Title: Anti-Müllerian hormone (*AMH*) in the European sea bass: Its gene structure, regulatory elements, and the expression of alternatively spliced isoforms

Key words: Müllerian inhibiting substance (MIS), *Dicentrarchus labrax*, transforming growth factor β family, promoter, transcription binding sites, transcript variants

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Abbreviations: A: adenosine, aa: amino acid(s); AMH: anti-Müllerian hormone; bp: base pairs; C: cytosine; cDNA: complementary DNA; G: guanine; nt: nucleotide(s); ORF: open reading frame; PCR: polymerase chain reaction; pfu: plaque forming unit(s); sb: sea bass; eSRS21: eel spermatogenesis-related substance; T: thymine; TGF- β : transforming-growth factor β

Abstract

In mammals, a multitude of studies have shown that anti-Müllerian hormone (AMH), apart from inducing Müllerian duct regression during male sexual differentiation, exerts inhibitory effects on male and female gonadal steroidogenesis and differentiation. However, in lower vertebrates like teleost fish, the function of AMH has been far less explored. As a first step to unravel its potential role in reproduction in teleost fish, we isolated and characterised the *AMH* gene in the European sea bass (sb), *Dicentrarchus labrax*, determined putative regulatory elements of its 5'-flanking region, and analysed its gene expression and those of alternatively-spliced transcripts. The characterisation of *sb-AMH* revealed distinct features that distinguishes it from mammalian and bird *AMH*, suggesting a high rate of diversification of *AMH* during vertebrate evolution. It contained 7 exons that were divided by 6 introns, of which the last intron (intron vi) was localised only a few nucleotides upstream of the putative peptide cleavage site. The guanine and cytosine content of the open reading frame (ORF) was 52.7 % and thus notably lower than that of bird and mammalian *AMH*. *Sb-AMH* cDNA was 2045 base pairs (bp) long, containing an ORF of 1599 bp encoding 533 amino acids. Deduced amino acid similarities of the conserved, carboxyterminal domain were highest with *AMH* in Japanese flounder (84.2 %) and lowest with chicken *AMH* (45.5 %). In the proximal promoter sequence of *sb-AMH*, a steroidogenic factor-1 (SF-1) binding site was present; however other regulatory sequences essential for transcriptional activation of *AMH* in mammals were absent. Likewise, there was no sequence homology to an *SF3A2* sequence within the first 3200 bp upstream of the *sb-AMH* translation start site. Gene expression of *sb-AMH* and of alternatively-spliced *sb-AMH* transcripts were analysed in male and female juvenile and adult gonads as well as in somatic tissues of juvenile males. *sb-AMH* expression was highest in juvenile testis, but still remarkably high in juvenile ovaries and adult testis, as well as in brain, pituitary, and heart of juvenile male sea bass. Apart from adult ovary, levels of alternatively-spliced *sb-AMH*exonII/-99 were marginal in comparison with *sb-AMH*. In contrast, the transcript variant *sb-AMH*exonVII/+5 was expressed to a similar extent as *sb-AMH* in all tissues examined. The results of this work have provided the basis for future studies concerning the regulation and function of AMH in this species.

1 Introduction

Anti-Müllerian hormone (AMH), (also called Müllerian-inhibiting substance (MIS)), a member of the transforming-growth factor- β family (TGF- β), has been named after its main function in mammals, birds and reptiles, which is the regression of the Müllerian duct in the male foetus during early testis differentiation (for review see Lane & Donahoe, 1998; Teixeira et al., 2001; Rey et al., 2003). Expression of AMH persists after completion of the reproductive duct system in males, and furthermore commences in females, where it is produced by ovarian granulosa cells from about week 36 of gestation in humans (Rajpert-De Meyts et al., 1999) and 6 days post-natally in mouse (Münsterberg and Lovell-Badge, 1991).

In the testis, AMH has been shown to inhibit the proliferation of Leydig cells and the expression of the steroidogenic enzymes cytochromes P450 side-chain cleavage (*CYP11*) and P450 17 α -hydroxylase,17-20-lyase (*CYP17*), which are key regulators in testosterone production (Trbovich et al., 2001). In females, AMH prevents the recruitment of primordial follicles and decreases the sensitivity of large preantral and small antral follicles to follicle stimulating hormone (FSH) (Durlinger et al., 2001, 2002). *In vitro* experiments have demonstrated that AMH action in the ovary is mediated by the down-regulation of luteinising hormone (LH) receptors and P450aromatase (Vigier et al., 1989; diClemente et al., 1992).

For nearly two decades, research on AMH had been focussed almost exclusively on mammals and birds. There were no reports of an *AMH* orthologue in teleost fish, and there might even have been some doubts about its existence given the name of this hormone and the fact that modern teleost do not have Müllerian ducts. Notwithstanding, in the summer of 2002, the isolation of a “spermatogenesis-related substance” (eSRS21) cDNA from the testis of Japanese eel was reported, and although bearing very low homology with mammalian and bird *AMH*, it showed remarkably similar expression patterns and functions as observed for mammalian AMH during gonadal development (Miura et al., 2002). Eel *eSRS21* was highly expressed in immature Sertoli cells, but strongly diminished after treatment with human chorionic gonadotropin *in vivo* and testosterone *in vitro*. Considering the low evolutionary conservancy of *AMH* between mammals and birds, it was assumed that this substance indeed could represent the teleost orthologue to *AMH* in mammals.

