Early Expression of AMH in Chicken Embryonic Gonads Precedes Testicular SOX9 Expression

EMMANUELLE OREAL,1 CLAUDE PIEAU,2 MARIE-GENEVIEVE MATTEI,2 NATHALIE JOSSO,1 JEAN-YVES PICARD,1 DANIELE CARRE-EUSEBE,1,4 AND SOLANGE MAGRE4

1Unité de Recherches sur l’Endocrinologie du Développement, INSERM U493, Ecole Normale Supérieure, Montrouge, France
2Laboratoire de Biochimie du Développement, Institut Jacques Monod, CNRS, Université Paris VII, Paris, France
3Laboratoire de Génétique Médicale et Développement, INSERM U406, Faculté de Médecine, Marseille, France
4Laboratoire de Physiologie de la Reproduction, CNRS URA 1449, Université Paris VI, Paris, France

ABSTRACT

In mammals, anti-Müllerian hormone (AMH) is produced by Sertoli cells from the onset of testicular differentiation and by granulosa cells only after birth. SOX9, a transcription factor related to the testis-determining factor SRY, is expressed in mouse testis 1 day before AMH. To determine the relationship between AMH and SOX9 in birds, we cloned the AMH promoter in search of SOX9 response elements, and we compared the expression of AMH and SOX9 in the gonads of chick embryos using in situ hybridization. Potential SOX response elements were found in the AMH promoter; however, AMH is expressed in both sexes at stage 25, 1 day before the first SOX9 transcripts appear in the male gonads. SOX9 is never expressed in the female. These results do not support the hypothesis that SOX9 could trigger the expression of testicular AMH in the chick but does not exclude a later role in testis development. Dev. Dyn. 1998;212:522–532.

Key words: chick embryo; AMH; MIS; SOX9; testis; ovary; sex differentiation

INTRODUCTION

Mammals and birds both have a chromosomal mechanism of sex determination, but the mechanisms differ in their implementation. In mammals, the male is heterogametic; the genetic switch inducing testis differentiation is located on the Y chromosome and has been equated to the SRY gene, which belongs to a family of transcription factors characterized by the presence of a high mobility group (HMG) box motif (reviewed in Greenfield and Koopman, 1996). In birds, the female is the heterogametic sex, and no homolog to SRY has been detected in genomic DNA. Birds, however, do express genes belonging to the HMG family, the so-called SOX genes (for SRY-like box), which are autosomal and share high structural and functional homology with SRY (Griffiths, 1991).

The SOX family of proteins appears to bind to the minor groove of the (A/T)(A/T)CAAAG motif (reviewed in Prior and Walter, 1996). Binding induces a sharp bend into DNA, which might promote association of other transcription factors. This is important because otherwise it is not clear how SOX genes could specifically activate their targets through a short, relatively ubiquitous, motif. Furthermore, in vitro experiments involving HMG proteins do not necessarily reflect the true in vivo situation (Lovell-Badge and Hacker, 1995). For instance, Sry significantly activates the transcription of a cotransfected reporter gene containing portions of the Fra1 promoter (Cohen et al., 1994), yet Fra1 is not expressed at any developmental stage of testis differentiation, proving that it is not a downstream target of Sry in the genetic cascade leading to testis differentiation (J eske et al., 1996).

In this context, great emphasis has been put on expression studies. Obviously, whatever the outcome of in vitro experiments, the product of a SOX, or indeed any other, gene cannot regulate another if it is not transcribed first in the appropriate setting. In mammals, one of the couples that has attracted the most attention is composed of SRY and anti-Müllerian hormone (AMH). AMH, also called Müllerian inhibiting substance (MIS), a member of the transforming growth factor-ß superfamily (Cate et al., 1986), induces the regression of Müllerian ducts in male fetuses and is subjected to tight developmental regulation (Münsterberg and Lovell-Badge, 1991). The mammalian AMH promoter contains a degenerated SRY response element, but in vitro experiments seeking to establish its involvement have yielded conflicting results (Haqq et al., 1994; Shen et al., 1994). In the mouse, testicular Amh is turned on 20 hours after the onset of Sry expression, at a time when Sry transcripts are at their peak. This result is formally compatible with a direct regulation of Amh by Sry, but the 20-hr delay leaves room for intervening steps (Hacker et al., 1995).

