Seabream ghrelin: cDNA cloning, genomic organization and promoter studies

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Abstract

Recent studies have indicated that ghrelin stimulates growth hormone release from the pituitary via the growth hormone secretagogue receptor (GHSR). We have previously isolated two GHSR subtypes from the pituitary of the black seabream *Acanthopagrus schlegelii*. In the present study, we have cloned and characterized ghrelin from the same fish species at both the cDNA and gene levels. The full-length seabream ghrelin cDNA, isolated from seabream stomach using a novel approach by exploiting a single conserved region in the coding region, was found to encode a prepropeptide of 107 amino acids, with the predicted mature ghrelin peptide consisting of 20 amino acids (GSSFLSPSQKPQNRGKSSRV). Embedded in this full-length cDNA is a putative fish orthologue of the recently reported mammalian obestatin peptide. The *ghrelin* gene in black seabream, obtained by genomic PCR, was found to encompass four exons and three introns, possessing the same structural organization as in tilapia and goldfish, but different from that in rainbow trout. In addition, a 2230-bp 5'-flanking region of the seabream *ghrelin* gene was obtained by genome walking. Sequence analysis revealed that, as in the case of the human *ghrelin* gene, there is neither a GC box nor a CAAT box present in the isolated 5'-flanking region. However, a number of putative transcription factor-binding sites different from the human counterpart were found in the 5'-flanking region of the seabream *ghrelin* gene, suggesting that different *cis*- and *trans*-acting

elements are involved in controlling their gene expression. Functional activity of this 5'-flanking region was examined by cloning it into the pGL3-Basic vector upstream of the luciferase reporter gene and transfected into various cell lines. Positive promoter activity could only be recorded in the colon-derived Caco-2 cells, suggesting that the cloned 5'-flanking region represents the functional promoter of the seabream *ghrelin* gene, which exhibits tissue-specific promoter activity. Using reverse transcriptase PCR analysis, expression of ghrelin was detected only in the seabream stomach, but not in the other tissues examined, including the brain, gill, intestine, kidney, liver and spleen. This stomach-specific expression of ghrelin in seabream is subject to regulation, as administration of growth hormone or ipamorelin to the fish *in vivo* was demonstrated to enhance its expression. Reminiscent of the homologous upregulation found in the transcriptional control of the seabream *GHSR* gene, a similar homologous regulatory mechanism might also exist in controlling the expression of seabream ghrelin. The identification of both GHSR and ghrelin from a single fish species would facilitate our subsequent studies on the elucidation of the physiological functions of the ghrelin/GHSR system in teleost. The possible existence of obestatin in teleost opens up new research avenues on the somatotrophic axis in fish.

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Introduction

Ghrelin, the endogenous ligand for growth hormone (GH) secretagogue receptor (GHSR), is involved in regulating diverse physiological functions in vertebrates, in addition to the stimulation of GH secretion (Gualillo *et al.* 2003). The cDNA encoding ghrelin was first isolated from mammalian stomach (Kojima *et al.* 1999) and subsequently from other non-mammalian species including bullfrog (Kaiya *et al.* 2001), chicken (Kaiya *et al.* 2002),

goldfish (Unniappan *et al.* 2002), eel (Kaiya *et al.* 2003b), tilapia (Kaiya *et al.* 2003c, Parhar *et al.* 2003), and rainbow trout (Kaiya *et al.* 2003a). Ghrelin possesses a unique structure in which the third serine residue is modified by an acylation that is essential for its biological activity (Kojima *et al.* 1999).

Previous studies on the isolation of ghrelin from fish species have indicated that the function of ghrelin in stimulating GH release from the pituitary appears to be conserved from fish to mammals (Kojima *et al.* 1999,

Table 1 Sequences of the primers used

Primer name	Sequence (5' to 3')	Purpose
FGD	GGCWCCAGCTTYYTNWSNCC	3'-RACE
GTD	GGYWCCAGCTTCYTSAGC	3'-RACE
oligo-dT	CCGAATTCTCGAGATCGA(T) ₁₈	3'-RACE
SGR1	GTCCCTCTGCTGCCTCTGTG	Library PCR
SGR2	ACGCTGAATGATCTCCTGCAGC	Library PCR
M13-R	AGCGGATAACAATTCACACAGG	Library PCR
SGFL-F	CACATGTGGGTCATATTGTTAGC	Library PCR and RT-PCR
SGFL-R	GTTTATCAATGCTTTATTTATATGGAC	Library PCR and RT-PCR
SGW-P	GCACCACAAAGTCAGAGAACAG	Genome walking
SGW-N	TGGTGTATTAAAGATGTAAGATGC	Genome walking
SGW-4	CGAAGAACTCGGTCACAGAAG	Genome walking
SGW-5	GCAACACAGATTATGCCGTAG	Genome walking
AP1	GTAATACGACTCACTATAGGGC	Genome walking
AP2	ACTATAGGGCACGCGTGGT	Genome walking
Actin-F	ACCCAGATCATGTTTCGAGACC	RT-PCR
Actin-R	ATGAGGTAGTCTGTGAGGTCTG	RT-PCR
SBG-PRO-F1	AGGCCTCTTCATAGCCTCCTC	Promoter cloning
SBG-PRO-F2	CTCGGTACCCCTTGTGTGCGAGTGTCT	Promoter cloning
SBG-PRO-F3	CTCGGTACCTTTGCTCATAGAGTTTGCC	Promoter cloning
SBG-PRO-F4	CTCGGTACCTATCAAGGTGTTCTGCTCC	Promoter cloning
SBG-PRO-R1	TCTTACATCTTTAATACACCA	Promoter cloning

Kaiya *et al.* 2003*b*, 2003*c*). Moreover, it has been demonstrated that ghrelin is capable of stimulating food intake in goldfish (Unniappan *et al.* 2002). These findings indicate that a ghrelin/GHSR system is also operative in fish. However, the cognate GHSR has not been cloned and characterized from those fish species in which their ghrelins have been isolated or studied. To date, fish GHSR has only been reported in two species, the pufferfish *Spheroideus nephelus* (Palyha *et al.* 2000) and the black seabream *Acanthopagrus schlegeli* (Chan & Cheng 2004), and is functionally characterized in black seabream only (Chan & Cheng 2004, Chan *et al.* 2004*b*). The putative *ghrelin* gene present in pufferfish, however, has not yet been isolated. We have recently identified and characterized two full-length GHSR cDNAs from the pituitary of the black seabream and demonstrated that these two receptor transcripts are derived from alternative splicing of a single *GHSR* gene (Chan & Cheng 2004). Herein we report the cloning and characterization of the *ghrelin* gene from the same fish species. Moreover, we have further demonstrated homologous upregulation of ghrelin expression in the seabream stomach. This work represents the first study on a teleost in which both the *GHSR* and *ghrelin* genes have been cloned.

