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Isolation of *Sparus auratus* prolactin gene and activity of the *cis*-acting regulatory elements

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Abstract

A sea bream prolactin (sbPRL) gene was isolated using a prolactin cDNA fragment, generated by PCR as a probe. The gene analyzed comprises 3.5 kb of DNA containing five exons as described previously for other fish PRL genes. Analysis of 1.0 kb of the proximal promoter sequence reveals a consensus TATAA box, up to seven $(A/T)_3NCAT$ consensus motifs for binding of the pituitary-specific factor Pit-1 and putative CREB and GATA binding sites. CHO culture cells co-transfected with a sbPRL promoter sequence and a sea bream Pit-1 cDNA expression plasmid showed expression of a linked luciferase reporter gene. Transient expression experiments with 5′-delection mutants reveals at least three regulatory regions on the sbPRL gene, two with a stimulatory effect on transcription and one with apparent inhibitory effect. From a comparative point of view, this study of PRL gene in *Sparus auratus*, correlates well with those previously published on tilapia and rainbow trout. The molecular data reported will be useful for comparative analysis of gene regulation in the GH/PRL gene family in teleosts.

Keywords: Prolactin; Gilthead sea bream; Gene promoter; *cis*-acting sequences; *Sparus auratus*

1. Introduction

Growth hormone (GH), prolactin (PRL), and somatolactin (SL) belong to a family of structurally related polypeptide hormones in fishes. On the basis of nucleotide and amino acid sequences, it has been suggested that the members of this gene family have evolved by divergence from a common ancestral gene (Miller and Eberhardt, 1983; Niall et al., 1971; Nicoll et al., 1986; Wallis, 2001). In vertebrates, PRL is a versatile hormone, involved in many physiological processes such as growth, differentiation, metabolism, osmoregulation, and reproduction (Hirano, 1986). In teleosts, the major function of PRL seems to be osmoregulation in freshwater to prevent the loss of Na^+ and electrolyte homeostasis (Ayson et al., 1994; Manzon, 2002). The protein structure of PRL is believed to be composed of four α-helices arranged in an anti-parallel four-helical bundle as described similarly for GH (Barsh et al., 1983). In fish, several PRL cDNAs have been characterized (Chan et al., 1996; Chen et al., 1991; Santos et al., 1999; Swennen et al., 1992; Xiong et al., 1992), and two PRL genes in tilapia have been reported (Rentier-Delrue et al., 1989). Also, regulatory elements of chinook salmon, tilapia, and rainbow trout prolactin genes have been studied previously (Argenton et al., 1996; Poncelet et al., 1996; Xiong et al., 1992). In the present study we have isolated and sequenced a *Sparus auratus* PRL gene and analyzed the transcription activity of the *cis*-elements under the control of the sea bream Pit-1 factor (Martínez-Barberá et al., 1997).

2. Methods

2.1. Cloning of sea bream prolactin gene

First, a sbPRL probe was generated by PCR using a forward primer designed within the 5′ end of the cDNA

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previously described (Santos et al., 1999) (primer PRL1 5′-GGCAGCGCATATGGCCGTGCCCATCAATGA CCT-3′) and a reverse primer based on the adaptor sequence of a pituitary cDNA expression library (Martínez-Barberá et al., 1994) (primer PRL2 5′-GGT GGCGACTCCTGGAGCCC-3′). The 1.3-kb PCR fragment isolated was used to screen 1×10^5 recombinant phages of the above library at 65 °C overnight, and filters were washed in 0.1× SSC/0.1% SDS. Five positive plaques were identified, isolated, and sequenced to corroborate the previous published PRL cDNA, except for two nucleotide differences that could make a single amino acid change. This sbPRL cDNA was used to screen a sea bream genomic library constructed by ligating *Bam*HI digested and partially filled sea bream genomic DNA (10–20 kb) (isolated from 1 ml of peripheral blood) into a partially filled *Xho*I site of λ FixII vector (Stratagene). From 4×10^6 recombinants used, four positive plaques were identified and analyzed by restriction mapping and Southern blot, to find if they were identical. Subcloning in the pGEM 3Z vector (Promega) was used to generate clone C1, of 2 kb containing the proximal promoter and the first and part of the second exon, and clone C2, of 4 kb containing the rest of second exon and exons 3, 4, and 5, and 3′ untranslated region of the PRL cDNA. Sequencing was performed in a 3100 Genetic Analyzer Sequencer (Applied Biosystem).

