used a synthetic primer (arrow E → B) corresponding to a part of tPrl already sequenced.

```
                                   -23         -20
                                   Met Ala Arg Arg Ser Gln Gly Thr Lys Leu His Leu
GAAUUCGAGCUCGCCCAAAAGAAGGAAAG      AUG GCU CGC CGA UCC CAG GGU ACC AAA CUC CAC UUA

    -10                                 1
Ala Val Leu Cys Leu Val Val Ser Cys His Ala Ile Gly Leu Ser Asp Leu Met Glu Arg
GCA GUU CUG UGU CUA GUU GUA UCC UGU CAU GCC AUU GGC CUU AGU GAC CUA AUG GAG AGA

10                                      20
Ala Ser Gln Arg Ser Asp Lys Leu His Ser Leu Ser Thr Ser Leu Thr Lys Asp Leu Asp
GCU UCC CAG CGA UCA GAC AAG CUU CAC UCA CUC AGC ACU UCC CUC ACC AAG GAC CUG GAC

30                                      40
Ser His Phe Pro Pro Met Gly Arg Val Met Met Pro Arg Pro Ser Met Cys His Thr Ser
UCU CAC UUC CCA CCA AUG GGA CGA GUG AUG AUG CCA CGC CCG UCU AUG UGU CAC ACC UCC

50                                      60
Ser Leu Gln Thr Pro Lys Asp Lys Glu Gln Ala Leu Lys Val Ser Glu Asn Glu Leu Ile
UCA CUC CAG ACA CCC AAG GAC AAG GAG CAA GCA CUC AAA GUA UCG GAG AAU GAG CUG AUC

70                                      80
Ser Leu Ala Arg Ser Leu Leu Leu Ala Trp Asn Asp Pro Leu Leu Leu Leu Ser Ser Glu
UCC CUG GCU CGC UCC CUC CUC CUG GCC UGG AAC GAU CCC CUG CUG CUG CUC UCC UCA GAG

90                                      100
Ala Pro Thr Leu Pro His Pro Ser Asn Gly Asp Ile Ser Ser Lys Ile Arg Glu Leu Gln
GCG CCC ACU CUG CCA CAC CCC UCC AAU GGC GAC AUC AGC AGU AAG AUC AGG GAA CUG CAG

110                                     120
Asp Tyr Ser Lys Ser Leu Gly Asp Gly Leu Asp Ile Met Val Asn Lys Met Gly Pro Ser
GAC UAC UCC AAG AGC CUG GGA GAU GGA CUG GAC AUA AUG GUC AAC AAG AUG GGA CCC UCC

130                                     140
Ser Gln Tyr Ile Ser Ser Ile Pro Phe Lys Gly Gly Asp Leu Gly Asn Asp Lys Thr Ser
UCC CAG UAC AUU UCU UCA AUC CCC UUC AAG GGU GGA GAC CUC GGC AAU GAC AAG ACC UCC

150                                     160
Arg Leu Ile Asn Phe His Phe Leu Met Ser Cys Phe Arg Arg Asp Ser His Lys Ile Asp
CGC CUC AUC AAC UUC CAC UUC CUC AUG UCC UGC UUC CGC AGG GAC UCC CAC AAA AUC GAC

170                                     180                         187
Ser Phe Leu Lys Val Leu Arg Cys Arg Ala Thr Lys Met Arg Pro Glu Ala Cys AM
AGU UUC CUC AAG GUC CUU CGA UGC CGG GCC ACC AAA AUG CGA CCA GAA GCA UGU UAG GAG

AAAAUGGCAGGCAUUUUGGUUCUGGAUUGUUCCAUUUUCAAACUGAUAGUGAAAAUGGGGUAGCCAUUUGAAGGAGAAU
UCAGGGAUUGUUUGUUGAGUUUGAUUUUGUGAAAUGACUAAUGCUGCCAUCUACAUCACAUUUGGACUAUUCAUAGACU
AUACGUUGUAUUCAACCUGUUAUCUGAACCACAUUUUCACCAUACAACUUAAGGUAGUUUUAUGUUCGGUAAUGCAUUU
AAUCUAAAAUAUGCAACCUAAUAUUGGGCACUUAUCAUAUUCAAUGAUUUAUAUAGAUAACAAAGGAUCCCUCCAAGUC
AUUCUUAGUAGAUACAGAGGGCUGAUAGUUGACCAAUGACUGUCAUGAUAACAUUUUAGAAUAUGAUUUCUCAAGUCAC
UUAUAACUUCUCUUAUACUAUACAUAAUGUACUGUUUCACUAAAUACCAAAUAAAAUCUUUAUUGCAUUAUAAAAAAAA
AAAA
```

FIG. 4. The 1,144-bp sequence of tPrl mRNA and primary structure of trout prePrl, deduced from the cDNA sequence. The translation initiation site, located at the first AUG codon (nucleotides 30–32), the UAG termination codon (nucleotides 660–662), and the polyadenylation signal AAUAAA (nucleotides 1,111–1,116) are boxed. The position corresponding to the probe (nucleotides 576–599) is underlined. The 209 amino acids include the peptide signal (amino acids −23 to −1) and the mature polypeptide chain (amino acids 1–187).

