Sox9: an Sry related gene involved in sex determination

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ABBREVIATIONS

ABP - Androgen binding protein
AHC - Adrenal hypoplasia congenita
AMH - Anti-Müllerian hormone
bp - base pair
BSA - Bovine serum albumin
°C - Celsius
CD - Campomelic dysplasia
COS - CV-1 origin, SV40
DAX1 - DSS-AHC critical region on the X, gene 1
DDS - Denys-Drash syndrome
DMEM - Dulbecco's modified Eagle's medium
DNA - Deoxyribonucleic acid
DSS - Dosage sensitive sex reversal
dpc - days post coitum
dpp - days post partum
EDTA - Ethylenediaminetetraacetic Acid
ELP - Embryonal long terminal repeat-binding protein
EMSA - Electrophoretic mobility shift assay
ES cells - Embryonic Stem cells
FCS - Fetal calf serum
FGF - Fibroblast growth factor
FITC - Fluorescein isothiocyanate
FS - Frasier syndrome
FSH - Follicle-stimulating hormone
FTZ-F1 - Fushi tarazu factor 1
g - gram
HHG - Hypogonadotropic hypogonadism
HMG - High mobility group
HOX - Homeo box
IgG - Immunoglobulin G
kb - kilobase
kDa - kiloDalton
l - liter
LEF-1 - Lymphoid enhancer-binding factor 1
LH-B - Luteinizing hormone-β subunit
M - Molar
ml - millilitre
mM - millimolar
Mat - Mating type genes
min - minute
MIS - Müllerian inhibiting substance
mg - milligram
NGFI-A - Nerve growth factor induced gene-A
OCT - Octamer binding protein
ORF - Open reading frame
PAX - Paired box
PBS - Phosphate-buffered saline
PCR - Polymerase chain reaction
PGC - Primordial germ cells
PMDS - Persistent Müllerian duct syndrome
PMSF - Phenylmethylsulfonyl fluoride
P-Mod-S - Peritubular modifies Sertoli
rpm - revolutions per minute
RT - Room temperature
RT-PCR - Reverse transcription-PCR
Sap62 - Spliceosome protein 62
sec - second
SF1 - Steroidogenic factor 1
SOX - SRY/Sry box
SRY - Sex determining region-Y chromosome
Ste11 - Sterility gene 11
STO - Swiss mice derived, Thymidine kinase, Oubian resistant
TBS - Tris-buffered Saline
TCF - T-cell specific factor
TDF - Testis determining factor - Y chromosome
Tdy - Testis determining Y gene
UBF - Upstream binding factor
V - Volt
WAGR - Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation
WT1 - Wilms' tumour 1
ZFY - Zinc finger - Y chromosome
ABSTRACT

The process of sex determination involves a developmental decision which gives rise to the choice of male or female specific gonadal differentiation. These events ultimately lead to sexual differentiation, resulting in testis or ovary formation and subsequent male or female development of the embryo. The choice of either forming a testis or an ovary by the bipotential indifferent gonad, depends on the presence or absence of the Y-linked testis determining gene, \textit{SRY} (Sex determining region on the \textit{Y} chromosome). \textit{SRY} is a member of a large family of embryonically expressed genes known as the \textit{SOX} gene family (SRY - box related genes). One member of this family, \textit{SOX9}, maps to chromosome 17 in humans and was recently shown to be the gene responsible for Campomelic dysplasia (CD). CD is a rare but frequently fatal syndrome, in which patients exhibit bowing of the long bones and other skeletal malformations. Of the reported CD cases that are chromosomally male, more than two thirds are phenotypically female.

To begin to understand the role of \textit{Sox9} in sex determination its expression was analysed in detail during gonadal development in the mouse and in two situations where testis differentiation occurs in the absence of \textit{Sry}. In the mouse, \textit{Sox9} was found to be expressed at a low level in the early genital ridge in both \textit{XX} and \textit{XY} embryos at a time
prior to *Sry* expression in males. Its expression increases to a high level in testis, specifically in Sertoli cells where it is maintained throughout fetal development and in the adult testis. However, in the developing ovary the gene is downregulated coincident with the onset of *Sry* expression in XY embryos. In the chick, where there is no evidence for an *Sry* gene, *Sox9* expression is also testis specific from the earliest sexually dimorphic stages. Also, in one type of experimental sex reversal in the mouse, *Sox9* expression begins coincident with the morphological appearance of Sertoli-like cells in grafts of fetal ovaries transplanted to adult kidney capsules.

Another pertinent question was to find genes that could be regulated by SOX9. The gene coding for the *Anti-Müllerian hormone (Amh)* is a good candidate. High levels of *Sox9* expression just precede the onset of *Amh* transcription. There are two important binding sites located in the 5' region of *Amh*: an element that shares homology with demonstrated *in vitro* binding sites for the testis determining factor SRY and the Lymphoid enhancer factor 1 (LEF-1), and an element that closely resembles the AGGTCA "half site" defined for nuclear hormone receptors. The last one has been shown to be a binding site for SF1 (Steroidogenic Factor 1). Co-transfection and electrophoretic mobility shift (EMSA) assays were performed; the results indicating a co-operative effect between SOX9 and SF1 to activate the *Amh* promoter.
A targeted mutation was introduced into the *Sox9* gene via homologous recombination in ES cells. Mouse chimaeras were generated using two different methods; conventional injection into normal host blastocysts and generation of chimaeras with tetraploid host embryos. Both techniques showed that the majority of mouse chimaeras resulting from *Sox9* +/- embryonic stem (ES) cells die around day 8.5 of gestation, presenting severe posterior malformations. These results are in agreement with the CD phenotype in which the patients show a variety of malformations, of which the posterior ones are the most severe, and a high mortality *in utero*.

All these results imply a very strong correlation in the gonad between high levels of *Sox9* expression and Sertoli cell differentiation. Given the timing of expression and the XY sex reversal phenotype of CD patients, *Sox9* may be a direct target for SRY in mammals, activate AMH production and be a critical Sertoli cell differentiation factor, perhaps in all vertebrates.
CHAPTER I.
GENERAL INTRODUCTION
I.1. First thoughts on sex determination

What determines the sexes? This question has been asked and debated since ancient times. For example Anaxagoras proposed that the "germ" came from the male while the female only provided the place in which it developed, and a male or a female would form depending from which testis a germ originates; male from the right and female from the left. Sex determination could also depend on the side of the uterus where the embryo would implant: male if the embryo would develop on the right side of the uterus, female on the left. Empedocles said that the determination would take place in the uterus: the hotter or colder the uterus, a male or female embryo would develop. Aristotle on the other hand believed that the heat of the love making was important to determine the sex of the child. He counselled the elderly to have intercourse during summer to produce male offspring. What all these theories had in common was that sex was determined by environmental factors coincidental to the act of fertilisation (Platt, 1972). Nowadays, we know that the environment has little to do with sex determination, at least in mammals!

Two main definitions arise in this field: sex determination itself, which is the decision that the embryo has to make during its development to became a male or a female, and sex differentiation which is the development of the secondary sexual characteristics.
I. II. The biology of sexual development

Sex determination and differentiation comprise a series of complex events during embryological development and result in the final differentiation of the gonads at around puberty stages. Understanding the biology of embryonic development of the urogenital derivatives is essential to discern what is happening at the molecular level. For this reason, the next subsections will describe the development of the sexual structures as well as the several cell types that form both the male and female gonads. Finally the cell-cell interactions that take place in the embryonic and adult gonad will be briefly reviewed.

I. II. 1. Embryonic development of the urogenital tract

The urogenital tract comprises the excretory system and the genital system. The excretory system develops through three consecutive primordial kidneys that develop during fetal life, pronephros, mesonephros and metanephros respectively. In mammals, the excretory anlagen, or nephric primordium, first appears between 11 to 13 somites stage. A condensation of intermediate mesoderm extends caudally from the pronephros and is separated from the dorsal mesoderm, forming the nephrogenic ridge. This ridge will give rise to the mesonephric nephrons and tubules. Several canals project from the
ceolomic epithelium to this ridge. The Wolffian duct develops during the interval between 15 to 20 somites stages. This arises as a cord which splits from the top of the nephrogenetic ridge and assumes a position lateral to it, and develops caudally starting around somite 12. This cord then hollows out to form a tube (Torrey, 1945).

The Müllerian ducts arise parallel to the Wolffian duct between 11.5 and 12.5 days post coitum (dpc) and develop as paired longitudinal invaginations of ceolomic epithelium. The Müllerian ducts do not open into the urogenital sinus until much later, when canalisation of the vagina occurs.

The gonads first arise at about 9.5-10 dpc in mice, as a thickening along the inner surface of the mesonephros (Fig.I.1.). Controversy has arisen when the cellular origin of the gonadal primordium is debated: some authors defend the production of cells from the ceolomic epithelium and some from the mesonephros. However, the current theory supports that the gonads are formed from cells which originate in both tissues (Byskov, 1986; Martineau et al., 1997 and section I.II.4.).

Immediately following sex determination, between 10.5 and 11.5 dpc, sex differentiation starts. This process is not synchronous but proceeds cranio-caudally, affecting the ducts first and then the external genitalia (Torrey, 1945). The Müllerian ducts will give rise to the uterus, oviducts and the upper portion of the vagina. The
Wolffian duct will give rise to the epididymides, vas deferens and seminal vesicles. Anti-Müllerian hormone (AMH) produced by the Sertoli cells of the fetal testes causes the regression of the Müllerian ducts and testosterone produced by Leydig cells induces the differentiation of the Wolffian duct system (see section I.II.3.1. and I.II.3.3.). The absence of both hormones during female fetal development permits the development of the Müllerian duct system while the Wolffian ducts passively regress (Josso, 1981; Fig.1.1.).

♦ I. II. 1. 1. Development of the gonads

The organisation of gonadal primordia into ovaries or testes is the first morphological event of sexual differentiation during fetal development. Four different cell lineages comprise the developing gonad, three somatic and one germinal, each of them bipotential: the supporting cells, the steroid-producing cells, the connective tissue cells and the germ cells. The supporting cells, differentiate into Sertoli cells in the testis and into follicle cells in the ovary. The steroid-producing cell lineage develops as Leydig cells and Theca cells in the testis and the ovary respectively. The connective tissue cells include the peritubular myoid cells in the testis as well as endothelial cells which form blood vessels both in testis and ovary. In the fetal testis, the germ cells develop into spermatogonia and arrest at mitosis around 13.5 dpc while in the fetal ovary at the
Fig. I. Development of the mouse urogenital tract. The Müllerian ducts give rise to the uterus, oviducts and the upper portion of the vagina. The Wolffian ducts give rise to the epididymes, vas deferens and seminal vesicles. The gonads arise along the inner surface of the mesonephros (adapted from Behringer, 1995).
Indifferent Stage

Mesonephros

Müllerian Duct

Wolffian Duct

Urogenital Sinus

Indifferent Gonads

Testis

Epididymis

Vas Deferens

Seminal Vesicle

Ovary

Oviduct

Uterine Horn

Body of Uterus
same time point, they progress into meiosis and begin to form oocytes (Byskov, 1981). The seminiferous tubules are formed by the Sertoli cells that provide support for the developing germ cells. Peritubular myoid cells surround the tubules and are in contact with the basal surface of the Sertoli cells. In the interstitium of the testis between the tubules are the Leydig cells, responsible for the production of androgen, intertubular blood vessels, macrophages and cells with fibroblast like appearance (Byskov, 1981).

The structure of a female gonad is recognised by its lack of cellular organisation as opposed to the male gonad at 12.5 dpc. At this stage, the germ cells gather in small clusters that are uniformly distributed throughout the ovarian tissue. Only at around 14 dpc, when the germ cells enter meiosis, the first signs of ovary differentiation occur. Subsequently, the follicle cells aggregate around germ cells to form follicles (Torrey, 1945).

I . II . 2 . Germ cells

The germ cells play no role in determining the structure of the testis. XY supporting cells in testes devoid of germ cells, as occurs with the mutants White spotting (W/W) or Steel (Ss/Ss) differentiate and form normal tubules (Chabot et al., 1988; Mintz and Russel, 1957; Zsebo et al., 1990).
The primordial germ cells are the only lineage whose origin is clearly established. A cluster of primordium germ cells (PGC) is first detected at around 7 dpc just posterior to the definitive primitive streak in the extraembryonic mesoderm, and only later in development do the cells move back to the embryo, to the mesoderm of the primitive streak and then to the endoderm (visceral and hindgut) (Ginsburg et al., 1990). Between 9 and 11 dpc, PGCs migrate out of the hindgut endoderm and through the dorsal mesentery, around the dorsal aorta and the celomic angles towards the genital ridges. By 12.5 dpc the PGCs are found aggregated in tight clumps in the gonad (Fox et al., 1981).

Male mouse germ cells enter mitotic arrest around 13 to 14 dpc but do not enter meiosis until after birth. Mitotic male germ cells are named prospermatogonia. Before puberty these cells restart a new wave of mitotic activity. At puberty a fraction of the germ cells, now called spermatogonia, begin the spermatogenic cycle by entering the first meiotic prophase, thus becoming spermatocytes. After the meiotic divisions, the spermatids differentiate into spermatozoa with either a Y or an X chromosome (Moens, 1987).

The mitotically dividing germ cells in a female gonad are termed oogonia. When multiplication ceases and the cells enter meiosis at around 14 dpc, they are named oocytes. The oocytes arrest in the last phase of the meiotic prophase, the diplotene stage and become surrounded by follicle cells and a basal lamina, forming a follicle. At
ovulation, the oocyte resumes meiosis, completing the second meiotic division after fertilisation. Oogenesis is thus the process that transforms an oogonium into an oocyte (Peters, 1978).

I. II. 3. Somatic cells

◆ I. II. 3.1. Sertoli cells

The embryonic Sertoli cells are thought to direct the differentiation of the indifferent gonad, having an intimate relationship with the formation of epithelial testicular cords. Anti-Müllerian hormone is produced by these cells causing the regression of the Müllerian ducts in the male embryo (Magre and Jost, 1991). Sertoli cells only proliferate during fetal and neonatal development (Jegou, 1992).

Sertoli cells in the adult testis provide support and sustenance to the germ cells as they transit the seminiferous epithelium (Griswold et al., 1988). Sertoli cells are bound to each other by means of linear tight junctions. This barrier is impermeable to certain macromolecules. The barrier also creates two distinct compartments within the seminiferous epithelium: basal and adluminal compartments each having a characteristic
cellular, molecular and ionic composition. Sertoli cells acts as "nurse cells" to create the proper structural support for the developing germ cells (Bardin et al., 1993).

◆ I. II. 3.2. Peritubular myoid cells

Peritubular myoid cells are a mesenchymal cell type. They surround the seminiferous tubule and, in co-operation with Sertoli cells, form the basal lamina. Therefore, one important peritubular cell function is to provide structural support for the seminiferous tubule and help to maintain the proper cytoarchitecture of the epithelium. They show some characteristics of vascular smooth-muscle cells and secrete several metalloproteinases, such as type IV procollagenase (Ailenberg et al., 1991). In vitro, Activin A, Activin receptor type II and P-Mod-S (testicular paracrine factor - see section II.4.) have also been reported to be produced by these cells (Winter et al., 1994).

◆ I. II. 3.3. Leydig cells

Leydig cells differentiate in the extratubular compartment and are responsible for the production of testosterone by the testis. The cytoplasm of these cells contains large amounts of tubular smooth endoplasmic reticulum and numerous large round
mitochondria with tubular cristae, features that are characteristic of steroid-producing cells (Ewing and Keeney, 1993). In addition it is also proposed that the Leydig cells are the "Astrocytes" of the blood testis barrier and function in part, to induce and/or maintain barrier features in order to prevent the free exchange of solutes between blood and tissue (Holash et al., 1993).

I. II 3 4. Follicle cells

Follicle cells, also named granulosa cells, share properties with the Sertoli cells of the testis. They are thought to derive from the supporting cell lineage and also form a basal lamina that surrounds the oocytes. They produce receptors for a gonadotrophin produced by the anterior pituitary, the Follicle-stimulating hormone (FSH). FSH stimulates the follicle cells to multiply and to produce estrogen. The follicle cells, however, require help to produce estrogen because they lack the ability to produce the androgens that are precursors for estrogen. This is supplied by the theca cells (Vander et al., 1990).
Theca cells form the outer layer of the follicle. They share most of the ultrastructural features of follicle cells although the mitochondria are more consistent in size and internal structure. Luteinizing hormone, another gonadotrophin produced by the anterior pituitary, stimulates Theca cells to proliferate and to synthesise androgens. Thus, Theca cells are analogous to Leydig cells in that they produce androgens (Vander et al., 1990; section I.II.4.).

Cell-cell interactions

Cell-cell interactions between different cell types have an important role in maintaining and regulating the differentiation function and growth of the cell. Cellular interactions in the testis or ovary are essential to ensure a correct cytoarchitecture, a nutritional environment and correct cell-cell signalling events.

A number of recent experiments suggest that migration of the cells from the mesonephros into the XY gonad is essential for cord formation in the testis (Buehr et al., 1993; Martineau et al., 1997; Merchant-Larios et al., 1993). Peritubular myoid cells and endothelial cells in response to an inductive signal secreted by the gonad, migrate
from the mesonephros into the gonad. In the absence of cell migration, Sertoli cells are not arranged into cords and no structural sex differentiation is evident.

In the adult testis, with a presence of an efficient blood-testis barrier, most of the components generally available to cells from the circulatory system must be produced or transported by the Sertoli cells for delivery to the developing germ cells. Sertoli cells produce energy metabolites, pyruvate and lactate, that subsequently can be used by germinal cells. Also Sertoli cells produce growth factors as well as proteins involved in the transport of essential components to germ cells such as transferrin (transport of iron) and ceruplasmin (transport of copper) (Bardin et al., 1993).

Inhibition of androgen produced by Leydig cells interferes with spermatogenesis. This observation lead to the proposal that testosterone acts on the seminiferous tubules to maintain spermatogenesis. Sertoli cells also contain androgen receptors and respond to testosterone. These cells also produce estrogens which are thought to act on Leydig cells to inhibit androgen production (Odell, 1989).

Primordial myoid cells and Sertoli cells aid in the production and formation of the basement membrane. Both cell types produce secreted and cell-surface associated proteoglycans. Co-culture of these cells stimulate Androgen binding protein (ABP) and transferrin production by Sertoli cells. Peritubular myoid cells secrete in response to
testosterone, a factor named P-Mod-S for "peritubular modifies Sertoli" that is proposed for this activation. Interactions between Peritubular myoid cells and Sertoli cells provide a classical example of mesenchymal-epithelial interactions (Skinner, 1991).

Germ cells are also known to interact with Sertoli cells. The effects on Sertoli cells are variable according to the stage of germ cell maturation. For example, production of FSH and ABP by the Sertoli cells vary with the different stages of spermatogenesis.

In the ovary, several analogies can be made between the cells that form the follicle, and the cells that form the seminiferous tubules in the testis. The follicle cells are similar to the Sertoli cells in controlling the microenvironment where the germ cell develops and matures and the theca cells are analogous to the Leydig cells in that they produce androgens that diffuse to the granulosa cells in order to be converted in estrogens. These two different cell types co-operate to metabolise cholesterol into estrogen. Follicle cells lack steroid 17α-hydroxylase (P450c17) activity which is involved in the conversion of progesterone to androgens, and theca cells lack P450 aromatase which catalyses the conversion of androgens into estrogens. Both cell types co-operate to metabolise progesterone into estrogens (Gore-Langton and Armstrong, 1988).

Another intriguing feature is the germ cell sex. The embryonic germ cells differentiate according to the sex of the gonad in which they reside, not according to their own sex.
chromosome constitution. It is known that the male gonadal environment promotes mitotic arrest and prevents meiosis. In the male embryo, germ cells that do not reach the gonad at the end of its migration and stay instead in tissues such as the adrenal primordium enter meiosis. This suggests that an inhibitor of meiosis is produced by the testis (McLaren, 1995).

