Molecular cloning, expression and immunological characterization of pejerrey (Odontesthes bonariensis) growth hormone

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Abstract

Growth hormone is an essential polypeptide required for normal growth and development of vertebrates. The pejerrey fish, Odontesthes bonariensis, is a South American atherinid freshwater fish considered as a promising species for aquaculture. Although growth hormone has been characterized in a number of fish, there are no published data on the structure of this hormone in atherinids, except that of a related species Odontesthes argentinensis. In this paper, the molecular cloning, expression and immunological characterization of pejerrey growth hormone (pjGH) is described. The predicted amino acid sequence of pjGH cDNA consisted of 204 amino acid residues with an estimated molecular mass of 23 kDa. Amino acid sequence was highly conserved among the two Atheriniformes where the growth hormone sequences are known (99% aa identity), highly to moderately conserve (75–92% aa identity) when compared to the other members of Acantopterigii superorder and clearly less conserved (49–66% identity) when compared to Salmoniformes (Protacanthopterygii), Cypriniformes and Siluriformes (Ostariophysi). A phylogenetic tree depicting the relationship of various teleost GH nucleotide sequences was inferred. Pejerrey GH was produced using recombinant DNA technology in a bacterial system, representing the first time an atherinid growth hormone protein was expressed as a recombinant protein in Escherichia coli. A specific antiserum of this hormone was raised in rabbits and its specificity tested by using Western blot and immunocytochemistry. The distribution of pjGH mRNA was also studied by RT-PCR and Southern blot analysis. The transcript was detected not only in the pituitary gland but also in the testis.

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Keywords: Growth hormone; Immunocytochemistry; mRNA; Odontesthes bonariensis; Pejerrey; RT-PCR; Southern blot; Phylogenetic tree

1. Introduction

Pejerrey, Odontesthes bonariensis (Atheriniformes), is a South American atherinid fish that, although considered a promising species for intensive aquaculture, its culture is hindered by a slow growth rate (Strüssmann et al., 1993).

Growth hormone (GH), mediated by specific receptors (Very et al., 2005), is an essential polypeptide required for normal growth and development of vertebrates (Chen et al., 1994; Forsyth and Wallis, 2002). In addition to the well known effects of GH on somatic growth in vertebrates (Isaksson et al., 1987), it has also been shown to have roles in mammalian (Chandrashekar et al., 2004; Hull and Harvey, 2002) and bony fish reproduction (Gomez et al., 1998, 1999; Le Gac et al., 1993) as well as in osmoregulation in euryhaline fish (Mancera et al., 2002; Sakamoto et al., 1993).

Although growth hormone has been characterized in a number of fish, there are no published data on the structure of this hormone in atherinid fish except for Odontesthes argentinensis (Marins et al., 2003). To overcome this limit, the knowledge of pejerrey growth hormone (pjGH) primary...
structure and the production of specific antibodies has become a prerequisite for any physiological and biotechnological study of growth in this species.

2. Materials and methods

2.1. Animals and tissues

Adult pejerrey (*O. bonariensis*, Actinopterygii: Atherinopsidae) of both sexes (30 cm total length) were collected from Cabra Corral Lagoon (Province of Salta, Argentina). The fish were rapidly decapitated, the pituitaries dissected out, immediately frozen in liquid nitrogen and stored at −70 °C for RNA extraction. Pejerrey brains, with their pituitary attached, were taken from adult pejerrey from the IIB-INTECH stock in early spawning season (September). They were also dissected out, fixed in 4% paraformaldehyde in phosphate buffer saline (PBS, pH 7.4), washed in PBS and then stored at 4 °C until processing.

The liver, kidney, heart, intestine, brain, pituitary, gills, skeletal muscle and gonads were dissected out from juvenile specimens (120 mm total length) at the beginning of spring (September), frozen in liquid nitrogen and stored at −70 °C.