Since then, in a fairly short period of time, cDNAs with homologies to mammalian, bird and reptile *AMH* have been cloned in a variety of fish species, i.e. in Atlantic salmon (GenBank accession number [AY722411](#)), Japanese flounder (Yoshinaga et al., 2004), zebrafish (Rodríguez-Marí et al., 2005), and Japanese medaka (GenBank accession number [AY899282](#)), and all of them have been named after their mammalian orthologue.

In mammals, the proximal promoter of AMH displays a number of evolutionary conserved binding sites suggesting a common mechanism in the regulation of AMH expression (for review see Lasala et al., 2004). Moreover, a gene encoding the spliceosome protein SF3A2 has been found immediately upstream of the AMH promoter in human, mouse, and also in chicken (Dressler et al., 1995; Lasala et al., 2004). In mouse, promiscuous expression of AMH has been observed as a consequence of a read-through transcription from *SF3A2* (Dressler et al., 1995).

As a first step in contributing to unravel the evolution and function of AMH in teleost fish, we set out to isolate and clone *AMH* in the European sea bass (*Dicentrarchus labrax*), characterise its 5'-flanking region, and analyse its gene expression, including such of alternatively spliced isoforms we encountered during the cloning process. The European sea bass (sb), a fish species widely used in aquaculture, shows a high percentage of precocious males in captivity, which is undesirable, because males exhibit reduced somatic growth compared to females as they mature earlier (Carrillo et al., 1995). Thus, the cloning of *AMH* in this species could potentially help to understand better the molecular mechanisms involved in sexual differentiation and development in the European sea bass in order to control sex proportions in cultured stocks.

2 Materials and Methods

2.1 Cloning of *sb-AMH*

2.1.1 cDNA library screening

A testis sb-cDNA library constructed into the Uni-Zap XR vector (Stratagene, La Jolla, CA, USA) was screened with a Japanese eel *eSRS21* probe which had been synthesised by PCR from 400 pg of eel *eSRS21* cDNA inserted into pSD(X) (Miura et al., 2001). The probe contained nucleotide positions 991-1851 of Japanese *eSRS21* cDNA (GenBank accession number **AB074569**), including the most conserved region of the *eSRS21/AMH* gene. One million pfu were screened with a ³²P-α-dCTP labelled probe, and hybridisation was performed under low stringent conditions (30 % formamide, 600 mM NaCl, 40 mM sodium phosphate buffer, pH 7.4; 2.5 mM EDTA, 1 % SDS) at 42 °C overnight. Membranes were washed with 2X SSPE, 0.1 % SDS at room temperature for 30 min and exposed to a film at – 80 °C for 4 days. After three rounds of screening, four positive clones were excised into pBluescript SK(-), rescued into *E. coli* SOLR cells and sequenced at the Sequencing service at the University of Valencia.

2.1.2 Polymerase chain reaction

Clones obtained from the cDNA library and containing *sb-AMH* were truncated at their 5'-end. To obtain the missing N-terminal end of *sb-AMH*, polymerase chain reaction (PCR) was performed on the phage stock of the testis cDNA library. Approximately 1.4×10^7 pfu were used in a PCR reaction, containing 1 μ M of SK primer (containing the sequence of the SK primer binding site of pBluescript) and an AMH-specific reverse primer (3-AMH1: nucleotides (nt) 704-724 of **AM232701**), 2.5 μ M MgCl₂, 200 μ M of each dNTP, 2 units of DFS-Taq polymerase, and the buffer supplied with the enzyme (Bioron GmbH, Ludwigshafen, Germany). PCR amplification was performed using touchdown PCR for 20 cycles, with a range of temperatures from 63 to 53, decreasing 0.5°C each cycle followed by an additional 15 cycles at 53 °C.

Sequencing of the library clones revealed that the *sb-AMH* mRNAs were only partially processed or completely unprocessed and contained introns iii-vi. To obtain the complete pre-RNA/genomic sequence for *sb-AMH*, a genomic *AMH* fragment including introns i and ii was amplified by PCR on *Dra*I digested sea bass genomic DNA using the Genome Walker kit (Clontech, BD Biosciences, Madrid). PCR reactions included 1 μ M of 3-AMH1 and the kit adaptor primer, 2.5 μ M MgCl₂, 200 μ M of each dNTP, 2 units of DFS-Taq polymerase, and the buffer supplied with the enzyme (Bioron). The PCR programme consisted of 7 cycles at 94 °C/15 sec and 72°C/3 min, and of 32 cycles at 94°C/15 sec, 67 °C/30 sec, and 72°C/3 min with a final extension at 72°C/7 min. cDNA and genomic PCR products were cloned into pGEM-T vector (Promega, Southhampton, UK) and their nucleotide sequences determined.

2.2 Determining the exon/intron structure of *sb-AMH*

For confirmation of the cDNA sequence and the gene structure of *sb-AMH*, both that had been deduced from its pre-RNA/genomic sequence, we performed PCRs on cDNA of sea bass juvenile testis and on genomic sea bass DNA. 1 μ l of sea bass juvenile testis cDNA was used in PCR of 20 μ l total volume, using *sb-AMH* specific primers (5-AMH1 (forward): nt. 29-50 and 3-AMH2 (reverse): nt. 3214-3234 of **AM232701**) to amplify a cDNA fragment of 1843 bp, including the open reading frame (ORF) and 225 base pairs (bp) of the 3'-untranslated region. The *sb-AMH* gene was amplified to yield two DNA fragments of 1701 and 1793 bp using primers 5-AMH1 and reverse primer 3-AMH3 (nt. 1708-1729 of **AM232701**) and forward primer 5-AMH4 (nt. 1442-1462 of **AM232701**) and 3-AMH2, respectively. PCRs were performed using DFS-Taq polymerase and reagents as described above.

