

## Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation

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### Summary

We describe here the isolation of cDNA and genomic clones corresponding to the mouse gene encoding anti-Müllerian hormone, and the use of these clones as molecular probes to study *AMH* gene expression. We constructed a 14.5 days *post coitum* (*dpc*) mouse fetal testes library and isolated a cDNA clone using bovine, human and rat partial cDNAs as probes. This clone contained a 1 kb insert, which was confirmed by sequencing to be the mouse homologue of *AMH*. Probes derived from the mouse cDNA clone were used to screen genomic libraries and a 12 kb DNA fragment containing the complete coding region of mouse *AMH* was isolated. *In situ* hybridisation was used to determine the precise timing and localisation of *AMH* expression in male and female embryos and postnatal testes and ovaries. *AMH* transcripts were first detected in fetal testes at 12.5 *dpc*

when differences between testes and ovaries first become visible. The signal was specific for the Sertoli cells of the testes. Other fetal tissues or female embryos were negative for *AMH* transcripts. During male development, *AMH* expression is shut off postnatally. In the female, the expression of *AMH* was first detected at day 6 after birth and is restricted to granulosa cells. We have correlated the pattern of *AMH* expression in both sexes with cellular events occurring in gonadal development and discuss some implications that this may have for its function and regulation.

Key words: anti-Müllerian Hormone, Müllerian inhibiting substance, sexual differentiation, *in situ* hybridisation, Sertoli cell, granulosa cell, testis, ovary.

### Introduction

During mammalian sex determination and differentiation, the gonads and the reproductive tract develop in fundamentally different ways. The genital ridge, which arises as a thickening on the mesonephros, is bipotential, developing as a testis if the Y chromosome is present due to the action of the testis-determining gene on the Y chromosome, *Tdy*. In the absence of the Y, it follows the default pathway giving rise to an ovary. In contrast the anlagen of the male and female reproductive tracts, the Wolffian and Müllerian ducts respectively, are both present within the mesonephroi prior to sex determination. These anlagen are unipotential and the survival and development of one *versus* the other depends on the type of gonad that differentiates. In the female, the Wolffian duct system degenerates and the Müllerian ducts give rise to the oviducts, uterus and upper vagina. This does not depend on any factors produced by the ovary and so can be considered part of the default pathway (Austin and Edwards, 1981). In the male, therefore, two processes have to occur. The Wolffian ducts have to be maintained and stimulated to differentiate into the male tract and accessory organs, the vas deferens, seminal vesicles and epididymides.

This is due to the influence of testosterone produced by Leydig cells in the testis. Additionally, the Müllerian duct system has to regress, and this is due to the action of anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS).

AMH is the first product known to be secreted by Sertoli cells in the developing testes (Tran and Josso, 1982). It is a member of the TGF- $\beta$  family of growth factors (Rentrop *et al.* 1986 for review). The carboxy-terminal part of the protein functions as a dimer (Pepinsky *et al.* 1988) through interaction with a receptor(s) that is as yet undefined. Its effect on Müllerian ducts can be reproduced *in vitro* with organ cultures of mesonephroi, and these cultures can also serve as a biological assay for the protein (Picard and Josso, 1984).

In addition to its function during Müllerian duct regression, AMH may play a role in testis differentiation itself as first suggested by observations of Freemartinism. Freemartins are masculinised female cattle embryos which are thought to arise due to the passage of AMH from a male twin *via* placental anastomoses (Jost *et al.* 1975). Freemartins not only show the absence of Müllerian duct derivatives but also exhibit masculinisation of their ovaries, namely the

formation of seminiferous tube-like structures, containing morphologically normal Sertoli cells. This effect can also be reproduced *in vitro* by exposing rat ovaries to either testis tissue or purified AMH (Vigier *et al.* 1987; Charpentier and Magre, 1990), or in transgenic mice overproducing human AMH (Behringer *et al.* 1990). It has been attributed to the action of AMH either directly on the somatic cells of the developing gonad, or indirectly by the elimination of germ cells entering meiosis (McLaren, 1990), since in females germ cells are thought to be necessary to ensure the normal differentiation and maintenance of follicle cells. In the complete absence of germ cells, ovaries fail to develop (Merchant, 1975; Merchant-Larios and Centeno, 1981).

In contrast to the masculinising effects described above, AMH may also have a normal role in females. Its activity can be detected in follicular fluid of the adult ovary, and immunohistochemistry suggests that it is produced by follicle cells (Vigier *et al.* 1984; Takahashi *et al.* 1986; Bezar *et al.* 1987). Its function here is not understood, but it is clear that the timing and dosage of AMH expression in the ovary is crucial, otherwise it could exert a masculinising effect on the female reproductive tract or ovaries themselves.

In order to understand more about AMH gene function in both male and female development, and as a preliminary to study its regulation, it is important to establish the precise timing and location of AMH transcription. We have decided to undertake this analysis using the mouse as a model system to facilitate future genetic manipulation. In this report, we describe the cloning and sequencing of the mouse AMH gene and present a detailed analysis of its embryonic and postnatal expression using *in situ* hybridisation. We also discuss the timing of AMH expression with respect to cellular events occurring in both male and female gonad development.

## Materials and methods

### Mouse stocks

Embryos and tissues for RNA isolation and *in situ* hybridisation studies were prepared from Parkes outbred mice and from mice homozygous for the extreme allele of the dominant white spotting mutation ( $W^c$ , Mintz and Russell, 1957). The  $W^c/W^c$  genotype of 14.5 days *post coitum* (dpc) embryos was confirmed by liver morphology (Borghese, 1959). The day of vaginal plug was taken to be 0.5 dpc, and the morphology of the hindlimb bud served as a reference for staging the embryo. Embryos at 11.5 dpc were sexed by staining for sex chromatin in amnion cells (Burgoyne *et al.* 1983).

### Probes and genomic libraries

The partial bovine and human cDNA clones (Picard *et al.* 1986) were obtained from Nathalie Josso (INSERM, Paris) and the partial rat cDNA clone was obtained from Richard Cate (Biogen, Boston). The mouse genomic libraries were obtained from A. M. Frischauf (ICRF, London) and Keith Willison (Chester Beatty, London).

