Gene Expression during Fetal Gonadal Development

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Sexual reproduction is...the masterpiece of nature.

(Erasmus Darwin, 1791)

We must not conceal from ourselves the fact that the causal investigation of organisms is one of the most difficult, if not the most difficult problem which the human intellect has attempted to solve, and that this investigation, like every causal science, can never reach completeness, since every new cause ascertained only gives rise to fresh questions concerning the cause of this cause.

(Wilhelm Roux, 1894)

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Declaration

The experiments described in this thesis were the unaided work of the author except where the acknowledgement is made by reference. No part of this work has been previously accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

Sexual differentiation and normal development of the testis and male genital tract are tightly regulated processes dependent upon a cascade of molecular and morphological events. In the past fifty years the incidence of reproductive problems in males including hypospadias, cryptorchidism and testicular cancer is reported to have increased progressively in many developed countries. Several studies have also presented controversial findings suggesting that the average sperm counts and/or semen quality have also declined over the same time period. It has been suggested that all these problems could arise from events occurring during fetal and/or neonatal life. Male offspring born to mothers who were given diethylstilbestrol (DES), a potent synthetic oestrogen, as a preventive to miscarriage, were found to have increased incidence of undescended testes, urogenital abnormalities and semen abnormalities compared with those from mothers not given DES. Similarities between these observations and the abnormalities being observed in the general population has led to hypothesis that one potential cause of the rise in male reproductive problems might be inappropriate exposure to oestrogens during fetal and/or neonatal life.

The aims of the present studies were to firstly examine the ontogeny of gene expression during normal development of the fetal rat testis and secondly to investigate if oestrogenic chemicals could affect this process as a first step towards elucidating the mechanisms which might account for the observed problems in male reproductive development.

In the first part of the thesis, temporal and spatial localisation of several different proteins has been studied using immunocytochemistry. Novel results regarding localisation of androgen receptor and inhibin subunits are reported which demonstrate differences in their patterns of expression in fetal and adult Leydig cells. Specifically, in contrast to their adult counterparts, fetal cells do not express androgen receptor suggesting that the negative feedback loop of testosterone on its own production, established in adult testis via the androgen receptor, is not functional in the fetus. Inhibin α and ßB subunits were localised to Leydig cells from days 14.5 and 16.5 of gestation respectively. In addition, functional differentiation of fetal Leydig cells has been studied by examining the expression of two steroidogenic enzymes 17α-hydroxylase/17,20-lyase (P450c17) and 3ß-hydroxysteroid dehydrogenase (3ß-HSD) and two orphan nuclear receptors, steroidogenic factor-1 (SF-1) and DAX-1. As expected 3ß-HSD and P450c17 immunolocalised exclusively to fetal Leydig cells.

Data on immunoexpression of DAX-1 has not been reported previously; DAX-1 protein was first detectable in the fetal rat testis on day 15.5 in the interstitial cells, simultaneously with the onset of testosterone production. Co-localisation of SF-1 and DAX-1 in the fetal testis demonstrated that both proteins are not exclusively co-localised in the same cell types, a finding at odds with suggestions that SF-1 regulates expression of DAX-1. The second part of the thesis describes experiments designed to elucidate potential mechanisms underlying the influence of oestrogens on the developing fetal testis. Studies have focused on the expression of the enzyme P450c17 and the transcription factor SF-1. A significant decrease in mRNA expression and enzyme activity of P450c17 occurred in fetuses of mothers treated with DES or the environmental oestrogen octylphenol (OP) but this was not mirrored by an obvious reduction in 3ß-HSD immunostaining. The reduction in P450c17 expression was paralleled by a reduction in SF-1 mRNA and protein expression. As SF-1 is known to act to regulate multiple genes in the pituitary - testicular axis, including P450 enzymes, a reduction in expression of this factor could have a significant effect on the development of the testis and genital tract.

In conclusion, the studies described in this thesis have elucidated the cellular site of expression of several gene products implicated in fetal testicular development and demonstrated that in some cases gene expression is reduced in fetuses from oestrogen-treated mothers.

List of abbreviations

ABC	Avidin-biotin complex
Ad4BP	Adrenal 4 binding protein (SF-1)
AHC	Adrenal hypoplasia congenita
AMH	Antimüllerian hormone
APEOs	Alkylphenol polyethoxylates
AR	Androgen receptor
ATP	Adenosine triphosphate
CD	Campomelic dysplasia
cDNA	Complementary DNA
CIS	Carcinoma in situ
cpm	Counts per minute
cRNA	Complementary RNA
CTP	Cytosine triphosphate
CYP17	cytochrome P450 17α-hydroxylase/17,20-lyase gene
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAX-1	DSS-AHC critical region on X chromosome, gene 1
DES	Diethylstilbestrol
DDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane
DHT	Dihydrotestosterone
DMSO	Dimethylsulphoxide
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
DSS	Dosage sensitive sex reversal
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELP	Embryonic long terminal repeat
ER	Oestrogen receptor
FITC	Fluorescein
FSH	Follicle stimulating hormone
Ftz F-1	Fushi tarazu factor-1
GnRH	Gonadotrophin releasing hormone
GTP	Guanosine triphosphate
hCG	Human chorionic gonadotropin
H&E	Haematoxylin and eosin

HMG	High mobility group
kb	Kilobase pairs
kD	Kilo Dalton
LH	Luteinizing hormone
mRNA	messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
OP	Octylphenol
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
P450scc	cytochrome P450 side chain cleavage
P450c17	cytochrome P450 17α-hydroxylase/17,20-lyase
P450arom	cytochrome P450 aromatase
P450c21	cytochrome P450 21ß-hydroxylase
RNA	Ribonucleic acid
rNTP	Ribonucleotide triphosphate
RT PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SF-1	Steroidogenic factor-1
SHBG	Sex hormone binding globulin
SOX9	Sry related hmg box gene 9
SRY	Sex determining region on Y chromosome
StAR	Steroidogenic acute regulatory protein
TBE	Tris Boric acid EDTA
TBS	Tris buffered saline
TDF	Testis determining factor
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-ß	Transforming growth factor ß
TLC	Thin layer chromatography
TRIS	Tris(hydroxymethyl)aminomethane
TRITC	Rhodamine
tRNA	transfer ribonucleic acid
TSH	Thyroid stimulating hormone
TTP	Thymidine triphosphate
T3	Triiodothyronine

UTP	Uridine triphosphate
WT-1	Wilms' tumour suppressor gene
3ß-HSD	3ß-hydroxysteroid dehydrogenase

1. Introduction

The mechanisms by which an individual's sex is determined has been one of the great questions of embryology since antiquity. Aristotle claimed that the sex of the offspring was determined by the heat of the male partner during intercourse. The more heated the passion, the greater was the probability of a male offspring. Aristotle also proposed that women are in fact men whose development is arrested prematurely due to the coldness of their mother's womb overcoming the heat of male semen. These theories were accepted by the Christian church and by Galen, remaining popular until the 16th and 17th century (Gilbert, 1994). The 20th century has seen a dramatic increase in our understanding of the processes underlying sexual differentiation, and it has become clear that this process is dependant on genetic information rather than on environment. Some of the most notable contributions were made by Professor Alfred Jost who in the 1940's and 50's suggested that in the mammals the default pathway in sexual determination is the development of the female phenotype. As a direct result of his experimental studies testosterone was identified as the key hormone essential for virilization of the male fetus (Jost, 1953). Furthermore, he also suggested the existence of an as yet undiscovered hormone responsible for degeneration of the female reproductive tract (the hormone was later isolated and named the Antimüllerian hormone (AMH; Jost, 1953). Another important milestone in studies on sexual determination was the identification of the testis determining factor (known as SRY/Sry) on the Y chromosome in the late 1980's (Berta et al., 1990; Sinclair et al., 1990). However, while the pieces of this complex mosaic are slowly assembling, many unanswered questions still need to be addressed.

In the past few years several publications have highlighted a trend towards an increase in male reproductive problems. Several studies from different European countries have shown a dramatic increase in the incidence of testicular cancer (today the most common cancer of young men in the Western world), cryptorchidism and hypospadias (Giwercman, 1995; Toppari et al., 1996). It has also been reported that the average sperm count in men from the Western world is falling by a rate of approximately 2% per year (Auger et al., 1995; Carlsen et al., 1992; Giwercman, 1995; Giwercman and Skakkebaek, 1992) although this is not universal (Fisch and Goluboff, 1996; Suominen and Vierula, 1993; Vierula et al., 1996). All these aforementioned problems in the male reproductive tract have one interconnecting point - they are believed to have a common origin in events during fetal testicular development (Sharpe and Skakkebaek, 1993). Firstly, cryptorchidism and

hypospadias are thought to be the consequence of inadequate virilization of the reproductive tract by testosterone. Secondly, cells causing the most common form of human testicular cancer, carcinoma in situ (CIS), are believed to arise from the uncontrolled growth of fetal germ cells (Giwercman, 1995). Thirdly, the apparent reduction in male sperm counts may also have its origin during fetal/neonatal life with smaller testicular size resulting from reduced numbers of Sertoli cells, the numbers of which are fixed at this stage of development (Sharpe and Skakkebaek, 1993). The aetiology for all these problems is not known, but some evidence suggests that exposure to oestrogens or chemicals mimicking the action of oestrogens could be involved. It has been suggested that changes in the lifestyle, diet and pollution of the environment may all result in a greater exposure of mankind to oestrogens and that this has contributed to the increase in male reproductive problems (Sharpe and Skakkebaek, 1993).

The aim of the experiments summarised in this thesis was to study the processes underlying normal sexual differentiation and to examine the effects of oestrogenic chemicals on these processes. Ontogeny studies have focussed on examining the pattern of expression of several key gene products in the fetal testis including the androgen receptor, two steroidogenic enzymes cytochrome P450c17 and 3ß-HSD, the inhibin/activin subunits and two nuclear orphan receptors SF-1 and DAX-1. The assumption was that pinpointing the time of onset and changes in the level(s) of these proteins would enhance our understanding of the role of these factors in testicular development and function. Studies on normal (untreated) animals were considered an essential prerequisite to an understanding of the possible causes of abnormalities in these processes.

The second part of this thesis describes studies designed to examine the possible influence of environmental oestrogens on fetal gonadal development and function. These studies have concentrated on the influence of oestrogens on one of the key steroidogenic enzymes P450c17 and on the expression of SF-1, another important factor regulating the development of the adrenal gland and the reproductive tract. Results indicate that the expression of both these genes is affected by inappropriate exposure to oestrogens during fetal life and provide a background from which to expand our understanding of the influence of oestrogens on male reproductive development.

In conclusion, this thesis provides new data about the expression of factors

influencing testicular development during fetal and neonatal life and the results obtained demonstrate that environmental oestrogenic chemicals may affect the expression of genes the products of which are essential to normal function of the Leydig cells at a time when production of testosterone is important for normal masculinisation.

2. Literature Review

2.1 Introduction

Sexual determination and development of the gonads is a tightly regulated process that requires a precise spatial and temporal interaction of many genes and proteins (reviewed by Bogan and Page, 1994; Capel, 1995; Ryner and Swain, 1995). In mammals, the sex of the offspring is determined at the moment of fertilisation by the sperm carrying either an X or a Y chromosome (Gilbert, 1994). Fertilisation of the ovum with a sperm containing an X chromosome results in a female phenotype whereas fertilisation with a sperm carrying a Y chromosome triggers development of the male phenotype (Gilbert, 1994). Differences in cell number and growth rates between male and female embryos can be seen very early in development, prior to differentiation of the gonads (reviewed by Mittwoch, 1993).

Gonadal development starts around midgestation in rodents (Kaufman, 1992) and at the middle of first trimester in humans (Gustafson and Donahoe, 1994). In mammals, the presence of a segment of the Y chromosome containing the gene for a Testis determining factor (TDF) triggers differentiation of the testis and subsequent development of the male phenotype (Koopman et al., 1991; Sinclair et al., 1990). Otherwise fetal development follows the default pathway resulting in the development of the female phenotype (Gilbert, 1994). A simplified diagram of sexual differentiation is presented in figure 2.1.

The gonads develop at the ventral side of the mesonephros, as a bulge of sexually undifferentiated mesenchymal tissue called the genital ridge (Gustafson and Donahoe, 1994; Pelliniemi et al., 1993; Satoh, 1985). Within the male genital ridge, Sertoli cells differentiate from mesenchymal cells and form testicular cords surrounding the germ cells (Gustafson and Donahoe, 1994; Kaufman, 1992). Sertoli cells start to produce Antimüllerian hormone (AMH, section 2.10.1) - the factor responsible for regression of the Müllerian ducts which would otherwise form female internal genitalia (reviewed by Lee and Donahoe, 1993). Following Sertoli cell differentiation, the Leydig cells differentiate from mesenchymal cells and start to produce testosterone, the androgen responsible for masculinisation of the male fetus (reviewed by Huhtaniemi and Pelliniemi, 1992 and Saez, 1994).

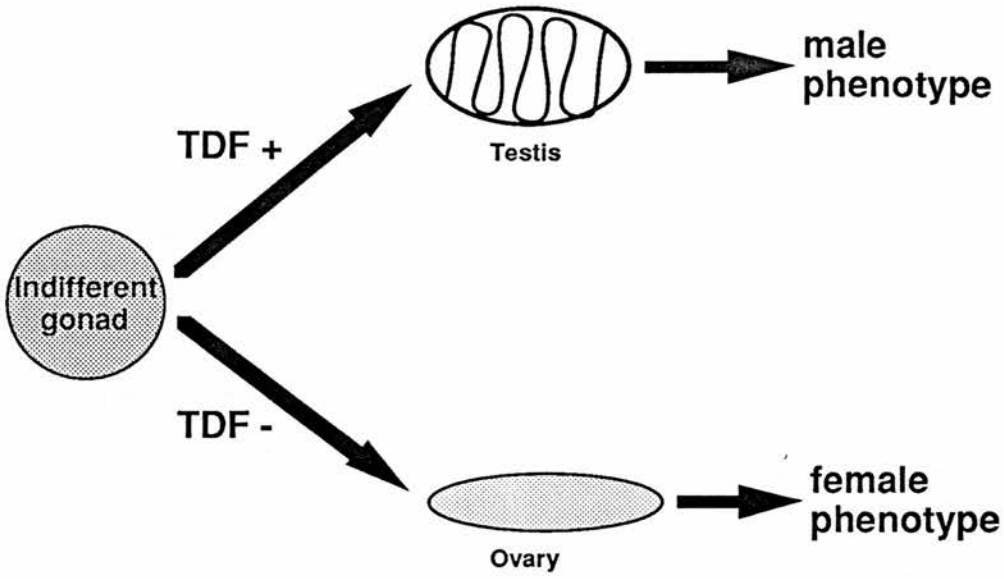


Figure 2.1: Development of the male or female phenotype

Identification of the product of the *SRY* gene (see section 2.5.2) as the TDF was a major breakthrough in studying the mechanisms of sexual differentiation (Koopman et al., 1991; Sinclair et al., 1990). This discovery together with the recent isolation of several other genes with potentially important function(s) in sexual differentiation and gonadal development, promise a greater understanding of these processes.

2.2 Primary sex determination

Primary sex determination concerns the determination of the sex of the gonads (Gilbert, 1994). In mammals, sex determination is strictly chromosomal and is not generally influenced by the environment (Bogan and Page, 1994; Gilbert, 1994; Ryner and Swain, 1995). Normally the female carries two X chromosomes and therefore each of her eggs contains a single X chromosome. In contrast, the XY genotype of the male results in sperm bearing either an X or Y chromosome. The X containing oocyte will develop ovaries and a female phenotype if it fuses with a X bearing sperm at the time of fertilisation. Alternatively, a male phenotype develops if the egg is fertilised by a sperm carrying a Y chromosome (Gilbert, 1994). It is the presence of the *SRY* gene of the Y chromosome, which is responsible for testis determination (Gilbert, 1994; Harley, 1993; Ryner and Swain, 1995). Even if an individual carries more than one X chromosome a male will develop in the presence

of a single functional Y chromosome. Similarly, an individual with only a single X chromosome (no second X or Y chromosome) develops as a female and forms ovarian tissue although these fail to maintain follicular development (Gilbert, 1994; Harley, 1993).

2.3 Secondary sexual determination

Secondary sex determination concerns the bodily phenotype outside the gonads (Gilbert, 1994). A male mammal develops a penis and secondary sexual glands (prostate, seminal vesicles, bulbouretric glands) together with sex specific size, vocal cartilage and musculature whilst a female mammal develops a vagina, cervix, uterus, oviducts and mammary glands. These secondary sexual characteristics are usually determined by hormones secreted from the gonads (Gilbert, 1994). However, in the absence of the gonads, a female phenotype results, emphasising the importance of the hormones produced by the fetal testis in the development of male secondary sexual characteristics (Bogan and Page, 1994; Gilbert, 1994; Jost, 1953). It is now widely accepted that all secondary sexual characteristics in eutherian mammals result from the action of two testicular hormones, AMH and testosterone. AMH is secreted by Sertoli cells and causes regression of the Müllerian ducts which would otherwise develop into a uterus, oviduct, cervix and upper portion of vagina (George and Wilson, 1994; Gilbert, 1994; Lee and Donahoe, 1993). The other hormone testosterone, secreted by Leydig cells (Huhtaniemi and Pelliniemi, 1992; Saez, 1994), induces the development of male structures derived from the Wolffian duct including the epididymis, vas deferens and seminal vesicles. In addition, the formation of male rather than female external genitalia requires the conversion of testosterone into dihydrotestosterone in the target tissue (George and Wilson, 1994).

A simplified diagrammatic representation of development of the testis and male phenotype is illustrated in figure 2.2.

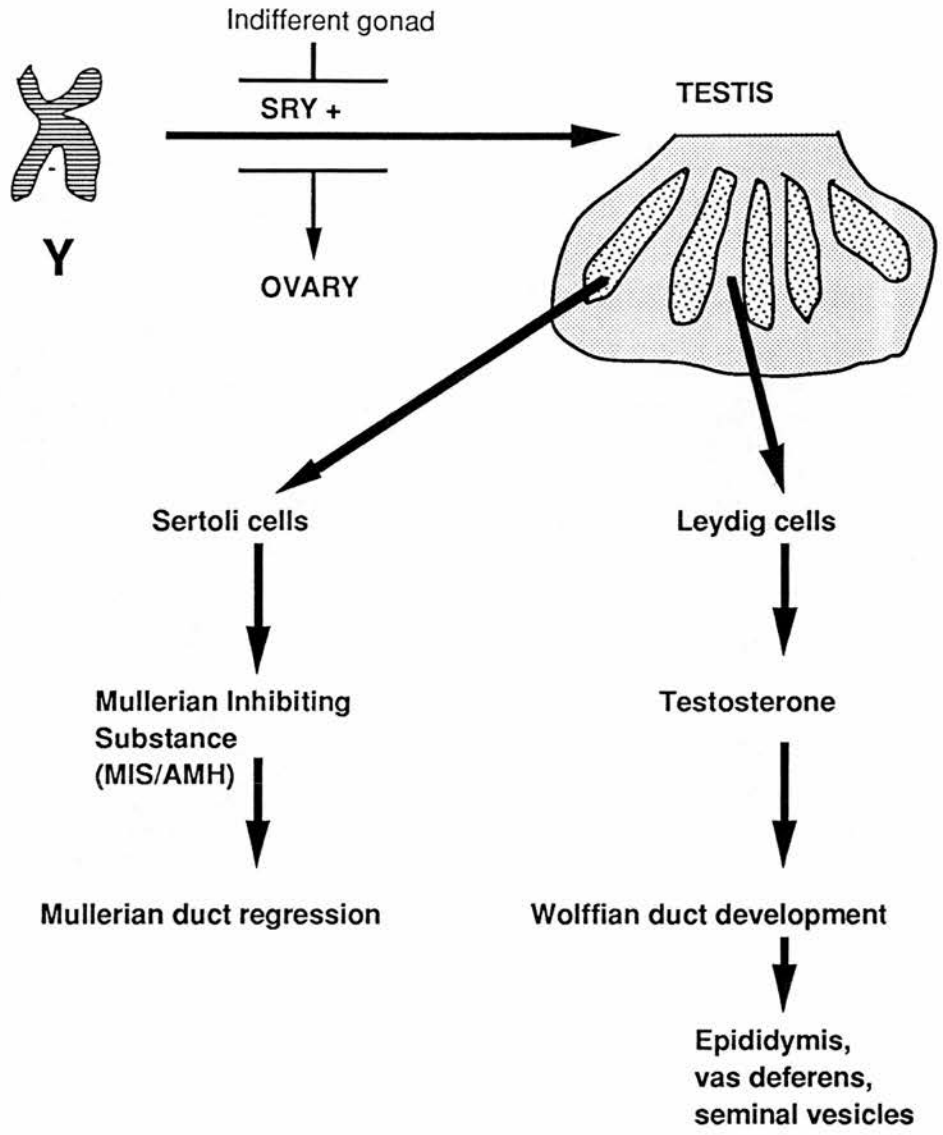


Figure 2.2: Development of the testis and male phenotype

2.4 Differences between XX and XY embryos during early development

The sex of the embryo is determined at the time of fertilisation, however, the gonads develop at a relatively late stage of organogenesis with the most obvious intersex differences evident only after puberty. Some differences between the sexes appear very early during development, at the blastocyst stage (Mittwoch, 1993). Several studies in mammalian species have shown differences in the size and number of cells in early preimplantation embryos (Avery et al., 1991; Burgoyne, 1993;

Tsunoda et al., 1985; Xu et al., 1992). Tsunoda and co-workers (1985) have reported that male embryos are more advanced at the blastocyst stage. Similarly, Seller and Perkins-Cole (1987) found that the number of somites present in a 9 day old mouse embryo varies between 0 and 12, with most of the embryos with 0 - 5 somites being female and most of the embryos with 6-12 somites being male. These results have raised the question as to whether the observed differences in development of early embryos result from a faster growth rate or an earlier time of fertilisation. The answer has been provided by studies using cattle embryos obtained by in-vitro fertilisation. These experiments showed that male cattle embryos, fertilised at the same time as female embryos, were already more advanced by the blastocyst stage (Avery et al., 1991; Xu et al., 1992). It has been suggested that the presence of the second X chromosome in the female embryo may be responsible for slower growth and development (Burgoyne et al., 1983). Conversely, it has been postulated that the Y chromosome carries a factor which accelerates the growth of early male embryos (Burgoyne, 1993; Tsunoda et al., 1985). However, the importance of these observations is unknown.

2.5 Genetic control of sexual determination

Sexual differentiation and development involve the action of many gene products. In recent years, several of these important genes have been identified and further studies are underway which will hopefully result in determining the action of these genes in sexual determination (Bogan and Page, 1994; Capel, 1995; Ryner and Swain, 1995). The current model of the involvement of some of these factors in the sexual determination cascade is illustrated in figure 2.3.

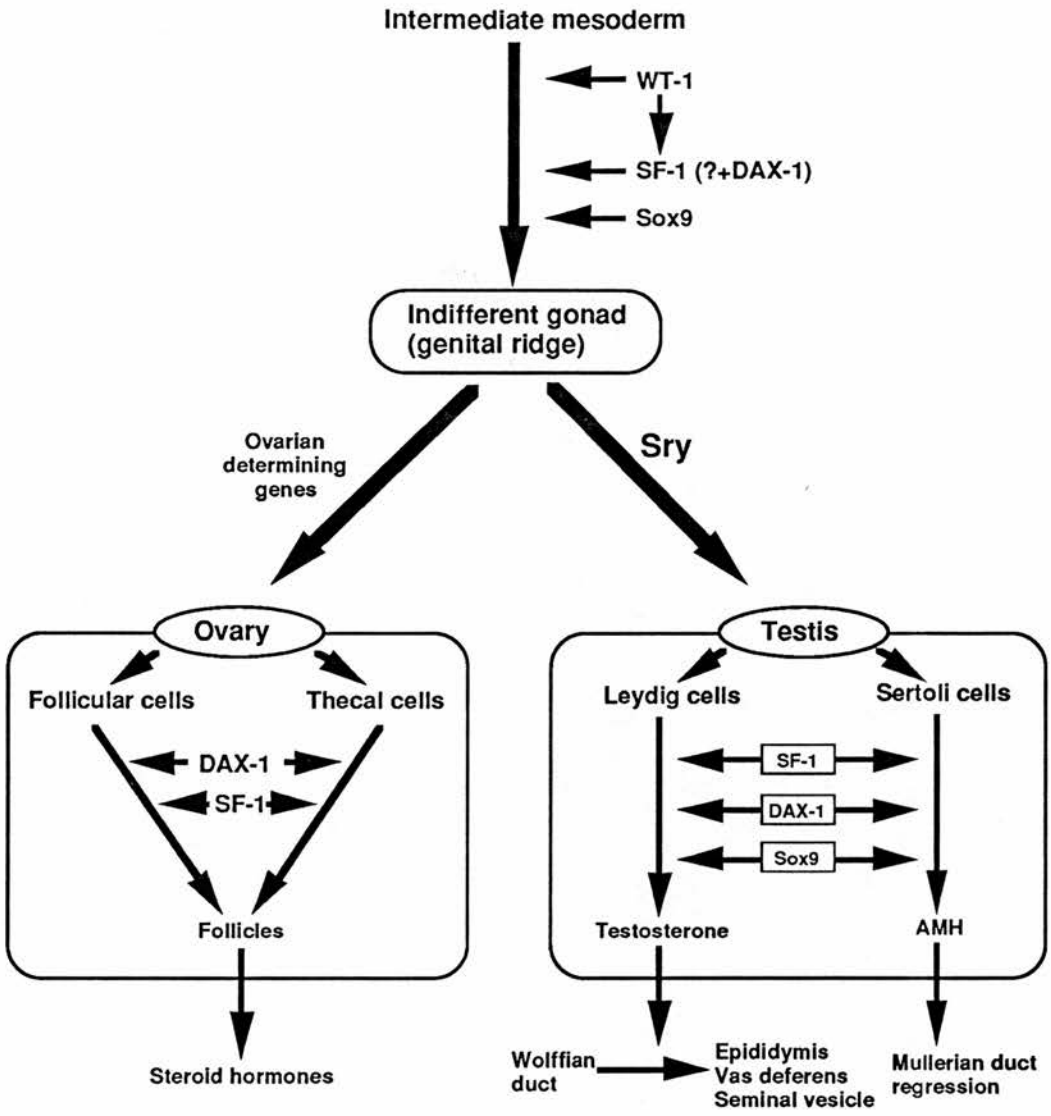


Figure 2.3: Genetic control of gonadal development

2.5.1 Testis determining factor

A small region on the short arm of the Y chromosome has been shown to contain the testis determining factor (TDF). This factor is responsible for testis determination, dominantly inducing the development of the testis and thus consequently the male phenotype (Bogan and Page, 1994; Goodfellow and Lovell-Badge, 1993). Extensive analysis and comparison of sex chromosomes of XX males containing small fragments of Y chromosome and XY females lacking fragments of Y chromosome finally resulted in identification of a single gene, named SRY (for sex

determining region on the **Y** chromosome) in humans and Sry in mice (Berta et al., 1990; Sinclair et al., 1990). Initial evidence implicating SRY/Sry as the TDF came from expression studies which showed *Sry* gene expression in the mouse genital ridges for a short time period prior to testis differentiation (Koopman et al., 1990). In addition, the identification of de novo mutations in the *SRY* gene of some XY females further corroborated SRY's involvement in sex determination (reviewed by Goodfellow and Lovell-Badge, 1993). Direct evidence that Sry is indeed the primary testis determining factor was provided by transgenic mice studies. A 14 kilobases long fragment of DNA containing the *Sry* gene and its flanking sequences was introduced into XX mice embryos and in some, but not all embryos, the fragment induced the formation of the testis and therefore a male phenotype (Koopman et al., 1991). Furthermore, these transgenic animals developed apparently normal testis as well as internal and external genitalia, suggesting that testosterone and AMH were produced during fetal development (Koopman et al., 1991). From these experimental studies it was thus apparent that Sry alone was capable of inducing testis development.

2.5.2 Structure and function of SRY/Sry

The SRY/Sry protein contains 204 and 395 amino acids in human and mouse respectively (Dubin and Ostrer, 1994) and is encoded by a small gene containing one exon (Behlke et al., 1993). This protein belongs to the high mobility group (HMG), a family of non-histone nuclear proteins (Grosschedl et al., 1994; Laudet et al., 1993). Members of this family are classified according to the number of DNA binding HMG domains they contain with proteins containing either multiple, low sequence specificity, or single, highly nucleotide specific domains. Common properties of the HMG domain proteins include interaction with the minor groove of the DNA helix, binding to irregular DNA structures and the capacity to modulate DNA by bending (Grosschedl et al., 1994; Laudet et al., 1993). The SRY/Sry protein contains a single HMG domain and binds DNA in a sequence specific manner (Harley and Goodfellow, 1994). The HMG box in the SRY/Sry protein selectively binds the sequence NACAAT, with the highest affinity for AACAAT (Dubin and Ostrer, 1994; Harley et al., 1992), and is capable of bending DNA up to 85° (Harley and Goodfellow, 1994). The DNA sequence encoding the HMG box region of the SRY/Sry gene is relatively conserved amongst different species (Sinclair et al., 1990; Whitfield et al., 1993). However, the remaining gene sequence shows no inter species homology and in fact differs even between two

subspecies of the mouse, suggesting rapid evolution of the gene (Tucker and Lundrigan, 1993; Whitfield et al., 1993). This observation together with the fact that all mutations in human XY females reside inside the HMG box (Goodfellow and Lovell-Badge, 1993) probably suggest that the HMG box is the only functional part of the SRY/Sry protein. Some studies have implied that SRY/Sry may directly regulate AMH gene expression, as both proteins are synthesised by Sertoli cells and AMH expression immediately follows that of Sry in the mouse embryo (Haqq et al., 1993; Koopman et al., 1990; Munsterberg and Lovell-Badge, 1991). However, the lack of any known transcriptional activation domains in human SRY might be consistent with an indirect action of SRY/Sry on the regulation of AMH gene expression. This is further supported by recent studies in vitro demonstrating an indirect interaction between these two factors (Haqq et al., 1994). It is possible that SRY/Sry acts solely by inducing changes in chromatin structure or by bending the DNA and therefore allowing interactions between distal regions of DNA and regulatory proteins (Bogan and Page, 1994).

In the mouse fetus, functional Sry is thought to be expressed only for a short time at the start of gonadal differentiation. Sry mRNA expression in the genital ridges is first detectable by PCR on day 10.5 p.c. and remains present until day 12.5 (Hacker et al., 1995; Jeske et al., 1995; Koopman et al., 1990). This finding suggests that the expression of Sry precedes Sertoli cell differentiation which occurs on day 12.5 p.c. In addition, Sry transcripts have also been identified in mouse blastocysts (Qiu et al., 1995; Zwingman et al., 1993) and differentiating adult germ cells (Capel et al., 1993). The transcripts in the blastocyst and adult germ cells appear to be unusual in that they are circular and it is unclear whether these mRNA transcripts are translated into functional proteins (Capel et al., 1993; Qiu et al., 1995).

The exact cellular localisation of SRY/Sry mRNA and protein have not yet been determined, partially due to the very low level of expression of the gene. However, Sry is presumed to be expressed in the somatic component of the developing gonad, as gonadal development is possible in the absence of germ cells (McLaren, 1991). Studies in chimeric XX/XY embryos have endorsed this view and shown that most but not all Sertoli cells are XY, while Leydig cells have a proportion of XX and XY cells similar to that observed in other organs. Likewise, in the developing ovary the granulosa cells are almost exclusively XX while other cells have a ratio of XX and XY cells similar to that observed in other tissues (Patek et al., 1991). In the testis, this finding suggests that Sry is autonomously expressed in the pre-Sertoli cells and

this event probably triggers differentiation of other cell types. Furthermore, the results of these studies imply that the initial steps in male/female gonadal differentiation occur in pre-Sertoli or pre-granulosa cells but as some XX Sertoli cells and XY granulosa cells have been detected in chimeric gonads this autonomous action must be supplemented by additional non-autonomous factors (Patek et al., 1991).

2.5.3 SOX9

SOX9 is the human homologue of the mouse gene *Sox9*, a member of a family of genes related to SRY at the level of their sequence (Foster et al., 1994; Wagner et al., 1994; Wright et al., 1995). The Sox gene family (Sry-type HMG box) is composed of more than 10 members which all share up to 60% homology in their HMG box domain. Unlike *SRY/Sry, SOX9/Sox9* is highly conserved between species showing 96% overall homology between the human and mouse genes (Wright et al., 1995), an observation which may be consistent with Sox9 having an important regulatory function. Sequence analysis and comparison suggest that Sox9 may function as a transcription factor and it is thought to be important in the development of the skeletal system (Foster et al., 1994; Wagner et al., 1994; Wright et al., 1995). In the mouse embryo, its expression predominates in mesenchymal condensations throughout the embryo before and during the deposition of cartilage (Wright et al., 1995). In humans, mutations in the *SOX9* gene are associated with the campomelic dysplasia (CD), a skeletal malformation syndrome (Foster et al., 1994; Wagner et al., 1994). The most obvious feature of CD is congenital bowing and angulation of the long bones which is often combined with other skeletal abnormalities. Moreover, two thirds of XY individuals with CD have a female phenotype or are intersex (Houston et al., 1983). At present, it is thought that SOX9 is required early in gonadal development as many cases of CD display only primitive gonadal histology with ovarian-like stroma, occasional primordial follicles, but with no recognisable testicular structures (Foster et al., 1994; Wagner et al., 1994). Sequence similarities between SRY and SOX9 suggest a possible evolutionary relationship between these two genes and the potential that SRY/SOX9 interactions may be required for full cell function in pre-Sertoli cells. SOX9 expression may also be required in other cell types such as the mesenchymal cells which migrate from the mesonephros and which are required for testis formation (Capel, 1995).

2.5.4 Steroidogenic Factor-1

Steroidogenic factor-1 (SF-1), also called Ad4BP, is a recently discovered transcription factor that was initially identified as a regulator of cytochrome P450 enzymes including P450scc, P450c17, P450arom and P450c21 (Clemens et al., 1994; Ikeda et al., 1993; Lynch et al., 1993; Morohashi et al., 1992; Morohashi et al., 1993; Zhang and Mellon, 1996). In addition, an SF-1 binding element is contained in the promoter regions of several other genes including AMH (Shen et al., 1994) and the common α-subunit of LH and FSH (Barnhart and Mellon, 1994). The SF-1 protein is encoded by the gene *Ftz-F1*, a mammalian homologue of the *Drosophila* Fushi tarazu factor 1 gene (Ikeda et al., 1993; Lala et al., 1992; Tsukiyama et al., 1992). The gene contains 5 introns, is highly conserved between mice and *Drosophila* (Ikeda et al., 1993), and is located on mouse chromosome 2 and human chromosome 9q33 (Taketo et al., 1995). Alternative gene splicing produces two different mRNA transcripts; one of the translation products is expressed at early stages of embryogenesis and named the embryonic long terminal repeat (ELP) (Ikeda et al., 1993; Morohashi et al., 1994), the other splicing variant encodes the 461 amino acid SF-1 protein. SF-1, a member of the nuclear receptor superfamily, contains a putative DNA binding domain at the amino terminus containing two zinc fingers and a putative ligand binding domain at the carboxy terminus (Honda et al., 1993; Tsukiyama et al., 1992). Its ligand has not yet been identified but some studies suggest that ligand-DNA interactions are necessary for transcriptional activity. Shen et al. (1994) cotransfected AMH and SF-1 genes in a HeLa cell line and failed to produce any transcriptional activation of AMH by SF-1. In contrast, transfection of primary Sertoli cells resulted in enhanced expression of the AMH gene. In the same study, the removal of the putative SF-1 ligand domain by deletion mutations resulted in creation of a constitutively active receptor, implying that a ligand is necessary for activation of SF-1 and that the requirement of SF-1 for a specific ligand may depend upon the cell type in which it is expressed. Additional experiments have shown that recombinant SF-1 protein binds with high affinity to a region of DNA with the sequence AGGTCA termed the SF-1 response element (Morohashi et al., 1992).

The construction of a transgenic mouse lacking a functional *SF-1* gene has demonstrated the importance of SF-1 in the development of steroidogenic tissues and reproductive organs as these SF-1 knock out mice lacked gonads and adrenal glands (Luo et al., 1994). In addition, they had a disorganised ventromedial nucleus

in the hypothalamus and did not express either LH or FSH in their anterior pituitary glands (Ingraham et al., 1994). Newborn mice homozygous for the SF-1 knock out died as a result of insufficient adrenal-derived steroid hormones but did survive if given replacement gluco- and mineralocorticoids (Luo et al., 1994). In addition, the hypothalamus of these mice produced GnRH but it was not transported to the pituitary and therefore the pituitary gonadotroph cells did not express LH, FSH or the GnRH receptor unless given GnRH exogenously (Ingraham et al., 1994). However, the lack of LH and FSH was not the cause of the observed agonadism since gonadal development (and adrenal development) was arrested prior to the onset of LH and FSH production. Studies have shown that these mice initially develop genital ridges within the mesonephric area but gonadal differentiation is soon halted and the cells undergo apoptosis (Ingraham et al., 1994; Luo et al., 1994).

Immunocytochemical and in situ hybridisation studies have shown that SF-1 is expressed in the mesonephric area, the ventromedial nucleus of hypothalamus and some pituitary cells in mouse and rat (Hatano et al., 1994; Ikeda et al., 1994; Ingraham et al., 1994). Initially, SF-1 is expressed in the genital ridges in both sexes but after initiation of gonadal differentiation, its continued expression is confined to the testis (Hatano et al., 1994; Ikeda et al., 1994). Within the testis, SF-1 mRNA and protein have been detected in both Leydig and Sertoli cells although expression levels are significantly higher in the Leydig cells (Hatano et al., 1994). This pattern of expression and the presence of an SF-1 response element in the promoter regions of the genes for P450scc, P450c17 and AMH provides evidence that SF-1 regulates the production of testosterone in Leydig cells and the expression of AMH in Sertoli cells. However, to substantiate this hypothesis additional in vitro and in vivo studies are required.

Only limited studies on the regulation of SF-1 gene expression have so far been presented. An E-box element, the binding site for the proteins with a helix-loop-helix motif, has been identified in the 5'-upstream region of the SF-1 gene (Nomura et al., 1995) but to date no specific factors controlling SF-1 gene expression have been identified. The Wilms' tumour (WT-1) gene product is a potential candidate as the phenotype of WT-1 knock out mice is similar to that observed in SF-1 knock out mice. However, the results of recent studies which show SF-1 expression in the urogenital ridges of WT-1 knock-out mice diminishes the possibility of WT-1 being a regulator of SF-1 (K.L. Parker, personal communication).

2.5.5 Wilms' Tumour (WT-1)

In humans, mutations of the tumour suppressor gene, WT-1, are associated with both Wilms' tumours and urogenital malformations (Baird et al., 1992; Bruening et al., 1992; Pelletier et al., 1991a, 1991b). Wilms' tumour is a childhood kidney malignancy which arises from abnormally persistent renal stem cells (Matsunaga, 1981) and can be associated with other congenital abnormalities such as those seen in WAGR syndrome (Wilms' tumour, aniridia, genitourinary anomalies and mental retardation) (Baird et al., 1992) and Denys-Drash syndrome (severe malformations including ambiguous genitalia and streak gonads; Bruening et al., 1992; Pelletier et al., 1991a). Individuals heterozygous for null mutations of the *WT-1* gene experience cryptorchidism and hypospadias, suggesting that these malformations are results of a gene dosage effect (Kreidberg et al., 1993). WT-1 encodes a protein with a proline/glutamine rich amino-terminal domain and four (Cys)2-(His)2 zinc fingers at the carboxy-terminus (Buckler et al., 1991; Call et al., 1990; Morris et al., 1991). Alternative RNA splicing produces four discrete transcripts but the functional role(s) of the protein products of these transcripts are unknown (Haber et al., 1991).

In mice the earliest expression of WT-1 is found in the intermediate mesoderm on day 9.0 and WT-1 mRNA is subsequently expressed in differentiating mesothelium, spinal cord and brain as well as derivatives of the urogenital ridge (Armstrong et al., 1992). WT-1 is expressed in the mesenchymal cells of the urogenital ridge and its expression continues into the epithelial cells of the differentiating meso- and metanephros. In the mouse fetal testis, the WT-1 gene is expressed in Sertoli cells at high levels from day 12.5 onwards (Armstrong et al., 1992; Pelletier et al., 1991c). Its expression stays maximal until after birth (day 8) when it starts to decline as the animal matures (Pelletier et al., 1991c). A knock-out mutation introduced into transgenic mice results in embryonic death in homozygotes. Examination of these embryos revealed a failure of kidney and gonadal development (Kreidberg et al., 1993). These abnormalities are thought to arise around day 11 of gestation as a result of apoptotic cell death of the metanephric blastema and failure of Wolffian duct development. WT-1 negative embryos also exhibited abnormal development of the heart and lungs (Kreidberg et al., 1993). Taken together this evidence suggest that WT-1 might be involved in the mesenchymal to epithelial transformation that occurs during development of the gonads and kidneys.

The exact function of the WT-1 gene products are unknown but it has been suggested that one or more may act as a repressor and not an activator of gene expression. One potential target for WT-1 is the *Pax-2* gene, another transcription factor involved in urogenital development (Ryan et al., 1995; Torres et al., 1995). The *Pax-2* gene, a member of a multigene transcription factor family (Gruss and Walther, 1992), is expressed during development of the metanephros and Wolffian/Müllerian ducts, but is not expressed in the gonads (Dressler et al., 1990). Transgenic mice lacking a functional *Pax-2* gene fail to develop kidneys, ureters and secondary sexual structures, but do contain gonads and, unlike WT-1 knock-out mice, the mutants survive until birth (Torres et al., 1995). In vitro experiments have shown that WT-1 can repress expression of the Pax-2 gene (Ryan et al., 1995) and WT-1 knockout mice do not express Pax-2 (Kreidberg et al., 1993), suggesting that the gene lies downstream from WT-1 in the urogenital development cascade.

2.5.6 DAX-1

Defects in the *DAX-1* gene have been identified as resulting in adrenal hypoplasia congenita (AHC; Guo et al., 1995; Muscatelli et al., 1994). AHC is a rare genetic X-linked disorder in which the patients have a disorganised nonfunctional adrenal cortex and which is often associated with the development of hypogonadotrophic hypogonadism (Grumbach and Styne, 1992). Duplication of the region of the X chromosome to which *DAX-1* has been mapped has been associated with the development of ovaries and a female phenotype in XY individuals despite the presence of SRY (Dosage sensitive sex reversal, DSS; Bardoni et al., 1994).

DAX-1 is a 470 amino acid protein which is encoded by a small gene containing a single intron. Sequence analysis has revealed homology to members of the nuclear hormone receptor superfamily (Zanaria et al., 1994). However, whilst a putative ligand binding domain has been identified, the putative DNA binding domain in the N-terminal portion of the protein is dissimilar to that seen in other members of the family. This DNA binding domain contains a 65-67 amino acid (glycine/alanine rich) repeat motif with cysteines located at conserved positions and may be organised in two zinc finger domains (Guo et al., 1995; Zanaria et al., 1994). Experiments in vitro have demonstrated the ability of the DAX-1 protein to bind DNA, however, the function of this factor in vivo is completely unknown (Guo et al., 1995; Zanaria et al., 1994).

2.5.7 Other genes involved in sexual differentiation

The available evidence suggests that other genes in addition to those described above are also involved in the regulatory cascade leading to gonadal differentiation. This assumption follows from observations that the majority of XY females with gonadal dysgenesis have an intact SRY gene and that in a minority of XX males no parts of the Y chromosome could be detected in their genome (Fechner et al., 1993a, 1993b; Pao et al., 1993). Apart from the *DAX-1* and *SOX9* genes, the autosomal locus on chromosome 9p has been implicated in sex reversal associated with chromosomal deletions in XY females (Bennet et al., 1993). However in this cases it is not known if sex reversal is due to monosomy for dosage sensitive gene(s) or whether the deletions reveal recessive mutations. In addition, a report by McElreavy et al. (1992) has shown a deletion of the Y chromosome more than 1.8 kb upstream from the SRY gene in a case of XY female sex reversal suggesting that additional genes on the Y chromosome are involved in sex determination or alternatively some important undiscovered regulatory parts of SRY reside in its upstream region. McElreavy et al. (1993) have suggested the existence of an additional gene, termed the Z gene, in the sexual differentiation cascade. They proposed that the Z gene is a negative regulator of male sexual differentiation and is functional in females. In males, the SRY product represses or otherwise negatively regulates Z and thereby allows male sex determination while in females, the absence of SRY allows the Z gene to be expressed and would consequently repress the expression of genes necessary for development of male phenotype (McElreavey et al., 1993).

2.6 Histogenesis of the gonads

Uniquely within the embryo, the gonadal rudiment has the potential to differentiate into either a testis or an ovary, an event dependent on the presence or the absence of the *SRY* gene as discussed above.

Gonadal rudiments first appear as a bulge on the ventral side of mesonephros resulting from the rapid proliferation of coelomic epithelium and the condensation of adjacent mesenchyme (Gilbert, 1994; Pelliniemi et al., 1993). At day 13.5 of fetal life in the rat (Satoh, 1985) and day 11.5-12.0 in the mouse (Kaufman, 1992) the gonadal rudiments appear as a ridge, known as the gonadal ridge, which is indistinguishable between the two sexes. Half a day later, (day 14.0 in rat, 13.0 in

mouse), epithelial strands extend into the underlying mesenchyme and give rise to the primitive sex cords in the gonad of the male fetus (Kaufman, 1992; Mittwoch et al., 1969). It is only at this stage that the sex of the fetus can be determined morphologically because at this time the ovary has a homogenous granular appearance while the testis contains wide, regularly arranged, bands of tissue, the testicular cords, which are destined to form the seminiferous tubules (Kaufman, 1992; Mittwoch et al., 1969). Within the testicular cords epithelial cells destined to become Sertoli cells surround the germ cells which have migrated from the wall of the yolk sac (Chiquoine, 1954; Witschi, 1948). Almost immediately after the formation of the testicular cords, Sertoli cells start to produce antimüllerian hormone, AMH (also known as Müllerian inhibiting substance, MIS), and continue to do so throughout fetal life (see 2.10.1; reviewed by Lee and Donahoe, 1993). During the subsequent days of gestation some of the mesenchymal cells between the testicular cords differentiate into steroid producing Leydig cells and around day 15.5 in the rat fetus, testosterone can be detected in the fetal testis (reviewed by Huhtaniemi and Pelliniemi, 1992 and Saez, 1994). On day 16.5, the testis and ovary are easily distinguished by their shape and size with the testis being a bigger and fairly ovoid shaped structure while the ovary is smaller with its craniocaudal axis almost three times as long as it is wide (Kaufman, 1992; Mittwoch et al., 1969). Functionally the testis develops early in fetal life into a major endocrine organ (Huhtaniemi, 1994). In marked contrast to the testis ovarian mesenchymal cells are quiescent until postnatal life when they differentiate into granulosa cells and start to produce steroids (Byskov, 1986; Gilbert, 1994).

