Cloning of a proopiomelanocortin cDNA from the pituitary gland of the sea bass (*Dicentrarchus labrax*) and assessment of mRNA expression in different tissues by means of real-time PCR

S Varsamos, S E Wendelaar Bonga, G Flik, R Queré¹
and T Commes²

Department of Animal Physiology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands

¹Skuld Tech, Université Montpellier II, Place Eugène Bataillon, CC 091, 34095 Montpellier, France

²IGH/UPR, CNRS 1142, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France

(Requests for offprints should be addressed to G Flik; Email: gertflik@sci.kun.nl)

Abstract

Proopiomelanocortin (POMC) cDNA was cloned from sea bass (*Dicentrarchus labrax*) pituitary gland. A 743 nucleotide sequence was obtained coding for the following sequences flanked by sets of proteolytic cleavage sites: ACTH (Ser⁸⁸-Met¹²⁷), α -MSH (Ser⁸⁸-Gly¹⁰²), CLIP (Pro¹⁰⁶-Met¹²⁷), β -LPH (Glu¹³¹-Gln²⁰⁸), γ -LPH (Glu¹³¹-Ser¹⁷⁵), β -MSH (Asp¹⁵⁹-Ser¹⁷⁵), and β -endorphin (Tyr¹⁷⁸-Gln²⁰⁸). No region homologous to γ -MSH/joining peptide (a tetrapod POMC feature) was found. Amino acid sequence identity was high with other teleostean species considered (tilapia: 73%) and lower with elasmobranchs (dogfish: 42%). However, the presumed

biologically active peptides were highly conserved within all species considered: α -MSH (93–100%), ACTH (80–95%) and β -endorphin (54–90%). Real-time PCR allowed us to quantify the expression of the POMC in different tissues of the sea bass: pituitary gland, liver, gonad and head kidney. No significant POMC expression was found in the integument. In pituitary gland, gonads, head kidney and liver, POMC expression was respectively, 1.26×10^{10} , 2.67×10^5 , 2.06×10^4 and 1.67×10^4 copies/ μ g mRNA.

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Introduction

The vertebrate brain controls the stress response through highly organized neuroendocrine structures. This control involves numerous pathways and humoral mediators that are characteristically activated in response to stressors, which are environmental or internal changes sensed as harmful and threatening. Activation of the hypothalamic–pituitary–adrenal axis stimulates pituitary proopiomelanocortin (POMC)-derived peptide production and secretion; these peptides are involved in the mediation and regulation of the stress response (Wendelaar Bonga 1997, Slominski *et al.* 2000). The POMC gene is expressed predominantly in the pituitary gland. For cattle it has been shown that roughly 30% of all mRNAs of the anterior pituitary gland are derived from the POMC gene (Inoue *et al.* 1979). POMC is produced in the corticotrope cells of the pars distalis and the melanotrope cells of the pars intermedia. POMC mRNA is translated into a single protein product, the precursor of adrenocorticotrophic hormone (ACTH), endorphins, melanotropins and lipotropins (Smith & Funder 1988). These neuropeptides are

differentially generated through successive cell-specific processing steps and post-translational modifications including endo- and exopeptidase cleavage, amidation and acetylation (for review see Slominski *et al.* 2000).

In general, mammals transcribe only one POMC gene per haploid genome (Drouin *et al.* 1989). In lower vertebrates such as fishes, some species were found to express two forms of the POMC gene that have different degrees of homology depending on species. In polyploid fishes (e.g. salmonids and carp) a second form of POMC is found (Salbert *et al.* 1992, Arends *et al.* 1998a) as in chondrosteian species such as paddlefish and sturgeon in which that gene was duplicated (Alrubaian *et al.* 1999, Danielson *et al.* 1999). In mammals, transcription and translation of the POMC gene have been detected in peripheral tissues such as placenta, gonads, adrenals, spleen and skin (for review see Slominski *et al.* 2000). However, for fishes so far data have been presented on only peripherally localized POMC-derived peptides (Balm & Pottinger 1995, Ottaviani *et al.* 1995, van den Burg *et al.* 2001), not on the messenger proper.

The present study focuses on the POMC of sea bass (*Dicentrarchus labrax*), a member of the Moronidae family, which includes species of great biological and economic interest and that occur in the Atlantic Ocean and the Mediterranean Sea. One of the aims of this study was to determine the POMC gene sequence, which is important for future studies focusing on the role of POMC-derived peptides in the stress response of this species. Most of the known teleost POMC sequences concern stenohaline freshwater (carp) and euryhaline species (salmon, trout). Information on a truly stenohaline marine teleost POMC sequence was not available and such information would add to our understanding of POMC phylogeny. Moreover, determination of the sea bass POMC sequence is necessary for further investigations concerning the detection and quantification of the expression of this gene. Little is known about peripheral expression of the POMC messenger in fishes. Hence, we set out to detect and compare the expression of the POMC gene in different tissues by means of a quantification method based on real-time PCR, which appears to be much more sensitive and precise compared with classical techniques based on hybridization.

