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Genomic Structure and Functional Analysis of Promoter Region of Somatolactin Gene of Sea Bream (*Sparus aurata*)

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Abstract: Somatolactin (SL) is a pituitary hormone belonging to the growth hormone–prolactin family and is produced in the intermediate lobe of teleosts. The SL gene was isolated from a sea bream genomic library and found to be composed of 5 exons distributed within a 9-kb length of DNA. Sequence analysis of the proximal promoter region showed the presence of a classical TATA box located 59 bp upstream from the initial start ATG codon, 5 consensus sequences corresponding to the Pit-1 binding element, and a putative CREB site. In CHO cells cotransfected with the DNA from 2 plasmids, one encoding sea bream Pit-1 under Rous sarcoma virus long terminal repeat regulation and one encoding the SL promoter driving the expression of luciferase, Pit-1 was found to enhance the expression of luciferase. Only one Pit-1 binding site was necessary for enhancement. Analysis by immunoblots of in vitro culture of pituitaries of *Sparus aurata* showed that several agents, including estradiol, verapamil, and phorbol myristate acetate, had different inhibitory effects on SL and growth hormone released to the culture medium.

Key words: somatolactin, sea bream, gene promoter, *Sparus aurata*.

INTRODUCTION

Somatolactin (SL) synthesis and secretion is limited to the somatotropic cells of the intermediate lobe of pituitary gland of teleosts. This term was based on the structural resemblance of SL to growth hormone (GH) and prolactin (PRL) (Rand-Weaver et al., 1991). During the past decade the structure and complementary DNAs of this novel pituitary hormone were determined for several fish species (Ono et al., 1990; Iraqi et al., 1993; Pendón et al., 1994; Astola et al., 1996; Amemiya et al., 1999). Although several reports have proposed SL activity to be associated with ion regulation (Olivereau et al., 1980; Kaneko and Hirano, 1993), reproduction (Rand-Weaver et al., 1992), background adaptation (Ball and Batten, 1981), and stress (Rand-Weaver et al., 1993), conclusive data on its physiologic role in teleosts have yet to be demonstrated. Characterization of the promoter region of chum salmon SL

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gene has shown that Pit-1, a pituitary-specific transcriptional factor for GH and PRL, also plays a major role in the expression of the SL gene in this tissue (Ono et al., 1994). Pit-1 cDNAs from 3 fish species including chum salmon (Ono and Takayama, 1992), rainbow trout (Yamada et al., 1993), and sea bream (Martínez-Barberá et al., 1997) have been isolated.

Sea bream is a fish species extensively cultured in the Mediterranean area with considerable economic significance in aquaculture. As a step toward understanding the regulation and physiologic roles of sea bream SL (sbSL), we have cloned and characterized the sbSL gene. We have also studied the activity of the promoter region of the gene under the species homologous Pit-1 protein factor. Further, initial studies showed in vitro culture of fish pituitaries to be a feasible system, indicating that although SL and GH are under the control of Pit-1, they follow different pathways for gene expression and secretion.

Materials and Methods

Isolation of Sea Bream SL Gene and Its Proximal Promoter

DNA from 1 ml of blood of sea bream was isolated, purified, and partially digested with *Bam*HI and fractionated to appropriate size (9–23 kb) by sucrose gradient centrifugation. Isolated DNA (140 μg) was precipitated with ethanol and analyzed by agarose gel before being cloned in a phage vector. A λ Fix II genomic DNA library was constructed and propagated in XL1 blue MRAP2 *Escherichia coli* cells. Recombinant phages (5×10^5) were screened with a probe generated by Polymerase chain reaction (PCR) corresponding to nucleotides 1 to 240 of the sbSL cDNA described previously (Astola et al., 1996). Three positive clones were identified, analyzed by Southern blot, and sequenced. These clones were found to represent the same sbSL genomic region. Subcloning of the phage DNA was in the pBS vector (Stratagene) after digestion with *Sac*I, which generated 2 fragments of 5.5 and 4.7 kb. Sequence analysis revealed that the 5.5-kb subclone contained the promoter, the first exon and part of the second exon of sbSL, while the 4.7-kb subclone covered the rest of exon 2 and exon 3. The 3′ end of the sbSL cDNA was missing in these genomic clones, and a PCR strategy was used for isolating the final exons and the 3′ untranslated region. A 800-bp area was amplified from sea bream genomic DNA using a forward primer, SLa 5′-AGG ACA AAT GGC TGC TTC ATT CTG-3′, designed to cover the 5′ end of the region missing the cDNA, and a reverse primer, SLb 5′-TTA TCG ACA GCT GTG CAT GTC ATT-3′, from the 3′ untranslated region of the sbSL cDNA (Astola et al., 1996).