### RNA isolation and northern analysis

Genital ridges and male and female gonads were dissected

from 11.5 dpc and 12.5 to 17.5 dpc embryos, respectively. Total RNA was isolated as described (Auffray and Rougeon, 1980). Tissues were homogenized in 6 M urea, 3 M LiCl. After overnight incubation at 4°C, the homogenate was centrifuged and the pellet obtained was washed twice in the same solution. The pellet was resuspended in 10 mM Tris pH 7.5, 0.5% sodium dodecyl sulphate (SDS) containing 50 µg ml<sup>-1</sup> proteinase K and incubated for 30–120 min at 37°C. After a number of phenol extractions, total RNA was ethanol precipitated and resuspended in water.

Total RNA (10 µg) was loaded on 1% agarose-formaldehyde gels, electrophoresed at 40 mA in 1×MOPS buffer and transferred to Hybond-N filters as described by Maniatis *et al.* (1982). The filters were baked at 80°C for 30 min and UV-crosslinked. Probes were labelled with <sup>32</sup>P using a multiprime labelling kit (Amersham) according to the manufacturer's instructions, denatured by heating, added to the filters in 7% SDS, 0.5 M sodium hydrogen phosphate pH 6.5, 1 mM EDTA and hybridised for 12–16 h at 65°C. Filters were washed at 65°C in 40 mM NaP, pH 6.5, 1% SDS and exposed to Kodak XAR film for 7 days.

### cDNA library construction and screening

Total RNA was prepared from approximately 500 pairs of testes, collected from 14.5 dpc embryos, giving a yield of 500 µg. Poly(A)<sup>+</sup>RNA was prepared using Hybond mAP paper (Amersham) according to the manufacturer's instructions. 1 µg poly(A)<sup>+</sup>RNA from 14.5 dpc fetal testes served as starting material for the synthesis of cDNA which was ligated into a bacteriophage lambda gt10 vector, using cDNA synthesis and cloning kits (Amersham). The resulting library contained 1×10<sup>6</sup> recombinant lambda clones of which approximately 200 000 were plated in 0.7% top agarose containing 10 mM MgSO<sub>4</sub> after adsorption to plating bacteria (*E. coli* strain NM538, Frischauf *et al.* 1983) for 15 min at 37°C. Plating bacteria were prepared by resuspending an overnight culture of NM538 in ice-cold 10 mM MgSO<sub>4</sub>. Phage DNA was transferred to Hybond-N filters, denatured in 0.5 M NaOH, 1.5 M NaCl, neutralised in 40 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> (NaP, pH 6.5), baked, UV crosslinked, and hybridised as follows: probes from partial cDNAs of human, bovine (Cate *et al.* 1986; Picard *et al.* 1986) or rat AMH were labelled with <sup>32</sup>P using a multiprime labelling kit (Amersham) according to the manufacturer's instructions, denatured by heating, added to the filters in 7% SDS, 0.5 M sodium hydrogen phosphate pH 6.5, 1 mM EDTA and hybridised for 12–16 h at 65°C. Filters were washed at 65°C in 40 mM NaP, pH 6.5, 1% SDS and exposed to Kodak XAR film. Positive clones were picked, replated and rescreened until a pure population was obtained.

### Sequencing

The entire coding region of the mouse AMH gene was sequenced using a random sequencing approach. A 2.5 kb *Sma*I fragment from the mouse AMH genomic clone XY-1 and the mouse AMH cDNA pBAM5 (see results), were self ligated and then sonicated on ice for 4×1 min. The ends were repaired using T4-polymerase and Klenow fragment (Boehringer). The DNA was separated by electrophoresis through a 1.5% agarose gel, and three different size fractions were isolated (200–400 bp, 400–600 bp, >600 bp) and cloned into M13mp19 (New England Biolabs) linearized with *Sma*I. After transformation into *E. coli* strain TG-1 (Gibson, 1984) recombinant M13 plaques were isolated. Single-stranded DNA was prepared and sequenced using the dideoxy chain termination method (Sanger *et al.* 1977) using T7 DNA polymerase (Pharmacia). Both strands were sequenced at

least once. The sequences were aligned using Staden-Plus software (Staden, 1980), and analysed using the University of Wisconsin Genetics computer group software package (Devereux *et al.* 1984).

#### In situ hybridisation

Transcription reactions were performed *in vitro* to generate sense and antisense RNA probes, labelled with  $^{35}\text{S}$ -UTP. The mouse *AMH* cDNA clone pBAM5 was linearized with *Pvu*II to serve as template for T7 RNA polymerase (Promega) giving rise to an *AMH* antisense RNA probe. To obtain the sense RNA probe, the plasmid was digested with *Xba*I and transcribed with T3 RNA polymerase (Promega). The transcription was performed in 20  $\mu\text{l}$  volume at 37°C for 1 h. The reaction mixture contained 40 mM Tris-HCl pH 8.25, 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM DTT, 0.25 mM each of GTP/ATP/CTP ribonucleotides; 50 units RNasin (Promega), 1  $\mu\text{g}$  DNA template and 5 units of either T3 or T7 RNA polymerase. The DNA template was removed by digestion with 40 units of RNAase-free DNAase I (Boehringer), for 15 min at 37°C. The RNA transcripts were hydrolysed to an average length of 0.1 kb in 40 mM NaHCO<sub>3</sub>/60 mM Na<sub>2</sub>CO<sub>3</sub>, at 60°C for  $t$  min (where  $t = (L - 0.1) / 0.011 \times L$ ; L = original probe length in kb; Cox *et al.* 1984). After neutralizing with an equal volume of 10% acetic acid, 1  $\mu\text{l}$  of yeast tRNA (10 mg ml<sup>-1</sup>) was added and the RNA probe was separated from unincorporated nucleotides by fractionation over a Sephadex G50 column (column buffer: 10 mM Tris-HCl, 1 mM EDTA pH 7.5, 10 mM DTT, 0.1% SDS). Fractions (200  $\mu\text{l}$ ) were collected and counted by liquid scintillation. The first peak was pooled and precipitated on dry ice by adding 0.75 volumes of 4 M ammonium acetate and 2.5 volumes ethanol. The pellet was dissolved in 10 mM DTT at a concentration of  $1 \times 10^6$  cts min<sup>-1</sup>  $\mu\text{l}^{-1}$ . After addition of 9 volumes hybridisation buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA pH 8.0, 10% dextran sulphate, 1xDenhardt's solution, 0.5 mg ml<sup>-1</sup> yeast RNA) the probes were stored at -70°C.

Embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C, and then successively incubated for at least 30 min in PBS (twice at 4°C), 50% ethanol/50% PBS, 70%, 70% (overnight, 4°C), 85%, 96% and 100% ethanol (twice). Next, the embryos were transferred through HistoClear (National Diagnostics, twice for 1 h) and paraffin wax (three changes of 25 min each at 60°C using Fibrowax, BDH). The embryos were orientated in a glass embryo dish under a dissection microscope before allowing the wax to set. Sections 5- or 7  $\mu\text{m}$  were mounted on slides treated with 3-aminopropyl ethoxysilane (TESPA; Rentrop *et al.* 1986).

Wax was removed from mounted sections by two 10 min treatments in HistoClear and the sections rehydrated through an ethanol series, and postfixed in 4% paraformaldehyde in PBS for 20 min. The sections were treated for 5 min with 10  $\mu\text{g}$  ml<sup>-1</sup> proteinase K, the postfixation was repeated and the slides were treated for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. Then the slides were dehydrated through an ethanol series and air-dried. Hybridisation under coverslips using  $^{35}\text{S}$ -labelled anti-sense or sense control RNA probes ( $2 \times 10^5$  cts min<sup>-1</sup>  $\mu\text{l}^{-1}$ ) was carried out at 55°C overnight in a moist chamber. The coverslips were removed in 5xSSC, 10 mM DTT at 55°C for 1 h and the slides subjected to a high-stringency wash in 50% formamide, 2xSSC, 10 mM DTT at 65°C for 30 min. The slides were then washed in 0.5 M NaCl, 10 mM Tris/HCl, pH 8.0, 10 mM EDTA prior to incubation at 37°C for 30 min with 20  $\mu\text{g}$   $\mu\text{l}^{-1}$  ribonuclease A in the latter buffer. The high-

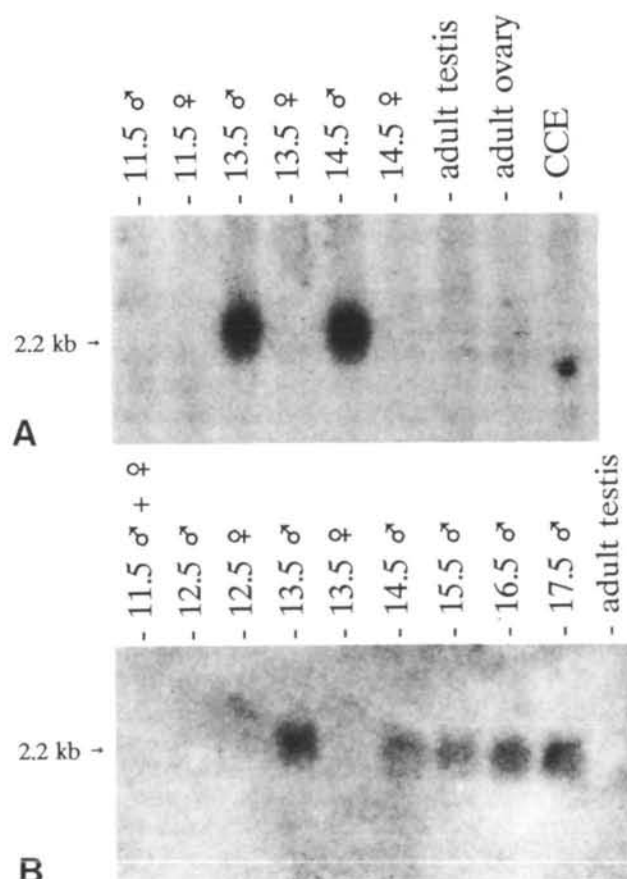
stringency washing was repeated once more before the slides were dehydrated through an ethanol series and air-dried.

The slides were dipped in Ilford K5 nuclear track emulsion diluted 1:1 with 2% glycerol at 42°C, and dried slowly at room temperature for 4 h before exposure at 4°C. Typically, slides were exposed for 6 days and then developed in Kodak D19 developer (2 min), rinsed in 1% acetic acid/1% glycerol (1 min) and fixed in 30% sodium thiosulphate (2 min). Following rinsing in H<sub>2</sub>O (twice for 10 min), the sections were stained with toluidine blue (0.02% solution, 1.5 min) and mounted using Permount (Fisher Scientific).

## Results

### Cloning of mouse *AMH* cDNA and genomic sequences

Screens of genomic libraries using partial bovine and human cDNAs as probes yielded no mouse *AMH*



**Fig. 1.** Northern blot analysis of *AMH* expression in mice. Total RNA from male (♂) and female (♀) gonads from 11.5 to 17.5 dpc, adult testis and ovaries and an embryonic stem cell line CCE, was used. (A) Filter probed with the human partial *AMH* cDNA showing a hybridizing band of 2.2 kb in 13.5 and 14.5 dpc testes. (B) Filter probed with the mouse cDNA clone pBAM5. A 2.2 kb band was detected from 13.5 to 17.5 dpc testes. The presence of approximately equal amounts of RNA in each lane was confirmed by ethidium bromide staining of the agarose gel before transfer to nylon membrane.

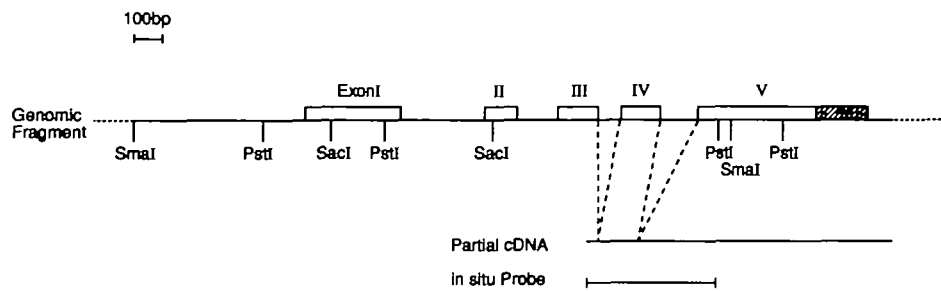


Fig. 2. Restriction map of a genomic fragment of XY-1. The positions of exons I to V and restriction enzyme recognition sites for *SmaI*, *PstI* and *SacI* are indicated. The homologies to the TGF- $\beta$  family of growth factors are found in the area which is shaded. The extent of the partial cDNA is shown; the exon-intron boundaries are indicated by stippled lines. The fragment that was used to derive sense and anti-sense RNA probes for *in situ* hybridisation experiments is indicated.

