Int. J. Dev. Biol. 40: 567-575 (1996)

Original Article

Characterization of ovine SRY transcript and developmental expression of genes involved in sexual differentiation

EMMANUEL PAYEN[1], ERIC PAILHOUX[1], RAGHIDA ABOU MERHI[1], LAURENCE GIANQUINTO[1], MAREK KIRSZENBAUM[3], ALAIN LOCATELLI[2] and CORINNE COTINOT[1]*

[1]*Laboratoire de Biologie Cellulaire et Moléculaire, INRA, Jouy en Josas,* [2]*Station de Physiologie de la reproduction, INRA, Nouzilly and* [3]*Laboratoire d'Immunoradiobiologie, Hopital St Louis, Paris, France*

ABSTRACT In mammals, the presence of SRY, the sex-determining gene located on the Y chromosome is required to induce the gonadal anlage to differentiate as a testis, whereas its absence leads to the development of an ovary. We report here the characterization by 5' and 3' RACE analysis of several SRY transcripts which are expressed in the ovine male developing gonads. These transcripts were not detected in any other fetal tissues and were expressed only in the genital portion of the urogenital ridge. The temporal profile of SRY expression analyzed by RT-PCR suggests that in the sheep fetus the role of SRY is not limited to initiating Sertoli cell differentiation as in mice. Indeed, SRY transcripts persist after the full differentiation of the testis. In addition to SRY, other genes are known to be involved in mammalian sex determination: Wilms' tumor gene WT-1, steroidogenic factor gene Ftz-F1 (SF-1) and anti-Müllerian hormone (AMH). We investigated the expression patterns of these genes by RT-PCR during fetal development in sheep gonads. Concerning WT-1 and SF-1, our results are consistent with those described in mice where the earliest expression was detected before the sexual differentiation in both sexes. In male, the ontogenesis of AMH transcription corresponds to the seminiferous cords formation (30 dpc). In female, we have observed the presence of SF-1 transcripts from the undifferentiated stage until birth. In addition, P450 aromatase expression is detected from 30 dpc and is correlated with the presence of 17-ß estradiol in sheep ovary. These data reveal significant differences between rodent and ruminant models concerning the sex-determining pathway.

KEY WORDS: *sex determination, gonadal differentiation, SRY, WT-1, SF-1, AMH, CYP19*

Introduction

The development of male or female phenotype is the result of a combination of sex determination and subsequent sexual differentiation. Sex determination in mammals depends upon the presence or absence of the Y chromosome (Welshons and Russell, 1959). Endocrinological and genetic data indicate that there must be a dominant gene on the Y chromosome required for testis development (Jost *et al.*, 1973; Eicher and Washburn, 1986; Page, 1986). This gene is a component of the pathway of genes implicated in testicular differentiation and acts as the switch in mammalian sex determination (Goodfellow and Darling, 1988). The pivotal event in determining the sex of an individual is the differentiation and specialization of the gonads. Once formed, the gonads control the subsequent sexual phenotype (Jost *et al.*, 1973). In males, the development of the testis is associated with differentiation of Sertoli and Leydig cells which secrete two essential hormones: anti-Müllerian hormone (AMH) and testosterone (Jost *et al.*, 1984; Josso and Picard, 1986).

In 1990, a gene named SRY (Sex determining Region of Y gene) was cloned from the human Y chromosome and proposed as a candidate for the testis determining factor (Sinclair *et al.*, 1990). Sequence analysis of this gene identified a region of 79 amino acids which showed homology to the HMG-box, a motif found in many different proteins (including the High Mobility Group proteins) known to bind DNA (Sinclair *et al.*, 1990; Nasrin *et al.*, 1991). In the mouse Sry is expressed in the genital ridge during the critical period of gonadal differentiation at days 10.5 to 12.5 (Koopman *et al.*, 1990; Hacker *et al.*, 1995; Jeske *et al.*, 1995). Finally, the causal link between Sry and male development has been provided by mice transgenic for Sry (Koopman *et al.*, 1991).

Following its discovery, the search is continuing to elucidate the role of SRY as transcription factor in the pathway of gene regulation involved in sex determination. Several autosomic genes which seem to be involved in the sex- determining cascade have

Abbreviations used in this paper: RT, reverse transcription; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends.

***Address for reprints:** Laboratoire de Biologie Cellulaire et Moléculaire, INRA, 78350 Jouy en Josas, France. FAX: 33.1.34652241. e-mail: corinne.cotinot@biotec.jouy.inra.fr

0214-6282/96/$03.00

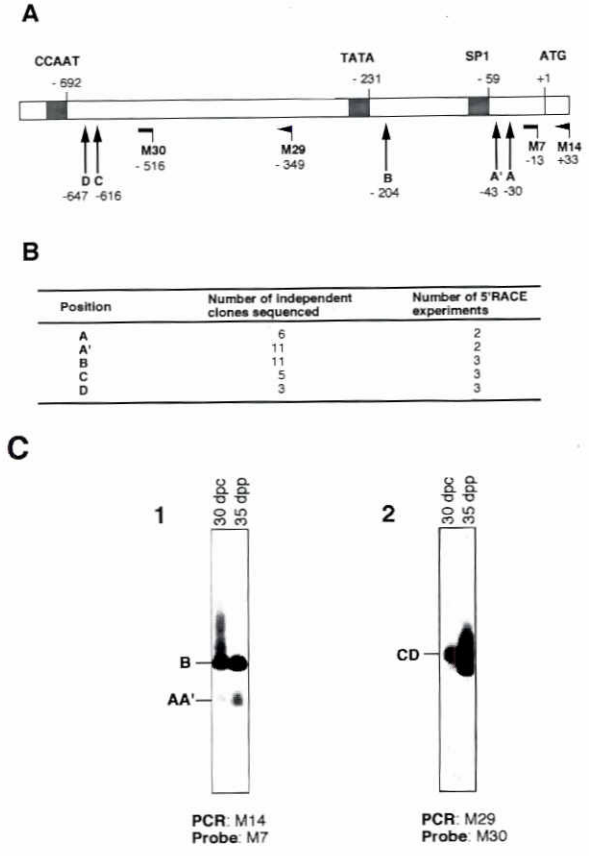


Fig. 1. Results of 5'RACE experiments. (A) *Diagrammatic representation of SRY transcription start sites. The synthesis of single-stranded cDNA was primed using random hexanucleotides and 5 µg of total RNA extracted from 30 dpc male fetal gonads and from 35 dpp testes.* **(B)** *Summary of 5' RACE experiments from fetal gonads.* **(C)** *Autoradiograms of 5' RACE products. After reverse transcription, PCR was performed using either M14 or M29 specific primers. PCR products were blotted and hybridized with ^{32}P-radiolabeled M7 or M30 probes respectively. Two stages were investigated 30 dpc and 35 dpp.*

