Anti-Müllerian Hormone and Androgens: Regulation of Receptors During Sex Differentiation and Gonadal Development

Anti-Müllerse Gang Hormoon en Androgenen: Regulatie van Receptoren Tijdens Geslachtsdifferentiatie en Gonadeontwikkeling

.

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WILLY MARIA BAARENDS

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PROMOTIE-COMMISSIE

Promotor:	prof. dr. J.A. Grootegoed
Co-promotor:	dr. ir. A.P.N. Themmen
Overige Leden:	dr. ir. P. de Boer
	prof. dr. S.L.S. Drop
	prof. dr. J.W. Oosterhuis

3β-HSD	3β hydroxysteroid dehydrogenase
17β-HSD	17β hydroxysteroid dehydrogenase
aa	amino acids
AC	adenylyl cyclase
ActRI	activin type I receptor
ActRII(B)	activin type II (B) receptor
Ad4BP	adrenal 4-binding protein
AF	atretic follicle
AHC	adrenal hypoplasia congenita
AIS	androgen insensitivity syndrome
Alk	activin receptor-like kinase
AMH	anti-müllerian hormone
AMHRII	AMH type II receptor
AR	androgen receptor
ARE	androgen response element
ATP	adenosine triphosphate
bp	base pairs
BMP	bone morphogenetic protein
BSA	bovine serum albumin
cAIS	complete androgen insensititvity syndrome
C. elegans	Caenorhabditis elegans
cAMP	adenosine cyclic-3':5'-monophosphate
CAT	chrloramphenicol acetyltransferase
cDNA	complementary DNA
CG	chorionic gonadotropin
CL	corpora lutea
CMPD1	campomelic dysplasia
COS	monkey kidney cell line (CV1, origin of SV40)
CRE	cAMP response element
D. melanogaster	Drosphila melanogaster
DAX-1	DSS-AHC critical region on the X chromosome, gene 1
DNA	deoxyribonucleic acid
DPP	decapentaplegic gene product
DSS	dosage sensitive sex reversal
E#	Embryonic day #
EB	estradiol benzoate
EDS	ethane dimethane sulfonate
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
EIS	estrogen insensitivity syndrome
ER	estrogen receptor
ERE	estrogen response element
FCS	fetal calf serum
FMPP	familial male precocious puberty
FSH	follicle-stimulating hormone
FSHR	FSH receptor
Ftz-F1	locus containing mouse gene that closely resembles Drosophila fushi tarazu factor 1
GAPD	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotropin releasing hormone
GnRHant	GnRH antagonist
GRE	glucocorticoid response element
GTP	guanosine triphosphate
h (prefix)	human
HF	healthy follicle
HHG	hypogonadotropic hypogonadism
HRE	hormone response element
HU	hydroxyurea
kb	kilo base pairs
Kd	equilibrium dissociation constant
kDa	kilo Dalton
LA	large antral follicle
LCH	Leydig cell hypoplasia
LH	luteinizing hormone
LHR	LH receptor
LNCaP	human lymph node carcinoma of the prostate (cell line)

LPF	large preantral follicle
LRPR1	leucine-rich primary response gene 1
m (prefix)	mouse
MAA	methoxyacetic acid
MEM	Eagle's minimal essential medium
MD	müllerian duct
MIS	müllerian inhibiting substance
mRNA	messenger RNA
Od	ovary determining genes
P-450c17	17α-hydroxylase P-450
P-450scc	cholesterol side-chain cleavage P-450
pAIS	partial androgen insensitivity syndrome
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMDS	persistent müllerian duct syndrome
PMSG	pregnant mare's serum gonadotropin
PTU	6-propyl-2-thiouracil
r (prefix)	rat
rab (prefix)	rabbit
RNA	ribonucleic acid
SA	small antral follicle
SAX	*Drosophila saxophone* gene product
sc	subcutaneous
SDS	sodium dodecyl sulfate
SF-1	steroidogenic factor 1
SOX genes	HMG-box containing genes that show >60% similarity to the SRY HMG box
SPF	small preantral follicle
SRA1	autosomal sex reversal locus
SRY	sex determining region Y (human)
Sry	sex determining region Y (mouse)
T	testosterone
T_3	triiodothyronine
TDF	testis determining factor (human)
Tdy	testis determining factor (mouse)
TKV	*Drosophila thick veins* gene product
TF	transcription factor
TGFβ	transforming growth factor β
TGFβRI	TGFβ type I receptor
TGFβRII	TGFβ type II receptor
TSH	thyroid-stimulating hormone
HMG	high mobility group
UTP	uridine triphosphate
UTR	untranslated region
WD	wolffian duct
WT-1	Wilms' tumor associated gene

VIII

General Introduction

1.1   Introduction

This chapter gives an outline of sex determination, sex differentiation, and gonadal development in mammalian species. In most studies described herein, rats and mice were used.

During embryonal development in mammals, sex differentiation is preceded by a bipotential stage. Indifferent gonads are formed that can develop into either testes or ovaries. The anlagen of the male and female internal genitalia, which are both present in embryos of either chromosomal sex, are called the wolffian and the müllerian ducts, respectively. The external genitalia are bipotential (Fig.1).

Male sex is determined in a subpopulation of somatic cells in the indifferent gonads. The presence of an intact Y chromosome initiates development of these cells along the male pathway. Further sex differentiation of the gonad into a testis is achieved via processes that involve cell-cell communication. All subsequent morphological differences between male and female eutherian mammals are hormonally regulated. The testis starts to synthesize two hormones that are essential for correct sex differentiation. The first hormone, anti-müllerian hormone (AMH) induces degeneration of the müllerian ducts. The second hormone, testosterone, stimulates development of the wolffian ducts into epididymides, vasa deferens and seminal vesicles. The testosterone metabolite dihydrotestosterone is essential for the development of the prostate and external genitalia from their respective primordia: urogenital sinus (prostate), genital tubercle (penis, urethra), and labioscrotal swellings (scrotum).

In the female, ovary differentiation takes place in the absence of an intact Y chromosome, and, in the absence of AMH which is not produced by fetal ovaries, the müllerian ducts develop into the fallopian tubes, the uterus and the upper part of the vagina. Moreover, in the virtual absence of testosterone, the wolffian ducts degenerate and the external genitalia develop along the female pathway.

The above described processes, dependent on either the presence or absence of AMH and testosterone, are described in detail in the first part of this chapter. An overview of the regulatory mechanisms in the gonads by which these hormones act, is given in the second part of this chapter, followed by the scope of this thesis.

INDIFFERENT STAGE

gonad
mesonephros
Mullerian duct
Wolffian duct

ovary
Fallopian tube
uterus
vagina
FEMALE

epididymis
testis
vas deferens
seminal vesicle
prostate
MALE
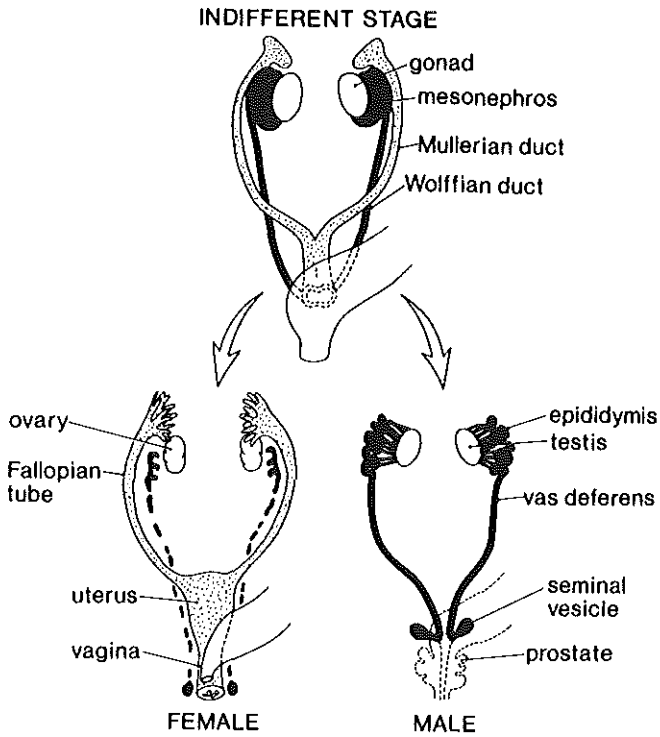
Fig. 1.1: Schematic representation of sexual differentiation of male and female internal genitalia during embryonic development (from Wilson et al. 1981).
During the indifferent stage, the gonads and the anlagen of the male and female internal genitalia, the wolffian and müllerian ducts, respectively, are formed. In the male embryo, the gonads develop into testes; the müllerian ducts degenerate, and the wolffian ducts develop into the epididymides, vasa deferens and seminal vesicles. In the female embryo, the indifferent gonads follow the ovarian differentiation pathway; the wolffian ducts degenerate, and the müllerian ducts develop into the fallopian tubes, uterus and upper part of the vagina.

1.2. Sex determination and differentiation

Many species exhibit an extensive range of dimorphic characteristics that distinguish the two sexes, when adult. Among different mammalian and non-mammalian species, various mechanisms have evolved that control the switch that initiates either the male or the female sex differentiation pathway. The signal for sex determination that precedes sex differentiation is environmental (temperature) in some species such as reptiles (reviewed by Crews et al. 1994), but the signal is chromosomal in mammals (reviewed by Goodfellow and Lovell-Badge, 1993; and George and Wilson, 1994), and in many other animal species. In the well studied species *Drosophila melanogaster* and *Caenorhabditis elegans*, sex determination is controlled by the ratio of the number of X chromosomes to the number of sets of autosomes (reviewed by Hodgkin, 1985; Cline, 1993; and Parkhurst and Meneely, 1994). This is referred to as the X:A ratio. The X:A ratio is counted in each individual cell of the developing organism. In the XX females, the ratio is 1, whereas the XY (*D. melanogaster*) or XO (*C. elegans*) males have an X:A ratio of 0.5 (for review see Parkhurst and Meneely, 1994). Sex determination in *D. melanogaster* is coupled to the mechanism that regulates dosage compensation. Dosage compensation corrects for the fact that males have only one copy of X-chromosomal genes, whereas females have two copies, and is achieved by doubling the level of X-chromosomal gene expression in males.

Mammalian sex determination depends upon the presence or absence of a Y chromosome, irrespective of the number of X chromosomes. Dosage compensation, an earlier and seemingly independent event, is achieved by inactivation of one X chromosome in females. If, in the case of chromosomal aberrations, more X chromosomes are present in males or females, all but one X chromosome are inactivated.

A single factor, encoded by the Y chromosome, dominantly induces the formation of a testis from the indifferent gonad, which otherwise would develop into an ovary. This factor is called the testis determining factor (TDF) (reviewed by Goodfellow and Lovell-Badge, 1993; and Bogan and Page, 1994).

1.2.1 Genetic sex determination in mammals: *TDF*

Identification of TDF

The region on the human Y chromosome that encodes TDF was defined through extensive analysis and comparison of the sex chromosomes of XX males containing small fragments of the Y chromosome, and XY females lacking fragments of Y. Finally, a single gene, named *SRY* (sex determining region Y) in human (Berta et al. 1990; Sinclair et al. 1990) and *Sry* in mouse (Gubbay et al. 1990; Sinclair et al. 1990), has been identified. Direct evidence that *SRY/Sry* is a primary sex determinant was obtained when the mouse *Sry* gene with its flanking sequences was shown to induce male sex differentiation, when introduced as a transgene into XX mouse embryos (Koopman et al. 1991). Furthermore, *SRY* point mutations have been detected in several human XY females (reviewed by Goodfellow, 1993).

SRY; structure and function

SRY encodes a protein of the high mobility group (HMG) family of DNA binding proteins. This family of proteins is characterized by a so-called HMG box. The HMG box of SRY can bind DNA in a sequence specific manner, and is predicted to bend the target DNA to perhaps as much as 130^0 (Nasrin et al. 1991; Giese et al. 1992; Harley et al. 1992). SRY binds also to cruciform DNA structures, but in a non sequence-specific manner (Ferrari et al. 1992).

None of the *SRY* point mutations reported in human XY females is located outside the HMG domain (Goodfellow and Lovell-Badge, 1993). This indicates that the biochemical functions of SRY mainly reside in the HMG domain. However, the human *SRY* gene with flanking sequences is incapable of inducing sex-reversal when expressed as a transgene in XX mice, despite the similar DNA binding characteristics of SRY and Sry *in vitro* (Koopman et al. 1991; Goodfellow and Lovell-Badge, 1993). Outside the HMG domain, there is little sequence conservation among mammals. Thus, the significance of the amino acid sequences outside the HMG domain is still unclear. Also the exact mechanism of action of the SRY protein needs further investigation, to determine whether SRY can actually induce transcription, perhaps through interactions with cofactors. It remains possible that SRY acts solely by inducing a change in chromatin structure and/or combining distant DNA sequences, allowing other transcription factors to bind (Bogan and Page, 1994).

Sex determining genes other than SRY

It is most likely that SRY function depends upon interactions with other autosomal or X-chromosomal genes involved in sex determination. Indications for the existence of such downstream genes come from the finding that ovotestes in mice are formed when the Y chromosome from Mus musculus (Y^{dom}) is placed onto the C57BL/6J (B6) background (Eicher and Washburn, 1983). Genetic evidence indicates that the interaction between tdy^{dom} and B6 autosomal or X-linked alleles involved in sex determination, may result in a delay of the onset of testis differentiation beyond a critical time point, allowing partial ovary differentiation to take place (Taketo et al. 1991). It has been found that the presence of a polymorphism of a CAG trinucleotide repeat in the *Sry* gene on the Y^{dom} chromosome correlates with the B6Y^{dom} partial sex reversal (Coward et al. 1994). The functional significance of this finding remains to be established.
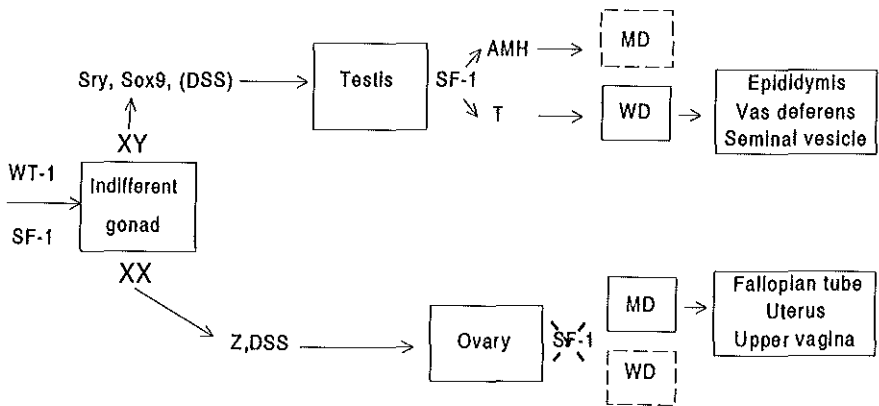
Fig. 1.2: Regulation of sex determination and differentiation in mammals (mouse).
The formation of the indifferent gonad depends on the action of at least two genes, Wilms' tumor locus-1 (WT-1) and Ftz-F1 (SF-1). In the male embryos (upper part of the figure) a single gene named Sry is able to induce testis differentiation, and to prevent the activation of ovary determining genes. If Sry is not present, as is the case in female embryos (lower part of the figure), ovary determining genes are activated, such as the gene located on the X-chromosomal Dosage Sensitive Sex reversal locus (DSS), and a possible negative regulator of male sex differentiation (Z). All other aspects of sex differentiation are regulated by gonadal hormones. An increase in SF-1 expression possibly results in a stimulation of AMH expression in the fetal Sertoli cells of the testis. AMH subsequently induces müllerian duct degeneration. The testicular Leydig cells start to synthesize testosterone, which stimulates the development of the wolffian duct. In the developing ovary, SF-1 expression decreases, and AMH is not expressed, allowing müllerian duct development. The wolffian duct degenerates, due to a lack of androgens.

In humans, certain chromosomal aberrations such as deletions (Bennett et al. 1993; Wilkie et al. 1993) or reciprocal translocation (Tommerup et al. 1993) may lead to XY female development. One such an autosomal sex reversal locus, *SRA1*, is associated with the skeletal malformation syndrome campomelic dysplasia (CMPD1). Recently, it has been shown that haploinsufficiency (deletion or mutation of only one allele) of the *SRY*-related gene *SOX9*, can cause both XY sex reversal and CMPD1 (Wagner et al. 1994; Foster, 1994). It is thought that *SOX9* acts very early in the sex determination pathway. Although nothing is known about the nature of the function of *SOX9* in sex determination, it is suggested that gene dosage may be involved in the mechanism of sex reversal in the case of haploinsufficiency of *SOX9* (Wagner et al. 1994; Foster, 1994). The dosage sensitivity of genes involved in sex determination may be a remnant of a primordial sex determination system, which has evolved into a dominant induction system, with Sry being the dominant inducer of the male phenotype (Foster, 1994; King et al. 1995).

The female pathway of mammalian sex differentiation is considered the default pathway. Development of the indifferent gonad into an ovary takes place when *SRY/Sry* is absent. To date, no genes that are involved in the genetic cascade that leads to ovary differentiation in the female have been identified. Studies of XX male individuals that lack *SRY* have led to the hypothesis that these individuals carry a recessive autosomal mutation in a gene named *Z* (McElreavey et al. 1993). In females, this gene could normally act as a negative regulator of male sex determination, and expression of *Z* would be repressed by SRY in males (McElreavey et al. 1993). Evidence for the existence of so-called ovary determining genes, comes from studies of several XY female patients with an intact *SRY* gene, carrying duplications of fragments of Xp (Bernstein et al. 1980; Scherer et al. 1989; Bardoni et al. 1994). Recently, it has been determined that a double active dosage of a locus at chromosome Xp21 is a cause of male to female sex reversal (Bardoni et al. 1994). This locus has been named *DSS* (Dosage Sensitive Sex reversal). 46,XY patients with deletions in this region have male external genitelia, suggesting that *DSS* is not required for testis differentiation. It is thought that in normal males, ovarian development and *DSS* function are repressed, thus allowing testis formation. Somehow, a double dosage of *DSS* in XY female patients hampers repression of the ovarian pathway, or activation of testis differentiation, although a normal *SRY* gene is present (Bardoni et al. 1994). Adjacent to, or overlapping with, *DSS*, a locus is mapped that is involved in adrenal gland development. Deletions in this region cause X-linked adrenal hypoplasia congenita (AHC) and also hypogonadotropic

hypogonadism (HHG). Mutations in a newly identified gene, called *DAX-1* (for DSS-AHC critical region on the X-chromosome, gene 1), give rise to both AHC and HHG (Zanaria et al. 1994; Muscatelli et al. 1994). Since this gene maps within the *DSS* critical interval, *DAX-1* is also a candidate gene underlying DSS (Zanaria et al. 1994). *DAX-1* encodes a new member of the nuclear hormone receptor superfamily, but displays a novel DNA-binding domain (Zanaria et al. 1994).

1.2.2 Development of the indifferent gonads

Morphological aspects

The indifferent gonad develops along the ventral cranial part of the mesonephros, which is the second of the three consecutive nephric structures (pro-, meso-, and metanephros). The primordial germ cells are first detected in the extraembryonic mesoderm at 7.5 days of embryonic development (E7.5) (days post coitum) (Ginsberg et al. 1990). Later during embryonic development, these cells migrate from the yolk sac into the developing gonads (reviewed by Byskov and Hoyer, 1994). The early nondifferentiated gonad consists of loose mesenchymal tissue, covered by the coelomic epithelium and supported by the developing mesonephric tissue (Byskov and Hoyer, 1994).

Genetic aspects

Before *Sry* can induce testis differentiation, genes common to both sexes have to regulate the formation of the indifferent gonad, and induce an intracellular environment in gonadal cells that allows the correct initiation of *Sry* expression.

Two such genes have recently been identified. Gene targeting experiments in mice have shown that the Wilms' tumor associated gene (*WT-1*), and a locus that is called *Ftz-F1* that encodes steroidogenic factor 1 (SF-1; also called adrenal 4-binding protein, Ad4BP), are both essential for development of intermediate mesoderm and formation of indifferent gonads (Kreidberg et al. 1993; Luo et al. 1994).

Targeted inactivating mutation of *WT-1*, resulted in a failure of kidney and gonad development. *WT-1* is a tumor suppressor gene that encodes a transcription factor that has four $(Cys)_2$-$(His)_2$ DNA-binding zinc fingers (Call et al. 1990).

In *Ftz-F1* null animals, gonad development is arrested at a very early stage, and also the adrenal glands are absent (Luo et al. 1994). Furthermore, SF-1 regulates the expression of several genes involved in steroid hormone biosynthesis, that are

essential for sexual differentiation of the internal and external genitalia (Lala et al. 1992; Honda et al. 1993; Shen et al. 1994) (see paragraph 1.1.4). SF-1 is an orphan nuclear receptor that shows the structural and functional characteristics of members of the steroid hormone receptor superfamily (see Chapter 2).

Thus, both *WT-1* and *SF-1* encode zinc-finger containing transcription factors that contribute to the establishment of the bipotential gonad. In the developing gonad of normal mouse embryos, the temporal and spatial expression patterns of these two genes overlap (Luo et al. 1994). However, it is not known whether WT-1 and SF-1 functionally interact.

1.2.3 Gonadal sex determination and differentiation

Differentiation of the gonadal cells

The first morphological sign of testis differentiation in the rat is the formation of testicular cords at the end of E13 (Jost et al. 1973). Cord formation depends upon interactions between the fetal Sertoli cells and the precursors of the peritubular myoid cells (Buehr et al. 1993). The testicular cords contain the prespermatogonia (also called gonocytes) and the fetal Sertoli cells, which regulate and support spermatogenesis in the adult testis. Differentiation of the steroid producing Leydig cells, located in the interstitium, is induced around E14 in the rat (Tapanainen et al. 1984).

Mesenchymal tissue, located between the outermost testicular cords and the coelomic epithelium develops into the tunica albuginea. Furthermore, the testis becomes rounded, thus minimizing further contact with the mesonephros (Byskov, 1986).

In the female gonad, the absence of *Sry* leads to ovary differentiation, which becomes morphologically visible at E16 in the rat, two days after testis formation is apparent in the male. The germ cells differentiate into oogonia that continue to divide mitotically. Subsequently, at E17.5 in the rat, meiosis is induced in cells that are located at the inner part of the cortex (Beaumont and Mandl, 1962). Many oocytes degenerate at different stages of meiosis, often in zygotene or pachytene. The oocytes become arrested in the diplotene stage of the prophase of meiosis I (dictyate). Then, folliculogenesis takes place, which depends upon the migration of mesonephros-derived cells, which surround the centrally placed oocytes and differentiate into granulosa cells (reviewed by Hirschfield, 1991, and Byskov and Hoyer, 1994).

Granulosa cells and Sertoli cells share many structural and functional characteristics, which supports the notion of their common embryonic origin. However, it has not been unequivocally established whether mesonephros-derived cells and cells from the coelomic epithelium both contribute to granulosa and Sertoli cell formation. Possibly, granulosa and Sertoli cells are solely mesonephros-derived in certain species (Patek et al. 1991; reviewed by Byskov and Hoyer, 1994). Mesonephros-derived cells have been shown to contribute also to the interstitial mesenchymal cell population from which Leydig cells and peritubular myoid cells differentiate in the mouse (Buehr et al. 1993).

Timing of Sry expression

Although genetic sex is set at fertilization, the first time point of Sry action that marks the onset of male sex determination and differentiation is not fully clear. Initial studies concerning the temporal mRNA expression pattern of *Sry* suggested that this gene is transcribed for only a very brief period starting at E10.5 in the mouse gonad, two days before testis formation becomes morphologically apparent (Koopman et al. 1990). However, recent studies have detected *Sry* mRNA expression in the mouse preimplantation embryo (Zwingman et al. 1993). Whether this pregonadal mRNA expression results in formation of functional protein remains to be established. After E12.5 in the mouse, *Sry* mRNA expression is detected only in the adult testis, in differentiating spermatids (Rossi et al. 1993). Most of this *Sry* mRNA is a circular transcript, and there is no evidence that it is actually translated into protein (Capel et al. 1993).

Cellular localization of Sry action

Since absence of germ cells from the indifferent gonad does not interfere with the formation of testis in XY individuals or ovaries in XX individuals (reviewed by Mclaren, 1991), sex determination must unfold in the somatic cell lineages of the gonad. Because Sry most likely functions in the cell nucleus, Sry is thought to act cell autonomously. Studies in chimeric XX/XY embryos have shown that in chimeras with testes, most, but not all, Sertoli cells are XY, whereas the proportions of XX and XY cells in the Leydig cell population are similar to those seen outside the gonad. In chimeric embryos with ovaries, the granulosa cells were predominantly XX (>90%) (Patek et al. 1991). These results indicate that the initial steps that commit the gonad to male or female differentiation occur in the pre-Sertoli or -granulosa cells. However, cell-autonomous action of Sry must be supplemented by non-autonomous Sry-induced

factors, because some XX Sertoli cells and XY granulosa cells have been observed in chimeric gonads (Patek et al. 1991). Consequently, factors that are involved in cell-to-cell communication must be expressed very early in the sex differentiation pathway of the gonad.

1.2.4 Endocrine differentiation of the gonads

The absence or presence of two fetal testicular hormones, AMH and testosterone, is essential for sex differentiation of all other internal genitelia, as well as the external genitalia.
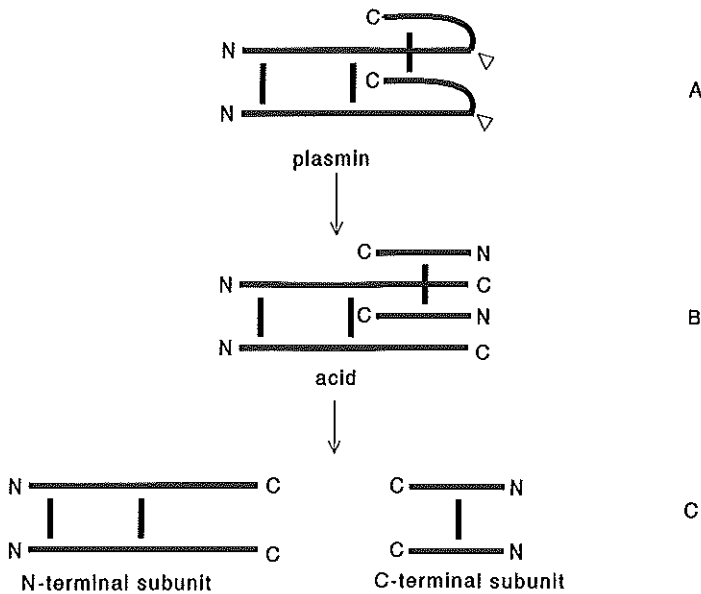
Fig. 1.3: Structural characteristics of AMH (adapted from Wilson et al. 1993).
AMH is synthesized as a 140 kDa homodimer. The vertical lines indicate that the dimer is disulfide linked (A). Plasmin cleavage between arginine 427 and serine 428 (indicated by the small arrows) generates N- and C-terminal subunits of 110 and 25 kDa, that remain noncovalently associated (B). The actual location of the disulfide bonds and the sites of N- and C-terminal interaction are currently unknown. Acid-dissociation generates the two separate subunits. Full bioactivity is obtained only when the cleaved subunits are associated. The C-terminus alone displays approximately 50% of the maximal bioactivity (C).

Structural and functional aspects of AMH

AMH is the first known product secreted by fetal Sertoli cells (Tran and Josso, 1982), and was identified as a member of the activin/TGFβ family of peptide growth and differentiation factors (Cate et al. 1986; Haqq et al. 1992) (see also Paragraph 1.2.3 and Chapter 2). In general, most members of this family are synthesized as large precursor molecules that form homodimers or heterodimers (Massagué, 1990). Each chain of the dimer is cleaved at approximately 110 amino acids from the C-terminus, generating the mature growth factor (the C-terminal subunit) and an N-terminal product (Massagué, 1990). AMH is a 140 kDa dimeric glycoprotein, which can be cleaved by plasmin to generate a 110 kDa N-terminal and a 25 kDa C-terminal subunit (Pepinsky et al. 1988). The C-terminal domain alone is biologically active. However, AMH differs from the other family members in that the N-terminus can potentiate the activity of the C-terminal domain, and appears to be required in order to obtain full bioactivity of AMH (Wilson et al. 1993) (see Fig. 3). The N-terminus possibly plays a role in maintaining the proper conformation of the C-terminus of AMH, and in preventing the formation of inactive aggregated C-termini (Wilson et al. 1993). Unlike AMH, reassociation of the N- and C-terminal domains of TGFβ results in latency, characterized by the inability of the C-terminus to bind to its receptor (Gentry and Nash, 1990).

Regulation of AMH expression during sex differentiation

It has been suggested that Sry directly regulates AMH expression through binding to the AMH gene promoter (Haqq et al. 1993). However, recent results (Shen et al. 1994; Haqq et al. 1994) indicate that Sry dependent activation of the AMH gene promoter is indirect. Shen et al. (1994) and Hatano et al. (1994) suggest that the expression of AMH is regulated by SF-1. This hypothesis is based on the observation that, after formation of the indifferent gonad, SF-1 expression decreases in the female and increases in the developing testis, which correlates with the absence of AMH from the fetal ovary and the onset and increase of AMH production by the testis (Shen et al. 1994; Ikeda et al. 1994). Indeed it has been shown that SF-1 binds to a conserved element in the AMH gene promoter (Hatano et al. 1994; Shen et al. 1994). Furthermore, activation of the AMH promoter in 15-day-old rat Sertoli cells *in vitro* required an intact SF-1 binding site (Shen et al. 1994). In order to stimulate AMH transcription, SF-1 most likely needs to be activated by an as yet unknown cofactor or ligand (Shen et al. 1994). This agrees very well with the structural similarity of SF-1 with members of the steroid hormone receptor family. Whether SF-1 actually

regulates AMH gene expression during sex differentiation remains a matter of debate, since in an *in vitro* assay system, in which Sry dependent transcription from the AMH gene promoter was operative, SF-1 acted as a transcriptional repressor (Haqq et al. 1994).

Regulation of testosterone synthesis during sex differentiation.

The other important testicular hormone, the steroid hormone testosterone, is synthesized by developing Leydig cells of the fetal testis. Several steroidogenic enzymes are involved in the steps that lead to the conversion of cholesterol into testosterone (see Fig.4). These enzymes are present in both male and female gonads. However, differences in the activity of only a few enzymes leads to testosterone synthesis in the male and estrogen synthesis in the female gonad. In the testis, the activity of 3β hydroxysteroid dehydrogenase activity (3β-HSD) increases, whereas in the ovary the aromatase activity increases (Wilson et al. 1981). This regulation can occur *in vitro*, independently of exogenous hormones or growth factors, in steroidogenic cells from rabbit testes (George et al. 1978; George and Wilson, 1980). SF-1 binding sites are present in the promoters of the genes encoding several steroidogenic enzymes, including P-450scc, P-450c17 and P-450 aromatase (Lala et al. 1992; Morohashi et al. 1992; Honda et al. 1993; Lynch et al. 1993). This implies that SF-1 may be involved not only in the regulation of AMH expression, but also in the regulation of expression of steroidogenic enzymes. The fetal production of testosterone reaches its maximum in rats at E18.5-E19.5, followed by a gradual decline (Warren et al. 1975).

Regulation and functions of the androgen receptor

Testosterone acts via the androgen receptor, a member of the steroid hormone receptor family (see also Chapter 2). The androgen receptor is a nuclear protein that specifically binds testosterone and dihydrotestosterone. After binding the ligand, the androgen receptor can regulate gene transcription through binding to an androgen response element (ARE) in the promoter of androgen regulated genes. The androgen receptor is involved in the regulation of many processes. Its function is crucial for the development and maintenance of internal and external sex organs.

Testosterone can down-regulate transcription of the gene encoding its own receptor in a number of tissues, including ventral prostate, kidney, epididymis, brain and coagulating gland (Shan et al. 1990; Quarmby et al. 1990; Blok et al. 1992a). However, in cultured immature Sertoli cells, testosterone did not affect androgen

receptor mRNA levels, whereas FSH stimulated androgen receptor expression (Verhoeven and Cailleau, 1988; Blok et al. 1989; Blok et al. 1992b). Testosterone deprivation *in vivo* resulted in up-regulation of testicular androgen receptor mRNA expression in one case (Sanborn et al. 1991), but no effect was observed in another study (Blok et al. 1992a). Thus, it is not clear whether downregulation of androgen receptor mRNA expression by androgens is operative in the testis.

Although it has been shown for many genes that their expression is up- or downregulated by androgens, it has been proven for only a few genes that this regulation is directly at the level of gene transcription (Riegman et al. 1991; Celis et al. 1993; Murtha et al. 1993; Rennie et al. 1993). Since primary androgen responsive genes in the testis have not been described, little is known about the mechanism by which testosterone exerts its effects on testicular functions.
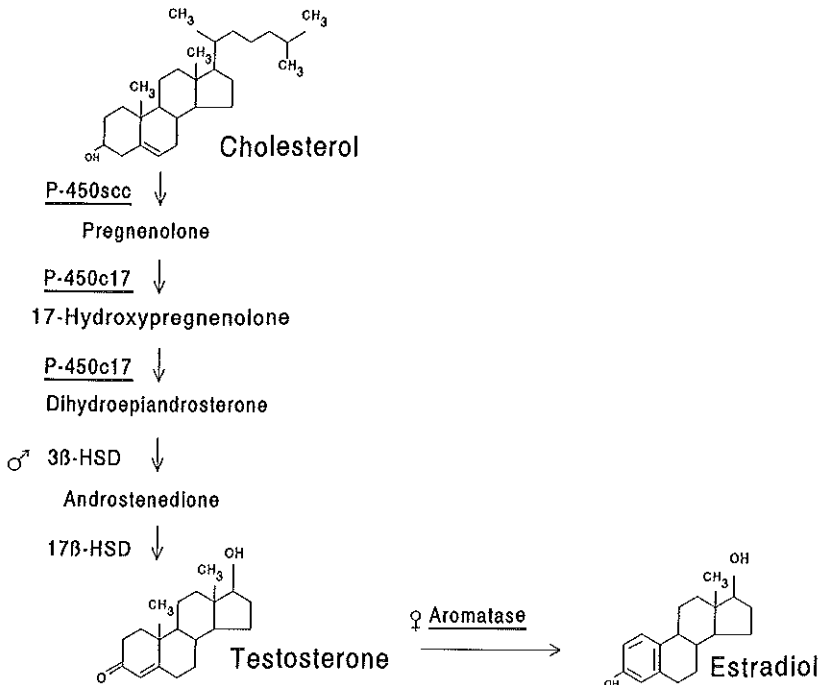
Fig. 1.4: Regulation of testosterone and estrogen synthesis during sex differentiation.
Four different enzymes are necessary to convert cholesterol into testosterone. One additional enzyme, aromatase, converts testosterone into estradiol. The enzymes of which it is known that the gene transcription is regulated by SF-1 are underlined. It is presently unknown whether 3β-HSD and 17β-HSD are also regulated by SF-1. In male embryos the activity of 3β-HSD in the testis increases during sex differentiation (indicated by ♂), whereas in the fetal ovary, aromatase activity increases (indicated by ♀).

Possible functions of AMH and testosterone in testis differentiation

AMH and testosterone are not essential for the differentiation of the testis itself. Mutations in the gene that encodes AMH in humans result in a phenotype that is characterized by the presence of a uterus and fallopian tubes in otherwise normally virilized males, a syndrome known as persistent müllerian duct syndrome (PMDS) (Josso et al. 1991; Josso et al. 1993; Imbeaud et al. 1994). The testes develop normally in these patients, but do not descent, resulting in infertility, which precludes conclusions about the role of AMH in spermatogenesis (see also Chapter 2). Similarly, mutations that disrupt the function of the androgen receptor in mice or man do not affect testis formation (Griffin and Wilson, 1989), and androgens cannot induce the formation of testicular tissue in differentiating ovaries during embryonic development (Jost, 1947a).

Contrary to the implications of these observations, there are some experimental results that indicate that AMH may perform certain functions during testis development. AMH can induce the formation of testis cord-like structures and endocrine sex reversal in ovaries cultured *in vitro* (Vigier et al. 1987; Vigier et al. 1989). Similarly, when AMH is ectopically expressed as a transgene in female embryos, testis cord-like structures occur in the developing ovaries (Behringer et al. 1990). Furthermore, in male mice that express extremely high levels of AMH, Leydig cell function is impaired (Behringer et al. 1990), and in the opposite situation, in AMH knock-out male mice, Leydig cell hyperplasia is observed (Behringer et al. 1994).

Possible role of estrogen in sex differentiation

In reptiles and birds, aromatase activity, and hence the level of estrogens, is a critical determinant in the regulation of gonadal sex differentiation (Elbrecht and Smith, 1992; Crews et al. 1994). In the mouse, estrogen receptors are present in male and female gonads throughout gonadal sex differentiation (Greco et al. 1993). Furthermore, fetal rat ovaries and testes can produce estrogens *in vitro* after gonadotropic stimulation (Weniger et al. 1993a; Weniger et al. 1993b). On the other hand, estrogen receptor (ER) knock-out mice show no abnormalities with respect to gonadal sex determination and differentiation (Lubahn et al. 1993). This may indicate that estrogens are not essential for correct gonadal sex differentiation. The fact that residual estrogen binding, to about 5% of the normal level and with normal binding affinity, could be detected in the ER-disrupted animals (Lubahn et al. 1993) implies that some reservation should be made. However, recent results suggest that this binding is due to either the presence of a non-ER estrogen-binding protein, or an

altered protein product transcribed from the disrupted ER gene that is biologically inactive (Korach, 1994).

1.2.5 The roles of AMH and testosterone in sexual differentiation of the genital ducts

The wolffian and the müllerian ducts are the anlagen of respectively the male and female reproductive tracts, but are present in the embryos of either sex. The wolffian duct is the excretory duct of the mesonephros. The müllerian duct, also called the paramesonephric duct, develops around E14 in the rat (Trelstad et al. 1982), as an invagination of the coelomic epithelium that starts lateral to the cranial end of the wolffian duct and develops in a cranial caudal direction along this duct (Byskov and Hoyer, 1994).

The wolffian ducts

The wolffian ducts become dependent on testosterone during the period immediately after gonadal sex differentiation. Further development of the wolffian ducts into epidydimides, vasa deferens and seminal vesicles is controlled by testosterone (Jost, 1947b; Jost, 1947a; Josso, 1970), although some action of dihydrotestosterone cannot be excluded (Tsuji et al. 1991; Tsuji et al. 1994). Initially, the mesenchymal cells surrounding the wolffian ducts are the testosterone target cells. During later stages of wolffian duct development, the epithelial cells also start expressing the androgen receptor (Cooke et al. 1991). This indicates the importance of mesenchymal/epithelial interactions during differentiation of the wolffian ducts (Cunha and Lung, 1979). In the female, the wolffian ducts regress due to the absence of testosterone.

The müllerian ducts

The müllerian ducts are sensitive to the degenerative action of AMH during a critical period that comprises E14.5-E15.5 in the rat (Picon, 1969; Donahoe et al. 1977; Tsuji et al. 1992). The first morphologic change that marks the beginning of müllerian duct regression, is the dissolution of the basement membrane that separates the epithelial cells, which form the actual duct, from the surrounding mesenchymal cells. Subsequently, contacts between the mesenchymal cells and the epithelial cells are frequently observed (Trelstad et al. 1982). Migration of both cell types takes place, which finally results in the formation of a circular whorl of cells at E16 in the rat

(Trelstad et al. 1982). Although some cells die during this process, müllerian duct degeneration seems to involve reorganization and functional changes of cells rather than programmed cell death (Trelstad et al. 1982). In analogy to the actions of testosterone on the wolffian ducts, AMH seems to act on the müllerian ducts via the mesenchymal cells that surround the ducts, rather than acting directly on the epithelial cells themselves. Evidence in support of this hypothesis comes from experiments on isolated epithelial and mesenchymal cells during müllerian duct regression in the rat (Tsuji et al. 1992). Using cells isolated from E15.5 fetuses, it was shown that AMH could reduce [^{3}H]-thymidine incorporation by müllerian duct mesenchymal cells, but not by epithelial cells (Tsuji et al. 1992).

If exposure of explanted rat müllerian ducts to AMH *in vitro* is delayed until after E15.5, müllerian duct regression does not occur (Picon, 1969; Donahoe et al. 1977; Tsuji et al. 1992). However, AMH induced the formation of linear bulges in the müllerian duct of urogenital ridges isolated from 17.5 to 18.5-day-old rat fetuses. This indicates that the müllerian ducts can still respond to AMH after the critical period, albeit in a different way (Tsuji et al. 1992).

In the female, the müllerian ducts develop into the fallopian tubes, the uterus and the upper part of the vagina. Early development and growth of the müllerian ducts are generally considered to occur autonomously (Jost, 1953; Lubahn et al. 1993; Byskov and Hoyer, 1994).

1.3 Gonad development and function

1.3.1 Testis development

Development of the testicular cell types

The neonatal testis consists of seminiferous tubules in which the prespermatogonia and Sertoli cells are located, and an intertubular space with Leydig cells and blood vessels. A basal membrane is located between the Sertoli cells and the peritubular myoid cells surrounding the tubules. The fetal and neonatal Leydig cell population differs from the adult Leydig cells in several aspects, illustrated by a much higher steroidogenic capacity of the fetal testis, which is important for the androgen dependent genital differentiation (Huhtaniemi et al. 1982; Tapanainen et al. 1984; Huhtaniemi, 1994). Fetal Sertoli cells divide rapidly, with their highest proliferative activity occurring on E16 in the mouse, followed by a gradual decline (Vergouwen et al. 1991). The Sertoli cells stop dividing around postnatal day 15 in the rat (van Haaster et al. 1992). A Sertoli cell can only support the development of a limited number of germ cells. Thus it is the number of Sertoli cells that is present at day 15 that determines the maximal output of sperm during adult life, and hence the final testis size (Orth et al. 1988; reviewed by Sharpe 1994). Sertoli cell maturation is characterized by a number of biochemical and morphological changes. A marked structural change is the formation of tight junctional complexes between adjacent Sertoli cells, which results in the formation of the so-called blood-testis barrier. Thus, a separated inner part of the testicular tubules is formed (the adluminal compartment) in which the meiotic and postmeiotic development of the germ cells takes place. Formation of the blood-testis barrier and cessation of Sertoli cell divisions occur more or less coincident (Russell et al. 1989).

The prespermatogonia have undergone series of mitotic divisions until E18 in the rat after which they enter a phase of mitotic arrest (Byskov and Hoyer, 1994). Between postnatal days 3 and 8 in the rat, reinitiation of prespermatogonia divisions marks the onset of the first wave of spermatogenesis (van Haaster and de Rooij, 1994). Subsequently, the first germ cells enter the prophase of meiosis and are translocated to the just formed adluminal compartment when they are in the preleptotene stage. The complete process of spermatogenesis, from undifferentiated spermatogonia into spermatocytes, that undergo two meiotic divisions, followed by the development of the haploid spermatids into mature sperm, culminates in the release of the first spermatozoa into the tubular lumen around day 45 in the rat (Clermont and

Perey, 1957).