clones. Genomic Southern analysis suggested (data not shown) that the mouse gene was fairly divergent from the probes used, and that other sequences in the genome crosshybridized. This was probably due to the GC-rich nature of the probes. To overcome this problem, we decided to construct a cDNA library from an appropriate stage of mouse testis development. To define the stage of peak levels of *AMH* transcripts, we performed northern analysis using the human probe on mouse RNA samples (Fig. 1A). *AMH* transcripts were first detected in embryonic testes at 13.5 *dpc* and the level of expression appeared to be similar at 14.5 *dpc*. No transcripts were found in developing ovaries or adult testes or ovaries. A cDNA library was constructed, using poly(A)<sup>+</sup> RNA from 14.5 *dpc* fetal testes. This cDNA library was screened using the bovine, human and rat *AMH* probes independently on triplicate filters. By this strategy, eight cDNA clones that hybridized to all three probes were isolated. The longest of the phage clones contained a 1 kb *EcoRI* insert. This was subcloned into Bluescript KS- (Stratagene) and named pBAM5. When probes derived from pBAM5 were used on a northern blot, they showed the expected pattern of *AMH* expression (Fig. 1B). Transcripts were found in fetal testes from 13.5 *dpc* up to 17.5 *dpc*, the last fetal stage examined. The hybridizing band was approximately 2.2 kb as expected for *AMH* by analogy with other species and was not detected in fetal ovaries.

The cDNA clone pBAM5 was used to derive probes for screening two genomic mouse libraries. Five independent phage clones were isolated. These fell into two different classes, which were largely overlapping. One of the phage clones, called XY-1, contained a 12 kb *SalI* insert. This was subcloned into Bluescript KS- and a detailed restriction map was derived, part of which is shown in Fig. 2.

#### Sequence analysis

From Southern analysis, the pBAM5 cDNA clone was shown to hybridize to a 2.5 kb *SmaI* fragment of XY-1 (data not shown). This fragment together with the pBAM5 cDNA were sequenced. It was established that the partial cDNA is missing the 5' end and starts within the third exon. The genomic clone contains all five

exons; exon-intron boundaries have been defined by comparison to the cDNA and the human gene (Cate *et al.* 1986). The start site of transcription has not been defined but a putative TATA-box is indicated (Fig. 3). This TATA-box is in a similar position to the one that was defined by S1 mapping in the bovine gene (Cate *et al.* 1986) and the human gene (Guerrier *et al.* 1990). The predicted amino acid sequence of the mouse protein was derived and compared to the bovine and human proteins (Fig. 4). The analysis showed an overall homology of 68% between mouse and human and 70% between mouse and bovine proteins. The carboxy terminal part of the proteins are extremely highly conserved with only 5 (mouse/human) and 6 (mouse/bovine) amino acid changes within the last 106 residues. This equals a 95% and 94% homology, respectively. Two possible N-glycosylation sites have been conserved in all three species. The cysteine residues and adjacent amino acids that are conserved within the TGF- $\beta$  family of growth factors (Cate *et al.* 1986) are also present in the mouse *AMH* protein.

#### *In situ* hybridisation studies of *AMH* expression

The sensitive method of *in situ* hybridisation was used to look precisely at the distribution of *AMH* transcripts in the developing embryo and in postnatal testes and ovaries. This technique allows the examination of many tissue types at once. In addition, it is possible to identify the cell type expressing the gene in complex tissues. The mouse *AMH* probe used in this study is described in Fig. 2.

*AMH* transcripts were not detected in 11.5 *dpc* genital ridges (data not shown), but were clearly present at high levels in 12.5 *dpc*, 13.5 *dpc* (data not shown) and 14.5 *dpc* testes (Fig. 5). The distribution of the signal over the testes suggested that it is the Sertoli cells of the testis that express *AMH*. No signal was found in the germ cells, which are surrounded by the cytoplasm of Sertoli cells. These are aligning into testis cords at this stage of development. No specific signal was seen outside the testis cords where interstitial cells and Leydig cells are found, or in any other tissue in the embryo (Fig. 6). Furthermore *AMH* transcripts were not detected in embryonic ovaries or in any other fetal