DNA sequence analysis was performed by the method of Sanger *et al.* (1977) after subcloning appropriate restriction fragments into M13 mp19 and mp18 (Yanisch-Perron *et al.*, 1985).

Northern analysis

Increasing amounts of denatured pituitary poly(A)$^+$RNA were electrophoresed on 1% agarose gels in the presence of 1 *M* glyoxal (Lehrach *et al.*, 1977; Goldberg, 1980) and transferred to nylon membranes (Pall, Biodyne A). Hybridization was carried out according to Thomas (1980), with a recombinant plasmid DNA labeled by nick-translation without removal of the vector sequences.

RESULTS

Isolation and translation of pituitary mRNA

Using the guanidium isothiocyanate/CsCl method, 13.1 mg of total cellular RNA was isolated from 5.30 grams of trout pituitaries; 5.6% of poly(A)$^+$mRNA was recovered after chromatography on oligo(dT)-cellulose. The translation products directed by 2 μg of poly(A)$^+$mRNA are shown in Fig. 2. A single band of about 23,000 daltons was immunoprecipitated by an antiserum to salmon Prl and no precipitation occurred in the presence of normal rabbit serum. This indicates the presence of intact prolactin mRNA in our polv(A)$^+$RNA preparation.

Isolation of cDNA coding for tPrl gene

The cDNA library was screened with the ^{32}P-labeled oligonucleotide probes complementary to amino acids 160–167 of chum salmon Prl. Twelve positively hybridizing clones were identified from 3,500 transformants and six clones with inserts of about 1.0 kb were isolated. Restriction endonuclease analysis indicated that only one tPrl mRNA species was present.

The complete nucleotide sequence of the tPrl mRNA was determined using the strategy shown in Fig. 3. The 1,144-bp sequence and the predicted amino acid sequence are shown in Fig. 4. The translation product of the tPrl mRNA, starting at the first AUG codon, is a polypeptide of 23,370 daltons. The 209 amino acids of pre-tPrl include a leader sequence of 23 amino acids. The mRNA sequence includes a long 3′-untranslated region of 470 nucleotides from the UAG termination codon (positions 660–662 on the cDNA), to the polyadenylated tail which starts at residue 1,133. The polyadenylation signal AAUAAA is located at positions 1,111–1,116. The amino acid sequence deduced from the nucleotide sequence of tPrl cDNA is very close to the amino acid sequence of chum salmon Prl, published by Yasuda *et al.* (1986). The only difference found is amino acid residue 185, where Thr in chum salmon Prl is replaced by Ala in tPrl.

Northern analysis

The size of tPrl mRNA was estimated by Northern blotting analysis using a ^{32}P-labeled recombinant plasmid as a probe. The plasmid contains a 735-bp insert corresponding to part of the 3′ end of the mRNA. The results (Fig. 5) show that tPrl mRNA is about 1.3 kb long (mean of five experiments).

