SRY and DAX1 in Mammalian Sex Determination

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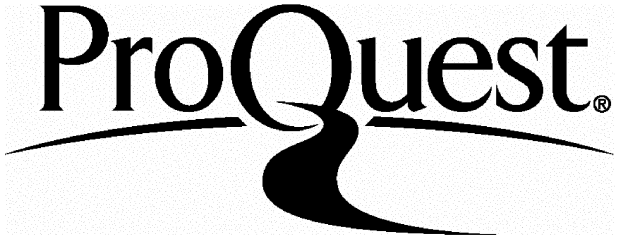

For Paul, Eileen and Tom, thanks for your love and support.

Abstract

Mammalian sex determination in its simplest form can be considered as a cell fate decision, dependent on *SRY/Sry*, the testis determining gene. SRY, a member of the HMG box family of transcription factors, is expressed in the indifferent XY gonad at around 10.5 – 12.0 dpc in mice and induces differentiation of the Sertoli cells which then influence other cell types in the gonad and the rest of the embryo to develop as male. In the absence of *SRY/Sry* expression, follicle cells develop and the fate of the bipotential gonad is switched to that of an ovary, and the embryo develops as female.

Sry is rapidly evolving gene, and the human and mouse genes share no homology outside of the HMG box DNA binding domain. Despite this sequence divergence, we describe that human SRY functions identical to its murine homologue, and causes male sex determination in XX transgenic mice. We have examined the expression and localisation of SOX9, a likely target of SRY, and show that in XX gonads transgenic for human *SRY,* SOX9 expression is identical to that observed in XY control gonads. The lack of sequence similarity outside of the HMG box domains questions how SRY functions in regulating target genes. Our results demonstrate that the CAG repeat domain present in the mouse SRY protein is not required for SRY function during sex determination and we propose that SRY may act simply as an architectural transcription factor.

In comparison to testis differentiation, little is known about female development and ovary differentiation, however it appears that an X-linked gene, *DAX1/Dax1,* may act as an antagonist of the male pathway. To address whether *Dax1* can function as an anti-testis factor, we chose the Estrogen Receptor ligand inducible system to misexpress *Dax1* before the onset of *Sry* expression. *In vitro* assays were established to test the function of this system and to ensure that DAX1 function was also retained. The initial Estrogen Receptor DAX1 fusion protein (DAX1:ER) was retained in the cytoplasm of transfected cells in the absence of ligand, and responded to ligand administration by localising to the nucleus. A reporter assay demonstrated that full length DAX1

repressed the transcriptional activation of the *Amh* promoter. However, DAX1:ER was unable to repress this activation and was therefore non functional. An additional fusion protein (ERglyDAX1) retained the repressive function of DAX1, however, it did not remain in the cytoplasm of transfected cells in the absence of ligand. Reasons why these two fusion proteins failed to function in the desired manner are discussed.

Acknowledgements

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Abbreviations

AMH – Anti Müllerian Hormone

Amp^r –Ampicillin resistance

AR - Androgen Receptor

bp – base pairs

CMV – Cytomegalo virus

DAPI – 4',6-diamidino-2-phenylindole

DAX1 – Dosage Sensitive Sex Reversal, Adrenal hypoplasia congenital, on X chromosome, factor 1

DEPC - diethylpyrocarbonate

DMEM – Dulbecco's Modified Eagle's Medium

DMSO – dimethyl sulphoxide

DNA – deoxyribonucleic acid

dNTP – deoxynucleoside triphosphate

dpc – days post coitum

ER – Estrogen Receptor

EtOH - Ethanol

EDTA – ethylenediaminetetraacetic acid

ER – Estrogen Receptor

FCS – Fetal calf serum

Fig. - Figure

g - gram

HSP – heat shock protein

hr - hour

kb - kilobase

kDa – kilo Dalton

IP – intra peritoneal

LBD – ligand binding domain

M – Molar

MEMFA – Mops, EGTA, $MgCl_2$, Formaldehyde

MeOH - Methanol

mins - minutes

mM – millimolar

NLS – nuclear localisation signal

4-OHT – 4 hydroxytamoxifen

O/N - overnight

ORF – open reading frame

PAR – pseudoautosomal boundary

PBS – Phosphate buffered saline

PGC – Primordial germ cells

RNA – Ribonucleic acid

rpm – revolutions per minute

RT - Room temperature

SF1 – Steroidogenic factor 1

SOX9 – Sry related HMG box protein 9

SRY – Sex determining region

TE – Tris EDTA

ts – tail somites

Tm - Tamoxifen

WT1 – Wilms Tumour 1

WT – Wild type

X-gal – 5-bromo-4-chloro-3-indolyl- β - D- galactosidase

Chapter I Introduction

I.I Sex Determination and Gonadal Development

Mammalian sex development is concerned primarily with three sequential processes: the inheritance of chromosomal sex at fertilisation, sex determination, and then differentiation of the gonads, the ductal systems and the external genitalia. Sex determination at its simplest level, is the decision of the indifferent gonad to respond to genetic cues to become either the testis in the male or an ovary in the female. Although chromosomal inheritance sets a precedence for the genetic sex of an individual, it is not until later in embryonic development that sex determination occurs. As we uncover the molecular and cellular events that occur during sex development, it has become clear that the processes of sex determination and differentiation are governed by a complex network of genes and no longer the once perceived simple linear pathway. Many factors are involved in the development of the gonads, and several of these have important roles at different times. Furthermore, both positive and negative feedback loops have been uncovered as well as a variety of antagonistic gene interactions.

Much work in the field of sex determination has focused on the role of *Sry*, the only gene on the Y-chromosome necessary to initiate male development (Koopman, Gubbay et al. 1991). Other genes, such as *Sox9, SF1* and *Amh*, are also involved in the differentiation of the male pathway. Little is still known, however, about the genes that play a role in female development, with the possible exception of *Dax1* which can be antagonistic to the action of *Sry*. The aim of the work described in this thesis is to focus on molecular players that may function in the early events of sex determination; first to gain some insight into the way *Sry* might work by comparing the ability of the mouse and human proteins to trigger male development in transgenic mice and second to explore events that may lead to female development.

I.II Biology of the gonad

The urogenital system arises from intermediate mesoderm and comprises the pronephros (which includes the adrenal primordium), the mesonephros, from which the gonad will arise, and the metanephros, from which the kidney will be derived. The gonadal primordium, probably arises via an inductive interaction between the underlying mesonephros and the overlying coelomic epithelium at about 10 days post coitum (dpc), but the initial trigger is not known. Following gonadal primordium development, it is the presence or absence of the Y chromosome gene, *Sry,* which determines the induction of the male or female developmental pathways (Fig.1). The term "uro-genital ridge" is usually used to describe the early mesonephros and the developing gonad, while "genital ridge" is just the gonadal portion. At the earliest stages of gonadal development, up to 11.5 dpc, no morphological distinctions are apparent between XX and XY genital ridges (Fig.2).

Fig. 1 A simple model describing sex determination in mouse.

The bipotential genital ridge begins to form at around 10 dpc. Expression of *Sry* at 11.5 dpc promotes development and differentiation of the embryonic testes, thus initiating the male pathway. Downstream events such as the secretion of Testosterone and Anti-Müllerian Hormone promote further development of the internal and external genitalia and secondary sexual characteristics. In the absence of *Sry* expression, female development occurs.

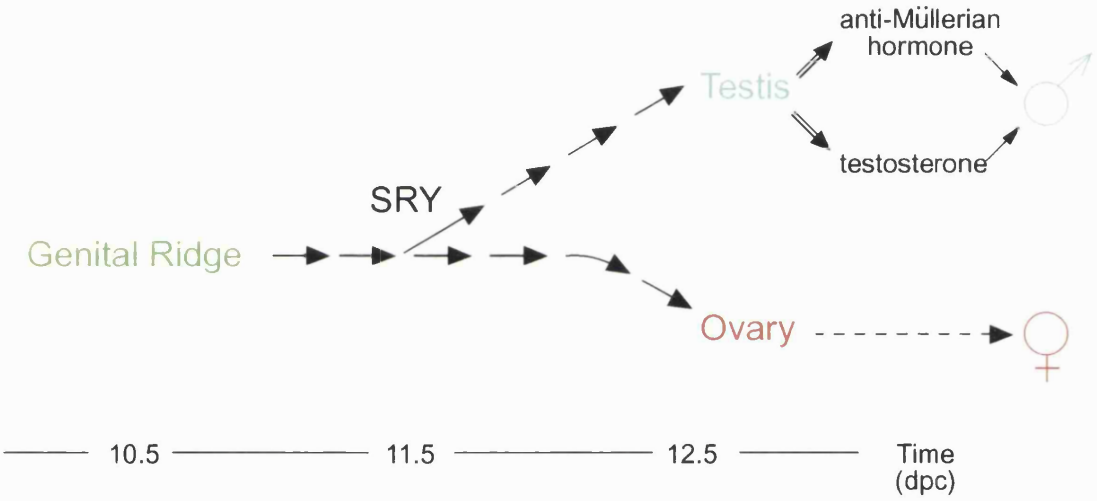
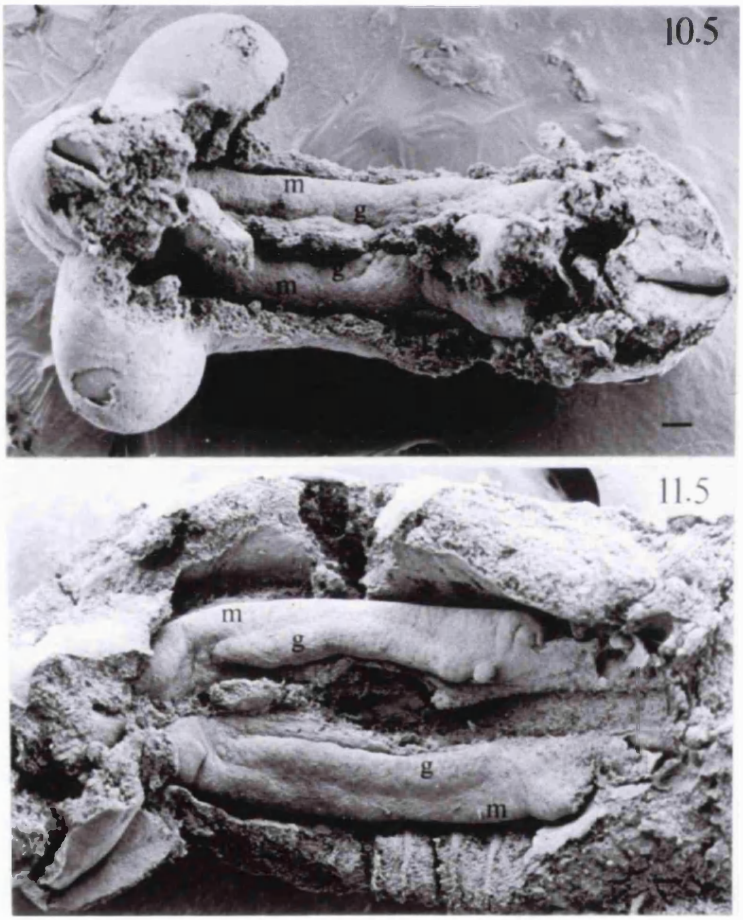

anti-Müllerian
hormone
Testis
testosterone
SRY
Genital Ridge
Ovary
10.5
11.5
12.5
Time
(dpc)

Fig.2 Scanning electron micrographs of a 10.5 dpc embryo (upper panel) and 11.5 dpc embryo (lower panel) showing the mesonephros (m) and genital ridge (g) within the coelomic cavity. No morphological distinctions can be made between XX or XY gonads at these stages (Modified from (Capel 2000)


10.5
m
g
g
m
11.5
m
g
g
m

I.II.I Müllerian and Wolffian Ducts

In mammals, prior to sex determination, the primordia for both the male and female ductal systems are initially present in the mesonephros. The Müllerian ducts (paramesonephric ducts) give rise to the uterus, the fallopian tube (or oviduct) and the cervix, whereas the Wolffian ducts (mesonephric ducts) are the embryonic precursors of the epididymis, vas deferens and seminal vesicles. Only one of the ductal systems will develop dependent on the fate of the gonad. During the early stages of testis differentiation two distinct secreted factors influence the development of the ducts. During male development Leydig cell derived testosterone acts to induce Wolffian duct differentiation whereas Anti-Müllerian Hormone(AMH), otherwise known as Müllerian Inhibiting Substance or MIS, a Sertoli cell secreted factor, induces regression of the Müllerian ducts. During normal female development, the absence of AMH permits differentiation of the Müllerian duct whereas the absence of testosterone leads to the passive regression of the Wolffian duct (Behringer, Finegold et al. 1994). This suggests that the signals controlling the female pathway are permissive and not instructive. The presence of testosterone and AMH impose a male pattern of differentiation on a system that would otherwise become female, as first suggested by Alfed Jost (Jost 1947). In a very classical set of experiments Jost demonstrated that sex differentiation is controlled by the action of hormones as well as by gene action. By depriving male rabbit foetuses of their testes, Jost showed that they developed female reproductive tracts and secondary sexual characteristics. Jost also showed that young male fetal Wolffian ducts regress, when removed from their testicular influence by castration, unless testosterone is implanted *in vivo*. Notably, implantation of a testosterone crystal into the female genital tract induces Wolffian duct stabilisation, but has no effect on Müllerian duct regression. Conversely, inhibition of Müllerian duct differentiation was induced by a fetal testis graft. Jost concluded that a specific Müllerian inhibitor, independent of testosterone, is secreted by the testis. This inhibitor was later named Anti Müllerian Hormone (AMH).

I.II.II Germ Cells

The indifferent gonads are essentially comprised of four distinct cell types, the germ cell lineage and three somatic cell lineages, each of which are bipotential and will contribute to structures either in the testis or the ovary. The primordial germ cells (PGC's) are specified between 6.5 dpc and 7.5dpc among a population of epiblast cells located close to the boundary with the extra-embryonic ectoderm, that migrate out of the posterior end of the primitive streak (Anderson, Copeland et al. 2000). These cells give rise to either extra-embryonic mesoderm or PGC's and only become committed to form one or the other cell type once in an extra-embryonic location. Primordial germ cells then migrate from their location at the base of the allantois into the embryonic endoderm of the hindgut. They continue migrating via the hindgut and dorsal mesentery and eventually populate the gonads via the mesonephros. In mouse, specification of primordial germ cells involves the action of members of the bone morphogenetic proteins, (BMPs). *Bmp4* homozygous null embryos contain no PGC's (Lawson, Dunn et al. 1999). In addition, the mutant mice lack an allantois, also derived from extraembryonic mesoderm. Heterozygous mutant mice have fewer PGC's than normal, due to a decrease in the population of precursors.

As PGC's are colonising the ventral region of the genital ridge, cells within the coelomic epithelium and the underlying mesonephros undergo migration into the gonad. Germ cells populate the gonads at 10.5 dpc and proliferate until around 12dpc in XX and XY gonads (Ginsburg, Snow et al. 1990; Gomperts, Wylie et al. 1994). When testis cords begin to form, the germ cells in an XY gonad become sequestered inside the cord where they arrest until mitosis resumes after birth. In the XX gonad, germ cells are required for the initial steps of follicle organisation and subsequent maintenance. These germ cells undergo a final mitosis at 13.5 dpc and then enter meiosis (McLaren and Southee 1997). The early entry into meiosis characterises the female pathway for a germ cell. It seems to be the default pathway as germ cells that fail to enter the genital ridge enter meiosis irrespective of gonadal or chromosomal sex. It is only if a PGC is surrounded

by the somatic cells characteristic of a testis that they are inhibited from entry into meiosis and become arrested in mitosis.

Receptor tyrosine kinases and their ligands function in the transduction of extracellular signals and are known to control processes such as cell proliferation, cell survival and differentiation. In mouse, mutations in the genes, *Steel (Sl)* and *W (dominant white spotting)* showed that they are important for the survival and migration of PGC's, and also play roles in hematopoiesis and melanocyte differentiation. The product of *W* , a proto-oncongene, encodes the transmembrane receptor tyrosine kinase, c-kit, that is expressed on the surface of migrating PGC's (Chabot, Stephenson et al. 1988, ; Zsebo, Williams et al. 1990). The *Sl* locus encodes mast cell growth factor, the *kit* ligand (KL) that is expressed on the surface of somatic cells in the environment of migrating PGC's. These two genes provide a ligand receptor pathway that facilitates the survival and migration of PGC's (Geissler, Ryan et al. 1988; Matsui, Zsebo et al. 1990; Motro, van der Kooy et al. 1991). Mutations in *c-kit* or *Sl* result in the absence of germ cells in the genital ridges, therefore outlining the importance of cell signalling along the migratory pathway to maintain germ cell viability.

Although required for fertility, testes can form in the absence of germ cells. In the female it is more difficult to address whether germs cells are required for early ovary formation, because of the lack of suitable markers. However, they are clearly required for follicle development which begins around 15-16 dpc. In the absence of PGC's, supporting cells in the ovary differentiate into pre-follicle cells but these cells eventually degenerate. However, in the presence of meiotic oocytes, the pre-follicle cells differentiate into follicle cells surrounding the oocyte. If the oocytes are subsequently lost, the supporting cells transdifferentiate into Sertoli cells and can form testis cord like structures (reviewed in (McLaren 1991; McLaren 2001)).

I.II.III Peritubular myoid cells and Leydig Cells

Peritubular myoid cells are a mesenchymal cell type that surround the Sertoli cells and give rise to the testis cords in the embryonic gonad. Peritubular myoid cells have been identified in all mammalian species, but their organisation in the interstitial space between testis cords differs from species to species. In mouse, the peritubular myoid cells comprise a single cell layer, whereas in human several layers exist. This cell type contributes to the contractile activity of the testis tubules and maintains mesenchymal-epithelial interactions with Sertoli cells, both by the deposition of extracellular matrix, and by secretion of paracrine agonists, for review see (Verhoeven, Hoeban et al. 2000). Fibronectin, collagens, and proteoglycans (extracellular matrix components) are secreted by peritubular myoid cells in addition to growth factors, such as basic Fibroblast growth factor (bFGF), TGF β molecules and Activins which may play a role in cellular differentiation and Sertoli cell function.

Leydig cells are an SF1 positive steroidogenic cell population that reside in the interstitial region of the developing male gonad. The main Leydig cell function during development is the production of Testosterone (among other factors) from cholesterol, to induce differentiation of the Wolffian ducts, external genitalia and other male characteristics. Leydig cell differentiation occurs after Sertoli cell differentiation and is likely to be indirectly influenced by Sertoli cell differentiation. Testosterone biosynthesis requires five steroidogenic proteins, steroid acute regulatory protein (StAR), cholesterol side chain cleavage enzyme (CYP11A), 3 β-hydroxysteroid dehydrogenase (3β-HSD), 17 α-hdroxylase (CYP17) and 17 β-hydroxysteroid dehydrogenase. Recently, it has been reported that Wnt 4 may act to repress the early differentiation of Leydig cells in the ovary (Vainio, Heikkila et al. 1999). In normal development, *Wnt4* is downregulated in the testes at about 12 dpc, shortly before Leydig cells differentiate, whereas it stays on in the ovary. In the absence of Wnt 4, due to a targeted disruption, steroidogenic fetal ovary cells express the steroidogenic enzymes, 3 ß-hydroxysteroid dehydrogenase (3 ß-HSD) and 17 α hydroxylyase (C_{17-20}) lyase

(P450c17), required for androgen synthesis, thereby promoting development of the Wolffian duct.

I.II.IV Follicle cells and Theca cells

Locally acting growth factors such as kit ligand (KL), bFGF, keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) play roles in epithelial mesenchymal interactions that are required for follicle development and folliculogenesis. At birth, females are born with a specific pool of oocytes, organised into primordial follicles. Granulosa cells within the developing follicles produce KL which can act on theca cells, that form the outer layer of the follicle. Follicle cells produce receptors for the gonadotropin, Follicle stimulating hormone (FSH), which stimulates follicle cell growth and the production of Estrogen {Vander, 1990}. The theca cells are also involved in the production of Estrogen and also Leutinizing hormone (LH). LH stimulates theca cells to proliferate and synthesise androgens. Although both hormones appear to increase growth *in vivo*, no proliferative response can been seen on isolated cells *in vitro*. As a result it has been postulated that gonadotropins and steroids indirectly stimulate follicular growth by influencing local mesenchymal-epithelial cell interaction in the developing ovary (Nillson and Skinner 2001)

I.II.V Sertoli cells and Granulosa Cells

The Sertoli cells in the testis and granulosa cells in the ovary both play critical roles to support and nourish germ cells. They are thought to have arisen from a common precursor lineage, termed the supporting cell precursor. During male gonadal development, the Sertoli cells coalesce to form the testis cords (also called the sex cords), the precursors of the seminiferous tubules, which enclose the germ cells (Pelliniemi, Frojdman et al. 1993). Similarly in the female, germ cells are surrounded by granulosa cells to form the follicles. Prostaglandin D synthase is expressed in the Sertoli cells and germ cells of the testis and it has been shown recently that

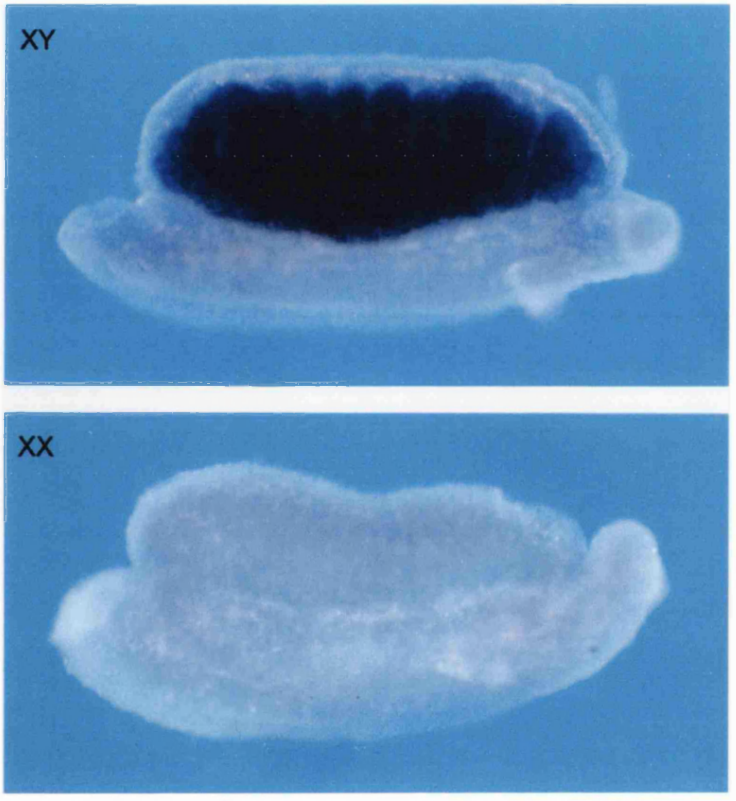
prostaglandin D_2 can partially masculinise female gonads in *in vitro* culture experiments (Adams and McLaren 2002).

Among somatic cells, the Sertoli cells are the first cell lineage to differentiate (Magre, Agelopoulou et al. 1980). Studies on gonads from XX-XY chimaeric animals revealed that of all types present, the Sertoli cells are the only lineage that show a bias in their sex chromosome constitution where they are almost exclusively XY (Palmer and Burgoyne 1991). From these pieces of evidence we can propose a simple model for sex determination. Our model suggests that the expression of *Sry* directs the supporting cell precursors to differentiate into Sertoli cells as opposed to granulosa cells, and that subsequent differentiation of all other lineages is dependent on Sertoli cell differentiation. A number of other genes are expressed in Sertoli cells, *SF1, Sox9, Amh* (discussed later) and *Dhh* to name but a few. *Dhh* encodes a secreted cell signalling molecule that shares homology with the *Drosophila* segment polarity gene *hedgehog* (*hh*), a key regulator in pattern formation in embryonic and adult structures. *Dhh* is expressed in the indifferent gonad at 11.5 dpc in pre-Sertoli cells, and subsequently in the differentiated Sertoli cell lineage through to adult stage, and is notably absent in the female gonad (embryonic and adult) (Bitgood, Shen et al. 1996).

Amh encoding anti-Müllerian hormone, is also expressed by Sertoli cells, and begins to be detected after 11.5 dpc in XY gonads and its strong expression is maintained until birth when levels decrease dramatically (Munsterberg and Lovell-Badge 1991; Hacker, Capel et al. 1995). *Amh* transcripts are absent in the embryonic female gonad, but expression begins around 6 days after birth in the ovary (Fig 3). Because of its Sertoli cell specific expression in the embryo, *Amh* is often used as a marker of testis differentiation, and is particularly useful as a tool for scoring sex reversal in the developing embryo. *Amh* is discussed in more detail later in the introduction, along with other factors that are expressed in Sertoli cells and play a role in testis differentiation.

Fig. 3 *Amh* is a male specific genital ridge marker.

Expression of *Amh* in the testis cords of a 13.5 dpc XY gonad (upper panel). Expression is absent in a similar stage XX gonad (lower panel).


XY
XX

I.III The origin of Sertoli cells

For some time it became an important question to clarify whether *Sry* was expressed in the Sertoli cell precursors, as expected, but an equally important question concerning the cell biology of the gonad was the origin of the Sertoli cell type. The origin of germs cells outside the gonad has been described independently by many groups (Ginsburg, Snow et al. 1990; Gomperts, Wylie et al. 1994; Anderson, Copeland et al. 2000). Many workers in the past have suggested that mesonephros derived cells contribute to the developing gonads (Upadhyay, Luciani et al. 1979) and in 1991 electron microscopy analysis showed that cells migrated from both the mesonephros and the coelomic epithelium into the developing gonad (Wartenberg, Kinsky et al. 1991). Elegant experiments where 11.5dpc isolated XY gonads were cultured with and without their mesonephroi demonstrated that the mesonephros was required for differentiated testes with organised cord structures (Buehr, McLaren et al. 1993). In similar culture experiments where the testis and mesonephros were separated by a filter membrane, the testis failed to differentiate properly suggesting the possibility that a diffusible molecule or cell migration was required for testis differentiation. However, when mesonephroi labelled with exogenous β-galactosidase were cultured with XY genital ridges, cells in the differentiated testes were shown to have originated in the mesonephroi. Peritubular myoid cells and interstitial cells frequently labelled strongly, however, β-galactosidase was never detected inside the testis cords. This suggested that Sertoli cells and germs cells most likely did not have their origin in the mesonephros or that they migrated from the mesonephros before 11.5 dpc. The coelomic epithelium had also been suggested as a source of Sertoli cells (Byskov 1986). By labelling single cells in the coelomic epithelium the fate of these cells was followed between tail somite(ts) stages 15-30 (approximately 11-12.5 dpc) (Karl and Capel 1998). Cells labelled at 15-17 ts migrated into the gonad and became Sertoli cells as well as giving rise to other cell types. This migration begins at a developmental time when *Sry* is maximally expressed. The labelled cells were also shown to be SF1 positive. After 18 ts, labelled cells no longer gave rise to Sertoli cells but instead contributed to a population of cells outside the testis cords. These are interstitial but appear not to be Leydig cells. Interestingly, at the time

when coelomic epithelial cells stopped giving rise to more Sertoli cells, SF1 was no longer detected in this layer.

I.IV Mesonephric cell migration

A consequence of *Sry* expression is the induction of somatic cell migration from the underlying mesonephros, via an unknown signal (Buehr, McLaren et al. 1993; Merchant-Larios, Moreno-Mendoza et al. 1993; Martineau, Nordqvist et al. 1997; Capel, Albrecht et al. 1999; Tilmann and Capel 1999). Within 24 hours after *Sry* expression the genital ridge has also undergone rapid cell proliferation. (Schmahl, Eicher et al. 2000). The subsequent cell-cell interactions organise the different cell types into the characteristic testis cord structure. After the onset of *Sry* expression, an increase in size of the XY gonad is observed in comparison to an XX gonad. BrdU labelling was used to compare cell proliferation in C57Bl/6 (B6) XX and XY gonads, where it was observed that cell proliferation was higher in the XY gonads. XX mice carrying a multi-copy *Sry* transgene, termed B6 XX/*Sry* develop as sex reversed males, due to the *Sry* transgene. Cell proliferation in the gonads of B6 XX/*Sry* mice was examined and shown to be indistinguishable from B6 XY gonads, suggesting that *Sry* is responsible for the increase in gonad size.

From 11.5 dpc onwards, cells from the mesonephros migrate specifically into XY gonads but not into XX gonads, independent of whether the mesonephros is XX or XY. This migration event occurs at such a time when levels of *Sry* expression are maximum. It has been proposed that downstream of SRY, some signalling event results in the deposition of peritubular myoid cells and endothelial cells into the XY gonad. Mesonephric cell migration occurs in a precise stage specific manner and has been shown to induce testis cord formation and further differentiation of the male gonad (Tilmann and Capel 1999). To test whether mesonephric cell migration into an XY gonad was dependent on the presence of *Sry,* Capel *et al* combined their gonad culture experiments with a genetic approach. Gonads were dissected from XY embryos, that have a Y chromosome deleted for *Sry* (Y^m, a derivative of Y^{TDYM}) and from XX *Sry*

embryos (XX embryos, carrying the *Sry* transgene). From these gonad culture experiments it was shown that both cell migration and cord formation were associated with the expression of *Sry,* observed only with XX *Sry* gonads, and not with XXY *Sry* deleted gonads (Capel, Albrecht et al. 1999). Additional data suggests that the steroid-producing cells (Leydig cells in the testis and theca cells in the ovary) originate from cells that migrate into the genital ridge from the mesonephros in a non-sex specific manner before 11.5 dpc (Hatano, Takakusu et al. 1996; Merchant-Larios and Moreno-Mendoza 1998; Capel 2000)

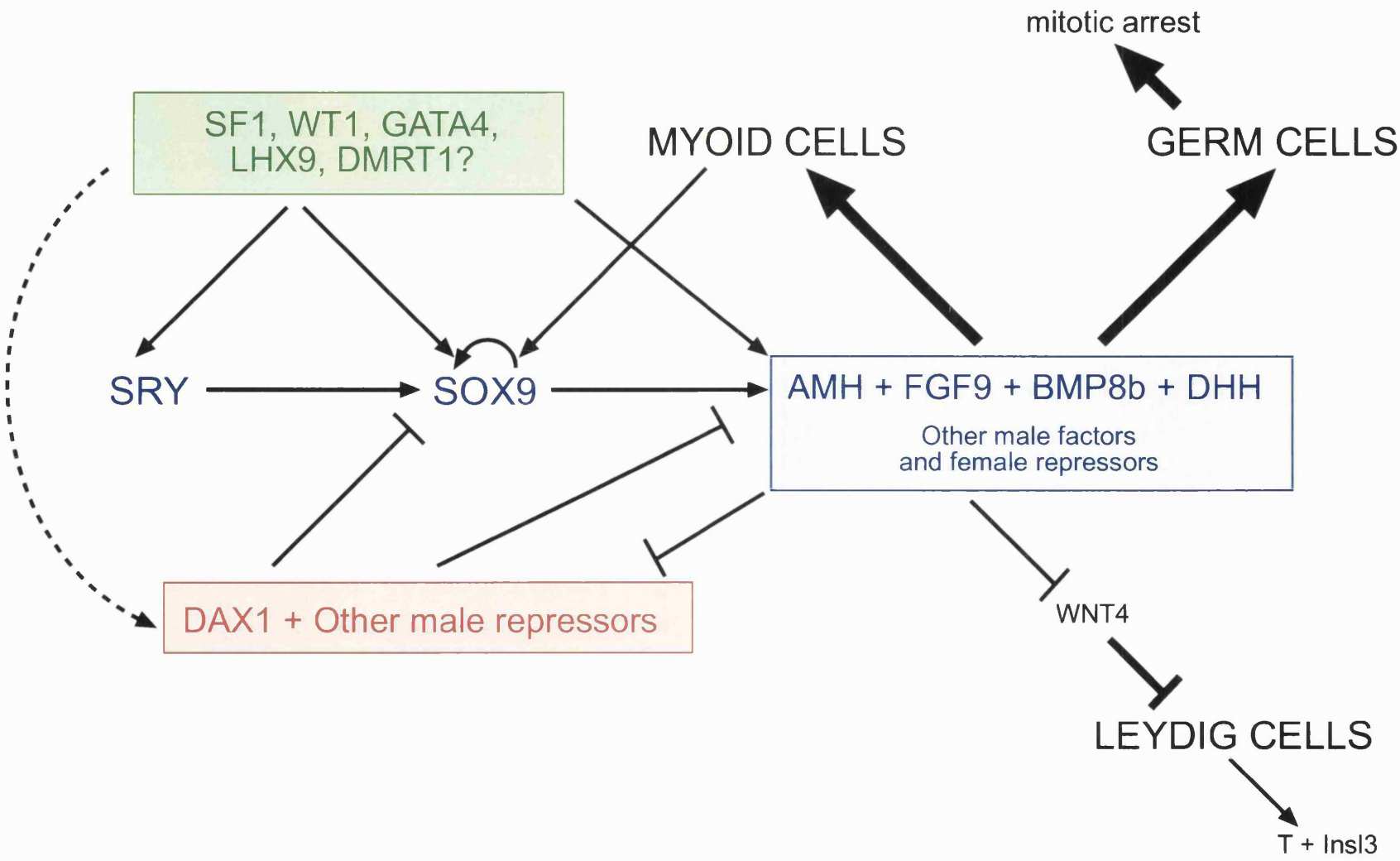
I.V. Testis descent

During development of the urogenital tract, the mesentery connects the gonads and Wolffian and Müllerian ducts to the abdominal wall. Development of two parts of the genital mesentery, the cranial suspensory ligament (CSL) and the caudal genital ligament (also called the gubernaculum) during male and female development is believed to be responsible for the sexual dimorphism of the position of the testis and the ovary (van der Schoot and Emmen 1996; Hutson, Hasthorpe et al. 1997). Bilateral cryptorchidism was shown to occur in mice lacking Insulin-like 3 (INSL3), because of impaired development of the gubernaculum. These results clearly demonstrate the role of INSL3 in testicular descent. INSL3, also designated Leydig insulin like, was first detected in the developing testis at 13.5 dpc and is restricted to Leydig cells (Adham, Burkhardt et al. 1993). No transcripts are detected in the fetal ovaries. SF1 has been shown to bind to and activate the *Insl3* promoter (Zimmermann, Schotler et al. 1997). Testicular descent occurs in two phases. During the first or transabdominal phase, the testes move from the higher to lower abdomen, which takes place between 15.5 and 17.5 dpc. The second phase involves the movement of the testes from the lower abdomen to the scrotum. During the first phase, development of the gubernaculum and regression of the CSL result in the movement of the testis to the inguinal region. In females, persistence of the CSL and lack of development of the gubernaculum results in the ovary remaining close to the kidney. INSL3 appears to be required for the outgrowth and differentiation of the primordium of the gubernaculum (Adham, Emmen et al. 2000).

Targets of INSL3 have yet to be identified and it is still unknown how the action of INSL3 is mediated.

Fig 4 . A model of genetic interactions during sex determination in mouse.

