Subcellular and Molecular Mechanisms Regulating Anti-Müllerian Hormone Gene Expression in Mammalian and Nonmammalian Species

CELINA LASALA,1 DANIÈLE CARRÈ-EUSÈBE,3 JEAN-YVES PICARD,3 and RODOLFO REY1,2

ABSTRACT

Anti-Müllerian hormone (AMH) is best known for its role as an inhibitor of the development of female internal genitalia primordia during fetal life. In the testis, AMH is highly expressed by Sertoli cells of the testis from early fetal life to puberty, when it is downregulated by the action of testosterone, acting through the androgen receptor, and meiotic spermatocytes, probably acting through TNFα. Basal expression of AMH is induced by SOX9; GATA4, SF1, and WT1 enhance SOX9-activated expression. When the hypothalamic–pituitary axis is active and the negative effect of androgens and germ cells is absent, for example, in the fetal and neonatal periods or in disorders like androgen insensitivity, FSH upregulates AMH expression through a non-classical cAMP-PKA pathway involving transcription factors AP2 and NFκB. The maintenance and hormonal regulation of AMH expression in late fetal and postnatal life requires distal AMH promoter sequences. In the ovary, granulosa cells express AMH from late fetal life at low levels; DAX1 and FOG2 seem to be responsible for negatively modulating AMH expression. Particular features are observed in AMH expression in non-mammalian species. In birds, AMH is expressed both in the male and female fetal gonads, and, like in reptiles, its expression is not preceded by that of SOX9.

INTRODUCTION

I

N EUHERIAN MAMMALS, sexual differentiation of the gonads is genetically determined, whereas hormones control sexual differentiation of the genitalia. Genetic sex, as determined by chromosome constitution, XY for male or XX for female, drives the indifferent gonad to differentiate into a testis or an ovary, respectively. The Y chromosome harbors the sex-determining region on the Y gene (SRY), whose expression is thought to trigger the differentiation of the Sertoli cell lineage, and in turn, the rest of the cell types in the testis (Tilmann and Capel, 2002; Harley et al., 2003). Once the gonads are differentiated as testes, they secrete two distinct hormones involved in normal male sexual differentiation: anti-Müllerian hormone (AMH) and testosterone. AMH, also called Müllerian inhibiting substance (MIS) or factor (MIF), is a Sertoli cell glycoprotein that causes regression of the Müllerian ducts, which in the normal female fetus develop into the uterus, Fallopian tubes, and upper portion of the vagina. This is AMH’s best known function, which takes place very early in fetal development (Picon, 1969; Taguchi et al., 1984). However, AMH also modulates Leydig (Racine et al., 1998; Rouiller Fabre et al., 1998; Sriraman et al., 2001; Trbovich et al., 2001; Laurich et al., 2002) and granulosa cell (Vigier et al., 1987, 1988, 1989; di Clemente et al., 1992; Durlinger et al., 1999, 2002; Grujters et al., 2003) differentiation and function, and is secreted in the male during the whole fetal life and infancy, only declining at puberty (Fig. 1) (Tran et al., 1977, 1981; Kuroda et al., 1990; Münsterberg and Lovell-Badge, 1991; Lee et al., 1992, 1994, 1996; Rey et al., 1993, 1996; Al-Attar et al., 1997; Rajpert-De Meyts et al., 1999). In the female, granulosa cells of the ovary express AMH only from late fetal life (Bézard et al., 1987; Ueno et al., 1989; Rajpert-De Meyts et al., 1999; Rey et al., 2000).

1Centro de Investigaciones Endocrinológicas (CEDIE-CONICET), Hospital de Niños R. Gutiérrez, Buenos Aires, Argentina.
2Centro de Investigaciones en Reproducción, Departamento de Histología, Biología Celular, Embriología y Genética, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.
3Unité de Recherches sur l’Endocrinologie du Développement (INSERM), Clamart, France.
AMH is a 140-kDa disulfide-linked glycoprotein dimer that belongs to the transforming growth factor beta (TGF-β) superfamily (Cate et al., 1986). The human protein is synthesized as a precursor of 560 amino acids (aa). The immature protein has a 25 aa leader containing a 16–18 aa signal sequence and a putative 7–8 residue pro-sequence, whose cleavage after a leucine residue generates a mature protein with a molecular weight of 57 kDa (Cate et al., 1986). As other members of the TGF-β superfamily, AMH must be cleaved to be fully active. A monobasic proteolytic cleavage site is located at 109 aa from the C-terminus, between arginine 427 and serine 428. The C-terminal fragment of the protein carries the biological activity, but addition of the N-terminus enhances the activity of the C-terminal moiety (Wilson et al., 1993), an unusual feature for a TGF-β superfamily member.