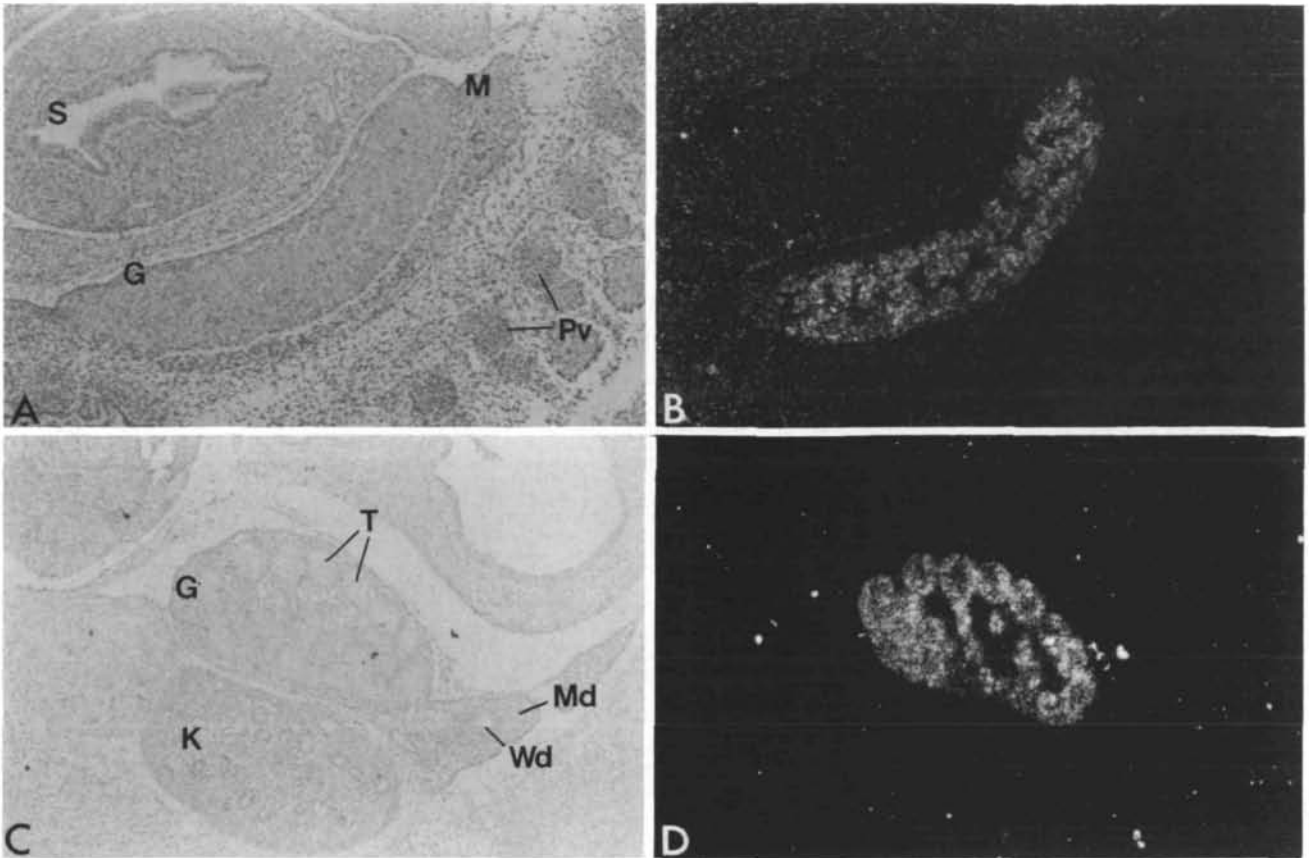


Fig. 5. *In situ* hybridisation to sections of 12.5 dpc (A,B) and 14.5 dpc (C,D) mouse testes using an anti-sense RNA probe specific for the mouse *AMH* transcripts. Bright-field illuminations (A,C) and dark-field illuminations (B,D) respectively. K, kidney; G, gonad; M, mesonephros; Md, Müllerian duct; S, stomach; Pv, pre-vertebrae; Wd, Wolffian duct.

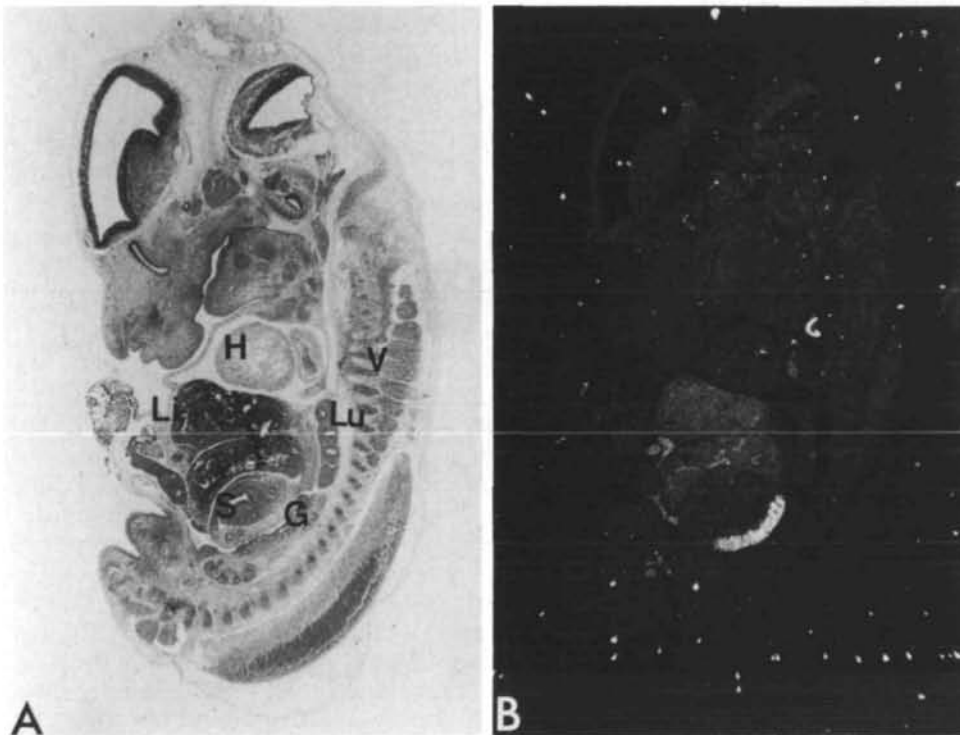


Fig. 6. *In situ* hybridisation to a section of a 12.5 dpc embryo. (A) Bright-field illumination. (B) Dark-field illumination. G, gonad; H, heart; Li, liver; Lu, lung; V, vertebrae.

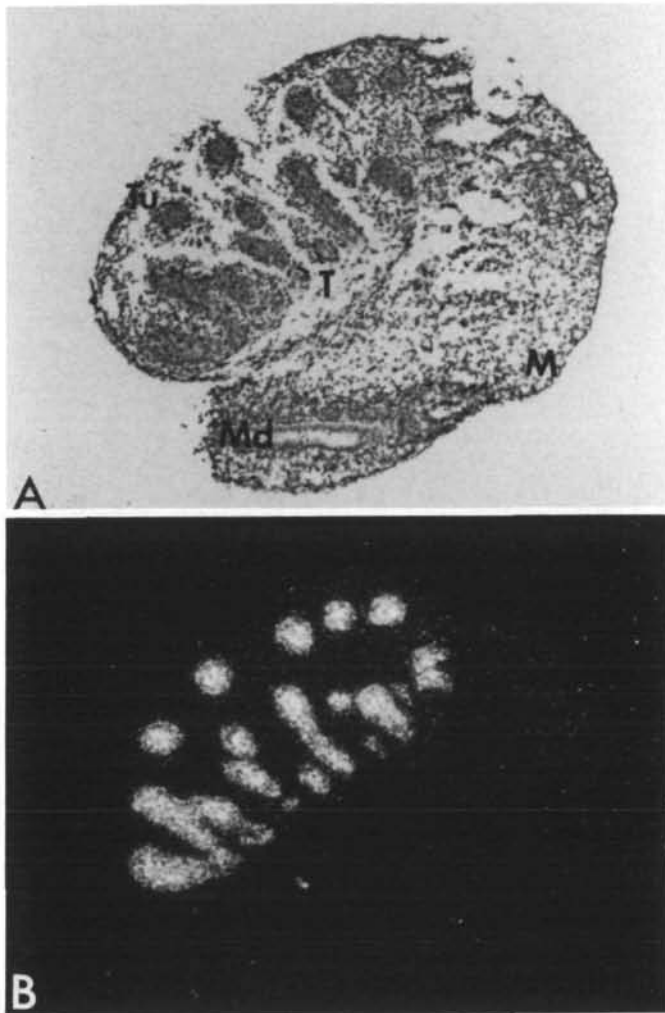


Fig. 7. *In situ* hybridisation to a section of a 14.5 dpc  $W^c/W^c$  testis. (A) Bright-field (B) dark-field illuminations. M, mesonephros; Md, Müllerian duct; T, testis cords; Tu, tunica albuginea.