been isolated. However, their precise interaction with each other and with SRY remains unknown. These include WT-1 — the gene implicated in WAGR and Denys Drash syndromes (Gessler *et al.*, 1992; Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991), SF-1 — the steroidogenic factor-1 that regulates expression of steroid hydroxylases (Lala *et al.*, 1992; Luo *et al.*, 1994), and AMH — the male key hormone which causes regression of Müllerian ducts and induces masculinization of fetal rat ovaries *in vitro* and female bovine freemartin (Vigier *et al.*, 1987, 1989; Behringer *et al.*, 1992). The differentiation of Sertoli cells and their organization into cord structures are the first sign of testicular morphogenesis. The genes studied here, WT-1, SF-1, SRY and AMH are all expressed in this cellular type and are involved in sex determination and/or early gonad differentiation (Münsterberg and Lovell-Badge 1991; Pelletier *et al.*, 1991; Hirobe *et al.*, 1992; Ikeda *et al.*, 1994). Study of their expression pattern in both male and female developing gonads is an interesting approach to elucidate their precise interactions. Such data have been obtained only in mouse and with different techniques (RT-PCR, RNAse protection and *in situ* hybridization). Here, we present results from another species of mammals where the process of gonadal differentiation is ten-fold longer in time than in mouse. All genes are simultaneously studied from the same fetal material and with the same technique of RT-PCR. To complete our study in female we have also analyzed expression of P450 aromatase (CYP19), the enzyme which converts testosterone into estrogen. Moreover, we have used 5' and 3' RACE to characterize ovine SRY transcript in the developing male gonads.

Results

Analysis of ovine SRY transcript

In order to characterize the SRY transcript in sheep, 5' RACE experiments were performed using fetal gonads (30 dpc) and prepubertal testis (35 dpp). The different clones obtained exhibited a diversity of 5' ends which fell into 3 clusters (Fig. 1A,B,C). The first cluster (A,A') consisted of 17 clones which began at nucleotides -43 and -30 from the ATG. An Sp1 binding site is located 16 and 29 bp downstream from these two sites. Eleven clones compose the second cluster B, all began at nucleotide -204, 27 bp downstream from a TATA-box sequence. The remaining 8 clones (C,D) were longer, beginning at nucleotides -647, -616. A GC rich region containing a CCAAT site is located 45 and 76 bp upstream from these last two sites. These results show the existence of several transcription start sites in both tested samples (30 dpc and 35 dpp). Analysis of first round PCR products (Fig. 1C) shows that in proximal region, site B seems to be more used than A,A' sites. Similar results were obtained from both stages. Concerning the distal region, the products resulting from the C, D start sites appear more abundant in testis after birth than during fetal life.

To locate the polyadenylation site of the ovine SRY transcript, 3' RACE was performed on male fetal gonads (40 dpc). Sequencing of cloned PCR products revealed two types of inserts (Fig. 2A,B). The first consists of the 3' end of the transcript, mapping to position 1872 (the position is numbered relative to the first ATG of the SRY ORF). This site is preceded 17 bp upstream by an AAUAAA sequence. The second group of clones stops at position 1529 and probably represents an artefact of reverse transcription due to non-specific hybridization of oligo(dT) in an A-rich region. Thus it is not possible to determine if the polyadenylation signal located at position 1540 is used. However, this site is not associated with a downstream GU- or U-rich region present in three quarters of mammalian polyadenylation signals (Chen *et al.*, 1995).

Time course of SRY expression

To define the window of SRY expression in sheep, we have tested developing gonads from "indifferent" genital ridge formation (23 dpc) until mature testis.

In sheep fetuses, the mesonephros and genital ridge can be identified at days 20 and 23 of gestation respectively (term= 145 days). Germ cells can be observed at the genital ridge as early as day 23. Until day 28, the genital ridge assumed the characteristics of a sexually indifferent gonad. At day 29, the differentiation of testis began with the formation of the tunica albuginea and the organization along a testis-to-mesonephros direction of seminiferous cords, tubuli recti, the cords of the rete

testis and the ductules efferentes (Zamboni and Upadhyay, 1982).

SRY expression was first detected in male fetuses at 23 dpc (the same period as genital ridge formation), peaked during 18 days between 27 and 44 dpc and became weak but not totally absent from 49 dpc until a few days after birth (Fig. 3). In female, at corresponding stages, no RT-PCR products were observed (data not shown). Moreover, to confirm the specificity of amplified bands during fetal male development, RT-PCR products were sequenced at two stages: 32 and 44 dpc. In the post partum testis, SRY was expressed from 12 dpp until adult stage (Fig. 4). After puberty (100-150 dpp), expression appeared lower (Figs. 3 and 4). In order to determine the cellular type where SRY is expressed we have isolated Sertoli cells from prepubertal 12 dpp testis. The signal obtained from purified Sertoli cells is more intense than in whole testis at the same stage (Fig. 4). Moreover, to determine the location of SRY expression during fetal life, the urogenital ridges from 35 to 75 dpc were dissected into their gonadal and mesonephric components and separately tested by RT-PCR. SRY expression was confined to the genital ridge portion of the urogenital ridge, no signal was detected from mesonephros (data not shown). Expression of SRY was also examined in whole fetuses (29 dpc) without urogenital ridges, and no transcripts were observed by RT-PCR.

Expression of WT-1, SF-1, AMH, P450 aromatase mRNA during ovine fetal development

Three to six fetal gonads samples were examined for each gene at each age and the results are shown in Figures 5, 6 and 7. To control and compare the quality of cDNA present within each sample after reverse transcription, we have used an ubiquitous gene as internal control ZFX/ZFY. This gene was expressed in all stages in both male and female animals. Expression profiles of different investigated genes are summarized in Table 2.

Messenger RNA for WT-1 was present at all stages in the testes and in the ovaries from the formation of the gonadal anlage. Expression of SF-1 was detected in female "indifferent" gonad at 25 dpc. From 30 dpc until adult state the expression was intense. In male, the signal before 28 dpc was very weak and then became more intense from 35 dpc until puberty. AMH transcripts were not detected before 30 dpc in male genital ridges (Figs. 5,7). Expression of AMH was intense from 35 dpc until puberty and

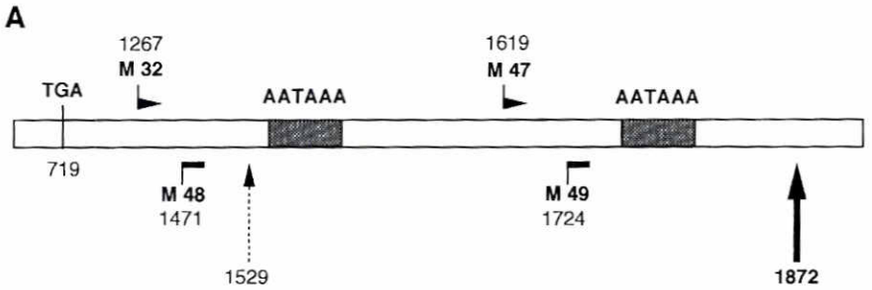


Fig. 2. 3'ends analysis of ovine SRY transcript. (A) *Diagrammatic representation of 3' RACE experiments. Position of different oligonucleotides (M32; M47) used to initiate reverse transcription and probes (M48; M49) was noted, and also, the presumed cleavage sites (≠).* **(B)** *DNA sequence of 3' untranslated region of the SRY transcript. The primers used for 3' RACE analysis are boxed and the two polyadenylation signals are shaded in grey. The site of polyadenylation is indicated by an arrow.*