2.2. Isolation of full length cDNAs and sequencing

Total RNA was extracted from 50 pituitary glands of adult pejerrey fish by a spin (SV) Total RNA Isolation System (Promega Corporation, Madison, WI, USA) following manufacturer’s instructions. Degenerate primers for pjGH reverse transcription–polymerase chain reaction (RT-PCR) were designed within conserved regions identified after multiple sequence alignments of growth hormone cDNA from teleosts of different orders. 3′-RACE (*Frohman et al.*, 1988) was utilized to clone a partial cDNA pjGH sequence. A total of 5 μg of pituitary RNA was reverse transcribed using an oligonucleotide annealing to the poly-A tail (Qt, Table 1) during 1 h incubation at 37 °C using AMV reverse transcriptase (200 U, Promega) in a final volume of 20 μL. Then, cDNA aliquots (1 μL) were amplified in a mixture containing 10× PCR buffer, deoxynucleotides (200 μM of each), 40 pmol each oligonucleotide primer (Q1, same as Qt but deprived of the poly-T segment, and E, Table 1) and 2 U Taq DNA polymerase (Promega Corporation, Madison, WI, USA) in 25 μL of final incubation volume. The amplifications were as following: 40 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min, followed by a final period of extension at 72 °C for 10 min. The PCR products were resolved by electrophoresis in 1.5% (w/v) agarose gels and visualized by ethidium bromide staining. The PCR product was digested with *BamHI* and *HindIII* (underlined), and cloned into the pBluescript SK(−)-vector (Stratagene). This product (pBluescript-pjGH3′) was then sequenced using universal primers within the flanking regions of the polylinker of the pBluescript vector. Once the 3′ end sequence was known, two reverse gene specific primers were designed (B2 and antiON1, Table 1) and used for the 5′ end PCR amplification. To generate 5′ end partial cDNA clones, reverse transcription was carried out as described above using 5 μg of total RNA and 2.5 μg of primer B2 in 25 μL final volume. After 1 h incubation at 37 °C, the reaction mixture was diluted to 1 mL with TE (10 mM Tris–HCl, pH 7.5/1 mM EDTA). Excess primer was removed using Centricom-100 (Amicon Corp), following manufacturer’s instructions. The material was then washed twice by spin filtration with TE and recovered in a final volume of 50 μL. A poly(A) tail was then appended using 0.5 μL terminal deoxynucleotidyltransferase (TdT, 30 U/μL, Promega), 4 μL of 5′ tailing buffer, 4 μL of dATP 1 mM and 11.5 μL of washed cDNA, and the reaction was incubated 60 min at 37 °C, followed by 5 min at 65 °C. The reaction mixture was diluted to 250 μL and the amplification achieved using primers Q1 (40 ng), Q1 (75 ng) and anti-ON1 (75 ng) and 10 μL of tailed cDNA. 50 μL PCR cocktail was heated 5 min at 95 °C, cooled to 75 °C and the Taq polymerase (2 U, Promega) was added. The reaction mixture was incubated 2 min at 50 °C, 40 min at 72 °C and then 30 cycles of amplification (1 min at 94 °C, 1 min at 54 °C and 2 min at 72 °C) followed by a 10 min final extension. The PCR products were resolved by electrophoresis in 2% (w/v) agarose gels and gel-purified with QIAEX kit (QIAGEN). The resulting PCR product was cloned in T-vector (pCR 2.1 TOPO, Invitrogen) and sequenced. Sequence data encoding pjGH was deposited in the GenBank database under accession number AY819758.

2.3. Sequence analysis, databases homology search and sequence alignment

The Database homology search was performed using the BLASTN and BLASTP programs (*Altschul et al.*, 1997). Multiple alignments were performed using the CLUSTAL X (*Chenna et al.*, 2003; *Thompson et al.*, 1997) program. For the construction of the phylogenetic tree, multiple alignments of deduced aa sequences excluding the signal peptide of various GH genes were aligned using a Gonnet matrix. The DNA alignment was achieved using Dnalign program, a computer program to convert polypeptides sequences alignment into a collinear DNA sequences alignment with specific codon positions. The phylogenetic tree was inferred using the Bayesian estimation of phylogenetic trees program MRBAYES (*Huelsenbeck and Ronquist*, 2001).