Materials and Methods

Animals

Adult sea bass (350 ± 45 g) originated from IFREMER (Palavas les Flots, France) and were maintained in the aquaculture facilities of the University of Nijmegen, The Netherlands. The fish were held in 200 liter tanks with filtered and aerated artificial sea water, obtained by addition of natural salt (Aquarium Systems, France) to tap water. The temperature was set at 20 °C and photoperiod to 12 h light:12 h darkness. The fish were fed commercial pellets, at a ration of 2% body weight per day. Animals were rapidly killed by decapitation. Tissues (pituitary gland, gonads, liver, head kidney and integument) for mRNA extraction were rapidly dissected, weighed and frozen in liquid nitrogen.

mRNA extraction and cDNA synthesis

Poly-adenylated RNA isolation was performed with Dynal's Dynabeads mRNA Direct kit (Dynal, Norway). Briefly, tissues were disrupted in a lysis buffer. Cellular lysate was centrifuged for 5 min at 800 *g* to eliminate cellular debris. The supernatant was transferred to a tube containing prewashed Dynabeads oligo(dT)₂₅, incubated for 20 min on a rotary agitator to anneal and washed (five times). For synthesis of first-strand cDNA annealed mRNAs were incubated for 2 min at 37 °C with a reverse transcription (RT)-Mix (11 µl H₂O, 4 µl RT buffer, 2 µl dithiothreitol, 1 µl dNTP) and then transferred to 37 °C for

1 min. Finally, 2 µl Superscript II reverse transcriptase (200 U/µl; Invitrogen) were added and the mixture was incubated at 37 °C for 1 h. The resulted cDNAs were stored at -20 °C.

PCR for gene-specific primers determination

To determine sea bass POMC-specific primers, PCR using a thermocycler (Hybaid, UK) was performed on cDNA obtained from sea bass pituitary mRNA with the following degenerated primers: forward 5'-ATG GAR CAY TTY GCN TGG GG-3' and reverse 5'-TGS YTC ATT TTG TAG GAG CC-3', designed on the basis of consensus POMC sequences. Template DNA (1 µl RT product) and 25 pmol of each primer were used in a final volume of 50 µl containing 1 × PCR buffer, 2.5 mM MgCl₂, 100 µM (each) dNTPs and 2.5 units *Taq* DNA polymerase (Promega). After an initial denaturing step at 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min were performed. A final extension step of 72 °C for 2 min was carried out. PCR products were analyzed by electrophoresis on 1.5% agarose/ethidium bromide gel, cloned and sequenced (see below). The obtained sequences (227 bp) were compared with POMC sequences of other species and sea bass POMC-specific primers were determined for the GeneRacer protocol (see below): 5'Dlp (5'-AAG CCT GTT GGA CGA AAG C-3'), 3'Dlp (5'-GAG CCA TCC TTC TTC TCG TG-3'), 5'Dlpnest (5'-CCG GTC AAA GTC TTC ACC TC-3') and 3'Dlpnest (5'-ACC TCC TGT GCC TTC TCC TC-3').

Cloning, transformation, purification and sequencing of PCR products

PCR products were ligated into pCR4-TOPO plasmid vector and transformed into chemically competent TOP 10 *Escherichia coli* cells (TOPO TA Cloning kit; Invitrogen). After selection on LB-ampicillin agar, transformed cells were screened for appropriate size inserts using a thermocycler and M13 sense and antisense primers (20 pmol each in a final volume of 50 µl). The PCR profile consisted of a denaturing step at 95 °C for 2 min, a step (35 cycles) comprising denaturing at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min and a final extension step at 72 °C for 2 min. Sequencing of PCR products was performed by the ddNTP procedure (Genome Express, France). For the needs of the RT-PCR (see below), PCR products were purified by MiniPreps (Promega) and isolated plasmids were stored at -20 °C.

Sea bass POMC sequence determination

Sea bass pituitary POMC sequence was determined using the GeneRacer protocol (GeneRacer kit; Invitrogen),

designed for full-length, RNA ligase-mediated amplification of 5' and 3' ends. Briefly, isolated sea bass pituitary mRNA was submitted to the following treatments according to the GeneRacer protocol: (i) dephosphorylated by treatment with calf intestinal phosphatase to eliminate truncated mRNA and non-mRNA, (ii) the 5' cap structure from intact full-length mRNA was removed, (iii) a GeneRacer RNA-oligo was ligated to the 5' end of the mRNA; this oligo provided known priming sites for GeneRacer PCR primers after transcription of the mRNA into cDNA, (iv) RT with a GeneRacer oligo dT primer (50 mM) (containing a dT tail to prime the first-strand cDNA synthesis in the RT reaction and known priming sites at 5', for GeneRacer PCR 3' primers). 5' and 3' PCR were performed using gene-specific and GeneRacer ligated primers (see above). Template DNA (1 µl RT product), 20 pmol gene-specific primer and 60 pmol GeneRacer primer were used in a final volume of 50 µl containing 1 × PCR buffer, 2.5 mM MgCl₂, 100 µM (each) dNTPs and 2.5 units *Taq* DNA polymerase. The PCR profiles included: (i) one cycle at 95 °C for 2 min, (ii) five cycles of denaturing at 95 °C for 30 s followed by 1 min at 72 °C, (iii) five cycles of denaturing at 95 °C for 30 s followed by 1 min at 70 °C, and (iv) 25 cycles of denaturing at 95 °C for 30 s, annealing at 65 °C for 30 s and elongation at 72 °C for 1 min. A final elongation step was carried out at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 1.5% agarose/ethidium bromide gel. One microliter of the amplified PCR product was used for 5' and 3' nested PCR to eliminate artifacts in the previous PCR. The same thermal profile and PCR mix composition were used except that in this case equal quantities (20 pmol) of gene-specific nested primer and GeneRacer nested primer were added in the PCR mix. PCR products were analyzed, cloned and sequenced (see above).