The human AMH (hAMH) gene is located on 19p13.2–13.3 (Cohen Haguenauer et al., 1987). It is a relatively short gene of 2.75 kilo bases (kb) divided into five exons that gives rise to a 1.7-kb long mRNA (Cate et al., 1986). The AMH gene also consists of five exons in the mouse (Münsterberg and Lovell-Badge, 1991; King et al., 1991), rat (Haqq et al., 1992), bovine (Cate et al., 1986; Picard et al., 1986) and wallaby (Pask et al., 2004). The sequence of exons 2 to 5 is highly conserved among species. The fifth exon bears the highest interspecies homology and is the only exon of the AMH gene to show homology with other members of the TGF-β family (Cate et al., 1986).

The bovine (Cate et al., 1986) and mouse (Hossain and Saunders, 2003) AMH promoters contain a canonical TATA box and a single transcription start site, located 10 bp upstream of the ATG codon, whereas the human promoter does not have consensus TATA or CCAAT box elements (Cate et al., 1986; Guerrier et al., 1990). In the human promoter, there is a degenerate TTAA motif at −26 base pairs (bp) and also a functional initiator (Inr) element that is recognized by transcription factor TFII-I (Morikawa et al., 2000).

The AMH proximal promoter displays a number of evolutionary conserved binding elements including two for steroidogenic factor 1 (SF1) (Shen et al., 1994; Watanabe et al., 2000), two for GATA (Tremblay and Viger, 1999; Watanabe et al., 2000), and one for SOX9 (de Santa Barbara et al., 1998; Arango et al., 1999). Recently, a binding site for Wilms’ tumor-associated-protein 1 (WT1) has also been described very close to the transcriptional start site (Hossain and Saunders, 2003). The proximal promoter of the AMH gene in eutherian mammals and wallaby show the same transcription factors binding sites, suggesting a common mechanism in the regulation of AMH expression in mammals (Lukas-Croisier et al., 2003; Pask et al., 2004).

The chronological and sexually dimorphic expression pattern of AMH is crucial in sex differentiation. During fetal life AMH must be expressed in the male before Müllerian ducts lose their responsiveness and should be absent in the female. AMH is the earliest Sertoli cell-specific marker in the developing testis. Its expression can be detected at the time of testicular cord formation in the fetus at 7 weeks in the human (Josso et al., 1993), 11.5 days postcoitum in the mouse (Hacker et al., 1995), and 13.5 days postcoitum in the rat (Tran et al., 1977).

In the bipotential gonad, SF1, SOX9, WT1, GATA4, and
DAX1 are expressed among other early genes. Once the testis has differentiated, SOX9, SF1, WT1, and GATA4 expression are upregulated and DAX1 is downregulated (Tilmann and Capel, 2002). AMH transcription is initiated by the binding of SOX9 to the conserved SOX site in the AMH proximal promoter (Arango et al., 1999). If this site is mutated, AMH transcription does not begin, leading to the retention of Mullerian ducts-derived tissues. Albeit at lower levels compared with SOX9, SOX8 has been found to synergize with SF1 to enhance AMH transcription through protein–protein interaction in mice (Schepers et al., 2003). The hAMH promoter has two functional SF1 binding sites (Shen et al., 1994; Giuili et al., 1997; Arango et al., 1999; Watanabe et al., 2000) and two GATA sites (Viger et al., 1998; Tremblay and Viger, 1999; Watanabe et al., 2000; Tremblay et al., 2001) (Fig. 2). The mutation of both SF1 binding sites completely abolishes AMH expression to a greater degree than does the mutation of either SF1 single site, and similar results are observed for GATA sites, suggesting that, in the context of the hAMH promoter, two sets of SF1 and GATA4 binding sites are required for the upregulation of AMH expression in the early developmental stage (Watanabe et al., 2000). WT1, first thought to increase AMH transcription only through physical contacts with SF1 (Nachtigal et al., 1998), has recently been shown to bind to a rich GC sequence in the murine AMH promoter, which is located between the TATA box and the transcriptional start site (Hossain and Saunders, 2003). It was also demonstrated that WT1, SOX9, and SF1 could synergistically activate AMH promoter when WT1 is bound to the DNA (Hossain and Saunders, 2003). DAX1 is a transcription factor that represses AMH expression by disrupting GATA4/SF1 and WT1/SF1 synergisms in Sertoli cells through a direct interaction with SF1 (Fig. 2) (Nachtigal et al., 1998; Tremblay and Viger, 2001b).

**FIG. 2.** Regulation of basal AMH expression in the fetal testis. (A) SOX9 initiates AMH expression through binding to a specific response element. SF1, GATA4, and WT1 also bind to the AMH promoter and enhance transcriptional activation. GATA4 and WT1 may also act by interacting with SF1, without binding to DNA. (B) DAX1 is capable of interacting with SF1 and disrupts GATA4/SF1 and WT1/SF1 synergisms in Sertoli cells.

### REGULATION OF AMH EXPRESSION IN THE POSTNATAL TESTIS

After birth, AMH testicular secretion is maintained at high levels until puberty when it declines, in coincidence with the elevation of androgen concentration within the testis and the onset of germ cell meiosis (Rey et al., 1996; Al-Attar et al., 1997; Rajpert-De Meys et al., 1999). The decrease of AMH secretion can be understood as an indication of Sertoli cell maturation at the beginning of puberty.