Materials and Methods

Cloning of seabream ghrelin cDNA

Initial attempts to clone the seabream ghrelin cDNA failed because of the difficulty in designing effective primer pairs

for reverse transcriptase (RT)-PCR owing to the small size of the peptide. A different approach was subsequently adopted and this involved the design of two overlapping forward degenerate primers from a single conserved region of ghrelin. A cDNA fragment towards the 3'-end of seabream ghrelin was obtained using these primers together with an oligo-dT primer in a 3'-rapid amplification of cDNA ends (RACE) approach. Subsequently, gene-specific reverse primers were designed from this partial sequence, allowing us to obtain the rest of the ghrelin cDNA using a library PCR approach. The experimental details are described below. Total RNA was first prepared from seabream stomach using TriPure Isolation Reagent (Roche Applied Science). First-strand cDNA was then made from 4 µg total RNA in the reverse transcription reaction. The reaction was carried out at 42 °C for 50 min using Superscript II RT (Invitrogen). Based on previously published fish ghrelin sequences (Unniappan *et al.* 2002, Kaiya *et al.* 2003*c*, Parhar *et al.* 2003), two degenerate primers, FGD and GTD (see Table 1 for their primer sequences), were designed and employed together with an oligo-dT primer in the first-round and nested PCR, respectively.

The PCR conditions were as follows: 5 min at 94 °C for initial denaturation, followed by 36 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing), and 1 min at 72 °C (extension), and then 7 min at 72 °C for the final extension. A dominant PCR product (with a size of about 800 bp) was obtained from the nested PCR. Cloning and sequencing of the PCR product revealed that it shared a high sequence homology with the tilapia

ghrelin cDNA. Subsequently, two gene-specific primers, SGR1 and SGR2 (see Table 1), were designed from the partial cDNA sequence and employed to amplify from a seabream stomach cDNA library constructed in the pCMV-SPORT6 vector (Invitrogen) in order to obtain the 5'-end sequence of the ghrelin cDNA. SGR1 and SGR2 were used together with the primer M13-R in the first round and nested PCR, respectively. The PCR conditions were the same as described above except that the annealing temperature was at 56 °C. The nested PCR yielded a distinct product with a size of about 400 bp, which was subsequently cloned and sequenced. Finally, a pair of gene-specific primers, SGFL-F and SGFL-R (see Table 1), were designed and used to amplify the full-length ghrelin sequence from the seabream stomach cDNA library. This final confirmation step was essential to demonstrate that the composite sequence obtained from the two separate fragments truly represents the actual full-length ghrelin sequence. Also, the nucleotide sequence of each PCR product obtained above was confirmed by the sequencing of at least five independent clones in each case.

Determination of the structural organization of the seabream ghrelin gene

Genomic DNA was extracted from seabream liver as previously described (Chan & Cheng 2004). Briefly, frozen seabream liver was homogenized in lysis buffer (10 mM Tris/HCl, pH 8.0, 50 mM EDTA and 0.5% SDS), followed by proteinase K digestion. The genomic DNA was then purified by phenol/chloroform extraction and ethanol precipitation. The gene-specific primers SGFL-F and SGFL-R were used to amplify the entire *ghrelin* gene in the genomic PCR. The PCR conditions were as follows: 5 min at 94 °C for initial denaturation, followed by 36 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing), and 2 min at 72 °C (extension), and then 7 min at 72 °C for the final extension. The PCR product was cloned and sequenced from both strands. The complete genomic sequence was obtained and confirmed by sequencing of at least five independent clones.

Isolation of the 5'-flanking region of the seabream ghrelin gene by genome walking

The 5'-flanking region of the ghrelin gene was isolated using the Universal GenomeWalker Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Five GenomeWalker libraries (independently generated by cutting with the following restriction enzymes: *Dra*I, *Eco*RV, *Pvu*II, *Sac*I, and *Stu*I) were constructed according to the manufacturer's instructions. First-round PCR was carried out using the adaptor primer 1 (AP1) and the gene-specific primer SGW-P (used in the first genome-walking experiment) or SGW-4 (used in the second genome-walking experiment;

see Table 1). The PCR conditions were as follows: 3 min at 94 °C for initial denaturation, followed by 38 cycles of 30 s at 94 °C (denaturation), 30 s at 62 °C (annealing), and 3 min at 72 °C (extension) for 38 cycles, and then 7 min at 72 °C for final extension. Nested PCR was performed using the adaptor primer 2 (AP2) and the gene-specific primer SGW-N (used in the first genome-walking experiment) or SGW-5 (used in the second genome-walking experiment; see Table 1) under the same conditions except that the number of cycles was reduced to 35. The nucleotide sequences of the PCR products obtained above were confirmed by sequencing of at least five independent clones in each case.

Construction of reporter gene plasmids

The 5'-flanking region of the seabream *ghrelin* gene was amplified by PCR using SBG-PRO-F1 and SBG-PRO-R1 as primers and 700 ng seabream genomic DNA as the template. Subsequent to its sequence determination, the amplicon of the PCR was then cloned into the promoterless pGL3-Basic vector (Promega) to generate the construct p(-2230/+1). Another reporter construct (p(+1/-2230)) containing the same 5'-flanking region as p(-2230/+1) but in the reverse direction was made in parallel. Deletion constructs with successive removal of the 5'-region were generated by PCR using the primers SBG-PRO-F2, SBG-PRO-F3, SBG-PRO-F4 and SBG-PRO-R1 (Table 1) to generate the constructs p(-1614/+1), p(-1422/+1), and p(-1289/+1). Reporter constructs p(-894/+1) and p(-450/+1) were made by digesting the 5'-flanking region amplified from SBG-PRO-F1 and SBG-PRO-R1 with *Sac*I and *Nhe*I respectively. All plasmid DNA preparations were isolated using the QIAprep Spin Miniprep Kit (Qiagen).