2.3 Cloning of the 5'-flanking region of *sb-AMH*

Putative transcription factor binding sites on the promoter region were determined by cloning about 3300 bp of the 5'-flanking region of *sb-AMH*, using the Genome Walker kit (Clontech). Four sea bass genomic libraries which had been constructed by adaptor-ligation and subsequent digestion of genomic DNA with *EcoRV*, *DraI*, *PvuII*, and *StuI* were used in PCR with specific primers derived from the 5'-end of *sb-AMH* and adaptor primers of the kit. Two runs of PCRs were performed, using a nested, *sb-AMH* specific primer in the second PCR and a 1/50 aliquot of the first PCR reaction. PCR reagents used were as described above, and the PCR programmes consisted of 7 cycles at 94 °C/15 sec and 72°C/3 min, followed by 32 cycles at 94°C/15 sec, 67 °C/30 sec and 72°C/3 min for the first round of PCR, and 5 cycles at 94°C/16 sec and 72°C/3 min, followed by 25 cycles at 94 °C/15 sec, 67 °C/30 sec and 72 °C/3 min in the second round of PCR. DNA fragments were cloned into pGEM-T vector (Promega) and sequenced. To exclude possible mutations introduced by *Taq* polymerase into the promoter sequence, we subsequently performed PCRs on genomic DNA with a high fidelity polymerase, *PfuTurbo* polymerase (Stratagene), and *sb-AMH* promoter specific primers to obtain the native sequence.

2.4 Gene expression of *sb-AMH* and of alternatively-spliced *sb-AMH* isoforms in gonadal and somatic tissues

Sb-AMH gene expression was analysed by RT-PCR and consecutive Southern hybridisation in gonads of juvenile and adult males and females, and in the pituitary, brain, liver, muscle, heart, and adipose tissue of juvenile males. RT was performed on 2 µg of total, DNase I-treated (1U/µg of total RNA; Promega) RNA, using 2.5 pmoles of a reverse *sb-AMH* specific primer (3-AMH4: nt. 3267-3288 of **AM232701**) and incubating the reactions at 55 °C for 1 hour with 1 U of SS III (Invitrogen). After enzyme deactivation, reactions were incubated with 2 U of RNase H (New England Biolabs Ltd., Hitchin, Hertfordshire, UK) for 20 min at 37 °C.

PCR reactions were performed to either amplify a 1843 bp *sb-AMH* cDNA fragment, including the complete ORF and about 230 bp of the 3'-untranslated region (as done under 2), or to amplify two alternatively spliced isoforms of *sb-AMH*, i.e. *sb-AMHexonII/-99* and *sb-AMHexonVII/+5*. To amplify *sb-AMHexonII/-99*, one forward (5-AMH1) and reverse primer (3-AMH5: nt. 496-515 of **AM232701**), flanking intron i, were used to amplify *sb-AMH exonII/-99* (201 bp) and/or “conventionally-spliced” *sb-AMH* (called *sb-AMH* in the following; 300 bp). To amplify *sb-AMHexonVII/+5* and/or *sb-AMH*, two forward primers, 5-AMH2 (nt. 1738-1755 and 2596-2598 of **AM232701**) and 5-AMH3 (nt. 1738-1755 and 2591-

2593 of **AM232701**), were used in combination with the reverse primer 3-AMH2 to yield cDNAs of 656 bp (*sb-AMH*) or 661 bp (*sb-AMH*exonVII/+5). To confirm the specificity of primers 5-AMH2 and 5-AMH3 for each cDNA we performed PCRs with both primer combinations on plasmid cDNA of clones 4 and 14 isolated from the cDNA library, each containing the respective variant (i.e. *sb-AMH* or *sb-AMH*exonVII/+5). PCR reactions for all PCRs were run in a total volume of 20 µl, using reagents and primer concentrations as described above. The PCR programme consisted of 35 cycles (for amplification of the 1843 bp cDNA fragment) or 24 cycles (for amplification of all other cDNA fragments) at 94°C/30 sec, 67°C/30 sec, and 72°C/2 min. All PCR reactions were run in an agarose gel, and subsequently transferred to nylon membranes. Hybridisation was carried out at high stringency (50 % formamide, 6X SSPE, 0.5 % SDS, 5X Denhardts, 0.1 mg/ml RNA type III from bakers yeast) with a 1843 bp, *sb-AMH*-specific, ³²P-α-dCTP labelled probe at 42 °C overnight. Membranes were washed with 0.1X SSPE, 0.1 %SDS at 65 °C and exposed to a film at -80°C.

2.5 Sequence analysis

Sequence analysis and comparisons of homology between *AMH* sequences were performed using European Molecular Biology Open Software Suite (EMBOSS). Phylogenetic analysis was done by multiple alignments of deduced amino acid (aa) sequences using the Neighbour-Joining method (Saitou and Nei, 1987) within the Clustal W programme. Evolutionary trees were constructed using MEGA, 3.1. Putative transcription factor binding sites were analysed using MatInspector, 7.4, Genomatix (Cartharius et al., 2005).