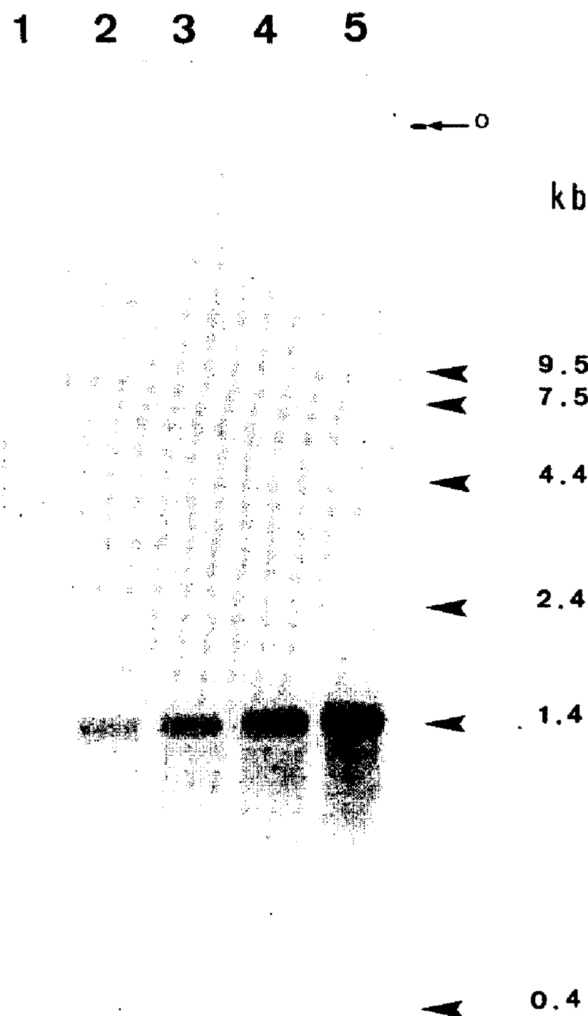

FIG. 5. Northern blot analysis of trout pituitary poly(A)$^+$ and trout liver RNA, denatured in 50% DMSO, 1 *M* glyoxal, 10 m*M* NaH_2PO_4 pH 6.5. Increasing amounts of pituitary poly(A)$^+$RNA (0.625 μg, 1.25 μg, 2.5 μg, and 5 μg), in lanes 2–5, respectively, and 10 μg of trout liver total RNA (lane 1) were electrophoresed on a 1% agarose gel in 10 m*M* phosphate pH 6.5. After transfer to nylon membranes (Pall, Biodyne A), RNAs were hybridized with a ^{32}P-labeled tPrl recombinant plasmid (SA, 3.5 × 10^8 dpm/μg DNA) and exposed to X-ray film (Kodak DEF 5) for 2 hr at −70°C. Positions of the RNA size markers (Bethesda Research Laboratories ladder) are indicated by arrows.

Comparison of tPrl amino acid sequence with other published sequences

Published Prl sequences are compared to tPrl in Fig. 6. When necessary, gaps were introduced in the sequences to obtain optimal alignment between chum salmon Prl (Yasuda *et al.*, 1986), carp Prl (Yasuda *et al.*, 1987), mouse Prl (Linzer and Talamantes, 1985), human Prl (Cooke *et al.*, 1981), bovine Prl (Miller *et al.*, 1981), rat Prl (Cooke *et al.*, 1980), whale Prl (Tsubokawa, 1985), ovine Prl (Li *et al.*, 1967), pig Prl (Li *et al.*, 1976), and tPrl, which all end with a cysteine residue. Comparison of these sequences shows at least two domains with strong similarity. The first has 73.3% similarity and extends from Cys-46 to Ala-60;