Transient Transfection Assays

A dual-luciferase reporter assay system (Promega) was used for analyzing the sbSL promoter. A fragment of the 5′-flanking region of sbSL (psbSL −1386/+131) was subcloned into a reporter plasmid pGL2-basic vector fused to the luciferase gene (see Figure 3). Deletion plasmids (psbSL −1386/−1; psbSL −1386/−382; psbSL −846/−1; psbSL −420/−1; psbSL −175/−1) were generated by PCR using Pfu polymerase (Invitrogen) and cloned into the *Sma*I site of the reporter vector. The accuracy of the plasmid constructions was confirmed by DNA sequencing, and the plasmid DNAs were purified by a plasmid midi purification kit (Qiagen, GmbH). The effector plasmid containing the entire coding region of sea bream Pit-1 cDNA (sb Pit-1) under the Rous sarcoma virus long terminal repeat (RSV-LTR) promoter had been described previously (Martínez-Barberá et al., 1997). CHO cells were seeded 24 hours prior to transfection at 60% to 70% confluency in DMEM medium supplemented with 7% fetal calf serum. Transfections were performed using the calcium phosphate transfection system (Life Technologies). CHO cells were cotransfected at 37°C with each reporter plasmid, and various amounts were tested as a control for selecting an appropriate one. Transfections were performed in triplicate, and constructs without sbSL promoter were used as negative controls. Cells were harvested 24 hours, after transfection with 1× passive lysis buffer, and luciferase assays were performed according to the manufacturer's instructions (luciferase reporter assay system, Promega). Luciferase activity from the freeze-thaw lysates was measured in a Lumat LB 9507 luminometer (EG & G Berthold). Light units were normalized with luciferase activity of cotransfected pRL-CMV containing the cDNA encoding Renilla luciferase. Values are expressed as percentage induction relative to the activity shown by psbSL −1386/−1 construct, and represent the mean plus or minus standard error of the mean for at least 3 determinations.

Release of SL from In Vitro Cultures of Sea Bream Pituitaries

Pituitaries were removed from freshwater sea bream of both sexes weighing from 100 to 150 g. The organs were cultured for 5 days in Eagle's minimal essential medium, supplemented with 10% fetal calf serum at 27°C. After 1 day in culture, the medium was removed and stored frozen for further analysis, and fresh medium was added to the culture for another 24 hours. Then, the medium was removed and stored as before, and fresh medium containing one of the following agents was added at a final concentration of 5 nM estradiol dissolved in water, 0.16 μM 4-β-phorbol 12-β-myristate 13-α-acetate (PMA) dissolved in DMSO, or 5 μM verapamil dissolved in water. After 24 hours of treatment the medium was removed and stored frozen, and the pituitaries were further incubated in fresh medium for 48 hours for recovery of hormone release. Experiments were performed using 4 pituitaries cultured individually for each reagent. As a control 4 pituitaries were cultured in vitro in medium without any treatment. SL and GH were both assayed by immunoblots in all batches of pituitary medium collected, for appropriate comparison of the results. For Western blotting analysis, culture media were analyzed by 12% Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose filter (Towbin et al., 1979). Antisera against sea bream SL and GH proteins had been described previously (Martínez-Barberá et al., 1994; Astola et al., 1996) and were used at 1:500 dilutions. After washing in phosphate-buffered saline containing 0.1% Tween, blots were incubated with peroxidase-conjugated antirabbit serum and developed by a chemiluminiscence detection kit (Amersham Biotec).