The AMH promoter: only 370-bp long?

For many years, it was believed that the *cis*-elements located in the proximal promoter were sufficient to regulate AMH expression. All the results described in the previous paragraphs were based on studies using less than 400 bp of the hAMH promoter. Furthermore, the SAP62 (SF3A2) gene, coding for a spliceosome protein that is ubiquitously expressed, lies 789 bp upstream of the start of translation site in the human gene and 434 pb in the mouse gene (Dresser et al., 1995). However, transgenic mice for various constructs of LacZ under the control of AMH regulatory sequences showed that a 370-bp proximal promoter, although sufficient for the onset of AMH expression in early fetal life, is not capable of maintaining it until puberty (Beau et al., 2001). We have used the prepubertal mouse Sertoli cell line SMAT1 (Dutertre et al., 1997) in transfection experiments with luciferase reporter constructs containing different lengths of the AMH promoter. These experiments showed for the first time that a 3-kb promoter is far more active than a 423-bp and a 202-bp promoter (Fig. 3A) (Lukas-Croisier et al., 2003). These results clearly indicate that distal 5′ sequences, whose importance has often been disregarded, are essential for the maintenance of the high AMH expression levels observed in Sertoli cells. Apart from the already described SOX9-, SF1-, and GATA-binding sites, we found a further GATA-binding site and consensus response elements for NFκB and AP2 more than 400 bp upstream of the transcription start site, lying even upstream of SAP62.

**Regulation of AMH by androgens and gonadotropins**

As already discussed, the expression of AMH at basal levels does not require gonadotropin stimulation of Sertoli cells, although a decrease in testicular AMH output has been observed in humans (Young et al., 1999, 2003) and rodents (Lukas-Croisier et al., 2003; Rey et al., 2003) with low or undetectable FSH levels. On the other hand, when the intratesticular concentration of androgens rises, the production of AMH declines, as a hallmark of the beginning of puberty (Rey et al., 1997). The first hint that androgens downregulate testicular AMH expression irrespective of gonadotropin action was given by a study in boys of prepubertal age in whom an activating mutation of the LH receptor in Leydig cells provoked a precocious elevation of testosterone levels while the gonadotropin axis was still quiescent: in these patients AMH was downregulated despite low FSH and LH levels (Rey et al., 1993). Furthermore, in patients with androgen insensitivity due to mutations in the androgen receptor, AMH expression was higher than in normal boys, suggesting that the lack of androgen negative effect results in upregulation of AMH expression (Rey et al., 1994).
During fetal life testosterone is produced at high levels; nevertheless, AMH is not repressed. To address this issue, which was contradictory with the affirmation that androgens downregulate AMH gene expression in Sertoli cells, immunocytochemistry experiments were performed and showed, in line with evidence obtained in other mammalian species (Bremner et al., 1994; Majdic et al., 1995; You and Sar, 1998; McKinnell et al., 2001; Weber et al., 2002), that the androgen receptor is not expressed in Sertoli cells of prepubertal mice (<7 days old). This can explain why androgens, although elevated in the fetal and neonatal testis, cannot downregulate AMH expression (Al-Attar et al., 1997). Thus, Sertoli cells are physiologically androgen-insensitive during the fetal and neonatal periods of development. The molecular mechanisms by which testosterone induces AMH repression has not been addressed. In general, after binding to testosterone, the androgen receptor regulates target gene expression through interaction with androgen response elements present in their promoters. However no androgen receptor element has been found in the AMH promoter (Rey and Josso, 1996), so the regulatory mechanism probably involves indirect or alternative pathways.

Interestingly, the elevation of AMH observed in the presence of androgen receptor mutations was more obvious at ages when gonadotropins raised (Rey et al., 1997). The previous observa-
tions in humans were in line with experimental observations in mice. Treatment of normal and androgen-insensitive (Tfm) prepubertal mice with a pharmaceutical preparation having both LH and FSH activities resulted in a dimorphic effect on AMH expression. LH provoked an increase of intratesticular testosterone concentration in both murine models; whereas a repression of AMH was observed—as expected—in normal mice, in the Tfm mice the levels of AMH unexpectedly rose. Further experiments lead us to the conclusion that not only did FSH not repress AMH expression but, in the absence of androgen actions, it could increase testicular AMH production (Al-Attar et al., 1997). However, this hypothesis needed further investigation. In fact, controversial results had been published concerning gonadotropin regulation of AMH expression. Whereas in vitro studies in calf Sertoli cells showed no effect of FSH on AMH secretion (LaQuaglia et al., 1986), in vivo studies by the same group in fetal (Kuroda et al., 1991) and neonatal (Kuroda et al., 1990) rats found an inhibitory effect of FSH on AMH expression. More recently, in vitro expression of AMH by cultures Sertoli cells was found to be inhibited by FSH (Arambepola et al., 1998). The understanding of the effect of gonadotropins on AMH production by Sertoli cells has been limited owing, on one hand, to the use of FSH preparations obtained by purification from biological extracts, which might carry undesirable biological activities, and, on the other, to the fact that both AMH and FSH receptor expression are rapidly lost in cultured Sertoli cells. In fact, in cultured human fetal Sertoli cells no effect on AMH expression could be observed after FSH addition but an increase of AMH mRNA was provoked when the FSH receptor was bypassed by directly supplying its second messenger cAMP (Voutilainen and Miller, 1987).

