Genetics and Molecular Biology, 31, 1 (suppl), 381-384 (2008)

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Short Communication

Molecular cloning and sequence analysis of growth hormone cDNA of Neotropical freshwater fish Pacu (*Piaractus mesopotamicus*)

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Abstract

RT-PCR was used for amplifying *Piaractus mesopotamicus* growth hormone (GH) cDNA obtained from mRNA extracted from pituitary cells. The amplified fragment was cloned and the complete cDNA sequence was determined. The cloned cDNA encompassed a sequence of 543 nucleotides that encoded a polypeptide of 178 amino acids corresponding to mature *P. mesopotamicus* GH. Comparison with other GH sequences showed a gap of 10 amino acids localized in the N terminus of the putative polypeptide of *P. mesopotamicus*. This same gap was also observed in other members of the family. Neighbor-joining tree analysis with GH sequences from fishes belonging to different taxonomic groups placed the *P. mesopotamicus* GH within the Otophysi group. To our knowledge, this is the first GH sequence of a Neotropical characiform fish deposited in GenBank.

Key words: Piaractus mesopotamicus, growth hormone, cDNA cloning, sequencing.

Received: September 15, 2006; Accepted: May 4, 2007.

Growth hormone (GH) is a protein of approximately 22 kDa secreted from the anterior pituitary gland. It plays important roles in the regulation of growth and development of vertebrates (Moore *et al.*, 1982). In fishes, GH is also involved in different physiological processes, such as reproduction (Trudeau, 1997) and osmoregulation (Sakamoto *et al.*, 1997). Genomic GH sequences and cDNAs from fishes have been characterized and used as a phylogenetic marker for different taxonomic groups (Koren *et al.*, 1989; Chang *et al.*, 1992; Lemaire *et al.*, 1994; Marins *et al.*, 2003; Clements *et. al.,* 2004).

Piaractus mesopotamicus (pacu) is an important South American freshwater fish, and a native species of the River Plate basin which comprises the Uruguay, Paraguay and Paraná Rivers. In the Paraguay basin, *P. mesopotamicus* is one of the most important fish for commercial and sports fishery. Moreover, it is the fourth most important species in terms of aquaculture production in Brazil (SEAP, 2006). Pacu belongs to the order Characiformes and, along with the orders Cypriniformes, Gymnotiformes and Siluriformes, is included in the Otophysi series (Fink and Fink 1981). At present, cDNA sequences from GH of nine species of Siluriformes and 27 species of Cypriniformes have been deposited in GenBank. In this report, we present the nucleotide sequence of the GH cDNA from *P. mesopotamicus* and compare the encoded polypeptide with GHs of other species of the Otophysi group.

Our strategy to isolate the GH cDNA was to PCR amplify it from a cDNA library using primers whose sequence was based on the respective cDNA sequence of other members of the Otophysi. The designed primers GHP- F (5'-TCAGACAACCAGCGGCTCTTC-3') and GHP-R (5'-CAGGGTGCAGTTGGAATCCAG-3') corresponded to the 5' and 3' regions of mature GH of *Cyprinus carpio.* (Chiou *et al.*, 1990; GenBank accession number M27000). For the construction of the cDNA library, the hypophysis of a 2 kg fish caught at a fish farm was removed, and total RNA was extracted from homogenized tissue with TRIZOL reagent (Invitrogen) following manufacturer's instructions. M-MULV reverse transcriptase (Invitrogen)

