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Expression and Subcellular Localization of SF-1, SOX9, WT1, and AMH Proteins During Early Human Testicular Development

PASCAL DE SANTA BARBARA, BRIGITTE MONIOT, FRANCIS POULAT, AND PHILIPPE BERTA*
Human Molecular Genetics Group, Institut de Génétique Humaine, Montpellier, France

ABSTRACT **Many transcription factors have been identified and implicated in male sex determination pathway. Specifically involved in Sertoli cell differentiation and subsequent anti-Müllerian hormone (AMH) secretion in eutherian mammals, they include steroidogenic factor-1 (SF-1), SOX9 (SRY HMG box related gene 9), WT1 (Wilms' tumor 1), and GATA-4 (a zinc finger transcription factor). These factors have been described to execute their function in the male sex determination pathway by controlling AMH transcriptional expression. To understand the hierarchies of these factors and their involvement in the developing testis, for the first time we show the expression and subcellular localization of these factors by immunohistochemistry in the early human testis during embryogenesis compared with AMH expression. If these studies do not refute their possible synergistic interaction to control AMH expression in human embryo, they also reveal a new sexual dimorphism in SOX9 expression during the sex determination process. We show that SOX9 sex specifically shifts from the cytoplasmic to the nuclear compartment at the time of testis differentiation and AMH expression. Putative models for this subcellular distribution are discussed.** ***Dev Dyn 2000;217:293–298.***

Key words: sex determination; human embryo; SF-1; WT1; SOX9; AMH; MIS; immunohistochemistry

INTRODUCTION

The mammalian gonad arises from coelomic epithelium adjacent to the mesonephros. Both germ and somatic cells will colonize this coelomic epithelium to form the gonad. The initially bipotential gonad first shows sexual dimorphism during midgestation to develop either a testis or an ovary. In the human, or more generally in mammals, male sex determination is initiated by activation of a Y chromosome-located gene SRY, within somatic cells of the genital ridge. When expressed at around 6 week's developmental age in human embryos (Poulat et al., 1995), SRY will trigger the testis-determining pathway by induction of the embryonic somatic cells into Sertoli cells and subsequent sex cord formation. Once Sertoli cells are determined, diverse genes contributing to Sertoli cell phenotype are transcribed and activated. A key factor in male gonadogenesis is anti-Müllerian hormone (AMH) also known as Müllerian inhibiting substance (MIS), a member of the transforming growth factor β family. Testicular secretion of AMH induces regression of the Müllerian duct. In the female, Müllerian ducts differentiate into oviduct, uterus, and upper part of the vagina. Since this first discovery, many transcription factors involved in the male sex determination cascade have been isolated (Schafer and Goodfellow, 1996; Swain and Lovell-Badge, 1999), yet their respective roles in the pathway have not been described. Among these factors, at least four have been suggested by different studies to contribute to the anti-Müllerian hormone production by Sertoli cells during fetal life. These candidate genes include the SRY-related gene SOX9, a member of the SOX family (Foster et al., 1994; Wagner et al., 1994; Kent et al., 1996; de Santa Barbara et al., 1998a), the steroidogenic factor-1 (SF-1), a member of the orphan nuclear receptor family (Shen et al., 1994; Giuli et al., 1997), the Wilms' tumor suppressor WT1 (Nachtigal et al., 1998), and GATA-4, a member of the GATA family of transcriptional regulators (Viger et al., 1998; Tremblay and Viger, 1999). To date, the few published expression studies involved only the candidate factor. A carefully described developmental expression pattern of these candidate factors at the subcellular level would provide insight onto their respective positions in a genetic pathway directing male sex determination. In this study, we describe the temporal pattern and subcellular localization of SF-1, SOX9, WT1, AMH, and GATA-4 proteins by using specific characterized antibodies. We find that SF-1 and

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The first two authors contributed equally to this work.

Dr. Pascal de Santa Barbara's present address is Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts.