I. III. In search of the testis determining factor

Classical embryological studies have helped us to a better understanding of sex determination. Alfred Jost, considered as the father of modern fetal endocrinology, discovered that the presence of testes during mammalian embryogenesis resulted in male differentiation. He castrated genetically male fetal rabbits at an early stage and observed phenotypical female differentiation (Jost, 1953). Jost thus deduced that the testis produced substances responsible for masculinizing the fetus. Testosterone was demonstrated to be the major effector, but because it was not responsible for the degeneration of the female structures, the existence of a second substance, the anti-Müllerian hormone (AMH), also called Müllerian Inhibiting Substance (MIS), was predicted (Jost, 1953). In summary, Alfred Jost concluded that the differentiation of the developing gonad determined sexual differentiation of the embryo, testis differentiation.
being the "dominant pathway". Therefore the testes appear to be the body sex differentiators, while the presence or absence of ovaries makes no difference.

In addition the presence of the Y chromosome determines whether an embryo will develop as a male. Individuals with a normal Y chromosome develop as males, irrespective of the presence of supernumerary X chromosomes. For instance, individuals that have Klinefelter's syndrome and are chromosomally XXY, develop the male phenotype whereas individuals chromosomally XO or with X polysomies have a female phenotype. This again shows the dominance of the Y chromosome over the female phenotype (Ford et al., 1959; Jacobs and Strong, 1959; Welshons and Russell, 1959).

I. III. 1. TDF/Tdy - The cloning of SRY

In combination, the facts described above lead to the concept of a gene or genes on the Y chromosome, encoding a signal that is expressed in the testis and thus is responsible for testis formation. This is referred to as the testis determining factor (TDF) in humans, or Tdy (Testis determining Y gene) in mice and other mammals. Several genes have been proposed as candidates for TDF/Tdy.
♦ I . III . 1 . 1 . H-Y antigen

For nearly ten years a hypothesis postulating that the male-specific histocompatibility antigen H-Y was the primary testis-inducer held sway (Ohno et al., 1979). H-Y antigen was first detected when male skin grafts were rejected by female mice within the highly inbred C57BL/6 strain (B6). Skin grafts transplanted from females to males were accepted and the same was true of skin grafts exchanged between males or between females. The incompatibility of male to female grafts was attributed to a male-specific transplantation antigen determined by a gene in the Y chromosome (Eichwald and Silmser, 1955). However some XY females with gonadal dysgenesis lacked H-Y antigens (Ghosh et al., 1978) and not all phenotypically normal males were H-Y positive, which started to add doubts to this theory (Teyssier et al., 1983). The lack of H-Y antigens in XX male mice carrying a minimal testis determining portion of the Y chromosome conclusively excluded it as a candidate for Tdy (McLaren, 1988) and the hypothesis was abandoned.

♦ I . III . 1 . 2 . ZFY gene

Chromosomal mapping of abnormalities linked with sex reversal in humans, for example the loss of part of the Y chromosome in XY females or gain of a Y chromosome segment
in XX males, allowed the testis determining gene (TDF) to be localised to the short arm of the Y chromosome.

Detailed mapping experiments relying on finding the smallest part of the Y chromosome able to give XX males and a XY female patient missing part of this region led to the identification of the ZFY gene. ZFY encodes a zinc finger protein, suggesting that it could be a transcription factor (Page et al., 1987). In the mouse there are two ZFY homologues on the Y chromosome: Zfy-1 and Zfy-2. However, the analysis of the two ZFY homologues were not consistent with ZFY being the testis determining factor. First Zfy-2 was not present in XXsxr\(^b\) males, which carry a small sex reversing translocation of the testis determining region (Page, 1988). These findings placed Zfy-2 as a very unlikely candidate for a gene involved in testis determination. And Zfy-1 expression is detected in XY\(^{dym1}\) mice in spite of their development as females (Gubbay et al., 1990). In addition Zfy-1 transcripts are confined to germ cells which have previously been demonstrated not to be essential for testis determination (Koopman et al., 1989).

Further evidence against the equivalence of TDY and ZFY comes from studies in marsupials where the formation of a testis is dependent on the presence of the Y chromosome. ZFY sequences are not found on the sex chromosomes but there are autosomal homologues (Sinclair et al., 1990).
ZFY was definitively excluded from being the testis determining factor when it was found to be absent in several XX male patients with just 35 kb of Y chromosome unique sequence (Palmer et al., 1989).

◆ I. III. 1. 3. SRY

Extensive mapping of this 35 kb region of the Y chromosome identified another open reading frame (Gubbay et al., 1990; Sinclair et al., 1990). In the mouse, this open reading frame encoded a gene transiently expressed in embryonic urogenital ridges, as expected for a testis determining factor (Koopman et al., 1990). In addition, a screen carried out on human patients with sex reversed phenotypes showed mutations in this open reading frame (Berta et al., 1990). The sex determining gene had been mapped. This gene was called SRY (for Sex determining region, Y chromosome).

The definitive proof for SRY being the testis determining factor came from further studies in mice. Sry expression was analysed and found to be limited to the period in which the indifferent gonad begins to differentiate (Koopman et al., 1990). When Sry was introduced into fertilised mouse eggs, chromosomal females were sex reversed and developed morphologically as males (Koopman et al., 1991).
In mice, *Sry* transcripts were detected in gonads at 10.5, 11.5 and 12.5 dpc by reverse transcription PCR (RT-PCR). *Sry* expression occurs in the somatic portion of the genital ridge as it is expressed in mice lacking germ cells. In the adult *Sry* is expressed in the testis by germ cells, as an unusual circular transcript for which no function has yet been determined (Capel *et al.*, 1993; Koopman *et al.*, 1990).

The *Sry* locus has a very unusual structure in that it consists of a long open reading frame (ORF) of 2739 base pairs (bp), flanked by a large inverted repeat of at least 17 kilobases (kb) (Gubbay *et al.*, 1992). The *Sry* ORF contains an HMG box binding motif of 237 bp. The majority of transcripts comprise a single exon of 4942 bp, which initiates 283 bp 5' of the HMG box and extends into the 3' arm of the inverted repeat. The large ORF of mouse *SRY* (395 amino acids) is composed of the 79 amino acid HMG box flanked by a short two amino acids N-terminal domain and 314 amino acid C-terminal domain characterised by a glutamine/histidine rich repeat region. The human *SRY* ORF differs from the mouse, it is considerably smaller, consisting of only 204 amino acids with the HMG box flanked by a 57 amino acids N-terminal domain and a 68 amino acid C-terminal domain and lacks the glutamine repeat (Hacker *et al.*, 1995).
The transient expression of Sry during embryogenesis and chimaera analysis suggests that it triggers the differentiation of supporting precursor cells into Sertoli cells characteristic of testes (Palmer and Burgoyne, 1991). Once Sertoli cells begin to differentiate they are thought to trigger the other cell lineages in the gonad to follow the male pathway (Lovell-Badge and Hacker, 1995). This results in the differentiation of Leydig cells from steroidogenic cell precursors, the arrest of germ cells in mitosis rather than early entry into meiosis characteristic of oocytes and the organisation of connective tissue cells into a testicular pattern.

I. IV. Abnormal sex development in humans

As it was described in the previous chapter, genetic and molecular studies of gonadal sex reversal syndromes have been essential in the identification and cloning of genes involved in sex determination. For that reason I shall briefly discuss the current understanding of human sex reversed syndromes and genes involved in sex determination in both humans and mice.
I. IV. 1. XY sex reversal

XY sex reversal has been loosely characterised as 46,XY females with complete gonadal dysgenesis, 46,XY females with partial gonadal dysgenesis and 46,XY true hermaphrodites. These concepts are flexible, due to the fact that in one given syndrome the range of gonadal dysgenesis may vary from patient to patient (Schafer, 1995). The concept of gonadal dysgenesis (Gonadendysgenesie) was introduced in 1912 by Kermauner (Opitz and Pallister, 1979). Until that period patients without well defined gonads were designated as "males without testes" or as "females without ovaries" on the basis of their genitalia.

This section, besides describing disorders of gonadal differentiation also describes disorders that lead to 46,XY pseudohermaphroditism, in which well developed vagina and uterus are associated with more or less well differentiated and even functional testes.

◆ I. IV. 1. 1. Swyer syndrome

Phenotypically female patients exhibiting a 46,XY karyotype with complete gonadal dysgenesis are categorised into the Swyer syndrome. The definition of gonadal
dysgenesis accounts for phenotypically female patients with a 46,XY karyotype that have a complete lack of functioning gonadal tissue. The resulting gonads often appear as white fibrous streaks, devoid of follicles and normal germ cells, and are endocrinologically inert. These patients have height above the average, eunuchoidal proportions, little or no mammary development, have pubic and axillary hair in normal amounts, vagina of normal length and a small uterus. All the mutations found in SRY in XY females cause a complete dysgenesis phenotype and account for 15% of 46,XY female phenotypes (Schafer, 1995). Evidence seems to support the fact that there is an increased prevalence of SRY mutations among 46,XY pure gonadal dysgenesis when compared with patients with the incomplete variety (Guidozzi et al., 1994). Deletions of material from the Y chromosome containing the sex determining region can cause phenotypical features that resemble Turner's syndrome (45,XO). The fact that a high percentage of 46,XY females with pure gonadal dysgenesis do not present mutations in SRY may be due to the techniques used to screen for mutations or even that the mutations may be located in regions of the gene which have not been analysed (Schafer, 1995). Alternatively, they may have mutations in other genes required for testis determination.
Denys-Drash, WAGR and Frasier syndromes display very similar phenotypes. Denys-Drash syndrome (DDS) is a progressive nephropathy which usually appears in the first year of life often coincident with the onset of Wilms' tumour (Mueller, 1994). The vast majority of patients appear at birth as phenotypically normal females or have ambiguous external genitalia. The gonads appear dysgenic, as streak gonads, with underdeveloped testicular tissue or both testicular and ovarian tissue. A high proportion of DDS sufferers have missense mutations in the Wilms' tumour suppressor gene (WT1) (Hastie, 1992).

The WAGR syndrome (Wilms' tumour, Aniridia, Genitourinary malformations and mental retardation) is also a nephropathy, characterised by Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation. Most patients carry a deletion at the 11p13 locus. This deletion comprises the Pax6 gene, which is responsible for the aniridia, as well as the Wilms' tumour suppressor gene (Heyningen and Hastie, 1992).

The Frasier syndrome (FS) has many similarities to DDS and WAGR: streak gonads, pseudohermaphroditism, renal failure and frequently develop gonadoblastoma (Moorthy, 1987). FS was recently demonstrated to be caused by donor splice site mutations in WT1 (Barbaux et al., 1997).
The occurrence of Wilms' tumour in association with genital abnormalities in WAGR, DDS and FS syndromes has provided evidence for the role of WT1 in genitourinary development (Barbaux et al., 1997; Heyningen and Hastie, 1992; Pelletier et al., 1991).

Wilms' tumour 1 (Wil) contains 10 exons and encodes a protein with a proline and glutamine rich domain and four zinc fingers domains. Wil is expressed in the differentiating stem cells of the kidney, the mesonephros, mesothelium, developing gonads (more specifically in the Sertoli cells), and all tissues whose development includes a mesenchyme-to-epithelium transition (Armstrong et al., 1992; Pelletier et al., 1991a). Targeted disruption of Wil (Kreidberg et al., 1993) results in embryonic lethality of homozygous mice. The ureteric bud of these mice does not develop and the metanephric mesenchyme dies through apoptosis. The mesonephros of homozygous embryos develop, although mesonephric tubules are not as numerous as in wild-type littermates. The mesonephroi lack caudal mesonephric tubules, but develop cranial ones. Thus, WT1 appears to regulate the development of only caudal mesonephric tubules (Sainio et al., 1997).

Dosage sensitive sex reversal (DSS)

Duplications of the short arm of the X chromosome have been implicated in male to female sex reversal. The minimal duplication was mapped to a 160 kb region of human
chromosome Xp21, which includes the adrenal hypoplasia congenita (AHC) locus (Bardoni et al., 1994). AHC is a developmental disorder of the adrenal gland, with profound hormonal deficiencies and is lethal if untreated. This disease is also characterised by the absence of the permanent zone of the adrenal cortex and hypogonadotrophic hypogonadism (HHG) at the time of pubertal maturation. This locus is not essential for testis formation as 46,XY individuals carrying deletions of the region are male. As two copies of the DSS region result in ovary formation, this suggests that DSS is normally necessary for ovarian development and may be repressed in XY individuals. The gene *DAX1* (DSS-AHC critical region on the X, gene 1) has been recently linked to both AHC (Zanaria et al., 1994) and DSS (Swain et al., 1998).

*DAX1* is a member of the nuclear hormone receptor superfamily (Zanaria et al., 1994). The DAX1 protein shows a peculiar structural organisation, the N-terminal portion, which defines a novel DNA-binding domain, and the C-terminal part, with the characteristics of a nuclear hormone receptor ligand biding domain. DAX1 is a transcriptional repressor, and binding sites are found in the *Dax1* and *StAR* promoters (Zazopoulos et al., 1997).

The *Dax1* expression pattern in mice reveals that it is present in XX and XY indifferent gonads as well as in the adrenals and hypothalamus. In the gonads, *Dax1* expression starts shortly after 10.5 dpc, its expression is then maintained in developing ovaries and
downregulated in developing testis after 12 dpc. In the adult testis, Daxl expression is confined to the Leydig and Sertoli cells, and in the ovary is restricted to the stromal cells (Swain et al., 1996; Tamai et al., 1996).

Evidence for Daxl being a feminizing factor came from studies in mice carrying more than one copy of the Daxl as a transgene. The transgenic XY mice show delayed testis development. When the transgene is tested against weak Sry alleles, sex reversal occurs (Swain et al., 1998).

I. IV. 1. 4. The persistent Müllerian duct syndrome (PMDS)

The persistent Müllerian duct syndrome (PMDS) is characterised by the persistence of the uterus, cervix and fallopian tubes in 46, XY phenotypic males. This syndrome is genetically transmitted implicating either anti-Müllerian hormone (AMH) or its type II receptor. The type of genetic defect can be predicted from the level of serum AMH which is very low or undetectable in patients with AMH mutations and at the upper limit of normal in receptor mutations (Guerrier et al., 1989). Whereas AMH mutations are extremely diverse, 25% of the patients with receptor mutations have a 27 bp deletion in exon 10 on at least one allele. All AMH and AMH receptor mutations were consistent with an autosomal recessive mode of transmission (Imbeaud et al., 1996)
The Anti-Müllerian hormone (AMH), also known as the Müllerian inhibiting substance (MIS), is a 560 amino acid glycoprotein dimer (Cate et al., 1986) member of the TGF-β family of growth factors. The C-terminal portion of the protein functions through interaction with its receptor. AMH is one of the first known secreted products made by the Sertoli cells (Tran et al., 1981). This hormone is produced as a 140 kDa dimer of two identical 70 kDa subunits linked by disulphide bonds, being cleaved by plasmin approximately 110 residues away from the C-terminus to yield a TGF-b-like fragment (Pepinsky et al., 1988).

The AMH gene has been cloned in human, bovine (Cate et al., 1986; Picard et al., 1986), chick (Eusebe et al., 1996), mouse (Munsterberg and Lovell-Badge, 1991) and rat (Haqq et al., 1992). The mouse Amh gene has been mapped to the distal part of chromosome 10 (King et al., 1991) and is transcribed as a 2.2 kb transcript, divided into 5 exons (Munsterberg and Lovell-Badge, 1991). Amh is first detected at 11.5 dpc in XY gonads and is upregulated in the testis until 7 days post partum (dpp) when the level of transcripts starts to decrease (Hacker et al., 1995). In females Amh is only detected at a low level, after birth, in the granulosa cells of the follicle.

Female mice carrying a human AMH transgene, driven by the mouse methallothionein promoter region, and thus expressing this hormone chronically, have no uterus or oviducts and their ovaries are depleted of germ cells with the somatic cells rearranged.
into cord-like structures, resembling the bovine freemartin model (Behringer et al., 1990). AMH-deficient male mice generated by gene targeting retain Müllerian duct-derived tissues, resembling the persistent Müllerian duct syndrome (PMDS).

To date, there is no definitive evidence for an AMH function other than regression of the Müllerian ducts, although its expression pattern and distribution of receptors has suggested several other roles. The production of AMH decreases with the terminal differentiation of Sertoli cells and the initiation of the first waves of spermatogenesis (Munsterberg and Lovell-Badge, 1991). This hormone can inhibit the biosynthesis of aromatase and the Luteinizing hormone receptor in postnatal ovaries (diClemente et al., 1994) and it can block epidermal growth factor-induced synthesis of progesterone in postnatal granulosa cells (Kim et al., 1992). These observations suggest a role for AMH in spermatogenesis and oogenesis in adults. Surprising results were obtained with the AMH-deficient mice (Behringer et al., 1994); the AMH-deficient female mice were fully fertile and the AMH-deficient male mice were infertile, not due to germ cell development, but to the presence of the female reproductive organs, which mechanically obstructed the transfer or the functional sperm along their normal pathway. However, older male mice testes showed focal Leydig cell hyperplasia, which is consistent with the proposed role for AMH in regulating Leydig cells (Behringer et al., 1990).
AMH type II receptor is a membrane-bound serine/threonine kinase. Its expression is localised to the mesenchymal cells adjacent to the Müllerian duct epithelium during embryogenesis and Sertoli cells and granulosa cells in fetal and adult testes and ovaries, respectively (Baarends et al., 1994; di Clement et al., 1994a; Teixeira et al., 1996). AMH receptor mutant mice have been generated by gene targeting in embryonic stem (ES) cells. Homozygous mutant males display internal pseudohermaphroditism, infertility, seminiferous tubule atrophy, and Leydig cell hyperplasia. Males that are otherwise normal have a uterus and oviducts, a phenotype identical to AMH ligand mutant male mice (Mishina et al., 1996).

◆ I . IV . 1 . 5 . Other disorders

The steroid hormone testosterone is secreted by the Leydig cells. This hormone is synthesised through a five step enzymatic process from cholesterol. This pathway is mediated by four enzymes: cholesterol side-chain cleavage enzyme, steroid 17α-hydroxylase/17,20 lyase, 3β-hydroxysteroid dehydrogenase-Δ4,5-isomerase and 17β hydroxysteroid dehydrogenase. Once testosterone is synthesised it can bind to the androgen receptor or it can be further converted into dihydrotestosterone by the enzyme steroid α-reductase. This also binds the androgen receptor but with a higher affinity. Testosterone promotes the differentiation of internal male reproductive tracts such as
the epididymis, vas deferens and seminal vesicles. On the other hand, dihydrostesterone promotes the differentiation of the male urethra, prostate and the external genitalia.

It is obvious to expect that mutations in genes that have a role in these processes will cause male differentiation abnormalities. Indeed, mutations in the enzyme 17β hydroxysteroid dehydrogenase, 5α-reductase and androgen receptor have been reported to cause pseudohermaphroditism phenotypes (Russel et al., 1996; Pinsky et al., 1996; Qin et al., 1996). In addition two chromosomal loci, 9p and 10q also account for some of the XY sex reversal cases (Schafer, 1995). No candidate genes have been found so far in these loci.

1. IV. 2. XX Sex reversal

Due to the lack of knowledge on the origin of most cases of XX sex reversal, the simplest way to classify this class of sex reversal syndrome is into 46, XX with or without translocated Y chromosome sequences. There are also reported cases of males with the karyotype 45, XO (due to translocations of Y chromosome sequences to autosomal chromosomes) and XX/XXY mosaicism.
46, XX sex reversal patients present a wide range of phenotypes from 46, XX males with and without ambiguous external genitalia to true hermaphrodites.