3 Results

3.1 Cloning of *sb-AMH*

In a BlastX search, three of the four clones isolated from the cDNA library showed highest identity with eel *eSRS2I*, and chicken, mouse, and human *AMH* (i.e. clones 4, 14 and 15), thus identifying these clones as *sb-AMH*. Sequencing of the complete cDNA of these clones revealed that clones 14 and 15 contained unprocessed introns, namely iii-vi. None of the clones was complete at the 5'-end; however employing PCR on the cDNA library and on genomic DNA, the missing 5'-end was determined, including the sequences and positions of introns i and ii. The pre-RNA/genomic DNA sequence for *sb-AMH* (GenBank accession number **AM232701**) consisted of 3408 bp, containing 7 exons divided by 6 introns (Fig. 1). Apart from intron vi (836 bp), introns were rather short, with lengths between 75 and 185 bp. All introns contained the highly conserved dinucleotides guanine (G) and thymine (T) and

adenine (A) and G at their 5' and 3' boundaries, respectively (Breathnach and Chambon, 1981).

The *sb-AMH* cDNA was 2045 bp long and possessed two potential initiation codons (i.e. ATG) 38 and 41 bp downstream of the 5'-end. The initiation codon at 41 bp was surrounded by nucleotides which according to Kozak (1986) provide a favourable context of initiation of translation. A stop codon (TAA) was present 349 nucleotides upstream of the 3'-end, which ended with a polyA-tail. There was a polyadenylation signal (ATTAAA) 20 bp upstream of the 3'-end, and in addition, a cytoplasmatic polyadenylation signal (CTTTTAT) was found 62 bp upstream of the 3'-end.

The ORF was 1599 bp long, encoding 533 aa. Its G and C nucleotide content was 52.7 %, and thus notably lower than that of mammalian and bird *AMH* (Table 1). Aligning the deduced amino acid sequence of *sb-AMH* with *AMH* in other species, at amino acid positions 423 and 426, in the immediate neighbourhood of the cleavage site for human *AMH* (Nachtigal and Ingraham, 1996), there were two potential proprotein convertase cleavage sites (consensus sequence: R-X-X-R, where R means arginine and X stands for any aa; Nachtigal and Ingraham, 1996) for *sb-AMH* (i.e. RGIR and RATR; Fig. 2). The TGF- β domain, which is highly conserved among members of the TGF- β family and characterised by seven conserved cysteine residues, was present in *sb-AMH* (Fig. 2). Deduced amino acid similarities between *sb-AMH* and *AMH* in other species were highest in the carboxyterminal domain, ranging between 84.2 % (Japanese flounder) and 45.5 % (chicken; Table 1). Regarding the whole protein, amino acid similarities were lower, and were between 77.6 % (Japanese flounder) and 36.3 % (mouse).

sb-AMH was phylogenetically most closely related to Japanese flounder *AMH* (Fig. 3) and grouped within a teleost clade of *AMH*. Chicken *AMH* was part of the (eutherian and marsupial) mammalian *AMH* clade. Evolutionary divergence between teleost *AMH*s was far greater than between (eutherian) mammalian *AMH*, namely 0.94 substitutions/site (between sea bass and zebrafish *AMH*) in contrast to only 0.27 substitutions/site (between mouse and pig *AMH*). All *AMH* were grouped in one main *AMH* clade and separated from human *GDF-1*, the latter forming an outgroup.

3.2 Regulatory elements on the promoter sequence of *sb-AMH*

By PCR-walking upstream of the putative translation start site of *sb-AMH*, 3326 nucleotides (relative to the ATG start codon) of the 5'-flanking region of *sb-AMH* were cloned (GenBank accession number **AM232702**). Putative core promoter elements were found at -46 to -62 and at -76 to -92 (TATA protein binding sites), at -50 to -68 (proximal sequence element of RNA

polymerase transcribed genes), and at -35 to -49 and at -163 to -177 (CCAAT protein binding sites). In close proximity to these core promoter elements, at -144 to -168 there was a nucleotide sequence that had (partially) overlapping binding sites for steroidogenic factor 1 (SF-1), estrogen related receptor (ERR), and progesterone receptor (PRE).

Among transcription factor binding sites that have been described for mammalian AMH, putative binding sites for members of the high mobility group (HMG) of proteins, i.e. SOX9 (-1419, -1687; -2300) and SRY (-327), as well as GATA proteins (-431; -476; -538; -608; -669; -779; -860; -873; -1572; -3096) and the nuclear orphan receptor SF-1 (-160; -2563) were also observed on the sea bass AMH promoter. Furthermore, there were binding sites for SOX5 (-1976; -2675 and -3259) and the progesterone receptor (-167; -891; -1623). In comparison with the first 3300 bp of the zebrafish AMH promoter, various transcription binding sites for homeodomain proteins were present in both sequences, namely for members of the ANTP (i.e. Hox, Gsh, NKx, Barx, and Cdx), PRD (i.e. Crx, Phox, and Pax), LIM (i.e. Pdx and Isl), TALE (i.e. Meis and Pbx), VENT (i.e. Xvent), OC-1 (i.e. HNF-6), and POU classes (Pit, Brn, Oct, and Clox). Common binding sites for bZip transcription factors included such for albumin D-box binding protein, chicken vitellogenin promoter binding protein, TEF (thyrotropic embryonic factor), MTF (melanocyte transcription factor) and E4BP (adenovirus E4 binding protein). Furthermore, there were consensus sequences for members of the forkhead transcription family, notably for Fox D1, FoxQ1, and FoxH1. There were also binding sites for members of the HMGP family, i.e. SOX5, SOX9, and SRY and for GATA (GATA-1, GATA-2, and GATA-3). Lastly, nuclear receptor binding sites were present on both sequences with similarities to retinoic acid, progesterone/androgen, and estrogen related receptor.