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was used for synthesis of first-strand cDNA. This reaction was carried out according to the manufacturer's instructions using approximately 2 µg of total RNA, 4 µL of RT buffer (5x), 2.5 µL of dNTPs (0.5 mM), 1 µL of RNase out (40 U/µL), 1 µL of Oligo dT12-18 (500 µg/mL), 1 µL of M-MLVRT. Following the cDNA synthesis, GH cDNA was amplified by PCR with the GHP-F and GHP-R primers. Amplification reactions were performed using 35 µL of cDNA, 0.3 UI of Taq Polymerase, 0.25 mM of each dNTPs, 0.3 µM of each primer, 2 mM $MgCl_2$. The PCR profile was as follows: 36 cycles of 96 °C for 1 min initial denaturation and 94 °C for 40 s denaturation, 60 °C for 1 min annealing, 72 °C for 2 min extension and a final extension at 72 °C for 5 min. A fragment of approximately 500 bp was cloned into pGEM-T Easy vector (Promega). Sequencing was carried out with universal primers (T7 and SP6) using BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems) according to the protocols provided by the manufacturer. Electrophoresis of the purified samples was performed on an ABI 377 DNA Sequencer (PE Applied Biosystems).

The sequence obtained comprised 534 bp which translated *in silico* to a polypeptide of 178 amino acids. Alignment of the putative polypeptide of the *P. mesopotamicus GH* (*pmGH)* with other GHs from the Otophysi group (Siluriformes and Cypriniformes) shows that the GH amino acid sequence of Pacu and that of other species of Siluriformes lack ten amino acids present in the GH hormone of Cypriniformes (Figure 1). This feature is also found in the flounder *Paralichthys olivaceus* (Watahiki *et al.*, 1989), a member of the superorder Acanthopterygii.

A neighbor-joining tree of GH nucleotide sequences of fishes from different taxonomic groups was inferred in the program MEGA version 3.1 (Kumar *et al.*, 2004), using different models. In this analysis, the *pmGH* sequence clustered with GH sequences from other Otophysi fishes, grouping with Siluriformes as expected (Figure 2).

Homology analysis of the mature amino acid GH sequence by BLAST (Altschul *et al.*, 1997) showed the highest identity values between *pmGH* and GH from species of the order Cypriniformes. Those identities ranged from 87% for *Ictiobus bubalus* (smallmouth buffalo fish) to 75% for *Catla catla* (catla). Although the amino acid alignment of *pmGH* and seven other Siluriformes species showed the lack of the same ten amino acids, the percentage of overall identity between the Characiformes *P. mesopotamicus* and the Siluriformes was lower than between the Characiformes *P. mesopotamicus* and the Cypriniformes species.

Appropriate folding and, consequently, the tertiary structure of GH requires the presence of paired Cys residues that participate in the formation of disulfide bonds (Paladini *et al.*, 1983). Four Cys residues are found in GH of several mammalian and in many fish species (Abdel-Meguid *et al.*, 1987; Sato *et al.*, 1988; Yamaguchi *et al.*, 1989). Clements *et al.* (2004) found two distinct GH cDNAs in the tetraploid small buffalofish (*Ictiobus bubalus*). One of these has the usual four Cys residues and the other GH has an additional Cys residue. This unpaired Cys residue is also found in other Otophysi species such as the goldfish *Carassius auratus* (Law *et al.*, 1996), the bighead carp *Hypophthalamichthys nobilis*, silver carp *Hypophthalamichthys molitrix*, grass carp *Ctenopharyngodon idella* and the common carp *Cyprinus*