Molecular pathways

To overcome the difficulties in interpreting the effect of FSH on Sertoli cell AMH production due to the experimental models that had been used, mice with knockouts of the β-subunit of FSH (Kumar et al., 1997) or of the FSH receptor (Abel et al., 2000) were thought to be an ideal model to unravel to the controversy. Johnston and colleagues (2004) found no differences between normal male mice and males lacking FSHβ or the FSH receptor in AMH mRNA levels in total testicular tissue preparations. However, these authors unexpectedly found no differences in Sertoli cell number between normal and FSH-null mice. Our group has found lower AMH levels in mice lacking FSHβ (Lukas-Croisier et al., 2003; Rey et al., 2003). As in men with hypogonadotropic hypogonadism showing low serum AMH in untreated conditions (Young et al., 1999, 2003) that increased after recombinant FSH administration (Young et al., submitted), serum AMH reached normal levels in prepubertal FSHβ knockout mice treated with recombinant FSH daily for a week (Lukas-Croisier et al., 2003). Furthermore, our results are in line with observations in a patient with McCune-Albright syndrome owing to an activating mutation of the Gα protein, involved in the FSH receptor signaling pathway, in whom elevated AMH production was observed (Coutant et al., 2001).

Experimental data showing that, in the absence of the negative effects of androgens, FSH increases AMH expression could be explained by the fact that FSH has a proliferative effect on prepubertal Sertoli cells (Orth, 1984, 1986; Almirón and Chemes, 1988; Sasaki et al., 1998; Atanassova et al., 1999; Sasaki et al., 2000; Sharpe et al., 2000; Wreford et al., 2001). As a matter of fact, the highest AMH expression coincides with mitotic activity in Sertoli cells (Hirobe et al., 1992). To evaluate whether the increase in AMH production by Sertoli cells is a consequence exclusively of proliferation in the Sertoli cell population, or whether there is also an upregulation of AMH gene expression in each Sertoli cell, we have evaluated the effect of FSH on the expression of AMH in various experimental models. First, using transgenic mice carrying a deletion in the FSHβ subunit gene, we confirmed that in the lack of FSH, although testicular AMH production is not abolished, it is significantly reduced, and regains normal levels after exogenous FSH administration. We found that a low level of AMH is coincident with a low Sertoli cell number, and concluded that in the prepubertal mice, AMH production is regulated by FSH and reflects the number of Sertoli cells actively expressing the AMH gene, as its proliferation is stimulated by FSH signaling (Lukas-Croisier et al., 2003).

To analyze whether AMH gene expression was also upregulated by FSH and to unravel the molecular mechanisms underlying the elevation of AMH expression following FSH administration, we used the prepubertal mouse Sertoli cell line SMAT1 (Dutertre et al., 1997) in transfection experiments. After ensuring FSH receptor expression in SMAT1 cells by transient transfection, we showed that the classic pathway was used by postnatal Sertoli cells to upregulate AMH expression in response to FSH, involving the heterotrimeric Gα-protein coupled to the seven transmembrane-domain FSH receptor. The resulting adenylyl cyclase activation, in turn, increases intracellular cAMP levels, that activate protein kinase A (PKA) (Lukas-Croisier et al., 2003). The involvement of this pathway in the elevation of AMH expression had already been suggested by the observation of an activating somatic mutation of the Gα protein, found in the testes of a patient with a McCune-Albright syndrome, resulting in elevated production of AMH (Coutant et al., 2001).

Usually, PKA phosphorylates a group of transcription factors that bind to a cAMP-responsive element (CRE) present in the promoter of FSH-responsive genes. When canonical CRE elements were searched in the AMH promoter, none could be found, suggesting that an indirect or alternative pathway must be involved (Rey and Josso, 1996). In transfection studies, we have recently shown in SMAT1 cells that a 3 kb AMH promoter has a more potent trans-activating capacity than shorter promoters not only in basal conditions but in response to cAMP as well (Fig. 3A) (Lukas-Croisier et al., 2003). The analysis of the sequence of this promoter allowed the identification of putative binding sites for NFκB and AP2 at −2208 and −1936, respectively. The prepubertal mouse Sertoli cell and SMAT1 cells express NFκB and AP2 proteins, which are translocated to the nucleus after FSH stimulation, as shown by immunohemistry. The functional importance of the putative NFκB and AP2 response elements was assessed by transfection and mutational analyses (Fig. 3B). Overexpression of NFκB or AP2 resulted in an enhanced activity of the AMH promoter, but there was no increase in the basal activity in the AMH promoter containing mutated NFκB or AP2 sites. On the other hand, when the inhibitor of κB (IκB) was overexpressed, the effect induced by NFκB was abolished. Furthermore, the mutation of the NFκB site or its deletion resulted in an important decrease of the AMH promoter response to cAMP stimulation (Fig. 3C). When the AP2 site was mutated, the response to cAMP also
decreased but, in the context of the full-length promoter, this effect seemed to be less pronounced. The concomitant mutation of both sites did not result in a complete abrogation of responsiveness to cAMP. These results, in addition to other experiments performed to study the FSH-dependent pathway involved, identify the transcription factors AP2 and NFκB as responsible, at least in part, for the enhancement of AMH expression induced by FSH via the cAMP-PKA pathway (Lukas-Croisier et al., 2003). NFκB transcription factor is kept in an inactive form in the cytoplasm by IκB. Our hypothesis postulates that PKA phosphorylates IκB, which would lead to its proteasome-mediated degradation and the release of NFκB for its translocation to the nucleus. AP2 would also be phosphorylated by PKA and translocated to the nucleus. Both transcription factors transactivate AMH gene transcription by binding to the specific enhancer elements located more than 1900 bp from the AMH transcription start site (Fig. 4).