B

```
TGACTTCCTTACTCTCGCTAACAAAGGCACTCTTTATCTCAATTTTACTACAGTTTCACCTGCGACTTAATTTTAAAATAAGCCAAATAA   90
GTACGCTTAACAAGTAAAGAATTTGGACTTTCCAAAATAACTGCTCCTCTGTTCATCACTTCTATCTGTAAGAGTACTTTTTTGTAAGAA  180
ATTATCTTAACAGCACCAAAACTGCTTGAGTATGAAGATCATCTGTTTTTCCTAGTAATGGCACAATTTTTATATTTCTAATTTTAATTG  270
TTCCAGAGATTGGCCATTAGTTAGATGGTAGCATATATTAATAACTTGGTAATAGCCACTATAGACAATATAACTTTTTATTTTAAATGC  360
TGTAACTCCAAACTATAGTACTTTCAGAAACACTCACAAATTCATGGTACAGAGGAAAAAATCTCATACTTGGATGGAAAGCTCCCTACC  450
TTCTAAAGCACTTTCTGGTACAAGCTCTGTTCTTTGGTCTTTCTAGCTACTTTCCACCTATTTGTAAATTGCAGGTAATTAGTGAATATA  540
                M 32
TATGCATATTCTATGTTCTTTCACAGCTTATAGCCTTTTGTTCCCACTGTGAAAAAGCCAGTTTTGCTGCCACTTGCTACTCAGTTCCTT  630
TAGTCATACAAAATTGTAAGGTTTTACTTGTGTTTTGAGGGGATTATAACATCCATCCAGTATTTAATTCACTCCTGTGAAAGCCAAGGT  720
                                M 48
TTCATCTCTTTTCTGAAAAGTTTCTTTTAATCACTGGCAATACACTTGTTTCCATTTTCACTTTACTTTTTGCATTCTTGGAGGGAGAAA  810
ACAAAAAAATAAAGTGATAGCATGTCAGGAATATAAACTATTCAGACTATGTGGCTTGGGGGATGGGATAAGCAAGATCCTGTCTGGTTA  900
          M 47
ATCTGATGAGGTATCAGTGAAAATGTAAGTCAAAGGTGTTTAAAAATGTGCAAACAGGCTAAAGTAGAAAAACTGGCAGCTTGCAAAGAA  990
                M 49
AGCCTACTTTTTTGGAATGAAAATAAGTCCATGGCCAAACTTTATGATATGATATGATATCATATGCTATATATTGCATATCAAAGTCAA 1180
CTTTCCAATATATATATGTACCAAACTCTTGAAAAACAGTACTTCAAAT AATAAATCTGAAAACTCAAAACAATATGTAGTGAAACGTGA 1270
```

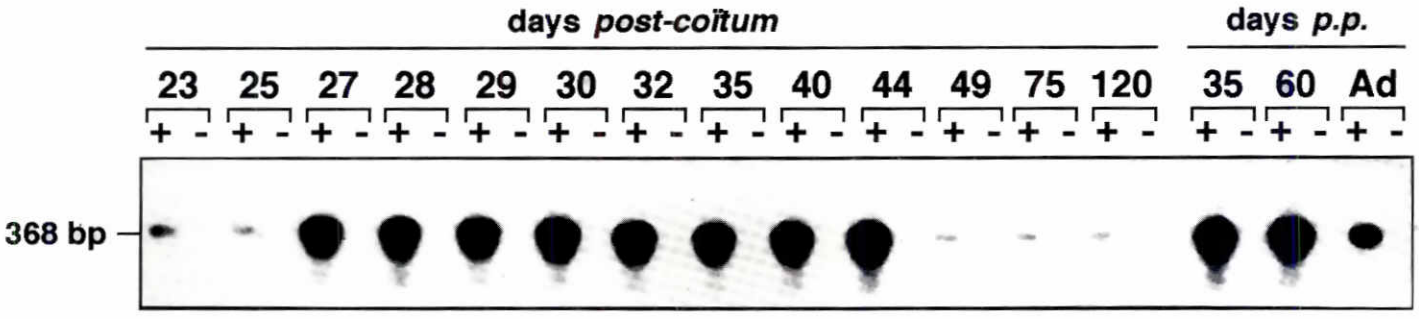


Fig. 3. Time course of sheep SRY expression. *Autoradiogram of RT-PCR products obtained from fetal and adult male gonads with M2-M9 primers after 30 cycles of amplification. The samples contained in an agarose gel were Southern blotted and the membrane hybridized with ^{32}P-labeled internal SRY probe (M11). Presence or absence of reverse transcriptase indicated by (+) and (-).*

then decreased at adult state (Fig. 8). In the ovary, AMH expression began before birth at 120 dpc (term= 145 dpc).

P450 aromatase mRNA was not detected in fetal testes at any stage. In contrast, amplified products of appropriate size were observed in fetal ovaries from day 30. Intensity of the signal was maximal between days 35 to 49. From 60 dpc until birth, expression was weaker. In addition, we have tested aromatase activity in 49 dpc ovaries to be ensured that the enzyme was functional. A high aromatase activity has been detected by the tritiated water technique (Fig. 9). The presence of functional P450 aromatase suggests the potential of fetal ovaries to convert androgens into estrogens.

Discussion

Because the physiological role of SRY occurs in the genital ridge during development, we have chosen this tissue to isolate and characterize SRY mRNA. We have constructed and screened a 29 dpc male fetal gonads cDNA library (2.5×10^5 clones) with an ovine specific SRY probe (see Materials and Methods). No positive colonies were identified, perhaps because of the low abundance of SRY transcripts. In absence of cDNA clones, we carried out 5' and 3' RACE analysis using male fetal gonads and prepubertal testis mRNA as template.

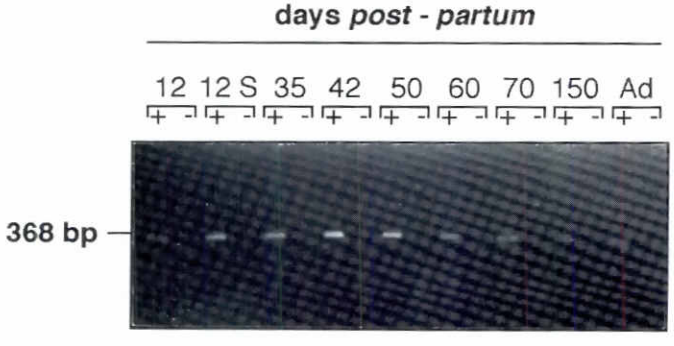


Fig. 4. SRY expression in postnatal testes. *RT-PCR amplification (30 cycles) from different testicular samples using sheep SRY primers (M2-M9). Lane 12 S corresponds to enriched Sertoli cells (>90%) from 12 day-old lambs. All (-) lanes represent the same samples in which reverse transcriptase was omitted from the reaction.*

We have shown the existence of several transcription start sites. These data agree with different studies performed in human and mouse where multiple transcription start sites were previously described (Vilain *et al.*, 1992; Behlke *et al.*, 1993; Clépet *et al.*, 1993; Su and Lau, 1993; Hacker *et al.*, 1995; Jeske *et al.*, 1995).