In the human fetus, the urogenital ridge first appears around day 28 of gestation and remains sexually indifferent until day 42 (Gustafson and Donahoe, 1994). After day 42, testicular cords are formed and Sertoli cells start to produce antimüllerian hormone; Leydig cells develop and start to produce testosterone between days 50 - 55 (Gustafson and Donahoe, 1994; Huhtaniemi and Pelliniemi, 1992; Siiteri and Wilson, 1974). Comparison of the crucial time points in testicular development in rat, mouse and human is summarised in the table 2.1.

	rat	**mouse**	**human**
appearance of genital ridge	13.0-13.5	11.5-12.0	28-42
testicular cords formation	14.0	13.0	42
start of AMH production	14.0	13.0	42-45
start of testosterone production	15.5	13.0-13.5	50-55

Table 2.1: Ontogeny of crucial points in testicular differentiation in rat, mouse and human fetus in days post coitum

2.7 Development of the testis

2.7.1 Development of Sertoli cells

Sertoli cells are the first differentiated cells that can be recognised morphologically in the testis. On day 14.0 in the rat fetus, the newly formed epithelial cells in the genital ridge surround the germ cells, start to produce antimüllerian hormone (AMH) and are now called Sertoli cells (Byskov, 1986; Pelliniemi et al., 1993). During fetal life the principle function of the Sertoli cells is to synthesise and secrete AMH (2.10.1). Several studies have also shown the presence of aromatase in fetal Sertoli cells thus enabling the conversion of testosterone to oestrogen (2.10.6) The significance of this oestrogen synthesis and the possible role of oestrogens in the fetal testis is unknown (reviewed by Dorrington and Khan, 1993).

Sertoli cells divide only during fetal life and for a limited period shortly after birth (Sharpe, 1994). In rats, the proliferation of Sertoli cells is complete by day 18 postnatally (Nagy, 1972; Orth, 1982) and a similar timespan applies to replication in mouse (Kluin et al., 1984) and rabbit (Sun and Gondos, 1981) whereas in the sheep (De Riviers et al., 1980), the boar (Kosco et al., 1989) and the bull (Berndtson et al., 1987) replication probably extends until 6 to 10 weeks postnatally. The available but limited evidence for Cebus monkey (Rey et al., 1993) and for man (Cortes et al., 1987) suggests that in primates, this period is longer and may continue at a low rate until puberty. However, the stallion appears to be an exception and some reports suggest that in this species Sertoli cells retain proliferative activity throughout adult life with seasonal changes in the total number of Sertoli cells (Johnson et al., 1991; Johnson and Nguyen, 1986). Once Sertoli cell proliferation ceases (with the

exception of the horse), it never resumes and cell numbers are fixed for life (Sharpe, 1994). Sertoli cells are the somatic component of seminiferous tubules and studies in rat suggested that an adult Sertoli cell can only support a finite number of germ cells (Berndtson and Thompson, 1990; Russell and Peterson, 1984). This observation means that any change in Sertoli cell number will be accompanied by a change in the total number of germ cells supported through the process of spermatogenesis in adult life (Orth et al., 1988). Consequently, the ultimate size of the adult testis and total potential sperm output are determined during fetal and neonatal life when Sertoli cells are able to proliferate. Any reduction in the rate of proliferation at this critical stage or premature maturation will result in a smaller total number of Sertoli cells and hence smaller testes and a reduced number of germ cells in adult life (Orth et al., 1988; Sharpe, 1994).

FSH is an important regulator of Sertoli cell function in adult life (Sharpe, 1994) and fetal Sertoli cells also express the FSH receptor (FSH-R) during the later stages of development. Studies by Ranniki et al. (1995) have shown expression of full length FSH-R mRNA in fetal rat Sertoli cells on day 16.5 using RT PCR whilst using northern blots, they were able to detect FSH-R mRNA only on and after day 18.5., consistent with an increased expression of FSH at this stage of development (Aubert et al., 1985; Watanabe and Daikoku, 1979). These results are in accordance with other studies (Warren et al., 1984) that demonstrated autoradiographic localisation of FSH-R on day 17.5 in fetal rat testis. They found low binding of FSH in fetal testis until day 19.5 p.c. after which it rose sharply until just before birth. Taken together it is assumed that FSH most likely acts in the fetal testis of the rat only during the later stages of gestation. One potential role of FSH in the perinatal period is in the stimulation of Sertoli cell proliferation. Orth (1984) has studied the role of FSH in the rat fetus on day 18.5 p.c. by either injecting fetuses with antisera against FSH or through fetal decapitation. Both treatments produced dramatic reductions in the percentage of dividing Sertoli cells as measured by (^{3}H)thymidine uptake. Similarly, the addition of recombinant FSH to day 18.5 fetal gonads in vitro, increased the number of Sertoli cells undergoing mitosis (Orth, 1984). Studies by Boitani et al. (1995) have demonstrated that FSH can increase mitotic activity in postnatal rat testis in vitro and that this activity could be further potentiated in the presence of activin.

Whilst the available data suggest that FSH stimulates proliferation of Sertoli cells during late fetal and early postnatal development, the mechanism involved in

cessation of their mitogenic activity is unknown although thyroid hormones have been implicated. Hypothyroidism during early postnatal life results in testicular enlargement (Cooke and Meisami, 1991) in adult life and studies by Hess et al. (1993) have shown that this enlargement is due to an increase in the number of Sertoli cells and consequently a higher germ cell number. Similarly, T3 inhibits the proliferation of Sertoli cells during postnatal development both in vitro (Cooke et al., 1994) and in vivo (Van Haster et al., 1993). Studies by Van Haster et al. (1993) have shown that in rats injections of T3 during postnatal life cause cessation of proliferation on day 12 (in normal animals this process continues until day 16). In addition, T3 also promotes the formation of lumens within the testicular cords and maturation of Sertoli cells as measured by the expression of inhibin-ßB (Cooke et al., 1994; Van Haster et al., 1993) and clusterin (SGP-2; Cooke et al., 1994). Taken together these observations suggest that thyroid hormones influence maturation of Sertoli cells but the mechanism and importance of this remain to be elucidated.

2.7.2 Development of Leydig and other interstitial cells

Leydig cells are important endocrine cells in both the fetal and adult testis. Fetal Leydig cells can be discriminated from undifferentiated mesenchymal cells on day 15.5 in the rat fetus (Magre and Jost, 1980). Leydig cells differentiate from interstitial cells. Precursors for Leydig cells increase their cytoplasmic content through the development of large mitochondria, lipid droplets and smooth endoplasmic reticulum and become morphologically distinct Leydig cells (Magre and Jost, 1980; Saez, 1994). Fetal Leydig cells usually appear as clusters of several cells positioned closely together (Huhtaniemi, 1994). Functional maturation of fetal Leydig cells proceeds in concert with morphological differentiation with testosterone production starting on day 15.5 in the fetal rat testis (Feldman and Bloch, 1978; Picon, 1976; Saez, 1994; Warren et al., 1973). Fetal Leydig cells have a larger steroidogenic capacity than their adult counterparts (Huhtaniemi et al., 1982; Tapanainen et al., 1984), a feature which is probably required to maintain the high levels of testosterone necessary for the masculinisation of the fetus (Huhtaniemi, 1994). The relative number of Leydig cells in the fetal rat testis reaches its maximum on day 18.5 when they represent about 9% of testis volume and decreases thereafter to 7% and 5% on days 20.5 and 21.5 respectively (Roosen-Runge and Anderson, 1959). After day 18.5, the relative and absolute number of Leydig cells starts to decline and after birth fetal type Leydig cells are slowly replaced by adult type Leydig cells (Lording and De Kretser, 1972). In the human

fetal testis, the number of Leydig cells increases sharply following the formation of the testis and reaches its peak around 14 - 15 weeks of gestation at which time they represent about half of the testicular volume (Saez, 1994). After the 16th week of gestation, the relative number of Leydig cells gradually decreases until birth (Saez, 1994).

The mechanism underlying the regulation of fetal Leydig cell differentiation is unknown. In a situation akin to the regulation of Sertoli cells by FSH, it is thought that LH is important in regulation of fetal Leydig cell function during the later stages of pregnancy. In the rat, LH receptor mRNA was detected in Leydig cells on or about day 16.5-17.5 p.c. (Zhang et al., 1994), and studies using ligand binding have demonstrated the ability of fetal Leydig cells to bind LH on day 16.5 (Warren et al., 1984). It is possible that LH stimulates the increase in Leydig cell number and testosterone production that occurs on and after this time. However, LH is unlikely to have an important function prior to this time as it is not detectable in fetal circulation or pituitary glands before day 17.5 of gestation (Aubert et al., 1985; Watanabe and Daikoku, 1979). Activin and inhibin have also been shown to modulate the function of fetal Leydig cells, with activin inhibiting (Lin et al., 1989), and inhibin stimulating, steroidogenesis in neonatal Leydig cells (Hsueh et al., 1987) under certain conditions. AMH may also regulate fetal Leydig cell function as transgenic mice lacking functional AMH show Leydig cell hyperplasia suggesting that AMH might be one factor inhibiting proliferation of Leydig cells (Behringer et al., 1994).

Apart from the Leydig cells, other non-steroidogenic cells are present in the interstitial compartment of the fetal testis including myoid peritubular cells surrounding the testicular cords. The function of these cells is not known, however, some of these interstitial cells are likely to be Leydig cells precursors (Hardy et al., 1989; Pelliniemi et al., 1993).

2.7.3 Development and migration of germ cells

While the somatic and steroidogenic cells of the gonads develop from mesonephric mesenchyme, germ cells develop from extraembryonic mesoderm in the wall of the yolk sac and subsequently migrate into the mesonephric region (Chiquoine, 1954; Lawson and Hage, 1994; McLaren, 1994; Witschi, 1948). Germ cells migrate along the allantois, the wall of the hindgut and dorsal mesentery and enter the mesonephric

region by day 12.5 in the rat or around the sixth week of gestation in humans (Byskov, 1986). Thereafter they become surrounded by developing Sertoli cells which appear to be necessary for their survival since germ cells that remain outside the testicular cords usually degenerate (Byskov, 1986). The tyrosine kinase receptor c-kit and its ligand have been implicated in ensuring germ cell survival within the gonad (Manova et al., 1993). C-kit is expressed by germ cells (Manova et al., 1990; Yoshinaga et al., 1991) and its ligand, known as the Kit ligand (KL) or Steel factor is expressed by Sertoli cells from day 17.5 of gestation (Rossi et al., 1993). Packer et al. (1995) have shown that injection of postnatal rats with antisera raised against c-kit induced a fivefold increase in death (apoptosis) of spermatogonia, suggesting that this factor is important for the germ cell survival.

In the male rat, after entering the mesonephric region, germ cells actively proliferate until day 16.5 when they became arrested in the G1 mitotic phase and remain as prespermatogonia until after birth (Byskov, 1986; Vergouwen et al., 1991). A few days after birth, mitosis of prespermatogonia resumes, they differentiate into spermatogonia A and B and at the start of the puberty enter meiosis (Clermont and Perey, 1957; Vergouwen et al., 1991). At the onset of spermatogenesis, many dividing germ cells degenerate (Roosen-Runge and Leik, 1968), suggesting that the mere presence of Sertoli cells is not enough to ensure their survival (Byskov, 1986). The reason the male germ cells within the testicular cords do not enter meiosis but arrest in G1 mitotic phase has been ascribed to the protective function of the cord itself or the presence of a meiosis preventing substance (MPS; Byskov and Saxen, 1976), however, to date no such factor has been isolated (Byskov, 1986).

2.8 Development of internal secondary sexual organs

The undifferentiated fetus contains precursors of male and female secondary sexual organs. Around the same time as the gonadal ridge is formed, two pairs of ducts are generated. The mesonephric (Wolffian) duct lies posteromedial to the genital ridge and is situated laterally to the mesonephros, entering the urogenital sinus caudally. The paramesonephric, or Müllerian ducts, develop as paired longitudinal invaginations of coelomic epithelium alongside the genital ridge (George and Wilson, 1994; Gilbert, 1994; Kaufman, 1992).

The mesonephric kidney together with the paramesonephric duct become non-functional soon after the formation of the gonadal ridge and their function is taken

over by the metanephros which forms the kidney (Gilbert, 1994). In the presence of testosterone the mesonephros does not regress but develops into the epididymis and the Wolffian duct develops into the vas deferens and seminal vesicles (George and Wilson, 1994; Gilbert, 1994). Concurrently, secretion of AMH by Sertoli cells within the testis causes the regression of Müllerian ducts (George and Wilson, 1994; Gilbert, 1994; Lee and Donahoe, 1993). In rats, development of the epididymis and vas deferens starts at the same time as production of testosterone on or around day 15.5 and Müllerian duct regression is usually complete on or around day 18.5.

In humans, the genital ducts (Wolffian and Müllerian) are developed by the 6th week of gestation. Regression of Müllerian ducts, induced by AMH, starts around day 51 and is completed by day 64 (Gustafson and Donahoe, 1994).

In the absence of the testes, the Wolffian ducts regress and the Müllerian ducts develop into the fallopian tubes, uterus and the upper portion of the vagina, with the urogenital sinus developing into the lower portion of the vagina (George and Wilson, 1994; Gilbert, 1994).

2.9 Development of the external genital organs

Development of the male external sexual organs is induced by dihydrotestosterone, a locally-produced hormone converted in target tissues from testosterone by the action of the enzyme 5α-reductase (George and Wilson, 1994; Siiteri and Wilson, 1974; Wilson and Lasnitzki, 1971). In rats, the external sexual organs are almost identical in both sexes throughout gestation. These bipotential structures include urogenital folds on either side of urogenital membrane, the labioscrotal swellings lateral to the urogenital folds, and the genital tubercle formed cranially by fusion of the urogenital folds. In the presence of dihydrotestosterone, the rapidly growing tubercule pulls forward the urogenital folds and eventually forms the elongated phallus with the tubercle forming the glans penis and the urogenital folds the penile shaft. These folds later fuse, while the labioscrotal swellings migrate caudally and fuse in the midline to form the scrotum and its septum. In humans, the external genitalia are identical until the 6th week of gestation. Around 7-8 weeks of gestation a similar process to that in rats begins and by the 12th week, the labioscrotal swellings have fused to form the scrotum and the formation of male external genitalia is completed (George and Wilson, 1994; Gilbert, 1994; Gustafson and Donahoe, 1994).

In the absence of testosterone, no major changes appear in the external genitalia with the tubercule developing into the clitoris and the urogenital folds and the labioscrotal swellings into the labia minor and labia major, respectively, forming female external reproductive organs (George and Wilson, 1994; Gustafson and Donahoe, 1994).

2.10 Endocrine function of the fetal testis

The fetal testis is endocrinologically distinct to the adult testis (reviewed by Huhtaniemi, 1994). Unlike the quiescent fetal ovary, the fetal testis secretes testosterone and AMH. These two hormones play key roles in sexual differentiation and all morphological differences between male and female mammals (apart from the gonads) result from the action of these two hormones (Bogan and Page, 1994). Several other hormones and/or growth factors including oestrogens, inhibin and activin are also produced by the fetal testis.

2.10.1 Antimüllerian hormone (AMH)

The existence of a testicular factor responsible for regression of the female reproductive ducts (Müllerian ducts) was first proposed by Professor Alfred Jost in the 1950's (Jost, 1953). In classical experiments, he transplanted a fetal rabbit testis into the vicinity of Müllerian ducts in a female fetus, observing regression of the Müllerian ducts. In contrast, the transplantation of fetal ovaries into a male fetus had no effect. This experiment implied the existence of a testicular hormone, apart from testosterone, synthesised during fetal life, a substance now known as antimüllerian hormone or Müllerian inhibiting substance (AMH/MIS; Josso et al., 1993). AMH is a member of a large multigene family that includes TGF-ßs, inhibins, activins, Xenopus Vg-1 and *Drosophila* decapentaplegia complex (Massague, 1990). Members of this family are homologous in their C-terminus, particularly around 7 conserved cysteine residues. Many of these proteins are produced as dimeric precursors and undergo posttranslational processing, cleavage and dissociation, to release a disulphide-linked bioactive C-terminal fragment (Massague, 1990).

The sequence of AMH is strongly conserved across species, particularly within its C-terminal domain (Lee and Donahoe, 1993). In the rat, the protein consist of 553 amino acids and is encoded by a gene containing five exons (Haqq et al., 1992).

The bovine, rat and human genes have 65-80% homology within their coding regions and 70-75% homology in the promoter region (Lee and Donahoe, 1993). Both the 5' and 3' untranslated regions are relatively short and probably do not exert any translational control (Lee and Donahoe, 1993). Mature AMH undergoes glycosylation and dimerisation and is secreted as a 140 kilodalton dimer of identical disulphide-linked subunits, called holo-AMH (Josso and Picard, 1986; Lee and Donahoe, 1993). Finally, the dimer structure is cleaved between amino acid residues Arg427 and Ser428 to produce a biologically active 25 kD C-terminal protein and a biologically inactive 110 kD N-terminal (Lee and Donahoe, 1993; Pepinsky et al., 1988).

AMH mRNA and protein can be detected in the developing rat fetal testis on day 14.0 (Lee et al., 1992) and in mouse fetal testis on day 12.5 (Munsterberg and Lovell-Badge, 1991). Expression remains high throughout fetal development dropping rapidly a few days after birth, although AMH is still present at low levels right up until puberty (Kuroda et al., 1990; Lee et al., 1992; Munsterberg and Lovell-Badge, 1991; Voutilainen and Miller, 1987). AMH has also been detected in the ovary (Munsterberg and Lovell-Badge, 1991; Voutilainen and Miller, 1987) where it is expressed postnatally and reaches its highest levels at puberty and during adulthood. The presence of AMH in the adult ovary and in the testis, long after regression of the Müllerian ducts, is suggestive of additional biological functions (Lee and Donahoe, 1993; Munsterberg and Lovell-Badge, 1991; Voutilainen and Miller, 1987). Recent studies indicate that rat AMH has two developmentally regulated mRNA transcripts that are differentially polyadenylated (Lee et al., 1992). A larger, 2.0 kilobase species is abundant by day 14.0, decreases markedly at birth and is barely detectable postnatally. A smaller, 1.8 kilobase species is detectable at day 18.5, prominent at day 21.5 and thereafter declines but remains detectable after birth (Lee et al., 1992; Lee and Donahoe, 1993). Although the biological significance of these two transcripts is unknown, differential polyadenylation may affect stability or translatability of the two mRNAs as has been described in other systems (Sachs, 1990).

Regulation of AMH expression has been studied extensively. The discovery of Sry was followed by suggestions that Sry might be the factor that directly induces the expression of AMH. This theory was supported by the timing of expression of both genes, with expression of Sry immediately preceding that of AMH (Hacker et al., 1995; Koopman et al., 1990), and by the finding that Sry can bind to a DNA

sequence within the 5' upstream region of the AMH gene (Haqq et al., 1993). However, the short window of expression of Sry compared to that of AMH does not support this hypothesis and recent evidence suggests that any interaction between SRY and AMH is more likely to be indirect (Haqq et al., 1994; Shen et al., 1994).

A 2 kb region upstream of the AMH mRNA start site appears to be sufficient for cell specific gonadal expression (Peschon et al., 1992). Recently, the binding site for Steroidogenic factor-1 (SF-1) has been shown at a site -180 (AMH RE1) within this region (Shen et al., 1994). Further experiments have demonstrated that SF-1 binds to this sequence and that an intact SF-1 binding site is required for AMH expression. Transfection studies in primary Sertoli cell cultures indicate that interactions between SF-1 and AMH RE1 are sufficient to promote activation of AMH. However, this activity was not present in HeLa cell lines transfected with both genes, suggesting that Sertoli cells contain the additional factors such as an SF-1 ligand and/or cofactors which are necessary for AMH expression (Shen et al., 1994). The suggestion that SF-1 regulates AMH expression is further supported by comparison of temporal, spatial and sex-specific profiles of both SF-1 and AMH transcripts (Ikeda et al., 1994; Munsterberg and Lovell-Badge, 1991).

The importance of extratesticular factors in the regulation of AMH gene expression is not clear. FSH has been shown to negatively regulate the production of AMH at the transcriptional level (Kuroda et al., 1990, 1991) while testosterone and LH may act posttranslationally, enhancing the cleavage of holo AMH into its N- and C-terminal fragments (Kuroda et al., 1991). None of these substances are likely to be involved in the induction and early regulation of AMH expression as they are not present (or are undetectable) in the fetus when AMH expression is induced. However, it is possible that FSH is involved in the postnatal decline in AMH expression within the testis (Kuroda et al., 1990).

2.10.2 Function of AMH

To date the only fully explored function of AMH is the regression of the Müllerian (paramesonephric) ducts. In organ culture experiments, purified AMH alone was able to induce regression of rat Müllerian ducts (Lee and Donahoe, 1993). Similarly, female mice chronically expressing AMH have no oviducts or uteri (Behringer et al., 1990), while male transgenic mice lacking a functional AMH gene develop both male and female reproductive organs (Behringer et al., 1994). In

addition in about half of human males with persistent Müllerian duct syndrome mutations in the AMH gene have been identified (Guerrier et al., 1989; Josso et al., 1991; Knebelmann et al., 1991). In some cattle twin fetuses, the pathological condition known as Freemartinism occurs when the AMH from male fetus crosses the placenta and causes the regression of the Müllerian ducts in the female fetus (Byskov, 1986; Jost et al., 1972; Lillie, 1916, 1917).

AMH acts on its target cells through a specific receptor, recently cloned and recognised as a member of serine/threonine kinase receptor II family (Baarends et al., 1994; Di Clemente et al., 1994). Serine/threonine kinase receptors are divided into three subtypes based on the observations made on the TGF-ß receptors. They are called receptors I, II or III according to their relative molecular masses of 55, 80 and 280 kD respectively (Chiefetz et al., 1987). The type III receptor is believed to be involved in presentation and binding of TGF-ß to the other receptor types (Lopez-Casillas et al., 1993). The TGF-ß type I receptor cannot bind TGF-ß in the absence of the type II receptor and association with the type I receptor is essential for the type II receptor to signal growth inhibition (Wrana et al., 1992). If the same organisation of receptor types/functions is true for the whole family this would suggest the existence of an as yet undiscovered AMH type I receptor.

The AMH receptor gene identified consists of 11 exons (Visser et al., 1995), encodes a 1.9 kilobases long mRNA, the open reading frame of which encodes a highly conserved 557 amino acid protein in rat (Baarends et al., 1994) which has 569 amino acids in rabbit (Di Clemente et al., 1994). In male and female rat gonads and adjacent ducts, the receptor is expressed in a tissue specific manner on day 14.5 (Baarends et al., 1994). Expression in the female gonads and associated ducts remains high throughout fetal life while expression in the male fetus becomes restricted to the small area around the degenerating Müllerian duct (Baarends et al., 1994).

Male mice lacking AMH (knock outs) still develop the male reproductive system and produce functional sperm but are usually infertile because of the presence of female reproductive organs that interfere with the delivery of sperm (Behringer et al., 1994). However, the testis of these mice exhibit Leydig cell hyperplasia, and occasionally Leydig cell neoplasia, suggesting a role for AMH in modulating Leydig cell development (Behringer et al., 1994). The ovaries of mice chronically expressing AMH become depleted of germ cells and their gonadal somatic cells

reorganise into testicular cord like structures (Behringer et al., 1990), a processes that has also been observed occasionally in female free-martin fetuses. These observations suggest that AMH may play a role in gonadal differentiation. Likewise, expression of AMH in the adult ovary and testis (Munsterberg and Lovell-Badge, 1991; Voutilainen and Miller, 1987) suggests additional roles for this hormone. Some of the proposed functions of AMH postnatally include regulation of spermatogenesis and oogenesis, testicular descent, lung maturation and Leydig cell development (reviewed by Lee and Donahoe, 1993).

2.10.3 Testosterone

Testosterone and dihydrotestosterone are the principle male sexual hormones and have important functions in both fetal and adult life (George and Wilson, 1994; Sharpe, 1994). Testosterone and dihydrotestosterone are steroid hormones derived from cholesterol by the action of several steroidogenic enzymes (Fig. 2.4; reviewed by Miller, 1988). The majority of steroid hormones are produced in the adrenal cortex and gonads, however, small amounts are also generated in other tissues such as the brain and gut. Despite their apparently similar structures, the different steroid hormones have very different functions. Adrenal glands mostly produce gluco- and mineralocorticoids, which are important in controlling the metabolism of carbohydrates, fat, proteins and minerals, regulating fluid transport in the kidney (and consequently control of blood pressure), and exhibit anti-inflammatory responses (O'Riordan et al., 1988; Orth et al., 1992).

Most sex steroid hormones are produced in the gonads with the ovary producing mostly oestrogen and progesterone and the testis mostly testosterone. High concentrations of testosterone are synthesised in the ovary, but the majority of this hormone is immediately aromatised into oestrogen (Carr, 1992; O'Riordan et al., 1988). Some oestrogens and testosterone are also produced in the adrenal gland (Carr, 1992; Griffin and Wilson, 1992; Orth et al., 1992).

The biosynthesis of testosterone and oestrogens is summarised in Fig. 2.4. Briefly, once inside steroidogenic cells, cholesterol is transported to the inner mitochondrial membrane, a process in which the recently discovered StAR protein has been implicated (Lin et al., 1995). The first step in the process of steroidogenesis is the conversion of cholesterol to pregnenolone, a reaction catalysed by the enzyme P450scc (cytochrome P450 side chain cleavage) which is located inside the

mitochondria. Pregnenolone then diffuses across the mitochondrial membranes and is further metabolised by enzymes associated with the smooth endoplasmic reticulum. In the smooth endoplasmic reticulum, pregnenolone is initially converted to progesterone by 3ß-HSD and then further catalysed by the cytochrome P450 enzyme, 17α-hydroxylase/C_{17-20} lyase (P450c17). P450c17 is a single protein that catalyses two distinct reactions; the hydroxylation of progesterone at C_{17} followed by the cleavage of the two-carbon side-chain to yield the C_{19} steroid, androstenedione - the immediate precursor of testosterone. The final reaction in the process is the reduction of androstenedione to testosterone by 17ß-hydroxysteroid dehydrogenase (reviewed by Miller, 1988).

In its target tissues, testosterone is usually further transformed by the 5α-reductase enzyme into 5α-dihydrotestosterone, which has a more potent androgenic activity than testosterone (O'Riordan et al., 1988).

As summarized in fig. 2.4, two different pathways are possible for conversion of steroids dependent on whether pregnenolone is converted by 3ß-HSD or P450c17. If pregnenolone is converted to progesterone by 3ß-HSD, the Δ^4 or progesterone pathway is used and if P450c17 transforms pregnenolone into 17α-hydroxypregnenolone, the Δ^5 or dehydroepiandrosterone pathway is used. Conversion of Δ^5 steroids to those of the Δ^4 pathway by 3ß-HSD can also occur. The choice of pathways is not random. Rats use the Δ^4 pathway predominantly if not exclusively, whilst pigs, rabbits and dogs use the Δ^5 pathway to varying degrees. Regulation of the choice of pathway could arise either as the result of the properties of the enzymes or by their arrangement within the membranes. If, for example, the C_{21} side-chain cleveage enzyme has a higher affinity for pregnenolone than for progesterone, the Δ^5 pathway would prevail (Hall, 1994.

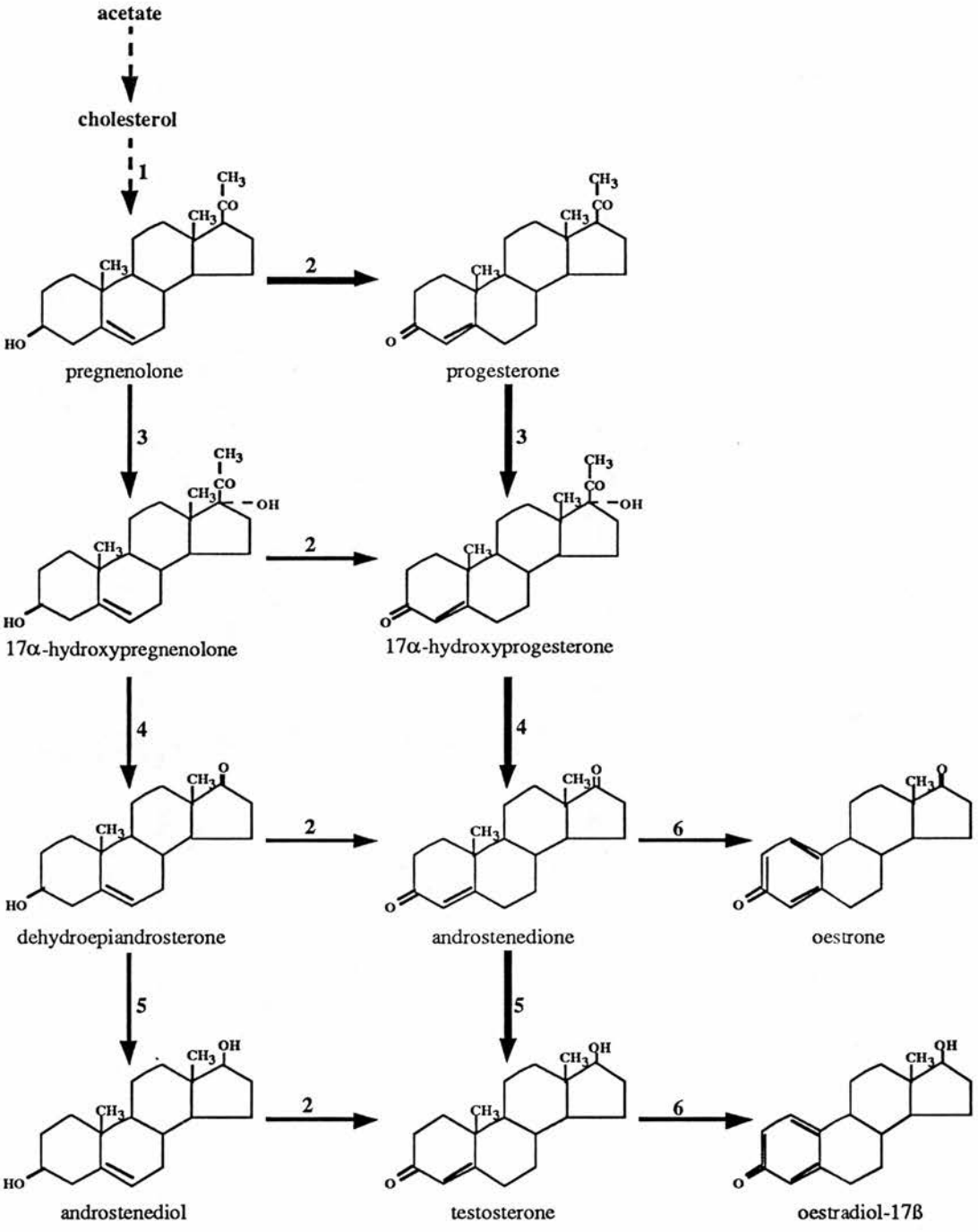


Figure 2.4: Pathways involved in biosynthesis of steroid hormones from cholesterol. Enzymes involved in each step are marked with the numbers and are as follows: 1. (P450scc); 2. 3ß-HSD; 3. P450c17 (17α-hydroxylase); 4. P450c17 ($C_{17/20}$-lyase); 5. 17ß-hydroxysteroid dehydrogenase; 6. P450aromatase. The Δ4 pathway, predominant in rat, is marked by arrows in bold.

2.10.4 Regulation of testosterone production

In fetal and adult life, the principle site of testosterone production are the Leydig cells within the testis. In the rat fetus, testosterone production in the Leydig cells starts around day 15.5 (Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973). This coincides with expression of P450scc which is first detectable using immunocytochemistry on day 15.5 (Rouiller et al., 1990). Similarly, studies in the mouse fetus have shown the presence of P450scc, P450c17 and 3ß-HSD mRNAs on day 13.5 (Greco and Payne, 1994), equivalent to rat day 15.5. Testosterone levels are low on day 15.5, rise sharply, reaching a peak on day 18.5, and remain high (or slightly decline) throughout the rest of fetal life (Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973). In the human fetus, testosterone production starts between 7 and 8 weeks p.c. (Siiteri and Wilson, 1974) and reaches a peak between 12 and 14 weeks of gestation followed by a gradual decline (Huhtaniemi and Pelliniemi, 1992; Tapanainen et al., 1981). Studies by Voutilainen and Miller (1986) have shown strong expression of P450scc and P450c17 mRNA's in the human fetal testis between 13 (earliest examined age) and 20 weeks with a rapid decline thereafter to only 35 and 19% of peak levels of P450scc and P450c17, respectively.

Regulation of testosterone production by fetal Leydig cells is still not fully understood. Luteinizing hormone (LH), the main regulator of testosterone biosynthesis by adult Leydig cells, has probably only a minor role during fetal life and is unlikely to be involved in the mechanism that triggers the onset of testosterone biosynthesis (Saez, 1994). In the rat fetus, binding of LH to fetal Leydig cells can be demonstrated for the first time on day 15.5 (Gangnerau et al., 1982; Warren et al., 1984). The mRNA for the extracellular domain of the LH receptor could be detected by RT PCR on day 15.5 with much stronger expression one day later (detectable by northern blot hybridisation) on day 16.5 p.c. (Zhang et al., 1994). However, there is apparently no detectable LH in the blood or pituitaries at this age (Aubert et al., 1985; Watanabe and Daikoku, 1979). For some time it was thought that hCG might be responsible for the activation of testosterone production, as this glycoprotein hormone binds to the fetal Leydig cells and stimulates testosterone production both in human and rat fetuses (Saez, 1994). It is possible that hCG is indeed involved in the onset of testosterone production in primates however hCG-like protein has not been isolated from the rat and a placental CG is unlikely to have the same role in rodents (Habert and Picon, 1990;

Wurzel et al., 1983). Two alternative explanations have been proposed in rodents; firstly that there is a non-gonadotrophic factor in the rat fetus that activates and maintains the initial period of fetal testicular steroidogenesis or secondly that onset of fetal testicular steroidogenesis is an autonomous process (Saez, 1994). This second proposal has been supported by studies showing that isolated genital ridges acquire the ability to synthesise steroids when cultured in vitro in the absence of gonadotrophins or any other additional factors (Agelopoulou et al., 1984; Gangnerau and Picon, 1987). A second piece of evidence supporting this hypothesis has arisen from an investigation using the hypogonadal (hpg) mouse (Cattanach et al., 1977). These animals have very low gonadotrophin levels due to a mutation in the GnRH gene rendering the gene product non-functional. Nevertheless, male hpg mice, despite their hypoplastic gonads are still normally masculinised, suggesting that their testis must start and maintain some testosterone production in utero without significant gonadotrophin stimulation (Cattanach et al., 1977). Recently, SF-1, discussed in section 2.5.4, appears as a possible candidate for an autonomous factor acting in the Leydig cells. SF-1 has been shown to regulate expression of the steroidogenic cytochrome P450 enzymes (Ikeda et al., 1993; Morohashi et al., 1992) and is expressed at the right temporal and spatial location (Hatano et al., 1994; Ikeda et al., 1994) to act as a trigger for the onset of steroidogenesis in the fetal testis. Further studies will be needed to clarify the function of SF-1 at this time and its possible involvement in onset of testosterone biosynthesis.

The regulation of testosterone production in fetal life once steroidogenesis is established is also unclear. Studies in vitro and in vivo have shown that fetal Leydig cells are responsive to LH stimulation (Feldman and Bloch, 1978; Gangnerau et al., 1982). As in the adult testis, LH appears to stimulate testosterone production in fetal Leydig cells (Feldman and Bloch, 1978; Gangnerau et al., 1982), however, differences between adult and fetal Leydig cells have been described. For example, in the adult testis, chronic exposure to LH (or hCG) results in down-regulation of LH receptors and in the blockade of the 17,20 lyase activity of the P450c17 enzyme finally resulting in lower testosterone production (Payne, 1990; Payne and Youngblood, 1995). This is not the case in the fetal Leydig cells where LH stimulates testosterone production continuously (Warren et al., 1987). Despite its ability to stimulate testosterone production in fetal Leydig cells, the physiological function of LH in the fetus is still questionable. LH is initially detectable in the pituitary (Watanabe and Daikoku, 1979) and in the fetal blood around day 17.5 (Aubert et al., 1985). LH levels stay very low (below 0.05μg; about 10% of adult

levels) until the day 19.5 (Aubert et al., 1985). However, the highest levels of fetal testicular steroid production occur on day 18.5 of gestation (Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973) and this means that the most marked increase in testosterone production occurs in the presence of very low levels of LH (Saez, 1994). This data together with the studies from the hpg mouse discussed above thus suggest the existence of other factors or autonomous mechanisms involved in the regulation of testosterone production in the fetus.

2.10.5 Action of testosterone

In the adult organism, testosterone and its metabolites have effects on the function of many different tissues. Testosterone is responsible for development and maintenance of secondary male sexual characteristics (deep voice, body hair, muscular body) and is essential for maintenance of fertility (O'Riordan et al., 1988; Sharpe, 1994). Testosterone is responsible for masculinisation of the male fetus, including stabilisation of the Wolffian ducts and hence is directly responsible for development of male secondary sexual organs discussed in sections 2.8 and 2.9. In the absence of testosterone, the Wolffian ducts regress and the external genital organs develops as in the female (George and Wilson, 1994). The importance of testosterone is most clearly shown in androgen insensitivity syndrome, the development of which is associated with mutations in the androgen receptor gene. Cases with complete androgen insensitivity syndrome develop female external genitalia despite the presence of developed testis and high levels of circulating testosterone (Griffin and Wilson, 1989; Quigley et al., 1995).

Once formed, testosterone is secreted from the Leydig cells into the blood. Most of the blood born testosterone is bound to sex steroid binding globulin (SHBG; Griffin and Wilson, 1992). At its site of action, testosterone dissociates from SHBG and passes through the cell membrane into the cytoplasm. Once in the cytoplasm, it is commonly transformed into the more potent metabolite 5α-dihydrotestosterone by the enzyme 5α-reductase (Griffin and Wilson, 1992). In the fetal male, testosterone and 5α-dihydrotestosterone play different roles during differentiation: testosterone is responsible for virilization of the Wolffian ducts while differentiation of the urogenital sinus and the external genitalia depends upon the action of 5α-dihydrotestosterone (George and Wilson, 1994; Siiteri and Wilson, 1974; Wilson and Lasnitzki, 1971).

In target cells, testosterone and 5α-dihydrotestosterone both act via the same specific nuclear hormone receptor, the androgen receptor (AR; George and Wilson, 1994). The androgen receptor gene is encoded by 8 exons and is located on the X chromosome (Lubahn et al., 1988; 1989). Deletion mutagenesis studies have identified three important domains within its structure: an amino terminal segment involved in transcriptional activation, a cysteine-rich DNA binding domain and a carboxy terminal hormone binding domain (Rundlett et al., 1990; van Steensel et al., 1995). The inactive androgen receptor resides in the cytoplasm bound to heat shock proteins, which prevent its diffusion into the nucleus. Upon testosterone binding the heat shock proteins dissociate and the androgen receptor together with its ligand are transposed into the nucleus where they bind to androgen response element(s) (ARE) in the promoter regions of androgen regulated genes (Parker, 1991; Rundlett et al., 1990).

The distribution of androgen receptors in the fetus has been studied by autoradiography and immunocytochemistry. Several studies (Bentvelsen et al., 1994; Cooke et al., 1991a; Kalloo et al., 1993) have shown the presence of the androgen receptor throughout development in the Wolffian ducts and urogenital sinus of the rat, mouse and human fetuses but there are no reports of androgen receptor localisation in the fetal testis (see chapter 5).

2.10.6 Oestrogens

The role of oestrogens together with their possible pathological role is further discussed in section 2.11. The physiological role of oestrogens during normal testicular development is still a matter of debate. The production of oestrogens by testis was proposed as early as 1934 from studies of the equine testis (Zondek, 1934). Subsequent studies have shown that Sertoli cells are the site of oestrogen production (Huggins and Moulder, 1945; Lacy et al., 1968), however, only in horses do adult Sertoli cells appear to have the capacity to aromatise steroids (Dorrington and Khan, 1993). In other species, the expression of P450arom and consequently ability to produce oestrogens is limited to immature Sertoli cells (Dorrington and Khan, 1993; Rommerts et al., 1978; Verhoeven et al., 1979). This function seems to be stimulated by FSH in immature rat Sertoli cells (Dorrington and Armstrong, 1975) but the ability of Sertoli cells to aromatise testosterone disappears with maturation of Sertoli cells around day 20 of life (Dorrington and Khan, 1993). In addition, oestrogen receptor (ER) have been identified in the fetal testis and

Wolffian ducts (Greco et al., 1992). However, transgenic mice with a non-functional oestrogen receptor gene show no abnormalities in fetal testicular development but display reduced fertility or even infertility in adult life (Korach, 1994). This finding suggests that oestrogens are not required for normal testicular development, but it is possible that oestrogen may have a negative effect on sexual differentiation.

Like the AR, the ER is a member of the nuclear receptor superfamily (Carson-Jurnica et al., 1990). Greco et al. (1992) have studied the presence and distribution of ER in the fetal mouse testis and associated duct system using frozen tissue and found strong expression in the gonads just after gonadal differentiation on day 13.5. The intensity of the staining decreased during the rest of the fetal life and became very weak at birth. Initially, positive nuclear staining appeared in the mesenchymal, peritubular and Sertoli cells but thereafter weak immunostaining was confined to the mesenchymal and Leydig cells (Greco et al., 1992, 1993).

2.10.7 Inhibin and activin

Inhibin and activin are members of the TGF-ß growth factor family (Wallach, 1996; Ying, 1988). In the adult testis, inhibin and activin are mainly produced by Sertoli cells and are able to modulate FSH expression by the pituitary gland (Schwall et al., 1989; Vale et al., 1988; Ying, 1988). Inhibin and activin are heterodimers formed by the possible interaction of three subunits, one α and two similar, but distinct, ß subunits (ßA and ßB). Inhibin is composed of an α-subunit covalently bound together with one of the two ß subunits (ßA and ßB). On the other hand, activins are dimers of the ß subunits and are classified as activin A (ßA/ßA), activin B (ßB/ßB) and activin AB (ßA/ßB) (Vale et al., 1988). The genes encoding all three subunits have a similar structure, containing one intron. In each case, the primary protein transcript is a large precursor molecule that undergoes post translational cleavage to form the individual functional subunits. Formation and structure of different inhibins and activins is shown in figure 2.5.

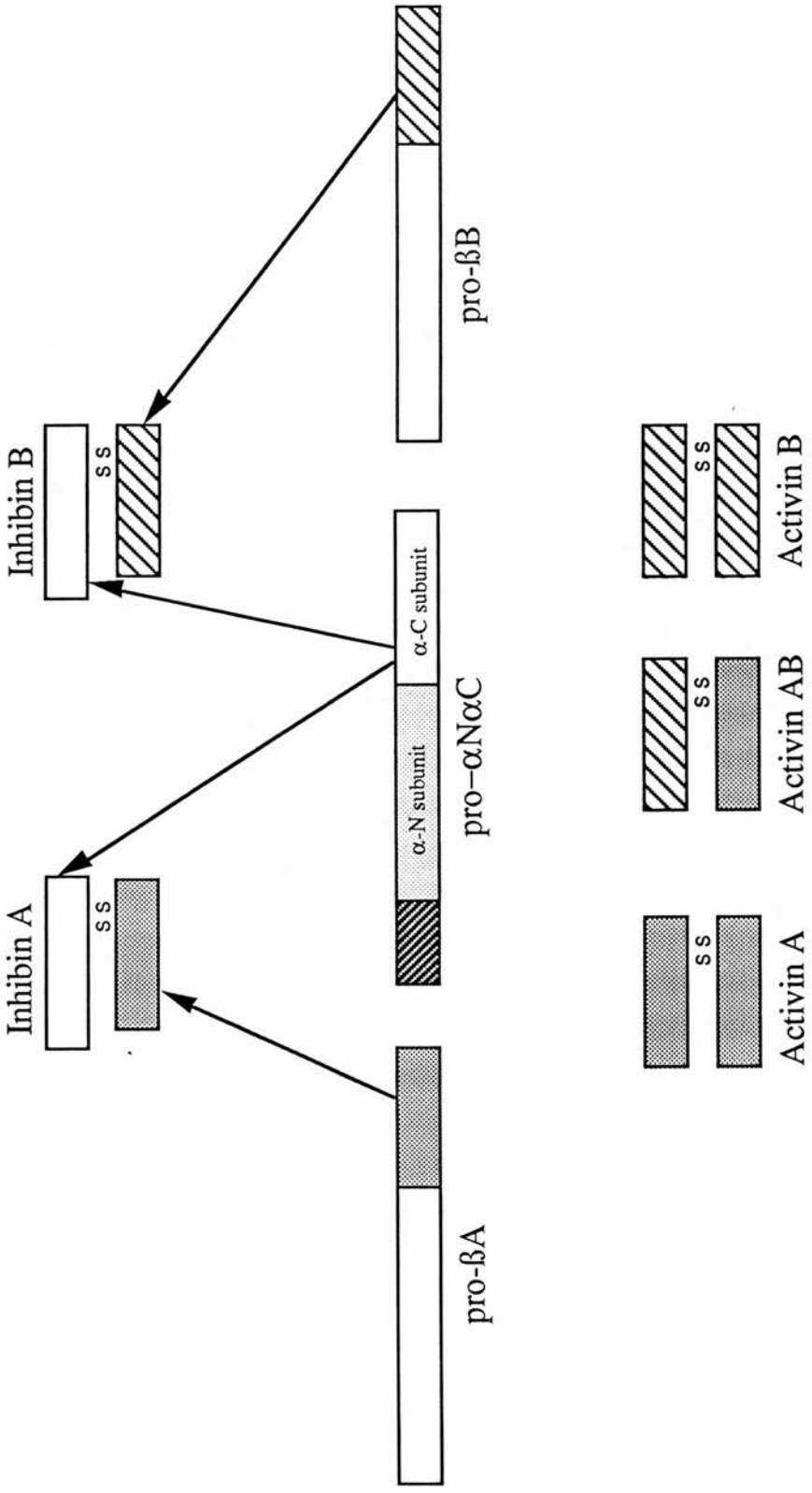


Figure 2.5: Schematic representation of the formation of different inhibins and activins from their precursors.

The precursor molecule for the α-subunit is 366 amino acids long and possesses a functional 133 amino acid domain in its carboxy terminus. ßA and ßB subunits are 116 and 115 amino acids respectively and are produced by proteolytic cleavage of larger precursor molecules of over 400 amino acids in length (Woodruf et al., 1987; Ying, 1988).

Apart from their influence on FSH secretion, inhibin and activin have been recently shown as important regulatory factors in the testis (Chen, 1993; Lin et al., 1989) and other tissues (Spencer et al., 1990). Roberts et al. (1991) have reported mRNA expression of the ßA subunit in embryonic heart, dermal layer of skin, whisker follicles and developing skeleton, suggesting a wide functional role for activin during development. Weak expression of ßA has also been detected in the fetal ovary, while the testis remained negative until day 21.5 when mRNA was detected in the interstitial region. The mRNAs for the ßB and α subunits were detected in an area of rapidly dividing cells surrounding the forebrain ventricle as well as in the testis and ovaries (Roberts et al., 1991). Studies by Kaipa and co-workers (1994) have demonstrated that activin can inhibit testicular cell proliferation during early testicular development (at day 14.5), but found no effect on cell proliferation at days 15.5 or 18.5. Furthermore, studies by Boitani et al. (1995) have shown that FSH stimulation of Sertoli cell proliferation in neonatal rat testis is greatly enhanced by the presence of activin. However, inhibin does not appear to be essential for fetal development since transgenic mice without a functional α subunit develop normally although these animals later develop Sertoli cell tumours with nearly 100% penetrance (Matzuk et al., 1992). In contrast, transgenic mice lacking functional ßA or both ßA and ßB subunits showed severe abnormalities and died within 24 h of birth, suggesting that activins are important for normal fetal development (Matzuk et al., 1995)

2.11 Influence of oestrogenic chemicals on fetal gonadal development

As detailed in the previous sections, fetal gonadal differentiation is a precise process and many different factors may interfere with the normal development of the gonads and/or the development of secondary sexual structures. In particular the role and importance of androgens, especially testosterone, in orchestrating normal endocrine reproductive function of the gonads is well known (section 2.10.3). However, the role of oestrogens has not been as well defined and the function of this steroid

hormone in mammalian male and female gonadal development has yet to be fully elucidated. Experimental studies carried out in reptiles, where the sex of the offspring is determined by the environmental temperature at the time of egg incubation, have shown that oestrogens applied on the egg shelf can change the sex of the offspring (Pieau, 1996). Additional studies have also suggested that temperature regulation of sex in these species is oestrogen dependent and that the aromatase enzyme, responsible for formation of oestrogens, is under the influence of a temperature sensitive promoter (Pieau, 1996).