The 46, XX males with ambiguous external genitalia, also referred as XX pseudohermaphrodites, account for 20% of XX male phenotypes. They present micropenis, male genital ducts and hypospadias. The testis are small, azoospermic with Sertoli and Leydig cell without germ cells. To date, no molecular evidence was found to explain the majority of these occurrences although a minority carry Y sequences including \textit{SRY} (Berta \textit{et al.}, 1990).

The frequency of 46,XX males without ambiguous external genitalia pathology is of 1:20 000 newborns. The majority carry Y chromosome sequences translocated to the X chromosome by aberrant recombination during meiosis. These patients show the most masculinised phenotype among the XX sex reversal patients, due to the presence of Y chromosome sequences having normal external genitalia with small azoospermic testes. The testes of these patients usually lacks germ cells but have normal Sertoli and Leydig cells. Infertility is due to the presence of two X chromosomes and to the absence of Y chromosomal genes involved in spermatogenesis (de la Chapelle, 1981).

The majority of true hermaphrodites present a 46, XX karyotype (Van Niekerk and Retief, 1981). Only a very few cases of this type of hermaphroditism are due to
translocations of Y chromosome sequences to the X chromosome. The phenotype in these cases can be explained by random inactivation of the X chromosomes and the spread of this inactivation to affect SRY expression. This leads to an analogous situation to the one seen in XX/XY chimaeras, with different cells following either testis or ovarian differentiation. The frequency of occurrence of 46, XX true hermaphroditism is 1:20,000. The outward phenotype of these individuals can be very similar to the one presented by XX males with ambiguous genitalia. However histological analysis of gonads of true hermaphrodites reveals the presence of both ovarian and testicular tissue in the same or opposite gonads.

No evidence of a molecular defect in the XX males or hermaphrodites has been found. McElreavey and colleagues proposed a model to explain these cases, where a repressor of male structures that normally is repressed by SRY, is mutated, allowing the activation of male-specific genes and the development of a male phenotype (McElreavey et al., 1993a).

As in the case of XY pseudohermaphrodites, disruptions in genes that play a role in the developing female structures will generate XX pseudohermaphrodites. Such is the case for P450 aromatase mutations, which is the enzyme required to convert androgens into estrogens (section I.II.4.).
I. IV. 3. Other genes involved in urogenital ridge development

Several genes have been linked to sex determination and differentiation in mice. One of them, Steroidogenic factor 1 (Sfl) is a member of the nuclear receptor family. Sequence comparisons indicate that Sfl is homologous to the Drosophila orphan nuclear receptor gene Fushi tarazu factor 1 (Ftz-f1), which regulates the expression of the Fushi tarazu homeobox gene in early development. Sfl encodes two different transcripts, just like Fushi tarazu factor 1 in Drosophila, Sfl and embryonal long terminal repeat-binding protein (Elp) (Ikeda et al., 1993; Lala et al., 1992; Tsukiyama et al., 1992). Sfl cDNA was isolated from an adrenocortical cDNA library and Elp transcripts were isolated from embryonal carcinoma cells. Sfl transcripts first appear at 9 dpc in the very early urogenital ridge. Until 12.5 dpc Sfl is detected both in the male and female gonads and adrenals. In the testes Sfl is expressed in the Sertoli and Leydig cells. After approximately 12.5 dpc, as morphological sexual differentiation occurs, Sfl expression persists at high levels in the testes but is downregulated in the ovaries. Sfl is also detected in the ventral diencephalon, which is the precursor to the endocrine hypothalamus, and in gonadotropes of the anterior pituitary. In adult mice, Sfl is expressed in cortical cells of the adrenal gland, Leydig cells in the testes, and theca and granulosa cells of the ovary. Elp transcripts were not detected from 8.5 dpc to adult, so it was postulated to have a role in early development (Ikeda et al., 1994).
SF1 is known to regulate genes encoding a range of steroidogenic enzymes responsible for the synthesis of androgens and estrogens (Ikeda et al., 1994; Lynch et al., 1993). However, the expression of SF1 in Sertoli cells and its sexually dimorphic pattern suggests that the role of SF1 in the gonad is more than just the regulation of steroidogenic enzymes.

Null mutations in the mouse SF1 lead to an absence of gonads and adrenals in both sexes (Luo et al., 1994). As DAX1 mutations in humans show a related adrenal defect, this raised the possibility of an interaction between these two molecules in endocrine differentiation and function. Both SF1 and Dax1 are expressed during embryonic development in the urogenital ridge, with SF1 expression preceding that of Dax1. The same pattern of expression of these two genes is observed in the hypothalamus and pituitary (Ikeda et al., 1996). Interestingly enough, in spite of the striking phenotype in null mice, no human syndromes have been linked to mutations in this gene so far.

It is obvious that many signalling molecules as well as other transcription factors must be involved in urogenital ridge development. Some of these genes have a clearly established role, while others are implicated indirectly. Among the later group are members of the Wnt gene family, Desert hedgehog, Lim1 and members of the Polycomb gene family.
The *Wnt* family of signalling molecules, seems to be also involved in sex differentiation. This family, comprising cysteine rich secreted glycoproteins, consists of at least 16 members in the mouse (Nusse and Varmus, 1992; McMahon, 1992). Three of them are expressed in the developing gonads; *Wnt4, Wnt7a* and *Wnt5a*, and are reported to have some role in urogenital ridge development. Targeted disruption of *Wnt4* results in partial masculinization of female gonads (A. McMahon, personal communication). *Wnt7a* expression is confined to the Müllerian ducts in the urogenital ridge. The targeted disruption of this gene results in males with both Müllerian and Wolffian ducts derivatives (A. McMahon, personal communication). *Wnt5a* is expressed, among other tissues, in the developing gonads. The targeted disruption results in postnatal lethality of homozygous embryos, with multiple defects. The skeleton displays a severe reduction in the length of the vertebral column along the A-P body axis, shortened limbs along the proximal-distal axis and reduced outgrowth of the frontonasal cartilage and mandible (Yamaguchi *et al.*, 1997). The role of *Wnt5a* in the developing gonad has not yet been assessed.

Desert hedgehog (*Dhh*) is one of the three mouse *hedgehog* genes (*Desert hedgehog, Indian hedgehog* and *Sonic hedgehog*), which share a striking homology with the *Drosophila* segment polarity gene *Hedgehog*, a key regulator of pattern formation in embryonic and adult structures (Echelard *et al.*, 1993). All encode secreted proteins thought to be involved in cell signalling. *Dhh* expression is initiated in Sertoli cells.
shortly after *Sry* expression and persists in the testis into the adult. Female mice homozygous for a *Dhh*-null mutation show no obvious phenotype, whereas males are viable but infertile, owing to a complete absence of mature sperm. Thus, *Dhh* plays an important role in the regulation of mammalian spermatogenesis. *Patched (Ptc)* is also expressed in the testicular Leydig cells. The expression of *Ptc* is lost in *Dhh* mutants, suggesting a conservation in the *hedgehog* signalling pathway between flies and mice, and indicating that Leydig cells may be a direct target of *Dhh* signalling (Bitgood *et al.*, 1996).

*Liml* encodes a LIM class homeodomain motif and two cysteine-rich LIM domains which were first recognised as conserved motifs in the protein encoded by the *lin-11* and *mec-3* in *Caenorhabditis elegans* (Freyd *et al.*, 1990) and *isl-1* in rat. *Liml*-null mice lacked anterior head structures, with a normal posterior body axis. These mutant mice also lack kidneys and gonads (Shawlot and Behringer, 1995).

*M33* is considered the mouse counterpart of the *Polycomb* gene since it is able to partially rescue the *Drosophila Polycomb* mutant phenotype (Muller *et al.*, 1995). *Polycomb* genes are required to maintain the correct expression of the *Hox* genes in *Drosophila* (Gould, 1997). *M33* mutant mice show abnormalities in the reproductive systems (Katoh-Fukui *et al.*, 1998, submitted). Some Homeobox genes are also expressed in the gonads like *Hoxa11* (Hsieh-Li *et al.*, 1995), *Hox-3.3* and -3.4 (Gaunt *et
al., 1990) and Hox-4.2, -4.3 and -4.4 (Izpisua-Belmonte et al., 1990), but their role in gonadal development has not been assessed.

I. V. Campomelic dysplasia and SOX9

Campomelic dysplasia (CD) is also referred to as Campomelic dwarfism and Campomelic syndrome. The name derives from the Greek "camptos", bent and "melos", limb and was first proposed by Maroteaux et al. and Bianchine et al., in separate reports, in 1971. Campomelic dysplasia, as its name implies, is characterised by congenital bowing and angulation of long bones, together with other skeletal and extraskeletal defects. The reported incidence of this syndrome is 0.05-0.09 per 10000, although this condition may vary between different populations. This number is probably an underestimate due to perinatal loss or because CD is easy to overlook or misclassify as other kinds of dwarfism, usually achondroplasia (Normann et al., 1993). Major diagnostic radiological features of CD include hypoplastic bones like the femora and tibia which are shortened and bowed, dislocated hips, hypoplastic scapulae and pedicles of many thoracic vertebrae. Common facial features include micrognathia, cleft palate, flat nasal bridge, hypertelorism and low set ears sometimes lacking cartilage. In addition, 11 pairs of ribs, small bell shaped chest, defective tracheo-bronchial cartilage, hypermobile joints, deep cutaneous dimples and bilateral talipes equinovarus
deformities. The severity of the skeletal defects increases from the anterior to the posterior, with the lower limbs more affected than the upper ones. Internally, the patients frequently show absence of olfactory bulbs, heart defects, renal hypoplasia and rarely renal cysts. The patients usually die during the neonatal period from respiratory distress (table I.1.). In addition, several parents of affected individuals have been reported to have suffered miscarriages, indicating that this syndrome may impair embryonic development (table I.3.). Two thirds of CD patients genetically XY are phenotypically female (table I.2.). The range of sex reversal varies from normal genitalia with descending testes, male pseudohermaphroditism, to female genitalia with streak-like gonadal rudiments (Houston et al., 1983).

In 1994, the SOX9 gene was demonstrated to be the gene responsible for CD (Foster et al., 1994; Wagner et al., 1994). The Sox9 expression pattern during mouse embryonic development and its localisation in human chromosome 17, adjacent to the translocation breakpoints in CD patients lead to the search for mutations in SOX9 (Tommerup et al., 1993). A variety of mutations at the SOX9 locus have been identified. These include translocations, deletions, missense mutations in the HMG box, frameshift and splice-acceptor mutations. CD patients are always heterozygous for the mutation (Foster et al., 1994; Wagner et al., 1994). There appears to be no correlation between the penetrance of the phenotype and the type of mutation. A good example are the cases described by Kwok and colleagues (1995) where two patients share the same SOX9
Table I.1. Main features of Campomelic dysplasia. The 32 cases were compiled from the literature (Cooke et al., 1985; Gillerot et al., 1989; Hall and Spranger, 1980; Houston et al., 1983; Lee et al., 1972; Lynch et al., 1993; Mansour et al., 1995; Normann et al., 1993; Pauli and Pagon, 1980; Storer and Grossman, 1974)

Table I.2. Ratio XY females/XY males in the same 32 patients with Campomelic dysplasia as in table I.1.

Table I.3. From the same literature as table I.1. and I.2. Several parents of Campomelic dysplasia patients have an history of miscarriages.
<table>
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<tr>
<th>CD Features</th>
<th>Yes</th>
<th>No</th>
<th>Not known</th>
<th>Total</th>
<th>%</th>
<th>Total patients</th>
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<td>Dwarfism</td>
<td>31</td>
<td>1</td>
<td></td>
<td>31/31</td>
<td>100</td>
<td>32</td>
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<td>Respiratory distress</td>
<td>27</td>
<td>1</td>
<td>4</td>
<td>27/28</td>
<td>96</td>
<td>32</td>
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<tr>
<td>Narrow trachea</td>
<td>19</td>
<td>3</td>
<td>10</td>
<td>19/22</td>
<td>86</td>
<td>32</td>
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<td>Absence of olfactory bulbs</td>
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<td>7</td>
<td>14</td>
<td>11/18</td>
<td>61</td>
<td>32</td>
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<tr>
<td>Macrocephaly</td>
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<td>4</td>
<td>5</td>
<td>23/27</td>
<td>85</td>
<td>32</td>
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<td>Micrognathia</td>
<td>24</td>
<td>8</td>
<td></td>
<td>24/24</td>
<td>100</td>
<td>32</td>
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<td>Cleft palate</td>
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<td>3</td>
<td>21/29</td>
<td>72</td>
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<td>Flat nasal bridge</td>
<td>24</td>
<td>2</td>
<td>6</td>
<td>24/26</td>
<td>92</td>
<td>32</td>
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<tr>
<td>Low set ears</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>16/26</td>
<td>61</td>
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<td>Hypoplastic scapulae</td>
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<td>32</td>
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<td>Small, bell shape chest</td>
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<td>5</td>
<td>25/27</td>
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<td>32</td>
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<tr>
<td>11 ribs</td>
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<td>9</td>
<td>6</td>
<td>17/26</td>
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<td>Non-mineralised thoracic pedicles</td>
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<td>Vertically narrow iliac bones</td>
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<td>24/32</td>
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<td>Dislocated hips</td>
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<td>2</td>
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<td>Bowed femora/tibia</td>
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<td>32</td>
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<td>30/30</td>
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<td>12/26</td>
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<td></td>
<td>6(moderate)</td>
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<td>1(Severe)</td>
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<td>8</td>
<td>12</td>
<td>12/20</td>
<td>60</td>
<td>32</td>
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<td>Enlarged heart</td>
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<td>19</td>
<td>9</td>
<td>4/23</td>
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<td>32</td>
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<td>Kidney defects</td>
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<td>15</td>
<td>13</td>
<td>4/19</td>
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<td>32</td>
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<td>16</td>
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Table I . 2

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<td>XYfemale</td>
<td>12</td>
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<td>% (XYf/XYt)</td>
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<td>Total</td>
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Table I . 3

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<th>previous miscarriages</th>
<th>miscarriages (%)</th>
<th>Affected (%)</th>
<th>Normal (%)</th>
<th>Total pregnancies</th>
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<tr>
<td></td>
<td>6(24%)</td>
<td>9(36%)</td>
<td>10(40%)</td>
<td>25(100%)</td>
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</table>
mutation, but one is an XY male and the other an XY female. However, studies by Sudbeck and colleagues (1996) indicate that C-terminal truncations lead to a more severe phenotype possibly due to dominant negative effects. There is also no evidence for SOX9 being responsible for sex reversal without Campomelic dysplasia (Kwok et al., 1995; Meyer et al., 1997; Schafer et al., 1996; Wagner et al., 1994).

SOX9, like SRY, is a member of the HMG box gene family (Koopman et al., 1990/1 and Pevny and Lovell-Badge, 1997 for a review). This family is divided in two groups based on their DNA binding characteristics; 1) the TCF/SOX family which comprises proteins with a single sequence-specific HMG box, like LEF1, TCF, SRY and the SOX family as well as the fungal mating type factors Mat Mc, Mat A1 and Ste11; 2) the HMG/UBF family containing relatively non sequence-specific DNA binding proteins with multiple HMG boxes (Laudet et al., 1993). However, unlike most classical transcription factors, they bind DNA in the minor groove and induce a sharp bend (Giese et al., 1992). For instance LEF1, a T-cell specific protein, is proposed to induce a bend in DNA so that transcription factors bound to recognition sequences flanking the LEF1 binding site can interact with each other. These observations and additional studies of this protein led to the proposal that LEF1 acts as an architectural component in the assembly of the T cell enhancer complex (Giese et al., 1995).
The SOX family of genes is comprised by more than twenty members, often conserved across evolution. SOX proteins bind to the consensus sequence A/TA/TCAAG and have been shown to activate transcription of reporter constructs in vitro. In addition, putative transcription activation domains have been mapped in a number of SOX proteins such as SOX4, SOX9 and SOX18 (Hosking et al., 1995; van de Wetering et al., 1993; Wright et al., 1995). SOX proteins appear to govern cell fate decisions during embryogenesis by acting as transcription factors and architectural components of chromatin.

It is also becoming apparent that SOX proteins act to regulate transcription in a context dependent manner. For example, it has been demonstrated that SOX2 plays a role in the regulation of Fgf4 by interacting with the OCT3/4 factor (Yuan et al., 1995) and in the regulation of ßCristallin genes (Kamachi et al., 1995; Kamachi et al., 1998).

Sox genes show diverse and dynamic patterns of expression during embryonic development and in several adult tissues. Interestingly, the expression pattern of many of the Sox genes seems to be correlated with early cell fate decisions. This is the case for Sry, where its expression is crucial to commit the supporting cell lineage of the developing gonads to develop as Sertoli cells and thus to promote testis development. The downregulation of Sox1, 2 and 3 in the developing neural system is linked to the differentiation of neural precursors (Pevny et al., 1998). Sox4 is expressed in the
immune system, in pre-B and pre T cells prior to terminal differentiation (Van de Wetering et al., 1993). In each case, expression patterns implicate these genes as playing a role in establishing cell fates.

*Sox9* is a member of the *Sox* genes group E (Pevny and Lovell-Badge, 1997). This group also comprises *Sox8* and 10 (Wright et al., 1993). *SOX9* maps to chromosome 17q25 in humans, 11q in mice and is also autosomal in chick (Foster et al., 1994; Kent et al., 1996; Wagner et al., 1994 and Wright et al., 1995). *Sox9* encodes a single transcript of approximately 5.5 kb in mouse and 4.5 kb in human. *Sox9* contains two introns, one of which interrupts the HMG box domain, a feature that is conserved in the chick *Sox9* gene (Healy et al., 1996). It has an open reading frame of 507 amino acids in mouse and 509 amino acids in human. The mouse and human SOX9 proteins are 96% identical and contain a transcription activation (TA) domain at the C-terminus. This domain is rich in proline, glutamine and serine residues (Sudbeck et al., 1996; Wright et al., 1995).

In humans, *SOX9* transcripts have been found in adult heart, brain, kidney, prostate, small intestine and pancreas. During development, *SOX9* is expressed in chondrogenic tissues, brain, liver, kidney and testicular tissue.

In mouse, *Sox9* has a widespread pattern of expression. However, its expression in mesenchymal condensation before and during cartilage deposition (Wright et al., 1995
and chapter IV) and in the developing gonad (Kent et al., 1996; Morais da Silva et al., 1996 and chapter II), is consistent with a role for this gene in skeletal formation and sex determination.

I. VI. Description of thesis

When mutations in Sox9 were found to be responsible for Campomelic dysplasia, it became imperative to analyse if this gene was expressed in the developing gonads and to analyse its role during embryonic development. In mouse, Sox9 shows a dimorphic pattern of expression in embryo gonads, being upregulated in males and downregulated in females at the onset of sex determination. This expression pattern is conserved in chick embryos where an Sry gene has not been found. In addition, Sox9 expression is in agreement with one of a gene that is involved in Sertoli cell differentiation (Chapter II). SOX9 also seems to be involved in Amh regulation, a downstream gene the product of which is responsible for the regression of the Müllerian ducts (Chapter III).

The targeted disruption of the Sox9 gene leads to severely affected embryos with phenotypes that range from impaired gastrulation, to posterior truncations presumably due to a deficient axial and paraxial mesoderm development (Chapter IV).
CHAPTER II. *SOX9* EXPRESSION DURING GONADAL DEVELOPMENT

IMPLIES A CONSERVED ROLE FOR THE GENE IN TESTIS DIFFERENTIATION IN MAMMALS AND BIRDS
II.1. Introduction

Because early Sertoli cells appear to have such a critical role in orchestrating the differentiation of the other cell lineages in the gonad, the problem of sex determination can essentially be reduced to one of understanding Sertoli cell differentiation. While Sry is important to initiate this event in mammals, it is clear that other genes must also play a role. For example, as Sry is expressed for such a brief period, at least in mice, it can not be required for maintenance of Sertoli-cell specific gene expression, but only for initiating it. The long term changes in gene activity responsible for Sertoli cell characteristics will be maintained by genes turned on as a result of SRY expression. However, these need not be immediate target genes as SRY could activate genes in the testicular pathway, repress genes in an ovarian pathway, or both (Lovell-Badge and Hacker, 1995; McElreavey et al., 1993).