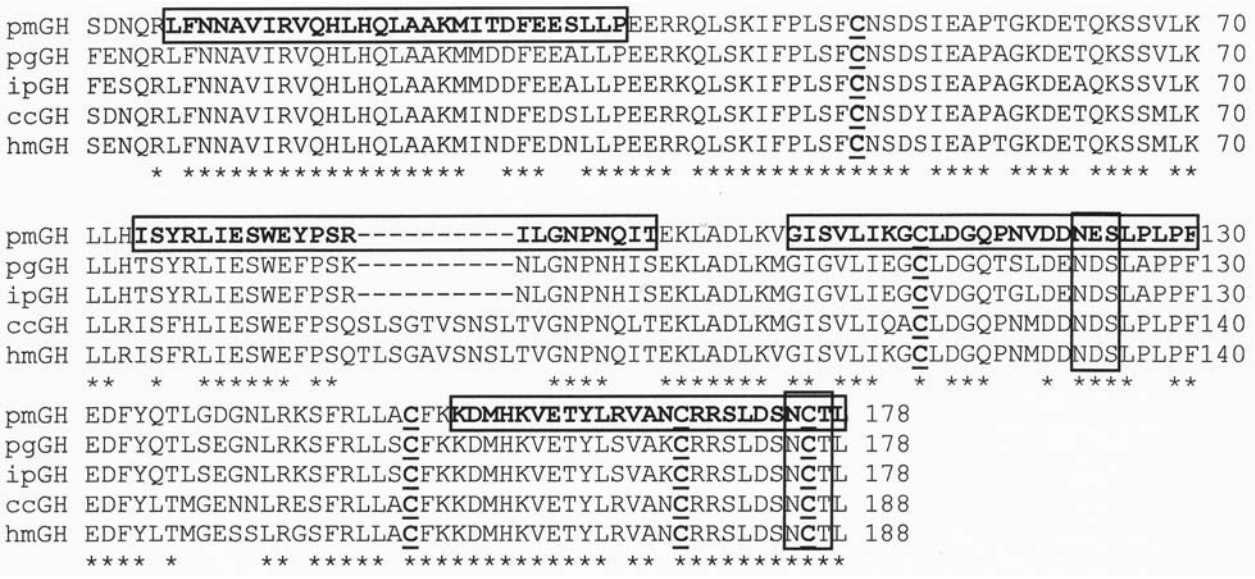

Figure 1 - Comparison of *Piaractus mesopotamicus* (*pmGH*) amino acid sequence to two other siluriform species (*pgGH* of *Pangasianodon gigas*, the giant catfish and *ipGH* of *Ictalurus punctatus*, the channel catfish) and two cypriniform species (*ccGH* of *Cyprinus carpio*, the common carp and *hmGH* of *Hypophthalamichthys molitrix*, the silver carp). A ten amino acid deletion in *pmGH, pgGH and ipGH* is shown. The conserved Cysteine residues are underlined. Vertical boxes indicate potential *N*-linked glycosylation sites and horizontal boxes are the four predicted alpha helixes for mature GH of pacu.

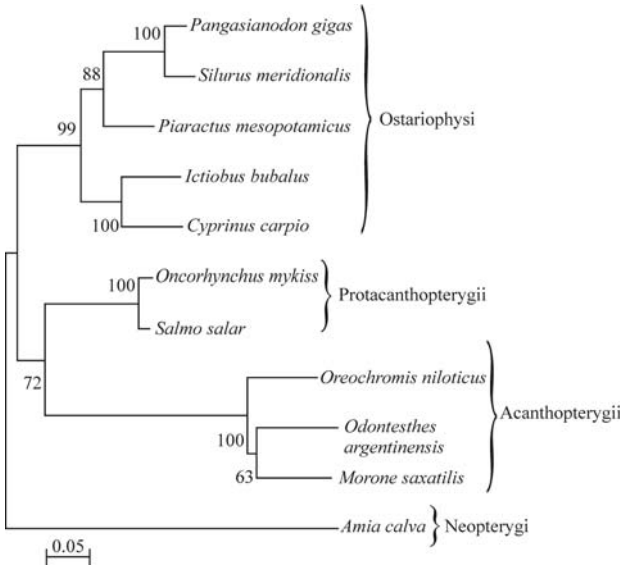

Figure 2 - Neighbor-joining tree generated from mature GH nucleotide sequences of several Telostomi species using the Kimura-two-parameter model. Numbers at branch points are bootstrap support percentages based on 5000 pseudoreplicates. Accession numbers for the nucleotides GH sequences are: AY375301 for *Ictiobus bubalus,* the smallmouth buffalo; M27000 for *Cyprinus carpio*, the common carp; DQ206404 for *Piaractus mesopotamicus*, the pacu, L27835 for *Pangasianodon gigas*, the giant catfish; AF530481 for *Silurus meridionalis*, the southern catfish; X14305 for *Salmo salar*, the Atlantic salmon; M24683 for *Oncorhynchus mykiss*, the rainbow trout; S78253 for *Morone saxatilis*, the striped sea-bass; M97765 for *Oreochromis niloticus*, the Nile tilapia; AF236091 for *Odontesthes argentinensis*, the Argentinean silverside; and S73969 for *Amia calva*, the bowfin.