In mammals, overexposure of the fetus to oestrogens results in abnormal development of the reproductive system, changes which may compromise fertility in adult life. For example, studies as early as 1939 showed that administration of high doses of diethylstilbestrol (DES), a synthetic non steroidal oestrogen, to pregnant rats had a detrimental effect on development of the offspring (Green et al., 1939). Experiments carried out by Green and co-workers (1939) demonstrated that pregnant female rats treated with DES delivered fetuses with exclusively female external genitalia together with visible nipples and that it was only possible to distinguish between the sex of the pups by internal examination of their gonads. Further examination of male fetuses from DES-treated females revealed that development of their seminal vesicles, epididymis, vas deferens and prostate glands was retarded. The female fetuses from the same mothers also developed abnormalities of their reproductive organs with extended uteri and remnants of Wolffian ducts. In another report, Boylan (1978) treated pregnant rats with much smaller doses of DES (1.2 - 120μg/animal). These experimental studies showed that the effect of DES-treatment of mothers on their female offspring was more pronounced than on their male pups. The females had abnormalities in their nipple development as well as premature opening of the vagina. Furthermore, DES treatment prevented parturition of mature fetuses and occasionally acted as an abortifacent. Studies by McLachlan and Newbold (1975) using mice have shown the DES treatment affects male offspring when injected daily (day 9 to 16 of pregnancy) at doses of 100μg/Kg to their mothers. Whilst the external appearance of new-born mice from treated mothers was comparable to that of controls from untreated mothers, the fertility of the treated male offspring was impaired. Examination of these males in adulthood revealed a high incidence of epididymal cysts, testicular abnormalities and in some cases abnormalities of accessory sexual organs. In experiments by Nomura and Kanzaki (1977), animals received a single dose of 10μg/Kg of DES between days 9 and 17 of pregnancy. The new-born

offspring showed no obvious external abnormalities, however, some females displayed premature opening of the vagina and an increased incidence of ovarian cancer. In addition, a proportion of the males had small undescended testes (examined at 12 months of age). No effects on the epididymis or other accessory sexual organs were reported. Similarly, the synthetic oestrogen RU 2858 (moxestrol) was reported to cause underdevelopment of Wolffian ducts and prostate glands, hypospadias and in some animals cryptorchidism when given to pregnant rats subcutaneously every day between days 15.5 to 19.5 p.c. in doses 0.4, 2, 10 or 50μg of RU 2858 per rat. The effects were most obvious in the group treated with highest dose of RU 2858 but were noticeable in all treated groups (Vannier and Raynaud, 1980). Similar effects of oestradiol were reported in the same study.

The influence of oestrogens, in particular the synthetic oestrogen DES, on the development of the human fetal reproductive tract was shown following use of this compound in the 1950's and 1960's. Clinical trials by Smith (1948) suggested that DES was beneficial in both the treatment of threatened abortions and in the prevention of abortions in patients who repeatedly miscarried. Between the late 1940's and 1971, when DES was finally banned as a therapeutic substance, an estimated 2-3 million women were prescribed this drug during pregnancy (Stillman, 1982). Unfortunately, control studies have proved that DES is not beneficial in treating these clinical conditions (Dieckmann et al., 1953) and in fact has serious adverse effects on fetal development. New-born babies (from mothers prescribed DES) exhibited no visible external abnormalities, however, follow-up studies have shown increased abnormalities in the reproductive tract of both male and female offspring of DES treated mothers. In the 1974, Herbst et al. (1974) reported the occurrence of vaginal clear cell adenocarcinoma in seven girls aged from 14 to 22 years. These cases exceeded the total of all previously reported cases in this age group and an epidemiological study has correlated development of this cancer to DES exposure *in utero*. Following on from this initial finding, several other serious long-term consequences of in utero exposure to DES have been described. Additional studies (Bibbo et al., 1977) have recorded a higher incidence of irregular menstrual cycles in offspring of DES treated mothers (compared to a control population) together with a lower incidence of pregnancies. Other reports have recorded a higher occurrence of anatomic abnormalities and fertility problems including an increase in spontaneous abortions, ectopic pregnancy and premature delivery in females (Stillman, 1982). In adult males exposed in utero to DES, epididymal cysts, hypotrophic testes and capsular induration were found in 25% of

examined males compared to an incidence of 6% in the control male population (Bibbo et al., 1977; Gill et al., 1979; Stillman, 1982). Semen analysis of these men revealed a two fold decrease in sperm motility and sperm density, although these numbers were still in the normal range. For example, 26% of DES exposed males produced ejaculate volumes under 1.5ml and "a severely pathological semen" score was found in 28% of exposed males. No such cases were observed in the control group and an association between pathologic semen quality and physical abnormalities was only observed in the group of exposed males (Bibbo et al., 1977; Gill et al., 1979; Stillman, 1982). Recently, Wilcox et al. (1995) have carried out a study to establish if the fertility or sexual function of men exposed in utero to DES is impaired. Surprisingly, their studies have shown that despite an incidence of congenital malformations three times higher in the exposed group compared with the control group, the males from both groups showed no significant differences in fertility based on their responses to a telephone survey.

The most common reproductive abnormalities occurring after in utero exposure to oestrogens are summarised in table 2.2.

Incidence of	**Animals**	**Humans**
Testicular maldescent	Increase	Increase
Hypospadias	Increase	Increase
Testicular cancer	no data	Increase
Small testes	Increase	Increase
Low sperm output	Increase	Increase

Table 2.2: Developmental abnormalities of the male reproductive system induced by administration of oestrogens to pregnant animals or humans

2.11.1 Mechanism(s) of oestrogenic action on the developing reproductive tract

The effect of oestrogens on male reproductive development may be mediated either directly at the level of the testis or indirectly via the pituitary/hypothalamus. Oestrogens usually act through interaction with specific oestrogen receptors. In the rat and mouse testes, oestrogen receptors have been identified in developing Leydig

cells using immunocytochemistry. Studies by Greco and co-workers (1992) have shown the presence of oestrogen receptors in mouse Leydig and peritubular cells and in Wolffian ducts between day 13.5 and 17.5 of fetal development using immunocytochemistry on frozen tissue sections. Furthermore, in adult rats exogenous administration of oestrogens results in a lowering of testosterone production, via the inhibition of an essential steroidogenic enzyme P450c17 (Kalla et al., 1980; Nozu et al., 1981b). It is therefore possible that exposure of Leydig cells to oestrogens or oestrogenic chemicals during fetal development might result in changes in testosterone production through a similar mechanism and thus ultimately have profound effects on masculinisation of the male fetus.

In addition, oestrogens are known to exert a negative feedback effect on the synthesis and secretion of LH and FSH in adult males (Griffin and Wilson, 1992) and this action is thought to result primarily from a decrease in GnRH release from the hypothalamus (Griffin and Wilson, 1992; Haisenleder et al., 1994). Oestrogen receptors have not been detected in the GnRH neurons (Herbison and Theodosis, 1992), but are present in some other neurons in the hypothalamus (Herbison and Theodosis, 1992; Sar et al., 1990) that could modulate the function of the GnRH neurons. It is possible to speculate that oestrogens might also influence gonadotrophin production and secretion during fetal life. However, the potential consequences of oestrogen mediated GnRH inhibition on the developing fetal gonads is unknown, although it is possible that a decrease in secretion of LH and FSH could reduce testosterone production and Sertoli cell proliferation during the latter stages of gestation. Possible sites of oestrogenic action in the reproductive axis are presented in figure 2.6.

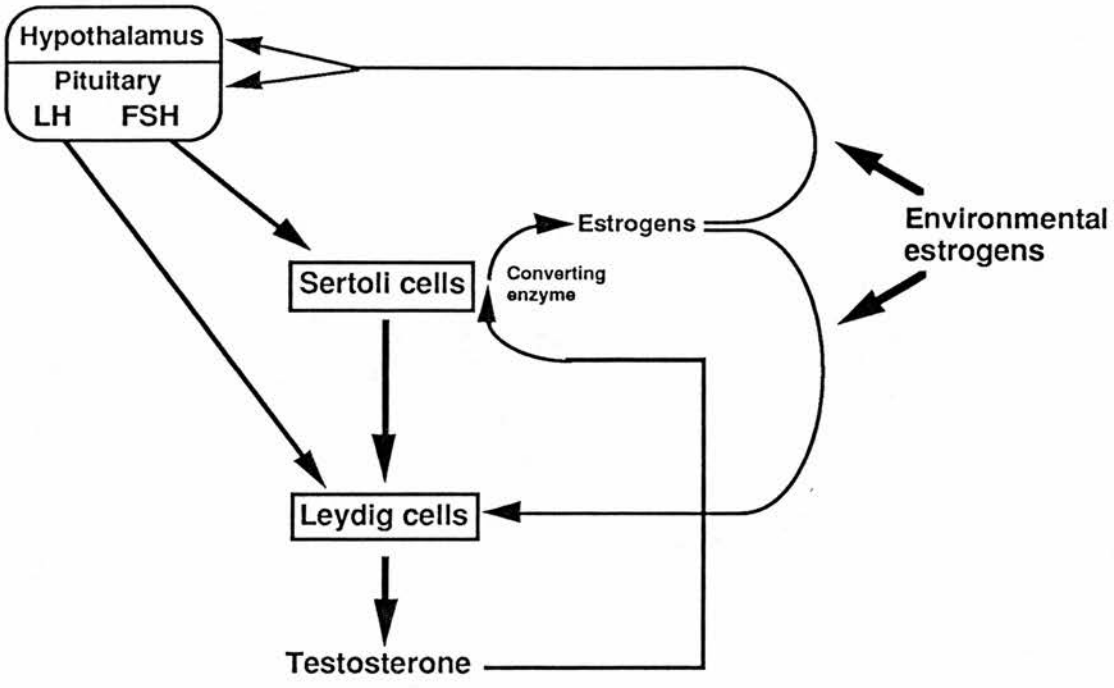


Figure 2.6: Possible sites of action for oestrogens and oestrogenic chemicals on the reproductive axis during fetal development

2.11.2 Evidence for an increase in the incidence of male reproductive problems

Over the last 5 years, scientific research has documented an increase in reproductive problems in men in the Western world. In particular, several independent studies have shown a sharp rise in the incidence of testicular (germ cell derived) cancer, currently the most common malignancy of young males (Giwercman, 1995; Giwercman and Skakkebaek, 1992). One of the most conclusive studies arises from analysis of the Danish Cancer Registry, which has revealed a 3-4 fold increase in the incidence of testicular cancer between 1940 and 1980 (Oesterlind, 1986). These findings have also been confirmed in studies carried out in Scotland (Boyle et al., 1987), the USA (Brown et al., 1986) and in Finland (Hakulinen et al., 1986). The incidence of male congenital malformations such as cryptorchidism and hypospadias, also appears to be on the increase although less research has been carried out in this area (Giwercman and Skakkebaek, 1992). Furthermore, changes are also reported to have occurred in the semen of normal males. It has been observed that semen quality in men in some but not all developed countries is

declining with reports of smaller ejaculates, less sperm per ejaculate and less viable sperm steadily increasing throughout the last five decades (Auger et al., 1995; Carlsen et al., 1992; Toppari et al., 1996; van Waeleghem et al., 1994). Evidence suggests that the mean sperm count has halved in the last 50 years, with people born after the 1960's having smaller ejaculates and less motile sperm than men born prior to 1950. These reports remain controversial especially as they may not have occurred in all countries (Fisch and Goluboff, 1996; Suominen and Vierula, 1993; Vierula et al., 1996), but increasing experimental evidence has endorsed these findings suggesting that the described trend is a real observation (Giwercman, 1995). Whilst little is known about the aetiology of these conditions and the factors contributing to the increase in male reproductive problems, Sharpe and Skakkebaek (1993) have proposed that oestrogens, or chemicals mimicking oestrogens, may affect male reproductive function. This hypothesis has been based primarily on the observations that males exposed in utero to the synthetic oestrogen DES (discussed above) exhibit similar patterns of reproductive abnormalities to those now increasing in other populations. Sharpe and Skakkebaek (1993) have reviewed the possible sources of oestrogenic exposure and have suggested that changes in diet, increases in body fat and obesity, together with increases in exposure to environmental pollutants with oestrogenic activity could all contribute to these changes. The diet of the individuals in many developed countries has radically changed over the last 50 years with an increase in the consumption of milk and dairy products. These may contain oestrogens due to intensive farming with milking occuring during pregnancy. A large increase in use of soya (soya is very rich in phytooestrogens) could also have contributed to an increased exposure to oestrogens in every day life. Furthermore, it is likely that we are all exposed to many oestrogens or chemicals mimicking oestrogen action due to environmental pollution. It has been speculated that usage of contraceptive pills containing synthetic oestrogens, the use of anabolic oestrogens in animal farming and widespread use of chemical agents that exhibit oestrogenic activity by industry may all have contributed to an increasing exposure to oestrogens (Sharpe and Skakkebaek, 1993).

2.11.3 Chemicals with oestrogenic activity

Analysis of environmental chemicals has resulted in the finding that many pollutants including DDT (and its metabolites) and PCBs have oestrogenic activities (Bittman and Cecil, 1970). Although these chemical agents have been banned in Western countries, their continued use in the third world together with their accumulative and

slow degradational properties has resulted in significant concentrations in the environment. Recent studies (Kelce et al., 1995) on the DDT metabolite p,p'-DDE have shown it can bind to the AR and it is therefore possible that DDE could prevent the action of endogenous androgens at critical times during fetal development. In addition to DDT and PCBs, weak oestrogenic activity has also been shown for several other chemicals, all of which have been previously considered environmentally safe (Jobling et al., 1995; White et al., 1994). The majority of these chemicals have a different chemical structure to endogenous oestrogens and at present it is not therefore possible to assess whether a compound is likely to have oestrogenic activity based on the knowledge of its chemical structure. In many instances the oestrogenic activity of these chemicals was discovered accidentally. For example, the observation that oestrogenic substances were released from laboratory plastic ware led to the identification of alkylphenols, in particularly nonylphenol and bisphenol-A as a chemicals with oestrogenic activity (Krishnan et al., 1993; Soto et al., 1991). In addition, oestrogenic activity has also been shown for phthalate esters, another group of chemicals that are ubiquitously found in the environment (Jobling et al., 1995).

Alkylphenol polyethoxylates (APEOs) were introduced in the 1940s and are the second largest group of nonionic surfactants in commercial production (Naylor et al., 1992). They are widely used in detergents, paints, herbicides, pesticides and many other products. The mixture of alkylphenol polyethoxylates is usually used in a 1:4 ratio (octylphenol:nonylphenol polyethoxylates). Over 300000 tons of APEOs are produced annually world-wide and it is estimated that about 60% of these end up in the aquatic environment, mostly via sewage treatment works. In the water APEOs usually form relatively stable metabolites like alkylphenols, nonylphenol and octylphenol and these by-products tend to accumulate in sewage sludge and river sediment (Naylor et al., 1992; White et al., 1994). Studies by White et al. (1994) have shown oestrogenic activity of nonylphenol and octylphenol in vitro and in vivo and in addition studies by Sharpe et al. (1995) have shown the effects of octylphenol exposure in utero and postnatally on the adult testis size in rats. This will be further discussed in chapter 8.

3. General Materials and Methods

The techniques outlined in this chapter are common to a number of studies in this thesis. Methods specific to individual experiments are described in the relevant chapters.

3.1 Chemicals and suppliers

Molecular biology grade chemicals were obtained from Sigma, Poole, Dorset and IBI, Cambridge. All radiolabelled nucleotides were obtained from Amersham, Little Chalfont, Bucks or DuPont-NEN, Stevenage, Hertfordshire. Enzymes were purchased from Boehringer Mannheim, Lewes, Sussex or Promega, Southampton. Phenol/chloroform was bought from CAMLAB, Cambridge, and was pre-buffered with Tris, pH 8.0. Autoradiography products were obtained from Eastman Kodak, Rochester, NY, USA, supplied by IBI.

3.2 Animals and treatments

3.2.1 Animals

Animals used for these studies were Wistar rats bred in the MRC Reproductive Biology Unit in Edinburgh. Rats were maintained in standard conditions of a 12 h light : 12 h dark cycle and an ambient temperature of 21°C. Food and water were available *ad libitum*. To obtain time mated females, the adult females were placed in the individual cages with one adult male. They were checked every morning for the presence of a copulating plug and if present, midday of that day was taken as day 0.5 of pregnancy (assuming that animals mated around midnight). Animals were killed by asphyxiation with CO_2 followed by cervical dislocation.

3.3 RNA extraction and separation

3.3.1 Dissection of tissue

Fetuses at day 17.5 and older were collected from the uterus and placed in warm PBS prior to dissection. Testes or ovaries were isolated from the fetuses under a dissecting microscope. Several testes or ovaries from the same litter were pooled together, transferred into 1 ml of TRI reagent (Sigma) and placed on ice.

3.3.2 Extraction of RNA from fresh tissue

Tissues were homogenised in TRI reagent (Sigma) in Eppendorf 1.5ml tubes by passing several times through a fine needle (23-26g) until the solution looked homogenous. To this homogenate, 0.2ml of chloroform was added and the mixture mixed thoroughly. The mixture was placed on ice for 15 min and then centrifuged at 10000 rpm for 20 min at 4°C. The upper, aqueous phase containing the RNA, was removed carefully to a new tube and an equal volume of cold (-20°C) isopropanol was added together with 2µl of polyinosinic acid (Pharmacia, Milton Keynes) to act as an inert carrier (Winslow and Henkart, 1991). The RNA was left to precipitate at -20°C for at least 1 h, pelleted by centrifugation (13000 rpm for 20 min at 4°C), washed in 0.5ml 75% ethanol, recentrifuged and air dried. The RNA pellet was dissolved in RNase-free water by warming to 65°C for 10 min and stored at -70°C.

RNA was scanned at 260 and 280nm on a spectrophotometer. The 260:280 ratio for each sample was calculated to give an estimation of the purity of the RNA. A ratio of 2.0 was taken to be pure. The concentration of the RNA was calculated from the 260nm value where an optical density of 1.0 is equal to 40µg/ml RNA.

3.3.3 Separation of RNA on denaturing agarose gels

RNA was separated on a 1.5% denaturing agarose gel. The gel was prepared by melting 2.25g agarose (Boehringer) in 127ml pure water. This was cooled to about 60°C and 15ml of 10 x running buffer (containing 200mM MOPS, 10mM EDTA and 50mM sodium acetate at pH 7.0) plus 8.1ml 37% formaldehyde added. The solution was mixed gently and poured into a gel tray (15 x 20cm) containing a 15 or 20 well comb in a fume hood. After setting, the comb was removed and the gel submerged in 1 x running buffer in a Sub-Cell electrophoresis tank (Bio-Rad, Hemel Hempstead, Hertfordshire).

RNA (5-10µg) in a volume of no greater than 5µl was denatured by adding 15.6µl of sample buffer and heating at 60°C for 5 min. Sample buffer contained 100µl 10 x running buffer, 500µl deionised formamide and 178µl formaldehyde (Sigma). After heating, 8µl of dye solution containing 7.5% w/v ficoll 400, 0.1% w/v bromophenol blue and 1µl of 1mg/ml ethidium bromide was added to each sample. Samples were loaded into individual wells of the gel and separated by electrophoresis for 16 h at 35V or for 5 h at 120V.

3.4 Tissue fixation and processing

3.4.1 Fixation

Fetuses were collected from the uterus (3.3.1.), decapitated and were either directly placed into Bouin's fixative or were dissected under a dissecting microscope to isolate the individual gonads which were then placed in the Bouin's. Whole fetuses were fixed from 4 to 8 h, depending on their size and isolated testes and ovaries were fixed for 1 to 2 h. After fixation, the tissue was processed immediately or stored in 70% ethanol.

3.4.2 Processing and sectioning of tissue

Tissue was processed through a graded series of alcohols (70%, 80%, 90%, 95%, 100% and xylene respectively) in an automatic 2LE Processor (Shandon Scientific Limited, Cheshire) using a standard 17.5 (for isolated gonads) or 24 h (for whole fetuses) cycle and embedded in paraffin wax.

Glass microscope slides to be used for in situ hybridisation were washed, dried and baked at 300°C for 8 h. Slides were washed in a 2% solution of 3-aminopropyl triethoxysilane (TESPA; Sigma) in acetone followed by a wash in acetone (BDH Ltd, Poole, Dorset) , rinsed in filtered distilled water and dried.

Tissues embedded in paraffin wax were sectioned to a thickness of 5μm using a hand operated Leitz (Wetzlar, Germany) microtome and a D-profile knife. Sections used for in-situ hybridization studies were floated on RNase-free water, transferred onto the treated slides and dried at 50°C overnight before use. Sections for use in immunostaining were sectioned as above but were floated on normal distilled water and dried onto untreated slides.

3.4.3 Staining of sections with haematoxylin and eosin

To examine the gross morphology of developing fetuses and gonads, sections were stained using haematoxylin and eosin. Briefly, sections were dewaxed in histoclear (National Diagnostics, Atlanta, GA, USA) and rehydrated in a decreasing series of alcohols (100%, 95%, 70% respectively). A water wash was followed by 1-3 min incubation in haematoxylin, a wash in distilled water and a short incubation in Scott's

tap water. If the staining with haematoxylin was too intense, it was reduced by placing the slides for a 5 - 10 sec in 1% acid alcohol. After the development of the blue colour, sections were placed for 5-20 sec in 1% eosin, washed in water and quickly dehydrated in an increasing series of alcohols. After clearing in histoclear, the sections were coverslipped using Pertex mounting medium (CellPath, Hemel Hempstead, Hertfordshire).

3.5 Immunocytochemistry

3.5.1 Preparation and immunodetection

Paraffin tissue sections were dewaxed for 5 min in xylene followed by rehydration in decreasing alcohols (100%, 96% and 70% respectively) and washed in the tap water followed by a 5 min wash in 0.05M Tris-HCl buffer pH 7.4 containing 0.85% NaCl (TBS). Sections were incubated for 30 min with normal serum from the same animal species as that in which the secondary antibodies were raised (usually rabbit or swine), diluted 1:5 in TBS. Sections intended for use with the horseradish peroxidase detection system were incubated in 1% hydrogen peroxide diluted in TBS for 30 min to block endogenous peroxidase and washed for 5 min in TBS prior to blocking with normal serum. After 30 min, the area of the slide around the tissue sections was dried and normal serum was replaced by 50µl of the primary specific antiserum at an appropriate dilution (specific dilutions are mentioned in the appropriate chapters). Sections were covered by gelbond film (Flowgene Instruments Ltd, Sittingbourne, Kent) and incubated in a humidified chamber at 4°C overnight. Next day the coverslips were removed and sections washed twice for 5 min in TBS, incubated with secondary antibodies for 30 min at room temperature (the appropriate secondary antibodies used are detailed in the individual chapters) and then washed twice for 5 min each in TBS. Avidin-biotin complex, peroxidase-antiperoxidase complex or alkaline phosphatase-anti-alkaline phosphatase complex (Dako, High Wycombe, Bucks) were used together with their appropriate substrates to detect bound antibodies and these are described in corresponding chapters. Development of the colour precipitate was followed by a wash in distilled water. Sections were usually counterstained with haematoxylin and dependent on the solubility of colour precipitate either directly mounted with Aquamount mounting medium (BDH) or dehydrated, cleared in xylene and mounted with Pertex mounting medium (Cellpath).

3.5.2 Antigen retrieval

Some proteins were only recognised by specific antisera following pretreatment of tissue sections. The method employed was that of microwave antigen retrieval (Shi et al., 1993). Briefly, after an initial wash in TBS and blocking in hydrogen peroxide (as in 3.5.1), the sections in a plastic rack were placed in a glass dish, covered with 0.01M sodium citrate buffer (pH 6.0), wrapped in cling film and microwaved at full power for 20 min in a 650W Sanyo microwave oven. If the buffer evaporated, preheated distilled water was added to maintain the buffer level. After microwaving, the sections were left undisturbed in sodium citrate for an additional 20 min, followed by a wash in TBS and incubation with blocking serum before proceeding with immunocytochemical detection (3.5.1).

3.5.3 Control of antibody specificity

The specificity of the antibodies was checked either by replacing the primary antibodies with normal serum derived from the same animal species as that in which the primary antibodies were raised in or by preabsorbing the antibodies with the antigen against which they were raised. Normal mouse or rabbit serum (Dako) was used in the same dilution as primary antibodies overnight at 4°C and the next day sections were processed under standard conditions (3.5.1). If the antigen (normally the peptide against which the antibodies were raised) was available (stated in corresponding chapters), the primary antibodies were incubated with 20-fold molar excess of the antigen overnight at 4°C and used the following day at the same dilution as for normal immunostaining.

3.6 Reverse transcription polymerase chain reaction (RT PCR)

3.6.1 Synthesis of oligonucleotide primers

Oligonucleotides (17-24mers) were synthesised using phosphoramadite chemistry on a Model 381 DNA synthesiser (Applied Biosystems (ABI), Warrington, Cheshire). Oligonucleotides were recovered into 1ml of pure concentrated ammonia, deprotected by incubating overnight at 55°C, recovered by two rounds of ethanol precipitation and resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8.0). The concentration of

the oligonucleotides were estimated by spectroscopy at 260nm where an optical density of 1.0 is equal to a concentration of 20µg/ml. Preprepared oligonucleotides were also purchased from Oswel, Edinburgh.

3.6.2 RT PCR Reaction

Reverse transcription was undertaken using 250-500ng of total RNA in 20µl volume containing 10mM Tris-HCl pH 8.3, 90mM KCl, 1mM $MnCl_2$, 200µM each dNTP, 5U Tth polymerase (ABI) and 0.75µM of the appropriate antisense downstream primer or random primer (Promega) containing a mixture of different hexanucleotides. The conditions used with antisense primer were usually 65°C 5 min, 50°C 5 min, 70°C 15 min and with random primers 65°C 5 min, 25°C 10 min, 48°C 10 min and 70°C 15 min and thereafter the samples were placed on ice. The reaction volume was increased to 100µl and PCR undertaken in buffer containing 1.5mM $MgCl_2$, 40% glycerol, 10mM Tris-HCl pH 8.3, 890mM KCl, 6mM EGTA, 0.4% Tween 20 ($MnCl_2$ in the first reaction solution was chelated by the EGTA) and 0.15µM of the upstream sense primer. If random primers were used in reverse transcription, upstream and downstream primers were both added to the PCR reaction at a concentration of 0.15µM. PCR conditions were adjusted according to the calculated annealing temperatures of the primers and the length of the DNA fragment to be amplified. Amplified product(s) were analysed by electrophoresis on 2% agarose gels run in TBE using standard methods.

3.6.3 Cloning of amplified cDNAs

If RT PCR reactions were conducted with aim of obtaining a probe for in situ hybridisation and/or RNase protection assay reactions, the amplified DNA fragments were subcloned into an appropriate plasmid vector. The vector used on most occasions was the TA vector, pCRII (InVitrogen, Abington). Amplification of the DNA was followed by purification of the fragments using spin columns (Clontech, Palo Alto, CA, USA) which had the ability to exclude contaminating cDNAs and excess oligonucleotide primers with sizes below 100bp or 400 bp dependent upon the column bed. Columns were prepared by centrifugation in a swing out rotor at 2100 rpm for 3 min. The PCR reaction mixture was pipetted to the centre of the dried column which was then recentrifuged at 2100 rpm for 3 min. Purified DNAs were used directly in ligation reactions.

Plasmid vector (25ng/µl) and 1µl of T4 DNA ligase were mixed with 50ng of purified PCR fragment and the mixture was incubated overnight on 12°C. Competent E. Coli cells (InvαF', InVitrogen) were thawed on ice and 2µl of ß-mercaptoethanol and 1µl of ligation mixture were gently mixed with the cells in a 2059 Falcon tube. The mixture was incubated on ice for 30 min, heatshocked by placing in waterbath at 42°C for 45 sec and placed again on ice for another 2 min. Thereafter 450µl of SOC medium prewarmed to 37°C was added to the cells which were incubated for 1 h at 37°C at 200 rpm. Aliquots (200µl and 50µl) were spread using an alcohol flamed glass rod onto LB agar plates containing 100µg/ml ampicillin and 25µl of X-gal (40µg/ml stock). Plates were incubated inverted overnight at 37°C. White bacterial colonies suggesting they contained recombinant plasmids were restreaked on LB agar plates. The plates were again incubated inverted overnight at 37°C; thereafter individual plasmid colonies were picked into broth and propagated as described in 3.7. The presence of the expected cDNA within the recombinant plasmids was checked by restriction digestion using Eco RI (when the plasmid used was pCRII) and checking the size of the released fragment on agarose gels. Additional investigations were carried out by sequencing (3.10).

3.7 Plasmid preparation and analysis

3.7.1 Growth of bacterial cultures

Most of the plasmid preparations used were kindly prepared by Julie Wilson.

Recombinant plasmids containing cDNA inserts were either obtained from colleagues or were prepared by ligating PCR products amplified from testicular cDNA pools into plasmid vectors containing amp^r gene. Bacteria transformed with recombinant plasmids were cultured in Luria Bertani (LB) broth containing 50µg/ml ampicillin. Typically 10ml of broth was inoculated with a single bacterial colony from an agar plate, or from a glycerol stock stored at -20°C, and incubated overnight at 37°C shaking at 225 rev/min.

3.7.2 Plasmid preparation from bacterial cultures

Plasmid DNA was isolated from 10ml bacterial cultures by the alkaline lysis method using the 'Magic Minipreps' and 'Wizard minipreps' DNA purification systems from Promega.

Briefly, bacterial suspensions were centrifuged at 1600g, the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl, pH 7.5, 10mM EDTA and 100µg/ml RNase and mixed with an equal volume of 0.2M NaOH containing 1% SDS. The resulting solution containing lysed bacterial cells was neutralised by the addition of 2.55M potassium acetate. The resulting cloudy solution was centrifuged at 12000g to sediment the majority of bacterial genomic DNA. The supernatant (approximately 600µl) was removed, mixed with a 1ml suspension of DNA purification resin and passed down a miniprep column which bound the plasmid DNA. The column was washed with washing buffer, 50µl TE buffer was added and then incubated at room temperature for 1min. Bound plasmid DNA was eluted from the column bed by centrifugation at 12000g for 20 sec and collected into a sterile tube.

3.7.3 Analysis of plasmid DNA quality

Purity of plasmid DNA was determined by analysis on a 0.8% (w/v) agarose minigels. This was prepared using agarose dissolved in 1x TBE buffer. The agarose was melted and approximately 200µg/ml ethidium bromide was added for visualisation of the DNA. The gel was poured into a 7 x 10 cm gel tray containing an 8 well comb and submerged in 1 x TBE buffer in submarine cell (Hoefer, Newcastle). Plasmid DNA (approximately 1µl) was run in a sample solution containing 1µl 'orange juice' (contains 0.25% w/v orange G, 15% w/v ficoll and 0.5M EDTA at pH 7.0) and 8µl water. Samples were separated in parallel with pGem DNA markers (range 36-2,645 bp, Promega) or Hae III digested øX174 markers (range 72-1353 bp, IBI) by electrophoresis at 100 volts for 1-2 h in 1 x TBE, viewed under UV light and photographed. Pure plasmid DNA appeared as two visible 'bands' of greater than 2kb (depending on plasmid size); one for the circular DNA and the other representing supercoiled DNA which migrates more rapidly through the gel due to its compact form.

3.8 Probe preparation for In situ hybridisation

3.8.1 Template preparation

Plasmid DNA prepared as described (see section 3.7) was linearised in a reaction containing 1µg DNA, reaction buffer (contains 10-50mmol/l Tris-HCl, 5-10mmol/l $MgCl_2$, 50-100mmol/l NaCl and 1mmol/l DTT, DTE or ß-mercaptoethanol), 10U restriction enzyme and pure water to a volume of 30µl. The enzyme used was

dependent on the plasmid vector being digested and the orientation of the cDNA relative to the RNA polymerase sites. The reaction was incubated at 37°C for 1-2 h. Thereafter, 3µl of reaction mix was separated on a minigel (0.8% agarose) and compared to an uncut sample of the same plasmid run in a parallel lane to test efficiency of digestion. Digested DNA was extracted once with Tris-buffered phenol/chloroform (100µl), centrifuged (13000 rpm, 5 min), the upper layer removed to a fresh tube and precipitated with 1/10th volume 3M sodium acetate pH 5.5 and 2.5 volumes absolute ethanol overnight at -20°C. The mixture was centrifuged at 13,000 x rpm for 20 min to pellet the DNA, the supernatant removed, the pellet washed with 75% ethanol, centrifuged, air dried and resuspended in 10µl pure water.

3.8.2 Preparation of radiolabelled riboprobe

Synthesis of riboprobes was carried out using 1µg linearised template in a reaction mix containing 10mM DTT, 20U RNase inhibitor (Promega), 1mM each rATP, rCTP and rGTP, transcription buffer (1 x transcription buffer contains 40mM Tris-HCl, pH 7.9, 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl) and 50µCi ^{35}S-UTP (400 Ci/mmol; Amersham). The reaction was catalysed by addition of 30U of the appropriate RNA polymerase (T3, T7 or SP6) and incubated at 37°C for approximately 1.5 h. The DNA template was removed by digestion with 40U RNase-free DNase for 15 min at 37°C and the radiolabelled cRNA was purified using RNA spin columns (IBI). Prior to use, the buffer was drained from columns by centrifugation for 1 min at 2500 rpm. Thereafter, the RNA labelling reaction mixture was carefully pipetted into the middle of the compacted column bed and the column recentrifuged in a swing out rotor for 2 min at 2500 rpm. The activity of 2 x 1µl aliquot of the liquid eluate of the column was determined by liquid scintillation spectroscopy. The average activity of the two samples was calculated and the volume of probe solution necessary to give 1 x 10^6 cpm was determined.

3.9 Radioactive In situ hybridisation

3.9.1 Pretreatment of tissue sections

Tissues were embedded, processed, sectioned and mounted on coated slides as detailed in 3.4.2. Sections were cleared in xylene for 5 min and rehydrated in a series of alcohols with decreasing concentrations (100%, 95% and 70% respectively). Tissue was placed in 0.2N HCl for 20 min followed by two 5 min washes in ultrapure

water. Sections were then incubated in 2µg/ml proteinase K (Sigma) in buffer containing 20mM Tris/HCl pH7.4 and 50mM EDTA at 37°C for 20 min followed by 0.2% glycine at 4°C for 10 min. Sections were washed briefly in 0.1M triethanolamine (TEA) pH 8.0 and acetylated in 0.25% acetic anhydride in 0.1M TEA pH 8.0 for 10 min. Finally, sections were prehybridised in buffer containing 4 x STE (1 x STE contains 150mM NaCl, 2,5mM Tris and 0.25mM EDTA), 1 x Denhart's solution (50 x Denhart's contains 5g BSA, 5g polyvinylpyrolidine and 5g ficoll in 500ml solution), 10mM DTT, 125µg/ml salmon sperm DNA, 125µg/ml yeast tRNA and 50% deionised formamide for 2-4 h at probe Tm-25°C.

3.9.2 Hybridisation of Probe to Tissue

Hybridisation was continued overnight with a probe concentration of 1 x 10^6 cpm probe in 40µl buffer/slide. Hybridisation buffer was prehybridisation buffer with 10% dextran sulphate. The incubation was carried out beneath coverslips prepared from gel bond film (Flowgen Instruments Ltd.) in a humidified chamber at Tm-25°C where Tm was calculated individually for each probe according to the equation:

$Tm = 79.8 + 58.4\ (F_{GC}) + 11.8\ (F_{GC})^2 + 18.5 \log(M) - 820/L - 0.35\ (\%F) - (\%M)$

where:

F_{GC} = mole fraction of GC content of probe, usually about 0.45

M = monovalent cation concentration (molarity of salt in hybridisation buffer)

L = length of duplexes formed during hybridisation (probe length)

%F = percentage of formamide in buffer

3.9.3 Post hybridisation washes

After incubation sections were washed in 4 x SSC for 10 min to remove the coverslips and treated with RNase A (Sigma) at a concentration of 20µg/ml in 0.5M NaCl, 0.1M Tris pH 8.0 and 1mM EDTA for 30 min at 37°C. Sections were washed in RNase buffer alone for 30 min at 37°C followed by two washes in 2 x SSC at room temperature for 5 min and finally in 0.1 x SSC/30% formamide at 40°C for 30 min.

3.9.4 Development of In situ hybridisation

After post hybridisation washes sections were dehydrated through alcohols. Slides were warmed to 45°C and dipped in NTB2 emulsion (Kodak) at 45°C in the dark. Emulsion coated slides were stored in a humidified, lightproof box overnight before

transfer to a polyacetyl black trough lightproof box (Lamb's laboratory supplies, London) with silica gel, and stored at 4°C for a minimum of 7 days (times of exposure were dependant on the abundance of the signal and are mentioned in appropriate chapters).

Silver grains formed by reaction of the hybridised, radiolabelled probe with the emulsion were developed using Kodak D19 developer as follows. Slides were placed in developer cooled to 15°C for 4 min, followed by a brief wash in pure water and grains were fixed by incubation for 10 min in Kodak unifix at room temperature. Sections were washed in water, stained with haematoxylin, dehydrated and mounted using Pertex mounting medium (Cellpath).

Slides were analysed under dark field using either BIOMED or LABORLUX 12 microscopes (Leitz) or the Olympus Provis Image analysis system (Olympus Corp., London) to visualise the silver grains indicating areas of hybridisation. Bright field microscopy was used to examine the morphology of the tissue sections.

3.10 Sequencing

Most sequencing reactions were carried out by Joe Gaughan. Automatic sequencing reactions were carried out using the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and were run on the Applied Biosystems Model 373A DNA sequencing system.

3.10.1 Sequencing reactions

A reaction premix was prepared containing the equivalent of 4µl 5x TACS buffer (400mM Tris/HCl, 10mM $MgCl_2$ and 100mM $(NH_4)_2SO_4$ pH 9.0), 1µl dNTP mix (containing 750µM dITP and 150µM each dATP, dTTP and dCTP), 1µl each ddATP, ddTTP, ddCTP and ddGTP terminators and 0.5-1µl AmpliTaq DNA polymerase for each sequencing reaction to be performed. For sequencing of PCR products 1µl DMSO was also added. A total of 11.5 (PCR template) or 9.5µl (plasmid DNA template) of the reaction premix was added to separate tubes containing 3.2 pmol primer and 5µl template DNA, the total volume adjusted to 20µl with water and overlaid with mineral oil.

Tubes were placed in a Perkin Elmer Cetus (Seer Green) thermal cycler Model 480, preheated to 96°C and 25 cycles of thermal cycling carried out as follows; rapid thermal ramp to 96°C, 96°C for 30 sec, rapid thermal ramp to 50°C, 50°C for 15sec, rapid thermal ramp to 60°C and 60°C for 4 min.

On completion of the sequencing reaction, the samples were extracted twice with water saturated phenol:chloroform and precipitated with 15µl 2M sodium acetate, pH 4.5 and 300µl cold absolute ethanol to remove unincorporated labelled terminators. Labelled DNA was pelleted by centrifugation, the supernatant discarded and the DNA washed using 75% ethanol, air dried and resuspended in formamide/EDTA buffer (5µl formamide, 1µl 50mM EDTA, pH 8.0) prior to loading onto the gel.

3.10.2 Automatic sequencing gel

A sequencing gel mix containing 50g urea, 15ml 40% acrylamide and water made up to a final volume of 80ml was prepared. Amberlite resin was added to remove acrylamide free acid and the mixture was stirred with gentle heating until the urea had dissolved, the solution was filtered through a 0.2µM cellulose nitrate filter (Nalgene, Hereford, UK) and 10ml 10x TBE added. The volume was then made up to 100mls with pure distilled water. The gel mix was polymerised using 45µl TEMED and 500µl 10% ammonium persulphate and poured carefully between clean glass plates.

The gel was prerun for 10-15 min at 500 volts. Samples resuspended in loading buffer (100% Formamide: 50mM EDTA, 5:1) were heated to 95°C for 5 min before loading onto the gel. The gel was run overnight using the automatic data collection and analysis programs.

3.11 RNase protection assay

3.11.1 Preparation of radiolabelled riboprobes

Radiolabelled antisense RNAs were prepared as described in section 3.8.2. except that ^{32}P-UTP was used in place of ^{35}S-UTP. As a control, the 18S antisense RNA (Ambion) was prepared as a specific RNA probe. In order to purify only full length cRNA, radiolabelled cRNA was mixed with loading dye (6x = 0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol, 50mM EDTA), heated at 95°C for 3 min and loaded on a 6% denaturing polyacrylamide gel and separated by electrophoresis at

50W (constant power) for 2 h. The gel was wraped in cling film and exposed briefly (1-2 min) to autoradiographic film (XAR-5, Kodak), the segment of the gel containing the full length probe cut out and placed in an Eppendorf tube together with 800µl of elution buffer (0.5M NH_4Ac, 1mM EDTA, 0.1%SDS and 10µg/ml yeast tRNA). The tube was placed in a water bath at 37°C overnight. The next day, the tube was centrifuged for 5 min at 13,000 rpm and the supernatant transferred into a fresh tube. The eluted probe was precipitated in the presence of 0.3M sodium acetate pH 5.5 and absolute ethanol for 20 min at -20°C and centrifuged for 20 min (13,000 rpm, 4°C). The resulting pellet was washed with 75% ethanol, air dried, dissolved in 20µl ultrapure water and the activity of 2 x 1µl aliquots determined by liquid scintillation spectroscopy. The average activity of the two samples was calculated and the volume of probe necessary to give 5 x 10^5 cpm was determined.

3.11.2 RNase protection assay reaction

Total RNA extracted from fetal tissues was mixed with 5 x 10^5 cpm of radiolabelled cRNA, dried in a freeze drier, dissolved in 20µl of hybridisation buffer (80% deionised formamide, 40mM PIPES (pH 6.7), 0.4M NaCl, 1mM EDTA), heated to 85°C for 10 min and incubated overnight at 45-50°C. The next day, single stranded RNA (which had not hybridised to radiolabelled cRNA probe) was digested by adding 10-20µg/ml RNase A (Sigma) and 20-100U/ml RNase T1 (Boehringer) into the mixture which was incubated at 37°C for 45 min. Proteinase K (50µg per reaction) and 20µl 10% SDS were added and the tubes were incubated at 37°C for a further 15 min. Each complete reaction mixture was then added to an equal volume of phenol chloroform (1:1, pH 8.0), mixed thoroughly by vortexing and centrifuged at 13,000 rpm for 5 min. The upper aqueous layers were transferred to fresh tubes, precipitated in the presence of 0.3M sodium acetate and absolute ethanol for 20 min at -20°C and centrifuged at 13,000 rpm for 20 min at 4°C. The resultant pellet was washed with 75% ethanol and air dried. RNA was dissolved in 10µl of loading buffer (90% formamide, 10mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol) heated to 95°C for 3 min and loaded on a 6% denaturing polyacrylamide gel. The gel was run for 2 to 3 h at 50W before drying at 80°C for 2 h and either exposed to autoradiographic film (Kodak) or a phosphorescent screen for use with the phosphorimager (Molecular Dynamics).

3.11.3 Preparation of polyacrylamide gel

A polyacrylamide gel composed of 9% acrylamide (30% stock of acrylamide : bis acrylamide 19:1) and 1.5 x TBE in 12M urea was prepared on Biorad Sequi-Gen Sequencing apparatus. Two gel mixes were prepared containing the ingredients outlined below.

Reagent

	Top	Base
Urea	27.6g	18.4g
Acrylamide (30%)	12ml	8ml
10 x TBE	6ml	10ml
Water	20ml	4ml

Urea was melted by heating the mixes in the microwave for 2 min at 30% power prior to the addition of 100µl each of 10% ammonium persulphate and N,N,N',N'-tetramethylethylenediamine (TEMED) to the base mixture which was poured into the casting tray. The bottom of the assembled gel apparatus was placed in the casting tray and the base mix was allowed to set to seal the bottom of the gel plates.

The 'top' mix was filtered using a 0.45µM millipore filter connected to a syringe prior to the addition of ammonium persulphate and TEMED (60µl and 20µl respectively) after which the gel was poured immediately between the gel plates using a 25ml pipette. A gel comb containing flat-bottom wells was placed into the liquid gel between the gel plates at the top of the apparatus and the gel was left to polymerise at room temperature for approximately 1h. The comb was removed from the gel, the individual wells were washed out with 1 x TBE and the gel was prerun in 1xTBE at 1800 volts for 30 min prior to sample loading.

4. Histogenesis of the gonads

4.1 Introduction

The gonads develop from embryonic mesoderm within the abdominal cavity. Gonadal rudiments first appear as a bulge on the ventral side of the mesonephros due to a rapid proliferation of coelomic epithelium and the condensation of adjacent mesenchyme (reviewed by Byskov, 1986; Kaufman, 1992; Pelliniemi et al., 1993). This process is first evident on day 13.0-13.5 p.c. in the rat fetus (Jost et al., 1973; Magre and Jost, 1980; Satoh, 1985) and between the 6th and 7th week of gestation in the human (Gustafson and Donahoe, 1994). The gonads in both the male and female fetuses are initially histologically indistinguishable. Half a day later, on day 14.0 in the rat, epithelial strands extend into the underlying mesenchyme, surround the germ cells and form primitive sex cords in the gonad of the male fetus (Jost et al., 1973; Magre and Jost, 1980; Satoh, 1985). The same process is evident around day 42 of gestation in the human fetus (Gustafson and Donahoe, 1994; Jost et al., 1973). At this developmental stage it is possible to distinguish between the testis and the ovary under the light microscope. The ovary has a homogenous granular appearance, while the testis appears to contain wide regularly arranged bands of tissue known as testicular cords, which are destined to form the seminiferous tubules after birth (Gustafson and Donahoe, 1994; Jost et al., 1973; Magre and Jost, 1980; Mittwoch et al., 1969).

Sertoli cells develop from epithelial precursors, which surround the germ cells residing in the indifferent gonads (Byskov, 1986; Pelliniemi et al., 1993; Satoh, 1985). The morphological differentiation of Sertoli cells is simultaneously accompanied by their functional differentiation with AMH production already detectable in the fetal rat testis on day 14.0 (Lee et al., 1992). The germ cells are the only cells within the gonads that are not derived from the embryonic mesoderm but migrate to the gonads from extraembryonic mesoderm in the wall of the yolk sac (Chiquoine, 1954; Witschi, 1948). After entering the gonads, the germ cells undergo mitotic arrest in G1 phase and resume proliferation only after birth (Byskov, 1986; Vergouwen et al., 1991). The first Leydig cells can be distinguished in the fetal rat testis around day 15.5 (Magre and Jost, 1980; Saez, 1994) and in the human fetus during the eighth week of gestation (Huhtaniemi and Pelliniemi, 1992; Saez, 1994). Leydig cells are easily recognisable using the electron microscope due to the presence

of large mitochondria, lipid droplets and a large amount of smooth endoplasmic reticulum (Magre and Jost, 1980). However, they are not easily distinguishable from other interstitial cells under the light microscope without specific immunostaining. In the next few days as the fetal development proceeds, the testis and the ovary become easier to distinguish morphologically and the testes grow faster. At day 16.5 in the rat, testis and ovary each have a distinct gross histological appearance with the testis appearing as a bigger and fairly ovoid structure while the ovary is smaller and has a much more elongated shape, with its craniocaudal axis being almost three times as long as it is wide (Kaufman, 1992; Mittwoch et al., 1969). At this time point, the testis begin their descent in a caudoventral direction while the ovaries remain in their original place lateral to the kidneys (Kaufman, 1992).