SRY is not, however, the best choice for an AMH regulator common to all vertebrates. It exists only in mammals (Graves, 1995) in contrast to AMH, which is present also in birds and reptiles (di Clemente et al., 1992). SOX genes have been conserved during evolution (Coriat et al., 1993) and are expressed in many...
developing, mostly neural, tissues but one, SOX9, closely related to SRY, in addition to its role in chondrogenesis, is expressed in the developing testis of mammals and chicken embryos (Kent et al., 1996; Morais da Silva et al., 1996; McBride et al., 1997). In human, its mutation leads to sex reversal in XY patients affected with campomelic dysplasia, a severe form of chondrodysplasia (Foster et al., 1994; Wagner et al., 1994). SOX9 has been considered for the role of AMH regulator in the mouse and would also be an attractive candidate in the chick (Morais da Silva et al., 1996). Having recently cloned the chick AMH cDNA (Carré-Eusèbe et al., 1996), we have used the probes at our disposal to clone the chick AMH promoter in search of a potential SOX9 response element and to localize the chick AMH gene on chromosomes. In parallel, to investigate a potential relationship between AMH and SOX9, we compared the early stages of expression of AMH and SOX9 in male and female embryonic gonads. The nucleotide sequence reported in this paper has been submitted to the Genbank/EMBL Data Bank with accession number AJ 225014.

RESULTS
Cloning and Nucleotide Sequence Analysis of the 5′ Flanking Region of the Chick AMH Gene

1.6 × 10^6 clones from a chick genomic DNA library were screened for AMH gene recombinants, using a labeled probe corresponding to the whole AMH cDNA. Nine independent positive clones were studied. Their identical restriction maps suggest that they are multiple isolates of the same recombinant phage containing 2.25 kb of the 5′ flanking region, 4.2 kb of the gene, and 6 kb of the 3′ flanking region (Fig. 1A). The 3-kb EcoRI-HindIII fragment containing the promoter region was subcloned in pBluescript KS (+) and sequenced.

The 1,050-bp sequence upstream of the transcription start site (+1) is shown in Fig. 1B. Very little general homology was found between this sequence and the mammalian AMH promoters (Cate et al., 1986; Guerrier et al., 1990; Haqq et al., 1992; Dresser et al., 1995); the spliceosome-associated protein SAP62 gene is not present in this region of the AMH promoter as it is in mouse and human genes (Dresser et al., 1995).

The main transcription initiation site (+1) has been previously identified from the 5′ RACE-polymerase chain reaction (PCR) results (Carré-Eusèbe et al., 1996) and by primer extension (Neepher et al., 1996). The existence of a minor transcription site at −2, suggested previously (Carré-Eusèbe et al., 1996), was confirmed by the present genomic DNA sequence. The sequence variability observed at position −2 was shown to correspond to a polymorphism by direct sequencing of PCR-amplified chick genomic DNAs.

The sequence was screened for potential promoter regulatory elements; only the most interesting ones will be described. (1) A degenerated TATA box, −31 TTAAAAG−24, was found within the expected distance from the transcription initiation sites. (2) The −45 TCAAGGCCA−37 sequence corresponds to the steroidogenic factor SF-1 consensus response element (C/T)CAAG(T/C)CA. SF-1 is a key regulator of expression of the steroidogenic enzymes (Morohashi et al., 1992). (3) A degenerated palindromic estrogen-responsive element (ERE) (Klein-Hitpass et al., 1986), −128 AGTTCCCTGACCCA−114, located in the proximal promoter, might interact directly with the estrogen receptor. Alternatively, part of this sequence, TGACCCA, is complementary to the halpinidromic ERE sequence TGGGTCA, found in the proximal region of the chick ovalbumin gene promoter, where it is a target for the transcription factor AP1 and mediates estradiol activation of the gene by the estrogen receptor (Gaub et al., 1990; Paech et al., 1997). (4) Last, but not least, the search for sites to which SOX9 protein has been shown to bind in vivo and in vitro (Bell et al., 1997), and for SRY response elements, has revealed two well-conserved motifs. The −604 CTTTTGTT−598 sequence is the inverse complement of the SOX consensus heptamer motif AACAAAG, which binds mouse SOX9 with good affinity (Südbek et al., 1996; Bell et al., 1997). The −194 TTTTGT−188 sequence is the complement of the SRY consensus response element (A/T)AACAA(T/A) (Harley et al., 1994).