Sequence pair distances of GH nucleotide and peptide growth hormone sequences were obtained with MegAlign 4.03

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sequences of oligonucleotide primers used in this study</th>
</tr>
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<tbody>
<tr>
<td>Qt</td>
<td>5′-GAGGACTCGAGCTCAAGC(TTT)3′</td>
</tr>
<tr>
<td>Q1</td>
<td>5′-GAGGACTCGAGCTCAAGCTT3′</td>
</tr>
<tr>
<td>E</td>
<td>5′-TATCGGATACGCTTTC(TC)CACCT(A/G)(A/T/C)TTC(T/C)TTTGAA-3′</td>
</tr>
<tr>
<td>B2</td>
<td>5′-TATCGGATACGCTTTCATTAGCCACCGTC3′</td>
</tr>
<tr>
<td>Anti-ON1</td>
<td>5′-TATCGGATACGCTTTCATTAGCCACCGTC3′</td>
</tr>
<tr>
<td>GH2</td>
<td>5′-ACCATATGACGACATTGGGATATGGAC-3′</td>
</tr>
<tr>
<td>GH7</td>
<td>5′-CTGTGCTGACATGGTCTTCTTGAA-3′</td>
</tr>
<tr>
<td>GH1</td>
<td>5′-ATGCAAGCCATACCGGAGACG-3′</td>
</tr>
<tr>
<td>Fwd-β-actin</td>
<td>5′-CCGAAGGCGTATTGCAGAG-3′</td>
</tr>
<tr>
<td>R-β-actin</td>
<td>5′-ACCCACACTTGCCATCATA-3′</td>
</tr>
</tbody>
</table>
(DNAstar Inc.) using Clustal method with PAM 250 residue weight table.

2.4. Plasmid construction for expression studies

The cDNA fragment encoding the mature hormone (without the signal peptide) was amplified by PCR with Pfu Polymerase, the forward (GH2, Table 1) and reverse (Q1: see above) primers and cDNA from the reverse transcription with Qt (see above). To facilitate in frame cloning and to provide an ATG codon that codifies one extra methionine at the N-terminus, a restriction enzyme site (NdeI, underlined) was introduce to the 5’ primer and a Hind III site was present in Q1. This PCR fragment (760 bp) was cloned in a pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The insert showed 100% identity to the expected sequence. The ORF fragment was subcloned into a pET-24 vector (Novagen) after digestion with NdeI and HindIII restriction enzymes. This plasmid construct was designed pET/pjGH and was used for transformation of BL21 Codon Plus (DE3) *Escherichia coli* cells (Novagen). The transformants were selected on LB agar plates containing chloramphenicol (20 mg/mL) and kanamycin (50 mg/mL). The colonies growing on the plates were selected and used for the expression.

2.5. Induction and expression of mature pjGH

A single colony from the above transformants was inoculated in LB medium containing Chloramphenicol and Kanamycin antibiotics to an A600 of 0.5 and then lactose was added to 20 g/L. The cells were grown overnight, pelleted for 15 min at 5000 g and resuspended in buffer TE (1/20 of the culture volume). The suspension was incubated 1 h at 37 °C with 1 mg/mL lysozyme (Roux-Ocefa) and 2 μg/mL of DNAse I type 4 (Sigma). Cells were lysed with a French press. The lysed suspension was diluted 1/4 with distilled water and pelleted for 15 min at 10,000 g. The pellet was resuspended in Triton X-100 0.5%, EDTA 20 mM solution, incubated 10 min and washed three times with distilled water. The inclusion bodies were maintained at −20 °C for later use.

2.6. Production of antiserum

Mature recombinant pjGH were used to produce polyclonal antiserum in rabbits. The antigen used for immunizations was purified by preparative electrophoresis from washed inclusion bodies under reducing and denaturing conditions on 15% polyacrylamide gels and electroeluted using the method described by Leppard et al. (1983). Electroeluted proteins were precipitated in 80% ethanol and resuspended in 200 μL of water. Approximately 100 μg of purified protein were injected to the rabbit at 0, 4, 8, 12 and 16 weeks. The blood was collected from each rabbit 10 days after injections, allowed to clot at 37 °C and centrifuged to separate the serum. The antiserum was stored at −20 °C. Pre-immune serum was obtained from the same animals before the first injection and stored in the same way. Antibodies titters were monitored by immunodetection of the protein in slot blots and were performed by incubating serial dilutions of the immune serum with two concentrations (0.1 and 1 μg) of nitrocellulose immobilized recombinant pjGH.