The central blue pathway outline the factors important for male development. Anti-male factors such as DAX1, that may also play a role in female development are depicted in red. Factors outlined in the green box are required for gonadal development but also have a positive effect on the central blue male pathway, and also on the repressive anti-testis genes. All of these factors act within the supporting lineage but some also signal to other lineages within the developing gonads. (Modified from (Lovell-Badge, Canning et al. 2002)).


mitotic arrest
SF1, WT1, GATA4,
LHX9, DMRT1?
MYOID CELLS
GERM CELLS
SRY
SOX9
AMH + FGF9 + BMP8b + DHH
Other male factors
and female repressors
DAX1 + Other male repressors
WNT4
LEYDIG CELLS
T + Insl3

I.VI Gene expression patterns and gene function during early gonadal development

I.VI.I *Wt1*

Gene targeting experiments in mouse and human mutation analysis have lead to the identification of a number of genes that are involved in development of the early indifferent genital ridge (Fig4) . The Wilms tumour suppresser gene *Wt-1* encodes a zinc finger protein, which when mutated results in childhood kidney tumours and sexual ambiguity. *Wt1* begins to be detected at around 9.5-10 dpc in the developing urogenital ridge (Armstrong, Pritchard-Jones et al. 1993). Mice carrying homozygous knockout mutations of *Wt1* lack kidney, gonad and adrenal glands, and also display heart defects (Kreidberg, Natoli et al. 1999; Moore, McInnes et al. 1999). Different isoforms of *Wt1* have been shown to act as transcriptional activators and repressors. The *Wt1* gene leads to the expression of at least 24 isoforms through alternative splicing, alternative start sites and RNA editing (Hammes, Guo et al. 2001). One very well conserved alternative splice donor site results in the insertion or deletion of a three amino acid sequence, Lysine Threonine Serine, (+/-KTS). Previously it had been suggested that the +KTS isoform, which localised in a speckled pattern in the nucleus with splicing factors, preferentially binds RNA, whereas the -KTS isoform played a role in transcriptional regulation binding DNA with a high affinity (Englert, Vidal et al. 1995; Larsson, Charlieu et al. 1995; Ladomery, Slight et al. 1999). Dominant negative mutations of *WT1* in humans gives rise to a syndrome known as Denys-Drash Syndrome, whereas Frasier syndrome is the result of defective alternative splicing of *WT1* leading to an altered ratio of WT1 +/-KTS isoforms. Denys-Drash Syndrome is a progressive nephropathy that usually appears early in life coincident with Wilms tumour. Sex reversal or hermaphroditism is common, with most patients developing phenotypically as females. Frasier syndrome has many similarities to Denys-Drash, with the development of streak gonads, renal failure and patients are often pseudohermaphrodites. Targeted mutation of the *Wt1* locus was carried out in ES cells and used to generate mouse models of the human Denys-Drash and Frasier syndromes. Mutations were introduced into the WT1 locus to generate mice that completely lacked

the +KTS isoform (these are described as –KTS mice as this is the only isoform they have) and alternatively mice were generated that only had the + KTS isoform (designated +KTS mice as they lacked the –KTS isoform). In general, the ratio of +/- KTS isoforms in wild type mice is kept constant and is usually 1:1. This *in vivo* approach revealed very different roles than those already proposed for the +/- KTS isoforms (Hammes, Guo et al. 2001). Analysis of these mutant animals revealed that development of the heart, adrenals and spleen were normal for both the +KTS and -KTS mice, indicating that these two isoforms had overlapping functions in these organs. In other words, deletion of one isoform was compensated for by the other. In these mutant mice, the absence of one KTS isoform results in a double dose of the other so that the overall level of WT1 protein is maintained. All of the XY -KTS homozygous mice developed as females and showed a reduction in *Sry* expression, suggesting a role for the + KTS isoform in male sex determination. This suggests that WT1 is upstream of *Sry* and may be involved in its regulation. Indeed it has been shown that human WT1 can activate *SRY* in *in vitro* transcription assays (Hossain and Saunders 2001). Gonads dissected from +KTS homozygous mice were much smaller than wild type and consisted mainly of undifferentiated tissue with a high number of apoptotic cells, suggesting a role for the –KTS isoform in cell survival. By deleting either KTS isoform these experiments imply a certain degree of overlapping function between the two isoforms, but in addition each appears to have significantly different roles in gonadal development. However, one caveat with these types of experiments is whether the phenotypes observed are due to a loss of one isoform or due to an overexpression of the other, as the levels of WT1 remain constant.

I.VI.II *Sf1*

Sf1 encoding Steroidogenic factor 1 (SF1) was initially identified as a regulator of tissue specific expression of cytochrome P-450 steroid hydroxylases; important mediators of steroid biosynthesis (Lala, Rice et al. 1992; Ikeda, Lala et al. 1993). SF1 is a typical member of the orphan nuclear receptor family with conserved DNA and ligand binding domains. SF1 contains a classic zinc finger DNA binding domain in the N-terminal

domain that enables it to bind as a monomer to its cognate response element with high affinity. *Sf1 is* expressed around 10 dpc in a cell population that separates into two, one which gives rise to the adrenal cortex and the other which contributes to the bipotential gonad. In order to characterise the function of SF1 in the hypothalamic-pituitary-adrenogonadal axis, gene targeting technology was carried out by three independent labs (Luo, Ikeda et al. 1994; Sadovsky, Crawford et al. 1995; Shinoda, Lei et al. 1995). *Sf1* null mice die of adrenocorticoid insufficiency at birth and were externally phenotypically female regardless of chromosomal sex. Although the gonads began to form, their development ceased at 11-12 dpc and through apoptosis the gonads completely regressed by 12.5 dpc. This suggested an important role for SF1 in gonadal development (Fig 4). SF1 expression remains higher in the testis than in the ovary, which suggested additional roles for the gene in the male pathway (Giuili, Shen et al. 1997; De Santa Barbara, Bonneaud et al. 1998; Arango, Lovell-Badge et al. 1999). SF1 has been shown to be a regulator of *Amh* transcription in synergy with SOX9 (discussed in detail later). *Sf1* null animals also displayed additional ventromedial hypothalamic and pituitary defects (Shinoda, Lei et al. 1995).

I.VI.III *Sox9*

Sox9 encodes a member of the SOX family of transcription factors characterised by the presence of an HMG box DNA binding domain similar to that of SRY. However, unlike SRY, SOX9 has a strong transactivation domain that maps to the C-terminus. *Sox9* is expressed in the gonads as well as in developing chondrocytes, the heart, pancreas, central nervous system and other sites. At 10.5 dpc, around the same time as *Sry* transcripts are first detected, *Sox9* is expressed at low levels in the developing gonads of both sexes (Morais da Silva, Hacker et al. 1996). By 11.5 dpc, *Sox9* is robustly expressed in the developing XY gonads but is no longer detected in the XX gonads. In humans, haploinsufficiency of *SOX9* is associated with a skeletal disorder called Campomelic Dysplasia (CD), in which 75% of affected XY individuals develop as females. A large proportion of CD patients exhibit partial or complete sex reversal. Genital morphologies have been shown to range from relatively minor variants such as

hypospadias, or cleft scrotum to complete female external and internal genitalia with streak gonads. Therefore, both expression analysis of *Sox9/SOX9* and human mutation analysis imply that it plays an important role in male sex determination and/or differentiation.

SOX9 was shown in cell transfection assays to transactivate reporter plasmids through binding a AACAAT motif (Ng, Wheatley et al. 1997) a sequence recognised by other HMG box transcription factors (Grosschedl, Giese et al. 1994). Deletion constructs were used to map the transcriptional activation domain and it was found to reside in the C-terminus. Two domains comprise the C-terminus of mouse SOX9, a region rich in proline and glutamine residues (PQ amino acids 329-421) and a second region which is rich in proline, glutamine and serine residues (PQT amino acids 424-507) both of which have been shown to modulate gene activity (Ng, Wheatley et al. 1997). It was also shown that the latter domain, PQT, is a very potent activator. Similarly it was shown that the terminal 103 amino acids of human SOX9 can activate gene transcription (Sudbeck, Lienhard Schmitz et al. 1996). Indeed many of the human mutations in *SOX9* are frameshift or truncations that alter the C-terminus of the protein. This suggests that the CD and sex reversal observed is due to loss of transactivation of genes downstream of SOX9. This further supports a role for SOX9 in the early stages of testis determination. Other mutations affect the HMG box, implying that it is also essential for the protein to interact with DNA, where it is assumed to have an "architectural" function, affecting the ability of other proteins to participate in an appropriate complex.

I.VI.III.I. SOX9 and XX male sex reversal

A single case of human XX sex reversal was reported with a duplication of chromosome 17q23-24, the region that contains the *SOX9* gene (Huang, Wang et al. 1999). This implied that an extra copy of *SOX9* may be sufficient to trigger male development. The effect of this duplication on timing and levels of *SOX9* expression cannot be predicted, but it is clear that the early developing gonads are sensitive to gene dosage, and threshold levels of gene expression exist to control entry into the male pathway. If the

level of SOX9 expression is altered above the threshold in the XX gonad, this may be sufficient to initiate testis development. However, it is possible that another unknown gene involved in sex determination could reside within the duplicated region, and it is the abnormal expression of this gene that is implicated in this case of sex reversal. Alternatively, the duplication could also disrupt a gene that has not yet been associated with sex determination.

An additional line of evidence supporting the idea that *Sox9* plays a critical role in testis determination comes from a mouse mutant called *Odsex (Ods)* (Bishop, Whitworth et al. 2000). In the *Odsex* mutation, a *Dct* (Dopachrome tautomerase-tyrosinase related 2, trp2) tyrosinase transgene has inserted 1 Mb upstream of the *Sox9* gene. The insertion event also lead to a 150kb deletion nearby. XX *Ods* mice are sex reversed and develop as males. *Sox9* expression is detected in the gonads of XX*Ods*/+ mice whereas in wild type XX gonads *Sox9* transcripts are not detectable. The authors postulate that the deletion may have removed a long range repressor element for *Sox9* in XX gonads. This was thought to lend support to a model for sex determination where SRY was proposed to work by repressing a repressor {McElreavy, 1993 #64}. An alternative explanation is that the transgene insertion has a positive regulatory effect on *Sox9* expression in the developing XX gonad, either by opening chromatin or by direct action of the enhancer elements of the transgene on the *Sox9* promoter. These data, like that from the sex reversal observed with the human *SOX9* duplication, were derived from a single mutation event. But many *Odsex* animals can be analysed and it is possible in this case to look at gene expression in the embryos. The results clearly imply that constitutive expression of *Sox9* in an XX gonad is sufficient to induce male development. Low levels of *Sox9* can be detected in the early developing gonads of both sexes at about 10.5 dpc but by 11.5 dpc they are no longer detectable in the ovary (Morais da Silva, Hacker et al. 1996). An interesting unresolved question is what factor acts to repress *Sox9* expression in the XX gonad?

Finally and most recently, it was reported that *Sox9* can induce testis development in mice when expressed under the control of the Wilms tumour 1 (WT1) regulatory

elements (Vidal, Chaboissier et al. 2001). Using a YAC targeting approach, *Sox9* was fused to the WT1 start codon in the context of a 160kb *Wt1* genomic region and this construct was used to generate transgenic mice. WT1 is expressed early in the gonadal primordium of XX and XY mice, and as a result, *Sox9* expression was induced before the onset of *Sry*. This expression was maintained throughout gonadal development in three out of three independent transgenics. This ectopic expression of *Sox9* in the XX gonad again resulted in testis development. The interpretation we can draw from the above data is that activation (or maintenance) of *Sox9* in the XX gonad is sufficient to trigger the male sex determining pathway.

The relationship between *Sry* and *Sox9,* two related genes at the top of the sex determination cascade, is still unknown. One hypothesis is that *Sox9* is the only critical gene downstream of *Sry,* perhaps even its immediate target, and its misexpression is sufficient to initiate the male pathway. SRY may simply be required to upregulate *Sox9* which is subsequently autoregulated, and this would offer a good explanation as to why we do not see prolonged expression of *Sox9* in the XX gonad. If this is the case then all downstream events in sex determination are initiated by SOX9 (see Fig. 4).

I.VI.IV *Amh*

I.VI.IV.I Overexpression of *Amh* in mouse

Amh encodes a homodimeric glycoprotein secreted specifically by the Sertoli cells of the embryonic and newborn testes and the granulosa cells of the postnatal ovary (Cate, Mattaliano et al. 1986). The product of *Amh* in the male gonad is responsible for regression of the Müllerian ducts, the anlagen of the oviducts, uterus and cervix (Josso and Picard 1986). Overexpression studies revealed that ectopic expression of human AMH in XX mice led to inhibition of Müllerian duct differentiation resulting in a blind vagina, and the absence of a uterus, oviducts and ovaries (Behringer, Cate et al. 1990). Variations of the phenotype were observed in different lines expressing lower levels of AMH, ranging from normal females to females lacking uteri but with ovaries. In order to address at what stages this overexpression of AMH affected ovarian development, gonads were dissected from females at different neonatal stages. At 2 dpp ovaries were present but the uterus was absent, but whilst the ovaries resembled wild type controls, germ cell numbers were greatly reduced. By 9 dpp the ovaries were completely devoid of germ cells and no follicles were present. By 16dpp the most pronounced effect was observed, the gonads were very small, they completely lacked germ cells and had taken on the appearance of cord like structures resembling seminiferous tubules. Whilst females were infertile, founder males were fertile and transmitted the transgene to their offspring. In the highest expressing lines a significant proportion of male offspring had external genitalia consisting of a vaginal opening and mammary gland development. These males also had very small undescended testes that were devoid of germ cells and had underdeveloped epididymides and no seminal vesicles. Usually feminisation observed in males and disrupted Wolffian derived structures can be explained by androgen insensitivity. High levels of *Amh* expression may have perturbed androgen biosynthesis, perhaps by affecting Leydig cell differentiation and function.

In certain species (cattle for example), a female twin that is exposed to a male twin's blood results in regression of her Müllerian ducts, and this condition has been termed

"freemartinism". Similar to the results outlined above, the ovaries cease to grow and lack germ cells and some even develop seminiferous tubules. I*n vitro* experiments where ovaries are exposed to AMH resulted in the same effects. These results demonstrate the importance of silencing *Amh* gene expression during female development and demonstrate a role for AMH in testis differentiation, although the latter is thought to be an indirect effect of eliminating oocytes.

I.VI.IV.II *Amh* deficient mice

To further characterise the role of *Amh* in gonadal development a targeted disruption was generated in ES cells to generate *Amh* deficient mice (Behringer, Finegold et al. 1994). All male and female heterozygous mice were phenotypically normal and fertile. Homozygous females had normal ovaries, oviducts and uterine development. Internal abnormalities were discovered in homozygous males. Testis size was normal and they had descended properly, and Wolffian duct differentiation appeared not to have been affected. However, all of these males also developed Müllerian duct derivatives, including a uterus and vagina, and uncoiled oviducts. These *Amh* deficient homozygotes are referred to as pseudohermaphrodites as they had testes and both Wolffian and Müllerian duct derivatives. Fertility defects were also observed with most *Amh* deficient males. Only a small proportion of homozygous males were able to sire litters and while the rest mated with females and produced a vaginal plug, no sperm was detected in the uteri of the females. However no spermatogenic defects were observed on examination of adult testes. Normal sperm were found in the vas deferens and epididymides and it was found that sperm isolated from these could fertilise eggs *in vitro*. Indeed these fertilised eggs were able to develop to term in pseudopregnant foster mothers. These results indicated that there were no detectable defects of the sperm from *Amh* deficient males, indicating that *Amh* was not required for normal spermatogenesis. So how was the lack of sperm observed in females that mated with pseudohermaphrodites explained? One theory is that the presence of female derivatives superimposed on male organs prevented the motility or sperm transfer into males. In order to address whether the normal sperm migrated through the female organs in the pseudohermaphrodites, uteri

and bladders of these animals were flushed. No sperm was detected in the bladders, however, sperm was reported to have been found in the uteri of some *Amh* deficient males. Although lack of *Amh* clearly did not affect early testicular differentiation, Leydig cell hyperplasia and a Leydig cell tumour were detected in older males. In conclusion, overexpression of *Amh* in mice was consistent with the phenotype observed in freemartin cattle and the male *Amh* deficient mice resembled human PMDS (persistent Müllerian duct syndrome) where mutations have been found in the *AMH* gene. Persistence of both ductal derivatives is deleterious to the proper function of each, and moreover, the loss of function mutation suggests a role for AMH in the regulation of Leydig cell proliferation.

X.VI.IV.III Regulation of *Amh*

Loss of function and gain of function mutations of the *Amh* gene have strongly demonstrated the importance of Müllerian duct regression. The next important question to resolve was how the production of AMH is regulated. *Amh* transcripts begin to be detected in the genital ridge from around 11.5 dpc and expression continues to be detected in Sertoli cells until birth and then decreases dramatically (Munsterberg and Lovell-Badge 1991; Dresser, Hacker et al. 1995). In females, *Amh* transcripts are first detected around 6 dpp in the granulosa cells of the ovary. Sequences within a 2kb 5' flanking sequence of the human *AMH* gene were sufficient to drive cell-specific expression of a reporter gene (Peschon, Behringer et al. 1992). Initially comparison between the human 2kb regulatory sequence and a 2kb region upstream of the mouse *Amh* gene identified a conserved sequence, named MIS-RE-1 that closely resembles a nuclear receptor half site (Shen, Moore et al. 1994). Primary Sertoli cell culture experiments were used to test the function of this conserved element in luciferase assays. Primary Sertoli cells isolated from 15dpp rats were cultured *in vitro,* as cells at this stage still express *AMH.* Deletion constructs were used to delineate the sequence required for Luciferase activity. A 180bp element in the proximal 5' flanking region of the mouse gene was thought to include all of the necessary regulatory elements and included MIS-RE-1. Subsequently it was shown that this 20bp MIS-RE-1 was a critical regulatory element and bound SF1, which co-localises with AMH in the Sertoli cells. Shen and co-authors suggested through *in vitro* and *in vivo* transgenic experiments, that this proximal promoter element was sufficient both for initiation and maintenance of *Amh* expression, in a sex and gonadal specific manner (Giuili, Shen et al. 1997). The *in vivo* transgenic experiments also provided evidence that the MIS-RE-1 element was specifically required and that the 180bp sequence targeted expression specifically to the gonads. However, this conclusion was based on data obtained from just three transgenics with the control sequence, where only one showed expression reminiscent of the endogenous sequence, and three transgenics with the mutant sequence, where none gave appropriate expression. Furthermore, their *in vitro* luciferase assays revealed a higher expression

level for a 650bp element and this region was not further characterised *in vitro* or *in vivo.*

During investigation of the 5' flanking region of the mouse *Amh* gene a region of homology with a human spliceosome factor (SAP62) was discovered (Dresser, Hacker et al. 1995). In mouse the *Sap62* stop codon lay only 434 bp upstream of the start codon of *Amh. Sap62* is a housekeeping gene and the strict specificity of *Amh* implied that all of its regulatory elements lay within the intervening 434 bp. These authors noted a high frequency of estrogen response elements and half sites within this region. In contrast to the experiments of Giuili *et* al (1997), Beau and colleagues showed that a minimal promoter of 370bp was sufficient to drive β-galactosidase specifically in the XY gonad, in several independent transgenic lines, but while expression was correctly initiated at about 11.5 dpc, it was not maintained beyond about 14.5 dpc. This suggests that additional control elements are required to maintain AMH expression until the onset of puberty (Beau, Vivan et al. 2001).

In subsequent years the *Amh* promoter has been further characterised and is clearly regulated by multiple factors in a more complex manner than initially described. Several factors that play different roles during sex determination and /or differentiation have been proposed to regulate *Amh* expression, including SRY, DAX1, WT-1, SF1, SOX9 and GATA-4. All with the exception of DAX1, which may not directly interact with DNA, and WT-1, have binding sites within the 180bp regulatory region. Sequence comparisons of a 269 bp 5' flanking sequence of the *Amh* gene from rat, human, mouse bovine and porcine origins revealed very conserved binding sites for GATA transcription factors, SOX9 and SF1. In addition to the already described proximal SF1 site, an additional distal site was reported as well as two GATA sites and a reverse GATA site. A 269bp human 5' flanking sequence was used to drive a luciferase reporter along with deletion constructs in their *in vitro* analyses. The authors found that the region between –192 and –169 was required for greater promoter activity. This region contains a distal SF1 site that was shown to bind SF1, and mutational analysis of both sites independently revealed that both SF1 sites are required for increased activity.

Most of the above data described came from *in vitro* analyses, which do not always match data obtained *in vivo.* One example of this comes from the different functions ascribed to the +/- KTS isoforms of WT1 interpreted from *in vitro and in vivo* experiments. *In vitro* experiments suggested that the -KTS isoform is able to synergise with SF1 in regulating *Amh* and that the +KTS isoform was an important RNA processing molecule. In fact, *in vivo* mutation analysis has revealed that the -KTS isoform played an earlier role in cell survival in the early indifferent gonad and the +KTS isoform plays an important role in male sex determination. In the absence of the +KTS isoform, *Sry* levels are very low and XY mice are sex reversed females. Not only does this experiment uncover new roles for the +/- KTS isoforms during gonadal development and sex determination but it is supportive of the necessity of *in vivo* transgenic/gene targeting strategies to uncover gene function.

While many factors have been associated *in vitro* with the regulation of *Amh*, such as SF1, GATA4 and SOX9, an *in vivo* approach was used to address regulation of the *Amh* promoter. Targeting mutations were generated in the SF1 binding site (R1-*neo*), and SOX9 binding site (R2-*neo*) and in both the SF1 and SOX9 binding sites *in cis* (R3-*neo*) (Arango, Lovell-Badge et al. 1999). The neomycin selectable cassettes were removed from positively selected ES cells as it was shown that the *neo* sequence, or the promoter driving it, had a negative effect on *Amh* expression. ES cells carrying the various types of mutation were used to inject blastocysts and contributed to the germline of resulting chimeras. R1/R1 homozygous males were shown to be normal with no retention of Müllerian duct derivatives. This result suggested that the SF1 binding site was not specifically required to activate *Amh in vivo.* This result immediately contradicted the data previously described for SF1 in regulating *Amh,* however, levels of *Amh* transcripts were reduced to about 70% of normal levels. The R1-*neo* allele, which is functionally equivalent to a null was combined with the R1 allele to further reduce *Amh* expression. The Müllerian ducts of R1/R1-*neo* mice were only partially regressed. This indicates that quite a low threshold level of AMH expression is sufficient to give complete regression of the Müllerian ducts. In order to quantitate levels of expression of *Amh*

from R1 alleles, RT-PCR and RNase protection analyses were performed which showed that *Amh* expression was detected at 12.5 dpc in R1/R1 mutant mice. Therefore the SF1 binding site was not required to initiate transcription. By examining testes isolated from 13.5dpc, 15.5 dpc, 9 dpp and 12 dpp R1/R1 males and wild type controls it appeared that the levels of expression detected from R1/R1 mice were lower than wild type. In addition these low levels of expression were detected until just before 12 dpp when it suddenly became undetectable compared to wild type expression which is still observed at this time.

The SOX9 binding site was shown to be essential for *Amh* regulation as R2 homozygous males were internal pseudohermaphrodites with complete retention of all Müllerian duct derivatives. RT-PCR analysis on embryonic R2/R2 testes at 12.5 dpc revealed that no *Amh* transcripts were detected indicating that the SOX9 binding site is required for initiation of *Amh* transcription. These results together show that SOX9 is required to initiate *Amh* transcription whereas SF1 is required as a quantitative regulator and may be necessary for postnatal maintenance of *Amh* expression. These data also fit expression profiles, with SF1 expression commencing at least 36 hours prior to the onset of *Amh*, whereas the onset of high levels of SOX9 is coincident with that of *Amh.*

I.VI.V The testis determining gene

Before the discovery of *Sry/SRY,* the testis specific male determining gene, it had been long accepted that the Y chromosome carries instructive genetic information required for testis determination (Jacobs and Strong 1959). Individuals with a single X chromosome are female whereas individuals with multiple X chromosomes and a Y chromosome are male. One of the most exciting questions in relation to the biology of gonads and sex of individuals was the search for this dominantly acting Y chromosome gene.

In 1975 it was proposed that the H-Y antigen (a Y-chromosome specific cell surface antigen) was the product of the testis-determining gene (Wachtel, Ono et al. 1975). This idea was supported when some human individuals with sex discordance presented with appropriate H-Y status. It was also reported that the H-Y antigen was absent in patients with 46 XY pure gonadal dysgenesis (Ghosh, Shah et al. 1978). A sex reversal chromosomal fragment (Sxr) when recombined onto an X chromosome was found to cause maleness in XXSxr mice (Evans, Burtenshaw et al. 1982; Singh and Jones 1982). This Sxr region also contained the gene encoding the H-Y antigen (Hya). However, the discovery of H-Y negative male mice ruled out this minor histocompatability antigen as the testis determining factor (McLaren, Simpson et al. 1984). In 1985, a major breakthrough was the analysis of XX individuals found to be male due to the presence of paternally derived Y sequences transposed onto an X chromosome (Page, de la Chapelle et al. 1985). *TDF* was found to map within the distal part of the Y chromosome adjacent to the pseudoautosomal region (PAR), by the analysis of Y chromosome fragments that were present in different XX males. A new gene called *ZFY,* encoding a Zinc finger DNA binding domain protein was found to map within 230kb of the PAR(Page, Mosher et al. 1987). *ZFY* was proposed as a candidate for *TDF* at this time. However in 1988, it became clear that *Zfy* sequences were autosomal in marsupials, raising doubt as to whether *ZFY* was the right gene (Sinclair, Foster et al. 1988). Additional XX sex reversed males were analysed for the presence of *ZFY*. Four individuals were found to carry Y specific sequences adjacent to the pseudoautosomal region, who lacked *ZFY,* demonstrating that the gene was not *TDF* (Palmer, Sinclair et al. 1989).

Further characterisation of the 4 XX males revealed them to have just 35kb immediately adjacent to the PAR boundary on the Y chromosome. A chromosome walk identified a single copy Y chromosome gene whose coding sequence was well conserved and Y-specific among a wide range of mammals, and moreover, the gene was expressed in the testes (Sinclair, Berta et al. 1990). The gene was termed Sex determining region on the Y chromosome (*SRY*). At the same time the murine homologue, *Sry,* was identified and shown to reside in the Sxr^b (sex determining region b), on the short arm of the mouse Y

chromosome (Gubbay, Collignon et al. 1990). The evidence that strongly supported *Sry* as a testis determining gene came from XY mice which developed as females due to mutation on the Y chromosome (Lovell-Badge and Robertson 1990). These mice had been shown to express *Zfy* (further evidence that it was not required for testis determination), but completely lacked the *Sry* coding region (Gubbay, Koopman et al. 1990). To confirm that *Sry* was the testis determining factor, a 14kb genomic DNA fragment carrying *Sry* was sufficient to induce testis development when introduced as a transgene in XX embryos (Koopman, Gubbay et al. 1991). This provided formal proof that *Sry* was indeed the testis determining gene and also showed that it was the only gene on the Y chromosome required to induce testis development.

I.VI.V.I SRY structure and function

Sry encodes a transcription factor, expressed in the indifferent XY gonad, whose DNA binding domain, the HMG box, is capable of binding to and bending DNA. HMG boxes are present in chromatin proteins, general transcription factors, nucleolar and mitochondrial RNA polymerases, and gene specific transcription factors (Ferrari, Harley et al. 1992). The HMG box of *Sry* encodes a 79 amino acid sequence which recognises a specific target sequence, AACAAA/T and can bend linear DNA through angles of 90^{o} (Harley, Lovell-Badge et al. 1994). These characteristics imply that although it may work as a transcription factor, it may act more as an architectural factor, co-ordinating local chromatin structure, rather than as a classical activator or repressor of gene activity. The mouse *Sry* gene encodes a 395 amino acid protein with a very short N-terminal domain of only two amino acids, the HMG box is then followed by a short linker region and a large degenerate glutamine repeat domain (encoded by a CAG repeat sequence). The CAG repeat domain of *M. m. musculus* is encoded by a 222 amino acid sequence arranged as 20 blocks of 2-13 glutamine residues interspersed with FHDHH or similar sequences. It has been postulated that this sequence arose when (Q_nFHDHH) sequence inserted into the *Sry* gene of an ancestral rodent and through mutations and deletions has resulted in the variety of length and sequence that exist in the SRY proteins of various mouse species and strains. In comparison to *M. m. musculus*, the CAG repeat

domain of *M. m. domesticus* is only 87 amino acids in length. The role for the CAG repeat has yet to be determined, but it has been reported that it can interact *in vitro* with putative SRY interaction proteins, SIP1, 2 and 3 (Zhang, Coward et al. 1999). In order to functionally characterise the CAG repeat domain, transgenic mice were generated which carried a truncated form of SRY that lacks the CAG repeat sequence (Bowles, Cooper et al. 1999). In their analysis, XX mice carrying the truncated *Sry* transgene failed to develop as males in comparison to XX mice carrying the wild type sequence which developed as sex reversed males. Their conclusion was a functional requirement of SRY for a CAG repeat domain. However, *Sry* is a rapidly evolving gene and among various mammals analysed, mice seem to be unique in their C-terminal CAG repeat domain. The only conserved sequence between the mouse and human SRY is the HMG box domain, and its importance in humans is further supported by the fact that mutations in human SRY, which lead to sex reversal, almost exclusively lie within the HMG box domain (Schmitt-Ney, Thiele et al. 1995; Koopman 2001). This raises the subject of rapid evolution of SRY sequences, and whether the critical functional domains are only those that are highly conserved. This is a focus of my thesis, addressing the functional domains within the *Sry/SRY* genes which may elucidate the mode of action of SRY. For example, whether it acts simply as an architectural transcription factor, or by specifically activating target gene(s) (Canning and Lovell-Badge 2002).

I.VI.V.II Expression of *Sry*

Looking at RNA expression by RT-PCR, RNase protection assays, and *in situ* hybridisation, it has been shown that *Sry* begins to be expressed in the indifferent XY gonad at approximately 10.5 dpc, levels peak at 11.5 dpc, and are no longer detected by 12.5 dpc (Koopman, Munsterberg et al. 1990; Hacker, Capel et al. 1995; Swain, Narvaez et al. 1998). Embryos homozygous for the W^e mutation completely lack germ cells in

the genital ridge, but show normal testis cord formation (Mintz and Russell 1957). *Sry* expression was analysed, by RT-PCR, in wild type and homozygous W^e genital ridges and it was clear that *Sry* was expressed in the absence of germ cells (Koopman, Munsterberg et al. 1990). This was the first evidence that *Sry* is expressed in a somatic cell lineage in the genital ridge. As mentioned previously, the Sertoli cell lineage is the first to differentiate in the developing XY gonad and it had been postulated that the testis determining gene may be expressed in this cell type. The Sertoli cells in the testis and the granulosa cells in the ovary, have long been proposed to arise from the same lineage.

Evidence from our lab suggests that *Sry* is expressed in the pre Sertoli cell lineage (Sekido R, *manuscript in preparation*). Endogenous *Sry* expression is no longer detected after 12.5 dpc, when overt testis differentiation is occurring. However, using a 14 kb *Sry* regulatory region to drive a human placental Alkaline phosphatase marker (hPLAP), *Sry* positive cells could be traced as hPLAP is a very stable protein and is detectable long after *Sry* transcription is switched off. Cells labelling positive for both hPLAP and SOX9 indicated that the cells in which *Sry* is expressed differentiate into Sertoli cells. This is the first direct evidence in the mouse that *Sry* directs the supporting cell precursors to become Sertoli cells. However, hPLAP is not sufficiently stable to be used as a lineage label in XX embryos, to address whether granulosa cells come from the same cell precursor as Sertoli cells.

To address the origin of granulosa cells and whether they share the same precursor as Sertoli cells, inducible DAX1:ER Cre transgenic lines were generated (Johnson, A. and Albazerchi, A., *unpublished data*). The inducible approach was chosen as it has been shown that DAX1 is expressed in the pre-implantation embryo (Johnson A, PhD Thesis, *pers comm*). By crossing the DAX1:ER Cre mice with Rosa 26 Floxed lacZ reporter lines, and administering Tamoxifen at approximately 10.5 dpc, LacZ could be used as a marker to address what cell types the supporting cell precursors give rise to in XX and XY embryos.

Albrecht *et al* generated an *Sry*-EGFP reporter mouse line, using a 200bp 5'sequence to drive EGFP followed by an SV40 polyA sequence, to address the hypothesis that Sertoli and granulosa cells arise from the same precursor lineage, (Albrecht and Eicher 2001). This construct was used with the expectation that the *EGFP* transgene expression would be identical to that of the endogenous *Sry* gene. However, most transgenic lines showed no expression, or ectopic expression. One transgenic line, analysed as a homozygote, did show expression restricted to the developing gonad that appeared to initiate at the correct time. However, *EGFP* RNA was still detected in the gonads at 15.5 dpc, three days after endogenous *Sry* is turned off. The authors used this line for subsequent experiments, even though the fact that it clearly did not mimic endogenous *Sry* expression was completely overlooked.

To examine EGFP in the indifferent genital ridges, protein expression was analysed using an anti-EGFP antibody (for early stages) as auto-fluorescence was undetectable. EGFP was first detected in a group of cells in the centre of the undifferentiated gonad at 11 dpc and at later stages was detected at the rostral and caudal poles of the gonad (Albrecht and Eicher 2001). A similar centre to pole expression of endogenous *Sry* was detected by *in situ* hybridisation (Bullejos and Koopman 2001).

EGFP expression was detected inside the testis cords at 12.5 dpc by autoflourescence, and was still detectable at 13.5-15.5 dpc using an anti-GFP antibody (Albrecht and Eicher 2001). No EGFP was detected in XY gonads after 18.5 dpc. By marker analysis, EGFP expression was detected in Sertoli cells. These authors also examined expression of EGFP in XX gonads as a lineage marker. Their analysis shows that EGFP expression is detected in XX gonads at 15.5 (a time when their construct is still being expressed, as shown by RT-PCR). EGFP was also detected in XX gonads at 18.5 dpc in the presumptive granulosa cells, a derivative of the supporting cell precursors. Classical

lineage labelling is normally based on marker analysis independent of gene transcription. However, the findings from this work are based upon analysis of a reporter construct, using the *Sry* promoter, that is being expressed long after the endogenous *Sry* expression is detected in the gonad. The EGFP expression does not reflect the expression of the endogenous *Sry* gene. This may be explained by the fact that a 200 bp sequence (part of the promoter/5'UTR) was used to drive EGFP, as opposed to the characterised mouse *Sry* regulatory elements, found in 14kb of sequence flanking the gene. However, the conclusions were that the Sertoli cells and granulosa cells were derived from the same lineage, the supporting cell precursors.

I.IX *Dax1* and its role in sex development

In the absence of *Sry*, or when *Sry* gene activity is defective, the bipotential gonad differentiates into an ovary, and therefore we can perceive the female as the default pathway. However, this does not mean that there are no factors that play a role in early ovary development and differentiation. Are there "ovary determining genes" that can instruct the XX bipotential gonad to follow the female pathway in the absence of *Sry* or are there simply "anti-testis" factors that function to block the male pathway or both? We can predict certain requirements for an "ovary determining or anti-testis gene". The first requirement is that it would be expressed in XX gonads at the same time or just after *Sry* would be expressed in XY gonads. Secondly, loss of function mutations in an ovary determining gene should not perturb testis development in an XY embryo, while gain of function mutations would give sex reversal in XY individuals causing them to develop as females. Third, loss of function mutation of an ovary determinant in an XX individual should lead to either agenesis or to testis differentiation, this would not occur if it was just an anti-testis gene.

I.IX.I *DAX1* and Dosage Sensitive Sex Reversal

In humans, a locus involved in sex determination had been mapped to Xp21, where a duplication of this region gives male to female sex reversal in XY individuals. X-inactivation normally occurs to silence one copy of X chromosome genes in the female, however, the duplication would lead to an increase in gene dosage. XY individuals would express two copies of genes within this region and hence the syndrome is called Dosage sensitive sex reversal (DSS). While a duplication of genes within this region is sufficient to cause sex reversal, deletion of this region in XY individuals leads to male development, implying that these genes are not required for testis development. However, such patients do show adrenal hypoplasia. The minimal duplicated region in DSS patients was mapped to 160 kb, and a gene, termed *DAX1* (Dosage sensitive sex reversal, Adrenal hypopoplasia congenita on the X chromosome) was identified within this region, that was found to have point mutations in XY patients with Adrenal

Hypoplasia Congenita. This gene became a candidate for DSS (Bardoni, Zanaria et al. 1994).

I.IX.II *Dax1*

Dax1 is a two-exon gene encoding an unusual member of the orphan nuclear receptor superfamily. Like most nuclear receptors it has a classical C-terminal ligand binding domain but it lacks the characteristic zinc finger DNA binding domain. Instead the N-terminal domain is composed of three and a half repeats of a 65 amino acid sequence that has not yet been identified in any other protein. The mouse protein sequence shows significant similarity with the human DAX1 sequence, 65% identity and 75% similarity overall, the identity being even higher in the ligand binding domain. This gene, like *Sry,* is a rapidly evolving gene. The N-terminal domain is cysteine rich and these residues (at amino acid positions 38, 41, 43, 66, 67, 69, 105, 1008, 110, 134, 135and 137) which are predicted to interact with Zn^{2+} ions are 100% conserved between mouse and human (Guo, Mason et al. 1995). This sequence has been proposed to encode a novel type of DNA binding domain.