3.3 The presence of *sb-AMH* alternatively spliced isoforms

During the cloning process and/or PCR analysis, two isoforms of *sb-AMH* were isolated, called *sb-AMH*exonII/-99 (GenBank accession number [AM232703](#)) and *sb-AMH*exonVII/+5 (GenBank accession number [AM232704](#)). While *sb-AMH*exonII/-99 was obtained partially and consisted of 665 bp of the N-terminal region of *sb-AMH*, *sb-AMH*exonVII/+5 was complete, consisting of 2050 bp. Both isoforms derived from an alternative splicing of intron/exon boundaries, recognising distinct 3'-end splice sites (i.e. dinucleotides AG) that were located either 99bp further downstream (*sb-AMH*exonII/-99) or 5 bp upstream (*sb-AMH*exonVII/+5) of the original 3'- intron/exon boundaries (Fig. 4A and B). Hence, exon II of *sb-AMH*exonII/-99 was 99 bp shorter, whereas exon VII of *sb-AMH*exonVII/+5 was 5 bp longer than the respective exon of *sb-AMH*. In the case of *sb-AMH*exonVII/+5, the uneven number

of inserted (intron vi-derived) nucleotides (i.e. 5) provoked a frame-shift in the ORF, leading to a premature stop codon (TGA) 36 nucleotides downstream of the intron/exon boundary (Fig.4B). Hence, *sb-AMHVII/+5* did not encode the bioactive part of *sb-AMH*.

3.4 Expression of *sb-AMH* and of *sb-AMH* alternatively-spliced isoforms

By RT-PCR and subsequent hybridisation of the PCR products, a 1843 bp *sb-AMH* cDNA was detected in juvenile and adult testis, juvenile ovaries and in juvenile male brain (Fig. 5). Although the PCR was not strictly quantitative, the result suggests that *sb-AMH* expression in juvenile testis was much higher than in adult testis and juvenile ovary, but similar to *sb-AMH* expression in juvenile male brain. There were some smaller, less-intensively hybridising bands of about 1450 and 560 bp.

There was expression of *sb-AMH exonII/-99* in all tissues studied; however, its levels were in most cases lower compared with *sb-AMH* (Fig. 6). In contrast, in the adult ovary, *sb-AMH exonII/-99* was the predominantly-expressed isoform. There was another band at 485 bp, which appeared most strongly in the liver.

Gene expression of *sb-AMH exonVII/+5* was found in juvenile testis, ovaries, and in juvenile male pituitary, brain, heart, and adult testis and its levels were very similar to *sb-AMH* expression in the respective tissues (Fig. 7A and B). Indeed, we roughly estimated it to account for about 50 % of total *AMH* transcripts. Plasmids carrying the respective *AMH* transcript were used as controls for verifying the specificity of the primers for each mRNA. There were some faint hybridisation signals in the negative controls, in particular for *sb-AMH exonVII/+5*, which were probably due to hybridisation of the probe with the *AMH* insert of the plasmid cDNA, the latter having migrated at various positions in the gel (Fig. 7A and B). When increasing the cycle number of the PCR programme, faint bands for *sb-AMH* and *sb-AMH exonVII/+5* were also detected for adult ovary (result not shown).

4 Discussion

In this study we report the cloning and characterisation of *sb-AMH* and the analysis of its expression including such of *sb-AMH* alternatively-spliced isoforms in gonadal and somatic tissue. The gene of *sb-AMH* contains unique features that distinguishes it from mammalian (Cate et al., 1986; Münsterberg and Lovell-Badge, 1991; Haqq et al., 1992) and bird *AMH* (Carré-Eusèbe et al., 1996), suggesting a high rate of diversification during (part of) *AMH* evolution. Mammalian and bird *AMH* consist of five exons, whereas *sb-AMH* (this study) and zebrafish *AMH* (Rodríguez-Marí et al., 2005) have seven. The two additional exons are created by the insertion of intronic sequences into exon I and V of mammalian and bird *AMH*,

creating exons II and VII in *sb*- and zebrafish *AMH* (compare Fig. 1). Most notably, interruption of (mammalian/bird *AMH*) exon V occurs only a few basepairs upstream of the beginning of the sequence coding for the bioactive part of the AMH protein. However, the remaining exons (i.e. exons II, III, and IV in mammalian/bird *AMH* and exons III, IV, and V in *sb*- and zebrafish *AMH*) encode homologous portions of the AMH protein, and thus introns dividing them are found at identical positions. Our own observations concerning the gene structure of *AMH* in Fugu (clone M000303 of the Fugu Genomics Project, <http://fugu.biology.qmul.ac.uk>), Tetradon (clone GSCT00010419001 of the Tetradon Genome browser, <http://www.genoscope.cns.fr/externe/tetraodon>) and Japanese medaka (GenBank accession number **AY899284**) point towards identical gene structures of *AMH* in these species. Nevertheless, since we do not possess any information about the *AMH* gene structure in more ancient teleost species (like eel) nor in any other group of fish, it is difficult to speculate whether the discordant number of introns between mammalian/bird and seabass/zebrafish *AMH* is due to a loss of ancestral introns or rather a gain of novel introns into a particular lineage.