*Correspondence to: Philippe Berta, Human Molecular Genetics Group, Institut de Génétique Humaine, UPR 1142 du CNRS, 141 rue de la Cardonille, 34396 Montpellier cedex 5, France. E-mail: berta@igh.cnrs.fr

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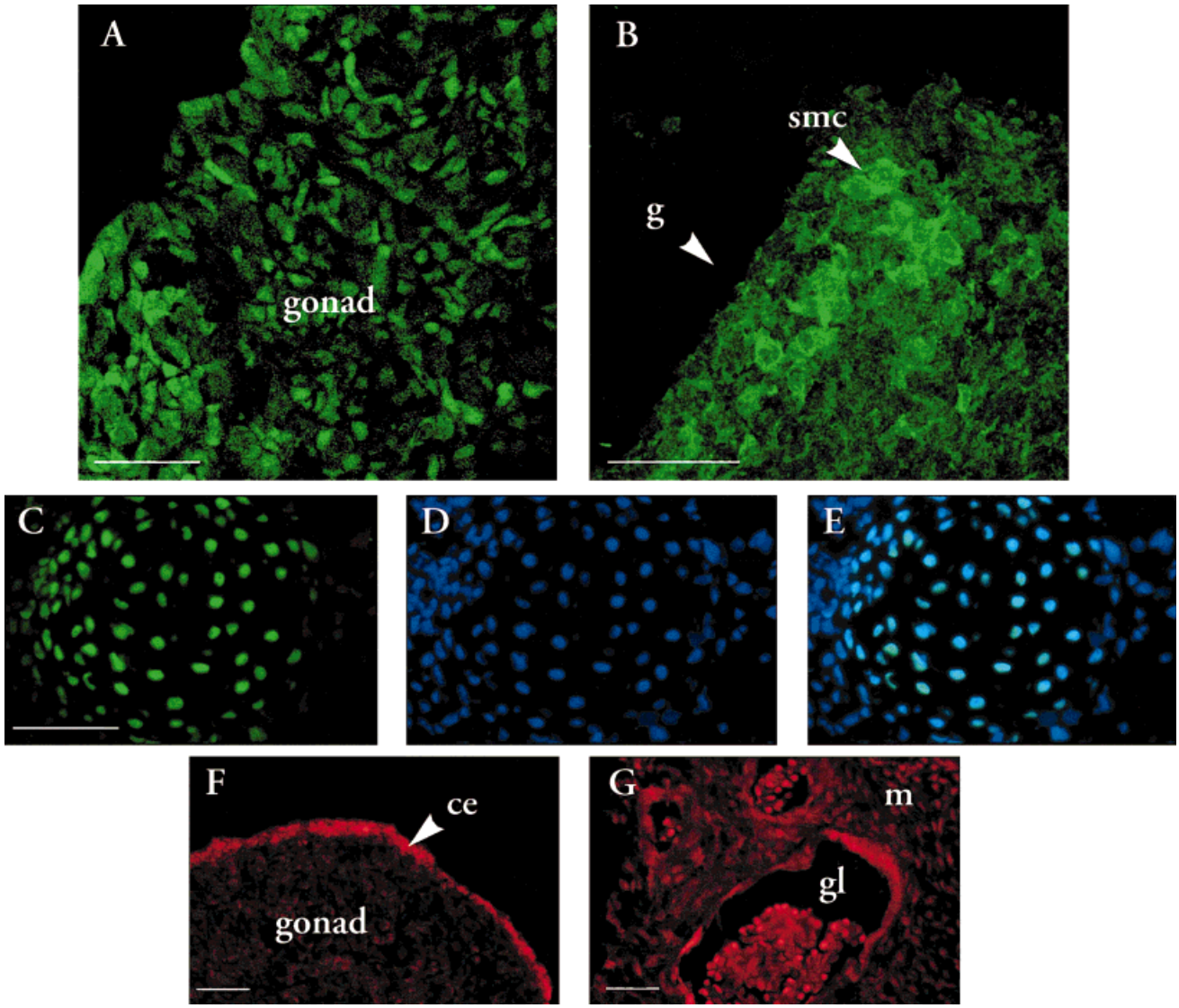

Fig. 1. Localization of human SF-1, SOX9, and WT1 proteins in a 6-week-old (CS17) male indifferent gonad. Transverse sections through undifferentiated gonadic blastema were stained with antibodies to SF-1 (**A**) or SOX9 (**B**). Internal control is provided by SOX9 expression (**C**), nuclear staining using Hoescht 33286 (**D**), and colocalization of SOX9 and Hoescht 33286 (**E**) in chondrocytes from the same embryo. Human WT1 protein immunolocalization (**F,G**), respectively, in undifferentiated gonad and mesonephric glomerules of a male embryo from the same stage. g, gonad; gl, glomerule; smc, somatic cells; ce, coelomic epithelium; m, mesonephros. Scale bars = 50 μm.