DNA sequences analysis

To minimize errors due to nucleotide mis-incorporation by *Taq* DNA polymerase, the full-length sequence represents a consensus of multiple overlapping clones. Nucleotide sequences and deduced protein sequences were checked for similarities to known genes using BLAST algorithms at NCBI, EMBL and SWISS-PROT databases. The nucleotide and amino acid sequences of sea bass POMC reported in our study were aligned with the POMC sequences obtained from the databases mentioned above.

Phylogenetic trees

The neighbor-joining method was used for the construction of phylogenetic trees. They were calculated using the programs PROTDIST and NEIGHBOR from the PHYLIP package (Felsenstein 1993). One thousand boot-

strap samples were created using SEQBOOT and PROTDIST, reconstructed with NEIGHBOR and a consensus tree was constructed with the CONSENSE program. Maximum-likelihood trees were constructed using the program PROTML from the MOLPHY package.

Detection and quantification of POMC expression in different tissues of sea bass

Detection and quantification of POMC expression was carried out on sea bass cDNAs from pituitary, head kidney, liver, gonads and integument, by means of real-time PCR with a Light Cycler (Roche). All measurements were performed in triplicate.

Control gene A control gene was necessary for the global calibration of the quantification by real-time PCR. The control gene of choice was the L17 ribosomal protein (AF139590), a sea bass housekeeping gene sequence downloaded from Genbank. Specific primers (5'DIL17rp (5'-GAG GAC GTG GTG GTT CAT CT-3') and 3'DIL17rp (5'-CTG GCT TGC CTT TCT TGA CT-3')) were determined on these sequences and tested by PCR on sea bass pituitary cDNA. A 201 pb PCR product was cloned and sequenced. After verification plasmids (L17rp) were purified by MiniPreps (Promega) and their concentration was determined by absorbance measured at 260 nm with a spectrophotometer (Safas 190 DES, Monaco) and on agarose/ethidium bromide gel. Dilutions of the L17rp plasmid were used in real-time PCR experiments on sea bass POMC to obtain the calibration curve. Twenty microliter reactions were run containing 2 µl 10 × PCR Mix (containing SYBR Green and purchased from Roche), 1.6 µl 25 mM MgCl₂ stock solution and 10 pmol of each primer and 1 µl template DNA (plasmid or cDNA from specific tissue). The thermal profile used for real-time PCR consisted of a step at 95 °C for 10 min and 40 cycles of denaturing at 95 °C for 15 s, annealing at 62 °C for 4 s and elongation at 72 °C for 8 s. After the last cycle, temperature in the Light Cycler chamber increased to 95 °C and then decreased to 62 °C for 30 s. Then it was increased gradually to 95 °C to obtain the melting curves of the amplified fragments. Absence of non-specific PCR products and primer dimers was checked by the melting curves (see Fig. 5) and by electrophoresis on 8% acrylamide/SYBR Green gel.

POMC gene For real-time PCR internal calibration, a fragment of 128 bp was amplified by classical PCR on sea bass pituitary cDNA with the following primers: 5'Dlpnest and 3'Dlpnest (see above). PCR product was cloned, sequenced and after verification plasmids were purified and its concentration was determined on agarose/ethidium bromide gel. Dilutions of this plasmid were used in real-time PCR experiments on sea bass POMC, to obtain

GAA GTG GAG GGG ACA GAA ATC TTT GTC CAA CAG CTG AAC AAC AAG AAA ACA ACA	54	
	←-----	
	M C P V W L L V A V	10
GAG AAA GAC AGG ACA GCG TGG GAA ATG TGT CCT GTG TGG TTA TTG GTG GCT GTG	108	
Signal peptide -----		
A L A G V A R G A V G Q C L E H P S	28	
GCG CTG GCG GGC GTG GCC AGA GGA GCT GTT GGT CAG TGT TTG GAA CAT CCC AGC	162	
----- N-Ter -----		
C Q E L N S E S N M L E C I Q L C R	46	
TGT CAG GAG CTC AAC TCT GAG AGC AAT ATG CTG GAG TGT ATC CAG CTC TGT CGC	216	