In human, the major sites seem to be those initially proposed by Vilain *et al.* (1992) located downstream from the first Sp1 binding site. A longer transcript, which initiates several hundreds of bases upstream of this site was also detected in adult testis and the NT2/D1 cell line (Clépet *et al.*, 1993). In mouse, there is no Sp1 binding site and a major transcription start site was detected 20 bp downstream from a TATA-box sequence. In sheep, since there are both Sp1 and TATA-box consensus sequences, several initiation sites are observed. As in human, a

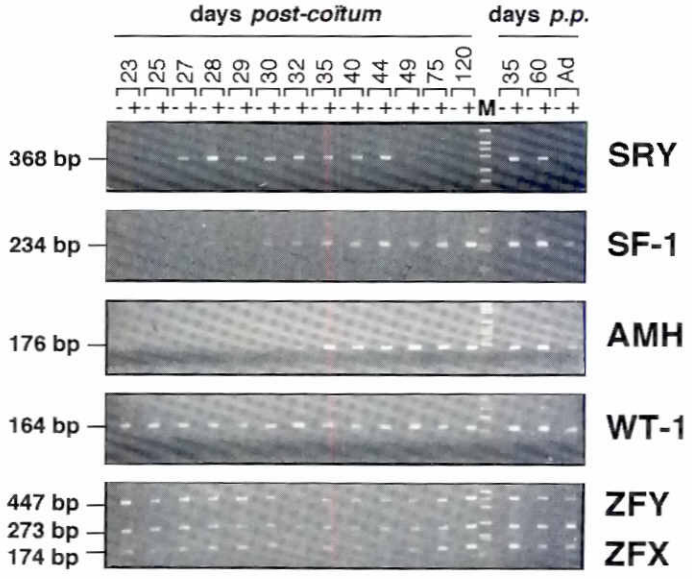


Fig. 5. Developmental expression of SRY, SF-1, AMH, WT-1 and ZFX/ZFY during male gonadal differentiation in sheep. *Total RNA preparations from fetal, postnatal and adult gonads were used in a reverse transcription reaction, with (+) and without (-) reverse transcriptase. Separate PCR reactions were performed using the same RT products with different specific primer pairs. Three control ZFX/ZFY bands were present in all RT (+) samples.*

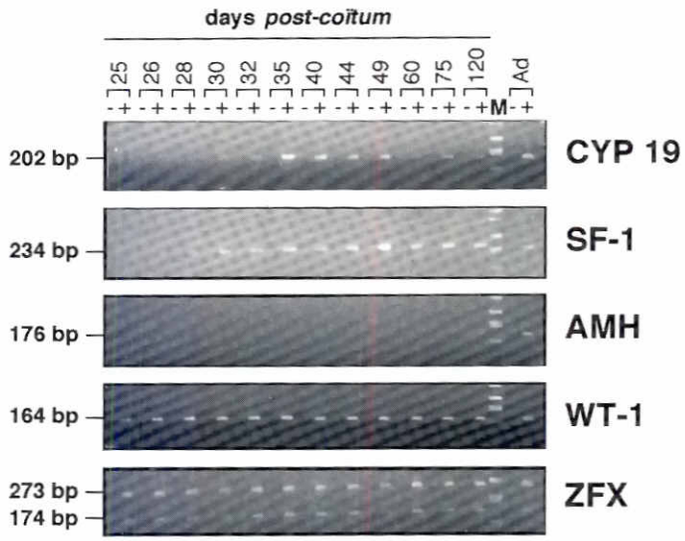


Fig. 6. Time course of P450 aromatase (CYP19), SF-1, AMH, WT-1 and ZFX expression during female gonadal development in sheep. *Ethidium bromide-stained agarose gels showing RT-PCR products obtained from fetal and adult female gonads with different primer pairs after 30 cycles of amplification. All samples were positive for ZFX, except when RT was omitted (-).*

longer transcript initiated further upstream was obtained from both gonadal samples. This type of transcript seems to be more abundant after birth than during fetal life and could reflected the preferential use of some sites according to the developing stage.

We have tried to determine the size of SRY mRNA by Northern blot analysis of poly(A) RNA taken from 29 dpc male gonads and prepubertal testes. After 15 days of film exposure, no hybridizing bands were seen (data not shown). A 3' RACE analysis was then performed and has permitted us to identify a termination site at position 1872 bp. A putative polyadenylation signal sequence AATAAA was found 17 bp upstream of this point. These results are inconsistent with those obtained in bovine, a species very close to sheep, where multiple sites of polyadenylation were described without consensus polyadenylation signal (Daneau *et al.*, 1995). These putative sites are probably the result of oligo(dT) hybridization in stretches of adenosines.

In sheep, the predicted size of the fetal SRY transcript is around 2100-2500 bp. It includes 647 to 204 bp of 5' upstream sequences, an open reading frame of 723 bp and 1153 bp of 3' downstream sequences. In human adult testes the SRY transcript is shorter, at about 1000 bp (Sinclair *et al.*, 1990; Clépet *et al.*, 1993) with 77 bp of 5'UTR, 612 bp of ORF and 137 bp of 3' UTR.

The developmental profiles of SRY expression in the only two eutherian mammals examined, the mouse and the sheep are different. In the mouse, the narrow window of Sry expression (10.5-12.5 dpc) in the genital ridge suggests a role limited to initiation of the cascade of testis differentiation genes (Koopman *et al.*, 1990; Hacker *et al.*, 1995; Jeske *et al.*, 1995).

In contrast, SRY expression in sheep is maintained until 44 dpc after the full differentiation of the fetal testis. It could be required not only for the differentiation of Sertoli cells, but also for the maintenance of the differentiated state.

The spatial profile of SRY expression also differs within mammals. In sheep during fetal life, SRY transcripts were only detected in the gonadal part of the urogenital ridge as in mice. In contrast, in adult human tissues (Clépet *et al.*, 1993) and embryonic marsupial tissues it is widely expressed (Harry *et al.*, 1995), suggesting that SRY may retain another function besides its sex determining role. Recently, murine Sry transcripts have also been detected in adult male hypothalamus and midbrain (Lahr *et al.*, 1995). However there is no evidence of translation of these transcripts or production of functional proteins.