Chromosomal Localization of the Chick AMH Gene

The female chicken karyotype is constituted of 78 elements, including nine pairs of morphologically distinguishable macrochromosomes (eight pairs of autosomes and the ZW sex pair) and 30 pairs of microchromosomes (Nanda and Schmid, 1994). We have performed fluorescence in situ hybridization (FISH) on chick fibroblast cells, using the chick AMH gene recombinant phage as a probe. A total of 50 metaphase cells were analyzed: 94% of them showed two fluorescent spots on both homologs of a microchromosome pair (Fig. 2).

Expression of AMH and SOX9 mRNA During Gonadal Differentiation

To compare the early expression of AMH and SOX9, we used in situ hybridization on gonadal sections rather than RNase protection or RT-PCR assays, because it provides precise localization of mRNAs at the cellular level and circumvents the risk of contamination by neighboring tissues, critical when the gene is widely expressed, as is the case for SOX9 (Morais da Silva et al., 1996, and our own results, see below). Furthermore, to compare accurately the timing of expression of the two genes, we performed parallel in situ hybridizations on frozen sections of the same gonads. Immunolocalization of cytokeratins and fibronectin on the very sections used for the in situ hybridization allowed us to monitor gonadal morphology and the evolution of the differentiation process. Differentiation stages (HH) are defined according to Hamburger and Hamilton (1951). Results shown for a given stage and sex are taken from the same embryo and are representative of all the embryos of similar sex and stage.
studied (five males and three females for stages 25–27 and for stages 28–29, four males and four females for stages 30–34).

**HH stages 25–27.** There was no structural difference either between male and female gonads or between left and right gonads, except that in both sexes the right gonad was smaller than the left. Gonads were constituted of two territories: a thick surface epithelium, positive for cytokeratins (Fig. 3B,F) and negative for fibronectin (Fig. 3A,K), and an inner cytokeratin-negative part, containing irregular groups of cells separated by strands of fibronectin-positive material.

Fig. 1. Structure of the chick anti-Müllerian hormone (AMH) gene. A: Restriction map of the cloned genomic DNA fragment showing the five AMH exons (boxes), the initiation and stop translation sites, and the position of restriction sites (E, EcoRI; B, BamHI; H, HindIII). B: Sequence of the region -1050 to +48 preceding the translation initiation codon (in bold). The main transcription initiation site is indicated by a large arrow, minor initiation sites by small arrows. Potential regulatory elements (TATA box, SF-1, ERE, and SOX) are shown.
A clear sex difference in the expression of AMH mRNA now became obvious. Many more positive cells were present in the male than in the female gonads (compare Fig. 4E with C). In both sexes, they were detected in the inner cytokeratin-negative part of the gonad (Fig. 4B,C,I,J). Localization of AMH transcripts did not yet reveal a clear testicular cord organization.

A very faint expression of SOX9 mRNA was detectable in the male gonads at both stages 28 and 29 (Fig. 4G,K). It was localized in the same area as AMH mRNA (compare Fig. 4E with G, and I with K). No SOX9 mRNA was found in female gonads (not shown).

**HH stages 30–34.** In male embryos (Fig. 5), testicular organization became progressively evident with well-delineated seminiferous cords appearing as fibronectin-negative structures, and a very thin surface epithelium (Fig. 5A,D). At stage 30, expression of SOX9 was detectable but still low (Fig. 5B), whereas that of AMH was strong (Fig. 5C). Strikingly, within a few hours, i.e., between stage 30 and stage 31, the expression of SOX9 became dramatically stronger (compare Fig. 5B and E). As testicular organization progressed, AMH and SOX9 expression were exclusively localized in the differentiating seminiferous cords (Fig. 5E,F).