In addition to the Sertoli, Leydig and germ cells, other types of cells are also present in the fetal testis (Pelliniemi et al., 1993). The function of these cell types is mostly unknown but at least some interstitial cells are most likely to be precursors of Leydig cells (Hardy et al., 1989). Peritubular cells surrounding the testicular cords contribute to the organisation of the testis by synthesising extracellular matrix components (Pelliniemi et al., 1993). Some studies have suggested that cells from the mesonephric area migrate into the testis and experiments by Buehr and co-workers (Buehr et al., 1993) have shown the importance of this migration for normal testicular development. In the absence of an influx of cells from the mesonephros, normal differentiation of the testicular cords did not occur, consistent with a role for peritubular cells in the organisation of the testis (Buehr et al., 1993).

Phagocytic cells in the fetal testis appear last of all the cell types. In rat, these cells differentiate around day 19.5 p.c. (Hutson, 1990) and in humans between 16 and 20 weeks (Dechelotte et al., 1989). The most probable precursor for these phagocytic cells are testicular mesenchymal cells although a hematogenous origin for some macrophages cannot be ruled out (Pelliniemi et al., 1993).

Immediately after birth, the rat testis still retains the same gross histological appearance as it did during fetal life. A few days after birth, the prespermatogonia resume mitosis and on day 4 postnatally, a few type A spermatogonia can be distinguished (Clermont and Perey, 1957; Vergouwen et al., 1991). In the following days, the spermatogonia as well as the Sertoli cells actively proliferate and on day 9 the first primary spermatocytes appear within the tubules (Clermont and Perey, 1957). In the rat on day 15 the first zygotene and pachytene spermatocytes are present in the

tubules (Clermont and Perey, 1957) and around days 16-18 a lumen is formed within the testicular cords/seminiferous tubules (Russell et al., 1989). While the spermatogonia continue to divide, the proliferation of Sertoli cells starts to decline and by day 18 no mitotic figures or uptake of (^{3}H)thymidine can be seen in Sertoli cells (Nagy, 1972; Orth, 1982; Vergouwen et al., 1991). In the subsequent days of life, the population of tubules by germ cells continues and by day 26 the first spermatids appear in some of the tubules with the first fully mature sperm detectable around day 45 (Clermont and Perey, 1957). Fetal-type Leydig cells persist in the rat testis for several days after birth, noticeable by their distinctive appearance in clusters (Saez, 1994). Between days 10 and 15 postnatally, the number of fetal Leydig cells declines and for a short period only a few steroidogenic cells can be observed in the testis (Lording and De Kretser, 1972). However, after day 15, the testis is slowly re-populated with adult type Leydig cells scattered between the tubules (Lording and De Kretser, 1972). In humans, the number of Leydig cells increases immediately after birth and reaches a second peak accompanied by a peak in testosterone production around 2 months of age (Codesal et al., 1990; Forest et al., 1973; Prince, 1990). Thereafter as in rat testis, the number of Leydig cells decreases and very few Leydig cells remain in the testis by the end of the first year of life (Codesal et al., 1990; Nistal and Paniagua, 1979). Similar to rat, the adult Leydig cells then start to repopulate the testis and are again numerous during puberty (Nistal and Paniagua, 1979).

The aim of this chapter is to give basic histological information on gonadal development in the rat as a background to the studies described in the rest of the thesis.

4.2 Experimental procedures

4.2.1 Collection and processing of tissues

Fetuses were collected, dissected, fixed and processed as described in Chapter 3. Tissue sections were cut and stained with H&E following the protocol described in sections 3.4.2 and 3.4.3.

4.2.2 Immunocytochemical staining

For examination of different cell types within the testis, immunocytochemical staining using different antibodies were used. Antibodies against AMH (donated by Dr.

Rudolfo Rey and Dr. Nathalie Josso, Montrouge, France) were used to visualise Sertoli cells, antibodies against 3ß-HSD (donated by Professor Ian Mason, University of Edinburgh, UK) were used as a marker for steroidogenic Leydig cells and antibodies against proliferating cell nuclear antigen (PCNA, purchased from Dako) were used as a marker for mitotically active (proliferating) cells.

Immunocytochemical stainings were performed as described in chapter 3. Polyclonal antibodies raised in rabbits against human AMH were used in dilution 1:500; antigen retrieval was necessary for successful staining (section 3.5.1). Bound antibodies were detected using swine anti-rabbit biotinylated immunoglobulins (Dako) at a dilution of 1:500 incubated for 30 min at room temperature. Two washes in TBS were followed by incubation with ABC-alkaline phosphatase complex (Dako) for 30 min at room temperature. After two further washes in TBS, sections were washed in Tris-Mg buffer (100mM Tris-HCL pH 9.5, 100mM NaCl, 50mM $MgCl_2$) for 5 min. Colour reaction product was developed by incubation in a solution containing 337.5 µg/ml nitro-blue tetrazolium, 175 µg/ml 5-bromo-4-chloro-3-indolyphosphate and 1mM levamisol in Tris-Mg buffer for 15-30 min. After a wash in distilled water, sections were counterstained with haematoxylin, dehydrated in alcohols, cleared in xylene and coverslipped using Pertex mounting medium (Cellpath).

Polyclonal antibodies raised in rabbit against human 3ß-HSD were used in dilution 1:1000 and incubated on sections overnight at 4°C. The following day, the sections were washed twice in TBS, incubated with biotinylated swine anti-rabbit immunoglobulins (Dako) for 30 min at room temperature, washed twice in TBS and incubated with ABC-horseradish peroxidase complex (Dako) for 30 min at room temperature. After two washes in TBS, colour reaction product was developed by incubating sections in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. After 5-15 min, sections were washed in water, counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (Cell path).

Proliferating cell nuclear antigen (PCNA) is a marker of dividing cells. It functions as an auxiliary protein to DNA polymerase delta (Bravo et al., 1987) and is strongly expressed in the cells during G1 and S phase of the cell cycle and weaker during M phase (Kurki et al., 1986). Monoclonal antibodies raised in mice against PCNA were used at a dilution of 1:1000 with biotinylated rabbit-antimouse secondary antibodies

(1:500) and ABC-horseradish peroxidase complex with DAB as a substrate for visualisation of bound antibodies as described for anti-3ß-HSD antibodies.

The specificity of the specific antibodies was checked by replacing them with normal serum from the same animal species from which the primary antibodies derived (section 3.5.2). None of these controls resulted in any signal above background.

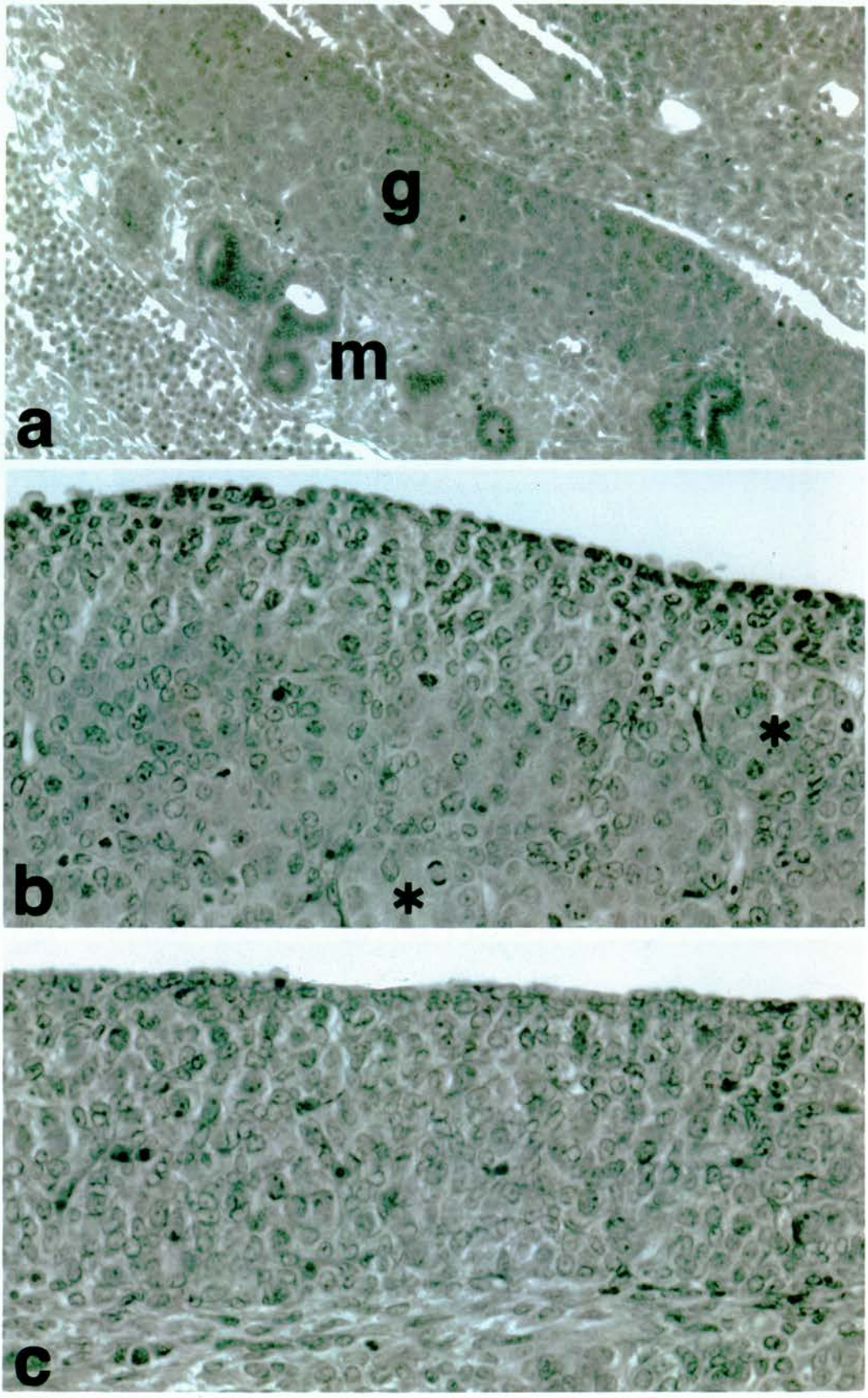
Sections were analysed using an Olympus Provis microscope and images captured onto computer using Nikon N90 camera with a Kodak DC digitalizer.

4.3 Results

4.3.1 General morphological appearance

Rat fetuses of different ages from day 11.5 until birth were fixed, sectioned and examined. Staining of sections with haematoxylin and eosin showed a well preserved tissue throughout the fetus which contained the expected tissue structures. There was no obvious gonadal ridge in the 12.5 p.c. old fetuses but at day 13.5, the gonadal tissue could first be identified (Fig. 4.1a). On saggital sections, the streak of the tissue lying ventromedial on the mesonephros was observed. At this age in the rat, the gonadal ridge is similar in appearance in both male and female and the gonads are indistinguishable under the microscope. Half a day later (day 14.0), the genital ridge became more prominent and for the first time, the testis could be distinguished from the ovary by its "stripy" appearance, resulting from organisation of testicular cords by Sertoli cells, which become more prominent on day 14.5 (Fig. 4.1b, c). At that age, the ovaries and testis are still similar in shape and size.

Figure 4.1: The genital ridges (g) in the rat fetus can first be identified on day 13.5 beside the mesonephros (m; panel a), however, at this time the male and female gonads are histologically indistinguishable. One day later, on day 14.5 (b), testicular cords formed by the newly developed Sertoli cells can be seen (asterisk) and the testis can be distinguished from the ovary (c) for the first time. Magnification x300 (a); x400 (b,c).


g
m
a
b
*
*
c

By day 15.5, the testicular cords within the testis were further developed and it was easy to distinguish the testis from the ovary. The difference in the size and shape of the gonads started to become obvious on day 15.5 and became more obvious on subsequent days; the testes were bigger and more ovoid in shape while the ovary remained elongated with its length being about three times that of its width (day 17.5; Fig. 4.2). Similarly, the position of the gonads in relation to other organs started to change on or about day 15.5/16.5. Whilst the ovary remained in its original position on the lateral side of the kidney, the testes started to descend ventrocaudally. On day 17.5 a change in the histological appearance of the ovary was apparent and small sexual cords, formed by theca cells surrounding germ cells, could be distinguished (Fig. 4.2b, arrowheads). However, these "cords" are much smaller and more evenly distributed throughout the ovary than the seminiferous cords of the testis (Fig. 4.2a, asterisks). For the remainder of gestation the histological appearance of the gonads remained similar to that on day 17.5 while the position of the testes within the abdominal cavity continued to change as they moved more ventrocaudally (Fig. 4.3). After birth, the cords within the testis gradually increased in size mainly due to a proliferation of germ cells which began to occupy a greater volume as development of the first wave of spermatogenesis commences. There was also an obvious reduction in the volume of the interstitial compartment (Fig. 4.4a) compared with that seen in fetal life (Fig. 4.2a). Sertoli cells cease to proliferate on day 18 (see section 4.3.4) and at around that age, a lumen appeared within the seminiferous cords (Fig. 4.4b). Postnatally, the germ cells that had previously resided in the centre of the testicular cords moved to their periphery and resumed mitosis (Fig. 4.4a). The prespermatogonia differentiated into type A spermatogonia and thereafter the different types of germ cells could be distinguished. The first pachytene spermatocytes were observed on day 15 (Fig. 4.5a, arrows), the first round spermatids around day 25 and the first mature elongate spermatids around day 45 (Fig. 4.5b).

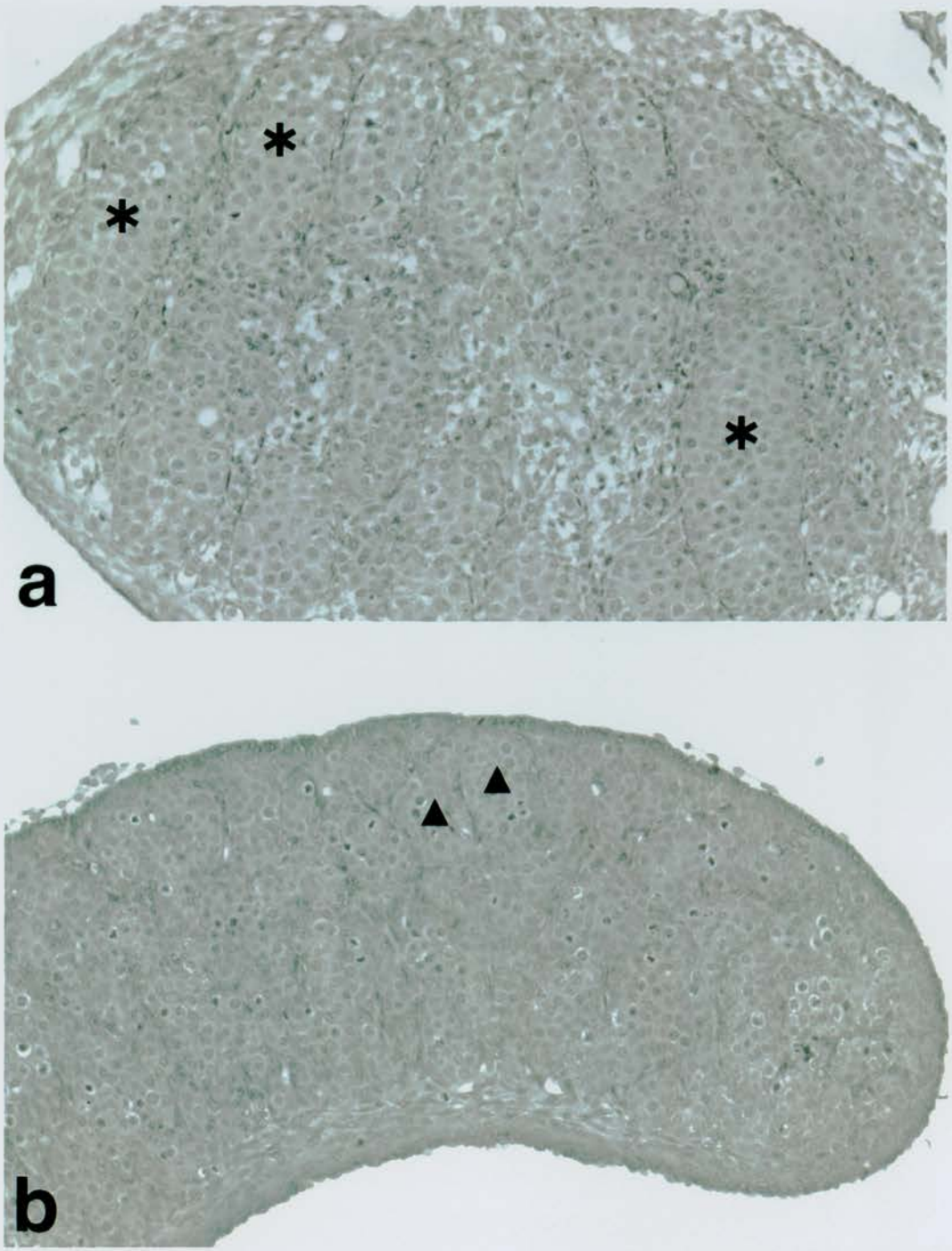


Figure 4.2: On day 17.5, the testis (a) could be easily distinguished from the ovary by its shape and the presence of prominent testicular cords (asterisks). At around this time, small sexual cords (triangles) are formed within the ovary (b), however, they are much smaller than the testicular cords. The shape of both organs is also obviously different at that age with the ovary having a much more elongated shape compared to the testis (compare b with a). Magnification x200.

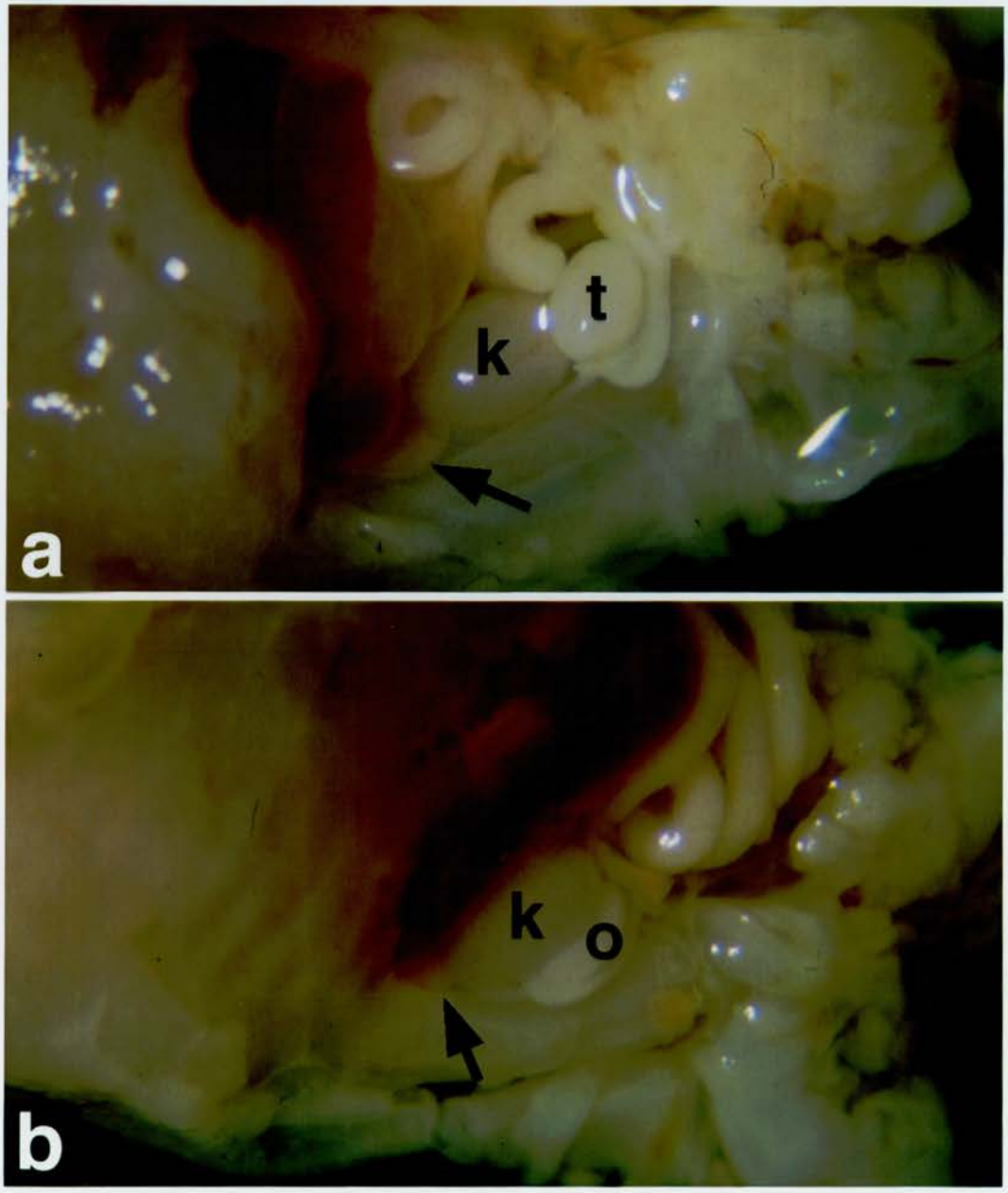


Figure 4.3: From day 15.5 onwards the testes gradually move ventrocaudally and on day 17.5 the testis (t; panel a), which is bigger than the ovary (o; panel b) lies more caudally than the ovary which remains in its original position close to the kidney (k) and adrenal gland (arrow).

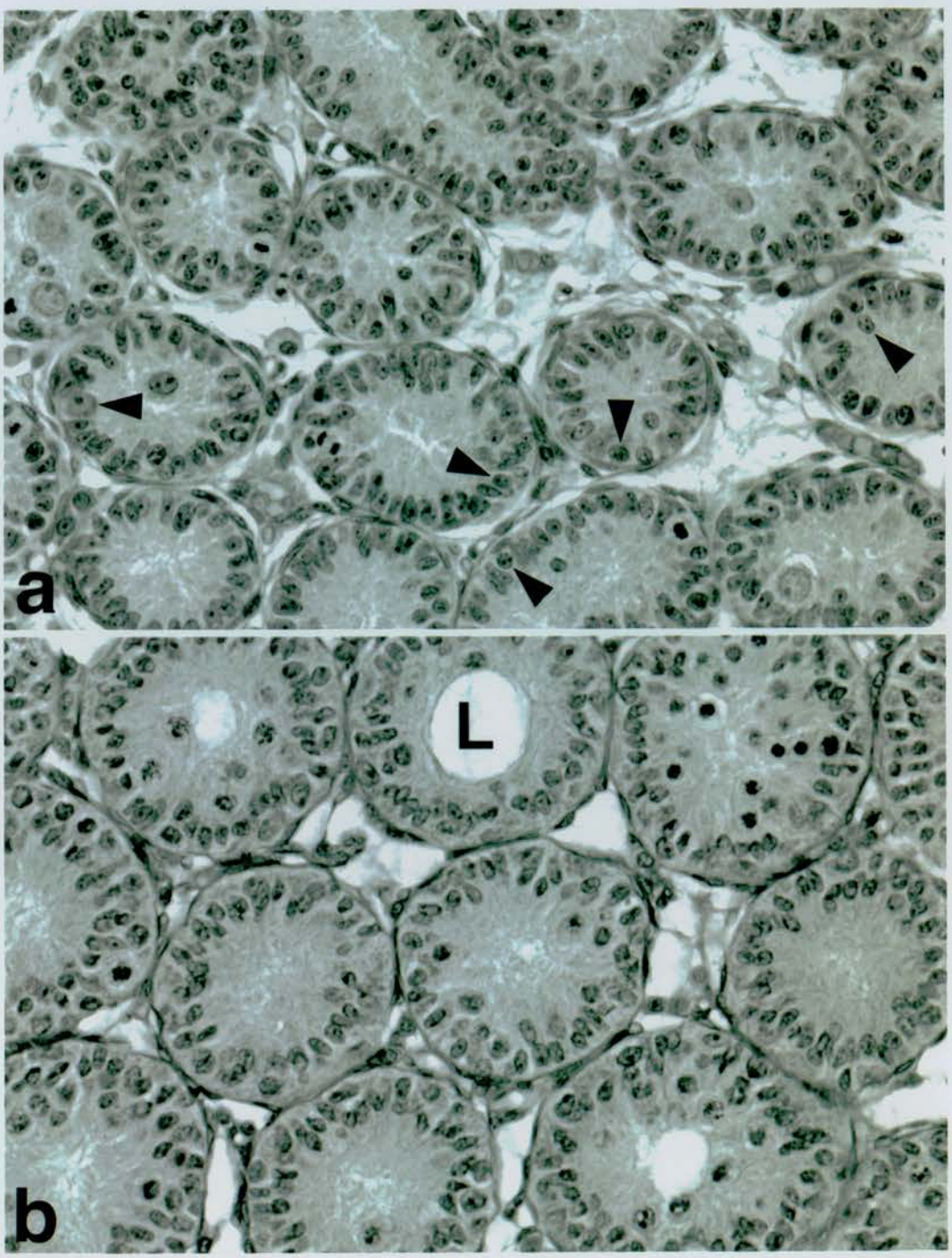


Figure 4.4: By day 7 after birth (a) the germ cells (arrowheads) within the testis resume mitosis and move to the periphery of the cords. The lumen (L) within the testicular cords first appears around day 16 (b). Magnification x300.

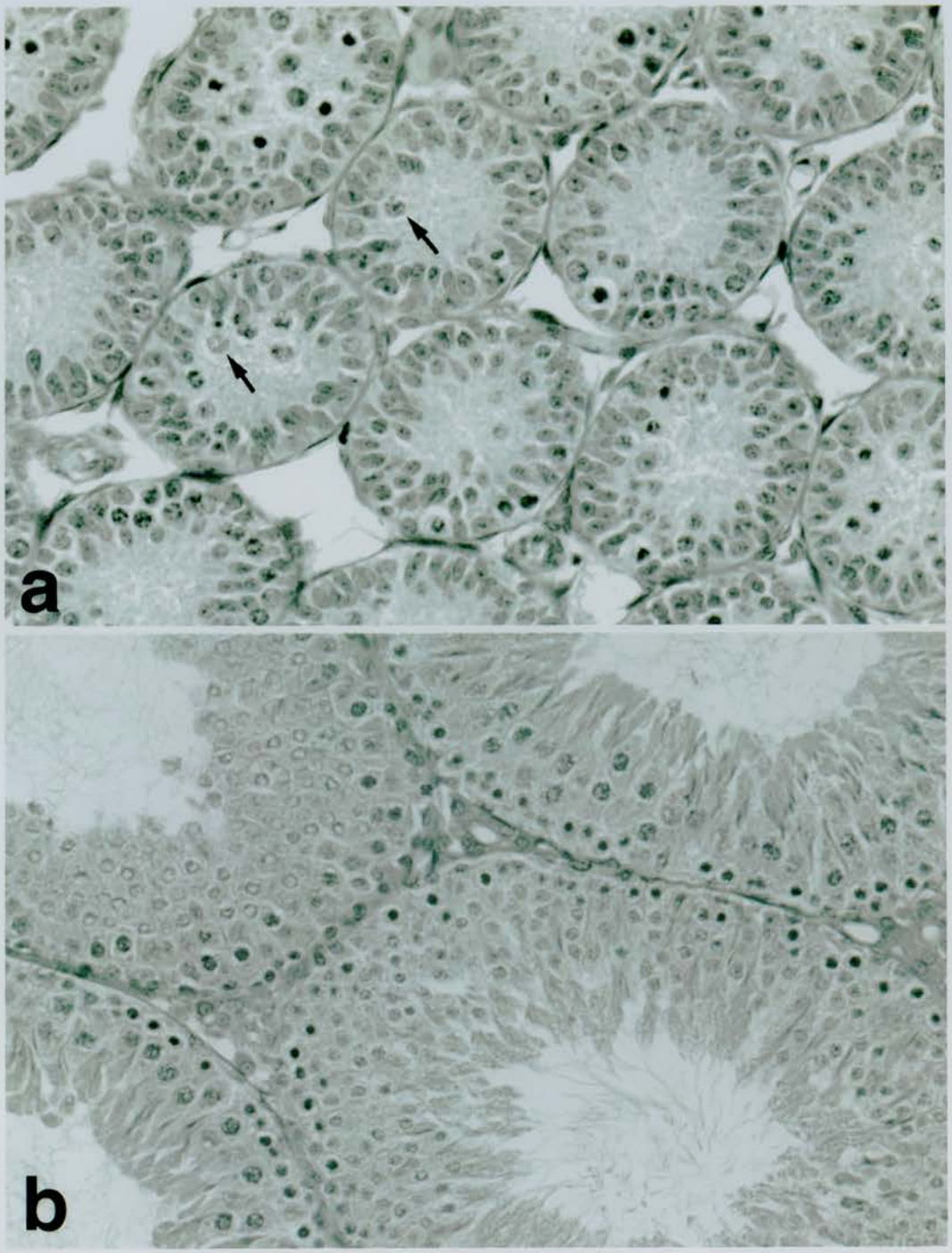


Figure 4.5: Postnatally the seminiferous tubules become larger as they are populated by successive generations of germ cells. The first pachytene spermatocytes (arrows) appear around day 15 (a), before the lumen is formed within the cords. The first wave of spermatogenesis is complete by day 45 at which time the first mature elongate spermatids can be distinguished (c, day 48). Magnification x300.

4.3.2 Staining with antibodies raised against AMH

Immunocytochemical staining with anti-AMH antibodies resulted in a strong immunopositive signal within the testicular cords which was confined to the cytoplasm of Sertoli cells. Positive immunostaining was first detected on day 14.0 (Fig. 4.6a), coincident with formation of testicular cords. The immunopositive staining remained strong throughout fetal life (Fig. 4.6a, b). Shortly after birth AMH immunostaining declined in the testis and by postnatal day 10 AMH was no longer detectable using immunocytochemistry.

4.3.3 Staining with antibodies against 3ß-HSD

3ß-HSD is an enzyme involved in steroidogenesis. Staining of fetal testis using antibodies against this enzyme resulted in cytoplasmic staining exclusively in some interstitial cells. Immunostaining was first apparent on day 14.5 p.c., however, on that day only a few cells were weakly immunopositive (Fig. 4.7a). One day later (15.5), the staining became much more prominent (Fig. 4.7b) and stayed strong throughout the remainder of fetal life (Fig. 4.7c) and for several days after birth (Fig. 4.7d). Within the interstitial compartment, only a subpopulation of interstitial cells within the fetal testis were immunopositive with anti-3ß-HSD antibodies. The role of this enzyme in steroidogenesis led to the conclusion that these were fetal type Leydig cells which are the site of testosterone biosynthesis but no conclusion can be drawn about the identity or function of the immunonegative interstitial cells. The immunostaining remained strong in clusters of Leydig cells after birth. Around day 10, the fetal type Leydig cells started to disappear and for several days between day 10 and 15, very few immunopositive cells were observed (Fig. 4.8a). After day 15, the numbers of immunopositive cells once more increased, however, an obvious difference in the distribution of these cells compared with younger ages was observed; immunopositive cells before postnatal day 10 were mostly grouped into clusters, however after day 15 immunopositive cells were scattered between the tubules (Fig. 4.8b).

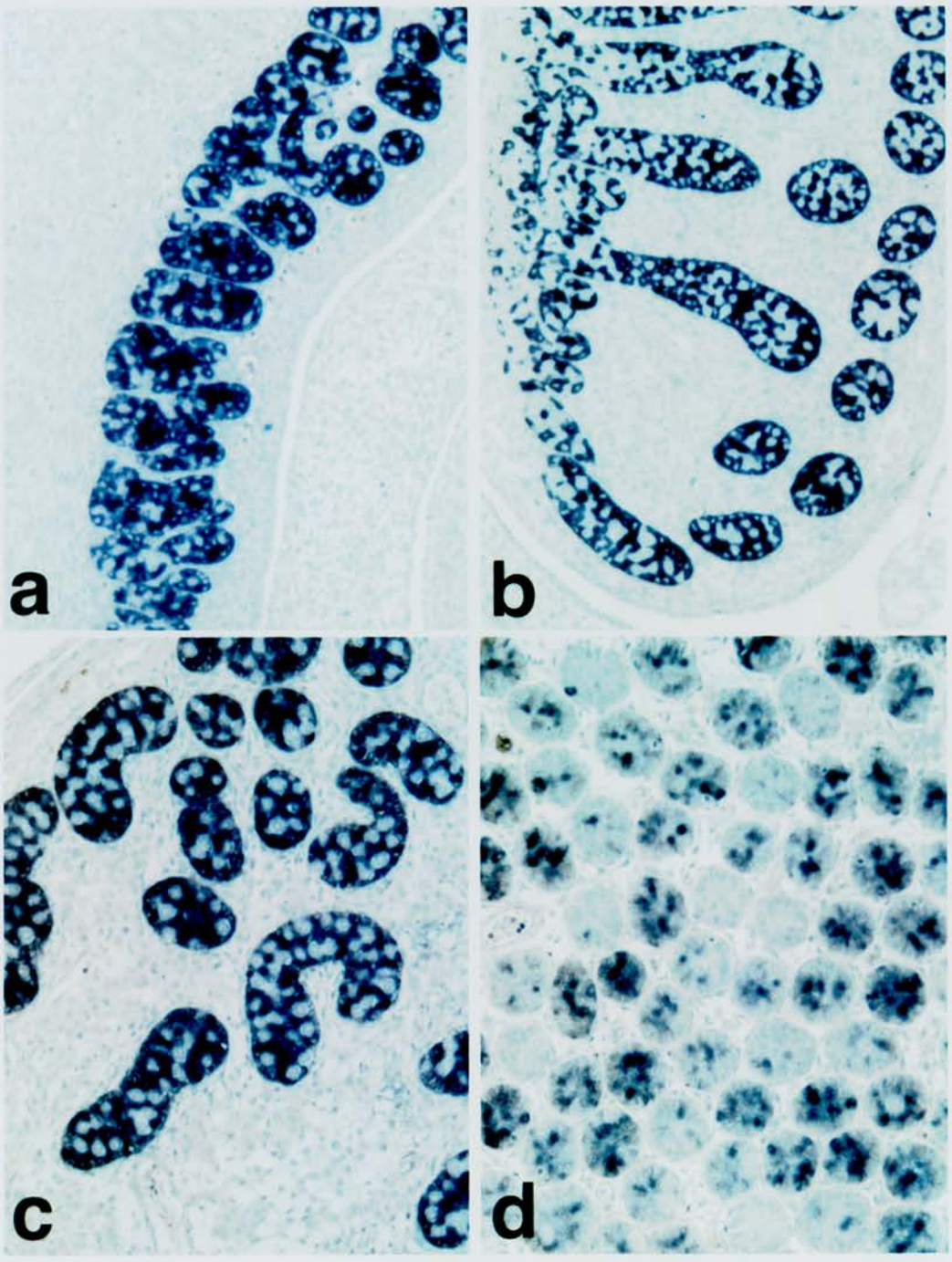
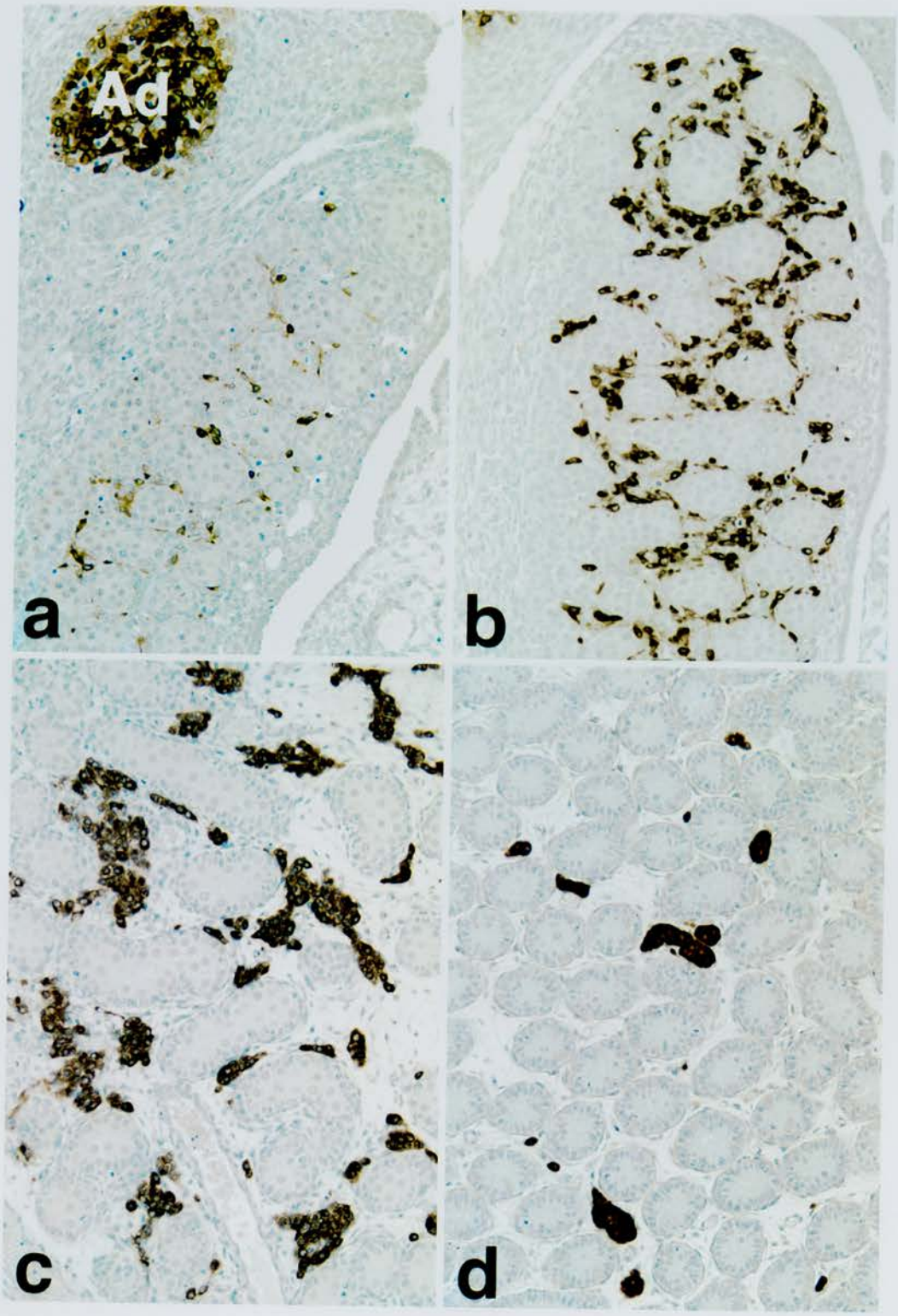


Figure 4.6: In the rat morphological and functional maturation of the Sertoli cells is a simultaneous process and positive immunostaining with antibodies raised against AMH was already detectable on day 14.0 (a). Immunostaining for AMH remained strong throughout fetal life (b, day 17.5; c, day 20.5) but started to decline shortly after birth (d, day 7 postnatally). Magnification x200.

Figure 4.7: Formation of Sertoli cells is followed by differentiation of steroidogenic Leydig cells within the interstitial compartment; few cells expressing 3ß-HSD can first be detected in the testis on day 14.5 (a), although at this age the adrenal gland (Ad) is strongly immunopositive. The numbers of immunopositive cells within the interstitial compartment of the testis had increased one day later (b, day 15.5) and thereafter immunopositive clusters of fetal-type Leydig cells could be identified throughout fetal life (c, day 20.5) and after birth (d, day 7 postnatal). Magnification x200.


Ad
a
b
c
d

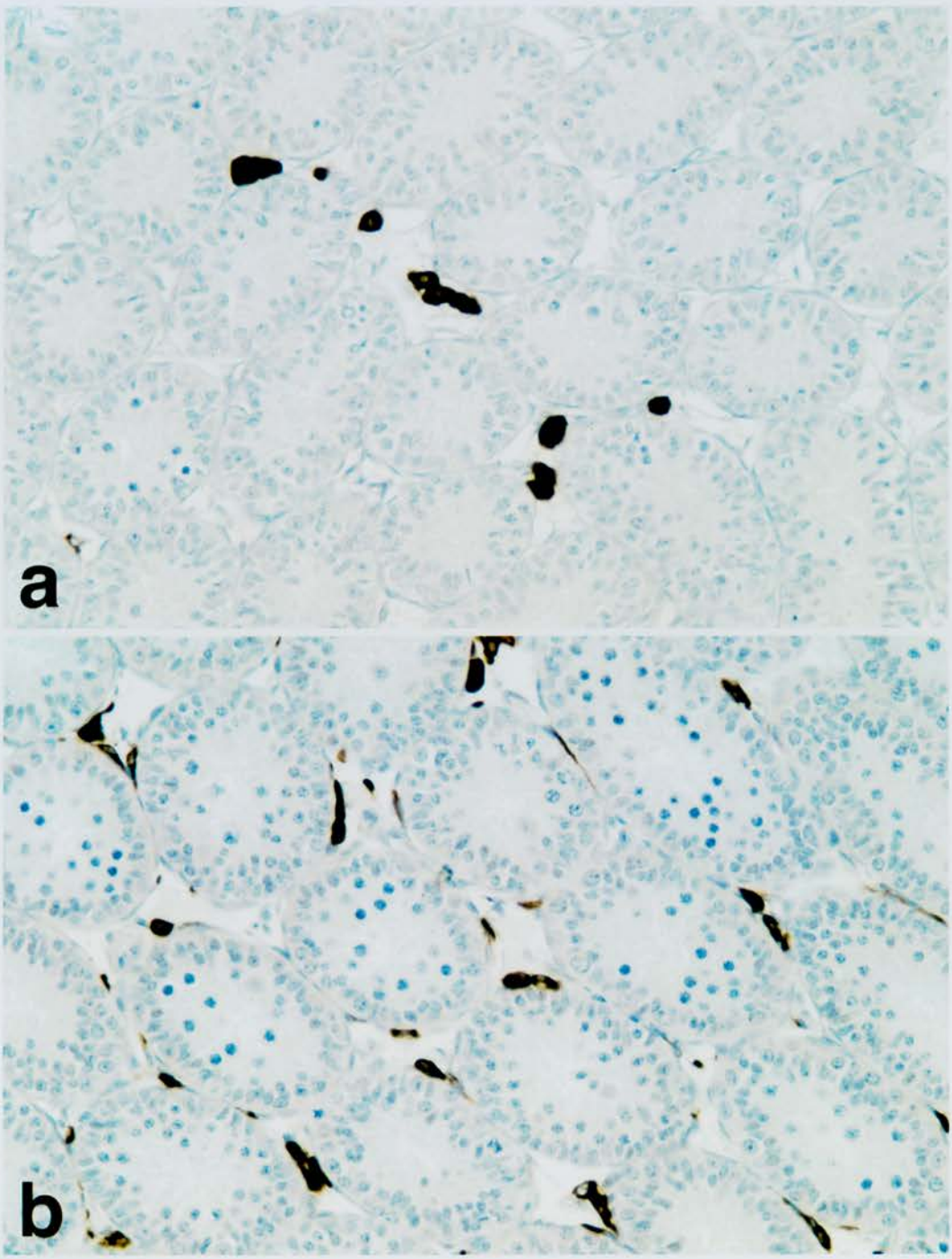


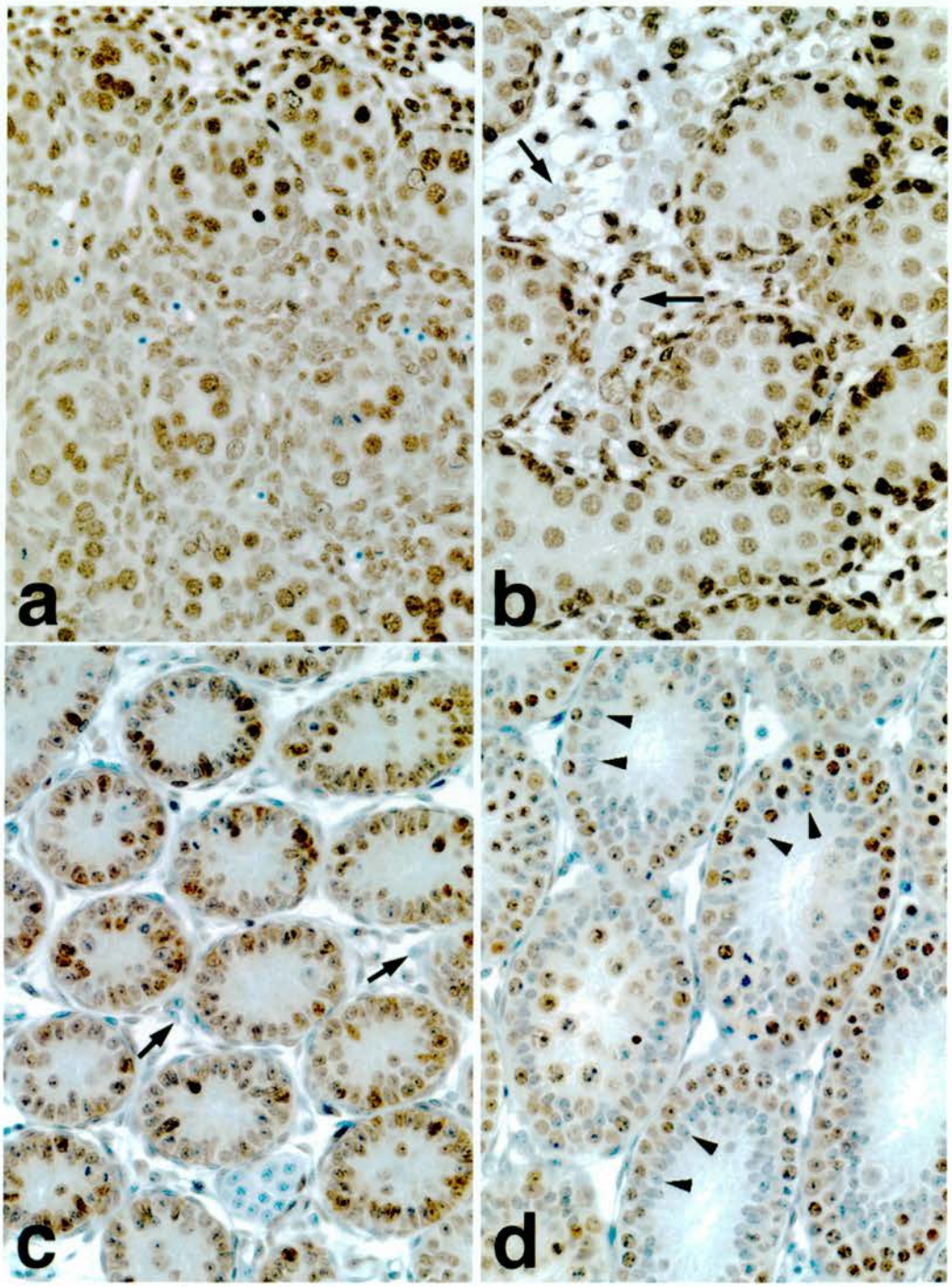
Figure 4.8: After the first week of life the numbers of 3ß-HSD immunopositive fetal-type Leydig cells reduced progressively (a, day 11) and thereafter were replaced slowly by adult-type Leydig cells (b, day 16) that were scattered between the tubules. Magnification x400.