2.2. Transient transfection assays

To characterize the transcription activity of the proximal sbPRL promoter sequences, a dual-luciferase reporter assay system was used. The 5′-flanking region of the sbPRL gene was subcloned into a reporter pGL2-basic vector fused to the luciferase gene (Promega), and this construct was named psb −1.0/0. Deletion constructs were generated by PCR using Pfu polymerase (Amersham Biotec). For that purpose, forward primers to −0.7 (5′-ACTTTAACATGACCTGGAG-3′), −0.15 (5′-AACCATCAGCCTTTACACT-3′), and −0.09 (5′-CATTACAGCTGAAGCCAAG-3′) regions of the sbPRL gene were used with a reverse primer (5′-TAGC TTTTGGCTTCTTTAC-3′). The PCR products were cloned in the *Sma*I site of the pGL2 reporter vector to generate constructs psbPRL −0.7/0, psbPRL −0.15/0, and psbPRL −0.09/0, respectively. These DNAs were purified by a Qiagen Plasmid Midi Purification Kit (Qiagen, Gmbh) and confirmed by sequencing.

For co-transfection experiments, CHO hamster cells were grown in DMEM supplemented with 10% fetal calf serum. Transfections were performed by the calcium phosphate procedure as described (Sambrook et al., 1989). A sea bream Pit-1 full-length cDNA cloned in a plasmid containing the RSV-LTR promoter was described in a previous report (Martínez-Barberá et al., 1997). CHO cells were co-transfected at 37 °C with sbPRL and sbPit-1 plasmids for 24 h. pGL2-basic vector without promoter was used as negative control and transfections were performed in triplicate. Cells were harvested with 1× reporter lysis buffer, assayed for luciferase according to the manufacturer's instructions (Promega), and luciferase activity was measured in a luminometer (Berthold Lumat LB 9507). In all transfections, pRL-TK was included to normalize the data to transfection efficiency. After 48 h of incubation, luciferase activities were measured. Values are expressed as percent activity relative to that shown by the psbPRL −1.0/0 construct.

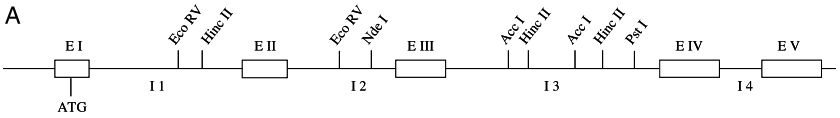
3. Results and discussion

3.1. S. auratus PRL gene cloning

A 1.3-kb PRL probe was generated by PCR from DNA isolated from a *S. auratus* expression library. The amplified product served to identify positive phages from the same expression library, to corroborate the sequence of sbPRL cDNA previously reported (Santos et al., 1999). Surprisingly we found two nucleotide differences in comparison with the reported sequence at positions 171 and 205. This finding could mean a change of amino acid Leu for Met at residue 69 of the cDNA. It is still not known whether this difference is merely a result of sequencing artefacts, or could represent a real amino acid change in the protein structure because different *S. auratus* strains were utilized. This DNA was used to isolate PRL clones from a sea bream genomic library, thus allowing us to identify a 6-kb genomic fragment containing the complete cDNA of sbPRL. Further, 1.0 kb of the proximal promoter of the sbPRL gene was also identified and sequenced. The gene is organized in five exons (Fig. 1A) as described previously for other fish PRLs including carp (Chen et al., 1991), salmon (Xiong et al., 1992), and tilapia (Swennen et al., 1992). Exon I encodes the initial 14 amino acids and the first letter of the 15th amino acid of the signal peptide. Exons II, III, and IV encode 37, 36, and 61 amino acids and finally exon V encodes 63 amino acid residues plus the TAA stop codon. This pattern of exon–intron junctions in *S. auratus* PRL gene (Fig. 1B) is similar to those previously described for other PRL genes in fish (Chen et al., 1991; Poncelet et al., 1996; Swennen et al., 1992; Xiong et al., 1992). Further, exon organization of PRL gene in *S. auratus* is similar to that of members of the GH gene family in other fish species, confirming the hypothesis that it derives from an ancestral common gene by duplication (Miller and Eberhardt, 1983; Niall et al., 1971).