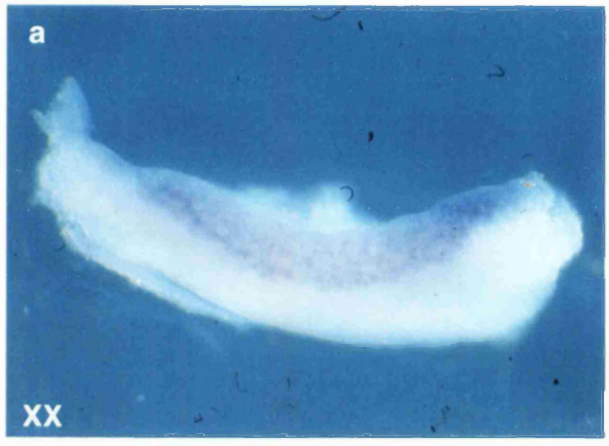
In mouse, *Dax1* is expressed at approximately the same time as *Sry,* at 10.5 dpc in XX and XY gonads. Expression levels peak at around 11.5 dpc in both sexes (Fig. 5), but by 12.5 dpc *Dax1* expression is almost completely absent in XY gonads but is maintained in XX gonads (Swain, Narvaez et al. 1998). DAX1 is co-expressed with the other orphan nuclear receptor, SF1, in the adrenal cortex, hypothalamus, pituitary gland and in the gonads.

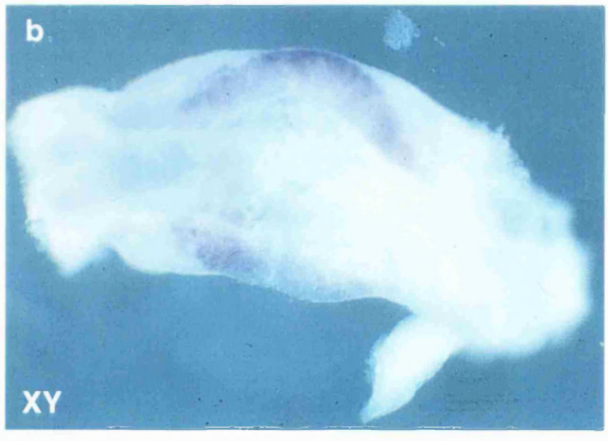
In humans, X-linked adrenal hypoplasia congenita (AHC) and associated hypogonadotropic hypogonadism (HH) are caused by mutations in the *DAX1* gene(Zanaria, Muscatelli et al. 1994). This disorder is characterised by adrenal insufficiency that usually presents in early infancy. The adult zone of the adrenal cortex fails to develop and the fetal zone fails to regress. The disorder, which is lethal if untreated, results in adrenal insufficiency in early infancy, with low serum concentration

of glucocorticoids, mineralocorticoids and androgens. Later in life, affected males fail to undergo puberty and are therefore infertile. Heterozygous carrier females are normal, and no females homozygous for AHC mutations have been reported, presumably because of the infertility associated with affected males. However, it is possible that a heterozygous female carrier could present some of the AHC phenotype due to X inactivation of the wild type allele, although this has not yet been reported in the literature. On the other hand, although X-inactivation is normally random, mutations in X-linked alleles often skew the pattern of X-inactivation seen to protect the non-mutated allele. Almost all of the mutations that result in AHC map to the C-terminal domain of DAX1. The developmental defects associated with AHC are similar to those observed in the mouse null mutant for SF1. The similarities of these abnormalities and their shared sites of expression implicate *SF1* and *Dax1* in the same genetic pathway.

Fig. 5 *Dax1* expression is identical in XX and XY gonads at 11.5 dpc.

(a) *In situ* hybridisation shows the expression of *Dax1* in 11.5 dpc XX gonads similar to that observed in (b) a pair of 11.5 dpc XY gonads. *Dax1* is absent from the mesonephros in both cases.


a
XX


b
XY

Exactly how DAX1 functions has been the centre of much debate. While no homology was found for the predicted N-terminal sequence, the C-terminal domain was very similar to domain E of the nuclear hormone receptor superfamily. Evidence was first presented to suggest that the novel N-terminal sequence of DAX1 could bind double stranded DNA, like the N-terminal DNA binding domains of the nuclear receptor superfamily (Zanaria, Muscatelli et al. 1994). However, some of the authors later showed that DAX1 preferably bound single stranded DNA in the form of hairpin structures (Zazopoulos, Lalli et al. 1997). These experiments were carried out *in vitro,* and it was proposed that DNA binding is efficient to stems composed of only 10-24 nucleotides. The sequence of the stem loop also appears to be important for DAX1 binding. DAX1 appeared to bind hairpin structures in its own promoter and in the promoter of another target gene, *StAR*, where it repressed transcriptional activation. It also seems that there are other mechanisms by which DAX1 can act as a repressor. One such mechanism involves the recruitment of corepressor molecules, such as N-CoR, to mediate transcriptional inactivation of targets such as SF1 (Crawford, Dorn et al. 1998). Alternative mechanisms of DAX1 function have been described from *in vitro* experiments where it has been shown that DAX1 and SF1 interact via direct protein-protein interaction (Ito, Yu et al. 1997; Natchigal, Hirokawa et al. 1998). Finally, deletion experiments carried out by two independent laboratories have identified transcriptional silencing domains for DAX1, mapping both to the N-terminal and C-terminal domains respectively (Ito, Yu et al. 1997; Lalli, Bardoni et al. 1997). The most likely mechanism, although not yet proven, is where SF1 and DAX1 heterodimers recruit co-repressors (such as NCo-R) and repress transcription of genes that would otherwise have been activated by SF1 alone.

Most recently, DAX1 has been proposed to act as a shuttling RNA binding protein (Lalli, Ohe et al. 2000). DAX1 was shown to be present in both the cytoplasm and the nucleus in both COS cells transfected with full length DAX1 and in a mouse Leydig tumour cell line. Zanaria, *et al* describe the nuclear localisation of DAX1 using Western blot analysis (Zanaria, Muscatelli et al. 1994). Very small amounts of protein were detected in the cytoplasmic fraction, but this may be explained by contamination. In

humans, mutations in DAX1 that impair transcriptional repression were also claimed to significantly impair RNA binding in the *in vitro* assays (Lalli, Ohe et al. 2000). However, there is no additional data to support these findings or any physiological or phenotypic consequences described *in vivo*. However, DAX1 was shown to be associated with actively translating polyribosomes in the cytoplasm, and complexed with poly(A)$^+$ RNA. Finally, DAX1 was reported to associate with ribonucleoproteins (RNPs) in the nucleus in addition to ribosomes in the cytoplasm. In an attempt to block energetic metabolism, by treatment with cyclohexamide and incubation of cells at 4°C for 4 hours, the authors found that DAX1 protein accumulated in the nucleus. Their conclusions were that DAX1 is shuttled out of the cytoplasm in a temperature sensitive manner, indicating that energy is required. They propose that DAX1 may be involved in the process of RNA export from the nucleus to the cytoplasm and that the protein shuttles between the two compartments. It still remains unclear whether DAX1 associates with specific RNA species or is involved in general RNA export. Although DAX1 has been shown to bind double stranded DNA, hairpin structures (single stranded DNA), RNA and protein (NCo-R, and SF1), we are still unclear as to how the protein functions in sex determination, although the latter, and its ability to antagonise SF1 action, provides a straightforward and logical explanation.

I.IX.VI *Dax1* and sex determination

As *DAX1* was a very strong candidate for DSS, it was decided to test this in mice using transgenic approaches. This study specifically addressed whether extra copies of murine *Dax1* could induce XY female sex reversal (Swain, Narvaez et al. 1998). A genital ridge specific *Dax1* regulatory element was isolated which gave gonad specific expression. This 11kb *Dax1* 5' flanking sequence when used to drive LacZ gene expression in both XX and XY gonads gave a pattern identical to that of endogenous *Dax1*. It did not give rise to expression at other sites, such as the adrenal, pituitary and hypothalamus. This promoter fragment was functionally tested by using it to drive expression of *Sry* (*Dax1:Sry*) in XX embryos, with the prediction that it should result in

female to male sex reversal. These mice are phenotypically male, and show no obvious differences when compared to wild type males. Four out of six independent liveborn *Dax:Sry* XX animals were sex reversed and developed as phenotypically normal looking males identical to mice transgenic for *Sry* itself. As expected, these mice were infertile as a result of a spermatogenic block at the pro-spermatogonia stage. This gonad specific *Dax1* promoter was used to drive *Dax1* expression (*Dax:Dax)* to test whether an extra copies of *Dax1* in XY gonads can induce male to female sex reversal. Of all of the liveborn transgenic animals, no XY mice developed as females.

To address whether ovotestes or delays in testis determination were occurring, genital ridges were dissected from transgenic mice and scored for testis cord structures and *Amh* expression. In one line, 1812, with five times the normal levels of *Dax1* transcripts and protein in the genital ridge, testis cord development and the onset of *Amh* expression were retarded but appeared to recover at later stages. This was a good indication that the over expression of *Dax1* had some effect on testis differentiation. Hemizygous animals from line 1812 were mated together to generate homozygous animals and although all XY homozygotes develops as males, their testes were approximately one third normal weight and they experienced a spermatogenic block. These studies revealed that overexpressing *Dax1* might mildly antagonise *Sry* function. As has been discussed previously it is believed that in order for *Sry* to act as the dominant trigger in sex determination, both levels and timing of *Sry* are critical factors. It was then decided to test the over expression of *Dax1* against a weaker than normal allele of *Sry*. This was genetically feasible as a *Mus domesticus poschiavinus* Y chromosome (Y^{POS}) on a C57BL/6 genetic background gives sex reversal, but on an outbred or mixed background gives a delay in testis cord formation, of about 14 hours, but normal fertile males result. This delay in testis cord formation is thought to be due to lower levels or a slight delay in expression of the *poschiavinus Sry* allele. Female mice from the *Dax:Dax* 1812 line were crossed to XY mice carrying the Y^{POS} allele. From this cross, several XY^{POS} transgenic mice that were born were sex reversed from male to female. In addition ovotestes were consistently found during development in XY embryos with the ovarian component ranging from a small portion to comprising almost all of the gonad. This

presented a very clear argument that against a weak allele of *Sry*, extra copies of *Dax1* can compete with *Sry* activity and induce ovary determination in XY animals.

We propose that under these circumstances *Dax1* may act in a dominant manner with respect to *Sry,* by competition in terms of timing and/or levels of gene expression within the bipotential gonad. To support this model, *Dax:Sry* XX mice were found to display a delay in testis development, when compared to wild type XY embryos. We propose that expression of *Dax1,* both from the 11kb promoter characterised, and presumably the endogenous promoter, occurs a short time (hours) after the onset of *Sry* expression. *Dax:Sry* line D:S23 gave 100% female to male sex reversal, however, when this line was crossed with the *Dax:Dax* line 1812, all XX double transgenic offspring developed as normal females. This abrogation of sex reversal implies that when driven by the same promoter elements, expression of *Dax1* could functionally antagonise SRY. The spatial and temporal expression of *Sry,* with respect to *Dax1,* in an XY gonad, results in *Sry* acting as the dominant factor at the beginning of the complex network of gene activity.

I.IX.III Regulation of *Dax1*

Multiple consensus SF1 binding sites have been mapped to the sequences upstream of the start site of *Dax1.* SF1 was shown to be able to bind to proximal binding sites and activate the *Dax1* reporter constructs containing these sites *in vitro* (Yu, Ito et al. 1998). *In vivo* evidence also demonstrated that SF1 is required for *Dax1* expression in the developing mouse gonad (Hoyle, Narvaez et al. 2002). Moreover these results differed from those obtained *in vitro.* Using transgenic approaches, a single SF1 site was identified more distal than already described in the *Dax1* 5' regulatory region, that was responsible for the initiation of *Dax1* expression specifically in the gonads. The proximal SF1 binding sites were not required for initiation but act as quantitative regulators of *Dax1* expression. Neither the distal nor proximal sites were required for maintenance of *Dax1* expression. This study demonstrated a role for SF1 in the initiation of *Dax1* expression and also showed that it is not specifically responsible for

the maintenance of expression. This is consistent with the difference in *Dax1* expression observed between XX and XY gonads. While it has long been anticipated that SF1 may play a role in the regulation of *Dax1* expression, high levels of *Dax1* expression are maintained later in XX gonads where *SF1* is downregulated. Conversely, SF1 is expressed in XY gonads at the time when *Dax1* is being down regulated, consistent with the finding that it is not involved in the maintenance of *Dax1* expression.

I.IX.V *Dax1* and its role in steroidogenesis

Steroid hormones are synthesised in a number of organs such as the adrenals, gonads, placenta and CNS. Despite the origin of synthesis, all steroid hormones have a common precursor, namely cholesterol. It is the delivery of cholesterol to the site of its first enzymatic reaction that is the rate limiting and hormonally regulated step in steroidogenesis. Cellular cholesterol, residing in the outer mitochondrial membrane in lipid droplets, or plasma membranes of steroidogenic cells, must be deposited to the inner mitochondrial membrane where cytochrome P450 side chain cleavage (P450scc) enzyme converts cholesterol to pregnenalone, the first steroid formed in all steroidogenic cells. StAR has been identified as the transporter that brings cholesterol to the inner mitochondrial membrane. Specifically, it has been shown that the C-terminal domain of StAR functions in cholesterol transfer from the outer to inner mitochondrial membrane (Arkane, Sugawara et al. 1996; Wang, Liu et al. 1998). As mentioned above, DAX1 has been shown to act as a repressor of transcriptional activity of SF1, either through direct interaction with SF1 or through recruitment of nuclear co-repressors. Additionally it was shown that through binding to DNA hairpin structures, DAX1 could inhibit the transcriptional activity of the *StAR* promoter in *in vitro* reporter assays (Zazopoulos, Lalli et al. 1997). This demonstrated that DAX1 could act as a negative regulator of steroidogenesis at the rate limiting step. This data was controversial as it had been previously proposed that DAX1 bound specifically to double stranded DNA. It has also been shown that apart from regulating the transcriptional activity of StAR, DAX1 can interfere with steroidogenesis at multiple levels along the pathway, by inhibiting

expression of P450scc and 3β-HSD enzymes. However, no hairpin structures within the critical regulatory regions have been described for these genes. Interestingly, it has been reported that SF1 can mediate the cAMP-dependant transcriptional activation of P450scc, P450c17, P450 aromatase, and StAR (Clemens, Lala et al. 1994; Michael, Kilgore et al. 1995; Zhang and Mellon 1996).

I.IX.VII A conditional Cre-mediated disruption of *Dax1*

To address the function of *Dax1* in mice, gene targeting was carried out (Yu, Ito et al. 1998). Initially, a standard strategy was used to generate a null allele, whereby a selectable marker, neomycin, replaced exon 1 of *Dax1*. However, although targeted clones were identified, they failed to grow and to generate undifferentiated clones of ES cells. This implied that there is a requirement for DAX1 expression in ES cells. Similar results were obtained in our lab, when a straightforward targeting strategy was used to mutate DAX1 and it was subsequently shown that DAX1 was expressed in ES cells (C. Wise and A. Swain *pers comm*). In order to overcome this lethality, a conditional approach was undertaken, whereby the second exon of *Dax1* was flanked by LoxP sites (Yu, Ito et al. 1998). Using Cre-recombinase, this second exon could be removed to test the function of DAX1. Exon two of *Dax1* encodes the latter half of the ligand binding domain. It has been shown that mutations in the ligand-binding domain of DAX1 result in AHC.

A similar adrenal defect to that which is observed in AHC patients was detected in the mouse conditional mutants, where the fetal zone of the adrenal cortex failed to regress. Targeted females crossed with homozygous CMV:*Cre* males generated knockout males ($Ahch^{\Delta 2/Y}$) which were hypogonadal with testis weights approximately 50% of normal wild type litter controls. Immature testes show a lack of stratification of the germinal epithelium. At later stages seminiferous tubules exhibit a wide range of epithelial dysgenesis and degeneration. By 14 weeks, germ cells were completely absent. DAX1 therefore appears not to be required in the early stages of spermatogenesis but may be required for the maintenance of germinal epithelium integrity in the adult. Homozygous mutant females showed no obvious defects in sexual maturation, ovulation or fertility. Ovary sections from homozygous mutant females revealed that some follicles contained multiple oocytes. This disorganisation may be functionally related to a similar defect in Sertoli cell interaction with germ cells.

In humans, most AHC patients report familial cases arising from a maternal origin, from heterozygous female carriers, although *de novo* mutations are also found. (Muscatelli, Strom et al. 1994). AHC males are infertile and therefore cannot transmit the mutated allele, explaining why AHC has not been detected in females. However, the process of X-inactivation implies that AHC could affect a female carrier, but if this did occur the phenotype may be so subtle that it could remain undetected.

If *Dax1* is an ovarian determining gene, we would expect this conditional mutation to affect primary sex determination, either to give XX males or at least some sign of testicular development. No such problem was reported, which leads us to interpret *Dax1* as an "anti-male" factor, rather than one that actively promotes female development. However, there are several caveats with the data and experimental designs. First, the authors did not look in detail at the developing gonads around the time of sex determination to see if there were any subtle perturbations of ovary development.

Secondly, the strategy used to generate this conditional mutant may not have resulted in a true null allele. Only exon two, which encodes the terminal half of the ligand binding domain, was floxed with *loxP* sites. This may have been the preferred choice so as to mimic the human AHC mutation, where most of the mutations are mapped to the ligand binding domain. The authors report that primers corresponding to exon one and exon two were used in an RT-PCR experiment to detect any remaining full length transcript. No primers were reported to have been used that correspond specifically to exon one or including the intronic sequences to check if any truncated transcript remained. In addition no immunohistochemistry was described to show complete elimination of DAX1 protein. We could therefore raise the question whether this conditional knockout represents a true DAX1 null allele. Finally, we could speculate that DAX1 is part of a redundant system, where more than one gene needs to be inactivated to reveal signs of XX male sex reversal.

I.IX.VIII Genetic Background effects of sex determination

Genetic Background is a very important parameter when observing cases of sex reversal with *Sry* transgenes. In approximately 30% of cases, XX mice transgenic for the 14kb *Sry* fragment developed as males. The animals were bred on a mixed CBA x C57 BL/10 (F1) genetic background. The failure of the remainder of these mice to show sex reversal is most likely due to site of integration of the transgene, otherwise known as a "position effect" leading to a low level of expression, below the threshold level needed to trigger Sertoli cell differentiation. One specific line of *Sry* mice, 32.10 was generated where the founder was an XX female. No sex reversal was detected among the offspring, but when these were crossed together, or when the males were bred onto other genetic backgrounds, XX transgenic males were found. This effect can be explained by genetic background. When the *Sry* transgene is on an outbred MF1 genetic background no sex reversal is observed. On a C57BL/6 background a low level of sex reversal is observed but on a CBA background approximately 60% is observed. Levels of gene expression were examined by RNase protection which revealed that on an MF1 background *Sry* transcripts were barely detectable but were close to 50% of the normal level on a CBA background (Hacker, Capel et al. 1995). Another case where genetic background specifically affects sex reversal, is observed when the Y Poschiavinus allele (Y^{POS}) of *Sry* is on a C57BL/6 (B6) background. XY^{Pos} embryos on this inbred background invariably show sex reversal, with most developing as females or hermaphrodites. However, some of the hermaphrodites appear phenotypically male at birth. This is a common phenomenon in mouse where ovotestes usually regress to either testes or ovaries, but usually the former (Eicher, Washburn et al. 1982). On an outbred or mixed background however, Y^{POS} gives a delay in testis cord formation, but normal fertile males result. It has been suggested that the sex reversal associated with Y^{POS} is associated with gene regulation, i.e. timing and levels of expression, and stability of SRY protein.

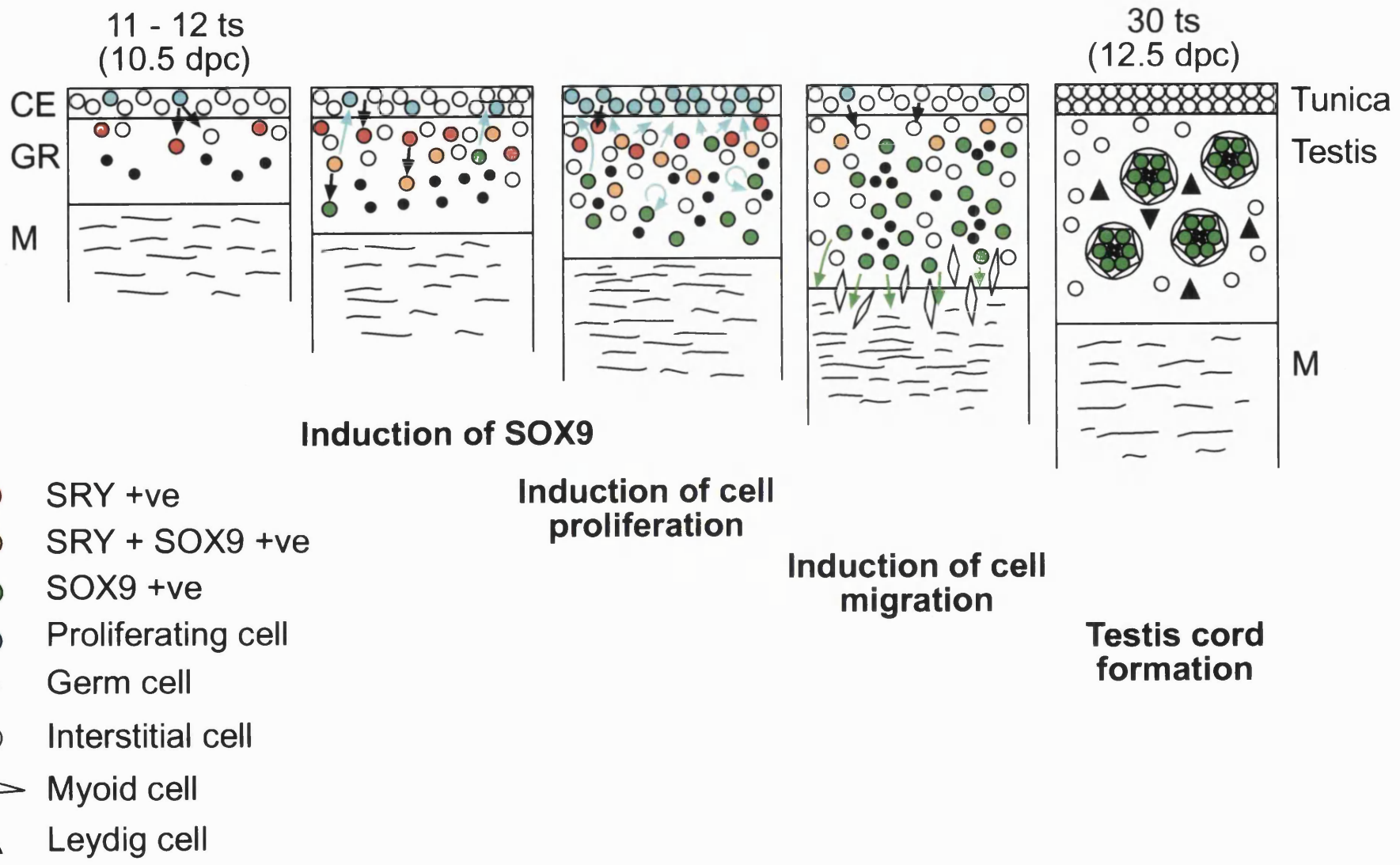
It was proposed that the sex reversal observed in C57Bl/6J (B6) mice carrying the Y chromosome of *Mus domesticus poschiavinus* was a result of the incompatibility between X-linked or autosomal genes in a B6 background and *Sry* from the Y^{Pos} chromosome (Eicher 1988). To map autosomal genes that may be involved in sex reversal on a Y^{Pos} B6 background, genetic crosses were conducted using B6 Y^{Pos} and DBA/2J (D2) mice (Eicher, Washburn et al. 1996). D2 mice were chosen as the Y^{Pos} chromosome gives XY males on a D2 background and in F1 offspring from matings between D2 females and B6 XY^{Pos} hermaphrodites. Three autosomal genes designated *tda* (testis determining, autosomal) 1, 2 and 3 were mapped and proposed to predispose XY^{Pos} embryos to ovarian development, but that no single locus or any combination of these loci alone were necessary and sufficient to cause sex reversal. *tda1* maps to the distal part of chromosome 4, *tda2* maps to the central region of chromosome 2 while the third gene, *tda3,* maps to chromosome 5. Statistical analysis and genetic linkage analysis demonstrated that the evidence was much stronger for *tda1* and *tda2*, but that a third gene might be implicated in C57Bl/6J Y^{Pos} sex reversal. It was suggested that genes within these loci might in fact regulate *Sry* or be involved in the formation of complexes with SRY to regulate target genes. Alternatively, *tda* genes might be regulated by SRY. Fine mapping analysis is currently underway to identify the *tda* alleles.

I.XII A model relating gene expression and cell biology of the XY gonad

The coelomic epithelium is a source of cells that migrate into the gonad and adopt a Sertoli cell fate upon the expression of *Sry* (Fig 6). *Sry* and *Dax1* are expressed at the same time during gonadal development and there seems to be a competition between these two genes to direct the bipotential gonad along either the male or female pathway. This competition may be explained by differences in timing and levels of gene expression. Once *Sry* accumulates above a threshold level it induces high levels of SOX9 expression. SOX9 initiates *Amh* expression and together with SF1 maintains expression in the embryonic gonad. High levels of *Sox9* expressing cells signal back to the ceolomic epithelium to induce proliferation and deposit more cells into the gonad that express *Sry*. The differentiating Sertoli cells, as a consequence of *Sry* expression, produce a long range signal(s) which induces cell migration from the mesonephros into the developing gonad. These cells include peritubular myoid cells and endothelial cells. Cell-cell signalling and interactions results in the organised testis cord structure. Sertoli cells come into direct contact with the germ cells and are surrounded by peritubular myoid cells to give rise to the characteristic stripy cord appearance. Interspersed between the cords are the interstitial Leydig cells.

Fig. 6 A model of the cellular events relating SRY and SOX9 expression.

At around 11-12 ts, cells are proliferating in the coelomic epithelium (blue cells) and can give rise to two populations of daughter cells that enter the genital ridge, one of which expresses SRY. Once SRY reaches a threshold level, it induces SOX9 expression (double positive cells are shown in orange). These cells can signal back to the coelomic epithelium and further contribute to cell proliferation that results in an increase in the population of pre-Sertoli cells with in the genital ridge. SOX9 expression initiates the expression of downstream targets such as *Amh* , and the subsequent differentiation of Sertoli cells results in the production of signals responsible for the migration of cells from the mesonephros. By 12.5dpc, the testis cords are beginning to form within the XY gonad, made up of the germ cells surrounded by Sertoli cells, which are arranged into tubular structures with the myoid cells around the perimeter. Interspersed between the testis cords are the Leydig and other interstitial cells.


11 - 12 ts
(10.5 dpc)
30 ts
(12.5 dpc)
CE
GR
M
Tunica
Testis
M
Induction of SOX9
Induction of cell proliferation
Induction of cell migration
Testis cord formation
SRY +ve
SRY + SOX9 +ve
SOX9 +ve
Proliferating cell
Germ cell
Interstitial cell
Myoid cell
Leydig cell

I.XIII A model describing the importance of timing and levels of DAX1 expression

Overexpression data from the mouse and human strongly suggest that *Dax1* plays a role in sex determination. However, several possibilities may be used to explain why DAX1, when overexpressed, only gave sex reversal against a weak *Sry* allele. First, *Dax1* was not solely or at all responsible for DSS. Secondly, other sex determination genes may reside within the duplicated 160 kb DSS locus that may act in concert with DAX1. Finally, levels of transgene expression might not have been sufficient to cause sex reversal. In humans, a double dose of *DAX1* activity may be sufficient to sex reverse XY patients, however, threshold levels and gene dosage may be different in mouse (see Fig 7c and f) . DAX1 may not be expressed at high enough levels when duplicated to overcome the critical threshold of *Sry* activity required for testis differentiation. Similarly, *Sry* may be expressed at much higher levels than the minimal threshold level to cause testis differentiation. Alternatively, it may be that timing of gene expression in addition to levels of activity may be important for the sex determination process. If in humans *SRY* behaves more like the *Poschiavinus* allele, it may be expressed at a level close to the threshold, such that a double dose of *DAX*1 can compete with *SRY* activity (Fig.7 c). Alternatively, if *Dax1* expression in mouse is delayed with respect to *Sry,* it may never reach the levels required to antagonise *Sry* before testis differentiation has begun (Fig. 7 f). If *Sry* expression is slightly delayed with respect to *Dax1*, then increased levels of *Dax1* might be able to interfere with testis differentiation (Fig. 7 g). Finally, the events that occur subsequent to overt testis differentiation may be different in mice and humans. In mice, it is well known that fetal ovotestes often resolve into mature testes before birth. Perhaps in human the reverse usually occurs, with ovotestes reverting to testes, or simply that patients with DSS are only identified when this happens. We propose the model that both levels and timing of *Sry* and *Dax1* expression are important for sex determination.

DAX1 is expressed in the XX gonad, along with other "anti-testis" genes, which leads to the repression of genes critical for testis development. Another transcription factor, *Gata*

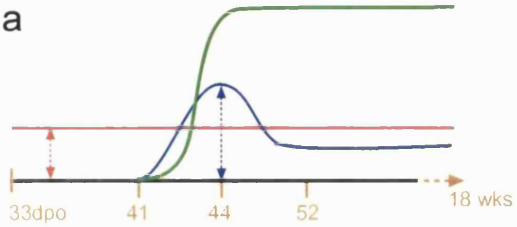
2 has recently been shown to be expressed in a sexually dimorphic manner, with expression being detected in the developing ovaries between 11.5 and 15.5 dpc (Siggers, Smith et al. 2002). In the case of female development, SF1 appears to be responsible for *Dax1* activation (Hoyle, Narvaez et al. 2002). Clearly more molecular players have yet to be identified in the female pathway before we predict a clear model for ovary determination. However, to definitively test the hypothesis that *Dax1* can act as an anti testis factor if expressed before *Sry,* and at high levels, we have aimed to use an inducible system to misexpress *Dax1* in the early undifferentiated gonad.

Fig 7. Timing and levels of gene expression are important for mammalian sex determination.

Expression patterns in human embryonic gonads are shown (left) and in mouse gonads (right). *SRY/Sry* is depicted in blue, *DAX1/Dax1* in red, and *SOX9/Sox9* in green. (a) Levels of *SRY* are higher than those of *DAX1* in human embryonic gonads and therefore it acts as a dominant trigger for testis differentiation. (d) Levels of *Sry* precede those of *Dax1* and therefore reach a threshold to induce testis differentiation. (b) *DAX1* expression in the absence of *SRY* promotes female development. (e) Similarly in mouse, where in the absence of *Sry*, *Sox9* is downregulated and the expression of *Dax1* results in female development. (c) A double dose of *DAX1* in humans can antagonise the effect of *SRY* expression and therefore promote XY female development. (f) A double dose of *Dax1* cannot antagonise *Sry* because of the slight delay of *Dax1* expression. *Sry* levels reach a threshold of expression to upregulate *Sox9* and trigger male development, before *Dax1* can antagonise *Sry*. (g) However, when *Sry* levels are lower and initiating later in embryonic development (as in the case of the Y^{POS} allele) an increased dose of *Dax1* can override the action of *Sry* and induce male to female sex reversal.

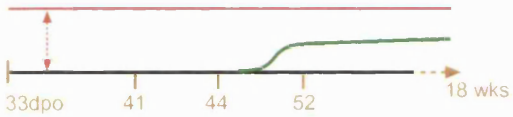
Human
XY

a



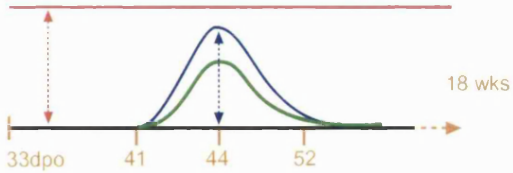
XX

b



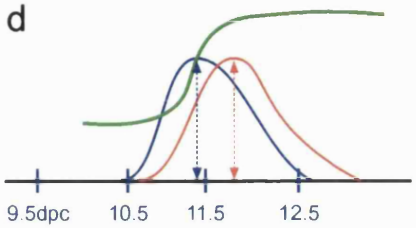
XY DSS ♀

c



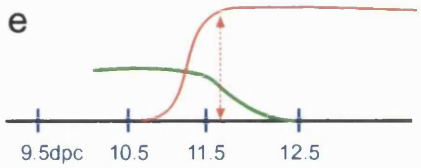
Mouse
XY

d



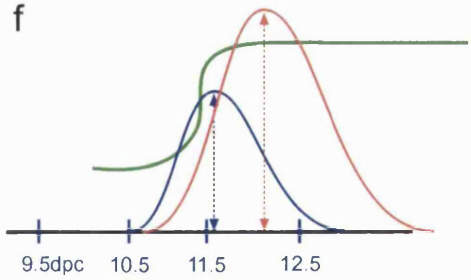
XX

e



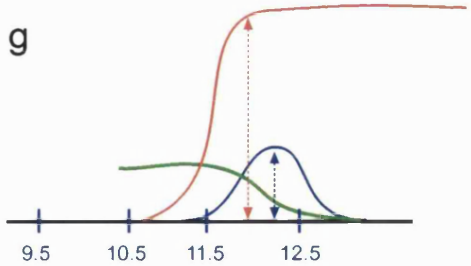
XY DAX:DAX ♂

f



XY^{POS} DAX:DAX ♀

g



Legend:

— *SRY*

— *DAXI*

— *SOX9*

dpo - days post ovulation

dpc - days post coitum

Chapter II Materials and Methods

II.I Commonly used Buffers and Reagents

LB medium per litre	-	10g tryptone, 5 g yeast extract, 5g NaCl, 1ml 1M NaOH
TE	-	10mM Tris-HCl, pH8.0, 1mMEDTA
Loading buffer 6x	-	0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol
50X TAE	-	Per litre, 242g Tris Base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA (pH 8.0)
10X TBE	-	108g Tris base, 54g Boric acid, 40ml 0.05M EDTA pH 8.0
10X PBS	-	1.3M NaCl, 70mM Na_2HPO_4, 30mM NaH_2PO_4
P1(Neutralisation buffer)		50mM Tris-HclpH 8.0, 10mM EDTA, 100µg/ml RNase A
P2 (Lysis buffer)		200mM NaOH, 1%SDS
P3 (Neutralisation buffer)		3.0M potassium acetate, pH 5.5
Tail Lysis Buffer	-	0.1M Tris, ph9.0, 1mM EDTA, 1% SDS
Ampicillin	-	50mg/ml stock solution, (500X), final conc. 100µg/ml

II.II Preparation of Plasmid DNA

Preparation of DNA (Alkaline Lysis Maxi Prep) was carried as per Qiagen instructions. Mini prep DNA (alkaline lysis) was modified from Manniatis and Sammbrook, using P1, P2, P3 as above, and precipitations were carried out in 0.7 vol. Isopropanol, followed by EtOH precipitation (Sambrook, Firitsch et al. 1982).

II.III Preparation of Plasmid DNA by CsCl Ethidium Bromide Equilibration Centrifugation

Plasmid was transformed and grown up from starter culture to a final 100 – 500ml culture volume. Cell pellets were harvested by centrifugation at 6,000 rpm and further prepared as per (Sambrook, Firitsch et al. 1982).

II.IV Cloning DNA constructs

All molecular steps used to clone DNA constructs were adapted from Maniatis and Promega Protocols and Applications Guide {Promega, 1996 #201;Sammbrook, 1982 #200}. Restriction enzymes were used according to manufacturers guidelines (NEB and Boehringer). T4 DNA polymerase was used to convert a 3' overhang to a blunt end, whereas Klenow polymerase was used to fill in a protruding 5' overhang. Vector was dephosphorylated using either Calf Intestinal Alkaline phosphatase (Promega) or Shrimp Alkaline phosphatase (Boehringer). Ligations were carried out at 16° C for 30 minutes using Takara ligase. Qiaquick Gel extraction kits (Qiagen) was used to gel purify DNA fragments < 10kb (Agarase digestion was used for larger fragments DNA constructs and for pronuclear injection, see below).

DH5α cells were used for routine bacterial transformations, using competent cells from a lab stock (cells were made using the Rubidium Chloride method, as outlined in the Promega Guide). In cases where cloning involved manipulating DNA that was likely to

recombine, Epicurian STBl 2 cells were used (Stratagene). Cells were thawed on ice for 5 minutes, DNA was incubated with cells on ice for 30 minutes and heat shocked for 25 seconds at 42°C. Cells were incubated at 30^0C shaking for 90 mins and plated on antibiotic resistant LB agar plates.