S D L T A E T P I I P G D G H L Q P	64	
TCC GAC CTC ACC GCA GAG ACA CCG ATC ATC CCG GGC GAT GGC CAC CTC CAA CCT	270	

L P P S E S L P P L S L F S S S S S	82	
CTT CCT CCG TCA GAG TCT CTG CCT CCC CTC TCC CTG TTC TCT TCC TCC TCC TCT	324	

P Q A K R S Y S M E H F R W G K P V	100	
CCA CAG GCC AAG CGC TCC TAC TCC ATG GAG CAT TTC CGC TGG GGG AAG CCT GTT	378	

----- ACTH -----		
G R K R R P V K V F T S N G CLIP V E E E	118	
GGA CGA AAG CGC CGC CCG GTC AAA GTC TTC ACC TCC AAC GGC GTG GAG GAG GAG	432	

S A E V F P E E M R R R E L A S E I	136	
TCA GCC GAG GTT TTC CCT GAA GAG ATG AGA AGG CGA GAG CTT GCC AGC GAG ATT	486	

----- γ-LPH -----		
I A A E D E E K A Q E V A E E E Q L	154	
ATA GCG GCA GAG GAC GAG GAG AAG GCA CAG GAG GTG GCG GAA GAG GAG CAG CTC	540	

H E K K D G T Y K M β-MSH K H F R W G G S	172	
CAC GAG AAG AAG GAT GGC ACG TAC AAG ATG AAG CAC TTC CGC TGG GGC GGG TCG	594	

P A S K R Y G G F M K S W D E R S Q	190	
CCG GCC AGC AAA CGC TAC GGC GGC TTC ATG AAG AGC TGG GAC GAG CGC AGC CAG	648	

----- β-endorphin -----		
R P L L T L F K N V I N K D G Q E Q	208	
AGG CCC CTG CTC ACG CTC TTC AAA AAT GTC ATC AAC AAA GAC GGA CAG GAG CAG	702	

K R E Q *	212	
AAG AGA GAG CAG TGA GGG AGG ACA	726	

Figure 1 Nucleotide and deduced amino acid sequences of sea bass POMC cDNA. Both sequences are numbered at the end of each line. The deduced POMC sequence is shown above the cDNA sequence. The POMC-derived peptides are shown: ACTH (Ser⁸⁸-Met¹²⁷), α-MSH (Ser⁸⁸-Gly¹⁰²), CLIP (Pro¹⁰⁶-Met¹²⁷), β-LPH (Glu¹³¹-Gln²⁰⁸), γ-LPH (Glu¹³¹-Ser¹⁷⁵), β-MSH (Asp¹⁵⁹-Ser¹⁷⁵) and β-endorphin (Tyr¹⁷⁸-Gln²⁰⁸). Potential proteolytic cleavage sites are shown in bold.