Results and Discussion

Cloning of Sea Bream SL Gene

Partial sequence of the sbSL gene was obtained from 3 positive phages isolated from a *Sparus aurata* genomic library screened with a 5′-end DNA probe generated by PCR from the sbSL cDNA (Astola et al., 1996). Restriction mapping and sequencing showed that all phages isolated were the same, covering 3 exons from nucleotide +1 to +321 of the sbSL cDNA previously described. To isolate genomic clones for the 3′ region of the sbSL gene, a PCR strategy was designed to amplify it from genomic DNA using a forward primer at nucleotide +322 of the cDNA and a reverse primer at +696 of the termination codon (see "Materials and Methods"). Cloning and sequencing of the genomic 800-bp product generated were used to provide a complete picture of the sbSL gene (Figure 1).

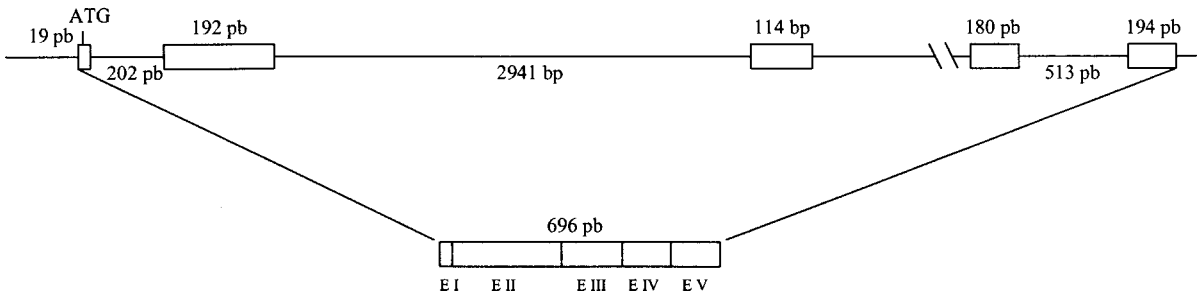
Sequence analysis of the sbSL gene showed it to consist of 5 exons, the sequences of which were identical to the corresponding cDNA (Astola et al., 1996). Several purine residues between the TATA box and the ATG start codon most probably represent the CAP site, although the transcription start site was not determined in this study. We estimated the length of the sbSL gene isolated to be 9 kb, although its final sizing would require the complete sequencing of intron 3 (estimated to be 3.3 kb by Southern hybridization) and the total length of exon V (containing the 3′-end nontranslated region), not determined in this study. Furthermore, additional data from Southern blots suggest to us the presence of a putative second SL gene in sea bream (data not shown).

We found the sizes of exons in sbSL very similar to those described in chum salmon SL, although the introns were significantly smaller in sbSL, which explains the difference in size between the 2 SL genes. Following the 5′ untranslated region of 1386 bases, exon I encodes the first 5 amino acids and the first letter (G) of the sixth amino acid of the signal peptide, and exon II covers a total of 192 nucleotides for 63 amino acid residues of sbSL (Astola et al., 1996). Exons III and IV encode 38 and 60 amino acid residues, respectively, and finally the sequence determined in exon V encodes 64 amino acids finishing at the stop codon TAA. The sbSL gene sequence analyzed was deposited in GenBank/EMBL under accession numbers AJ272342 and AJ508042.

From comparison of the amino acid sequences of SLs with GHs in teleosts, the SL gene had been considered to derive from an ancestor common to GH and PRL by gene duplication (Takayama et al., 1991b; Ford, 2000). Thus the exon-intron junctions of the sbSL gene were separated by both class 1 and class 0 splice sites (Figure 1) and showed a similar pattern to those described previously for SLs and GHs, confirming that prediction.