SOX9 proteins expressed in male gonadal somatic cells before AMH expression and sex cord formation. Moreover, SOX9 harbors a sex-specific subcellular translocation from the cytoplasm to the nucleus in male genital ridge cells at the time of testis differentiation and AMH secretion. This strongly supports a role of SOX9 upstream of AMH. Others transcription factors such as WT1 and GATA-4 are shown strongly expressed in Sertoli cells later, only after sex cords' organization and suggest that these factors are not involved in AMH expression onset.

RESULTS AND DISCUSSION

In a 6-week-old developmental age male human embryo, the sexually undifferentiated gonads (gonada primordia) begin to develop as a swell on the mediolateral surface of the mesonephros protruding into the coelomic cavity. At this stage, AMH is not detected (data not shown), yet numerous SF-1-immunoreactive cells are observed both in the coelomic epithelium and in the somatic component of the gonada primordia (Fig. 1A). SF-1 expression in each case appears localized to the nucleus. Another section from the same XY genital ridge also reveals in the center of undifferentiated gonad that somatic cells are positively labeled when using antibodies specific for SOX9 (Fig. 1B). SOX9 is present in the cytoplasmic compartment of the somatic cells in male and female gonads (data not shown), confirming previous observations made in mice (Morais

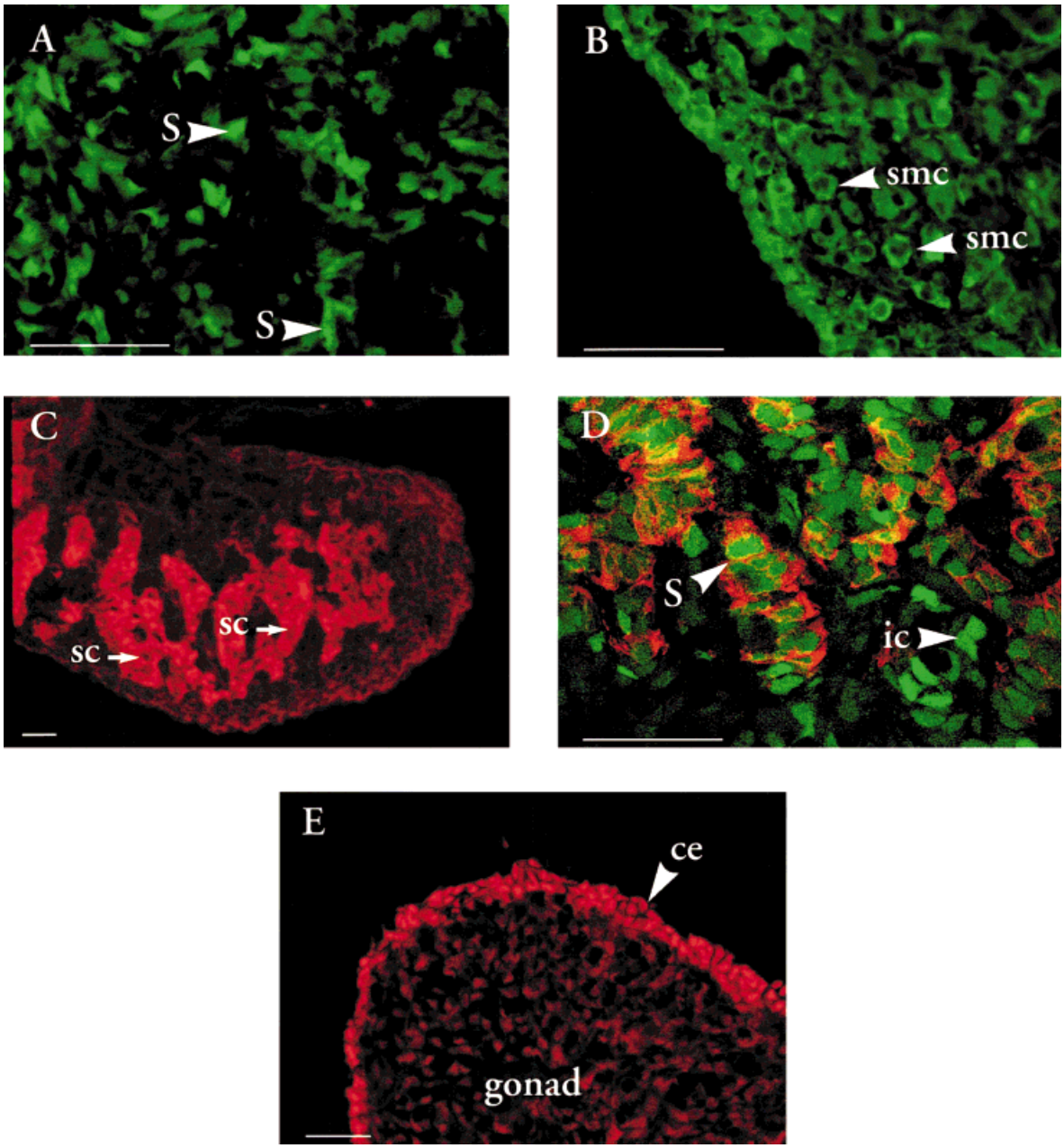