In female embryos (Fig. 6), up to stage 31, there were no significant differences between right and left gonads for either gonadal structure or expression of AMH mRNA (Fig. 6A,B,D). AMH transcript-positive cells were scattered throughout the gonads. The hybridization signal was much weaker than in differentiating testes. At stage 34, an ovarian cortex is developing at the surface of the left gonad (Fig. 6E). AMH mRNA was expressed in the inner part of the gonad, in dispersed cells or in small patches of cells preferentially localized below the differentiating cortex (Fig. 6F) as previously shown for 17-day ovaries (stage 43) (Carré-Eusèbe et al., 1996). SOX9 mRNA expression was not detectable in female gonads either at stage 30 (Fig. 6C) or at stage 34 (not shown).

**DISCUSSION**

The relative role of HMG proteins in sex differentiation is still a debated subject, which, as stated above, cannot be resolved by DNA binding experiments alone. One of the arguments brought forward to stress the importance of SOX9 is its early expression by Sertoli cells of different vertebrate classes (Kent et al., 1996; Morais da Silva et al., 1996). The present report shows that, in the chicken, although the AMH promoter contains putative response elements for SOX9, AMH is expressed before SOX9 in the early embryonic gonads, disproving the hypothesis that, in this species, SOX9 can trigger AMH expression.

Despite the low homology between the chick and mammalian promoters (Cate et al., 1986; Guerrier et al., 1990; Haqq et al., 1992; Dresser et al., 1995), common regulatory elements were found, including SF-1 and potential estrogen and SOX response elements. In mammals, the conserved SF-1 response
Fig. 3. Embryonic gonads at HH stages (differentiation stages defined according to Hamburger and Hamilton) 25 and 27. A–E: Male at stage 25 (same gonad). F–H: Female at stage 25 (same gonad). I,J: Males at stage 27. K,L: Female at stage 27. B,C and F,G and K,L are double-labeled sections. All figures, except I, are at the same magnification. Positive cells for anti-Müllerian hormone (AMH) mRNA are localized in the inner part of the gonads in both male and female embryos; the number of positive cells appears to be higher in males. No expression of SOX9 mRNA is observed in gonads (g) of either sex, whereas a strong reaction is observed in some mesonephric tubules (mt), dorsal mesentery (dm), neural tube (n), and sclerotomal cells surrounding the nervous tube and the chorda (c). Scale bars = 100 µm.
element, located approximately 60 bp upstream of the TATA box, is required for AMH promoter activity in rat (Shen et al., 1994) and mouse (Giulii et al., 1997) Sertoli cells. Despite a closer location of the SF-1 element relative to the TATA box, the same function can be expected in the chick, in which the SF-1 factor has recently been cloned (Kudo and Sutou, 1997). The presence of a potential estrogen response element in the AMH promoter is interesting in keeping with the major role played by estrogens in avian sex differentiation (Scheib, 1983; Elbrecht and Smith, 1992).

The pattern of expression of AMH is very different in chick and in mammals, apart from the fact that in both, the hormone is produced exclusively by somatic cells of the gonad. In mammalian fetuses, AMH is expressed only in males, whereas in chick embryos, it is expressed only in males, whereas in chick embryos, it is expressed...
in both sexes. In the mouse fetal testis, Amh transcripts are detected, by in situ hybridization, in Sertoli cells at 12.5 days postcoitum (Münsterberg and Lovell-Badge, 1991), when distinct cord organization is observed, and at 11.5 days postcoitum using the extremely sensitive RNAse protection assay (Hacker et al., 1995). Mammalian granulosa cells express AMH only after birth. In chicken, previous studies had detected AMH transcripts (Carré-Eusèbe et al., 1996) and bioactive (Hutton et al., 1981; di Clemente et al., 1992) or immunoreactive (Teng, 1987) AMH protein in embryonic gonads of both sexes. The use of frozen rather than paraffin sections greatly increased the sensitivity of in situ hybridization technique and allowed us to detect the presence of transcripts at earlier stages than in previous studies (Carré-Eusèbe et al., 1996). AMH mRNA was expressed in female as well as in male gonads, from the earliest stage studied, i.e., stage 25, 2 days