In addition, testes or testicular like structures with apparently normal Sertoli cells can develop in the absence of Sry in a number of naturally occurring or experimental situations in mammals. For example there are cases of XX males who lack Sry in humans, dogs, voles, and other mammals (Berkovitz et al., 1992; Just et al., 1996; McElreavey et al., 1993; Meyers-Wallen et al., 1995). These can be explained by either a gain of function mutation in a downstream testis determinant or a loss of function mutation in an ovarian determinant. There are also cases where gonadal environment is
important. In Freemartin cattle, AMH from a male twin results in masculinisation of the female twin. This masculinisation includes apparent loss of oocytes and transdifferentiation of ovaries into testes (Jost et al., 1972). A similar process can also be demonstrated experimentally \textit{in vitro}, through the addition of AMH to fetal ovary cultures (Vigier et al., 1987), or \textit{in vivo} by grafting fetal ovaries to the kidney capsule of adult male or female mice (Taketo and Merchant-Larios, 1986).

Furthermore, there is no evidence that an \textit{Sry} homologue is involved or even exists in non-mammalian vertebrates. \textit{Sry} appears to have evolved as a dominant male determinant coincident with the evolution of mammals (at least the metatheria and eutheria)(Collignon et al., 1996; Graves, 1995). In contrast, birds employ a ZZ/ZW system where the female is the heterogametic sex (Krishan et al., 1965; Ohno et al., 1960). Moreover, in many reptiles and fishes, males and females are genetically identical, sex being determined by environmental factors such as temperature (Johnston et al., 1995). It seems instead that the gene employed as the control point in sex determination can vary between species. Nevertheless, since the basic cellular organisation of the gonads is similar in different classes of vertebrates, it is likely that there will be genes in common which are responsible for specifying the particular cell types and these genes ought to be highly conserved amongst all vertebrates. The identification of these genes is therefore of particular importance to a general understanding of sex determination.
If Sox9 is to be a good candidate for being involved in sex determination it must satisfy several criteria: notably, its expression must overlap both temporally and spatially with Sry and it must be different between females and males. Sox9 expression was therefore analysed during gonadal development in both mouse and chick, which is a system where Sry is not present. In both cases studied, Sox9 has a conserved expression pattern. One type of experimental sex reversal in the mouse has also been studied. In all situations high levels of Sox9 expression were correlated with testicular development, in a manner suggesting that it is required for Sertoli cell differentiation. The timing of Sox9 expression during normal mouse development suggests it could be a direct target for Sry action, however it may also have general importance as an ancestral sex determining gene.

II. II. Materials and methods

II. II. 1. Animals and tissues

Mouse embryos and tissues were obtained from Parkes outbred mice (maintained at NIMR) unless otherwise stated. The testis from the XXY male (XXY cunning) used in
Figs. 4f and 4h was a gift from Tristan Rodriguez. This animal was obtained by mating an XYY*^ male (Hunt and Eicher, 1991) with an XXY''^ female (Lovell-Badge and Robertson, 1990; Mahadevaiah et al., 1993). These were on an MF1 outbred background from stocks maintained by Paul Burgoyne at NIMR. For embryo staging, midday on the day of appearance of the vaginal plug is taken as 0.5dpc. More accurate staging of embryos was carried out by counting tail somites posterior to the hind limb bud or by hind limb morphology as described by Hacker et al. (1995). Chick embryos were incubated at 38°C and staged according to Hamburger and Hamilton (1951). Chick embryos were sexed by PCR using W-specific primers as described by Clinton (Clinton, 1994).

II. II. 2. RNase Protection Assays

Total RNA was prepared from embryonic and adult gonads as described by Chomczynski et al. (Chomczynski and Sacchi, 1987). RNase protection assays were carried out using 10μg of total RNA from adult tissues or RNA prepared from four pairs of genital ridges, as described by Hacker et al. (Hacker et al., 1995). A genomic fragment of Sox9, Int-H-Sox9, suitable for making probes to distinguish RNA from DNA, was obtained by PCR between nucleotides 372 of the first exon and 44bp of the first intron. This was cloned into pCR™II (Invitrogen) by TA cloning. The primer
sequences were 5'-CCACCATGTCGGAGGACTCGG-3' and 5'-TGAATTCTCGGTGAGTACAGCCCGCG-3' (Wright et al., 1995) (Edwina Wright, personal communication). The Sap62 probe used as a loading control was probe A, as described previously (Dresser et al., 1995). Transcription reactions were performed in vitro using T7 RNA polymerase (Promega) in both cases to generate antisense RNA probes labelled with 32P-UTP.

II. 11. 3. pbSox9 probe

This probe was obtained by RT-PCR between nucleotides 758 to 1256 using cDNA from 13.5 dpc embryonic limb RNA. At the time the mouse sequence was not yet available, therefore comparison of chick, rat and human Sox9 sequences allowed the design of degenerate oligonucleotides (5'-CCCTTCGTGGAGGAGGCA/G/C/TGAG-3' and 5'-GAC/G/A/TGA/TGCTGCTGATGCC/TGTA-3'). The product was cloned into EcoRI and XbaI sites within the polylinker of pbBluescript (Stratagene) and sequencing was used to verify its identity.
II . II . 4. In situ hybridisation

To detect mouse Sox9 expression, an antisense RNA probe used was generated from a cDNA clone, pbSox9. For the whole mount in situ hybridisation performed on chick gonads the cSox9 cDNA clone 20-1 inserted into pbBluescriptSK (a gift of Paul Sharpe) was linearised with XbaI. Transcription reactions were performed in vitro using T7 RNA polymerase or T3 RNA polymerase (Promega) to generate sense (control) and antisense RNA probes labelled with digoxygenin-UTP. The complete procedure is as described in Wilkinson et al. (Wilkinson and Nieto, 1993). No signal was seen with the sense probe (data not shown). For sections, tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, and successively passed through solutions of 0.83% NaCl, 0.83% NaCl: Ethanol, 70%, 85%, 95% and 100% Ethanol. The tissues are then placed in histoclear (BDH) and embedded in paraffin wax (BDH). Sections of 6μ were cut in a microtome (Bright) and mounted using Aquamount (BDH).

II . II . 5. Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), then equilibrated in 30% sucrose overnight, before freezing and cryosectioning. Tissue sections were washed for 10 minutes in PBS, permeabilised in 1% Triton X-100 in PBS for 10 min, RT, rinsed in TBS, then blocked
using 5% goat serum in incubation buffer (TBS, 3% globulin-free BSA, 0.5% Triton). The sections were then incubated overnight in VAH2 antibody (Morais da Silva et al., 1996) at 1:500 dilution in incubation buffer. After washing, the sections were treated with FITC-goat anti-rabbit IgG (Vectorshield), 1:100 in incubation buffer, for 30 min at RT, rinsed and mounted using Citifluor (UKC chem. lab.)

II. II. 6. Transplantation of fetal gonads

Fetal ovaries and testes at 13.5 dpc, dissected away from mesonephros, were transplanted beneath the kidney capsule of 6-8 week old male mice as described by Taketo and Merchant-Larios (1986). 1-3 gonads of the same sex were transplanted to separate locations of one kidney only in each host. The gonadal grafts were dissected out between the 1st and 30th day after transplantation, and processed for whole mount in situ hybridisation and immunohistochemistry as described above.
II. III. Results

II. III. 1. Sox9 expression during gonadal development in the mouse

Each gonad begins to develop from about 9.5-10.0 dpc in mice as a ridge of distinct cells on the medio-lateral surface of the mesonephros. Up until about 11.5 dpc, the genital ridges of XY and XX embryos are morphologically indistinguishable and are comprised of the primordial germ cells and one or more somatic cell types which give rise to the supporting cells (Sertoli or follicle cells) and to steroidogenic cells (Leydig or theca). Other lineages of the gonad, notably connective tissue cells, such as the myoid cells and endothelial cells, migrate into the genital ridge from the mesonephros after 11.5 dpc (Buehr and McLaren, 1993; Martineau et al., 1997; Merchant Larios et al., 1993), coincident with the first morphological differences between testis and ovary. Whole mount *in situ* hybridisation shows Sox9 expression in the genital ridge of both XY and XX embryos from the earliest stage observed, about 10.5 dpc (Fig.II.1.a,b). This corresponds to a time when Sry expression is only just being activated in XY embryos. Although many parts of the embryo express Sox9, notably developing cartilage and neural tube, expression is not seen in the mesonephros at early stages (Fig.II.1.a,b,c). By 11.5 dpc, Sox9 expression is very abundant in genital ridges from XY embryos but it is absent from those of XX embryos (Fig. II.1.c,d,e). Sections of whole mounts of XY
Fig.II.1 Whole mount *in situ* hybridisation to reveal *Sox9* transcripts during gonadal development in the mouse. **a-b**, Dissected gonads and mesonephros from XX and XY mouse embryos, at 10.5dpc (**a,b**), 11dpc (**c**), 11.5dpc (**d,e**), 12.5dpc (**f**), 13dpc (**g**), and 13.5dpc (**h**). Note the anterior spot of *Sox9* expressing cells arrowed in **f, i,j,k**. Sections of testis whole mounts at 11.5dpc (**i**) and 13.5dpc (**j,k**), visualised by Nomarski optics.
genital ridges at these stages indicate that the majority, if not all of the somatic cells within the centre are positive, whereas cells of the coelomic epithelium are negative (Fig.II.1.i).

Sox9 continues to be expressed at a high level in developing testes, with the transcripts being localised to the cords (Fig.II.1.f,g,h). Sections of whole mounts at 13.5 dpc reveal the signal to be within Sertoli cells and absent from all other lineages (Fig.II.1.j,k). On the other hand, Sox9 transcripts continue to be completely absent from developing ovaries (Fig.II.1.f). The only exception is a small patch of positive cells located anteriorly, just within the mesonephros (Fig.II.1.f). This is also seen in males and is reminiscent of a patch of Dax1 expression seen in both sexes after 12.5 dpc (Swain et al., 1996) which may represent a population of relatively undifferentiated cells.

II . III . 2 . Changes in Sox9 transcript levels coincide with onset of Sry expression

To confirm the sexually dimorphic nature of Sox9 expression in developing gonads and to provide some estimate of how transcript levels change over time, quantitative RNase protection assays were performed with urogenital ridge RNA obtained from accurately staged embryos (Fig.II.2.). These assays confirm that Sox9 is expressed in both sexes prior to Sry, but then, coincident with the onset of Sry transcription, Sox9 is
Fig. II.2 Timecourse of Sox9 expression in the developing gonad, ovary and testis. a, RNAse protection assays used total RNA from urogenital ridges dissected from embryos staged by tail somite number (10-31), where 12 somites corresponds to about 11.0 dpc, 18 somites to 11.5 dpc and 30 somites to 12.5 dpc, or by limb morphology (13.5 and 14.5 dpc) and from adult testis and spleen. b, The relative amounts of Sox9 transcripts were plotted by first normalising against a loading control (Sap62 probe) and then taking expression of Sox9 at 31 tail somites in the XY embryo to be 100%. The 10 tail somite sample was left off this analysis as it was dissected in a separate experiment, care being taken to avoid non-genital ridge Sox9 expressing cells, unlike the remainder which would have had some positive cells in the anterior mesonephros and in dorsal mesentery (see Fig.I.d,e). Data obtained in a similar way for Sry and Amh expression are plotted for comparison. (Te)adult testis, (Sp)spleen.
downregulated in XX genital ridges and upregulated in XY genital ridges. Apart from
*Sry* itself, the changes seen in *Sox9* expression represent the earliest difference yet seen
in gene expression between males and females. *Amh*, which is a very early marker of
Sertoli cells, is not transcribed until after 11.5 dpc, when the difference in *Sox9*
expression has already been established. In these assays, *Sox9* transcripts are still
detected in XX samples, however, these will have included the anterior patch of
positive cells and, at early stages, perhaps some cells of the dorsal mesentery and aorta
which show a low level of expression (see Fig.II.1.e,f).

**II . III . 3 . SOX9 protein is nuclear in Sertoli cells**

While RNA localisation gives a good indication where a gene may be functional, this is
revealed in a more rigorous manner by looking at protein distribution. Antibodies
against SOX9 were therefore used on sections of mouse gonads throughout their
development. At about 11.5 dpc, SOX9 is detected within cells of the genital ridge,
with consistently higher levels in XY versus XX embryos (Fig.II.3.a,b). The finding
that some protein is still seen in XX genital ridges at a time when transcripts are absent,
suggests that the protein is quite stable. However, we have not detected SOX9 protein
at any subsequent stage in development (Fig.II.3.d). In developing testes, SOX9 is
found at high levels specifically within Sertoli cells lining the testis cords (Fig.II.3.c). In
Antibody localisation of SOX9 in cryostat tissue sections of mouse gonads. 

a-d, XY(a,c) and XX(b,d) embryos at 11.5dpc (a,b) and 13.5dpc (c,d). e-h, immunostaining and haematoxylin staining of similar sections from normal XY adult testis (e,g) and from XXY adult testis lacking germ cells (f,h). i-j, Cellular localisation of SOX9 protein in XY gonads at 11.0 dpc (i) and 14.5 dpc (j).
the adult, SOX9 protein is still detected at a high level in cells surrounding testis tubules. From their position and appearance these are likely to be Sertoli cells (see Fig.II. 3.e,g), and this can be confirmed by looking at testes of males with two X chromosomes which completely lack germ cells (Fig.II.3.f,h). This latter result also shows that SOX9 expression in adult testis is independent of the presence of germ cells.

SOX9 protein is generally seen to be localised to the nucleus, in agreement with the presence of two nuclear localisation signals in the HMG domain (Sudbeck and Scherer, 1997). However, in genital ridge sections prior to 11.5 dpc the antibody detects protein within the cytoplasm of many cells (although not all) (Fig.II.3.i). This cytoplasmic location, which tends to be perinuclear, is independent of chromosomal sex. After 11.5 dpc the protein is nuclear in all positive cells within the developing testis (Fig.II.3.j).

II . III . 4 . Sox9 shows testis specific expression in the chick

The homologues of Sox9 from mouse, chick, alligator and fish, such as Fugu, show a high level of protein conservation (Coriat et al., 1994; Wright et al., 1995; P.N.Goodfellow unpublished observations). This suggests that SOX9 has conserved functions, and consistent with this, Sox9 has been shown to be expressed in developing cartilage of chick as well as mice (Healy et al., 1996; Wright et al., 1995) (Fig.II.4.).
Fig.II.4 Whole mount *in situ* hybridisation to detect Sox9 transcripts in a mouse embryo at 11.5 dpc (a) and in a chick embryo at stage 25 (b). c and d Sox9 limb expression in the mouse at 12.5 dpc (c) and (d) in the chick at stage 29 (Hamburger and Hamilton, 1951)
However, genes controlling sex determination are notorious for being rapidly evolving or completely absent between species and there is no \textit{a priori} reason to suppose that \textit{Sox9} has a conserved role in sex determination. Its expression in developing gonads of chick embryos was therefore examined.

c\textit{Sox9} transcripts are present in the genital ridges of both ZW and ZZ embryos at stage 25 (Fig.II.5.a,b), a stage at which the genital ridges look morphologically identical. The levels are much lower than those seen in developing testes at later stages. c\textit{Sox9} is also transiently expressed in mesonephric tubules, which are functional in the chick, at these early stages. This is reminiscent of expression seen in tubules of the definitive kidneys (metanephros) of mouse embryos as they begin to develop (A. Swain, personal communication) and it is worth noting that CD patients often show renal defects. In ZZ embryos at stage 28, both gonads look identical and are beginning to differentiate as testes. They both show very strong expression of c\textit{Sox9} (Fig.II.5.c). In ZW embryos at this stage, the left gonad is beginning to form an ovary and the right gonad is arrested in development. Both gonads are negative for c\textit{Sox9} expression (Fig.II.5.c). The same pattern of expression is seen at stage 32 when sexual dimorphism is even more apparent (Fig.II.5.d). Sections show the expression to be within cells that have begun to align into epithelial cords (Fig.II.5.f).
Fig. II.5 Whole mount *in situ* hybridisation to detect *Sox9* transcripts in dissected chick gonads and mesonephros from ZW and ZZ embryos at stage 25 (a,b), stage 28 (c), stage 32 (d). Transverse sections through the developing ovary (e) and a testis (f) of the specimens in d, visualised by Nomarski optics.
II. III. 5. Sox9 expression marks experimental XX sex reversal in the mouse

The expression data above reveals a strong correlation between Sertoli cell differentiation and high levels of Sox9 expression. To test this further, a case of experimental sex reversal in the mouse was examined, where fetal ovaries are grafted to the kidney capsule of adult mice. Previous studies by others, notably Taketo and colleagues (1985), have shown that the best “transdifferentiation” is obtained when 13.5 dpc fetal ovaries which have been dissected away from mesonephros are used as grafts. In a first series of experiments, a total of 30 ovaries were grafted to 13 animals and, as positive controls, 16 testes of the same age were grafted to 6 animals. In a second series, 21 ovaries and 8 testes were grafted to 7 and 3 hosts respectively. A proportion of grafts of both sexes degenerated, possibly due to immune rejection (the grafts and hosts were from outbred mice), however, the majority were viable and showed good development over time. The control testis grafts maintained an obvious cord structure.
Fig.II.6 Ovary and testis grafts from 13.5 dpc embryos to adult kidney capsule. a-h, Whole mount \textit{in situ} hybridisation to detect Sox9 transcripts. a, Ovary graft after 6 days still within the kidney capsule and b, dissected free. c, Testis grafts after 6 days within the kidney capsule. d, f and h, ovary grafts after 18, 26 and 30 days respectively; e and g, testis grafts after 18 and 28 days. i-j, Antibody localisation of SOX9 in sections of a testis graft (i) and an ovary graft (j) after 26 days.
and Sox9 expression was clearly visible by whole mount in situ hybridisation at all stages looked at (Fig.II.6.c,e,g). The ovary grafts showed no organisation into cords and were negative for Sox9 expression at early time points, e.g. at 6 days after grafting (Fig.II.6.b). By 18 days after grafting, Sox9 expression is detected in isolated cells in some grafts (Fig.II.6.d). However, by 26 - 30 days, there is considerable organisation of cells into cord like structures, and many of the cells show quite strong Sox9 expression (Fig.II.6.f,h). Sections show some of these positive cells to be tightly adherent to each other and to have morphological characteristics of Sertoli cells. Cells of similar morphology show nuclear staining with anti-SOX9 antibody in both ovary and testis grafts (Fig.II.6.i,j).

II. IV. Discussion

To begin to address the question whether Sox9 has a role in sex determination, Sox9 expression was analysed in developing gonads. Sox9 starts being differentially expressed, coincidentally with the differentiation of the gonads. At that time it is upregulated in males and downregulated in females. Sox9 transcripts and SOX9 protein were confined to the Sertoli cells in mouse as in chick. This study suggests that Sox9 is directly involved in sex determination.
The expression pattern seen for Sox9 is in agreement with one of a gene that is involved in Sertoli cell differentiation. The sex reversed phenotype of human CD patients is entirely consistent with lack of Sertoli cell differentiation. In most cases, where an essentially female phenotype is apparent, they show normal Müllerian duct development, which strongly suggests that AMH was not being made by Sertoli cells. This is a very early product of Sertoli cells. There also appear to be in some cases primary follicles with oocytes, suggesting normal follicle cell differentiation, and early entry of germ cells into meiosis. Leydig cell differentiation must also be affected as signs of masculinization of internal and external genitalia can be absent. This all suggests that Sertoli cell differentiation and signalling to other cell lineages has been prevented at a very early stage.