Upstream of SRY function, morphogenetic processes take place to initiate the urogenital ridge formation which may not be sex-specific. Both WT-1 and SF-1 appear to be required for early commitment and maintenance of the gonadal anlage because in WT-1 and SF-1-null mice, gonadal development is arrested at an early stage (Kreidberg *et al.*, 1993; Luo *et al.*, 1994) and because they are expressed both in male and female. Concerning WT-1 expression, our results are consistent with those obtained in mice where the earliest expression is detected on embryonic day 9, which is before the time of gonadal differentiation. For SF-1, expression in mice is first detected at embryonic days 9-9.5 when the gonadal anlage is still indifferent (Ikeda *et al.*, 1994). In sheep, the time interval between genital ridge formation (23 dpc) and significant SF-1 expression (28 dpc) is longer than in mice. SF-1 seems to have multiple roles in gonadal differentiation. Evidence has recently been provided that AMH expression is regulated by a direct interaction of SF-1 with the promoter region of the AMH gene (Shen *et al.*, 1994). The spatio-temporal expression of SF-1

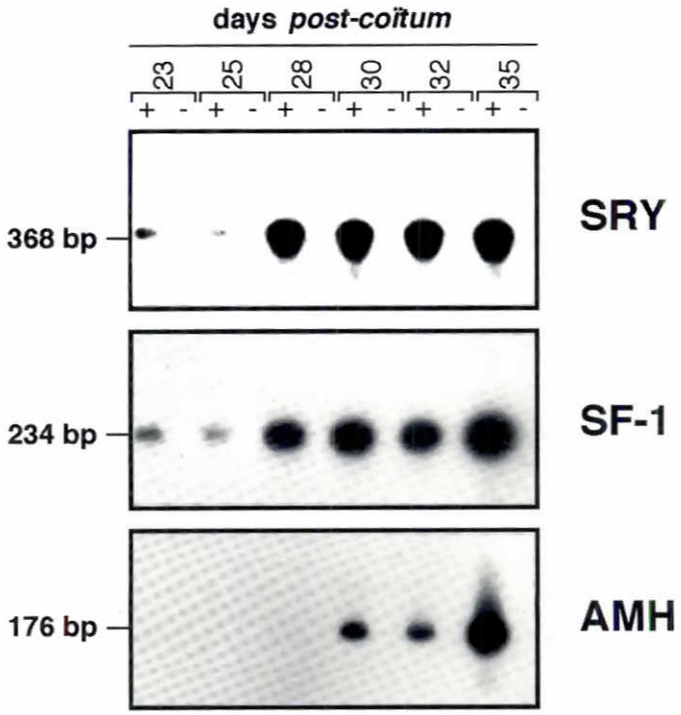


Fig. 7. Expression patterns of SRY, SF-1 and AMH in developing male gonads. *RT-PCR products obtained from gonadal RNA extracted at 23, 25, 28, 30, 32 and 35 dpc were Southern blotted and hybridized with a $\gamma^{32}P$ radiolabeled internal oligonucleotide corresponding to each investigated gene. Autoradiograms obtained from each membrane confirm the specificity of the amplification and precise the onset of SRY, SF-1 and AMH expression.*

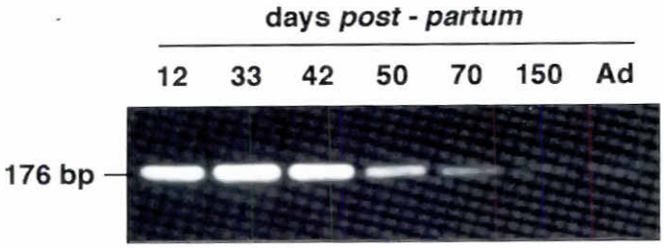


Fig. 8. AMH expression in postnatal testes. *Samples were taken at different stages of development from birth until adult. RNA was extracted, reverse transcribed and then amplified by PCR with AMH specific primers. An aliquot of each PCR reaction (10 µl) was electrophoresed on 4% agarose gel.*

and AMH genes observed in ovine male developing gonads is consistent with a direct interaction between these two genes. As in other investigated mammals, AMH in sheep is expressed from the beginning of the formation of the seminiferous tubules (30 dpc).

SF-1 also has a key role in the expression of cytochrome P450 steroid hydroxylases which convert cholesterol to various steroid products (Rice *et al.*, 1991; Lynch *et al.*, 1993; Hatano *et al.*, 1994; Ikeda *et al.*, 1994). In the sheep, we have observed the presence of SF-1 transcripts in ovary during all fetal life from the indifferent stage (25 dpc) until birth. The situation is completely different in mice where SF-1 expression disappears from the ovary between 13.5 and 16.5 dpc and reappears only during late gestation (Ikeda *et al.*, 1994). Moreover, in sheep ovary, P450 aromatase transcripts are detected from 30 dpc until birth with a maximal level between 35 to 50 dpc. The presence of P450 aromatase transcripts is correlated with the presence of 17-β estradiol. This hormone has been dosed in ovine ovary as early as 31 dpc and until at least 50 dpc. Then, at 62 dpc, the synthesis is very weak (Mauléon *et al.*, 1977). The synthesis of estrogens in ovary during the fetal period varies according to the species. In mice, there is no steroid synthesis before birth (Greco and Payne, 1994). In the sheep, gonadal sex becomes morphologically recognizable from 29-30 dpc, but meiosis in ovary does not begin before day 55 of gestation. In the ovary, the first germ cells begin meiosis at day 55 (McNatty *et al.*, 1995). The period between gonadal sex differentiation and onset of female meiosis has been termed the "delay period" (Byskov, 1979). It is during this period that estrogen synthesis occurs. In other species, e.g. mouse, meiosis in the female begins almost simultaneously with gonadal sex differentiation (Peters, 1970) without a delay period and there is no estrogen synthesis. These two phenomena (estrogen synthesis and delay of onset of meiosis) seem to be related but we can not come to a conclusion of cause-effect relation. Female gonads cultured in the presence of aromatase inhibitors will make it possible to answer this question.

The present study describes expression profiles which differ from those observed in mice for several key genes involved in the sex-determining pathway; particularly for SF-1 and P450 aromatase in female and SRY in male (Table 2). The mouse model does not appear to be the universal model for all mammals as regards sex determination, and the ruminant model could provide an alternative model system in which fetal material is easily available and where the interactions between different "actors" of sex-determining pathway can be scrutinized owing to the long period of development of these species. Moreover, with regard to the SRY gene structure, the ruminant model seems to be closer to humans than mice.

TABLE 1

SEQUENCES OF PRIMERS AND PCR CONDITIONS

GENES	PRIMERS	PCR CONDITIONS	SPECIES	REFERENCES
WT-1	5' - GACTGTTTTTAAGTAACTTAAG - 3' 5' - GTAAATAATAAATTCCCTCC - 3'	94°C - 1min. 45°C - 1min. 72°C - 1min.	HUMAN	Gessler *et al.* (1992)
SF-1	5' - GATCGCCAGGAGTTCGTCTG - 3' 5' - GTGCTTGTGGTACAGGTACTC - 3'	94°C - 1min. 53°C - 1min. 72°C - 1min.	COW	Honda *et al.* (1993)
SRY	5' - GCAATCGTATGCTTCTGCTA - 3' 5' - TGTCTCGGTGTATAGCTAG - 3'	94°C - 1min. 55°C - 1min. 72°C - 1min.	SHEEP	Payen and Cotinot (1994)
AMH	5' - CTATGAGCAGGCCTTCCTGG - 3' 5' - CCTCCAGGTGCAGGACCACC - 3'	94°C - 1min. 58°C - 1min. 72°C - 1min.	COW	Cate *et al.* (1986)
CYP 19 aromatase	5' - CTGTCGTGGACTTGGTCATG - 3' 5' - GGGGCCCAAAGCCAAATGGC - 3'	94°C - 1min. 58°C - 1min. 72°C - 1min.	HUMAN	Means *et al.* (1989)
ZFX / Y	5' - ATAATCACATGGAGAGCCACAAGCT - 3' 5' - GCACTTCTTTGGTATCTGAGAAAGT - 3'	94°C - 1min. 58°C - 1min. 72°C - 1min.	HUMAN	Aasen and Medrano (1990)