II.IV.I Constructs

*Amh:*Luc

pδ51LucII (from Ryo Sekido) was linearised with Hind III, and blunt ends were generated with Klenow small fragment. This fragment was also digested with Bam HI, generating a BamHI , blunt HindIII open plasmid. A 395bp 5' AMH plasmid was digested with Nco1, and filled in using T4 DNA polymerase. The 395 bp AMH 5' sequence was released with Bam HI, and this NcoI (blunt) BamHI promoter sequence was cloned upstream of pδ51LucII , to generate *Amh:*Luc.

*CMV:*DAX1

A 1.4kb mouse DAX1 open reading frame was cloned into pCS2+, a ubiquitous Cytomegalovirus (CMV) expression plasmid.

*CMV:*DAX1:ER

As described in chapter 3.

(i) TAG ATC TAT GGC GGG TGA GGA CCA CCC G
TAG ATC TCC CAG CTT TGC ACA GAG CAT CTC

Primer pairs (i) used to destroy the stop codon of DAX1 and to introduce a BglII site to the 3' sequence.

*CAGGS:*ER gly DAX1

As described in chapter 4.

(i) CCG GAA TTC CGG GCC CCC TCC GCC CCC TCC GCC CCC TCC GCC CCC TCC GCC CCC CAG TGT GGC AGG GAA

TCC CCCGGG GGA ATG CTC GAG CCA TCT GCT GGA GAC

(ii) CCG GAA TTC ATG GCG GGT GAG GAC CAC CCG TGG CAG

TCC CCC GGG GGA TCA CAG CTT TGC ACA CAG CAT CTC

Primer pairs (i) to destroy the stop codon of the human ER LBD and generate the glycine hinge region and (ii) to destroy the start codon of DAX1 and to introduce compatible restriction enzyme sites as follows:

741hSRY

As described in chapter 5.

II.V Cell Culture

II.V.I Cell Culture Media

Dulbeccos Modified Eagle Medium (DMEM) – Sigma

Supplemented with *

			Final Concentration per 500ml
Fetal Calf Serum (heat inactivated) (GIBCO BRL)			10%
Penicillin (GIBCO BRL)	100x	10000 U/ml	100U/ml
Stretpomycin (GIBCO BRL)	100x	10000µg/ml	100µg/ml
L-Glutamine (GIBCO BRL)	100x	200mM	2mM
*COS7 cell media was also supplemented with Sodium Pyruvate		100mM (100X)	1mM

Cells were grown at 37°C with 5% CO_2, and were washed with PBS w/o calcium and magnesium (GIBCO). Cells were passaged by treating with 0.25% Trypsin 0.02% EDTA, for 5 minutes at 37°C, and pelleted at 1000rpm for 5 mins at RT. Cells were counted in a hemocytometer, and resuspended in Cell Culture Media, and were fed every 24-48 hours. PBS 0.01% Gelatin was used to gelatinise wells. Cells were frozen in DMEM supplemented with 10% FCS and 20% DMSO

II.V.II Transient or Stable Transfection of Adherent cells by Lipofection

For immunohistochemistry, cells were plated onto gelatinised coverslips in 6-well plates, and for all other procedures cells were plated directly onto 6-well plates. In each case, COS7, NTD2 or NIH3T3 cells were counted and plated at a density of $3x10^{-5}$ on the night before transfection and incubated at 37°C in 5% CO_2. Transfections were routinely carried out under the same conditions. Cells were fed again the morning of transfection.

For each transfection , Soln I; a total of 2 μg DNA was diluted in 100 μl of Opti-MEM (reduced serum media) with Glutamax (GIBCO BRL) and Soln II; 7 μl of Lipofectamine Reagent or Lipofectamine 2000 (both from GIBCO BRL) was mixed with 100 μl Opti-MEM were set up in sterile conditions. These two solutions for each experiment were combined and mixed gently for 30-45 mins to allow DNA-liposome complexes to form. Before adding the complexes to cells, cells were washed twice with serum free medium. For each transfection, 0.8 ml Opti-MEM was added to each well and DNA –liposome complexes (200 μl) were then added. Cells were incubated for 6 hours and 1 ml of cell culture media was added to each well, containing 2x (20%) FCS. Cells were fed the following day with normal complete cell culture media.

II.V.III Transfection using $CaPO_4$-DNA precipitates formed in BES

The following protocol is based on a N,N-bisΣ (2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffered system and was performed as described in Current Protocols in Molecular Biology (Ausubel, Brent et al. 1994). Note pH of 2X BBS is critical, and must be recalibrated each time a fresh batch is prepared. All solutions were filter sterilized.

Solutions required:

2.5M $CaCl_2$, filter sterilized, (stored at –20°C)

2X BBS [BES Buffered Solution] pH 6.95- 6.98

50 mM BES

280 mM NaCl

1.5 mM Na_2HPO_4

Titrate to pH 6.95 with 1N NaOH

2X HEPES Buffered Saline (HeBS) solution (stored at –20°C)

0.28M NaCl

1.5mM Na_2PHO_4

Titrate to pH 7.0 with 1N NaOH

DNA (2μg total per well of 6-well plate) was precipitated and resuspended in 450 μl sterile water and 50 μl 2.5M $CaCl_2$. 500 μl of 2X HeBS was added to sterile 15 ml conical tube, and the DNA/$CaCl_2$ was added dropwise while the HeBS was pipetted up and down to precipitate the calcium phosphate. Precipitate was allowed to sit for 20 mins at RT. Precipitate was distributed evenly over the cells in 6-well plates and incubated at 37°C in 5% CO_2 for 4-16 hours. Media was removed and cells were washed in PBS 2X and fed with normal complete media. For transient analysis, cells were harvested 24-72 hours post transfection.

II.V.IV Tamoxifen addition to cells

4-hydroxytamoxifen (Sigma) was suspended at 10^{-3} M in 100% EtOH and kept at 4°C. Tamoxifen was generally added to cells 6 hours after transfection at a final concentration of 10^{-7}M in cell culture media.

II.VI Pronuclear Injection

DNA was digested O/N with restriction enzymes to remove plasmid backbones, and run on 0.7% Low Melting Point (LMP) agarose gels at 35V O/N at 4°C. DNA constructs were purified from LMP agarose with Agarase (Roche), 5-10 U per 100µl melted gel for 1- 2 hrs at 50°C. Precipitation with 3M Sodium Acetate was used to pellet the oligosacharides, and the supernatant (DNA) was precipitated and resuspended in 0.1X TE at 2-5 ng-µl, for injection.

Fertilised eggs at one cell stage were harvested from superovulated mice (CBA X C57 B/l 10) in M2 media, and treated with hyaluronidase (5 mins) to remove the surrounding cumulous cells. Cells were washed 5x successively in M2 before being transferred to M16 media and incubated at 37°C with 5% CO_2 until injection apparatus is set up.

Injection apparatus, injection needles and holding pipettes were prepared as described in (Hogan, Beddington et al. 1994). Eggs were injected in M2 media, using the chamber method. Injected eggs were cultured O/N at 37°C with 5% CO_2 and were transferred into pseudopregnant recipient mothers.

II.VII Primers used to genotype mice for the presence of transgenes and for sexing of mice

XES1 (A6/A9)
AGA TCA GCA AGC AGC TGG GAT ACC
XES2
TGT AGC GGT CCC GTT GCT GCG GTG

GPSry3 (741hSRY)
GGG ATA AAT ATT TTC TTA CAC ACG
GPhSRY1
AGT GCA AAG GAAGGA AGA GC

OMIA (Myogenin)
TTA CGT CCA TCG TGG ACA GCA T
OMIB
TGG GCT GGG TGT TAG CCT TAT G

ZFY (ZFY)
GAC TAG ACA TGT CTT AAC ATC TGT CC
YNLS
CCT ATT GCA TGG ACA GCA GCT TAT G

CDE1 (CMVDAXER)
CTA TCT GAA AGG GAC CGT GC
CDE2C
CAT CCA ACA TCT CCA GGA GC

II.VIII Concomitant RNA and Nuclei Isolation from Tissue

Buffer N

15mM Tris-HCl, pH 7.5

60mM KCl

15mM NaCl

5mM $MgCl_2$

2mM $CaCl_2$

1mM dithiothreitol (DTT)

250 mM sucrose

5μg/ml Protease inhibitor cocktail (Sigma)

1mM phenylmethylsulfonyl fluoride (PMSF)

Tissues or cells were harvested in ice cold PBS and homogenized in a 1ml Dounce homogeniser and pelleted for 5 min at 3,000 rpm at 4°C. Pellets were washed in 250μl of Buffer N, and a further 500 μl was added containing 0.6% NP-40. Samples were mixed gently, incubated on ice for about 5 mins, and pelleted at 3,000 rpm for 5 mins at 4°C. The supernatant (cytoplasmic fraction) was kept aside in a 2 ml eppendorf for isolation of RNA. The pellets (nuclei) were resuspended in 1.0 ml buffer N, and pelleted at 3,000 rpm for 5 min at 4°C . This step was repeated. Nuclei were resuspended in 500μl buffer N and quantitated for use (approx 5-10 μl nuclei suspension was added to 1ml 2M $MgCl_2$) by the UV absorption . (3 A260 units correspond to approximately to 100μg DNA).

1 μl RNasin (Promega) was added to the supernatant kept aside from above. Two phenol/chloroform/isoamyl alcohol (25:24:1) extractions were carried out by vortexing and spinning at 13,000 rpm for 5 minutes at 4°C. 50μl 3M NaOAc, pH 5.2, 1μl glycogen (20μg/μl), and 1.2 ml ice cold 100% EtOH were added to each sample and placed at –80°C for 30 mins. Samples were pelleted at 13, 000 rpm for 15 min at 4°C

and washed with 85% cold EtOH, dried briefly and resuspended in DEPC H_2O. RNA was stored at –80°C.

II.IX Western Blot Analysis

II.IX.I Preparation of total Protein Extract

Buffer S (Cell Suspension)	Buffer A-Total Extract and nuclear extract
10mM Tris-HCL pH 7.8	20mM Tris-HCl pH 7.8
1.5mM $MgCl_2$	420 mM NaCl
10mM NaCl	0.2mM EDTA
0.5 mM DTT	0.5mM DTT
0.5 mM PMSF	0.5mM PMSF
	1.5mM $MgCl_2$

Tissues were dissected in ice cold PBS and then homogenised in 1-200µl of Buffer S in a 1 ml Dounce homogeniser and the cells were pelleted by spinning briefly at 3,000 rpm at 4°C. Cells were resuspended in 500µl of buffer A and incubated on ice for 5 mins. Total protein extracts were frozen in dry ice and stored at –80°C

II.IX.II 6x SDS Protein Loading Dye

per 100 ml

35 ml of 1 M Tris-HCl pH 6.8
10.28g SDS
41.4 ml 87% glycerol (v/v)
9.25g DTT
0.012 g Bromophenol blue (BPB)

II.IX.III Protein Gel

10% Resolving Gel		5% Stacking gel	
H_2O	4.0 ml	H_2O	2.7 ml
30% acrylamide(BioRAD)	3.3 ml	30% acrylamide	0.67 ml
1.5M Tris (pH 8.8)	2.5 ml	1.5M Tris (pH 6.8)	0.5 ml
10% SDS	0.1 ml	10% SDS	0.04 ml
10% ammonium persulphate(aps)	0.1 ml	10% aps	0.04 ml
TEMED	0.004 ml	TEMED	0.004 ml

II.IX.IV 10X Tris/Glycine Transfer Buffer

	per 1000ml	To make 1L of 1X Working stock
Tris Base	58g	700ml Elga H_2O
Glycine	29g	100 ml 10X Tris/glycine transfer buffer
		200 ml Ice Cold MeOH
Anti mDAX1	1:1000 dilution	
Anti hSRY	1:3000 dilution	

Gels were run in BioRad Mini Protean II apparatus, using 8 x 10 cm and 7.2 x 10 cm size plates to form gels. Combs and spacers were 0.75mm. 6x loading buffer was added to protein extracts and samples were incubated at 90-100°C for 5 minutes prior to loading. Rainbow molecular weight markers from Amersham (High Range, 10-250kDa) were run along side samples, in 1x running gel buffer (BioRAD). Gels were run at 30mA/gel for approximately 45mins – 1 hr or until BPB was at the bottom of the gel. Gels were electrophoretically transferred onto Nitrocellulose membrane, sandwiched

between whatman paper and transfer pads, at 100V (150mA) for 1 hr at RT. Position and orientation of the gel was marked on nitrocellulose membrane using a ballpoint pen. Blots were rinsed 3 X briefly with PBS/0.1% Tween-20 (PBT) and blocked for 1 hr RT or 4°C O/N in 3% Carnation Milk in PBT. Blots were rinsed 4 x, 15mins and incubated with primary antibody in 0.3% Carnation milk in PBT. Note, Carnation milk was replaced with Blocking Reagent (Boehringer) when the human SRY antibody was used. All primary antibody incubations were carried out at O/N at 4°C. Blots were rinsed 4 x, for 15 mins and secondary antibody was added in 0.3% Carnation milk in PBT for 1hr at RT. Blots were rinsed 4x as before and incubated with ECL reagent (Pierce) for 5 mins. Blots were developed for 1 min, 30 sec, 15 sec, 5 sec, using Kodak Biomax film.

II.X Immunohistochemistry on cells

Cells were grown on gelatinised cover slips and rinsed with PBS w/o calcium or magnesium and fixed in 4% Paraformaldehyde (PFA) in PBS on ice for 15 mins. Cells were rinsed in PBS 2 x and then in PBS containing 0.5% Triton and 1% heat inactivated goat serum (AB staining solution). Cells were blocked in AB staining solution for 30 mins at RT. Primary antibody was incubated on cells O/N at 4°C. Cells were quickly rinsed in AB staining solution 3x and secondary antibody was incubated for 1 hour at RT. Cells were washed quickly 3x and overlaid with Vectashield with DAPI when mounting.

II.XI Immunohistochemistry on cryosectioned material

Antibody Dilutions

Anti-HSRY (ascites)	1:1000
Anti-mSOX9 serum	1:1000
Anti-cMYC (Santa Cruz)	1:250
All secondary antibodies (Cy5, FITC etc)	1:300

PBSTx

0.1% Tritonx-100 in PBS

Blocking Reagent

1.5% Blocking Reagent (Boehringer) in PBS

MEMFA	Stock	50ml
1x MEM	10x	5ml
4% Formalin	40% (Sigma)	5ml
H_2O		40ml

10x MEM; 1M MOPS, 20mM EGTA, 10mM $MgCl_2$ (pH 7.35-7.4)

Embryonic gonads were dissected in PBS and a tip of tail was kept for genotyping. Gonads were fixed in MEMFA for 30-60 mins on ice and then washed 3 x in PBS for 10mins at RT. Gonads were incubated O/N in 30% sucrose in PBS at 4°C. The next day gonads were rinsed in OCT compound twice at RT and embedded in OCT and chilled on dry ice. Embedded tissue was then kept at –80°C until sectioned. Gonads were cut into 10μm thin sections and placed onto superfrost plus slides (BDH) and airdried for approximately 30 mins. Sections were then stored at –80 °C or used immediately for immunohistochemistry. Sections were washed with PBSTx, 2x for 5 mins and blocked for 1 hr at RT. Primary antibody was incubated O/N at 4°C and slides were washed the following day with PBSTx for 30 mins at RT x5. If double immunostaining was performed, the first secondary antibody was incubated O/N at 4°C, and washed again the next day with PBSTx for 30 mins at RT x5. Sections were microwaved for 5 mins (this step was omitted when single immuno was performed). The sections were blocked as before and the second primary antibody was incubated O/N at 4°C, followed by washing in PBSTx 5x for 30 mins. The second secondary antibody was incubated as before. Slides were washed again as before and mounted with vectashield DAPI.

CHAPTER III Human SRY functions similarly to mouse SRY despite their sequence dissimilarities

III.I Introduction

The search for the testis determining factor *TDF* in humans, led to a 60kb region of the short arm of the Y chromosome proximal to the pseudoautosomal boundary (the region of homology between the X and Y chromosome, PAR) (Palmer, Sinclair et al. 1989). This conclusion was reached based on the analysis of XX sex-reversed individuals, that inherited Y sequences through abnormal XY interchange during meiosis. Subsequently, more detailed analysis of these chromosomal rearrangements showed that the breakpoints were clustered around a region that is approximately 35kb proximal to the PAR boundary, thus narrowing down the minimal portion of the Y chromosome able to confer maleness. This 35 kb sequence was used to carry out a "chromosome walk" to identify genes within the region (Sinclair, Berta et al. 1990). Subclones from this region were used to probe Southern blots of DNA extracted from human male and females, bovine and murine males and females. One particular subclone hybridised strongly to Y specific fragments in human, bovine and murine genomic DNA. The sequence of this clone was found to be conserved across male eutherian species. When translated, this 0.9 kb HincII fragment encoded a protein that shared some homology to a portion of the *S. pombe* Mc protein, the product of one of the mating–type genes, and had striking similarity to a conserved motif in several non-histone proteins related to HMG1 and HMG2. This clone was named *SRY* (sex-determining region Y) and was shown to encode a testis specific transcript. The conserved motif is now what we know to be the HMG box or DNA binding domain common to all known SRY proteins and SOX (SRY related HMG Box) proteins. The gene was proposed to be a good candidate for the elusive testis determining gene.

Coincident with the identification of *SRY,* the murine counterpart, *Sry* was also isolated and found to reside in a region on the Y-chromosome known to be involved in sex determination, and to be missing in a line of XY female mice known to be mutant in *Tdy* (Gubbay, Collignon et al. 1990). The most striking feature identified within the open reading frame was a conserved 237bp sequence, with an amino acid sequence homology of 80% compared to the conserved motif identified in human *SRY.* This region of

similarity was later shown to encode the HMG box DNA binding domain. *Sry* was shown to be expressed in the XY genital ridge, between 10.5 dpc and 12.0 dpc.

Expression of *Sry* was also detected in W^e mutant mice that completely lack germ cells in the genital ridge. Hence, soon after the discovery of *Sry,* it was confirmed to be expressed in somatic cells of the developing testis. *Sry* was subsequently shown to be the definitive testis determining gene, when XX mice, carrying the *Sry* transgene and 14kb of regulatory sequences, developed as phenotypic males (Koopman, Gubbay et al. 1991).

III.I.III *Sry* can induce testis development in XX female mice

XX mice carrying the *Sry* transgene (XX*Sry*) develop as males, with no obvious visible differences compared to wild type males. XX*Sry* males were caged with normal female animals, and although copulation appeared normal, no pups were ever born. Vaginal plugs were detected in females mated with XX *Sry* males, but the absence of mature sperm in the plug explained their infertility. The presence of two X chromosomes always results in sterility in male mice, as germ cell are arrested at the prospermatogonia stage. Moreover other genes from the Y chromosome are known to be required for spermatogenesis.

Sex reversed transgenic mice were examined internally, and no hermaphrodites were found. The vas deferens appeared normal, however the testes appeared smaller than normal males. Testis weights of sex reversed animals were about one third that of normal wild type controls. Adult testis sections revealed a recognisable testis tubule structure, but the lumen of the testis tubules were devoid of germ cells and were vacuolated, most likely due to a disruption of the cell contacts that would normally occur between germ cells and Sertoli cells.

III.I.II Comparison between mouse and human SRY.

Comparison of *SRY* sequences from different mammalian species, including humans, rodents (*M. musculus*) and rabbits, all of which are eutherian mammals, and finally a marsupial, the striped face dunnart (*Sminthopsis macroura),* indicates that the only region showing conservation is that encoding the HMG box, which binds and bends DNA (Whitfield, Lovell-Badge et al. 1993). Comparing the mouse and human SRY coding sequences, regions outside the HMG box show very poor conservation both at the nucleotide and amino acid level. Mouse SRY comprises 395 amino acids whereas the human counterpart is 204 amino acids (Fig 8). In mouse, the N-terminal domain is restricted to the first two amino acids followed directly by the 79 amino acid HMG box domain. In comparison, the human N-terminal domain is comprised of 57 amino acids followed by the 79 amino acid HMG box. These figures demonstrate the considerable differences in size and therefore sequence between mouse and human N-terminal domains. In addition, the C-termini of the two species have diverged completely. The mouse 314 amino acid C-terminal domain encodes a short linker domain followed by a long degenerative glutamine repeat domain. No similar glutamine repeat region is present in the human C-terminus, which is much shorter than that of mouse, encoded by only 68 amino acids. The HMG box domains of human and mouse SRY show 89% conservation in amino acid similarity based on automated sequence analysis and are 72% identical at the amino acid level (Koopman 2001). As the HMG box is highly conserved across mammalian species, and sequences outside of the HMG box appear to be rapidly evolving, much emphasis has been placed on the functional relevance of this domain.

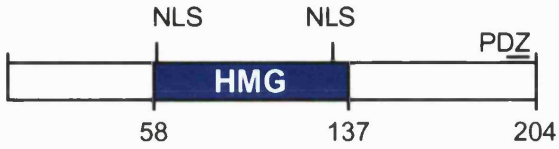
Fig 8 Comparison of Human and Mouse SRY sequences.

A. Human SRY is 204 amino acids, the HMG box domain is shaded in blue and a 7 amino acid PDZ protein interaction domain is located at the distal C-terminus. Mouse SRY is comprised of 395 amino acids, with the HMG box shaded in blue, and the large 222 amino acid Q(glutamine) rich sequence is shaded in yellow.
B. Sequence comparison on the human and mouse HMG box domains, with the conserved amino acids in boxes. The two nuclear localisation signals NLS are bracketed: these are well conserved between mouse and human. Two human mutations, defined by asterixes, perturb nuclear localisation of human SRY, leading to sex reversal.

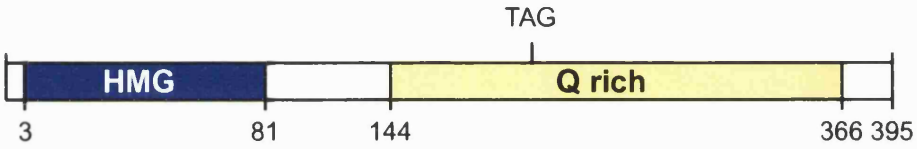
Comparison of Human and Mouse SRY Sequences

A.

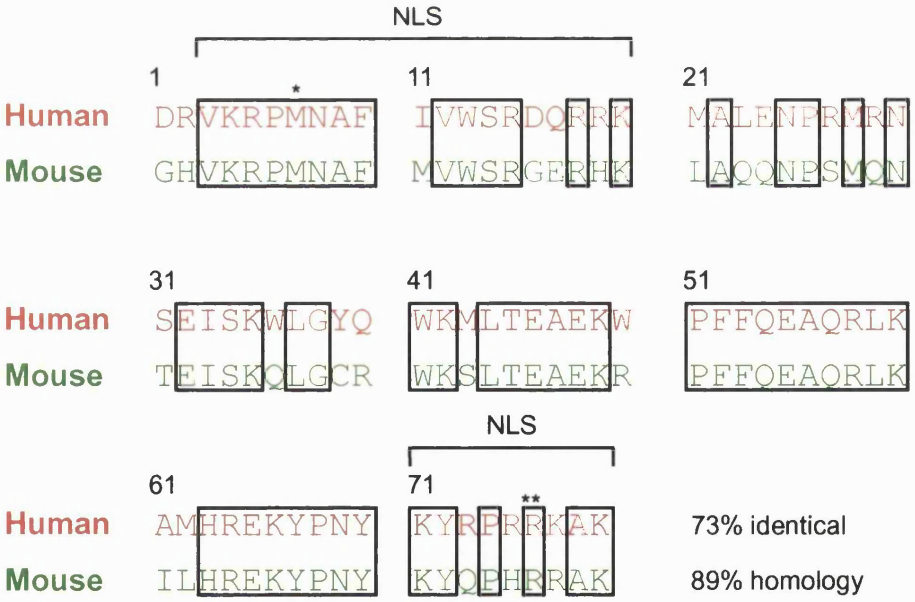
Human SRY



Mouse SRY



B.



III.I.III SRY and transcriptional activation

Random oligo selection was used to show that the preferred DNA binding site(s) of mouse SRY is A/TACAAT. Multiple copies of this site were cloned upstream of a weak promoter driving chloramphenicol acetyl transferase (a CAT reporter), and transfected along with a ubiquitously driven SRY expression plasmid into HeLa cells. It was shown that mouse SRY could activate transcription of the CAT reporter and that this depended on the presence the CAG repeat C-terminal sequence (Dubin and Ostrer 1994). The human SRY protein was also tested for the presence of a transcriptional activation domain, but, despite being able to bind the same target sequences, the analysis revealed that it did not stimulate transcription. The lack of transcriptional activity of human SRY, compared to mouse, could be explained by the lack of a CAG repetitive sequence in the human ORF. Nevertheless, the mechanism of transcriptional activation observed with the mouse protein is still unknown, although it has been proposed to involve interacting partners. The finding that the human SRY sequence did not show any transcriptional activity could explain why the human SRY sequences did not give sex reversal in transgenic mouse experiments (Koopman, Gubbay et al. 1991)

IV.II.II Human SRY and testis determination

Although genetic data argued that both the mouse and human *Sry/SRY* genes were responsible for testis determination, it was interesting to address whether the two genes were functionally identical given the lack of sequence conservation outside of the HMG box. To do this, it was important to address which domains within SRY were required for testis determination and also how the two genes were regulated.

The 14kb genomic region containing the mouse *Sry* gene, as used in the transgenic experiments, clearly carried regulatory elements sufficient to drive *Sry* expression in the developing gonad and to induce testis development in XX mice. To test whether human *SRY* could work in mice, a 25kb portion of a cosmid clone was isolated that contained the *SRY* gene flanked both upstream and downstream by putative regulatory elements

(Fig 9). When present as a transgene in mice, this human *SRY* fragment did not give XX sex reversal. By this assay, the human and mouse *Sry/SRY* genes were therefore not functionally interchangeable. However, the lack of sex reversal could be interpreted in a number of ways. First, mouse and human SRY may exhibit subtle differences in their ability to interact with DNA. Indeed, it has been suggested that the HMG boxes of mouse and human SRY may have different DNA binding and bending properties (Giese, Pagel et al. 1994). Secondly, the two proteins may interact with distinct protein partners. The latter may or may not be conserved between the two species. Third, the human gene may not be correctly transcribed in mice. Two lines, A6 and A9, were analysed and found to be expressing the transgene in the genital ridge. Although *SRY* transcripts were detected both by RT-PCR and RNase protection assays, it was not clear if these were the correct size or abundance, as there is no good data on the endogenous transcript in humans. Perhaps there were insufficient regulatory elements present within the 25 kb clone for correct transcription or post transcriptional modification. Finally, the mouse and human mouse *SRY* genes could require different regulatory factors such that even if the appropriate regulatory regions were present, the human *SRY* gene could not be correctly expressed in mice. It is notable that the regions flanking the mouse and human genes show no obvious sequence conservation.

III.II.IV Mouse and human DNA binding domains are functionally conserved

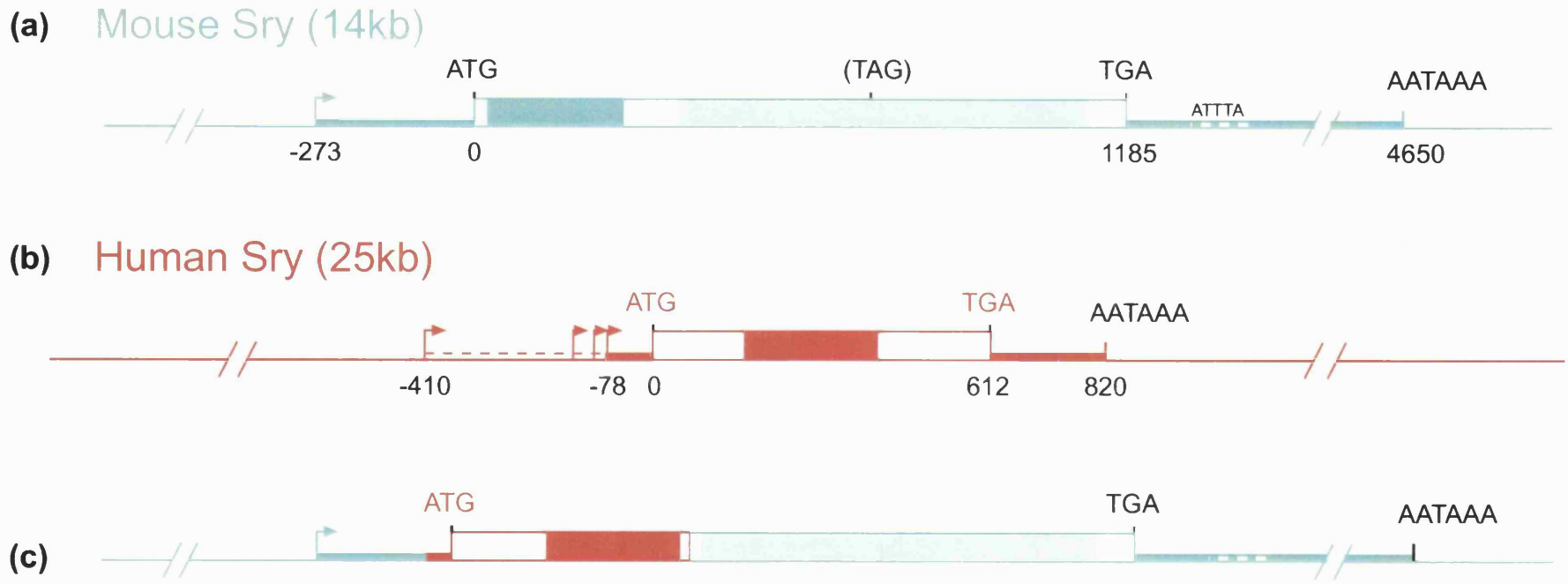
Given that the HMG box domains of mouse and human SRY are well conserved, but not identical, it was decided to test whether the DNA binding domains of mouse and human were interchangeable. Transgenic constructs were generated using the 14 kb regulatory sequences from mouse *Sry*. The human N-terminal domain and HMG box sequence were fused in frame with the entire mouse C-terminal domain (construct generated by Isabelle Barr) (Fig 9). This human/mouse chimaeric construct (named 14kb m/hHMG) was injected as a transgene to give three lines from three independent founders. Two lines gave XX sex reversal in transgenic offspring with the third giving an incomplete sex reversal phenotype. This was similar to the frequency of the original 14kb transgenic line. This result indicated that the human HMG box is functionally

interchangeable with that of mouse SRY. Moreover, the human N-terminal domain can substitute for the very short 2 amino acid mouse N-terminus, and does not interfere with function. However, this experiment did not rule out a distinct role for the C-terminal domains of the mouse and human proteins.

To test this, the entire human SRY coding sequence was placed under the control of the mouse promoter. The primary goal of this experiment was to address whether the human SRY protein could function in mouse to cause sex reversal.

Fig 9. Comparison of the SRY sequences used in transgenic sex reversal assays.

A 14kb genomic mouse *Sry* sequence that gave sex reversal in transgenic mice (a), a 25kb human SRY sequence(b) that was unable to give sex reversal, and (c) a construct where the HMG box of mouse SRY was replaced with the human HMG box domain, that was able to give sex reversal. ATG, start codon, TGA, TAG stop codons, ATTTA, an RNA instability site, AATAAA, polyadenylation site.


(a)
Mouse Sry (14kb)
ATG
(TAG)
TGA
AATAAA
ATTTA
-273
0
1185
4650
(b)
Human Sry (25kb)
ATG
TGA
AATAAA
-410
-78
0
612
820
(c)
ATG
TGA
AATAAA

III.III Results

III.III I Human SRY can functionally substitute for mouse SRY

To test whether the mouse and human SRY proteins function in a similar manner in the context of mouse testis differentiation, a construct was generated to include the open reading frame of human SRY, under the control of mouse regulatory sequences, and used to generated transgenic mice. A 0.9 kb HincII human SRY sequence, containing the entire open reading frame including its own stop codon, was engineered into the EcoRV sites of the 14 kb mouse regulatory sequences and called 741 hSRY, (the construct was generated originally by Graeme Penny, although further verified and characterised by myself) (Fig10) . This construct contained all of the previously described regulatory sequences necessary to drive mouse *Sry* in the gonad and to induce sex reversal in XX embryos. Although the mouse HMG box coding region was removed, the sequence encoding the C-terminal glutamine rich domain was left in place downstream of the human stop codon. This C-terminal mouse sequence was left intact in order to ensure that all regulatory sequences were present. Previous transgenic experiments from our lab and that of Peter Koopman and Eva Eicher that have given sex reversal have retained this sequence. Moreover, reporter constructs containing only 5' flanking sequences do not express (Barr, I, unpublished data). However it was expected that the human stop codon would prevent any downstream mouse sequences from being translated.

Fig 10. A schematic comparing 741hSRY with previously described truncation constructs that did not give sex reversal .

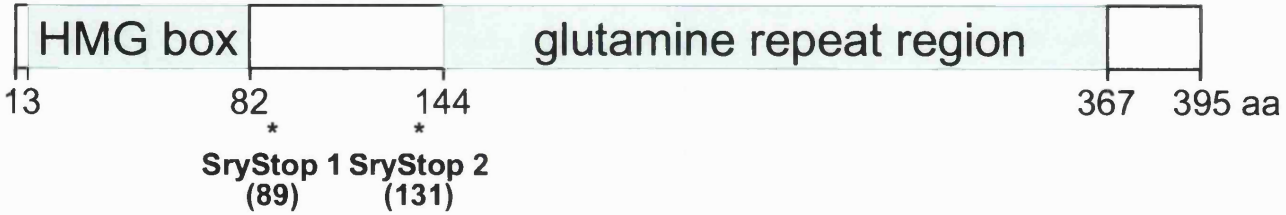
A. The 741hSRY construct expressing human SRY that gave sex reversal in transgenic XX mice, and B. the mouse SRY truncation constructs that did not give sex reversal (Bowles *et al*).

A

741 hSRY

Human SRYseq - under control of mouse elements

B



III.II.II Sequence Analysis

Sequence analysis was carried out confirming that the human stop codon was present and in frame, and that it preceded the mouse glutamine rich domain. Although no sequences downstream of the human stop codon were expected to be translated, the CAG repeat sequence would still be present in the transcript, as the first available poly A sites are those normally used by the mouse *Sry* transcript.

III.II.III Generation of Transgenic mice

The 741 hSRY construct was injected into the pronucleus of fertilised eggs harvested from CBA x C57 BL/10 superovulated mice to generate five independent transgenic lines. Two lines, 741 hSRY line 1 and 741 hSRY line5, expressed the human *SRY* transgene and gave sex reversal. Transgene expression was shown by Western blot (Fig 11) and RT-PCR analysis (data not shown). Transgenic offspring were analysed from both lines. In the case of line 1, several of XX transgenic mice were found to be sex reversed and developed as males, but not all. Line 1 was therefore an incompletely penetrant line (8 liveborn XX sex reversed mice from 32 XX transgenic animals). RT-PCR analysis showed that at 11.5 dpc not all of the gonads dissected from transgenic embryos expressed *SRY*. However, in the case of line 5, all XX transgenic mice developed as sex reversed males (17 from 17 XX liveborn transgenic mice were sex reversed males). The phenotype of these animals was completely identical to that of the original XX sex reversed mice carrying the mouse *Sry* transgene.

III.II IV Western Blot analysis of human SRY sex reversing and non sex reversing lines

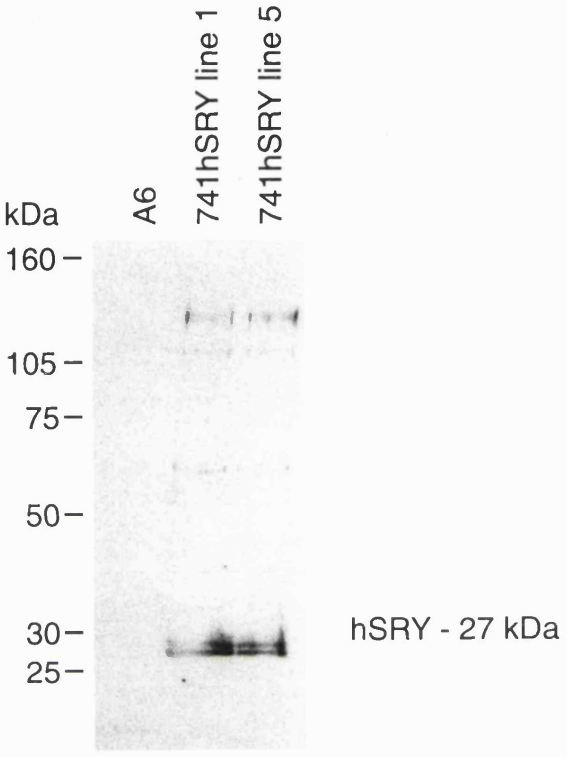
Genital ridges were dissected from embryos at 11.5 dpc from line 1 and line 5, and total protein was extracted. Western blot analysis was carried out to detect the size of the translated message to ensure that the human stop codon was used. It was expected that

only the human SRY sequences, and not the glutamine rich sequence of mouse SRY would be translated. A polyclonal antibody raised against human SRY (a gift from Ken McElreavy) detected a band of 27 kD, which is the predicted size of human protein product (Fig 11). This indicated that the stop codon was indeed functional and that no 3' mouse sequences were translated. The sex reversal observed in these lines of mice was therefore due to human SRY alone. These experiments showed that when under the control of the mouse promoter, human SRY could function identically to its murine counterpart and induce testis development in XX transgenic embryos.