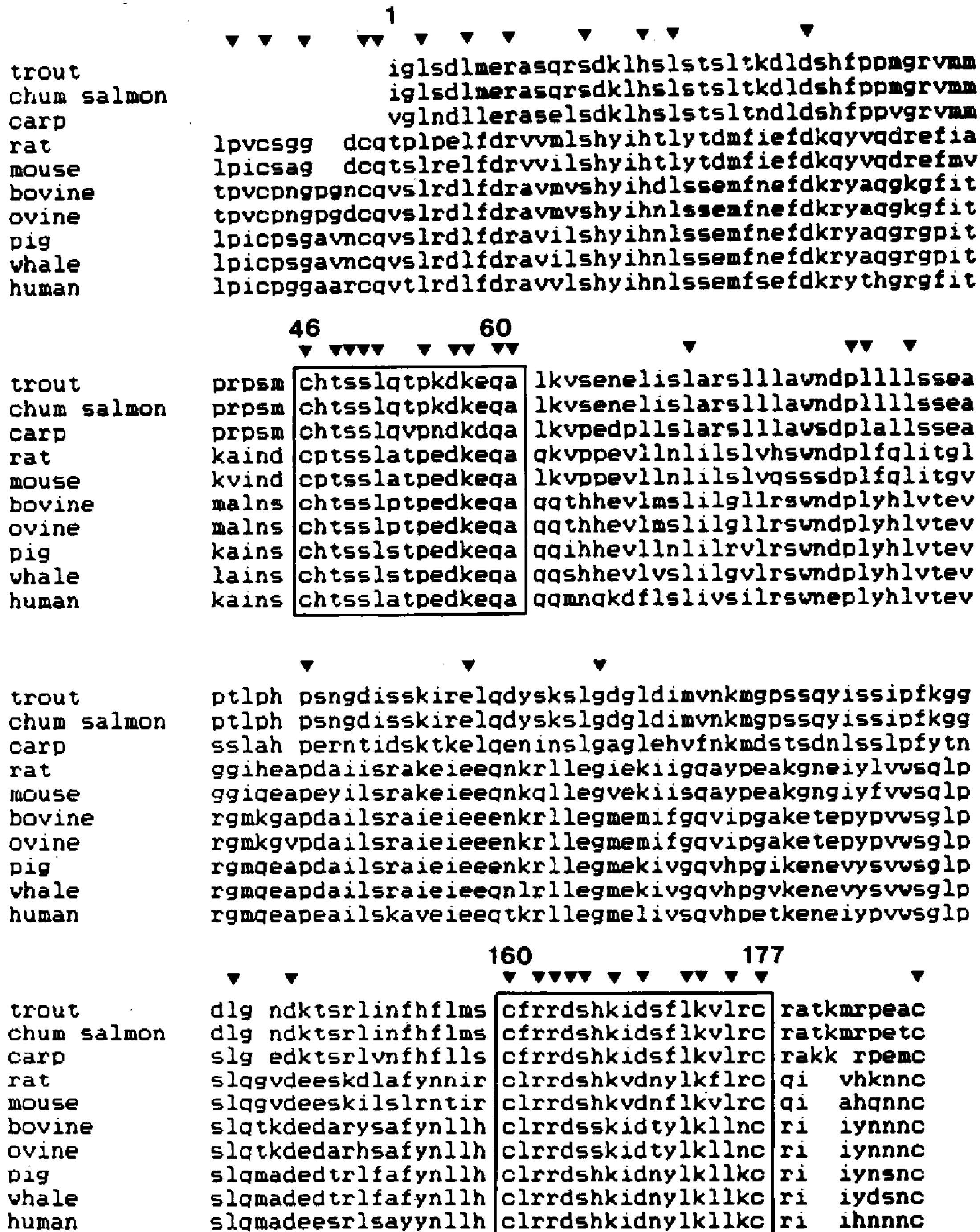

FIG. 6. Optimal alignment map obtained by comparison of prolactin amino acid sequences from vertebrates. The regions with striking similarity are boxed. The numbers indicated on the upper lines refer to the amino acid sequence of tPrl. Gaps were introduced to obtain the optimal alignment; the same amino acid found in the same position is indicated by a dark triangle.

the second has 61% similarity and extends from Cys-150 to Cys-177 of tPrl. These two conserved domains may correspond to parts of the polypeptide chain involved in biological activities that are common to the Prls from the different vertebrate species.

The strongest similarity was obtained with fish sequences, since carp Prl shares 144 amino acid positions with tPrl and salmon Prl, resulting in 77.4% similarity when considering the entire polypeptide sequence.

DISCUSSION

We have cloned and sequenced trout Prl cDNA. The amino acid sequence of tPrl deduced from the nucleotide sequence of the cDNA was very similar to the chum salmon Prl amino acid sequence (Yasuda *et al.*, 1986), since only the residue located at position 185 differs in these two proteins. This agrees with our finding that antibodies raised against salmon Prl cross-react with tPrl. Prunet *et al.* (1985) used such an antiserum to measure plasma tPrl by radioimmunoassay. We have also used it for immunoprecipitation of our cell-free translation products. Moreover, we found that the primary structure of prolactin is highly conserved in teleosts, as shown by comparison of trout, chum salmon, and carp Prl sequences, which have 77.4% similarity.

Recently, Song *et al.* (1988) have cloned and sequenced a cDNA encoding chinook salmon (*Oncorhynchus tschawytscha*) Prl. From their deduced amino acid sequence, it appears that the difference between chinook and chum salmon Prl (189 and 187 amino acids, respectively, plus 4 amino acid substitutions) is fairly high, particularly when compared to the single substitution (amino acid 185) which differentiates trout and chum salmon Prl.

Despite our extensive analysis of the tPrl clones by restriction mapping and sequencing, only one form of cDNA was found. This contrasts with results reporting isolation of two forms of prolactin in chum salmon (Yasuda *et al.*, 1987) and in tilapia (Specker *et al.*, 1985). This might be related to our use of pituitaries from rainbow trout raised in seawater. Prunet *et al.* (1985) showed that plasma Prl concentration and pituitary Prl content decrease when rainbow trout are transferred from freshwater to seawater. This suggested that Prl gene expression might be affected by changes in water salinity. If trout, like salmon, possess two genes for Prl, one of these could be unexpressed in seawater or expressed at such low level that its cDNA is not represented in our cDNA library.

To test this hypothesis, we constructed a second cDNA library starting from pituitary mRNA extracted from trout raised in freshwater. No second form of tPrl cDNA was found. This could be explained if, as for trout GH cDNA (Rentier-Delrue *et al.*, 1989) and salmon GH cDNA (Sekine *et al.*, 1985), one form of cDNA is clearly predominant over the other, and if we take into account that, for both of our cDNA libraries, fewer Prl clones than GH clones were obtained.

The final answer to the number of Prl genes in trout will be provided by the analysis of the Prl sequences isolated from a genomic library.

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Address reprint requests to:
Dr. L. Mercier
INSERM U 298, CHR
49033 Angers Cédex
France

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