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

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Abstract

In mammals, a multitude of studies have shown that anti-Müllerian hormone (*AMH*/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*/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), *Dicentrachus 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-AMHexonII/−99* were marginal in comparison with *sb-AMH*. In contrast, the transcript variant *sb-AMHexonVII/+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*/AMH in this species.

Keywords: Müllerian-inhibiting substance (*MIS*/MIS); *Dicentrarchus labrax*; Transforming-growth factor-β family; Promoter; Transcription binding sites; Transcript variants

1. Introduction

Anti-Müllerian hormone (*AMH*/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 and Donahoe, 1998; Teixeira et al., 2001; Rey et al., 2003). Expression of *AMH* persists after completion of the reproductive duct system in

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.

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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 (*CYP*11) and P450 17α-hydroxylase,17-20-lyase (*CYP*17), 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 P450 aromatase (Vigier et al., 1989; Di Clemente 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 (Dresser et al., 1995; Lasala et al., 2004). In mouse, promiscous expression of *AMH* has been observed as a consequence of a read-through transcription from *SF3A2* (Dresser 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., 2002). 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 ^{32}P-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 2× 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 μM of SK primer (containing the sequence of the SK primer binding site of pBluescript) and a *sb-AMH*-specific reverse primer (3-AMH1: nucleotides (nt) 704–724 of AM232701), 2.5 μM $MgCl_2$, 200 μM of each dNTP, 2 U of DFS-Taq polyermase, 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 μM of 3-AMH1 and the kit adaptor primer, 2.5 μM $MgCl_2$, 200 μM of each dNTP, 2 U of DFS-Taq polymerase, and the