4.3.4 Staining with antibodies against PCNA

Immunocytochemical staining using antibodies specific for PCNA resulted in strong nuclear staining in the testis, ovary and surrounding areas throughout fetal life. As expected, all cells in the fetal testis on day 15.5 strongly expressed PCNA (Fig. 4.9a), and a similar pattern was observed throughout fetal development with the exception of some interstitial cells which appeared to be immunonegative (Fig. 4.9b, arrows).

For several days after birth the majority of the cells within the testis were immunopositive (Fig. 4.9c) with the exception of some interstitial cells which were immunonegative. Immunostaining of Sertoli cells was reduced from day 15 onwards and by day 20 no Sertoli cell nuclei with positive immunostaining were detectable (Fig. 4.9d).

Figure 4.9: Staining with antibodies raised against PCNA revealed that the majority of cells were dividing at the earlier stages of testicular development (a, day 15.5), and the situation remained similar throughout fetal development with the exception of some interstitial cells which appear immunonegative (arrows; b, day 17.5). For several days after birth the majority of cells in the testis expressed PCNA with the exception of some interstitial cells (c, day 7 postnatally). However, thereafter the numbers of Sertoli cells proliferating starts to decline and on day 18 (d) Sertoli cells (arrowheads) were no longer immunopositive for PCNA. Magnification x400.


a
b
c
d

4.4 Discussion

As previously described, the gonadal rudiments are reported to appear in the rat fetus around day 13.0-13.5 of gestation as a thickening of the tissue on the ventral side of the mesonephros (Mittwoch et al., 1969; Satoh, 1985). Our results do not differ from those previously described and we first noticed the presence of an obvious gonadal ridge in 13.5 day old fetuses. At this age, it is impossible to distinguish the testis from the ovary following staining with H&E. However, this observation does not exclude the probability that the ovaries and testis already differ at this age at the molecular level. Experiments in the mouse have demonstrated that the mRNA for the testis determining factor, Sry, is present in male genital ridges prior to a time when they are morphologically distinguishable from those of females (Jeske et al., 1995; Koopman et al., 1990). Following the formation of the genital ridge, differentiation of the testis is swift and within half a day, the formation of the testicular cords containing Sertoli cells is evident (Kaufman, 1992; Magre and Jost, 1980; Mittwoch et al., 1969). Initially the Sertoli cells differentiate by mesenchymal to epithelial cell transformation, a process the regulation of which is not yet fully understood. Some studies have suggested that the Wilms' tumour (WT1) gene product could be involved in this process since in mice lacking a functional WT1 gene testicular cords fail to develop and the gonadal rudiments subsequently regress (Kreidberg et al., 1993). Recently, another gene called SOX9 has been implicated in this process (Foster et al., 1994; Wagner et al., 1994; Wright et al., 1995). Mutations in the SOX9 gene are responsible for development of a pathological condition called campomelic dysplasia, which is associated with failure of differentiation of cartilaginous tissue resulting in severe abnormalities in the skeletal system which are usually fatal soon after birth (Foster et al., 1994; Wagner et al., 1994). Notably, campomelic dysplasia is often associated with 46,XY male to female sex reversal. Development of cartilaginous tissue requires transformation of mesenchyme suggesting that SOX9 may be essential for this process. The suggestion that SOX9 may also be involved in mesenchymal differentiation in the testis is further supported by its pattern of expression in the fetal gonads. SOX9 is expressed in the gonadal ridge before sexual differentiation (Wright et al., 1995); thereafter SOX9 continues to be expressed in the Sertoli cells within the fetal testis but is switched off in the ovary (R. Lovell-Badge, personal communication). Whilst its expression in the testis throughout development suggests that it may have additional functions, it is possible that SOX9 is involved in the transformation of Sertoli cells from their precursors.

From day 15.5 onwards, the size of the gonads increases rapidly and the difference between testis and ovary becomes more obvious. The data described in this chapter show very similar results to those described previously for mice and rats regarding the size and morphological appearance of both gonads (Kaufman, 1992; Magre and Jost, 1980; Mittwoch et al., 1969).

AMH is reported to be one of the earliest protein products synthesised by differentiated Sertoli cells (Lee et al., 1992; Munsterberg and Lovell-Badge, 1991). Antibodies directed against AMH were used successfully to demonstrate the development and organisation of Sertoli cells within the testicular cords. Positive immunostaining was first detected on day 14.0, coinciding with the morphological differentiation of recognisable testicular cords, confirming that morphological and functional differentiation of Sertoli cells in the rat proceeds simultaneously. Immunostaining for AMH in Sertoli cells remained strong throughout fetal life in the rat, a finding consistent with the important role of AMH in inducing regression of the Müllerian ducts (Josso and Picard, 1986; Lee and Donahoe, 1993). However, as the regression of the Müllerian ducts is mostly complete by day 18.5 in the rat fetus, strong expression of AMH in the testis after that age would suggest additional functions for this hormone. In male fetuses in the absence of production or function of AMH the Müllerian ducts persist, as illustrated by the human pathological condition, Persistent Müllerian Ducts Syndrome (Lee and Donahoe, 1993). Male patients suffering from this condition develop both male and female internal reproductive organs due either to lack of bioactive AMH (Knebelmann et al., 1991; Lee and Donahoe, 1993) or mutations in the AMH receptor preventing regression of Müllerian ducts during fetal life (Lee and Donahoe, 1993).

Positive immunostaining with 3ß-HSD antibodies consistent with development of functional Leydig cells was first detectable on day 14.5 in male rat fetuses where it was confined to a very few interstitial cells. This result is surprising since previous studies have reported the appearance of morphologically typical steroidogenic cells only on day 15.5 (Magre and Jost, 1980), the first day on which testosterone production has been detected (Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973). However, Bloch (1979) has reported the presence of 3ß-HSD enzyme activity on day 14.5 in the rat testis, suggesting that functional differentiation of the Leydig cells starts before their morphological differentiation. The importance of this finding is not known, however, it is possible that Leydig cell precursors slowly acquire steroidogenic capacity and the expression of some of the enzymes within the cascade

starts a little earlier than the capacity to synthesise testosterone. This hypothesis is supported by studies on expression of P450c17 which was found to be detectable only from day 15.5 (Chapter 6).

Immediately after birth, the overall appearance of the testis remains unchanged for the first week of life, however, the testicular cords grow fast resulting in a reduction in the proportion of the testis occupied by the interstitial compartment. By day 10, the immature testis appears similar to the adult testis with small interstitial spaces between the tubules. In accordance with other reports, the lumen in the cords appear on or around day 16-18 postnatally (Russell et al., 1989) and the data gathered from animals examined during the course of the present study agrees with this.

Immunostaining using antibodies directed against PCNA resulted in strong uniform, nuclear staining in the majority of cells in the fetal testis throughout development. This result was surprising since prespermatogonia are reported to be quiescent during fetal development following their initial proliferation on or about day 14.5 (Byskov, 1986; Vergouwen et al., 1991). However, these cells are reported to be arrested in the G1 phase of the cell cycle and as previous studies have demonstrated the expression of PCNA at this stage (Kurki et al., 1986), it is possible that PCNA is present in the prespermatogonia arrested in G1. The only cells in the fetal testis that did not contain detectable PCNA were some of the interstitial cells. It has been suggested that functional Leydig cells do not proliferate but are formed by differentiation from precursors and it is possible that the immunonegative cells are functional, non-proliferative Leydig cells.

Immediately after birth, the pattern of immunocytochemical staining using antiserum directed against PCNA showed a strong positive reaction in most cells within the testis. However, as development proceeded, the immunostaining of Sertoli cells was reduced such that by day 18 Sertoli cells were all immunonegative. This finding is in accordance with other reports, claiming that in the rat and mouse Sertoli cells divide only during fetal life and for short time after birth, after which they cease to divide and their numbers remain fixed throughout life (Kluin et al., 1984; Nagy, 1972; Orth, 1982; Sharpe, 1994). While the same appears to be true for most species, horses appear to be an exception with several reports suggesting that their Sertoli cells retain proliferative activity throughout life with seasonal changes in cell numbers (Johnson et al., 1991; Johnson and Nguyen, 1986). The available, but limited evidence, suggests that in primates (e.g. Cebus monkey and human) Sertoli cell proliferation continues at

a low rate until puberty (Cortes et al., 1987; Rey et al., 1993) and studies by Schlatt and Weinbauer (1994) have detected some PCNA immunopositive Sertoli cells in testis from prepubertal Rhesus monkeys. It is possible that the specialised function(s) of Sertoli cells in the adult testis which are essential for maintenance of germ cell development are incompatible with proliferative activity. At about the same time as the Sertoli cells differentiated and stopped dividing they also stopped expressing AMH. In the present study immunostaining for AMH was still present on day 7 after birth although it was already much weaker than during fetal life and by day 10 postnatally it had completely disappeared.

For the first few days after birth, the Leydig cells appeared similar in distribution and appearance to those in the fetal testis; the cells were organised in clusters and strongly expressed steroidogenic enzymes. However, thereafter, in the rat the number of Leydig cells in the testis starts to decline so that between day 10 and 15 postnatally, only a few cells were immunopositive for 3ß-HSD. After day 15 the numbers of steroidogenic Leydig cells increased but these cells remained scattered between the tubules and did not aggregate into clusters, as had typically been seen during fetal life. These adult-type Leydig cells seem to differ from their fetal counterparts not only in appearance but also functionally. Whilst fetal Leydig cells do not express androgen receptor suggesting they are not responsive to the action of testosterone (Chapter 5), they contain inhibin α and ß subunits (Chapter 7) while in the adult the Leydig cells contain only very low levels of these proteins (Roberts et al., 1989; Shaha et al., 1989). The morphological and physiological differences between fetal and adult type Leydig cells, confirmed by the findings in this thesis support studies that suggest adult Leydig cells are not formed from fetal type Leydig cells but represent a completely different population of steroidogenic interstitial cells which develop independently from mesenchymal precursors.

The appearance of the testicular cords starts to change soon after birth; the prespermatogonia differentiate to spermatogonia, migrate to the periphery of the tubules and resume cell division whilst Sertoli cells stop dividing (Vergouwen et al., 1991). As germ cell division and differentiation proceeds, the new types of cells start to populate the testis (Clermont and Perey, 1957). During this initial wave of spermatogenesis, many of the developing germ cells within the seminiferous tubule degenerate (Roosen-Runge and Leik, 1968). It is possible that this is because the Sertoli cells are still developing and have not yet acquired all the functions necessary to fully support and nourish the developing germ cells. As germ cells numbers

increase there is a dramatic increase in testicular size despite the presence of a fixed number of Sertoli cells.

In conclusion, the data described in this chapter generally agree with previously published observations and provide useful background information for the following chapters.

5. Ontogeny of androgen receptor in the fetal testis and associated ducts

5.1 Introduction

Androgens are essential for masculinisation of the reproductive tract and external genitalia during development in utero (George and Wilson, 1994). Androgen action is mediated by a specific intracellular receptor expressed in target tissues which binds to testosterone and dihydrotestosterone with high affinity (Carson-Jurnica et al., 1990). The androgen receptor is a member of the nuclear receptor superfamily (Carson-Jurnica et al., 1990; Rundlett et al., 1990). Deletion mutagenesis has identified three important domains within its structure, the carboxy terminal domain responsible for hormone binding, a cysteine rich DNA binding domain and an amino terminus which contains sequences essential for transcriptional activation (Rundlett et al., 1990; van Steensel et al., 1995). Upon binding with androgens, the conformation of the receptor changes and the hormone-receptor complex becomes able to bind to specific regions of DNA (androgen response elements) resulting in up or down regulation of the transcription of target genes (Parker, 1991; Rundlett et al., 1990).

The importance of a functional androgen receptor for development of the male reproductive tract is clearly illustrated by the condition known as androgen insensitivity syndrome (AIS; Quigley et al., 1995). Patients suffering from this disorder develop testes and produce androgens, however mutations in the gene encoding androgen receptor which make the receptor totally or partially non-functional prevent the normal action of androgens on their target tissue. The consequence of this is the incomplete masculinisation of the fetus and in extreme cases, when a functional AR is absent, these patients develop female external genitalia despite the presence of TDF and a developed testis. The phenotype of AIS patients varies greatly depending upon the nature of the mutation in their AR and consequently the degree by which the function of the AR is impaired (Quigley et al., 1995).

The location of the androgen receptor in fetal mouse reproductive organs has been studied by several groups (Bentvelsen et al., 1995; Cooke et al., 1991a; Kalloo et al., 1993) who have used autoradiography or immunocytochemistry to document the ontogeny of AR in reproductive tissues. In the rat fetus Bentvelsen et al. (1995)

recorded that the first immunoexpression of AR occurred on day 14.5 in the efferent ductules and epididymis, thereafter in the vas deferens and finally in the seminal vesicle. The first AR immunopositive cells in the prostate were detected on day 16.5 while mesenchymal cells of the urogenital tubercule expressed AR from day 14.5 onwards (Bentvelsen et al., 1995). Cooke et al. (1991a) have shown similar results in mice using autoradiography. They found that radiolabelled androgens bound to mesenchymal cells in the efferent ductules, Wolffian ducts, urogenital sinus, epididymis, ductus deferens, seminal vesicles, prostate and bulbourethral glands from day 13.5 of fetal development. However, the binding to epithelial cells was only detectable in efferent ductules on day 16.5, in epididymis and ductus deferens on day 19.5 and in seminal vesicle, prostate and bulbourethral glands after birth. Similarly, Kalloo et al. (1993) have reported the expression of androgen receptor in human external genitalia to be strong in mesenchymal cells at 18-22 weeks of gestation while epithelial cells did not show positive immunocytochemical staining for androgen receptor at this time.

In the adult testis, testosterone is an important regulator of testicular function (reviewed by Sharpe, 1994). Recent reports have documented the presence of androgen receptors in several cell types within the testis. Immunocytochemical staining revealed a positive signal in Sertoli, Leydig, peritubular cells and cells lining arterioles (Bremner et al., 1994). In the rat, staining in the nuclei of Sertoli cells appears to be stage dependent consistent with the reported role of testosterone in supporting the process of spermatogenesis (Bremner et al., 1994; Vornberger et al., 1994).

At the time the study reported in this chapter was undertaken there were no reports detailing the pattern of expression of androgen receptor in the fetal testis. In view of the important role of androgens in development of the normal male phenotype and testicular function in the adult we decided to use immunocytochemistry to determine the ontogeny of androgen receptor in the rat testis and associated ducts.

5.2 Experimental procedures

5.2.1 Collection and processing of tissues

Tissues were collected from time mated animals, fixed and processed into paraffin wax as described in chapter 3 (section 3.4). Tissue sections were cut and processed as described in section 3.4.2.

5.2.2 Immunocytochemical staining

Immunocytochemical staining was performed essentially as described in chapter 3. A specific antiserum raised against a peptide containing the first 17 aminoacids of the human androgen receptor was purchased from Novocastra (Newcastle). Primary antibodies were used in a dilution of 1:20 overnight at 4°C. Prior to incubation with primary antibodies, the sections were subjected to antigen retrieval technique (section 3.5.1). The following day, sections were washed twice in TBS, incubated with biotinylated swine anti-rabbit immunoglobulins (Dako) for 30 min at room temperature, washed twice with TBS and incubated with ABC alkaline phosphatase complex (Dako) for 30 min at room temperature. After two washes in TBS, sections were washed in Tris-Mg buffer (100mM Tris-HCL pH 9.5, 100mM NaCl, 50mM $MgCl_2$) for 5 min. Colour reaction product was developed by incubation in a solution containing 337.5µg/ml nitro-blue tetrazolium, 175µg/ml 5-bromo-4-chloro-3-indolyphosphate and 1mM levamisol in Tris-Mg buffer for 30 - 120 min. After washing in distilled water, sections were counterstained with haematoxylin, dehydrated in alcohols, cleared in xylene and coverslipped using Pertex mounting medium (Cellpath).

To detect androgen receptor and the steroidogenic enzyme 3ß-hydroxysteroid dehydrogenase (3ß-HSD) simultaneously in one tissue section, double immunostaining using antibodies directed against androgen receptor and anti 3ß-HSD was performed. Antibodies against human 3ß-HSD (Lorence et al., 1990), raised in rabbits, were kindly donated by Professor Ian Mason (University of Edinburgh, UK). Immunostaining with anti-androgen receptor antibodies (as described above) was followed by a wash in TBS before blocking with normal swine serum. Thereafter the sections were reincubated with anti-3ß-HSD used at a dilution of 1:1000 in TBS overnight at 4°C. The next day, the sections were washed twice for 5 min each time in TBS, incubated with swine anti rabbit antibodies (Dako) at a

dilution of 1:60 for 30 min at room temperature, rewashed twice in TBS and incubated with rabbit peroxidase-antiperoxidase complex (Dako) at a dilution of 1:100 at room temperature for 30 min. After two washes in TBS, colour reaction was developed by incubating the sections in a mixture of 0.02% 3-amino-9-ethylcarbazole (Sigma) in 10ml 0.02M sodium acetate buffer (pH 5.2) containing 1.2ml dimethylsulphoxide (DMSO; BDH) and 0.01% hydrogen peroxide. After 5 - 10 min, the sections were washed in double distilled water and coverslipped using Aquamount (BDH) mounting medium.

The specificity of the antibodies was tested by either replacing the primary antiserum with the normal serum derived from the same animal as primary antibodies or by preabsorbing the antibodies with the peptide against which the antibodies were raised (section 3.5.2).

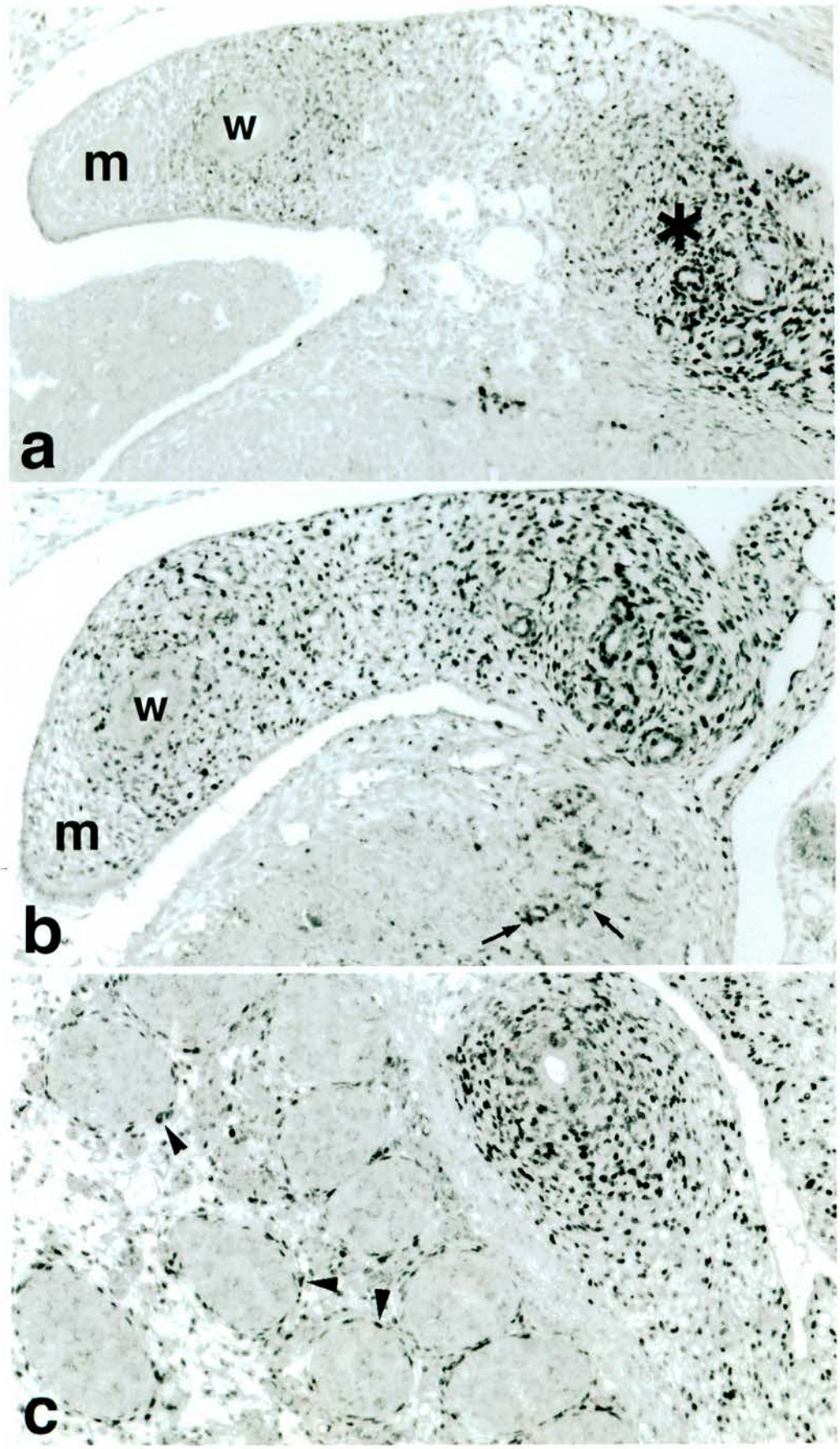
The sections were analysed using Olympus Provis image analysis system and images captured onto Macintosh computer using Nikon N90 camera with Kodak DC digitalizer.

5.3 Results

In the testes and associated structures, there was no signal detected on days 14.5 or 15.5 (earliest examined days) using anti-AR antibodies. On day 16.5 (Fig. 5.1a), immunopositive staining was present in the nuclei of the mesenchymal cells surrounding Wolffian ducts and mesonephric tubules, but the staining was clearly absent from the region around the regressing Müllerian duct. Weak nuclear staining was also noticed in the epithelial cells of the Wolffian duct. Half a day later, on day 17.0, the immunostaining in the mesenchymal cells and epithelium of the Wolffian duct became stronger while the cells around the Müllerian duct remained immunonegative. At that stage, few immunopositive cells were detected within the testes (not shown) but were confined to interstitial regions in the vicinity of the mesonephros. On day 17.5 (Fig. 5.1b), the situation remained similar to that observed half a day earlier, with strong immunostaining in the nuclei of mesenchymal cells around Wolffian ducts and mesonephric tubules but none in the area around the Müllerian duct. Within the testis a few immunopositive cell nuclei were detected in the interstitial region adjacent to the mesonephros. On day 18.5, staining in the nuclei of cells adjacent to the ducts became very strong (Fig. 5.1c). Within the testis, immunopositive cells were now spread throughout the interstitial

region of the testis. Cells forming a narrow layer around the individual testicular cords (presumably peritubular myoid cells) contained positive nuclear staining (Fig. 5.1c, arrowheads). For the remainder of gestation and for several days after birth the pattern of immunostaining observed on day 18.5 p.c. remained unchanged with strong nuclear immunostaining of mesenchymal cells in developing epididymis (mesonephric tubules), and interstitial and peritubular cells surrounding testicular cords within the testes (Fig. 5.2a,b).

Figure 5.1: Androgen receptor immunostaining was first detectable on day 16.5 (a) in some mesenchymal cells within the mesonephros (asterisk) and in the area around the Wolffian duct (W). On day 17.5 (b), the staining in the mesonephric cells and around the Wolffian duct became stronger. Some epithelial cells of the Wolffian ducts started to show a positive signal but immunostaining was clearly absent from the area around Müllerian duct (m). For the first time some AR positive cells appeared within the testis in the interstitial region close to the mesonephros (small arrows). On day 18.5 (c), the immunostaining in the mesonephros and Wolffian ducts remained strong while within the testis, the staining was now widespread and confined to the interstitial cells and peritubular cells (arrowheads) surrounding testicular cords. Magnification x200.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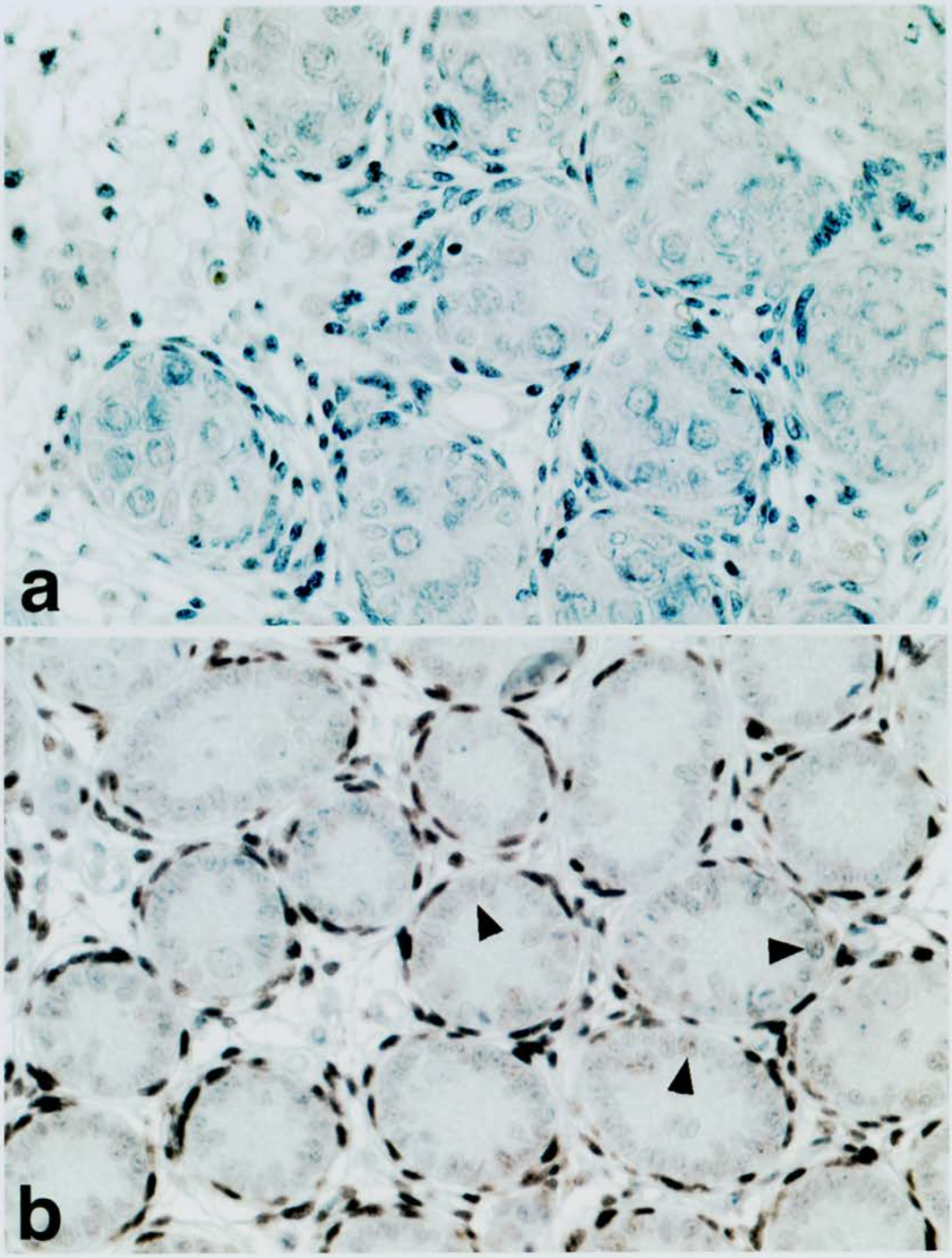


Figure 5.2: The pattern of immunostaining on day 20.5 p.c. (a) was similar to that observed on day 18.5 with positive nuclear staining in interstitial and peritubular cells. Postnatally on day 5 (b), the interstitial and peritubular cells remain strongly positive and weak immunostaining appeared in Sertoli cell nuclei (arrowheads) for the first time. Magnification x400.

In order to address whether AR immunopositive cells within the interstitial population of the testis were members of the fetal Leydig cell population double immunostaining with antibodies directed against AR and 3ß-HSD was undertaken. Immunostaining with anti-3ß-HSD resulted in a strong immunopositive signal in the cytoplasm of steroidogenically active Leydig cells which make up a proportion of the total interstitial cell population. AR immunostaining was localised to cell nuclei as described above. A comparison between the immunopositive cells identified by these two antisera revealed that the interstitial cells expressing androgen receptor are not exclusively the same cells that express 3ß-HSD (fetal Leydig cells) and that only a few cells express both proteins (Fig. 5.3).

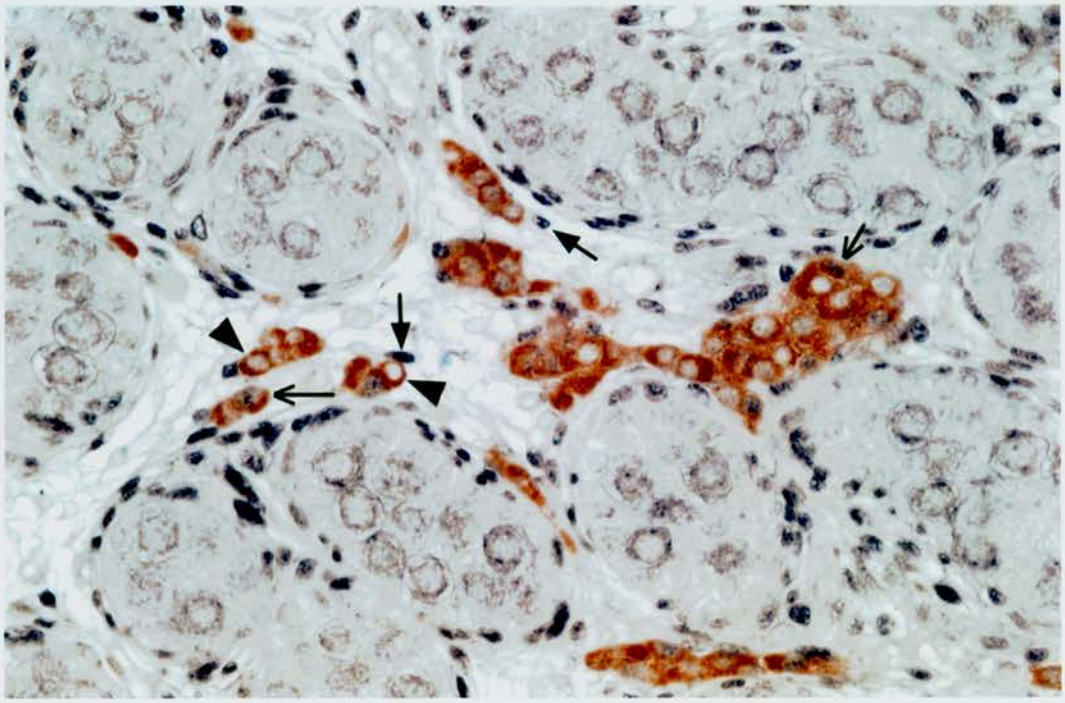


Figure 5.3: On day 19.5, the fetal Leydig cells contained a strong immunopositive staining using antibodies directed against 3ß-HSD (arrowheads), however double immunostaining clearly shows that different populations of cells express 3ß-HSD and AR (arrows). Few cells also expressed both proteins (open arrow). Magnification x400.

5.4 Discussion

In the male fetus testosterone synthesis is essential for masculinisation of the reproductive tract (George and Wilson, 1994). Production of testosterone in the rat fetal testis starts on day 15.5 p.c. and reaches its peak on day 18.5 (Feldman and Bloch, 1978; Warren et al., 1973). Androgens, and a functional androgen receptor, are essential for normal development of male genitalia and a number of studies have addressed the timing and distribution of androgen receptors in the developing urogenital system using binding assays (Cooke et al., 1991a) or immunocytochemistry (Bentvelsen et al., 1995; Kalloo et al., 1993). These studies and those using in vitro approaches have implicated the cells of the mesenchyme as the primary targets for androgen action during fetal life. The studies described above have identified clear and abundant nuclear AR in mesenchymal cells surrounding the Wolffian duct on and after day 16 of pregnancy. However, AR were not distributed uniformly throughout the mesenchymal cells within the mesonephric area being absent from the cells surrounding the Müllerian duct which is destined to regress under the influence of anti-Müllerian hormone (Lee and Donahoe, 1993) and sparse in the central region directly adjoining the testis. Some cells within the epithelial layer of the Wolffian duct, especially in the area which will develop into the epididymis, contained nuclear AR on day 16.5 and the nuclei of nearly all the epithelial cells of the ducts were positively stained for AR on and after day 17. The present results are consistent with the location of AR described in earlier reports using ligand binding but seem to place expression of AR in the epithelial cells of the ducts one to two days earlier than might have been expected from reports in the mouse (Cooke et al., 1991a) reflecting the greater sensitivity of the immunohistochemical technique and show that expression of AR within the mesenchymal cells of the mesonephric region is not uniform.

In the adult testis the primary target for androgen action is believed to be the Sertoli cells within the seminiferous epithelium (reviewed by Sharpe, 1994) although both peritubular myoid cells and Leydig cells also express AR. In the rat only the Sertoli cells were found to exhibit stage-dependent expression of nuclear AR (Bremner et al., 1994) which correlates with the reported stage-dependent effects of testosterone in this species (Sharpe et al., 1992), but the immunoexpression of AR does not appear to be stage specific in primates (marmoset and human; Saunders et al., 1996). In the fetal rat we failed to find convincing immunolocalisation of nuclear AR in the testis before day 17 p.c., at which time a few cells of the interstitial cell population

did appear positive. As testicular development progressed the number of cells in the interstitial cell population with AR nuclear immunostaining increased. Based on their location within the testis and lack of staining with an antibody against 3ß-HSD these AR positive cells are likely to be peritubular and interstitial mesenchymal cells, both of which have a fibroblastic appearance. In the adult testis peritubular myoid cells around the tubules have been shown to contain AR (Verhoeven, 1980) and in other studies conducted in Edinburgh nuclear AR were found in adult peritubular cells (Bremner et al., 1994). In an interesting series of experiments in vitro, Buehr and co-workers (1993) found that cells from the mesonephric region migrated into the testis on or after day 11.5 p.c. in the mouse and contributed to the interstitial cell population including those in the peritubular layer. In the absence of an influx of cells from the mesonephros normal differentiation of testis cords did not occur, consistent with the synthesis of components of the extracellular matrix by peritubular cells contributing to organisation of the testis. In the context of the current findings it is interesting to speculate that the cells in the fetal testis containing AR have developed from the same cell population which express AR in the mesonephros and which we were able to detect on day 16.5 p.c. Previous studies have suggested that a peritubular sheath of cells is present at birth but these do not become 'myoid' cells until puberty when they do so under the influence of testosterone (Bressler and Ross, 1972; Skinner, 1991). These results which demonstrate abundant immunostaining of AR around the testicular cords would indicate that peritubular cells have the potential to be responsive to androgens during fetal life and this may have implications for development of the fetal testis. Peritubular cells from immature rats (postnatal days 15-25) have recently been shown to secrete activin-A (de Winter et al., 1994) and to respond to androgens with increased expression of an androgen response element reporter (Ku et al., 1994). Previous studies have shown that at this time they can secrete a range of growth factors and regulatory agents (reviewed by Skinner, 1991) the production of some of which may be testosterone dependent. Peritubular cells may also demonstrate similar functions during fetal life and could influence development of the fetal testis. Positive staining of AR protein within the cells of the seminiferous cords was unconvincing prior to birth. Previous reports have demonstrated an increase in the expression of AR in immature Sertoli cells of the rat over the period when spermatogenesis is becoming established between days 14 and 28 of postnatal life and establishment of stage-dependent immunostaining of Sertoli cells in the rat (Bremner et al., 1994; Ku et al., 1994).

To detect the AR protein in our sections of fixed tissue it was necessary to undertake antigen retrieval of the samples by microwaving in citrate buffer (Shi et al., 1993). In the absence of this pretreatment, which was recommended by the supplier of the antiserum, no immunostaining was detected. In separate studies using adult rat testes AR immunostaining in Sertoli cell nuclei at stages IV-VII has been reported both following antigen retrieval (Bremner et al., 1994) and in samples embedded in polyester wax and not subjected to antigen retrieval (Vornberger et al., 1994). The immunogen used to raise the antiserum used in this study is located at the extreme N terminal end of the receptor protein in the region associated with ligand-independent transcriptional activation (Jenster et al., 1991). Antigen retrieval, due to the high temperatures used, is believed to cause 'unmasking' of antigenic sites and has been particularly applicable to proteins with a nuclear location, and can sometimes be mimicked by treatment of sections with proteolytic enzymes (Shi et al., 1993). ARs are known to associate with other proteins both before and after ligand binding (reviewed by Carson-Jurnica et al., 1990) and a recent study has shown retention of receptor-bound androgen is enhanced by an interaction between the AR N terminal and steroid binding domains (Zhou et al., 1995). In the present study whether the detection of AR by the antibody directed against the N terminal epitope is dependent upon the conformation of the AR protein was not investigated but in a previous study it was assumed that immunohistochemical localisation of AR reflected the presence of an active ligand-bound receptor because of the correlation with known sites of androgen action (Bremner et al., 1994).

In conclusion, some cells within the mesonephric mesenchyme contain AR as early as day 16 of gestation, consistent with these cells being a primary target of androgen action, but cells around the Müllerian duct remain receptor negative. In the fetal rat testis AR detected by immunohistochemistry is confined to cells of the interstitial population which may have migrated from the mesonephros but is mostly absent from Leydig cells. In the rat androgen action within the testis during fetal life seems likely to be confined to the peritubular and interstitial mesenchymal cell population.

6. Functional differentiation of Leydig cells during fetal and neonatal life

6.1 Introduction

Androgens are essential for normal differentiation and virilization of the male fetus. Leydig cells in the developing testis are the main source of testosterone during fetal life (George and Wilson, 1994). Cells containing large mitochondria with tubular cristae, lipid droplets and smooth endoplasmic reticulum, all characteristics of steroidogenic cells, have been observed in fetal rat testis on day 15.5 of gestation (Magre and Jost, 1980). This timing coincides with the start of testosterone production, which is first detectable on day 15.5 and thereafter rises to reach its peak around day 18.5 of gestation (Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973). While the testis is a very active endocrine organ throughout fetal life, steroidogenesis in the ovary does not start until several days after birth (Juneau et al., 1993).

Conversion of cholesterol to testosterone requires the activity of four steroidogenic enzymes: cholesterol side chain cleavage (P450scc), 3ß-hydroxysteroid dehydrogenase (3ß-HSD), 17α-hydroxylase/17,20-lyase (P450c17) and 17ß-hydroxysteroid dehydrogenase. Both testosterone and androstendione can be converted to oestrogen by the enzyme aromatase (P450arom) while testosterone is often converted to DHT by the enzyme 5α-reductase in its target cells (Miller, 1988). Several investigators have studied the pattern of production of steroid hormones and the presence of steroidogenic enzymes in fetal rat testis by measuring different hormones and their precursors (Bloch, 1979; Feldman and Bloch, 1978; Picon, 1976; Rouiller et al., 1990; Warren et al., 1973). The presence of P450scc in the fetal rat testis was demonstrated using immunocytochemistry on day 15.5 p.c. by Rouiller et al. (1990), but there are no other reports containing immunocytochemical evidence for the expression of the other steroidogenic enzymes in the fetal rat testis prior to day 18.5 p.c. Greco and Payne (1994) have identified mRNAs for P450scc, 3ß-HSD, P450c17 and P450arom in fetal mouse gonads using reverse transcription plus polymerase chain reaction (RT PCR). They found evidence of expression of the genes encoding P450scc, 3ß-HSD and P450c17 in fetal testis from day 13.5 p.c. (the earliest examined day, equivalent to day 15.5 in rat) while aromatase gene expression appeared only later in development on day 17.5 p.c. Expression of all three steroidogenic enzymes remained high in all fetal testis examined. In contrast, the

developing ovaries did not show expression of steroidogenic enzymes with the exception of 3ß-HSD that was detected in about one third of examined samples.

Two recently discovered members of the nuclear hormone receptor superfamily are thought to have essential roles in development of the gonads and adrenal glands. Firstly, steroidogenic factor 1 (SF-1; Lala et al., 1992; Morohashi et al., 1992) has been shown to regulate expression of several cytochrome P450 steroidogenic enzymes (Ikeda et al., 1993; Morohashi et al., 1992) as well several other genes including AMH (Shen et al., 1994) and αGSU (Barnhart and Mellon, 1994). A functional SF-1 gene is required for development of gonads, adrenal glands, hypothalamus and pituitary gonadotrophs (Ingraham et al., 1994; Luo et al., 1994). Secondly, defects in the *DAX-1* gene have been identified as responsible for the pathological condition adrenal hypoplasia congenita which is often associated with hypogonadotrophic hypogonadism (Guo et al., 1995; Muscatelli et al., 1994; Zanaria et al., 1994). DAX-1 lies in a region on the X chromosome (Zanaria et al., 1994), identified as responsible for XY sex reversal when it is duplicated (dosage sensitive sex reversal, DSS; Bardoni et al., 1994). The position of DAX-1 in this region suggests that duplications of DAX-1 could be responsible for DSS, however this data is not yet conclusive. The function of the *DAX-1* gene is as yet unknown but similarities between SF-1 knock out mice (which lack gonads and adrenals) and patients with adrenal hypoplasia congenita suggest a possible interaction between these two genes or their products. While the expression of SF- 1 in the fetal gonads has been explored (Hatano et al., 1994; Ikeda et al., 1994), little is known about the cellular site and timing of expression of DAX-1.

6.2 Experimental procedures

6.2.1 Collection and processing of tissues

Tissues were collected from time mated animals, fixed and processed into paraffin wax as described in chapter 3 (section 3.4). Tissue sections were cut and processed as described in section 3.4.2.

6.2.2 Immunocytochemistry

Immunocytochemical staining was essentially performed as described in chapter 3. Polyclonal rabbit antiserum directed against recombinant human P450c17 (Imai et al.,

1993), used at a 1:1000 dilution was a gift from Professor Michael Waterman (Vanderbilt University, Nashville, Tennessee), polyclonal rabbit anti-human 3ß-HSD (Lorence et al., 1990) antiserum (used at a dilution of 1:1000) was a gift from Professor Ian Mason (University of Edinburgh, UK) and polyclonal rabbit antiserum raised against bovine SF-1 (Morohashi et al., 1993) was donated by Dr. Ken Morohashi (Kyushu University, Japan) and used at a dilution of 1:200. Polyclonal rabbit antibodies against human DAX-1 were purchased from Santa Cruz Biotechnology (San Diego, CA, USA) and used at 1:100 dilution. All diluted primary antisera were incubated on tissue sections at 4°C overnight. Staining with anti-P450cl7, anti-SF-1 and anti-DAX-1 antibodies required antigen retrieval prior to immunostaining to detect a positive signal (section 3.5.1). The incubation with primary antibodies was followed by two washes in TBS and incubation with biotinylated swine anti-rabbit immunoglobulins (Dako, 1:500) for 30 min at room temperature. Sections were washed again twice in TBS, incubated for 30 min at room temperature with ABC-horseradish peroxidase complex (Dako) and washed twice in TBS. Colour reaction product was developed by incubation in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. The development of the colour reaction was monitored under the microscope and after 5 -15 minutes, the slides were placed in distilled water to stop the reaction. Slides were counterstained in haematoxylin, dehydrated in graded alcohols, cleared in xylene and coverslipped using Pertex mounting medium (Cellpath).

The specificity of the antibodies was checked by replacing the primary antibodies with the normal serum from the same species that the primary antibodies were raised in. Specificity of the DAX-1 antiserum was also checked by preabsorbing the antibodies with the peptide. In none of the controls was a positive signal detected.

In some cases, double immunostaining was performed as follows: staining with anti-SF-1 antibodies was performed as above and development of a colour reaction was followed by a wash in distilled water and a wash in TBS. Sections were then blocked with normal swine serum diluted 1:5 in TBS for 30 min at room temperature prior to incubation with anti-3ß-HSD or anti-DAX-1 antibodies overnight at 4°C. The following day sections were washed twice in TBS, incubated for 30 min at room temperature with swine anti-rabbit antibodies (Dako) used at a dilution 1:60, washed twice in TBS and incubated for 30 min at room temperature with rabbit alkaline phosphatase-anti alkaline phosphatase complex diluted 1:100 (Dako). After two

washes in TBS, colour reaction product was developed by incubating the sections in 1µg/ml solution of Fast blue (Sigma) in Fast blue buffer (0.1M TRIS pH 8.2, 2% dimethylformamide, 0.02% naphtol). Slides were washed in distilled water, counterstained with haematoxylin and embedded using aquamount (BDH) mounting medium.

6.3 Results

6.3.1 Detection of 3ß-HSD in the fetal gonads

Staining of fetal testes with antibodies against 3ß-HSD resulted in a strong immunocytochemical positive signal in the cytoplasm of many of the interstitial cells between testicular cords (fetal Leydig cells). No positive staining has been detected on day 14.0 p.c., the earliest stage at which it was possible to morphologically distinguish the testis from the ovary by its "stripy" appearance (see chapter 4). Half a day later, on day 14.5, strong immunostaining was apparent in the adrenal glands and moderate staining in some of the Leydig cells within the testis (Fig. 6.1a). On day 15.5, staining in the testis became much stronger (Fig. 6.1b) when compared with day 14.5 and thereafter, this immunostaining remained strong for the rest of the fetal life (Fig. 6.1c, d). No positive staining was detected in the fetal ovaries at any stage of development.

6.3.2 Detection of P450c17 in the fetal gonads

Cytoplasmic staining with antibodies against P450c17 was first detectable on day 15.5, in a few weakly stained fetal Leydig cells (Fig. 6.2a). On day 16.5, the staining became stronger with more immunopositive cells within the interstitium (Fig. 6.2b). As with 3ß-HSD, Leydig cells remained strongly immunopositive throughout fetal life (Fig. 6.2c, d). The staining with antibodies directed against 3ß-HSD usually resulted in a stronger colour reaction than staining with antibodies against P450c17. No positive staining was detected in fetal ovaries at any stage of gestation.