	1	15	16	30	31	45	46	60	61	75	76	90
1 Seabass	-----	MCPVWLIV	AVALAGVARGAVGQC	LEHPSQCELNSESNM	LECIQLCRSDLTAE	PIIPGDGHLQPLPPS	ES----	LPPLSLF--				
2 Tilapia	-----	MCPVWLIV	ALVVVGAGAREAVSQ	WEHPSQCELSSESNM	MECIQLCHSDLTAE	PVIPGNAHLQPAVPS	D-----					
3 Salmon	-----	MLCPAWLL	AVAVVGVRGVKGC	WENPRCHDLSSSENSI	LECIQLCRSDLTTKS	PIFVKVHLQPPYPS	DS-----	PPLYLPLSL				
4 Carp		MVRGERMLCPAWLLA	LAVLCAAGSEVRAQC	MEDARCRDLTTDENI	LDCIQLCRSDLTDET	PVYPGESHLQPPSEL	EQ----	TEVLVPLSP				
5 Sturgeon		---MLRPVWGCVVAV	MGVLWFYCSGVQSQ	WEHSQCRDLSSSEANI	LECIQACKVDLSAES	PLFPGNGHLQPTSED	IEGASTTSLVLEAL					
6 Lungfish		---MLKPVWRHLFVL	SAVLMITYGTGVHSQC	WETSQCRDLSTESNL	LECIKSKCSDLAES	PVYPGNGHMQPLSED	IEDGYKTPILLSIIPA					
7 Dogfish		--MQQSMWRSVLV	LCMVVARSSGQLREC	WDHTKCRQLTSAPKL	MECTEACKVEKTLES	PIYPGNGHTEPIAES	LEDEPVRAFLNHLPA					
8 Human		--MPRSCSSRGALL	LALLLQASMEVRGWC	LESSQCQDLTTESNL	LECI RACKPDLSAET	PMFPGNGDEQPLTEN	PEDVSAGEDCGPLPE					
9 Bovine		--MPRLCSSRGALL	LALLLQASMEVRGWC	LESSQCQDLTTESNL	LACIRACKPDLSAET	PVFPGNGDEQPLTEN	PEEEVAVGEG----					
10 Rat		--MPRFCNSRGALL	LALLLQTSIDVWSWC	LESSQCQDLTTESNL	LACIRACRLDLSAET	PVFPGNGDEQPLTEN	PAEEETAGGD----					
11 Chicken		---MRGALCHSLPVV	LGLLLCHPTTASGPC	WENSKCQDLATEAGV	LACACACRAELSAEA	PVYPGNGHLQPLSES	IEEVAGLALPAASPH					
12 GiantToad		---MLQPGWRCILTI	LGAFIFHVGEVKSQC	WESGKCADLTSEDGI	LECI KDC KMYLSAES	PVFPGNGHMQPLSEN	IEDIANYP IFNLFPPT					
	91	105	106	120	121	135	136	150	151	165	166	180
1 Seabass	-----	-----	SSSSSPQAKRSYSM	EHFRWGKPVGRKRRP	VKVFTSNGVEEESAE	VFPEEMRRRELASEI	IAAED-----					
2 Tilapia	-----	-----	ASSSPSQAKRSYSM	EHFRWGKPVGRKRRP	VKVYTSNGVAEESAE	VFPEEMRRRELTNEL	LAEEG-----					
3 Salmon	LSPSSPLYPGEQ---	---	QNSVSPQAKRSYSM	EHFRWGKPVGRKRRP	VKVYT-NGVEEESSE	AFPSEMRRRELGTDNA	MYPSL-----					
4 Carp	---AALAPAEQMDP---	---	ESSPQHEHKRSYSM	EHFRWGKPVGRKRRP	IKVYT-NGVEEESSE	TLPAMRRRELATNEI	DYPQ-----					
5 Sturgeon	SQPRDEVERESEEEE	---	GLQQHRRDDKRSYSM	EHFRWGKPVGRKRRP	VKVYP-NEVEEESAE	SYPAEIRRDLSLKLD	YPQG-----					
6 Lungfish	LESHNNLDMEED---	---	SLSRQDDRRSYSM	EHFRWGKPVGRKRRP	IKVYP-NGMEDESAE	SYSPDLRRDMPMEFD	YP-----					
7 Dogfish	VVSQTSQMEDEE---	M	ETLFPQDGRKRSYSM	EHFRWGKPMGRKRRP	IKVYP-NSFEDESVE	NMGPELKREASVDFD	YPVVE-----					
8 Human	GG---PEPRSDGAK---	---	PGPREGKRSYSM	EHFRWGKPVGRKRRP	VKVYP-NGAEDESAE	AFPLEFKRELGTGRL	REGDGPDPGADDGAG					
9 Bovine	---PGPRGDAAE---	---	TGPREDKRSYSM	EHFRWGKPVGRKRRP	VKVYP-NGAEDESAA	AFPLEFKRELGTGERL	EQARGPEAQAESAAA					
10 Rat	---GRPEPSP---	---	REGKRSYSM	EHFRWGKPVGRKRRP	VKVYP-NVAENESAE	AFPLEFKRELEGE---	-----					
11 Chicken	HP---AGEEEDGEG---	---	LEREEGKRSYSM	EHFRWGKPVGRKRRP	IKVYP-NGVDEESAE	SYPMEFREMAPDGD	PFG-----LS-----					
12 GiantToad	NDNQNTQDGNMEE---	---	LRRQDNKRSYSM	EHFRWGKPVGRKRRP	IKVFP-DAEESSE	IXPTEYRRELSVEFD	YP-----DTN-----					
	181	195	196	210	211	225	226	240	241	255	256	270
1 Seabass	---	EKAQOEVAEE	Q-----	-----	-----	-----	---	LHEKDD	GTYSKMHFRWGGSPA	SKRYGGFMKSWDERS		
2 Tilapia	---	EKAQEMVEGAE	EE-----	-----	-----	---	---	QQLNGVQEKDD	GSYKMHFRWGGPPA	SKRYGGFMKSWDERS		
3 Salmon	---	EAGTAEGGEAE	GT-----	-----	---	---	---	EG-VFSLQEKDD	GSYKMHFRWGGPPA	SKRYGGFMKSWDERS		
4 Carp	---	EE-----	GALN	---	---	---	---	QDKDD	GSYKMSHFRWSPPA	SKRYGGFMKSWDERS		
5 Sturgeon	---	EELLEVFGGEN	DL-----	---	---	---	---	LN-----	LQEKDD	GSYKMHFRWGGPPK	DKRYGGFMKSWDERS	
6 Lungfish	---	EPSSEEKSEEN	DI-----	---	---	---	---	MN-----	LLEKDD	RNYRMQHFRWNSPPK	DKRYGGFMKSWDERS	
7 Dogfish	---	TSEAGEEEMLE	DAGPKGSAQSWGPDR	TQPMQFTNLEDMLQE	SMDNDLPEEEVKKDG	DDYKFGHFRWSVPLK	DKRYGGFMKSWDERS					
8 Human	---	AQADLEHSLLV	AA-----	---	---	---	---	EKKDE	GPYRMEHFRWSPPK	DKRYGGFMTS--EKS		
9 Bovine	---	RAEL EYGLVAEAEAE	AA-----	---	---	---	---	EKKDS	GPYKMEHFRWSPPK	DKRYGGFMTS--EKS		
10 Rat	---	QP-DGLEQVLE	PD-----	---	---	---	---	TEKAD	GPYRVEHFRWGNPPK	DKRYGGFMTS--EKS		
11 Chicken	---	EEEEEEEEEEG	EE-----	---	---	---	---	EKKDG	GSYRMRHFRWHAPLK	DKRYGGFMSL--EHS		
12 GiantToad	---	SEEDMDDSMMLM	ES-----	---	---	---	---	PNRKD	RKYKMHFRWEGPPK	DKRYGGFMTS--ERS		
	271	285	286									
1 Seabass	QRPLLTFLFKNVINKD	GQEQKREQ--		212								
2 Tilapia	QKPLLTFLFKNVINKE	GQQQK----		208								
3 Salmon	QKPLLTFLFKNVIKD	GQQ--KREQ--		230								
4 Carp	QKPLLTFLFKNVINKE	HQ--KKDQ--		222								
5 Sturgeon	QKPLLTFLFKNVMIKD	GHE--KKGQ--		238								
6 Lungfish	QKPLMTFLFKNVMIKD	AYE--KKGQ--		231								
7 Dogfish	QKPLLTFLFRNVIVKD	GHE--KKAQSQ		275								
8 Human	QTPLVTFLFKNIAIKN	AY--KKGE--		237								
9 Bovine	QTPLVTFLFKNIAIKN	AH--KKGQ--		234								
10 Rat	QTPLVTFLFKNIAIKN	VH--KKGQ--		207								
11 Chicken	QTPLMTFLFKNIAIVKS	AY--KKGQ--		226								
12 GiantToad	QTPLMTFLFKNIAIKN	AH--KKGQ--		229								