The results above also confirmed that an antibody raised against human SRY could reliably detect full length human SRY expression in transgenic mice, by Western blot analysis. This was used as a positive control to address whether human SRY was expressed in the non sex reversing A6 and A9 transgenic lines. Genital ridges were dissected from 11.5 dpc embryos from the A6 and A9 lines carrying the 25kb human *SRY* clone (Fig 11). Protein was extracted and Western blot analysis was carried out to address whether the human SRY transgene was translated. No protein was detected in genital ridge extracts from these transgenic mice, explaining the lack of sex reversal. Therefore it is likely that regulatory problems, assumed to be either incorrect transcriptional initiation or post transcriptional, prevented the human SRY sequence from functioning in mouse. It is now clear that human SRY can function as its murine counterpart by inducing testis development in XX mice, but it is dependant upon specific regulatory elements not present in the original 25kb human genomic clone. These data also confirms that the 14kb mouse regulatory sequences upstream and downstream of the mouse *Sry* gene contains the necessary elements to drive genital ridge expression of exogenous genes.

Fig. 11 Detection of human SRY in sex reversing transgenic lines

Western blot analysis showing human SRY protein in 741hSRY line 1 and line 5, as shown by a 27kDa band (lane 2 and 3). These results confirm that no mouse sequences are translated. Protein extracts from A6 transgenic gonads were also used to address whether human SRY was present in the 25kb human SRY transgenics. The absence of a band (lane1) implies that no SRY protein was translated. Coomassie staining confirmed the presence of protein loaded in each lane (data not shown).

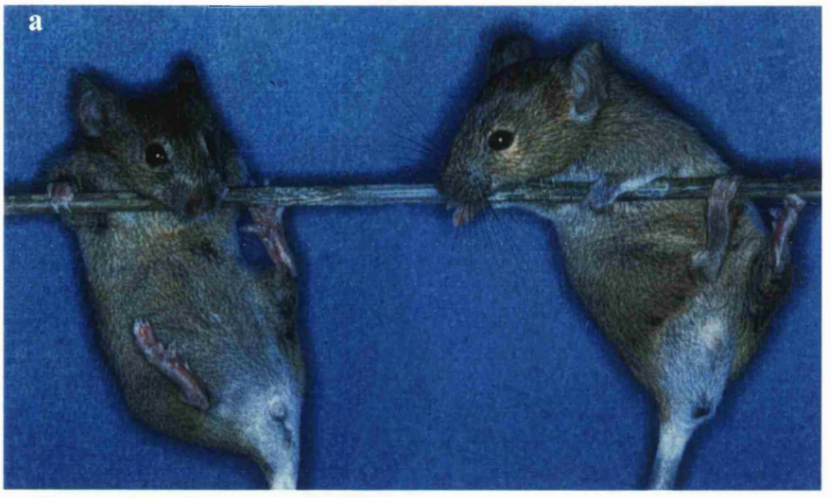

kDa
A6
741hSRY line 1
741hSRY line 5
160
105
75
50
30
25
hSRY - 27 kDa

III.II.V Morphology of the testis of XX hSRY mice

Sex reversal observed in 741 hSRY mice appeared to be completely identical to that observed in the original *Sry* transgenic experiments, but to verify this XX transgenic males were mated with females, to test fertility, and external and internal genitalia were examined. Phenotypically, the XX sex reversed males appeared identical to XY control males, but they had small testes and were infertile (Fig. 12). Adult testes were dissected from line 1 and line 5 XX transgenic males and compared to wild type testes. In all cases, wild type testes (average weight 90g) were about 3 times the weight of XX hSRY testes (average 30g). WT and sex reversed testes were sectioned and stained with hematoxylin and eosin to examine morphology (Fig 13). In comparison to normal XY controls, the structure of XX sex reversed testes was disorganised, germ cells were absent from the lumen of the testis tubules and a vacuolation appeared in the tissue surrounding the testis tubule. Sertoli cells were visible and the vacuolation may have been due to disruption of cell contact that normally occurs between germ cells and Sertoli cells. In addition, there appeared to be an increase in the number of interstitial cells in between testis tubules. Leydig cell hyerplasia can occur as a result of the breakdown of signalling between the germ cells and somatic cells of the testis. However, this can also be an illusion, because the testis tubules are much smaller than normal. The testis histology is consistent with an arrest of spermatogenesis at the prospermatogonia stage. This is due to the presence of two X chromosomes, which is incompatible with germ cell maturation, and not a result of SRY misexpression. Additionally, all other Y chromosome factors required for spermatogenesis are absent. The phenotype is identical to XX males with the mouse *Sry* transgene (data not shown, but see Koopman et al, 1991 for comparison).

Fig 12 Fig. XX control and XX hSRY sex reversed mice, and testis from XY control and XX hSRY sex reversed transgenic mice.

Upper panel compares the external genitalia of an XX control female (left) and an XX sex reversed male transgenic for human SRY (right). Lower panel compares the size of an adult testis dissected from a control XY male (left) in comparison to a testis from an XX sex reversed male (right).


a

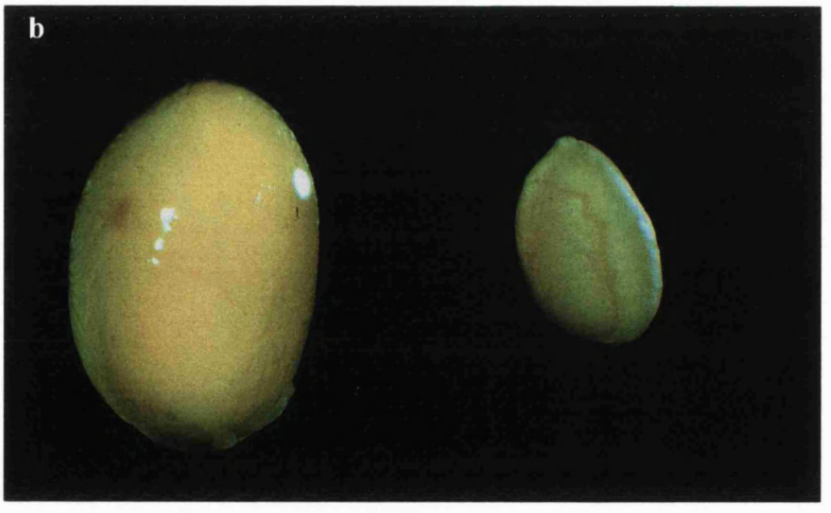
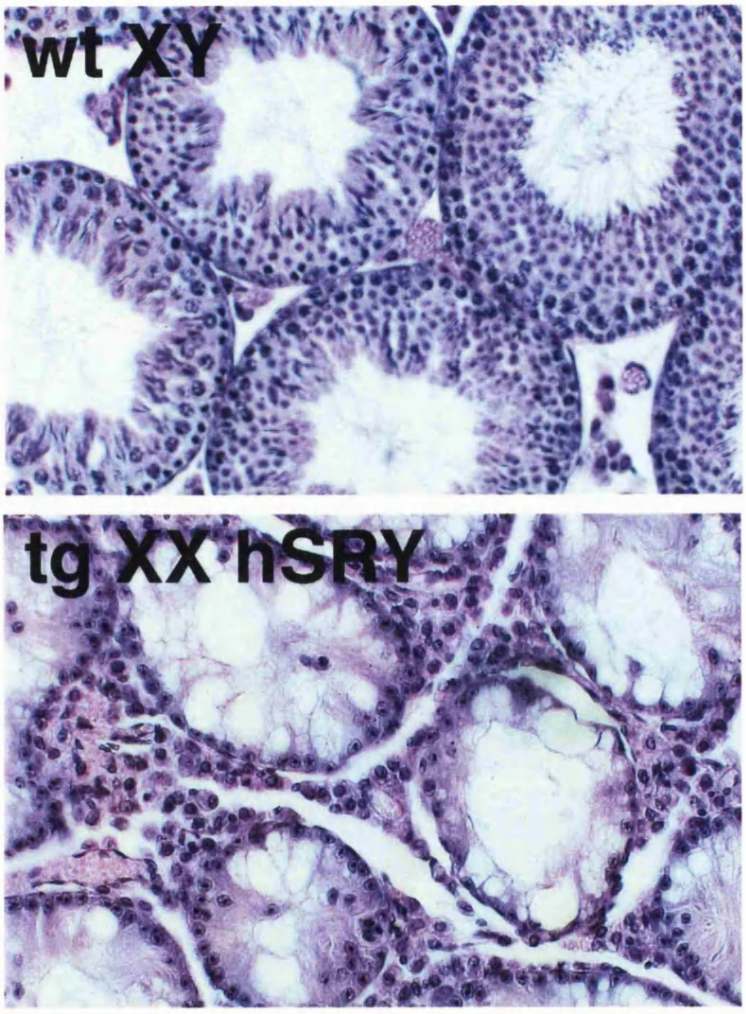

b

Fig. 13 Sections of wild type and sex reversed adult testes from mice transgenic for human SRY.

Adult testis section of 12 week old WT XY males (upper panel) stained with Hematoxylin and Eosin show well structured testis tubules, with sperm entering the lumen of the tubules, Sertoli cells are enclosed in the tubules close to the periphery, and interstitial cells lie in between the tubules. In comparison, the lower panel shows hematoxylin and eosin stained sections through the testis of a similar stage XX sex reversed animal. Testis tubules are smaller in comparison, and germs cells are absent in the lumen of the testis tubules. Vacuolation is also apparent in addition to Leydig cell hyperplasia.


wt XY
tg XX hSRY

III.II.VI Genetic Background did not have an effect of the percentage of sex reversal

Line 1 was maintained on the original mixed CBA x C57 BL/10 background but was also backcrossed onto the CBA/Ca strain to address whether genetic background affects the penetrance of the sex reversal phenotype. Transgenic XY males and XX transgenic females were crossed to CBA/Ca females and males respectively. XX transgenic offspring were scored for sex reversal and breeding was maintained through subsequent generations. No change in the penetrance of the phenotype was observed between offspring on F1 or CBA/Ca backgrounds. In addition, intercrosses were also carried out between pairs of XX transgenic females and XY transgenic males, but no increase in the penetrance of the transgene was observed. Therefore, an increase in transgene dosage did not appear to alter the penetrance of the phenotype.

III.II.VII Localisation of the human SRY protein in the embryonic testis

Human SRY was detected in total protein extracts from 11.5 dpc genital ridges, dissected from line 1 and line 5, by Western blot analysis. As line 5 gave 100% sex reversal, these mice were used to investigate protein expression in more detail. It has been shown by Sekido *et al*, (manuscript in preparation), that MYC6 epitope-tagged mouse SRY co-localises with endogenous SOX9 in XY embryos between 18ts and 28ts. These results show that mouse SRY is expressed in the supporting cell precursor lineage that gives rise to the Sertoli cells. The regulatory sequences used to drive expression of *Sry* ^{MYC6} was the same as those used here, however, it was important to check the localisation of human SRY in the early embryonic gonad, as it could have different stability and/or subcellular localisation compared to the mouse protein.

Matings were set up between line 5 hSRY XY males and XX females from a non sex reversing mouse *Sry*^{MYC6} line, to generate embryos positive for both human SRY and SRY^{MYC6}. Females from a partially sex-reversing SRY ^{MYC6} line were used, as XX mice from a fully penetrant sex reversing line are infertile males. The number of embryos recovered from these matings was low, on average between two and three

embryos per breeding pair. There was no obvious reason for the small litter sizes, but this limited the number of double transgenic embryos recovered. Embryos were staged by counting tail somites (ts), and gonads from 20ts embryos were fixed in MEMFA. The yolk sac was kept to genotype chromosomal sex of the embryos and the presence of the transgene. Transgenic embryos were then embedded, frozen and sectioned on a cryostat.

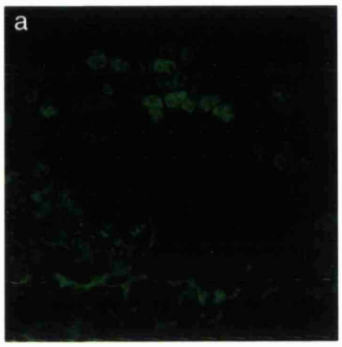
As both antibodies were raised in different species, antibody incubations could be carried out simultaneously. SRY ^{MYC6} positive cells were detected in the gonad, however, no cells within the gonad appeared to be positive for human SRY. Instead, staining with the anti-hSRY antibody was detected in the mesonephric portion of the gonads, but this appeared to be trapping in the tubular structures. Immunohistochemistry for double antibody staining was repeated under different conditions, but a similar pattern was detected in all cases.

Immunohistochemistry was therefore repeated on line 5 transgenic gonads, with the anti-hSRY antibody alone. Some cells within the gonad were positive for human SRY expression, and this appeared nuclear as expected (Fig. 14). However, staining was still detected in the mesonephros, within the lumen of tubular structures. As a control, wild type male gonad sections were incubated with the anti-hSRY antibody (Fig 15). These showed an almost identical pattern of staining in the mesonephric tubules to the transgenic gonads, but they did also show what appeared to be some nuclear staining in the gonad itself, when examined at higher magnification (Fig 16). Therefore it may be that the human SRY antibody cross reacts with mouse SRY in wild type XY gonads, although this is not entirely convincing.

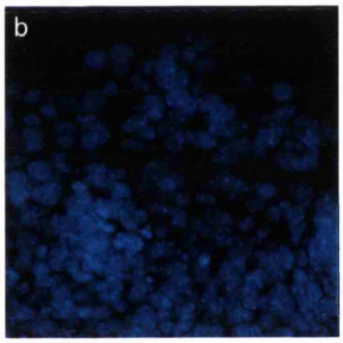
Fig. 14 Human SRY expression in line 5 transgenic gonads

Section through a 741hSRY transgenic gonad showing SRY staining in green (upper panel. Nuclei are stained with DAPI in blue (middle panel). The gonad is to the top, and the mesonephros is beneath.

hSRY



DAPI



Merge

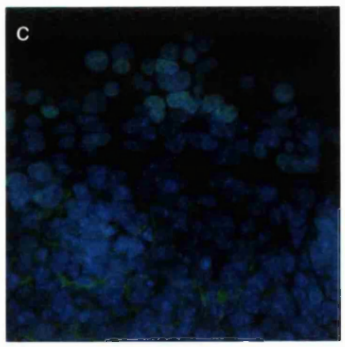


Fig. 15 Comparison of a saggital section and cross section of XY control gonads showing non specific staining of hSRY.

Background staining of human SRY(red) in the mesonephros of a saggital section and cross section of two XY control gonads (b) and (e). DAPI staining of nuclei is shown in blue (a) and (d).

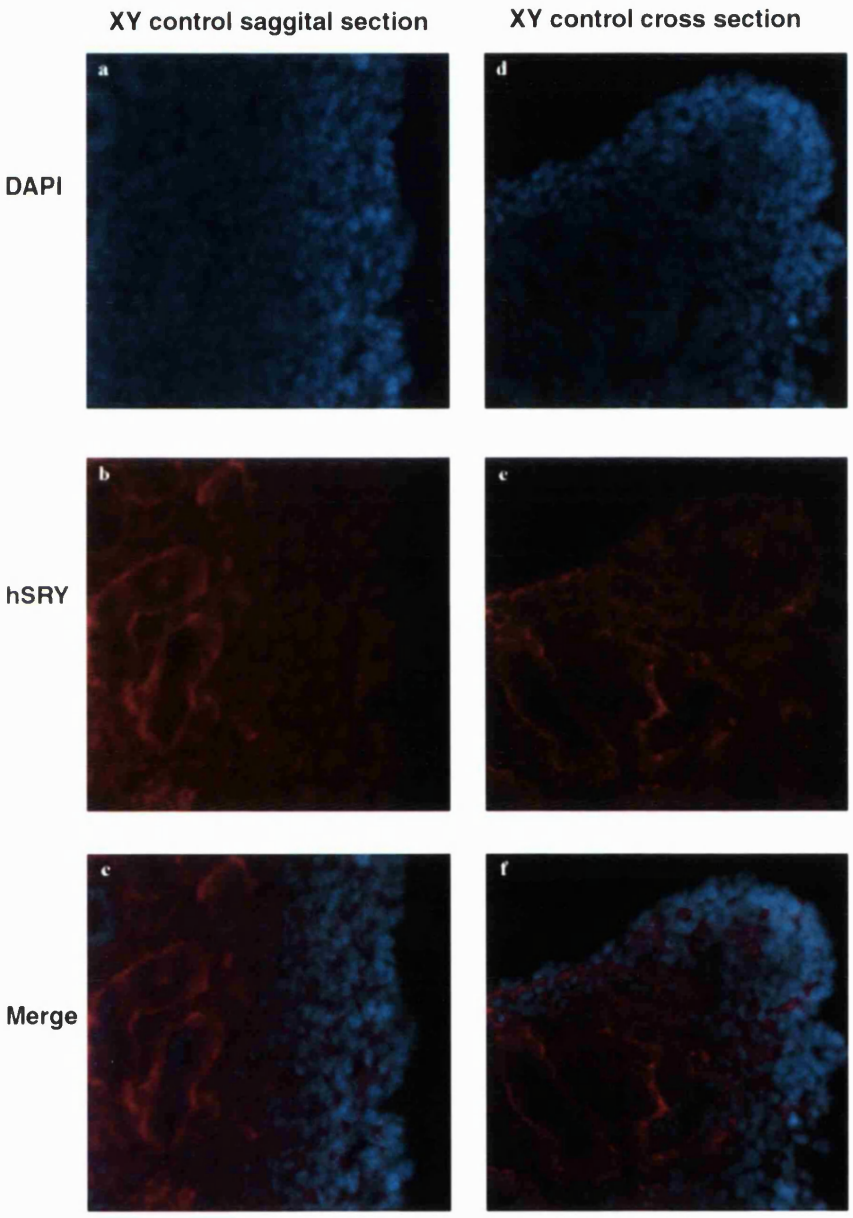
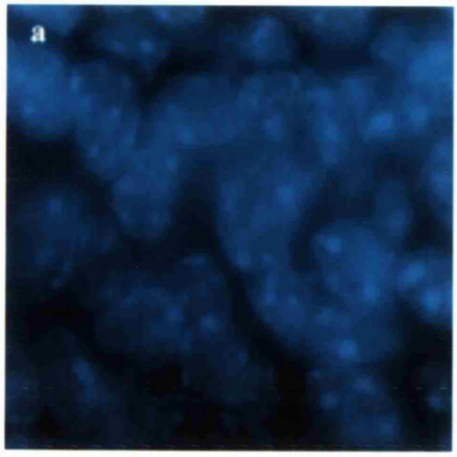

XY control saggital section
XY control cross section
DAPI
hSRY
Merge
a
b
c
d
e
f

Fig. 16 Higher magnification of non specific staining of human SRY in cross sections of an XY control gonad.

(a) DAPI staining of nuclei (blue). Staining with the human SRY antibody in red, with apparent nuclear staining(b). DAPI and human SRY signals are merged in (c).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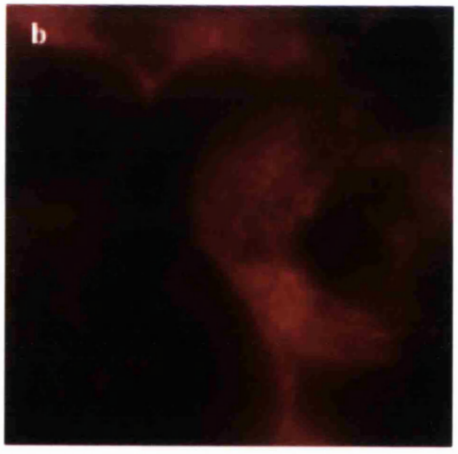
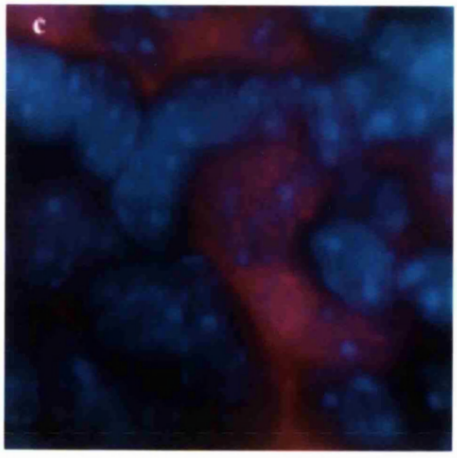

b


c

Although the embryos positive for both human SRY and SRY^{MYC6} were infrequent, gonads from 741 hSRY line 5 could be used to detect both human SRY and endogenous SOX9. As before, mostly non-specific staining was observed with the anti-hSRY antibody, while cells within the gonad were positive for SOX9. Antigen retrieval was carried out by incubating sections in 0.1M EDTA pH 5.0, but no gonad specific expression of human SRY was detected. It is clear that the anti-hSRY antibody does not work well under the range of conditions tried for immunohistochemistry on sections of mouse gonads. However, the antibody did work well in Western blot analysis. As all XX embryos and mice analysed from this line gave 100% sex reversal, we can almost certainly rule out the possibility that the transgene did not express in the particular litters of embryos analysed.

II.II.VIII SOX9 expression in wild type gonads and hSRY transgenic gonads

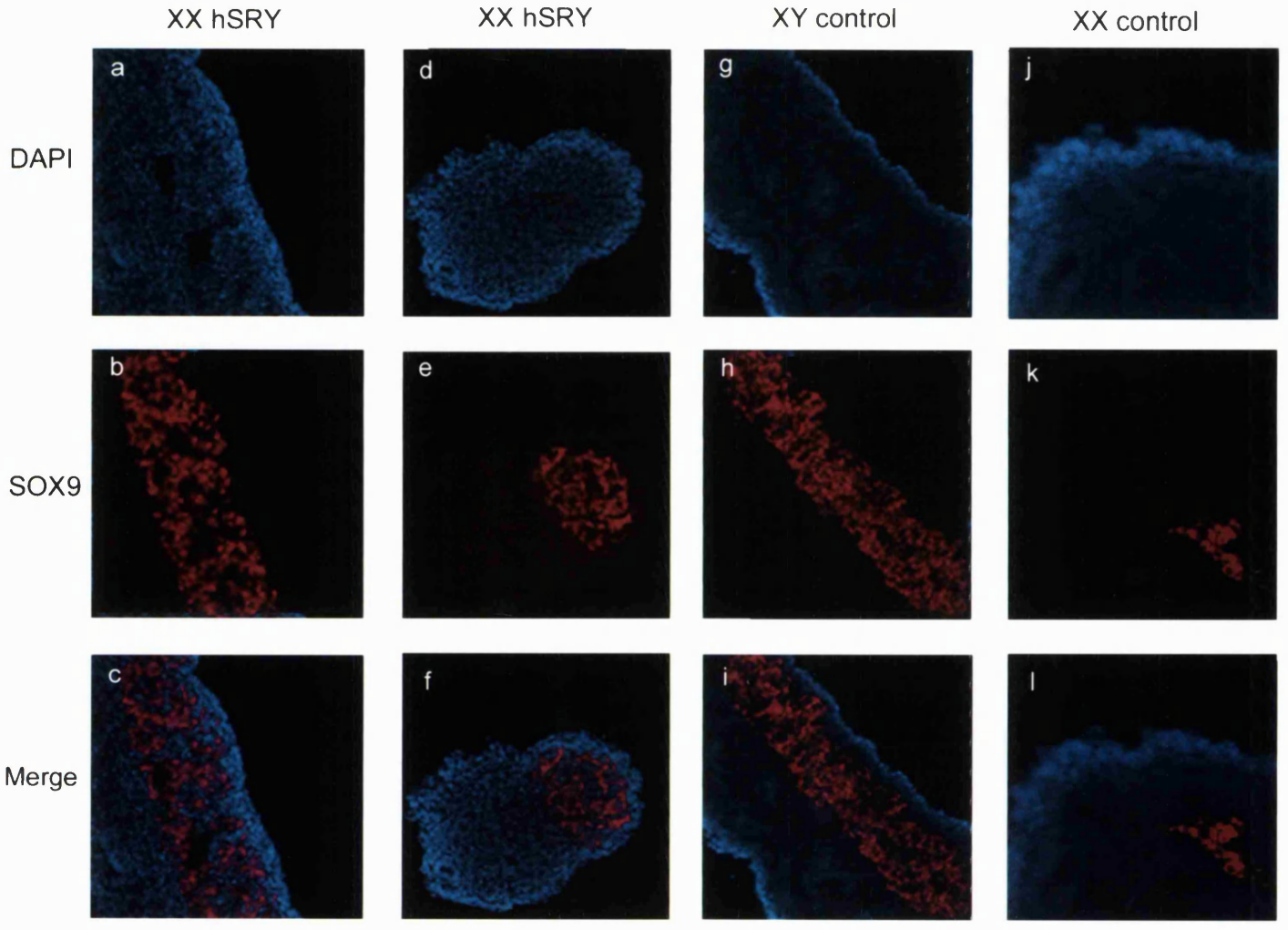
SOX9 is upregulated in XY gonads shortly after *Sry* expression and within the same lineage: the supporting cell precursors that give rise to the Sertoli cells in the testis. Indeed, it is the best candidate for an immediate target of SRY. RNA *in situ* hybridisation shows that *Sox9* is expressed at low levels in both XX and XY gonads at around 10.5 dpc, whereas by 11.5 dpc it is no longer detected in XX gonads and high levels are seen in XY gonads, (Morais da Silva, Hacker et al. 1996). We wished to address whether a similar pattern of SOX9 expression was seen in XX gonads transgenic for hSRY.

At 20ts, corresponding to approximately to 11.5 dpc, SOX9 expression was found to be present at high levels in somatic cells of wild type XY gonads. SOX9 localised to the nucleus of cells within the gonad and was completely absent in the mesonephros. In XX gonads, however, there appeared to be clumps of cells positive for SOX9, but fewer in number compared to normal XY gonads. Expression was not as strong as that observed in XY gonads and SOX9 staining appeared to be localised to the cytoplasm.

SOX9 protein was found to be strongly expressed in the gonads of XX hSRY embryos (Fig. 17). This staining was identical to that observed in wild type XY gonads at the same stage, with all the protein being localised to the nucleus and none in the cytoplasm. Therefore expression of human SRY appears to result in activation or upregulation of *Sox9* in XX hSRY transgenic gonads and to the SOX9 protein becoming nuclear rather than cytoplasmic, where the latter is characteristic of both sexes earlier. It is not possible to say whether either the upregulation or nuclear localisation of SOX9 is a direct effect of hSRY expression. The mechanism of SOX9 nuclear localisation is still unknown, but it relies on two nuclear localisation signals within the HMG box (Sudbeck and Scherer 1997). It may simply be that SOX9 expression at high levels is sufficient to overcome factors tending to retain it in the cytoplasm. Alternatively, there could be a mechanism operating at precisely this stage that promotes SOX9 translocation to the nucleus. Nevertheless, human SRY protein can trigger the correct expression of SOX9 in a similar way to that seen in normal WT XY controls.

Fig. 17 Expression of SOX9 in XX human SRY gonads compared to XX and XY control gonads.

(a), (b) and (c), Nuclear localisation of SOX9 (red) in a saggital section of an XX gonad transgenic for human SRY. (d), (e) and (f), Nuclear localisation of SOX9 in a cross section of an XX gonad transgenic for human SRY. (g), (h) and (i), Nuclear localisation of SOX9 in a saggital section of a control XY gonad, and (j),(k) and (l), Cytoplasmic localisation of SOX9 in a cross section of a control XX gonad. (j) (k) and (l) are taken at higher magnification (20X) than (a)-(f) (10X magnification.


XX hSRY
XX hSRY
XY control
XX control
DAPI
SOX9
Merge
a
b
c
d
e
f
g
h
i
j
k
l

III.III Discussion

The initial step in mammalian sex determination, which triggers cells of the supporting cell lineage to differentiate along the Sertoli cell pathway, is governed by the transient expression of *SRY*. However, it is still not clear exactly how the *SRY* gene and protein functions at the molecular level. Detailed comparisons between homologues may offer clues as to which parts of the gene are important for its function. However, the *Sry* gene appears to have evolved so rapidly that merely comparing sequences is uninformative. Comparisons between the mouse and human SRY proteins reveal that only the HMG box domain is conserved, and the same is true for any two distantly related species. Nevertheless, we know that both mouse and human genes act to initiate testis development. To understand more about the genes and the proteins they produce, it is therefore necessary to carry out functional assays, both for regulatory regions and for protein domains. Beginning with the observation that a mouse 14 kb *Sry* genomic fragment (m741) was able to cause sex reversal in XX transgenic mice, whereas a 25kb human genomic clone containing *SRY* failed to do so, we set out to ask if this was due to differences in regulation or protein structure.

III.III.I Human SRY functions during sex determination in mouse

When the HMG box of mouse SRY is swapped with that of human SRY (together with their N-terminal sequences), sex reversal was obtained, indicating that at least the DNA binding domains , and N-terminal sequences are functionally equivalent. We have now shown that the entire human SRY protein can function in the mouse to induce testis development when expressed from mouse *Sry* regulatory sequences. The XX sex reversal observed with the human *SRY* transgene is identical to that seen with the mouse *Sry* transgene.

Although the human cosmid clone used to derive the A6 and A9 transgenic mouse lines contained 25kb of flanking sequences upstream and downstream of the *SRY* gene, no sex reversal was ever observed (with several hundreds of offspring scored over many

generations and with differing genetic backgrounds). The lack of sex reversal occurred despite the presence of transcripts in the genital ridge. We have now shown that the two proteins are functionally interchangeable. It therefore seems likely that the regulatory elements within the 25 kb sequence were not sufficient to give correct expression of human SRY within the developing mouse testis. Consistent with this, Western blot analysis showed that no human SRY protein was present in the genital ridges of A6 and A9 embryos. This provides a clear reason for the lack of sex reversal, although the problem could be transcriptional, posttranscriptional, such as mRNA stability, or at the level of translation.

III.III.II Misregulation of a 25kb human SRY clone may have prevented sex reversal

Analysis, by RNase protection assays, of 11.5 dpc gonads from A6 and A9 mice revealed a number of different size *SRY* transcripts in the genital ridge (Hacker, A. *unpublished data)*. These are likely to reflect the use of multiple initiation sites and/or the use of multiple poly adenylation sites. However, it is not known if any of the resulting mRNAs are able to be translated efficiently. At least four different transcription initiation sites have been described in the literature for human *SRY*, however, none of these studies looked at the endogenous gene in genital ridges because of the difficulty in obtaining intact tissue from 6 week embryos. A major initiation site was found to be 91 nucleotides upstream of the ATG start codon, using human adult testis as a source of RNA (Vilain 1992). Two additional, more distal transcriptional start sites were found by a second group, but in this case an *in vitro* system was used, where the human *SRY* gene was transfected into mouse cell lines (Su and Lau 1993). To address in more detail the possibility that *SRY* transcription may involve multiple start sites, a range of fetal and adult tissues were examined (Clepet, Schafer et al. 1993). It was shown that *SRY* is indeed expressed in multiple tissues as well as in the fetal and adult gonads, but additional novel transcripts were also detected, at – 410 bp upstream of the start codon . It is not clear if any of these transcripts are biologically relevant for sex determination – indeed there is no obvious reason why SRY should continue to be expressed in fetal and adult testis in the human.

Perhaps the human *SRY* sequences fail to give sex reversal in transgenic mice because none of the transcripts allow correct translation. There are at least four reasons why this might be so. (i) Transcripts with an abnormally long 5' UTR might simply be translated very inefficiently. (ii) If transcripts originating from any of the initiation sites are incorrectly polyadentylated, then mRNA instability may prevent any translation. (iii) If transcripts initiated distal to a suitable ATG (or CTG) initiator codon, then translation from this might lead to either a truncated or otherwise non-functional protein, especially if it was out of frame with the normal SRY coding sequence. (iv) SRY is a single exon gene, but if the abnormal transcripts included a splice donor site, they might lead to splicing to a cryptic acceptor site, again leading to an mRNA that could not encode a protein. Something related to this occurs in the adult testis of the mouse, where an unusual splicing event occurring in a long primary transcript, leads to a non-functional circular transcript (Capel, Swain et al. 1993) Whatever the reason, the absence of human SRY protein as shown by Western blot analysis implies that transcriptional errors prevented the correct protein from being translated in mouse.

But why would the transcripts be abnormal in the A6 and A9 transgenic lines ? There are essentially two possible explanations. Critical regulatory sequences may be missing from the 25kb clone. These could include sequences that would normally prevent readthrough from sequences adjacent to the site of transgene insertion. The *SRY* genomic region may be quite sensitive to position effects as the 4 XX male patients found with just 35 kb of Y-unique sequence (used in the original positional cloning of *SRY*), showed a wide range of phenotypes from only slightly to almost completely masculinised (Palmer, Sinclair et al. 1989). Alternatively, there could be differences between the two species in the set of regulatory factors present within the early genital ridges that are required for *Sry/SRY* expression. These differences, which could be more or less subtle, may lead to preferential selection of the inappropriate human *SRY* transcriptional initiation site in the mouse genital ridge.

III.III.III A glutamine rich sequence is not required for human SRY function

The 741 hSRY construct was sequenced to verify that the human stop codon was present and in frame with the rest of the human SRY coding sequence. Sequence analysis also showed that the human poly A site was absent. The mouse sequences downstream of the human stop codon should therefore be transcribed but not translated as the transcript should finish at the mouse poly A site(s) downstream of the CAG repeat sequence. Protein extracts from transgene positive gonads, used in Western blot analysis, confirmed that only the human SRY ORF was translated, as observed by the expected 22 kDa band corresponding to the predicted size of the human SRY protein. This result ruled out the possibility that any sequence downstream of the mouse CAG repeat was translated. The ability of the 741 hSRY transgene to induce XX male sex reversal must therefore be due to the expression of the human SRY protein alone.

Bowles *et al* , in their transgenic experiments, suggested that the CAG repeat of mouse *Sry* is required during mouse sex determination (Bowles, Cooper et al. 1999). The authors presented evidence that the constructs designed to truncate SRY before the CAG repeat region, gave rise to proteins of the expected size after in vitro translation(discussed in more detail in general discussion). However, due to a lack of specific antibodies against mouse SRY, they were not able to show that any protein was present within the genital ridges of transgenic embryos from either of the two constructs.

III.III.IV Penetrance of sex reversal in transgenic mouse lines

When the human SRY coding sequences were driven by the mouse regulatory elements present in the 14 kb *Sry* genomic clone, sex reversal was observed in two independent lines. One transgenic line (741 hSRY line 5) gave 100% sex reversal, with all XX transgenic mice developing as males. In comparison, the other line (741 hSRY line 1) had a partially penetrant phenotype with only 25% of XX transgenic offspring developing as sex reversed males. No hermaphrodites or ovo-testes were ever detected.

Endogenous *Sry* expression is normally detected within a small time window between 10.5 dpc and 12.0 dpc in XY gonads. Data from R Sekido *et al* demonstrate that pre-Sertoli cells may only express SRY protein during a very short period of time, and therefore it is considered to have a very short half life. Swain and colleagues have reported waves of expression of *Sry* along the gonad from anterior to posterior, whereas Koopman and co-workers report that *Sry* expression begins in the centre of the gonad, and then is expressed towards the poles of the genital ridge (Swain, Narvaez et al. 1998; Bullejos and Koopman 2001). It is therefore conceivable that only a few cells at any given time during this 24 hour window actually express *Sry*.