Fig. 5. Male embryonic gonads at HH stages (differentiation stages defined according to Hamburger and Hamilton) 30 and 31. A–C: Testis at stage 30. D–F: Testis at stage 31. A,B and D,E are double-labeled sections. SOX9 mRNA expression is still weak at stage 30 and becomes stronger at stage 31. Anti-Müllerian hormone (AMH) mRNA expression already very strong at stage 30, is increasing at stage 31. At stage 31 SOX9 and AMH transcripts are localized in well-delineated testicular cords. Scale bar = 100 µm in F (applies to A–F).
before any histologic sign of differentiation were detected (Carlon and Stahl, 1985). At stages 25 and 27, the number of positive cells in the male gonads is greater than in female ones, making it an early indicator of gonadal sexual dimorphism; however, the level of expression of the mRNA per cell appears to be the same in both sexes. From stage 28 thereon, AMH expression increases dramatically in the male.

If SOX9 is implicated in the triggering of AMH expression, a prerequisite is that SOX9 should be expressed before AMH in the same cell lineage. Both genes are expressed in the same testicular cells; nevertheless, AMH transcripts were detectable at stage 25, a full day before a faint expression of SOX9 mRNA appeared at stage 28. Furthermore, in the female embryo, in which ovarian SOX9 transcripts were never detected, AMH was expressed nonetheless.

Two previous studies of gonadal SOX9 expression in chicken did not include evaluation of AMH transcripts and resulted in conflicting results. Kent et al. (1996) reported results similar to ours, i.e., no SOX9 expression observed in the male genital ridges at day 5.5
(likely to correspond to stage 27–28), and no expression in the female. On the other hand, Morais da Silva et al. (1996) detected a low level of SOX9 expression in both sexes at stage 25, followed by an up-regulation in the male and a down-regulation in the female at later stages. In both studies, whole-mount in situ hybridization with digoxigenin-labeled RNA probes was used at the youngest stages. The discrepancy could possibly arise from differences in probe specificity, our own probe being similar to the one used by Kent et al. (1996). More importantly, our in situ hybridization was performed on sections, a technique that provides a more resolutive approach than whole-mount, showing the exact cellular localization of the label with no possible interference by underlying tissues.

The important sex-dependent differential expression of AMH from stage 28 onward corresponds to an up-regulation of AMH expression restricted to the male gonad. It cannot be explained by a direct gene dosage effect because, as shown here by FISH analysis, the chick AMH gene is autosomal, like the chick SOX9 gene (Kent et al., 1996). Could SOX9 be implicated in this up-regulation? This mechanism cannot be ruled out because SOX9 transcripts begin to be detected in the male gonads precisely at stage 28. However, the level of these transcripts is very low compared with later stages. In keeping with the suggestion that SOX9 is involved in Sertoli cell differentiation (Kent et al., 1996; Morais da Silva et al., 1996), we observed a dramatic increase in chick SOX9 mRNA expression, colocalized with AMH in well-organized seminiferous tubules of the testes, at stage 31.

In conclusion, the precedence of AMH over SOX9 expression seems to be a characteristic of chick gonadal differentiation. In the mouse, separate studies have monitored Amh and Sry expression (Hacker et al., 1995) on one hand, and Sox9 expression on the other (Morais da Silva et al., 1996). They concluded that high levels of Sox9 expression precede the onset of Amh transcription by about 1 day and made the hypothesis that SOX9 might control AMH expression. This mechanism may apply to the mammalian embryonic testis, but, even in mammals, SOX9 is not absolutely required for AMH expression because AMH is produced in granulosa cells of the adult ovary in the absence of SOX9 expression (Kent et al., 1996). Our observation that, in the chick, the early sexually dimorphic expression of AMH precedes testicular SOX9 expression challenges the primacy of SOX9 as the common ancestral sex-determining gene in vertebrates and leaves open the question of the role of this factor in testicular differentiation.