Figure 2 Alignment of POMC amino acid sequences from different species. Amino acids identical to the sea bass sequence are shown in bold. The accession number of the sequences used in the comparison and the percentage of their similarity with the sea bass sequence were: tilapia (*Oreochromis mossambicus*, AF116240; 73%), salmon A (*Oncorhynchus nerka*, Okuta et al. (1996); 65%), carp I (*Cyprinus carpio*, CAA74968; 57%), sturgeon (*Acipenser transmontanus*, P87352; 54%), lungfish (*Protopterus annectens*, 50%), dogfish (*Squalus acanthias*, Amemiya et al. (1999); 42%), human (*Homo sapiens*, P01189; 44%), bovine (*Bos taurus*, P01190; 49%), rat (*Rattus norvegicus*, P01194; 44%), chicken (*Gallus gallus*, BAA34366; 46%), giant toad (*Bufo marinus*, AAF06345; 45%). γ - and δ -MSH sequences were removed (when present) from the sequences before alignment.

the calibration curve. Real-time PCR profile and mix composition were equivalent to those described for the control gene.

Quantification and analysis Quantification and analysis of the results were performed with the computer device of the Light Cycler (Light Cycler Relative Quantification

Table 1 Amino acid sequence similarity between sea bass and POMC sequences of other species

	Accession number	Similarity (%)
Species		
Tilapia	AF116240	73
Carp I	CAA74968	55
Sturgeon	P87352	55
Lamprey	I51117	29
Lungfish	AAD29144	48
Giant toad	AAF06345	44
Chicken	BAA34366	45
Rat	P01194	45
Bovin	P01190	46
Human	P01189	45

Software 1.0; Roche) and calculations were done according to Rasmussen (2001). The POMC messenger quantification was expressed in a number of copies/ μ g mRNA.

Results

POMC sequence

A single form of functional POMC gene was found to be expressed in sea bass. Using the sea bass pituitary cDNA library as template, the GeneRacer protocol resulted in the amplification of a 522 (5' RACE) and a 349 (3' RACE) nucleotide product. Five clones of each product were sequenced to minimize erroneous nucleotide incorporation by *Taq* DNA polymerase. The 5' product contained the signal peptide, N-terminal and α -melanocyte-stimulating hormone (α -MSH) sequences, the 3' product contained the corticotropin-like intermediary peptide (CLIP) sequence from ACTH, β -MSH, β - and γ -lipotropin (γ -LPH) and β -endorphin sequences as well as a stop codon. Alignment of the overlapping sequences resulted in a final 743 nucleotide product containing an open reading frame encoding a 212 amino acid protein (Fig. 1). The open reading frame contained the following sequences flanked by sets of proteolytic cleavage sites indicating potential post-translational processing: ACTH (Ser⁸⁸-Met¹²⁷), α -MSH (Ser⁸⁸-Gly¹⁰²), CLIP (Pro¹⁰⁶-Met¹²⁷), β -LPH (Glu¹³¹-Gln²⁰⁸), γ -LPH (Glu¹³¹-Ser¹⁷⁵), β -MSH (Asp¹⁵⁹-Ser¹⁷⁵), and β -endorphin (Tyr¹⁷⁸-Gln²⁰⁸) (Fig. 1). Each of these regions contains small modifications, which seem to be unique to sea bass concerning amino acid substitution or number. No region homologous to γ -MSH/joining peptide was present. Figure 2 shows alignment of the sea bass POMC sequence with POMC sequences from other species. Amino acid sequence identity between sea bass POMC and POMC from other species varies according to the groups considered (Table 1). Similarity was high with known sequences of teleostean species (tilapia: 73%) and low with those of mammals (human: 45%). The biologically active peptides

α -MSH (93–100%), ACTH (80–95%), and β -endorphin (54–90%) are highly conserved among all species considered. Sea bass β -endorphin sequence displayed two additional amino acids compared with species other than fish, the one characteristic for ray-finned fish, i.e. the paired Trp¹⁸⁵Asp¹⁸⁶ and the Glu²⁰⁷ residue, as well as a monobasic consensus sequence Glu¹⁸⁷ArgSerGln¹⁹⁰. The ACTH sequence displayed a putative phosphorylation consensus sequence Ser¹¹⁹AlaGlu¹²¹.