Fig. 2. Localization of SOX9, SF-1, WT1, and AMH proteins in 6.5-week-old (CS 18–19) developing gonad. Comparison of SOX9 expression in a section through a male (**A**) or a female (**B**) gonad. Immunofluorescence analysis of AMH alone (red) (**C**) or in combination with SF-1 (green) (**D**). WT1 labeling in a male gonad at the same developmental stage (**E**). S, Sertoli cells; smc, somatic cells; sc, seminiferous cords; ic, interstitial cells; ce, coelomic epithelium. Scale bars = 50 μm.

da Silva et al., 1996). The cytoplasmic localization of SOX9 appears specific to the gonadal tissue. Nuclear localization of SOX9 in chondrogenic cells is present, confirming previous observations (Fig. 1C) (Lefebvre and de Crombrugghe, 1998). WT1 appears highly expressed in both the coelomic epithelium (Fig. 1F) and mesonephric glomerules (Fig. 1G).

At 6.5 weeks male embryo, Sertoli cell differentiation, and sex cord formation begin. During this stage, SOX9 expression becomes restricted to the nuclei of Sertoli cells (Fig. 2A) but remains cytosolic in a female embryo of the corresponding stage (Fig. 2B). Cytosolic expression of the AMH protein is only observed in the Sertoli cells (Fig. 2C), not in the ovary (data not shown). SF-1 and AMH are colocalized within Sertoli cells (Fig. 2D), but SF-1 is also present in other interstitial cells (Fig. 2D). These interstitial cells are likely Leydig cell precursors (Hatano et al., 1994; Ikeda et al.,