2.7. SDS-PAGE and Western blot analysis

Proteins were analysed on 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Tris-glycine buffer (Laemmli, 1970) and the gels were stained with 0.1% Coomassie Brilliant Blue R 250. For Western blot analysis, the protein bands were electroblotted onto a nitrocellulose membrane. The strips were equilibrated in PBS pH 7.2 and blocked for 2 h in 5% non-fat dry milk in PBS (blotto) at room temperature. The membrane was then incubated in different dilutions of the antiserum for 1 h in blotto, fully washed and allowed to react for 1 h with HRP-labelled goat anti-rabbit IgG (1/1500 dilution in blotto). After washing in blotto (3 × 15 min), the strips were rinsed briefly in PBS and finally developed by incubation for 15–20 min in a freshly prepared substrate solution of 20 mL of 100 mM Tris–HCl pH 7.2 containing 2 mg of 3,3-diaminobenzidine, 8 mg NiCl2 and 7.5 μL of ice cold 30% hydrogen peroxide. Colour development was stopped by washing the strips in water (Harlow and Lane, 1988). Native gels were done on 10% polyacrylamide gel in the same way as described above, but without SDS in the gel preparation.

2.8. Tissue distribution of pjGH by RT-PCR and Southern blot

Tissue total RNA was extracted by using the acid guanidinium thiocyanate phenol/chloroform method of Chomczynski and Sacchi (1987). Extreme care was taken to avoid specimen contamination during the extraction procedure. The integrity of RNA was assessed by analysis of 28S and 18S rRNA on ethidium bromide/stained 1.2% agarose gels. cDNA was synthesised from total RNA from liver, kidney, heart, intestine, brain, pituitary, gills, skeletal muscle, testis and ovary. About 10 μg total RNA was incubated with 1 μg of poly dT primer at 80 °C for 3 min and quenched on ice. Reverse transcriptase mix (0.5 U RNase inhibitor, 800 μM dNTPs, 1× RT buffer and 15 U AMV-Reverse Transcriptase) was added to the quenched sample. First strand cDNA was synthesised by incubating the above mix at 37 °C for 2 h. The mix was diluted with sterile distilled water (1:10) and stored at −20 °C.

Using the first strand cDNA as template, a fragment of GH was amplified by PCR with GH1 (forward) and GH7 (reverse) primers (Table 1) chosen on both sides of pjGH coding region (500 bp). The same reaction was performed using primers for β-actin (Biodynamics) cDNA amplification (Table 1). PCR reaction was performed using 10 μL of cDNA in a final volume of 25 μL under the following conditions: 1 U Taq DNA polymerase (Promega), 1× reaction buffer, 2.5 mM MgCl2, 0.2 mM dNTPs and 10 pmol of each primer. After initial denaturation for 2 min at 94 °C, 40 cycles of amplification were performed with 30 s denaturation at 94 °C, 1 min annealing at 52 °C and 1 min extension at 72 °C in a Perkin Elmer thermocycler. The last cycle had an elongation time of 7
min at 72 °C. PCR products were run on a 1.5% TAE/agarose gel, stained with ethidium bromide and blotted onto nitrocel-
lulose membrane (Hybond H+, Amersham). Southern blot was
carried out with digoxigenin-labelled probe. The probe was
generated by PCR from 100 ng plasmid pBluescript-pjGH3′ (see above) using DIG High Prime DNA labelling kit
(Boehringer Mannheim) and primers E and B2 (480 pb of the
coding region of GH cDNA). Hybridization was performed
overnight at 65 °C in a solution containing 5× SSC, 0.1%
sodium lauryl sarcosine (SLS), 0.02% sodium dodecyl sulfate
(SDS), 1% blocking reagent (DIG High Prime DNA labelling
kit, Boehringer Mannheim) and 20 μL of the labelled probe.
Membranes were washed 2×5 min in 2× SSC, 0.1% SDS at 65
°C and 2×15 min in 0.1× SSC, 0.1 SDS at 65 °C, revealed with
Detection Starter Kit II (Boehringer Mannheim) and exposed to
Kodak X/OMAT AR5 film for various periods of time at room
temperature.