**Regulation of AMH by germ cells**

The decline of AMH and the meiotic entry of germ cells are both androgen-dependent processes that can be interpreted as two different consequences of androgen action. However, even if testosterone concentration increases rapidly in the testis at pubertal onset, the decrease in AMH expression does not occur synchronously in all seminiferous tubules. Interestingly, a patchwork pattern is observed: AMH expression is repressed only in those tubules that enter meiosis both in humans (Rey et al., 1996; Rajpert-De Meyts et al., 1999) and mice (Al-Attar et al., 1997). Whether meiotic germ cells could play a direct role on AMH repression was evaluated in Tfm mice and in XXsxrb male mice. In androgen-insensitive Tfm mice, we expected to find high levels of AMH expression throughout adulthood, based on our observations in human patients with androgen insensitivity (Rey et al., 1994). However, in Tfm mice, although AMH expression remains at high levels until older ages than in normal males, it is downregulated to a certain extent in the adult (Al-Attar et al., 1997; Johnston et al., 2004). Interestingly, downregulation of AMH expression in Tfm coincides with the occurrence of early primary spermatocytes, which cannot progress through meiosis (Al-Attar et al., 1997). XXsxrb mice display normal androgen production and sensitivity, but lack germ cells owing to the presence of two X chromosomes and to the absence of Y-chromosome genes involved...
in spermatogenesis. Although AMH is partially repressed in XXsor² mice at the time of puberty, significantly higher expression levels are observed than in control XY mice, with normal spermatogenesis (Al-Attar et al., 1997). Altogether, these observations support the hypothesis that meiotic germ cells downregulate AMH expression independently of androgen action.

A recent study suggests that TNFα might mediate AMH downregulation occurring when meiotic germ cells appear in seminiferous tubules at puberty (Hong et al., 2003). TNFα is secreted by germ cells only after they have entered meiosis (De et al., 1993), which is a sign of pubertal onset. NFκB is an intracellular mediator of TNFα action and is expressed in Sertoli cells (Delfino and Walker, 1998; Lukas-Croisier et al., 2003). Upon binding to its receptor on Sertoli cell membrane, TNFα activates NFκB, which translocates to the nucleus. The hypothesis to explain AMH downregulation by meiotic germ cells (Hong et al., 2003) postulates that the p50 subunit of NFκB acts as a corepressor of SF1 on AMH transcriptional activity—without interacting with the AMH promoter—after binding to SF1 at aa 116–125. Although protein–protein interaction between NFκB and SF1 does not affect SF1 binding to its response elements in the AMH promoter, the complex recruits histone deacetylases 4 (HDAC4) and 5 (HDAC5), resulting in further repression of SF1 transactivation of the AMH gene.

The opposite effects postulated for NFκB on AMH transcription following FSH (Lukas-Croisier et al., 2003) or TNFα (Hong et al., 2003) action are not contradictory. On one hand, the negative effect postulated to mediate meiotic germ cell action via TNFα was observed in transfection assays using a short AMH promoter of ~180 bp lacking NFκB binding sites. On the other hand, the positive FSH-dependent effect was observed in a testicular context compatible with normal prepuberty or with abnormal pubertal conditions where meiotic spermatocytes and androgen action are absent. It is conceivable that when both FSH and germ cell-dependent stimuli are present, for example, at normal puberty, the negative effect of the latter could be predominant.

Finally, another member of the GATA family of transcription factors, GATA1, is specifically expressed in Sertoli cells at the time of AMH downregulation, and is capable of binding to the GATA sites of the AMH promoter. While these observations suggested that GATA1 might be involved in the decrease of AMH expression at puberty (Beau et al., 2000), a recently published study on a tissue-specific GATA1 knockout mouse model concludes that, although GATA1 may be involved in the physiological downregulation of AMH expression, it does not seem to be a pivotal regulator of the process, since AMH is still inhibited at puberty in the absence of GATA1 expression in Sertoli cells (Lindeboom et al., 2003).