Cell culture, transfection and promoter assay

Cell culture materials were purchased from Invitrogen unless otherwise stated. Cell lines were purchased from ATCC (Manassas, VA, USA; NIH-3T3, HepG2, HEK-293, Caco-2) and RIKEN Bioresource Center (Ibaraki, Japan; ECC10, GAKS). NIH-3T3, HepG2, and HEK-293 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Caco-2 cells were kept in DMEM at 37 °C containing non-essential amino acids and 10% FBS. GAKS cells were maintained at 37 °C in DMEM containing 5% FBS. ECC10 cells were maintained in RPMI containing 10% FBS at 37 °C. All media were supplemented with antibiotics (10 U/ml penicillin and 100 µg/ml streptomycin).

In the transfection experiments, Lipofectamine reagent (Invitrogen) was used as recommended by the manufacturer. Twenty hours before transfection, 1.5×10^5 cells/well were seeded onto 24-well tissue-culture plates.

Five hundred ng seabream ghrelin promoter-reporter constructs and 50 ng pRL-CMV (for normalization of transfection efficiency) containing *Renilla* luciferase reporter gene were co-transfected into the cells in 250 µl serum-free medium. After 5 h of transfection, the reaction mixture was replaced with 20% FBS and the cells were incubated for 24 h. The firefly luciferase activities driven by various seabream ghrelin promoter regions were assayed using the Dual-Luciferase Assay System (Promega).

Analysis of ghrelin gene expression in seabream tissues by RT-PCR

First-strand cDNA was prepared from various tissues as described above. The gene-specific primers SGFL-F and SGFL-R were used in the RT-PCR to amplify the full-length ghrelin transcript. The PCR conditions were as follows: 5 min at 94 °C for initial denaturation, followed by 36 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing), and 1 min at 72 °C (extension), and then 7 min at 72 °C for the final extension. Mock RT controls were performed to exclude the possibility of genomic DNA contamination in the RNA preparations. Moreover, PCR using a pair of β-actin primers, Actin-F and Actin-R (see Table 1), was also performed in parallel to indicate the quality of the cDNA templates synthesized. The PCR conditions were as follows: 5 min at 94 °C for initial denaturation, followed by 26 cycles of 30 s at 94 °C (denaturation), 30 s at 58 °C (annealing), and 30 s at 72 °C (extension), and then 7 min at 72 °C for the final extension.

Regulation of ghrelin expression in seabream stomach in vivo

Black seabream (body length, 24.3 ± 0.5 cm; body weight, 311.5 ± 21.7 g) of mixed sex purchased from a local marine fish farm were kept in fully aerated seawater at ambient temperature and fed *ad libitum* once daily with commercial diet. Fishes were injected with either synthetic ipamorelin (NeoMPS, Strasbourg, France) or recombinant seabream GH (GroPep, Thebarton, Australia) intraperitoneally once daily for 4 days. Physiological saline was injected in parallel as the control group. The fishes were then killed by decapitation and their stomachs removed and frozen in liquid nitrogen. The tissue was kept at -70 °C until further analysis.

RT-PCR was carried out to assess the expression of ghrelin in seabream stomach as described above. The number of cycles used in each case was determined first by a validation test in which the PCR was performed as described but terminated at different cycle numbers. A kinetic profile of the amount of PCR product generated at different cycle numbers was constructed and the cycle number to be adopted for the gene-expression assessment was chosen within the linear portion of the amplification

curve. This validation step is essential to ensure that the amount of the PCR product reflects the amount of template in the original sample. The PCR products were then resolved on an 1.5% agarose gel and stained with ethidium bromide. The intensity of the amplicon was determined by densitometric scanning using Multi-Analyst software (Bio-Rad).

Statistical analyses

Where applicable data were analysed by one-way analysis of variance followed by Tukey's test using PRISM software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when $P < 0.05$.

Results

Cloning of seabream ghrelin cDNA

Using degenerate primers (FGD and GTD) designed from the most conserved region of ghrelins (i.e. the core region of the mature peptide, G(S/T)SFLSP) together with an oligo-dT primer, a partial seabream ghrelin cDNA of about 800 bp was obtained in a 3'-RACE approach. Subsequently, two gene-specific reverse primers (SGR1 and SGR2) were designed and employed to isolate the 5'-end sequence from a stomach cDNA library by a library PCR approach. Finally, the full-length ghrelin cDNA (Fig. 1) was amplified from the same library using two specific primers (SGFL-F and SGFL-R) designed at both ends of the full-length sequence. The complete cDNA was found to encode 107 amino acids, with 56 bp in the 5'-untranslated region (UTR) and 564 bp in the 3'-UTR. Amino acid sequence comparison of the seabream ghrelin with other vertebrate ghrelins (Fig. 2) indicates that it shares the highest homology with the tilapia ghrelin (Fig. 3).

The nucleotide sequences of the ghrelin cDNA and gene reported in this paper have been submitted to the GenBank database under the accession nos. AY643808 and AY643809, respectively.

Structural organization of the seabream ghrelin gene

The structural organization of the seabream *ghrelin* gene was determined by genomic PCR using the two gene-specific primers SGFL-F and SGFL-R. Sequence analysis of the amplified product (Fig. 4A) revealed that the *ghrelin* gene in black seabream consists of four exons and three introns (Fig. 4B). The DNA sequences of the exon/intron junctions are shown in Table 2. The exon/intron junctions were found to conform to the GT/AG rule (Chabot 1996). Exon 1 (170 bp) encodes the 5'-UTR and 38 amino acids (including the first 12 amino acids of the

Isolation of the 5'-flanking region of the seabream ghrelin gene

As a first step towards understanding the transcriptional regulation of the seabream *ghrelin* gene, the 5'-flanking region of the gene was also isolated in the present study. In the first genome-walking experiment, the longest