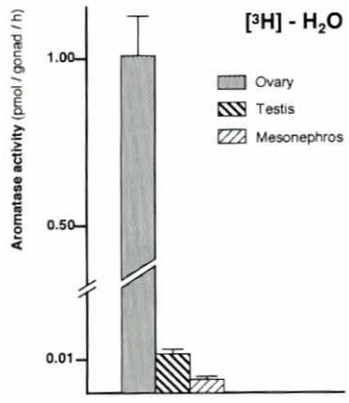
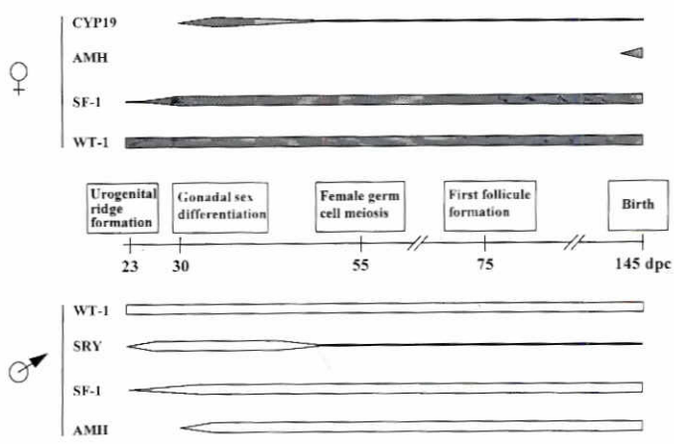


Fig. 9. Analysis of the total aromatase activity by the tritiated water technique on 49 dpc-old gonads. *Gonads and mesonephros of both sexes were incubated (1 per tube) in CMRL 1066 at 37°C in a Dubnoff incubator under a 95% O_2/ 5% CO_2 atmosphere for 5 h in the presence of 0.7 µM [1b-^{3}H] androstenedione. At the end of the incubation, tritiated steroids were removed by chloroform extraction followed by dextran-charcoal adsorption. The aromatization rate is expressed as the mean±SEM of the amount of precursor aromatized per gonad per hour. Two assays were performed for each tissue sample.*

TABLE 2

DYNAMIC REPRESENTATION OF RT-PCR DATA OBTAINED IN GONADS OF BOTH SEXES FROM UROGENITAL RIDGE FORMATION UNTIL BIRTH



Materials and Methods

Animals

Cyclic Prealpes and Ile de France ewes were synchronized with a prostagen-PMSG treatment (Cognié *et al.*, 1970). Animals were inseminated 24 h (day 0) and 36 h post sponge removal. Pregnant female tracts were recovered at slaughter and rapidly dissected to extract fetuses. From each fetus, different organs were taken. The gonads were first detached and frozen immediately in liquid nitrogen.

Sexing of fetuses

Before 50 days of gestation, all fetuses were sexed by PCR. A part of liver was used to obtain genomic DNA. Bry and ZFY, two markers of the Y chromosome were used to amplify liver genomic DNA. Primers and PCR conditions were described by Peura *et al.* (1991) and Aasen *et al.* (1990) respectively.

RNA extraction

Total RNA was extracted from frozen tissues using RNA-plus solution (Bioprobe Systems). All samples were treated with 50 U of DNase I, RNase-free (Boehringer) for 2-4 h at 37°C to avoid genomic DNA contamination.

RNA filter hybridization

Total RNA (15 µg) was denatured and electrophoresed on 2.2 M formaldehyde/agarose gels in MOPS buffer according to Sambrook *et al.* (1989). The RNA was transferred to Zetaprobe membrane (Bio-Rad). After UV irradiation, prehybridization and hybridization were performed at 65°C in a mixture containing 5xSSPE, 5xDenhardt, 0.1% SDS and 0.1 mg/ml salmon sperm DNA. Washings were performed in 2xSSC, 0.5% SDS at 65°C.

Construction and screening of cDNA library

Five micrograms of poly A^+ RNA isolated from male gonads of 29 day-old sheep fetuses were reverse transcribed using a kit (cDNA synthesis system- Amersham) and oligo(dT) as primer. EcoRI adapters were ligated to the cDNA and then were cloned into λgt10 phage arms (Promega) using standard procedure (Sambrook *et al.*, 1989). Packaging kit (Gigapack gold-Stratagene) was used according to the manufacturer's instructions. A library of $5.7x10^6$ recombinant clones was generated and amplified on *E. coli* strain C600 (Promega). For screening of the library, approximately $2.5x10^5$ phages were plated onto 22cmx22cm dish and plaque lifts were performed. Membranes were hybridized with ^{32}P-labeled probe specific for ovine SRY.

Reverse transcription- PCR

Three micrograms of DNase treated-RNA were reverse transcribed in 20 µl at 44°C for 50 min using 200 U of Superscript II (Gibco-BRL) and 7.5 µM random hexamers (Pharmacia) in the presence of 20 U of RNase Inhibitor (Boehringer). Negative controls were performed by omitting reverse transcriptase. One tenth of RT reaction was added to a 100 µl PCR reaction containing 200 µM each dNTP, 150 µM each primer, 2 U of Taq polymerase in the supplied buffer containing 2.5 mM $MgCl_2$ (Perkin Elmer-Cetus). Thirty cycles were performed in a Perkin Elmer apparatus. From each reaction, 10 µl aliquot was electrophoresed on 2% agarose-TBE gel. DNA was visualized by ethidium bromide staining.

For SRY, SF-1 and AMH, RT-PCR products were Southern blotted and probed with a γ-^{32}P radiolabeled internal oligonucleotide (M11: 5' ATGTTAGGGAGAGCAGCCAG 3' for SRY; AD4BP7: 5' GTTCCTCATTC-TCTTCAGCC 3' for SF-1 and AMH probe: 5': TGTGCCCCGCTGGCAA-CGGG 3').

For each investigated gene, RT-PCR products were cloned and sequenced to confirm the specificity of the bands observed.

Primers

No sequences were previously described in the sheep. For each, we have tested several primers and PCR conditions. For WT-1 we have previously shown the existence of an evolutionary conserved microsatellite in 3' untranslated region of the transcript (Vaiman *et al.*, 1995). Ovine specific primers were synthesized from this region. CYP19 primers arise from comparison between human and rat sequences and correspond to exon 9 and 10. For AMH, they were chosen in human-bovine conserved region and are located in exon 1. SF-1 primers surround intron 6. The sequences and PCR conditions are summarized in Table 1. Concerning SRY, primers amplify a specific region upstream HMG-box. Complete sequence of the ovine SRY open reading frame was previously published (Payen and Cotinot, 1994).