In the case of line 1, it may be that the temporal expression is misregulated or that levels are too low to be detected. Differences in penetrance of a phenotype between independent transgenic lines may often be explained by transgene position effect variegation (PEV) or site of integration of a transgene. PEV may lead to mosaicism, and can therefore have a negative effect on transgene expression. As the integration of a transgene is a random process, transgene insertion into a locus may result in a phenotype that may be due to disruption of an endogenous locus, as opposed to a direct effect of the transgene expression. Negative regulatory elements nearby may result in silencing expression of a transgene, and similarly enhancers nearby may give ectopic expression of a transgene. Transgene copy number also varies upon each random insertion and may result in different levels of transgene expression in different independent transgenic lines. PEV, copy number or mosaicism, may contribute to the lack of sex reversal observed in all XX transgenic embryos, as described for line 1. When gonads from line 1 were analysed, not all of the transgene positive embryos expressed human *SRY* at 11.5 dpc , by RT-PCR analysis. A number of possibilities may be used to explain this result. Firstly, mosaicism as a result of PEV could give rise to either no expression or very low levels of gene expression within the developing gonads. However levels were not addressed in a quantitative manner. Secondly, there may be a problem with timing of gene expression, whereby *Sry* expression may have already been downregulated or not yet initiated at the time when gene expression was analysed. Precise timing of gene

expression was not analysed further, as some embryos did show expression at 11.5 dpc. Regardless of the explanation, the lack of *Sry* expression in a proportion of transgenic embryos analysed explains why only a subset of XX transgenic mice were sex reversed.

III.III.V Expression of human SRY and endogenous SOX9

Using an antibody against human SRY, we wished to examine in which cell type SRY was expressed. An anti-MYC antibody and an antibody against human SOX9 that cross-reacts with mouse SOX9 were used to address whether human SRY co-localised with SRY^{MYC6} in double transgenic embryos or with endogenous SOX9 in hSRY line 5 embryos. However, it was not possible to see expression of both antigens together, even though SRY^{MYC6} and SOX9 expression were detected when looked for individually. Experimental conditions were modified including varying blocking conditions, antigen retrieval by incubation with EDTA and microwaving samples, incubation temperature and duration for both primary and secondary antibodies, and finally dilution factors, however, no gonadal specific staining above background was ever observed. In one single experiment, cells within the gonad appeared to stain positive for human SRY, however, there was also non specific staining within the mesonephros. However, as a control, the human SRY antibody was used on genital ridge sections from WT embryos dissected at 20-22 ts stage. Non-specific staining was observed in the gonadal portion, as well as strong staining in the mesonephros that appeared as trapping in the tubules. As the human SRY antibody did not appear to work well in our hands, under varying conditions, we decided to examine the expression of SOX9 in 741hSRY line 5 transgene positive XX gonads at around 11.5 dpc or after (22 ts).

SOX9 appeared to be more strongly expressed in the gonads of wild type XY embryos than in XX gonads. In XY gonads, SOX9 expression was localised to the nucleus whereas in XX gonads the expression appeared to be cytoplasmic. These results are consistent with previous data from the mouse and human. In mouse, SOX9 is expressed in the cytoplasm of both XX and XY gonads before 11 dpc, and is nuclear in XY gonads

during Sertoli cell differentiation (Morais da Silva, Hacker et al. 1996). The cytoplasmic localisation of SOX9 appears specific to the gonadal tissue and is not detected within the mesonephros. Prior to Sertoli cell differentiation in the human, SOX9 in observed in the cytoplasm in both sexes. As the time of Sertoli cells differentiation, SOX9 becomes restricted to the nuclei of Sertoli cells but remains cytoplasmic in the female gonad at 6.5 weeks (De Santa Barbara, Moniot et al. 2000). In 741hSRY line 5 strong nuclear expression of SOX9 was observed in XX transgenic gonads identical to that seen in XY wild type gonads at the same stage (Fig 17). These results imply that although human SRY was not detected, but was assumed to be expressed, SOX9 expression was upregulated and detectable in the nucleus around 11.5 dpc.

The major classes of SOX9 mutations found in CD sex reversed patients are amino acid substitutions in the HMG box and truncations or frameshift mutations that affect the C-terminal domain, where two domains required for transactivation reside. A novel SOX9 mutation has been found in a human patient with CD that is a sex reversed XY female. This mutation can be found in the third helix of the HMG box domain and has been shown to reduce nuclear import of SOX9. This is followed by a reduction of DNA binding and reduced transcriptional activation (De Santa Barbara, Moniot et al. 2000). This mutation analysis outlines the importance for correct nuclear localisation of SOX9 during normal Sertoli cell differentiation.

Similarly, two nuclear localisation signals have been identified for human SRY, one at the N-terminal region of the HMG box and a second at the C-terminus of the HMG box (Poulat, Girard et al. 1995; Sudbeck, Lienhard Schmitz et al. 1996). A *de novo* sex reversal mutation in the C-terminal part of the HMG box of human SRY has now been shown to impair nuclear localisation but does not appear to impair DNA binding or bending *in vitro*. Therefore, sex determination and Sertoli cell differentiation appear to rely on multiple facets of gene expression, including specific timing, levels of gene expression and cellular localisation to name but a few.

III.IV. General Discussion

III.IV.I The importance of the HMG box domain

Despite the complete lack of conservation outside of the HMG box, the mouse and human SRY proteins were functionally interchangeable during mouse sex determination. This leads us to ask how these two proteins mediate their function. *SRY/Sry* genes are considered to be rapidly evolving, and the similarities of the HMG box sequences between mouse and human strongly implies that the DNA binding domain is important for SRY function. In support of this, almost all of the SRY mutations in humans leading to sex reversal can be found within the HMG box sequences. Therefore, it is possible that SRY functions primarily through its HMG box DNA binding domain, acting as an architectural transcription factor. It has been proposed that by directing DNA bending, architectural transcription factors can regulate the assembly of higher order DNA–multiprotein complexes required for transcriptional activation or repression. Structural and kinetic studies of SRY proposed that a bent DNA: protein complex may regulate transcriptional potency, implying that SRY may function in architectural gene regulation. The structure of the HMG box domain has been characterised in a *de novo* sex reversing mutation, an amino acid substitution in the HMG box domain, M64I (Methionine to Isoleucine at amino acid 64) (Murphy, Zhurkin et al. 2001). DNA binding is only minimally reduced in this mutation analysis whereas DNA bending is significantly reduced compared to the wild type HMG box, by 13°. This 13° difference in bend angle may seem modest, but it translates to large displacements of distances involved in the formation of transcription complexes. The authors predict that this mutation in SRY would significantly alter the formation of a nucleoprotein complex required for transcriptional activity.

If SRY acts as an architectural transcription factor, then the HMG box of *SRY* related genes that show considerable conservation, might substitute for the equivalent SRY sequence. The HMG box domains of mouse SOX3, and SOX9, are 84% and 76% similar to the HMG box of SRY respectively, at the amino acid level. *Sox3* is the most

closely related *Sox* gene to *Sry,* and similarly lacks an intron, and *Sox9* , like *Sry,* is involved in testis determination. Because of these similarities, the HMG box sequences of *Sox3* and *Sox9* were introduced into the 14kb mouse *Sry* sex reversing clone, to replace the *Sry* HMG box sequence(Bergstrom, Yound et al. 2000). Transgenic mice, analysed for the expression of either construct, gave either 100% sex reversal, or in some lines, homozygous animals resulted in sex reversed XX animals. These results show that the HMG boxes of both SOX9 and SOX3 can functionally substitute for the corresponding DNA binding domain of mouse SRY.

Alternatively, we cannot rule out that the HMG box of *Sry* may also function as a protein interaction domain, and the similarity between the mouse and human SRY and SOX3 and SOX9 HMG box domain sequences is sufficient to maintain a significant degree of conservation throughout evolution.

Recently it has been shown that human SRY interacts with the Androgen Receptor (AR) and can negatively regulate the transcriptional activity of AR (Yuan, Lu et al. 2001). This interaction is direct, as shown by mammalian one- and two-hybrid assays, and is mediated through the AR DNA binding domain and the SRY HMG box domain. This report indicates that SRY can interact with other factors through the HMG box domain, although the mechanism by which SRY over expression represses AR transcriptional activity is not yet understood. Although human SRY has not been shown to encode autonomous Tran activation or repressor domains, it is clear that SRY, in cooperation with other factors, may play a role in transcriptional repression. The above results are obtained from *in vitro* assays and it is unclear whether there is any interaction between human SRY (or mouse SRY) and the AR *in vivo.* However, these results underlie the importance of the HMG box domain of SRY, demonstrate that it may function as a protein interaction domain as well as a DNA binding domain. Indeed, it was also shown previously, by *in vitro* analysis, that human SRY can interact with Calmodulin (CaM) albeit with low affinity (Harley, Lovell-Badge et al. 1996). This interaction has also been shown to occur through the HMG box domain of SRY, although CaM can also interact with the HMG box domain of HMG1, although this domain is only

25%identical to that of SRY. Since the first half of the HMG box domain of SRY includes as stretch of basic amphiphilic amino acids, this may be sufficient to display weak CaM interaction, as CaM binding proteins share similar basic amphiphilic sequences. Again, no physiological role has been determined for an interaction between human SRY and CaM *in vivo*.

SOX proteins that require specific protein partners also have protein interaction domains that overlap the HMG box. SOX2 in particular is a good example, as it cooperates with the octamer binding protein OCT3 (also known as OCT4 and recently renamed to as POU5F1) though a protein: protein interaction involving the HMG box domain of SOX2 and together, they synergistically activate the FGF4 enhancer (Yuan, Corbi et al. 1995; Ambrosetti, Basilico et al. 1997; De Santa Barbara, Bonneaud et al. 1998).

IIII.IV.II Does SRY require a CAG repeat sequence encoding a Q-rich domain?

Apart from the HMG box domain of mouse SRY, the only other obvious sequence is the Q-rich domain (encoded by a partially degenerate CAG repeat sequence) which is unique to rodents. However, we have shown that human SRY functions during mouse sex determination in the absence of a glutamine rich domain, begging the question of functional relevance for this sequence during mouse sex determination. It has been previously suggested that the CAG repeat was required for *Sry* function during sex determination (Bowles, Cooper et al. 1999). The authors showed that the 14 kb genomic *Sry* sequence caused sex reversal in XX transgenic mice. By generating *Sry* constructs deleted for the CAG repeat sequence, the authors did not see sex reversal in XX transgenic embryos. Two truncated constructs were generated, introducing premature stop codons directly after the HMG box domain, or immediately before the CAG repeat sequence. However, neither of the truncated transgenes gave sex reversal in XX mice, although transcripts were detectable. *In vitro* transcription and translation experiments demonstrated that these two constructs could be translated into proteins of the appropriate size. The lack of sex reversal, however, indicated that the CAG repeat encoded Q-rich region was required for SRY function in mouse sex determination. It was not possible, however, to examine whether either truncated transcript gave the

expected translated product *in vivo,* as no mouse SRY antibody was available. Therefore, we cannot rule out the possibility that the truncated constructs did not produce proteins *in vivo.* Many laboratories have experienced problems in generating a reliable antibody against mouse SRY. This may be due to the lack of optimal sequence against which to raise an antibody, as the N-terminus and HMG box sequences are both unsuitable, the former due to the fact that it is encoded by only two amino acids and the latter may result in cross reactivity to other SOX proteins. This leaves only the glutamine rich region to raise antibodies against, however this have never proved successful. Epitope tagged fusions are therefore an alternative option to analyse SRY protein *in vivo* using transgenic approaches.

The CAG repeat domain however, may be required for RNA and/or protein stability. In our transgenic analysis described above, the CAG repeat sequence should be transcribed but we have shown that it is not translated. To address the possibility that this sequence is required for *SRY* mRNA stability, a 741 h SRY CAG repeat deletion construct could be made, and tested in mice for XX sex reversal.

Bowles *et al* also suggest an alternative role for the Q-rich domain and its interspersed FHDHH polar sequences; the formation of ß-strands that dimerise with interaction proteins. This type of interaction may be necessary to mediate transcriptional activation by recruiting other factors through this domain.

III.IV.III The Q-rich domain and SRY interaction proteins (SIP's)

It has been described that the Q-rich domain of mouse SRY binds three SRY interaction proteins (SIP 1, 2 and3) that were initially found to be expressed in the adult testis (Lau and Zhang 1998). Mouse SIP 1, 2 and 3 are also detected in the embryonic gonads at 11-13dpc and so far are not found in any other mammal apart from mice. Surprisingly, the authors also report that the majority of protein bindings, using Far-Western techniques, were detected in the interstitium and the periphery of the seminiferous tubules of adult

mice. In the adult testis, *Sry* transcripts are present as circular RNA's, and are continuous with no stop codon and appear not to be translated , in comparison to the linear transcript expressed in the mouse genital ridge(Capel, Swain et al. 1993; Clepet, Schafer et al. 1993; Jeske, Bowles et al. 1995). SRY is expressed in no cell type other than Sertoli cells in the developing embryonic gonad, and is not a secreted factor or membrane bound. Therefore the evidence that SIP binding to mouse SRY appears stronger in the interstitial regions of the mouse adult testis may be due to non-specific binding detected in this *in vitro* assay. However, if the Q-rich domain of mouse SRY is a functional domain required for interaction with SIP's, this interaction must be mouse specific, as the Q-rich domain is mouse specific and these particular SIP's are not found in any other mammal.

Perhaps human SRY has specific co-factors or interaction partners that are expressed in both the mouse and human embryonic gonad, and therefore it can activate the same targets and mediate the same function as its murine counterpart. Alternatively, it may be that human SRY does not require any interaction partners at all but can functionally replace mouse SRY simply be through its HMG box domain, as this has been shown to function ,*in vitro,* as both a DNA binding domain and a protein interaction domain.

III.IVIV Identification of a human SRY SIP

As is the case for mouse SRY, interaction proteins have also been identified for human SRY. A nuclear factor, containing two PDZ protein interaction domains, has been isolated that can bind the distal 7 amino acids of the hSRY C-terminus(Poulat, de Santa Barbara et al. 1996). No comparisons exist between the mouse SIP's and this human PDZ domain SRY interaction protein (this will be PDZ SIP, as to distinguish it between mouse SIP1,2 and 3). Similarly, there appears to be no homology between the terminal 7 amino acids of hSRY and the Q-rich domain of mouse SRY. Unlike the case in mouse, PDZ SIP is expressed in a wide variety of different tissues apart from the gonad, suggesting that the function of this interaction protein may not be restricted to the sex determining process. This also fits with data suggesting that hSRY is expressed in other

tissues apart from the developing testis. It has been shown that PDZ SIP can interact with SRY proteins forma variety of different species with the exception of rodents. Therefore, if an interaction protein is required for human SRY function, then it must also be expressed in the developing mouse gonad. To test whether PDZ SIP is specifically required for human SRY function, this 7 amino acid sequence has been deleted from human SRY. This type of deletion construct could then be tested fro its ability to cause sex reversal in transgenic XX mice.

Neither SIP 1, 2 or 3 or PDZ SIP have yet been shown to be functionally required for SRY in mouse or human testis determination. However, it is possible that these distinct SRY interaction partners can bind differentially to mouse and human SRY, and serves as adaptor molecules to regulate the same downstream targets.

III.IV.V SRY and SOX9 during sex determination and testis differentiation

The most likely function of *Sry*, in mouse testis determination, is the activation or upregulation of a downstream gene that is responsible for maintaining the developing male pathway. *Sox9* remains a likely target of *Sry* as it is expressed in the same cell type at a similar time point, and Sekido *et al* has shown a genetic interaction between *Sry* and *Sox9*, as *Sox9* expression is altered in SRY null mice(R Sekido, manuscript in preparation). It has also been shown that *Sox9/SOX9* plays an important role in testis determination both in mouse and human (Huang, Wang et al. 1999; Bishop, Whitworth et al. 2000; Vidal, Chaboissier et al. 2001). When SOX9 is expressed early in the genital ridge, before the onset of *Sry* then male sex determination and differentiation is initiated in the absence of *Sry*. Two possibilities can be used to explain this result. First, SOX9 has been suggested to auto regulate its expression implying that a short burst of SOX9 expression is sufficient to initiate and maintain the male pathway. Second, SOX9 may mimic SRY function by virtue of the similarities between their HMG box domains, implying that all that is required of SRY is architectural transcriptional regulation.

III.IV.VI A novel function for SRY and SOX proteins

Most recently, Ohe *et al* proposed that SRY and two SOX proteins, SOX9 and SOX6, were proposed to be involved in pre mRNA splicing (Ohe, Lalli et al. 2002). They proposed that SRY , SOX6 and SOX9 are localised in splicing factor nuclear speckle domains, and were shown to be co-localised with general splicing factors. These localisation patterns are also detectable in embryonic tissues, although this data was not shown. Furthermore, by *in vitro* splicing assays, SRY, SOX6 and SOX3 were shown to have pre-mRNA splicing activities. However, these results are somewhat controversial as SOX proteins have been shown to bind to DNA in the promoter regions of target genes. It has never previously been shown, either *in vivo* or *in vitro,* that SOX proteins or SRY can bind to RNA. However, the authors suggest that SOX proteins operate through a common biochemical mechanism, pre-mRNA splicing, in regulating mammalian sex determination and other developmental processes.

III.IV.VII Conclusions

The importance of *SRY* genes in sex determination has not prevented it from strong selective forces that have caused it to evolve rapidly in mammals. *SRY* genes have evolved at least 10 times more rapidly than other SOX genes, and outside of the DNA binding domains, their sequences show little conservation. This raises the question as to whether the non-conserved sequences have evolved to acquire specific functions, or whether the conserved HMG box domain remains relatively unchanged because of a conserved functional requirement during mammalian testis determination. We favour the latter, and propose that SRY functions most likely as an architectural transcription factor, and through binding and bending DNA, SRY can recruit complexes required for transcriptional activation or repression. In addition, it is possible that the HMG box domain may also play a role in protein: protein interactions.

We have shown that despite the lack of sequence conservation outside of the HMG box domain, human SRY is able to trigger testis determination in XX mice, in a similar manner to mouse SRY. Evidence is still required to show that SOX9 is a direct target of SRY, but it remains a likely candidate. In XX hSRY transgenic gonads, it appears that SOX9 is upregulated and therefore maintains the male pathway of testis differentiation in XX mice, which would otherwise be switched off. The most likely function of SRY is therefore the initiation of the male pathway, and because of its short temporal expression is dependant on downstream factors to maintain the pathway of male development.

Chapter IV An Inducible System to Misexpress DAX1

IV.I Background

In order to achieve temporal and spatial control of transgene expression, much work has made use of tissue specific promoters. Where a promoter region has not been well characterised, or there is a lack of a suitable regulatory region to drive expression in a specific spatial and temporal manner, misexpression studies often rely on gene targeting into a defined locus. This is achieved by homologous recombination in ES cells, followed by blastocyst injection and the generation of chimaeric mice. The use of a ubiquitous promoter, such as cytomegalovirus (CMV) or β-actin, provides an alternative method to misexpress a transgene. However, this is not ideal for genes that may give rise to developmental defects at sites of expression other than those being investigated. In particular, this type of misexpression, which can often be at inappropriately high levels, may mask an expected phenotype, especially if it occurs early in the embryo. The generation of an inducible system permits the introduction of mutations within a given gene, using perhaps a Cre-Lox strategy, or the misexpression of a gene in a temporally and spatially controlled manner.

The Estrogen Receptor (ER) inducible system is a very useful strategy currently used in gene targeting studies and over expression analysis both *in vitro* and *in vivo.* We chose the ER inducible system to address whether misexpression of *Dax1* before the onset of *Sry* expression could interfere with the function of the latter in sex determination, and thereby lead to female development in XY embryos. The ER is a member of a large family of ligand inducible nuclear hormone receptors whose activities normally depend on binding of a hormone ligand. The natural ligand for the Estrogen Receptor is Estradiol (but more commonly referred to as the agonist, Estrogen), but many synthetic agonists have been manufactured that can bind to the ER under normal physiological conditions. Mutations have also been introduced in the ligand binding domain of the mouse ER that block binding of the endogenous estrogen but permit binding of synthetic ER ligands, such as Tamoxifen (Tm) or a derivative, 4-hydroxytamoxifen (4-OHT) (Danielian, White et al. 1993; Littlewood,

Hancock et al. 1995). Under normal conditions, in the absence of ligand, estrogen receptors are found associated with heat shock proteins (HSPs) in an oligomeric complex in the cytoplasm, and are therefore transcriptionally inactive. Only after ligand binding can the ER become active. It has been shown that in the absence of estrogen the ER specifically binds HSP 90 in the cytoplasm, and in response to ligand binding undergoes a conformational change. This change in conformation dissociates the complex to permit receptor dimerisation, migration into the nucleus, high affinity DNA binding and transcriptional activation (Chambraud, Redeuilh et al. 1990). Therefore, the activity of a particular protein, which is expected to function within the nucleus, may be controlled in a temporal manner by fusing the coding sequence to a nuclear hormone receptor ligand binding domain (LBD), such as that from the ER. The LBD is the only ER sequence present, and therefore no downstream effects of estrogen activation are obtained. Moreover, by using a mutated form of the LBD with high specificity for a synthetic ligand, activation of endogenous ER can be avoided. Indeed a Glycine residue was mutated at position 525 (mERG525R) that resulted in the preferential binding of 4-OHT as opposed to the naturally occurring estrogen ligand, and optimal DNA binding and transcriptional activity was observed (Danielian, White et al. 1993) .

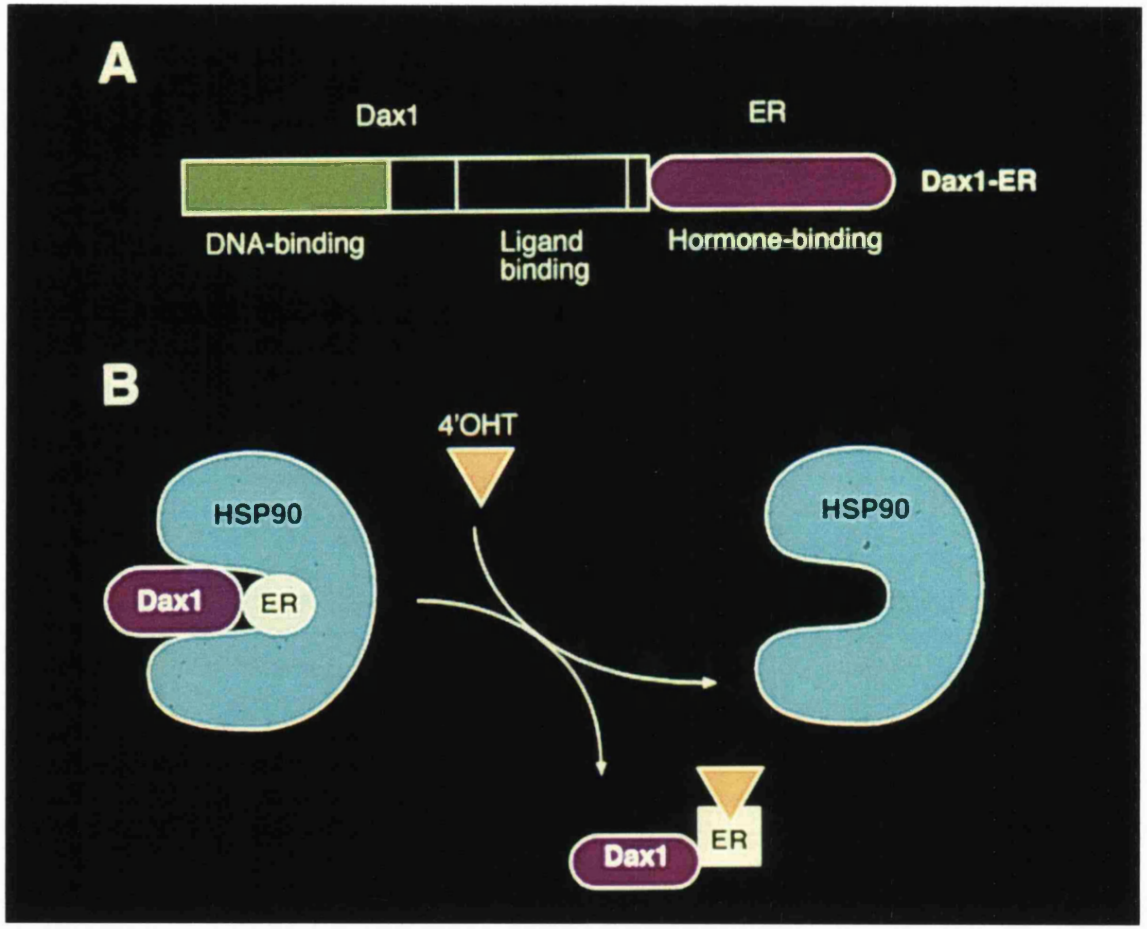
The most frequently cited application of ER ligand binding domain inducible systems is the activation or removal of gene function, by site-specific Cre-mediated recombination. This system is based upon the recombination between loxP sites by P1 phage derived Cre integrase. Cre-mediated recombination is usually achieved with a bipartite system, whereby a promoter driven Cre-recombinase can excise a specific sequence flanked by loxP sites. Alternatively, Cre-mediated recombination can be used to remove a functional barrier (e.g. a loxP flanked stop codon) thereby activating gene function (Danielian, Muccino et al. 1998; Indra, Warot et al. 2000; Takeshi, Jiang et al. 2001).

A more direct approach can be used by fusing the coding sequence of a gene of interest (such as DAX1) to the mutated ER ligand binding domain (ER LBD) (Fig. 18). This fusion protein should be retained in the cytoplasm in the inactive state

bound to the HSP 90 complex, in the absence of 4-OHT. Upon addition of 4-OHT, a conformational change is induced which permits dissociation of the ER from the heat shock protein complex, followed by translocation of the fusion protein to the nucleus. The first reported use of this strategy *in vivo* used mRNA from a MyoD-ER fusion construct injected into *Xenopus laevis* embryos, and targets of MyoD were activated specifically by 4-OHT administration (Kolm and Sive 1995).

Fig. 18 An inducible system to temporally control misexpression of DAX1

A. The DAX1 full length sequence is fused to the ligand binding domain of the Estrogen Receptor. B. In the absence of an estrogen ligand (such as 4-OHT), the ER fusion protein will be maintained in the cytoplasm in a complex with heat chock proteins (HSP90). Upon the addition of ligand, a change in conformation dissociates the ER from the HSP complex, allowing DAX:ER to enter the nucleus.


A
Dax1
ER
Dax1-ER
DNA-binding
Ligand binding
Hormone-binding
B
4'OHT
HSP90
Dax1
ER
HSP90
Dax1
ER

Our model of sex determination suggests that both levels and timing of *Sry* expression are important. When *Sry* is expressed at a low level there are three possible outcomes: (i) A delay in testis cord formation. This can be up to about 24 hours, yet still recover. (ii) The development of ovotestes. These may persist into the newborn or even the adult, but frequently one or other component is lost during foetal development. (iii) A complete failure of testis determination (primary sex reversal), resulting in essentially normal ovary formation. All three outcomes have been found in transgenic experiments with the 14kb mouse *Sry* genomic fragment, where position effects sometimes compromise levels of expression. The delay of cord formation may reflect a flattening out of the peak of *Sry* transcripts/protein, which will mean that it takes longer to reach a critical threshold. Alternatively a similar delay has been seen when an 11 kb 5' *Dax1* regulatory sequence was used to drive *Sry* expression (*Dax:Sry*). In this case all transgenic lines showed the delay, suggesting that expression from these regulatory sequences begins after those of *Sry*.

The same three outcomes were also seen in situations when *Dax1* was overexpressed. Thus a transgene expressing 5 times the normal level of *Dax1* on a background with a *Mus musculus musculus* Y chromosome (and *Sry* allele), showed a delay in cord formation; ovotestes and complete sex reversal were found when the same *Dax1* transgene was bred onto a background with a *Mus domesticus poschiavinus* Y, known to express *Sry* at lower levels. This suggested that *Dax1* was somehow antagonistic to *Sry*, but did not reveal mechanisms. Nor did it say why sex reversal is seen in DSS patients with only a double dose of *DAX1*, whereas it was necessary to have both high levels of *Dax1* and weak or delayed *Sry* expression in mice.

The proposal that I wished to test is that the relative timing of *Dax1* and *Sry* expression is critical to their antagonistic action. In other words, if higher than normal levels of DAX1 are achieved before the onset of *Sry* expression, this may be sufficient to override the male pathway and induce ovary development in XY embryos. The ER inducible approach was chosen to misexpress *Dax1* during early gonadal development, before the onset of *Sry* expression. In the absence of a specific

promoter, that would drive *Dax1*expression in the bipotential gonad before the onset of *Sry,* a ubiquitous CMV promoter was used to drive expression of a DAX1:ER fusion construct. This approach would also ensure high levels of DAX1 expression, with respect to SRY, but in a temporally controlled manner.

IV.II Results

The mouse ER LBD (mERG525R) was cloned into the BamH1 – EcoR1 sites of Bluescript KS (Stratagene) (Littlewood, Hancock et al. 1995). The DAX1 ORF was cloned into the EcoRV site of Bluescript SK. The stop codon of DAX1 was mutated by PCR, and Bgl II sites were introduced at both the 5' and 3' ends. Sequence analysis confirmed that the stop codon was destroyed. The ER plasmid was linearised with BamH1, and the Bgl II DAX1 coding sequence was cloned into the BamHI site (BglII and BamHI have compatible cohesive ends). This ligation generated the DAX1:ER fusion construct. This fusion construct was released by NotI/ApaI digestion, and cloned directionally into pCDNA3, behind the CMV promoter. An SV40 poly A site was present on the vector at the 3' end of DAX1:ER.

This construct was used to generate six independent transgenic founders. As transgenic mice were breeding, *in vitro* assays were established to test the function of both DAX1 and the ER LBD domains. Sub cellular localisation was used to test the functional interaction of the ER LBD with heat shock proteins *in vivo,* that would retain the fusion protein in the cytoplasm. In the absence of 4-OHT, DAX1 should be localised to the cytoplasm, whereas 4-OHT administration should promote DAX1 localisation into the nucleus.

IV.II.I Protein Localisation

The CMV-DAX:ER construct was transfected into NIH 3T3 fibroblasts and the subcellular localisation of DAX1 was examined in the absence and presence of 4-OHT, using an anti-DAX1 antibody (a gift from Ken Morohashi) (Fig 19) . In experiments carried out in the presence of 4-OHT, this was normally added to cell culture media immediately after transfection, when cells were washed to remove liposome-DNA complexes. 4-OHT was the ligand used in these analyses as it is soluble in Ethanol and can then be diluted in cell culture media. 4-OHT (referred to as Tm) is only soluble in a viscous oil such as Corn oil and is therefore used for IP injections when administered to mice.

In the absence of 4-OHT, DAX1 expression was detected in the cytoplasm of transfected cells. However, after a 24 hour exposure of the cells to 4-OHT, DAX1 protein was localised to the nucleus (Fig 19). Untransfected cells were used as a negative control, and no staining above background was observed, either in the presence or absence of 4-OHT, demonstrating that the anti-DAX1 antibody could recognise the DAX1 epitope in the DAX1:ER fusion protein. These results also indicated that the ER LBD was functional in the retention of the fusion protein in the cytoplasm of transfected cells in the absence of ligand. However, in the presence of 4-OHT, DAX1 was efficiently transported to the nucleus. These experiments were repeated in COS7 cells and NTD2 cells and the results were the same for both (Fig. 20). Therefore, an *in vitro* assay was set up to address the function of DAX1, initially using the full length protein.

Fig 19. DAX1:ER protein localisation in the absence and presence of 4-OHT.

Upper panels show DAX1 expression in green, and nuclei are stained in blue (lower panels). Intact DAX1 is shown in (a), and expression is detected in the nucleus. DAX1 expression in the absence of 4-OHT is cytoplasmic (c) and is found in the nucleus in the presence of 4-OHT (e).

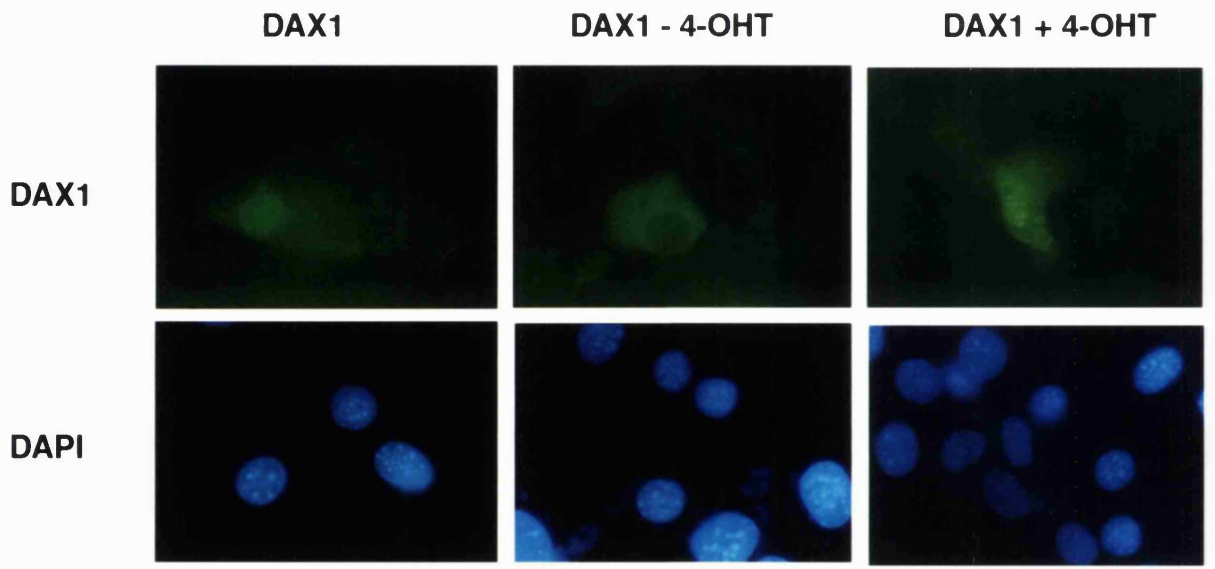
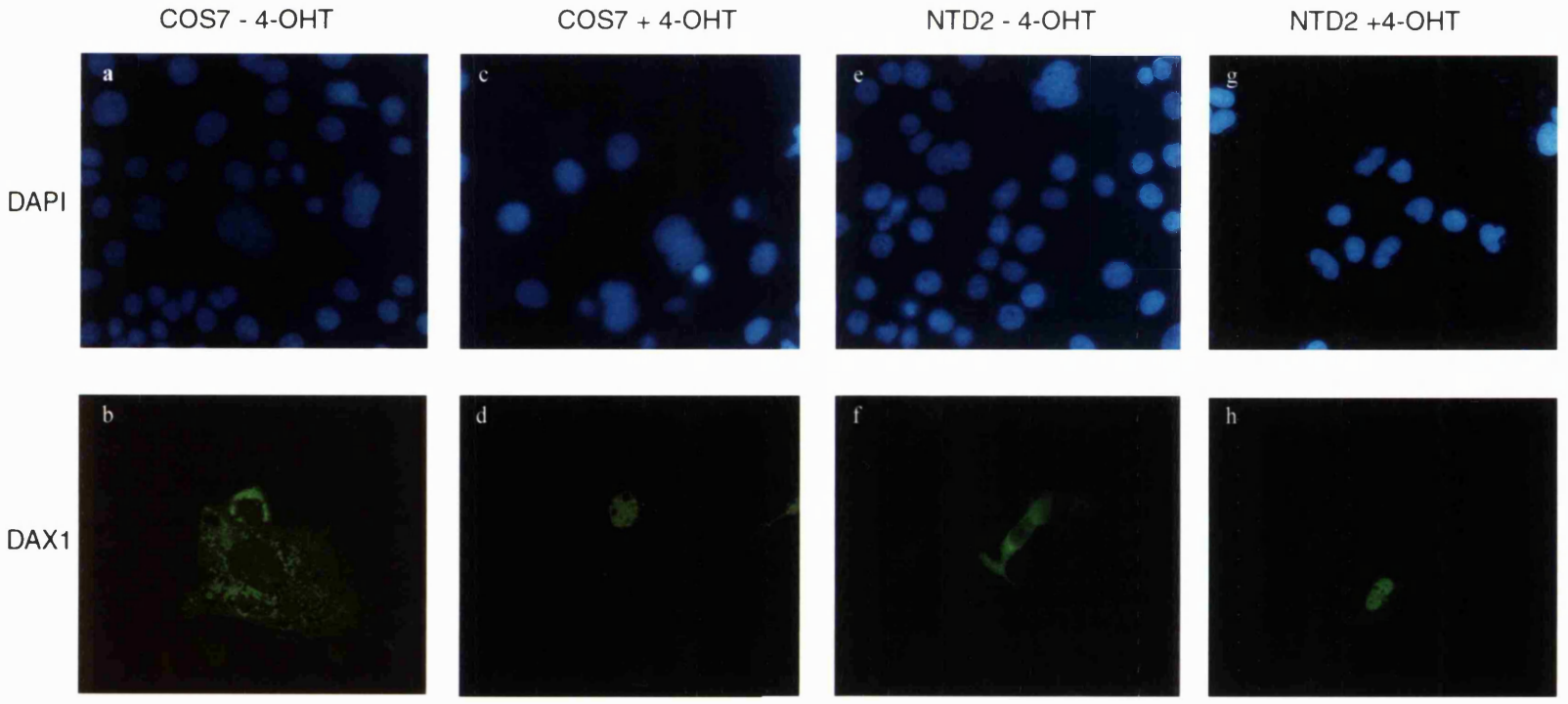

DAX1
DAX1 - 4-OHT
DAX1 + 4-OHT
DAX1
DAPI

Fig 20 Comparison of DAX1 localisation in the absence and presence of 4-OHT in COS7 cells and NTD2 cells.