	10	20	30	40	50
Seabream	MFLKRNTYLL	VFLFCSLTLW	CKSTSA	GSSF LSPSQ-K--P	Q----NRGKS
Tilapia	MLLKRNTCLL	AFLLCSLTLW	CKSTSA	GSSF LSPSQ-K--P	Q----NKVKS
Eel	MRQMKRTAYI	ILLVCVLALW	MDSVQA	GSSF LSPSQ-R--P	Q GK-DKK--P
Rainbow trout	MPLKRNTGLM	ILMLCTLALW	AKSVSA	GSSF LSPSQ-K--P	QVR-QGKGKP
Goldfish	MPLRRRASHM	FVLLCALSLC	VESVKG	GTSF LSPAQ-K--P	QGR-----RP
Bullfrog	MNFGKAAIFG	VVLFCLL--W	TEGAQA	GLTF LSPADMQKIA	ERQSQNKLRH
Chicken	MFL-RVILLG	ILLLSILG--	TETALAG	GSSF LSPTY-KNIQ	QQK-DTRKPT
Turkey	MFL-RLALLG	ILLLSILG--	TETAQA	GSSF LSPAY-KNIQ	QQK-DTRKPT
Emu	MFL-RGALLV	ILLFSVLW--	TETTLAG	GSSF LSPDY-KKIQ	QRK-DPRKPT
Human	MPS-PGTVCS	LLLLGMLWL-	-DLAMAG	GSSF LSPEH-QRVQ	QRK-ESKKPP
Rhesus monkey	MPS-PGTVCS	LLLLGMLWL-	-DLAMAG	GSSF LSPEH-QRAQ	QRK-ESKKPP
Mouse	MLS-SGTICS	LLLLSMLWM-	-DMAMAG	GSSF LSPEH-QKAQ	QRK-ESKKPP
Rat	MVS-SATICS	LLLLSMLWM-	-DMAMAG	GSSF LSPEH-QKAQ	QRK-ESKKPP
Dog	MPS-LGTMCS	LLLFSLWV--	-DLAMAG	GSSF LSPEH-QKLQ	QRK-ESKKPP
Pig	MPS-TGTICS	LLLLSVLLM-	ADLAMAG	GSSF LSPEH-QKVQ	QRK-ESKKPA
Cow	MPA-PWTICS	LLLLSVLCM-	-DLAMAG	GSSF LSPEH-QKLQ	-RK-EAKKPS

	60	70	80	90	100
Seabream	SRVGRQV---	-----MQEP	QQPTDDKHIT	ISAPFEIGIS	MTEEDYDEYG
Tilapia	SRIGRQA---	-----MEEP	NQANEDKTIT	LSAPFEIGVT	LRAEDLADYI
Eel	PRVGRRSDG	ILDLF-MRPP	LQDEDIRHIT	FNTPFEEIGIT	MTEELFQQYG
Rainbow trout	PRVGRRDIES	FAELF--EGP	LHQED-KHNT	IKAPFEMGIT	MSEEEFQYEG
Goldfish	PRMGRRDVAE	-----PEI	PVIKEDDQFM	MSAPFELSVS	LSEAIEYKYG
Bullfrog	GNNMRRGVED	DLAGEE----	-----IGVTFPLDMK	MTQEQFQKQR	
Chicken	ARLHRRGTES	FWDTDET-EG	EDDNNSVDIK	FNVPFEEIGVK	ITEREYQYEG
Turkey	ARLHRRGTES	FWDTDET-AG	EDDNNSVDIK	FNVPFEEIGVK	ITEREYQYEG
Emu	TKLHRRGVEG	FSDTDEA-WA	EDDNNSIEIK	FNVPFEEIGVK	ITEEQYQYEG
Human	AKLQPRALAG	WLRPEDGGQA	EGADELEVR	FNAPFDVGIK	LSGVQYQQHS
Rhesus monkey	AKLQPRALGG	WLRPEDGDQA	EGADELEIQ	FNAPFDVGIK	LSGVQYQQHS
Mouse	AKLQPRALEG	WLHPEDRGQA	EETEELEIR	FNAPFDVGIK	LSGAQYQQHG
Rat	AKLQPRALEG	WLHPEDRGQA	EAELEIR	FNAPFDVGIK	LSGAQYQQHG
Dog	AKLQPRALEG	SLGPEDTSQV	EAELEIR	FNAPFDVGIK	LSGPQYHQHG
Pig	AKLKPRALEG	WLGPEDSGEV	EGTEDKLEIR	FNAPCDVGIK	LSGAQSDQHG
Cow	GRLKPRITLEG	QFDPEVGSQA	EGADELEIR	FNAPFNIGIK	LAGAQLQHG

	110	120	130
Seabream	VVLQEIIQRL	LGG-----	TEAAEGPPQL
Tilapia	VELQEIVQRL	LGN-----	TETAERPSPR
Eel	EVMQKIMQDL	LMD-----	TPAKE-----
Rainbow trout	AVLQKILQDV	LGD-----	TATAE-----
Goldfish	PVLQKVLVNL	LGD-----	S-----PLEF
Bullfrog	AAVQDFLYSS	LLSLGSVQDT	EDKNENPQSQ
Chicken	QALEKMLQDI	LAE-----	-NAEETQTKS
Turkey	QALEKMLQDI	FEE-----	-NAKETQTKD
Emu	QMLEKVLGDI	LEE-----	-NTKETRMKN
Human	QALGKFLQDI	LWE-----	-EAKEAPADK
Rhesus monkey	QALGKFLQDI	LWE-----	-EAKEAPADK
Mouse	RALGKFLQDI	LWE-----	-EVKEAPADK
Rat	RALGKFLQDI	LWE-----	-EVKEAPANK
Dog	QALGKFLQEV	LWE-----	-DTNEALADE
Pig	QPLGKFLQDI	LWE-----	-EVTEAPADK
Cow	QTLGKFLQDI	LWE-----	-EAEETLANE

Figure 2 Protein sequence alignment of the seabream ghrelin with other vertebrate ghrelins. The region corresponding to the mature ghrelin peptide is boxed. The region corresponding to the obestatin peptide in mammals is indicated by a thick black line underneath the aligned sequences. The alignment was generated using the DNASIS program (Hitachi Software Engineering Co., Yokohama, Japan). The amino acid sequences shown were obtained from the NCBI GenBank database (accession numbers: seabream, AY643808; tilapia, AB077764; eel, AB062427; rainbow trout, AB096919; goldfish, AF454389; bullfrog, AB058510; chicken, AB075215; turkey, AY497549; emu, AY338467; human, AB029434; rhesus monkey, AY372274; mouse, AB060078; rat, AB029433; dog, AJ298295; pig, AF308930; cow, AF350329).

Figure 3 A phylogenetic tree generated from protein sequence alignment of the vertebrate ghrelins using the DNASIS program. The tree was created based on amino acid conservation over time between the different species using the default settings of the program.