The proximal *AMH* promoters (approximately 230 bp) of eutherian mammals and marsupial *AMH* contain evolutionary conserved binding sites, including two binding sites for SF-1 (Watanabe et al., 2000), two for GATA (Tremblay and Vigier, 1999; Watanabe et al., 2000) one for SOX9 (de Santa Barbara et al., 1998; Arango et al., 1999) and one for WT-1 (Hossain and Saunders, 2003). These binding sites are found at homologous parts of the *AMH* promoter in different species, and *in vitro* and *in vivo* studies have proven their functionality in *AMH* transactivation (de Santa Barbara et al., 1998; Arango et al., 1999; Tremblay and Vigier, 1999; Watanabe et al., 2000; Hossain and Saunders, 2003). In contrast, the sequence and organisation of the chicken *AMH* promoter (1050 bp) is drastically divergent from the sequence of mammalian *AMH* promoters (Oréal et al., 1998). There is only one SF-1 binding site, which is located just 5 bp upstream of a TATA-box, and putative SOX-binding sites are positioned more upstream compared with mammalian *AMH* promoters. GATA binding sites are completely absent.

Analysing the *sb-AMH* promoter we similarly found that the proximal region was very distinct from mammalian *AMH* promoters. Although several putative binding sites for SOX and GATA were scattered along the *sb-AMH* promoter, they were neither located in the proximal region nor were they found in an organisational context similar to mammalian *AMH* in any other (more upstream) region. Nevertheless, as observed for chicken *AMH*, there was an SF-1 binding site in the proximal *sb-AMH* promoter, which furthermore could be aligned to a SF-1/ERE binding site in the proximal promoter of zebrafish *AMH* (clone zK211F22 of

zebrafish genomic database) and mammalian *AMH*. In the mammalian male foetus, *SOX9* initiates *AMH* expression (and thus Müllerian duct regression) whereas *SF-1* has been shown to be a potent up-regulator of *AMH* expression (Arango et al., 1999). However, *AMH* is also expressed in tissues where *SOX9* is not expressed, like ovarian granulosa cells, and studies in the chicken and alligator have shown that *AMH* transcripts are present before the commencement of *SOX9* transcription (Oréal et al., 1998; Western et al., 1999). Furthermore, the expression pattern of *SF-1* in chicken suggests an important role for the initiation of *AMH* transcription (Smith et al., 1999). Therefore, there is the possibility that in some species of vertebrates, *SF-1* is the key regulator of *AMH* transcription. In this context, recently in zebrafish a nuclear orphan receptor homologous to *SF-1* and termed *ff1d* has been shown to have an identical expression pattern to *AMH* during embryonic development, and to be highly expressed in adult testis, where it was localised in Sertoli and Leydig cells (von Hofsten et al., 2005).

Lastly it should be emphasised that *in vitro* studies in mammals have shown that a range of transcription factors, including GATA (Tremblay and Viger, 1999), *SOX9* (de Santa Barbara et al., 1989), and WT-1 (Nachtigal et al., 1998) can interact and synergise with *SF-1* without binding to their consensus binding site on the *AMH* promoter. The absence of putative binding sites for these factors on the sea bass (and other teleost/vertebrate) *AMH* promoter therefore does not necessarily exclude the possibility that at least some of them participate in the transcriptional activation of the *AMH* promoter.

In mammals and chicken, a gene encoding the house-keeping, spliceosome protein *SF3A2* has been localised at about 400 and 1200 bp upstream of the transcription start site of *AMH*, respectively (Dressler et al., 1995; Lasala et al., 2004). Since *SF3A2* is constitutively expressed, and a number of *SF3A2* transcripts moreover seem to fail to polyadenylate and are thus transcribed through *AMH*, it is likely that the *AMH* locus is in a permanent open chromatin stage in these species (Dressler et al., 1995). Genomic regions that possess high transcriptional activity and thus an open chromatin structure are often characterised by a high GC content including coding sequences (Bernardi, 2000; Cruveiller et al., 2000). It has been hypothesised that the high GC content is needed to stabilise the DNA of the open chromatin (Saccone et al., 2002). In line with these observations, we noted a high GC content of the ORF in *AMH* of species which are known to possess *SF3A2* upstream of the *AMH* promoter (i.e. human, mouse, and chicken). In contrast, the GC content of *AMH* in sea bass and zebrafish (as well as in Atlantic salmon and Japanese flounder) was notably lower. Interestingly, the results of a BLASTX search of about 3200 bp of the 5'-flanking region of *AMH* in sea bass and zebrafish did not reveal any homologies to *SF3A2*. Hence, someone

could argue that the high GC content observed for *AMH* in mammals and birds has evolved as a consequence of the presence of *SF3A2* upstream of the *AMH* promoter in these species. Curiously, Japanese eel *eSRS21* is characterised by a high GC content; however since its promoter has, to our knowledge, not been characterised this hypothesis will await further clarification.