2.9. Immunohistochemistry assay

Antibodies to different piscine pituitary hormones raised in
rabbits were used to immunodetect the hormones on pituitary
section as previously described (Miranda et al., 2001). Briefly,
the immunocytochemical (ICC) analysis was carried out using
the avidin–biotin peroxidase method with a commercial Kit
(Vector, Burlingame, USA). The final reaction product was
generated by PCR from 100 ng plasmid pBluescript-pjGH3
(see above) using DIG High Prime DNA labelling kit
(Boehringer Mannheim) and primers E and B2 (480 pb of the
coding region of GH cDNA). Hybridization was performed
overnight at 65 °C in a solution containing 5× SSC, 0.1%
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temperature.

3. Results

3.1. cDNA sequence and phylogenetic analyses

The cDNA of 965 pb with poly(A) tail containing the open
reading frame encoding pejerrey growth hormone was charac-
terized from pituitary mRNA. The coding region (615 pb) and
the 5′ and 3′ untranslated sequences (153 and 195 nt,
respectively) were sequenced. The 3′UTR contains two
polyadenylation consensus sequences. The predicted amino
acid sequence of pjGH cDNA consisted of 204 amino acid
residues with an estimated molecular mass of 23 kDa (Fig. 1)
corresponding to the precursor protein with the signal peptide
(17 amino acid residues). The comparative analysis with other
fish mature growth hormones revealed that there were
numerous conserved amino acids among Acanthopterygii
superorder (Fig. 2). pjGH is highly homologous to GH of
Hemiruphus brasiliensis (Beloniformes, 92%), another member
of Atherinomorpha; Thunnus thynnus (Perciformes, 91%),
Tetraodon nigroviridis (Tetraodontiformes, 85%) and Hippo-
glossus hippoglossus (Pleuronectiformes, 73%). Although the
homology of GH is high within members of Acanthopterigii
superorder, the divergence is mainly situated between amino
acids 120 and 143 (Fig. 2, bold letters). Pejerrey GH is less
homologous to members of Paracanthopterigii superorder
(Gadus morhua, Gadiformes) and Protacanthopteri superor-
der (Salmo salar, Salmoniformes). The most divergent groups
are Cypriniformes and Siluriformes (Cypriniformes) and Siluriformes
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Kodak X/OMAT AR5 film for various periods of time at room
temperature.

Fig. 1. The complete nucleotide sequence of the pejerrey GH cDNA encoding
for the GH peptide. The pjGH peptide contains the signal peptide (residues 1 to
and 143 (Fig. 2, bold letters). Pejerrey GH is less
homologous to members of Paracanthopterigii superorder
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SDS-PAGE analysis of proteins expressed in the control and
induced E. coli cultures showed the over expression of a 21 kDa
protein upon induction with lactose (Fig. 3, compare lanes 1 and 2). The protein was mainly present in the pellet of the culture extracts as inclusion bodies (Fig. 3, lanes 3 and 4). Washed inclusion bodies did not modify significantly the purity of the main protein (78% compared to 83% of purified inclusion bodies) (Fig. 3, lanes 3 and 5).

### 3.3. Antiserum specificity

Preparative SDS-PAGE was carried out to obtain pure recombinant protein in order to raise antibodies in rabbits. The titer of the antisera obtained after the four and five injections was measured by slot blots assays using 0.1 and 1 μg of recombinant pjGH and 1 μg of BSA as negative control. The resulting titers were 1:2000 and 1:5000, respectively, for 0.1 μg of recombinant protein.

In order to test the specificity of this antiserum, Western blot analysis using denaturing and native gels was performed using the purified recombinant protein and BSA as negative control.
total pituitary extracts. The antiserum could recognize a single band in both conditions (Fig. 4, panels A and B). As expected, the band of the recombinant protein was also recognized by the antiserum (Fig. 4A, lane 3). A slightly difference in molecular mass could be seen between recombinant and pituitary protein in the SDS gel probably due to the presence of an extra methionine in the recombinant pjGH.

3.4. Immunohistochemical characterization

The pituitary distribution of GH cells was performed using the pjGH antiserum produced in this study. The anti-pjGH antiserum produced a strong and specific staining of somatotropes in proximal pars distalis (PPD) with no reaction in those pituitary areas where prolactin and somatolactin are expressed. It is known that SL cells are clearly located in pars intermedia (PI) and PRL cells appeared mainly in the rostral pars distalis (RPD) (Vissio et al., 1997). Interestingly, the ventral part of the PPD contained cells that cross-reacted with anti-PRL antiserum but did not stained with pjGH antiserum (Fig. 5).