PCR product (~800 bp) was obtained from the *PvuII* GenomeWalker library. In order to isolate a longer 5'-flanking region for transcriptional analysis, a second genome walking was performed using the two gene-specific primers SGW-4 (for first-round PCR) and SGW-5 (for nested PCR). Distinct PCR products of sizes of about 1500 bp were obtained from both the *StuI* and *EcoRV* GenomeWalker libraries. The PCR products from the *StuI* library were subsequently cloned and sequenced. From the two consecutive and overlapping genome walks, a 2230-bp 5'-flanking region of the seabream *ghrelin* gene was finally isolated. Sequence analysis using TFSEARCH software (<http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html>) revealed a number of putative transcription factor-binding sites on the 5'-flanking region of the seabream *ghrelin* gene (Fig. 5).

Functional mapping of the seabream *ghrelin* promoter

The promoter activity of the 5'-flanking region of the seabream *ghrelin* gene was then tested by transfecting the plasmid p(-2230/+1) into various cell lines and measuring the putative promoter-driven luciferase activity. A 6-fold increase of luciferase activity over and above the

promoter-less control (pGL3-Basic) was recorded in the colon epithelial Caco-2 cells (Fig. 6A). This result suggested that the cloned 5'-flanking region represents the functional promoter of the seabream *ghrelin* gene. Moreover, this promoter activity was orientation-dependent as no detectable luciferase activity could be measured in Caco-2 cells transfected with the construct p(+1/-2230), which has an identical nucleotide sequence to p(-2230/+1) but in a reverse orientation. Furthermore, the seabream *ghrelin* promoter is cell-type-specific as no promoter activity could be detected in the other cell lines originated from embryo fibroblast (NIH-3T3), liver (HepG2), kidney (HEK-293) and stomach (ECC10), nor from fish scale fibroblast (GAKS; Fig. 6B).

To further characterize the seabream *ghrelin* promoter, progressive 5'-deletion mutants were constructed and their activities were assessed in transfected Caco-2 cells. A significant reduction ($P < 0.001$) in promoter activity was observed in the construct p(-1422/+1). Promoter activity was completely abolished when construct p(-894/+1) was used and further deletion also gave no measurable luciferase activity. These results suggested that the core promoter region of the seabream *ghrelin* gene lies within the region -1289 to -894 and an enhancer element may be present within the region -1614 and -1422.

Figure 4 Structural organization of the seabream *ghrelin* gene. (A) An agarose gel showing the result of the genomic PCR (with the product size of 1771 bp) using the primers SGFL-F and SGFL-R. Full-length ghrelin transcript (941 bp) was also amplified from a stomach cDNA library using the same primers. (B) A schematic diagram showing the genomic organization of the seabream *ghrelin* gene. Exons and introns are represented by boxes and lines, respectively. The region encoding the 20 amino acids of the ghrelin mature peptide is shown in black. The sizes of the exons and introns are also indicated.

Analysis of ghrelin gene expression in seabream tissues by RT-PCR

The expression of the *ghrelin* gene in various seabream tissues was analyzed by RT-PCR using a pair of gene-specific primers (SGFL-F and SGFL-R) that amplified the

full-length transcript (941 bp). Using this pair of primers, which span three introns of the seabream *ghrelin* gene, we could only obtain a single distinct band of about 950 bp on the gel, indicating that there was no genomic DNA present in the first-strand cDNA samples since any such contamination would give rise to a much larger amplicon

Table 2 DNA sequences of exon/intron junctions of the seabream *ghrelin* gene

	5' splice sequence	Intron size (bp)	3' splice sequence
Intron			
1	AAAACCTCAGgtaagagct	631	ttctctgtagAACAGGGGAA
2	ACACATCACAgtgagtggaa	87	ctctaagcagATTAGCGCTC
3	GAGGCAGCAGgttcgcaaaa	112	cacctccagAGGGACCGCC

Intron sequences are shown in lower case. The conserved GT and AG nucleotides of the introns are underlined.