SOX9 protein is likely to be a transcription factor and is therefore expected to be nuclear. SOX9 protein is seen within the nucleus of differentiating and mature Sertoli cells and is absent from other testicular cell lineages. However, in the indifferent gonad of both XX and XY embryos, only some cells show nuclear localisation and the majority show SOX9 protein within the cytoplasm. Other members of the SOX family including SRY appear to be nuclear in most instances. Nevertheless SOX2, which is present within nuclei of uncommitted cells of the central and peripheral nervous systems, has a cytoplasmic form derived from an alternative transcript in some differentiated neuronal cell types (S. Sockanathan, personal communication).
Alternatively, Sox9 cellular localisation could be concentration dependent, where low levels are retained in the cytoplasm by interaction with another protein, but at high levels this protein is overwhelmed and SOX9 can enter the nucleus.

The chick is a system where Sry is not present. Therefore there must be another way to trigger sex determination in birds. Since Sox9 is highly conserved throughout the open reading frame in several species (Coriat et al., 1994; Wright et al., 1995; P.N. Goodfellow unpublished observations) it would be logical to see if its expression was also conserved. During normal development of the chick, we see cSox9 expression in genital ridges at a low level in early stages and again, coincident with the first signs of morphological differences between the sexes, cSox9 expression is down regulated in ZW gonads and upregulated in ZZ gonads. As in the mouse, the high level in ZZ gonads precedes overt cord formation. Sox9 transcripts seem to be confined to the Sertoli cells in a similar pattern to the one described very recently for cAmh (Eusebe et al., 1996). Presumably, development of the testicular cords occurs in a similar fashion to the mouse. In ZW embryos the expression is down regulated in both the left gonad, which normally develops as the ovary, and in the right gonad which is retained in an indifferent or immature state. This suggests that whatever represses cSox9 is active in both gonads and that it can function independently of the signal that leads to ovarian development. The genes responsible for the normal decision between ovarian and testicular development in chicken are not known. cSox9 is the first gene found to show such early
sex-specific expression in chicks and should be an important lead to begin to dissect sex
determination in birds at a molecular level.

If Sertoli cells play an important role in orchestrating cellular organisation of the testis,
Sox9 can also play a part in that role. To test this hypothesis, Sox9 expression was
analysed in a case of experimental sex reversal in the mouse. In this case the appearance
of Sertoli cells is an accompaniment of ovary transdifferentiation and clearly
independent of Sry. No signal responsible for testicular development from ovaries
grafted to the kidney capsule is known. This situation demonstrates that there must be
a fine balance between testis and ovary differentiation and that the loss of oocytes, or
direct effect of exogenous factors must suppress ovary differentiation and result in the
activation of secondary testis determining genes. At the stage when the ovaries are
dissected for grafting, they are completely negative for Sox9 expression, both at the
RNA and protein levels. While there is a small anterior patch of Sox9 expressing cells,
this is within the mesonephros and will have been dissected away. The appearance of
Sox9 expressing cells in the grafts over time therefore represents either invasion of
positive cells from the host kidney or it can be associated with "transdifferentiation" of
Sertoli cells from follicle cells. It is conceivable that the Sox9 expressing cells invade
from the host tissue. Indeed, Sox9 expression in developing tubules in the early fetal
kidney is seen (A. Swain, personal communication). However, in the adult kidney only
very low levels of Sox9 are detectable by RNAse protection assays (A. Hacker,
personal communication) and the host kidney tissue gives no detectable signal by in situ hybridisation (Fig.II.6. a,c). Also, while it seems likely that some host cells will enter the grafts, all available evidence suggests that these give rise to connective tissue cells (Martineau et al., 1997; Taketo-Hosotani et al., 1985). The control grafts with fetal testes also show no Sox9 positive cells in addition to those which are clearly arranged in cords and have Sertoli cell morphology. We can therefore conclude that the Sox9 positive cells within the ovary grafts are most likely a result of transdifferentiation of follicle cells to give Sertoli-like cells. This strengthens the correlation and argues that Sertoli cell differentiation is closely associated with Sox9 expression.

A model can be presented for the role of Sox9 during sex determination and differentiation. Sox9 could be the determining gene for Sertoli cell differentiation. This can be supported by a number of facts: the lack of Sertoli cells is a main feature in CD phenotypes. Sox9 transcripts are seen in Sertoli cells of both mouse and chick male gonads and appear coincidentally with the "transdifferentiation" of ovaries. The mouse SOX9 protein has a nuclear localisation in differentiated Sertoli cells, which can reflect its role as a transcription factor in this cell type.

A repressor of male structures could be present in the indifferent gonads. Once Sry is activated, this molecule would then be prevented from acting or downregulated and Sox9 could be activated. Sox9 could be directly repressed by this molecule. A good candidate
for this proposed repressor is DAX-1. This is encoded by a gene found in the DSS (Dosage Sensitive Sex reversal) region that is localised on the X chromosome and that when in double copy female development occurs in XY individuals. The *Dax-1 in situ* data (Swain *et al.*, 1996) and its overexpression in transgenic mice (Swain *et al.*, 1998) further supports the possibility of DAX1 being the proposed repressor.

It is also difficult to exclude that SRY can be directly regulating *Sox9* expression. Several predictions can be made concerning SRY target genes: they must be expressed in the same cell lineage as *Sry*, there should be a change in the expression of the target gene which correlates with the presence of SRY and a difference between males and females. There should be a preferred binding site (consensus site) for SRY within the minimal promoter region able to confer sex specific expression. *Sox9* clearly satisfies all these criteria, apart from the latter for which no data is yet available. The only problem is to explain the lack of interaction between *Sry* and *Sox9* in the grafting experiments. But this could be a different situation. The sex reversal in this case is different. Other mechanisms could explain transdifferentiation at a time which is later than usual, and where the tissue is reverted from the female to the male pathway, instead of an indifferent tissue to a specific male or female pathway. The host environment could also somehow induce an intermediate process leading to testicular organisation.
Sox9 could be regulated by SRY and/or DAX-1. What about downstream genes? Amh is a good candidate for a molecule directly regulated by SOX9. AMH is so far the earliest marker of Sertoli cells. High levels of SOX9 expression just precede the onset of Amh transcription, perhaps Amh is regulated by SOX9 rather than SRY as has been proposed (Haqq et al., 1994). The next chapter will address this question.
CHAPTER III. SOX9 WITH SF1 PROMOTES Amh TRANSCRIPTION IN EMBRYONIC TESTIS: ANOTHER LINK IN THE SEX DETERMINATION CASCADE
III.1. Introduction

Amh gene expression was shown to be both temporally and spatially restricted to the gonads of the developing embryo. Amh transcription is first detected at 11.5 dpc, the expression being restricted to the Sertoli cells, and is shut off postnatally. In the female, the expression of Amh is first detected at day 6 after birth and is restricted to granulosa cells. The levels of AMH in the ovary after birth are 0.1% of the levels produced by the fetal testes (Josso and Picard, 1986; Munsterberg and Lovell-Badge, 1991).

Other genes are expressed in gonadal development at the time sex determination occurs like Sry, Sfl, and Sox9. These three genes are expressed in a sexually dimorphic pattern, being present at high levels in Sertoli cell precursors, prior to the onset of Amh transcription. They are therefore reasonable candidates to regulate Amh transcription.

Previous studies have proposed that SRY, the testis determining factor, activates Amh (Haqq et al, 1994). However, detailed analysis of Sry and Amh RNA levels during testis development, showed that Amh transcription begins 20 hours after the onset of Sry. These expression studies raised the possibility that another factor may be involved in Amh activation (Hacker et al, 1995). The expression pattern shown by Sox9 during testis development lead us to believe that this transcription factor, rather than SRY, could be a regulatory factor of Amh transcription.
The aim of this work, is to begin to understand the transcription regulatory mechanisms responsible for the spatial and temporal restricted expression of the Amh gene. Previous sequence comparisons of the human, bovine, chicken, rat and mouse 5' regulatory region of Amh (-180 to +1 bp), have revealed several conserved regions. This analysis is focused on two of these sites (Fig.III.1.). The first (BOX B) represents a putative binding element for Steroidogenic factor 1 (SF1). The second conserved motif in the Amh 5' region (BOX A) is a SOX/SRY binding motif.

In this chapter, it is proposed that SOX9 binds to the SOX/SRY element, in the Amh promoter and moderately activates the transcription of Amh. In addition it is shown that the activation of Amh by SOX9 is enhanced by the addition of SF1. The results suggest a synergistic co-operation between SOX9 and SF1 to regulate Amh transcription.
**Fig. III.1.** Comparison of the -215bp *AMH* promoter. **a** Alignment of the human (Guerrier *et al.*, 1990), pig (Genbank SSU80853), rat (Haqq *et al.*, 1992) and mouse (Munsterberg and Lovell-Badge, 1991) sequences upstream of the ATG are given (adapted from Shen *et al.*, 1994). Several conserved sites are highlighted in colour: putative SRY/SOX binding site (red), SF1 binding site (blue) and TATAA box (green). Sequences highlighted in yellow and white denote other conserved sites in the regulatory region of *AMH*. **b** Probes used in this chapter with the wild type and mutated binding sites for SRY/SOX (wt-red and mutated-pink) and SF1 (wt-light blue and mutated-dark blue).
### Human, Pig, Rat, and Mouse Consensus

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### SRY/SOX

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Materials and methods

1. Animals and tissues

Embryonic mouse tissues were obtained from Parkes outbred mice (maintained at NIMR). For embryo staging, midday on the day of appearance of the vaginal plug is taken as 0.5 dpc.

2. Plasmid constructs

Mouse Sf1 and Sox9 cDNA were subcloned into the EcoRV restriction site on the pcDNA3 expression vector (Promega). One copy of each of the oligonucleotides SOX/SF1, MUT/SF1, SOX9/MUT and MUT/MUT (Fig. III.1.) was cloned into the EcoRV restriction site of the pGL3 basic vector (Promega). The reporter plasmids were named SOX/SF1luc, MUT/SF1luc, SF1/MUTluc and MUT/MUTluc.
Embryonic gonads were dissected at 11.5 dpc and homogenised. Tissue extracts were prepared by three cycles of freeze-thaw lysis in Buffer A (20mM Hepes, 450mM NaCl, 25% Glycerol, 250mM EDTA, 0.5mM PMSF, 0.5 μg/ml Leupeptin, 0.5μg/ml Aprotinin, 40 μg/ml Pestatin, 10mM β-Glicerophosphate). Tissue extracts were prepared by centrifugation at 10,000rpm for 15min at 4°C, and supernatants were stored at -70°C (Scholer et al., 1989). Protein concentrations were determined using the Bio-Rad protein assay. Synthetic oligonucleotides, probe SRY/SOX, probe SOX/SE1, probe MUT/SE1, probe SOX/MUT, MUT/MUT (Fig.III.1.) were labelled with $[^{32}P]dCTP$ (Amersham) by using T4 polynucleotide kinase (Promega). Tissue extracts (10μg) were incubated with 10-20 fmol labelled oligonucleotides for 30 min at room temperature in 10 μl of retardation mix 2X (20mM TrisHCl, pH 8, 0.2mM EDTA, 20mM MgCl₂, 4mM Dithiothreitol, 35% glycerol, 4mg/ml BSA, 50 mM NaCl, 0.5mM PMSF) containing 1 μg salmon sperm DNA. For supershifts, incubations of VAH2 antibody (Morais da Silva et al., 1996) with tissue extracts was performed for 45 min prior to the addition of the labelled oligonucleotides. For competition analysis, 100-fold excess of end-filled unlabelled oligonucleotides were incubated 15min prior to addition of labelled oligonucleotides. The DNA-protein complexes were resolved on 5% polyacrylamide gels in 0.5X Tris-Borate-EDTA buffer.
III. II. 4. Cell culture and transient expression assays

COS cells were grown in DMEM supplemented with 10% FCS, L-glutamine and streptomycin/penicillin in a 5% CO$_2$ atmosphere at 37°C. The cells were transfected with the lipofectamine method (Gibco). Cells were harvested 48 hours after transfection. Luciferase assays were performed with the Luciferase kit (Promega), according to the manufacturers protocol. The data shown are from one experiment and each value represents the mean of three separate wells, with similar results obtained in three independent experiments. Bars represent the standard error.

III. III. Results

III. III. 1. SOX9 binds a conserved CCTTTGA motif in the *Amh* promoter

The alignment of the *Amh* sequences reveal two conserved elements within the *Amh* promoter: the first element (CCTTTGA) is similar to sites recognised by HMG box type proteins. The second element, designated MIS-RE-1 (AGGTCA) matches the half-site for a number of nuclear hormone receptors (Umesono *et al.*, 1991) (Fig.III.1.).
Electrophoretic mobility shift assays (EMSA) were performed, using protein extracts from 11.5 dpc testis with the probe SRY/SOX which contains a SOX binding site (Fig.III.1.). These reveal a pattern of bands that are effectively competed by addition of an excess of unlabelled probe (Fig.III.2.a, lanes 1,2). In order to address if this shift is caused by SOX9 binding to the probe, an antibody raised against SOX9 (VAH2) was added (Morais da Silva et al., 1996). The disappearance of the bands with the addition of SOX9 antibody indicates that the shift is caused by SOX9 protein contained in the extracts (Fig.III.2.a, lanes 3,4). No change was observed with addition of preincubated VAH2 with SOX9 peptide 486 (Morais da Silva et al., 1996; data not shown) The same experiment was performed with protein extracts from 14.5dpc testis with similar results (data not shown).

III . III . 2. SF1 and SOX9 bind to the Amh promoter region

SF1 protein has been shown to regulate Amh transcription (Shen et al., 1994). 11.5 dpc protein extracts were analysed with probe SOX/SF1 by EMSA. Two major bands were detected, which are dependent on each of the binding sites SRY/SOX and MIS-RE-1. When the experiment is performed with a probe in which the SOX site is mutated (MUT/SF1) only one band is detected. Conversely when the SF1 site is mutated (LEF/MUT) one of the bands almost disappears while the other band is still detected.
Fig III.2. SOX9 binds to the SRY/SOX binding site in the *Amh* region. EMSA was performed with labelled SRY/SOX probe and 11.5 dpc testis extracts as described in the materials and methods. **Lane 1.** 100-fold excess probe was used as a competitor. **Lane 2.** Labelled SOX/SRY probe with 11.5 dpc testis extracts. Addition of 1 mg (Lane 3) and 1.5 mg (Lane 4) of VAH2 antibody to the labelled SOX/SRY probe and 11.5 dpc testis extracts. (Unshifted probe not shown).
Fig III.3. Two major bands are detected which are dependent on each of the binding sites SRY/SOX and MIS-RE-1. **Lane 1.** EMSA performed with 11.5 dpc testis extracts and labelled probe SOX9/SF1. **Lane 2.** 11.5 dpc testis extracts with the MUT/SF1 labelled probe. **Lane 3.** 11.5 dpc testis extracts with the SOX/MUT labelled probe. **Lane 4.** 11.5 dpc testis extracts with the MUT/MUT labelled probe.
The mutation of both sites (MUT/MUT) results in the abolishment of the lower band while the upper band is still observed, although with less intensity (Fig.III.3.).

III. III. 3 SOX9 and not SRY interacts with SF1 to activate Amh

As shown in Fig. III.4, when the Sox9 expression vector is separately transfected with vector SOX/SF1luc (see materials and methods), a low activation is observed. The relatively low activation caused by SOX9 alone of the Amh promoter (Fig.III.4) in addition with previous studies of contact dependent SOX activation leads to the hypothesis that SOX9 acts with another factor to fully activate Amh transcription. This factor is likely to be SF1, which has already been shown to activate Amh (Shen et al., 1994). In fact, if both Sox9 and Sf1 are co-transfected with the vector SOX/SF1luc, a high activation is seen. When the same experiment is performed with the MUT/SF1luc, SOX/MUTluc or MUT/MUTluc vectors the high activation observed when Sox9 and Sf1 are co-transfected is abolished, although the values obtained for individual tranfections with either of these genes are only slightly diminished. These results show that the low activation seen when Sox9 or Sf1 are transfected separately with SOX/SF1luc is likely to be due to the fact that both proteins can bind to other motifs within the Amh 5' sequences used. However, the mutations would abolish the interaction between SOX9 and SF1, which probably need to bind to adjacent sites.
Fig. III.4. SOX9 and SF1 co-operate to promote *Amh* activation. Transient transfection assays were performed in COS cells using a luciferase reporter vector containing the probes SOX/SF1, MUT/SF1, SOX/MUT and MUT/MUT (Fig. III.1.). The cooperation between SOX9 and SF1 is abolished when one or the two binding sites are mutated.
Luciferase activity (Light units)

SOX9 (1000ng) - + + + + + + + + + + + +
SF1 (1000ng) - + + - + - + + + + + + +

MUT/SF1 (500ng) MUT/MUT (500ng)

MUT/SF1 (500ng) SOX/MUT (500ng)

MUT/SF1 (500ng) SOX/SF1 (500ng)
Another interesting result of this experiment is the fact that activation of the SOX/SF1 reporter construct is observed in COS cells. Previous studies have demonstrated that the \textit{Amh} promoter region shows a cell-restricted expression (Shen \textit{et al.}, 1994). The SOX/SF1 reporter construct contains only the region between -177 and -79bp. These results show that a region that gives additional tissue specificity to Amh expression is deleted, enabling the expression of the SOX/SF1 reporter construct in cells other than Sertoli cells.

If both SRY and SOX9 are capable of binding to the 5' region of \textit{Amh}, what mechanism initiates \textit{Amh} expression? Why is not \textit{Amh} highly transcribed with the onset of SRY expression? To try to assess this problem, titration of activation levels caused by SOX9 and SRY in the presence of SF1 were performed (Fig.III.5.). The results show that SRY activates very weakly \textit{Amh} in the presence of SF1. In the case of SOX9 a strong activation of the reporter vector is observed. These results can explain why there is a 20 hour delay between the onset of \textit{Sry} versus \textit{Amh} transcription. It seems that it is not SRY that activates \textit{Amh}, but SOX9.
Fig. III.5. Titration of activation levels caused by SOX9 and SRY in the presence of SF1 on the Amh promoter. Transient transfection assays were performed in COS cells using a luciferase reporter vector containing the SOX/SF1 probe. SOX9 and not SRY activates Amh transcription.
III. IV. Discussion

Since the control of eukaryotic gene transcription appears to be the result of a multitude of combinations of a finite number of transcription factors, it may be safe to say that any imaginable alternative is likely to be found to exist at some gene locus. The Amh gene is expressed in a specific developmental stage in the mouse and plays an essential role in the regression of the Müllerian ducts, it must not be expressed in female development, therefore it must be tightly regulated.

In this study, the combination of factors that promote Amh transcription are shown to be SOX9 and SF1. Although these are in vitro studies, they tend to suggest a co-operation between the two proteins. Other authors have suggested that SRY and SF1 activate Amh transcription (Haqq et al., 1994; Shen et al., 1994). In this case, the binding site proposed for Sry (-45 to -75bp) is not the same as the CCTTTGA element (-150 to -144 bp), and is not conserved between species (Fig.III.1.). Two recent reports by Nachtigal and colleagues (1998) and Viger and colleagues (1998) show further Amh regulation by WT1, DAX1 and GATA-4.

The Amh 5' region studied in this work extends from -171 until -73bp. The fact that this region does not show cell-specific expression, like the -180bp MIS (Shen et al., 1994), suggests that an element that confers specificity to Amh expression lies in the
region not included in the SOX/SF1 construct. The sequence used in this work contains a LEF/SRY and a SF1 element that are conserved in several species. The specific activation by SOX9 and SF1 described in this work represents the first demonstration of a co-requirement from these two important developmental families: the SOX and the orphan nuclear receptor families.