growth of the oocyte in medium-size follicles (stages 3b, 4 and 5a), which are found in the centre of the early postnatal ovary, whereas the small follicles are found mainly in the periphery. It was clearly the granulosa cells in the growing follicles that were expressing *AMH*. No silver grains were detectable over theca cells or the oocyte itself. Whilst maturing, follicles migrate from the centre of the ovary to its periphery. The oocyte in these follicles is still arrested in meiosis but is increasing in size and transcribing many genes. It is surrounded by many layers of granulosa cells and *AMH* transcripts were evenly distributed over all the granulosa cells. In large antral follicles (stage 6 and 7) *AMH* was still expressed at high levels. These follicles contain a fully grown oocyte, which is arrested in the first meiotic division, and a cavity filled with follicular fluid. This cavity divides the granulosa cells into two distinct populations: some layers at the periphery of the follicle and some around the oocyte. *AMH* transcripts were clearly present in granulosa cells surrounding the oocyte; however, the signal was significantly lower in

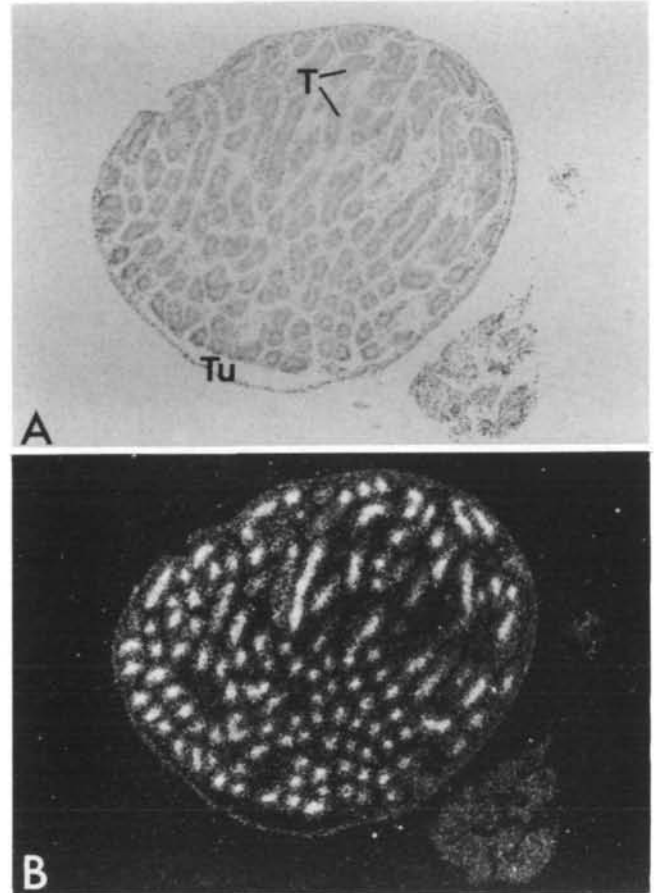


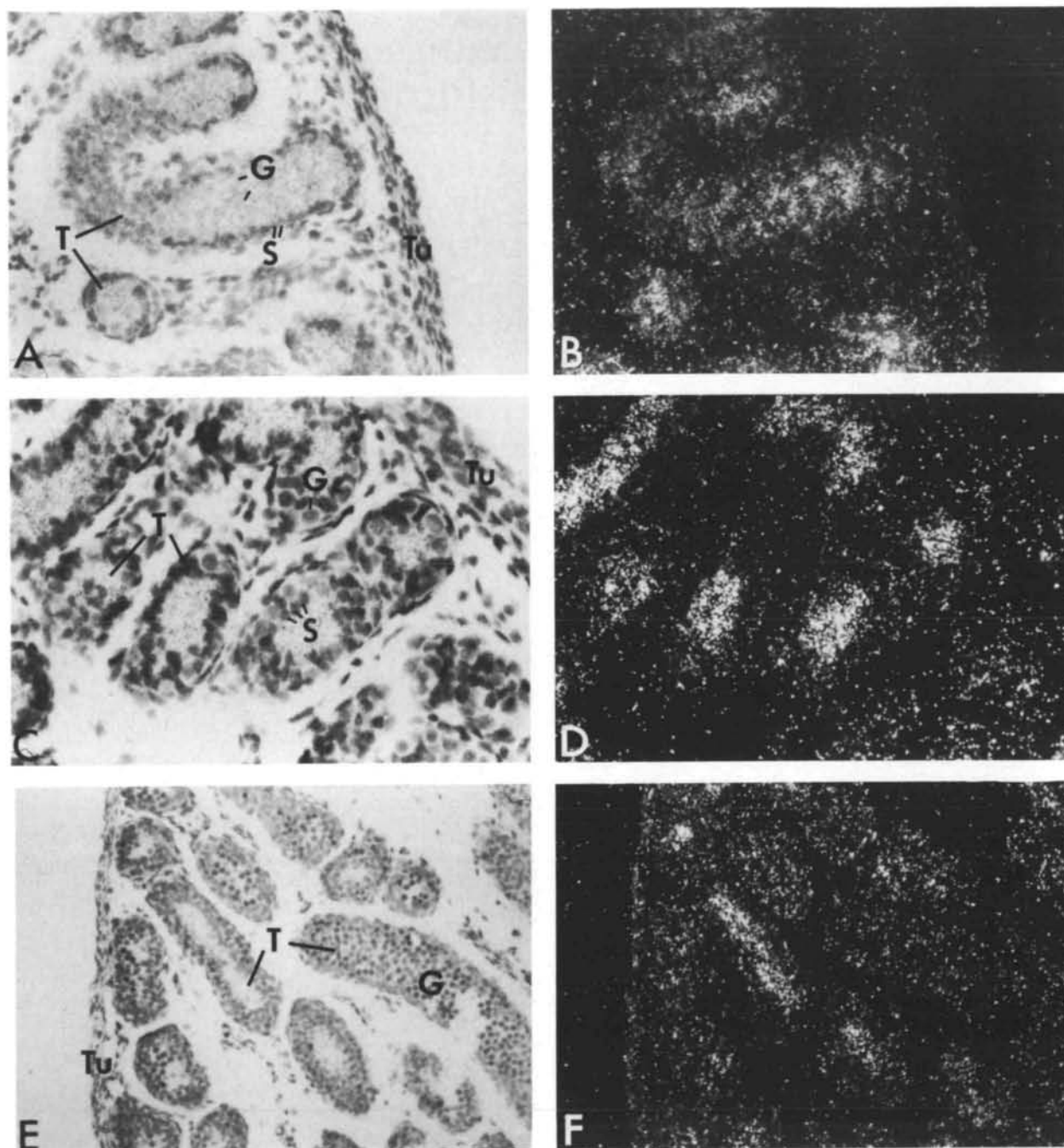
Fig. 8. *In situ* hybridisation to a section of a 7 day testis. (A) Bright-field and (B) dark-field (b,d) illuminations. T, testis cords; Tu, tunica albuginea.