A TATAA box identified in sbPRL gene (Fig. 2) matches the position of that found in PRL genes of salmon (Xiong et al., 1992) and tilapia (Swennen et al.,

A



Exon	Exon size	cDNA position	Exon-intron junctions	Intron	Intron size
I	84 pb	1–84	…GAGATGGCTCA....CATTACGG**gt**gagatg	1	717 pb
II	113 pb	85–197	atcctc**ag**TGTTGTGC....AAGATCTG**gt**gcccgt	2	389 pb
III	108 pb	198–305	ccttcc**ag**AGCAATCA....AATTATCA**gt**gcgtaa	3	979 pb
IV	183 pb	306–488	ttctgc**ag**GAGTCGGA....CTGGCAAG**gt**aagcaa	4	147 pb
V	>192 pb	489-680	tttgtc**ag**ATGGGTCC....TGTGCTAA…		

B

	Gene size	Exon I	Intron 1	Exon II	Intron 2	Exon III	Intron 3	Exon IV	Intron 4	Exon V
Tilapia	**2899**	**97**	**1023**	**113**	**363**	**108**	**701**	**183**	**119**	**192**
Common Carp	**2076**	**88**	**118**	**122**	**138**	**108**	**993**	**183**	**137**	**189**
Chinook Salmon	**2555**	**96**	**89**	**113**	**1087**	**108**	**518**	**183**	**172**	**189**
Gilthead Seabream	**2912**	**84**	**717**	**113**	**389**	**108**	**979**	**183**	**147**	**192**