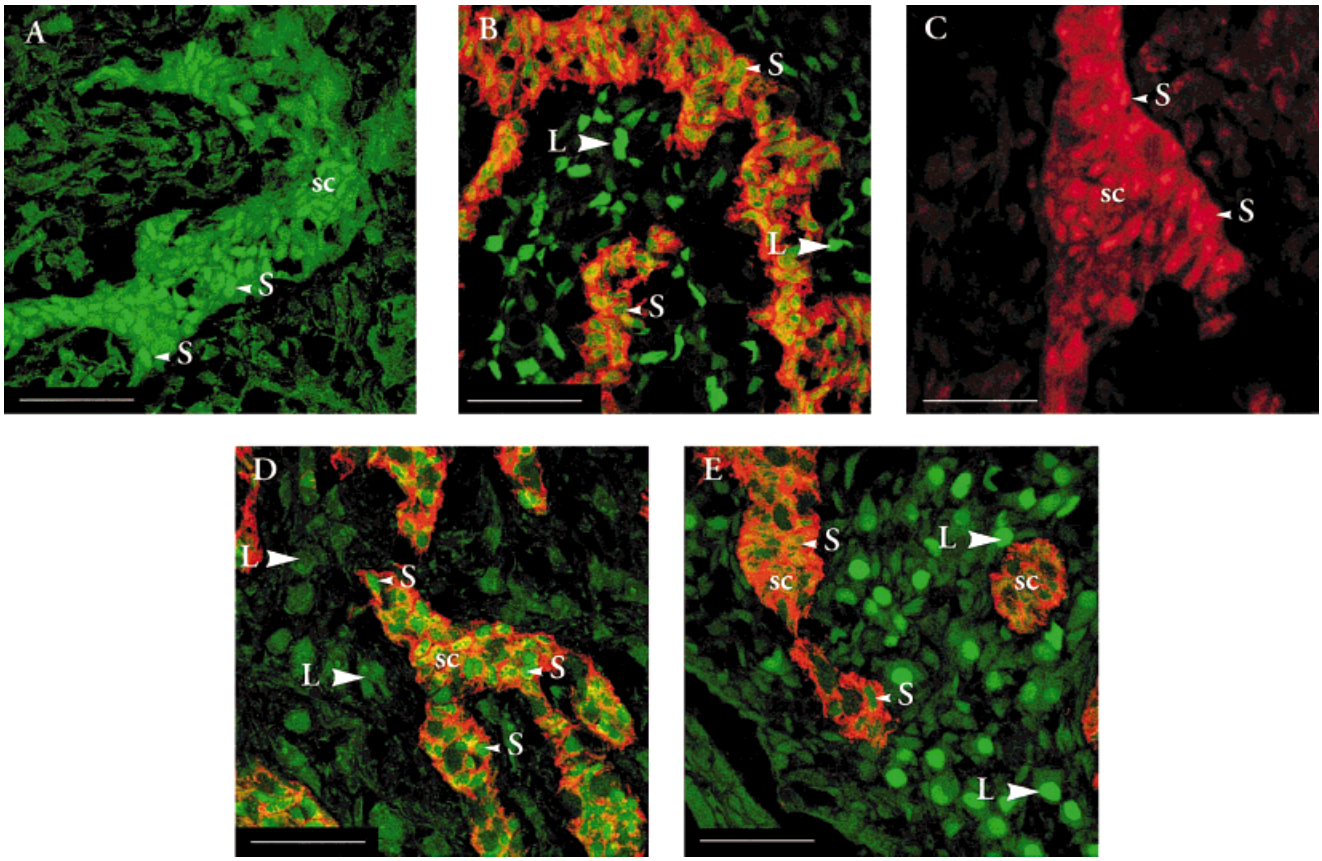

Fig. 3. Localization of SOX9, SF-1, WT1, and AMH proteins in a 7.5-week-old (CS 21) developing testis (A–C). Immunofluorescence analysis of SOX9 (**A**), of both SF-1 (green) and AMH (red) (**B**), and of WT1 (**C**). Localization of human SOX9, SF-1, and AMH proteins in an 8-week-old (CS 23) male embryo (D,E). **D:** Double immunolocalization of SOX9 (green) and AMH (red). **E:** Double immunolocalization of SF-1 (green) and AMH (red). sc, seminiferous cords; S, Sertoli cells; L, Leydig cells. Scale bars = 50 μm.

1994). At this stage, WT1 expression remains strong in the coelomic epithelium with weak WT1 expression in the testis (Fig. 2E). A similar WT1 expression is observed in female gonad from the same stage (data not shown).

At 7.5 or 8 weeks, after overt sexual differentiation, SOX9 and AMH expression remain unchanged (Fig. 3A,B,D), but the levels of SF-1 protein apparently decrease in Sertoli cells compared with younger stages and Leydig cell expression (Fig. 3B,E). WT1 expression is now stronger throughout the seminiferous cords being restricted to the Sertoli cell nuclei (Fig. 3C). We found, as have others (Ketola et al., 1999), GATA-4 protein expression in Sertoli and Leydig cells only after AMH and sex cord formation (data not shown).