To find those elements involved into the regulation of expression of *S. aurata* SL gene, nucleotide sequencing was conducted up to 1386 bp from the start ATG codon. A potential TATA site was identified at nucleotide −59 (Figure 2), which matches the position of those found in the salmon SL gene (Takayama et al., 1991a) and the

A



B

Exon	Exon size	cDNA position	Exon-intron junctions	Intron	Intron size
I	19 pb	1 – 19	ACAATGCGCATGATAAGAG**gt**aatgtg	1	202 pb
II	192 pb	20 – 211	gtcctc**ag**CAATAAAG....CTTTGTTT**gt**gagtaa	2	2941 pb
III	114 pb	212 – 325	gattgt**ag**GAGGAGAT....AGATATCT**gt**gagtac	3	N.D.
IV	180 pb	326 – 505	**ag**GACAAATG....TCAAGAAG**gt**ggggcc	4	513 pb
V	>194 pb	506 – 699	ctgtgc**ag**ATGCTGGA....GTCGATAA.....		

Figure 1. Genomic organization of *Sparus aurata* SL gene. **A:** The 5 exons are shown in boxes. The start ATG codon was found in exon 1. Intron sizes were determined from genomic sequencing, except for intron 3, which was estimated by restriction analysis and gel electrophoresis. **B:** Exon-intron junctions of sbSL gene. Exonic sequences are shown in uppercase letters and intronic sequences are in lowercase letters. Acceptor and donor nucleotides are shown in bold face. Start and termination codons are boxed.

yellowtail GH gene (Ohkubo et al., 1996). *Cis* elements responsible for the pituitary-specific expression of the sbGH gene were identified in the 5′-flanking region of the SL gene, representing putative binding sites for the trans-acting factor Pit-1 (Figure 2). The sbSL proximal promoter contains 5 Pit-1 binding sites, as described previously for the rainbow trout GH gene (Agellon et al., 1988), which closely resemble the 4 sites described for the chum salmon SL (Takayama et al., 1991a). The consensus sequence of these sites closely matches the 8-bp motif T/ANCTNCAT described for fish Pit-1 (Ohkubo et al., 1996), although there are some differences to be considered (Figure 2).

Stimulation of SL Gene Expression by Pit-1

Pit-1 protein binds to elements positioned in the upstream region of GH, PRL, and SL genes and stimulates their expression (Ingraham et al., 1990). The availability of the genomic sequences for sbSL (this report) and the cloning of sbPit-1 (Martínez-Baberá et al., 1997) have enabled us to investigate the activity of Pit-1 in *Sparus aurata* SL gene expression. The results of multiple transient cotransfection experiments of sbSL and sbPit-1 plasmid constructs in CHO cells are shown in Figure 3. Similar analyses were performed in HeLa and 293 cells, although CHO showed a higher level of transfection activities (data not shown). In control experiments transfection of either sbSL promoter constructs or sbPit-1 alone resulted in no significant stimulation of luciferase activity (data not shown). However, when the luciferase gene linked to a 1.4-kb genomic fragment upstream of the *S. aurata* SL gene (construct psbSL −1386/−1) was cotransfected into CHO cells with sbPit-1 effector plasmid, a severalfold increase in stimulation of luciferase gene expression was observed, compared with control experiments. This value of activity was used as the reference for all other constructs assayed (Figure 3).

We have found a 420-bp sequence upstream of the SL gene (psbSL −420/−1, Figure 3) sufficient for transac-