**EXPERIMENTAL PROCEDURES**

**Screening of Genomic Library**

Clones containing the chick AMH promoter were isolated by screening a chick genomic DNA library in Charon 4A phage prepared by Dodgson et al. (1979). Recombinant phages (1.6 × 10⁵ clones) from the amplified library were adsorbed onto LE392 Escherichia coli host, plated (3 × 10⁶ plaque-forming units per 22 cm × 22 cm plates), and grown for 4 hr at 42°C. Replicas of the plates on Hybond-N membranes (Amersham, Arlington Heights, IL) were treated successively with 0.5 M NaOH, 1.5 M NaCl (2 min), 0.5 M Tris/HCl, pH 7.4, 1.5 M NaCl (5 min), and 3× citrate-buffered saline (SSC) (5 min) and cross-linked with ultraviolet light (0.6 kJ/cm²). Screening was performed by hybridization with a [α-³²P]dCTP-labeled random-hexamer generated probe ( Megaprime DNA labeling system, Amersham) corresponding to the full-length cDNA, overnight at 42°C in a 6× SSC, 5× Denhardt's solution, 10% polyethylene glycol (PEG), 1% sodium dodecyl sulfate (SDS), 50% formamide solution, containing 0.1 mg/ml denatured sonicated herring sperm DNA. Membranes were washed twice at 65°C with 2× SSC, 0.1% SDS for 20 min and autoradiographed. Nine positive clones, purified to homogeneity by three successive rounds of rescreening under identical conditions, were further used for restriction mapping and subcloning.

**Cloning and Sequencing of Inserts**

For sequencing, DNA inserts from positive plaques were subcloned, after digestion with EcoRI and HindIII, into pBluescript KS (+) (Stratagene, La Jolla, CA) cleaved with the same enzymes. Plasmid DNA was purified using Wizard minipreps (Promega, Madison, WI) and sequenced with the Sequenase V2.0 kit (United States Biochemical, Cleveland, OH), using the denaturation method of Hsiao (1991). Ambiguities were resolved by inosine substitution. Two independent DNA clones were sequenced, on both strands. Sequences were analyzed on 6% Hydrolink long range (Bioprobe systems, Montreuil, France), 8 M urea sequencing gels, with 0.6× TBE [54 mM Tris-borate, 1.2 mM ethylenediaminetetraacetic acid (EDTA)] as running and gel buffers.

**Sequence Analysis**

The sequence was analyzed manually and with TFASTA and MatInspector (Quandt et al., 1995) online for motifs present in the TRANSFAC database.

**Chromosome Localization of the AMH Gene**

Chicken chromosome spreads were obtained from cultured female embryo fibroblasts according to standard procedures. Before hybridization, slides were pre-treated with RNaseA, then denatured in 70% deionized formamide in 2× SSC for 2 min at 70°C and dehydrated through an ice-cold ethanol series. Lambda phage DNA containing the AMH gene (12.5-kb insert) was biotinylated by nick translation with biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN) as indicated by the manufacturer. Hybridization to chromosome spreads was performed as described (Pinkel et al., 1986). The biotin-labeled DNA was mixed with hybridization solution at a final concentration of 10 µg/ml and used at a concentration of 150 ng/slide. Before hybridization, the labeled probe was annealed with a 500-fold excess amount of total
chicken DNA (previously cleaved with Sau3A I), to compete with the aspecific repetitive sequences. The hybridized probe was detected by means of fluorescent isothiocyanate-conjugated avidin (Vector Laboratories, Burlingame, CA). Chromosomes were counterstained with propidium iodide diluted in antifade solution.

Riboprobes

In situ hybridization was performed using digoxigenin-labeled riboprobes (DIG-RNA). The chick AMH probe has been previously described (Carré-Eusèbe et al., 1996). It corresponds to part of the fourth and fifth exons (821 nucleotides) and lacks the region coding for the last 78 C-terminal amino acids, a sequence displaying some degree of conservation within members of the transforming growth factor (TGF)–β superfamily. No hybridization was observed with chick heart, a tissue rich in TGF-β.