Fig. 1. Genomic organization of *S. auratus* prolactin gene. (A) The five exons of the sbPRL gene are shown in boxes. The start ATG codon was found in exon I as indicated. Exon V contains the stop TAA codon and a poly(A) signal. Unique restriction sites are indicated. Exon–intron junctions of the sbPRL gene are shown below. Exon sequences are in upper case letters and intron sequences in lower case letters. The start and stop codons are boxed. (B) Comparison of PRL genes in several fish species. Based on the data available for each PRL gene, the analysis is presented considering from the initial nt of the cDNA up to the stop codon of each gene. Similar size and exon–intron PRL gene organization is shown in tilapia, carp, salmon, and sea bream.

```
-1010 atgacttt aacatgacctggaggacttagaactttcatcaacatcagtagaggttgtgtt  -950
-949  cctgtactgtaccctgaagtatataatataaaattagtatgtatgcatgaattaatccat  -890
-789  aattaatcatgcacattattctaaatgtagtcctgccgaatgagtccttttacttcttgt  -830
-829  acttttaatacagattattttgtacctttacataaactggcaacagacacattttcggcg  -770
-769  tcagggtgtcattttgaagtaaatgttaattgcacaattaaatggatacataataatgaa  -710
-709  accatcagcctttacactggttccttgtaaattttgctttcaccaaacagttttgtctgc  -650
-649  ttcaaggcaccgccactgcttctattacaaactaaatggtttgtccttagttgtatttac  -590
-589  tacccagtcgatcgatgtcatcatccagttgtcttggcgaaagtggtgccaacaaaccac  -530
-529  ttggaaaagttgtctgtcgccgctttaatgaaaattccaatgtaggtgtttttttctttct  -470
-469  tggatcagagtatttctacatttccatgccacttttaccttggtaaatgatctgaatag  -410
-409  gtctttcattactgactgtacatttaaaaggatcagttgagttatggactttgatggtca  -350
-349  tcagtgatttaaccataaaatgaattaaaaccgatgtcacggtaaacgtcaatggcttta  -290
-289  ttcatccatccaattaagatggaaaaaacttttccacttccatgtcacacgcacacaca  -230
-229  cacacacacacacacacttatcaacctgttacatccattcaagactcctcgaccatgttt  -170
-169  ctcattataaagtggaccaaacaggtttcaagcaatcagcacacactgacaaagctgctg  -110
-109  catgaccattacagctgaagccaagacactataaaaatgatggaccacagagaatgagaga  -50
-49   gaaagagcgACGAGGTAAAGAAGCCAAAAGCTAGCGAAAAAACAACTGAGATGGCTCACA  +10
+11   GAGAAACCAATGGAAGCAAACTCTTCATTACGGgtgagatgatctccgcttggctattta  +70
+71   atctctgaaattattttaaaacacaacatttaaaaagaatattttgtgtaaaatgtattt  +130
+131  gtttaattcccgtgtactgatacttttttaaaaatctgtttttgtgaacttaacaagaac  +190
+191  atgtatcgcattcaaataataataaataaatctattccacaatgttaataggtttcttaa  +250
+251  aaagaaaaaaagggttttctgaacaaatcttattctctcagtgaaaaatatttccctatg  +310
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Fig. 2. Promoter sequence of *S. auratus* PRL gene. Numbers begin with the initial start ATG codon. Upper case letters show nucleotides of exon I of sbPRL cDNA. Seven consensus motifs $(A/T)_3NCAT$ for putative Pit-1 binding are boxed. A consensus sequence for a GATA site is underlined and a putative CREB binding site is underlined in bold letters. The TATAA box is indicated in lower case bold letters. The full genomic sequence of sbPRL gene is at NCBI sequence database under Accession No. AJ509807.

1992). Putative *cis*-elements responsible for the specific expression of pituitary GH genes were identified in the 5′-flanking region of PRL gene, representing binding sites for the transacting factor Pit-1 (Fig. 2). The 1.0-kb proximal promoter of sbPRL contains up to seven motifs that closely resemble the consensus sequence $(A/T)_3$NCAT described for Pit-1 binding in mammals (Elsholtz et al., 1992) (Fig. 2). The sequences found in the sbPRL gene are identical to that core or exhibit only minor mismatch. Comparison of these Pit-1 sites observed in sbPRL promoter with those described previously in different fish PRL promoters suggest a similar distribution. Yet, another site found in the sea bream prolactin promoter is the heptamer sequence known as cAMP-responsive element (CRE) to be recognized by CRE-binding protein (CREB) (Habener, 1990). This site at position nt −1.090 could play some role on the stimulation activity found in the transient transfection analysis of sbPRL promoter (Fig. 3, below). Further experimentation will be required, such as a detailed footprinting analysis, to reach any conclusion on this putative Creb site on sbPRL promoter.

3.2. Stimulation of sbPRL gene expression by sbPit-1