(a),(c), (e) and (g), nuclei stained with Dapi, (b), (d), (f) and (h), transfected cells expressing DAX:ER.
(b), DAX1 expression in the cytoplasm of COS7 cells in the absence of4-OHT, (d), DAX1 expression in the nucleus of COS7 cells in the presence of TM, (f), cytoplasmic DAX1 expression in NTD2 cells in the absence of 4-OHT and (h), nuclear DAX1 expression in NTD2 cells in the presence of 4-OHT.


COS7 - 4-OHT
COS7 + 4-OHT
NTD2 - 4-OHT
NTD2 +4-OHT
DAPI
DAX1
a
b
c
d
e
f
g
h

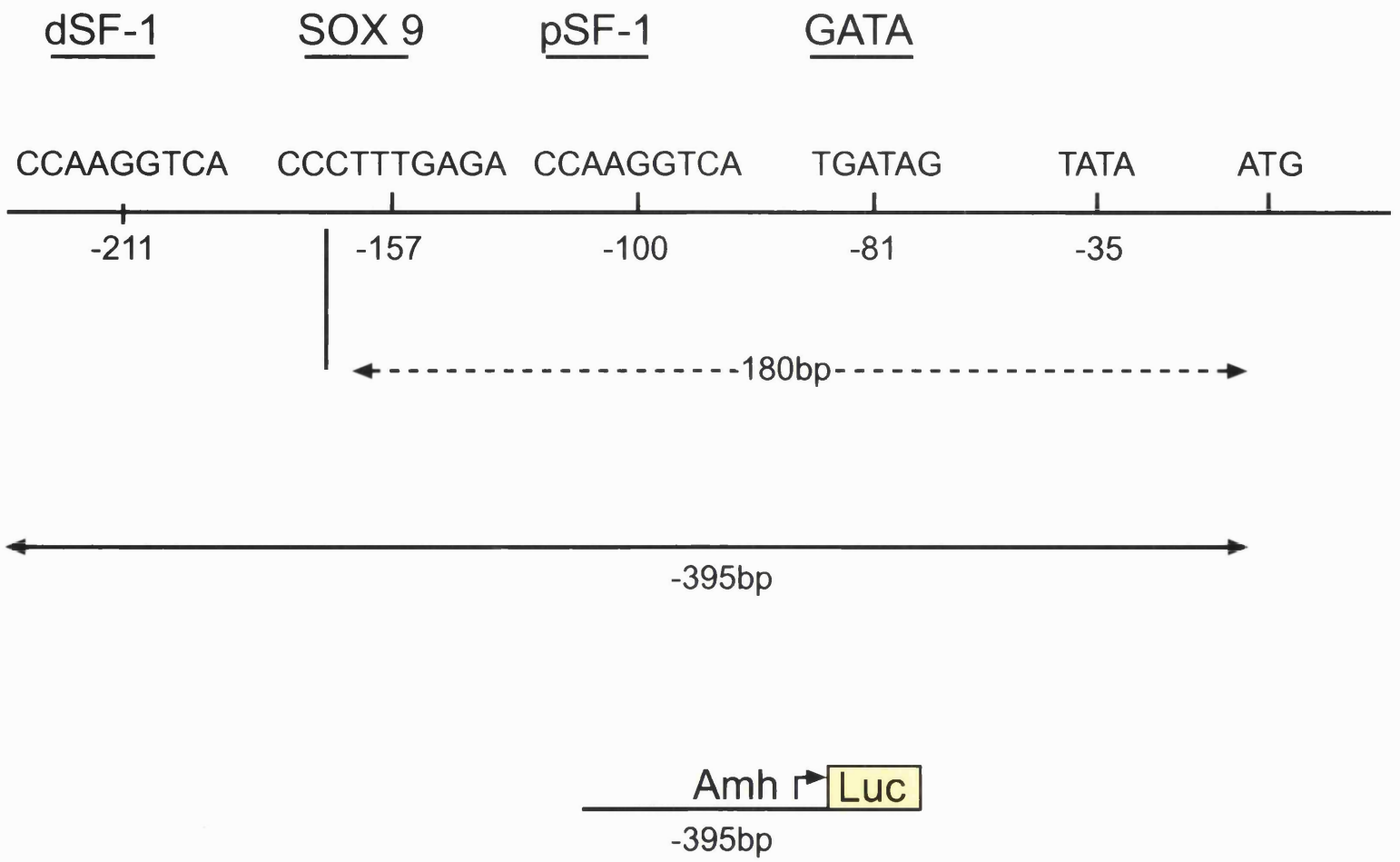
IV.II.II A DAX1 functional assay

A reporter assay system was chosen to test the effect of the full length DAX1 protein on the regulation of a specific reporter gene. It has been described, *in vitro,* that SF1 and SOX9 co-operatively activate the *Amh* promoter (De Santa Barbara, Bonneaud et al. 1998). DAX1 has previously been described to act as a potent inhibitor of SF1 mediated transcriptional activation probably through direct interaction with SF1 (Ito, Yu et al. 1997). It also has been reported that DAX1 antagonises the synergistic activity of WT1and SF1 in regulating the *Amh* promoter, presumably through direct interaction with SF1 (Natchigal, Hirokawa et al. 1998). Therefore, it was decided to use this assay to test whether DAX1 was functional within the DAX1:ER fusion protein, as it should interfere with the activation of *Amh* by SF1 and SOX9.

A ubiquitously expressing SF1 plasmid, CMV SF1, (a gift from Keith Parker), and a similar ubiquitously expressing SOX9 plasmid, CMV SOX9, (Sara Morais Da Silva, PhD Thesis, 1998) were chosen for the co-transfection assays. A 395bp 5' *Amh* sequence (depicted in Fig. 21) driving EYFP expression, (*Amh*:EYFP) was initially used as a reporter construct in this assay (the *Amh*:EYFP construct was generated by Tamara Caspary), so that the effect of SOX9 and SF1 on the expression from the *Amh* regulatory elements could be measured by quantitating EYFP expression. It would then be possible to address whether DAX1 could interfere with the SOX9 and SF1mediated activation of *Amh:EYFP.*

Fig 21. The 395bp *Amh* promoter sequence used in reporter assays

395bp of 5'*Amh* sequence was cloned upstream of a luciferase reporter gene, containing a GATA binding site, a SOX9 binding site, and two SF1 binding sites (proximal and distal).


dSF-1
SOX 9
pSF-1
GATA
CCAAGGTCA
CCCTTTGAGA
CCAAGGTCA
TGATAG
TATA
ATG
-211
-157
-100
-81
-35
180bp
-395bp
Amh
Luc
-395bp

COS7 cells were cotransfected with *Amh:*EYFP, CMV:SOX9 and CMV:SF1 plasmids, however, no expression of *Amh* EYFP was ever detected either in transient or stable co-transfections. Transient transfections were repeated multiple times under different conditions, using both the Calcium Phosphate precipitation and lipofection methods, but with no success.

Activation of the *Amh* promoter may have been weak, making it difficult to detect EYFP expression. The 395 bp regulatory sequence used to drive EYFP expression has been shown to be sufficient to initiate expression both *in vitro* and *in vivo* in a manner similar to endogenous expression (Beau, Vivan et al. 2001, Sara Morais Da Silva, PhD Thesis). Previous *Amh* promoter analysis and reporter constructs have generally used a 180bp sequence, that includes a single SOX9 binding site, a GATA binding site and a SF1 binding site (also called MIS-RE-1) (Shen, Moore et al. 1994; Giuili, Shen et al. 1997; De Santa Barbara, Bonneaud et al. 1998; Natchigal, Hirokawa et al. 1998). However, the *Amh* regulatory sequence chosen for the reporter assays contains a second SF1 binding site and 200bp of additional 5' sequence. This regulatory sequence has been shown to give more reliable expression *in vivo* than the 180bp sequence described by others (Beau, Vivan et al. 2001) and was therefore chosen for the following reporter assays (Fig. 21).

As EYFP expression was undetectable, an alternative reporter gene construct was generated using the same 395 bp *Amh* 5' sequence to drive expression of a firefly luciferase reporter gene (Luc). This reporter was chosen as Luciferase activity is both reliable and easily quantifiable. A reporter construct (*Amh: Luc)* was generated by inserting the 395bp *Amh* promoter sequence upstream of a δ-crystallin minimal promoter driving firefly Luiferase (*Photinue pyralis*) followed by an SV40 intron and polyA site (pδ51 LucII; a gift from Ryohei Sekido).

COS 7 cells were again transiently co-transfected with *Amh:Luc* , CMV:SOX9 and CMV:SF1 plasmids, and luciferase activity was detected 48 hours post transfection. As a control *Amh:Luc* was transfected alone, and did not give any background

luciferase activity. Cells were harvested and lysed two days after transfection and luciferase values were measured using a luminometer. A *Renilla:*luciferase reporter plasmid was cotransfected in each experiment as an internal standard, which served as a baseline control (based on a dual Luciferase reporter assay kit, Promega). All experimental values were normalised with *Renilla* luciferase activity values. Firefly and *Renilla* luciferase's, because of their distinct evolutionary origins, have different enzyme structures and substrate requirements, and can therefore be measured sequentially from a single cell lysate sample. Individual transfections and luciferase assays were always carried out under the same conditions and all experiments were carried out in duplicate or in triplicate.

Amh:Luc activity was detected when CMV:SOX9 was transfected in the absence of CMV:SF1 and *vice versa* (on average, 1-3 fold above basal) (Fig 22). However, when SF1 and SOX9 were coexpressed, activation of the *Amh* promoter was greater, suggesting an additive effect (on average 5 fold above basal). These results are consistent with data describing the additive effect of SOX9 and SF1 in activating the human *AMH* promoter (De Santa Barbara, Bonneaud et al. 1998). Therefore the assay was successfully established and could provide the basis to test for a repressive function of DAX1.

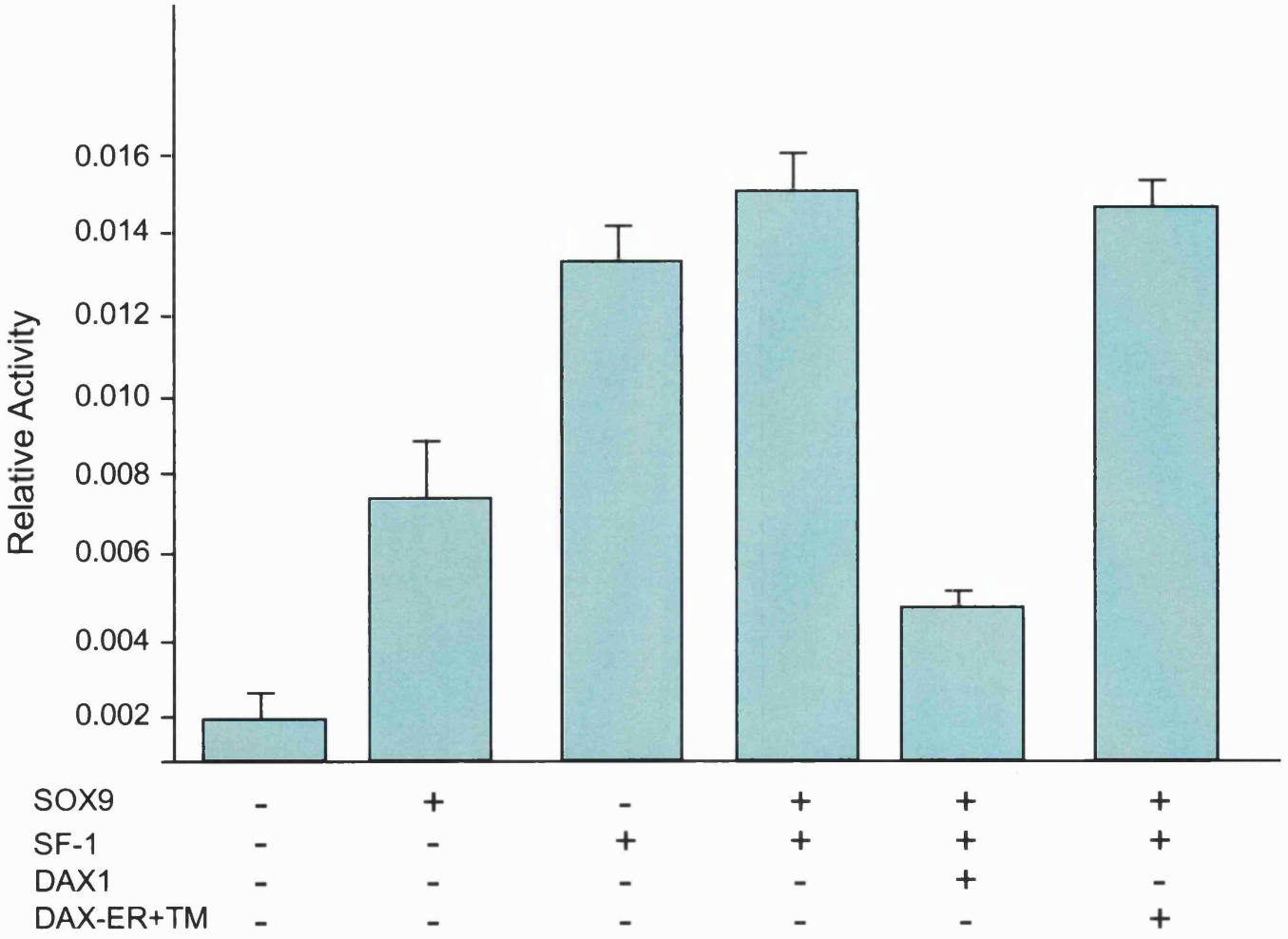
To test whether DAX1 could inhibit SF1 mediated activation of *Amh,* a DAX1 expression plasmid was used in similar assays outlined above. The DAX1 ORF was cloned downstream of a ubiquitous promoter (CMV) in a PCS2 plasmid and called WT DAX1. Under similar conditions outlined above, CMV:SOX9, CMV:SF1 and *Amh:luc* were cotransfected into COS 7 cells. In parallel, WT DAX1, SF1, SOX9, and the *Amh* reporter gene were co-transfected to study the effect of DAX1 on *Amh* activation. In the presence of SOX9 and SF1, a substantial activation was recorded as before, whereas when the full length DAX1 sequence was co-transfected, luciferase values were lower (Fig 22). These results suggested that DAX1 could inhibit the cooperative effect of SOX9 and SF1 activation of the *Amh* promoter. This functional assay could therefore be used to test whether CMV-DAX1:ER, in the presence of 4-

OHT, could also repress the effect of SF1 and SOX9 on activating the *Amh* promoter. Transfections were repeated as above, but CMV-DAX1:ER was transfected instead of CMV-DAX1, to test whether the DAX1:ER fusion protein has the same properties as the full length DAX1 protein . Transfections were carried out both in the presence and absence of 4-OHT, and it was shown that the ligand alone had no effect on either luciferase activity (*Renilla* controls were at the usual levels) or the activation of the *Amh* promoter (data not shown).

As expected, in the absence of 4-OHT, DAX1:ER had no effect on the activation of *Amh* by SOX9 and SF1(Fig 22). Surprisingly, in the presence of 4-OHT, DAX1:ER , which was shown to localise to the nucleus, under such conditions, was unable to inhibit activation of the *Amh* reporter. These assays were repeated multiple times and gave reproducible results. It was therefore concluded that while wild type DAX1 could interfere with the SF1 and SOX9 mediated activation of the *Amh* promoter, the DAX:ER fusion protein was no longer functional as a repressor.

Fig 22. DAX1, but not DAX:ER represses the cooperative activation of SOX9 and SF1 on the *Amh* promoter.

COS7 cells were transiently transfected with 100ng TKrl (a Thymidine kinase *Renilla* Luciferase reporter gene) as an internal standard and 100ng *Amh:*Luc in each experiment. 500ng SOX9, 500ng SF1, and 500ng DAX1 or DAX1:ER in the presence of Tamoxifen as indicated on the X axis. Relative Luciferase activity is represented on the Y axis. In the presence of SOX9, the *Amh* promoter is activated above basal levels (3 fold), and in the presence of SF1 this activation is increased (to 6 fold), whereas an additive effect on the *Amh* promoter is detected in the presence of both SOX9 and SF1. In the presence of full length intact DAX1, the activation of SOX9 and SF1 on the *Amh* promoter was repressed to near basal levels. However, in the presence of DAX1:ER, no repression was detected.


Relative Activity
0.016
0.014
0.012
0.010
0.008
0.006
0.004
0.002
SOX9 - + - + + +
SF-1 - - + + + +
DAX1 - - - - + -
DAX-ER+TM - - - - - +

IV.III Discussion

IV.III.I The ER Domain of the Inducible system appears to be functional

Inducible systems are often reported to give "leaky" expression, meaning that fusion proteins may not remain exclusively in the cytoplasm in the absence of ligand. This means that activity would be detectable both in the absence and presence of the activating ligand. This was not the case in the experiments described above, in which the subcellular localization of the fusion protein was not impaired. DAX1 was mostly detected in the cytoplasm of transfected cells in the absence of 4-OHT, whereas in the presence of 4-OHT, DAX1 was detected in the nucleus in the majority of cells analysed (Fig 19,20). These data provide good evidence to suggest that the inducible system tightly controlled tethering of DAX1 within the cytoplasm in the absence of ligand, whereas the presence of ligand permitted DAX1 to localise to the nucleus.

IV.III.II A functional assay to test DAX1

Construction of any fusion protein carries a risk that some functional domain might be masked, due to impaired folding. Correct protein folding is necessary to permit the normal interaction of functional domains with their binding partners. DAX1 is a nuclear transcription factor that has been shown to interact either directly, or indirectly, with DNA and with a number of other proteins, including SF1, and it has also been shown that these interactions are required for its activity (Ito, Yu et al. 1997). This was why it was necessary to set up a functional assay for the DAX:ER fusion protein.

An assay was first set up to test the function of the full length intact DAX1 protein. Coexpression of SOX9 and SF1, along with an *Amh:*Luc reporter gene, resulted in activation of a 395bp murine *Amh* promoter sequence. These *in vitro* findings were in line with *in vivo* data that demonstrates roles for both SOX9 and SF1 in the activation and maintenance, respectively, of the *Amh* promoter (Arango, Lovell-Badge et al. 1999). In the *in vitro* experiments described above, SOX9 and SF1

appear to be acting co-operatively to activate *Amh.* When the co-transfection assays also included CMV:DAX1, the expression of the *Amh*:Luc was much lower, showing that DAX1 can act as a repressor in this system.

In XY embryos at the time when *Amh* is highly expressed, DAX1 is absent from the differentiating testis presumably because it would have a negative effect on *Amh,* by antagonizing the activity of SF1 and/or SOX9. Indeed, DAX1 expression declines in the XY genital ridge coincident with the onset of *Amh* transcription. It is possible that the early expression of DAX1 in the indifferent gonad, at a time when SF1 levels are quite substantial, serves to ensure that no *Amh* can be produced until gonadal sex is established.

IV.III.III DAX:ER function in an *in vitro* reporter assay

When DAX:ER was coexpressed instead of the full length DAX1 sequence, it was unable to repress transcriptional activation of the *Amh* promoter sequence in the absence and even in the presence of 4-OHT. The former result fits with the observation that in the absence of 4-OHT, DAX1 was retained in the cytoplasm. However, the latter suggests that the DAX:ER fusion protein is unable to function even when present in the nucleus. In conclusion, the function of the ER domain was unperturbed, whereas the repressive function of DAX1 was lost. The fusion protein was therefore unlikely to be useful for *in vivo* experiments.

It is difficult to say how functional properties can be altered, in the absence of biochemical or structural analyses, but some predictions can be made on the basis of known functional domains within the DAX1 protein. DAX1 has been shown to recruit NCo-R, a nuclear receptor co-repressor that results in the further recruitment of a complex of factors that act to mediate repression (Crawford, Dorn et al. 1998). Similarly, it has also been shown that DAX1 can interact with another corepressor, Alien, through its C-terminal domain (Altincicek, Tenbaum et al. 2000). Alien is a novel human nuclear co-repressor that was cloned and characterised on the basis of

its amino acid identity to the *Drosophila* Alien orthologue (90%). Alien belongs to a class of corepressor distinct from NCoR, and interacts with nuclear repressors in a hormone sensitive manner, implying that a ligand for DAX1 may exist. The interaction of Alien with DAX1 differs from its interaction with other nuclear hormone receptors, such as the thyroid hormone receptor (TR). Therefore, it appears that DAX1 can bind to more than one corepressor, and that Alien uses distinct protein interaction domains to interact with different silencing domains. As a result of fusing the ER domain to DAX1, an induced secondary structure dissimilar to that of the full length protein, may have disrupted the interaction potential of DAX1 either with SF1 directly or with corepressors such as NCo-R or Alien.

IV.III.IV DAX1 has two transcriptional silencing domains

DAX1 has been suggested to inhibit SF1 mediated transactivation *in vitro,* either through a direct interaction between DAX1 and SF1, preventing SF1 from binding to its target gene, or via the recruitment of co-repressors such as NCo-R (Ito, Yu et al. 1997; Lalli, Bardoni et al. 1997; Crawford, Dorn et al. 1998). Sequential deletion of human DAX1 reveals the presence of a transcriptional silencing domain in the terminal 28 amino acids of the coding sequence (Ito, Yu et al. 1997). All natural occurring DAX1 deletions, reported in humans to date, remove this inhibitory domain. By deleting the SF1 DNA binding site, DAX1 repression of SF1 was still observed, indicating that repression of SF1 can occur independent of DNA binding. In this particular study DAX1 was found to interact directly with SF1, however deletion of the C-terminal domain did not interrupt protein binding, indicating that the N-terminal domain of DAX1 contains a protein interaction domain. Deletion of the N-terminal domain showed a decrease in DAX1 binding to SF1, indicating that an interaction domain was present in the three and a half repeat N-terminal sequence. It is therefore possible that DAX1 can bind directly to SF1 but may require interaction partners to mediate transcriptional repression.

Subsequent to the discovery of a transcriptional silencing domain in the C-terminus of DAX1 by Ito *et al,* structural analysis of human and mouse DAX1, in comparison to the LBD of RXRα, has revealed the presence of an additional transcriptional silencing domain (Lalli, Bardoni et al. 1997). This second transcriptional silencing domain has been mapped to the N-terminal DAX1 sequence, immediately before the ligand binding domain of DAX1. In comparison to other nuclear hormone receptors, DAX1 has an unusual N-terminal sequence. Instead of the characteristic Zinc finger DNA binding domain, the DAX1 N-terminus is comprised of three and a half repeats of a 65-67 amino acid motif. Database searches and sequence comparisons suggest that this is an unusual sequence not found in any other protein of the nuclear receptor family. The C-terminal three dimensional structure of RXRα and RARγ, corresponding to α helices 1-12 were compared to the mouse and human DAX1 C-terminal domains (Lalli, Bardoni et al. 1997). The two transcriptional silencing domains mapped to date reside in helices H1 and H12, the latter corresponding to the previously described C-terminal silencing domain (Ito, Yu et al. 1997). The former, helix H1, corresponds to the last and incomplete repeat sequence connecting the N-terminal and C-terminal domains. It was important therefore, to generate a DAX1 fusion protein, in which the structure of the C-terminal helices remain intact and are folded correctly. This would enable corepressors such as NCoR and Alien, and indeed any interaction partner, to bind to their respective DAX1 interaction domains, and recruit components required for transcriptional repression.

The generation of a non-functional DAX1:ER fusion protein may have resulted from fusing the ER sequence to the C-terminal repressor domain. This may have altered protein folding in such a way that SF1 binding, or interaction of DAX1 with any of its co-factors was disrupted.

In redesigning a functional DAX1 inducible system, a number of modifications were included, the most obvious being a switch in the order of sequence of the DAX1 and ER sequences.

Chapter V An alternative DAX1 inducible approach

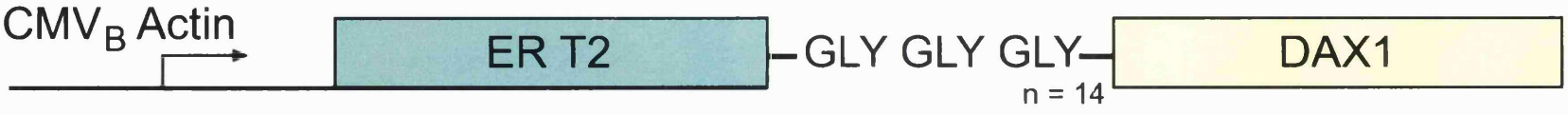
V.I Background

The fusion protein described in the above functional assays is based upon a mutation in the ligand binding domain of mouse ER (MERG525R) (Littlewood, Hancock et al. 1995). Another specific mutation in the LBD of human ER, called ER^{T2} has been recently shown to confer higher sensitivity to the ER to 4-OHT. It has been reported that when administered to pregnant mice this ER^{T2} mutation has no adverse effects on embryo or maternal development (Indra, Warot et al. 2000). ER^{T2} was therefore chosen to replace mERG525R in the generation of a modified inducible construct.

The incorporation of spacer, or linker sequences, between domains in fusion constructs can permit independent folding and therefore separation of distinct functional domains. A C1 λ repressor sequence has frequently been used to generate a hinge region between domains in fusion proteins (Amstroff and Ptashne 1995; Dubois, Lecourtois et al. 2001). Similarly, glycine residues can also be used to create flexible linkers (Robinson and Sauer 1998). Glycine based peptide linkers are usually designed to span the minimum distance between the C-terminus of one domain and the N-terminus of the following domain (usually between 12-15 residues). The absence of a β Carbon in the glycine residue permits a greater flexibility of the polypeptide backbone that may be energetically impossible for other amino acids. Therefore a glycine linker was chosen to separate the DAX1 and ER^{T2} sequences, as it should be more flexible than a linker generated from non-glycine residues (Fig. 23).

Fig 23 . A modified ER DAX1 fusion protein.

A human ER LBD sequence (ERT2) is placed 5' to the mouse DAX1 ORF , and these two domains are separated by a $glycine_n$ (n=14) hinge region.


CMV$_B$ Actin
ER T2
GLY GLY GLY
n = 14
DAX1

V.II Results

V.II.I Generation of Inducible Construct

ER^{T2} was fused to the N-terminal domain of DAX1, as opposed to the previous construct where it had been fused to the C-terminal domain. A $Glycine_n$ (n=14) hinge region was inserted between the ER^{T2} and DAX1 sequences. The length of the glycine hinge (n=14) was based on previous fusion constructs generated for *in vitro* and *in vivo* misexpression studies (C.Alexandre and L.Dubois, personal communication).

First, the Glycine hinge was fused to the 3' ER^{T2} sequence by PCR, and in doing so, mutated the ER stop codon. Sequence analysis of the resulting construct confirmed the presence of the mutation and verified the open reading frame. The start codon of DAX1 was then destroyed, enabling it to be directionally fused to the ER-^{T2} glycine tail .This cloning resulted in the generation of an ER^{T2}-gly-DAX1 fusion protein. This fusion construct was cloned into XhoI site (that was blunt ended) in pCAGGS, a plasmid containing a chimaeric promoter of the CMV immediate early enhancer and chick β-actin promoter/enhancer (Niwa, Yamamura et al. 1991). This plasmid also contained an SV40 polyA site on the 3' end of the vector, downstream of the ERglyDAX1 sequence. This will be referred to as ERglyDAX1 for the remainder of these discussions (Fig. 23).

V.II.II DAX1 subcellular localisation

The ERglyDAX1 fusion construct was transiently transfected into COS7 cells to examine the localisation of DAX1 protein, both in the absence and in the presence of 4-OHT . Transfections were performed as before, with the exception of the lipofection reagent used in transient transfection assays. Lipofectamine 2000, which is a more efficient transfection reagent compared to the Lipofectamine reagent previously used (according to manufacturers guidelines, GIBCO BRL), was chosen for the following series of experiments.

Transfected cells grown in the presence or the absence of 4-OHT, as before, at a final concentration of 10^{-7}M. After 24-36 hours incubation time, cells were washed and fixed for immunohistochemistry.

No DAX1 expression was observed in untransfected cells as expected (Fig.24), and as a further control immunohistochemistry was carried out on cells exposed only to 4-OHT (data not shown). In cells transfected with the ERglyDAX1 expression plasmid, cultured in the in the absence of 4-OHT, DAX1 was detected. However, in the majority of positive cells, the protein was unexpectedly localised to the nucleus (Fig 24 and 25). In a minority of DAX1 positive cells, the expression was detected both in the cytoplasm and in the nucleus but the protein was never detected exclusively in the cytoplasm (Fig 24 and 25). The addition of Tamoxifen made essentially no difference to the proportion of cells showing exclusive nuclear localisation.

Fig. 24 Localisation of ERglyDAX1 expression in the absence and presence of 4-OHT.

Transient transfections of ERglyDAX1 in COS7 cells showing localisation of DAX1 in green (e) and (h) and nuclei stained with DAPI in blue (a), (d) and (g). (a),(b) and (c) represent untransfected cells and no DAX1 staining is detected in (b). (d),(e) and (f), represent cells transfected with ERgly DAX1 in the absence of Tamoxifen, with nuclear DAX1. (g),(h) and (i) show cells transfected with ERgly DAX1 in the presence of tamoxifen, and DAX1 localises to the nucleus, in the same pattern as in the absence of tamoxifen.

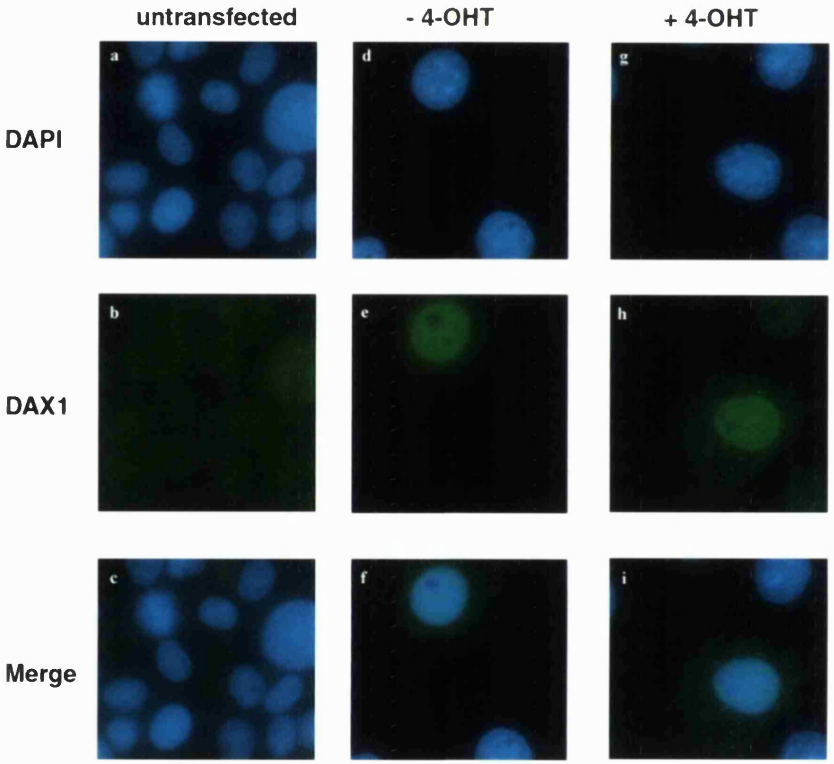
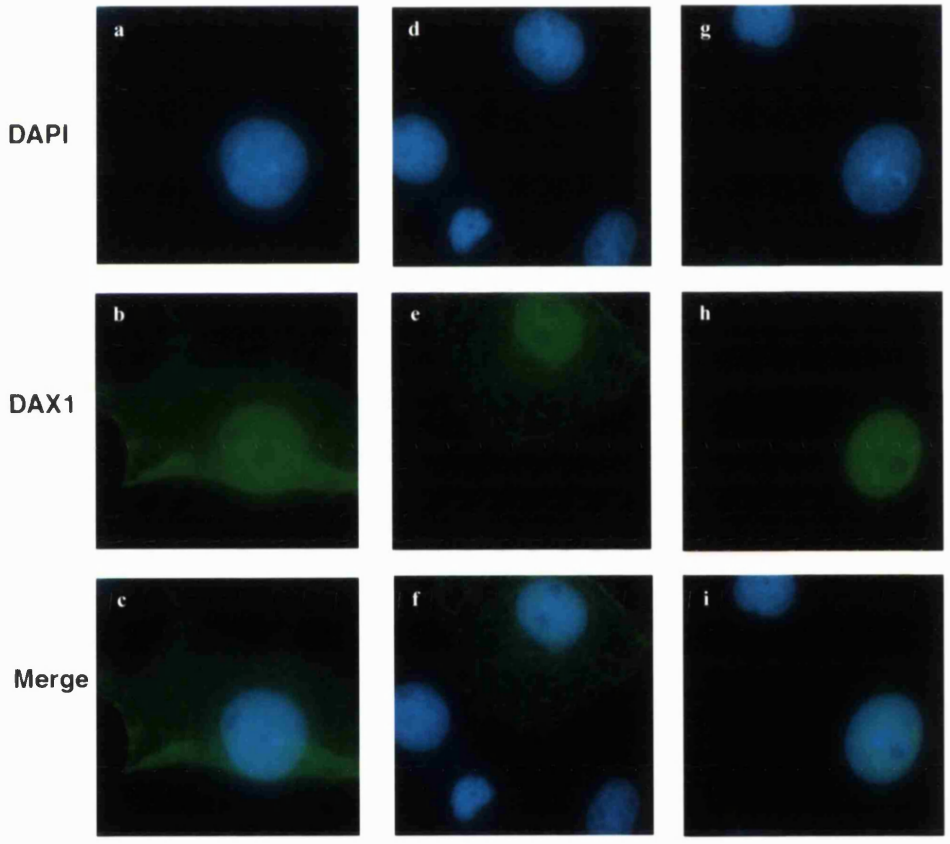

untransfected
- 4-OHT
+ 4-OHT
DAPI
DAX1
Merge
a
b
c
d
e
f
g
h
i

Fig. 25 A comparison of the different cellular localisation of DAX1 observed in the absence of 4-OHT.

COS7 cells transfected with ERglyDAX1 show mostly nuclear localisation of DAX1 (in green), in the absence of 4-OHT. Nuclei are stained with DAPI (in blue). DAX1 localisation is mostly nuclear (with some cytoplasmic staining) in (b) and in (e), but is almost exclusively nuclear in panel (h).