the granulosa cells in the periphery of the antral follicles (Fig. 10E,F). This result is in agreement with immunohistochemical studies in adult sheep ovaries where the authors describe heterogeneity in the distribution of *AMH* protein in the granulosa cell population (Bezard *et al.* 1987). Just before ovulation, when the oocyte completes the first meiotic division and becomes a secondary oocyte *AMH* transcripts are no longer detectable (Fig. 10E,F).

### Discussion

To help understand the role of *AMH* in sex differentiation, we wished to look precisely at the timing and the sites of *AMH* expression during male and female development. Previous biochemical and immunohistochemical experiments had shown that *AMH* protein is found in Sertoli cells and in testicular and follicular fluid (Tran and Josso, 1982; Vigier *et al.* 1984). However, we were interested in determining when *AMH* is first transcribed during testis formation and whether the Sertoli cell is the only site of expression in male embryos. We wanted to clarify further the timing of *AMH* expression in the female. It was hoped that correlating *AMH* expression with cellular events in the





**Fig. 9.** *In situ* hybridisation to a section of a 1 day postnatal mouse testis (A,B), a 7 day mouse testis (C,D) and a 14 day mouse testis (E,F). Bright-field (A,C,E) and dark-field (B,D,F) illuminations, respectively. G, germ cells; T, testis cord; S, Sertoli cell; Tu, tunica albuginea.

developing gonads would shed some light on how *AMH* functions.

Using *in situ* hybridisation, we have shown that *AMH* transcripts are present in the Sertoli cells of the testis when they first differentiate at 12.5 *dpc*. At this time testis and ovaries show the first morphological differences due to the Sertoli cells beginning to align into testis cords. Within the next few days of development, the effect of *AMH* on the epithelial cells of the Müllerian ducts becomes visible and they degenerate. If

*AMH* is not expressed in the male at the right time during gestation the Müllerian duct persists (Guerrier *et al.* 1989).

These studies also confirmed that the Sertoli cells are the only site of expression in male embryos. Extragonadal sites were negative for *AMH* mRNA. *In situ* hybridisation to 14.5 *dpc*  $W^e/W^e$  testis (Fig. 6) confirmed the localisation of *AMH* transcripts to Sertoli cells, and demonstrated that expression of *AMH* is independent of gene action in the male germ cells.