An example of an interaction between SF1 and another factor is the activation of the Luteinizing hormone-β subunit transcription. When the LH-β promoter linked to a reporter gene is co-transfected with NGFI-A and SF1, a stronger activation is observed than when the transfection is with each of the effectors separately. The combination of NGFI-A and SF1 resulted in synergistic activation of the LH-β gene (Lee et al., 1996).

Other members of the SOX gene family are reported to interact with other factors to promote activation of a given target. Such is the case of SOX2. This can interact with the OCT3/4 transcription factor to promote Fgf4 transcription, and with δEF1 to activate the δCristallin gene (Kamachi et al., 1995; Kamachi et al., 1998; Nishiguchi et al., 1998; Yuan et al., 1995).

The observation that SF1 acts specifically with SOX9 but probably not with SRY illustrates one mechanism by which selective gene activation can be achieved by individual members within these families. Maybe the transactivation domain plays an
important role in this interaction, although it has been reported that it is through the HMG domain that the interaction between POU domains and high mobility group proteins occur (Zwilling et al., 1995).

Several questions arise from this work. First, is there protein-protein interaction between SOX9 and SF1. One way to find out would be to perform an immunoprecipitation with either recombinant proteins or testicular extracts. Another way would be to increase the distance between the two conserved sites and determine if the activation caused by SOX9 and SF1 is disrupted in the same way that is observed when the conserved sites are mutated.
IV. TARGETED DISRUPTION OF THE MOUSE Sox9 GENE
IV. I. Introduction

Sox9 genes have been cloned from human, mouse, chick, alligator and fugu, and show a high level of conservation suggesting that Sox9 has conserved functions (Foster et al., 1994; Wright et al., 1995; Wagner et al., 1994; P.N.Goodfellow, personal communication). In addition its expression pattern is highly conserved during development, at least in mammals and birds (Chapter II; Kent et al., 1996).

Its critical role in human development is indicated by the phenotype of CD patients. In addition to severe skeletal malformations and sex reversal, some mortality in utero has also been described, giving emphasis to a possible critical role of this gene during embryogenesis. The expression pattern of a gene during embryonic development often give clues about its possible function. To help understand the role of Sox9 during embryonic development and the CD phenotype, Sox9 expression during mouse embryonic development was analysed by in situ hybridisation.

Sox9 transcripts are first detected by the late streak stage at 7.5 dpc, being confined to the node, the mouse organiser, and to cells in the prechordal mesoderm. Later, the expression is mainly in neural crest cells, somites and sites in the developing embryo where chondrogenesis is occurring (Ng et al., 1997; Wright et al., 1995; Zhao et al., 1997; section IV. III. 1.).
To determine the function of *Sox9* during mouse development, the gene was inactivated by homologous recombination in embryonic stem (ES) cells. Embryonic stem cells were first isolated by culturing the inner cell mass cells of blastocyst stage embryos on fibroblast feeder cell layers (Martin, 1980; Evans and Kaufman, 1981). Exogenous DNA containing the sequence to be targeted in the host genome and selective markers, can be introduced into ES cells where homologous recombination will take place (Ramirez-Solis *et al.*, 1993).

The phenotypic effects of the inactivation of *Sox9* were studied directly in ES cell chimaeras obtained by two methods; injection into host blastocysts and aggregation with cleavage stage embryos. In the former technique, 10-15 cells from an ES cell clone are injected into a host blastocyst stage embryo. The resulting chimaeras can be crossed to normal animals to test for germ line transmission of the mutation. If heterozygous offspring are viable, they can be used to breed homozygotes (Papaioannou and Johnson, 1993).

The aggregation technique used is a modification devised to produce embryos that are totally ES cell derived (Nagy and Rossant, 1993). Tetraploid embryos resulting from electrofusion of two-cell stage embryos, are aggregated with 10-15 ES cells. These embryos are cultured and transferred at blastocyst stage to pseudopregnant recipients.
ES cells and tetraploid embryos can complement each other in aggregation chimaeras: the fetuses are ES cell-derived and most of the extraembryonic tissues are provided by the tetraploid component.

While a few live born chimaeras were obtained, they failed to transmit the mutation to their offspring. The majority of the chimaeras obtained by the two techniques described above died as embryos, showing posterior truncations. The most severe chimaeras did not gastrulate properly and do not present any recognisable structures or tissues.

The results obtained in this chapter indicate that the role of Sox9 during development is essential for proper embryo formation, possibly in the elongation of the anterior-posterior axis. It is also possible that there is a correlation between the severity of the phenotype and the percentage of chimaerism, in that the majority of the aggregation chimaeras show impairments in gastrulation, whereas the ones resulting from blastocyst injection vary in severity, possibly reflecting the variability of chimaerism.
IV . II . Materials and methods

IV . II . 1 . In situ hybridisation

Animals and tissues, pbSox9 probe and in situ hybridisations were obtained as described in section II.II. For sections and flatmounts, tissues were treated as described in section II.II.4. Whole embryos were mounted with 100% glycerol to produce flatmounts.

IV . II . 2 . Gene targeting

Targeting was done by standard means (Robertson et al., 1987; Wurst and Joyner, 1993) in the laboratory of Peter Goodfellow.

IV . II . 3 . Blastocyst injection

CCE (Bradley et al., 1984) ES cells were grown on monolayers of mitomycin treated STO cells. The tissue culture medium was Dulbecco's modified Eagle's medium
(DMEM) (Gibco) supplemented with 15% Fetal Calf Serum (FCS), 2mM L-glutamine, 50 mg/ml penicillin and 50 mg/ml streptomycin (Gibco). Blastocyst stage embryos recovered from C57BL/6 3.5 dpc females, were injected with 10-15 ES cells and transferred to 2.5 days pseudopregnant female mice (F1(CBA/C57BL6)) (Papaioannou and Johnson, 1993).

IV . II . 4. Electrofusion and aggregation chimaeras

Two cell stage embryos were collected the day after vaginal plug (1.5 dpc) from superovulated C57BL/6 female mice and washed in M2 medium (Sigma). These embryos were placed in an electrode chamber containing a 0.3 M mannitol solution. 15-20 embryos at a time were subjected to an electric field of 1 V. A fusion pulse of 100 V for 25 msec was applied. The embryos were then placed in M16 (Gibco) medium and incubated at 37°C, 5%. 30-60 minutes after application of the electric pulse, perfectly fused tetraploid embryos are selected and transferred in groups of 15-20 to new M16 media and cultured overnight. For aggregation the zona pellucida of the tetraploid embryos was removed by acid Tyrode's solution (8 mg/ml NaCl, 0.2 mg/ml KCl, 0.24 mg/ml CaCl₂·2H₂O, 1 mg/ml glucose, 4 mg/ml polyvinylpyrrolidone (PVP), adjusted with HCl to pH 2.5). ES cell aggregations are made by "sandwiching" 10-15 Sox9 targeted ES cells between two tetraploid embryos in an aggregation plate (Nagy and
Rossant, 1993). The aggregated sandwiches are cultured in M16 until they reach the early blastocyst stage and are transferred to 2.5 dpc pseudopregnant F1(CBA/C57BL6) female mice. Embryos were recovered at 8.5 dpc.

**IV. III. Results**

**IV. III. 1. Sox9 expression during embryonic development**

*Sox9* transcripts are first detected at late streak stage at 7.5 dpc in the node, the mouse organiser (Fig.IV.1.a-d). The expression in the node is first seen at the late streak stage, at a time where the node is already regressing posteriorly. This structure acts to organise the body plan. Cells from the organiser can differentiate into axial mesoderm, neuroectoderm, gut endoderm and somites. During gastrulation the cells recruited to the primitive streak undergo an epithelial to mesenchymal transition, then ingress in-between the epiblast and endoderm to become incorporated in either the mesoderm or the definitive endoderm germ layers (Tam and Behringer, 1997). At this stage, the cells expressing *Sox9* are likely to be mesodermal cells (Fig.IV.1.c,d).
Fig.IV.1. Whole mount \textit{in situ} hybridisation to reveal Sox9 transcripts during mouse early embryonic development. a-f 7.5 dpc mouse embryos, at late streak stage (a-d) and head fold stage (e-f). c and d Sections of the embryo showed in a. g 8.5 dpc mouse embryo. h flat mount of the embryo showed in g. The arrow points the Sox9 expression in the node and primitive streak. Note that the signal detected in a and b outside the streak is due to trapping of the Sox9 probe as can be confirmed by the sections shown in c and d.
By the beginning of the head fold stage, Sox9 is expressed in the node and in cells within the prechordal mesoderm, anterior to the node cells (Fig.IV.1.e) (Ng et al., 1997). The expression of Sox9 during early head fold stage is extended anteriorly from the node. Later, Sox9 expression can be observed in the prospective midbrain/hindbrain region (Fig.IV.1.f).

At 8.5 dpc Sox9 expression can be observed in the lateral edges of the neural folds (Fig.IV.1.g and Fig.IV.2.a,b). Some of the labelled cells appear to migrate ventrally, consistent with them being neural crest. There is also high expression in the notochord and node. In the node, the expression is higher in the anterior part which may be the cells that contribute to notochord and paraxial mesoderm, promoting axis elongation (Wilson and Beddington, 1996) (Fig.IV.1.h).

At 9 dpc the rostral somites are expressing Sox9. The first branchial arch and the forehead also show Sox9 signals resulting from the ventrally migrating cells, possibly neural crest, from the forebrain/midbrain region (Fig.IV.2.c).

At 9.5 dpc Sox9 expression can be seen at the optic vesicle, head mesenchyme, the first branchial arch and in the rostral somites. Sox9 expression seems to follow the migrating neural crest from the neural folds to the somites, head mesenchyme and frontal nasal mass (Fig.IV.2.d).
Fig. IV.2. Whole mount *in situ* hybridisation to reveal Sox9 transcripts during mouse development. *a,c,d,e,g* lateral views of 8 dpc (*a*), 8.5 dpc (*b*), 9 dpc (*c*), 9.5 dpc (*d*), 10.5 dpc and 11.5 dpc (*g*). *f,h* Sox9 is expressed in dorsal root ganglia and sclerotome in the 10.5 dpc embryo shown in *e* (*f*) and 11.5 dpc embryo shown in *g* (*h*).
By 10.5 dpc both rostral and caudal somites show Sox9 transcripts (Ng et al., 1997; Zhao et al., 1997). In addition the second branchial arch also shows staining (Fig.IV.2.e,f). These observations indicate that the ventral migration of these cells progresses in a rostral to caudal manner.

By 11.5 dpc Sox9 expression is observed at sites where chondrogenesis is occurring, like the limbs, the scapulae and the vertebra (Ng et al., 1997; Wright et al., 1995; Zhao et al., 1997). It is also seen in the otocyst and dorsal root ganglia. The expression at this stage in the somites is confined to the sclerotome (Fig.IV.2.g,h).

IV. III. 2. Gene targeting

Constructing of the targeting vector (Fig.IV.3.) and the targeted disruption of the Sox9 gene in ES cells was performed by Jamie Foster and Alan Shafer while in Peter Goodfellows' laboratory. All the subsequent experiments were performed by myself. The targeting efficiency was 1:50. Six clones with the correct homologous recombination event at the Sox9 locus were obtained: C78, B14, E117, B55, B67 and C191.
Fig. IV.3. Strategy for disrupting the mouse Sox9 gene. Pink indicates exons and red the HMG box. The neo selectable marker and thymidine kinase (TK) gene were inserted into a plasmid containing the indicated Sox9 genomic sequences. The neo gene replaced the first exon and part of the HMG box. Restriction endonuclease sites are E, EcoRI; P, PstI; B, BamHI; S, SstI; X, XbaI; H, HindIII; Xh, XhoI. The position of the probe used for Southern blotting analyses is indicated.
Clones C78, B14 and E117 were injected into blastocysts to generate chimaeric mice. The chimaerism of the live born mice was judged by the percentage of the Agouti coat colour. The mice obtained with these ES cell clones presented 10-80% chimaerism (table IV.1). The male chimaeras were test mated to C57BL6 females but no newborn animals were obtained with agouti coat colour, expected if there is germ line transmission from the ES cells. To investigate this further, 80 embryos ranging from 12.5 to 14.5 dpc resulting from the crossing of the resulting chimaeras to C57BL6 mice were analysed phenotypically under a microscope and genotypically by Southern blot to look for the targeted allele (data not shown). All the embryos were normal and none carried the mutation.

The failure to transmit the mutation suggests a problem of fertility or very early embryogenesis. To overcome this, tetraploid host embryos were used to give chimaeras where 100% of the embryo should be ES cell derived, i.e. Sox9 heterozygotes. Clone C78 was initially used to produce aggregation chimaeras. 27% of the transferred blastocysts to pseudopregnant female mice were recovered (table IV.2). The most severely affected presented a strange round shape, as if gastrulation was impaired. The others show retarded growth with severe posterior truncations, lacking posterior mesoderm.
Table IV . 1 Adult chimaeras obtained by blastocyst injection.

Table IV . 2 Analysis *in utero* of embryos obtained by the aggregation technique.
Number of resorbtions versus number of embryos.

Table IV . 3 Analysis *in utero* of embryos obtained by blastocyst injection. Number of resorbtions versus number of embryos.
Table IV . 1

<table>
<thead>
<tr>
<th>Chimaera</th>
<th>Sex</th>
<th>% Chimaerism</th>
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<tbody>
<tr>
<td>C78#1</td>
<td>male</td>
<td>5%</td>
</tr>
<tr>
<td>C78#2</td>
<td>female</td>
<td>5%</td>
</tr>
<tr>
<td>C78#3</td>
<td>male</td>
<td>80%</td>
</tr>
<tr>
<td>C78#4</td>
<td>female</td>
<td>60%</td>
</tr>
<tr>
<td>C78#5</td>
<td>male</td>
<td>80%</td>
</tr>
<tr>
<td>B14#1</td>
<td>female</td>
<td>60%</td>
</tr>
<tr>
<td>B14#2</td>
<td>male</td>
<td>10%</td>
</tr>
<tr>
<td>B14#3</td>
<td>female</td>
<td>50%</td>
</tr>
<tr>
<td>B14#4</td>
<td>male</td>
<td>25%</td>
</tr>
<tr>
<td>B14#5</td>
<td>male</td>
<td>25%</td>
</tr>
<tr>
<td>B14#6</td>
<td>male</td>
<td>25%</td>
</tr>
</tbody>
</table>

Table IV . 2

<table>
<thead>
<tr>
<th></th>
<th>Resorptions(%)</th>
<th>Embryos(%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantations</td>
<td>63(79%)</td>
<td>17(21%)</td>
<td>80(100%)</td>
</tr>
</tbody>
</table>

Table IV . 3

<table>
<thead>
<tr>
<th></th>
<th>Resorbtions(%)</th>
<th>Embryos(%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantations</td>
<td>6(19%)</td>
<td>25(81%)</td>
<td>31(100%)</td>
</tr>
</tbody>
</table>
In order to confirm these results and to try to correlate the severity of the phenotype with the percentage of chimaerism, analysis of chimaeras resulting from blastocyst injections were performed. With this technique, 79% of the transferred embryos were recovered (table IV.2). The phenotypes range from normal embryos, embryos showing retarded growth with severe posterior truncations to embryos showing severe gastrulation defects. All the embryos shown in Fig. IV.4. were collected at 8.5 dpc. As can be seen in Figs. IV.4.a,b gastrulation seems to be severely affected. No patterning or recognisable structures are observed. The embryos in Figs. IV.d,e present posterior truncations. There seems to be no somites present and the allantois failed to contact the chorion. In Fig. IV.d, the embryo seems to lack axial and paraxial mesoderm. Fig. IV.f shows a normal embryo resulting from the injections for comparison. All these results were confirmed when two other clones, B14 and E117 were injected. However, normal embryos were recovered when wild type CCE cells were used for injection or aggregation chimaeras.
Fig IV.4. Chimaeras obtained at 8.5 dpc both by aggregation technique (a, d) and blastocyst injection (b, c, e). a. b. c. No recognisable structures are observed and gastrulation seems to be severely affected. d.e. The embryos present posterior truncations, there seems to be no somites present. In f, a normal embryo is shown for comparison.
IV. IV. Discussion

There are clearly three major events in the Sox9 expression pattern during the embryonic stages described in this chapter. The first one concerns the expression in the node and cells anterior to it, from the late streak stage and still visible by 8.5 dpc. The second major observation is that Sox9 expression seems to be in neural crest cells prior to and during their migration. The third event is the expression in the somites and sclerotomal derivatives.

Formation of the anteroposterior body axis is tightly coupled to the formation of mesoderm during gastrulation (Tam and Behringer, 1997). Sox9 expression is observed in the node at the late streak stage and in cells anterior to it. This pattern is observed still at 8.5 dpc. It is possible that the labelled cells are the ones that contribute to the axis and unsegmented mesoderm and later, to the somites (Selleck and Stem, 1992; Wilson and Beddington, 1996).

In mammalian embryos, cranial neural crest cells migrate before the neural tube is closed and give rise to the facial mesenchyme. In humans, the crest cells that originate from the forebrain and midbrain contribute to the nasal process, jawbones, soft palate and to bones in the middle ear. The crest cells originating in the hindbrain region generate the
facial cartilage, and the ones from the cervical region contribute to the neck bones and muscles.

At the early headfold stage, Sox9 expression is first detected in the midbrain/hindbrain region. Later the expression is in the lateral edges of the neural folds. This type of expression is coincident with neural crest cells. In later stages, the in situ show widespread expression of Sox9 in the head, which may reflect cephalic neural crest that will form craniofacial mesenchyme.

The first somites appear in the anterior paraxial mesoderm, and new somites arise at regular intervals from the rostral end of the unsegmented mesoderm. Somites are transient structures extremely important in organising the segmental pattern of vertebrate embryos. These structures will give rise to the cells that form: (1) the vertebrae and ribs (2) the dermis of the dorsal skin, (3) the skeletal muscles of the back, and (4) the skeletal muscle of the body wall and limbs.

As the somites mature, the various regions of the somite became committed to form only certain cell types. The ventral medial cells of the somite undergo an epithelial-mesenchymal transition and form the sclerotome. These mesenchymal cells ultimately become chondrocytes responsible for constructing the axial skeleton (vertebrae, ribs,
cartilage and ligaments). The cells of the lateral portion of the somite also migrate giving rise to the muscles of the limbs and body wall.

$Sox9$ expression in the somites is localised to the sclerotome. This is consistent for a gene having a role in chondrogenesis. The expression becomes restricted to the sclerotome in a rostral-caudal sequence during embryonic development.

By 10.5 dpc in situ hybridisation reveals strong $Sox9$ expression in the regions undergoing chondrogenesis. Structures like the vertebrae, scapulae, hips, limbs and cranial cartilage show $Sox9$ transcripts. In addition SOX9 is required for Collagen type II expression (Bell et al., 1996). $Sox9$ expression is downregulated as chondrogenesis is completed.

The phenotype seen in the chimaeric embryos is what one might expect from a gene that contributes to the formation of axial and paraxial mesoderm. If one takes into account that in humans there are several parents of CD patients with a history of miscarriages (section I.V., table I.III.), then the severe phenotype in mice could reflect the importance of $Sox9$ in early development.
Although viable adult chimaeras were produced, some of them with relatively high contribution for the ES cells, at least in the coat, no germ line transmission was observed. The live born chimaeras presented a normal distribution of coat colour and were breeding normally producing viable offspring. However, the existence of some chimaeric females with a high contribution for the XY Sox9+/- ES cells indicates that either the cells did not contribute to the gonads or, that sex reversal occurred due to the absence of normal levels of Sox9 giving impaired Sertoli cell development.