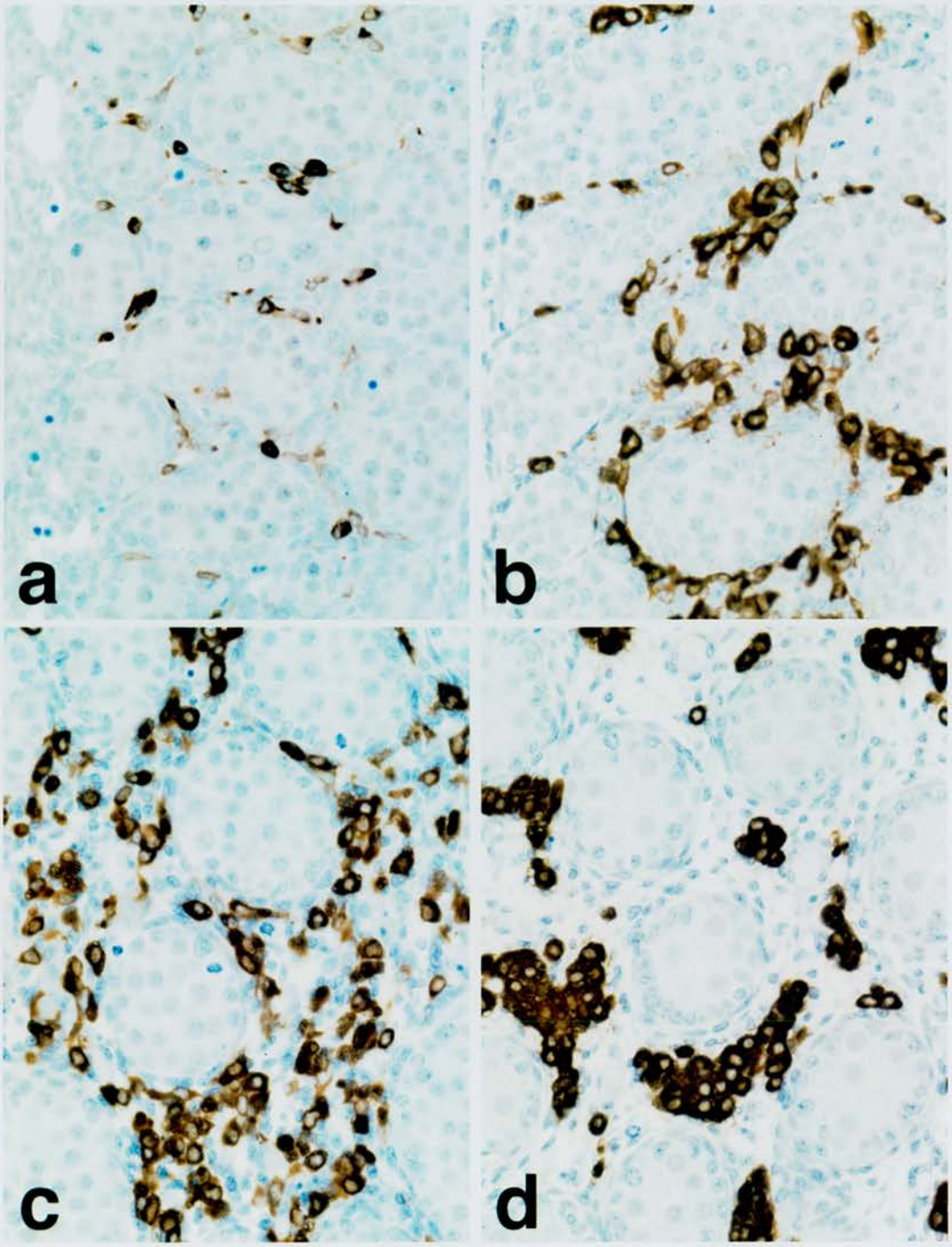


Fig. 6.1: 3ß-HSD immunostaining was first detectable in fetal testis on day 14.5 (a) but became much stronger and more widespread one day later, on day 15.5 (b). The staining remained strong throughout the fetal life (c, day 17.5; d, day 20.5). Magnification x400.

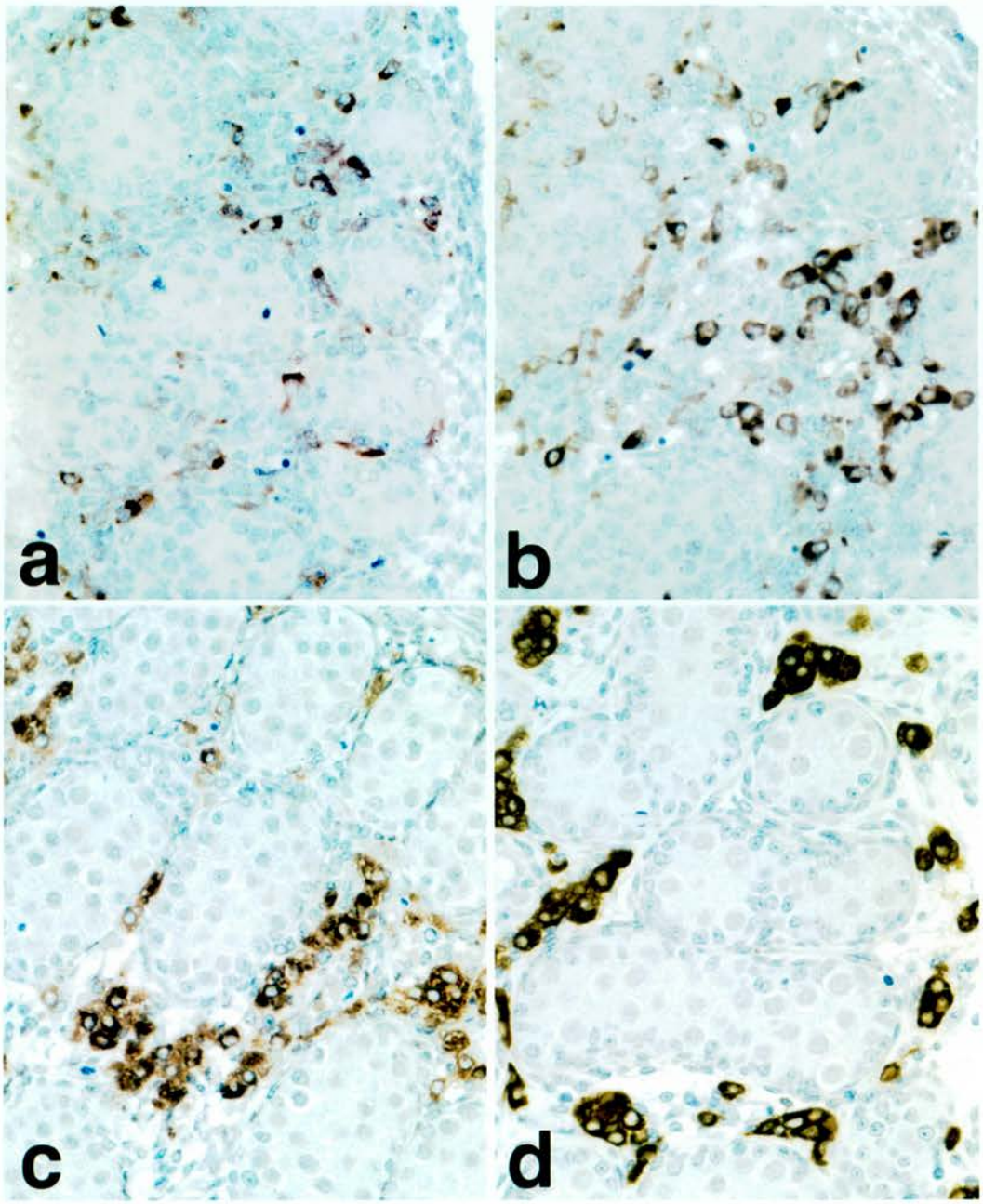


Fig. 6.2: Few interstitial cells showed positive immunostaining for P450c17 in the fetal testis on day 15.5 (a). The amount of protein increased one day later (b, day 16.5) and thereafter cells remained strongly immunopositive throughout the fetal life (c, day 17.5; d, day 20.5). Magnification x400.

6.3.3 Detection of SF-1 in the fetal gonads

SF-1 was immunolocalised to nuclei of cells within the genital ridge from day 13.5 p.c. (not shown). Immunostaining at that age and on day 14.0 was weak and present in both ovaries and testes. On day 14.5, the intensity of immunostaining increased and was still present at a similar intensity in cells within both testes and ovaries (Fig. 6.3a, b). In the testis, SF-1 was present in both the interstitial cells and Sertoli cells; staining of interstitial cells was not uniform and cells with both strong and weak expression of SF-1 could be clearly distinguished (Fig. 6.3a). During subsequent days of gestation, the staining in the fetal ovaries (Fig. 6.3d) declined until the only cells that showed a relatively strong immunostaining were those of the germinal epithelium (arrows). In the testis, the immunoexpression of SF-1 remained in Sertoli and interstitial cells throughout fetal development (Fig. 6.3c).

6.3.4 Detection of DAX-1 in the fetal gonads

DAX- 1 protein was first detectable in rat gonads within cellular cytoplasm on day 15.5 p.c. Weak immunostaining was detected in the interstitial cells in the testis (Fig 6.4a, arrows) and in the germinal epithelium of the ovary. Immunoexpression within the testis increased as pregnancy progressed (Fig. 6.4b), however it remained weak throughout fetal life.

6.3.5 Detection of 3ß-HSD and SF-1 in fetal testis

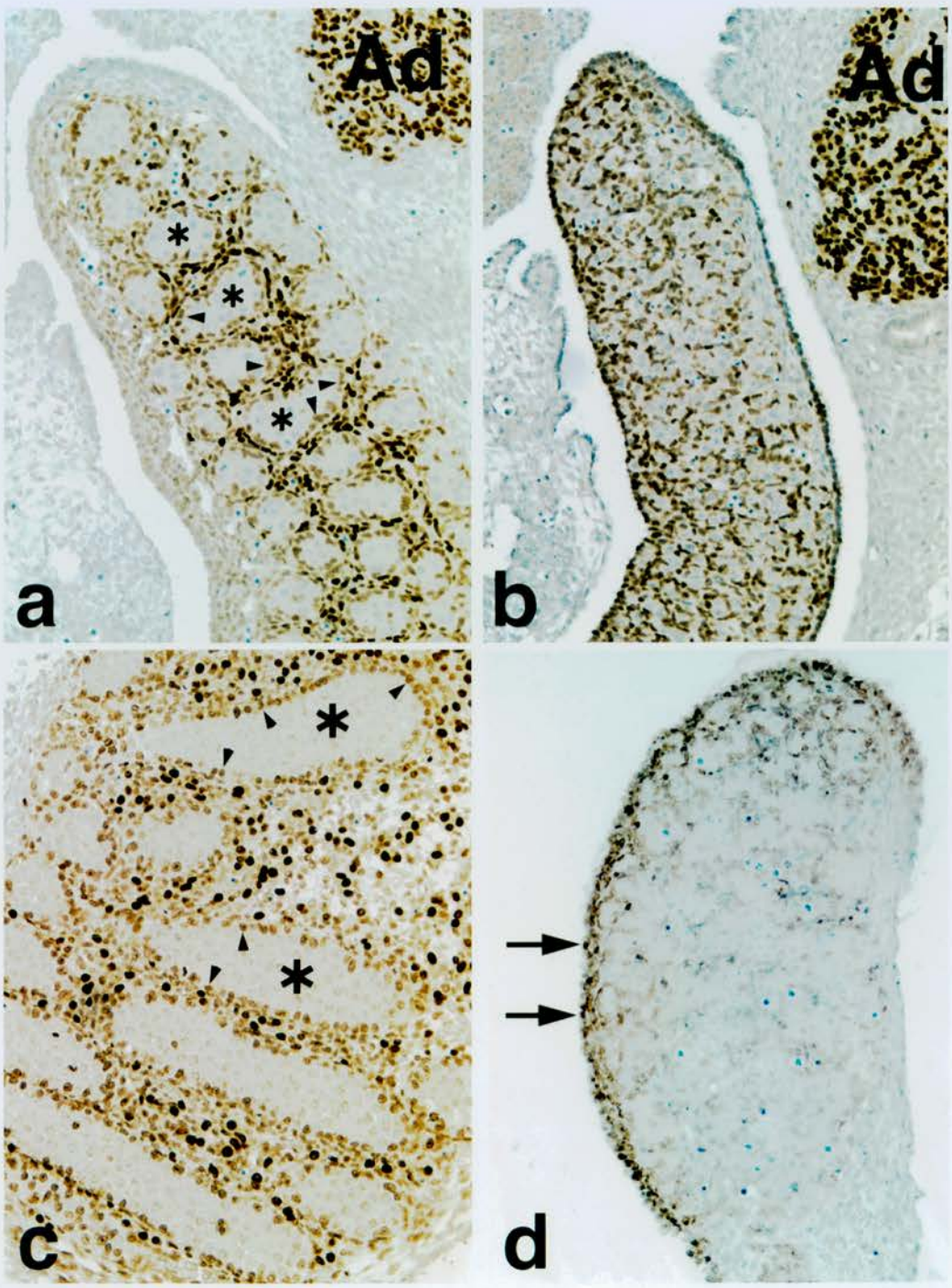
Double immunostaining using antisera raised against 3ß-HSD and SF-1 resulted in strong immunostaining in both cases. The results obtained clearly demonstrated that the interstitial cells with abundant nuclear immunostaining for SF- 1 also expressed 3ß-HSD (Fig. 6.5a, arrows) and are therefore steroidogenically active fetal Leydig cells. In contrast, interstitial cells with "weak" expression of SF-1 were not immunopositive for 3ß-HSD (Fig. 6.5a, arrowheads).

6.3.6 Detection of SF-1 and DAX-1 in fetal gonads

Double immunostaining with antibodies raised against SF-1 and DAX-1 proteins revealed only partial colocalisation of SF-1 and DAX-1 in the interstitial cells (Fig. 6.5b). Some DAX-1 positive cells did not appear to express SF-1 and while the

majority of cells with strong SF-1 expression also showed positive immunostaining for DAX-1, some were also immunonegative for DAX-1.

Fig. 6.3: Weak immunostaining for SF-1 was detectable on day 14.0 but the staining became much stronger half day later on day 14.5 in the testis (a) and ovary (b). In the testis, SF-1 was present in interstitial and Sertoli cells (arrowheads) within the testicular cords (asterisks). Among interstitial cells, two different populations could be distinguished; cells with strong immunoexpression of SF-1 and cells with weak SF-1 expression. The staining in the testis remained strong throughout the fetal life (c, day 17.5) but declined in the ovary so that on day 17.5 (d) only cells of the germinal epithelium still strongly expressed SF-1 (arrow). Strong immunostaining was also detected in adrenals (Ad). Magnification x200.


Ad
Ad
a
b
c
d

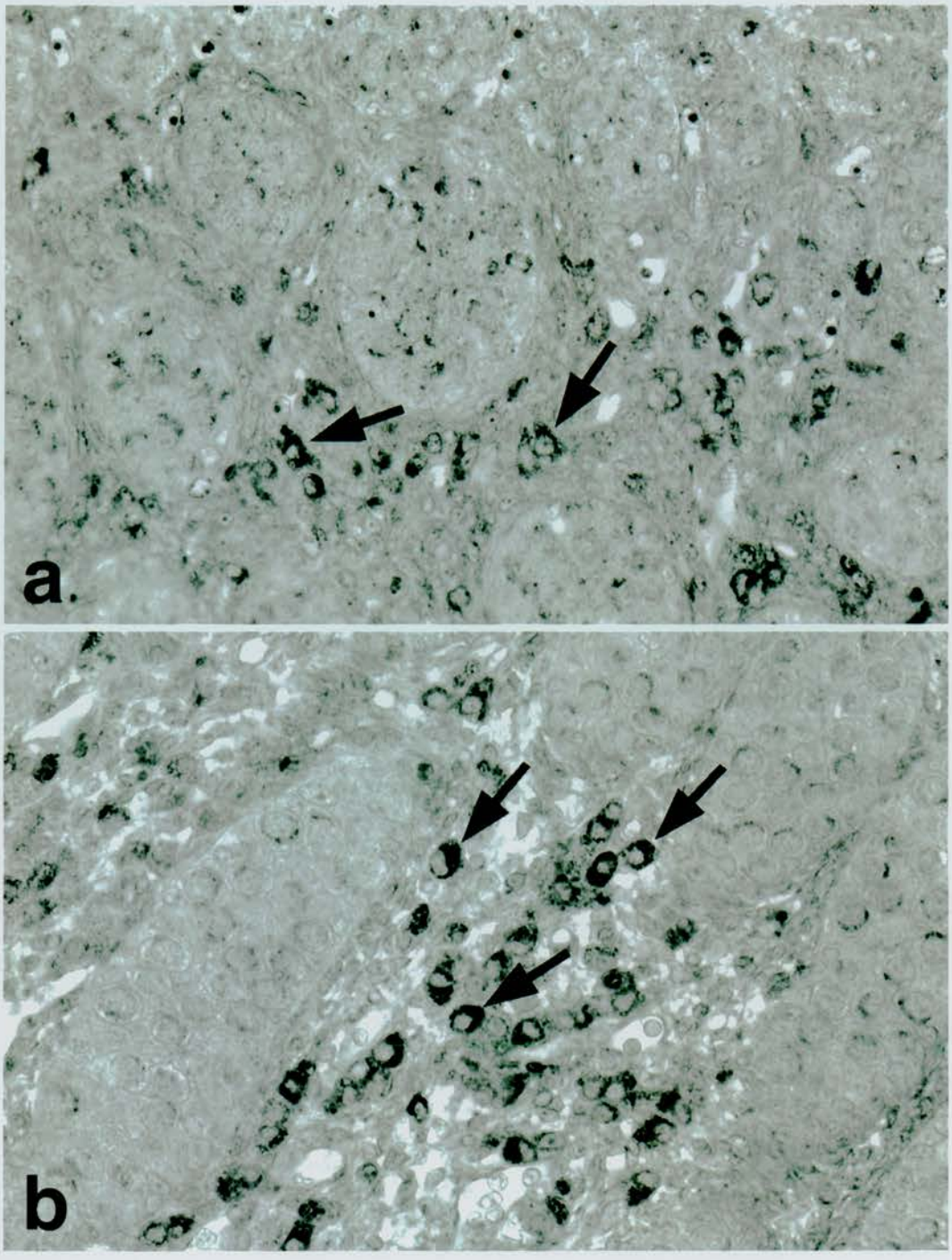
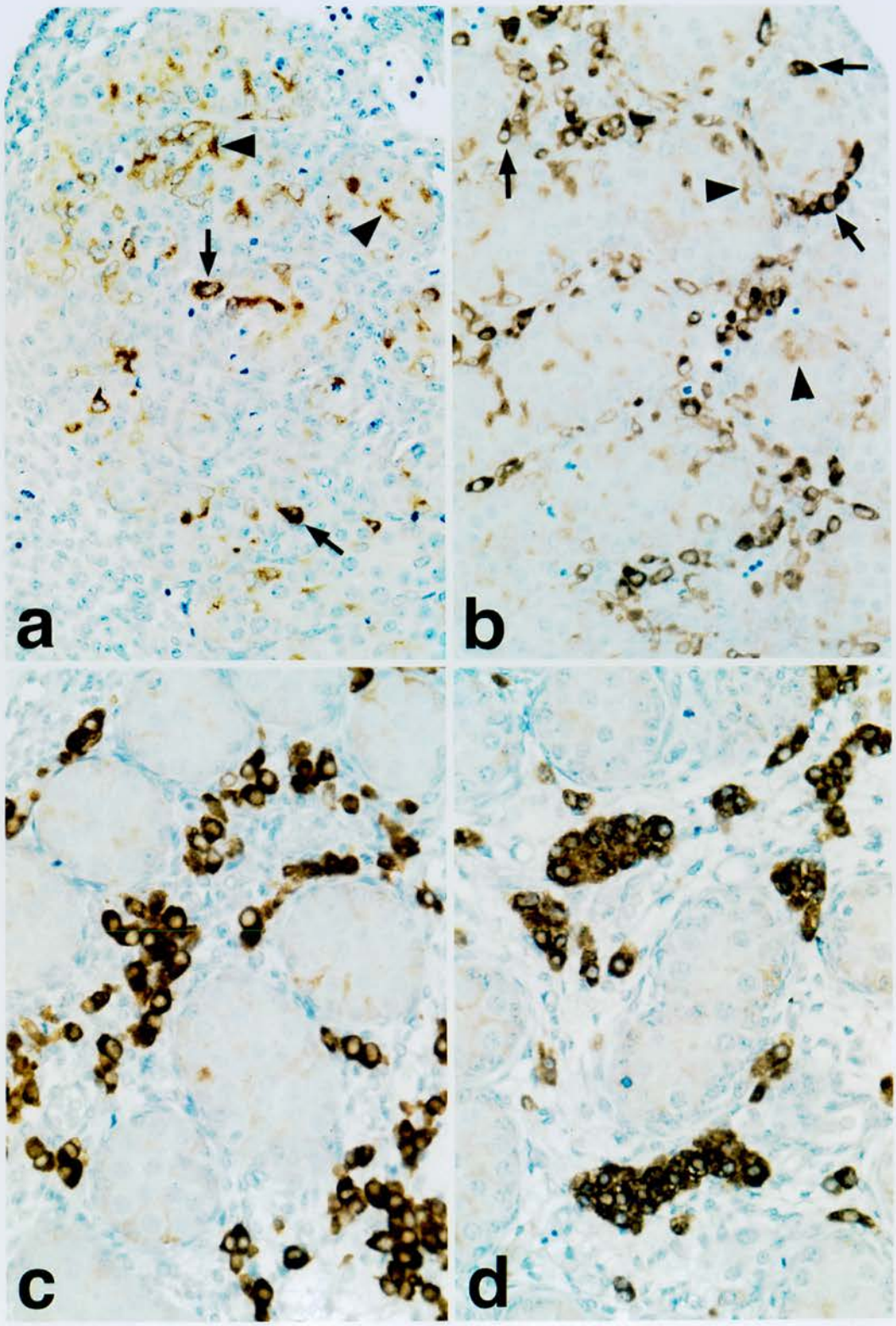


Fig. 6.4: DAX-1 immunostaining was detected in the fetal rat testis on day 15.5 (a) in cytoplasm of some interstitial cells (arrows). During the following days of gestation, the amount of protein increased (b, day 18.5). Postnatally, immunostaining was very weak and as in fetal life, was confined to a subpopulation of interstitial, presumably Leydig cells (not shown). Magnification x400.


a
b
c
d

6.4 Discussion

Using immunocytochemistry with specific antibodies, the patterns of expression of two steroidogenic enzymes and two orphan nuclear receptors with important functions in sexual differentiation and gonadal development was demonstrated in the fetal rat testis. Previous data on the ontogeny of steroidogenic enzymes has been principally based on measurements of different steroids and their precursors (Bloch, 1979; Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973) but with the exception of P450scc (Rouiller et al., 1990), there is no data on cellular localisation of the individual enzyme proteins in the fetal testis. Studies by Rouiller et al. (1990) have detected the presence of P450scc in fetal rat testis using immunocytochemistry on day 15.5 of gestation, in agreement with our observation on the onset of synthesis of another cytochrome P450, P450c17, suggesting that a similar mechanism for initiating expression of those two cytochromes may exist. In addition, several studies have reported that the onset of testosterone production begins on day 15.5 p.c. (Feldman and Bloch, 1978; Picon, 1976; Warren et al., 1973). Whilst the testosterone content in the fetal testis is reported to be low on day 15.5, it rises sharply thereafter to reach a peak on day 18.5 (Feldman and Bloch, 1978; Warren et al., 1973). After that, testosterone levels in the rat testis remain at a similar level or even slightly decline during the rest of fetal life. The present findings on the pattern of immunoexpression of P450c17 are in accordance with these reports with only weak immunostaining in some Leydig cells on day 15.5 but an increase in the numbers of immunopositive cells one day later and abundant immunostaining thereafter. However, while reports on the onset of synthesis of P450scc and P450c17 coincide with the reported start of testosterone production, that of another steroidogenic enzyme, namely 3ß-HSD, was detected in the testis one day earlier, on day 14.5. Studies by Magre and Jost (1980) have reported that morphological differentiation of steroidogenic cells in the fetal rat testis occurs on day 15.5, simultaneously with the reported onset of testosterone production. However, studies by Bloch (1979) have recorded 3ß-HSD activity in the rat testis on day 14.5 of gestation in agreement with the current study. The reported absence of other steroidogenic enzymes (Feldman and Bloch, 1978; Warren et al., 1973) in the fetal testis on that day raises the question of what the role of 3ß-HSD might be at this age. There are no reports of production of progesterone by the fetal testis and this would be unlikely as neither P450scc protein (Rouiller et al., 1990) nor enzyme activity (Warren et al., 1973) are detectable before day 15.5. P450scc is involved in the first step of steroidogenesis, converting cholesterol to pregnenolone

(Miller, 1988) and as it is not present at day 14.5 p.c., no pregnenolone would be available to act as a substrate for 3ß-HSD.

The regulation of the expression of steroidogenic enzymes and the production of testosterone in fetal testis have not been fully elucidated. Oestrogens (Bartke et al., 1977; Moger, 1980) and androgens (Darney et al., 1996; Pointis et al., 1984) both inhibit production of steroid hormones, mainly by reducing the activity of the 17α-hydroxylase enzyme in fetal (Majdic et al., 1996) and adult testis (Darney et al., 1996; Kalla et al., 1980). LH, secreted by pituitary gonadotrophs, is believed to be the primary regulator of testosterone production in the adult testis but its function during fetal life is not clear (Saez, 1994) although several studies have shown that LH is able to stimulate testosterone production in the fetal testis (Feldman and Bloch, 1978; Habert, 1993). LH is probably involved in the rise of testosterone production that occurs up to day 18.5 p.c. in the rat (Saez, 1994), but it is unlikely that LH is essential to trigger production of testosterone on days 15.5 to 16.5 since LH is only detectable in the fetal pituitary (Watanabe and Daikoku, 1979) and circulation (Aubert et al., 1985) on day 17.5. Some reports suggest that, at least in primates, placental hCG might play a role in initiating testosterone synthesis (Saez, 1994). Whilst hCG is able to increase production of testosterone both in fetal and adult rat testis in vitro and it is possible that hCG is indeed the triggering factor in the primate testis, studies have so far failed to identify a factor similar to hCG in rat placenta (Habert and Picon, 1990; Wurzel et al., 1983). Reports that 3ß-HSD protein and enzyme activity (Bloch, 1979) are present one day before the onset of P450scc and P450c17 expression suggest that different regulatory mechanisms may exist for determining expression of 3ß-HSD and the cytochrome P450s. Whether regulation occurs at a transcriptional or posttranscriptional level (or both) currently remains unanswered.

Steroidogenic factor-1 (SF-1) has been recently discovered as a common regulator of expression of genes encoding several steroidogenic enzymes, including P450scc and P450c17 (Ikeda et al., 1993; Lala et al., 1992; Morohashi et al., 1992; Zhang and Mellon, 1996). Further studies have demonstrated the importance of SF-1 in development of the gonads and adrenal glands since transgenic mice lacking a functional SF- 1 gene do not develop either gonads or adrenal glands and die shortly after birth, probably due to lack of gluco- and mineralocorticoids (Luo et al., 1994). SF-1 is expressed very early in the mesonephric area, before the gonads differentiate (Hatano et al., 1994; Ikeda et al., 1994) and hence well before steroidogenesis starts, consistent with a role of that factor in early gonadal differentiation. The present study

identified expression of SF-1 in the gonads on day 14.0, however, immunoexpression was increased half a day later on day 14.5, coinciding with the appearance of 3ß-HSD protein in the fetal testis. Whether these two findings are connected is unclear, since so far there have been no reports of SF-1 regulating 3ß-HSD expression. In contrast, the genes encoding for P450scc and P450c17 have been reported to be regulated by SF-1. Double immunostaining using anti SF-1 and anti-3ß-HSD antibodies showed clear colocalisation of SF-1 and 3ß-HSD in those interstitial cells with strong SF-1 expression. However, within the interstitium of the fetal testis, other population(s) of SF-1 positive cells were detected, which had lower SF-1 expression and did not express 3ß-HSD and therefore are not steroidogenically active fetal Leydig cells. At present, it is only possible to speculate on the identity of this non-Leydig cell population of cells and what the function of SF- 1 in this cell population might be. One obvious possibility is that these cells are Leydig cell precursors and that SF- 1 plays a role in differentiation of these cells into functional Leydig cells.

DAX-1 is a recently discovered orphan nuclear receptor (Guo et al., 1995; Zanaria et al., 1994). The gene lies in the region of the X chromosome (Zanaria et al., 1994), duplications of which are associated with XY female sex reversal (Bardoni et al., 1994). Similarities between the phenotype of SF-1 knock out mice and patients with AHC has led to suggestions that there is an interaction between SF-1 and DAX-1. This hypothesis was further supported by the finding of a putative SF-1 binding element in the *DAX-1* upstream region (Burris et al., 1995) but it is not yet known if SF-1 directly regulates DAX-1 expression. The precise role(s) of DAX-1 in the gonad have yet to be elucidated although it has recently been reported that DAX-1 mRNA is expressed in the gonadal ridge and the interstitium of the testis of adult mouse (Swain et al., 1996). The presence of a putative SF-1 binding site on *DAX-1* led us to anticipate that SF-1 might co-localise with DAX-1 in the fetal testis and/or be expressed in a less mature cell type. In contrast to SF-1 which was detected in the nuclei of both interstitial and Sertoli cells, specific DAX-1 immunostaining was present only in cells within the interstitium. DAX-1 protein was first detectable on day 15.5 of gestation in the rat testis and was localised to the cytoplasm of interstitial cells in fixed tissue sections. This finding is somewhat surprising as the presence of putative DNA and ligand binding domains in DAX-1 suggest this protein, like SF-1, may function as a transcription factor. In the tissue sections SF-1 was localised exclusively to cellular nuclei consistent with previous data using frozen tissue sections. We have as yet no explanation for the cytoplasmic localisation of DAX-1 but have seen a similar pattern of staining using antisera directed against peptides of the

oestrogen receptor (unpublished observations) but not the androgen receptor (Majdic et al., 1995) on fixed sections of fetal testis processed in an identical way to those in the current thesis. Oestrogen receptors have been reported to undergo nucleocytoplasmic shuttling which is energy dependent (Dauvois et al., 1993). Previous investigations using frozen tissue sections have reported that monoclonal antibodies localise to nuclei of target cells (King and Greene, 1984) and we speculate that in the case of ER, and possibly DAX-1, the fixation of the tissue may have influenced the subcellular localisation of the protein in keeping with results reported for the glucocorticoid receptor (Brink et al., 1992).

A recent study (Swain et al., 1996) detected DAX-1 mRNA in mice using RNase protection and whole mount in situ hybridization. DAX-1 mRNA was present in the interstitial cell compartment in adult mice and was presumed to be in the adult Leydig cells. However in the fetal mouse DAX-1 mRNA was present on day 11.5 p.c. (approximately equivalent to day 13 in the rat) and declined after day 12.5, a finding apparently at odds with the present study although no protein data are included in the study (Swain et al., 1996) and a direct comparison between species is therefore not possible. In a preliminary experiment we have been able to detect significant amounts of DAX-1 mRNA in fetal rat testis on day 17.5 by Northern blotting.

SF-1 immunostaining was detected in several cell populations within the testis. Sertoli, Leydig and other interstitial cells all expressed SF-1 protein while only some interstitial cells show positive immunostaining with anti-DAX-1 antibodies and double immunostaining demonstrated that DAX-1 and SF-1 are not exclusively localised in the same cell types. The importance of this finding is not known, however it would question the role of SF-1 in regulation of DAX-1 as was suggested earlier (Burris et al., 1995). While similarities in the phenotypes as a result of mutations in the DAX-1 and SF-1 genes firmly suggest the interaction of those two factors, lack of exclusive colocalisation may suggest an indirect rather than a direct connection between these two proteins. However, in the present study, investigations were confined to immunocytochemistry and it is possible that this method is not sensitive enough to detect a very low level of protein expression and that expression of DAX-1 protein occurs in additional cells within the testis at levels undetectable using immunocytochemistry with the present antiserum. Direct regulation of DAX-1 expression by SF-1 cannot be ruled out at the present time and further studies will be needed to clarify the function and possible interaction of these proteins.

7. Immunoexpression of inhibin and activin subunits in the fetal and neonatal rat testis

7.1 Introduction

Inhibins and activins are structurally related dimeric gonadal glycoproteins that were initially characterised by their ability to alter FSH secretion from the pituitary (Vale et al., 1986, 1988; Ying, 1988). Inhibin selectively suppresses FSH secretion (Vale et al., 1988) while activin stimulates pituitary FSH release (Schwall et al., 1989). Inhibin and activin are members of the TGF-ß superfamily (Wallach, 1996; Ying, 1988), a large family of growth factors that regulate cell proliferation, differentiation and function. The group also includes AMH and the decapentaplegia gene product in *Drosophila* (Massague, 1990). Inhibins are composed of an α-subunit and one of two similar, but distinct, ß subunits (ßA and ßB). Activins are dimers of two ß subunits and the three possible combinations of activin dimers have been designated activin A (ßA/ßA), activin B (ßA/ßB) and activin AB (ßA/ßB; Vale et al., 1988). The genes for all three subunits have a similar structure containing one intron; translation of their mRNAs produces large precursor molecules which are subsequently cleaved to produce the functional subunits (Ying, 1988). The precursor molecule for the α-subunit in rat is 366 aminoacids long and the mature subunit which is 133 aminoacids long is located at the carboxyterminal. ßA and ßB subunits which are 116 and 115 aminoacids long are produced by proteolytic cleavage of the larger precursor molecules, 424 and 407 aminoacids long, respectively (Woodruf et al., 1987; Ying, 1988).

It is widely accepted that inhibin is produced in the adult testis, mainly by Sertoli cells (Ying, 1988). Some reports have also suggested that the production of inhibin and activin also occurs in Leydig and peritubular cells (Maddocks and Sharpe, 1989; Roberts et al., 1989; Shaha et al., 1989). Similar patterns of expression have been described in the fetal and neonatal testis, although, immunostaining of the Leydig cells did appear to be more prominent than in adult testis (Roberts et al., 1989; Shaha et al., 1989). A similar pattern of expression has also been reported in the primate (rhesus monkey and human) fetal and neonatal testis (Rabinovici et al., 1991).

The main biological function of both inhibin and activin is believed to be the control of FSH secretion from the anterior pituitary (Wallach, 1996; Ying, 1988). Inhibin is a potent suppressor of FSH release and reduces the cellular content of FSH in cultured

rat pituitary cells but does not affect the other pituitary hormones including LH and TSH (Ying, 1988). The time course of action of inhibin differs from that of GnRH. Suppression of FSH release by inhibin occurs within 4-18 hours whereas the response to GnRH requires only seconds to a few hours (Ying, 1988). In contrast, activins have a strong stimulatory effect on release of FSH from the anterior pituitary. Activin is a more potent stimulator of FSH release than GnRH, however, in the presence of inhibin, the stimulatory effect of activin is completely abolished (Ying, 1988). Like inhibin, activin does not exhibit any effect on secretion of LH or other pituitary hormones (Ying, 1988). Apart from their role in modulating FSH secretion, both inhibin and activin are believed to have important autocrine and paracrine functions within the gonads (Chen, 1993; Lin et al., 1989) and other tissues (Spencer et al., 1990). Studies by Boitani and co-workers (1995) and Kaipia and co-workers (1994) have demonstrated that activin stimulates Sertoli cell proliferation whilst Lin et al. (1989) have reported that activin can inhibit hCG-stimulated testosterone production by cultured Leydig cells. In the same study, Lin et al. failed to detect any influence of inhibin on Leydig cell function, however some earlier studies have reported that inhibin has a stimulatory effect on testosterone production in isolated immature and adult Leydig cells (Hsueh et al., 1987). Transgenic mice lacking a functional inhibin α subunit develop normally and are born without any obvious abnormalities. However, postnatally these animals develop testicular tumours with almost 100% penetrance suggesting that inhibin is not required for normal fetal development but may act as a tumour supressor in the testis (Matzuk et al., 1992).

In 1987, another group of proteins with the ability to suppress FSH secretion was identified (Robertson et al., 1987; Ueno et al., 1987). This were named follistatins. Several bioactive peptides were detected in bovine and porcine follicular fluid, and the cloning of the cDNA showed that all derived from a single copy gene (Michel et al., 1993). The apparent ability of follistatin to suppress FSH secretion was only 10-30% of that of inhibin, resulting in the suggestion that follistatins were weak, and perhaps redundant, inhibin agonists with uncertain function (s) (Michel et al., 1993). However, studies by Kogawa and Nakamura and their colleagues (Kogawa et al., 1991; Nakamura et al., 1990) have shown that follistatins bind to activins and to a lesser extent to inhibins (Shimonaka et al., 1991). The role of follistatin is not as yet completely elucidated, but the available data suggest that it may function primarily as a regulator of activin bioavailibility rather than as a simple carrier molecule (Michel et al., 1993; Moore et al., 1994). Follistatin is reported to be expressed both in the pituitary gland (Kogawa et al., 1991) and in the adult testis (Shimasaki et al., 1989)

but has not been identified in the fetal testis (Roberts and Barth, 1994).

7.2 Experimental procedures

7.2.1 Collection and processing of tissues

Tissues were collected from time mated animals, fixed and processed into paraffin wax as described in chapter 3 (section 3.4). Tissue sections were cut and processed as described in section 3.4.2.

7.2.2 Immunocytochemical staining

Immunocytochemical staining was performed as described in chapter 3 (section 3.5). Specific monoclonal antibodies raised in mice against all three inhibin/activin human subunits and against human follistatin were gift from Dr. Nigel Groom (Brooks University, Oxford, UK). Antibodies directed against the α-subunit were used at a dilution of 1:500, those against ßB subunit at a dilution of 1:5000, those against ßA in dilutions 1:500 - 1:5000 and those against follistatin at a dilution of 1:50. Sections were subjected to antigen retrieval (section 3.5.1), blocked with normal rabbit serum and then incubated overnight at 4°C in a humidified chamber. The following day, sections were washed twice in TBS, incubated with biotinylated rabbit anti-mouse immunoglobulins (Dako) for 30 min at room temperature, washed twice with TBS and incubated with ABC-horseradish peroxidase complex (Dako) for 30 min at room temperature. The colour reaction product was developed by incubation in a mixture of 0.05% (w/v) 3,3'-diaminobenzidine tetra-hydrochloride(DAB, Sigma) in 0.05M Tris-HCl, pH 7.4 and 0.01% hydrogen peroxide. Development of the colour reaction was monitored under the microscope and after 5 -15 min, slides were placed in distilled water to stop the reaction. After a wash in distilled water, sections were counterstained with haematoxylin, dehydrated in alcohols, cleared in xylene and coverslipped using Pertex mounting medium (Cellpath).

To detect α-subunit and the steroidogenic enzyme 3ß-hydroxysteroid dehydrogenase (3ß-HSD) simultaneously in one section, double fluorescent immunostaining was performed. Tissue sections were blocked with normal goat serum and incubated with the mixture of both primary antibodies (anti 3ß-HSD were used at a dilution 1:1000 and anti α-inhibin at a dilution 1:500) overnight at 4°C. Incubation with primary antibodies was followed by two washes in TBS and incubation with mixture of

fluorescent secondary antibodies (goat anti-mouse FITC conjugated and goat anti-rabbit TRITC conjugated, Sigma) at a dilution of 1:20 for 1 h at room temperature. Sections were washed again three times in TBS and coverslipped using glycerol gelatine. Sections were examined under UV light using an Olympus Provis microscope.

Specificity of antibodies was checked by either replacing primary antibodies with the normal mouse serum or by preabsorbing the antibodies with the peptide against which they were raised (section 3.5.2). None of the controls showed any positive signal above background.

7.3 Results

7.3.1 α-subunit

Positive cytoplasmic immunostaining for α subunit was first detectable in fetal testis on day 14.5 (Fig. 7.1a), the earliest age at which the testis could be morphologically distinguished from the ovary. At this age the colour reaction product was equally strong in both Sertoli and interstitial cells but one day later, on day 15.5 (Fig. 7.1b), the immunostaining appeared much stronger in the interstitial cells. Thereafter, immunostaining remained very strong in the fetal Leydig cells but was barely detectable in the fetal Sertoli cells from day 17.5 onwards (Fig. 7.1c, d). Double immunofluorescent immunostaining with antibodies directed against the α-subunit and 3ß-HSD clearly showed colocalisation of 3ß-HSD and α-subunit in the fetal testis from the 17.5 p.c. days old fetuses (Fig. 7.2).

Fig. 7.1: Inhibin α-subunit was first detected on day 14.5 p.c. (a). Immunostaining was present in both interstitial (arrow) and Sertoli (arrowhead) cells. As pregnancy proceeded (b, day 15.5; c, day 17.5; d, day 20.5), the immunostaining remained strong in the fetal Leydig cells but became almost undetectable in Sertoli cells. Magnification x300.

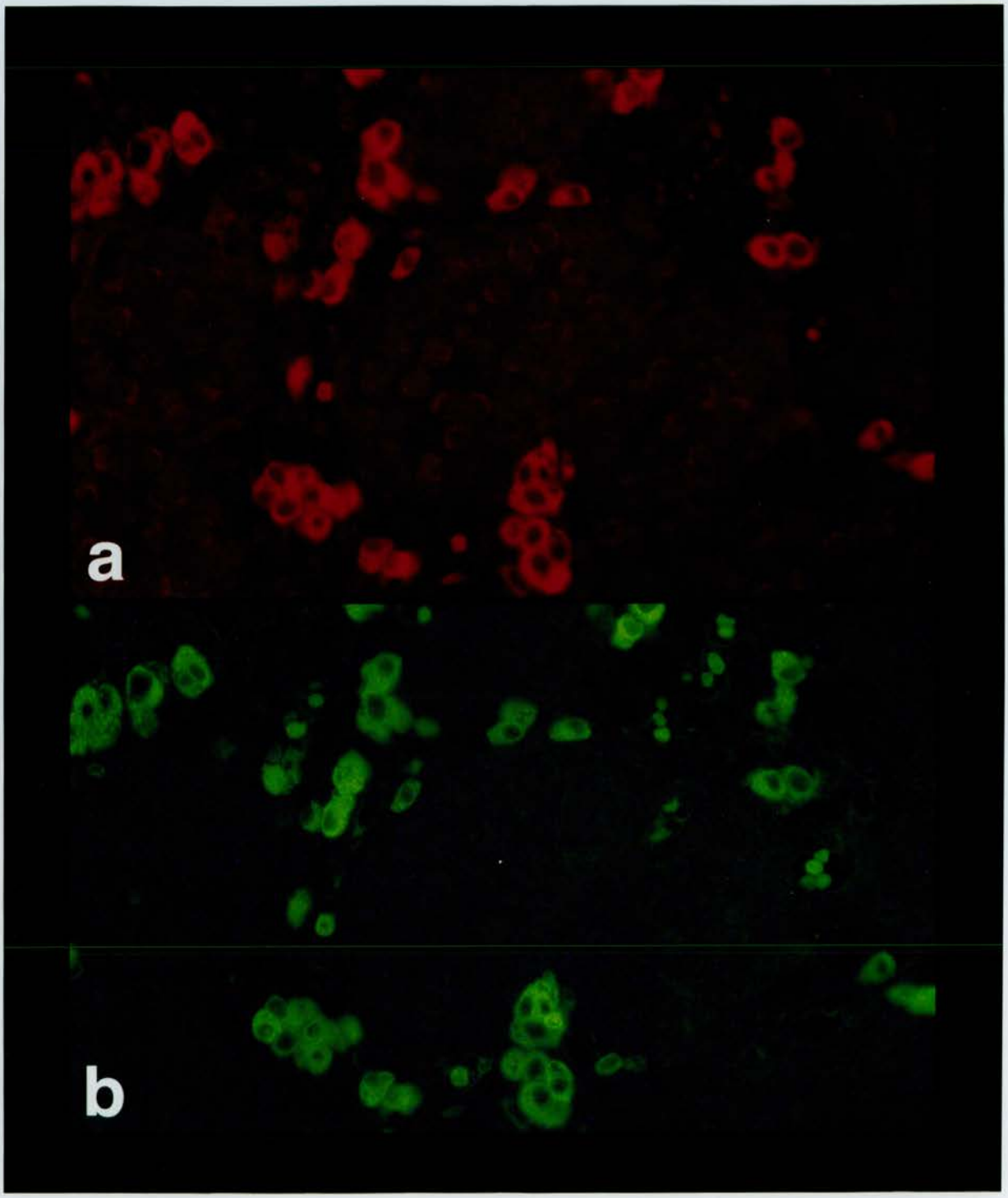
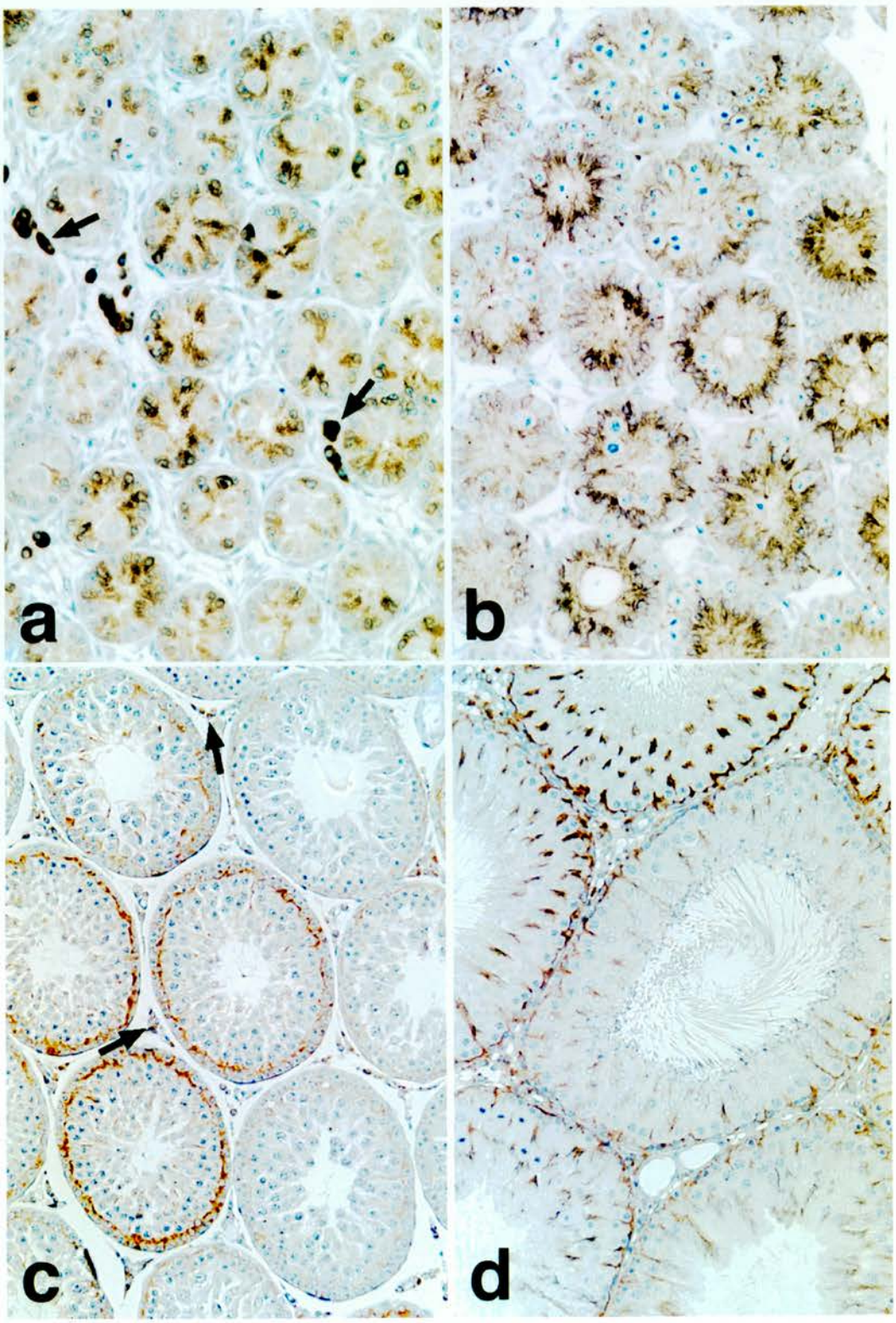
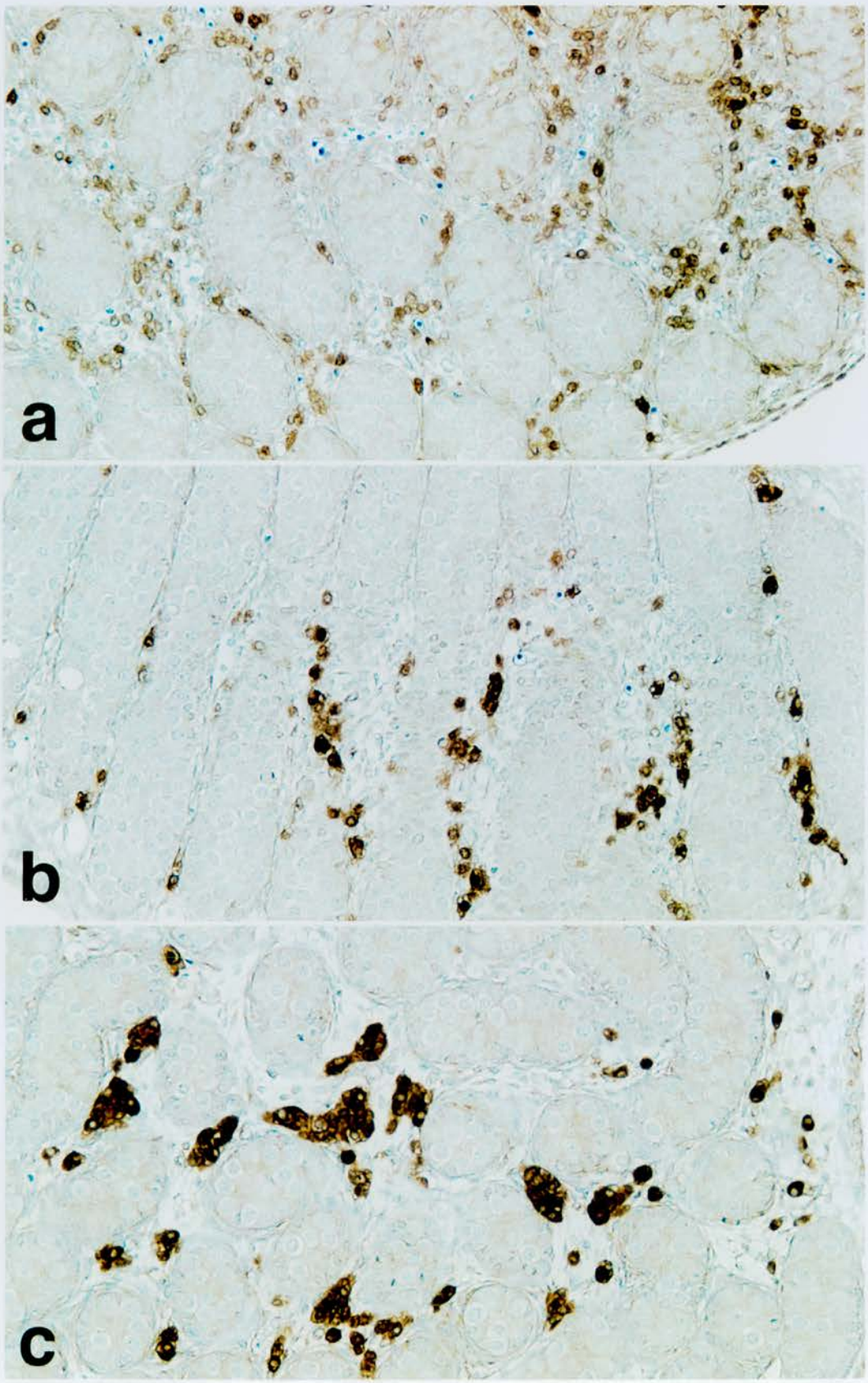


Fig. 7.2: Double fluorescent immunostaining with antibodies directed against 3ß-HSD and inhibin α-subunit demonstrate clear and exclusive colocalisation of 3ß-HSD enzyme (a) and inhibin α-subunit (b) in the fetal Leydig cells. Magnification x400.


a
b
c
d

Inhibin α subunit continued to be expressed in clusters of fetal Leydig cells for several days after birth (Fig. 7.3a). As development of the testis continued postnatally with replacement of fetal Leydig cells with those of the adult type, the amount of α subunit declined (Fig. 7.3b). In contrast, the amount of α subunit in Sertoli cells increased immediately after birth with strong immunostaining detectable by day 3 of postnatal life (Fig. 7.3a). Immunostaining in Sertoli cells remained strong throughout postnatal development and became stage specific around day 27 (Fig. 7.3c). In the adult testis, the immunostaining was stronger in Sertoli cells, however, weak immunostaining was also detectable in interstitial cells (Fig. 7.3c, d).