AGGCCTCTTCATAGCCTCCTCTTCTCCATCTCCCACTCCTGCCTCTGAAGCTCCCGCCGATGTCCTCTGAGAGTA	-2154
ACGTCTTGTGAGCTGAGACTTTTCTACACAGTAAAGTCATATCAAAAACAGTTCAAGTTCCCAACATGATCCCAATA	-2077
<u>AP-4</u>	
TCCAGTCTCCAACCTTCATAACCTGTAGTAGAATATTTACCCTTTTCTCTTAAGGTCTTGGTCCATTTTCTTAAATC	-2000
TGGCAGGCTTTTCTTATGCATTTTCGAGATCGGCCAAAGCGTCAACATAGTCCACCTACCGACAAACAACGTGA	-1923
<u>GATA-1</u>	
AGGAGAAGAGTTAACAATGAAAGGTTTCAAACCTGTAGAGCATCATCACATATACATTTTGAATTATTTTTTTCAT	-1846
<u>Oct-1</u>	
TTGTACTGTTTTCAATTATAACTTGCATTCTTAAGATTAGAAGCGGTGGTGGGAGTAGTCCTAGTGATTGTTCATT	-1769
<u>TGTACAGATATTGTATATACAATTTAACAACACACTACTTTAGCTATATTTTCTTAACTGTTGATAAAGAATGAAT</u>	-1692
<u>GATA-1</u> <u>c-Myb</u> <u>AP-1</u>	
<u>CAGACGTTACCATTCTCATCTGGGTTTTGCGGAGGGGGGATAGGAGATGAGCTGTCTCTTCTTCATCATCTCTAT</u>	-1615
<u>GATA-2</u>	
CCTTGTGTGCGAGTGTTTCTCTCTTTTGTCAATAATCTTCTGGGTGAAGTCAACCAGGAACAGTCCTTCTGTCTCT	-1538
<u>CREB</u>	
TCATCTGACAAATTACAAGACAAGTTTATTACAACTAAACATACCTGTGCATACAACAAAATATCAGAGAGGTGTC	-1461
ATTTAGGCTTCACCACCTGGAAGTCTCCTTTTGTCAATTTGCTCATAGAGTTGGCCTTCTCCTCCAACCTGCGTC	-1384
TGTTATAGGCAGATAAATGAGGAGTCAGAGGTTGTGATCACCATTTTATTATGACAGTAAATCTGCTGTATGATGT	-1307
<u>GATA-1</u> <u>AP-1</u>	
<u>GTTGAAGTGAATTTGCTGTATCAAGGTTGTTCTGCTCCTCAGCCAGCTGCTCTGCATCTTTCTCAGCTCTTGTCTGAG</u>	-1230
<u>c-Myb</u> <u>AP-4</u> <u>AP-4</u>	
ACACCAGCATTTTGTGTGCTCCAACATTAGGTTTCTACACCAGAGCACGCAGACAGGACACAGTTAGTGATCTAAA	-1153
TGTGCTGTATACAAGCAGGTCTAAATATCTCTCTAAATGACACAACTGTACCTTGACTTTCTGATGAGATTTTGA	-1076
TGTGAACCCAGCACCAGAGTTTTCTCGCCCAAGTTATCCTGTTTGAAGTGGTCTTCTGTAAAGTTCAGCTT	-999
TAAGGTCCACTAGCTGTAACAAAGAAGGCATTATCGACACATAAAATCAACAGCGAACTGTTTATATTATTCTTTAG	-922
CTACTAAACAACCTTTAACTGTGTTTGTAGCATTTAACGTTATAGCCTGACAGCAGGTTAGCTAACGTTAGCTGTT	-845
<u>CRE-BP</u> <u>AP-4</u> <u>CRE-BP</u>	
<u>ATAATCATTGAATTAACGTCGCCGAAGCCAAACAGAATCATGTAATAATGGTTGTACAGCTGTTTTTTGTAATGTAAC</u>	-768
<u>Oct-1</u> <u>C/EBPb</u>	
TCACCGATGAAGCAGTCACATCAAAGGGTTTCTCTTTTTTCCATGCTTACTGGTTATTTTCTACGGCATAATCT	-691
GTGTTGCTACTTCTGTGACCGACTTCTTCGTTGGTGGCGCAGCTCTCGCGAGAAAACCTGTCGCTTTACCGCCGACT	-614
<u>AP-4</u>	
AGTGGCGTTTGTAGTCCAGCTCTGTTTAGATACACTTGTCTCTGATTACAAAAATAAGTAAACCTCAAGGCCT	-537
<u>C/EBPa</u>	
GTTGAAAGGTTTTACCACATAAAACCAAATGTATTGGTGTGGATTTTGTCAAATTCCTCAGATAAGACTCATTTCA	-460
<u>GATA-1</u>	
AAAAGAGCTCTATTGTGTTAAGCATTGTTATCGTCAAATATCAACCTTAGTATTTGACATTGCATATTCCTGGGTA	-383
<u>C/EBP</u>	
TATTAACCTCAACACTGGTATGCTGCTGGAGAGACATACATGCATAACAGGCTGAATTAGTTCCTCATCAGTGCTCT	-306
CTATGACAGCAATACATTACCTGCATGACTTTCATTACCACATTGAACAGATAAGATGATCAGCAGGTTGAAACAG	-229
<u>GATA-1</u> <u>AP-4</u>	
AGAAATGCCTACTTTGTTATCAACTTAAACATTTGAACAGTCCAACGCAACAGATATGACAGCACACAGCACCAT	-152
TATTATTACTGGATGCATTTTAAACCAAAATGAAAGCGAATGGAAGCTATATATAGGTATGAACCGGTGAGAAG	-75
CTGAACTCAGGTTGGTGTACATGTGGGTATATTGTTAGCAGTGAACGAGCATCTTACATCTTTAATACACCA	+1

Figure 5 The complete nucleotide sequence of the 5'-flanking region of the seabream *ghrelin* gene. The numbering of the sequence shown on the right is relative to the ATG translation start site (designated as +1). Putative transcription factor binding sites identified in the 5'-flanking region are underlined and labeled.

of 1771 bp. This result was in line with the mock RT-PCR controls where no bands were observed. Ghrelin mRNA expression was only detected in the stomach but not in all the other tissues examined including

the brain, gill, intestine, kidney, liver and spleen (Fig. 7). A control PCR using two β -actin primers (Actin-F and Actin-R) was also performed. The β -actin transcripts (with the expected size of 212 bp) were detected in all the

Figure 6 Functional analysis of seabream *ghrelin* gene promoter. The 5'-flanking region of the seabream *ghrelin* gene was cloned into the pGL3-Basic vector to generate plasmid p(-2230/+1) and transfected into various cell lines (A). The putative promoter-driven luciferase activity was assayed using the Dual Luciferase Assay System. Further analysis of the functional region in the seabream *ghrelin* promoter was performed by transfecting luciferase reporter-plasmids carrying successive 5'-deletion of the seabream *ghrelin* promoter into CaCo-2 cells (B). Results are expressed as means $\pm$ S.E.M. ($n=9$). *** $P<0.001$ compared with p(-2230/+1).

tissues examined (Fig. 7), indicating that the absence of a ghrelin transcript in most tissues could not be attributed to the quality of the cDNA templates.

Regulation of seabream ghrelin expression in stomach

Hormonal regulation of ghrelin expression in seabream stomach was also studied *in vivo*. As shown in Fig. 8, ghrelin expression could be enhanced in fish injected with recombinant seabream GH or with the synthetic GH secretagogue ipamorelin in a dose-dependent manner. A significant increase ($P<0.05$) in ghrelin expression was

observed when 1 $\mu\text{g/g}$ seabream GH or 100 ng/g ipamorelin were administered to the fish.

Discussion

The ghrelin cDNA has been isolated from a number of fish, yet the cognate GHSR has not been cloned from these species. We have previously identified and functionally characterized two GHSR subtypes from the pituitary of a teleost fish, the black seabream *Acanthopagrus schlegelii* (Chan & Cheng 2004). In the present study, as part of our

Figure 7 Assessment of *ghrelin* gene expression in seabream tissues by RT-PCR. The full-length ghrelin transcript (941 bp) was amplified using the primers SGFL-F and SGFL-R and analyzed on an agarose gel. An internal control PCR (with the expected product size of 212 bp) using two β -actin primers was also performed.

ongoing work on the elucidation of the physiological functions of the fish ghrelin/GHSR system, we have cloned and characterized the ghrelin from the same fish species at both the cDNA and gene levels.

Initial attempts to design degenerate primers from known ghrelin sequences had encountered difficulties because there is only a single conserved region within the short sequence where primers could be reasonably designed. Thus the conventional method of RT-PCR using a pair of degenerate primers failed. Alternatively we have adopted a novel method in the present study. This entails the design and deployment of two overlapping forward degenerate primers from the single conserved region to obtain a cDNA fragment of the target by a nested 3'-RACE approach. This has successfully generated a cDNA fragment from which gene-specific reverse primers could be designed to enable the successful cloning of the 5'-end sequence using a library PCR approach in a nested manner. This method, which we have successfully employed in obtaining the full-length seabream ghrelin cDNA in the present study, could be equally applicable to other target genes where the design of degenerate primers is severely restricted by the limited conserved regions among the known sequences.