DNA sequencing

RT-PCR products were purified and cloned into pBluescript KS II+ (Stratagene). DNA sequencing was performed by using U.S.B. Sequenase, version 2.0 DNA sequencing kit (U.S.B.).The reaction mixture was run on a 8 M urea-6% polyacrylamide gel which was then dried and exposed to an X-ray film overnight

RACE-PCR

5' RACE-PCR

Total RNA was extracted from 30 dpc male fetal gonads and 35 dpp testes. Five micrograms of total RNA were reverse transcribed using random hexanucleotides (Pharmacia) and SuperScript II enzyme (Gibco-BRL). First-strand cDNA was treated by RNase H (Boehringer), purified on GlassMax-DNA isolation Spin cartridge system (Gibco-BRL) and poly-C tailed using dCTP and terminal transferase (Gibco-BRL). Forty PCR cycles were performed using APG anchor primer (poly G in addition to an universal amplification primer-Gibco-BRL) and SRY specific primers M14(TAGCAGAAGCATACGATTGC) or M29 (AGCTGAATACACA-GGATACC).

An aliquot of these PCR products were blotted and hybridized with an internal probe M7 (TTGCACCAAGTCAGTCTCTG) or M30 (TGTTGT-ATCTGAGATTGACC) respectively (Fig. 1A,C). The remaining part of the PCR reactions were electrophoresed on a 1% agarose gel. The DNA fragments corresponding to the size of hybridized products were then purified, cloned into pBluescript KS II+ and sequenced. The totality of the experiment was repeated three-fold and 46 clones were sequenced (Fig. 1B).

3' RACE-PCR

Total 40 dpc male fetal gonads RNA was reverse transcribed using NN^{-1} oligo(dT) primer and SuperScript II enzyme (Gibco-BRL). First round of asymmetric PCR using specific primers M32 (ATTCTATGTTCTTTCACAGC) or M47 (TCTGATGATGAGGTATCAGT) was performed during 20 cycles in 50 µl reaction. The totality of this reaction was used for PCR with M32 or M47 and APT primer (anchor sequence linked to the 5' end of the oligo(dT)-Gibco-BRL) for 10 cycles. Ten µl of this reaction was spotted on 2% agarose gel and after transfer, the membrane was hybridized with an internal probe M49 (CCATGGACTTATTTTCATTCC) or M48 (CTGGCAATACACTTGTTTCC). After purification on Tip 5 column (Qiagen) , the remaining 90 µl of PCR products were cloned into pBluescript KS II+ and sequenced. Six clones for each primer were sequenced.

Acknowledgments

We thank N. LePennec for his technical help, B. Vigier for his help in aromatase activity measurement and P. Mauléon for his constant interest and encouragement. We are also grateful to M. Fellous for helpful discussion and K. McElreavey for critical comments. This work was supported by INSERM grant (930705) and INRA AIP (4862).

References

AASEN, E. and MEDRANO, J.F. (1990). Amplification of the ZFY and ZFX genes for sex identification in human, cattle, sheep and goats. *BioTechnology 8:* 1279-1281.

BEHLKE, M.A., BOGAN, J.S., BEER-ROMERO, P. and PAGE, D.C. (1993). Evidence that the SRY protein is encoded by a single exon on the human Y chromosome. *Genomics 17:* 736-739.

BEHRINGER, R.R., CATE, R.L., FROELICK, G.J., PALMITER, R.D. and BRINSTER, R.L. (1990). Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. *Nature 345:* 167-170.

BYSKOV, A.G. (1979). Regulation of meiosis in mammals. *Ann. Biol. Anim. Biochem. Biophys. 19:* 1251-1261.

CATE, R.H., MATTALIANO, R.J., HESSION, C., TIZARD, R., FARBER, N.M., CHEUNG, A., NINFA, E.G., FREY, A.Z., GASH, D.J, CHOW, E.O., FISHER, R.A., BERTONIS, J.R., TORRES, G., WALLNER, B.P., RAMACHANDRAN, K.L., RAGIN, R.C., MANGANARO, T.F., MACLAUGHLIN, D.T. and DANAHOE, P.K. (1986). Isolation of the bovine and human genes for Müllerian Inhibiting Substance and expression of the human gene in animal cells. *Cell 45:* 685-698.

CHEN, F., McDONALD, C.C. and WILUSZ, J. (1995). Cleavage site determinants in the mammalian polyadenylation signal. *Nucleic Acids Res. 23:* 2614-2620.

CLÉPET, C., SCHAFER, A.J., SINCLAIR, A.H., PALMER, M.S., LOVELL-BADGE, R. and GOODFELLOW, P.N. (1993). The human SRY transcript. *Hum. Mol. Genet. 2:* 2007-2012.

COGNIÉ, Y., MARIANA, J.C. and THIMONIER, J. (1970). Etude du moment de l'ovulation chez la brebis normale ou traitée par un prostagène associé ou non à une injection de PMSG. *Ann. Biol. Anim. Biochem. Biophys. 10:* 15-24.

DANEAU, I., HOUDE, A., ETHIER, J.F., LUSSIER, J.G. and SILVERSIDES, D.W. (1995). Bovine SRY gene locus: cloning and testicular expression. *Biol. Reprod. 52:* 591-599.

EICHER, E.M. and WASHBURN, L.L. (1986). Genetic control of primary sex determination in mice. *Am. Rev. Genet. 20:* 327-360.

GESSLER, M., KÖNIG, A. and BRUNS, G.A.P. (1992). The genomic organization and expression of the WT-1 gene. *Genomics 12:* 807-813.

GOODFELLOW, P.N. and DARLING, S.M. (1988). Genetics of sex determination in man and mouse. *Development 102:* 251-258.

GRECO, T.L. and PAYNE, A.H. (1994). Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3beta-hydroxysteroid dehydrogenase, P450 17alpha-hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. *Endocrinology 135:* 262-268.

HACKER, A., CAPEL, B., GOODFELLOW, P. and LOVELL-BADGE, R. (1995). Expression of Sry, the mouse sex determining gene. *Development 121:* 1603-1614.

HARRY, J., KOOPMAN, P., BRENNAN, F.E., MARSHALL-GRAVES, J. and RENFREE, M.B. (1995). Widespread expression of the testis- determining gene SRY in a marsupial. *Nature Genet. 11:* 347-349.

HATANO, O., TAKAYAMA, K., IMAI, T., WATERMAN, M.R., TAKAKUSU, A., OMURA, T. and MAROHASHI, K.I. (1994). Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. *Development 120:* 2787-2797.

HIROBE, S., HE, W.W., LEE, M.M. and DONAHOE, P.K. (1992). Müllerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. *Endocrinology 131:* 854-862.

HONDA, S.I., MOROHASHI, K.I., NOMURA, M., TAKEYA, H., KITAJIMA, M. and OMURA, T. (1993). Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *J. Biol. Chem. 268:* 7494-7502.

IKEDA, Y., SHEN, W.H., INGRAHAM, H.A. and PARKER, K.L. (1994). Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid Hydroxylases. *Mol. Endocrinol. 8:* 654-662.

JESKE, Y.W.A., BOWLES, J., GREENFIELD, A. and KOOPMAN, P. (1995). Expression of a linear Sry transcript in the mouse genital ridge. *Nature Genet. 10:* 480-482.

JOSSO, N. and PICARD, J.Y. (1986). Anti-Müllerian hormone. *Physiol. Rev. 66:* 1038-1090.