The spermatogenic cycle

      The location of all spermatogenic cell types within the seminiferous epithelium of the adult rat is highly organized. A single Sertoli cell is always associated with one of fourteen defined sets of spermatogenic cells. The regular occurrence of these fourteen cell associations is called the spermatogenic cycle. In the adult rat, waves of differentiating spermatogonia enter the cycle at regular time intervals (see Fig 1.5).
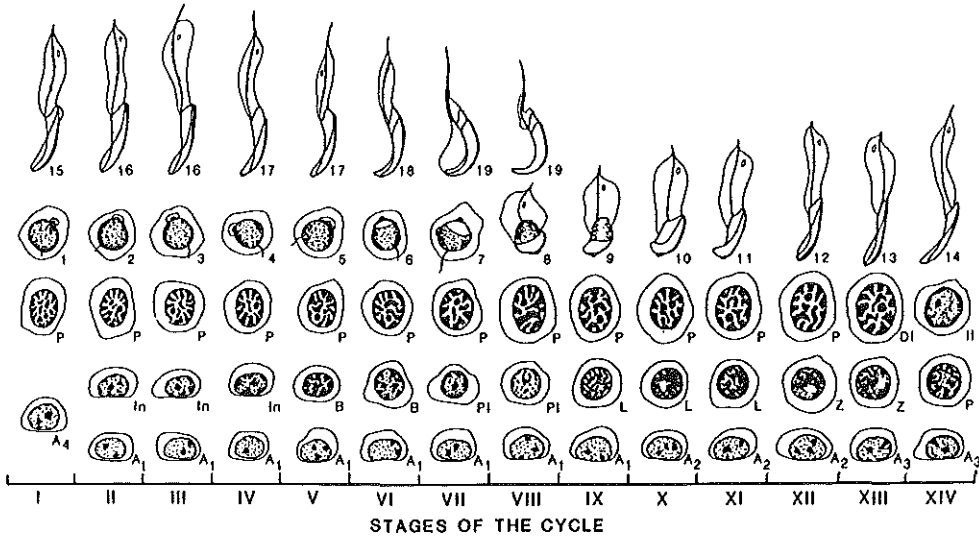
Fig. 1.5: Germ cell associations at the different stages of the spermatogenic cycle in the rat (adapted from Sharpe, 1994).
Each vertical column or stage describes the fixed complement of germ cells associated with Sertoli cells at that particular stage, with the lumen of the tubule to the top and the basement membrane to the bottom of the diagram. Each Stage of the cycle lasts for a fixed period of time. A single germ cell goes through each step of spermatogenic development from left to right and bottom to top through the diagram until it reaches Step 19 of spermatid development at Stage VIII and is released as mature sperm. Thus, each germ cell from A_1 passes through the cycle 4.5 times before it is released. The complete process of spermatogenesis can be divided into phases based on the major developmental events that occur. A_1, A_2, A_3, A_4: spermatogonia type A; In: intermediate spermatogonia; B spermatogonia type B; PL: preleptotene spermatocytes; L: leptotene spermatocytes; P: pachytene spermatocytes; DI: diplotene spermatocytes; II: secondary spermatocyte undergoing meiotic division II; 1-19: steps of spermatid differentiation. Note: The undifferentiated spermatogonia are not represented in this diagram. The undifferentiated spermatogonia are named according to their topographical arrangement in the seminiferous tubules. Clones of one cell are named A_{single} (A_s), two cells A_{paired} (A_{pr}), and four or more cells $A_{aligned}$ (A_{al}). The A_s cells are the stem cells of spermatogenesis and are present during all stages of the cycle. Stem cell renewal and differentiation take place in a cyclic manner. A period of active proliferation spans Stages X-II, whereas during Stages III-IX the stem cell proliferation is inhibited and the A_{al} enter the spermatogenic cycle as spermatogonia type A1 during Stage VII (Huckins, 1971).

Hormonal regulation of testis development and function

FSH and LH are the most important regulators during prepubertal testis development. LH stimulates Leydig cell development and steroidogenesis, while FSH stimulates the proliferation of Sertoli cells and also regulates Sertoli cell maturation (reviewed by Sharpe, 1994). Leydig cell development and Sertoli cell development are interactive processes. FSH receptor binding can be detected at E17.5 in the rat, and increases parallel to the degree of testicular growth, indicating that the number of FSH receptors per Sertoli cell is relatively constant (Warren et al. 1984).

Leydig cells secrete large amounts of testosterone around the time of birth, followed by a rapid decrease which is due to changes in the steroidogenic pathway (Warren et al. 1984; Huhtaniemi, 1994). Full length mRNA encoding the LH receptor can be detected from E16 in the rat (Zhang et al. 1994). However, it is thought that the peak of testosterone synthesis in the rat during fetal/early postnatal life is not caused by LH, since levels of LH in the rat are too low during this period (Huhtaniemi, 1994). The level of LH receptor mRNA expression is maximal at E21.5 and decreases after birth (Zhang et al. 1994).

After an initial decline in testosterone synthesis shortly after birth, the levels of testosterone rise again around puberty, resulting in high adult levels (Podesta and Rivarola, 1974). Testosterone is absolutely required for maintenance of spermatogenesis (reviewed by Sharpe, 1994), but no androgen receptor, essential for testosterone action, is expressed in the spermatogenic cells of the testis (Grootegoed et al. 1977; Buzek and Sanborn, 1988; Bremner et al. 1994). Therefore, testosterone must exert its effects through the androgen receptor positive peritubular myoid cells and Sertoli cells. There is an increase in testicular androgen receptor levels during sexual maturation in the rat (Buzek and Sanborn, 1988). This increase may be partly caused by FSH, which has been shown to up-regulate androgen receptor expression in immature Sertoli cells *in vitro* (Verhoeven and Cailleau, 1988; Blok et al. 1989; Blok et al. 1992b). Total testis androgen receptor mRNA expression (per given amount of total RNA) decreases from postnatal day 26 onwards, but this is most likely due to the increasing amount of androgen receptor negative germ cells (Lubahn et al. 1988).

The role of FSH in adult male animals is not clear, and seems to be species dependent. In the rat, conflicting results have been reported. On the one hand, there is an age dependent decline in responsiveness of Sertoli cells to FSH in terms of secretion of several FSH regulated proteins, which appears to result from an increased activity of cAMP phosphodiesterase activity (Ritzen et al. 1989). On the other hand, studies using hypophysectomized rats substituted with FSH and/or androgen, indicate that the combination of the two hormones is necessary to maintain normal testicular

weight (Bartlett et al. 1989). Also, in Sertoli cells isolated from 20-day-old rat testis and adult testis, similar levels of FSH receptor mRNA are detected (Heckert and Griswold, 1991). Furthermore, the expression in adult Sertoli cells of both FSH receptor and cAMP phosphodiesterase is strictly regulated during the spermatogenic cycle (Kangasniemi et al. 1990; Heckert and Griswold, 1991; Parvinen, 1993). The highest responsiveness of Sertoli cells to FSH during Stages XIV to VI (Parvinen, 1993) of the spermatogenic cycle, is consistent with the hypothesis that FSH plays a role in the regulation of survival of differentiated spermatogonia that undergo the last four mitotic divisions during this period (Sharpe, 1994).

1.3.2 Ovary development

Follicle growth

Primordial follicles, which are all formed around birth in the rat, consist of an oocyte surrounded by a single layer of pregranulosa cells, that form the precursors of the granulosa cells that will finally populate the follicle. From the pool of primordial follicles, groups of follicles will enter the growing phase throughout life. The pregranulosa cells that surround the oocyte in the primordial follicle cease to divide and enter a period of quiescence. Theca cells are not immediately observed, but differentiate during early stages of follicle growth in the rat and arise from the undifferentiated mesenchymal interstitial cells throughout reproductive life (reviewed by Hirschfield, 1991).

Each follicle that begins to grow either goes on to ovulate or degenerates, a process which is called atresia. Follicular atresia can occur at all stages of follicle development (Fig. 6A). When follicle growth is initiated, the oocyte, still arrested in the late prophase of meiosis I (dictyate), grows rapidly in size. The granulosa cells of these small preantral follicles (Fig. 1.6A) proliferate extensively, also after the oocyte has ceased to grow. A basement membrane separates the granulosa cells on the inside from the theca interna (steroidogenic cells) and theca externa (connective tissue cells) outside. During the following phase of growth, fluid-filled spaces appear between the granulosa cells, eventually merging into a single large antral cavity; these follicles are called antral follicles (Fig 1.6A) (reviewed by Hirschfield (1991), and Byskov and Hoyer, 1994). The complete process of follicle development from a small preantral follicle into a preovulatory follicle takes approximately 15-17 days (Hage et al. 1978). During the final steps of follicle differentiation (follicle maturation), the

granulosa and theca cells differentiate further and the antral cavity enlarges. After the LH surge, resumption of oocyte-meiosis takes place (oocyte meiotic maturation), resulting in formation of the first polar body and arrest of meiosis in metaphase II. Then, ovulation occurs.

The estrous cycle

In the adult rat, the surge of FSH at estrus stimulates a group of preantral follicles to become antral. Subsequently, 12-14 of these follicles are selected, reach the preovulatory size, and ovulation is induced by the LH surge at proestrus. This process, which is called the estrous cycle, repeats every 4-5 days depending on the strain. After ovulation the remaining follicle luteinizes and develops into a corpus luteum. The corpus luteum will degenerate after 4-5 days unless the ovum is fertilized; then the corpus luteum is rescued from luteolysis.

A)
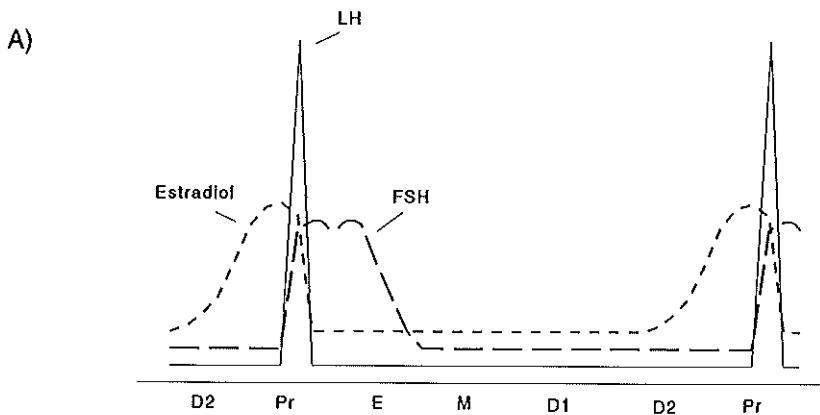
Fig. 1.6: Follicle development and the estrous cycle in the rat.
A) Schematic representation of hormone concentrations in serum during the rat estrous cycle. E: Estrus; M: Metestrus; D1;D2: Diestrus 1 and 2; Pr: Proestrus.
B) (next page) Schematic illustration of follicular development and atresia (adapted from Findlay (1994). The amount of primordial follicles is set during the first days of postnatal development when follicle formation takes place. Primordial follicles consist of an oocyte surrounded by a single layer of pregranulosa cells. Once follicle growth is initiated, the committed follicles either go on to ovulate or become atretic. In a committed follicle, the oocyte grows rapidly, and the granulosa cells proliferate extensively. These follicles are surrounded by a basement membrane and a theca cell layer. Small antral follicles develop fluid-filled spaces between the granulosa cells, and they become responsive to gonadotropins, and large antral follicles are gonadotropin dependent. The preovulatory follicles are mature and ovulate in response to the LH surge. Atresia can take place during all steps of follicle development. The highest rate of atresia occurs in the population of large antral follicles.

B)

Primordial follicles

Committed follicles

Small/large antral follicles

Pre-ovulatory follicles

Atresia
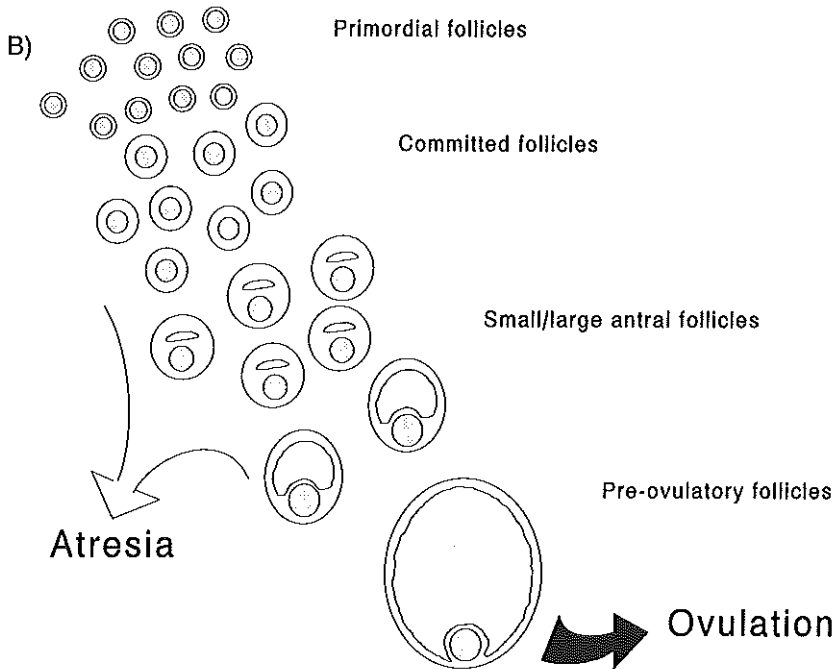
Ovulation

Hormonal regulation of ovary development and function

During the prepubertal period, follicle growth occurs, but this does not lead to formation of mature preovulatory follicles, and the follicles slowly degenerate. Ovulation does not occur until after the first LH surge (35-45 days after birth, reviewed by Ojeda and Urbanski, 1994). During the prepubertal period, establishment of the hypothalamus-pituitary-gonad axis takes place, and negative and positive feedback mechanisms develop.

The level of circulating FSH in the rat increases after birth to a maximal value at day 12, after which there is a gradual decline (Ojeda and Ramirez, 1972; Meijs-Roelofs et al. 1973). Also LH levels are higher during the first two weeks of postnatal development than during later phases of prepubertal development (Ojeda and Ramirez, 1972; Meijs-Roelofs et al. 1973). Binding of FSH and LH to their respective receptors cannot be detected until postnatal day 7 in the rat (Smith-White and Ojeda, 1981; Sokka et al. 1992). During the second week of postnatal ovary development their is an increase in sensitivity of the growing follicles to FSH and LH which is illustrated by a concomitant increase in gonadotropin receptor numbers (Smith-White and Ojeda, 1981; Uilenbroek and van der Linden, 1983; Sokka and Huhtaniemi, 1990). In the female, granulosa cells specifically express FSH receptors

(like Sertoli cells in the male). LH receptors are expressed by theca cells and, during the final phases of follicle development, also by granulosa cells (Zeleznik et al. 1974). The initiation of follicular growth does not depend on gonadotropins, FSH is required for continued follicle growth and development (reviewed by Hirschfield, 1991).

In the adult cycling female rat, the elevated level of FSH at estrus stimulates growth and differentiation of the granulosa cells. FSH induces aromatase activity (Dorrington et al. 1975; Fitzpatrick and Richards, 1991) and LH receptors (Piquette et al. 1991) in granulosa cells of maturing follicles. An important functional characteristic of these follicles is the increased output of estrogens. The theca cells are stimulated by LH to produce androgens. In granulosa cells, these androgens are subsequently converted to estrogens by aromatase. This is known as the "two-cell, two-gonadotropin" model (reviewed by Hillier et al. 1994). FSH secretion is decreased by the increased estrogen output and also by inhibin, a member of the activin/TGFβ family of growth/differentiation factors (see also Paragraph 1.2.3). The decrease in FSH secretion results in atresia of large follicles that are in less than optimal condition, and thus in selection of follicles that can reach the final stages of follicle maturation and will ovulate at estrus. The high estrogen levels eventually elicit the FSH and LH surges (Fig. 1.6B) that result in ovulation and subsequent luteinization of the remaining follicle (positive feedback mechanism). A secondary FSH surge starting at early estrus recruits follicles that will undergo the final stages of follicle maturation in the next cycle. Estrogens are not only important as a feedback regulator, but are thought to act as an autocrine/paracrine factor as well. For example, estrogens have been reported to stimulate granulosa cell proliferation and to prevent follicular atresia (reviewed by Hirshfield, 1991).

1.3.3 Local regulatory actions in the gonads

During the last decade, it has become more and more obvious that besides gonadotropins and steroids, local growth factors also play important roles in the regulation of gonadal functions. Many different growth factors have been reported to be locally expressed, and/or to have effects upon gonadal functions. These include fibroblast growth factors, transforming growth factor-α, insulin-like growth factors I and II, nerve growth factor, interleukins, and members of the activin/TGF-β family of growth/differentiation factors (reviewed by Adashi et al., 1991; Lamb, 1993; Spiterigrech and Nieschlag, 1993; Findlay, 1993 and 1994; and Tsafriri and Adashi

1994).

In general, the actions of growth factors may not be as marked as the effects of gonadotropins and steroid hormones. In many cases, their major role seems to be local fine-tuning of hormone action. Several features, inherent to local regulatory mechanisms, complicate the assignment of specific gonadal functions to a certain growth factor. First, these factors often function in many extragonadal processes that are essential for the overall viability of the organism, thus making it impossible to study the effect of their absence. Second, if the absence of a factor is not lethal, redundancy phenomena may hide the physiologic function of that factor, for example in gene knock-out experiments (Shastry, 1994). Third, species-specific effects and/or species-specific expression patterns of growth factors may preclude the development of general hypotheses. Finally, the interpretation of *in vitro* experiments that address the effects of exogenously added growth factors, may be complicated if these factors are also produced by the isolated cell types or tissues in culture; local production may result in high local levels. Thus, although many results on expression and effects of growth factors have been reported, little progress has been made concerning their actual functions *in vivo*. This paragraph focusses on the available data concerning the gonadal expression and effects of some members of the activin/TGFβ family of growth factors. The existing concepts on their *in vivo* actions in the gonads will be described.

TGFβs

The synthesis and effects of the three known mammalian TGFβs (TGFβ1, TGFβ2 and TGFβ3) in the gonads vary depending on the species (reviewed by Benahmed et al., 1993). It is not known whether the different TGFβs each perform specific functions, or whether their functions overlap. Many cell types in the rat ovary and testis have been reported to express protein or mRNA representing one or more of the TGFβs (Skinner and Moses, 1989; Mullaney et al. 1991; Benahmed et al. 1993; Schmid et al. 1994; Gautier et al. 1994). Gonadotropins most likely control TGFβ expression in their target cells (Benahmed et al. 1993; Mullaney and Skinner, 1993). During the estrous cycle in the ovary, interstitial cell TGFβ production varies little, whereas the granulosa cells start expressing TGFβ 1 and 2 at the preovulatory stage of follicle development (Teerds and Dorrington, 1992). Mullaney and Skinner (1993) report marked changes in the TGFβ expression patterns in the somatic cells of the testis around puberty. During adult spermatogenesis, there are different levels of TGFβ expression also in spermatogenic cell types (Teerds and Dorrington, 1993).

Receptors for TGFβ have recently been cloned (see Chapter 2). However, little

is known about their expression in the gonads. TGFβ type II receptor mRNA has been detected mainly in the theca cells of preantral and antral follicles of the mouse ovary (Schmid et al. 1994), and in the peritubular myoid cells of the rat testis, with much lower levels in the other somatic cell types, and being not detectable in spermatogenic cells (Le Magueresse et al. 1994). TGFβ type III receptor is expressed in all testicular cell types. In addition, spermatogenic cells express high levels of an abberrant mRNA of 3.5 kb (Le Magueresse et al. 1994).

All these expression data have not led to a comprehensive model that fits all the effects of TGFβ in a general mechanism by which TGFβ might influence the regulation of gonadal functions. It is clear that most forms of TGFβ are locally synthesized in the gonads. Also, TGFβ can regulate important functions of most gonadal cell types *in vitro* (Hutchinson et al. 1987; Morera et al. 1988; Skinner and Moses, 1989; Hernandez et al. 1990; Dorrington et al. 1993; Hakovirta et al. 1993). Yet, it is not clear whether these actions are important *in vivo*.

A step towards the development of a general concept was made by Dorrington et al. (1993), who have proposed that FSH, estradiol and TGFβ positively interact to stimulate cell proliferation and differentiation, both in granulosa and Sertoli cells. In this model, TGFβ is thought to mediate the stimulatory actions of estradiol.

AMH

AMH is one of the very few factors whose functions during postnatal development are most likely restricted to the gonads (see also Chapter 7). In females, AMH is synthesized only by the granulosa cells of preantral and antral follicles, but not by large preovulatory follicles, in rat (Hirobe et al. 1994) and mouse (Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992) (see also Chapter 4). In males, the Sertoli cells specifically express AMH, of which the level decreases after birth, but low levels of AMH mRNA can be detected throughout adult life in various species (Kuroda et al. 1990; Baker et al. 1990; Lee et al. 1992; Lee et al. 1994) (see also Chapter 5). No convincing binding data of AMH to potential target cells in the gonads have been reported (see also Chapters 3 and 7). Several effects of AMH on gonadal cell functions have been described. AMH represses the biosynthesis of aromatase, decreases the number of LH receptors (di Clemente et al. 1994), and opposes induction of progesterone biosynthesis by epidermal growth factor, in granulosa cells from several species (Kim et al. 1992). It has also been reported that bovine AMH can inhibit meiotic maturation of both denuded and cumulus-enclosed rat oocytes *in vitro* (Takahashi et al. 1986). However, Tsafriri et al. (1988) have not been able to

reproduce these results. In the male, AMH has been postulated to cause mitotic arrest of prespermatogonia (Münsterberg and Lovell-Badge, 1991). This hypothesis is based on prenatal and neonatal expression of AMH, which is high around the time of mitotic arrest and decreases during the first 2-3 weeks in rat and mouse, concomitant with the first wave of spermatogenesis (Kuroda et al. 1990; Münsterberg and Lovell-Badge, 1991). Also, there is a strong correlation between germ cell arrest at the prespermatogonia stage and the expression of AMH in adjacent somatic cells in the ovotestis of B6Y^{dom} mice (Taketo et al. 1991). However, results from *in vitro* culture experiments of neonatal mouse testes in the presence or absence of AMH, showed no inhibitory effects of AMH on spermatogonial numbers, but instead indicated that differentiation of prespermatogonia into spermatogonia A requires AMH (Zhou et al. 1993).

Experiments using transgenic mice (overexpression of AMH and AMH knock-out) indicate that AMH may regulate Leydig cell development (Behringer et al. 1990; Behringer et al. 1994). Thus, although numerous observations suggest that AMH functions in the testis during fetal and neonatal development, the nature of this function remains to be established. No effects of AMH on the adult testis have been reported.

Activins and inhibins

Similar to TGFβ and AMH, activins and inhibins are dimeric proteins. Inhibin consists of an inhibin-α and an inhibin-βA or -βB subunit, named inhibin A and inhibin B, respectively, whereas inhibin-β subunits (A or B) dimerize to form activin A, B or AB (see also Chapter 2). The fact that inhibin-β subunits contribute to both inhibin and activin formation, complicates the interpretation of inhibin subunit immunohistochemical and mRNA expression data.

The inhibin subunits are expressed in many ovarian and testicular cell types in the rat (Meunier et al. 1988; Kaipia et al. 1992; Klaij et al. 1994). Bioassays and/ or selective immunoassays that specifically identify inhibin and/or activin are important tools in determining the cell types that produce these growth factors (Eddie et al. 1979; Robertson et al. 1988; Grootenhuis et al. 1989; Shintani et al. 1991; de Winter et al. 1994). Synthesis and possible functions of inhibins and activins in the gonads have recently been reviewed (Findlay, 1993; Hillier and Miró, 1993; Moore et al. 1994).

Based upon the expression of inhibin subunits in granulosa cells during follicle development, follicular activin production is thought to increase during early stages of follicle development, followed by an inhibition of activin secretion during follicle

maturation (Woodruff et al. 1988; Meunier et al. 1988; Findlay, 1993; Hillier and Miró, 1993), whereas inhibin production by granulosa cells increases when follicles reach the preovulatory size, but decreases again after the LH surge (Woodruff et al. 1989; Hillier and Miró, 1993).

In the testis, Sertoli cells produce both inhibin and activin. Immature Leydig cells produce activin, whereas adult Leydig cells produce only free α-subunits (de Winter et al. 1992b; de Winter et al. 1993; Moore et al. 1994). Immature peritubular myoid cells have also been reported to produce activin *in vitro* (de Winter et al. 1994).

The expression of the inhibin subunits varies during the spermatogenic cycle (Kaipia et al. 1992; Klaij et al. 1994). Furthermore, germ cell preparations enriched in early spermatids have been found to increase the level of α-subunit mRNA expressed in cultured Sertoli cells isolated from 20-day-old rats (Pineau et al. 1990). These results indicate that the relative levels of inhibin and activin secreted by Sertoli cells depend upon interactions with specific spermatogenic cell types.

Two activin receptor type II genes (ActRII and ActRIIB) and two activin receptor type I genes (Alk-2 and Alk-4) have recently been identified (see Chapter 2). Inhibin can bind to the ActRIIB with approximately ten-fold lower affinity than activin. Whether there exists a separate inhibin receptor remains an enigma, because most effects of inhibin can be explained by neutralization of activin actions. However, recent binding experiments indicate that Leydig cells may express specific inhibin receptors, whereas activin was shown to bind specifically to cells in the basal compartment of the seminiferous tubules and to Step 7 and 8 round spermatids (Krummen et al. 1994). In the testis, ActRII mRNA is expressed in Sertoli cells, peritubular myoid cells, pachytene spermatocytes, round spermatids and also, albeit at a very low level, in Leydig cells (de Winter et al. 1992a; Cameron et al. 1994). ActRIIB mRNA is expressed in interstitial cells including Leydig cells (Cameron et al. 1994). mRNAs encoding type I receptors for activin are found in Sertoli cells (Alk-2 and Alk-4), Leydig cells (Alk-2), spermatocytes and spermatids (Alk-4) (de Jong et al., personal communication). Less is known about activin receptor expression in the ovary. ActRII mRNA is detected at high levels in oocytes and at a low level in corpora lutea (Cameron et al. 1994). Granulosa cells express low levels of ActRII and ActRIIB mRNAs (Cameron et al. 1994).

Follistatin is a protein that is functionally, but not structurally, related to inhibin and activin (reviewed by Michel et al., 1993). Follistatin is expressed in the gonads and can bind inhibin and activin, and thereby acts as an inhibitor of their actions.

In the ovary, most data concerning the expression of activin and inhibin and

their receptors, as well as reported effects on cell functions, seem to fit in a model that implicates activin in the stimulation of early follicular development, and inhibin in preovulatory follicle selection and maintenance of follicle dominance. It is postulated that activin is involved in inducing FSH sensitivity, by increasing FSH receptor numbers, in undifferentiated granulosa cells from preantral follicles (Findlay, 1993), whereas the increasing levels of inhibin during the late phases of follicular development may stimulate the LH-induced androgen synthesis in theca cells, thereby positively influencing the final follicular estrogen output. However, this model of activin and inhibin action is not supported by Woodruff et al. (1990), who found that inhibin stimulated follicular growth, whereas activin had atretogenic effects *in vivo*. In a more recent report, Li et al. (1995) show that activin promotes ovarian follicle development *in vitro*, and these authors suggest that the findings of Woodruff et al. (1990) might be explained by an activin-induced overproduction of follistatin, which counteracts activin.

Several effects of activin and inhibin on spermatogonial cell proliferation, Leydig cell steroidogenesis and Sertoli cell functions have been reported (van Dissel-Emiliani et al. 1989; Lin et al. 1989; Mather et al. 1990; Mauduit et al. 1991; Hakovirta et al. 1993; de Winter et al. 1993). However, there is no model that could explain effects of activin and inhibin on testicular cell types. One important function of activin and inhibin could be the maintenance of a constant rate of spermatogonial proliferation during the spermatogenic cycle in adult animals, as suggested by Moore et al. (1994). Furthermore, inhibins and activins are likely to be involved also in the control of somatic cell growth in the gonads, since transgenic mice that carry a targeted deletion in the α-inhibin gene develop gonadal stromal tumors (Matzuk et al. 1992) (see also Chapter 2).

1.4   Scope of this thesis

Gonadotropins (LH and FSH), together with steroid hormones (in particular estradiol and testosterone), are the principal regulators of gonadal development and function. Members of the activin/TGFβ family of growth and differentiation factors, including AMH, are probably some of the most important local factors that affect gonadal functioning. Of the different growth factors of this family, only AMH functions are specifically restricted to sex differentiation and the gonads. This thesis is focussed on the regulation of the postnatal functions of AMH and testosterone in the gonads, and on the interactions of these two hormones with other regulators of gonadal function.

Chapter 2 describes the mechanism of action of steroid hormones, gonadotropins, and members of the activin/TGFβ family. Furthermore, the importance of these regulatory hormones and their receptors is illustrated by the disturbances in sex differentiation and/or gonadal functioning, caused by mutations that disrupt their functioning.

Due to the fact that no good binding assays for AMH could be developed (Donahoe et al. 1977), the receptors that bind AMH have remained elusive. In Chapter 3, the first cloning of a receptor for AMH is described. The levels of AMH and AMH receptor mRNA in gonads before and after birth were studied using sensitive molecular techniques (*in situ* hybridization and RNase protection). In the male rat, the testicular levels of AMH and AMH receptor mRNAs were determined during postnatal testis differentiation and also in the adult rat. Regulatory effects of certain germ cell types upon AMH and AMH receptor mRNA expression in Sertoli cells of adult rats were determined by elimination of germ cells that are at specific steps of spermatogenesis (Chapter 4). In Chapter 5, AMH and AMH receptor expression is studied in ovarian follicles of different stages, during postnatal development of the ovary and during the estrous cycle, and the effect of various hormonal treatments was evaluated.

Chapter 6 describes the first cloning of the androgen receptor promoter from the rat. The availability of this genomic DNA region that determines the expression of the androgen receptor has led to important advances in understanding the control mechanisms of this key regulator of testicular function.

In the General Discussion (Chapter 7), a hypothesis concerning the postnatal functions of AMH is presented. The current knowledge on the AMH receptor(s) and related receptors is integrated, to discuss the mechanism of action of AMH. An overview of recent results concerning regulation of the androgen receptor promoter is

presented, and the functional implications of these data are described. Finally, some directions are given for future research.

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Structural and Functional Aspects of Hormone Receptor Mechanisms Involved in Sex Differentiation and Gonadal Development

2.1   Introduction

This chapter describes structural and functional aspects of gonadotropins, steroid hormones, and members of the activin/TGFβ family of growth and differentiation factors (which also can be considered as hormones).   These three groups of hormones and their receptors each display specific structural characteristics that allow for their biological functions (see Figs. 2.1, 2.2).   Clinical and experimental data concerning mutations in some of these hormones or their receptors illustrate their importance in sex differentiation and/or gonadal development and function (see Table 2.1).

2.2   Gonadotropins

2.2.1   Hormone and receptor structure.

LH and FSH are large heterodimeric glycoprotein hormones that consist of a common α-subunit and a hormone specific β-subunit (Gharib et al. 1990).   They are members of a distinct family of glycoprotein hormones which also includes thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG), and their receptors have a similar overall structure (Frazier et al. 1990; McFarland et al. 1989; Sprengel et al. 1990) (Fig. 2.2A).   A large extracellular domain encompasses the ligand binding domain, followed by a transmembrane domain that contains seven membrane-spanning segments.   The short intracellular C-terminal domain and the intracellular loops can interact with G proteins.   Both LH and FSH receptors couple to adenylyl cyclase through G_s, although it has been found in *in vitro* studies that coupling to phospholipase C, resulting in phosphoinositide breakdown, is also possible (Gudermann et al. 1992).   The LH and FSH receptors belong to the large family of G protein-coupled receptors (Segaloff and Ascoli, 1992), but the presence of the large extracellular domain places the LH and FSH receptors together with the TSH receptor in a separate subfamily.   Another difference is that, while most G protein-coupled receptors are encoded by a single exon, the extracellular domains of the LH and FSH receptors are encoded by respectively 10 and 9 exons (Tsai Morris et al. 1991; Heckert et al. 1992).   These exons contain several "leucine-rich repeats", that occur in a large and diverse group of functionally non-related proteins, collectively known as leucine rich glycoproteins (Leong et al. 1992).   The leucine rich repeats are probably involved in formation of amphipatic helices that may contact other proteins.
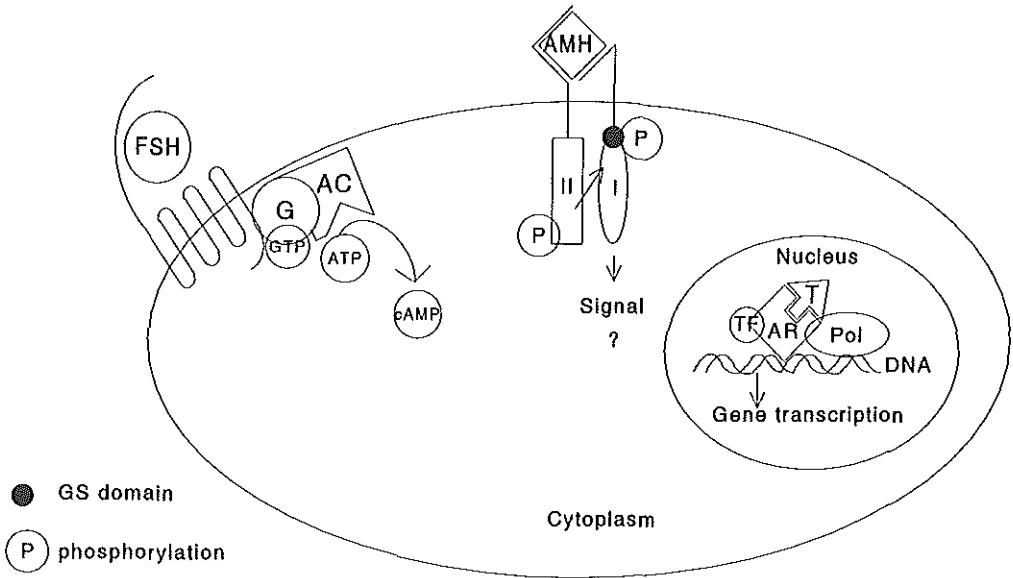
Fig. 2.1:Schematic drawing of a Sertoli cell expressing three receptor types that are essential for correct sex differentiation and/or gonadal development.
FSHR and AMHR are specifically expressed only in Sertoli cells (or in granulosa cells in females), whereas the AR is also expressed in a number of other cell types. The FSHR is a member of the large family of G protein-coupled receptors. It contains a large extracellular ligand binding domain. The transmembrane domain contains seven membrane spanning regions. The intracellular loops, together with the C-terminal intracellular domain, interact with intracellular G proteins. G proteins (G), when bound to GTP, activate adenylyl cyclase (AC), which converts ATP into the second messenger cAMP. AMH most likely binds to a receptor complex that contains two different receptor types (I and II). Both receptors are members of the family of transmembrane serine/threonine kinase receptors (see also Chapter 7). Probably, the type II receptor binds AMH, then recruits the type I receptor into the complex, and subsequently the constitutively active kinase domain of the type II receptor phosphorylates the GS domain of the type I receptor. This results in further propagation of the signal to unknown downstream substrates. Testosterone (T) enters the cell through diffusion and binds to the AR that is located in the nucleus. T binding to the AR results in dissociation of heat-shock proteins, and binding of the AR to specific response elements in the DNA. Certain regions of the AR are capable of interacting with other transcription factors (TF), and components of the basic transcription machinery, containing RNA polymerase II (Pol). Together, these events lead to transcription of androgen responsive genes.

The transmembrane and intracellular domains of the LH and FSH receptors are encoded by a single exon, and are homologous to the other members of the G protein-coupled receptor family.

The deduced molecular weight from the cDNA of the FSH receptor is approximately 75 k. Using a polyclonal antibody to a synthetic peptide derived from the rat FSH receptor, and a mammalian cell line transfected with an FSH receptor cDNA construct, Quintana et al. (1993) detected the FSH receptor on Western blots as a 58-83 k protein or a 69-81 k protein, under non-reducing and reducing conditions, respectively. However, a solubilized, isolated membrane FSH receptor complex of approximately 240 k, that dissociates into four subunits upon exposure to dithiothreitol, has also been described (Dattatreyamurty et al. 1992; Sperbeck et al. 1993; Dattatreyamurty and Reichert Jr, 1993). Thus, some controversy exists about whether the FSH receptor forms large (possibly FSH dependent) homomeric complexes *in vivo*, or whether it exists as a single peptide in the membrane.

The predicted molecular weight of the LH receptor is 75 k and the difference between this calculated number and the apparent molecular weight (93 k) on SDS-PAGE gels is thought to be due to the glycoprotein nature of the molecule (Segaloff and Ascoli, 1993). Multiple species of LH and FSH receptor mRNAs have been reported. Many of these are the result of differential splicing, often resulting in mRNAs that encode truncated proteins. Whether these receptor forms have any functional significance is not clear and needs further investigation (reviewed by Themmen et al., 1994). It has been suggested that alternative splicing of gonadotropin receptor pre-mRNAs is used as a means to regulate the expression of the receptors in the fetal gonads (Huhtaniemi, 1994)

After binding of gonadotropin, the receptor changes its conformation, and starts to activate G proteins. The receptor does not stay in this activating conformation, but mechanisms such as phosphorylation by kinases acting on the receptor, will lead to a process called desensitization, resulting in lower ligand affinity, less efficient coupling to G proteins, and a lower receptor number at the plasma membrane (LaPolt et al. 1991; Themmen et al. 1991; Heckert et al. 1992).

2.2.2 Mutations in LH, FSH, and their receptors

FSH deficiency is an uncommon cause of infertility, producing amenorrhoea and hypogonadism in women, and oligo- or azoospermia with normal testosterone levels in men. In one such case, a woman with primary amenorrhoea and infertility, a frame-shift deletion in both genes encoding the β-subunit of FSH has been reported (Matthews et al. 1993). Homozygeous mutation of the β-subunit of the LH gene (single amino acid substitution) has been reported in one case (Weiss et al. 1992). This male patient failed to undergo spontaneous puberty, and testicular biopsy revealed an arrest of spermatogenesis and absence of Leydig cells.

The clinical symptoms of other patients, with hypogonadism and Leydig cell hypoplasia, and certain forms of pseudohermaphroditism, suggest that in some cases the defect may be caused by a loss-of-function mutation of the LH receptor (David et al. 1984; Wu et al. 1984). Kremer et al. (1995) have recently identified a mutation in the LH receptor of 2 siblings who presented with female external genitalia and a 46,XY karyotype. *In vitro* expression studies showed that this mutated receptor binds hCG with a normal Kd. However, in contrast to the wild type receptor, ligand binding did not result in increased production of cAMP (Kremer et al. 1995).

Several constitutively activating mutations of the LH receptor have been reported in man (Kremer et al. 1993; Shenker et al. 1993). These mutations lead to a syndrome that is called familial male-limited precocious puberty. This gonadotropin-independent disorder is inherited in an autosomal dominant manner, and thusfar no female phenotype has been described. It is thought that constitutive cyclic AMP production in Leydig cells of the affected boys causes Leydig cell hyperfunction, and subsequently increased testosterone production, and signs of puberty at a very young age. The male-limited inheritance pattern of precocious puberty may be explained by the fact that in females FSH is required for LH receptor expression in the postpubertal cycling ovary, and thus for subsequent ovarian steroidogenesis (Shenker et al. 1993).

No naturally occurring mutations of the FSH receptor have been reported yet, but are expected to be present in the human population. Constitutively activating mutations might lead to Sertoli and granulosa cell hyperplasia. Loss of function mutations may be found in infertile males and females with supranormal FSH levels.

Fig. 2.2: (Next page) Linear representation of gonadotropin receptors, steroid receptors, and transmembrane serine/threonine kinase receptors.

A) Both the rat FSHR and LHR contain a large extracellular ligand binding domain that consists of 14 imperfect leucine-rich repeats of approximately 20 aa each (1-14). The transmembrane domain contains seven membrane spanning segments (I-VII), followed by the short intracellular C-terminal domain. Regions that show a high degree of sequence similarity are denoted by the lines below the figure; the numbers indicate the percentage similarity between the two receptors. Regions in which more than 90% of the aa residues is conserved are indicated by black boxes. The numbers of aa residues of the rat FSHR (Sprengel et al. 1990) and LHR (McFarland et al. 1989) are indicated (Fig. adapted from Sprengel et al., 1990).

B) The general structural domains of steroid receptors are shown. The N-terminal domain contains regions that are essential for transcription activation, the DNA binding domain contains the two zinc fingers, and the C-terminal ligand binding domain contains several conserved aa residues that form a hydrophobic hormone binding pocket. The numbers in the DNA binding domain and hormone binding domain indicate the percentage of sequence similarity of the ER compared to the AR in these domains. The number of aa residues of the human AR (Chang et al. 1988) and ER (Green et al. 1986) are indicated.

C) Members of the transmembrane serine/threonine kinase receptor family show a similar overall structure. The extracellular ligand binding domain contains a cysteine rich region; the location of most cysteines is conserved. A single transmembrane domain is followed by a large intracellular domain that consists almost entirely of a kinase domain, that contains two inserts that are characteristic for members of the family (shown as black boxes). The transmembrane serine/threonine kinase receptor family can be divided in two subfamilies, based on structural and functional characteristics of the receptors. The type II receptors contain a longer C-terminal tail than the type I receptors, and lack the GS domain that is present in type I receptors. The GS domain is a conserved region of approximately 29 aa residues that contains a GSGSG sequence. The numbers in the cysteine rich domain and in the kinase domain indicate the percentage of sequence similarity of the receptor domain compared to the corresponding domain of the ActRII (type II receptors) or ActRI (type I receptors). The number of aa residues of the mouse ActRII (Mathews and Vale, 1991), human TGFβRII (Lin et al. 1992), rat AMHRII (see Chapter 3), human ActRI (Attisano et al. 1993), and human TGFβRI (Franzén et al. 1993) are indicated.
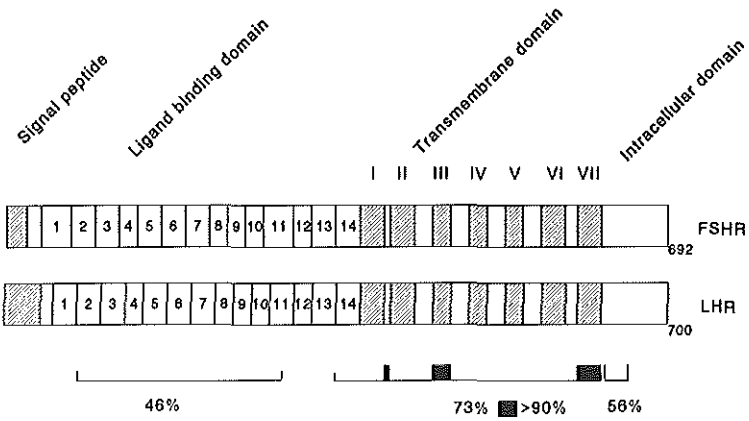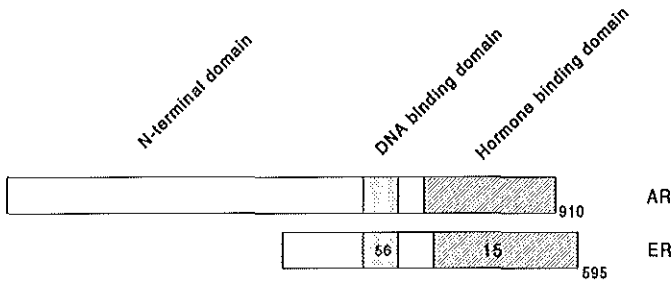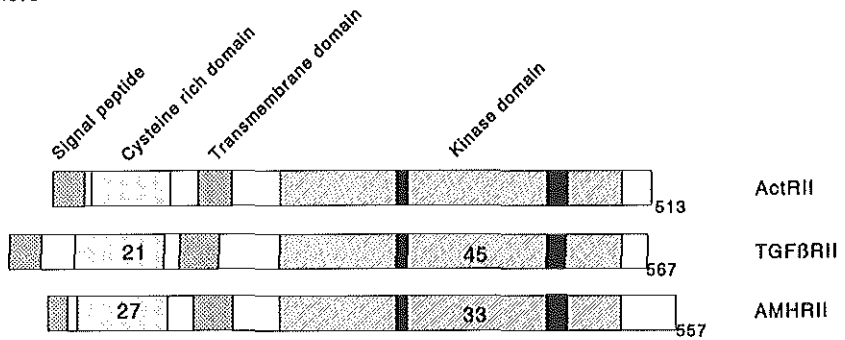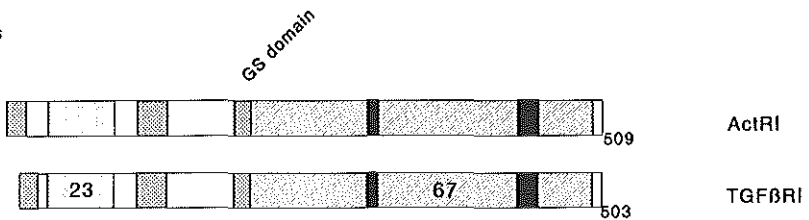
2.3 Androgen and estrogen receptors

2.3.1 Functional and structural aspects of members of the steroid receptor superfamily

Estrogens and androgens can enter the cell through diffusion and act via intracellular receptors (the existence of active transport systems and/or plasma membrane receptors is not excluded). The ER and the AR belong to a large family of receptors that exert their regulatory effects by acting as ligand-activated transcription factors (Evans, 1988; Beato, 1991). Members of this family include the receptors for all steroid hormones, thyroid hormone, retinoids, vitamin D3, and an increasing number of orphan receptors. The human AR gene contains 8 exons (Kuiper et al. 1989; Faber et al. 1991), and is transcribed into a mRNA of approximately 11 kb which encodes a protein of 902 amino acids (Chang et al. 1988; Lubahn et al. 1988; Tilley et al. 1989; Trapman et al. 1988). The relative molecular weight on SDS-PAGE is approximately 110 k. The estrogen receptor (ER) is encoded by 9 exons, and the 6 kb mRNA encodes a 595 aa protein of 66 k (Green et al. 1986).