Fig. 7.3: Postnatally, strong expression of inhibin α-subunit appeared in Sertoli cells immediately after birth (a, day 3 postnatal). The staining remained strong throughout postnatal development (b, day 16; c day 27; d, day 90) and became stage specific at around day 27 (c). Immunostaining remained strong in the fetal type Leydig cells (a, arrow) but was weaker in adult-type Leydig cells (c, arrow). Magnification x200.


a
b
c

7.3.2 ßB-subunit

The ßB subunit was first detectable by immunocytochemistry with specific antibodies in fetal Leydig cells on day 16.5 (Fig. 7.4a). Immunostaining in the Leydig cells remained strong throughout fetal life (Fig. 7.4b, c) and, like the α-subunit, remained strong in fetal-type Leydig cells present neonatally but was weaker in their adult counterparts (Fig. 7.5c). Within the tubules inhibin ßB subunit was detected in the Sertoli cells, however, immunostaining was very weak throughout fetal life. Postnatally, immunostaining in the cytoplasm of Sertoli cells remained weak until day 27 (Fig. 7.5a, b, c). As with inhibin α-subunit, immunostaining for inhibin ßB subunit in the adult testis was strongest in Sertoli cell cytoplasm (Fig. 7.5).

Fig. 7.4: Inhibin ßB-subunit was first detectable by immunocytochemistry in the fetal testis on day 16.5 (a), two days after α-subunit was detected. Like the α-subunit, the immunostaining was most prominent in fetal Leydig cells. The staining pattern remained similar throughout the fetal life (b, day 17.5; c, day 20.5). Magnification x200.

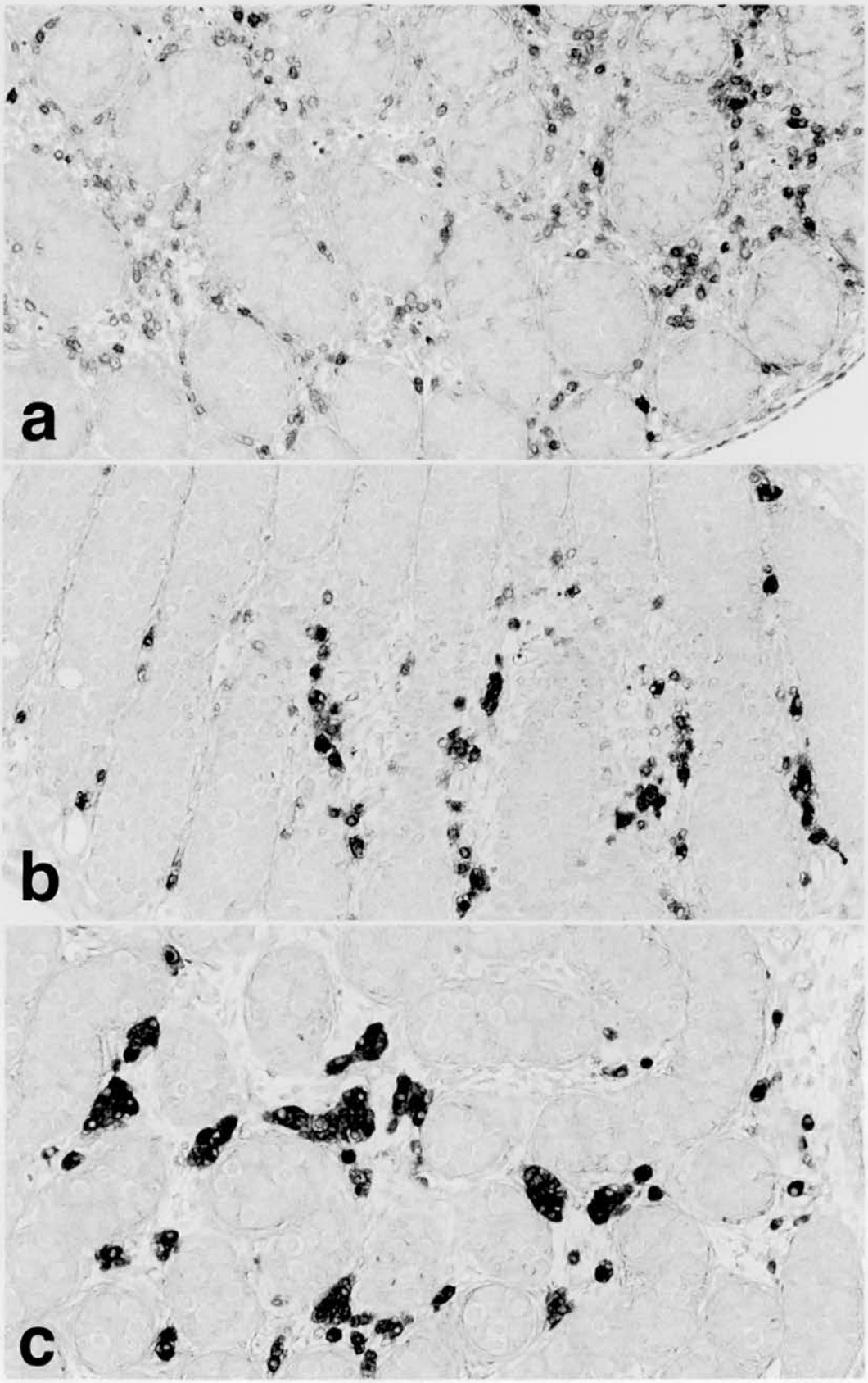
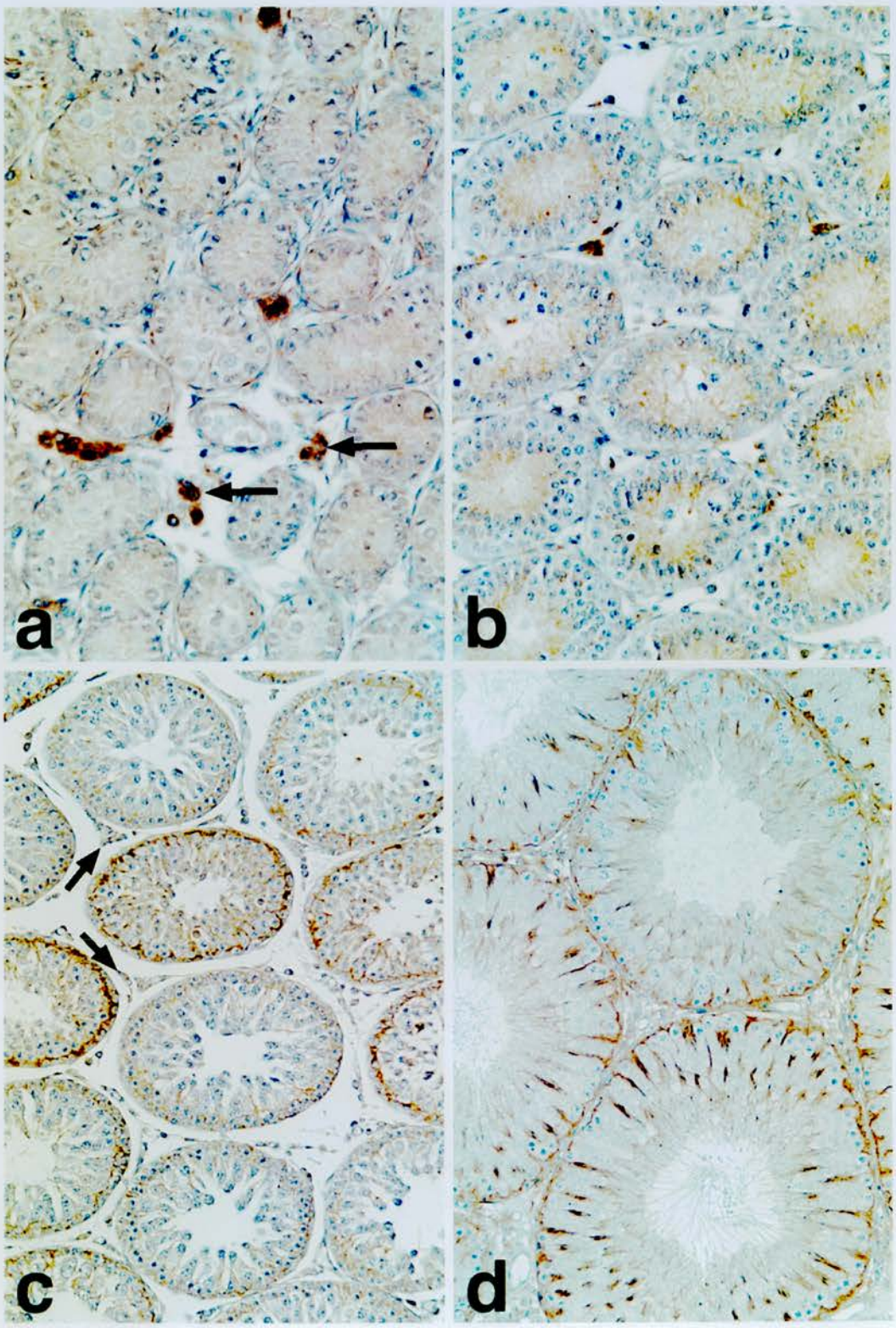

a
b
c

Fig. 7.5: Like inhibin α-subunit, immunostaining for ßB subunit was detected in Sertoli cells immediately after birth (a, day 3) but was much weaker than immunostaining for α-subunit. The amount of ßB subunit had increased in Sertoli cells by day 16 (b) and thereafter remained high throughout postnatal development (c, day 27) and in the adult testis (d, day 90). Immunostaining in the Leydig cells remained strong postnatally in the fetal type Leydig cells (a, arrow) but was much reduced in adult type Leydig cells (c, arrow). Magnification x200


a
b
c
d

7.3.3 ßA-subunit

Incubation with antisera specific for inhibin ßA-subunit did not result in any specific immunolocalisation in the rat testis during fetal and neonatal life. Similarly, no specific immunoexpression has been detected in adult rat testis.

7.3.4 Follistatin

No follistatin immunoexpression was detected in the fetal testis at any time prior to birth. However, immediately after birth, immunostaining was detected in the Leydig cells (Fig. 7.6a). Both the fetal and adult type Leydig cells remained immunopositive throughout postnatal development (Fig. 7.6b) and in the adult testis.

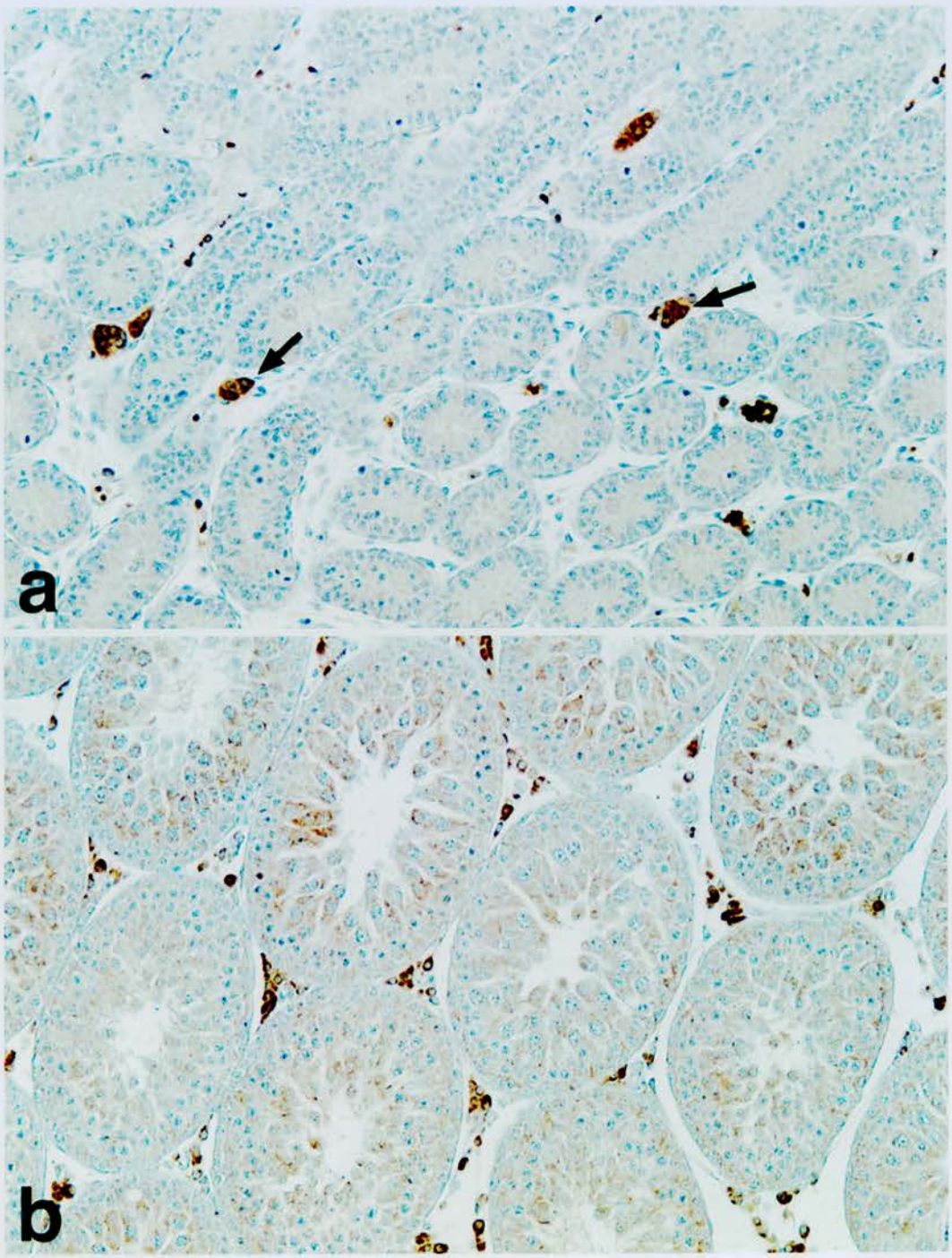


Figure 7.6: Follistatin immunoexpression was detectable immediately after birth in the fetal type Leydig cells (a, day 7, arrows) and immunostaining in the Leydig cells remained strong throughout life (b, day 27). Magnification x200.

7.4 Discussion

Inhibins and activins are dimeric glycoproteins, produced mainly in the gonads (Vale et al., 1988) which were initially identified as hormonal regulators of FSH secretion by the pituitary (Vale et al., 1988); Schwall, 1989). Several studies have subsequently demonstrated that in addition to their action on FSH secretion inhibins/activins play an important regulatory role at a paracrine level in several tissues/organs during fetal, neonatal and adult life (Chen, 1993; Wallach, 1996). The expression of inhibin/activin is not confined to the gonads; during embryonic development, the ß subunits are expressed in wide range of tissues including embryonic heart, salivary glands, neural tissues and the adrenal glands, as well as in the gonads (Roberts et al., 1991; Roberts and Barth, 1994). The inhibins/activins were initially isolated as Sertoli and granulosa cell products, modulating FSH secretion (Schwall et al., 1989; Vale et al., 1988). However, several studies have also reported the expression of inhibin and activin subunits in testicular interstitial cells pre- and postnatally in rat (Roberts et al., 1989; Shaha et al., 1989) and primate (Rabinovici et al., 1991). In addition, studies by de Winter et al. (1994) have shown expression of activin-A by peritubular cells in immature testis. Studies by Roberts and co-workers (1989) have detected α-subunit protein using immunocytochemistry in both Sertoli and interstitial cells in 12 day-old neonatal rats with the stronger immunostaining being in the Sertoli cells. Immunostaining with antibodies directed against ßB subunit produced a similar intensity of immunostaining in both interstitial and Sertoli cells but no staining was detected in clusters of Leydig cells. In addition, Shaha et al. (1989) did not detect any significant expression of α or ßB subunit proteins in interstitial cells of fetal and neonatal testis. In contrast, Roberts and co-workers (Roberts et al., 1991; Roberts and Barth, 1994) observed expression of α-subunit mRNA in interstitial and intratubular cells but detected ßB subunit mRNA expression only in the intratubular compartment and ßA mRNA only in the interstitial compartment later in gestation. However, Rabinovici et al. (1991) reported strong immunoexpression of α and ßB subunit proteins in the interstitial cells of the fetal testis at midgestation in the human and rhesus monkey with α-subunit also detected in Sertoli cells while ßB subunit appeared to be only in Leydig cells. The results described in this chapter demonstrate strong immunostaining by specific antisera of both α- and ßB subunits in cytoplasm of some of the interstitial cells within the fetal rat testis. Double fluorescent immunostaining using antibodies directed against 3ß-HSD clearly showed complete colocalisation of α subunit and 3ß-HSD confirming that steroidogenically active Leydig cells are the cells which also express inhibin α-

subunits. Preliminary observations suggest that the inhibin ßB subunit is also co-localised in the same fetal Leydig cell population. The first detectable immunoexpression of α-subunit was evident at a very early stage of testicular development, immediately following the formation of testicular cords. Immunoexpression of ßB subunit was detectable two days later and ßA subunit was undetectable at the time points studied. These results question the role of α-subunit expression at this early stage since the lack of expression of either ß subunits means no biologically active inhibin could be formed. It is possible that the early onset of synthesis of the α-subunit is part of a protective mechanism ensuring the formation of inhibin rather than activin once ß subunit expression commences. Recently it has been suggested that the N-terminal peptide of the α-subunit could have a regulatory paracrine function in the adult ovary (Russel and Findlay, 1995) and a similar role during fetal testicular development cannot be excluded. Alternatively, it is possible that small amounts of ßB subunit are produced at the same time as α-subunit but at levels that are undetectable by immunocytochemistry.

The detection of ßB immunostaining in fetal testis on day 16.5 p.c. would be consistent with the formation of inhibin B or activin B within the fetal gonad. However, at this time the expression of both subunits was confined to the fetal Leydig cells and occurred at a time when FSH expression is not detectable (Aubert et al., 1985; Watanabe and Daikoku, 1979) so that the role(s) of inhibin/activin in the fetus appear initially not to be confined to regulation of FSH secretion. Several studies have demonstrated potential effects of inhibins/activins on Sertoli cell proliferation (Boitani et al., 1995; Kaipia et al., 1994). A number of different studies agree that neither inhibin nor activin influence basal testosterone production by Leydig cells but could regulate hCG stimulated steroidogenesis although the action of inhibin and activin seems to be dependent on the time of exposure as well as the presence of LH or hCG (Hsueh et al., 1987; Lin et al., 1989). All these studies suggest possible autocrine and/or paracrine roles for inhibins/activins within the fetal testis. However, inhibin at least, does not appear to be essential for testicular development during fetal life as transgenic mice lacking a functional α subunit do not show any abnormalities at the time of birth (Matzuk et al., 1992).

The pattern of expression of both α- and ßB subunits changed dramatically after birth. While the level of immunostaining in the fetal type Leydig cells remained unchanged for several days, the expression of α-subunit in Sertoli cells had increased dramatically by day 3 of postnatal life. The cause of this increase is unknown but it could well be

connected with the reported increase in the levels of circulating FSH which occurs just prior to birth and continues postnatally in the rat (Aubert et al., 1985). FSH is known to stimulate inhibin/activin production (Ying, 1988); in the fetus, FSH can be detected around day 19.5 p.c. (Aubert et al., 1985; Watanabe and Daikoku, 1979) but its concentration is initially very low and only starts to rise just before birth. In light of the well established interrelationship between levels of inhibins/activins and FSH in the adult it is likely that the increased levels of FSH results in stimulation of α-subunit gene expression in Sertoli cells immediately after birth.

Follistatins were originally identified as proteins with the ability to suppress FSH secretion from the pituitary similarly to inhibins but with much lower potency (Robertson et al., 1987; Ueno et al., 1987). However, additional studies have shown their ability to bind activin (Kogawa et al., 1991; Nakamura et al., 1990) and it is thought that their primary function is the binding of bioactive activins to prevent their biological effects (Michel et al., 1993; Moore et al., 1994). Follistatins are expressed in the adult testis (Shimasaki et al., 1989) and pituitary (Kogawa et al., 1991) but studies by Roberts and Barth (1994) have failed to detect mRNA expression in the fetal testis. The immunocytochemical study presented in this chapter confirmed their finding with no immunodetection of follistatin in the fetal testis at any age. This would suggest that activin is required in the fetal testis for normal development or alternatively that no bioactive activin is produced in the fetal testis and therefore there is no need for follistatins to prevent its action. Strong immunoexpression of inhibin α subunit two days before the appearance of ßB subunit and very strong expression throughout gestation would support the second hypothesis, suggesting that α subunit is produced in excess to the ßB subunit and thus prevents the formation of activin. Several studies in vitro have shown the effects of activin in fetal testicular cells (Boitani et al., 1995; Kaipia et al., 1994), however, it is not known whether these effects are important for normal testicular development in vivo since bioactive activin may not be present in the fetal testis. Postnatally, the expression of follistatin was detectable in the Leydig cells immediately after birth. This is rather surprising since at that age the Leydig cells are still of the fetal type, found in the fetal testis. What triggers the expression of follistatin in these cells is not known. It is unlikely to be LH, which regulates the function of Leydig cells during late fetal life (Saez, 1994) since LH is already present in the fetus on day 17.5 of gestation (Aubert et al., 1985). FSH could have a role in stimulating the expression of α subunit in the Sertoli cells immediately after birth but as no FSH receptors are present on the Leydig cells, the same is unlikely to be true for follistatin expression. Therefore, the question of the

regulation of onset of follistatin immunoexpression remains unanswered and will require further studies. Studies by Boitani and co-workers (1995) have suggested that Sertoli cell proliferation is stimulated by activin in the presence of FSH. Postnatally, Sertoli cell proliferation in the rat testis starts to decline and ceases by day 18 postnatally. Therefore, the expression of follistatin in the testis postnatally could be involved in the regulation of the effects of activin on Sertoli cells by binding activin and therefore preventing its mitogenic action on Sertoli cells.

Fetal type Leydig cells differ from their adult counterparts in several respects (Saez, 1994). It is believed that postnatally, the fetal Leydig cells do not transform into their adult type counterparts but degenerate and are replaced by a new population of adult type Leydig cells which differentiate from interstitial cells and repopulate the developing testis (Saez, 1994). The data reported in this chapter suggest yet another difference between fetal type and adult type Leydig cells, the expression of inhibin subunits. Both the α and ßB subunits show a similar pattern of expression in the Leydig cells during neonatal life with immunostaining for both subunits remaining strong immediately after birth in clusters of Leydig cells. By around day 10-15 of life, very few Leydig cells are present within the testis and thereafter when the new Leydig cells repopulate the testis they are scattered between the tubules and not in clusters as seen during fetal life. The immunoexpression of α and ß subunits in this newly differentiated population of Leydig cells is much weaker than that seen in the clustered fetal-type cells during early life. The significance of this finding is unknown, but as discussed earlier, may be consistent with a role for inhibin/activin in the fetal Leydig cells as a paracrine or autocrine factor acting locally within the testis.

8. Effect of oestrogenic chemicals on the function of fetal Leydig cells

8.1 Introduction

Testosterone synthesis is a multistep process that requires the action of several enzymes to transform cholesterol into testosterone (Miller, 1988). The first step in the process is the transport of cholesterol into the inner mitochondrial membrane; a protein called StAR (**St**eroidogenic **a**cute **r**egulatory protein) has been recently identified as a factor involved in this step. StAR is thought to function as a carrier of cholesterol to facilitate its transport from the outer to the inner mitochondrial membrane and this appears to be the limiting step in acute regulation of steroidogenesis (Lin et al., 1995). The first enzymatic step in steroidogenesis is the conversion of cholesterol to pregnenolone, a reaction catalysed by the enzyme cytochrome P450 side chain cleavage (P450scc). Pregnenolone then diffuses across the mitochondrial membranes and in the smooth endoplasmic reticulum is converted to progesterone by the action of 3ß-hydroxysteroid dehydrogenase (3ß-HSD). The next step in the enzymatic cascade is catalysed by another cytochrome P450 enzyme, 17α-hydroxylase/C_{17-20} lyase (P450c17), a single protein that catalyses two distinct reactions, the hydroxylation of progesterone at C_{17} followed by the cleavage of the two-carbon side-chain to yield the C_{19} steroid, androstenedione, the immediate precursor of testosterone. The final enzymatic reaction is the reduction of androstenedione by 17ß-hydroxysteroid dehydrogenase (Miller, 1988). The expression of the enzymes involved in testosterone synthesis appears to be regulated by different mechanisms; testosterone production seems to be regulated principally by regulation of the expression and activity of P450scc and P450c17 while the enzymes 3ß-HSD and 17ß-hydroxysteroid dehydrogenase appear to be constitutively expressed (Keeney et al., 1988; Payne and Youngblood, 1995). Expression of P450c17 in adult Leydig cells is a tightly regulated process, dependent upon the stimulation of the cells by LH via its specific receptor and mediated by cAMP (Payne and Youngblood, 1995). In the absence of LH the expression of P450c17 ceases, in contrast to that of P450scc which is constitutively expressed at a low level even in the absence of LH (Payne and Youngblood, 1995). Testosterone (Darney et al., 1996; Hales et al., 1987; Pointis et al., 1984) and oestrogens (Bartke et al., 1977; Moger, 1980) both exert inhibitory effects on steroidogenesis in adult rat Leydig cells but oestrogens appear to have no such effect on mouse testis (Hales et al., 1987). Several studies have shown that in adult animals the inhibitory effects of testosterone (Darney et al., 1996; Hales et al., 1987) and oestrogens (Kalla et al.,

1980; Nozu et al., 1981a) on testosterone biosynthesis are largely explained by reduced expression of cytochrome P450c17 enzyme.

In the fetal testis, expression of P450c17 appears to be locally regulated. While LH modulates testosterone production in the fetal testis (Feldman and Bloch, 1978; Habert, 1993) at the later developmental stages, it is not expressed when the onset of testosterone production and P450c17 expression occurs (Aubert et al., 1985; Watanabe and Daikoku, 1979). The factor(s) that trigger testosterone production and/or expression of P450c17 are not known. Studies on isolated gonads have shown that testosterone production starts autonomously; when the indifferent genital ridges were dissected and cultured in vitro, testosterone production occurred in the absence of any external factors (Agelopoulou et al., 1984; Gangnerau and Picon, 1987). One candidate as a local regulator of cytochrome P450s enzymes is steroidogenic factor-1 (SF-1) (Lala et al., 1992; Morohashi et al., 1992). Sequences which bind SF-1 have been found on several cytochrome P450 genes: P450scc, P450c17, P450c21 and P450aromatase (Clemens et al., 1994; Lynch et al., 1993; Morohashi et al., 1992; Zhang and Mellon, 1996). The SF-1 gene is expressed in fetal and adult steroidogenic tissues (Hatano et al., 1994; Ikeda et al., 1994; Morohashi et al., 1994) and studies in vitro have demonstrated its importance in regulation of expression of the cytochrome P450s (Clemens et al., 1994; Lynch et al., 1993; Zhang and Mellon, 1996). SF-1 mRNA and protein are present in the fetal gonads, adrenal glands, hypothalamus and pituitary gland (Hatano et al., 1994; Ikeda et al., 1994; Ingraham et al., 1994). SF-1 is expressed in the genital ridges of both sexes but following gonadal differentiation expression in the ovary almost disappears. Expression of SF-1 in the testis remains strong throughout the fetal life (Hatano et al., 1994; Ikeda et al., 1994) in Leydig cells, other interstitial cells and Sertoli cells (Hatano et al., 1994). The exact functions of SF-1 as well as the regulation of its expression and action are not known, however its ability to influence the expression of steroidogenic enzymes in vitro makes it a likely candidate for a tissue specific regulator of steroidogenesis and as a possible mediator of the action of extracellular factors. Consistent with its pattern of expression and an important role in development mice lacking a functional SF-1 gene fail to develop gonads or adrenals and have a disorganised hypothalamus and nonfunctional pituitary gonadotrophs (Ingraham et al., 1994; Luo et al., 1994)

A possible link between the reported increase of reproductive abnormalities in Western men and oestrogens has been outlined in chapter 2. Some of the

abnormalities which have been recorded, including hypospadias and cryptorchidism, might be the result of reduced or inappropriate exposure to testosterone during periods of fetal life critical for normal masculinisation of the male fetus. It is therefore possible that an increased exposure to oestrogens or chemicals that mimic oestrogen action during fetal life could interfere with regulation of steroidogenesis in the fetal Leydig cells and consequently result in impaired production of testosterone.

Octylphenol polyethoxylate is one example of a group of chemicals known as alkylphenol polyethoxylates that were introduced in the 1940s and which are the second largest group of nonionic surfactants in commercial production. These chemicals are widely used in detergents, paints, herbicides and pesticides. Large amounts of octylphenol polyethoxylate are produced worldwide and a significant amount of this substance ends up in the aquatic environment, where APEOs usually form relatively stable metabolites like nonylphenol and octylphenol (OP) which tend to accumulate in sewage sludge and river sediment (Naylor et al., 1992). Several studies have shown that both nonylphenol and OP have a weak oestrogenic activity. They can stimulate growth of breast cancer cells in vitro, bind to the oestrogen receptor, and stimulate transcriptional activity of reporter genes containing an oestrogen response element (White et al., 1994). Furthermore, when tested in vivo on fish, OP has been shown to inhibit testicular growth in male trout and to stimulate the production of vitellogenin, an oestrogen inducible protein normally produced only by female fish and incorporated in egg yolk (Jobling et al., 1996). Sharpe et al. (1995) have used rats to study the effect on male offspring of OP-treated mothers. Female rats were treated with OP via drinking water for 8-9 weeks in the period before mating, throughout gestation and postnatally until weaning. In these experiments, treatment with low doses of either DES or OP resulted in a small but significant effect on testicular size and daily sperm production in adult offspring, however, none of the treatments had any adverse effect on testicular morphology.

The aim of the studies described in this chapter was therefore to assess the effects of oestrogen exposure at the level of gene expression in the developing fetal testis as a first step towards understanding the mechanism(s) underlying the observed effects of oestrogenic chemicals.

8.2 Experimental procedures

8.2.1 Animals, treatments and tissue recovery

Female rats were time mated as described in chapter 3. Pregnant females were injected subcutaneously on day 11.5 and 15.5 p.c. with diethylstilbestrol (DES; 100 or 500μg/kg) or 4-octylphenol (OP; 100 or 600 mg/kg) in corn oil (1ml/kg) or with the vehicle alone (controls). Pregnant females were killed on day 17.5 p.c. by inhalation of carbon dioxide and subsequent cervical dislocation. Fetuses were examined under a dissecting microscope and the testes recovered. Testes were either immersion fixed in Bouin's solution for 5-6 h prior to processing for immunocytochemistry and in situ hybridisation or used for extraction of RNA (sections 3.3.2 and 3.4) or determination of enzyme activity (section 8.2.6).

8.2.2 Immunocytochemistry

Immunocytochemical staining was performed as described in chapters 3 and 6.

8.2.3 Reverse transcription plus polymerase chain reaction

Primers based on the sequence of rat 17α–hydroxylase [upstream 5'-CTTGTCGGA CCAAGGAAAAGGCGT (bases 344-367, sense strand) and downstream 5'-AGCC AGCTGATCGTGCAGC (bases 669-692, antisense strand)] and primers based on the bovine SF-1 sequence [upstream 5'-GACCAGATGACACTGCTGC (bases 996-1014, sense strand) and downstream 5'-AACTCCTGGCGGTCCAGTT (bases 1204-1222, antisense strand)] were synthesised on an Applied Biosystems PCR mate (ABI). Reverse transcription was undertaken as described in chapter 3 (section 3.6.2) using the RNA samples from 17.5 days old fetal testes. The conditions used with both sets of primers were 65°C 5 min, 53°C 5 min, 70°C 15 min and samples were then placed on ice. The reaction volume was increased to 100μl and PCR undertaken as described in chapter 3. PCR conditions for both sets of primers were denaturation at 95°C for 3 min, followed by 30 cycles of amplification 94°C 0.5 min, 55°C 1 min, 70°C 2 min with a final elongation at 70°C for 10 min. The expected size of the amplified products was 348 bp for 17α-hydroxylase and 226 bp for SF-1. Amplified product(s) were analysed by electrophoresis on a 2% agarose gel run in TBE using standard methods.

The resultant cDNAs were subcloned into the pCRII vector according to the manufacturer's instructions (InVitrogen) as described in chapter 3 (section 3.6.3). The identity of cloned cDNAs was confirmed by sequencing using an ABI automated sequencer and fluorescent primers as described in chapter 3 (section 3.10).

8.2.4 Riboprobe synthesis

To synthesise sense and antisense riboprobes 17α-pCRII or SF-1-pCRII were linearised with Xho I and Bam H1 restriction enzymes and cRNAs transcribed with SP6 or T7 RNA polymerase (Boehringer) respectively. Single stranded RNA synthesised from 17α-pCRII was labelled with ^{35}S-UTP (400 Ci/mmol; Amersham) and purified as described in chapter 3 (section 3.8) using RNA spin columns (IBI) and the efficiency of the labelling determined using a beta counter (LKB). Antisense RNA synthesised form SF-1-pCRII was labelled with ^{32}P-UTP (NEN-Dupont) and purified on a polyacrylamide gel (section 3.11.1)

8.2.5 In situ hybridisation

Paraffin sections (5μm) were mounted on slides coated with TESPA (Sigma), dried overnight at 50°C, dewaxed in xylene and rehydrated in graded ethanols. In situ hybridisation was performed essentially as described in chapter 3 (section 3.9). Sections were hybridised with 10^6 cpm ^{35}S-cRNAs in 40 μl hybridisation buffer at 50°C overnight. Next day, sections were washed in 4X SSC for 10 min at room temperature, incubated in RNase A (Sigma) solution (2mg/ml in 0.01M Tris-HCl, 0.05M NaCl, 5mM EDTA pH 8.0) for 30 min at 37°C, washed 2 times for 5 min each in 2X SSC at room temperature and finally washed in 0.1X SSC/30% formamide for 30 min at 40°C. Sections were dehydrated in graded ethanols, air dried, dipped in undiluted photographic emulsion (NTB-2, Kodak) and exposed at 4°C in a light tight box for 2 weeks and processed as described in chapter 3. To ensure that all the sections were hybridised and washed under the same conditions, sections from control and treated animals were mounted on the same slide.

Tissue sections were observed under bright field illumination using an Olympus BH2 microscope and a x40 objective. Images were captured into computer using a datatranslation quick capture card and a Macintosh IIcx computer and quantified using NIH image 1.57 computer software. To determine the abundance of P450c17

mRNA, the number of silver grains in 10 fields in the interstitial region of the testis was counted. Background counts were determined by counting 10 random fields in regions of the same sections that were outside the interstitium, e.g. mesonephros, and the average of the grain counts in these regions was deducted from the grain densities determined for the interstitial regions. The silver grains were counted in 2-3 testes from 3 different litters for each treatment group, and the group mean ± SEM were calculated. Sections from the same testes were also probed with sense strand probes and no differences between grain densities in the different regions of the sections were observed.

8.2.6 RNase protection assay

The RNase protection assay was performed as described in chapter 3 (section 3.11); 2.5µg of total RNA was mixed with 5 x 10^5 cpm of purified probe and hybridised overnight at 50°C. Any single stranded RNA was digested, remaining double stranded RNA precipitated and run on a 6% polyacrylamide gel as described in chapter 3. The gel was dried and exposed to autoradiographic film or a phosphorescent screen; results were quantified and analysed using a Phosphorimager (Molecular Dynamics). The quantified data from four experiments (RNAs from four different litters for each treatment group) were pooled together and analysed.

8.2.7 Measurement of 17α-hydroxylase enzyme activity

The activity of the 17α-hydroxylase enzyme was measured as described previously (O'Shaughnessy and Murphy, 1991). Fetal testes from two animals (4 testes) were pooled and homogenised in 0.4ml phosphate buffer saline (0.05M PBS, pH 7.6). As a substrate for the enzyme, 10^6 cpm [^{3}H] progesterone (88Ci/mmol, Amersham) were dissolved in 35 µl DMSO and mixed with 0.87ml phosphate buffer (0.05M, pH 7.2) containing NADPH (1mmol/L, Sigma). Homogenised sample (100µl) was mixed with tritiated progesterone in phosphate buffer and incubated at 37°C for 20 min. Nonradioactive progesterone (Sigma) was added to the incubation mixture to give a final substrate concentration of 2µM, which previous studies have shown to be saturating (O'Shaughnessy and Murphy, 1991). Reactions were stopped by adding 100µl of 1M NaOH into each tube on ice. To allow determination of steroid recovery, ^{14}C-labelled testosterone (50Ci/mmol) and androstendione (50Ci/mmol, Amersham) were added (2000 cpm of each to each reaction) and the steroids extracted from the solution using 5ml volume of toluene (AnalaR, BDH). The

organic phase was recovered, dried by heating to 45°C under a stream of nitrogen, dissolved in few drops of methanol and applied to plastic backed silica gel plates (Whatman, Maidstone, Kent). Steroids were separated by TLC in chloroform-ether (7:1, vol/vol). Fluorescent bands (detecting under UV light) corresponding to 17-hydroxy-progesterone, androstendione and testosterone were cut from the plates, placed in pony vials (Packard Instrument Company, Caversham) and mixed with 6mls of scintillation fluid (Ecoscint A, National Diagnostic). Samples were left for 30 min at room temperature to allow steroids to elute into the scintillation fluid. The amount of each radiolabelled steroid was determined on a beta counter using a program to detect both ^{3}H (counting range 5-304KeV) and ^{14}C (counting range 325-765KeV) simultaneously. The activity of 17α-hydroxylase was expressed as pmol of radiolabelled progesterone converted by one testis in 1 min. Controls were run in each experiment using PBS without added tissue. The 17α-hydroxylase activity was measured in eight separate pools of four testes from five different litters for the control group and in six separate pools of four testes from three different litters for each of the treatment groups.

8.2.8 Statistical analyses

Data from each experiment were subjected to ANOVA to determine whether there were significant effects of treatment. Where this was indicated, subgroup comparisons between means for control and individual treatment groups were then made using the variance from the experiment as a whole as the measure of error.

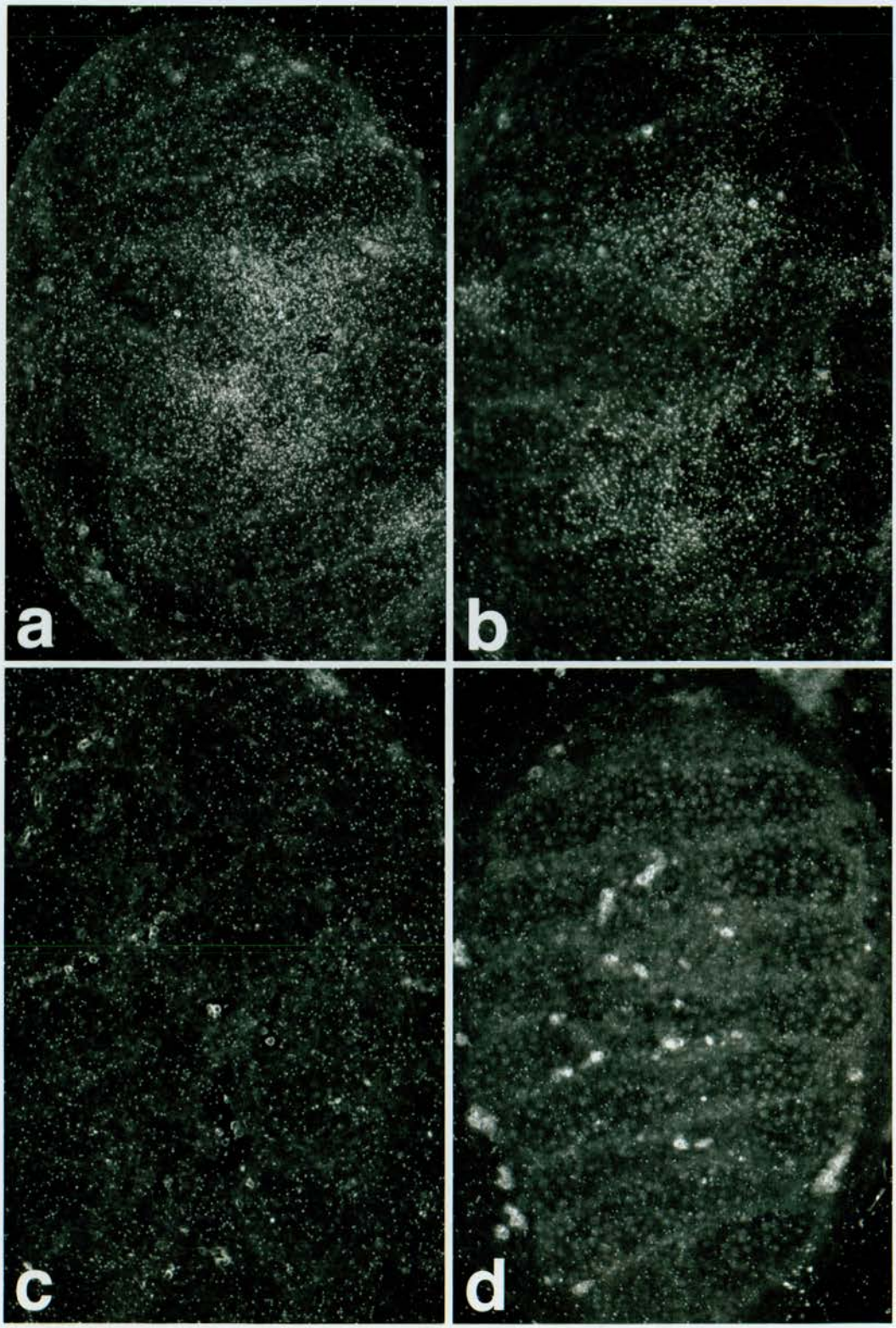
8.3 Results

8.3.1 Expression of mRNAs for P450c17 and SF-1

In situ hybridisation using a specific radiolabelled cRNA directed against rat P450c17 resulted in detection of abundant silver grains, as expected, in the interstitial region of the day 17.5 fetal testis after exposure to photographic emulsion for 2 weeks (Fig. 8.1). Less silver grains were detected in fetal testis from the animals treated with DES (Fig 8.1c) when compared with controls (Fig. 8.1a) or with low dose OP (Fig. 8.1b). Sense controls did not show any signal above background (Fig. 8.1d). The results were confirmed and extended by image analysis quantification and were subjected to statistical analysis (Fig. 8.2). Quantification of results showed a statistically significant ($p<0.001$) reduction of expression in

animals treated with 500μg/Kg of DES but no significant difference between control animals and animals treated with low dose of OP.