**REGULATION OF AMH EXPRESSION IN THE MAMMALIAN OVARY**

In the ovary, anti-Müllerian hormone is expressed in granulosa cells from primary to preantral stages and becomes restricted to a low level in cumulus cells in preovulatory follicles (Bézard et al., 1987; Münsterberg and Lovell-Badge, 1991; Raipert-De Mefts et al., 1999; Rey et al., 2000). The mechanism whereby AMH expression is regulated in granulosa cells has been far less studied than in Sertoli cells. SF1 (Hatano et al., 1994; Ikeda et al., 1996), WT1 (Mundlos et al., 1993; Chun et al., 1999) and GATA4 (Heikinheimo et al., 1997; Anttonen et al., 2003) are present in granulosa cells, and may participate in the AMH transcription complex (Table 1). On the contrary, SOX9, the key regulator of AMH expression in Sertoli cells, is not expressed in the ovary. It is possible that another SOX factor might substitute for SOX9 in the ovary, as it has been suggested for SOX8 who shares with SOX9 the property to activate the AMH promoter in vitro, albeit with less efficiency (Schepers et al., 2003). Another explanation for the sexual dimorphism in AMH expression in the gonads was suggested by a difference of GATA4 cofactor FOG2 antagonism between Sertoli and granulosa cells. While GATA4 is expressed in both Sertoli and granulosa cells throughout development, FOG2 is not expressed in Sertoli cells but it is expressed in granulosa cells, where it is believed to counteract AMH gene transcription.

**AMH REGULATION IN NONMAMMALIAN SPECIES**

In nonmammalian vertebrates, embryonic regression of the male Müllerian ducts under the action of a testicular hormone is only documented in birds and reptiles. Regression is not the rule in amphibian and, in fishes, Müllerian ducts exist only in primitive species such as the sturgeon (Acipenser), albeit with less efficiency (Schepers et al., 2003). Another explanation for the sexual dimorphism in AMH expression in the gonads was suggested by a difference of GATA4 cofactor FOG2 antagonism between Sertoli and granulosa cells. While GATA4 is expressed in both Sertoli and granulosa cells throughout development, FOG2 is not expressed in Sertoli cells but it is expressed in granulosa cells, where it is believed to counteract AMH gene transcription by GATA4 (Anttonen et al., 2003). In addition, AMH expression in cultured granulosa cells is upregulated by coculture with oocytes from preantral or preovulatory follicles (Salmon et al., 2004). This effect may explain the observed differential level of AMH expression between cumulus and mural follicular cells in preovulatory follicles. Control of AMH expression by oocytes might provide a way to control interfollicular inhibition of follicular recruitment.

**Birds**

In birds, the sex is genetically determined, with males being homogametic (ZZ) and females heterogametic (ZW). The mechanism of this determination is unknown and may involve a W-chromosome gene such as Wpkci, or the dosage of genes located on Z chromosome, such as DMRT1 (reviewed in Ellegran, 2001).

The chick AMH gene, located on the microchromosome 28 (Oréal et al., 1998; Schmid et al., 2000), is 4200 bp long and has a five-exon structure as in mammals. The main transcription initiation site is found 45 bp from the protein initiation codon. The predicted 644-aa protein contains, as in mammals, a monobasic prolylolytic cleavage site, 106 aa upstream from the C-terminus. Amino acid conservation with mammalian AMH is low, 30 and 50%, respectively for the N- and C-terminal domains (Carré-Eusèbe et al., 1996; Neepuer et al., 1996).

The chick AMH 5’ proximal DNA region has been sequenced (Oréal et al., 1998; D. Carre-Eusèbe, unpublished results). The gene coding for the spliceosome protein SAP62 (SF3A2) is found 1227 bp upstream from the AMH transcription start, that is somewhat further than in human, mouse, and rat genomes. This synathy with a housekeeping gene, con-
served over 300 million years of evolution, might underline the requirement for a special open chromatin structure. The AMH promoter region displays a degenerated TATA box (-31 TT-TAAAAG-24), a well-conserved SF1 site (-45 TCAAGGCCA-37), functional in bandshift experiments, two potential SOX9 responsive elements (-195 TTTTGTTTT -187 and -606 CCTTTGTTCCT -597) and three GATA sites (at -365, -566 and -1035), which functionality remains to establish (Oréal et al., 1998; D. Carré-Eusèbe, unpublished results). No in vitro chick AMH expression studies are available to date by lack of a good expressing cell line, and data on the regulation of AMH expression are essentially derived from comparative in situ hybridization studies.

Unlike in eutherian mammals, AMH is transcribed and translated in male and female chick embryonic gonads (Hutson et al., 1981). Expression starts in both sexes at the same time and with similar intensity in a few dispersed medullar cells of indifferent gonads, from stage 25 (4.5 days of incubation), before any detectable gonadal SOX9 expression (Oréal et al., 1998). Thus, unlike in mammals, SOX9 is not required for the onset of AMH transcription in chick gonads. At this time, SF1, WT1, GATA4, Wnt-4, and DMRT1 are expressed in the gonad medullar area, but their expression is not restricted to the AMH-producing cells (Oréal et al., 2002). The sexual dimorphism of DMRT1, more highly expressed in male than female gonads, starts to be apparent around this period (Raymond et al., 1999; Smith et al., 1999).