The seabream ghrelin cDNA encodes a total of 107 amino acids, with the predicted mature peptide consisting of 20 amino acids (Fig. 1). Protein sequence alignment of different vertebrate ghrelins (Fig. 2) reveals that the seabream ghrelin shares the highest homology with the tilapia counterpart, as indicated by the phylogenetic tree shown in Fig. 3. This finding of a close evolutionary relationship between seabream and tilapia is in agreement with phylogenetic analysis of other fish hormones such as the type II form of gonadotropin-releasing hormone (Ikemoto & Park 2003). In fact, a similar evolutionary relationship among teleost species is observed in the present and other studies (Zhu *et al.* 1999, Nilsson *et al.*

Figure 8 Seabream GH (sbGH; A) and ipamorelin (B) upregulates seabream ghrelin expression in stomach. Hormones of different concentrations were administered intraperitoneally into the fish as described in the Materials and Methods section. The expression level of ghrelin in different treatment groups were normalized by their corresponding β -actin expression and were expressed as means $\pm$ S.E.M. ($n=6$). ** $P<0.01$; *** $P<0.001$.

2001, Ikemoto & Park 2003), indicating that seabream is more closely related to tilapia than to rainbow trout, and that goldfish is more distantly related to these fish species (Fig. 3). The structural organization of the seabream *ghrelin* gene (Fig. 4) is also similar to that of tilapia (Parhar *et al.* 2003). The sizes of the cloned seabream and tilapia

ghrelin genes are 1771 and 1416 bp, respectively. Both genes are consisted of four exons and three introns. This pattern is in fact similar to that of goldfish, in which the genome size is only 980 bp (Unniappan *et al.* 2002). However, the genomic organization in rainbow trout (Kaiya *et al.* 2003a) is different from that in other known fish species. It is consisted of five exons and four introns, which is more similar to that of the mouse *ghrelin* gene (Tanaka *et al.* 2001).

It is interesting to note that gene duplication has been observed for some components of the somatotrophic axis in certain teleosts. For example, it has been reported that tilapia has two *GH* genes (Ber & Daniel 1993). Cardoso *et al.* (2003) also suggested the presence of two *GH-releasing hormone receptor* genes in the *Fugu rubripes* genome. Furthermore, we have recently reported the identification of two *GH* receptors from the black seabream (Jiao *et al.* 2006). From the present study however, there is no evidence for the existence of multiple *ghrelin* genes in seabream. Whether *ghrelin* gene duplication occurred during teleost evolution awaits further investigation. In this context though, it is interesting to note that we have previously shown that there exists only a single gene for *GHSR* in the seabream genome (Chan & Cheng 2004).

Although the *ghrelin* genes have been previously identified from a number of fish species, little is known about their promoter structures and functions. The transcriptional regulation of the teleostean *ghrelin* genes therefore remains poorly understood. To date, the 5'-flanking regions of the *ghrelin* genes from human and rat only have been isolated and characterized (Kishimoto *et al.* 2003, Kanamoto *et al.* 2004, Wei *et al.* 2005). Hence in the present study we have isolated a 2230-bp 5'-flanking region of the seabream *ghrelin* gene (Fig. 5) as a first step towards understanding its transcriptional regulation. Our results revealed that, similar to the human situation (Kishimoto *et al.* 2003), there is neither a GC box nor a CAAT box present in the isolated 5'-flanking region. A putative transcription start site is located at position -56 (Fig. 5), corresponding to the most 5'-end of the *ghrelin* transcripts (Fig. 1) identified from the seabream stomach cDNA library. Although a TATATAA element was found in the human *ghrelin* gene, deletion and mutation studies have shown that it is not important for promoter activity (Kishimoto *et al.* 2003). However, the seabream *ghrelin* gene does not contain any TATA-like element upstream of the putative transcription start site (Fig. 5). These observations therefore suggest that both mammalian and fish *ghrelin* genes are TATA-less. Incidentally this is the same as in the *GHSR* genes (Kaji *et al.* 1998, Petersenn *et al.* 2001, Tanaka *et al.* 2003, Yeung *et al.* 2004). However, the 5'-flanking region of the seabream *ghrelin* gene is found to contain a number of putative transcription factor-binding sites (Fig. 5) different from the human counterpart (Kishimoto *et al.* 2003), indicating that they are probably regulated in different manners. The functional

analysis of the seabream *ghrelin* promoter gave further support to this notion since its cell-type specificity is different from that of human. The human *ghrelin* promoter was active in ECC10 cells (Kishimoto *et al.* 2003) but the seabream *ghrelin* promoter was totally inactive in this cell type. Instead, the seabream promoter exhibited strong activity in human colon epithelial adenocarcinoma Caco-2 cells (Fig. 6). Limited by the unavailability of intestine-derived cell line from teleost species, it is not known whether the seabream promoter is also active in the same tissue type in fish. Nevertheless, no promoter activity was recorded in the fish cell line GAKS derived from scale epithelial cells.

Another difference between fish and human *ghrelin* genes is that there is only one transcription start site identified in seabream that is different from the situation in human where two transcription start sites were found (Kanamoto *et al.* 2004, Wei *et al.* 2005). Moreover, studies have suggested that E-box elements are involved in the promoter activation of the human *ghrelin* gene (Kanamoto *et al.* 2004) but no E-box elements were found in the seabream *ghrelin* promoter. Although *ghrelin* expression in both human and rat could be modulated by upstream stimulatory factor and glucagon stimulation, the regulation of *ghrelin* expression varied significantly between human and rat even though a 65% sequence identity was found between the core promoter regions of the human and rat *ghrelin* genes (Wei *et al.* 2005). These observations further suggest that the regulatory mechanisms of *ghrelin* expression are very different among species. Further studies on the transcriptional control of *ghrelin* expression in fish are highly warranted.