Phylogenetic tree

A consensus tree of 1000 bootstrap samples (Fig. 3) of the neighbor-joining program allowed us to determine the position of sea bass POMC sequence within the POMC family. All methods employed showed a configuration in which teleosts branched off separately. The bootstrap value of the node clustering the euryhaline sea bass and tilapia was 96.2%.

POMC expression

Real-time PCR allowed us to quantify the expression of the POMC in different tissues of the sea bass: pituitary gland, liver, gonad and head kidney (Figs 4 and 5). Values were calibrated by means of a housekeeping gene (control) present in all the tissues. The efficiencies of the real-time PCR procedure for the control gene and the POMC gene were high: 1.904 and 1.901 respectively. The lowest POMC expression was found in the liver. In pituitary, gonads and head kidney, POMC expression was respectively, 7.5×10^5 -, 1.6- and 1.2-fold higher compared with that in the liver. Expressed in terms of number of copies/ μ g mRNA, POMC expression was 1.26×10^{10} (pituitary), 2.67×10^5 (gonads), 2.06×10^4 (head kidney) and 1.67×10^4 (liver). The values measured for the integument (not shown) were below the background values of the standard curve. The real-time PCR approach allowed detection of less than ten copies of POMC mRNA.

Discussion

The GeneRacer technique allowed us to clone a single functional form of POMC from the pituitary gland of the European sea bass. A single form of that prohormone was also found in tilapia (*Oreochromis mossambicus*) (Lee *et al.* 1999a). However, in tetraploid teleost species such as carp (*Cyprinus carpio*) (Arends *et al.* 1998a), chum salmon (*Oncorhynchus keta*) (Kitahara *et al.* 1988) and trout (*Oncorhynchus mykiss*) (Salbert *et al.* 1992) two forms of POMC were found. In general, also in mammals only one form of POMC occurs (Drouin *et al.* 1989), although the mouse genome contains two non-allelic POMC α - and β -genes (Uhler *et al.* 1983). In some lower vertebrates such as chondrosteans (Alrubaihan *et al.* 1999) two forms of POMC are found, but this is not a general phenomenon as the phylogenetically old diploid fishes (lung fishes) appear

Figure 3 Phylogenetic tree of the POMC family based on amino acid sequences. γ - and δ -MSH sequences (when present) were removed from the sequences before alignment. Numbers in branches indicate the bootstrap values calculated with the neighbor-joining method. For accession numbers and Latin names of the species used in this study see Fig. 2.

Figure 4 Real-time PCR analysis (Light Cycler) of sea bass POMC mRNA isolated from pituitary gland (P), liver (L), gonad (G) and head kidney (Hk). One microliter of cDNA from each tissue was used as template for amplification of the POMC gene fragment. First the control gene L17rp was run and then the POMC templates were calibrated to the control gene in each of the samples. The number of PCR cycles required to obtain measurable amplification (P: 13,9 cycles; G 31,59 cycles; Hk: 34,9 cycles; L: 35,62 cycles) is related to the initial concentration of target POMC mRNA. Clearly, the results show that POMC is much more highly expressed in the pituitary gland than in other tissues. Water was used as negative control (red line). SYBR Green was used as fluorochrome.

to express only one form (Dores *et al.* 1999, Lee *et al.* 1999b).

Several recent studies have focused on the evolution of the POMC gene in vertebrates. In sea bass the POMC end product regions flanked by sets of basic amino acids were ACTH, α - and β -MSH, β - and γ -LPH and

β -endorphin. The observed structure of the sea bass POMC corresponds to the ones reported in other teleost species. Overall, the substitutions, additions and deletions observed for the ACTH, β -MSH and β -endorphin regions are relatively few. However, the absence of γ -MSH sequence and most of the joining peptide region,

Figure 5 Melting curves of the PCR products from the RT-PCR displayed in Fig. 4. When temperature increases, double-stranded DNA dissociates, SYBR Green is liberated and fluorescence signal decreases. After the last cycle, temperature in the Light Cycler chamber was increased to 95 °C and then decreased to 62 °C for 30 s (maximum annealing). Then it was increased gradually to 95 °C to obtain the melting curves of the amplified fragments. When 50% of the double-stranded DNA is denatured, fluorescence decreases abruptly; the corresponding temperature is the T_m (melting temperature) of the PCR product. The T_m corresponds to the maximum of the curve: $-d(\text{Fluorescence})/d(\text{Temperature})$. All the PCR products of sea bass POMC from different tissues display the same T_m , indicating the absence of non-specific PCR products and primer dimers.