In this study, we describe the localization of several proteins involved in male sex determination: SF-1, WT1, SOX9, GATA-4, and AMH. SOX9 and SF-1 expression patterns before AMH are consistent with their possible synergistic interaction to control AMH expression. The low level of WT1 and SF-1 labeling, respectively, prior (Hanley et al., 1999), and after the onset of AMH expression do not exclude their respective contribution in the onset and in the sustained AMH expression, maybe in association with a GATA factor. The observed expression pattern of SOX9 and its cellular translocation from the cytoplasm to the nucleus in male embryos provides new insights into the position of SOX9 in the male sex determination pathway. Developmental analysis of gene expression coupled with immunohistochemical analysis at the subcellular levels reveals regulation mechanisms not evident with either technique alone. Similar observations have been reported with the *fish-hook*/*dichaete* protein. This Sox protein is localized in Drosophila initially in both nuclear and cytosolic compartments. Later, during embryonic stages, this protein is restricted to the nucleus (Ma et al., 1998). This result with our findings suggests subcellular pathway may be a general phenomenon for all SOX proteins. The presence of both a leucine motif located within the SOX9 DNA-binding domain (Soullier et al., 1999) and a nuclear localization signal (Poulat et al., 1995; Südbeck and Scherer, 1997) support this theory. The leucine motif is one attractive candidate for a nuclear extrusion signal (Wen et al., 1995). The present data highlight the sexual dimorphism us-

ing SOX9 cell distribution during early male sex determination and raise the question of its mode of control. The sex specificity of this subcellular localization suggests that it could be directly or indirectly under the control of SRY. An alternative possibility is that SOX9, like LEF/TCFs, other transcription factors of the high mobility group superfamily (Cox and Peifer, 1998), mediates a nuclear response of a signal molecule. First, SOX9 may be trapped in the cytoplasm by protein interactions, which under control of the first messenger contributes to the assembly of multiprotein enhancer complexes integrating this signaling pathway. In this respect, the recent implication of SOX proteins in the regulation of Wnt signaling pathways open new fields of investigations (Zorn et al., 1999).

Recently, some studies examined expression of different genes in embryonic chicken gonads and revealed AMH expression in both sexes before male-specific SOX9 expression (Oreal et al., 1998; Smith et al., 1999). If SOX9 is not involved in the onset of AMH in chicken, it may be involved in the up-regulation of AMH expression in testis. Our study brings to light the differences between human (and more generally eutherian mammals) and avian species. In mammals, SRY activates the genetic pathway leading to the testis differentiation. Although no *Sry* homolog has been identified in avian species, others genes involved in mammalian sex determination have been isolated, supporting the idea of one conserved sex-determining pathway (Morais da Silva et al., 1996; Kent et al., 1996). Nevertheless, mammalian and avian expression profiles suggest a divergent pathway using common genes. These differences may be due to a delay in mRNA translation, posttranslational protein modifications, or involvement of avian-specific unknown genes.

In summary, the present data provide some insights to the respective contribution of candidate control factors of AMH expression. Moreover, we described for the first time a sexual dimorphic subcellular expression of SOX9, which suggests a new mechanism to modulate SOX9 activity. Finally, our observation of the subcellular localization of SOX9 provides an explanation for the delay between SOX9/SF-1 expression and the onset of AMH production observed during embryonic life.

EXPERIMENTAL PROCEDURES

Obtaining Human Embryonic Gonadal Tissues

Human embryonic gonadal tissues were obtained from surgical abortions as part of a program approved by both the ethics committee from CNRS and by the French National Ethics Committee. Embryos were staged according to the recognized Carnegie stages (O'Rahilly, 1983). DNA was extracted from embryonic limbs, and sex was determined by polymerase chain reaction as described (Josso et al., 1993).

Immunohistochemistry

The five antibodies used in the experiments were obtained as follows: polyclonal human SF-1-specific rat antiserum raised against amino acids 121–232 from the human SF-1 protein (de Santa Barbara et al., 1998a), polyclonal human SOX9 rat serum raised against the bacterially expressed SOX9 TA domain (de Santa Barbara et al., 1998a), specific GATA-4 antibody (C-20 antibody; Santa Cruz Biotechnology, Santa Cruz, CA), WT1 antibody (C-19 antibody, Santa Cruz Biotechnology), and rabbit anti-AMH antibody, which was a generous gift from Dr. R. Rey (INSERM, Montrouge). Tissue sections were probed and processed as previously described (de Santa Barbara et al., 1998a, 1998b). To define the primordial germ cells, some sections were processed for alkaline phosphatase activity before incubation with the antibody. Cell nuclei were visualized by using Hoeschst 33286. Steroidogenically active Leydig cells were determined by enzymatic detection of 3β-hydroxysteroid dehydrogenase activity (not shown). Images were collected and processed on a Biorad confocal microscope or on a Zeiss Axiophot.

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