For the first time in a Perciform fish, we studied from a comparative point of view, the transcription activity of the proximal regulatory elements of sbPRL gene. We approached this, with a series of co-transfection assays with *S. auratus* Pit-1 pituitary factor isolated previously (Martínez-Barberá et al., 1997). The pituitary-specific transcription factor Pit-1 binds *cis*-elements of the GH, PRL, and SL genes in fish and stimulates their transcription (Argenton et al., 1996; Bernardini et al., 1999; Nelson et al., 1988; Yamada et al., 1993). To characterize those *cis*-elements, nucleotide sequencing was conducted up to 1.0 kb from the start ATG codon (Fig. 2). The results of CHO co-transfected cells are shown in Fig. 3. In control experiments, single transfection assays of sbPRL promoter without the sbPit-1 plasmid resulted in basal stimulation of luciferase activity (data not shown). However co-transfection of several sbPRL promoter constructs and sbPit-1 significantly increases luciferase activity in all cases analyzed. In control experiments, the homologous rat Pit-1 showed less but significant stimulation toward sbPRL promoter constructs corroborating previous findings (Martínez-Barberá et al., 1997) (data not shown). Further, similar results were obtained when HeLa and 293 human cells were used, although in these cells, lower levels of transfection activity were found (data not shown). Our results first indicated that a 0.15-kb (psbPRL −0.15/0) upstream region of the PRL gene was sufficient for trans-activation by sbPit-1 when introduced in CHO cells. Since a single putative sbPit-1 binding site is found in this region (Fig. 2) and it resembles the consensus core sequence described for other Pit-1 factors (Ohkubo et al., 1996), it should mediate the function of sbPit-1. Our transfection constructs suggest other Pit-1 sites (on psbPRL −1.0/0), which may contribute to the complete expression of sbPRL experimentally observed. Detailed analysis of the 1.0-kb sequence of the sbPRL proximal promoter, showed to us up to a total of seven $(A/T)_3$NCAT motifs for Pit-1 binding (Fig. 2). This observation is in agreement to those reported previously by others for tilapia and trout PRL genes (Argenton et al., 1996; Poncelet et al., 1996). Further, psbPRL −0.7/0 construct showed less stimulation than both psbPRL −1.0/0 and psbPRL −0.15/0 (Fig. 3), suggesting a putative negative control site between nt −150 and −700. Similar observations have also been reported for other fish PRL and GH promoters in transient transfected cells (Argenton et al., 1996; Bernardini et al., 1999; Poncelet et al., 1996).

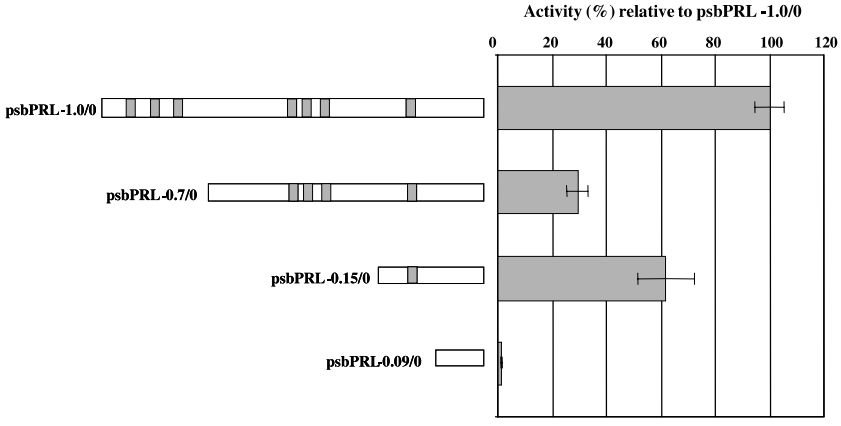

Fig. 3. Analysis of proximal promoter activity of sbPRL gene. On the left, the length of each construct is shown with first and last nucleotide enumerated being the nt +1 of the start ATG codon. Shaded areas represent the positions of consensus $(A/T)_3$NCAT motifs for putative Pit-1 binding. On the right, the data represent means ± SEM of the luciferase activity expressed in percent activity relative to the psbPRL −1.0/0 construct and correspond to three independent experiments.

Although essential, Pit-1 is not sufficient either to determine completely GH/PRL/SL gene expression in fish or to explain their differential regulation. However, it is well known that the pituitary-specific factor acts as a homodimer form and as heterodimer with other factors such as estrogen receptors (Day et al., 1990). In this regard, the high activity of the psbPRL −1.0/0 construct (Fig. 3) could be explained as a contribution of both various Pit-1 sites (Fig. 2), but also by the contribution of a putative CREB site located at position nt −1011 as occurs in rainbow trout PRL gene (Argenton et al., 1996). Studies of GH and PRL genes have consistently implicated DNA binding sites for the POU homeodomain of Pit-1 as mediating a cAMP response (Delegeane et al., 1987; Peers et al., 1991). Thus it seems conclusive that the participation of other DNA-binding proteins or auxiliary factors, both positive and negative in PRL gene regulation, is needed. Accordingly, putative GATA and CREB sites identified in the PRL promoter may play a role in regulating PRL gene expression in *S. auratus*.

The structure of a first Perciform PRL gene promoter described in this report provides the molecular information necessary for further study of the factors controlling prolactin synthesis in *S. auratus*.

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