The members of the steroid hormone receptor family show a similar overall structure with several functional domains (Fig. 2.2B). First, the C-terminal region of the receptors constitute the hormone binding domain. Several conserved amino acids in this region are essential for the formation of a hydrophobic pocket (Evans, 1988).

Second, the zinc finger-containing DNA binding domain, which is the most conserved domain, is located N-terminal to the ligand binding domain. Zinc-fingers are present in the DNA binding domain of many transcription factors (Schwabe and Rhodes, 1991). In the steroid hormone receptor superfamily, the C-terminal located zinc-finger is assumed to be involved in protein-protein interactions, whereas the N-terminal zinc finger is able to recognize and interact with specific DNA sequences (Freedman and Luisi, 1993). These DNA sequences are so-called hormone response elements (HREs), and are located in the promoters or enhancers of the genes that are regulated by the steroid hormone receptors. Both the ER and the AR bind as homodimers to HREs consisting of two halfsites, that are inverted repeats separated by a 3 base pair gap. The consensus androgen response element is 5'AGAACAnnnTGTTCT3', whereas the ER binds to the 5'AGGTCAnnnTGACCT3' consensus sequence (Forman et al. 1992; Freedman and Luisi, 1993).

Finally, the N-terminal domain is highly variable in size and amino acid composition among the different family members. It contains regions that are

essential for transcription activation (Jenster et al. 1991). The N-terminal domain of the AR is relatively long, whereas the ER has a much shorter N-terminal domain (see Fig. 2.2B).

In the absence of hormones, the AR and the ER are bound to so-called heat-shock proteins. Heat-shock proteins are important for several cellular house-keeping functions. Their expression is enhanced under stress conditions, and they are thought to function as molecular chaperones (Craig et al. 1993). When a steroid receptor is complexed with heat-shock proteins, it is unable to bind DNA. Binding of a steroid hormone to its receptor results in dissociation of heat-shock proteins and other changes that result in transformation of the receptor to the tight nuclear binding form (Veldscholte et al. 1992; Smith and Toft, 1993). An increase in the degree of receptor phosphorylation is one of the changes that occur after ligand binding. Several possible physiological roles of phosphorylation, concerning the capacity of the receptors to act on gene transcription, have been suggested (reviewed by Kuiper and Brinkmann, 1994).

2.3.2 Mutations in the AR and ER

Mutations that disrupt AR function will affect male but not female individuals, due to the X chromosomal localization of the AR gene (Lubahn et al. 1988). Depending on the degree of AR functional impairment, a partial or complete androgen insensitivity syndrome (pAIS or cAIS) is observed (Griffin and Wilson, 1989). Patients with cAIS (sometimes called testicular feminization), are 46,XY females with undescended testes instead of ovaries, and lacking fallopian tubes, uterus and upper part of the vagina.

Many naturally occurring mutations of the AR have been described, varying from single base pair mutations to complete deletion of the AR gene (Quigley et al. 1992). Single base pair mutations can lead to either amino acid substitutions, the introduction of premature stopcodons or an aberrant pre-mRNA splicing pattern (for examples see McPhaul et al., 1993). All these mutations result in complete or partial AIS. Although *in vitro* mutagenesis studies have shown that truncated AR receptors, from which the hormone binding domain is deleted, can activate gene transcription independent of androgens (Jenster et al. 1991), naturally occurring mutations, leading to such constitutively active receptors, have not been reported.

The human ER gene has been localized to chromosome 6 (Walter et al. 1985).

It has been described that estrogens may perform essential functions during blastocyst implantation (George and Wilson, 1978), and that this function of the ER precludes the existence of an estrogen insensitivity syndrome analogous to AIS. However, one recent report indicates that an estrogen insensitivity syndrome exists in man (Smith et al. 1994). Analysis of the ER genes of this individual revealed a homozygous mutation in exon 2, resulting in a premature stop codon. The phenotype of this male patient indicated the importance of estrogen in the pubertal growth spurt, maturation of the epiphyses, and mineralization of the skeleton.

Furthermore, it has been shown that targeted disruption of the ER gene in the mouse is not lethal (Lubahn et al. 1993). These ER knock-out mice showed no abnormalities with respect to sex differentiation, but the females were infertile and had hemorrhagic cystic ovaries. Fertility of the males also was much reduced; less than 10% of the normal sperm count was detected. Although residual estrogen binding could still be detected in the ER knock-out mice, no ER protein could be detected by Western blot using a monoclonal antibody that recognizes an epitope in the C-terminus, near the ligand-binding domain of the receptor (Korach, 1994). There are currently two possible explanations for the observed residual estrogen binding; the presence of a non-ER estrogen-binding protein, or the presence of an altered protein product, transcribed from the disrupted ER gene which is biologically inactive (Korach, 1994).

ER mutations leading to constitutive activity have been reported to exist together with expression of a wild type ER gene in human breast cancer tissue (Fuqua et al. 1991).

Table 2.1: (Next page) Schematic overview of several hormone/receptor mutations that cause aberrant sexual differentiation.
All mutations are complete loss of function mutations, unless otherwise indicated. Symbols and abbreviations: AR,androgen receptor; ER,estrogen receptor; AMH,anti-müllerian hormone (receptor); LH,luteinizing hormone; LHR,LH receptor; FSH,follicle-stimulating hormone; FSHR,FSH receptor; cAIS,complete androgen insensitivity syndrome; EIS,estrogen insensitivity syndrome (presumably complete); PMDS,persistent müllerian duct syndrome; LCH,Leydig cell hypoplasia; FMPP,familial male precocious puberty; Chromos. sex,chromosomal sex; Phenot. sex,phenotypic sex; Int. Genit.,internal genitalia; WD,wolffian duct; MD,müllerian duct; Fert.,fertility; ♂,male external genitalia; ♀,female external genitalia; -,absent; +,present; T,testes; O,ovaries.

Mutated gene	Syndrome	Genet. sex	Phenot.sex	Int. genit. derived from MD	WD	Gonads	Puberty	Fert.
AR	cAIS	XY	♀	-	-	T	Normal breast dev. Sparse pubic and axillary hair Primary amenorrhea	-
ER	EIS	XY	♂	-	+	T	No pubertal growth spurt No maturation of epiphyses	?
		XX	Unknown					
AMH	PMDS	XY	♂	+	+	T	Normal puberty	-
		XX	♀	+	-	O	Unknown	
AMHRII	Unknown							
LH	LCH	XY	♂	-	+	T	No puberty	-
	Unknown	XX	Unknown					
FSH	Unknown	XY	Unknown					
	Amenorrhoea	XX	♀	+	-	O	Primary amenorrhoea	-
LHR	LCH	XY	♀	-	+/-	T	No breast dev. Primary amenorrhoea	-
		XX	♀	+	-	O	Primary amenorrhoea	-
	FMPP (constitutive LHR)	XY	♂	-	+	T	Precocious puberty	+
FSHR	Unknown							

2.4   Structural and functional aspects of TGFβs, activins, inhibins, AMH and their receptors

2.4.1   General structure of the activin and TGF-β family of growth/differentiation factors

All activin and TGFβ family members are synthesized as large precursors, that form disulphide linked dimers which are subsequently cleaved. This results in an N- and a C-terminal domain (each composed of two subunits), the latter being the active hormone (AMH has somewhat different characteristics, see Paragraph 1.1.4). The spacing of seven cysteines in both chains of the active C-terminal domain is the most conserved feature among the different family members (Massagué, 1990). The elucidation of the crystal structure of TGFβ2 has revealed that six of these cysteines form a rigid central structure known as the cysteine knot. The seventh cysteine forms a disulphide bridge with the other folded chain of the dimer (Daopin et al. 1992; Schlunegger and Grütter, 1992).

In mammals, three closely related genes are known that encode respectively homodimeric TGFβ1, 2, and 3. As mentioned in Paragraph 1.1.4, AMH is also a homodimeric protein. Activins consist of two inhibin-β subunits (βA or βB, forming activin A,B, or AB), whereas inhibin is a heterodimer of an inhibin-α and an inhibin-βA or βB subunit.

2.4.2   Serine/threonine kinase transmembrane receptors

Members of the activin/TGFβ family of growth and differentiation factors act through heteromeric receptor complexes. For TGFβ, type I, type II and type III receptors (TGFβRI, TGFβRII, TGFβRIII) have been defined, based upon their relative molecular weights of 55, 80 and 230 k, respectively (Massagué, 1990). This nomenclature is also used for receptors of the other family members. Genetic evidence from cell mutants resistant to TGFβ actions suggests that TGFβ binding to receptor type I requires the presence of receptor type II. Furthermore, both receptors are required for signalling of any response (Laiho et al. 1991). Expression cloning was used to identify the genes encoding the human TGFβRII and TGFβRIII, and mouse activin receptor type II (mActRII) (Wang et al. 1991; Mathews and Vale, 1991; Lin et al. 1992). The TGFβRIII is a betaglycan that has no signalling motive, and is thought to be involved in presenting the ligand to the type I and II receptors (Lopez-Casillas et al. 1993). The

type II receptors for TGFβ and activin are structurally similar and, together with the *C. elegans* orphan receptor Daf-1 (Georgi et al. 1990), they were the first identified members of a new family of transmembrane serine/threonine kinase receptors (Lin et al. 1992). Subsequently, PCR-strategies resulted in the cloning of similar receptors from other species (Donaldson et al. 1992; Hemmati-Brivanlou et al. 1992; Legerski et al. 1992; Matzuk and Bradley, 1992; Ohuchi et al. 1992; Shinozaki et al. 1992; Childs et al. 1993; Tsuchida et al. 1993a; Barnett et al. 1994), a second type II receptor for activin (ActRIIB) from mouse (Attisano et al. 1992), and a number of orphan receptors from rat (R1-4) and human (Alk-1-5 and SKR1) (He et al. 1993; Matsuzaki et al. 1993; ten Dijke et al. 1993). In later studies, type I receptors where identified for TGFβ (Alk-5, TSR-I/Alk-1), activin (ActR-1/Alk-2/R1/Tsk-7L, Alk-4/R2), and bone morphogenetic proteins (ALK-3 and Alk-6) (Attisano et al. 1993; Ebner et al. 1993; Franzén et al. 1993; Tsuchida et al. 1993b; Koenig et al. 1994; ten Dijke et al. 1994b; ten Dijke et al. 1994c). Some of these receptors were initially called orphan receptors, due to their inability to bind ligand in the absence of type II receptors. Genetic studies combined with PCR-based strategies, have led to the identification of a type II receptor in *C. elegans* that can bind bone morphogenetic proteins (Daf-4) (Estevez et al. 1993), and of two receptors for the decapentaplegic gene product, which also is a member of the activin/TGFβ family, from *D. melanogaster* (Brummel et al. 1994; Nellen et al. 1994; Xie et al. 1994).

Both inhibin and AMH are notorious for loss of binding and bioactivity upon iodination. This has made it extremely difficult to develop good binding assays for these hormones, so that specific inhibin and AMH receptors have remained elusive.

General structure of serine/threonine kinase receptors

This large family of transmembrane serine/threonine kinases consists of receptors that have the following overall structure: an extracellular ligand-binding domain with conserved cysteine residues, a single hydrophobic transmembrane domain, and an intracellular domain with intrinsic serine/threonine protein kinase activity, containing two inserts that are characteristic for the family (Fig.2.2C). The degree of sequence similarity between the kinase domains is higher among the type I receptors compared to the type II receptors (ten Dijke et al. 1994a). The type I receptors have a distinct distribution of the cysteine residues in the extracellular domain and a shorter C-terminal tail compared to the type II receptors. Furthermore, the kinase domain of type I receptors is preceded by a so-called GS domain which contains a short glycine/serine repeat. This domain is highly conserved among type

I receptors (Attisano et al. 1994; Lin and Moustakas, 1994; Massagué et al. 1994).

Mechanism of receptor activation

Type II receptors can bind hormone in the absence of type I receptors, but both receptor types are needed for transduction of the signal (Laiho et al. 1991; Wrana et al. 1992). In the case of the TGFβ receptors, the mechanism of signal transduction has been elucidated (Fig. 2.3) (Wrana et al. 1994). The kinase domain of TGFβRII is constitutively active, and the receptor exists as an autophosphorylated protein. Autophosphorylation of TGFβRII increases two-fold upon hormone binding, but this is not the receptor activating event. Hormone binding to type II receptor is followed by recruiting a type I receptor molecule, and the formation of a highly stable heteromeric complex. Subsequently, the type II receptor phosphorylates the GS domain of the type I receptor, which results in activation of the kinase domain of the type I receptor. This series of events allows the signal to be propagated to downstream substrates (Wrana et al. 1994). Thus, the nature of the biological response to ligand is specified by the type I receptor that is engaged in the complex (Carcamo et al. 1994; Wrana et al. 1994). Homo-oligomers of TGFβRI, TGFβRII and TGFβRIII have also been observed in transfected cells *in vitro* (Henis et al. 1994; Yamashita et al. 1994). This may indicate that TGFβ induces formation of heterotetrameric, rather than heterodimeric complexes, containing two type I and two type II receptor molecules (Yamashita et al. 1994; Okadome et al. 1994). Whether the above described mechanism of receptor activation applies to the receptors for all members of the activin/TGFβ family awaits further study.

Transfection studies have shown that one species of type I receptor may interact with different species of type II receptors, but it is unclear whether this also occurs *in vivo* (Attisano et al. 1993; ten Dijke et al. 1994b). The high level of receptor expression in transfected cells may lead to aberrant interactions. However, recent experiments using non-transfected cell lines, in which receptor levels are more physiological, have shown that ALK-2 can be detected in a heteromeric complex with a type II receptor for bone morphogenetic proteins, and also, in a different cell line, can form a heteromeric activin binding complex with ActRII (ten Dijke et al. 1994c). This indicates that some type I receptors may function together with different type II receptors, depending on the hormone, cell type or cell differentiation state, *in vivo*. Thusfar, ligand-activated signalling of receptor complexes could only be shown with specific type I and type II receptors in combination with one specific ligand (Attisano et al. 1993).

TGFß binding   RI recruitement   RI phosphorylation
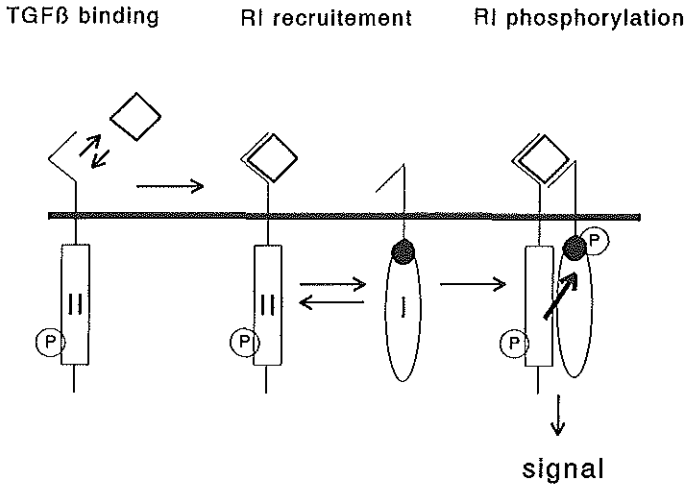
signal

Fig. 2.3: Mechanism of activation of the TGFß receptor complex
A general model for the initiation of signalling by the TGFß receptor complex. The type II receptor is
a constitutively active serine/threonine kinase that recruits the type I receptor by means of bound TGFß
(diamond). Subsequent phosphorylation of the GS domain (striped circle) by the type II receptor allows
the kinase of the type I receptor to propagate the signal to downstream substrates (Fig. adapted from
Wrana et al., 1994)

2.4.3 Functional disruption of members of the activin and TGFß family of growth/differentiation factors

No natural occurring mutations of genes encoding members of the TGFß and activin family of growth factors or their receptors in mammals have been reported, with the exception of mutations in the gene encoding AMH (see below). In mice, targeted disruption of genes encoding several of these growth factors and of the ActRII has provided some information on their functioning.

The TGFß1 gene was the first member of the family, for which genetic knock-out mice were generated (Shull et al. 1992; Kulkarni et al. 1993). Based on the embryonal expression pattern of TGFß1, it was expected that this protein would have a vital role during embryogenesis. However, TGFß1 null neonates were indistinguishable from heterozygous or wild type littermates. Soon after birth, the absence of TGFß1 led to diffuse and lethal inflammation. The animals died before breeding age, and this precluded thorough investigation of gonadal function. It is thought that redundancy in the expression of the various isoforms of TGFß, and

maternal support lead to fetal rescue of the TGFβ1 null mice (Letterio et al. 1994). Dexamethasone treatment of the TGFβ1 null animals allowed prolonged survival and a successful pregnancy of a single homozygous null female. Pups homozygous for the mutated allele showed a much more impaired phenotype than TGFβ null pups that were derived from heterozygous mothers, and died within one day (Letterio et al. 1994).

Targeted disruption of the α-inhibin gene in mice leads to the development of mixed or incompletely differentiated gonadal stromal tumours (Matzuk et al. 1992). However, in these homozygous α-inhibin deficient animals (males and females), initial gonadal development seems to have occurred in a normal manner, since mature oocytes and spermatozoa were initially present. There is an arrest of spermatogenesis and folliculogenesis as the tumours grow and become more destructive. The interpretation of these results is complicated by several factors. First, all homozygous animals were derived from heterozygous mothers, thus maternal sources of α-inhibin may (partially) rescue the α-inhibin null fetuses, as has been shown in the case of targeted disruption of the TGFβ1 gene. Second, the absence of α-inhibin may have an effect on the synthesis of activins, either through the presence of excess β-inhibin subunits, incapable of forming inhibin, or through a disturbance of the regulatory mechanisms that may result in changes in the level of β-inhibin subunit expression. Recent results have shown that this is the case. The mRNA of βB-inhibin is increased more than 200-fold in the testes of α-inhibin deficient mice compared to wild type controls (Trudeau et al. 1994), and the serum levels of activin are 10-fold elevated in the mutated animals (Matzuk et al. 1994). Together with the report that the growth of gonadal tumour cell lines derived from mice deficient in both α-inhibin and p53 depends on activins (Shikone et al. 1994), these results indicate that overexpression of activin significantly contributes to the observed phenotype in α-inhibin deficient mice.

Taking these considerations into account, it is clear that the balance between inhibins and activins is a critical determinant in the control of Sertoli cell and granulosa cell proliferation. Indications for direct effects of the absence of inhibin, or the presence of excess activin, on spermatogenesis come from the observation that in some male α-inhibin knock-out mice there is an arrest of germ cell maturation in the contralateral gonad, where tumours have not appeared (Matzuk et al. 1992).

Mice homozygous for a null mutation of βB-inhibin are viable and fertile (Vassalli et al. 1994; Schrewe et al. 1994). These mice have a defect in eyelid development, and homozygous mothers neglect and consume their progeny. Tissue

sections of the gonads of mutated and wild-type animals at six weeks of age did not reveal any significant differences (Schrewe et al. 1994). βA-inhibin gene knock-out mice develop to term, but die within 24 h of birth (Matzuk et al. 1995b). These mice have a number of defects, that are all different from those observed in βB-inhibin gene knock-out mice. The phenotype of mice deficient in both βA- and βB-inhibin show the defects of both individual mutants, but no additional abnormalities (Matzuk et al. 1995b). This does not exclude the possibility that inhibin A and activin A may partially compensate for the absence of inhibin B and activin B proteins in the βB gene knock-out transgenic mice, since the synthesis of βA-inhibin subunit was upregulated in the βB deficient animals (Vassalli et al. 1994). If the gene encoding one of the known activin type II receptors, ActRII, is disrupted, a phenotype unlike that of any of the inhibin subunit deficient mice develops (Matzuk et al. 1995a). The observed phenotypes suggest a role for ActRII signalling during embryonic mandible development, through a mechanism that does not involve action of activin A or B. The recently cloned βC-inhibin gene (Hötten et al. 1995) might play a role in this process. The observed reduced fertility in ActRII gene knock-out males, and infertility in females (Matzuk et al. 1995a) are consistent with the known gonadal ActRII expression patterns (de Winter et al. 1992; Cameron et al. 1994), but mainly concern the reported stimulatory effect of activin on FSH secretion (reviewed by De Kretser and Robertson, 1989).

Although the gene knock-out experiments have provided new insights into the functions of TGFβs, activins and inhibins, redundancy phenomena might mask additional functions of these proteins.

In the case of AMH, the situation is slightly different, at least during embryonic development where redundancy cannot compensate for loss of AMH, since it is known that lack of AMH function leads to the persistent müllerian duct syndrome (PMDS) in humans. This is a rare form of inherited male pseudohermaphroditism, characterized by the presence of a uterus and fallopian tubes in otherwise normally virilized males. The molecular basis for PMDS is heterogenous. AMH positive and AMH negative cases have been described (Guerrier et al. 1989). In the latter case, mutations in the gene encoding AMH have been identified in several patients as the cause of the syndrome (Guerrier et al. 1989; Imbeaud et al. 1994). AMH positive cases of PMDS could be caused by a receptor defect, although mutations in downstream genes cannot be excluded. Targeted disruption of the gene encoding AMH leads to the development of müllerian duct derived female reproductive organs in males (Behringer et al. 1994), similar to the above described phenotype in human PMDS patients.

Apart from the degenerative effect of AMH on müllerian duct development, the phenotype of the AMH knock-out mice points to a possible role of AMH in testis development (Behringer et al. 1994). The AMH-deficient males had testes that were fully descended and produced functional sperm. However, some testes showed Leydig cell hyperplasia, suggesting that AMH is a negative regulator of Leydig cell proliferation. The combined presence of male and female reproductive organs interfered with sperm transport, rendering most of these males infertile. In human PMDS patients the testes do not descent, which results in infertility. There are no data on the numbers of Leydig cells in the testes of these patients. Female AMH knock-out mice showed no abnormalities and were fertile. Thus, the phenotype of the AMH knock-out mice is in agreement with impairment of müllerian duct regression, the classical function of AMH. However, some of the possible functions of AMH may be shared with other members of the activin and TGFβ family, and consequently may not be apparent from the phenotype of the AMH knock-out mice. Generation and analysis of mice that have mutations in more than one member of the activin/TGFβ family, may provide more information about possible functional overlaps between the different hormones.

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A Novel Member of the Transmembrane Serine/Threonine Kinase Receptor Family is Specifically Expressed in the Gonads and in Mesenchymal Cells Adjacent to the Müllerian Duct

Willy M. Baarends, Marjolein J.L. van Helmond, Miriam Post, Pieter J.C.M. van der Schoot, Jos W. Hoogerbrugge, Johan P. de Winter, Jan Th. J. Uilenbroek, Bas Karels, Laurens G. Wilming, J.H. Carel Meijers, Axel P. N. Themmen and J. Anton Grootegoed

A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the müllerian duct

Willy M. Baarends[1*], Marjolein J. L. van Helmond[1], Miriam Post[1], Pieter J. C. M. van der Schoot[1], Jos W. Hoogerbrugge[1], Johan P. de Winter[1], Jan Th. J. Uilenbroek[1], Bas Karels[1], Laurens G. Wilming[2], J. H. Carel Meijers[2], Axel P. N. Themmen[1] and J. Anton Grootegoed[1]

[1]Department of Endocrinology and Reproduction, Faculty of Medicine and Health Sciences, and [2]Department of Pediatric Surgery, Sophia Children's Hospital, Erasmus University, P.O. Box 1738, 3000 OR, Rotterdam, The Netherlands

*Author for correspondence

SUMMARY

The activin and TGF-β type II receptors are members of a separate subfamily of transmembrane receptors with intrinsic protein kinase activity, which also includes the recently cloned TGF-β type I receptor. We have isolated and characterized a cDNA clone (C14) encoding a new member of this subfamily. The domain structure of the C14-encoded protein corresponds with the structure of the other known transmembrane serine/threonine kinase receptors. It also contains the two inserts in the kinase domain that are characteristic for this subfamily. Using in situ hybridization, C14 mRNA was detected in the mesenchymal cells located adjacent to the müllerian ducts of males and females at day 15 (E15) of embryonic development. Marked C14 mRNA expression was also detected in the female gonads. In female E16 embryos, the C14 mRNA expression pattern remained similar to that in E15 embryos. However, in male E16 embryos C14 mRNA was detected in a circular area that includes the degenerating müllerian duct. The expression of C14 mRNA was also studied using RNase protection assays. At E15 and E16, C14 mRNA is expressed in the female as well as in the male urogenital ridge. However, at E19, a high C14 mRNA level in the female urogenital ridge contrasts with a lack of C14 mRNA in the male urogenital ridge. This correlates with the almost complete degeneration of the müllerian ducts in male embryos at E19. C14 mRNA expression was also detected in embryonic testes at E15, E16 and E19 using RNase protection assays, but at much lower levels than those found in the developing ovaries. In eleven other tissues no C14 mRNA was observed.

The results point to anti-müllerian hormone (AMH) being the most likely candidate ligand for C14. The embryonic C14 mRNA expression pattern in the urogenital ridge correlates with the expected site of AMH action, and C14 mRNA expression in the fetal ovary is in agreement with known effects of AMH on gonadal differentiation.

Postnatal C14 mRNA expression in rats was found to be confined mainly to the gonads. In the testis, C14 mRNA expression occurs in Sertoli cells. This testicular expression markedly increases during the first 3 weeks after birth, concurrent with the onset of spermatogenesis.

Key words: anti-müllerian hormone, activin, TGF-β, receptor, sex differentiation, testis, ovary

INTRODUCTION

The cloning of receptors for the peptide growth factors activin (Mathews and Vale, 1991; Attisano et al., 1992) and TGF-β (Lin et al., 1992, Ebner et al., 1993) has provided information about their overall structure and the molecular mechanism of signal transduction. The activin receptor types II and IIB and the TGF-β receptor types I and II, together with the *C. elegans* orphan receptor Daf-1 (Georgi et al., 1990) and several recently cloned rat orphan receptors (He et al., 1993), constitute a new subfamily of transmembrane protein kinase receptors. Based upon its primary structure, the kinase domain has a predicted specificity for serine and threonine residues.

However, the mouse activin receptor type IIB has been shown to contain serine, threonine and tyrosine kinase activity (Nakamura et al., 1992).

The terms type II and type I refer to the nomenclature that is used for the different TGF-β receptors that have been described (Cheifetz et al., 1987). Crosslinking of radiolabelled TGF-β to cellular proteins allowed the identification of at least three different TGF-β binding proteins at the cell surface, named TGF-β receptor types I, II and III according to their relative molecular masses of 55, 80 and 280×10^3, respectively. The type III receptor is a betaglycan without a signalling motif (Wang et al., 1991), and is thought to play a role in the presentation and binding of TGF-β to the other receptor types

(López-Casillas et al., 1993). The TGF-β type I receptor cannot bind TGF-β in the absence of the type II receptor (Wrana et al., 1992, Ebner et al., 1993), and association with the type I receptor is essential for the TGF-β type II receptor to signal growth inhibition (Wrana et al., 1992). It is thought that this obligatory association between type I and type II receptors is a functional characteristic for all members of the serine/threonine kinase receptor family (Wrana et al., 1992). However, there are also indications that for some cellular responses to TGF-β the presence of only the type I or type II TGF-β receptor might be sufficient (Chen et al., 1993).

In this report, we describe the cloning and characterization of a rat cDNA, termed C14, which encodes a new member of the serine/threonine kinase receptor family. Based upon the cell-/tissue-specific C14 mRNA expression pattern, described herein, it will be discussed that the most likely candidate ligand of C14 is anti-müllerian hormone (AMH) (also called müllerian inhibiting substance; MIS).

AMH is a member of the activin and TGF-β family of peptide growth factors (Cate et al., 1986). These signalling molecules share numerous structural similarities. They are synthesized as large precursor molecules that form homo- or hetero-dimers. Each chain of the dimer is cleaved at approximately 110 amino acids from the C terminus, which generates the mature subunit and an N-terminal proregion. Sequence identity between members of this growth factor family is found mainly in the C-terminal mature subunits (25-80%; for review see: Massagué et al., 1990).

The known action of AMH in sex differentiation is very specific, when compared with the variety of functions that are performed by most other growth factors of the same family. For example, activin may regulate the secretion of follicle-stimulating hormone (FSH) by the pituitary gland (Vale et al., 1988), but also plays different roles in erythroid cell differentiation (Yu et al., 1987), neural cell survival (Schubert et al., 1990), and during embryogenesis (Thomsen et al., 1990; Hemmati-Brivanlou and Melton, 1992).

AMH is the earliest protein product known to be secreted by Sertoli cells in the fetal testis (Tran and Josso, 1982). Around day 15 of embryonic development in the male rat, AMH induces the regression of the müllerian ducts, which form the antagen of the uterus, oviducts, and upper vagina. The Leydig cells in the developing testis produce testosterone, which stimulates the differentiation of the wolffian ducts into epididymides, vasa deferens and seminal vesicles. The window of sensitivity of the müllerian duct to AMH in the rat has been shown to be between E14.5-E15.5. After E16, exposure to AMH does not result in müllerian duct regression (Josso et al., 1977; Tsuji et al., 1992). In female embryos, the lack of androgens leads to wolffian duct regression, and the müllerian duct persists because the ovarian cells do not produce AMH. All these events form part of the earliest steps in sex differentiation following gonadal sex determination. In addition to its effect upon the müllerian duct, AMH may have a role to play in the development of ovaries and testes (Münsterberg and Lovell-Badge, 1991; Hirobe et al., 1992). The cellular mechanism by which AMH induces müllerian duct regression is poorly understood. Recent results show that AMH elicits its effect upon the müllerian duct epithelium most likely via the surrounding mesenchymal cells (Tsuji et al., 1992).

The expression of C14 mRNA during embryonic sex differ-

entiation, described in the present report, is in accordance with the hypothesis that C14 encodes a type I/II receptor for AMH.

MATERIALS AND METHODS

Tissue and RNA preparations

Sertoli cells and peritubular myoid cells were isolated from 21-day-old rats and cultured as described previously (Themmen et al., 1991; Blok et al., 1992). Round spermatids were isolated from 35-day-old rats as described by Grootegoed et al. (1986). Total tissues, isolated cell fractions, or cultured cells were snap frozen in liquid nitrogen and stored at −70°C until used for RNA isolation. Total RNA was isolated using the LiCl/urea method (Aufray and Rougeon, 1980).

Isolation of C14 from a rat Sertoli cell cDNA library

Sertoli cells were cultured for 6 hours without (−T) or with 0.6 μM testosterone (+T), in the presence of 50 μg/ml cycloheximide. These cells were used to prepare −T and +T cDNA libraries in Lambda ZAPII (Stratagene, Westburg, Leusden, The Netherlands), using standard molecular biology techniques (Sambrook et al., 1989). All radiolabelled agents were from Amersham ('s Hertogenbosch, The Netherlands). A +T-enriched subtracted probe was prepared (Sive and St John, 1988), using in vitro synthesized cRNA from the −T cDNA library as driver RNA. RNA was synthesized using an in vitro RNA transcription kit (Stratagene) according to the instructions of the manufacturer. Clones from the +T cDNA library that showed a stronger hybridization signal with the subtracted probe than with a −T cDNA probe were isolated, and possible induction by androgens was tested on northern blots containing +T and −T total RNA. Northern blotting and random-primed labelling of the C14 cDNA with [^{32}P]dATP was performed according to Sambrook et al. (1989). C14 mRNA expression initially seemed to be androgen responsive, but this was not confirmed in later experiments (Fig. 1).

Sequencing

The complete 1.9 kb C14 cDNA clone was sequenced on both strands using the dideoxy chain termination method (Sanger et al., 1977). The sequence was analyzed using the sequence analysis program Microgenie (Beckmann, Mijdrecht, The Netherlands). Computer searches were carried out using the UWGCG program (Devereux, 1992).

RNase protection assay

A *Pst*I fragment containing bp 1243-1640 from C14 was subcloned in pBluescript KS (Stratagene) and used to generate [^{32}P]UTP-labelled anti-sense transcripts in vitro. The follicle-stimulating hormone receptor (FSHR) RNA probe was obtained using a 386 bp *Hind*III/*Pvu*II fragment from the rat cDNA (Sprengel et al., 1990). The activin receptor type II (ActRII) RNA probe was obtained using a 569 bp rat ActRII cDNA fragment (de Winter et al., 1992). The control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA probe was synthesized using a construct containing a 291 bp *Xba*I/*Sau*3AI fragment from rat GAPDH cDNA (Fort et al., 1985). Approximately 5×10^4 cts/minute of C14, FSHR, or ActRII probe, together with 5×10^4 cts/minute of GAPDH probe, was mixed with 5 or 10 μg of total RNA in a total volume of 30 μl hybridization mixture containing 40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide. The hybridizations were performed overnight at 55°C. The RNase protection assay was performed as described by Sambrook et al. (1989).

In situ hybridization

The C14 subclone that was used for RNase protection assays was also used to prepare sense and anti-sense ^{35}S-UTP-labelled transcripts in vitro. The probes were dissolved in 5 μl 0.2 M DTT. Immediately prior to use, the probe solution was mixed with 5 μl of 30 μg/μl S-

ATP (Calbiochem, Omnilabo, Breda, The Netherlands), incubated at 100°C for 1 minute and diluted in prewarmed (55°C) hybridization mixture to a final concentration of approximately 2×10^5 cts /minute per µl. The hybridization mixture contained: 50% (v/v) deionised formamide, 0.3 M NaCl, 10 mM Tris pH 8, 1 mM EDTA, 1× Denhardt's solution, 1 mg/ml yeast tRNA, 50 mM DTT and 10% (w/v) polyethylene glycol 6000.

The lower body region of embryos at E15 and E16 was separated from the head region and fixed in fresh Bouin's fixative overnight at 4°C. All in situ hybridization procedures (tissue embedding, slide preparations, pretreatments, hybridization and posthybridization incubations) were performed as described by Zeller and Rogers (1991). The tissue was embedded in paraffin, and 8 µM sections were mounted on slides which were coated with poly-L-lysine. Adjacent sections where mounted and stained with Mayer's haematoxylin and eosin for tissue and cell identification. The hybridizations were performed at 55°C overnight in moist chambers. The slides were dipped in 1:1 (v/v) dilution of Kodak NTB-2 emulsion and dried at room temperature for 2 hours, followed by exposure at 4°C for 2 weeks. After developing, the sections were counterstained with Nuclear fast red and mounted.

Genomic DNA isolation and PCR procedures

The head region of the embryos at E15 and E16 was snap frozen in liquid nitrogen and used for genomic DNA isolation according to Davis et al. (1986). This DNA was used in a DNA amplification reaction (Saiki et al., 1988) using primers for mouse Sbx and Sby (Mitchell et al., 1991, Kay et al., 1991) as described by Mitchell et al. (1991). The product obtained with the Sbx primers (approximately 250 bp) was used as a positive control to test the quality of the DNA, and the presence or absence of a product with the Sby primers (approximately 1000 bp) was used to determine the sex of the embryos (results not shown).

RESULTS

Selection of C14

C14 was isolated from a Sertoli cell cDNA library as described in Materials and methods. The 1.9 kb C14 cDNA probe hybridizes on northern blots to a major transcript of approximately 2.5 kb, which is expressed at high levels in cultured Sertoli cells (Fig. 1). However, there was no significant stimulation of the expression by testosterone. The tissue specificity of C14 mRNA expression was determined using an RNase protection assay. Fig. 2 shows that C14 mRNA expression in the adult rat is mainly restricted to the gonads. Based upon this marked tissue specificity of C14 mRNA expression, C14 was selected for further investigation.

C14 sequence analysis

The complete 1.9 kb C14 cDNA was sequenced. It contains a single open reading frame that encodes a 557

Fig. 1. C14 mRNA expression in cultured Sertoli cells. C14 cDNA probe was hybridized to a northern blot containing 20 µg total RNA isolated from Sertoli cells cultured in the absence (–) or presence (+) of testosterone (see also Materials and methods). Clone 14 indicates the 2.5 kb band of C14 mRNA, abundantly expressed under both culture conditions. The GAPDH hybridization indicates that equal amounts of mRNA were loaded in each lane. 18S and 28S indicate the locations of the respective rRNA bands.

amino acid protein, starting from the first methionine codon at residue 60 (Fig. 3A). This ATG codon and its context form a potential functional initiation site (Kozak, 1987). Analysis of the protein sequence indicated the presence of a signal sequence with a predicted cleavage site before Gln[16] (von Heijne, 1986). A hydrophobic region between amino acid residues 142-168 represents the single putative transmembrane domain (TMD). Thus, the mature protein consists of an extracellular domain of 126 amino acid residues, a TMD of 27 amino acid residues, and an intracellular domain of 389 amino acid residues. The extracellular domain is cysteine rich and contains 2 potential N-glycosylation sites.

EMBL and GenBank database searches revealed that the intracellular domain of C14 is most closely related to the intracellular domains of the mouse activin receptor types II (ActRII; Mathews and Vale, 1991) and IIB (ActRIIB; Attisano et al., 1992), the human TGF-β type II receptor (TGF-βRII; Lin et al., 1992), the mouse TGF-β type I receptor (TGF-βRI; Ebner et al., 1993), and the C. elegans orphan receptor Daf-1 (Georgi et al., 1990) (Fig. 3B). The ActRII, ActRIIB, TGF-βRII, TGF-βRI and Daf-1 together form a separate subfamily of receptors with intrinsic protein kinase activity (Mathews and Vale, 1991; Lin et al., 1992). Recently, several rat orphan receptors have been cloned, that are also members of this newly defined receptor family (He et al., 1993).

The C14 intracellular kinase domain contains the 12 subdomains, which are found in all protein kinases, in the proper order (Hanks et al., 1988), and also contains the two inserts located between subdomains VI-A and VI-B and between subdomains X and XI, that are characteristic for the subfamily (Mathews and Vale, 1991). The percentage identical amino acids of the kinase domain of C14 compared to the ActRII, TGF-βRII, TGF-βRI and Daf-1 kinase domains is 34%, 32%, 33% and 29%, respectively.

The sequences in the kinase subdomains VI-B and VIII indicate substrate specificity (Hanks et al., 1988). The relevant sequences in C14 within these subdomains are more in accordance with the serine/threonine kinase consensus sequence than with the tyrosine kinase consensus sequence, with the exception of serine residue 333. At this position in the kinase domain, a lysine residue is found in almost all known serine/threonine kinases including ActRII, ActRIIB, TGF-βRII, TGF-βRI and Daf-1.

The extracellular domains of C14, ActRII and TGF-βRII show a relatively low level of sequence identity (Fig. 3C). However, the position of most cysteine residues is conserved.

Fig. 2. Tissue specificity of C14 mRNA expression. RNase protection assays were used to study the expression of C14 mRNA in different tissues. From 4-week-old rats: Te, testis; Oa, ovary; Ut, uterus. From adult rats: Oi, oviduct; Pr, prostate; Ep, epididymis; Se, seminal vesicle; Br, brain; Pi, pituitary gland; He, heart; Lu, lung; Sp, spleen; Li, liver; Ki, kidney; In, intestine; Mu, muscle. 10 µg of total RNA was used for each lane. C14 indicates the position of the protected RNA fragment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was present in all samples (not shown).