carpio (Chang *et al.*, 1992), the giant catfish *Pangasianodon gigas* (Lemaire *et al.*, 1994) and the channel catfish *Ictalurus punctatus* (Tang *et al.*, 1993).

Five conserved Cys residues were also found in the pacu GH (Cys^{49}, Cys^{113}, Cys^{151}, Cys^{168} and Cys^{176}). Based on the proposed structure of GH, four of these are likely to participate in the formation of disulfide bonds. As shown in Figure 1, these are in the same amino acid positions when compared to other Otophysi species. The additional Cys residue is located in position 113 in Siluriformes and Characiformes, and in position 123 in Cypriniformes. One possible role of this fifth Cys residue might be the formation of oligomeric complexes, which might affects the appropriate refolding of GH (Fine *et al.*, 1993).

Glycosylation has been shown to occur in GH of salmon (Wagner *et al.*, 1985). This post-translational modification may also take place in two *N*-linked glycosylation motifs (N-X-S/T) found in pacu GH (N^{123} and N^{175}). These same motifs are also present in other Cypriniformes and Siluriformes species, as shown in Figure 1. Anathy *et al.* (2001) showed that for the siluriform species *Heteropneustes fossilis* (an Indian catfish), the first motif for *N*-linked glycosylation is Ala instead of Thr/Ser at position 147 in the mature GH peptide sequence, which eliminates this *N*-linked glycosylation motif.

A possible secondary sequence structure of pacu GH was predicted using NNPREDICT Protein Secondary Structure Prediction package. This analysis indicated the formation of four antiparallel α-helixes (data not shown). The first α-helix was positioned on amino acid residues 6 to 34, the second on amino acid residues 74 to 106, the third on amino acid residues 115 to 139, and the fourth α-helix on aminoacid residues 174 to 188 (Figure 1). Cunningham and Wells (1989) verified that the C-terminal helix of GH may act in receptor binding. This fourth α-helix may have important functional activity since there is a high similarity of this domain when *pmGH* and other fish GH are compared.

Acknowledgments

We thank João Davidson, Neyton Barrabás and Renata Martins for their technical assistance. This work was partly supported by grants from FAPESP and IFS.

References

Abdel-Meguid SS, Shieh HS, Smith WW, Dayringer HE, Violand BN and Bentle LA (1987) Three-dimensional structure of genetically engineered variant of porcine growth hormone. Proc Natl Acad Sci USA 84:6454-6437.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res 25:3389-3402.

Anathy V, Venugopal T, Koteeswaran R, Pandian TJ and Mathavan S (2001) Cloning, sequencing and expression of cDNA encoding growth hormone from Indian catfish (*Heteropneustes fossilis*). J Biosci 26:315-324.

Chang Y, Liu C, Huang F and Lo T (1992) The primary structures of growth hormones of three cyprinid species: Bighead carp, silver carp, and grass carp. Gen Comp Endocrinol 87:385-393.

Chiou C-S, Chen HT and Chang W-C (1990) The complete nucleotide sequence of the growth-hormone gene from the common carp (*Cyprinus carpio*). Biochim Biophys Acta 1087:91-94.

Clements MD, Bart Jr HL and Hurley DL (2004) Isolation and characterization of two distinct growth hormone cDNAs from the tetraploid smallmouth buffalofish (*Ictiobus bubalus*). Gen Comp Endocrinol 136:411-418.

Cunningham B and Wells J (1989) High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenis. Science 244:1081-1085.