ER gly DAX1 in the absence of 4 - OHT



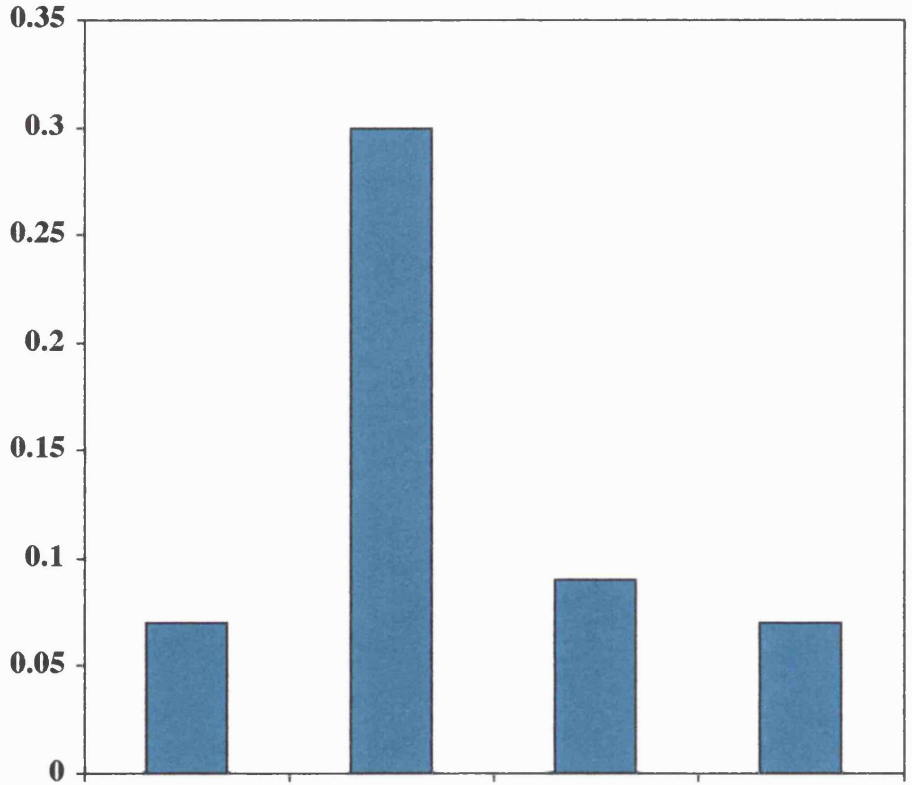
V.II.III Luciferase Assays

The dual luciferase assay was used again, incorporating *Renilla luciferase* as an internal control, to address the function of the DAX1 sequence in the modified ERglyDAX1 fusion protein. As shown previously, wild type DAX1 can interfere with the activity of SOX9 and SF1 on the *Amh* promoter driving luciferase gene expression. To test whether the DAX1 and ER^{T2} domains were functional, *AmhLuc,* CMV:SOX9, CMV:SF1 and ERgly DAX1 were cotransfected in COS7 cells or NTD2 cells in independent experiments, and analysed before and after 4-OHT induction. The values observed were compared to those obtained in parallel experiments where *Amh;Luc* , CMV:SOX9 and CMV:SF1, were cotransfected in the absence of DAX1. As before, all transfections were carried out in triplicate under the same conditions.

Repression of SOX9 and SF1 mediated activation of the *Amh* promoter was observed when ERglyDAX1 transfected cells were cultured in the absence of 4-OHT (Fig 26). This was consistent with the ligand independent nuclear localisation of DAX1 and suggested that ERglyDAX1 was not being retained sufficiently in the cytoplasm. Similar results were obtained for transient transfections cultured in the presence of ligand, but in comparison, repression by DAX1 was often slightly increased (Fig 26).

Fig. 26 ERglyDAX1 represses the activity of SOX9 and SF1 on the *Amh* promoter, but repression is observed both in the presence and absence of 4-OHT.

COS7 cells were transiently transfected with 100ng TKrl, 250ng *Amh*:Luc, for each experiment. Subsequently, 250ng CMVSOX9 and 250ng CMV SF1 were transfected as indicated by (+) symbols. In the presence of SOX9 and SF1, activation of the *Amh* promoter was observed above basal activity. However, when ERglyDAX1 was cotransfected, both in the presence and absence of 4-OHT, repression of the *Amh* promoter was observed, indicating that the ER component was non functional. Relative Luciferase units are depicted on the Y-axis.



Amh Luc	+	+	+	+
TKrl	+	+	+	+
CMVSOX9	-	+	+	+
CMVSF1	-	+	+	+
ERglyDAX -4-OHT	-	-	+	+
ERglyDAX +4-OHT	-	-	-	+

In these functional assays, transcriptional silencing mediated by DAX1 was obtained independent of 4-OHT addition. This is consistent with the observation that DAX1 was almost always observed in the nucleus, even in the absence of inducing ligand. While the first DAX1:ER fusion protein was responsive to 4-OHT, but was shown to be non functional, this new ERglyDAX1 fusion protein can mediate transcriptional repression of the *Amh* promoter, similar to the wild type protein, but it is constitutively active, being able to move into the nucleus independent of ER ligand administration.

V.III Discussion

The ERglyDAX1 fusion protein was detected in the nucleus of cells even when cultured in the absence of Tamoxifen, and was able to repress the SOX9 and SF1 mediated transactivation of the *Amh* promoter. Although the DAX1 component appeared to be functional, its inhibitory effects were observed both in the presence and in the absence of the Tamoxifen ligand. This may be due to either "leaky" activation of the ERglyDAX1 fusion protein, or that the ER domain was no longer functional.

Leaky activation of an inducible fusion protein may occur if it is expressed at unusually high levels within a cell, resulting in a saturation of the HSP complex. In this case expression of the fusion protein would be detectable both in the cytoplasm and in the nucleus of uninduced cells. If ERglyDAX1expression levels are very high, then not all of fusion protein may be complexed with HSP, permitting some to move into the nucleus in the absence of Tamoxifen. One notable difference between the original DAX1:ER construct and the ERglyDAX1 construct is the choice of promoter driving both sequences. In the former fusion construct, a relatively strong ubiquitous promoter (CMV), was used to express DAX1:ER. This promoter however, has been reported not to give reliable ubiquitous expression *in vivo*. The chimaeric CMV chick β-actin promoter (CAGGS), however, appears to be give stronger ubiquitous expression *in vivo* (Niwa, Yamamura et al. 1991). Perhaps the CAGGS promoter is stronger than the CMV promoter previously used, and strong over expression of ERgly DAX1 results in the migration of the protein into the nucleus. However, two pieces of evidence can be used to argue against the idea that strong misexpression results in leaky activity. Firstly, DAX1 expression was mostly detected in the nucleus, and not did not appear to be distributed equally between the cytoplasm and the nucleus. Secondly, the luciferase values observed in the functional assay are the only quantitative measure of DAX1 expression. The activation of the *Amh* reporter gene was not considered high (as the luciferase values were not as high as observed before) and although DAX1 repressed this transcriptional activation, it is difficult to address the strength of this activity.

To test whether over expression of the fusion protein is promoting translocation to the nucleus, a minimal promoter could be used to drive expression of the fusion construct. Alternatively, a Sertoli cell specific promoter could be used to drive ERglyDAX1 expression in primary Sertoli cell cultures, to test the function of DAX1 in inhibiting the SF1/SOX9 mediated activation of *Amh* promoter, in a manner closer to the *in vivo* situation. However, there could be problems in terms of finding a Sertoli cell specific promoter that will not have any effects on the assay itself, or on any of the factors being expressed.

Alternatively, the human ER component may have lost its function in this modified fusion protein. In the native situation the ligand binding domain of human and mouse ER proteins is located towards the C-terminus. In the first fusion construct generated, where the ER domain was functional, the position of the ER LBD was at the C-terminus of the fusion protein. However, in the modified fusion protein, the ER domain was placed at the N-terminus. Perhaps to maintain function of both DAX1 and ER, a linker domain is required to separate the two functional components in conjunction with the ER domain being placed at the very C-terminus.

Finally, as DAX1 has been suggested to be an RNA binding protein that shuttles between the nucleus and the cytoplasm, there may be some uncharacterised factors specifically required to mediate its sub cellular localisation. Non-native protein folding, and conformational changes, induced by generating fusion proteins, may mask interaction domains or motifs involved in protein localisation. For the initial fusion protein, the ER domain was functional, and in the absence of Tamoxifen DAX1 remained tethered in the cytoplasm. Full length DAX1 is normally detectable in the nucleus, but the ER domain of the first construct succeeded in binding the HSP complex thereby retaining the DAX1:ER in the cytoplasm and only permitted entry into the nucleus upon ligand addition.

However, for the latter fusion protein, the conformational changes induced by fusing the ERgly sequence to the DAX1 ORF may permit spontaneous DAX1:ER localisation to

the nucleus. Protein folding may have prevented the ER from complexing with heat shock proteins in the cytoplasm, therefore allowing the fusion protein to go directly into the nucleus in the absence of ligand. Alternatively, DAX1 nuclear localisation signals may simply have been exposed to the machinery required for entry into the nucleus, and there may be competition between the ER LBD interacting with the HSP complex, and DAX1 being imported into the nucleus. These explanations are highly speculative as the nuclear localisation of DAX1 or the process of nuclear cytoplasmic shuttling has not yet been fully characterized (Lalli, Ohe et al. 2000). However, a very recent report demonstrates that mutations in DAX1 associated with Adrenal Hypoplasia Congenita (AHC) result in DAX1 cytoplasmic localization, which impairs its ability to be involved in transcriptional repression (Lehman, Lalli et al. 2002). However, there appears to be some inconsistencies with this report. Some of the same authors previously report that DAX1 is a shuttling RNA binding protein associated with polyribosomes, via mRNA (Lalli, Ohe et al. 2000). These authors claimed that DAX1 is found in both the cytoplasm and the nucleus, however, Lehman *et al*, show that wild type DAX1 is almost exclusively localised to the nucleus. This latter result is much more consistent with the findings described above in the cytoplasmic to nuclear localization experiments. These results question the functional requirements of DAX1 in RNA processing, as the authors suggest that human DAX1 mutant sequences result in cytoplasmic localisation of the protein. Secondly, all of the mutations found in human AHC patients alter the protein C-terminus, and the deletions and mutation analysis carried out *in vitro* resulted in DAX1 cytoplasmic localisation. However, the nuclear localisation signal (NLS) is predicted to reside in the N-terminal sequence of DAX1, and is intact in these mutation studies. To date there has been no functional analysis of any predicted DAX1 NLS, nor have they been shown to specifically map to the N-terminal domain. It may be that there are some regions in the C-terminal domain also required for correct subcellular localisation.

Neither of the two fusion constructs designed to control the activity of DAX1 in an inducible manner, worked correctly. The ER domain but not DAX1 appears to have functional integrity in the first set of experiments, whereas the DAX1 domain but not ER

is functional in the other fusion, in these *in vitro* analyses. In the latter construct, where DAX1 was functional in the repression of SOX9 and SF1 mediated activation of the *Amh* promoter, it is hard to assess whether the system is simply non-functional, and even more difficult to anticipate how this fusion protein would behave *in vivo*. One caveat of using *in vitro* assays, is that misexpression and transcription *in vitro* may not be a true reflection of the *in vivo* situation. If tighter regulation could be achieved *in vitro,* then this inducible system could be tested *in vivo,* by generating transgenic mice expressing the fusion construct. We would expect that pregnant transgenic mice, expressing ERgly DAX1, could be administered Tm, which can cross the placental barrier, to activate the fusion protein in transgenic embryos.

Doses of Tamoxifen used previously in inducible experiments, were approximately 1 mg per 40 g mouse (Danielian, Muccino et al. 1998; Imai, Jiang et al. 2001). A recent report outlines the doses required to activate the ER in ubiquitously expressing Cre-ER mouse lines crossed to Rosa 26 loxP reporter lines (Hayashi and McMahon 2002). The authors propose that a single intraperitoneal (IP) injection of Tamoxifen to a pregnant mouse, at 8.5 dpc, results in detectable recombination and activation of a *LacZ* reporter gene within 6 hours. Activation in all three germ layers is efficient within 12 hours post injection. Hayashi *et al* report that single doses of between 3-9 mg of TM per mouse gives a dose dependant activation of the Cre-ER(Hayashi and McMahon 2002). However as the dose is increased above 3 mg, embryonic lethality is observed although most embryos are viable at 13.5 dpc. This may limit inducible misexpression analysis and site directed recombination experiments to transient analysis of phenotypes before 13.5 dpc.

If optimal activation of the ER could be observed between 6 and 12 hours post IP injection of Tm, then to misexpress DAX1 at the right time Tm should be administered to pregnant transgenic mice a number of hours before 10 dpc. This would ensure activation of ERglyDAX1 just before 10.5 dpc when *Sry* is beginning to be expressed. Optimisation of timing of TM administration may require more precise consideration to ensure DAX1 activation occurs just before the onset of *Sry* expression.

In the event that the second fusion protein described gives very leaky activation of the ERgly DAX1 protein *in vivo* , then this particular system is not suitable for understanding the role of DAX1 during sex determination and embryogenesis. The functional assay described is based upon the ability of DAX1 to silence transcriptional activation by SF1. The exact nature of this repression is not completely understood *in vivo,* but it is possible that DAX1 either binds directly to SF1 and represses transcriptional activity, and/or that nuclear corepressors may be required for gene silencing by DAX1. Leaky expression of DAX1 driven by a ubiquitous promoter such as pCAGGS may give strong expression of DAX1 during the early stages of gonadal development where SF1 has been shown to be required. DAX1 repression of SF1 might therefore result in the same phenotype as that reported for the SF1 knockout, where male and female SF1 null mice completely lack gonads (Luo, Ikeda et al. 1994; Sadovsky, Crawford et al. 1995).

These findings described above, concerning the two DAX1 fusion proteins, outline the risks associated with the generation of fusion proteins in general, and the generation of inducible misexpression systems. These may simply be a consequence of attempting to tether a transcription factor within the cytoplasm of a cell. Clearly more modifications to the inducible system are required before we can address the question of misexpression of DAX1 in the early developing gonad, before overt sex determination.

V.IV General Discussion

As well as gain of function analysis, loss of function mutations may also be used to address the function of DAX1 during mammalian sex determination. In a conditional targeting approach, XY mice, null for DAX1, developed as males but were infertile and hypogonadal, with testis weights approximately 50% of normal control testes (Yu, Ito et al. 1998). XX homozygous null mice were fertile and showed no obvious defects in reproductive organ development. In addition, the adrenal phenotype was not identical to that found in human AHC patients, although adrenal abnormalities were described. If DAX1 plays a role in female development then one would expect a loss of function mutation to result in perturbation of sex determination and or differentiation. However, the phenotype of DAX1 null mice came as a surprise. No defects in primary sex determination were observed either in XX or in XY animals. One possibility is that the mutation generated was not a complete null and that the transcripts detected arising from exon I could be translated into a partially functional protein. However, in this report, no detailed analysis was carried out during early gonadal development and sex determination, and it is possible that weak effects on either process could have been recovered during late embryogenesis. Alternatively, DAX1 may be part of a redundant system where other genes may play a similar role as DAX1 during sex determination.

Certain defects, however, were reported in the XY null mice. At birth, testes appeared normal, but soon showed progressive epithelial dysgenesis and sloughing of germ cells. By 14 weeks, germ cells were completely absent from the adult testis. Leydig cell hyperplasia was also observed adjacent to the degenerate tubules of the mutant adult testes. DAX1 is normally expressed both in the Sertoli and Leydig cells of the adult testis, although its function in these cell types is not completely understood (Ikeda, Swain et al. 1996; Swain, Zanaria et al. 1996; Tamai, Monaco et al. 1996). It is therefore possible that DAX1, like many factors involved in gonadal development and differentiation, has multiple roles at different stages during sex development. Perhaps DAX1 is involved in the early stages of sex differentiation but also plays a later role during spermatogenesis and steroidogenesis. DAX1 may be required in the Sertoli cell

for the integrity of the germinal epithelium. Sertoli cells are known to provide nourishment for the germs cells and although DAX1 did not appear to be required for the early stages of spermatogenesis, it may play a role in its maintenance. In addition, Leydig cell hyperplasia may be a direct consequence of the absence of DAX1 in Leydig cells, or an indirect effect of disrupted cell signalling downstream of DAX1 either in Leydig or Sertoli cells.

Recently, a number of reports have focused on the spermatogenic defects and Leydig cell hyperplasia observed in DAX1 mutant male mice. When testis and ductal pathology was closely examined in *Dax1* deficient males, structural disorganisation of the testis appeared to diminish sperm fertilising ability and may have resulted in the infertility observed(Jeffs, Meeks et al. 2001). In brief, examination of the testes of 12 week old *Dax1* deficient males revealed that there was a progressive degeneration of the germinal epithelium and a dilation of the seminiferous tubules. Analysis of sperm production revealed low epididymal sperm counts and decreased sperm motility. Fewer sperm were able to undergo an acrosome reaction and fewer eggs were fertilised *in vitro* when compared to wild type sperm. This is reminiscent of a blockage of the testicular duct system. The rete testis is the region where the seminiferous tubules converge in the testis to enter the ductal system which gives rise to the vas deferens where sperm mature. Leydig cells in the rete were hyperplastic with large mitochondria whereas Leydig cells in the seminiferous tubules were much smaller with small mitochondria. The peritubular tissue surrounding the seminiferous tubules was also abnormal. The peritubular myoid cells were undifferentiated and there was a lack of basement membrane between the myoid cells and the Sertoli cells. This suggested a possible role for Sertoli cells and Leydig cells in regulating other cell types either through cell interactions and/or cell signalling.

DAX1 expression is regulated in Sertoli cells during spermatogenesis and is at its highest levels during the androgen sensitive phase (Tamai, Monaco et al. 1996). It was suggested therefore that DAX1 might function in Sertoli cells as well as Leydig cells and play an important role during spermatogenesis. However, it appears that DAX1 is not required for the initiation of spermatogenesis, but may be required for its maintenance. To test this hypothesis, rescue experiments were performed by expressing a FLAG epitope tagged human DAX1 specifically in Sertoli cells under control of the *Amh* promoter. A 180bp *Amh* 5' regulatory sequence was used to drive expression of DAX1, as it was proposed to mimic the temporal and spatial expression of endogenous *Amh* (Giuili, Shen et al. 1997; Jeffs, Ito et al. 2001). The Sertoli cell specific rescue of DAX1 appears to restore fertility to some degree in *Dax1* deleted XY mice, however, no apparent improvements were observed in testis morphology. The germinal epithelium was vacuolated and Leydig cell hyerplasia was still abundant, indicative of a breakdown of Sertoli cell junctions with other cell types. Therefore, Sertoli cell expression of DAX1 could only partially rescue the *Dax1* null phenotype. Perhaps DAX1 is also required in Leydig cells and cooperation between Leydig and Sertoli cells is required for normal testis development and function during spermatogenesis.

Another possibility is that the *Amh* promoter used could only give expression of DAX1 later during testis formation. The 180 bp region maybe only have been sufficient to give expression in the adult testis and not in the embryonic gonad as proposed (Giuili, Shen et al. 1997). Expression of FLAG tagged DAX1 was only ever examined in adult testis by RT-PCR and immunohistochemistry, and not in the embryos where it has been shown to be expressed (Munsterberg and Lovell-Badge 1991). Only a small proportion of Sertoli cells actually expressed DAX1 when sections were examined after FLAG antibody staining. Perhaps the partial rescue observed is due to a late effect of DAX1 expression, whereas if DAX1 was expressed earlier a more complete rescue of the phenotype may have been observed. A longer 395 bp *Amh* 5' fragment has been shown to give more reliable expression of a LacZ marker, *in vivo.* This may have been more suitable for use in these rescue experiments, however, it is clear that additional elements are still required to mimic exact endogenous *Amh* expression (Beau, Vivan et al. 2001). In

mouse, *Dax1* is expressed between 10.5 and 12.5 dpc in XY gonads and is being down regulated at the time when *Amh* is being turned on. It is still unclear whether DAX1 is required for early gonadal development, but it is clear that this time window of expression is important for Sertoli cell differentiation. A reliable inducible system allowing the ectopic expression of DAX1 at different times during gonadal development would perhaps offer a better strategy for these rescue experiments.

It appears however, that DAX1 may also function in other somatic cells types within the testis as it has been shown to be expressed in both fetal and adult Leydig cells (Ikeda, Swain et al. 1996). Close examination of the expression of steroidogenic genes in DAX1 null mice, lead to the finding that *Cyp19* expression was upregulated (Wang, Jeffs et al. 2001). *Cyp19* encodes aromatase, the enzyme responsible for the conversion of testosterone to estradiol. This enzymatic reaction is reversible and therefore regulates the relative amounts of testosterone to estrogen. Primary Leydig cell cultures were generated from the testes of 12 week old XY *Dax1* deficient mice. RT-PCR and Western blot analysis revealed that of the five enzymes required for testosterone biosynthesis and the conversion of testosterone to estradiol, *Cyp19* was the only gene whose expression was altered. *Cyp19* mRNA levels were 4 times higher in *Dax1* deficient cells than in control cultures of wild type cells, suggesting that upregulation of aromatase in *Dax1* mutant males may be a critical factor in the disruption of Leydig cell function. This over expression of aromatase in mutant males resulted in a 40-fold increase in intratesticular Estradiol. Estradiol signalling occurs through the ER, and synthetic ER ligands, such as Tamoxifen, are known to bind the ER and prevent binding of endogenous estradiol, thereby blocking estrogen signalling. Tamoxifen pellets were implanted into DAX1 null males, that release the drug over a 90 day period. Over this course of Tamoxifen treatment, fertility was completely restored in mutant males, and several other improvements were observed. Testis weights increased significantly, but not quite to normal controls, and sperm count and motility also improved. Another notable improvement was the decrease in Leydig cell number isolated from mutant males, indicating that Leydig cell hyperplasia was also dramatically improved. These

findings outline the importance of DAX1 expression in Leydig cells, and the importance of estrogen in normal male reproduction.

The above results demonstrate how fertility can be rescued to some degree in DAX1 mutant males by two different methods, by rescue of DAX1 expression in the adult Sertoli cells and by blocking estrogen receptor signalling in Leydig cells. It is becoming clear that DAX1 plays important roles in both cell types and perhaps a cooperation between the somatic cells and germ cells is important for normal testis development, spermatogenesis and therefore fertility. We cannot rule out the fact that DAX1 may also play a role in sex determination, and clearly more experiments are needed to understand this process fully both in the male and female gonad.

So far, the female pathway of gonadal development has been more difficult to understand mostly due to the lack of cell markers. However one gene in particular, *Wnt* 4 has been proposed to play a role during female development both in mouse and in humans (Vainio, Heikkila et al. 1999; Jordan, Mohammed et al. 2001). Wnt 4 is a member of the Wnt family of secreted signalling proteins and disruption of *Wnt4* in mouse results in masculinisation of females (Vainio, Heikkila et al. 1999). *Wnt4* is expressed in multiple tissues in the embryo including the kidney, mesonephros, the gonads and the Müllerian ducts. Mice homozygous for a *Wnt4* null allele die of kidney failure after birth, however XX pups examined shortly before and at birth revealed a masculinisation of internal genitalia. Male development appeared to be normal in the absence of *Wnt4*, however the internal organs of homozygous XX mice were masculinised. Ovaries were still located in their normal abdominal position, but developed in association with a fat body similar to that found in normal males. In addition, the ovaries were round and unencapsulated as are the testes. The single remaining duct was highly coiled and convoluted, resembling the structure of the Wolffian duct. Marker analysis of this duct revealed that it expressed Wolffian duct specific factors, such as *Shh* and *Pax6* that are not normally expressed in the developing Müllerian duct. These results suggested that some reversal of sex development occurred

in *Wnt 4* mutant females. However, external genitalia of homozygous mutant females were normal.

Wolffian duct development in the male depends on steroid biosynthesis, and it was suggested that expression of Wnt 4 in females acts to control steroidogenesis. 3β-hydroxysteroid dehydrogenase (3β-HSD), the hormone responsible for the conversion of pregnenalone to progesterone in the testosterone biosynthetic pathway, is expressed strongly in Leydig cells but is completely absent in the developing ovary. However, it was found that *3β-HSD* was upregulated in the ovaries of *Wnt4* mutant females. Therefore in the absence of *Wnt4* steroidogenesis is initiated in the ovary. These findings are reminiscent of the relationship between *Dax1*and aromatase discussed above. Similar to *Dax1*, *Wnt4* is down regulated in the male gonad after 11.5 dpc, whereas expression persists in the developing female gonad (Vainio, Heikkila et al. 1999). In the original *Wnt4* targeted mutation analysis, no change in *Dax1* expression was observed in *Wnt4* mice, but on closer examination it has been found that *Dax1* levels of expression are down regulated in the ovary in the absence of *Wnt4* (Jordan, Mohammed et al. 2001). It is possible that there is some relationship between DAX1 and Wnt 4 and that this may exist at the steroidogenic level. To understand the relationship between DAX1 and Wnt4, the role of DAX1 needs to be addressed both at the level of sex determination and during later gonadal development and steroidogenesis.

Perhaps some functional redundancy or cooperativity exists between DAX1 and Wnt4 that may explain why a defect in primary sex determination was not observed in the *Dax1* mutant mice. The human *WNT4* gene has been isolated and shown to be highly conserved to its mouse orthologue, having 98.9% sequence identity and 99.7% sequence similarity at the amino acid level (Jordan, Mohammed et al. 2001). *WNT-4* was shown to map to human Chromosome 1 at the cytogenetic band 1p35. This region of chromosome 1 is highly syntenic to mouse chromosome 4q where *Wnt4* localises. An interesting connection between *WNT4* and *DAX1* comes from analysis of 4 XY patients with a duplication of part of chromosome 1p, including the Wnt4 locus, These two patients are described as having sexual phenotypes ranging from cryptorchidism to sex

reversal. Despite the extent of the duplication, Wnt4 may be considered a candidate for this dosage dependant sex reversal. Fibroblasts were obtained from a human XY sex reversed patient, carrying a duplication of 1p31-1p35. This individual had severe hypospadias, fibrous gonads with rudimentary tubules, and had remnants of both Müllerian and Wolffian ducts. A rudimentary uterus and vagina was also present. FISH analysis demonstrated that the *WNT4* locus was duplicated, and *WNT4* was expressed in fibroblasts from this patient, but was not detected in fibroblasts from a normal XY male. Although this may not be physiologically relevant, it does indicate that at least in one cell type analysed *WNT4* expression could be upregulated in these patients.

It was also shown that Wnt4 over expression in primary culture of mouse Leydig and Sertoli cells could lead to upregulation of DAX1, suggesting that by upregulating DAX1 expression, WNT4 could be important for primary sex determination.

It is becoming clear that many factors involved in sex determination and or differentiation, such as DAX1, SOX9 and possibly WNT4, may be sensitive to gene dosage. The subtle differences in gene dosage sensitivity, between mice and humans, may be responsible for differences in sex phenotypes when comparing gain of function and loss of function mutations. Finally, it appears that most genes that act during sex determination and gonadal development play multiple roles at different levels along the pathway, with the obvious exception of *Sry*. These observations could imply that *Sry* has a defined role at a critical step during sex determination and functions only to initiate the male pathway by regulating a single target, with SOX9 being the likely candidate. Perhaps the role of DAX1 is to antagonize this event, but clearly more analysis is required to determine exactly what role(s) DAX1 plays during early sex determination and gonadal development.

Chapter VI Final Comments and Conclusions

VI.I DAX1 and a role during sex determination in mouse

The model we propose for sex determination suggests that the relative timing and levels of gene expression of *Sry,* with respect to *Dax1,* is important for the cell fate decision which determines whether or not Sertoli cell differentiation occurs. It has been the central dogma of sex determination for some time that the expression of *Sry* acts as a dominant trigger to initiate sex determination and development of the testis. In the absence of *Sry* expression, ovary development occurs. The inducible systems outlined in chapters IV and V were set up to address whether DAX1, when expressed before the onset of *Sry* and at high levels, could completely interfere with SRY and cause sex reversal of XY mice. However, in both cases described, neither of the overexpression constructs was completely functional. In the first case, the ER domain was functional in that the DAX1:ER fusion protein was retained in the cytoplasm in the absence of ligand, and only localised to the nucleus in the presence of Tamoxifen. However, the DAX1 sequence was no longer functional as a repressor, in comparison to the intact full length sequence. In the second case, the ER domain appeared to be non-functional in that localisation of the fusion protein to the nucleus occurred independent of ligand administration.

A third attempt could be made to generate an inducible mechanism of misexpressing DAX1, however, this may give similar results that were associated with the previous experiments. Perhaps as in the first construct, the ER domain could be fused 3' of the DAX1 sequence, and as in the second attempt, the two domains could be separated by a linker sequence.

The results discussed in chapters IV and V outline the difficulties of trying to retain a transcription factor in the cytoplasm, and may simply reflect the problems associated with this type of overexpression system. Indeed, no reports have been published where this type of straightforward inducible system has been used to successfully misexpress a transcription factor, although this approach has been tried in multiple developmental

systems. However, a bipartite system has been used efficiently to introduce both gain of function and loss of function mutations into the mouse germline.

This system also makes use of the ER LBD, but involves retaining a Cre (prokaryotic recombinase) ER fusion protein in the cytoplasm, in the absence of Tm. Perhaps it is more feasible to control the subcellular localisation of a prokaryotic factor that appears to have no functional requirement within the cytoplasm of eukaryotes, in comparison to a transcription factor. Tissue specific promoters are generally chosen to drive expression of a Cre-ER cassette. This type of construct may then be used to generate transgenic mouse lines (often referred to as inducible Cre lines). The second part of the bi-partite system relies on the introduction of LoxP sites (which can undergo Cre-mediated recombination) into a targeted locus, depending on whether gain or loss of function analysis is of interest.

An alternative inducible system based on this bipartite approach could be considered to give temporally controlled *in vivo* misexpression of DAX1. For example, a Cre:ER fusion protein could be targeted into the SF1 locus or into a Wt1 BAC regulatory sequence, to generate Cre:ER mice. To control misexpression of DAX1, a LoxP flanked (Floxed) stop codon cassette (LoxP TAG LoxP) could be inserted into a DAX1 promoter driving the full length DAX1sequence, and again used to generate transgenic mouse lines. In this situation, translation of the DAX1 transgene sequence will be prevented by the upstream Floxed stop codon. However, when the two transgenic lines are interbred, this will permit a temporally controlled expression of Cre that will mediate recombination and excision of the Floxed stop codon, and overexpression of DAX1. Therefore using different promoters sequences to drive expression of the Cre:ER cassette, temporal expression of DAX1 could occur in specific cell types to address the roles of DAX1 during different stages of gonadogenesis and indeed spermatogenesis.

However, prior to generating a new inducible misexpression system, it may be more important to investigate more closely the DAX1 loss of function experiments, whereby a conditional approach was used to generate *Dax1* deficient mice. The questions still

remains as to whether this approach generated a true null allele. A number of experiments could be used to try to address this issue. Firstly, different primers could be designed for RT-PCR analysis to test whether any transcript was generated from the remaining first exon. Secondly, *in vitro* transcription and translation experiments could be carried out to address whether a protein could be translated from this targeting construct in the presence of Cre. Thirdly, immunohistochemistry could be used both in *in vitro* and *in vivo* experiments to address whether any protein is made. Of course, this would depend on a specific antibody being able to recognise a shortened form of DAX1 if it were indeed translated.

VI.II SRY and the functional domains required during sex determination

To test whether the CAG repeat domain has any RNA stability effects, it would be interesting to remove this sequence downstream of the human stop codon and to assay again for sex reversal in XX transgenic mice. In parallel, this could be done for the mouse *Sry* sequence, by deleting part or all of the CAG repeat to address, but leaving the unique C-terminal sequence. However, this may give the same result as the previous experiments, where stop codons were introduced into the *Sry* sequence.

We have shown that the human SRY requires no Q rich domain to interact with partners or adapter molecules in activating the male sex determining pathway in mouse. To address the requirement of a PDZ interaction protein for human SRY, we have deleted the 7 amino acid binding sequence at the distal C-terminus of human SRY. Additionally, this experiment has been designed in such a way that the final cloning step will generate a 7 amino acid deleted human SRY protein that will either use the human or mouse poly adenylation site. In the case of the former, only human coding sequences will be transcribed and translated, whereas in the latter case, as in the human SRY experiments discussed in chapter V, the CAG repeat region of mouse will be transcribed but not translated. These constructs (currently being generated by C.Canning and R.Houghton) will be used in transgenic sex reversal assays, firstly to address whether

SRY can function in sex determination in the absence of the PDZ interaction domain, and secondly to address whether the CAG repeat has any stability functions.

Finally, in collaboration with Ryohei Sekido, Hu-Hui Lee, and Richard Behringer, a marsupial *Sry* clone has been isolated from a *S. macroura* genomic library, and the corresponding cDNA has been cloned into the mouse *Sry* promoter. This is currently being injected to test again whether marsupial SRY can function in mouse. Unlike other *Sry* genes isolated so far, marsupial *Sry* contains an intron and a conserved HMG box domain, but lacks any conserved sequences outside of the DNA binding domain. To date, no SIP's have been isolated for marsupial SRY. If marsupial *Sry* can function in mouse to cause sex reversal then this will further support our model that the HMG box is the only important domain required for SRY function during testis determination.

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Appendix **Luciferase Assay Values**

Luciferase Assay results. (Averages of triplicate or duplicate experiments) for CMVDAX-ER +Tm

Luciferase Assays	firefly	Renilla	Ratio
-395bp Amh Luc, 100ng SOX9/SF1	47.38	1847	0.024
-211bp AmhLuc "	91.74	2753	0.033
-180bp AmhLuc "	110.4	2909	0.038
-395bp Amh Luc, 500ng SOX9/SF1	30.78	913.4	0.034
-211bp Amh Luc "	65.81	3628	0.018
-180bp Amh Luc "	103.1	3258	0.032
-395 Amh luc SV40rlLuc	1.9	237	0.008
Amh Luc + SOX9 +"	6.074	217.3	0.028
Amh Luc + SF1 +"	22.78	599.2	0.038
" " +SOX9 +SF1	22.69	405	0.056
" " " + WT DAX1	18.75	534	0.035
" " " +CMV DAX-ER +Tm	9.711	124.1	0.078

Untransfected	0.000	0.001	0.000
Amh-Luc +CMV rlLuc (10ng)	9.78	9000	0.001
+SOX9	6.489	5987	0.001
+SF1	16.19	7596	0.003
+SOX9 +SF1	54.12	6937	0.008
+SOX9 +SF1+DAX (WT)	15.99	5520	0.002
+DAX-ER	10.56	1437	0.007
AmhLuc + CMV rl	12.4	3745	0.003
AmhLuc +rl+ SOX9	54.2	6905	0.008
AmhLuc +rl+ SF1	61.6	5298	0.012
AmhLuc +rl+SOX9 +SF1	68.4	5037	0.014
AmhLuc +rl+SOX9 +SF1+DAX1	24.6	4387	0.005
AmhLuc +rl+SOX9+SF1+DAXER	57.61	4307	0.014

Luciferase Values for ERglyDAX1

AmhLuc +TKrl	1.7	81.29	0.002
+SOX9+SF1	0.47	77.9	0.006
+ERglyDAX1-Tm	1.532	92.45	0.017
+ERglyDAX1+Tm	1.148	84.19	0.015
AmhLuc +TKrl	0.492	86.79	0.005
+SOX9+SF1	1.084	58.49	0.019
+ERglyDAX1-Tm	1.727	106.4	0.016
+ERglyDAX1+Tm	1.452	107.4	0.013
AmhLuc +TKrl	0.355	72.81	0.004
+SOX9+SF1	1.82	92.66	0.02
+ERglyDAX1-Tm	1.33	112.5	0.012
+ERglyDAX1+Tm	1.596	102.0	0.015

AmhLuc +TKrl	0.834	33.84	0.025
+SOX9+SF1	1.533	28.07	0.05
+ERglyDAX1-Tm	4.37	699.9	0.006
+ERglyDAX1+Tm	1.277	54.45	0.023
AmhLuc +TKrl	0.767	90.62	0.008
+SOX9+SF1	1.487	9.225	0.16
+ERglyDAX1-Tm	1.062	54.72	0.019
+ERglyDAX1+Tm	6.313	72.01	0.089
AmhLuc +TKrl	0.657	9.011	0.07
+SOX9+SF1	2.635	8.502	0.3
+ERglyDAX1-Tm	1.312	13.14	0.09
+ERglyDAX1+Tm	0.463	6.484	0.07

AmhLuc +TKrl	36.51	8566	0.004
+SOX9+SF1	126	4039	0.03
+ERglyDAX1-Tm	0.591	169.1	0.003
+ERglyDAX1+Tm	1.685	842.7	0.002
AmhLuc +TKrl	5.201	721.5	0.007
+SOX9+SF1	108.9	4936	0.022
+ERglyDAX1-Tm	1.22	257.8	0.004
+ERglyDAX1+Tm	0.592	119.7	0.005
AmhLuc +TKrl	5.669	7197	0.0007
+SOX9+SF1	127.4	4703	0.03
+ERglyDAX1-Tm	1.23	134.1	0.009
+ERglyDAX1+Tm	0.39	326.1	0.001