In consistency with the function of *AMH* during testicular development, we observed high *AMH* expression in the testis of juvenile sea bass. *Sb-AMH* gene expression was also detected in juvenile and adult ovary, although in the latter it was very low. The presence of *AMH/AMH* in gonadal tissue, concretely in the Sertoli cells of the testis and the granulosa cells of the ovary, has been well-described in mammals (Cate et al., 1986; Münsterberg and Lovell-Badge, 1991; Haqq et al., 1992; Juengel et al., 2002; Pask et al., 2004), birds (Eusèbe et al., 1996; Oréal et al., 1998; Vaillant et al., 2001), and reptiles (Wibbels et al., 1998; Western et al., 1999). Similarly, in teleost fish, i.e. Japanese eel, zebrafish and Japanese flounder, *AMH* transcripts have been found in gonadal tissue of one or both sex, with highest levels in the testis (Miura et al., 2002; Yoshinaga et al., 2004; Rodríguez-Marí et al., 2005; von Hofsten et al., 2005).

In this study we also observed expression of *sb-AMH* in extra-gonadal tissues derived from juvenile males. Although *sb-AMH* transcript levels in adipose and muscle were barely detectable (and only when augmenting the cycle number of the PCR and/or amplifying small cDNA fragments thereby increasing PCR efficiency), considerable levels of *sb-AMH* expression were observed for juvenile male brain, heart, and pituitary. In mammals, continuous expression of *AMH* after Müllerian duct regression has been mainly associated with its role during gonadal development, including effects on steroidogenesis (di Clemente et al., 1992; Racine et al., 1998; Laurich et al., 2002), Leydig cell function (Wu et al., 2005), and follicle recruitment (Baarends et al., 1995; Durlinger et al., 2001, 2002). Nevertheless, proper gonadal function and development is dependent on a functional hypothalamic-pituitary-gonadal axis. *In vivo*, addition of *AMH* to L β T2 cells (a murine gonadotrope derived cell-line) induced *FSH β* mRNA levels and activated transcription of *FSH β* and *LH β* gene promoters (Bédécarrats et al., 2003). Interestingly, L β T2 cells also expressed the *AMH* receptor and the same was observed for adult rat pituitaries. Hence, the presence of *sb-AMH* transcripts in the pituitaries might point towards an autocrine role of *AMH* in the regulation of gonadotropin synthesis.

Expression of *sb-AMH* was particularly high in the brain of juvenile male sea bass. In mammals the inhibitory effect of *AMH* on P450 aromatase (*CYP19/CYP19*) expression and activity in the ovary and testis *in vitro* has been reported (Vigier et al., 1989; di Clemente et

al., 1994; Rouiller-Fabre et al., 1998). Given the high levels of P450 aromatase mRNA transcripts and the high P450 aromatase activity found during certain developmental stages in the brain of teleost fish (Gelinas and Callard, 1993), we could speculate that *AMH* is involved in the regulation of P450 aromatase expression and activity in the brain. In the European sea bass, P450 aromatase activity in the brain is 10-fold higher in adult animals compared to juveniles (González and Piferrer, 2003). However, the exact biological function of the high amounts of neuroestrogens produced by P450 aromatase in the brain is still an unresolved issue. In teleost fish it has been suggested that the high production of neuroestrogens is related to the remarkable neuroplasticity and regenerative potential of the adult fish central nervous system (Callard et al., 2001). Notably, in recent times *AMH* and the *AMH* receptor have been reported to be simultaneously expressed in adult mice motor neurons (Wang et al., 2005). As the concurrent presence of *AMH* and its receptor would enable intercommunication between neighbouring motor neurons, the authors suggested that *AMH* could be important for neuroregeneration after injury, as interactions between motor neurons are known to be more prevalent after damage to motor axons/nerves. Future studies employing *in situ* hybridisation and immunocytochemistry for exact cell localisation of *sb-AMH*/*sb-AMH* in the brain as well as the isolation and localisation of the *sb-AMH* receptor would be needed to shed more light on this issue.

During the cloning process and PCR analysis, we encountered two alternatively-spliced *sb-AMH* transcript which we named, according to their altered exon structure in comparison with *sb-AMH*, *sb-AMH*exonII/-99 and *sb-AMH*exonVII/+5. To our knowledge, the presence of alternatively-spliced *AMH* transcripts has not been reported before. In some mammalian species and in chicken, more than one transcript hybridised to a *AMH*-specific probe in Northern analysis, however their identities remained unknown (Cate et al., 1986; Carré Eusèbe et al., 1996; Juengel et al., 2002;). *AMH*, like other members of the TGF- β family, is synthesised as a large precursor protein with a cleavage site approximately 110 aa from the C-terminus (compare Fig. 2). Cleavage of this site results in the proregion, the N-terminal domain, and the mature, bioactive protein, the C-terminal domain (for review see Ingraham et al., 2000; Josso and di Clemente, 2003). In eel, there is evidence that eSRS21 is produced as a 60 kDa precursor protein but secreted by proteolysis as a monomeric 30 kDa protein (Miura et al., 2002).

For most members of the TGF- β family, the biological activity resides exclusively in the C-terminal domain. The N-terminal domain, apart from being probably required for folding and secretion of the C-terminal domain, has no own biological activity, and can even, as in the case of TGF- β , inactivate the mature protein (Massagué, 1990). In contrast, the bioactivity

of human AMH has been demonstrated to be dramatically increased by association with its N-terminal domain *in vitro* (Wilson et al., 1993). It is therefore tempting to speculate that *sb-AMH* variants comprising different N-terminal domains (as in the case of *sb-AMHexonII/-99*) or those whose C-terminal domain is not translated (as in the case of *sb-AMHexonVII/+5*) might exert distinct effects on the bioactivity of *sb-AMH*. Finally, it should be emphasised that the results of the hybridisation of PCR products indicate that *sb-AMH* splice variants others than *sb-AMHexonII/-99* and *sb-AMHexonVII/+5* exist. In fact, the size (about 485 bp) of a *AMH*-specific band that appeared when amplifying *sb-AMHexonII/-99* would be compatible with an *AMH* transcript having retained (part of) intron i.