A

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  1   GCGTTCACGAGGGTAGCTGTGCTGGTGTGTGCTGTCCTGCTGACATCCTTTAGTAGGATGCTGGGGACACTGGGGCTTTGGACACTGCTTCCTGCAGCTGCACAAGTATCCCCAAACAGG
  1                                                    M  L  G  T  L  G  L  W  T  L  L  P  A  A  A  Q  V  S  P  N  R

121   AGGACCTGTGTGTTCTTTGGAGGCTCCTGGGAGTTCGGGGAAGCACAAAAACACTGGGGGAAGTGGTAGATGCAGGACCAGGCCCCCGAAGGGTATCCGCTGCCTCTACAGCCATTGCTGC
 22    R  T  C  V  F  F  E  A  P  G  V  R  G  S  T  K  T  L  G  E  V  V  D  A  G  P  G  P  P  K  G  I  R  C  L  Y  S  H  C  C

241   TTTGGGATCTGGAATCTGACCCATGGCCGGGCACAAGTGGAGATGCAAGGATGCCTAGACAGTGATGAACCGGGCTGTGAGTCCCTCCACTGTGACCCCGTGCCCCGAGCGCACCCCAGC
 62    F  G  I  W  N  L  T  H  G  R  A  Q  V  E  M  Q  G  C  L  D  S  D  E  P  G  C  E  S  L  H  C  D  P  V  P  R  A  H  P  S

361   CCCAGCTCCACACTCTTCACATGCTCCTGCGGTACCGACTTCTGCAATGCCAACTACAGCCATCTGCCTCCTTCAGGAAACCGAGGGGCTCCCGGCCCCCAGGAGCCTCAGGCTACCCCA
102    P  S  S  T  L  F  T  C  S  C  G  T  D  F  C  N  A  N  Y  S  H  L  P  P  S  G  N  R  G  A  P  G  P  Q  E  P  Q  A  T  P

481   GGTGGGCCCATCTGGATGGCGCAGCTGCTGCTGGGAGTGTTCCTTGTGCTGCTGCTGCTCAGTATCATCATCTTGGCCCTGCTTACAACGAAAAGCCTGTAGAGTGCAAGGTGGGTCAGACCCG
142    G  G  P  I  W  M  A  Q  L  L  L  G  V  F  L  V  L  L  L  S  I  I  I  L  A  L  L  Q  R  K  A  C  R  V  Q  G  G  S  D  P

601   GAGCCGGAGGCCAGGCTCAGGAGGAGAGACTGCAGTGAAGAACTGCCCCGAGTTGGCTGAGCTGCCGCTTCTCCCAGGTAATCCAGGAAGGAGGTCATGCAGTCGTCTGGGGCTGGGAGGCTCCAG
182    E  P  E  P  G  G  G  D  C  S  E  E  L  P  E  L  A  E  L  R  F  S  Q  V  I  Q  E  G  H  A  V  V  M  A  G  R  L  Q

721   GGTGAGATGGTAGCCATCAAGGCCTTCCCCCCAAGGGCTGTGGCCCAGTTCCGAGCTGAGCGGAGCTGTGTACCAGCTGCTAGGCCTACAGCATAACCACATTGTTCGCTTTATCACTGCT
222    G  E  M  V  A  I  K  A  F  P  P  R  A  V  A  Q  F  R  A  E  R  A  V  Y  Q  L  L  G  L  Q  H  N  H  I  V  R  F  I  T  A

841   GGCCAGGGAGGCCCTGGCCCCTTGCCCTCTGGGCCTTTGCTGGTACTGGAACTGTACCCTAAGGGCTCCTTATGCCACTACTTGACTCAGTACACGAGCGACTGGGGCAGTTCCTTGAGG
262    G  Q  G  G  P  G  P  L  P  S  G  P  L  L  V  L  E  L  Y  P  K  G  S  L  C  H  Y  L  T  Q  Y  T  S  D  W  G  S  S  L  R

961   ATGGCTCTGTCCCTGGCCGAGGGCCTGGCGTTTCTCCACGGGGGAGCGGTTGGCAGGATGGCCAGTACAAACCAGGTATCGCTCACCGAGACCTGAGCAGCCAGAATGTGCTCATTCGGGAA
302    M  A  L  S  L  A  E  G  L  A  F  L  H  G  E  R  H  Q  D  G  Q  Y  K  P  G  I  A  H  R  D  L  S  S  Q  N  V  L  I  R  E

1081  GATAGGTCATGTGCCATCGGAGACCTGGGCCTTGCCTTGGTACTCCCTGGCCTTGCTCAGCCCCCTGCCTTGGCCCCTACTCAGCCCCGAGGCCCAGCTGCCATTTTGGAGGCTGGCACC
342    D  R  S  C  A  I  G  D  L  G  L  A  L  V  L  P  G  L  A  Q  P  P  A  L  A  P  T  Q  P  R  G  P  A  A  I  L  E  A  G  T

1201  CAGAGATACATGGCTCCGGAGCTCTTGGACAAGACTCTAGACCTGCAGGACTGGGGGCACAGCTCTCAGAGACCGGATGTTTACTCTGGCTCTACTACTGTGGGAGATCCTGAGCCGGC
382    Q  R  Y  M  A  P  E  L  L  D  K  T  L  D  L  Q  D  W  G  H  A  T  A  L  Q  R  A  D  V  Y  S  L  A  L  L  L  W  E  I  L  S  R

1321  TGTTCCGATTTGAGGCCTGACCACAGACCACCACCTTTTCAATTGGCTTATGAGGCAGAACTGGGCAGCAATCCCAGTGCCTGTGAGCTCTGGGCCCTGGCGGTCGCGGAGAGGAAGCGC
422    C  S  D  L  R  P  D  H  R  P  P  F  Q  L  A  Y  E  A  E  L  G  S  N  P  S  A  C  E  L  W  A  L  A  V  A  E  R  K  R

1441  CCCAACATCCCTTCCTCTTGGAGCTGCTCTGCCACAGACCCTAGGGGGCTGAGGGAGCTCCTGGAAGATTGCTGGGACGCAGACCCAGAAGCAAGGCTGACAGCTGAGTGTGTGCAGCAG
462    P  N  I  P  S  S  W  S  C  S  A  T  D  P  R  G  L  R  E  L  L  E  D  C  H  D  A  D  P  E  A  R  L  T  A  E  C  V  Q  Q

1561  CGCCTGGCAGCCCTGGCTTATCCTCAGGTGGCCTTCCTCCTTCCCAGAGAGGCTGTCCACAGGGCTGCCCAGAGAACTACCCTGCAGCGCCTGCCTCTGCGGCTTCCCCTGCAGGCCTCAG
502    R  L  A  A  L  A  Y  P  Q  V  A  S  S  F  P  E  S  C  P  Q  G  C  E  N  Y  P  A  A  P  A  S  A  A  F  P  V  Q  Q

1681  CAGAGTTCTTGTCTCCAGTGTTCAGCAAGGCTCTGGCTCTAAGTCCTGACCCTGTAAGTGCCCACTGTTCAGGTGTATGTAAATGAGCAGCTCATATGAACTCTGTACAGGCTGACATG
542    Q  S  S  C  L  L  S  V  Q  Q  G  S  G  S  K  S  *

1801  CGTGTGGCCGACATGCGTGTGTCGGGTGTCAGCCTCATGCCTGTTGATGTCATTCTGTGTGGGGTTTCTAACCCATCGGGTAGCTATGCTCAGGAAAGAGAATAAAGCAAGATTCCCACTC
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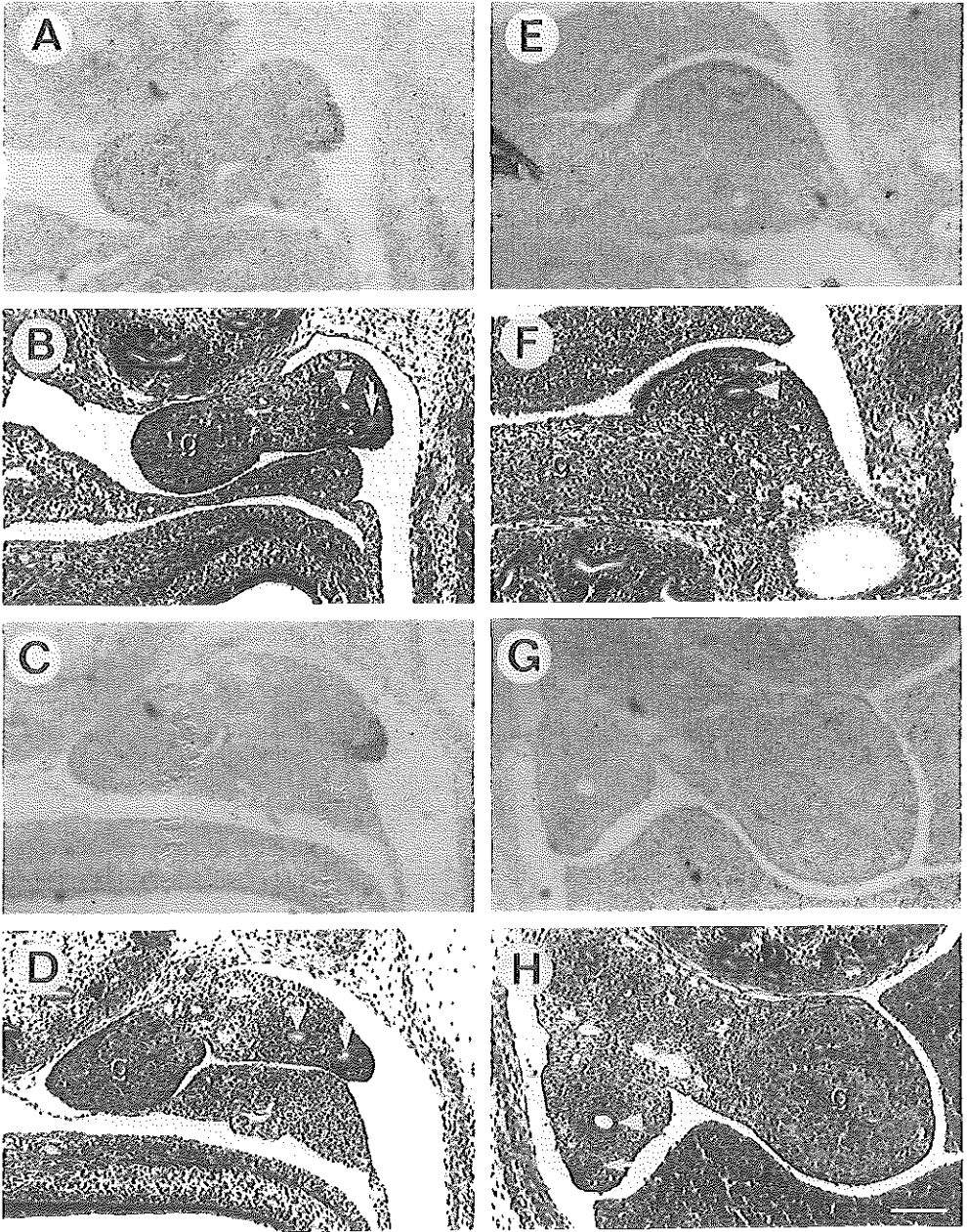
B

C

Fig. 3. Analysis of C14 cDNA. (A) The complete 1920 bp cDNA clone sequence encoding a protein of 557 amino acid residues is shown. The kinase domain is in bold type. The hydrophobic signal sequence and transmembrane region are underlined, and the potential *N*-glycosylation sites are double underlined. (B) Comparison of the kinase domain of rat C14 to the kinase domains of mouse ActRII and human TGF-βRII. The Roman numerals under the sequence indicate the kinase subdomains. The inserts between subdomains VI-A and VI-B and between subdomains X and XI are underlined. The asterisk denotes the location of serine residue 333. (C) Comparison of the extracellular domain of C14 to the extracellular domains of ActRII and TGF-βRII. In B and C reversed script is used to highlight amino acid residues that are found in at least two of the three kinase receptors.

C14 expression during embryonic sexual differentiation

The fact that C14 encodes a member of the activin and TGF-β type I/II receptor family, indicates that the C14 ligand will be a member of the activin and TGF-β-family of peptide growth factors. C14 is expressed mainly in the gonads in adult rats (present results), where anti-müllerian hormone (AMH) may perform specific functions (Hirobe et al., 1992; Münsterberg and Lovell-Badge, 1991). This pointed to AMH as an important candidate ligand for C14. To investigate this possi-

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Fig. 4. Expression of C14 mRNA at E15 and E16. Bright-field photomicrographs from in situ hybridization of the antisense C14 RNA probe to paraffin sections (A,C,E,G) and from haematoxylin/eosin stained adjacent sections (B,D,F,H) of female embryos at E15 (A,B), E16 (C,D), and of male embryos at E15 (E,F) and E16 (G,H). Embryonal sex was determined as described in Materials and methods. g, gonad; arrowhead, wolffian duct; arrow, müllerian duct. Hybridization of the sense control C14 RNA probe did not result in signals above background (not shown).

bility, the expression pattern of C14 mRNA in the urogenital ridge of rat embryos during the induction of müllerian duct regression was studied.

Using in situ hybridization, marked C14 mRNA expression was detected in the female gonads, but not in the male gonads, of embryos at E15 and E16 (Fig. 4A-H). Furthermore, C14 mRNA is expressed in the female urogenital ridges at E15 and E16, in a sickle-shaped area that includes the mesenchymal cells between the müllerian duct and the coelomic epithelium (Fig. 4A-D). In the male urogenital ridges at E15, the expression of C14 mRNA is identical to the E15-E16 female pattern (Fig. 4E,F). However, this male expression pattern has clearly changed at E16, when C14 mRNA is present in a small circular area that includes the degenerating müllerian duct (Fig. 4G,H). No other sites of C14 mRNA expression above background were detected in the lower body halves of the embryos at E15 and E16.

RNase protection assays were used to study the expression of C14 mRNA in isolated urogenital ridges and gonads at E15, E16 and E19 (Fig. 5). C14 mRNA was detected at much higher levels in ovary than in testis at all embryonic stages tested. At E15, C14 mRNA expression in the urogenital ridge was detected at approximately equal levels in both male and female embryos. However, at E16, the C14 mRNA level in the male urogenital ridge is lower than in the female urogenital ridge. Finally, at E19, C14 mRNA was not detected in the male urogenital ridge, whereas the C14 mRNA level remains high in the female urogenital ridge. No C14 mRNA was detected in total RNA isolated from E19 intestine, skin, lung, liver, kidney, adrenal, stomach, heart, thymus, muscle, and brain (not shown).

Postnatal expression of C14 mRNA in testis and ovary

Different testicular cell types were isolated, to study the cellular location of C14 mRNA expression in the testis using RNase protection assay. It was observed that C14 mRNA is specifically expressed in Sertoli cells, at equal levels in cells isolated from 21-day-old or mature rats (Fig. 6A). No C14 mRNA was detected in round spermatids and peritubular myoid cells. ActRII mRNA, which is expressed at a high level in round spermatids (de Winter et al., 1992), was used to verify the integrity of the round spermatid mRNA. FSHR mRNA, which is expressed exclusively in Sertoli cells (Heckert and Griswold, 1991), was used to show the cellular colocalization of FSHR and C14 mRNAs and the absence of Sertoli cell contamination from the other isolated cell types.

C14 mRNA expression in total testis is very low at birth, and increases to a maximum at day 21 of postnatal development (Fig. 6B). Since we found similar levels of expression in Sertoli cells from immature and adult rats (Fig. 6A), the relative decrease in C14 mRNA expression in total testis between days 21 and 63 can be explained by the increasing population of C14-negative spermatids.

In the ovary of adult rats, marked expression of C14 mRNA was detected, using in situ hybridization, in the granulosa cell layers of small antral follicles (not shown).

DISCUSSION

Based upon the observation that in adult rats C14 mRNA expression is most abundant in the gonads, C14 was selected

and characterized. C14 encodes a novel member of the activin and TGF-β type I/II serine/threonine kinase receptor family. Including C14, nine members of this gene family are presently known: Daf-1 encoding an orphan receptor (Georgi et al., 1990), TGF-βRII (Lin et al., 1992), TGF-βRI (Ebner et al., 1993), R2, R3 and R4, which are three orphan receptors cloned through PCR amplification of rat fetal urogenital ridge cDNA with a relatively high percentage of similarity to TGF-βRI in the kinase domain (He et al., 1993), and two genes encoding the different activin type II receptors, ActRII and ActRIIB (Mathews and Vale, 1991; Attisano et al., 1992). The sequence identity between ActRII and ActRIIB is 51% in the extracellular domain and 75% in the intracellular domain, and four different splice variants of ActRIIB have been described (Attisano et al., 1992).

There is complete structural homology between C14 and the other members of the serine/threonine kinase receptor family. The C14 kinase domain has the highest degree of amino acid identity with the kinase domain of ActRII (34%). However, the ActRII and TGF-βRII kinase domains are more closely related, showing 45% amino acid identity to one another. It has been shown by Nakamura et al. (1992) that ActRIIB is a dual specificity kinase, which can phosphorylate serine/threonine as well as tyrosine residues. It remains to be determined whether the change from the conserved lysine to a serine at position 333 in the C14 kinase domain has any significance with respect to substrate specificity or kinase activity.

Our hypothesis that AMH could be an important candidate ligand of C14 implies that C14 mRNA should be expressed in the urogenital ridge during the induction of müllerian duct degeneration by AMH, around day 15 of embryonic development in the rat (Jost, 1947; Tsuji et al., 1992). It was observed that C14 mRNA is expressed in an area that includes the mesenchymal cells between the müllerian duct and the coelomic epithelium at E15, in both male and female embryos. Recent results have shown that AMH can reduce DNA synthesis of cultured urogenital ridge mesenchymal cells but not of cultured müllerian duct epithelial cells (Tsuji et al., 1992). This suggests that AMH most likely elicits its effect upon the müllerian duct epithelium via the surrounding mesenchyme
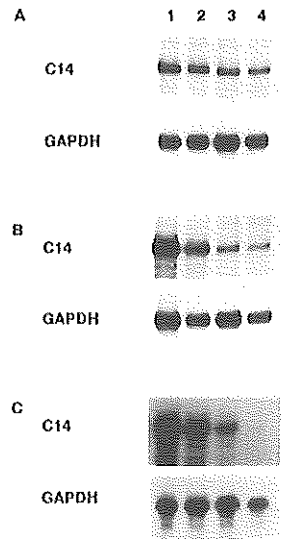

Fig. 5. Expression of C14 mRNA in different embryonal tissues at E15, E16 and E19. Embryos were collected at E15 (A); E16 (B) and E19 (C), and C14 mRNA expression was studied using RNase protection assays in: Lane 1, ovary; 2, female urogenital ridge; 3, testis; 4, male urogenital ridge. C14 and GAPDH indicate the positions of the respective protected fragments.

(Tsuji et al., 1992). Light and electron microscopic studies have demonstrated that the regression of the müllerian duct epithelium is closely associated with changes in the surrounding mesenchymal cells and changes in the epithelial-mesenchymal interface. One of the observed changes during müllerian duct regression is the formation of a dense circular whorl of mesenchymal cells around the epithelial duct (Trelstad et al., 1982). These observations on the cellular sites of AMH action and histological changes associated with müllerian duct regression, correlate with C14 mRNA expression.

The level of C14 mRNA in the urogenital ridges at E16 is relatively low in male compared to female embryos. At E19, C14 mRNA is expressed at a high level in the female urogenital ridge whereas at this stage it is no longer detected in the male urogenital ridge. This correlates with the gradual degeneration of the müllerian ducts in male embryos between E16 and E19 (Trelstad et al., 1982). Furthermore, in the female, the AMH receptor indeed persists after the critical period of induction of müllerian duct regression as can be concluded from the observation that AMH induces the formation of bulges in the cranial portion of the müllerian duct, in cultured urogenital ridges that were isolated from female embryos at E18.5 (Tsuji et al., 1992).

Using in situ hybridization at E15 and E16, marked C14 mRNA expression was detected in developing ovaries but not in testes. However, using RNase protection assays at E15, E16 and E19, C14 mRNA was detected at low levels in the testis (and at a high level in the ovary); the RNase protection assay appears to be more sensitive than in situ hybridization. In view of this C14 mRNA expression in fetal testis, AMH and its receptor might be involved in normal testis differentiation in the male. Overexpression of AMH in transgenic mice can induce formation of testis cord-like structures in fetal ovaries (Behringer et al., 1990). Furthermore, AMH exerts an inhibitory effect on aromatase activity in cultured fetal ovaries (Vigier et al., 1989). Aromatase catalyzes the conversion of testosterone to estradiol during female gonadal development, and inhibition of ovarian aromatase activity by AMH leads to production of testosterone rather than estradiol. These biological responses to AMH suggest that the AMH receptor indeed is present in fetal ovaries, where it is probably inactive due to the absence of AMH. Following day 3 of postnatal development, AMH is expressed in the ovary where it might play a role in oocyte maturation (Hirobe et al., 1992; Münsterberg and Lovell-Badge, 1991). In agreement with this, we observed postnatal ovarian expression of C14 mRNA in the granulosa cells of small antral follicles. Future studies will have to provide more information about the pattern and regulation of C14 mRNA expression in the ovary.
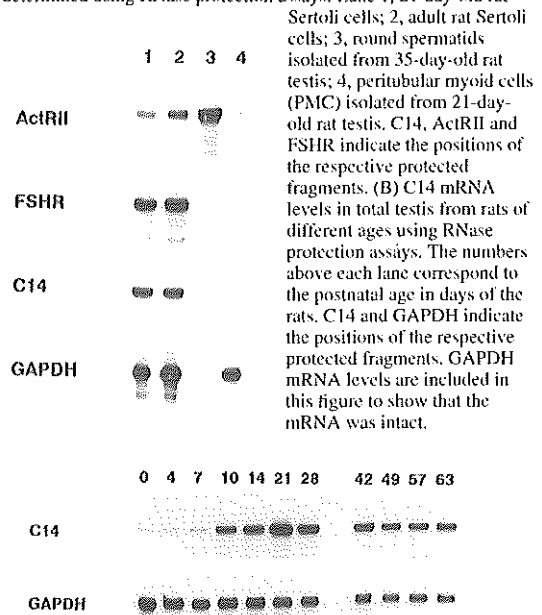
It is not known whether AMH plays a physiological role in the testis after birth. We have shown that C14 mRNA expression increases in Sertoli cells to a maximum during postnatal testis development, and remains high in the adult rat testis. With respect to testicular production of the ligand, it is known that testicular AMH mRNA expression decreases after birth, but persists at a low level throughout adulthood (Lee et al., 1992). Low levels of AMH may exert autocrine actions upon Sertoli cells in immature and mature testis. It is also possible that testicular AMH production varies at different stages of the spermatogenic cycle and hence involves local

concentration gradients. Inherited defects in the biosynthesis of AMH do not seem to have a major effect on male fertility (Josso, 1992), but this does not exclude a quantitative defect in spermatogenesis. Moreover, other testicular factors (possibly members of the activin and TGF-β family of peptide growth factors) may partly compensate for a lack of AMH by binding to their specific receptors and activation of similar intracellular pathways.

The present results are in accordance with - but do not provide conclusive evidence for - the hypothesis that C14 encodes an AMH receptor. Evidence might be provided by showing AMH binding to cells that are transfected with a C14 expression vector. However, attempts that have been made in the past to show binding of AMH to its receptor on AMH-responsive cells have been hampered by the fact that radiolabelling of AMH seems to abolish its ability to bind to the receptor (Donahoe et al., 1977). Furthermore, conclusive results that identify a receptor ligand through a binding assay using a cell line that stably expresses the relevant receptor, might be difficult to obtain if this receptor behaves as a type I receptor; such a receptor would require cooperation with a specific type II receptor which may not be endogenously expressed in the cell line used. To resolve these problems, it will be necessary to develop a binding assay for AMH. Then the possible existence of different types of AMH receptors can be studied, and suitable model cell lines can be developed to test candidate receptors for ligand binding.

The persistent müllerian duct syndrome (PMDS) is a rare form of male pseudohermaphroditism that is characterized by

Fig. 6. C14 mRNA expression in testis. (A) The expression of C14, activin type II receptor (ActRII) and follicle-stimulating hormone receptor (FSHR) mRNAs in different testicular cell types, determined using RNase protection assays: Lane 1, 21-day-old rat Sertoli cells; 2, adult rat Sertoli cells; 3, round spermatids isolated from 35-day-old rat testis; 4, peritubular myoid cells (PMC) isolated from 21-day-old rat testis. C14, ActRII and FSHR indicate the positions of the respective protected fragments. (B) C14 mRNA levels in total testis from rats of different ages using RNase protection assays. The numbers above each lane correspond to the postnatal age in days of the rats. C14 and GAPDH indicate the positions of the respective protected fragments. GAPDH mRNA are included in this figure to show that the mRNA was intact.

the presence of uterus and fallopian tubes in otherwise normally virilized males (Josso et al., 1991). In some of these patients it has been shown that the phenotype is caused by a mutation in the AMH gene (Knebelmann et al., 1991), but other patients express a normal amount of bioactive testicular AMH (Guerrier et al., 1989). The existence of this type of PMDS indicates that mutation of the AMH receptor gene(s) can result in a complete loss of responsiveness to AMH. In the future, ultimate proof that C14 encodes an AMH receptor can be obtained through genetic analysis of PMDS, or when knock-out transgenic mice are generated that show the characteristics of PMDS.

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Anti-Müllerian Hormone and Anti-Müllerian Hormone Type II Receptor mRNA Expression During Postnatal Testis Development and in the Adult Testis of the Rat

Willy M. Baarends, Jos W. Hoogerbrugge, Miriam Post, Jenny A. Visser, Dirk G. de Rooij, Martti Parvinen, Axel P.N. Themmen, and J. Anton Grootegoed.

Summary

Anti-müllerian hormone (AMH) induces degeneration of the müllerian ducts during male sex differentiation, and may have additional functions concerning gonadal development. In the immature rat testis, there is a marked developmental increase in AMH type II receptor (AMHRII) mRNA expression in Sertoli cells, concomitant with the initiation of spermatogenesis. AMHRII mRNA is also expressed at a high level in Sertoli cells in adult rats. To obtain information about the possible functions of AMH in the testis, we have investigated the postnatal expression patterns of the genes encoding AMH and AMHRII in the rat testis in more detail.

Using RNase protection assays, AMH and AMHRII mRNA expression was measured in total RNA preparations from testes or testicular tubule segments, isolated from control rats and from rats that had received various treatments. The testicular level of AMHRII mRNA was found to be much higher than that of AMH mRNA in adult rats. AMH mRNA was detected at a maximal level at stage VII of the spermatogenic cycle, and at a low level at the other stages. AMHRII mRNA increases from stage XIII, is highest at stages VI and VII, and then rapidly declines at stage VIII to almost undetectable levels at stages IX-XII. It was found that the increase in testicular AMHRII mRNA expression during the first three weeks of postnatal development also occurs in sterile rats (prenatally irradiated), and hence, is independent of the presence or absence of germ cells. Yet, the total testicular level of AMHRII mRNA was decreased in sterile adult rats (prenatally irradiated, and experimental cryptorchidism), as compared to intact control rats. However, treatment of adult rats with methoxyacetic acid or hydroxyurea, which resulted in partial germ cell depletion, had no effect on total testicular AMHRII mRNA expression. It is concluded that a combination of multiple spermatogenic cycle events, possibly involving changes of Sertoli cell structure and/or Sertoli cell-basal membrane interactions, regulate autocrine AMH action on Sertoli cells, in particular at stage VII of the spermatogenic cycle.

Introduction

Anti-müllerian hormone (AMH), also called müllerian-inhibiting substance (MIS), is a member of the activin and TGFβ family of peptide growth/differentiation factors (Cate et al. 1986). During male embryonic sex differentiation, AMH induces the degeneration of the müllerian ducts, which form the anlagen of the uterus, the oviducts and the upper part of the vagina. AMH is the earliest product known to be

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secreted by fetal Sertoli cells, starting at day 13 of embryonic development in the rat (Tran and Josso, 1982; Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992). Sertoli cells are the exclusive source of AMH in the male. Granulosa cells also can produce AMH, but there is no ovarian AMH production during fetal development (Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992).

Recently, we have isolated a cDNA clone named C14, that encodes the candidate rat AMH type II receptor (Baarends et al. 1994). During embryonic development, C14 mRNA is specifically expressed in mesenchymal cells located adjacent to the müllerian duct epithelium (Baarends et al. 1994), which is in agreement with the expected site of AMH action during müllerian duct regression (Tsuji et al. 1992). Furthermore, this gene is expressed in fetal ovaries and testes, and in granulosa cells and Sertoli cells in postnatal developing and adult gonads (Baarends et al. 1994). C14 is a member of the family of transmembrane serine/threonine kinase receptors, which also includes the TGFβ and activin receptors (reviewed by Lin and Moustakas, 1994). Members of the activin and TGFβ family signal through heteromeric complexes composed of type I and type II receptors (Wrana et al. 1994). The structural characteristics of C14 indicate that it is a type II receptor. di Clemente et al. (1994) have shown that the rabbit homologue (H1) of C14 specifically binds AMH. This provides further evidence that C14 and H1 encode, respectively, the rat and rabbit AMH type II receptor. Herein, this receptor will be referred to as the AMH type II receptor (AMHRII), in analogy to the nomenclature that is used for the TGFβ receptors. An AMH type I receptor may also exist. Moreover, the existence of additional AMH type II receptors is not excluded.

In addition to its function during müllerian duct regression, AMH may play an intratesticular role during gonad differentiation. This hypothesis is based on several observations. First, overexpression of the cDNA encoding AMH in transgenic mice, not only leads to müllerian duct regression in female embryos, but also induces the formation of testis cord-like structures in fetal ovaries (Behringer et al. 1990). In transgenic males, high levels of AMH can perturb normal testicular development and testosterone secretion, resulting in partial feminization of genitalia (Behringer et al. 1990). Second, the phenotypic sex reversal of fetal ovaries induced by AMH *in vivo*, is confirmed by the hormonal sex reversal that has been observed in cultured fetal ovaries, where exogenous AMH inhibits aromatase activity (Vigier et al. 1989). Aromatase catalyses the conversion of testosterone to estradiol during female gonadal development, and inhibition of ovarian aromatase activity by AMH leads to production of testosterone rather than estradiol. Third, the prenatal and neonatal expression of

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AMH is high around the time of mitotic arrest, and decreases during the first 2-3 weeks after birth in rat and mouse, concomitant with the initiation of spermatogenesis (Kuroda et al. 1990; Münsterberg and Lovell-Badge, 1991). Also, there is a strong correlation between mitotic arrest at the prespermatogonia stage and expression of AMH in adjacent somatic cells, in ovotestis which occur in B6Y[dom] mice (Taketo et al. 1991). Based on these data, it has been suggested that AMH plays a role in the mitotic arrest of gonocytes (Cate and Wilson, 1993). Results from tissue culture experiments of neonatal mouse testes in the presence or absence of exogenous AMH indicate a related but different function, namely that the maturation from gonocytes to type A spermatogonia requires AMH (Zhou et al. 1993).

Thus, although there are many indications for a role of AMH in the testis, further investigation is needed to clarify the exact nature of testicular AMH actions. The cloning of the AMHRII has provided a new tool to study the targets of AMH action. There is a marked increase in rat testicular AMHRII mRNA expression, derived mainly or exclusively from Sertoli cells, concomitant with the initiation of spermatogenesis between birth and postnatal day 21 (Baarends et al. 1994). Furthermore, the level of AMHRII mRNA in Sertoli cells remains high during adult life (Baarends et al. 1994). This postnatal upregulation of AMHRII is in contrast to the reported decrease in AMH expression during postnatal testis development (Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992; Kuroda et al. 1990). No effects of AMH on adult testis function have been reported.

To gain more insight in the possible roles of AMH and its type II receptor in postnatal testis development, we have studied the testicular mRNA expression patterns of AMH and AMHRII during testis development in immature rats and during the spermatogenic cycle in adult rats.

Materials and Methods.

Animals and treatments
Wistar rats were maintained under standard animal house conditions. Four different experiments were performed to test the effects of germ cell depletion on AMH and AMHRII (C14) expression in the testis. In Experiment 1, pregnant rats were γ-irradiated (1.5 Gy) at day 19.5 post coitum, as described by Beaumont (1960). This treatment results in a total or near total loss of gonocytes. Testes from control and irradiated rats were isolated at postnatal days 9, 14, 21, and 70. One testes of each animal was used for RNA analysis and the other was used for histological observation.

In Experiment 2, ten adult rats (9-11 weeks of age) were made experimentally cryptorchid by suturing both testes to the abdominal wall. At 2, 4, 8, 12, and 14 days after the operation, two cryptorchid animals were anesthesized (the experiment included also two control rats). One testis of each rat was removed and snap frozen for RNA isolation, prior to perfusion of the rat with physiological

saline followed by Bouin's fixative via the aorta. The testis was postfixed overnight in Bouin's fixative and used for histological examination.

In Experiment 3, twelve adult rats were used, ten of which were treated with a single oral dose of methoxyacetic acid (MAA) (Janssen Chimica, Beerse, Belgium) (650 mg in 2.9 ml physiological saline/kg body weight) according to Bartlett et al. (1988). MAA is a germ cell toxicant that leads to selective loss of pachytene and later spermatocytes at all stages of the spermatogenic cycle other than early-to-mid stage VII. In MAA-treated rats, spermatogenesis proceeds with normal kinetics, which results in the absence of specific germ cell types at different time points after MAA treatment, due to maturation depletion. Two control rats received physiological saline alone. At 3, 7, 14, 21, and 28 days after treatment, two MAA-treated rats (control rats at 3 and 28 days after saline injection) were sacrificed and the testes were processed as described for Experiment 2.

In Experiment 4, four adult rats were given three i.p. injections with 500 mg/kg body weight hydroxyurea (Aldrich, Sigma, Bornem, Belgium) in physiological saline (HU) or physiological saline alone (controls) every 16 hours. HU affects cells in the S-phase except for a 4h delay in the G_1-S transition (Oud et al. 1979). This HU injection protocol depletes the testis of some undifferentiated spermatogonia and all differentiating spermatogonia types A, Intermediate and B (Oud et al. 1979). Five days (115h) after the last injection the animals were sacrificed, and the testes were processed as described for Experiment 2.

Histology

After perfusion fixation and overnight post-fixation in Bouin's fixative, testes were dehydrated and embedded in paraffin. Eight-μm sections were made and stained with periodic acid-Schiff and Gill's haematoxylin No. 3 (Polysciences Inc., Warrington, USA). From each animal, numerous tubular cross-sections were examined using light microscopy, to determine which spermatogenic cell types were present or depleted.

Tissue and RNA isolation

Seminiferous tubule segments at defined stages of the spermatogenic cycle were collected according to Parvinen and Ruokonen (1982).

Total tissues or pooled tubule segments were snap frozen in liquid nitrogen and stored at -80 C until used for RNA isolation. Total RNA was isolated using the LiCl/urea method (Auffray and Rougeon, 1980).

RNase protection assay

A *PstI* fragment containing bp 1243-1640 from C14 was subcloned in pBluescript KS (Stratagene, Westburg, Leusden, The Netherlands) and used to generate [^{32}P]-UTP-labelled anti-sense transcripts *in vitro*. All radiolabelled agents were from Amersham ('s Hertogenbosch, The Netherlands). A rat AMH DNA template for *in vitro* transcription was generated using a DNA amplification reaction (Saiki et al. 1988). Rat liver was used for genomic DNA isolation according to Davis et al. (1986). The primers 5'GCTGCTGCTAGCGACTATG3' (forward primer) and 5'AGATGTAGGCTAGCAACTG3' (reversed primer) were used to amplify bp 38-400 of the first exon of the rat AMH gene (Haqq et al. 1992). An amount of 200 ng of each of the primers was added to the DNA amplification reaction using 700 ng genomic DNA as a template in a standard reaction mixture containing 1.5 mM $MgCl_2$. The reaction conditions were as follows: a hot start (5 min 95 C), followed by 35 cycles of 1 min 94 C, 2 min 58 C, 2 min 72 C. The DNA amplification reaction generated a fragment of 362 bp, which was cut with *NheI* and subcloned in the *SmaI* site of Bluescript KS. The control glyceraldehyde 3-phosphate dehydrogenase (GAPD) RNA probe was synthesized using a construct containing a 291 bp *XbaI/Sau3AI* fragment from rat GAPD cDNA (Fort et al. 1985). Approximately 5×10^4 cpm of either C14 or AMH probe, together with 5×10^4 cpm of GAPD probe, was mixed with 5 μg of total RNA in a total volume of 30 μl hybridization mixture containing 40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide. Care was taken to use equal amounts of mRNA in each sample through OD measurement at 260/280 nm of the RNA preparations immediately prior to the RNAse protection experiment. The hybridizations were performed overnight at 55 C. The RNase protection assay was performed as described by Baarends et al. (Baarends et al. 1994). The relative amount of protected mRNA fragments was quantified through exposure of the gels to a phosphor screen (Molecular

Dynamics, B&L Systems, Zoetermeer, The Netherlands), followed by a calculation of the relative density of the obtained bands using a phospho-imager (Molecular Dynamics) and Image Quant computer analysis software. The ratio between the arbitrary units obtained for the AMHRII mRNA level and for the GAPD mRNA level (AMHRII/GAPD) was determined. Control samples of each experiment were set at a ratio of 1.0.

Results

AMH and AMHRII mRNA expression during postnatal development in control and prenatally irradiated rat testes

Histological observation confirmed (van Haaster and de Rooij, 1993) that spermatogenesis advanced from the differentiating spermatogonia type A present at day 9, to zygotene and early pachytene at day 14 and mid-to-late pachytene spermatocytes at day 21 in normal testes of immature control rats. In the prenatally irradiated testes from the immature rats, no germ cells were observed, with the exception of a few degenerating gonocytes on day 9. In the prenatally irradiated adult rats, normal spermatogenesis took place in approximately 6% of the tubular cross-sections in one rat, and no germ cells were observed in tissue sections of another rat.

Using RNase protection assay, AMH mRNA was detected at low levels at days 9, 14, and 21 in control and in prenatally irradiated testes (Fig 4.1). The pattern of induction of AMHRII mRNA expression between days 9 and 21 was similar in total testis RNA extracted from control and prenatally irradiated rats (Fig. 4.1). The major increase in testicular AMHRII mRNA expression was detected between days 9 and 14, the AMHRII mRNA level on day 9 seemed somewhat higher in prenatally irradiated rats compared to control rats. There was no difference in the AMHRII/GAPD mRNA ratio between control and irradiated rats at day 21 (Fig. 4.2), but the AMHRII/GAPD mRNA ratio in adult prenatally irradiated testes was decreased compared to the ratio in control adult rat testes (Fig. 4.2). The level of GAPD mRNA is used as an approximate measure for the relative amount of somatic cell-derived mRNA in the total testis RNA preparations (see Discussion).
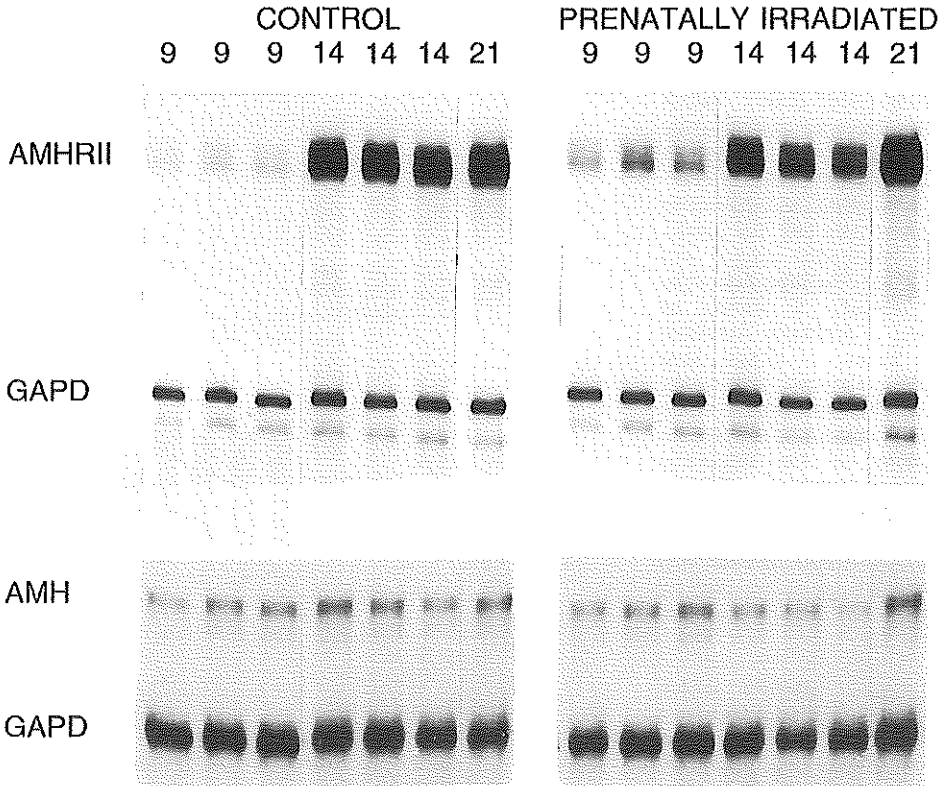
Fig. 4.1: AMHRII and AMH mRNA expression in testes from control and prenatally irradiated immature rats of different ages.
AMHRII, AMH and GAPD mRNA expression was studied using RNase protection assays on total testicular RNA from: control and prenatally irradiated 9-day-old, 14-day-old, and 21-day-old rats. The numbers above the lanes indicate the age (in days) of the rats. Each lane represents at least one rat. AMHRII, AMH and GAPD indicate the positions of the respective protected fragments.

Effect of experimental cryptorchidism on AMHRII mRNA expression in adult rat testes

Histological observation of the Bouin's fixed testes showed that cryptorchid testes at 2 days post-operation had normal spermatogenesis, but 5 days post-operation a depletion of many round and elongating spermatids was observed. This depletion had become more complete after 8 days of cryptorchidism, when most tubules contained only spermatogonia and spermatocytes. At 12 and 14 days after the operation, no further depletion of germ cell types was observed. No difference in the AMHRII and GAPD mRNA levels in the cryptorchid testes were observed at 2 days post-operation, compared to control testes. Concomitant with the depletion of germ cells at later time points, the GAPD mRNA level increased (per constant amount of total RNA). This resulted in a decrease of the AMHRII/GAPD mRNA ratio (Fig. 4.3).

Fig. 4.2: AMHRII expression in testes from control and prenatally irradiated immature and mature rats.
AMHRII and GAPD mRNA expression was studied using RNase protection assays on total testicular RNA from: 21-day-old control and prenatally irradiated rats (left four lanes), and 70-day-old control and prenatally irradiated rats (right four lanes). C, control rat testes; I, prenatally irradiated rat testes. Each lane represents on rat. Top: results of the RNAse protection assay. AMHRII and GAPD indicate the positions of the respective protected fragments. Bottom: quantitative analysis of the AMHRII/GAPD mRNA ratios. The AMHRII/GAPD mRNA ratio in the 21-day-old control rat testis in the first left lane was set at 1.0.
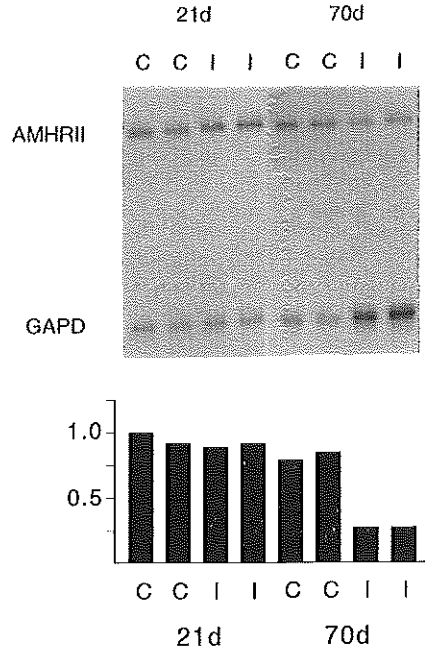
Fig. 4.3: AMHRII mRNA expression in total testes RNA isolated from control rats and experimentally cryptorchid rats. AMHRII and GAPD mRNA expression was studied using RNase protection assays on total testicular RNA from: control adult rats (C), and experimentally cryptorchid adult rats at 2, 5, 8, 12, and 14 days post-operation. The numbers above the lanes and below the histogram indicate the number of days post-operation. C, control rat testis. Each lane represents one rat. Top: results of the RNAse protection assay. AMHRII and GAPD indicate the positions of the respective protected fragments. Bottom: quantitative analysis of the AMHRII/GAPD mRNA ratios. The AMHRII/GAPD mRNA ratio in the control adult rat testis in the first left lane was set at 1.0.
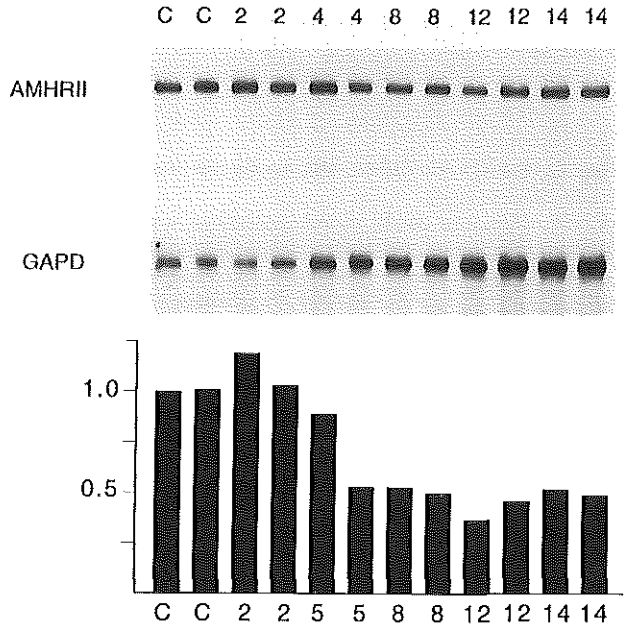
AMH and AMHRII mRNA expression during the spermatogenic cycle
The expression of AMH and AMHRII mRNAs showed a marked regulation during the different stages of the spermatogenic cycle (Fig. 4.4). The expression of AMHRII mRNA increased from stage XIII to a maximum at stages VI and VIIab, followed by a sudden drop in the expression level at stage VIII to a very low level at stages IX-XII.