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-1386 aaacagaacagtggtaatgacagtctcaaggtttgagacactttcacgcatcgacatgtg -1327
-1326 caaatagtgattttgaaactaaatgtgggtgctttttttttttttgtttgtagccacctt -1267
-1266 aattacctcaaatgtgaatataaagaatctagcatgaacaagaaatttggttaaatgttg -1207
-1206 tgctttatccaaatctgactagattgagcctaacaatacagaaaacagtcacggccatga -1147
-1146 cttgagtcctttcagaataattgggatgtttgtgaatttaccaacaaacagtttttacgg -1087
-1086 ttctcttcaatcccctgtacatttagtagaggtggttgaagaaaaacaagatgtttttgt -1027
-1026 tgttgtttttggagaggtgctcttcaattgtgagaatgtacaaacctgccaaagaggtg  -967
 -966 gtgagggaaaaagcgaaggagttgaagaaaggtgataagaagagaacactggttcatatg  -907
 -906 aaacaggctgacggacaggtggcttctgttgttttgacaatgtgacgtcgagttcagtaa  -847
 -846 tatgacccccctgtagccacaactctctgatcccccatctgtcttagctcatccttaaaaa  -787
 -786 tgccttactaaggcaaaatgacatttgagcacatgcctctttattgaaaaaaaatatcct  -727
 -726 tgcttttactgaacaatgatacatgattatattcaacttgatgtgcgagaggcagaaatg  -667
 -666 tccctgaaccatctggcttctgggttgacctactctcacacacccatggaaaatggaatta  -607
 -606 tggatgtcaaagtaaaaaaaaaaaaaattacaagaagaagatttgaggggttttgtgaga  -547
 -546 ttccagtgatttccgggtctcattcttaccgtccctttttgttgcgtctattacagttct  -487
 -488 atggtgtcttttctcacagccctctctctgccatgcgctgtttaaatctacattacgtgc  -427
 -426 ttcctcagctgaaaggagggatttttctgctcgttcttttatttgaagcttttgtttgt  -367
 -366 taaatctaatcaatctcagtcttagttcttagattgttggccaatggaatggattgagga  -307
 -306 cgcagggtggaataaaaatccaacaacattttttgggggtttatttttaaatcaagtaggtt  -247
 -246 gagacagatttcgtacagtgaactttaaatctggaacaccaagatatttacacagatgca  -187
 -186 tgcacgcctagttaccataattgtactatgcatgtgtaaagaaatacgttgtttctgaat  -127
 -126 gaccagtacctttttaacgcaacctgcgcaaaggtgacctttcctaaaaacatggcctgttg  -67
  -66 gaggcttTATAAAagaggtcagcagtgacagaaaggctacgaagatttgaagactcaacc  -7
   -6 gacACAATGCGCATGATAAGAGgtaatgtgtcacgcatcacgacagaaacagcactgacg  +54
  +55 gatataaagttatcaagctgagcactgattttggttcatgtcgctctgaagaaggtagat  +114
 +115 taagaaaattaagatct                                           +131
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Figure 2. Sequence analysis of *Sparus aurata* SL promoter. Numbering begins with the initial start ATG codon (uppercase boldface letters). Uppercase letters refer to nucleotides of exon 1 of SL cDNA. Five potential binding sites for gilthead sea bream Pit-1 transcription factor are marked in gray. Three additional binding sites for rat Pit-1 factor are boxed. Consensus sequences for putative binding of transcription factor GATA (underlined), a CREB site (underlined in black), and NF1 (dashed underline) are shown. The TATAA box is indicated in boldface lowercase letters at –59 nucleotides.

tivation by sbPit-1 when introduced in CHO cells. Since 2 potential sbPit-1 binding sites are in this region and their consensus sequences closely resemble that recognized by other Pit-1 factors previously described (Ohkubo et al., 1996), they should mediate the function of sbPit-1 on SL gene expression. Our transfection constructs suggest other sites that may contribute to the overall expression of sbSL. Specifically, the psbSL −846/−1, construct showed less stimulation than psbSL −1386/−382 and psbSL −420/−1, suggesting a putative negative control site between nucleotides −420 and −382. Similar findings have been described for other promoters (Kaji et al., 1998; Liang et al., 2002). The shorter construct assayed (psbSL −175/−1, Figure 3), which maintains a high level of stimulation of luciferase gene expression, is probably explained by the presence of a Pit-1 binding site at nucleotide −83 and the absence of any inhibitory site.

In summary, we conclude that the proximal −175-bp sequence of sbSL promoter is a minimal *cis*-element for controlling specific SL expression under the control of sbPit-1, although other sequences containing extra binding sites for Pit-1 could modulate the SL gene expression in