3.5. GH expression in extrapituitary tissues

To determine whether the mRNA of GH is detectable in extra pituitary tissues of pejerrey, RT-PCR and Southern blot analysis of mRNA from different tissues were assessed. As shown in Fig. 6, an amplified fragment of the same length was detected in pituitary and testis.

4. Discussion

In the present work, the cloning and expression of pjGH in E. coli is described. The nucleotide sequence of this hormone predicts the complete amino acid and signal peptide sequence of pjGH. No variation could be detected within the clones sequenced using different amplification strategies suggesting that there exist a single copy of GH gene. The nucleotide sequence showed 94.9% identity to O. argentinensis, a South American marine silverside; however, the highest divergence is located in the 5’UTR that is larger in O. bonariensis (150 nt against 110 nt in O. argentinensis) and also divergent (Fig. 7).

The putative signal peptide is 17 amino acid long as in all Acantopterigii but shorter than those of Salmoniformes (22 AA) or human beings (26 AA). Amino acid sequence were highly conserved among the two Atheriniformes where the growth hormone sequences is known (99% identity), highly to moderately conserved when compared to Beloniformes, Perciformes and Mugiliformes (85–92%), and clearly less conserved when compared to Salmoniformes, Cypriniformes

Fig. 4. Western blot analysis of pituitary and recombinant pjGH. PAGE under denaturing (panel A) and non-denaturing (panel B) conditions were carried out with total crude pituitary protein (lanes 1 and 2) and inclusion bodies containing recombinant pjGH (lane 3). The gel was stained with Coomassie brilliant blue (lane 1, panels A and B) or electroblotted onto nitrocellulose membrane and revealed with anti-pjGH antibodies (lanes 2 and 3, panel A; lane 2, panel B).

Fig. 5. Mediosagittal sections through the pituitary of adult specimen of O. bonariensis showing the different distribution of immunoreactive PRL, GH and SL cells. The sagittal sections were stained using anti-chum salmon PRL (1:1000), anti-red-drum SL (1:1000) and anti-pejerrey GH (1:2000).

Fig. 6. Detection of GH mRNA in extrapituitary tissues of adult fish. RNA samples were isolated from various tissues. One microliter of first strand cDNA was used as the template for PCR amplification of GH (panels A and B) or β-actin cDNAs fragments (panel C) in separate reactions. The PCR products were fractionated on 1.5% agarose gels in TAE buffer and stained with ethidium bromide (β-actin) or blotted onto nylon membranes (GH). Southern blotting of PCR products amplified with oligonucleotide primers GH1 and GH7 (500 bp) using a DIG-labelled pituitary GH probe revealed after 30 min (panel A) or 14 h (panel B). Lane C, no template DNA; P, pituitary; B, brain; H, heart; L, liver; M, muscle; I, intestine; K, kidney; G, gill; T, testis; O, ovary.
and Siluriformes (53–66%). A Phylogenetic tree depicting the relationships of various teleosts GH nt sequences is shown in Fig. 8. In this tree, teleosts belonging to the same superorder following Nelson (1994) can be grouped together, supporting the present classification of 11 major orders of teleost. Other orders belonging to different superorders should be included at the time they became available in the gene bases. At this time, three superorders have GH cDNAs fully sequenced and submitted to the web. *O. bonariensis* is located nearby *O. argentinensis* and in the same branch of *H. brasiliensis*, which is the Atherinomorpha series branch. Acanthonopterygii superorder is more closely related to Protacanthopterigii, represented by Salmoniformes, than to Ostariophysi. This is in accordance with the data reported by Venkatesh and Brenner (1997) that shows not only an identity differentiation between sequences but a change in the number of introns between Protacanthopterigii (four introns) and Ostariophysi (five introns). Whether these differences have only an evolutionary distance meaning or they

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**Fig. 7.** Percentage of sequence identity and divergence of the translated region (TR), 5′ untranslated region (5′UTR) and 3′ untranslated region (3′UTR) of the GH cDNAs of *Odontesthes bonariensis* and *Odontesthes argentinensis*.