set sea bass POMC and that of other teleostean fishes apart from the general organization of the prohormone. γ -MSH is also absent in jawless fishes such as the lamprey (Heinig *et al.* 1995) but it is present in gnathostomes, such as cartilaginous fishes (*Squalus acanthias* (Amemiya *et al.* 1999); *Dasyatis akajei* (Amemiya *et al.* 2000)), chondrosteans (as a remnant in *Acipenser transmontanus* (Amemiya *et al.* 1997)), neopterygians (as a remnant in *Lepisosteus osseus* (Dores *et al.* (1997))), amphibians (*Rana ridibunda* (Hilario *et al.* 1990)) and mammals (Nakanishi *et al.* 1979). When the internal duplication event occurred that gave rise to the γ -MSH sequence has not been determined so far. The absence of this MSH in the lamprey and its presence in species from the three major gnathostome radiations suggests that the γ -MSH duplication event occurred in an early Devonian ancestral gnathostome (Lee *et al.* 1999b). Since carp, salmon and sea bass first appeared during the Miocene, the important differences observed in POMC sequence of these species could be related to different ecological or physiological requirements and thus to different environmental constraints.

Although we found some significant differences, the sea bass POMC sequence displays a high degree of amino acid sequence identity with other groups when regions coding for biologically active peptides are considered. For example, the overall degree of amino acid identity with mammals was approximately 44%, but this was much higher (67%) when regions coding for ACTH, β -MSH and β -endorphin were considered. The β -endorphin sequence in sea bass displays two amino acid insertions compared with amphibians, avians and mammals. This feature appears to be characteristic of all fish species examined so far. The physiological relevance of these additional amino acids in fish β -endorphin remains unknown. Five single basic amino acids (three lysines and two arginines) are present in the sea bass endorphin sequence, suggesting a putative post-translational truncation by trypsin-like enzymes. This could give rise to several different endorphin signals as was reported in other fish species (van den Burg *et al.* 2001).

The phylogenetic analysis indicates that, although teleosts appeared long after the divergence of the main groups of gnathostome vertebrates, the sequence of the sea bass POMC has retained several features that are conserved in other vertebrates. The teleostean species considered in this study cluster in the phylogenetic tree. Among the ray-finned fish, the highest degree of speciation is observed in the teleosts (more than 30 000 species identified up to today), which have settled in all kinds of aquatic niches. A role of a prohormone such as POMC in that successful radiation would implicate that it has been subject to selective evolutionary pressure.

Few studies have focused on POMC localization and expression in fish brain (Arends *et al.* 1998a,b, Ficele

1998). POMC-derived peptides are known to be involved in the development of the immune response (Weyts *et al.* 1999), as well as in the reproductive cycle (Mosconi *et al.* 1994). However, remarkably little is known about peripheral expression of POMC in fish. In mammals, transcription and translation of the POMC gene have been detected in a variety of peripheral tissues including placenta, uterus and gonads. There is evidence for involvement of locally produced POMC-derived peptides in the skin pigmentation and in the maintenance of skin integrity (Slominski *et al.* 2000), as well as in the regulation of the feto-maternal unit of the mammalian placenta (Petraglia *et al.* 1998). We were able to detect and quantify POMC mRNA expression in head kidney, liver and gonad (besides pituitary tissue) through the sensitive real-time PCR approach. Compared with other (semi-)quantitative techniques such as hybridization, capillary electrophoresis etc., the real-time PCR is much more convenient; most importantly its sensitivity allows the detection of roughly ten copies of the messenger under consideration. POMC expression was detected in all tissues but the integument; for the latter the values recorded were below the background values of the calibration curve. As anticipated, the concentration was found to be very much higher (roughly 10^6 -fold) in the pituitary gland than in the other tissues. To the best of our knowledge this is the first report on expression rate of POMC in the pituitary (as well as in other tissues) of a fish. The high rate of expression displayed by the prohormone gene in the pituitary gland was anticipated because of the involvement of this gland (via the pleiotropic POMC-derived peptides) to a variety of physiological processes including the stress response (Wendelaar Bonga 1997). On the other hand the expression rates measured in the peripheral tissues may reflect POMC expression activity of only a small but relevant subpopulation of cells in these tissues. Evidence is accruing for other (higher) vertebrates that both immune and neuroendocrine systems are able to produce POMC (reviewed in Blalock 1999). In that case, the presence of a POMC transcript in the head kidney of sea bass can be of great physiological relevance since this tissue constitutes, together with the thymus and the spleen, the major source of immune cells in fish. Recent work suggests that also in lower vertebrates (frog, *Rana esculenta*) extra-pituitary, peripherally produced and regulated POMC production contributes to the normal physiology, e.g. of the ovary (Nabissi *et al.* 2001). Thus, further studies on fishes with their phenomenal adaptive radiation and endless and subtle physiological adaptations are warranted to broaden our view on POMC-dependent processes.

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