The AMH mRNA level was very low compared to AMHRII mRNA expression. However, at stages VIIab and VIIcd, AMH mRNA expression was relatively high, compared to a low expression level at the other stages of the cycle.

Expression of AMHRII mRNA in adult testes depleted of specific germ cell types
Three days after MAA treatment, histological examination showed that all pachytene, diplotene and meiotically dividing spermatocytes (with the exception of pachytene spermatocytes that were associated during stage VI), and all round spermatids at steps 1 and 2 of spermiogenesis, were lost. Seven days after MAA administration, pachytene spermatocytes associated with stages VI-VII, and XI-XII were lost, and also the round spermatids at Steps 1-7 of spermiogenesis were depleted. Fourteen days after MAA administration, Step 1 spermatids, and Steps 5-15 spermatids were lost from the seminiferous epithelium. Steps 7, 8, 13 and 16-19 spermatids were lost at 21 days after MAA administration, but one week later only Steps 17-19 spermatids were absent. In contrast to the clear effects of MAA on the composition of the spermatogenic cell population, there was no major change in the AMHRII/GAPD mRNA ratio at any of the time points studied (Fig. 4.5).

Hydroxyurea treatment resulted in the loss of all Intermediate and type B spermatogonia, and preleptotene, leptotene and almost all zygotene spermatocytes, 4.8 days after the last injection of HU. However, at that time point, we found no change in the expression of AMHRII and GAPD mRNAs in total testes from HU-treated rats compared to control rats (Fig. 4.6).
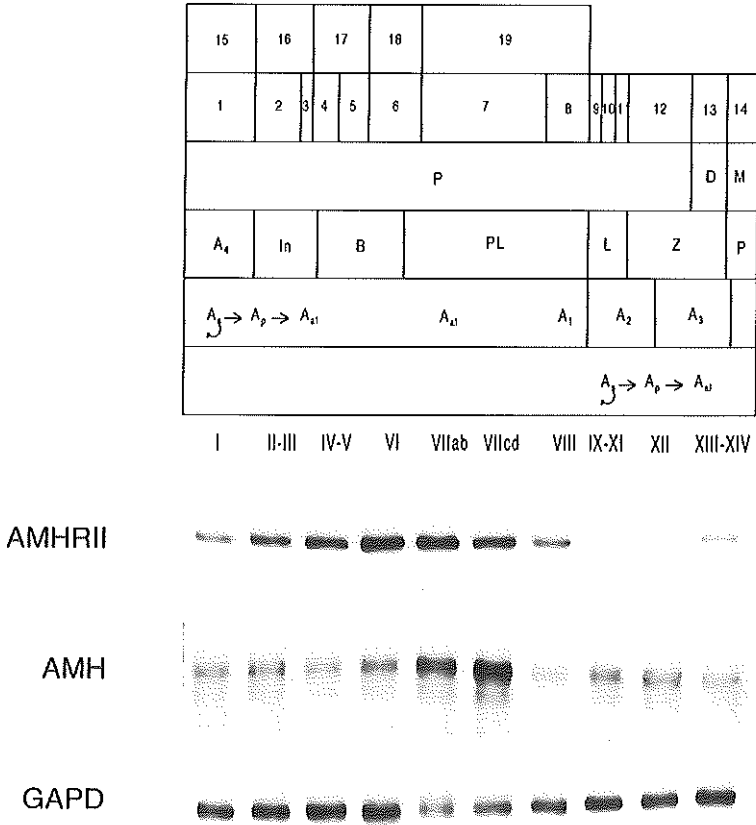
Fig. 4.4: AMHRII, AMH and GAPD mRNA expression at different stages of the spermatogenic cycle. AMHRII, AMH and GAPD mRNA expression was studied using RNase protection assays on tubule segments that were sectioned and separated according to the stages of the spermatogenic cycle. The Roman numerals above the lanes indicate the stage numbers that were collected together in each sample. As a reference to the cell types that are associated at the different stages of the spermatogenic cycle, and the relative duration of the stages (represented by the width of boxes 1-14), a schematic drawing is presented (adapted from van Pelt, 1992) . A_s, A_p, A_{al}, single, paired, and aligned undifferentiated type A spermatogonia; A_{1-4}, In, B, different types of differentiating spermatogonia; PL, L, Z, P, D, spermatocytes at the preleptotene, leptotene, zygotene, pachytene, and diplotene stages of the meiotic prophase; M, spermatocytes undergoing meiotic divisions; 1-19, spermatids at subsequent steps of spermiogenesis. Note: the observed AMH mRNA level results from exposure of the gel to radiographic film for 14 days, whereas the signal that represents AMHRII mRNA expression was obtained after exposure for 24 h.

Discussion

In the testis, AMH is known to be secreted specifically by Sertoli cells (Tran and Josso, 1982), and also AMHRII expression mainly or exclusively occurs in Sertoli cells. Using RNase protection, we have shown in previous studies (Baarends et al. 1994) that AMHRII mRNA is absent from total RNA prepared from isolated cell preparations highly enriched in pachytene spermatocytes and spermatids, or in interstitial and peritubular myoid cells. Furthermore, preliminary *in situ* hybridization results of 21-day-old rat testes demonstrated that the AMHRII mRNA expression patterns were similar in testes from control as compared to sterile (prenatally irradiated) immature rats (not shown), indicating that AMHRII mRNA expression in spermatogonia is low or absent. Thus, it can be suggested that primary effects of AMH/AMHRII on testis function are autocrine effects on Sertoli cells.

The expression of the mRNA encoding the ligand AMH did not show a major quantitative change between postnatal days 9 and 21 in control rat testes, and the absence of germ cells in prenatally irradiated rats did not result in a different developmental expression pattern. These results are in accordance with the fact that the most dramatic decrease in AMH mRNA level occurs during the first week of postnatal testis development in the rat (Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992; Kuroda et al. 1990).

AMHRII mRNA expression occurs in fetal and neonatal testes, but the expression markedly increases during the first three weeks of postnatal development in the rat (Baarends et al., 1994; and present results). It was found that the developmental AMHRII mRNA expression pattern in the testis, was not distinctly influenced by induction of sterility by prenatal irradiation, and hence, is independent of the presence or absence of germ cells.

Concerning the development of Sertoli cell characteristics in testes from prenatally irradiated rats, it is known that some events are delayed, such as formation of the blood-testis barrier and lumen formation (Means et al. 1976). The present results indicate that the timing of the postnatal increase in AMHRII mRNA expression in prenatally irradiated rats seems to be slightly advanced rather than delayed.

In adult prenatally irradiated rats, the absence of germ cells results in in a testis that is relatively enriched in somatic cell types, including Sertoli cells. We have previously reported that the GAPD mRNA that is detected with the probe used in our studies is not expressed in rat spermatocytes and spermatids (Baarends et al. 1994).
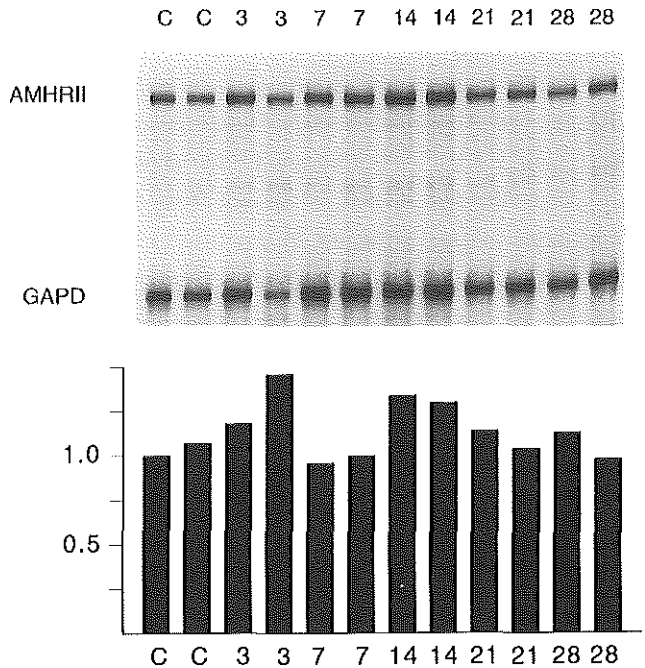
Fig. 4.5: AMHRII mRNA expression in total testes from control and methoxyacetic acid-treated rats.
AMHRII and GAPD mRNA expression was studied using RNase protection assays on total testicular RNA from: control adult rats (C), and adult rats that had received a single oral dose of methoxyacetic acid (MAA) at 3, 7, 14, 21, or 28 days after MAA administration. The numbers above the lanes and below the histogram indicate the number of days after MAA treatment (C, control rat testis). Each lane represents one rat.
Top: results of the RNAse protection assay. AMHRII and GAPD indicate the positions of the respective protected fragments.
Bottom: quantitative analysis of the AMHRII/GAPD mRNA ratios. The AMHRII/GAPD mRNA ratio in the control adult rat testis in the first left lane was set at 1.0.

Fig. 4.6: AMHRII mRNA expression in total testes from control and hydroxyurea-treated rats
AMHRII and GAPD mRNA expression was studied using RNase protection assays on total testicular RNA isolated from a control adult rat (lane 1), and adult rats that were injected with hydroxyurea (HU) and killed 4.8 days after the last HU injection (lanes 2 and 3).
Top: results of the RNAse protection assay. AMHRII and GAPD indicate the positions of the respective protected fragments.
Bottom: quantitative analysis of the AMHRII/GAPD mRNA ratios. The AMHRII/GAPD mRNA ratio in the control adult rat testis in the first left lane was set at 1.0.
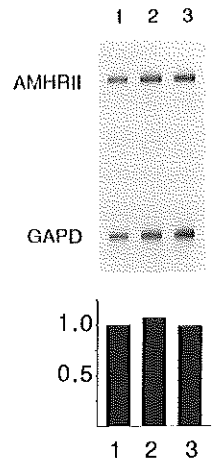
This is in accordance with results from other investigators (Mori et al. 1992; Welch et al. 1992) who reported that the germ cells express a testis specific GAPD-s gene. Only the early germ cells express GAPD, whereas this gene is silenced in the more advanced germ cell types that make up the bulk of the germ cells in the adult testis. Therefore, the GAPD mRNA level can be viewed as an approximate measure for the

relative amount of somatic cell-derived mRNA. Following this argument, we have used the ratio between AMHRII mRNA and GAPD mRNA levels, to study the level of AMHRII mRNA corrected for the amount of somatic cell-derived mRNA. Expressed in this manner, the present results show that the level of AMHRII mRNA expression in adult Sertoli cells is quantitatively reduced in sterile testes from prenatally irradiated rats.

Similar to the difference in AMHRII mRNA expression in adult control and prenatally irradiated rats, pronounced derangement of spermatogenesis upon experimental cryptorchidism also resulted in a relative decrease of AMHRII mRNA expression. The decreased AMHRII mRNA expression in cryptorchid rat testes may reflect a direct effect of the abdominal temperature on Sertoli cells (Kerr et al. 1979) or, may be caused by structural changes that occur in Sertoli cells as a result of the disorganization of the spermatogenic epithelium.

To study the possible regulation of AMH and AMHRII expression in Sertoli cells by spermatogenic cells in more detail, the mRNA levels were determined in isolated tubule segments that were separated according to the stages of the spermatogenic cycle. It was found that both AMH mRNA and AMHRII mRNA show a marked regulation during the spermatogenic cycle. Maximal AMH and AMHRII mRNA levels were found at stage VII. AMHRII mRNA levels are at a minimum during stages IX-XII. It has been shown that FSH receptor mRNA (Heckert and Griswold, 1991) and binding (Kangasniemi et al. 1990) are at a minimum at stage VII. Since FSH has been shown to down-regulate AMH expression during early postnatal development (Kuroda et al. 1990), the nadir in FSH action at stage VII might be involved in the observed up-regulation of AMH mRNA expression. Another candidate factor that may modulate the pattern of AMH mRNA expression during the spermatogenic cycle is steroidogenic factor-1 (SF-1) (Honda et al. 1993; Lala et al. 1992), which is an orphan nuclear receptor that is crucial for adrenal and gonad formation, and regulates the expression of a number of steroidogenic enzymes (Morohashi et al. 1992; Lynch et al. 1993). This factor is capable of binding to an upstream conserved element in the AMH promoter *in vitro* (Shen et al. 1994; Hatano et al. 1994). The expression pattern of SF-1 in rat embryos suggests that it is involved in a positive regulation of the prenatal expression of AMH (Shen et al. 1994), although conflicting data are reported indicating a stimulatory effect of SF-1 on AMH gene transcription in one study (Shen et al. 1994) and an inhibitory effect in another study (Haqq et al. 1994), using different experimental approaches *in vitro*. It will be of interest to study whether postnatal expression of SF-1 in Sertoli cells correlates with AMH expression.

Sertoli cells support spermatogenesis, and at each stage of the spermatogenic cycle different combinations of spermatogenic cell types are present that require distinct Sertoli cell actions. The cyclic expression of many Sertoli cell proteins has been established (reviewed by Parvinen, 1993), providing evidence that spermatogenic cells influence Sertoli cell function. Therefore, we have studied the effect of depleting the adult testes of various germ cell types on AMHRII mRNA expression. Treatment with methoxyacetic acid (MAA) or hydroxyurea (HU) resulted in selective loss of several types of spermatogenic cells. However, in both experimental models, there was no major change in the testicular AMHRII/GAPD mRNA ratio after treatment. These results do not identify a single defined group of germ cell types that is involved in the up- or down-regulation of AMHRII mRNA expression in Sertoli cells. It is of interest to note that immunohistochemical studies of androgen receptor expression in Sertoli cells of adult rats showed a marked pattern of androgen receptor protein expression during the spermatogenic cycle, which was very similar to the pattern of AMHRII mRNA expression described herein, and which also was not influenced by treatment of the rats with MAA (Bremner et al. 1994).

Since both AMH and AMHRII mRNA levels are high at stage VII, actions of AMH on Sertoli cell function might occur mainly at that stage. There are various events that take place at stages VII-VIII of the spermatogenic cycle in which AMH could have a function. Leptotene spermatocytes leaving the basal membrane starting to migrate through the blood-testis barrier, the onset of spermatid elongation, and spermiation of Step 19 spermatids, all occur at stages VII and VIII (reviewed by de Kretser and Kerr, 1994).

The mechanism of AMH-induced regression of the müllerian ducts may involve a secondary effect on the extracellular matrix (Trelstad et al. 1982; Tsuji et al. 1992), induced by the surrounding mesenchymal cells. Since the spermatogenic events that take place at stage VII involve major changes in the structure of Sertoli cells and their interaction with the basal membrane, it is tempting to suggest that testicular AMH action may involve some effect on the extracellular matrix components in the basal membrane. As part of a feedback mechanism, major structural changes in the tubules, rather than specific germ cell products, may regulate the cyclic AMH and AMHRII mRNA expression patterns.

A possible function of AMH can be inferred from the inverse relationship between AMHRII mRNA expression and [^{3}H]-thymidine incorporation during the spermatogenic cycle, showing minimal [^{3}H]-thymidine incorporation at stage VII (Soder et al. 1991). In concordance with this, mitotic divisions of differentiating spermatogonia

take place at stages IX-VI, but not at stages VII and VIII (Huckins, 1970; Huckins, 1971). Factors that have been postulated to stimulate spermatogonial cell proliferation (interleukin-1, EGF, Kit ligand, activin), show their lowest expression at stage VII (Soder et al. 1991; Manova et al. 1993; Bartlett et al. 1990; Klaij et al. 1994). Since AMH and its receptor are at a maximum at this stage, the inhibition of mitotic divisions of undifferentiated and/or differentiated type A spermatogonia could be one of the possible effects of AMH on spermatogenesis.

If AMH plays a role in determining the amount of spermatogonia that enter spermatogenesis, lack of AMH action may result in a subtle change in the balance between stimulatory and inhibitory effects on spermatogonial proliferation and degeneration. Activin and inhibin, two members of the activin and TGFβ family of growth factors, have also been reported to regulate spermatogonial proliferation (Mather et al. 1990; van Dissel-Emiliani et al. 1989). Several members of the activin and TGFβ family of growth factors show overlapping or counteracting actions. A redundancy phenomenon may prevent clear loss of function effects when a single factor is missing.

Natural mutations that block AMH action lead to the persistent müllerian duct syndrome (PMDS), that is characterized by the presence of a uterus and fallopian tubes in otherwise normal males (Josso et al. 1991). The testes develop normally in these patients, but do not descent, which results in infertility. Behringer et al. (1994) have recently generated AMH gene knock-out mice. Analogous to the PMDS human males, male AMH-deficient mice were born with both müllerian duct- and wolffian duct-derived reproductive organs. The testes in these animals produced functional sperm, and Leydig cell hyperplasia was the only testicular abnormality that was observed (Behringer et al. 1994). These results indicate that, through an autocrine action on Sertoli cells, AMH may negatively regulate Leydig cell proliferation and/or maturation. The existence of other type II receptors for AMH on Leydig cells, however, cannot be excluded. With respect to the cyclic regulation of AMH and AMHRII mRNA expression, it is of interest to note that the morphology of Leydig cells surrounded by stage VII seminiferous tubules differs from the morphology of those located near other stages (Bergh, 1983; Bergh, 1985), and also the expression of certain genes in Leydig cells depends on the stages present in the neighbouring tubules (Gizang-Ginsberg and Wolgemuth, 1985).

The results presented herein indicate that there may be a function of AMH in the adult rat testis. In order to identify the nature of this function, future studies will be directed towards the development of strategies that allow modulation of AMH and

AMHRII action at certain time points during postnatal testis development.

In conclusion, a high level of AMHRII mRNA is an intrinsic feature of mature rat Sertoli cells, which is down-regulated at stages IX-XII of the spermatogenic cycle. AMH mRNA is expressed at a low level in adult Sertoli cells, but specific up-regulation of this mRNA at stage VII of the cycle would permit a local autocrine action of AMH. The postulated functions of AMH with respect to proliferation of spermatogonia and/or Leydig cells might be explained by effects of AMH on the extracellular matrix, thereby influencing the communication between the different testicular cell types.

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Anti-Müllerian Hormone and Anti-Müllerian Hormone Type II Receptor mRNA Expression in Rat Ovaries During Postnatal Development, the Estrous Cycle, and Gonadotropin-Induced Follicle Growth

Willy M. Baarends, Jan Th. J. Uilenbroek, Piet Kramer, Jos W. Hoogerbrugge,
Liesbeth van Leeuwen, Axel P.N. Themmen, and J. Anton Grootegoed.

Summary

During fetal development, anti-müllerian hormone (AMH) is produced only by Sertoli cells, but postnatally granulosa cells also produce this peptide growth/differentiation factor. We have recently identified a candidate AMH type II receptor (AMHRII) (Baarends et al. 1994). In the present study, postnatal ovarian AMH and AMHRII mRNA expression was studied by *in situ* hybridization and RNase protection. In ovaries from adult rats, AMH and AMHRII mRNAs were found to be mainly expressed in granulosa cells from preantral and small antral follicles. Corpora lutea and large antral follicles express little or no AMH and AMHRII mRNA, and primordial follicles and oocytes appeared to be AMH and AMHRII mRNA negative. Theca and interstitial cells express no detectable amount of AMH mRNA, and little or no AMHRII mRNA. The colocalization of AMH and AMHRII mRNAs in granulosa cells of specific follicle types, suggests that actions of AMH via AMHRII are autocrine in nature. There is a decreased level of AMH and AMHRII mRNA expression when follicles have become atretic. Both mRNA species are eventually lost from atretic follicles, although AMHRII mRNA expression seems to persist somewhat longer than AMH mRNA. During the estrous cycle, no marked changes in the patterns of AMH and AMHRII mRNA expression were detected, except at estrus, when expression of both mRNA species in preantral follicles was decreased compared to the other days of the cycle.

At postnatal day 5, total ovarian AMH mRNA expression is low, and is located in small preantral follicles. During the first weeks of postnatal development, AMH mRNA expression in preantral follicles increases, and the later formed small antral follicles also express AMH mRNA. In contrast, AMHRII mRNA is expressed at postnatal day 5 at a higher level than AMH mRNA, but cannot be localized to specific cell types. From postnatal day 15 onwards, AMHRII mRNA expression becomes more restricted to the preantral and small antral follicles.

Treatment of prepubertal rats with GnRH antagonist (Org 30276) and human recombinant FSH (Org 32489), or with GnRH antagonist and estradiol benzoate, resulted in follicle growth, and in an inhibition of AMH and AMHRII mRNA expression in some, but not all preantral and small antral follicles. These results indicate that FSH and estrogens may play a role in the downregulation of AMH and AMHRII mRNA expression *in vivo*, when small antral follicles differentiate into large antral follicles. Furthermore, the FSH surge on the morning of estrus may inhibit AMH and AMHRII mRNA expression in preantral follicles. It is discussed that autocrine action of AMH on granulosa cells of preantral and small antral follicles might result in an inhibition of follicle maturation and/or play a role in follicle selection.

Introduction

Anti-müllerian hormone (AMH), also called müllerian inhibiting substance, is a member of the activin/TGFβ family of peptide growth and differentiation factors (Cate et al. 1986). During male embryonic/fetal sex differentiation, AMH is synthesized by the fetal Sertoli cells, and induces the degeneration of the müllerian ducts, which are the anlagen of the uterus, the oviducts and the upper part of the vagina. There is no ovarian AMH production during normal female fetal development (Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992). However, overexpression of the gene encoding AMH in transgenic mice, leads to müllerian duct regression also in female embryos, and in addition induces the formation of testis cord-like structures in fetal ovaries (Behringer et al. 1990), indicating that the fetal ovary is sensitive to AMH. In cultured fetal ovaries, AMH inhibits oogonial replication (Vigier et al. 1987) and granulosa cell aromatase activity (Vigier et al. 1989; di Clemente et al. 1992). Aromatase catalyses the conversion of testosterone to estradiol, and inhibition of ovarian aromatase activity by AMH leads to production of testosterone rather than estradiol. The effects of AMH on the fetal ovary may reflect some function of AMH during prenatal testis development, rather than a function during prenatal ovary development, since the ovary is normally not exposed to AMH prior to birth.

In the female rat, AMH mRNA expression is detected in ovarian granulosa cells from postnatal day 3 onwards (Hirobe et al. 1992). Immunohistochemical (Ueno et al. 1989b; Ueno et al. 1989a) and mRNA *in situ* hybridization (Hirobe et al. 1992; Hirobe et al. 1994) data have shown specific expression of AMH in granulosa cells during defined stages of follicle development in the rat. It has been shown that exogenous AMH inhibits biosynthesis of aromatase (di Clemente et al. 1994a), and decreases the number of LH receptors (di Clemente et al. 1994a) in cultured rat granulosa cells. Furthermore, AMH opposes EGF-induced progesterone biosynthesis and proliferation of cultured human granulosa-luteal cells (Kim et al. 1992; Seifer et al. 1993). Conflicting data have been reported concerning possible effects of AMH on oocyte maturation; inhibition of meiosis by AMH was observed in one case (Takahashi et al. 1986), but no effect of AMH could be found in another study (Tsafriri et al. 1988).

The marked regulation of AMH expression and the reported effects of AMH in *in vitro* culture systems, indicate that AMH may perform certain functions during follicle development in the postnatal ovary. More insight in these functions could come from studies on ovarian expression of the receptor for AMH. Recently, we have cloned a cDNA, named C14, that encodes the candidate rat AMH type II receptor (Baarends

et al. 1994). C14 is a member of the family of transmembrane serine/threonine kinase receptors to which also the TGFβ and activin receptors belong (reviewed by Lin and Moustakas, 1994). Members of the activin/TGFβ family of growth and differentiation factors signal through heteromeric complexes composed of type I and type II transmembrane serine/threonine kinase receptors (Wrana et al. 1994). The structural characteristics of C14 indicate that it is a type II receptor. di Clemente et al. (1994b) have cloned the rabbit homologue (H1) of C14, and have shown that H1 binds AMH. Together with the observed expression of C14 mRNA in AMH target cells during embryonic sex differentiation, these data provide evidence that C14 and H1 encode, respectively, the rat and rabbit AMH type II receptor (AMHRII). The existence of an AMH type I receptor can be anticipated, but this receptor has not been identified yet. Herein, C14 will be referred to as the AMH type II receptor, in analogy to the nomenclature that is used for the TGFβ receptors.

In the present study, mRNA *in situ* hybridization and RNase protection were used to study in detail the expression of both AMH and AMHRII mRNA in rat ovaries. The cellular localization, temporal expression pattern, and response to hormonal treatment of AMH and AMHRII mRNA expression were determined, to identify ovarian AMH target cells, and to study the regulation of AMH and AMHRII mRNA expression during postnatal development, the estrous cycle, and gonadotropin-induced follicular growth.

Materials and Methods

Animals and treatments

Wistar rats, locally bred, were maintained under controlled conditions (20-23 C, lights on from 05.00 h to 19.00 h). AMH and AMHRII mRNA expression was studied in ovaries collected from rats at various reproductive stages. Animals were killed by an overdose of ether between 09.00 -10.00 h. Four different experiments were performed. In Experiment 1, female rats were killed at 5 (8 animals), 10 (4 animals), 15 (4 animals), 20 (2 animals), 25 (2 animals), 30 (2 animals), and 35 days (2 animals) after birth. From each animal, one ovary was snap-frozen in liquid nitrogen and used for RNA isolation, and the other ovary was fixed overnight at 4 C in Bouins' fixative and used for histological examination and *in situ* hybridization purposes. Frozen ovaries isolated at postnatal days 5, 10, and 15 were pooled.

In Experiment 2, folliculogenesis and ovulation was induced by injecting (s.c.) 26-day-old female rats with 10 IU PMSG followed 56 h later by injection of 10 IU hCG. Ovaries were isolated 0, 1, 2, 3 and 6 days after PMSG injection. Two animals were used at each time point, and the ovaries were isolated; one ovary was used for RNA isolation, the other ovary was fixed overnight at 4 C in Bouins' fixative and used for histological examination.

In Experiment 3, ovaries were collected from adult rats at different stages of the 5-day estrous cycle. The stage of the cycle was determined through analysis of daily vaginal smears. Only animals with a regular 5-day cycle were used. Two animals were studied for each day of the cycle. The ovaries were fixed overnight at 4 C in Bouins' fixative and used for histological examination and *in situ* hybridization.

In Experiments 4 and 5, effects of FSH, hCG and estradiol benzoate (EB) on AMH and AMHRII mRNA expression were studied in immature rats. GnRH antagonist (GnRHant) (Org 30276), human recombinant FSH (FSH) (Org 32489), and hCG (Pregnyl) were obtained from Organon International (Oss, The Netherlands). Endogenous levels of gonadotropins were reduced by administration of GnRHant (500 μg/100 g body weight) at days 22, 24, 26 and 28 (Meijs-Roelofs et al. 1990). FSH, hCG and EB were given at days 26, 27, 28 and 29. FSH and hCG were given twice daily at 09.00 h and 17.00 h; FSH in a total daily dose of 10 IU, and hCG in a total daily dose of 1 IU. EB was given once daily in a dose of 100 μg/injection. The animals were killed at day 30. In Experiment 4, rats were treated with GnRHant alone, with GnRHant plus FSH, or with GnRHant plus FSH plus hCG. In Experiment 5, rats were treated with GnRHant alone, with GnRHant plus FSH, with GnRHant plus EB, or with GnRHant plus FSH plus EB. Two animals were used for each treatment group, and two untreated 30-day-old female rats were included in both experiments. The ovaries were isolated as described in Experiment 1.

Isolation of follicles and corpora lutea
From adult rats, the 10 largest follicles at estrus, and the 10 largest follicles and corpora lutea at proestrus were isolated under a dissection microscope in phosphate buffered saline. The follicles isolated at estrus are small antral follicles, and those isolated at proestrus are large preovulatory follicles. Twenty corpora lutea, twenty large antral follicles, and forty small antral follicles were pooled in two fractions each, and snap-frozen in liquid nitrogen for RNA isolation. Half of the total amount of total RNA that was isolated for each sample was used in a single RNase protection assay.

RNA isolation and RNase protection assay
Total RNA was isolated using the LiCl/urea method (Auffray and Rougeon, 1980). A *Pstl* fragment containing bp 1243-1640 from C14 (AMHRII) was subcloned in pBluescript KS (Stratagene; Westburg, Leusden, The Netherlands) and used to generate [^{32}P]-UTP-labelled anti-sense transcripts *in vitro*. All radiolabelled agents were from Amersham ('s Hertogenbosch, The Netherlands). A rat AMH DNA template for *in vitro* transcription was generated using a DNA amplification reaction (Saiki et al. 1988). Rat liver was used for genomic DNA isolation according to Davis et al. (1986). The primers 5'GCTGCTGCTAGCGACTATG3' (forward primer) and 5'AGATGTAGGCTAGCAACTG3' (reverse primer) were used to amplify bp 38-400 of the first exon of the rat AMH gene (Haqq et al. 1992). An amount of 200 ng of each of the primers was added to the DNA amplification reaction using 700 ng genomic DNA as a template in a standard reaction mixture containing 1.5 mM MgCl$_2$. The reaction conditions were as follows: a hot start (5 min 95 C), followed by 35 cycles of 1 min 94 C, 2 min 58 C, 2 min 72 C. The DNA amplification reaction generated a fragment of 362 bp, which was cut with *Nhel* and subcloned in the *Smal* site of Bluescript KS. The control glyceraldehyde 3-phosphate dehydrogenase (GAPD) RNA probe was synthesized using a construct containing a 291 bp *Xbal/Sau3Al* fragment from rat GAPD cDNA (Fort et al. 1985). Approximately 5x10^4 cpm of either C14 or AMH probe, together with 5x10^4 cpm of GAPD probe, was mixed with 5 μg of total RNA (unless otherwise indicated) in a total volume of 30 μl hybridization mixture containing 40 mM Pipes pH 6.4, 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide. Care was taken to use equal amounts of mRNA in each sample through OD measurement at 260/280 nm of the RNA preparations immediately prior to the RNAse protection experiment. The hybridizations were performed overnight at 55 C. The RNase protection assay was performed as described (Baarends et al. 1994). The relative amount of protected mRNA fragments was quantified through exposure of the gels to a phosphor screen (Molecular Dynamics; B&L Systems, Zoetermeer, The Netherlands), followed by a calculation of the relative density of the obtained bands using a phospho-imager and Image Quant (Molecular Dynamics) as computer analysis software. The ratio between the arbitrary units obtained for the AMHRII mRNA level and for the GAPD mRNA level was determined. The ratios of control samples of each experiment were set at 1.0.

In situ hybridization
The AMH and AMHRII subclones that were used for RNAse protection assays were also used to generate sense and antisense [^{35}S]-UTP-labelled transcripts *in vitro*. *In situ* hybridization procedure was performed as described by Zeller and Rogers (Zeller and Rogers, 1991) with some modifications

(Baarends et al. 1994). Ovaries were embedded in paraffin, and 8 μm sections were mounted on slides which were coated with 3-aminopropyl-ethoxysilane. After deparaffination sections were treated with 0.2 N HCl (20 min), digested with proteinase-K (1 μg/ml in 0.2 M Tris, 2 mM $CaCl_2$; incubation 15 min at 37 C) and postfixed in 4% paraformaldehyde in 0.1 M PBS. After treatment with dithiothreitol and blocking non-specific binding with 0.1 M triethanolamine, followed by 0.1 M triethanolamine and acetic anhydride, sections were incubated with ^{35}S-labelled antisense and sense AMH and AMHRII cRNA probes in a final concentration of 5×10^5 cpm/μl. Hybridization was carried out overnight at 55 C in moist chambers. The hybridization mixture contained: 50% (v/v) deionised formamide, 0.3 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 1x Denhardt's solution, 1 mg/ml yeast tRNA, 50 mM DTT and 10% (w/v) polyethylene glycol 6000. Slides were washed four times in 2x SSC, 55% (v/v) formamide, at 55 C for 15 min, then treated with RNAse solution (40 μg RNAse A/ml, 700 U RNAse T/ml, in 0.3 M NaCl, 5 mM EDTA, 10 mM Tris) for 15 min at room temperature, and washed again two times in 2x SSC (55 C, 30 min), and two times in 1x SSC, 55% (v/v) formamide (55 C, 30 min). After dehydration, slides were dipped in Kodak NTB-2 emulsion and dried at room temperature for 3 hours, followed by exposure at 4 C for 1 week. After developing, the sections were counterstained with haematoxylin and mounted.

Histology
Histological examination of the follicles was based on sections, stained with haematoxylin and eosin, adjacent to sections used for *in situ* hybridization. Healthy and atretic follicles were grouped in primordial follicles (< 50 μm), small preantral follicles (50-200 μm), large preantral follicles (201-274 μm), small antral follicles (275-450 μm) and large antral follicles (>450 μm). Follicular size was calculated from two perpendicular diameters of the follicle. Atretic and healthy follicles were distinguished according to criteria described by Osman (Osman, 1985).

Results

AMH and AMHRII mRNA expression in prepubertal rat ovaries
At day 5 of postnatal development, the ovaries contained only primordial follicles and follicles with a single layer of granulosa cells (primary follicles) (Fig. 5.1A). The granulosa cells of some primary follicles that were present at day 5 expressed AMH mRNA (Fig. 5.1B). At days 10 and 15, AMH mRNA expression became more pronounced in granulosa cells of small preantral follicles. Oocytes and interstitial tissue were AMH mRNA negative (below the level of detection). AMHRII mRNA expression was detected in an evenly distributed pattern at days 5 and 10 (Fig. 5.1C). From day 15 onwards, AMHRII mRNA expression became mainly restricted to the granulosa cells of primary and preantral follicles (not shown).

At day 20, AMH mRNA positive follicles were mainly found in the peripheral region, whereas AMHRII mRNA positive follicles were also present in the center of the ovary (Fig. 5.2B,C). At days 25-35, AMH and AMHRII mRNA expression was high, and most of this expression colocalized to the same follicles. The expression of both mRNAs was highest in preantral follicles. Small antral follicles also expressed some AMH and AMHRII mRNA from day 25 onwards (Table 5.1). The signal was lost in larger antral follicles (Fig. 5.2E,F).
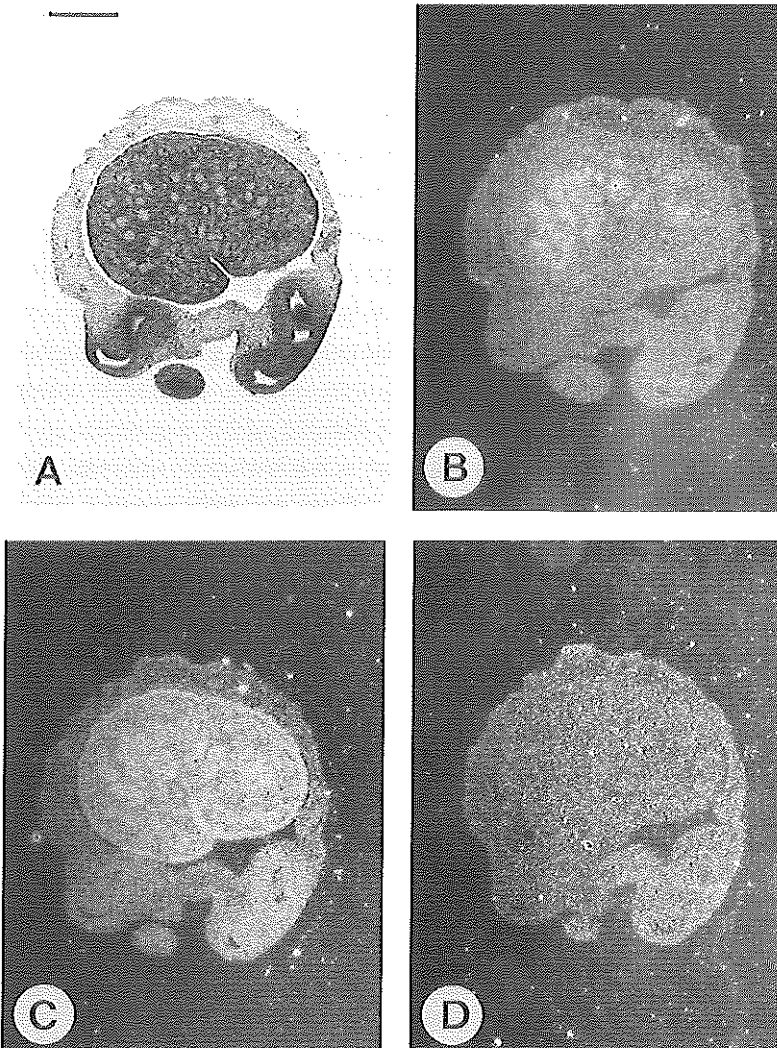
Fig. 5.1: *Localization of AMH and AMHRII mRNA expression in ovaries of 5-day-old rats, using in situ hybridization.*
A: ovarian section stained with haematoxylin and eosin; B: AMH mRNA expression pattern; C: AMHRII mRNA expression; D: negative control, hybridized with AMHRII sense RNA (B,C, and D are darkfield views). Note the presence of AMH mRNA in primary follicles and the even distribution of AMHRII mRNA over the whole ovary. Bar represents 200 μm.

Preantral atretic follicles expressed less AMH and AMHRII mRNA than healthy follicles of the same size class, but the expression seemed to increase somewhat with increasing age (Table 5.1).

In general, AMH mRNA produced stronger signals than AMHRII mRNA. The AMH and AMHRII cRNA antisense probes are comparable with respect to probe length and percentage of G/C residues. However, it is difficult to compare the absolute levels of AMH and AMHRII mRNA due to two major differences between the expression patterns of these mRNAs First, in small preantral follicles, AMHRII mRNA is evenly expressed in the granulosa cells of single positive follicles, whereas AMH mRNA expression is not always evenly distributed, but sometimes highest in the granulosa cells immediately surrounding the antrum and/or the oocyte (compare Fig. 5.2E with Fig. 5.2F). Second, a low level of AMHRII mRNA expression in interstitial cells at some stages of ovarian development is not excluded, whereas there was a clear contrast between AMH mRNA positive follicles and AMH negative interstitium in all studied ovarian sections.

RNase protection results showed, that there was a small increase in total ovarian AMHRII mRNA expression between days 10 and 15 (Fig. 5.3). From day 15 onwards, the total ovarian AMHRII mRNA expression was maintained within the same range. Compared to AMHRII mRNA expression, there was a more pronounced increase in the level of total ovarian AMH mRNA expression between days 5 and 15, and subsequently between days 25 and 30 (Fig. 5.3).