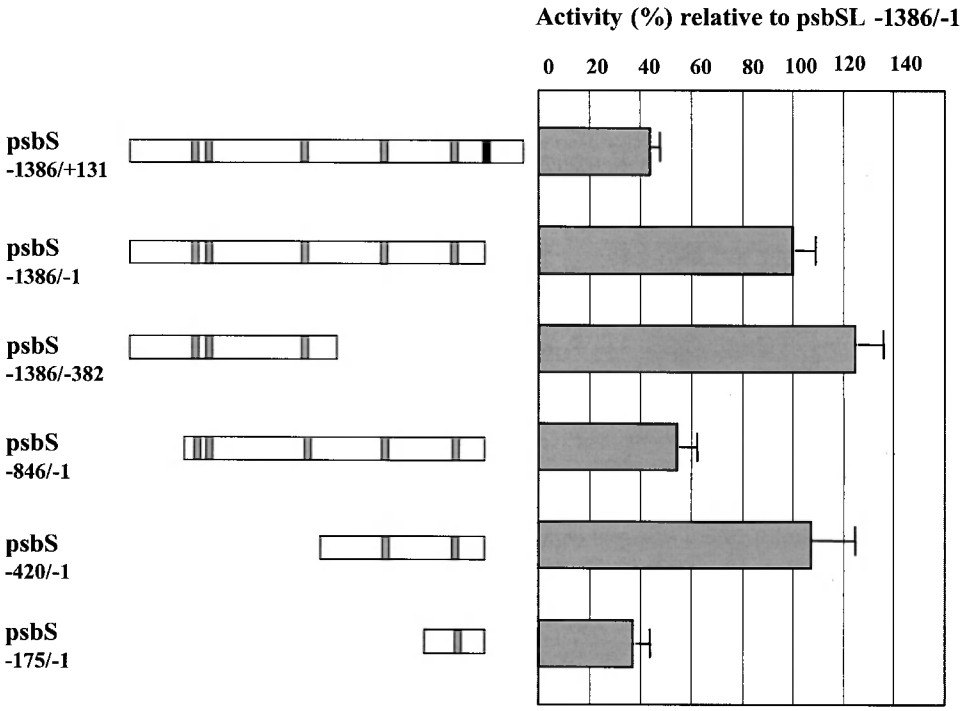

Figure 3. Analysis of *Sparus aurata* SL proximal promoter activity. The length of each construct is indicated, with first and last nucleotides enumerated on the left. Numbering used is equivalent to the sequence shown in Figure 2. The larger construct analyzed (psbSL −1386/+131) contains the first exon of sbSL cDNA (vertical black bar). Pit-1 sites are shown in the constructs as a gray vertical boxes. At the right, promoter activity was normalized with Renilla luciferase activity and expressed as percentage induction relative to the activity of −1386/-1 psbSL construct. The data are the means ± SEM of triplicate determinations. Similar results were obtained in 2 independent experiments.

S. aurata. Although essential, Pit-1 is not sufficient to completely determine sbSL gene expression and its differential regulation. The participation of other DNA-binding proteins or auxiliary factors, both positive and negative, is needed. Most probably included among these are GATA 1, CREB, and NF-1, the binding sites of which were found on the sbSL promoter (Figure 2).

Secretion of SL from Pituitary Culture In Vitro

In addition to mediating pituitary-specific expression, the GH/PRL/SL promoter regions also contain *cis*-elements modulating transcription in response to hormonal signals such as thyroid hormone, growth hormone releasing factors, and glucocorticoids (Karin et al., 1990). Thus the participation of other regulatory factors, together with Pit-1, in the expression of SL genes is widely accepted. In this context *S. aurata* pituitaries were cultured in vitro so that we might examine the effects of several different agents on SL secretion (Specker et al., 1985). The effects of estradiol, PMA, and verapamil on the release of SL and GH into the culture media were determined by immunoblots. Analysis of sbSL and sbGH with fish-specific sera showed that, at 5 nM estradiol, SL release was inhibited while GH release was apparently stimulated (Figure 4). However, 5 μM verapamil treatment inhibited GH release with no apparent effect on SL release, and finally, incubation with 0.16 μM PMA inhibited SL release slightly and stimulated GH secretion (Figure 4). Immunoblot controls using media from pituitaries cultured without treatment demonstrated the specificity of the effects obtained with the agents used (data not shown).