Fine M, Sakal E, Vashdi D, Daniel V, Levanon A and Lipshitz O (1993) Recombinant carp (*Cyprinus carpio*) growth hormone: Expression, purification, and determination of biological actity *in vitro* and *in vivo*. Gen Comp Endocrinol 89:51-61.

Fink SV and Fink WL (1981). Interrelationships of the ostariophysan fishes (Teleostei). Zool J Linn Soc 72:297-353.

Kumar S, Tamura K and Nei M (2004) MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings Bioinf 5:150-163.

Koren Y, Sarid S, Ber R and Daniel V (1989) Carp growth hormone: Molecular cloning and sequencing of cDNA. Gene 77:309-315.

Law MS, Cheng KW, Fung TK, Chan YH, Yu KL and Chan KM (1996) Isolation and characterization of two distinct growth hormone cDNAs from the goldfish, *Carassius auratus.* Arch Biochem Biophys 330:10-23.

Lemaire C, Writ S and Panyin S (1994) Giant catfish *Pangasianodon gigas* growth hormone-encoding cDNA cloning and sequencing by one sided polymerase chain reaction. Gene 49:271-276.

Marins LF, Jose AL, Josep MF and Armand S (2003) A growth hormone-based phylogenetic analysis of euteleostean fishes including a representative species of the Atheriniformes Order, *Odontesthes argentinensis*. Genet Mol Biol 26:295-300.

Moore D, Walter M, Diamond D, Conkling M and Goodman H (1982) Structure, expression and evolution of growth hormone genes. Rec Prog Hormone Res 38:197-225.

Paladini AC, Pena C and Poskus E (1983) Molecular biology of growth hormone. Crit Rev Biochem 15:25-26.

Sakamoto T, Shepherd BS, Madsen SS, Nishioka RS, Siharath K, Richman NH and Grau EG (1997) Osmoregulatory actions of growth hormone and prolactin in an advanced teleost. Gen Comp Endocrinol 106:95-101.

Sato N, Watanabe K, Murata K, Sakaguchi M, Kariya Y, Kimura S, Nonaka M and Kimur AA (1988) Molecular cloning and nucleotide sequence of tuna growth hormone cDNA. Biochim Biophys Acta 949:35-42.

SEAP (2006) Secretaria Especial de Aqüicultura e Pesca/PR, Produção brasileira da Aqüicultura Continental por estado e espécie para o ano de 2003. http://www.presidencia.gov.br/seap/ (May 15, 2006).

Tang Y, Lim CM, Chen TT, Kawauchi H, Dunham RA and Powers DA (1993) Structure of the channel catfish (*Ictalurus punctatus*) growth hormone gene and its evolutionary implications. Mol Mar Biol Biotechnol 4:198-206.

Trudeau VL (1997) Neuroendocrine regulation of gonadotropin II release and gonadal growth in the goldfish *Carassius auratus*. Rev Reprod 2:55-68.

Wagner GF, Fargher RC, Brown JC and McKeown BA (1985) Further characterization of growth hormone from the chum salmon (*Oncorhynchus keta*). Gen Comp Endocrinol 60:27-34.

Watahiki M, Yamamoto M, Yamakawa M, Tanaka M and Nakashima K (1989) Conserved and unique amino acid residues in the domains of the growth hormones: Flounder growth hormone deduced from the cDNA sequence has the minimal size in the growth hormone prolactin gene family. J Biol Chem 264:312-316.

Yamaguchi K, Yasuda A, Lewis UJ, Yokoo Y and Kawauchi H (1989) The complete amino acid sequence of growth hormone of an elasmobranch, the blue shark (*Prionace glauca*). Gen Comp Endocrinol 73:252-259.

Internet Resources

Protein Secondary Structure Prediction package (NNPREDICT), http://www.cmpharm.ucsf.edu/~nomi/nnpredict.html (May 20, 2006).

Associate Editor: Cláudio Oliveira