Fig. 5.2: (Next page) Localization of AMH and AMHRII mRNA expression during postnatal ovary development, and in adult cycling rats, using in situ hybridization.
The left figures (A,D,G) are ovarian sections stained with haematoxylin and eosin. The middle and right figures show darkfield views of AMH (B,E,H) and AMHRII (C,F,I) mRNA expression in adjacent sections. A,B,C: 20-day-old rat ovary, showing AMH mRNA expression in preantral follicles, mainly located in the peripheral area, and AMHRII mRNA expression in preantral follicles, both in the peripheral as well as in the central ovarian region; D, E, F: 30-day-old rat ovary, showing colocalization of follicular AMH and AMHRII mRNA expression. Note the presence of AMH and AMHRII mRNA expression in preantral follicles, and the absence in large antral follicles. Also, small antral follicles are indicated that show expression of AMH mRNA mainly in granulosa cells surrounding the oocyte; G,H,I: adult rat ovary (at proestrus), showing absence of AMH and AMHRII mRNA expression in preovulatory follicle and corpus luteum. HF, healthy follicle; AF, atretic follicle; SPF, small preantral follicle; LPF, large preantral follicle; CL, corpus luteum. Bar represents 200 μm.
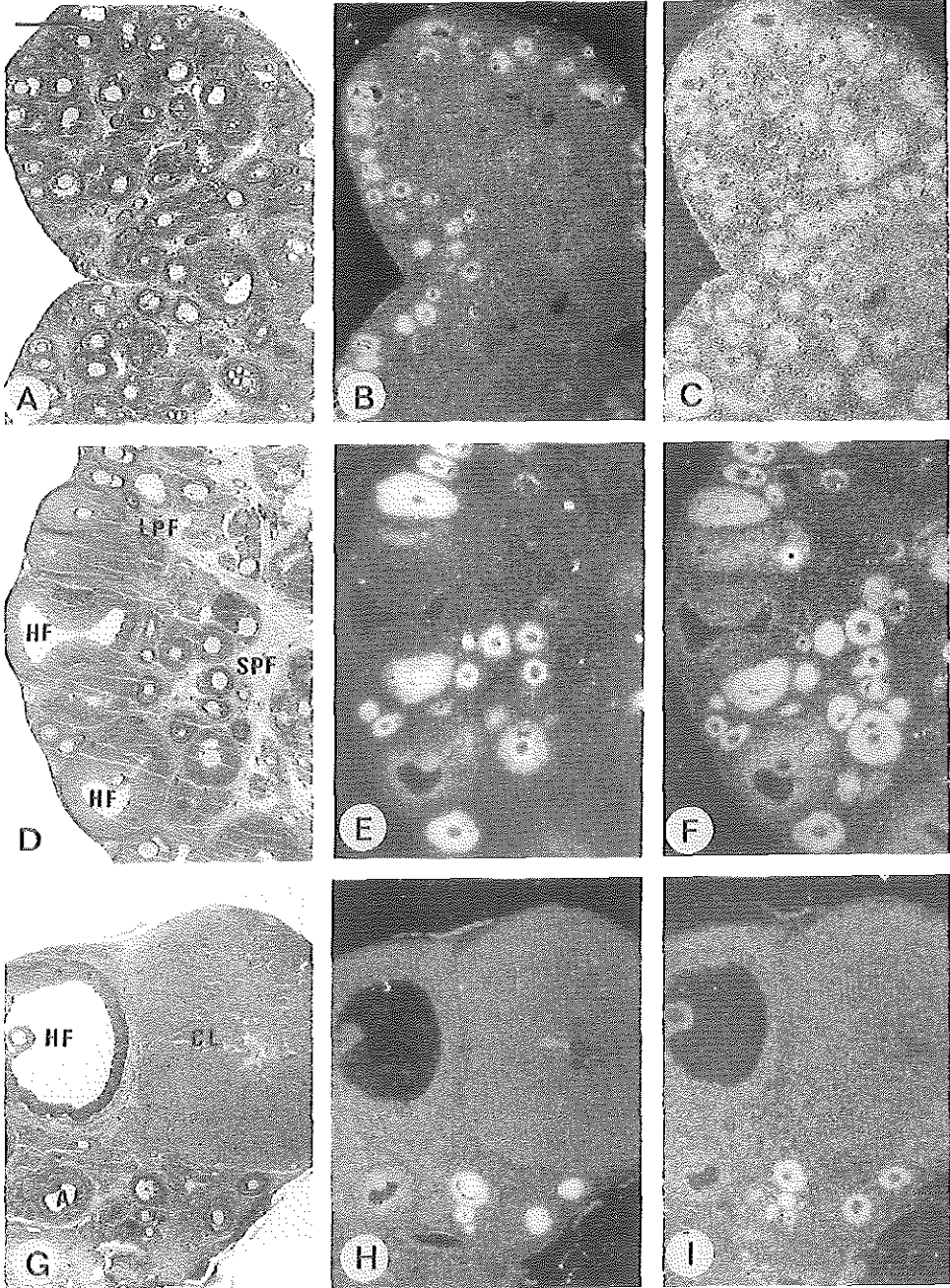
Table 5.1: Expression of AMH and AMHRII mRNAs in ovaries of prepubertal rats, as measured by *in situ* hybridization

		Day 20			Day 25			Day 30			Day 35		
		n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII
Healthy	preantral	(30)	0/+	+	(31)	+	+/++	(17)	+/++	+/++	(24)	++	+/++
	small antral	(2)	0	0	(10)	0/±	0/±	(4)	0/±	+	(4)	-	+/++
	large antral		-	-		-	-	(2)	0	0	(3)	0	0
Atretic	preantral	(1)	0	0	(11)	0	0/±	(8)	±	+	(8)	±/+	±/+
	small antral		-	-	(5)	0	0	(6)	0/±	0/±	(4)	0	0
	large antral		-	-		-	-		-	-		-	-

- = follicle not present

0 = no silver grains above background, ± = moderate expression, + = clear expression, ++ = highest expression

Number of follicles (n) is given in parentheses

Expression of AMH and AMHRII mRNAs during the estrous cycle

In situ hybridization results showed, that in adult rats, AMH and AMHRII mRNAs colocalized mainly in granulosa cells of healthy preantral and small antral follicles (Table 5.2, Fig. 5.2H,I). In large antral follicles the AMH and AMHRII mRNA signal was not above background, and corpora lutea and primordial follicles also were negative (Fig. 5.2H,I). Both AMH and AMHRII mRNA expression was gradually lost from atretic follicles, with AMHRII mRNA persisting somewhat longer than AMH mRNA (Table 5.2). There were no marked changes in the follicle types that express AMH and AMHRII mRNA during the estrous cycle (Table 5.2), except at estrus, when the granulosa cells of some of the preantral follicles expressed little, or no, AMH and/or AMHRII mRNA.
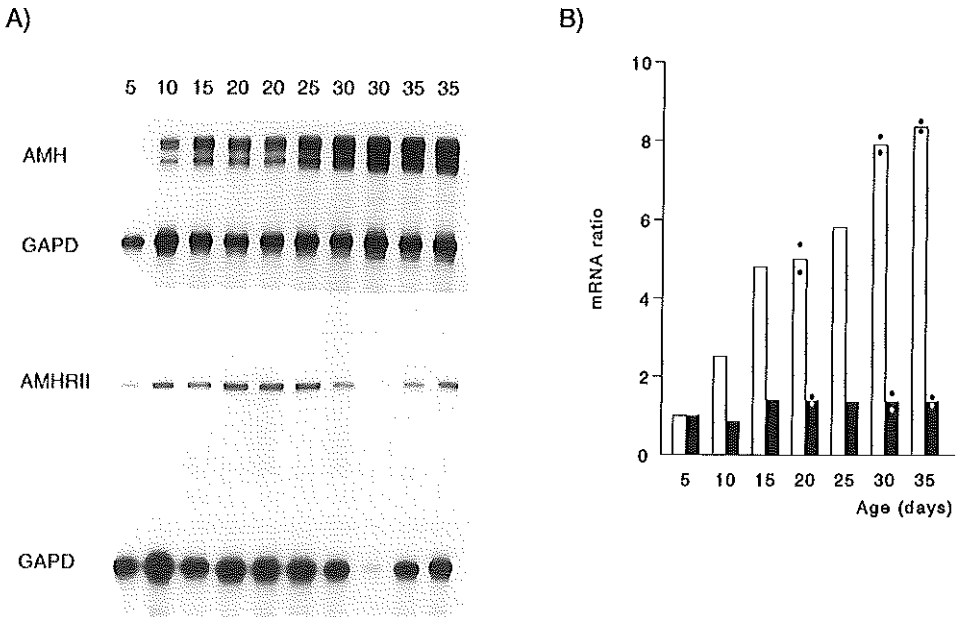

Fig. 5.3: Expression of AMH and AMHRII mRNAs in total ovaries during postnatal development. AMH, AMHRII, and GAPD mRNA expression was studied using RNase protection assays on total RNA from whole ovaries, from rats of different age. The numbers above each lane in (A) and below the histogram in (B) correspond to the age of the rats in days.
A: results of the RNAse protection assay. AMH, AMHRII, and GAPD indicate the positions of the respective protected fragments. Each lane represents at least one rat. B: quantitative analysis of the AMH/GAPD (open bars) and AMHRII/GAPD (closed bars) mRNA ratios. Values at days 5, 10, 15, and 25 are derived from one rat, or from a number of rats whose ovaries were pooled before RNA isolation; values at days 20,30, and 35 are the mean and individual values from 2 rats. The AMH/GAPD and AMHRII/GAPD mRNA ratios in the 5-day-old rats were set at 1.0.

RNase protection assays, performed with total RNA from isolated antral follicles and corpora lutea, showed that AMH and AMHRII mRNA is clearly expressed in small antral follicles. Using this method, AMH and AMHRII mRNA expression was also detected in large antral follicles as well as in corpora lutea, albeit at a very low level, not detectable by in situ hybridization (Fig. 5.4).

Expression of AMH and AMHRII mRNAs during PMSG-induced follicle growth and after subsequent hCG-induced ovulation, in ovaries of immature rats
Treatment of 30-day-old rats with PMSG followed 56 h later by hCG injection, resulted in the presence of a large number of antral follicles at 24 and 48h, recently ovulated follicles at 72h, and fully developed corpora lutea 7 days after start of PMSG treatment. AMH and AMHRII mRNA levels in total ovaries were decreased four to five-fold at 24h and at 48h, compared to untreated rats. A further decrease (two to three-fold) was seen at 72 h, followed by a small increase in the levels of AMH and AMHRII mRNAs at 7 days (Fig. 5.5).
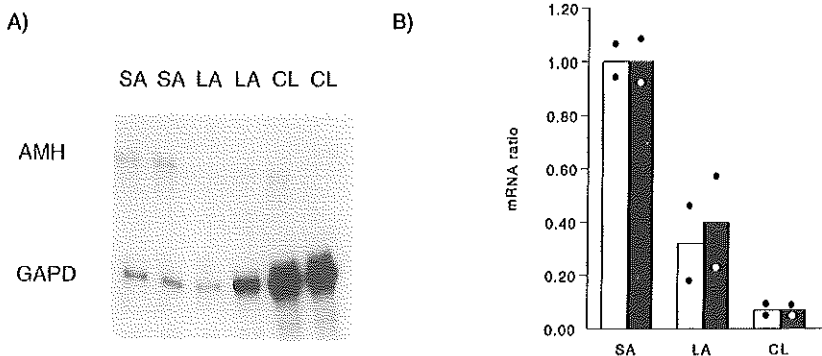

Fig. 5.4: Expression of AMH and AMHRII mRNAs in isolated antral follicles and corpora lutea.
AMH, AMHRII, and GAPD mRNA expression was studies using RNase protection assays on total RNA from isolated corpora lutea (CL), small antral follicles (SA) and large antral follicles (LA). The source of the RNA is indicated above the lanes in (A) and below the histogram in (B).
A: results of the RNase protection assay. Each lane represents a number of pooled follicles or corpora lutea. AMH, AMHRII, and GAPD indicate the positions of the respective protected fragments. B: quantitative analysis of the AMH/GAPD (open bars) and AMHRII/GAPD (closed bars) mRNA ratios. The bar/dots represent the mean and individual values of two different isolated follicle fractions. The AMH/GAPD and AMHRII/GAPD mRNA ratios in the small antral follicles were set at 1.0.

A)

B)

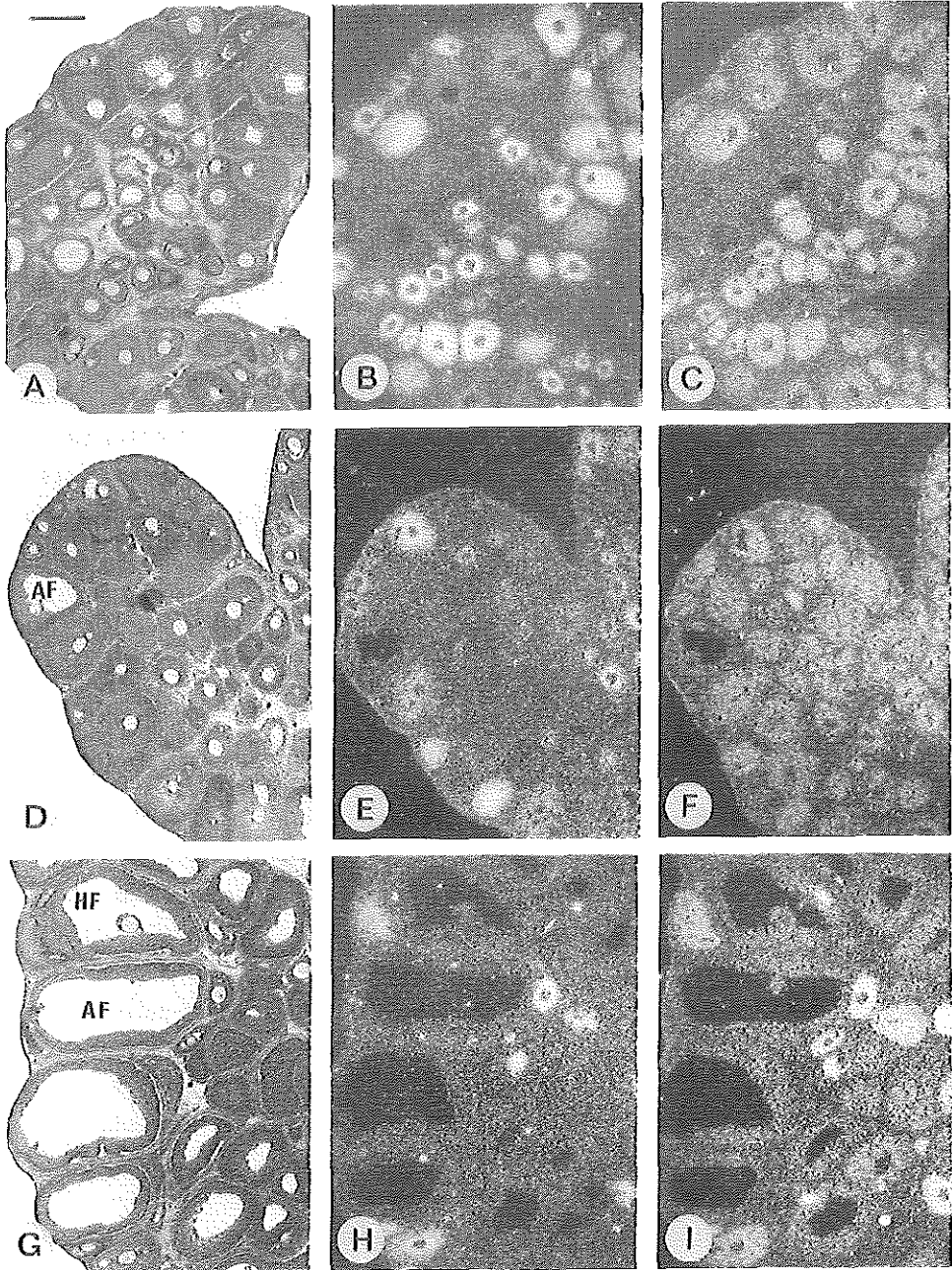*Fig. 5.5: Expression of AMH and AMHRII mRNAs in total ovaries from immature rats treated with PMSG and hCG.*
AMH, AMHRII, and GAPD mRNA expression was studied using RNase protection assays on total RNA from whole ovaries, from rats at different time points after PMSG treatment (at t=0h) followed by hCG treatment (at t=56h). The numbers above each lane in (A) and below the histogram in (B) correspond to number of hours after start of the treatment.
A, results of the RNAse protection assay. Each lane represents one rat. AMH, AMHRII, and GAPD indicate the positions of the respective protected fragments. B: quantitative analysis of the AMH/GAPD (open bars) and AMHRII/GAPD (closed bars) mRNA ratios. The bar/dots represent the mean and individual values of two rats. The AMH/GAPD and AMHRII/GAPD mRNA ratios at t=0 were set at 1.0.

Hormonal regulation of AMH and AMHRII mRNA expression

GnRHant treatment (see Materials and Methods) resulted in an inhibition of follicular development. The ovaries of GnRHant-treated rats contained mainly preantral follicles and a few small antral follicles (Fig. 5.6A). Combined treatment with GnRHant and 10 IU FSH resulted in the presence of small antral follicles (not shown), while combined treatment with GnRHant and 100 µg EB resulted in follicle growth with little antrum formation (Fig. 5.6D). Treatment with GnRHant plus FSH plus hCG, or GnRHant plus FSH plus EB, resulted in ovaries containing many large antral follicles (Fig. 5.6G).

The patterns of AMH mRNA localization in healthy follicles were similar in ovaries isolated from GnRHant-treated rats and control 30-day-old rats, whereas the AMHRII mRNA signal in the healthy preantral follicles was slightly decreased in the GnRHant-treated rat ovaries (Table 5.2 and Table 5.3). AMH and AMHRII mRNA expression in preantral atretic follicles was lower in GnRHant-treated rats compared to control 30-day-old rats. Moreover, no, or very little, AMH and AMHRII mRNA expression was detected in atretic follicles in ovaries of rats from all other treatment groups. In some ovaries, AMH mRNA expression was restricted to follicles located in the periphery of the ovary, similar to the expression pattern found in 20-day-old rats. This pattern was found in several ovaries from different treatment groups, but no correlation between this expression pattern and hormonal treatment was found (see for example Fig. 5.6E,F).

In ovaries from rats treated with GnRHant plus FSH, small preantral follicles clearly expressed AMH mRNA, whereas the expression was inhibited in large preantral follicles, and/or located in the granulosa cells closest to the oocyte. AMHRII mRNA expression in both small and large preantral follicles was highly variable; some follicles still expressed a high level of AMHRII mRNA whereas other follicles were AMHRII mRNA negative. In healthy small antral follicles no AMH mRNA was observed. AMHRII mRNA expression in small antral follicles was lower, and showed more variation, than in similar follicles present in ovaries from GnRHant-treated rats.

Fig. 5.6: (Next page) Localization of AMH and AMHRII mRNAs in immature rat ovaries after hormonal treatment using in situ hybridization.
The left figures (A,D,E) are ovarian sections stained with haematoxylin and eosin. The middle and right figures show darkfield views of AMH (B,E,H) and AMHRII (C,F,I) mRNA expression in adjacent sections. A,B,C: ovary from GnRHant-treated rat (AMH and AMHRII mRNA expression is colocalized in preantral and small antral follicles); D,E,F: ovary from GnRHant plus EB-treated rat (AMH and AMHRII mRNA expression is lost from a number of healthy preantral follicles; note the peripheral localization of AMH positive follicles and even distribution of AMHRII mRNA expressing follicles); G,H,I: ovary from GnRHant plus FSH plus hCG-treated rat (numerous large antral follicles are present, showing no AMH and AMHRII mRNA expression). AF, atretic follicle. Bar represents 200 µm.

Combined treatment with GnRHant plus EB resulted in an inhibition of AMH and AMHRII mRNA expression in a number of preantral and small antral follicles. Both AMH and AMHRII mRNA expression were highly variable, and no consistent morphologic difference between AMH and/or AMHRII mRNA positive and negative follicles was observed.

Ovaries isolated from rats treated with GnRHant plus FSH plus hCG, or GnRHant plus FSH plus EB contained many large follicles which did not show any AMH and AMHRII mRNA expression. The few preantral follicles that were present had no or variable AMH and AMHRII mRNA expression, similar to the mRNA expression detected in GnRHant plus FSH-treated rat ovaries (Fig. 5.6G-I).

RNase protection assays of total RNA isolated from ovaries from the different treatment groups showed that the total ovarian levels of AMH and AMHRII mRNAs were increased in ovaries from GnRHant-treated rats compared to control 30-day-old rats. All combined hormonal treatments resulted in decreased levels of total ovarian AMH and AMHRII mRNA expression, compared to GnRHant treatment alone (Fig. 5.7); the relative decrease of AMH mRNA was more pronounced than the decrease of AMHRII mRNA expression.

Discussion

The specific patterns of AMH mRNA and protein expression during postnatal ovary development in mouse and rat (Münsterberg and Lovell-Badge, 1991; Hirobe et al. 1992; Ueno et al. 1989a; Ueno et al. 1989b; Hirobe et al. 1994) and the expression of AMHRII mRNA in adult rat ovary (Baarends et al. 1994; di Clemente et al. 1994b), have provided indications that AMH may play a role in the hormonal network that regulates ovarian functions.

In the experiments reported herein, we have made use of mRNA *in situ* hybridization and RNase protection assay, to determine the ovarian AMH and AMHRII mRNA expression patterns in detail. The *in situ* hybridization results provide information about the cellular location of AMH and AMHRII mRNA expression. RNase protection assay is more sensitive and quantitative than *in situ* hybridization, and was used to determine the relative levels of AMH and AMHRII mRNAs in isolated follicles and corpora lutea, and to study quantitative changes in total ovarian levels of AMH and AMHRII mRNAs during ovarian development and in immature rat ovaries following hormonal treatment. Comparison of *in situ* hybridization results and RNase protection results, combined with the morphologic characterization of the ovaries, are essential

to obtain complete information about the AMH and AMHRII mRNA expression patterns and their regulation.

In situ hybridization revealed that AMH and AMHRII mRNAs in the rat ovary are specifically expressed in granulosa cells. Oocytes, theca and interstitial cells, express no, or a very low amount of AMH and AMHRII mRNAs. In analogy to the proposed autocrine action of AMH on Sertoli cells in postnatal rat testes (Baarends et al. 1994), these results indicate that most actions of AMH via AMHRII in the ovary will be autocrine in nature.
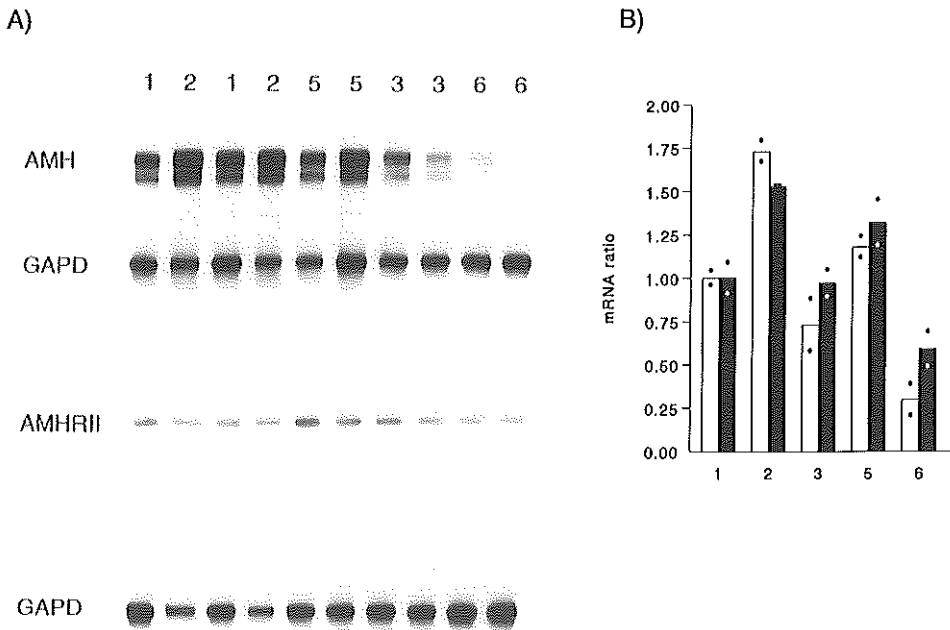
Fig. 5.7: Expression of AMH and AMHRII mRNA in total ovaries from immature rats after hormonal treatment.
AMH, AMHRII, and GAPD mRNA expression was studied using RNase protection assays on total RNA from whole ovaries, from immature rats after different treatments. Ovaries were isolated from: 1, control 30-day-old rats; 2, GnRHant-treated rats; 3, GnRHant plus FSH-treated rats; 4, GnRHant plus FSH plus hCG-treated rats (not shown; results of this group were very similar to the mRNA expression data obtained for group 6); 5, GnRHant plus EB-treated rats; 6, GnRH plus FSH plus EB-treated rats. A: results from the RNase protection assays. Each lane represents one rat. AMH, AMHRII, and GAPD indicate the positions of the respective protected fragments. B: quantitative analysis of the AMH/GAPD (open bars) and AMHRII/GAPD (closed bars) mRNA ratios. The bar/dots represent the mean and individual values of two rats. The AMH/GAPD and AMHRII/GAPD mRNA ratios from control 30-day-old rats were set at 1.0.

Table 5.2: Expression of AMH and AMHRII mRNAs in follicles during the estrous cycle in ovaries of adult rats, as measured by

in situ hybridization

		Proestrus			Estrus			Metestrus			Diestrus-1			Diestrus-2		
		n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII
Healthy	preantral	(18)	+/++	+/++	(21)	0/+	0/++	(41)	+/++	+/++	(16)	+/++	+	(28)	+/++	+
	small antral	(5)	+	+	(8)	±	±/+	(13)	0/±	±/+	(6)	±/+	±/+	(4)	0/±	0/±
	large antral	(10)	0	0		-	-		-	-	(2)	0	0	(2)	0	0
Atretic	preantral	(3)	0	0	(3)	0	±	(1)	0	+	(2)	±	±		-	-
	small antral	(4)	0	0	(7)	0	0/±		-	-	(4)	0	±	(5)	0	0/±
	large antral		-	-	(1)	0	0		-	-		-	-		-	-

- = follicle not present

0 = no silver grains above background, ± = moderate expression, + = clear expression, ++ = high expression

Number of follicles (n) is given in parentheses

During follicle development, AMH and AMHRII mRNAs are expressed mainly in preantral and small antral follicles, resulting in a marked colocalization of the two mRNAs. Primordial and large antral follicles, as well as corpora lutea, are AMH and AMHRII mRNA negative (below the limit of detection). AMH mRNA is expressed in some preantral atretic follicles, but rarely in antral atretic follicles. Also AMHRII mRNA expression gradually disappears in atretic follicles, although this mRNA persists somewhat longer than AMH mRNA. It is of interest to note, that AMHRII mRNA is always evenly distributed in the granulosa cell layer, whereas AMH expression is often restricted to granulosa cells surrounding the oocyte and/or the antrum in some follicle types. This pattern of AMH mRNA expression is found mainly in follicles that start expressing AMH mRNA (small preantral follicles) and in follicles that loose AMH mRNA (large preantral follicles, atretic follicles), in prepubertal as well as in adult rat ovaries. This AMH mRNA distribution pattern may reflect functional differences between the granulosa cells surrounding the antrum/oocyte, and the peripheral granulosa cells, such as differences in proliferation capacity and steroidogenic activity (reviewed by Hirschfield, 1991; and Richards, 1994).

During the first two to three weeks of postnatal development, AMH and AMHRII mRNA expression patterns differ slightly from the above described adult patterns. AMH mRNA is not expressed prior to birth, and becomes detectable after birth when the first preantral follicles have developed (Hirobe et al. 1992; and present results). Thereafter, AMH mRNA expression gradually increases in preantral follicles. When small antral follicles appear, these show low AMH mRNA expression. AMHRII mRNA expression is high in fetal ovaries (Baarends et al. 1994), and is expressed in an evenly distributed manner, covering the ovary, at postnatal day 5. From day 15 onwards, AMHRII mRNA expression becomes restricted to granulosa cells, and is found in the follicle types that also express AMHRII mRNA in the adult ovary.

Hirobe et al. (Hirobe et al. 1992) have also reported data concerning AMH mRNA expression data in 35-day-old rat ovaries, that are consistent with our findings. However, in a more recent report (Hirobe et al. 1994), Hirobe and co-workers show an intense hybridization signal representing AMH mRNA, in cumulus cells and luminal granulosa cells in follicles larger than 500 µm, which is lost just prior to ovulation. We also find that AMH mRNA, when its expression decreases, becomes restricted to luminal granulosa cells and the granulosa cells closest to the oocyte, but we observe that this signal is lost in follicles larger than 450 µm. Decreased AMH and AMHRII mRNA expression in large antral follicles compared to small antral follicles was also observed using RNase protection assay. Residual AMH and AMHRII mRNA

expression observed in large antral follicles and corpora lutea using RNase protection, may represent low levels of mRNA that are evenly distributed and therefore not detected using *in situ* hybridization. It is not clear what causes the disagreement concerning AMH mRNA expression in large follicles, although it cannot be excluded that a difference in the part of the AMH gene that was used as a probe, in the present experiments and by Hirobe et al. (Hirobe et al. 1994), may result in detection of different spliced forms of AMH mRNA.

Based on the results presented in this paper, we conclude that both AMH and AMHRII mRNA expression are turned on in granulosa cells of small preantral follicles, increase to maximal levels in large preantral follicles, and decrease in small antral follicles. Subsequently, both AMH and AMHRII mRNA expression decrease to undetectable levels as the follicles develop a large antral cavity and start to undergo the final stages of follicular maturation. Using RNase protection, which is more sensitive than *in situ* hybridization, it was shown that the expression levels of AMH and AMHRII mRNAs in large antral follicles are approximately 2.5 and 3-fold lower, respectively, than in small antral follicles, and are even further reduced, but still detectable, in corpora lutea.

Marked coregulation of AMH and AMHRII mRNAs is also observed in total RNA preparations from ovaries isolated from immature rats that were treated with PMSG and hCG. After PMSG treatment, the ovaries consist mainly of large antral follicles (at 24 h) and preovulatory follicles (at 48h), which results in relatively low levels of AMH and AMHRII mRNAs at these two time points. After ovulation (72h after PMSG), the signal decreases even further, reflecting the low levels of AMH and AMHRII mRNAs in corpora lutea. Total ovarian AMH and AMHRII mRNA expression have somewhat increased again after 7 days, which may reflect AMH and AMHRII mRNA expression in small preantral follicles that have started to grow.

The expression of AMH and AMHRII mRNAs appears to be tightly coupled to follicle differentiation. This indicates that local changes in the environment surrounding the follicle, and/or in the responsiveness of granulosa cells to certain factors, may play a major role in the regulation of both AMH and AMHRII mRNA levels. During the estrous cycle, a change in the patterns of AMH and AMHRII mRNA expression is observed at estrus, when AMH and AMHRII mRNA expression is lost from several but not all preantral follicles. FSH has been reported to downregulate AMH mRNA expression in neonatal rat testis (Kuroda et al. 1990). Furthermore, results obtained in this report using GnRHant plus FSH-treated female rats, indicate that FSH may also downregulate ovarian AMH as well as AMHRII mRNA expression (see below).

Table 5.3: Expression of AMH and AMHRII mRNAs in ovaries from rats treated with GnRH antagonist in various combinations with FSH, estradiol benzoate (EB), and hCG.

		GnRHant			GnRHant +FSH			GnRHant +EB			GnRHant +FSH +hCG			GnRHant +FSH +EB		
		n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII	n	AMH	AMHRII
Healthy	preantral	(61)	+/++	±/+	(64)	0/+	0/+	(52)	0/+	0/±	(35)	0/+	0/+	(20)	0/+	0/±
	small antral	(5)	±	±	(38)	0	0/±	(8)	0	0/±	(26)	0	0	(12)	0	0
	large antral		-	-	(2)	0	0		-	-	(16)	0	0	(3)	0	0
Atretic	preantral	(21)	0/±	0/±	(18)	0	0	(14)	0	±	(5)	0	0	(3)	0	0
	small antral	(4)	0/±	0/±	(19)	0	0	(3)	0	0	(17)	0	0	(8)	0	0
	large antral		-	-	(8)	0	0		-	-	(14)	0	0	(4)	0	0

- = follicle not present

0 = no silver grains above background, ± = moderate expression, + = clear expression, ++ = highest expression

Number of follicles (n) is given in parentheses

Thus, it could be postulated that the FSH surge on the morning of estrus may cause a decrease of ovarian AMH mRNA expression and perhaps also of AMHRII mRNA expression. Estrogens might also be involved in downregulation of AMH and/or AMHRII mRNA expression in the ovary. Estrogen is locally produced, and the results presented in this paper show that AMH and AMHRII mRNA levels during follicular development decrease at the time that follicular estrogen output increases (Uilenbroek and Richards, 1979). Furthermore, it has been reported that estrogens regulate AMH actions during sex differentiation in chickens (Hutson et al. 1982), and, mice treated with high doses of synthetic estrogens *in utero* develop abnormalities (Newbold et al. 1984), that may be partly caused by impaired AMH function. Since AMH inhibits estrogen synthesis in cultured granulosa cells through inhibition of expression of aromatase mRNA and protein (di Clemente et al. 1994a), estrogenic regulation of AMH and/or AMHRII mRNA expression could be part of a feedback mechanism.

The possible roles of estrogens and gonadotropins in regulation of AMH and AMHRII mRNA expression in the ovary were investigated. For this, 30-day-old rats were treated with GnRHant to inhibit endogenous gonadotropin secretion (Meijs-Roelofs et al. 1990). Ovaries from these rats contain numerous preantral follicles; such follicles express high amounts of AMH and AMHRII mRNAs in control rat ovaries. *In situ* hybridization results showed that there was a small decrease of AMHRII mRNA expression in preantral follicles of GnRHant-treated rats, compared to control rats. This may indicate that gonadotropins are required for the maintenance of AMHRII mRNA expression in preantral follicles. Alternatively, the decreased AMHRII mRNA expression may be due to a direct action of GnRHant upon ovarian cells by blocking the GnRH receptor, which is expressed in the granulosa cells of most follicle types (Whitelaw et al. 1995).

The results from combined treatment of GnRHant with FSH and/or EB, indicate that high doses of either FSH or EB are capable of inhibiting AMH and AMHRII mRNA expression in preantral and small antral follicles. Inhibition of AMH mRNA expression may result from a direct effect of estrogens on AMH gene transcription. The AMH promoter has been characterized in several species (Guerrier et al. 1990; Haqq et al. 1992; Cate et al. 1986; Shen et al. 1994), and a number of possible regulatory sequence elements have been described (Haqq et al. 1993; Guerrier et al. 1990; Shen et al. 1994). The human AMH gene promoter contains a putative estrogen responsive element (Guerrier et al. 1990). When several copies of this 35 bp fragment are inserted in front of a basal promoter-reporter gene construct, the estrogen receptor binds to this construct, and activates transcription *in vitro* (Guerrier et al. 1990).

However, these results could not be obtained with a 182 bp fragment, containing the putative estrogen responsive element. Whether this element and surrounding sequences are involved in downregulation of AMH mRNA expression *in vivo* remains to be determined. No putative FSH-regulated AMH gene promoter elements have been described.

The increased AMH and AMHRII mRNA levels in GnRHant-treated rats, as determined by RNase protection assay, are in accordance with the observed enrichment, when compared to control rat ovaries, in AMH and AMHRII mRNA positive follicles. Also, RNase protection assay results of the other hormonal treatment groups reflect the sum of the effects on follicle population and mRNA downregulation by FSH and EB.

In adult ovaries, AMH and AMHRII mRNA expression decrease in granulosa cells of those follicles, which are at a decisive step of their development; many small antral follicles become atretic, and only a few survive and are selected to differentiate to the preovulatory stage. During this period, the sensitivity of the follicles to FSH increases (Richards, 1980), which results in increased aromatase activity and increased synthesis of estrogens. Based on the present results, it could be suggested that increased sensitivity of granulosa cells to FSH in small antral follicles, is the initial cause of downregulation of AMH and AMHRII mRNAs, resulting in a diminished inhibitory effect of AMH on aromatase activity. The subsequent increased estrogen production could then result in a further decrease of AMH and AMHRII mRNA expression.

Total ovarian AMH mRNA expression increases during the first 15 days of postnatal development. Concomitantly, FSH levels increase to a high maximal level around day 15 (Meijs-Roelofs et al. 1973). Binding of FSH to granulosa cells is very low until day 17, and increases thereafter (Uilenbroek and van der Linden, 1983). Thus, it cannot be excluded that a low sensitivity of immature granulosa cells to FSH during the first two weeks of postnatal development allows AMH mRNA expression to increase. Between days 25 and 30, although FSH binding increases, FSH levels decrease, which might result in a further increase in AMH mRNA expression. The change in the pattern of AMHRII mRNA expression around postnatal day 15, from an evenly distributed pattern to a more restricted pattern, cannot be explained by changes in FSH and/or estrogen levels, or by known changes in the sensitivity of ovarian cells to these hormones, and is thus most likely caused by other factors.

Recently, Behringer et al. (Behringer et al. 1994) have generated AMH knockout mice. Female AMH deficient mice develop normally, with ovaries that are

morphologically similar to the ovaries of control animals, and the mice are fertile and produce phenotypically normal progeny. Although this indicates that AMH may not be essential for ovarian function, functional redundancy between AMH and other members of the activin and TGFβ family of growth and differentiation factors may mask some of the ovarian functions of AMH in the AMH knock-out mice. Indeed, follicular expression patterns of activins, inhibins, TGFβs, and some of their receptor types, are partially overlapping with AMH and AMHRII mRNA expression (Meunier et al. 1988; Woodruff et al. 1988; Teerds and Dorrington, 1992; Cameron et al. 1994). Furthermore, detailed studies of multiple parameters related to ovarian function throughout the reproductive life-span of the AMH knock-out female mice, might provide some clues concerning long-term ovarian functions of AMH.

The results presented herein provide evidence that granulosa cells of preantral and small antral follicles are major target cells of AMH. Together with the reported inhibitory effects of AMH upon granulosa cell proliferation (Kim et al. 1992; Seifer et al. 1993), aromatase activity, and LH receptor expression (di Clemente et al. 1994a), it is an intriguing possibility that AMH is involved in preventing premature follicle maturation, rather than playing a direct role in oocyte meiotic maturation. It is also possible that AMH function involves a role in selection of follicles that do not undergo atresia. Little is known about the mechanism that determines whether a follicle will undergo atresia, or eventually ovulate (reviewed by Hsueh et al., 1994). Many follicles become atretic when they reach the small antral stage, and the FSH-surge at estrus is thought to be essential for survival of those follicles at the large preantral/small antral stage which are destined to ovulate at the next proestrus. FSH-regulated survival of follicles may involve downregulation of AMH and AMHRII mRNA expression at estrus. In this respect, it is of interest to note that androgens are thought to be atretogenic, and that androgen receptor immunohistochemical staining data indicate that the ovarian androgen receptor expression pattern is markedly similar to the AMH and AMHRII mRNA expression patterns in adult rats (Tetsuka et al. 1995). Future experiments can be directed towards the study of functional interactions in the ovary, between AMH and other members of the activin/TGFβ family, and between AMH, estrogens, and androgens.

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The Rat Androgen Receptor Gene Promoter

Willy M. Baarends, Axel P.N. Themmen, Leen J. Blok, Petra Mackenbach, Albert O. Brinkmann, Dies Meijer, Peter W. Faber, Jan Trapman and J. Anton Grootegoed

The rat androgen receptor gene promoter

Willy M. Baarends [1], Axel P.N. Themmen [1], Leen J. Blok [1], Petra Mackenbach [1],
Albert O. Brinkmann [1], Dies Meijer [2], Peter W. Faber [3], Jan Trapman [3]
and J. Anton Grootegoed [1]

Departments of [1] Biochemistry II, [2] Genetics and [3] Pathological Anatomy, Erasmus University Rotterdam, Rotterdam, The Netherlands

Summary

The androgen receptor (AR) is activated upon binding of testosterone or dihydrotestosterone and exerts regulatory effects on gene expression in androgen target cells. To study transcriptional regulation of the rat AR gene itself, the 5' genomic region of this gene was cloned from a genomic library and the promoter was identified. S1-nuclease protection analysis showed two major transcription start sites, located between 1010 and 1023 bp upstream from the translation initiation codon. The area surrounding these start sites was cloned in both orientations in a CAT reporter plasmid. Upon transfection of the constructs into COS cells, part of the promoter stimulated transcription in an orientation-independent manner, but the full promoter showed a higher and unidirectional activity. In the promoter/reporter gene constructs, transcription initiated from the same positions as in the native gene. Sequence analysis showed that the promoter of the rat AR gene lacks typical TATA and CCAAT box elements, but one SP1 site is located at about 60 bp upstream from the major start site of transcription. Other possible promoter elements are TGTYCT sequences at positions -174 to -179, -434 to -439, -466 to -471, and -500 to -505, resembling half-sites of the glucocorticoid-responsive element (GRE). Furthermore, a homopurine stretch containing a total of 8 GGGGA elements and similar to sequences that are present in several other GC-rich promoters, is located between -89 and -146 bp upstream from the major start site of transcription.

Introduction

Following differentiation of the testis, androgen action is essential for development of the male reproductive tract, virilization and full initiation and maintenance of spermatogenesis (Griffin and Wilson, 1989). The androgenic hormones testosterone and dihydrotestosterone exert their effects on gene expression through binding to the androgen receptor (AR). The AR belongs to the family of ligand-activated transcription factors that includes the other steroid hormone receptors as well as the thyroid hormone, the retinoic acid and the vitamin D_3 receptors. The progesterone and glucocorticoid receptor are structurally most closely related to the AR (Tilley et al., 1989).

Recently, the cDNAs encoding the human and rat AR have been cloned (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988). The rat AR mRNA is approximately 10 kb, containing large 5′ and 3′ untranslated regions (UTRs) of approximately 1 and 7 kb respectively. The open reading frame encodes a protein with 902 amino acid residues. The rat and human AR mRNAs show a high degree of sequence similarity in the coding regions and the 5′ UTRs.

The regulation of AR protein levels may constitute an important level of control at which the physiological effects of testosterone can be modulated. In the testis, AR protein is expressed in Sertoli cells, Leydig cells and peritubular cells, but not in the developing germ cells (Grootegoed et al., 1977; Buzek and Sanborn, 1988). Recently, it has been shown that both follicle-stimulating hormone (FSH) and testosterone can stimulate AR protein levels in cultured Sertoli cells from immature rats (Verhoeven and Cailleau, 1988; Blok et al., 1989). However, only FSH stimulated the amount of AR mRNA, whereas testosterone had no effect on mRNA expression (Blok et al., 1989). In other tissues, including prostate, kidney, brain and epididymis, the AR mRNA content is down-regulated by testosterone (Quarmby et al., 1990).

The cellular amount of AR mRNA may be regulated at the level of transcription, but also through alteration of its stability. To study a possible regulatory effect of testosterone and FSH on the rate of AR gene transcription, the AR gene promoter should be defined. Here we report the identification and characterization of the rat AR gene promoter.

Materials and methods

Isolation of 5′ genomic rat AR clones

A rat genomic library was constructed by cloning rat DNA that had been partially digested with *Mbo*I into the *Bam*HI site of λEMBL3. This library (1.7×10^6 independent plaques) was screened using human AR probes corresponding to the 5′ region of the human AR first exon (Faber et al., 1989). Hybridization was carried out using standard methods (Maniatis et al., 1982) under conditions of low stringency. Positive clones were selected and rescreened to obtain purified

single plaques. Five independent clones were isolated: GrAR2, 3, 4, 6, 7.

Subcloning and sequencing

Several restriction fragments derived from GrAR2 were subcloned into pGEM7 (Promega, Madison, WI, U.S.A.) using standard techniques (Maniatis et al., 1982). Partial overlapping clones were sequenced by dideoxy-chain termination (Sanger et al., 1977) in two orientations using T7 polymerase (Pharmacia, Uppsala, Sweden). Double-stranded plasmid DNA was used as a template. The promoter/reporter gene constructs were prepared as follows: The genomic *Hin*dIII fragment ($-296/+120$) was cloned in the *Hin*dIII site of the polylinker from the pCATENH vector (Promega) in the antisense direction (HASCAT). pGEM7H3.1, containing the *Hin*dIII fragment ($-296/+120$), was partially cut with *Nhe*I, treated with Klenow enzyme and dNTPs to prepare blunt ends, followed by a complete *Hin*dIII digestion. Subsequently, the appropriate *Hin*dIII-*Nhe*I fragment ($-296/+97$) was isolated from low-melting-point agarose. The fragment was ligated into pCATENH which had been cut with *Xba*I and blunt-ended with Klenow enzyme followed by a second digestion with *Hin*dIII (HSCAT). The *Pst*I fragment ($-507/+912$) was cloned into the *Pst*I site from pCATENH in the antisense direction (PASCAT). pGEM7E3, containing the *Eco*RI fragment ($-570/+1005$), was cut with *Kpn*I, treated with T4 polymerase and dNTPs to create blunt ends, followed by digestion with *Pst*I. The *Kpn*I-*Pst*I fragment was isolated from low-melting-point agarose and cloned into pCATENH (PSCAT), which had been cut with *Hin*dIII, blunt ended with Klenow and digested with *Pst*I. The insertion of the correct fragment in the desired orientation was checked by restriction enzyme digestion.

RNA isolation

Frozen tissues and cultured cells were lysed and homogenized in 3 M LiCl/6 M ureum, and left on ice for several hours. The RNA was pelleted through ultracentrifugation (25×10^3 rpm, 25 min, SW40 rotor). The pellet was dissolved in 0.1% (w/v) sodium dodecyl sulfate (SDS) and proteins were removed by repeated phenol extraction and a

single proteinase K treatment in 0.1% (w/v) SDS. After precipitation the RNA was dissolved in a small volume of water and the amount of RNA was determined by spectrophotometric optical density (OD) measurement at 260 nm. RNA was stored in 0.3 M NaAc/70% ethanol at $-20°C$.

S1-nuclease protection assay

pGEMH3.1 was cut with *Hin*dIII, dephosphorylated and end-labeled with T4 polynucleotide kinase and $[\gamma\text{-}^{32}P]ATP$ according to Maniatis et al. (1982). The *Hin*dIII fragment was subsequently isolated from low-melting-point agarose and dissolved in 0.3 M NaAc (pH 5.2). Approximately $0.5\text{-}1 \times 10^5$ cpm were precipitated with the appropriate amount of total RNA. After centrifugation, the pellet was dissolved in hybridization buffer containing 80% (v/v) deionized formamide, 40 mM Pipes (pH 6.3), 0.4 M NaCl and 1 mM EDTA according to Favaloro et al. (1980). The samples were denatured for 3 min at 90°C fol-

lowed by an overnight hybridization at 55°C. S1-nuclease (Boehringer Mannheim, Mannheim, F.R.G.) digestions (Favaloro et al., 1980) were carried out for 1 h at 37°C or for 3 h at 20°C, with similar results. The protected DNA fragments were analyzed on a 6% or 8% polyacrylamide sequencing gel.