Consistent results were obtained with the injection of three different clones into host blastocysts. Injections and aggregations with the parental cell line produced normal embryos. However the production of chimaeras derived from ES cell clones with a random integration of the targeting construct is necessary. This experiment will confirm that we are observing a phenotype caused by the disruption of one of the Sox9 alleles in the targeted ES cells and not caused by alterations in the wild type ES cells.

In humans, CD shows an increase in the severity of malformations in a cranio-caudal manner: the upper limbs are less severely affected when compared with the lower limbs. This is similar to the more severe posterior defects seen in the chimaeric mouse embryos. Perhaps a problem arises when the cells derived from the somites migrate into the developing hindlimbs where chondrogenesis is more affected. The flat forehead, flat nasal bridge and cleft palate can be interpreted as a neural crest derived deficiency.
Another plausible explanation for the bowing of the limbs involves muscle development: a primary shortness of hamstrings and calf muscles will result in straining the tibia or femur, which being still cartilaginous, will naturally bend (Middleton, 1934; Houston et al., 1983).

In light of the Sox9 expression pattern, Campomelic dysplasia could be described as a combination of neural crest deficiency, linked to deficient sclerotome development, which is more pronounced in the posterior, during early development, followed by deficient cartilage differentiation.

Many more studies need to be done. More chimaeric analyses are required to understand the correlation of chimaerism with phenotype. However, penetrance may be variable as it is observed in CD patients, as there are cases of siblings with the same SOX9 mutation where the severity of the phenotype is different.

Markers should be used to pinpoint the missing structures. For instance, in situ hybridisations with the T gene to label mesoderm and notochord (Wilkinson et al., 1990), Hnf3β and nodal for node labelling (Ang and Rossant, 1994; Conlon et al., 1994) and Shh for notochord (Echelar et al., 1993), would give a better idea of the deficient developing structures. To try to assess defects in the developing neural crest and
somites in particular the sclerotome markers *mox1* and *mox2* could be used (Candia et al., 1992).

The correlation between percentage of chimaerism and phenotype can be used to try to obtain older embryos and then try to see if other deficiencies in later development like sex reversal, can be observed.
V. DISCUSSION
In 1993, the Sox9 gene was cloned (Wright et al., 1993). Its expression pattern during mouse development was studied and gave emphasis to a possible role in skeletal development. Expression in mesenchymal condensation before and during cartilage deposition was consistent with a role for this gene in skeletal formation (Wright et al., 1995). The authors in this work pointed out that Sox9 expression could be causally related to chondrocyte differentiation, rather than being a consequence of it, mainly due to the fact that Sox9 expression precedes the deposition of cartilage in all skeletal elements and that its expression may be the earliest marker of sclerotomal cells.

Two groups at this time (Goodfellow's and Scherer's groups) were trying to find the gene disrupted in the translocations exhibited by some CD patients. SOX9 was already known to be in the vicinity of the translocations breakpoints. The work by Wright and colleagues directed them to search and find SOX9 mutations in several CD patients with no translocations (Foster et al., 1994; Wagner et al., 1994).

The translocations presumably affect SOX9 regulation (Wunderle, 1997), whilst the other types of mutations have been found in different parts of the gene including the HMG box and in the transactivation domain, leading to simple aminoacid substitutions, splicing defects, frame shifts or truncated proteins (Meyer et al., 1997). In all cases described so far the patients showing CD with or without sex reversal are heterozygous
for the mutation. It therefore seems most likely that the phenotypes observed are due to haploid insufficiency.

The results shown in this work are in agreement with those published by Wright and co-workers (1995). In chapter IV, the Sox9 expression pattern during mouse embryonic development was analysed. Sox9 expression pattern may be subdivided into five major events: (1) expression in the node and during notochord formation, (2) Sox9 expression in neural crest cells prior and during its migration, (3) Sox9 expression in the somites, (4) Sox9 expression before and during cartilage deposition and 5) Sox9 expression in gonadal development. Sox9 is first detected in the late streak stage in the node and mesoderm of the streak. Later, at 8.5 dpc Sox9 expression is still observed in the node, the notochord and neural crest cells. As the somites form, Sox9 expression can be found in the sclerotome. By 10.5 dpc, the in situ reveals a strong Sox9 expression in the regions undergoing chondrogenesis. Structures like the vertebrae, scapulae, hips, limbs and cranial cartilage show Sox9 transcripts. This expression is downregulated as chondrogenesis is completed. The Sox9 pattern of expression is consistent to one of a gene that plays a role in Campomelic dysplasia. Although SOX9 has been shown to regulate the major component of cartilage, Collagen type II (Zhao et al., 1997; Ng et al., 1997; Bell et al., 1997), CD can not be reduced to a simple cartilage deficiency syndrome. CD presents a phenotype that is derived from problems that arise in different time points during embryonic development.
When the *SOX9* gene was demonstrated to be the gene responsible for Campomelic dysplasia and the XY sex reversal associated with it (Foster *et al.*, 1994; Wagner *et al.*, 1994), *SOX9* expression in the developing gonads and its consequent role in sex determination became one of the obvious issues to be studied. In chapters II and III the role for *Sox9* in mouse sex determination was addressed. *Sox9* expression in the developing gonads was first detected at 10.5 dpc. At this stage, the gonads are still indifferent and *Sox9* expression is identical both in males and females. Between 10.5 and 11.5 dpc (the time when *Sry* is though to act), *Sox9* expression is upregulated in testes and downregulated in ovaries. In testes, *Sox9* expression is confined to the developing Sertoli cells. This pattern of expression is conserved in birds, where *Sry* is not present. To test the hypothesis of *Sox9* involvement in orchestrating cellular organisation of the testis, expression was analysed in a case of experimental sex reversal in the mouse. Sertoli cell differentiation seems to be closely associated with *Sox9* expression.

Another question was the downstream genes of SOX9. *In vitro* studies reveal that SOX9 has a transactivation domain (Sudbeck *et al.*, 1996). In addition, SOX9 is likely to be a transcription factor due to its HMG box domain and its nuclear localisation. Thus, SOX9 is likely to activate downstream genes. The expression pattern of other genes expressed in the gonad reveal that *Amh* was a likely candidate for a gene activated by SOX9. *In vitro* studies described in chapter III reveal that *Amh* is activated by SOX9 in co-operation with SF1. Although other work suggests that SF1 is a critical *Amh*
regulator in developing gonads (Giuli et al., 1997), the results obtained in chapter III show that SF1 alone can not activate Amh to high levels. The fact that SOX9, together with SF1, activate Amh gives another link in the sex determination cascade. However the gene or genes that activate Sox9 have not yet been determined.

Several genes are known to have a role in sex determination. Sf1 and Wt1 are expressed before sex determination occurs in the gonads. Null mutations in either of these two genes result in mice with gonadal agenesis (Kreidberg et al., 1993; Luo et al., 1994). These results show that these genes act upstream of Sry, perhaps involved in the cellular development of the gonad rather than sex determination itself. However, in addition to this early role, Sf1 is also involved in activating steroidogenic enzymes (Giuli et al., 1997; Ikeda et al., 1994). Sf1, like Sox9, is expressed prior to Sry and has a dimorphic expression pattern, being upregulated in males and downregulated in females. Another important member of this group of intervening genes in gonadal development is Dax1. Dax1 expression is first detected at 11 dpc and is then upregulated in ovaries and downregulated in testes. In vitro studies show that DAX1 is a repressor molecule (Ito et al., 1997; Zanaria et al., 1994; Zazopoulos et al., 1997). Two mechanisms are hypothesised for DAX1 mediated inhibition: 1) direct interactions between DAX1 and SF1 or 2) DAX1 interacts with a co-activator for SF1 or DAX1 recruits a repressor (Ito et al., 1997), the latter seeming the most likely. Upstream binding sites for SF1 and DAX1 itself are found in the regulatory region of Dax1. In addition, mice transgenic for
Dax1 when mated with mice that contain weaker alleles of Sry produce XY females among the offspring (Swain et al., 1998).

One may think that SF1 may activate Sox9 and DAX1 may repress it. But if these molecules are present before the onset of Sry expression with no change in Sox9 transcription levels, this interaction can not be alone or may not exist at all. Several works propose that SRY could be an activator (Dublin and Ostrer, 1994) as well as a repressor (McElreavey et al., 1993a). The likeliest is that SRY functions as a local organiser of chromatin structure. In humans, SRY lacks the transactivation domain described in mice (Sinclair et al., 1990). If SRY functions as an activator then Sox9 is a good candidate for a gene directly regulated by SRY. In addition Sox9 fulfils almost all the requisites for a gene directly activated by SRY: 1) it is expressed in the same lineage as Sry; 2) it is upregulated in males at a time when SRY is present; 3) Sox9 is important for testis differentiation. However, it is necessary to show that there are binding sites for SRY in a critical enhancer region of Sox9. Once Sox9 transcription is upregulated an autoregulatory loop would ensure that the levels of SOX9 are maintained (Fig V.I.).

Another reasonable prediction is that SOX9, SF1, WT1 and DAX1 act to control Amh transcription (Haqq et al., 1994; Viger et al., 1998). However, if this was the only role, the phenotype in the gonads of null mutations for these genes would resemble the null XY mice for Amh or Amh type II receptor: the presence of both male and female
Fig. VI.I. One possible model for Sox9 regulation.
Minor  [SF1]  Sox9  10.5 dpc
Major

Minor  [SRY]  DAX1  SF1  Sox9  XX 11.0 dpc
Major

Minor  [SOX9]  Sox9  XY 11.0 dpc
Major  [SF1]

Maybe low affinity SOX9 binding site  need high levels
derivatives from the Wolffian and Müllerian ducts, but with normal, descending testes presenting normal spermatogenesis (Behringer et al, 1994; Mishina et al, 1996). In this case there is still Leydig cell differentiation and testosterone production. The fact that XY mice for Amh or Amh type II receptor: the presence of both male and female derivatives from the Wolffian and Müllerian ducts, but with normal, descending testes presenting normal spermatogenesis (Behringer et al, 1994; Mishina et al, 1996). In this case there is still Leydig cell differentiation and testosterone production. The fact that XY sex reversal is observed in human syndromes caused by mutations in SOX9 (CD) and WT1 (Denys-Drash, WAGR and Frasier syndromes) present gonadal dysgenesis, reveals that the role of these genes is also linked to the differentiation of the testis cell types. They are probably linked to the role of Sertoli cells in orchestrating testis development. The lack of these cells will cause complete sex reversal. The absence of AMH could be a consequence of the failure to commit the supporting cell lineage to the Sertoli cell fate.

If Sox9 is the only critical gene required for Sertoli cell differentiation, one prediction would be that the misexpression at high levels in XX supporting cell precursors should lead to XX male development. Although this argument is plausible, it is also possible that Sox9 expression in females does not interfere with ovary development, as in humans, duplications of regions containing the SOX9 gene on chromosome 17q do not
cause sex differentiation problems although skeletal defects are seen (Lenzini et al., 1988). Sox9 missexpression in females is under study (Swain, personal communication).

The scenario is far from complete. It is obvious that not all of the participants in the sex determination cascade are known. A recent report describes XY sex reversal in mice with a targeted disruption in the m33 gene, a member of the Polycomb family of genes, known in Drosophila as homeotic gene repressors (Katoh-Fukui et al, 1998 ). Also the transcription factor GATA-4 has been shown to regulate the Amh gene (Viger et al., 1998) Cases of XX males with no SRY sequences in their genome or XY females with no alterations in SRY also point to the existence of other genes in the sex determination cascade. The human gene DMT1 contains a DM binding motif that is found in both the Drosophila melanogaster male sexual regulatory gene doublesex and Caenorhabditis elegans mab-3 which was recently cloned (Raymond et al, 1998). This gene is expressed in the testis and maps to the distal short arm of chromosome 9, a location implicated in human XY sex reversal. The search for genes involved in sex determination is far from the end.

The targeted disruption of the mouse Sox9 gene did not help in the understanding of the role of Sox9 in sex determination. Instead, this experiment indicated that Sox9 has a critical role from very early on development. The phenotype observed in the chimaeras involved deficiencies in the cells that form the axial mesoderm and somites in particular.
the posterior ones. Obviously many more experiments are needed to understand the phenotype observed in the chimaeras.

Sox gene action seems to be context dependent, relying on both the direct interactions with DNA binding sites as well as protein interactions with other transcription factors (Pevny and Lovell-Badge, 1997). In the case of SOX9, its widespread expression pattern in mice may be explained if one thinks that the SOX protein may interact with different factors in different contexts. Sox9, as are other Sox genes, may be an early marker of cell fate. The fact that a given cell is expressing Sox9 does not mean that this cell has a definitive fate, but means that is predisposed to a certain fate. The neural crest cells labelled in the Sox9 in situ are going to give rise to a variety of neural crest derivatives. However Sox9 does not label all the neural crest cells and derivatives. Sox9 is predisposing a subpopulation of neural crest cells that will give rise to different tissues according to environmental signals that this cells may encounter during their migration pathways in the developing embryo. The same may be happening in the gonads. The cells expressing Sox9 in the indifferent gonads are predisposed to became Sertoli cells, but they will only fulfil their fate if a specific factor that interacts with SOX9 is expressed in these cells. The data presented in this thesis together with the findings about other members of the Sox gene family suggest that Sox genes are involved in governing cell fate decisions in several developmental processes.
At this time several approaches can be made to assess the role of the \textit{Sox9} gene in embryonic development. The first general approach would be to generate a tissue specific knock out. The cre-lox system could be used in the targeting of \textit{Sox9}, and the resulting mice could be crossed with a variety of mice expressing cre-recombinase in different tissues. A mouse expressing cre under the promoter of genes such as the \textit{T}-gene, \textit{HNF3\beta} or \textit{Shh} which are expressed in tissues that give rise to axial structures could be crossed with a loxP \textit{Sox9} mouse to try to assess the role of \textit{Sox9} in the node and notochord. A mouse expressing cre under the promoter of genes such as \textit{Sfl}, \textit{Sry} or \textit{Dax1} could address its role in sex determination. These experiments require that the regulatory regions within the promoters of these genes that confer tissue specific expression are mapped. These are already known for \textit{Sry} and \textit{Dax1}.

The knock out described in this thesis may be used to study further the role of \textit{Sox9} in mouse embryonic development. Maybe the rescue of the phenotype by re-introduction of \textit{Sox9} with several lengths of upstream regulatory region in the ES cells will allow mapping of tissue specific mouse regulatory regions and analysis of function in specific tissues. Also the production of mice with several degrees of chimaerism will allow the study of other later effects in development, caused by the \textit{Sox9} disruption, such as sex reversal. The induction of teratocarcinoma formation in mice, will allow to assess if \textit{Sox9} +/- ES cells are as capable to produce the several different tissues as the wild type ES cells do (Robertson, 1987).
To examine further the role of Sox9 in skeletal development it would be interesting to find if there is a regulatory relationship between Sox9 and members of the FGF family of genes involved in limb formation (Cohn and Tickle, 1996), with Hox genes involved in forelimb/hindlimb development (McGinnis and Krumlauf, 1992), with members of the Bmp family (Francis et al., 1994), with some T-box genes like Tbx-5 (restricted expression to the forelimb buds) and Tbx-4 (restricted expression to the hindlimb buds)(Chapman et al., 1996) and a bicoid-related homeoprotein, Ptx1 expressed in the hindlimb (Lanctot et al., 1997). Perhaps the infection in chick embryos of the posterior lateral mesoderm or the developing hindlimb with a retrovirus containing a dominant negative form of SOX9 (for example with the transcriptional activation domain swapped by a strong repressor such as Drosophila engrailed (Badiani et al., 1994)), would result in disruption of hindlimb identity or development. Misexpression of Sox9 in developing mouse or chick embryos would possibly give some clues about tissues induced as a result of the ectopic expression of Sox9, like cartilage formation for instance.

To further analyse Sox9 function in the developing gonads, mapping of the Sox9 promoter region responsible for gonadal expression is essential. Transgene analysis of the Sox9 flanking regions linked to a reporter gene should allow the mapping of the regulatory elements responsible for gonadal expression. The aim being to discover
conserved binding sites which will indicate candidate molecules for Sox9 regulation. A similar study was already done but using a YAC containing the human SOX9 gene with up to 600 kb of regulatory regions. Although the expression pattern was comparable to the endogenous Sox9 expression in skeletal tissues, no expression was observed in the gonads suggesting that either the transgene was missing the specific regulatory elements or species specific factors are involved in this pathway (Wunderle, 1997). The misexpression of Sox9 in chick developing gonads may give insights into the Sox9 role in birds where Sry is absent.

The data collected during this study has revealed that Sox9 plays roles in sex determination as well as in many different stages in embryonic development. This gene will keep many people busy for a long time!
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APPENDIX 1 - Values for the Luciferase assays shown in Chapter III
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Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds

Sara Morais da Silva¹, Adam Hacker¹,³, Vince Harley²,⁴, Peter Goodfellow²,⁵, Amanda Swain¹ & Robin Lovell-Badge¹

Heterozygous mutations in SOX9 lead to a human dwarfism syndrome, Campomelic dysplasia. Consistent with a role in sex determination, we find that Sox9 expression closely follows differentiation of Sertoli cells in the mouse testis, in experimental sex reversal when fetal ovaries are grafted to adult kidneys and in the chick where there is no evidence for a Sry gene. Our results imply that Sox9 plays an essential role in sex determination, possibly immediately downstream of Sry in mammals, and that it functions as a critical Sertoli cell differentiation factor, perhaps in all vertebrates.

The Y-linked gene Sry is central to the process of sex determination in mammals¹–⁴. The SRY protein possesses a HMG box type of DNA binding domain, and has properties consistent with its acting as a transcriptional regulator through local effects on chromatin structure⁵–⁸. The gene is expressed for a brief period in developing male embryos, between 10.5 and 12 days post coitum (dpc) in the mouse, specifically in somatic cells of the indifferent gonad (or genital ridge)⁹–¹¹. Evidence from expression studies and from chimaeras indicates that SRY functions within cells of the supporting cell precursors to trigger their differentiation into Sertoli cells characteristic of testes¹². In the absence of SRY, these cells give rise to granulosa or follicle cells in the ovary. Once Sertoli cells begin to differentiate they are thought to trigger the other cell lineages in the gonad to follow the male pathway¹³–¹⁵. Two factors produced by the developing testis effectively export the male determining signal to the rest of the embryo: anti-Mullerian hormone (AMH, otherwise known as Mullerian inhibiting substance or MIS) from Sertoli cells and testosterone from Leydig cells¹⁶,¹⁷.

Because early Sertoli cells appear to have such a critical role in orchestrating the differentiation of the other cell lineages in the gonad, the problem of sex determination can essentially be reduced to understanding Sertoli cell differentiation. While Sry is important in initiating this event in mammals, it is clear that other genes must also play a role. For example, the maintenance of Sertoli cell characteristics cannot be due to SRY itself, as it is expressed too briefly, but to genes turned on as a result of SRY action. Such genes need not necessarily be immediate targets of SRY as the latter can activate genes in the testicular pathway, repress genes in an ovarian pathway, or both¹⁸–²².

In addition, testes or testicular like structures with apparently normal Sertoli cells can develop in the absence of Sry in a number of naturally occurring or experimental situations. For example there are cases of XX males who lack Sry in humans, dogs, voles, and other mammals²³–²⁵. These can be explained by either a constitutive mutation in a downstream testis determinant or a loss of function mutation in an ovarian determinant. There are also cases where gonadal environment is important. In Freemartin cattle, AMH from a male twin results in masculinisation of the female twin. This masculinisation includes apparent loss of oocytes and transdifferentiation of ovaries into testes²⁶. A similar process can also be demonstrated experimentally in vitro, through the addition of AMH to fetal ovary cultures²⁷, or in vivo by grafting fetal ovaries to the kidney capsule of adult male or female mice²².