Our experiments also consistently demonstrated that 2 forms of SL (glycosylated and nonglycosylated) were released at similar rates (Figure 4). The physiologic significance of these forms of SL with similar rates of release remains to be elucidated (Valdivia et al., 1998). In addition, only a nonglycosylated form of *S. aurata* GH was released to the medium under identical culture conditions (Figure 4). Although the experiments described were limited in the number and concentration of agents studied, and the number of glands used (a total of 24 pituitaries), unequivocal differences were found in the pattern of SL and GH released to the culture media.

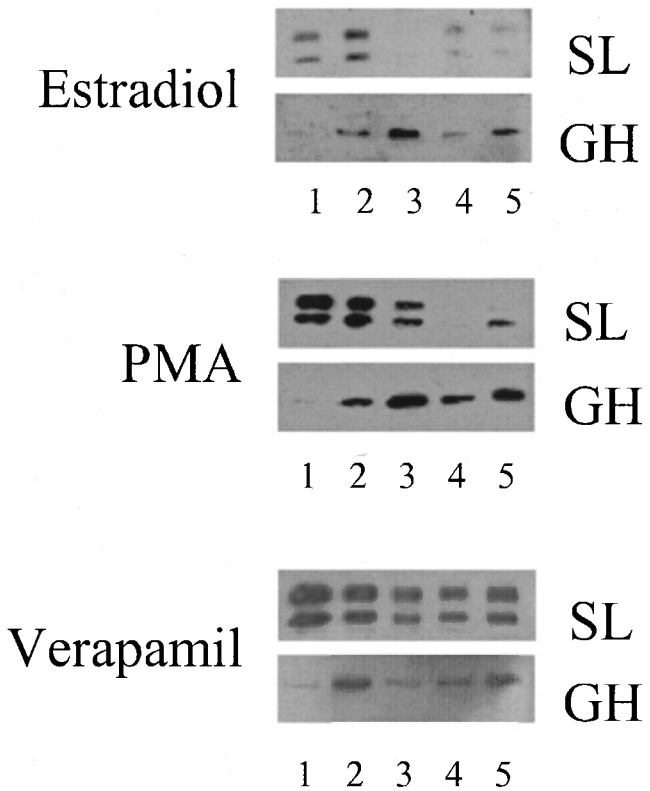

Figure 4. Analysis of SL released from in vitro culture of *Sparus aurata* pituitaries. The glands after 2 days of stabilization in culture media (lanes 1, represent media after 24 hours, and lanes 2, after 48 hours) were treated for 24 hours with 5 nM estradiol, 5 μM verapamil, or 0.16 μM PMA as final concentrations (lanes 3). Lanes 4 and 5 represent the media after releasing the agent treatment for 24 and 48 hours, respectively. The effect of each agent was assayed in 4 pituitaries cultured individually. Samples of each pituitary-cultured medium were tested by immunoblots with fish-specific anti-SL and anti-GH sera. Note that SL was released in 2 forms, with the upper band being glycosylated form as previously described (Valdivia et al., 1998). At the concentration tested, while estradiol clearly inhibited SL and stimulated GH, verapamil and PMA showed different inhibitory effects on SL and GH released to the culture media.

Our immunoblot results suggest that in vitro culture of pituitaries is a feasible approach to study the different pathways involved in GH and SL secretion in fish. In this regard it had been shown previously that cooperation between Pit-1 and estrogen receptor is affected by protein kinase A and protein kinase C (Day et al., 1990). The estradiol results observed in sea bream pituitary cultures suggest that Pit-1 drives different hormone signaling cascades in the estrogen responses of the SL and GH genes in *S. aurata*. Also, phorbol esters are known protein kinase C activators and have been shown to induce Pit-1 phosphorylation in pituitary cells, which might then stimulate gene expression (Kim et al., 1993). Again, our results with PMA suggest that modifying the Pit-1 phosphorylation stage could be part of the mechanisms driving SL and GH gene expression in different ways in response to hormonal stimulation. Alternatively, other factors could become inactive with PMA, and this inactivation may result in an inhibition of Pit-1 binding to SL promoter. Taken together these preliminary results suggest the existence of independent pathways for regulating SL and GH gene expression in *S. aurata*. In view of the inducible nature of the SL gene in fish, research is in progress to unravel some of the pathways involved in Pit-1-driving SL secretion in fish.

Acknowledgments

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