Cell culture and transient transfection assays

COS cells were grown in Dulbecco's modification of Eagle's medium (Gibco BRL, Grand Island, NY, U.S.A.), supplemented with 10^5 IU/l of penicillin, 100 mg/l streptomycin and 5% fetal calf serum. All cultures were maintained at 3°C under an atmosphere of 5% CO_2 in air. Before transfection, cells were plated in 6 cm diameter culture dishes (Nunclon, Roskilde, Denmark) at approximately 10% confluence and cultured for 24 h. Transfections were carried out according to Graham and van der Eb (1973) using 10 μg of plasmid DNA per dish. The cells were shocked 24
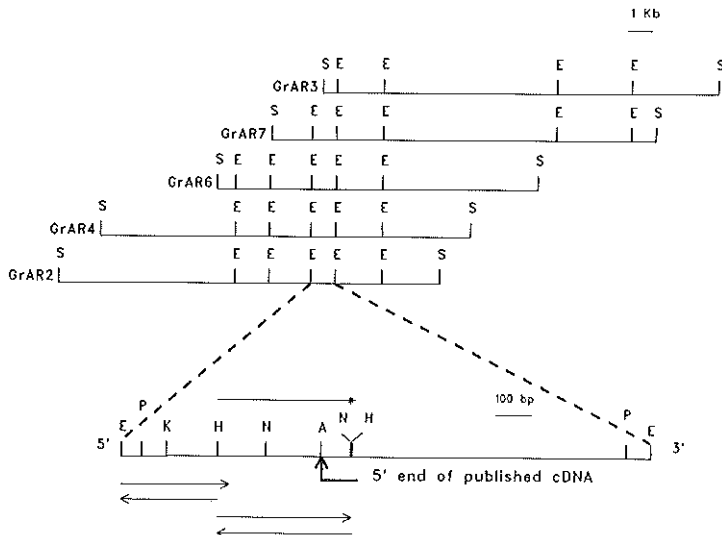

Fig. 1. Schematic representation of isolated lambda clones, containing genomic rat AR sequences (GrAR2, 3, 4, 6, 7). Part of GrAR2 is shown in more detail. The 5′ end of the published rat AR cDNA (Lubahn et al., 1988) is indicated. The horizontal arrows show the sequence strategy. The horizontal line above the restriction map of GrAR2 represents the probe that was used for the S1-nuclease protection assays. The asterisk indicates the site used for end-labeling by $[\gamma\text{-}^{32}P]$ ATP. A = *Sau*3A, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, N = *Nhe*I, P = *Pst*I, S = *Sal*I.

h later for 2 min with 15% glycerol in phosphate-buffered saline (PBS), and 48 h after transfection the cells were harvested and broken by three cycles of freezing and thawing. After centrifugation, supernatants were assayed for CAT activity as described by Gorman et al. (1982). A transfection with pSV2CAT was included in each experiment as a control to compare the transfection efficiencies of the different experiments.

Results

Isolation and characterization of 5' genomic rat AR clones

A rat liver genomic library was screened with human AR cDNA probes representing the 5' end of the AR mRNA. Five independent overlapping clones were isolated as presented in Fig. 1. GrAR2 contained the farthest 5' extending sequences and this clone has been used for subsequent studies.

Based upon hybridization results, we have subcloned and sequenced several restriction fragments derived from GrAR2. The sequencing strategy is also shown in Fig. 1 and the obtained sequence is presented in Fig. 2. The 3' end of this sequence (117 bp) is identical to the published 5' end of the rat AR cDNA (Lubahn et al., 1988).

Identification of the 5' end of the rat AR mRNA

In order to locate the cap site of the rat AR mRNA we have performed S1-nuclease protection assays. A genomic *Hind*III fragment that sur-

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-570   GAATTCCCCATCTACGCTACTGGAGGATCTCAAAGGTTTCTGCAAGAGTTGCTTTGGCTG


-510   CAGCTTGTTCTTTAATCTCTTGGGACTCTCCCTTCTGCTTGTCCTGGTGGGCCCTGGGGA


-450   GAGGGTACCTAAGAACAATTGGTAGCCGGTACTTCTAATGCCCCTTCCTCCTCGGAGAAT


-390   CTGTTTTGGGATTGGGTTCAGGAATGAAATCCGGCCTGTGCTAACCTTTTGAGGAGCCGG


-330   TAGGCTTGTCTGTTAAAAAATCGCTCCAAGTTAAAGCTTCTGCTTTGGAGTCTAAAGCCC


-270   GGTTCCGAAAAACAAGTGGTATTTGGGGAAAAGGGGTCTTCAGAGGCTACAGGGAGTCCT


-210   TCCAGCCTTCAACCATACTACGCCACGACTATGTTCTCTAAAGCCACCCTGCGCTAGCTT
              HOMOPURINE-STRETCH
-150   GCGTGAGGGCGAGGGCGAGAAGCAAGGGCGAGGGCGAGGGCGAGGGCGAGGGCGAGGGCGAGGGCAGAG
                        GC-BOX
-90    AGGAAAGGAGGTGGGAAGGCAGGGAGGCGGGCGGGGGGCGGGACCGACTCACAAACTGTTG
                  **---------**----
-30    CATTTGCTTTTCCACCTCCCAGCGCCCCCTCGGAGATCCCTAGGAGCCAGCCTGCTGGGA
                           +1
                           L___

+31    GAACCAGAGGGTCCGGAGCAAACCTGGAGGCTGAGAGGGCATCAGAGGGGAAAAGACTGA


+91    GCTAGCCACTCCAGTGCCATACAGAAGCTT
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Fig. 2. Sequence of the 5' genomic region of the rat AR gene containing putative promoter elements. The +1 position is defined at the major transcriptional start site. The asterisks above the sequence indicate the other start sites. Putative promoter elements are bold-faced: TGTYCT and AGAACA, the homopurine stretch and the GC box. The possible splice acceptor site is underlined and the transcribed sequence is overlined.
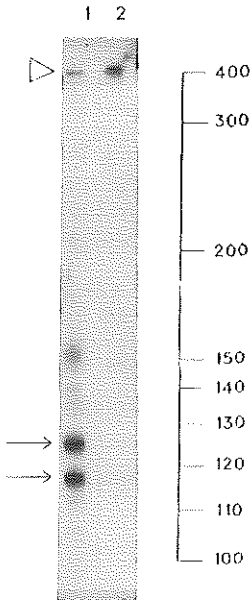
Fig. 3. S1-nuclease protection assay of the 5′ genomic *Hind*III fragment. Protected fragments obtained with 50 µg of total testis RNA (lane 1), and 50 µg of total spleen RNA (lane 2) are shown. The triangle indicates intact probe, and arrows indicate the major bands. The numbers on the right represent the lengths of the marker DNA in bp.

rounds the 5′ end of the cDNA was used as a probe (Fig. 1). Total RNA preparations from 21-day-old rat testis or spleen were hybridized to the end-labeled DNA probe, and subsequently the S1-nuclease digestion was carried out; Fig. 3 shows the resulting protected DNA fragments after analysis on a denaturing polyacrylamide gel.

Using total testis RNA, two pronounced bands were observed (Fig. 3; lane 1). The 5′ ends of the two major bands map just upstream from the 5′ end of the published rat AR cDNA which is located in Fig. 2 at position + 5. Using total RNA derived from spleen, an AR-negative tissue (Lubahn et al., 1988), no labeled DNA fragments protected from S1-nuclease digestion were detected. The S1-nuclease digestion has been carried

out at 37 ° C as well as at 20°C, and similar results were obtained at both temperatures (not shown).

We have also performed primer extension analysis as described by Krug and Berger (1987), to identify the transcription start sites using another method. However, this method did not yield results, most likely because the RNA was present in a conformation which interfered with AMV reverse transcriptase activity.

Structure of the 5′ genomic region of the rat AR gene

The finding that different bands were obtained with the S1-nuclease protection assay strongly implies the presence of multiple start sites of transcription, a feature that is common to most promoters that lack a TATA box. In concordance with this, sequence analysis of the 5′ genomic region shows that no typical TATA or CCAAT box is present (Fig. 2). However, several structural elements indicative of a promoter can be indicated. The genomic region from − 1 to − 300 has an overall G/C content of 58%, and one SP1 site (GGCGGG) is located around position − 60 within an uninterrupted stretch of G/C residues. Furthermore, a homopurine stretch containing a total of eight GGGGA elements is located between − 89 and − 146 bp upstream from the major start site of transcription. These GGGGA sequences might represent promoter elements (see Discussion).

Other possible promoter elements in this region are the TGTYCT consensus elements that are present at positions − 174 to − 179, − 434 to − 439, − 466 to − 471, and − 500 to − 505, and which can be considered as glucocorticoid-responsive element (GRE) 'half sites'. The GRE consists of a palindromic pair of hexameric TGTYCT sequences separated by three nucleotides (Klock et al., 1987). It should be noted that the 5′ genomic region also contains a possible splice acceptor site (Fig. 2).

Functional promoter activity in the 5′ genomic region of the rat AR gene

From the above described results, it is not clear whether the protected bands are derived from different start sites of transcription, or might re-
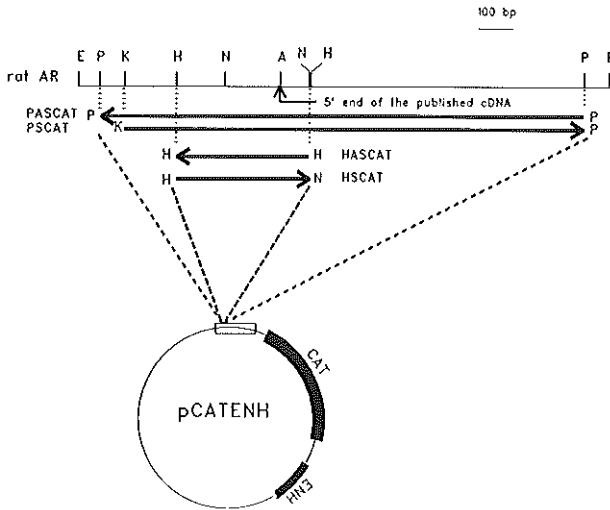
Fig. 4. Promoter constructs cloned into the pCATENH vector. The promoter fragments and the genomic region from which they were derived are shown. Arrows pointing to the right indicate the sense orientation. A schematic drawing of the pCATENH vector shows the relative positions of the polylinker (open box), the CAT gene, and the SV40 enhancer (closed boxes). Restriction enzyme abbreviations are as in Fig. 1.

sult from the use of a splice acceptor site: The sequence 5′TTTCCACCTCCAG3′ is located in the area that contains the supposed start sites of transcription (Fig. 2) and is in accordance with a possible intron/exon splice acceptor site. Consequently, further experimental evidence was needed to determine that the region shown in Fig. 2 indeed represents the rat AR gene promoter.

Promoter trap constructs were used to test the functional capacity of the putative promoter. The cloning vector pCATENH (described in Materials and Methods) contains the CAT reporter gene and
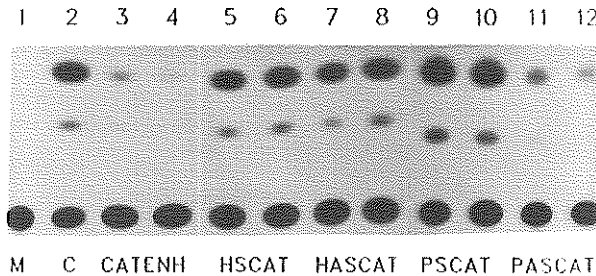
Fig. 5. Rat AR promoter activity in COS cells. CAT activity in COS cells which were transfected with: no DNA (M; lane 1), PSV2CAT (C; lane 2), pCATENH (lanes 3 and 4), HSCAT (lanes 5 and 6), HASCAT (lanes 7 and 8), PSCAT (lane 9 and 10), PASCAT (lanes 11 and 12). The S denotes the position of the substrates in the chromatogram, and the A is located at the position of the acetylated products of the enzymatic reaction.

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a SV40 enhancer sequence, but no promoter. Two types of constructs were made, which contained the putative promoter sequences and flanking regions in either the sense or antisense orientation with respect to the CAT reporter gene. A schematic representation of the different constructs is shown in Fig. 4. After transfection into COS cells, the stimulatory effect of the different 5′ genomic fragments on the rate of CAT gene transcription was determined by performing CAT assays (Fig. 5). pSV2CAT, the positive control plasmid that contains a SV40 enhancer and promoter, showed a
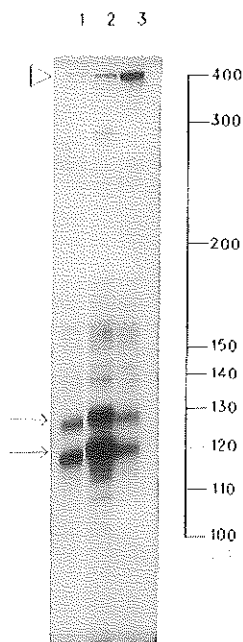
Fig. 6. Analysis of transcriptional start sites in promoter/reporter gene constructs. The probe shown in Fig. 1 was hybridized to RNA extracted from: COS cells transfected with PSCAT (lane 1; 20 μg), COS cells transfected with PASCAT (lane 2; 20 μg), and total testis (lane 3; 60 μg). The triangle points to the intact probe and the arrows indicate the major protected fragments. The numbers on the right indicate the lengths of the marker DNA in bp.

high CAT activity as compared with pCATENH. The construct HSCAT as well as the antisense analogue HASCAT stimulated the transcription of the CAT gene several fold, as compared to the background activity of the parent vector pCATENH. These constructs contain about 300 bp of possible promoter sequences and approximately 100 bp of the 5′ UTR. The larger construct, PSCAT, that extends 150 bp further upstream and 850 bp further downstream than HSCAT and HASCAT, resulted in a somewhat higher CAT expression relative to HSCAT and HASCAT. In contrast, PASCAT, identical to PSCAT but containing the promoter in the antisense orientation, did not show stimulation of CAT activity.

To investigate whether transcription of the promoter/CAT reporter gene constructs starts at the same sites as the transcription of the wild type AR promoter, we performed S1-nuclease protection assays with RNA extracted from the transfected COS cells, using as a probe the genomic *Hind*III fragment that had also been used to locate the cap site in our previous experiment. The assay was performed on total cellular RNA derived from COS cells transfected with either the PSCAT (sense) or PASCAT (antisense) construct (Fig. 6).

Using the sense and anti-sense rat AR genomic sequence constructs, stable transcripts were obtained in both orientations, although CAT-gene derived sequences are only included in the PSCAT transcripts. This experiment was performed to show that the 5′ ends of the protected fragments in RNA from the transfected cells mapped at exactly the same positions as those observed using RNA from rat testis (Fig. 6; lane C). It can be concluded, therefore, that the promoter region in PASCAT as well as PSCAT directs transcription from the correct initiation sites and on the correct DNA strand.

Discussion

Structural features of the rat AR gene promoter

From the present results it is concluded that the minimal promoter of the rat AR gene is located within the area from position +100 to −300 in the 5′ genomic region of the rat AR gene. The sequence upstream of position +1 is not effi-

ciently transcribed into mRNA as shown by S1-nuclease protection analysis. A regulatory function of the largely untranscribed sequence −1 to −300 is reflected in the presence of known sequences in this area that have been proven to be functional elements of other promoters.

First, a GC box (GGCGGG) is located around position −60. This sequence is identical to a recognition site for the transcription factor SP1 that has been shown to activate the transcription of several genes (Saffer and Singer, 1984).

Second, a homopurine stretch of 67 bp, located between positions −79 and −146. Within this region a total of eight repeats of the sequence GGGGA is present. Several other genes also contain purine-rich sequences in the promoter (Christophe et al., 1989; Claessens et al., 1989; Watson and Milbrandt, 1989; Young et al., 1989), some including the GGGGA element (Watson and Milbrandt, 1989; Young et al., 1989). No functional role has yet been assigned to this sequence motive, although homopurine stretches are known to contain sites that are sensitive to S1-nuclease digestion indicating an irregular DNA structure (Claessens et al., 1989; Young et al., 1989). The GGGGA element could very well represent a regulatory protein binding site. For example, the transcription factor ETF can bind to sequences that contain CCCC or GGGG repeats separated by one nucleotide. Through binding to these consensus repeats, ETF specifically enhances transcription from promoters which do not contain a TATA box (Kageyama et al., 1989). The P2 promoter of the c-*myc* gene also contains a GGGGA element which is involved in protein binding and promoter function. The sequence element GGGGAGGGA, located 48 bp upstream of the start site of transcription in the c-*myc* P2 promoter, can be specifically bound by protein in a gel retardation assay and is capable of increasing the promoter activity (Hall, 1990).

Finally, TGTYCT elements are present at four different positions between −174 and −505 in the promoter region. A glucocorticoid responsive element (GRE) consists of a palindromic pair of the hexameric TGTYCT sequence, separated by three nucleotides (Klock et al., 1987). In a random distribution, the TGTYCT sequence would be expected to occur approximately once in every 10^3

bp. It might be suggested that this non-palindromic sequence, either as repeat, or together with flanking sequences, might also have some function in steroid hormone responsive promoters in general. In this respect, it is of interest that TGTYCT sequences are present in the promoter of the androgen-dependent prostatic binding protein genes C1, C2 and C3 (Claessens et al., 1989a). Furthermore, fragments from the promoter and first intron of the C3 gene that contain this consensus sequence have been shown to bind AR-steroid complex (Rushmere et al., 1987). Only the intron-derived sequence could confer androgen responsiveness to a heterologous promoter and this effect could be annulled by a single point mutation in one of the two TGTYCT motives that were located in this area (Claessens et al., 1989b).

Recently, we have also cloned the human AR gene promoter (Faber et al., manuscript in preparation). Comparison of the rat and human sequences indicates that the AR promoter shows a similar structural organization in these two species. The major start sites of transcription are located at the same positions within a region that shows a high degree of sequence similarity. The human AR gene promoter also contains one SP1 site and a homopurine stretch, but no other conserved elements are located within 570 bp upstream from the major transcription start site.

Two major start sites of transcription and a putative splice acceptor site are located within the same region

The results of the S1-nuclease protection assay indicated the presence of two major start sites of transcription between 1010 and 1023 bp upstream from the translation initiation codon. However, it is important to exclude that the results reflect the presence of an intron/exon boundary (splice acceptor site), or that the results are derived from non-specific hybridization. This is indicated by the following. First, the two major bands were not found when spleen derived RNA was used. Second, RNA from COS cells expressing the promoter-trap constructs PSCAT and PASCAT, resulted in the same S1-nuclease protection pattern as that obtained using total testis RNA. In PSCAT and PASCAT, the possible splice acceptor site is separated from any possible splice donor site in the

genome. It can be concluded therefore, that the two double bands observed in the S1-nuclease assay are derived from hybridization of the probe to AR mRNAs, and represent two start sites of transcription.

The minimal promoter of the rat AR gene is located within the 5' genomic region between positions +100 and −300

Promoter trap constructs were used to test the functional capacity of the DNA sequences surrounding the start sites of transcription to act as a promoter. By cloning 5' genomic sequences in either the sense or antisense orientation, additional information about the direction of transcription initiation should be obtained. The fragment that spans the region from −296 to +120 (constructs HSCAT and HASCAT) activates transcription in an orientation-independent manner. This is not surprising from what is currently known about the characteristics of promoters lacking typical TATA or CCAAT box sequences, also called housekeeping or GC-rich promoters. The transcription factors SP1 and ETF can activate transcription on both DNA strands (Saffer and Singer, 1984; Kageyama et al., 1989) and the upstream half of the calcium-dependent protease (CANP) gene promoter shows activity in either orientation (Hata et al., 1989).

However, a larger construct of the rat AR 5' genomic region appears to function in one direction only. This can be concluded from the observation that the transcriptional activity is stimulated by PSCAT that contains the promoter fragment in the sense orientation, but not by the PASCAT antisense construct. PSCAT and PASCAT contain a promoter fragment which includes additional regulatory up- and downstream sequences as compared to the H(A)SCAT constructs.

The start sites of transcription that were functional in the promoter/reporter gene constructs in the transfected COS cells were found to map at exactly the same positions as the start sites that were identified using testis RNA. Hence, the protected DNA fragments that were obtained with RNA transcribed from both the sense and antisense constructs can only be the result of transcription initiation. Intron/exon splicing could not

have resulted in the same protected fragments from either orientation of the promoter fragment, unless the splice donor site would be present in the genomic fragment. However, this is very unlikely not only because we have shown that the different constructs resulted in transcription starting at the rat AR gene promoter, but also because there is no splice donor consensus sequence present in the tested promoter reporter gene constructs.

No consensus cyclic AMP responsive element (CRE) is located within or near the AR gene promoter region. This may point to a complex and indirect regulatory mechanism for the effect of FSH on AR mRNA expression in Sertoli cells; the transcription of several CRE-containing genes in Sertoli cells is stimulated by FSH and/or dbcAMP (Hall et al., 1988; Klaij et al., 1990). By transfection of AR promoter/reporter gene constructs into Sertoli cells, the upstream regions and possible regulatory elements of the rat AR promoter will be functionally analyzed.

In conclusion, the rat AR gene promoter lacks TATA and CCAAT box elements, but it contains one SP1 site and several other possible binding sites for transcription factors. Part of the promoter can function in an orientation-independent manner, but the full promoter shows a higher and unidirectional activity.

Acknowledgement

Note added in proof

The findings described in the present paper are in agreement with recently published data concerning the promoter of the human androgen receptor gene (Tilley et al., J. Biol. Chem. 265 (1990) 13776–13781).

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General Discussion

7.1   Introduction

It was Jost who defined, almost 50 years ago, that two testicular hormones, viz. testosterone and another factor (now known as AMH), are required for sex differentiation during male fetal development (Jost 1947). The fetal functions of AMH and testosterone in sex differentiation, have been elegantly demonstrated using animal model systems and naturally occurring mutations in men. The phenotype that is caused by inactivating androgen receptor mutations has been analysed in the testicular-feminized mouse (He et al. 1991) and also in humans, where the condition is known as the (complete) androgen insensitivity syndrome (see also Chapter 2). Lack of AMH action leads to the persistent müllerian duct syndrome, which is a rare cause of male pseudohermaphroditism. Overexpression of the human AMH gene in transgenic mice also generated much information about possible functions of AMH in males and females (Behringer et al. 1990; Lyet et al. 1995). More recently, Behringer et al. (Behringer et al. 1994), generated AMH knock-out mice, and mice that are both AMH and androgen receptor deficient. In the AMH knock-out mice, the müllerian ducts persist not only in females but also in males. If, in addition, the androgen receptor is deficient, both XX and XY mice show persistent müllerian ducts, and absence of wolffian ducts. Furthermore, through these experiments it was shown that correct and full differentiation of the wolffian and müllerian ducts along the male or female pathway, requires complete regression of the müllerian or wolffian ducts, respectively (Behringer et al. 1994). Thus, full development of either the wolffian or the müllerian ducts are mutually exclusive processes, regulated by AMH and testosterone.

There are several other observations that support a functional interaction between AMH and testosterone during sex differentiation (Heller et al. 1992; Ichas et al. 1994). However, little is known about possible postnatal interactions between AMH and testosterone. It is generally accepted that synthesis of androgens by Leydig cells of the testis and by theca cells of the ovary is essential for postnatal gonadal development and adult gonadal functions. However, the postnatal role of AMH in the gonads is not clear.

Through the cloning of the anti-müllerian hormone receptor type II (AMHRII) (Chapter 3), and subsequent study of the regulation of expression of AMH and this receptor (Chapters 4 and 5), results have been obtained that indicate that AMH may function in the gonads of both sexes after birth. The cloning of the androgen receptor promoter (Chapter 6) has generated information about the DNA regions that may be

important in the regulation of androgen receptor expression.

This chapter aims to integrate the results which have been described in the previous chapters, with data from the literature, to define the putative mechanism of AMH/AMH receptor interaction and possible functions of AMH in the gonads. Furthermore, the roles of the principal regulators of gametogenesis, LH, FSH, testosterone and estrogen, in the regulation of AMH, AMHRII, and androgen receptor expression in the gonads will be discussed. Finally, aspects of AMH expression and function that may be of clinical significance are described.

7.2 AMH and AMH receptor interaction

The existence of AMH was first suggested by Jost (Jost, 1947), and the cloning of the cDNA in 1986 (Cate et al. 1986) revealed that AMH is a member of the activin/TGFβ family. Initially it was thought that, in contrast to the other family members, AMH existed as a homodimer of two 70 kDa subunits which were not cleaved. Subsequently, Pepinsky et al. (1988) showed that 5-20% of recombinant human AMH expressed in COS cells was cleaved at 109 amino acids from the carboxyl terminus, generating a dimeric 110 kDa N-terminal fragment, and a dimeric 25 kDa C-terminal fragment, that remain non-covalently associated. Plasmin treatment of purified recombinant human AMH generates the same cleavage products. The N- and C-terminal fragments have to remain associated to obtain full bioactivity (Wilson et al. 1993). This is in contrast to the functional characteristics of TGFβ, of which the C-terminal fragment is the active hormone. If associated, the N- and C-terminal TGFβ fragments form a latent complex (Gentry and Nash, 1990). The dimeric C-terminal AMH fragment is capable of inducing müllerian duct regression, and exhibits about half so-called maximal activity (activity observed with the combined N- and C-terminal fragments) in the fetal aromatase assay (Wilson et al. 1993). It has been suggested that *in vivo*, AMH is not cleaved until it binds to the receptor complex in the membrane (Wilson et al. 1993). In the past, these aberrant characteristics of AMH may have contributed to the difficulties that were observed when attempts were made to iodinate AMH in order to identify the AMH receptor type(s). Iodination of AMH resulted in loss of bioactivity of the hormone, indicating that the molecules were no longer intact (Lee and Donahoe, 1993). Other strategies, involving the production of anti-idiotypic antibodies, or biotinylation of AMH, were reported to be unsuccessful with respect to binding to known AMH-target cells (Lefèvre et al. 1989). Generation of AMH receptor

antibodies was hampered by difficulties in obtaining purified AMH receptor positive cell preparations (Lefèvre et al. 1989). However, AMH binding to mammalian cells has been reported in several cases. MacLaughlin et al. (1992b), reported binding of an AMH anti-idiotypic antibody to tumor cell lines (including A431) which previously had been claimed to be AMH-responsive (Chin et al. 1991) (see also Paragraph 7.4). MacLaughlin et al. (1992b) also showed displacement of iodinated AMH binding to a 200 kDa receptor on A431 cells with unlabelled AMH and with the anti-idiotypic antibody. Using a different technique, Catlin et al. (1992) showed binding of AMH to the müllerian ducts in cultured rat urogenital ridges, and to cultured fetal rat lungs. More recently, this group (Catlin et al. 1993) used fluorescein isothiocyanate labelling of AMH to show binding of AMH to A431 cells, and iodinated C-terminal AMH to identify a 88 kDa receptor on A431 cells with a Kd of 5.8 nM. All these reports lacked data showing specificity of binding of AMH with respect to other members of the TGFβ family. Thus, the specificity of AMH binding to these cells remains disputable.

The cloning of a candidate AMH receptor as reported in this thesis (Chapter 3), and, more recently, also by di Clemente et al. (1994b), has provided a new tool to study AMH-receptor interaction. Based on the amino acid sequences of the rat and rabbit AMH receptors, the cDNAs most likely encode type II receptors. Binding of AMH to the type II receptor, independent of the presence or absence of a type I receptor, would be expected to be possible, if the known binding characteristics of the TGFβ and activin type II and type I receptors (see Chapter 2) are generally applicable.

Fig. 7.1. (Next page) Hypothetical evolutionary relationship dendrograms of several members of the activin/TGFβ family of growth and differentiation factors, and their type I and type II receptors.
The dendrograms were constructed according to Fitch and Margoliash (Fitch and Margoliash, 1967) using the PHYLIP software package.
*A) Hypothetical evolutionary relationship dendrogram of the C-terminal mature fragments of human AMH (Cate et al. 1986), TGFβ1,2,3 (Derynck et al. 1985; de Martin et al. 1987; Derynck et al. 1988), inhibin-α (Inh),-βA (ActA),-βB (ActB) and -βC (ActC) (Mason et al. 1986; Mason et al. 1989; Hötten et al. 1995), and bone morphogenetic proteins 2,3,4,5,6,7 (BMP2-7) (Wozney et al. 1988; Celeste et al. 1990) and D. melanogaster Decapentaplegic (DPP) (Padgett et al. 1987).*
B) Hypothetical evolutionary relationship dendrogram of the rat and rabbit AMH type II receptors (ratAMHRII and rabAMHRII) (Baarends et al. 1994; di Clemente et al. 1994b), human TGFβ type II receptor (TGFBRII) (Lin et al. 1992) and activin receptor-like kinases 1,2,3,4,5,6 (Alk1-6) (type I receptors) (ten Dijke et al. 1993), mouse activin receptor type II (ActRII) (Mathews and Vale, 1991) and IIB (ActRIIB) (Attisano et al. 1992), C. elegans DAF1 (type I receptor) (Georgi et al. 1990), and DAF4 (type II receptor, binds bone morphogenetic proteins) (Estevez et al. 1993) and D. melanogaster SAX and TKV (type I receptors for decapentaplegic) (Nellen et al. 1994).

A)
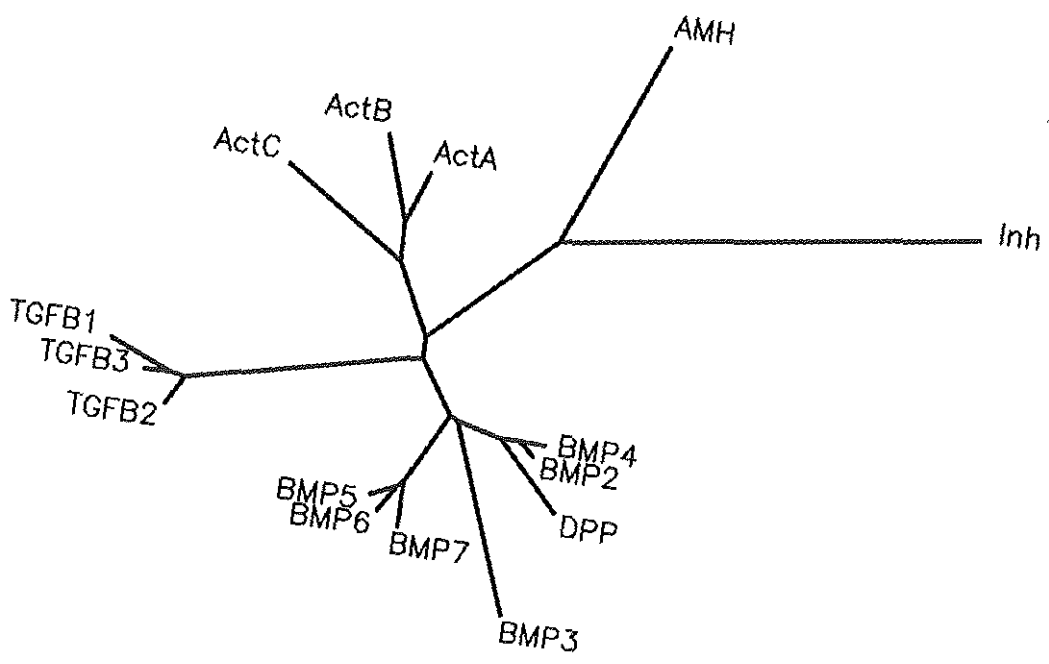
B)
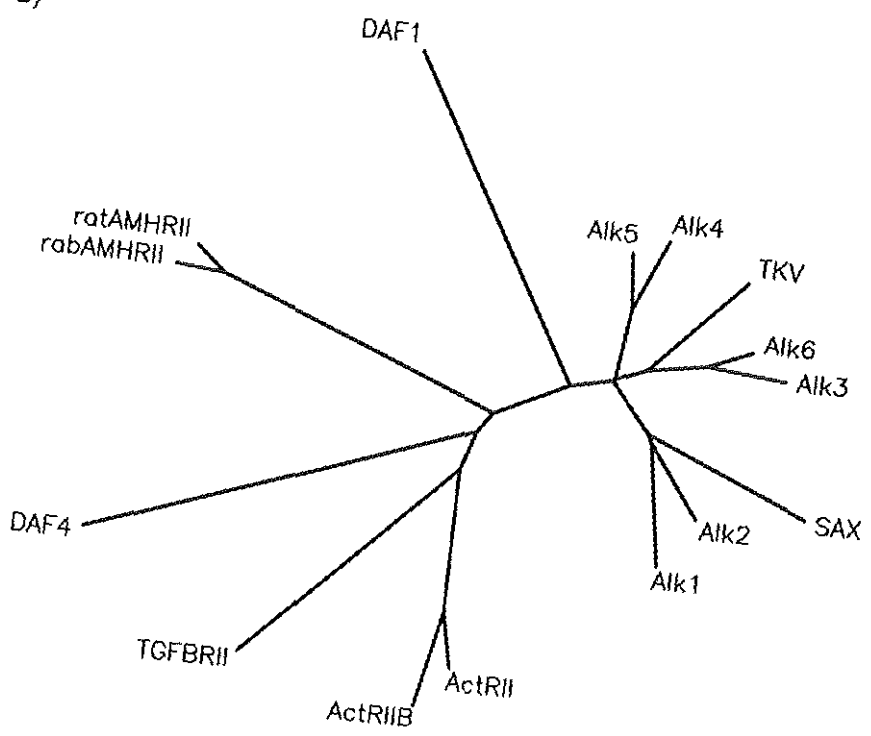
We have generated CHO cell lines expressing large amounts of rat AMH type II receptor mRNA, and used iodinated AMH (recombinant, plasmin-cleaved) in a routine binding assay. No specific binding of AMH could be detected using up to 1nM of labelled AMH (non-specific binding was determined using a 100-fold excess of non-labelled AMH). Since we had no means to determine the bioactivity of the radiolabelled ligand, no definite conclusions could be drawn. Di Clemente et al. (1994b) performed a similar experiment, on COS cells transiently expressing the rabbit AMH type II receptor, using iodinated AMH that still contained 70% of its ability to repress aromatase activity of fetal rat ovaries. A low level of specific binding of labelled AMH was observed at concentrations ranging from 0.5-2.0 nM. Saturation of binding was not obtained at reasonable ligand concentration, making meaningful estimates of binding parameters problematic. However, these data were substantiated by showing that expression of a truncated receptor form, which lacked the ligand binding domain, did not result in binding. Furthermore, incubation of cells transiently expressing rabbit AMHRII with iodinated AMH, followed by autoradiography, revealed specific binding. No AMH binding was observed in control cells, or when TGFβ type II receptor or truncated AMH type II receptor were used for transfection. Thus, although no normal binding curve of AMH to its type II receptor has been generated to date, the data of di Clemente et al. (1994b), together with the structural characteristics and the expression patterns of the rat and rabbit AMH type II receptor, indicate that this receptors is competent for AMH binding.

The aberrant structural characteristics of AMH described herein, may result in a receptor binding mechanism that differs from TGFβ and activin. Binding of these latter two factors to their type II receptors can unequivocally be demonstrated with Kds ranging from 100-800 pM (Attisano et al., 1992 and 1993). Aberrant characteristics of AMH and AMHRII may be deduced also from the relatively distant positions of AMH and AMHRII in hypothetical evolutionary relationship dendrograms (Fig. 7.1).

It remains to be shown that an AMH type I receptor also exists. It has been suggested (He et al. 1993; Wang et al. 1994) that one of the activin type I receptors, Alk-2, might also function as an AMH type I receptor. This assumption is based upon the observation that Alk-2 mRNA is expressed in the mesenchymal cells that surround the müllerian ducts in the rat, during the critical period of induction of müllerian duct regression (He et al. 1993). Since Alk-2 can interact with activin as well as bone morphogenetic protein type II receptors *in vivo* (ten Dijke et al. 1994), it seems likely that this type I receptor may associate with different type II receptors, depending on the cell type in which it is expressed. However, postnatal expression of Alk-2 mRNA

in the rat ovary (specific expression in oocytes) (He et al. 1993) does not correlate with AMH type II receptor expression (specific expression in granulosa cells; Chapter 5). Also, activin and AMH have opposite effects upon FSH-induced aromatase activity in granulosa cells (Hutchinson et al. 1987; di Clemente et al. 1994a), which makes it unlikely that they share the same type I receptor in this cell type, since it is the type I receptor that most likely determines the intracellular signal specificity (Wrana et al. 1994). These data indicate that a separate AMH type I receptor most likely exists, although it cannot be excluded that Alk-2 may function as an AMH type I receptor during müllerian duct regression. Candidate AMH type I receptors may be identified in AMH target cells (Sertoli/granulosa cells) through PCR-based strategies using conserved sequences of the known type I receptors. The development of a bioassay to monitor AMH receptor complex activation is important for the subsequent identification of AMH type I receptors. Such a bioassay may be obtained by expressing a construct of an AMH responsive promoter linked to a reporter gene, in a cell line that also expresses the AMH type II receptor. Cotransfection of cDNAs encoding candidate type I receptors, followed by addition of AMH to the cells, could then lead to identification of possible AMH type I receptors. The aromatase gene or LH receptor gene promoters may be useful as AMH responsive promoters, since AMH inhibits mRNA expression of these two genes in cultured granulosa cells (di Clemente et al. 1994a).

The present discussion of the possible mechanism of AMH receptor activation is based on current knowledge of activin and TGFβ receptors. However, in view of the specific characteristics of AMH and its type II receptor (see above) unexpected findings concerning AMH receptor activation are anticipated.

7.3   AMH and androgens in the gonads

Gonadotropins and steroid hormones are principal regulators of gonadal functions. Apart from these hormones, a vast number of locally synthesized peptide growth/differentiation factors provide specific micro-environments, and probably allow differential effects of gonadotropins and steroids on gametogenesis depending on the stage of development or cyclic changes. Such a regulatory network may show much redundancy, resulting in compensation of the absence of a single factor by the establishment of a different balance between the actions of the remaining regulators, that still allows the formation of mature eggs and sperm. AMH may be one of the

growth/differentiation factors that contributes to the regulation of postnatal gonadal functions. The study of the regulation of AMH and AMHRII mRNA expression during postnatal gonadal development (Chapters 4 and 5) has revealed possible sites of gonadal actions of AMH. This paragraph compares the expression patterns of AMH, AMHRII, and the androgen receptor, in testis and ovary. Analogies in expression patterns may provide additional clues, not only about the targets of AMH, but also about the possible nature of AMH actions, and about functional interactions with androgens. Furthermore, the regulation of AMH, AMHRII and the androgen receptor by gonadotropins and steroids is discussed.

7.3.1 Comparison of AMH, AMHRII, and androgen receptor expression in ovary and testis

During fetal development in the rat, AMH mRNA expression is high in testis but absent from the ovary (Hirobe et al. 1992), whereas AMHRII mRNA is low in testis but high in ovary (Baarends et al. 1994). Although the presence of another AMH-like ligand that interacts with AMHRII in fetal ovaries cannot be excluded, it seems unlikely that the high fetal AMHRII mRNA expression in ovary has any functional significance. This may indicate that a common factor, present in both testis and ovary, initially stimulates fetal AMHRII mRNA expression, and that the fetal testicular AMHRII mRNA level is downregulated by a male specific factor.

During early postnatal development in the rat, AMH mRNA expression is oppositely regulated in ovary versus testis, resulting in relatively high levels of AMH mRNA towards puberty in ovary and a low level in testis (Hirobe et al. 1992; Hirobe et al. 1994) (Chapter 5) (Fig. 7.2). Total ovarian AMHRII mRNA expression remains similar during postnatal development (Chapter 5), whereas total testicular AMHRII mRNA levels markedly increase during the first three weeks of postnatal testis development (Chapters 3 and 4) (Fig. 7.2). Detailed observation of AMHRII mRNA expression in ovary and testis by means of *in situ* hybridization revealed that an even distribution of AMHRII mRNA expression during the first weeks of postnatal development, in granulosa and Sertoli cells, precedes a more restricted expression pattern in specific ovarian follicles and during specific stages of the spermatogenic cycle (Chapter 5, and unpublished results). A similar biphasic pattern has been described for expression of androgen receptor protein in testis (Bremner et al. 1994).

In the adult rat, there is a striking coregulation of AMH mRNA, AMHRII mRNA, and androgen receptor protein in both granulosa cells and Sertoli cells during follicle development and the spermatogenic cycle, respectively; although, the androgen receptor, is also expressed in other gonadal cell types (Chapters 4 and 5; Bremner et al. 1994; Tetsuka et al. 1995).
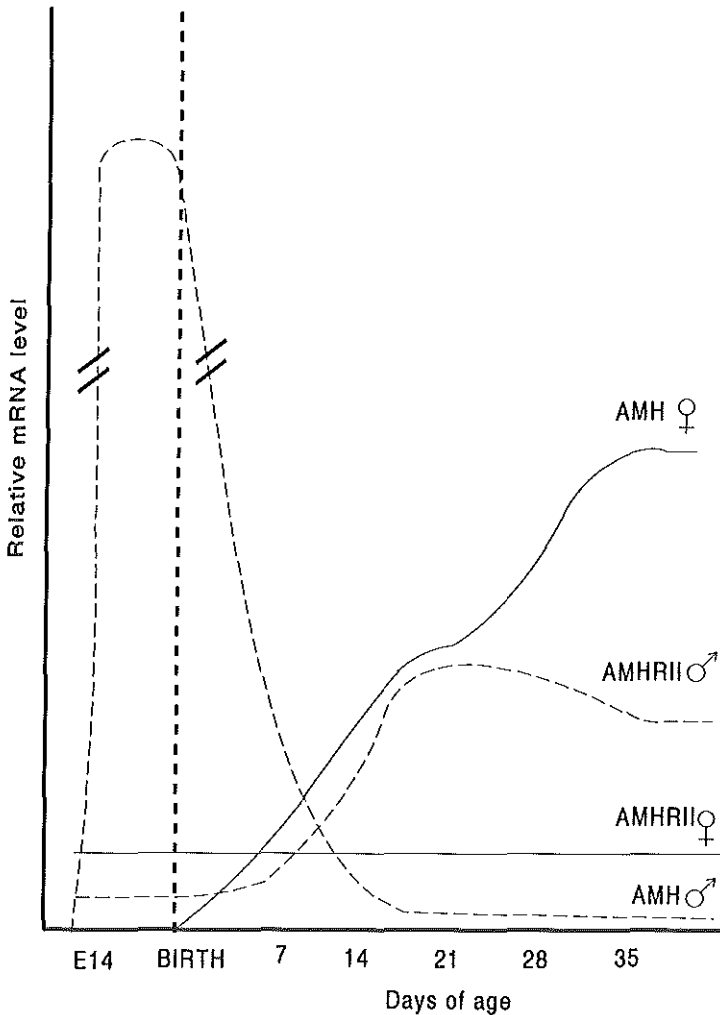

Fig. 7.2 Relative levels of AMH and AMHRII mRNA expression during gonadal development in the rat. The mRNA levels are estimated per constant amount of total ovarian (indicated by ♀) or testicular (indicated by ♂) RNA. The figure gives an impression of data described in Chapters 3, 4 and 5, and of data described by Kuroda et al. (1990), Haqq et al. (1992), and Hirobe et al. (1992).

7.3.2 Hormonal regulation of AMH, AMHRII, and androgen receptor expression in ovary and testis

In the testis, the expression of a number of genes in Sertoli cells is known to be regulated by FSH and testosterone. Only a few genes, such as α-inhibin, c-fos, jun-B, tissue plasminogen activator, and TSC-22, are known to be directly regulated by FSH at the transcriptional level (Hall et al. 1988; Klaij et al. 1990; Hamil et al. 1994; Hamil and Hall, 1994). c-Myc mRNA expression is induced by testosterone in immature rat Sertoli cells *in vitro*, and this induction also occurs when protein synthesis is inhibited (Lim et al. 1994), indicating that c-myc is a candidate androgen responsive gene in immature Sertoli cells. No other primary androgen responsive genes in the testis have been described. In order to get more insight in the mechanism by which FSH and testosterone regulate spermatogenesis, we have tried to isolate candidate androgen- and/or FSH-regulated cDNAs from Sertoli cells, by using different molecular techniques, such as subtractive hybridization and differential screening. One FSH-regulated cDNA (LRPR1) (Slegtenhorst-Eegdeman et al. 1995) was identified. The AMHRII cDNA was isolated as a candidate androgen-regulated gene (Chapter 3). The promoter of the androgen receptor was isolated to study genomic regions that might be responsible for regulation of androgen receptor mRNA by testosterone and FSH (Chapter 6).