One day later, AMH expression undergoes a male-specific upregulation concomitant with the occurrence of a very low level of SOX9 transcripts. Clear upregulation of SOX9 takes place only at 7 days of incubation, when AMH-producing cells get organized into testicular cords (Oréal et al., 1998). Thereon, AMH, SOX9, SF1, WT1, GATA4, and DMRT1 are clearly coexpressed in the same cells, the Sertoli cells (Oréal et al., 2002), and AMH regulation might be similar to what it is in mammalian Sertoli cells. However, the role of SOX9 in testicular

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<th>Factor</th>
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| SOX9   | ↑      | Not expressed | de Santa Barbara et al., 1998  
|        |        |       | Arango et al., 1999 |
| SOX8   | ↑      | Not expressed | Schepers et al., 2003 |
| SF1    | ↑      | ↑      | Shen et al., 1994  
|        |        |       | Nachtigal et al., 1998  
|        |        |       | Giuli et al., 1997  
|        |        |       | Arango et al., 1999  
|        |        |       | Tremblay and Viger, 1999  
|        |        |       | Watanabe et al., 2000 |
| GATA4  | ↓      | ↑      | Viger et al., 1998  
|        |        |       | Tremblay and Viger, 1999  
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|        |        |       | Tremblay and Viger, 2001a |
|        |        |       | Anttonen et al., 2003 |
| GATA1  | ↓      | Not expressed | Beau et al., 2000  
|        |        |       | Lindeboom et al., 2003 |
| FOG2   | ↓      | ↓      | Anttonen et al., 2003 |
| WT1    | ↑      | Not studied | Nachtigal et al., 1998  
|        |        |       | Hossain and Saunders, 2003 |
| DAX1   | ↓      | ↓      | Nachtigal et al., 1998  
|        |        |       | Swain et al., 1998 |
| TNFα   | ↓      | Not studied | Hong et al., 2003 |
| cAMP   | ↑      | Not studied | Lukas-Croisier et al., 2003 |
| AP2    | ↑      | Not studied | Lukas-Croisier et al., 2003 |
| NFκB   | ↑      | Not studied | Lukas-Croisier et al., 2003 |
chick AMH regulation is still questionable. Indeed, electroporation of a SOX9 expressing vector into female indifferent gonads does not induce a higher AMH transcription level, whereas aromatase expression is decreased (R.H. Sekido and R. Lovell-Badge, personal communication). These results show that SOX9 alone is unable to upregulate AMH expression. Sox8, whose expression is not sexually dimorphic during gonadogenesis, is therefore not responsible for sex-dependent differences in AMH expression (Takada and Koopman, 2003).

While upregulated in testis cords in the male gonads, AMH continues to be expressed, at a much lower level, in dispersed cells of the medulla in female gonads during the embryonic life. Gonadal differentiation is not symmetrical in female embryos. In the left gonad, which will form the only functional ovary, the surface epithelial region covering the medulla differentiates into a growing cortex harboring groups of germ cells surrounded by somatic cells that express Lhx9, a LIM homeodomain factor (Oréal et al., 2002). In contrast, the right gonad remains limited to the medulla covered by a thin epithelium. This gonad gains its maximum weight around day 12 of incubation, then regresses and disappears after birth. Asymmetry may be explained by a left–right difference in estrogen action. From day 6 of incubation, the estrogen synthesizing enzyme, aromatase, is present in the medulla of both female gonads and estrogens are produced. However, the estrogen receptor-α is expressed only in the cortex of the left gonad and to a lower extent in both medullas (Andrews et al., 1997; Smith et al., 1997; Nakabayashi et al., 1998). The mechanism controlling estrogen receptor-α asymmetry is unknown. During the embryonic period, AMH remains expressed in dispersed cells of the medulla with a predominant location in the juxtacortical region in the left gonad, and throughout the medulla in the right gonad. This medullar AMH expression is not controlled by SOX9, which is not expressed in female gonads. In addition, it does not require high levels of SF1, DMRT1, WT1, GATA4, or Wnt-4, which either disappear or are not clearly colocalized with AMH-expressing cells (Oréal et al., 2002).

Estrogens are critical for female gonadal sex differentiation in birds. Early treatment of female embryos by aromatase inhibitors results in masculinization of the gonads. An increase in DMRT1 expression is first observed (Smith et al., 2003), followed by the induction of SOX9 (Vaillant et al., 2001; Smith et al., 2003). AMH expression is upregulated and testicular cords are formed (Elbrecht and Smith, 1992; Vaillant et al., 2001). The precise timing of AMH upregulation is not established, nor is it known if the effect of estrogen on its expression is direct or not. A degenerated estrogen responsive element found in the AMH promoter region (Oréal et al., 1998) appears too degenerated to be functional (Driscoll et al., 1998) and is not recognized by human or chick estrogen receptors in bandshift experiments (D. Carré-Eusèbe, unpublished results). However, estrogen action may be mediated without physical interaction of the estrogen receptor with DNA.