The expression of *ghrelin* and its receptor (*GHSR*) have been demonstrated in a number of human cells and tissues including immune cells, thyroid gland, pancreas, spleen, myocardium, adrenal gland, and ovary (Hattori *et al.* 2001, Gnanapavan *et al.* 2002, Volante *et al.* 2002, Gaytan *et al.* 2003). Their expression is also found in the mouse kidney (Mori *et al.* 2000) and embryo (Kawamura *et al.* 2003), and rat testis (Tena-Sempere *et al.* 2002, Barreiro *et al.* 2003) and adrenal (Barreiro *et al.* 2002, Andreis *et al.* 2003). These findings indicate that *ghrelin* is widely expressed and may play important physiological roles in different mammalian tissues. However, a recent study has shown that *ghrelin* is not critically required for the viability, fertility, growth, appetite, bone density, and fat deposition in a *ghrelin*-null mouse model (Sun *et al.* 2003). In spite of the diverse physiological functions that have been proposed for *ghrelin* in mammals, the *ghrelin*-null mice are visibly healthy and could reproduce normally (Sun *et al.* 2003). This study suggested that compensatory mechanisms probably accommodate some of the actions of *ghrelin* in the mutant mice. By analyzing *ghrelin*-deficient mice, another study (Wortley *et al.* 2004) has also indicated that endogenous *ghrelin* is not an essential regulator of food intake. However, it has been

demonstrated that ghrelin plays a prominent role in determining the type of metabolic substrate employed for maintenance of energy balance (Wortley *et al.* 2004). More recently, another hormone called obestatin was found to be encoded by the *ghrelin* gene (Zhang *et al.* 2005). Obestatin appears to act as an anorexic hormone by decreasing food intake via activation of an orphan G-protein-coupled receptor GPR39. It has therefore been suggested that the reason why mutant mice with a deletion of the *ghrelin* gene do not exhibit impaired appetite or growth is that these animals lack both the orexigenic ghrelin and the anorexic obestatin (Zhang *et al.* 2005). Although no report has been published so far on the identification of teleostean obestatin, our sequence alignment shown in Fig. 2 indicates a putative region of low similarity to mammalian obestatin in the teleostean ghrelin prepropeptide, suggesting that an orthologue of mammalian obestatin might be present in fish. Further studies to substantiate this possibility are highly warranted. Previous studies have indicated that the function of ghrelin in stimulating GH release from the pituitary appears to be conserved from fish to mammals (Kaiya *et al.* 2003b, 2003c, Parhar *et al.* 2003). In particular, ghrelin has been shown to be capable of stimulating food intake in goldfish (Unniappan *et al.* 2002). The expression of ghrelin mRNA has also been demonstrated in the kidney and gill of some fish species (Kaiya *et al.* 2003b, 2003c), suggesting that it may have an autocrine or paracrine function in these organs. In the present study, ghrelin was found to be expressed significantly in the seabream stomach (Fig. 7). This result is similar to the findings in Nile tilapia (Parhar *et al.* 2003), rainbow trout (Kaiya *et al.* 2003a), eel (Kaiya *et al.* 2003b), Mozambique tilapia (Kaiya *et al.* 2003c) and catfish (Kaiya *et al.* 2005), but somewhat different from that in goldfish (Unniappan *et al.* 2002, Unniappan & Peter 2005). Similar to the situation in Nile tilapia (Parhar *et al.* 2003), we did not detect ghrelin expression in all the other tissues examined. This might be due to a low expression level beyond the detection limit of RT-PCR. Actually, in eel and Mozambique tilapia, the authors could only detect ghrelin expression in the brain and gill of Mozambique tilapia and in other tissues of the eel using an extended number of PCR cycles. When a more sensitive method of real-time PCR was used, ghrelin expression could be detected in various tissues at very low levels in catfish (Kaiya *et al.* 2005). Further investigations on the expression of ghrelin using a more sensitive and quantitative method would be warranted to study the tissue-specific and developmental pattern of expression in seabream. In light of our previous finding that the seabream GHSR is mainly expressed in the central nervous system but not in the peripheral tissues (Chan & Cheng 2004), it is envisaged that ghrelin secreted from the seabream stomach might have to traverse the blood-brain barrier to exert its physiological actions on the brain.

It has been demonstrated that ghrelin expression in the mammalian stomach is regulated by nutritional and energy status (Kim *et al.* 2003, Hanada *et al.* 2004, Sanchez *et al.* 2004). Moreover, it was found that the ghrelin expression in the rodent stomach was upregulated by estradiol, leptin and insulin (Toshinai *et al.* 2001, Matsubara *et al.* 2004), and downregulated in hyperthyroidism (Caminos *et al.* 2002). In fish, ghrelin expression was reported to be regulated by feeding conditions in which starvation increased ghrelin mRNA content in goldfish (Unniappan *et al.* 2004). This regulation of expression is sex-dependent in that an elevated ghrelin mRNA level could only be detected in the female cichlid fish (Parhar *et al.* 2003). However, information regarding its regulation of expression by other hormones is not available in any fish species. In the present study, we have demonstrated that ghrelin expression in the seabream stomach could be upregulated by both GH and the synthetic GH secretagogue ipamorelin (Fig. 8). This is indeed the first report on the homologous regulatory nature of this peptide in teleosts. While the physiological significance of this homologous upregulation in fish is not understood at present, conflicting results have been reported in mammals. Qi *et al.* (2003) have reported that GH exerts a negative-feedback effect on stomach ghrelin production in rats. On the other hand, however, Nass *et al.* (2004) have provided evidence that chronic changes in peripheral GH levels do not affect ghrelin stomach mRNA expression in mice.

Taking into consideration that ipamorelin is a potent GH secretagogue of GH secretion in seabream (Chan *et al.* 2004a) and that injection of GH *in vivo* could also upregulate ghrelin expression in the seabream stomach, it is possible that this homologous upregulation is GH-mediated. However, the other possibility that ipamorelin exerts a direct action in stimulating ghrelin expression in stomach could not be excluded since both ghrelin and GHSR could be detected in the seabream stomach (Chan & Cheng 2004). Further studies are needed to dissect the exact role of ipamorelin and GH in controlling the expression of ghrelin in the fish stomach. Interestingly, we have previously reported that the seabream GHSR promoter could be enhanced by another GH secretagogue (Yeung *et al.* 2004), indicating that this homologous upregulation mechanism is applicable to both the ligand and the receptor of the ghrelin/GHSR system in seabream.

In summary, we have isolated the cDNA and gene for ghrelin in a fish species in which its cognate receptor has been identified. Moreover, we also isolated and characterized the first *ghrelin* gene promoter in teleost. Similar to its receptor, the expression of seabream ghrelin could be enhanced by a GH secretagogue analogue. The identification of both the GHSR and *ghrelin* genes from seabream would facilitate our subsequent studies on the physiological functions and regulation of the fish ghrelin/GHSR system.

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