Regulation of AMH and AMHRII expression in the testis

FSH injection decreases testicular AMH mRNA and protein levels in neonatal rats (Kuroda et al. 1990), indicating that FSH may, at least in part, be responsible for the postnatal decrease in testicular AMH. In adult rats, downregulation of AMH protein at defined stages of the spermatogenic cycle by FSH is compatible with expression patterns of AMH mRNA (Chapter 4) and FSH receptor mRNA (Heckert and Griswold, 1991). The negative correlation between AMH and testosterone levels during human postnatal development (Rey et al. 1993), may lead to the suggestion that AMH production at puberty in boys is negatively regulated by testosterone also. Furthermore, in patients suffering from the androgen insensitivity syndrome (see also Chapter 2), AMH levels are elevated during the first postnatal year and after puberty (Rey et al. 1994). In testis of rats treated with testosterone at day 19 of fetal development, AMH mRNA levels were not changed when estimated two days later, and AMH immunohistochemical data indicated that testosterone may regulate AMH cleavage, rather than AMH synthesis (Kuroda et al. 1991).

No effects of testosterone on AMH mRNA or protein in adult animals or humans have been described.
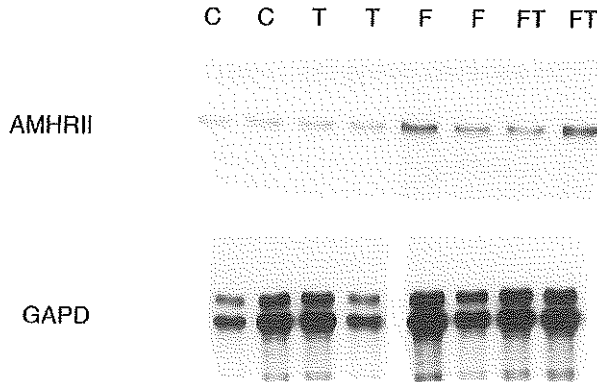

Fig. 7.3 Regulation of AMHRII mRNA expression by FSH and testosterone in cultured immature Sertoli cells.
Partially purified Sertoli cells were isolated according to Themmen et al. (1991) from 14-day-old rats and cultured for three days in Eagle's minimal essential medium (MEM) + 1% (v/v) fetal calf serum. At day four, medium was changed into MEM, 0.1% (w/v) bovine serum albumin, insulin (5µg/ml) and retinol (0.3µM) (control medium), with or without 1µM testosterone and/or 500 ng/ml FSH (NIH S16), and the cells were kept in culture for an additional 24 h. Subsequent RNA isolation and RNase protection assay were performed as described in Chapter 3. RNase protection was performed using total RNA from Sertoli cells cultured in control medium only (C), plus FSH (F), plus testosterone (T), and plus FSH and testosterone (FT). AMHRII and GAPD indicate the positions of the respective protected fragments. A GAPD probe was used to verify whether equal amounts of mRNA were used in each lane.

The expression of AMHRII mRNA in cultured Sertoli cells, isolated from 21-day-old rats, initially seemed to be androgen responsive, but this could not be confirmed in later experiments (Chapter 3). Also in vivo, depletion of testosterone through the use of the Leydig cell toxicant ethane dimethane sulfonate (EDS), did not result in any major change in total testicular level of AMHRII mRNA as compared to control adult rats, at three or five days after EDS administration (unpublished results). AMHRII mRNA expression in cultured Sertoli cells isolated from immature or adult rats rapidly decreases during the first few hours after isolation of the cells. After culturing testis tubules from 14-day-old rats for several days, AMHRII mRNA is still detectable, and stimulation with FSH for 24 h results in an increase in the AMHRII mRNA level. Again, no effect of testosterone was observed (Fig. 7.3). These results indicate that AMHRII mRNA expression is stimulated by FSH *in vitro*. Whether FSH also regulates testicular AMHRII mRNA expression *in vivo*, is not clear. During the prepubertal phase in male rats, FSH levels increase (de Jong and Sharpe, 1977; Ketelslegers et

al. 1978), concomitant with the most pronounced increase in AMHRII mRNA expression in Sertoli cells (Chapters 3 and 4). We also observed a normal pattern of AMHRII mRNA expression during the first three weeks of postnatal testis development in sterile (prenatally irradiated) rats (Chapter 4), that have been reported to have a normal pattern of FSH secretion during this period (Means et al. 1976).
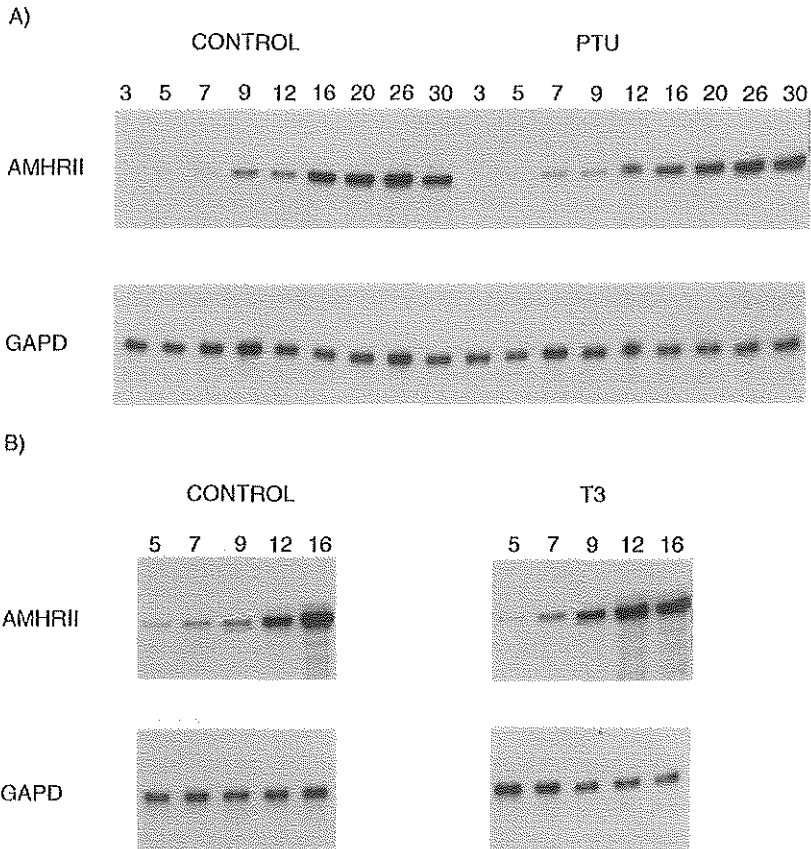

Fig. 7.4 Effects of experimentally induced neonatal hypothyroidism and hyperthyroidism on the developmental expression pattern of AMHRII mRNA in rat testis.
Hypothyroidism was induced through administration of 6-propyl-2-thiouracil (PTU) from birth until 26 days of age, as described by van Haaster et al. (1992). To induce hyperthyroidism, newborn rats were treated with T3 from birth to 16 days of age, as described by van Haaster et al. (1993). Total testicular RNA was isolated at 3, 5, 7, 9, 12, 16, 20, 26, and 30 days after birth of control and PTU-treated rats, and at 5, 7, 9, 12, and 16 days after birth of control and T3-treated rats. A) RNase protection assay results from control and PTU-treated rat testes, B) RNAse protection results from control and T3-treated rat testes. The numbers above each lane correspond to the age in days of the rats. AMHRII and GAPD indicate the positions of the respective protected fragments. A GAPD probe was used to verify whether equal amounts of mRNA were used in each lane.

Hypothyroidism, induced during the prepubertal period by administration of 6-propyl-2-thiouracil (PTU), retards morphological differentiation of Sertoli cells, and prolongs the period of proliferation of these cells (van Haaster et al. 1992). The postnatal induction of total testicular AMHRII mRNA expression was delayed in PTU-treated rats as compared to controls (Fig. 7.4a). Furthermore, in the opposite situation, when high neonatal triiodothyronine (T_3) levels are present, Sertoli cell differentiation is advanced (van Haaster et al. 1993), and AMHRII mRNA expression reached its maximum at day 16, as compared to day 20 in control rats (Fig. 7.4b). The altered temporal AMHRII mRNA expression patterns after PTU and T_3 treatment are most likely not caused by actions of FSH, since a moderate reduction of the FSH level was observed, both after PTU and after T_3 treatment (van Haaster et al. 1992; van Haaster et al. 1993). However, FSH may be required to induce the expression of sufficient amounts of AMHRII mRNA. It will be of interest to study AMHRII mRNA expression *in vivo*, after FSH treatment of immature male rats, which are known to respond to an extra dose of FSH (Blok et al. 1992b; Slegtenhorst-Eegdeman et al. 1995). In the adult rat testis, the situation is less clear. The stage specific expression of AMHRII mRNA during the spermatogenic cycle is at a maximum when FSH receptor expression is minimal, indicating an inhibitory rather than a stimulatory effect of FSH on AMHRII mRNA expression in adult rat Sertoli cells.

Regulation of androgen receptor expression in the testis
Although testosterone can downregulate transcription of the gene encoding its own receptor in a number of tissues (Shan et al. 1990; Quarmby et al. 1990; Blok et al. 1992a), it is not clear whether this also occurs in testis (see also Chapter 1). FSH stimulates the transcription of the androgen receptor gene in Sertoli cells in culture (Blok et al. 1989; Blok et al. 1992b), and high doses of forskolin or dibutyryl cAMP can stimulate androgen receptor gene transcription in other cell lines (Mizokami et al. 1994; Lindzey et al. 1993). The cloning of the rat (Chapter 6; Song et al. 1993) human (Tilley et al. 1990; Faber et al. 1991b) and mouse (Faber et al. 1991a) promoters of the respective androgen receptor genes has provided tools to study the mechanism of regulation of androgen receptor gene transcription in different animal species. The androgen receptor promoter contains two major transcriptional start sites in rat, mouse and human, that each require specific nearby sequence elements. Downregulation of androgen receptor transcription by testosterone in a number of human cell lines concerned both transcriptional start sites in a similar manner (Wolf et al. 1993). For each species, sequence elements that are essential for basal

transcription have been identified (Tilley et al. 1990; Faber et al. 1991a; Faber et al. 1991b; Blok et al. 1992c; Song et al. 1993; Mizokami et al. 1994) (summarized in Fig. 7.5). Furthermore, different sequence elements that are responsible for the stimulation of transcription by cAMP/FSH have been localized in the mouse, rat and human androgen receptor promoters (Blok et al. 1992c; Lindzey et al. 1993; Mizokami et al. 1994). No consensus CRE sequences are present in any of the studied promoters, but the human and mouse promoters contain sequences that are very similar to the consensus CRE, and those sequences are thought to mediate at least part of the stimulatory effects of cAMP (Mizokami et al. 1994; Lindzey et al. 1993). However, these elements are not conserved between the mouse, rat, and/or human androgen receptor promoters (Fig. 7.5).
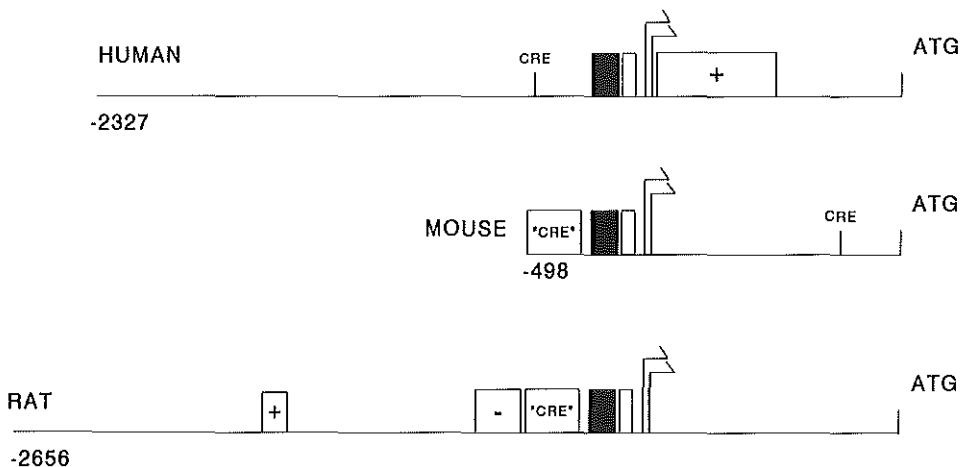

Fig. 7.5. Comparison between the androgen receptor gene promoters of human, mouse and rat. The promoters of the human (Tilley et al. 1990; Faber et al. 1991b), mouse (Faber et al. 1991a) and rat (Chapter 6; Song et al. 1993) androgen receptor genes are shown. The arrows indicate that all three promoters contain two major transcriptional start sites. Each of these promoters contains several GGGGA repeats (indicated by the black boxes; the function of these repeats is unknown). The SP1 binding-site, indicated by open bars, is thought to be an important element of the basal promoter. Other elements that have been shown to stimulate (box marked +) or inhibit (box marked -) the basal level of transcription are indicated. Some of the identified cAMP/FSH response elements resemble the consensus CRE (TGACGTCA) (indicated by CRE), and other DNA regions that are also involved in mediating (part of) the response to FSH and/or cAMP are indicated by "CRE".

Little is known about the regions of the androgen receptor promoter that are responsible for the downregulatory action of testosterone in certain tissues. Transfection of a rat androgen receptor promoter-reporter construct, comprising approximately 8 kb of promoter sequence and almost the complete 5' UTR, into the human prostate cancer cell line LNCaP, followed by addition of testosterone, did not result in any changes in reporter gene expression (Blok et al. 1992c). However, cotransfection experiments of a mouse androgen receptor promoter-reporter construct and a mouse androgen receptor expression vector in a quail cell line, showed that dihydrotestosterone could reduce basal and forskolin-induced expression of the mouse androgen receptor (Lindzey et al. 1993). Deletion of a putative androgen response element had no effect on this suppression. Thus, it is unclear which regions in the androgen receptor promoter of the different species confer androgen-mediated transcriptional downregulation.

The above described functional analysis of the androgen receptor promoter indicates that the postnatal increase in testicular AR mRNA level may be an FSH regulated event. In contrast, the expression patterns of the FSH receptor and the androgen receptor in adult Sertoli cells are negatively correlated during the spermatogenic cycle (Kangasniemi et al. 1990; Heckert and Griswold, 1991), suggesting that stimulatory effects of FSH on androgen receptor expression in adult rat Sertoli cells might be unlikely. With respect to the *in vivo* regulation of testicular androgen receptor mRNA expression by androgens, the situation is also complicated. In adult rats, testosterone withdrawal through the use of EDS does not affect testicular androgen receptor mRNA levels and total androgen binding (Blok et al. 1992a), but androgen receptor immunostaining is reduced (Bremner et al. 1994).

Regulation of AMH, AMHRII, and androgen receptor expression in the ovary
Since manipulation of the levels of gonadotropins and steroids in the ovary often results in rapid changes in follicle development, it is difficult to decide whether changes in ovarian levels of gene products are due to direct hormonal effects, or to a change in the numbers of follicle types that are present. Therefore, *in situ* hybridization and immunohistochemistry are of great importance to study effects of hormones on follicular development and gene expression. The expression patterns of AMH, AMHRII, and androgen receptor in granulosa cells during follicular development (Chapter 5; Tetsuka et al. 1995) are consistent with a role of these gene products during the follicular phase that precedes the FSH-induced follicular maturation. For AMH and AMHRII mRNAs, we have shown that their expression can be

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downregulated by FSH and estradiol benzoate in large preantral follicles (Chapter 5). Fig. 7.6 illustrates possible interactions between AMH and FSH, that could be involved in regulation of aromatase and LH receptor expression during different phases of follicular development. This model is based on the observation that the FSH-sensitivity of granulosa cells increases as a follicle starts to develop a large antrum (reviewed by Richards, 1994). Little is known about the significance of androgen receptor expression in the ovary, and no effects of androgens on the ovarian expression of androgen receptor, or any other gene, have been described.

Future studies of the AMHRII gene promoter (Visser et al., personal communication) may provide additional insight into the mechanism of regulation of AMHRII gene transcription in ovary and testis. Comparison between the AMH, AMHRII, and androgen receptor gene promoters may reveal possible common regulatory sequences that are responsible for the unique coregulation of these three genes (see 7.3.1) in adult Sertoli and granulosa cells.

7.3.3 Possible functional significance of AMH expression in the gonads, and interactions between AMH and androgens

Granulosa and Sertoli cells are thought to be derived from a common precursor cell (Chapter 1). In this respect, it is not surprising that there are a number of analogies in the expression of AMH, AMHRII, and androgen receptor in these two cell types. Whether the analogies also extend to the functions of the genes in these cells, remains a matter of speculation. The fact that the three genes are coregulated in adult Sertoli and granulosa cells, indicates that the functions of AMH and testosterone may be interdependent and may even overlap. The developmental change in the AMHRII mRNA expression pattern from an even distribution to a more specific pattern in both ovary and testis, may indicate separate functions of AMH during the prepubertal and adult phases. The same suggestion can be made with respect to distinct functions of the androgen receptor, since this gene displays a similar biphasic developmental expression pattern in rat testis (Bremner et al. 1994).

AMH may be involved in regulation of Leydig cell proliferation, through an effect on Sertoli cells, possibly involving the extracellular matrix (Chapter 4). There are two distinct growth phases of Leydig cells, leading to two different Leydig cell populations, termed fetal and adult Leydig cells (reviewed by Huhtaniemi, 1994). Fetal Leydig cell proliferation is maximal between E17.5-E19.5 (Tapanainen et al. 1984) in the rat.

From postnatal day 15 onwards, the adult Leydig cell population is formed and Leydig cell numbers increase steadily until 60 days after birth (Tapanainen et al. 1984). An inhibitory effect of AMH on the adult number of Leydig cells, is exemplified by the Leydig cell hyperplasia in AMH knock-out mice (Behringer et al. 1994), and by the severely decreased androgen production by Leydig cells of adult transgenic mice expressing a relatively high level of human AMH (Lyet et al. 1995). Whether these effects reflect a function of AMH during the immature or the adult phase, remains to be established.

In the adult testis, maximal levels of AMH mRNA, AMHRII mRNA, and androgen receptor protein during the spermatogenic cycle are found at the stages that have been defined as being androgen dependent (reviewed by Sharpe, 1994). Possibly, AMH contributes to one of the Sertoli cell functions that depend on androgen.

In the adult ovary, there might be a role for AMH in the inhibition of follicular maturation or in follicle selection, through autocrine mechanisms. This assumption is based on the expression patterns of AMH and AMHRII mRNAs, and on the *in vitro* effects of AMH on granulosa cells (Chapter 5) (Fig. 7.6). It is not known whether AMH has effects on the number of theca cells during follicle development in the ovary, analogous to the possible effects of AMH on Leydig cell numbers in the testis. The expression pattern of the androgen receptor in granulosa cells during follicular development indicates that any effect of androgens on granulosa cell functions also would take place prior to final follicle maturation.

In order to gain more insight into the possible effects of AMH on Leydig cell and/or theca cell development, the ontogeny of these cell numbers in AMH overexpressing and AMH knock-out mice should be studied, to determine during which phase of postnatal testis and/or ovary development AMH may influence Leydig and/or theca cell numbers. Indications about possible effects of AMH on adult Leydig cell numbers may be obtained through study of long-term AMHRII mRNA expression using *in situ* hybridization, or observation of the effect of manipulation of AMH action, during Leydig cell regeneration in EDS-treated adult rats.

Manipulation of the levels of AMH and/or AMHRII at precise time points during postnatal gonad development may provide additional information about effects of AMH on Leydig or theca cells. Furthermore, other genes that are involved in gonadal function, such as androgen receptor, FSH receptor, LH receptor, activin, inhibin, and aromatase, may be affected by AMH, and must be studied as well.
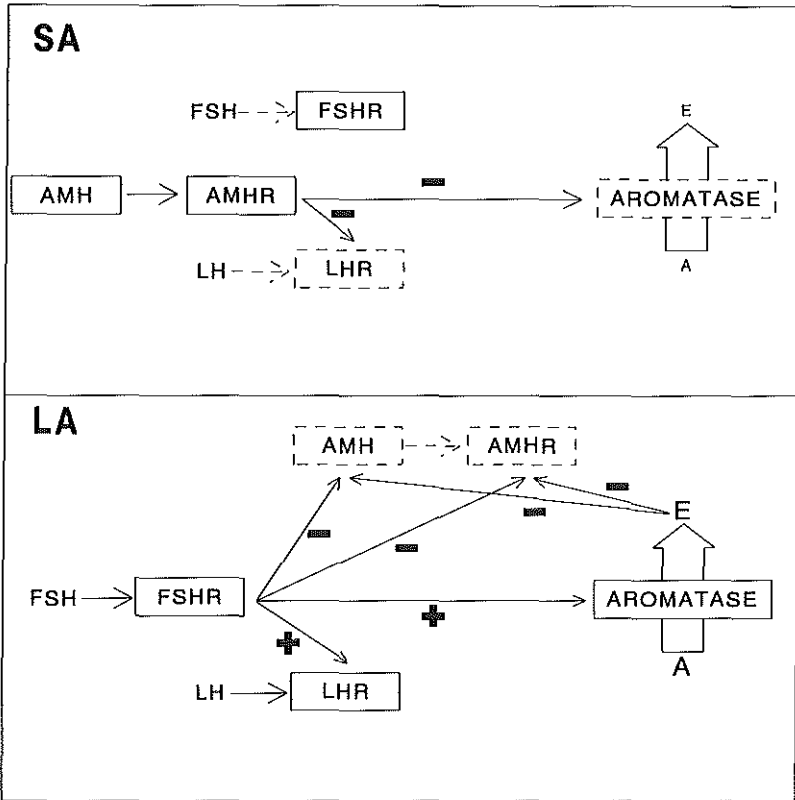
Fig. 7.6 Schematic illustration of hypothetical regulatory interactions between AMH, FSH, and estrogens in granulosa cells during follicle development.
In this hypothetical model, the following processes may occur:
SA: In large preantral and small antral (SA) follicles, both AMH and AMHRII mRNAs are expressed at a high level. The AMHRII participates in repressing aromatase activity and LH receptor expression during these stages of follicle development.
LA: The granulosa cells of large antral (LA) follicles have become more sensitive to FSH, thus allowing downregulation of AMH and AMHRII mRNA expression by FSH. Aromatase activity and LH receptor expression are no longer downregulated, and FSH stimulates both aromatase and LH receptor expression. Furthermore, androgen synthesis by the theca cells has increased. Together this results in an increased estrogen output, which leads to a further downregulation of AMH and AMHRII mRNA expression via an autocrine effect of estrogens on the granulosa cells.
Granulosa cell-derived proteins are boxed, stippled lines indicate that the amount of protein is relatively low. -, +, negative, positive regulation of the factor indicated by arrow; arrows also indicate hormone/receptor interaction; stipled arrow, hormone signal is not (or not efficiently) transduced; open arrow, enzymatic conversion of androgen (A) into estrogen (E); AMH, anti-müllerian hormone; AMHR, AMH type I/type II receptor complex; FSH, follicle-stimulating hormone; FSHR, FSH receptor; LH, luteinizing hormone; LHR, LH receptor.

The possible role of the extracellular matrix in AMH action deserves further study. It is known that the basement membrane (composed of extracellular matrix components) that surrounds the spermatogenic epithelium plays an important role in maintenance of the differentiated function of Sertoli cells. Furthermore, a number of growth factors are sequestered in basement membranes, and their action may be dependent on interaction with extracellular matrix components (reviewed by Dym, 1994). Our observation that cultured Sertoli cells rapidly lose AMHRII mRNA, indicates that the expression of this gene may be dependent on the maintenance of the columnar shape and polarity of the Sertoli cell, and/or the presence of an extracellular matrix. The production rate of several Sertoli cell gene products is known to be increased when the cells are cultured in the presence of extracellular matrix (reviewed by Dym, 1994). Immature or adult Sertoli cells cultured on top of reconstituted basement membrane may provide an adequate model system to investigate the interaction between the extracellular matrix and AMH action.

The extracellular matrix may also affect local concentrations of AMH. The concentration of AMH in small bovine follicles is 0.29 nM (Vigier et al. 1984), whereas the ED_{50} of AMH effects on aromatase activity and LH receptor expression *in vitro* is 7 nM (Wilson et al. 1993; di Clemente et al. 1994a). In this respect, it will be of interest to study whether local accumulation of AMH occurs, and whether the presence of extracellular matrix can influence AMH dose-response curves for cultured cells.

7.4   Clinical applications of AMH and AMHRII

Mutations in the gene encoding AMH are an important cause of the persistent müllerian duct syndrome (PMDS) (Chapter 2). It will be of interest to study whether any of the AMH positive cases of PMDS are due to mutations in the human AMHRII gene.

The development of sensitive immunocytochemical techniques has allowed detailed measurements of normal levels of AMH between birth and adulthood in humans (Baker et al. 1990; Josso et al. 1990; Hudson et al. 1990; Baker and Hutson, 1993). Between 4 and 12 months of age, relatively high serum AMH levels are detected in boys. The levels gradually decrease during childhood and become very low around puberty in males. In girls, AMH becomes first detectable around puberty, but the serum AMH concentrations remain low during the reproductive years in females, and are comparable to those observed in the adult male (Hudson et al. 1990). Measurement of serum AMH levels can be helpful in the diagnosis and

management of intersex and gonadal abnormalities (Gustafson et al. 1993). For example, serum AMH concentrations can be used to confirm the presence of testicular tissue in patients with anorchia and/or intersex anomalies (Josso et al. 1991; Gustafson et al. 1993). Also, elevation of serum AMH appears to be a marker of androgen resistance in sexually ambiguous genetic male infants (Rey et al. 1994). In cases of certain gonadal tumors, such as AMH secreting granulosa cell tumors, it has been shown that serum AMH concentrations decrease after surgical removal of tumor tissue, and if tumor recurrence occurs AMH levels rise correspondingly (Gustafson et al. 1992). Thus, AMH measurements may serve as a predictive marker of persistent or recurrent disease. AMHRII mRNA is expressed at a high level in the mesenchyme surrounding the müllerian duct during fetal development and in fetal and adult ovaries and testes (Chapters 3-5). Study of the expression of AMHRII mRNA or protein in gonadal tumors, or in tumors from müllerian duct derived tissues, may provide information about the possibilities to use AMHRII expression to identify the cellular origin of a tumor, and may help to select tumors that might be responsive to growth inhibitory actions of AMH.

AMH has been reported to act as an antitumorigenic agent. Partially purified bovine AMH inhibited the growth of certain cancer cells *in vivo* and *in vitro* (Donahoe et al. 1981; Fuller Jr et al. 1982; Fuller Jr et al. 1985). However, when purified recombinant AMH became available, these results could not be reproduced, and only minimal antiproliferative effects were observed (Wallen et al. 1989). Subsequently, it was reported (Chin et al. 1991; Ragin et al. 1992) that modification of the purification protocol of the recombinant AMH resulted in the elimination of factors produced by the AMH expressing CHO cells that stimulate tumor growth, and that recombinant AMH purified in this manner inhibited growth of a number of tumor cell lines of müllerian duct/coelomic epithelium origin (Chin et al. 1991) and ocular melanoma cell lines (Parry et al. 1992). However, partially purified recombinant AMH is generally a more potent inhibitor (Chin et al. 1991), and the antiproliferative activity of AMH remains preparation dependent. MacLaughlin et al. (1992a) suggest that this may be due to the fact that the tumor cell lines lack the ability to cleave AMH to a biologically active conformation (see Paragraph 7.2). These authors report that the purified 25 kDa C-terminal AMH dimer induces reproducible dose-dependent inhibition of the growth of A431 cells (derived from a squamous cell carcinoma of the vulva) (MacLaughlin et al. 1992a), but extremely high concentrations (1µg/ml–40µM) of the C-terminal AMH were used in these experiments. Thus, consistent inhibition of tumor growth by purified recombinant AMH at concentrations comparable to those needed for müllerian

duct regression has not been shown. Recently, di Clemente et al. (1994b) have reported that A431 cells do not express the human homologue of the rabbit and rat AMHRII. It will be of interest to study whether any of the other tumor cell lines that have been reported to be AMH-responsive, express AMHRII. If such cell lines exist, it will be possible to study whether the antiproliferative effects of the AMH-preparations are mediated via the AMHRII, or whether cross-reaction with another receptor is involved.

The possible implications of the postnatal AMH and AMHRII mRNA expression data, as discussed in this chapter, support the concept that AMH functions not only during fetal sexual differentiation, but also during postnatal gonad development. This should stimulate research that aims to define the exact nature of these functions, and to identify regulatory interactions between AMH and FSH, LH, testosterone, and/or estrogens.

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The fetal testes produce two hormones that are essential for correct male sex differentiation. The first hormone, anti-müllerian hormone (AMH), is a dimeric glycoprotein, which induces the regression of the müllerian ducts (the anlagen of the fallopian tubes, the uterus and upper part of the vagina). The other hormone, the steroid hormone testosterone, stimulates development of the wolffian ducts into epididymides, vasa deferens and seminal vesicles. The situation is reversed in females: ovaries develop instead of testes, and the absence of AMH and testosterone leads to müllerian duct development and wolffian duct regression. The principal regulators of postnatal gonadal development are follicle-stimulating hormone (FSH) and luteinizing hormone (LH). LH stimulates the gonadal synthesis of androgen (testosterone) which is converted to estrogen in the ovary. The gonadal actions of gonadotropins (FSH, LH) and steroid hormones (androgen, estrogen) are modulated by a vast number of locally acting factors. Members of the activin/TGFβ family of growth and differentiation factors, including AMH, are probably some of the most important local factors. This thesis focuses on structural and regulatory aspects of the receptors for AMH and testosterone.

In the General Introduction (Chapter 1) the regulation of sex determination, sex differentiation, and pre- and postnatal gonadal development are described, with emphasis on the roles of testosterone and AMH in these processes. Chapter 2 gives an overview of the receptor mechanisms that are used by gonadotropins, steroid hormones, and members of the activin/TGFβ family of growth and differentiation factors. Furthermore, some naturally occurring, or experimentally induced mutations in the genes encoding these hormones or their receptors are described, to illustrate their importance in sex differentiation and gonadal development.

The cloning of a candidate rat AMH type II receptor (AMHRII, initially named C14) is described in Chapter 3. This receptor is a member of the family of transmembrane serine/threonine kinase receptors, and is specifically expressed from embryonic day 14 in the rat in the gonads and in the mesenchymal cells surrounding the müllerian duct. Thereafter, the expression gradually disappears from the male urogenital ridge, and persists in the female. After birth, expression of AMHRII is mainly confined to ovary and testis. The structure of the receptor, and its specific expression in AMH target cells, strongly suggest that AMH is the ligand.

In Chapters 4 and 5, the postnatal expression patterns of AMH and AMHRII mRNA in the gonads are studied in detail, using RNase protection assays and *in situ*

hybridization. In the male, AMH and AMHRII mRNA are specifically expressed in the Sertoli cells of the testis. The total testicular AMHRII mRNA expression level increases during the first three weeks of postnatal development, whereas during the first week of this period, there is a sharp decline in the amount of AMH mRNA. This leads to an AMHRII mRNA level in the adult testis that is much higher than that of AMH mRNA. However, the expression pattern of both genes in Sertoli cells during the spermatogenic cycle shows a maximum at Stage VII. Thus, autocrine actions of AMH on Sertoli cells may be of particular importance during Stage VII. Depletion of specific groups of germ cell types, ranging from intermediate spermatogonia to mature sperm, does not affect the total testicular level of AMHRII mRNA expression. However, AMHRII mRNA levels are decreased in testes from adult prenatally irradiated adult rats, and also in experimentally cryptorchid rat testes. This indicates that a combination of spermatogenic cycle events, possibly involving changes of Sertoli cell structure and/or Sertoli cell-basal membrane interactions, regulate autocrine AMH action on adult rat Sertoli cells.

In the ovary (Chapter 5), AMH and AMHRII mRNA expression is found to be mainly restricted to the granulosa cells of preantral and small antral healthy follicles, except during the first two weeks of postnatal ovary development, when AMHRII mRNA is expressed in an evenly distributed manner, and AMH mRNA expression is lower than in adult rat ovary. Estrogen and FSH can downregulate AMH and AMHRII mRNA expression in granulosa cells of gonadotropin-releasing hormone antagonist-treated immature rat ovaries. The results suggest that, similar to the above described autocrine action of AMH on Sertoli cells of the testis, AMH acts in an autocrine manner on granulosa cells of the ovary. The pattern of AMH and AMHRII mRNA expression during follicular development may be regulated in part by the combined action of FSH and estrogen. As follicles reach the small antral stage, the sensitivity of granulosa cells to FSH and estrogen production increase, which subsequently may result in downregulation of AMH and AMHRII mRNA expression. The function of AMH in preantral and small antral follicles may involve inhibition of follicle maturation, and/or AMH may play a role in follicle selection.

Chapter 6 describes the isolation of the rat androgen receptor promoter. This promoter contains two major transcriptional start sites, separated by 11 bp, which are preceded by an SP1 site located approximately 60 bp upstream. Androgen can downregulate androgen receptor gene transcription in several tissues, and FSH can stimulate androgen receptor gene transcription in immature rat Sertoli cells. However, no consensus androgen/glucocorticoid response elements or cyclic AMP-response

elements are present. The possible localization and functional significance of promoter elements that may confer androgen or cAMP/FSH regulation on the androgen receptor gene is discussed in Chapter 7.

Chapter 7 also describes the possible mechanism of action of AMH, based on the results described in this thesis, and the current knowledge about the mechanism of action of other members of the activin/TGFβ family of growth and differentiation factors. Furthermore, the expression patterns of AMH, AMHRII and the androgen receptor in the gonads are compared and discussed in the light of regulatory interactions between AMH, gonadotropins, androgen and estrogen. Suggestions about the possible postnatal functions of AMH are given. Finally, the possible clinical significance of AMHRII and AMH is discussed.

Tijdens de ontwikkeling van een zoogdier-embryo worden structuren gevormd waaruit in een latere fase de geslachtsorganen ontstaan. Eén van deze structuren is de ongedifferentieerde gonade, het orgaan waaruit een eierstok (ovarium) of een zaadbal (testis) wordt gevormd. De geslachtsbepaling vindt in deze ongedifferentieerde gonaden plaats: wanneer de cellen een X en een Y chromosoom bevatten, zal zich een testis ontwikkelen. Echter, wanneer het Y chromosoom afwezig is, wordt uit de ongedifferentieerde gonade een ovarium gevormd. Eén gen op het Y chromosoom is verantwoordelijk voor het in gang zetten van testisdifferentiatie. Vervolgens produceren de foetale testes in mannelijke embryo's twee hormonen waarvan de werking essentieel is voor een juist verloop van de mannelijke geslachtsdifferentiatie. Het eerste hormoon, het anti-müllerse gang hormoon (AMH), is een eiwithormoon dat zorgt voor het ten gronde gaan van de müllerse gangen. In het vrouwelijke embryo wordt geen AMH gevormd, en daar ontwikkelen de müllerse gangen zich tot de eileiders, de baarmoeder, en het bovenste deel van de vagina. Het tweede hormoon uit de testis, testosteron (een steroidhormoon), bewerkstelligt de vorming van de bijballen, de zaadleiders en de zaadblazen, uit structuren die de wolffse gangen genoemd worden. De wolffse gangen degenereren in de vrouwelijke embryo's, omdat daar vrijwel geen testosteron aanwezig is.

De verdere ontwikkeling van ovarium en testis na de geboorte is ook afhankelijk van hormonen. In de hypofyse, een aanhangsel van de hersenen, worden de gonadotropinen follikel-stimulerend hormoon (FSH) en luteïnizerend hormoon (LH) gevormd. Via de bloedbaan bereiken deze hormonen de testes of ovaria (de gonaden). FSH en LH oefenen hun werking uit op specifieke cellen in de gonaden. FSH stimuleert de Sertoli cellen in de testis en de granulosa cellen in het ovarium. Sertoli cellen ondersteunen en reguleren de vorming van zaadcellen, terwijl granulosa cellen een groot deel van de follikel vormen waarin de eicel zich bevindt. LH is belangrijk voor de ontwikkeling van de steroidhormoon producerende cellen; de Leydig cellen in de testis en de theca cellen in het ovarium. LH stimuleert deze celtypen tot het maken van androgenen, waaronder testosteron. In het ovarium worden androgenen omgezet in oestrogenen. De werking van LH en FSH wordt waarschijnlijk beïnvloed door een aantal lokaal werkende stoffen, zoals AMH en soortgelijke groeifactoren (leden van de activine/TGFβ familie van groei- en differentiatiefactoren).

Het in dit proefschrift beschreven onderzoek omvat studies naar de regulatie van de werking van AMH en testosteron in de gonaden na de geboorte. Hierbij is vooral onderzoek gedaan aan de structuur en regulatie van de receptoren voor deze

hormonen. Receptoren zijn eiwitmoleculen die zich op of in een lichaamscel bevinden en het hormonale signaal in een cellulaire respons vertalen.

De Algemene Introductie (Hoofdstuk 1) omvat een beschrijving van de regulatie van geslachtsbepaling, geslachtsdifferentiatie, en de ontwikkeling van de gonaden, zowel voor als na de geboorte. Hierbij is extra aandacht geschonken aan de rol van AMH en testosteron bij deze processen. In Hoofdstuk 2 worden de verschillende mechanismen beschreven waarvan de hierboven beschreven hormonen gebruik maken om signaal-overdracht in cellen te bewerkstelligen. Het belang van elk van de hormonen en/of hun receptoren wordt geïllustreerd door het feit dat verandering (mutatie) in één van de genen die coderen voor deze hormonen of hun receptoren kan leiden tot ernstige verstoringen van de gonadeontwikkeling en/of geslachtsdifferentiatie.

Hoofdstuk 3 beschrijft de clonering van een stukje DNA (cDNA) dat codeert voor een kandidaat AMH type II receptor (AMHRII) in de rat. De structuur van deze receptor en de specifieke aanwezigheid van het boodschapper RNA (mRNA) dat codeert voor het AMHRII eiwit in de cellen rondom de müllerse gang en in de foetale gonaden, geven sterke aanwijzingen dat AMH inderdaad de belangrijkste activator van deze receptor is.

De aanwezigheid (expressie) van AMH en AMHRII mRNA in ovaria en testes van de rat na de geboorte is nader onderzocht in experimenten beschreven in de Hoofdstukken 4 en 5. Hierbij werd gebruik gemaakt van technieken zoals RNase protectie en *in situ* hybridizatie. In de testis komen het AMH en AMHRII mRNA specifiek tot expressie in Sertoli cellen. Dit wijst op een mogelijk autocriene werking van AMH. Kort na de geboorte neemt de hoeveelheid AMH mRNA in de testis af, terwijl de AMHRII mRNA expressie toeneemt. Dit leidt tot een hoog AMHRII mRNA niveau en een laag AMH mRNA niveau in testes van een volwassen rat. De expressie van beide genen is echter niet constant, maar hangt af van de aanwezigheid van bepaalde met de Sertoli cel geassocieerde spermatogene cellen, tijdens de spermatogenese. Veranderingen in de associatie tussen Sertoli cellen en spermatogene cellen zijn cyclisch, en er zijn 14 (I-XIV) verschillende stadia gedefinieerd in deze zogenoemde spermatogenetische cyclus. AMH en AMHRII mRNAs zijn beiden maximaal tijdens Stadium VII. Alhoewel totale afwezigheid van spermatogene cellen leidt tot een verlaging van de AMH en AMHRII mRNA expressie in de volwassen testis, bleek het niet mogelijk om een specifieke groep spermatogene celtypen te definiëren die betrokken is bij de (cyclische) regulatie van AMH en AMHRII

mRNA expressie. Mogelijk zijn vooral de structuur van Sertoli cellen en de interactie met de basale membraan, regulerende factoren voor AMH en AMHRII mRNA expressie in de Sertoli cellen.

In het ovarium van de rat komen AMH en AMHRII mRNAs voornamelijk tot expressie in de granulosa cellen in bepaalde stadia van follikelontwikkeling, in preantrale en kleine antrale follikels (Hoofdstuk 5). Tijdens de eerste twee weken na de geboorte is het expressiepatroon iets afwijkend: AMHRII mRNA is over het gehele ovarium aanwezig, terwijl de AMH mRNA expressie van zeer laag stijgt to een hoog niveau in het ovarium van een volwassen rat. Overeenkomstig met de werking in de testis, oefent AMH mogelijk ook in het ovarium een autocriene functie uit, in dit geval op de granulosa cellen. Twee hormonen, FSH en oestrogenen, bleken de expressie van AMH en AMHRII mRNAs te kunnen verlagen in ovaria van immature ratten die behandeld waren met een antagonist van het luteiniserend hormoon-releasing hormoon. Deze antagonist verlaagt de endogene FSH en LH niveaus en daardoor ook de produktie van oestrogenen. Deze resultaten suggereren dat FSH en oestrogenen mogelijk betrokken zijn bij de regulatie van AMH en AMHRII mRNA expressie in ovaria van normale ratten. Functies van AMH in het ovarium kunnen betrekking hebben op processen zoals follikelselectie en de remming van follikelmaturatie.

Hoofdstuk 6 beschrijft de clonering van de promotor van het androgeen receptor gen. De plaatsen in het DNA van de promotor waar transcriptie kan opstarten zijn vastgesteld, en tevens werd het functioneren van de promotor *in vitro* bepaald. Verder is op basis van de DNA base volgorde van de promotor onderzocht, welke gebieden betrokken zouden kunnen zijn bij de regulatie van transcriptie van het androgeen receptor gen door androgenen en door FSH.

In de Algemene Discussie (Hoofdstuk 7) wordt het mogelijke werkingsmechanisme van AMH beschreven, op basis van gegevens uit dit proefschrift en gegevens uit de literatuur omtrent het moleculaire werkingsmechanisme van andere leden van de activine/TGFβ familie van groei- en differentiatiefactoren. Verder worden de expressiepatronen van AMH, AMHRII en de androgeen receptor in de gonaden vergeleken, en besproken in het kader van regulerende interacties tussen AMH, gonadotropinen, androgenen en oestrogenen. Er worden enkele suggesties gedaan omtrent de mogelijke postnatale functies van AMH. Tot slot wordt de klinische relevantie bediscussiëerd van AMH en de AMHRII.

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Willy Maria Baarends werd geboren op 12 februari 1965 te Steenbergen (Noord-Brabant). In 1983 behaalde zij het VWO diploma aan de Scholengemeenschap Dukenburg te Nijmegen. In datzelfde jaar begon zij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. Tijdens deze studie werd bij de Afdeling Genetica van de Landbouwuniversiteit Wageningen onderzoek verricht aan succinaatdehydrogenase activiteit als parameter voor eicel-vitaliteit bij de muis (Dr. P. de Boer). Tijdens een stage bij het Department of Zoology, University of Tennessee, Knoxville, USA (Prof. Dr. Mary Ann Handel), werd een *in situ* hybridisatie techniek opgezet met als doel de expressie van genen in de testis van de muis te bestuderen. Bij het Hubrecht Laboratorium te Utrecht (Dr. O.H.J. Destrée/ Prof. Dr. S.W. de Laat) werd de structuur van verschillende genen onderzocht die een belangrijke rol spelen tijdens de ontwikkeling van *X. laevis*. In mei 1989 werd het doctoraalexamen behaald (*cum laude*).

Vervolgens was zij van mei 1989 tot juli 1994 werkzaam bij de Vakgroep Endocrinologie & Voortplanting van de Erasmus Universiteit Rotterdam, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd. Momenteel is zij bij voornoemde afdeling aangesteld als tijdelijk wetenschappelijk medewerker, en onderzoekt moleculaire aspecten van spermatogenese en mannelijke infertiliteit.

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