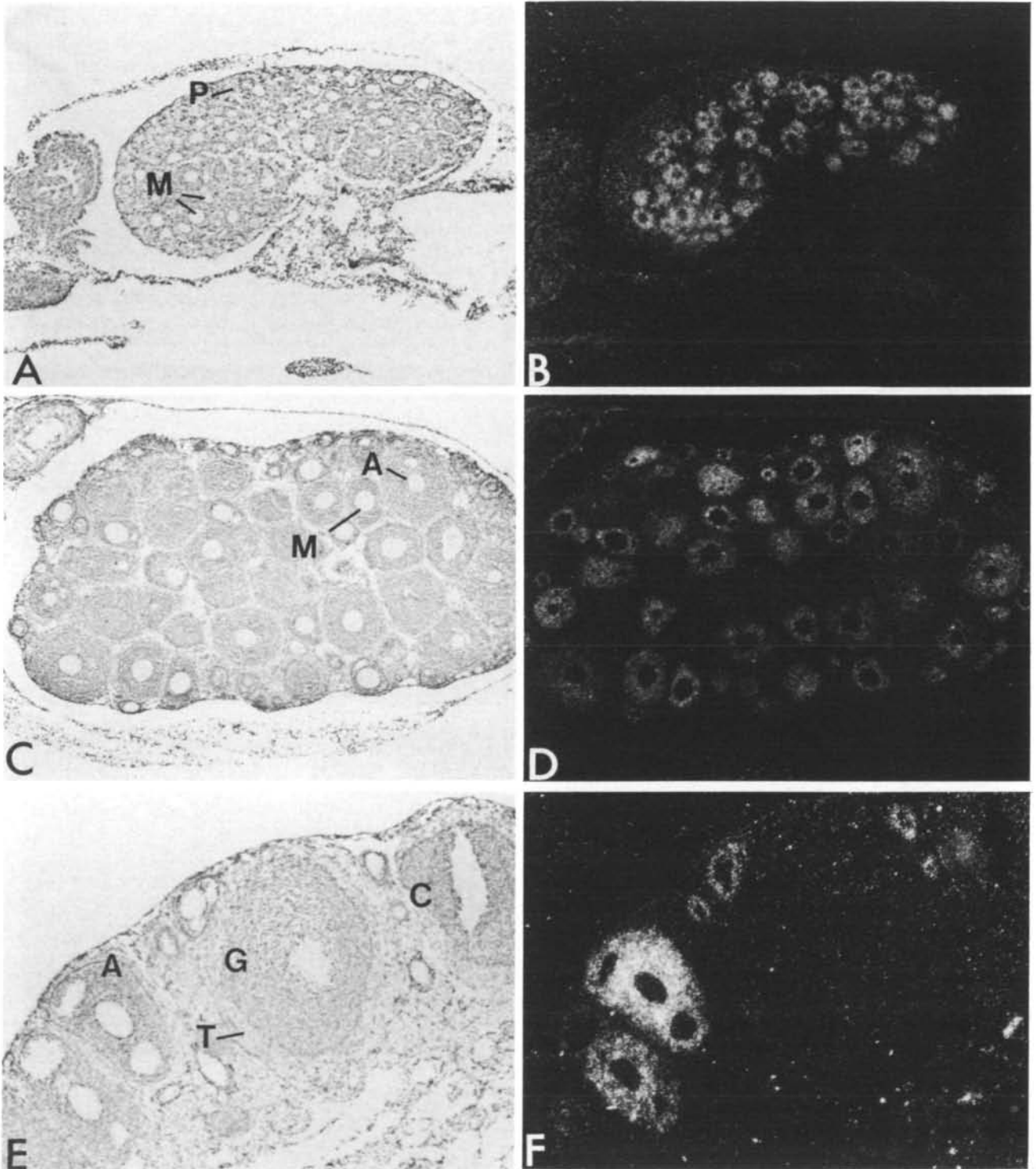


Fig. 10. *In situ* hybridisation to a section of a 6 day mouse ovary (A,B), a 3 week mouse ovary (C,D) and a 7 week mouse ovary (E,F). Bright-field (A,C,E) and dark-field (B,D,F) illuminations, respectively. A, antral follicle; C, corpus luteum; G, Graafian follicle (contains secondary oocyte); M, medium size (=growing) follicle; P, primordial follicle; T, theca cells.

The level of *AMH* expression in the Sertoli cells remains roughly constant until several days after birth. As regression of the Müllerian ducts is completed during fetal life, the persistence of *AMH* expression implies additional functions for *AMH*. During later fetal stages of testis development, the Sertoli cells

continue to differentiate and form seminiferous tubules harbouring the maturing germ cells in their lumen. In the first stages of spermatogenesis, the germ cells undergo multiple rounds of mitosis, before the first generation enters meiosis. It is interesting to note that *AMH* expression is switched off between 2 and 3 weeks

after birth, coincident with the first wave of spermatids undergoing meiosis. *AMH* may interfere with male germ cells entering and completing meiosis. A possible function of *AMH* in fetal testes might be to eliminate any germ cells that enter meiosis precociously despite the inhibiting effect of the testis cords (McLaren, 1990). Evidence from female transgenic animals ectopically expressing human *AMH* at very high levels supports this idea. Behringer *et al.* (1990) found that the ovaries of these transgenic mice had fewer germ cells at birth than normal. The oocytes were then lost during the next few weeks and the somatic cells developed as structures resembling testis cords. This suggests that timing, site and levels of *AMH* expression are very important. Early in development, and at high levels, *AMH* appears to be toxic for germ cells in meiosis (Vigier *et al.* 1989). However, at moderate concentrations and at a later stage in development, *AMH* may be required to keep the oocyte in meiotic arrest as it starts growing. The onset of *AMH* expression in the female is correlated with the onset of oocyte growth postnatally. *AMH* transcripts are only detected in granulosa cells and expression continues through maturation of the follicle until the oocyte completes the first meiotic division just before ovulation. A better understanding of *AMH* function will be achieved by introducing mutations into the gene by homologous recombination.

It has been suggested that Sertoli cells and granulosa cells differentiate from the same precursor cell lineage (Vigier *et al.* 1984). Both types of cells carry a common lineage-specific surface antigen (Cicarese and Ohno, 1978), form a diffusion barrier shielding germ cells from the somatic environment, and release their secretion products into a fluid that bathes germ cells during normal maturation. The fact that both Sertoli and granulosa cells express *AMH* supports this assumption. However, the transcriptional regulation in both cases must be different since expression in the female occurs with a significant time delay, and strictly controlled expression is very important in order to achieve appropriate male and female gonadal and genital tract development.

The pattern of *AMH* expression allows us to speculate on its possible regulation. It is clear that the presence of *AMH* is absolutely required to give rise to the complete male phenotype, and it has been thought for a long time that *AMH* is a very likely target for direct regulation by the Y-linked testis-determining gene *Tdy*. *Tdy* is thought to be autonomously expressed in Sertoli cells (Burgoyne *et al.* 1988; Palmer and Burgoyne, 1991), and a candidate gene for *Tdy* known as *Sry* has recently been cloned (Gubbay *et al.* 1990; Sinclair *et al.* 1990). This has proven to be sufficient to cause sex-reversal in transgenic mice (Koopman *et al.* 1991). The *Sry* gene product contains a putative DNA-binding domain. *Sry* is expressed in the somatic portion of the genital ridge just prior to testis differentiation from 10.5 *dpc* to 12.5 *dpc* (Koopman *et al.* 1990). *AMH* transcripts were first seen using *in situ* analysis at 12.5 *dpc*. The timing and location of expression of the two genes so far does not exclude the possibility of *AMH*

being a target for regulation by *Sry*. However, the delay of 48 h between the first appearance of *Sry* and *AMH* transcripts suggests an indirect rather than a direct interaction. *In vitro* studies can be used to distinguish these possibilities. If *Sry* does play a role in inducing *AMH* expression in male embryos, it is certainly not required for its maintenance, since *Sry* expression is switched off at 12.5 *dpc* whereas *AMH* continues to be expressed.

During embryonic development of the female, it is very important that *AMH* expression is not induced. One way to ensure the silencing of *AMH* in females would be the lack of an essential transcription factor, for example *Tdy*. However, *AMH* seems to be required at a later stage in female development and the inductive function of *Tdy* may be substituted by a factor released by the oocyte. A palindromic element that is nearly identical to the consensus sequence of estrogen response elements (*ERE*) has been identified upstream of the transcriptional start site of the human *AMH* gene (Guerrier *et al.* 1990). The authors conclude from *in vitro* studies that this element may play a regulatory role in granulosa cells. The cloning of mouse *AMH* will now allow *in vivo* analysis of regulatory elements in transgenic mice.

We thank A. McLaren and M. Buehr for allowing us the use of *W<sup>c</sup>* mouse stocks, B. Skene for help in initiating the project, D. Wilkinson for assistance with *in situ* hybridisation, R. Buxton for help with the compilation of the sequence data, P. Koopman, J. Gubbay, B. Capel and D. Guerrier for helpful comments on the manuscript and our colleagues for discussion and encouragement. We thank N. Josso and R. Cate for probes and A. M. Frischauf and K. Willison for genomic libraries. A. M. is supported by the Gottlieb Daimler and Carl Benz Stiftung.

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(Accepted 12 July 1991)