Primary follicles are formed in the cortical area of the female left gonad a few days after hatching. Follicular granulosa cells express AMH at a high level, in conjunction with SF1, WT1, GATA4, Wnt-4, and, transiently, Lhx9. Granulosa cells do not appear to derive from the medullar cells previously expressing AMH, but rather from the Lhx9-positive somatic cells of the cortex that surrounded the germ cell clusters (Oréal et al., 2002). Control of AMH expression in granulosa cells, in which SOX9 is not expressed, may be compared to what occurs in mammalian primary follicles. Thus, in birds, different combination of transcription factors must be considered for AMH regulation in Sertoli cells, granulosa cells, and medullar cells of indifferent and female embryonic gonads.

Reptiles

In reptiles, anti-Müllerian activity has been reported in species with strict genotypic sex determination (GSD) and species with temperature-dependent mechanisms of sex determination (TSD). As in birds, in the American alligator (Alligator mississippiensis), a species with TSD, the onset of AMH expression does not depend on the presence of SOX9. AMH starts to be expressed at stage 22 in gonads of embryos incubated at a male-promoting temperature, prior to morphological testicular differentiation. SOX9 is not detected in male gonads before stage 23.5 and is upregulated between stages 24 and 25, when testicular cords are formed. At male-promoting temperatures, SOX9 and AMH are not expressed in the embryonic gonads (Western et al., 1999). DMRT1 does not appear either responsible for the onset of male-specific AMH expression, since it is initially detected in gonads of both sexes and is upregulated in the male gonads only around stage 24 (Smith et al., 1999). WT1 and SF1 are also present in male and female differentiating gonads (Western et al., 2000). In turtles, AMH expression has not been studied. However, in Trachemys scripta and Lepidochelys olivacea, two species with TSD, SOX9 does not appear to be an early trigger of male differentiation. It is expressed similarly at male- and female-promoting temperatures during the period of male commitment and is later downregulated in females (Spotila et al., 1998; Moreno Mendoza et al., 1999). The expression of SOX9, similar in male and female, leads to exclude also this factor (Takada and Koopman, 2003). Conversely, DMRT1 expression levels are higher at male-determining temperature, even at early stages (Kettlewell et al., 2000; Torres Maldonado et al., 2002). SF1 and WT1 may participate in AMH regulation in the testis. SF1 expression is higher in gonads undergoing a male-promoting temperature (Fleming and Crews, 2001), and WT1 is highly expressed in proliferating medullar cells, precursors of the Sertoli cells, at male-promoting temperature (Schmahl et al., 2003). As in birds, estrogens play an important role in turtles, since gonadal sex differentiation may be reversed by treatment with estrogens at masculinizing temperature and treatment with antiestrogens or aromatase inhibitors at feminizing temperature (Pieu et al., 1999).

Fishes

Modern teleosts have no Müllerian ducts, but separate ovarian excretory pathways for their ova. However, a Sertoli-specific protein sharing 43% similarity with chick AMH in the C-terminal domain, has been found in the Japanese eel (Anguilla japonica), and named spermatogenesis-preventing substance. This protein blocks spermatogonial proliferation under freshwater conditions. Its expression, like that of mammalian testicular AMH at puberty, is negatively regulated by gonadotropins (hCG) and 11-ketotestosterone (Miura et al., 2002). A protein with close sequence homology also exists in...
the rainbow trout (*Oncorhynchus mykiss*) (accession number BX865284). These proteins might represent a primitive form of AMH, sharing with the mammalian hormone some testicular function and regulation.

CONCLUDING REMARKS

While basal expression of AMH is triggered by SOX9, and enhanced by GATA4, SF1, and WT1 in Sertoli cells of eutherian mammals, in ovarian granulosa cells SOX9 is not expressed, and in the gonads of birds and reptiles AMH expression precedes that of SOX9. The issue raises a controversy that still needs to be elucidated. The 5'-flanking sequences of the AMH gene involved in the initiation of AMH transcriptional activity seem to be limited to less than 400 bp, yet more distant regulatory cis-elements are necessary for the maintenance and hormonal regulation of AMH expression in Sertoli cells during late fetal and postnatal life. The increasing knowledge of the hormonal, cellular, and molecular mechanisms explaining the differential expression of AMH between species, cell types and developmental stage provides insight into the understanding of the phylogeny of gonadal development and of normal and pathological aspects of the physiology of Sertoli and granulosa cells.

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DRESSER, D.W., HACKER, A., LOVELL-BADGE, R., and GUER-


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Address reprint requests to:
Rodolfo Rey, M.D., Ph.D.
CEDIE-Endocrinología, Hospital de Niños
Gallo 1330
C1425EFD Buenos Aires, Argentina
E-mail: rodolforey@cedie.org.ar

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