In summary, in this study we have isolated and characterised the gene for *AMH* in the European sea bass. Its distinct gene structure (including the 5'-flanking region) and composition together with its low deduced amino acid similarity compared with mammalian *AMH* suggests a high rate of diversification of *AMH* during vertebrate evolution. Furthermore the presence of alternatively-spliced isoforms of *sb-AMH* and their putative translation into distinct proteins points towards a complex regulatory mechanism of *AMH* transcription and protein activity. Lastly, the presence of *sb-AMH* in brain and pituitary opens up the possibility of an autocrine control of gonadotropin and steroid synthesis of AMH in these tissues, and together with the presence of *sb-AMH* in the heart additionally suggest a role for AMH in functions other than in reproduction.

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Legend

Figure 1: Gene structure of *sb-AMH* in comparison with zebrafish and mouse *AMH*. Exons (I-V in mouse and I-VII in zebrafish and sea bass) and introns (i-iv in mouse and i-vi in zebrafish and sea bass) are depicted by boxes and lines, respectively. The 5'- and 3'-untranslated regions are grey-shaded and the region coding for the bioactive protein of *AMH* is dotted. For zebrafish *AMH*, the lengths of the 5'- and 3'-untranslated regions are unknown.

Figure 2: Alignment of the deduced amino acid sequences of *AMH* in European sea bass, Japanese flounder, Japanese eel, zebrafish, human, mouse, wallaby and chicken.. The signal peptide sequence and the beginning of the N-and C-terminal domain for AMH in human are shown with arrows. The peptide cleavage site in human as well as potential cleavage sites for AMH in other species are boxed and dotted. The TGF- β domain is grey-shaded and the 7 conserved cysteines of this domain are boxed. Identical and similar amino acids are marked by asterix and dots (: = strong score, . = weak score according to comparison matrix Gonnet Pam250, respectively).

Figure 3: Phylogenetic tree based on a deduced amino acid alignment using the Neighbour-Joining method for *AMH* in eutherian and marsupial mammals, birds, and teleost fish. Human *GDF-1* is used as an outgroup. Bootstrap values (%) are indicated (1000 replicates). Branch lengths are drawn in proportion to evolutionary distance.

Figure 4: *sb-AMH* alternatively-spliced isoforms in comparison with “conventionally-spliced” *sb-AMH*. A: *sb-AMH*exonII/-99; B: *sb-AMH*exonVII/+5. Both isoforms result from an alternative splicing of introns i and vi, respectively, utilising 3'-splice sites (ag) that are positioned either 99 bp downstream (for *sb-AMH*exonII/-99) or 5 bp upstream (for *sb-AMH*exonVII/+5) of the original 3'-splice sites. Introns are depicted by lines and exons by boxes. The highly conserved gt and ag dinucleotides, marking the beginning and the end of an intron, are shown. The 3'-untranslated region is grey-shaded, and the part coding for the bioactive region (in the case of *sb-AMH*) of the protein is dotted. Stop codons are marked with an asterix.

Figure 5: Southern hybridisation of RT-PCR products generated with *sb-AMH* primers to amplify a 1843 bp *sb-AMH* cDNA fragment, containing the complete ORF and 225 bp of the 3-UTR. Juv: juvenile; ad: adult; te: testis; ov: ovaries; pit: pituitary; br: brain; liv: liver; mu: muscle; he: heart; ad: adipose. Somatic tissues were derived from juvenile males.

Figure 6: Southern hybridisation of RT-PCR products generated with *sb-AMH* primers to amplify 201 and 300 bp *sb-AMH* cDNA fragments, representing *sb-AMH*exonII/-99 and *sb-AMH*, respectively. Juv: juvenile; ad: adult; te: testis; ov: ovaries; pit: pituitary; br: brain; liv: liver; mu: muscle; he: heart; ad: adipose. Somatic tissues were derived from juvenile males.

Figure 7: Southern hybridisation of RT-PCR products generated with *sb-AMH* primers to amplify either (A) a 656bp *sb-AMH* cDNA fragment or (B) a 661 bp *sb-AMH* cDNA fragment, representing *sb-AMH*exonVII/+5. Juv: juvenile; ad: adult; te: testis; ov: ovaries; pit: pituitary; br: brain; liv: liver; mu: muscle; he: heart; ad: adipose. Somatic tissues were derived from juvenile males. *psbAMH*: plasmid containing *sb-AMH*; *psbAMH*exonVII/+5: plasmid containing *sb-AMH*exonVII/+5. Somatic tissues were derived from juvenile males.

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Table 1: Amino acid similarities (%) of the C-terminal and entire protein sequence of sea bass AMH with AMH in other species and GC content (%) of nucleotide sequences of the open reading frame.

	C-terminal domain	Complete deduced amino acid sequence	GC content of ORF
human	48.6	38.0	73.3
mouse	47.0	36.3	65.2
tammar wallaby	53.5	39.0	62.3
chicken	45.5	39.7	66.8
Japanese eel	67.7	52.1	68.1
zebrafish	67.3	55.4	50.6
Atlantic salmon	73.3	61.4	55.2
Japanese flounder	84.2	77.6	56.4
European sea bass	100	100	52.7

Figure(s)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 7