**Fig. 8.** Multiple alignments (Clustal W) of deduced aa sequences excluding the signal peptide of various GH genes were aligned using a Gonnet matrix. The DNA alignment was achieved using DNAAlign program, a computer program to convert polypeptides sequences alignment into a collinear DNA sequences alignment with specific codon positions. Phylogenetic tree was inferred using the Bayesian estimation of Phylogenetic trees program MRBAYES (Huelsenbeck and Ronquist, 2001). Values at the branch point are the probability of that point. Four probabilities are shown; no other probability of the tree branch points was under 0.90. The GH aa sequences other than *O. bonariensis* were retrieved from GenBank. The GenBank accession numbers of the sequences used are: *O. argentinensis*, AF236091; *H. brasiliensis*, AY775149; *P. flavescens* AY007303; *T. thynnus*, X06735; *S. aurata*, AY038038; *S. kneri*, AY155227; *E. coioides*, AY038606; *O. niloticus*, M26916; *M. plantanus*, AY775148; *S. mormoratus*, LA3627; *C. kazika*, AB079553; *O. mykiss*, X122731; *S. talar*, X14305; *C. carpio*, X51969; *C. australis*, AF069398; *I. punctatus*, S69215; *P. pangasius*, M63713. Numbers represent the orders of the particular species: 1, Perciformes; 2, Atheriniformes; 3, Beloniformes; 4, Scorpaeniformes; 5, Synbranchiformes; 6, Mugiliformes; 7, Pleuronectiformes; 8, Tetraodontiformes; 9, Salmoniformes; 10, Siluriformes; 11, Cypriniformes.
have a profound functional signification is still a matter of future studies.

Pejerrey GH was produced using recombinant DNA technology in a bacterial system, representing the first time an atherinid growth hormone protein that is expressed as a recombinant protein in E. coli. Recombinant pjGH was expressed as inclusion bodies in accordance with other bacterial recombinant proteins having disulfide bonds (Patra et al., 2000).

A specific antiserum of this hormone was raised in rabbits. In a Western blot analysis, this antiserum recognized native and recombinant pjGH showing no cross-reactivity to other pituitary proteins. Pituitary pjGH showed the expected molecular mass predicted from the pjGH sequence.

The specificity of the antiserum was also tested using immunocytochemistry comparing the cells labelled with different antisera raised against those pituitary hormones belonging to the same family: GH, PRL and SL (Chen et al., 1994). Using this technique, three distinct hormone-producing cells types were identified in pejerrey pituitary gland by using one homologous antiserum (anti-pjGH) and two heterologous antisera. None of them produced any visible reaction where the other hormones had been previously detected in pejerrey (Vissio et al., 1997), suggesting that all of them are very specific in staining the corresponding pituitary cells. Immunoreaction against PRL was detected in the RPD and in some cells in the anteroventral section of the PPD. The anti-pjGH serum produced a strong and specific staining in the PPD in the same region already reported to contained somatotropes by using other antiserum (Vissio et al., 1997). Somatolactotropes were identified in the PI.

Growth hormone is primarily produced in the pituitary gland, although secretion and expression are not necessarily correlated (Chan et al., 2004). However, it is well established that some other tissues also express the GH gene (Yang et al., 1999). Immunoreactivity to GH has been demonstrated in chicken testis (Luna et al., 2004). On this respect, the testis is thought to be a site of GH synthesis since GH transcripts have been detected in human testis (Untergasser et al., 1996, 1997) and in domestic chicken (Harvey et al., 2004). In fish, GH seems to be also related with proliferative activity of trout spermagonia (Loir, 1999). IGF-I transcription in trout testis is well established (Biga et al., 2004; Le Gac et al., 1996) and gonad IGF-I mRNA was reported to be affected by recombinant bovine somatotropin in rainbow trout. Although we demonstrate the presence of GH mRNA in pejerrey testis, the localization and the protein existence have not been examined by in situ hybridization and immunohistochemistry respectively in order to ascribe to GH a possible paracrine/autocrine action. Interestingly, the variation in testicular GH-R concentration did not appear related to changes in plasma GH concentrations (Gomez et al., 1998). In this context, it is possible to hypothesize that probably the decrease in testicular GH-R concentration observed during spermatogenesis could be ascribed to local GH production.

Previous results suggest that GH gene is also expressed in the fish ovary (Li et al., 2005). Our analysis fails to detect GH mRNA in the pejerrey ovary possibly because different GH mRNA/total RNA rate between the testis and ovary exist.

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