Furthermore, there is no evidence that a Sry homologue is involved or even exists in non-mammalian vertebrates. Sry appears to have evolved as a dominant male determinant coincident with the evolution of mammals (at least the metatheria and eutheria)²³,²⁴. In contrast, birds employ a ZZ/ZW system where the female is the heterogametic sex²⁵,²⁶. Moreover, in many reptiles and fishes, males and females are genetically identical, sex being determined by environmental factors such as temperature²⁷. It seems instead that the gene employed as the control point in sex determination can vary between species. Nevertheless, since the basic cellular organisation of the gonads is similar in different classes of vertebrates, it is likely that there will be genes in common

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which are responsible for specifying the particular cell types. Identification of these genes will be particularly important for a general understanding of sex determination.

One way to find additional sex determining genes is to examine cases of sex reversal and to use positional cloning or candidate gene approaches. The SRY related gene SOX9 was identified in this way.28-30 Mutations in SOX9 lead to a severe dwarfism syndrome in humans termed Campomelic dysplasia (CD). This is characterised by general defects in cartilage and bone development, plus a variety of non-skeletal abnormalities of the olfactory system, central nervous system, heart and kidneys. In addition, approximately 75% of XY patients show sex reversal. Examination of the gonads from these sex reversed patients reveals a range of phenotypes from gonads with some testicular differentiation, to gonads positioned as ovaries with some testicular features (the latter phenotype being the most common). In all cases described so far, the patients showing CD with or without sex reversal are heterozygous for the mutations.31 Analysis of the patients showed a wide variety in the type of mutation at the SOX9 locus. It therefore seems most likely that the phenotypes observed are due to haploid insufficiency rather than to dominant negative effects.

The mutation analysis gives no indication of how SOX9 fits into the tests determining pathway. It could be upstream of SRY, in a parallel pathway, or downstream. Unlike SRY, SOX9 is highly conserved in vertebrate evolution32-35 (our own unpublished observations). We have therefore looked at Sox9 expression in detail during normal gonadal development in both mouse and chick. We have also examined one type of experimental sex reversal in the mouse. In all situations we find high levels of Sox9 expression correlated with testicular development, in a manner suggesting it is required for Sertoli cell differentiation.

Sox9 expression during gonadal development

Each gonad begins to develop from about 9.5-10.0 dpc in mice, as a ridge of distinct cells on the medio-lateral surface of the mesonephros. Up until about 11.5 dpc, the genital ridges of XY and XX embryos are morphologically indistinguishable and are comprised of primordial germ cells and one or more somatic cell types which give rise to supporting cells (Sertoli or follicle cells) and to steroidogenic cells (Leydig or theca). Other lineages of the gonad, notably connective tissue cells, such as myoid and endothelial cells, migrate into the genital ridge from the mesonephros after 11.5 days (Capel et al., unpublished data), coincident with the first morphological differences between testis and ovary. Whole mount in situ hybridization shows Sox9 expression in the genital ridge of both XY and XX embryos from the earliest stage observed, about 10.5 dpc (Fig. 1a-c). This corresponds to a time when Sry expression is only just being activated in XY embryos. Although many parts of the embryo express Sox9, notably developing cartilage and neural tube, expression is not seen in the mesonephros at these stages (Fig. 1b-d). By 11.5 dpc, Sox9 expression is very abundant in genital ridges from XY embryos but is absent from those of XX embryos (Fig. 1d-f). Sections of whole mounts of XY genital ridges indicate that the majority, if not all of the
somatic cells within the centre are positive, whereas cells of the coelomic epithelium are negative (Fig. 1k).

Sox9 continues to be expressed at a high level in developing testes, with the transcripts being localized to the cords (Fig. 1g–i). Sections of whole mounts at 13.5 dpc reveal the signal to be within Sertoli cells and absent from all other lineages (Fig. 1m). On the other hand, Sox9 transcripts continue to be completely absent from developing ovaries (Fig. 1g,h). The only exception is a small patch of positive cells located anteriorly, just within the mesonephros (Fig. 1h). This is also seen in males and is reminiscent of a patch of Dax1 expression seen in both sexes after 12.5dpc which may represent a population of relatively undifferentiated cells.

**Sox9 levels change with onset of Sry expression**

To confirm the sexually dimorphic nature of Sox9 expression in developing gonads and to provide some estimate of how transcript levels change over time relative to Sry expression, we performed quantitative RNAse protein assays with urogenital ridge RNA obtained from accurately staged embryos (Fig. 2). These assays confirm that Sox9 is expressed in both sexes prior to Sry, but then, coincident with the onset of Sry transcription, Sox9 is downregulated in XX genital ridges and upregulated in XY genital ridges. In these assays, unlike the whole mounts (Fig. 1), we still detected Sox9 transcripts in XX samples, however, these will have included the anterior patch of positive cells and perhaps some cells of the dorsal mesentery and aorta which show a low level of expression (see Fig. 1e–h). Apart from Sry itself, the changes we observed in Sox9 expression represent the earliest difference yet seen in gene expression between males and females. Amh, which is a very early marker of Sertoli cells, is not transcribed until after 11.5 dpc, when the difference in Sox9 expression has already been established. Differences in SF1 levels between testis and ovary are also not apparent until after 11.5 dpc (our own unpublished observations).

**SOX9 protein is nuclear in Sertoli cells**

While RNA localization gives a good indication where a gene may be functional, this is revealed in a more rigorous manner by looking at protein distribution. We therefore used antibodies against SOX9 on sections of developing mouse gonads. At about 11.5 dpc, SOX9 is detected within cells of the genital ridge, with consistently higher levels in XY versus XX embryos (Fig. 3a,b). Our finding that some protein was still seen in XX genital ridges at a time when transcripts are absent, suggests that the protein is quite stable. However, we have not detected SOX9 protein at any subsequent stage in developing or adult ovaries (Fig. 3d and data not shown). In developing testes, SOX9 is found at high levels specifically within Sertoli cells lining the testis cords (Fig. 3e). In the adult, SOX9 protein was detected at a high level in cells surrounding testis tubules. From their position and appearance these may be other Sertoli cells (see Fig. 3g–i), and this can be confirmed by looking at testes of males with two X chromosomes which completely lack germ cells (Fig. 3f). This latter result also shows that SOX9 expression in adult testis is independent of the presence of germ cells.

We generally observed SOX9 protein localized to the nucleus. However, in genital ridge sections prior to 11.5 dpc the antibody detected protein within the cytoplasm of many cells (although not all) (Fig. 3h). This cytoplasmic location, which tends to be perinuclear, was independent of chromosomal sex. After 11.5 dpc the protein was nuclear in all positive cells within the developing testis (Fig. 3i).

**Testis specific expression in the chick**

The homologues of SOX9 from human, mouse, chick, alligator and Fugu, show a high level of protein conservation (P.N.G. unpublished observations). This sug-
suggests that SOX9 has conserved functions, and consistent with this, Sox9 has been shown to be expressed in developing cartilage of chick as well as mice (Sharpe et al., personal communication and our own unpublished observations). However, genes controlling sex determination are notorious for being rapidly evolving or completely absent between species and there is no a priori reason to suppose that Sox9 has a conserved role in sex determination. We therefore examined its expression in developing gonads of chick embryos.

We found that cSOx9 transcripts were present in the genital ridges of both ZZ and ZZ embryos at stage 25 (Fig. 4a,h), a stage at which the genital ridges look morphologically identical. The levels are much lower than those seen in developing testes at later stages. cSOx9 is also transiently expressed in mesonephric tubules, which are functional in the chick, at these early stages. This is reminiscent of expression seen in tubules of the definitive kidneys (mesonephros) of mouse embryos as they begin to develop (data not shown) and it is worth noting that CD patients often show renal defects. In ZZ embryos at stage 28, both gonads look identical and are beginning to differentiate as testes. They both show very strong expression of cSOx9 (Fig. 4c). In ZW embryos at this stage, the left gonad is beginning to form an ovary and the right gonad is arrested in development. Both gonads stain only weakly for cSOx9 expression (Fig. 4c). The same pattern of expression is seen at stage 32 when sexual dimorphism is even more apparent (Fig. 4d). Sections through an ovary at this stage (Fig. 4e) show no expression within the gonad, although a few positive cells are seen at the surface. Testis sections show strong expression within cells that have begun to align into epithelial cords (Fig. 4f).

**Sox9 and experimental XX sex reversal**

The expression data above reveals a strong correlation between Sertoli cell differentiation and high levels of Sox9 expression. To test this further, we examined one case of experimental sex reversal in the mouse where fetal ovaries are grafted to the kidney capsule of adults. Previous studies by others, notably Taketo et al., have shown that the best "transdifferentiation" is obtained when 13.5-dpc fetal ovaries which have been dissected away from mesonephros are used as grafts. In a first series of experiments, we grafted a total of 30 ovaries to 13 animals and, as positive controls, 16 testes of the same age were grafted to six animals. In a second series, 21 ovaries and eight testes were grafted to seven and three hosts respectively. A proportion of grafts of both sexes degenerated, possibly due to immune rejection (the grafts and hosts were from outbred mice), however, the control testis grafts were viable and showed good development over time. The control testis grafts maintained an obvious cord structure and Sox9 expression was clearly visible by whole-mount in situ hybridization at all stages looked at (Fig. 5c,f). The ovary grafts showed no organization into cords and were negative for Sox9 expression at early time points, for example at six days after grafting (Fig. 5b). By 18 days after grafting, we detected Sox9 expression in isolated cells in some grafts (Fig. 5d). However, by 26–30 days, there is considerable organization of cells into cord like structures, and many of the cells showed weak staining with anti-Sox9 antibody at early stages and strong staining by day 30. The ovary grafts grafted to the kidney capsule of adults maintain Sox9 expression throughout development and in the adult testis. This suggests a role for Sox9 in Sertoli cell differentiation and in the maintenance of Sertoli cell function.

**Discussion**

To begin to address the role of Sox9 in sex determination we examined the expression of the gene during the normal process of gonadal development in both mouse and chick, and in one situation of experimental sex reversal in the mouse. In the mouse gonad, Sox9 RNA and SOX9 protein are clearly found within Sertoli cells and not within cells of any other lineage, both during development and in the adult testis. This suggests a role for Sox9 in Sertoli cell differentiation and in the maintenance of Sertoli cell function.

The sex reversal phenotype of human CD patients is entirely consistent with a failure of Sertoli cell differentiation. Most of these patients exhibit an essentially normal female phenotype.

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**Fig. 5** Ovary and testis grafts from 13.5 dpc embryos to adult kidney capsule. a–h, Whole mount in situ hybridization to detect Sox9 transcripts. a, Ovary graft (arrowed) after 6 days still within the kidney capsule and b, dissected free. c, Testis grafts (arrowed) after 6 days within the kidney capsule. d,f,h, ovary grafts after 18, 26 and 30 days respectively: e,g, testis grafts after 18 and 28 days. i,j, Antibody localization of SOX9 in sections of a testis graft (i) and an ovary graft (j) after 26 days.
Mullerian duct development into uterus and oviducts, consistent with failure to produce AMH, no signs of masculinisation of internal or external genitalia, suggesting no testosterone synthesis by Leydig cells, and, in some cases the ovaries contain primary follicles with oocytes. This suggests that some cells have followed a normal follicle cell pathway resulting in a very early failure of signalling associated with Sertoli cell differentiation.

SOX9 protein is likely to be a transcription factor and is therefore expected to be nuclear. At most stages we observed this to be the case with the exception of the early indifferent gonad of both XX and XY embryos, where only some cells show nuclear localization and the majority show SOX9 protein within the cytoplasm. There could be an active mechanism to regulate its nuclear localization. We have found other SOX proteins to be nuclear (unpublished observations). However, SOX2, which is present within nuclei of uncommitted cells of the central and peripheral nervous systems, has a cytoplasmic form derived from an alternative transcript. In some differentiated neuronal cell types (S.ock Throwin, unpublished data). Thus, the regulation of SOX9 target genes is likely to involve other transcription factors which will themselves be critical for sex determination.

The control grafts with fetal testes also showed no Sox9 positive cells in addition to those which were clearly arranged in cords and had Sertoli cell morphology. We therefore conclude that the Sox9 positive cells within the ovary grafts are most likely a result of transdifferentiation of follicle cells to Sertoli-like cells. This strengthens the correlation and argues that Sertoli cell differentiation is closely associated with Sox9 expression.

What is the relationship between Sox9 and Sry? The protein products of the two genes contain very similar HMG box DNA binding domains. Such domains bind DNA in a sequence specific manner with moderately high affinity and cause an acute bend in the DNA. Studies of mutant SRY proteins, found in cases of XY female sex reversal, and on related proteins such as LEF1, have led to the suggestion that SRY acts at its target sites as a local organizer of chromatin structure (or as an architectural factor) to allow or prevent the interaction of other transcription factors bound nearby. SRY is a rapidly evolving gene where the HMG box is the only conserved region and its function therefore may depend solely on its architectural properties. If this is the case, the regulation of SRY target genes is likely to involve other transcription factors which will themselves be critical for sex determination. Sox9 is highly conserved throughout the open reading frame and contains a putative activation domain. Some cases of CD with or without sex reversal are due to mutations within this domain, suggesting it has an important function, in addition to that provided by the HMG box. It is notable that high levels of Sox9 expression just precede the onset of Amh transcription, perhaps Amh is directly regulated by Sox9 rather than SRY as has been proposed. Recently, the chick Amh gene has been cloned and its expression studied. While low levels were seen in ovaries, much higher levels were found within Sertoli cells of the fetal testes, but again only after the stage when we observed upregulation of Sox9.

In mammals it is conceivable that SRY is directly involved in the regulation of Sox9 transcription. Initiation of Sox9 expression presumable depends on other factors as transcripts are already evident prior to those of Sry in both sexes. However, SRY could be required at a critical period for its maintenance (see below). Several predictions can be made concerning SRY target genes: they must be expressed in the same cell lineage as Sry, there should be a change in expression of the target gene which correlates with the presence of SRY and a difference between males and females. There should be a preferred binding site (consensus site) for SRY within the minimal promoter region able to confer sex specific expression. Sox9 clearly satisfies all these criteria, apart from the latter for which no data is yet available.

During normal XY genital ridge development a high level of Sox9 precedes morphological differentiation of Sertoli cells and induction of Sertoli cell specific gene expression such as Amh and Desert hedgehog (Dhh). This implies that Sox9 is instructive for Sertoli cell differentiation. But this process clearly depends on a relatively fine balance between several genes. Thus in humans two copies of SOX9 are required for testis differentiation, one is generally insufficient, one copy of the gene responsible for the dosage sensitive sex reversal (DSS) phenotype (possibly DAX1) is compatible with testis differentiation whereas two leads to XY sex reversal, and there are several instances in mice...
where levels of Sry can be shown to be critical (Golding and Gill, unpublished transgenic experiments). Based on gene expression, it is possible to propose a molecular scheme where a high level of Sox9 is central to the differentiation of a subset of the somatic cells of the gonad into Sertoli cells, but that Sox9 is repressed by Dax-1 during normal ovary development. The role of Sry in the male gonad would then be to prevent the repression brought about by Dax-1. This is clearly an oversimplified scheme and other factors such as SF1 and WT1 are likely to be involved.

The sex-specific expression of cSox9 expression in chick gonads suggests that it may also have general importance as an ancestral sex determining gene. However, as lower vertebrates have no obvious Sry homologue other factors would have to ensure Sox9 expression in males. Understanding how Sox9 is regulated during early gonadal development in the chick and in mice may lead to a more general understanding of vertebrate sex determining mechanisms.

Methods

Animals and tissues. Mouse embryos and tissues were obtained from Parkes outbred mice (maintained at NIMR) unless otherwise stated. The tests from the XXY (XYXY/NIMR) used in Fig. 3i, h, and k was a gift from T. Rodriguez. This animal was obtained by mating an XXY male (XXXY/NIMR) female (XYY/NIMR) with an XXY male (XXXY/NIMR). These were on an MF1 outbred background from stocks maintained by P. Burgess at NIMR. For embryo staging, midday on the day of appearance of the vaginal plug was taken as 0.5 dpc. More accurate staging of embryos was by counting tail somites posterior to the hind limb bud or by hind limb morphology as described. Chick embryos were incubated at 38 °C and staged according to Hamburger et al. Chick embryos were sexed by PCR using W-specific primers as described.

RNase protection assays. Total RNA was prepared from embryonic and adult gonads as described. RNase protection assays were carried out using 10 µg of total RNA from adult tissues or RNA prepared from four pairs of genital ridges, as described. A genomic fragment of Sox9, Int 1-Sox9, suitable for making probes to distinguish RNA from DNA, was obtained by PCR between nucleotides 372 of the first exon and 44 bp of the first intron. This was cloned into pcR3HT (Invitrogen) by TA cloning. The primer sequences were 5'-CCACATTGCGAG-ACTGCCG-3' and 5'-GAAATCTTCGTCGATACCCGGATTCCG-3' (ref. 30) (Edwina Wright, personal communication). The Sox9 probe used as a loading control was probe A, as described. Transcription reactions were performed in vitro using T7 RNA polymerase (Promega) in both cases to generate antisense RNA probes labelled with [32P]-UTP. Gels were exposed on PhosphorImage screens and quantitative analysis used ImageQuant software as described. The results in Fig. 2 are mostly from one complete time course, but a number of partial experiments gave comparable results.

In situ hybridization. To detect mouse Sox9 expression, the antisense RNA probe used was generated from a cDNA clone, pbsox9. This was obtained by RT-PCR between nucleotides 758 to 1256 (ref. 30) using cDNA made from 13.3-dpc embryonic limb RNA. The product was cloned into EcoRI and XhoI sites within the polylinker of pBluescriptSK (Stratagene) and sequenced to verify its identity as Sox9. For the whole mount in situ hybridization performed on chick gonads the cSox9 cDNA clone 20-1 inserted into pBluescriptSK (a gift of P. Sharpe) was linearized with XhoI. Transcription reactions were performed in vitro using T7 RNA polymerase or T3 RNA polymerase to generate sense (control) and antisense RNA probes labelled with digoxigenin-UTP. The complete procedure is as described. No signal was seen with the sense probe (data not shown).

Immunohistochemistry. Sox9 peptide 486 (HSPQHWE-QPA'TQLT) represents amino acids 486 to 500 close to the C-terminus of human Sox9. The peptide was coupled to KLH and injected into rabbits (Zeneca according to their standard protocol and serum collected at 56 d. From 25 ml of serum, non-IgG protein was removed by caprylic acid precipitation and the soluble IgGs was then collected by ammonium sulfate precipitation, resuspended in TBS and affinity purified over a CNBr-sephrose column coupled to Sox9-486 peptide. Bound IgGs was eluted at low pH, concentrated by ammonium sulfate precipitation and resuspended at 2mg/ml in TBS/0.05% sodium azide. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), then equilibrated in 30% sucrose overnight, before freezing and cryosectioning. Tissue sections were washed for 10 minutes in PBS, permeabilized in 1% Triton X-100 in PBS for 10 min, RT, rinsed in PBS, then blocked using 5% goat serum in incubation buffer (TBS, 3% gelatin-free BSA, 0.5% Triton X-100). The sections were then incubated overnight in V50 antibody at 1:500 dilution in incubation buffer. After washing, the sections were treated with FITC-goat anti-rabbit IgG (Vectorshield), 1:100 in incubation buffer, for 30 min at RT, rinsed and mounted using Citifluor (UK chem. lab.)

Transplantation of fetal gonads. Fetal ovaries and testes at 13.5 dpc, dissected away from mesonephros, were transplanted beneath the kidney capsule of 6-8-week-old male mice as described. Gonads of the same sex (1-3) were transplanted to separate locations of one kidney only in each host. The gonadal grafts were dissected out between the 1st and 30th day after transplantation, and processed for whole mount in situ hybridization and immunohistochemistry as described above.

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