The chick SOX9 probe was obtained by PCR amplification of a 623-bp fragment of chick genomic DNA chosen in an intronless region, downstream of the HMG box, corresponding to nucleotides 748 to 1370 of the chick SOX9 cDNA sequence (accession No. U12533). Sense and antisense primers were 5′-CGGGCAAGCAGGACCTGAAG-3′ and 5′-CGTGGGGTTCATGTAGGTGA-3′, respectively. The blunt-ended, 5′ phosphorylated PCR product was digested at the EcoRV site within the poly linker of Bluescript KS (+) vector, and the identity of the insert was verified by sequencing. The plasmid was linearized by digestion with EcoRI or HindIII and transcribed with T3 or T7 RNA polymerases, using the digoxigenin labeling kit (Boehringer Mannheim) as indicated, to synthesize sense or antisense riboprobes, respectively. Homology searches in sequence databases detected only chick and human SOX9 genes and the trout SoxP1 gene (accession No. D83256).

Tissue Preparation

The chicken strain used was characterized by a sex-linked imperfect albinism (s al), allowing the recognition of the genetic sex (males with pigmented eyes, females with albino eyes). Staging of the embryos was performed according to Hamburger and Hamilton (1951). This chicken strain presents a delay of about half a day in embryonic development compared with wild type (Mérat et al., 1986). Under our incubation conditions at 38°C, stages 25, 27, 28, 29, 30, 31, and 34 corresponded to 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, and 9.0 days of incubation, respectively.

Gonads with the associated mesonephros were fixed 1 hr in 2% paraformaldehyde phosphate buffered saline (PBS). After washing in PBS with increasing concentrations of sucrose (0, 12, 15, and 18%), specimens were embedded in Tissue-Tek O. C. T. Compound (Miles, Inc., Kankakee, IL) and frozen at −20°C. Cryostat sections (5-µm thickness) were mounted on slides coated with 2% 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, A-3648) and stored at −20°C.

In Situ Hybridization

After thawing, sections rehydrated in PBS were fixed in 4% paraformaldehyde-PBS (20 min), rinsed in PBS and treated by 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8). Prehybridization was performed for 2 hr at 55°C in the following mixture: 50% formamide, 2× SSC, 5× Denhart’s solution, 50 µg/ml yeast tRNA, 250 µg/ml salmon sperm DNA, 4 mM EDTA, pH 8.0, 2.5% dextran sulfate. Digoxigenin-labeled antisense and sense riboprobes were diluted in the same mixture without salmon sperm DNA or EDTA. The optimal probe concentration was determined empirically. Hybridization was carried out overnight at 55°C in a humid chamber. After treatment with ribonuclease A (20 µg/ml), sections were washed in 0.1× SSC-30% formamide, 1 hr at 55°C. Detection of DIG-RNA was performed as described previously (Fridmacher et al., 1995) with an alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim, 1093274). The duration of the reaction with the alkaline phosphatase substrates varied from 2 hr for AMH mRNA to overnight for SOX9 mRNA. In some cases, the duration was homogenized in using more diluted AMH riboprobes. The reaction was stopped in 10 mM Tris, 1 mM EDTA, pH 8.0. Hybridizations with sense and antisense probes were carried out on the same slide.

Immunofluorescence

To identify gonadal structures, immunofluorescence was performed using as primary antibodies either a rabbit anti-human plasma fibronectin serum (Life Technologies, Grand Island, NY, 6071 SA) or a mouse monoclonal anti-cytokeratin pan antibody (clone lu-5) reacting with an epitope common to most cytokeratins and preserved from amphibian to man (Franke et al., 1987). The reaction was performed on sections previously treated by in situ hybridization (double labeling) or on adjacent sections. In both cases, sections were washed in PBS, and indirect immunofluorescence detection was performed as described (Fridmacher et al., 1995).

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