JOST, A., PATSAVOUDI, E., MAGRE, S., CASTANIER, M. and SHOLLER, R. (1984). Relation entre organogénèse testiculaire et sécrétion de testostérone par le testicule foetal *in vitro*. *Pathol. Biol. 32:* 860-862.

JOST, A., VIGIER, B., PREPIN, J. and PERCHELLET, J.P. (1973). Studies on sex differentiation in mammals. *Recent Prog. Horm. Res. 29:* 1-41.

KOOPMAN, P., GUBBAY, J., VIVIAN, N., GOODFELLOW, P.N. and LOVELL-BADGE, R. (1991). Male development of chromosomally female mice transgenic for Sry. *Nature 351:* 117-121.

KOOPMAN, P., MÜNSTERBERG, A., CAPEL, B., VIVIAN, N. and LOVELL-BADGE, R. (1990). Expression of a candidate sex determining gene during mouse testis differentiation. *Nature 348:* 450-452.

KREIDBERG, J.A., SARIOLA, H., LORING, J.M., MAEDA, M., PELLETIER, J., HOUSMAN, D. and JAENISCH, R. (1993). WT-1 is required for early kidney development. *Cell 74:* 679-691.

LAHR, G., MAXSON, S.C., MAYER, A., JUST, W. PILGRIM, C. and REISERT, J. (1995). Transcription of the Y chromosomal gene, Sry, in adult mouse brain. *Mol. Brain Res. 33:* 179-182.

LALA, D.S., RICE, D.A. and PARKER, K.L. (1992). Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol. Endocrinol. 6:* 1249-1258.

LUO, X., IKEDA, Y. and PARKER, K.L. (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell 77:* 481-490.

LYNCH, J.P., LALA, D.S., PELUSO, J.J., LUO, W., PARKER, K.L. and WHITE, B.A. (1993). Steroidogenic factor 1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. *Mol. Endocrinol. 7:* 776-786.

MAULÉON, P., BÉZARD, J. and TERQUI, M. (1977). Very early 17 beta- estradiol secretion by fetal sheep ovary. *In vitro* study. *Ann. Biol. Anim. Biochem. Biophys. 17:* 399-401.

McNATTY, K.P., SMITH, P., HUDSON, N.L., HEATH, D.A., TISDALL, D.J., O,W.S. and BRAW TAL, R. (1995). Development of the sheep ovary during fetal and early neonatal life and the effect of fecundity genes. *J. Reprod. Fertil. 49 (Suppl.):* 123-135.

MEANS, G.D., MAENDROO, M.S., CORBIN, C.J., MATHIS, J.M., POWELL, F.E., MENDELSON, C.R. and SIMPSON, E.R. (1989). Structural analysis of the gene encoding human aromatase cytochrome P-450, the enzyme responsible for oestrogen biosynthesis. *J. Biol. Chem. 264:* 19385-19391.

MÜNSTERBERG, A. and LOVELL-BADGE, R. (1991). Expression of the mouse anti-Müllerian hormone gene suggests a role in both male and female sexual differentiation. *Development 113:* 613-624.

NASRIN, N., BUGGS, C., KONG, X. F., CARNAZZA, J., GOEBL, M. and ALEXANDER-BRIDGES, M. (1991). DNA-binding properties of the product of the testis-determining gene and a related protein. *Nature 354:* 317-320.

PAGE, D.C. (1986). Sex reversal: deletion mapping the male- determining function of the human Y chromosome. *Cold Spring Harbor Symp. Quant. Biol. 51:* 229-235.

PAYEN, E.J. and COTINOT, C.Y. (1994). Sequence evolution of SRY gene within Bovidae family. *Mammal. Genome 5:* 723-725.

PELLETIER, J., SCHALLING, M., BUCKLER, A.J., ROGERS, A., HABER, D.A. and HOUSMAN, D. (1991). Expression of the Wilms tumor gene WT1 in the murine urogenital system. *Genes Dev. 5:* 1345-1356.

PETERS, H. (1970). Migration of gonocytes into the mammalian gonad and their differentiation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci. 9:* 91-101.

PEURA, T., HYTTINEN, J.M., TURUNEN, M. and JANNE, J. (1991). A reliable sex determination assay for bovine preimplantation embryos using the polymerase chain reaction. *Theriogenology 35:* 547-555.

PRITCHARD-JONES, K., FLEMING, S., DAVIDSON, D., BICKMORE, W., PORTEOUS, D., GOSDEN, C., BARD, J., BUCKLER, A., PELLETIER, J., HOUSMAN, D., VAN HEYNINGEN, V. and HASTIE, N. (1990). The candidate Wilm's tumour gene is involved in genitourinary development. *Nature 346:* 194-197.

RICE, D.A., MOUW, A.R., BOGERD, A.M. and PARKER, K.L. (1991). A shared promoter element regulates the expression of three steroidogenic enzymes. *Mol. Endocrinol. 5:* 1552-1561.

SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. (1989). *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

SHEN, W.H., MOORE, C.C.D., IKEDA, Y., PARKER, K.L. and INGRAHAM, H.A. (1994). Nuclear receptor steroidogenic factor 1 regulates the Müllerian inhibiting substance gene: a link to the sex determination cascade. *Cell 77:* 651-661.

SINCLAIR, A.H., BERTA, P., PALMER, M.S., HAWKINS, J.R., GRIFFITHS, B.L., SMITH, M.J., FOSTER, J. W., FRISCHAUF, A.M., LOVELL-BADGE, R. and GOODFELLOW, P.N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature 346:* 240-244.

SU, H. and LAU, Y.F.C. (1993). Identification of the transcriptional unit, structural organization, and promoter sequence of the human sex-determining region Y (SRY) gene, using a reverse genetic approach. *Am. J. Hum. Genet. 52:* 24-38.

VAIMAN, D., PAILHOUX, E., PAYEN, E., SAIDI-MEHTAR, N. and COTINOT, C. (1995). Evolutionary conservation of a microsatellite in the Wilms' tumour (WT) gene : mapping in sheep and cattle. *Cytogenet. Cell Genet .70:* 112-115.

VIGIER, B., FOREST, M.G., EYCHENNE, B., BÉZARD, J., GARRIGOU, O., ROBEL, P. and JOSSO, M.G. (1989). Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries. *Proc. Natl. Acad. Sci. USA 86:* 3684-3688.

VIGIER, B., WATRIN, F., MAGRE, S., TRAN, D. and JOSSO, N. (1987). Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it *in vitro. Development 100:* 43-55.

VILAIN, E., FELLOUS, M. and McELREAVEY, K. (1992). Characterization and sequence of the 5' flanking region of the human testis- determining factor SRY. *Methods Mol. Cell. Biol. 3:* 128-134.

WELSHONS, W.J. and RUSSELL, L.B. (1959). The Y chromosome as the bearer of male determining factors in the mouse. *Proc. Natl. Acad. Sci. USA 45:* 560-566.

ZAMBONI, L. and UPADHYAY, S. (1982). The contribution of the mesonephros to the development of the sheep fetal testis. *Am. J. Anat. 165:* 339-356.

Received: March 1996
Accepted for publication: April 1996