Fig. 8.1: In situ hybridisation using ^{35}S-labelled cRNA to rat P450c17 mRNA on sections of fetal rat testis. Panel a, control, panel b, OP treatment (100 mg/Kg) of mother, panel c, DES treatment (500 μg/Kg) of mother. A strong concentration of grains was present in the control rat testis and brightfield illumination revealed that they were concentrated mainly within the interstitial region of the testis. The specific hybridisation signal appeared reduced in the testes of males deriving from mothers treated with 500 μg/Kg DES (c). The sections probed with the sense probe did not show any signal above background (d). Magnification x200.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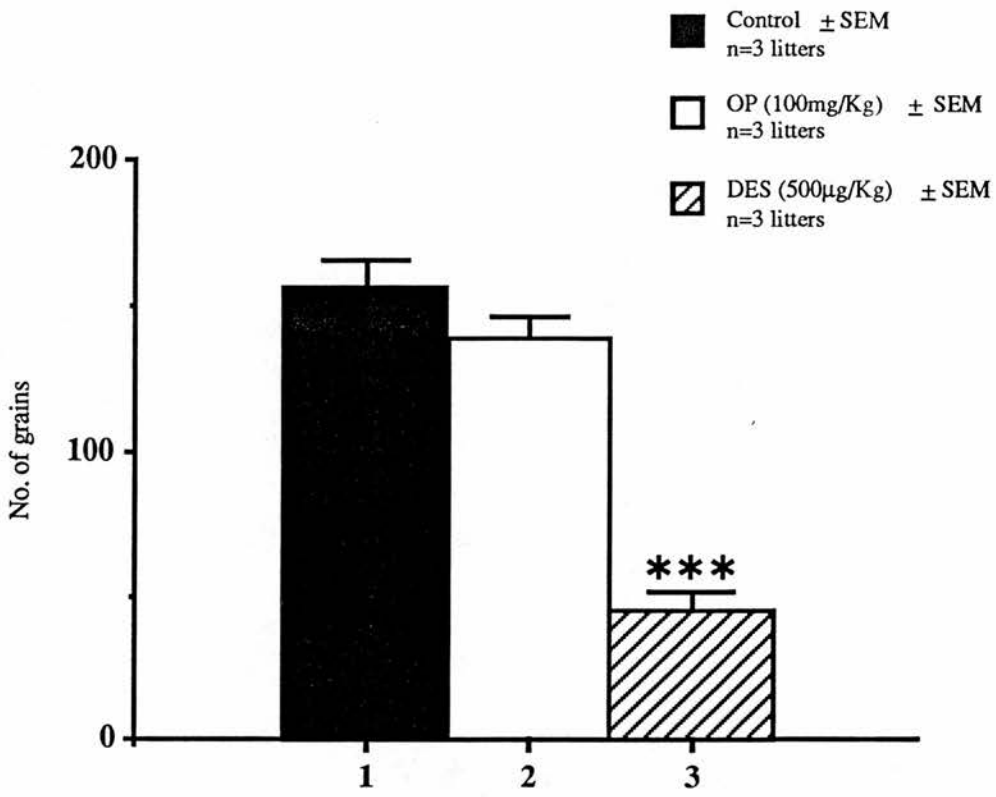


Fig. 8.2: Quantification of the results obtained by in situ hybridisation for P450c17 in fetal rat testis. The number of silver grains detected was reduced significantly in animals from mothers exposed to 500 µg DES (column 3, $p<0.001$) compared with controls (column 1). Maternal treatment with 100mg/Kg OP resulted in a slight reduction in grain counts but this did not reach significance (column 2). Testes from three animals from each litter were analysed and the mean for number of grains in each field of view calculated.

RNase protection assay using a specific radiolabelled cRNA directed against rat SF-1 resulted in bands of double stranded RNA after electrophoresis and exposure to autoradiographic film (Fig. 8.3). The amount of SF-1 mRNA appeared to be reduced in the testes from both OP and DES treated animals compared with controls. In contrast, the amount of mRNA in the ovary appeared to be increased following the exposure to OP but remain unchanged in the ovary from animals treated with DES (Fig. 8.3, upper panel). As a control, 18S mRNA was simultaneously detected in RNase protection reactions and was used to adjust the results of quantification

(Fig. 8.3, lower panel). Quantification of the results showed a significant reduction ($p<0.05$) in the amount of SF-1 mRNA in testes from both treated groups when compared to controls (Fig. 8.4). The increase in the expression of SF-1 mRNA in the ovary, however, did not reach statistical significance (Fig. 8.5).

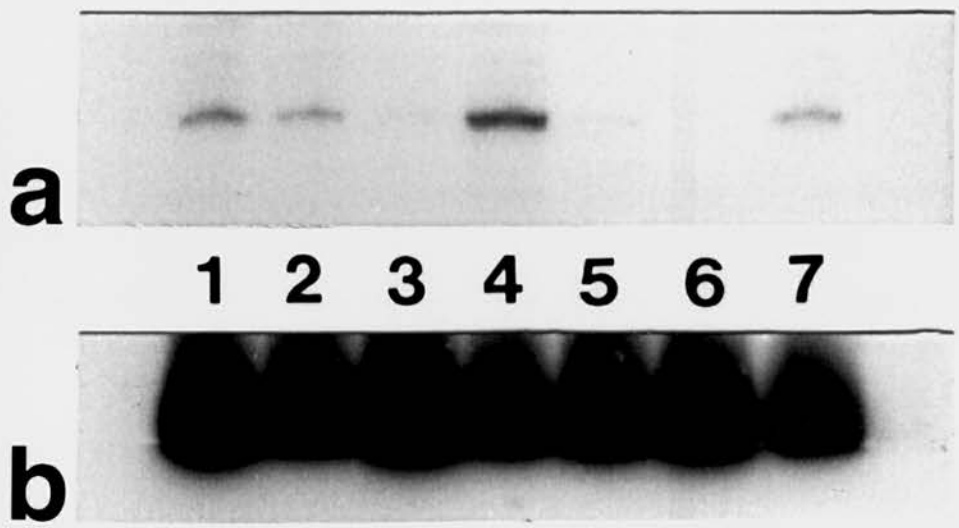


Fig. 8.3: Results obtained by RNase protection assay using SF-1 cRNA (a) and 18S cRNA (b) revealed that whilst a similar amount of total RNA was present in all samples (b) the amount of SF-1 mRNA varied with treatment (a). SF-1 mRNA was higher in fetal testes from controls (lane 1) than in those from DES- or OP-treated mothers (lanes 2 and 3 respectively). In contrast, whilst the amount of SF-1 mRNA in fetal ovaries from control (lane 5) or DES-treated (lane 6) mothers was lower than that in the testis (lane 1), ovaries in fetuses in OP-treated mothers showed increased SF-1 mRNA expression (lane 7) compared with control ovaries (lane 5). A sample of adult rat adrenal (lane 4) was included as a positive control. Pregnant rats were treated with either 500µg/Kg of DES or 100mg/Kg of OP.

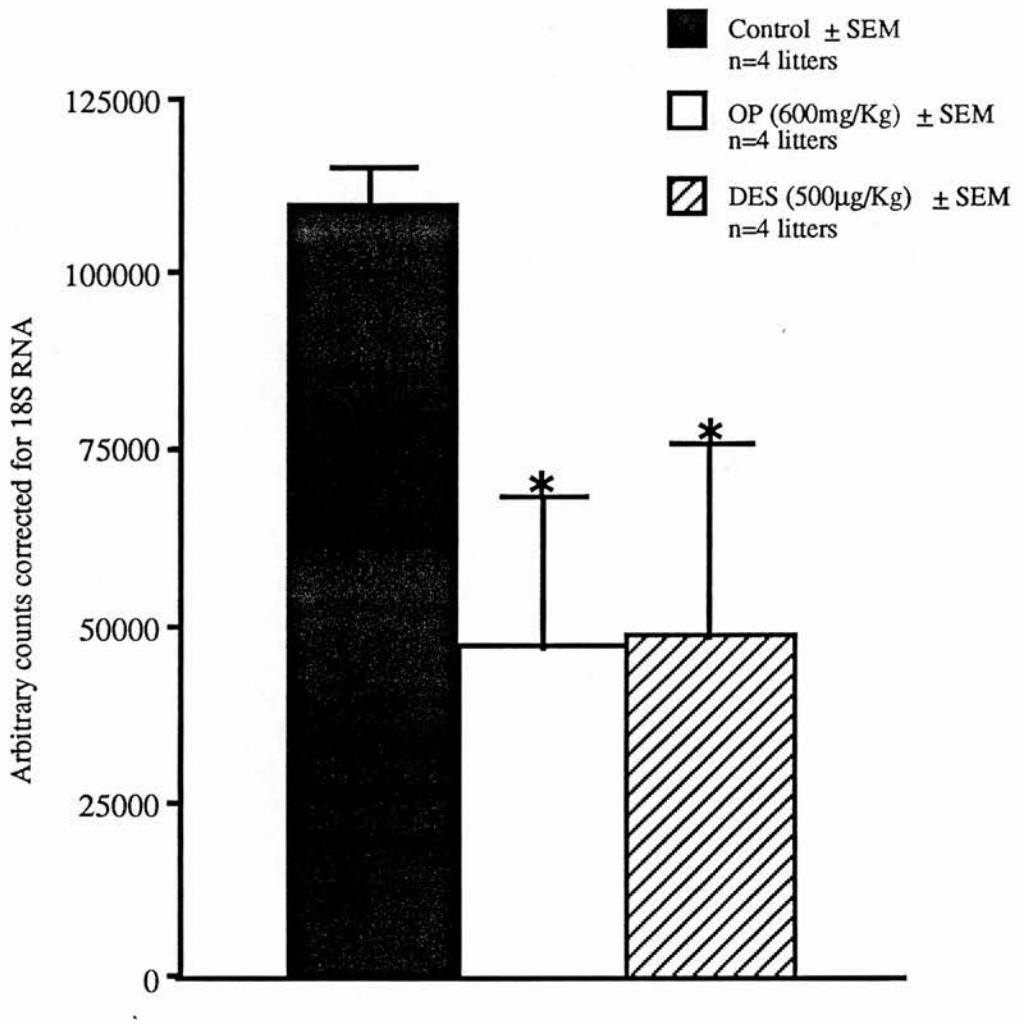


Fig. 8.4: SF-1 mRNA in testes from the fetuses of OP and DES-treated mothers were reduced significantly ($p<0.05$; *) compared with controls. The amount of specific signal for SF-1 mRNA was quantified using a phosphorimager (Molecular Dynamics) and corrected for total RNA by comparison to the signal obtained for 18S ribosomal RNA.

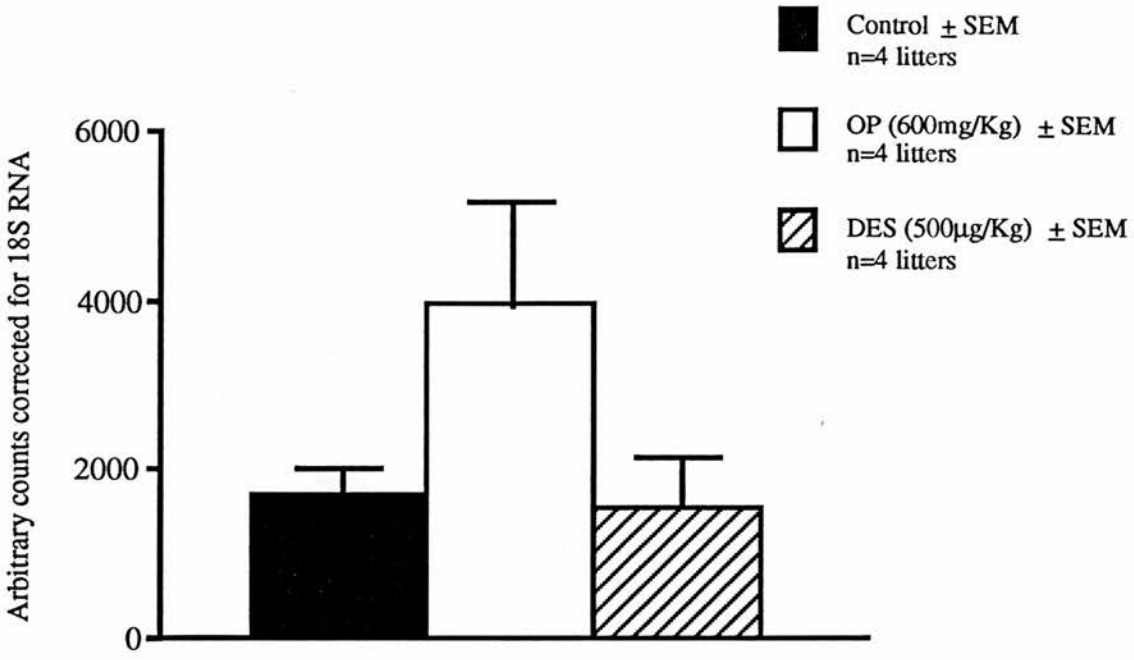


Fig. 8.5: SF-1 mRNA in ovaries from the fetuses of control and DES-treated mothers were comparable, however that in the ovaries from the fetuses of OP-treated mothers appeared to be increased, though this just failed to reach statistical significance.

8.3.2 Detection of P450c17 and 3ß–HSD proteins in the fetal testis

The gross histology of the testes recovered on day 17.5 of gestation from treated and control animals appeared similar. Testes from fetal males recovered from untreated mothers contained numerous Leydig cells which were stained strongly following immunohistochemistry with primary antibodies directed against the steroidogenic enzymes P450c17 (Fig. 8.6) and 3ß-HSD (Fig. 8.7). Specific immunostaining of P450c17 within fetal Leydig cells appeared to be the same in fetal testes of untreated control mothers (Fig. 8.6a) and those from mothers treated with 100mg/Kg OP (Fig. 8.6b) or 100µg/Kg DES (Fig. 8.6d). However there was a marked reduction in immunostaining when testes were recovered from fetuses of mothers given 600mg/Kg OP (Fig. 8.6c) or 500µg/Kg DES (Fig. 8.6e). In contrast, when fetal testes were immunostained for 3ß-HSD there was no apparent reduction in positive

immunostaining in testes of fetuses from treated mothers (Fig. 8.7b and c) compared with controls (Fig. 8.7a).

Fig. 8.6: Immunocytochemical staining of Leydig cells in fetal rat testis using antibodies directed against P450c17. Panel a, testis from control group, panels b and c, testes from fetuses of mothers treated with 100mg/kg and 600 mg/Kg OP respectively, panels d and e, testes from fetuses of mothers treated with 100µg/kg and 500 µg/Kg DES respectively. Specific immunostaining of P450c17 was reduced in fetuses from the higher dose treatment groups (c, e) compared with controls (a). Magnification x200 (a), x120 (b-e).

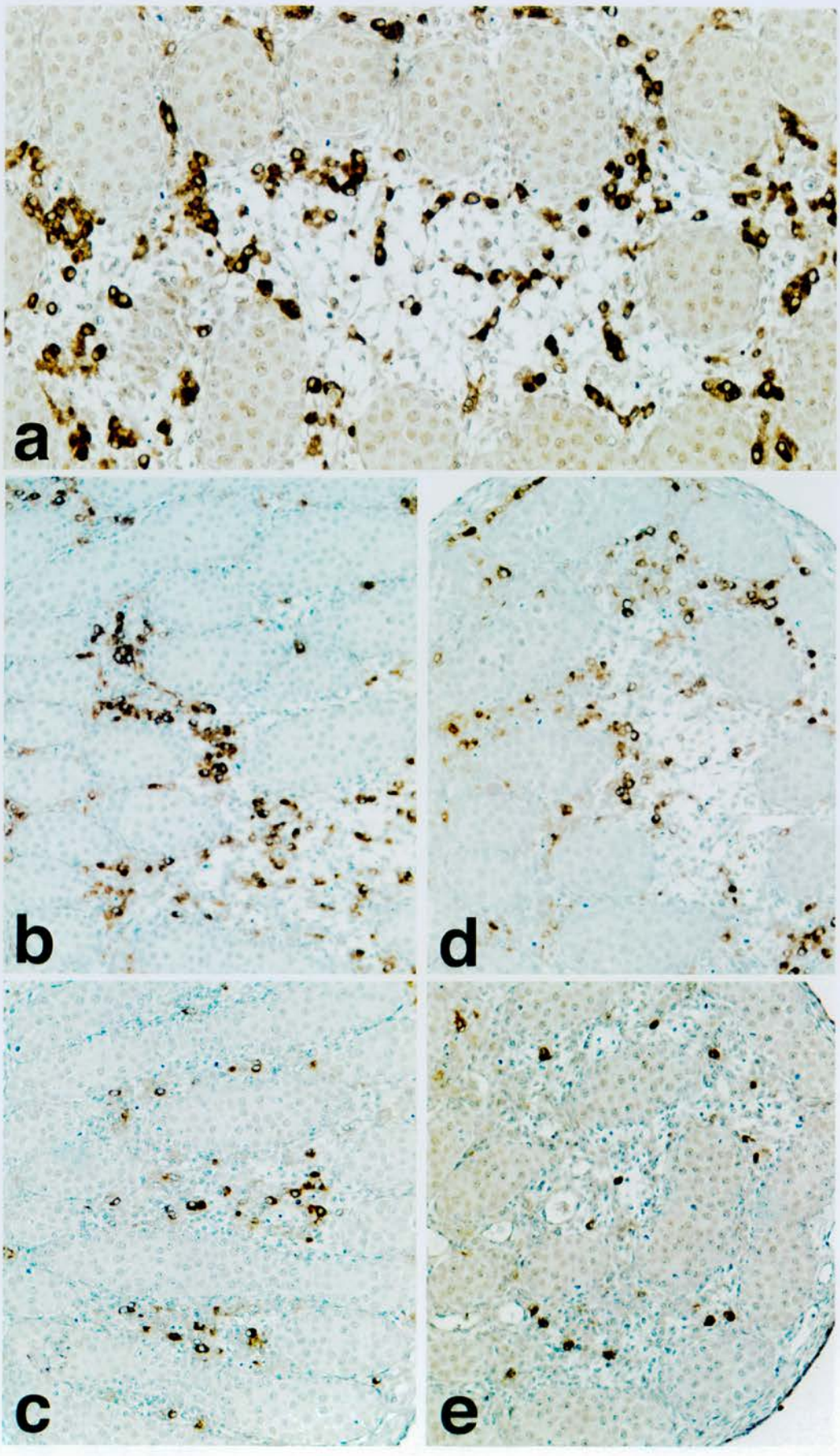
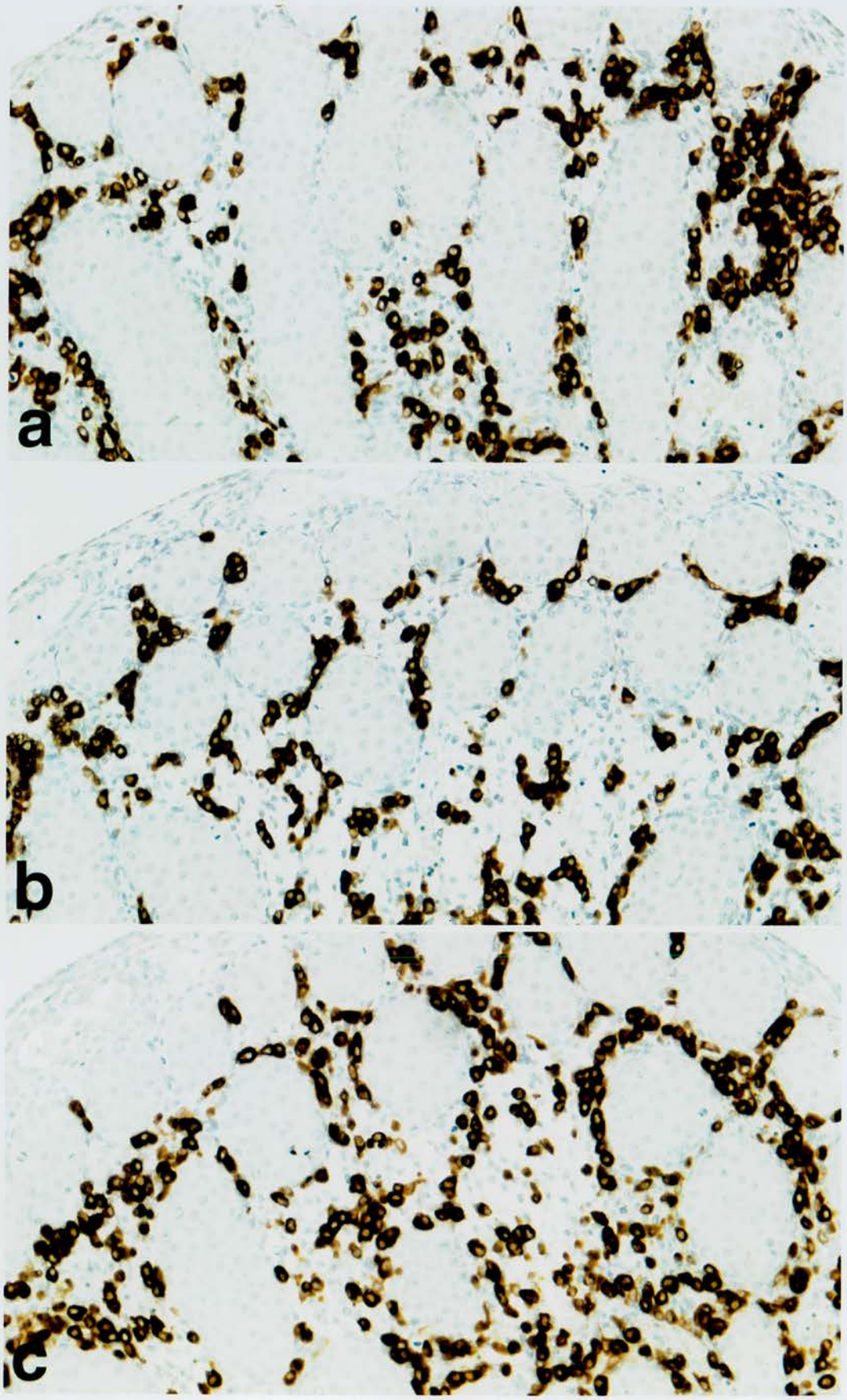

a
b
d
c
e

Fig. 8.7: Immunocytochemical staining of Leydig cells in fetal rat testis using antibodies directed against 3ß-HSD. Panel a, testis from control, panel b, from OP 600mg/Kg treatment group, panel c, testis from DES 500 µg/Kg treatment group. Note that numbers of Leydig cells and the abundance of 3ß-HSD appears the same in all groups. Magnification x200.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8.3.3 Enzymatic activity of 17α-hydroxylase in fetal testes from control and treated mothers

17α-hydroxylase activity was measured in 4 testes pooled from two fetuses. Eight such pools from five litters were analysed for the control group and 6 pools from 3 litters were analysed for each treatment group.

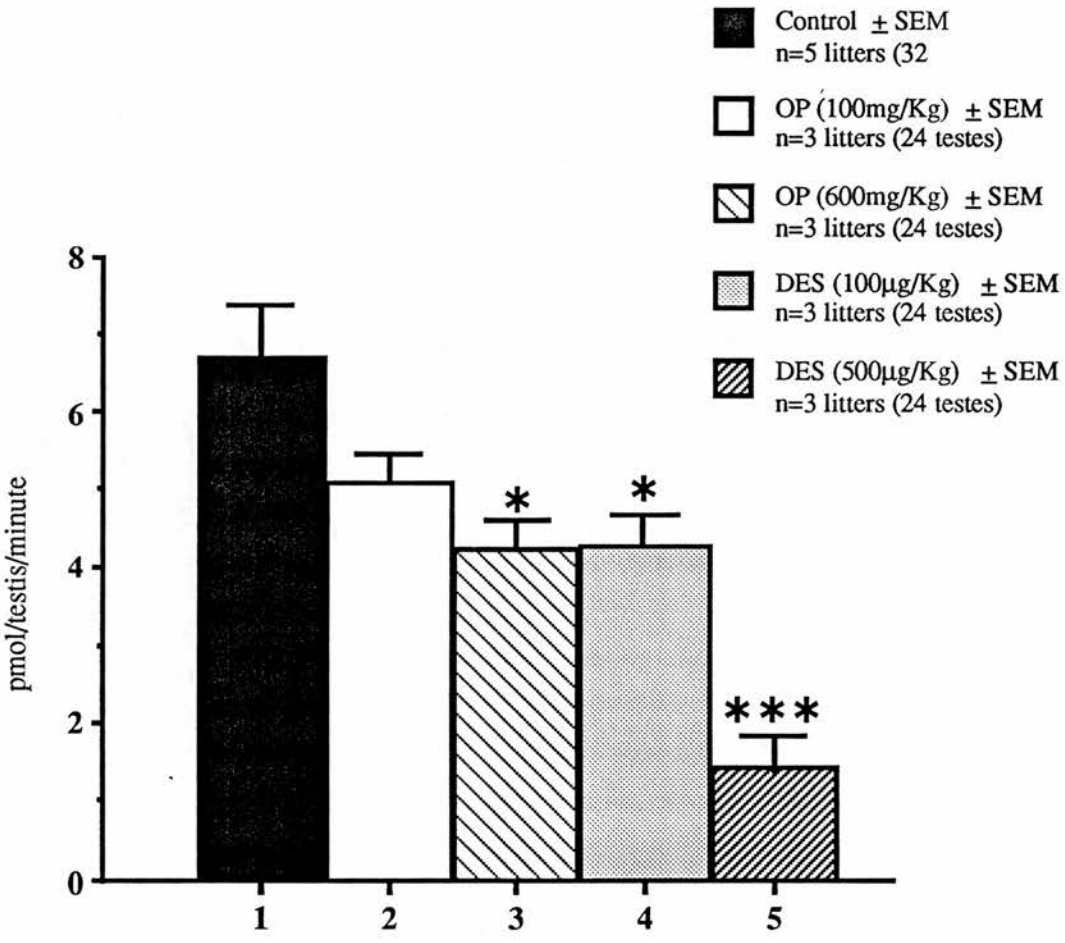


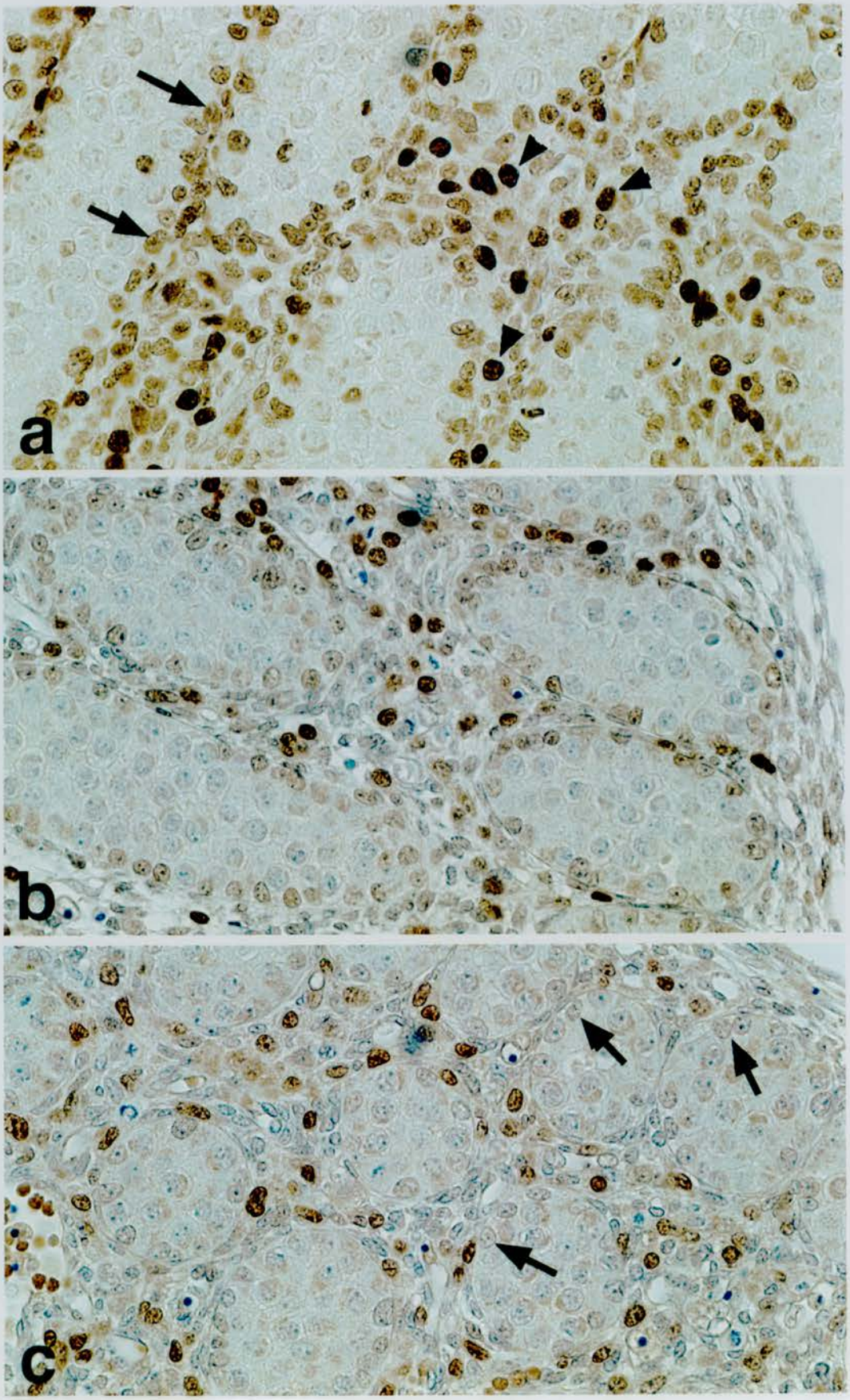
Fig. 8.8: Level of 17α-hydroxylase enzyme activity in fetal testes isolated on day 17.5 of gestation from mothers treated with vehicle (=control, column 1), OP (600 mg/Kg or 100 mg/Kg; columns 2, 3) or DES (500 µg/Kg or 100 µg/Kg; columns 4, 5) show significantly reduced activity in the animals treated with DES or high dose of OP. Each bar is the mean ± SEM for 8 (control group) or 6 (all treated groups) determinations using tissue pooled from 2 male fetuses per determination.
* $p<0.05$, *** $p<0.001$, in comparison with control group.

Enzyme activity in animals treated with the high (500μg/Kg) or low (100μg/Kg) doses of DES was reduced by 78% ($p<0.001$) or 30% ($p<0.05$) respectively, when compared to enzyme activity in control animals. In animals treated with the high (600mg/Kg) dose of OP, enzyme activity was reduced by 30% ($p<0.05$) while the lower dose (100mg/Kg) of OP resulted in an 18% reduction ($p<0.07>0.05$) in enzyme activity compared to control animals (Fig. 8.8).

8.3.4 Detection of SF-1 protein in the fetal testis

Staining with antibodies directed against SF-1 detected a positive signal in the nuclei of most cells within the testis including fetal Leydig and Sertoli cells (Fig. 8.9; see also chapter 6). There was an obvious reduction in intensity of immunostaining in animals treated with 600mg/Kg of OP (Fig. 8.9b) or 500μg/Kg of DES (Fig. 8.9c) when compared to controls (Fig. 8.9a). The reduction in immunostaining was most obvious in the Sertoli cells which appeared to be immunonegative in the testes from animals treated with DES.

Fig. 8.9: Immunolocalisation of SF-1 in the gonads removed from the fetuses of control and oestrogen-treated mothers on day 17.5 of gestation. In testes of fetuses from control mothers immunostaining was present in Sertoli cell nuclei (arrows) within the cords and in the majority of interstitial cells some of which were very heavily stained (arrowheads). When the amount of SF-1 was determined in sections from the testes of fetuses from DES- (c) or OP-treated (b) mothers processed at the same time on the same slides a marked decrease in the level of immunostaining was noted in the treated animals (compare b and c with a). The effect was most marked when the Sertoli cells were examined (arrows) as these appeared immunonegative in DES-treated animals (c). Within the interstitium immunostaining was reduced but not absent from all cells. Magnification x400.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8.4 Discussion

Testosterone is essential for maintenance of fertility in adult life (Sharpe, 1994) and for the development of the male reproductive tract during fetal life (George and Wilson, 1994). In the rat, synthesis of testosterone in the Leydig cells of the fetal testis starts on day 15.5 p.c. and then increases rapidly over the next few days to reach a peak on day 18.5 (Feldman and Bloch, 1978; Warren et al., 1973). The results obtained in the present study in which oestrogenic chemicals were administered to mothers at around this time demonstrate that oestrogens could interfere with steroid production in the testis during fetal life by inhibiting expression and activity of P450c17 and expression of SF-1. The long term consequences of these reductions are not known but deficits in various aspects of masculinisation are an obvious possibility.

The reduction in 17α–hydroxylase enzyme activity in fetal testes resulting from oestrogen administration to the mothers, was accompanied by reduced expression of P450c17 mRNA as determined by RT PCR (not shown) and in situ hybridisation, consistent with the effect of the oestrogenic chemicals being mediated at the level of transcription of the *CYP17* gene. In the same tissue sections in which reduced immunostaining for 17α-hydroxylase was observed, the number of Leydig cells expressing 3ß-HSD did not appear to have changed. Studies on steroidogenesis by adult Leydig cells have demonstrated that expression of P450c17 is acutely regulated by LH stimulation and that levels of enzyme fall rapidly in the absence of cAMP whilst expression of 3ß-HSD remains unchanged under the same circumstances (Keeney et al., 1988; Payne and Youngblood, 1995). In rats, plasma LH levels remain low until fetal day 19.5 (Aubert et al., 1985) and it has been concluded that although LH receptors are present in the fetal rat testis on or about day 15.5 (Warren et al., 1984), activation of expression of steroidogenic enzymes and fetal testicular testosterone production involves factors other than LH, presumably originating within the testes (Pakarinen et al., 1994). Recently a factor termed SF-1, present in fetal gonads and adrenals during and after sexual differentiation, has been shown to regulate expression of steroidogenic enzymes and this may therefore be the missing regulator of fetal Leydig cell steroidogenesis (Hatano et al., 1994; Lala et al., 1992; Morohashi et al., 1992). As SF-1 has been reported to regulate the expression of P450c17, we looked at the expression of this transcription factor. Like that of P450c17, the expression of SF-1 appeared to be reduced in the fetal testes from animals whose mothers were treated with either DES or OP. Reduced expression of

SF-1 may provide an explanation for the reduced expression of P450c17 as SF-1 binding sites are present on the *CYP17* gene and SF-1 was shown to regulate P450c17 expression in vitro (Zhang and Mellon, 1996). However SF-1 is expressed not only in Leydig cells but also in other interstitial cells and Sertoli cells (Hatano et al., 1994; Ikeda et al., 1994) and studies on SF-1 "knock out" mice have shown that SF-1 is essential for normal development of the reproductive system (Luo et al., 1994). The data obtained by immunostaining suggest that a reduction in SF-1 expression occurs in all the cell types in the fetal testis. The most striking reduction appeared to be in the Sertoli cells of animals from mothers treated with DES, however it is possible that these cells still express SF-1 but at a level undetectable by immunocytochemistry. The way in which SF-1 acts as a transcriptional regulator on its target genes is not yet fully elucidated. Some studies suggest that it requires a ligand, which has not yet been identified, to be fully functional (Shen et al., 1994). It is possible that different mechanisms may operate in different cells and that in some situations SF-1 may be active in the absence of ligand (Zhang and Mellon, 1996) or modulate cell function by dimerisation with other members of the steroid/thyroid hormone superfamily (Beato et al., 1995).

Oestrogen receptors (ER) have been detected in genital tracts of male mice using binding assays (Cooke et al., 1991b) and immunocytochemistry (Greco et al., 1992). Within the mouse testis, ER immunostaining has been detected in interstitial cells including Leydig cells on fetal days 15 to 19 (Greco et al., 1993). Tsai-Morris and co workers (1986) reported that exposure of fetal rat Leydig cells (day 21) in vitro to oestradiol resulted not only in a decrease in the synthesis of testosterone but also in an increase in ER detected by binding assays. They concluded that the action of oestrogens was mediated at the level of the nucleus, consistent with the action of oestrogen being mediated via its receptor, because incubation of isolated microsomes with oestrogen failed to affect enzyme activities (Nozu et al., 1981b). In the mouse, P450 aromatase mRNA was detected in testes on and after day 17.5 p.c. (Greco and Payne, 1994) suggesting that fetal and neonatal testes have the capacity to produce oestrogens locally. Recent studies of mice in which expression of the ER gene was disrupted selectively found that the mature adult males were infertile because of impaired spermatogenesis but otherwise appeared to have anatomically normal accessory organs (Korach, 1994).

DES is a potent oestrogen, and previous observations in man (Gill et al., 1979) and animals (McLachlan and Newbold, 1975) have indicated that it is likely to exert

adverse effects during the time of masculinisation and reproductive tract development. In contrast, there are no published studies of the effects of OP on mammalian development, although it has been shown to be weakly oestrogenic in vitro (White et al., 1994). OP is an alkylphenolic compound formed as a metabolite of some non-ionic surfactants and which are used widely in industrial and some household detergents, as plastic and petrol additives, spermicides and as dispensers for insecticides (Naylor et al., 1992). However, the level of human exposure is largely unknown. The present data are the first to show that maternal exposure to high levels of such compounds can suppress 17α-hydroxylase activity. OP has been shown previously to bind to the ER and to stimulate oestrogen-dependent growth of breast cancer cells and gene expression in vitro to a similar extent to 17ß-estradiol, albeit with approximately 1000-fold less potency (White et al., 1994). Male trout exposed to low levels of OP (38.5 µg/l) in the surrounding water synthesise vitellogenin, an oestrogen inducible protein normally only expressed in the female, and as a result had reduced testis size (Jobling et al., 1996). Studies in our group have shown that exposure of male rats to OP during gestation and neonatal life, whether administered by injection or via the drinking water, results in small but significant reductions in testicular size and sperm production in adulthood (Sharpe et al., 1995). In these experiments the doses added to the drinking water were very low, comparable to those to which humans may be exposed. Similarities between the effects of treatment with DES and OP suggest that the potentially deleterious action of OP on developing mammalian testis may be due to its oestrogenic activity, however, the mechanism underlying these observations remains to be elucidated. Reduced sperm output has been interpreted as being a consequence of reduction in Sertoli cell number rather than alteration of Leydig cell function but it is possible that the suppression of 17α-hydroxylase activity and/or reduced expression of SF-1 could have contributed to changes in Sertoli cell function.

It is presumed that the present effects of DES and OP on P450c17 and SF-1 are mediated via the oestrogen receptor, although it has been reported recently that the major and persistent metabolite of DDT, p,p'-DDE, which had previously been implicated in induction of abnormalities in male sex development in wildlife, bound more efficiently to the androgen receptor than it did to the oestrogen receptor (Kelce et al., 1995) and that DES could also bind to the androgen receptor. In the present study we do not believe that the effects on Leydig cell 17α-hydroxylase activity observed following treatment with DES or OP reflect interaction at the level of the AR as the few AR which are present within the testis at this time are not present in

Leydig cells (Majdic et al., 1995) and chapter 5. The pattern of expression of ER in the fetal testis has also been examined during the present studies. On day 17.5 p.c., ER were present only in interstitial cells and double immunostaining with antibodies against 3ß-HSD demonstrate that these are mostly Leydig cells (Majdic and Saunders, in preparation), but no difference was observed between control and treated animals either in intensity or distribution of staining. These results are in accordance with previously published observations (Greco et al., 1992) where authors noted expression of ER in the interstitial cells. Whilst it seems logical to conclude that the reduced expression of P450c17 and SF-1 in Leydig cells may reflect direct action of oestrogenic chemicals via the ER, this could not account for the parallel decrease in expression of SF-1 in Sertoli cells in which no ER were detected. It is therefore presumed that the effect of oestrogens on these cells is indirect or mediated via a different receptor system. However, very recently, a second type of ER (beta) has been cloned from the prostate and shown to be expressed in granulosa cells of the ovary (Kuiper et al., 1996). Sequence analysis revealed that ERß had significant sequence differences to the ER (alpha) to which the used antiserum was raised. The possibility therefore exists that the effects observed on SF-1 expression might also be mediated via a different ER subtype but this will require additional investigations. To date, the information on the control of expression of the SF-1 gene is restricted to the identification of a putative binding site with homology to the consensus E-box element which binds proteins with a helix-loop-helix motif. Mobility shift analysis has suggested that a protein(s) capable of binding to this element is present in the fetal testis (Nomura et al., 1995), however, the protein has not yet been identified. No data on the presence of a putative binding site for ER on the SF-1 gene has been presented.

While it is feasible to assume a direct action of oestrogens via ER on fetal Leydig cells, the possible role of LH in regulation of P450c17 expression cannot be ignored. In adult animals, oestrogens and androgens both exert negative feedback effect(s) on production and release of pituitary gonadotrophins (Griffin and Wilson, 1992). In the fetal rat, LH is first detectable on day 17.5 (Aubert et al., 1985; Watanabe and Daikoku, 1979) and while it is unlikely that LH is important at the initiation of steroidogenesis in Leydig cells it is probably involved in stimulating the rise in testosterone production between days 17.5 and 18.5 p.c. (Saez, 1994). Preliminary studies using immunocytochemistry to detect LHß protein in fetal pituitary cells from the control and treated animals described above did not show any major changes in the intensity of immunostaining or in the number of LHß positive cells

between groups. However, differences in the rate of LH release and/or production cannot be excluded without further analyses which should include an examination of the level of LHß mRNA and measurement of levels of LH in fetal circulation.

The importance and effect(s) of reduced 17α-hydroxylase enzyme activity induced by exogenous oestrogens during fetal life and the consequent reduction in production of testosterone are not clear. Long term effects on male reproductive function have been reported following maternal treatment with DES in both humans (Gill et al., 1979) and rodents (McLachlan and Newbold, 1975), although a recent survey did not find any evidence that long term male fertility had been affected (Wilcox et al., 1995). However, the question remains as to whether extended exposure to low doses of oestrogens throughout life could have a long term and deleterious effect on Leydig cell steroidogenesis. Evidence that the effects of intra-uterine exposure to low doses of oestrogen might be reflected in altered Leydig cell function in adulthood is provided by an intriguing report from Nonneman and co-workers (1992) in which they found that mice differed in their adult reproductive characteristics according to whether they developed in utero between two male fetuses, which have higher testosterone levels, or between two female fetuses which have higher estradiol levels. They recorded lower levels of 17α-hydroxylase activity in adult male mice which had been adjacent to the female fetuses than those next to the males and higher androgen receptor in the prostates of these same mice leading to the conclusion that low level oestrogen exposure exerts a significant influence on androgen metabolism and responsiveness.

Similarly, the importance of reduced expression of SF-1 is not known. In the present study we treated animals only after gonadal differentiation starts, however, in some parallel studies, pregnant females received their first injection of OP on day 9.5 p.c., prior to gonadal differentiation. The gonads of these animals did not show any gross abnormalities at the time of birth. It is possible that the fetus develops a "defensive" mechanism by overexpressing certain important proteins/hormones and thereby ensures that normal development occurs despite some deleterious external influences. Lower levels of SF-1 expression may still be sufficient to initiate the normal development of the gonads. However, any long term effects of inappropriate gene expression during fetal life that might become evident only during adult life cannot be excluded.

In conclusion, these data show that maternal exposure to oestrogens such as OP and

DES inhibits expression of SF-1 and the activity of 17α-hydroxylase in the fetal testis. The mechanisms which bring about this reduction and its consequences remain to be elucidated. We cannot yet conclude that the reduction in SF-1 expression and P450c17 enzymatic activity necessarily explain the incidence of reproductive problems in male offspring from DES-treated mothers or the rise in the reported incidence of similar problems in the general population. However, the present observations suggest that the effect of long term exposure to oestrogenic chemicals, especially environmental oestrogens, on Leydig cell function needs to be more fully explored.

9. General discussion

Sexual differentiation is one of the most intriguing and interesting processes of developmental biology. Discoveries in recent years have broadened our understanding of the events underlying development of the gonads and sexual phenotype but many questions still remain unanswered. Notably, there have been increasing numbers of reports suggesting that a decline in male reproductive health is occurring which has been highlighted by an increase in the incidence of abnormalities of development of the male reproductive tract. For example, the reported incidence of testicular cancer, hypospadias and cryptorchidism (Giwercman, 1995; Toppari et al., 1996) has increased drastically and statistical analysis of sperm concentration data for normal men from over 60 publications during the period 1938-1990 found that mean sperm concentrations had declined from an average of $113x10^6$/ml to $66x10^6$/ml over this time period (Carlsen et al., 1992). These data have been supported by the results of other studies (Auger et al., 1995; Irvine, 1994; van Waeleghem et al., 1994), however, a decline in sperm counts does not appear to have occurred in all developed countries (Suominen and Vierula, 1993). It has been suggested (Giwercman, 1995; Sharpe and Skakkebaek, 1993) that the development of testicular cancer, hypospadias, cryptorchidism and eventual sperm output are all predetermined during development of the male reproductive system during fetal and neonatal life. Understanding how and why development of the male urogenital system may be adversely affected is therefore dependent on elucidating the molecular, biochemical and endocrine mechanisms that operate during this critical period.

9.1 Physiological development of the fetal gonads

Development of the testis is a precisely regulated process that requires accurate temporal and spatial expression of many different genes/proteins. Several genes, the products of which appear important during gonadal development, have recently been identified and it is likely that many more are still undiscovered (Bogan and Page, 1994; Capel, 1995; Ryner and Swain, 1995). To aid in understanding the mechanisms underlying normal testicular development it is important to know the temporal and spatial localisation of expression of the genes involved.

Expression of a normal, functional androgen receptor (AR) is necessary to enable testosterone, the main male sexual hormone, to act on its target cells. Lack of a

functional androgen receptor in humans is associated with androgen insensitivity syndrome, a pathological condition where XY individuals develop testes which remain undescended and so have a female or intersex phenotype (Griffin and Wilson, 1989; Quigley et al., 1995). Several studies have shown the presence of androgen receptors in the developing genital tract in humans (Kalloo et al., 1993) and rodents (Bentvelsen et al., 1995; Cooke et al., 1991a) using either ligand binding or immunocytochemistry but there have been no previous reports on the presence and cellular localisation of AR within the fetal testis. In this study, the AR were clearly shown to be present in nuclei of interstitial cells from day 17.5 onwards, however, rather surprisingly, during fetal life the AR were not localised to the Leydig cells, as in the adult testis, but in other types of interstitial cells and in peritubular cells. In addition in contrast to the situation in the adult testis, the AR was not present in nuclei of fetal Sertoli cells. The implications of this finding are not known, however, a clear conclusion could be drawn that testosterone cannot influence steroidogenesis in the fetal Leydig cells. In contrast feedback inhibition of testosterone production by testosterone acting via AR has been shown in adult testis (Darney and Ewing, 1981; Hales et al., 1987; Pointis et al., 1984). The absence of AR from fetal Leydig cells may be associated with the high steroidogenic capacity of these cells (Huhtaniemi, 1994; Saez, 1994), which is necessary to maintain the high testosterone levels required for virilisation of the male genitalia (George and Wilson, 1994). The lack of AR in fetal Leydig cells could therefore prevent the negative feedback effect of testosterone and consequently prevent inhibition of testosterone production. The lack of another feedback inhibition loop, namely desensitisation of fetal Leydig cells following stimulation with LH, has been reported (Payne and Youngblood, 1995; Warren et al., 1987) and it is feasible that fetal Leydig cells have developed mechanisms to prevent negative effects on testosterone production ensuring maintenance of testosterone production at levels sufficient for normal masculinisation of the fetus. In our studies, the first AR immunopositive cells within the testis were detected on day 17.5 in an area of the gonad close to the mesonephros. During the next few days of gestation, the AR immunopositive cells became dispersed throughout the interstitial and peritubular compartment of the testis. Studies by Buehr et al. (1993) have demonstrated that migration of cells from the mesonephros into the fetal testis occurs and have highlighted the importance of this migration for normal testicular development. The presence of the first AR immunopositive cells in the area adjacent to the mesonephros and strong expression of AR in the cells within the mesonephros one day before the appearance of the AR positive cells in the testis make it tempting to conclude that AR positive cells migrate from the mesonephros and gradually populate the testis.

However, at present this idea is purely speculative and further studies will be needed to establish if it is true.

Fetal rat Leydig cells start to produce testosterone on day 15.5 p.c. (Feldman and Bloch, 1978; Habert and Picon, 1982; Picon, 1976), simultaneously with their morphological differentiation (Magre and Jost, 1980). However, studies by Bloch (1979) and the results presented in this thesis suggest that the functional differentiation of these cells starts earlier since 3ß-HSD, one of the steroidogenic enzymes essential to testosterone synthesis, was detectable on day 14.5 p.c. The importance, or implications, of this finding are not known and clearly different mechanisms are involved in the regulation of expression of the different steroidogenic enzymes because two other enzymes, P450scc (Rouiller et al., 1990) and P450c17, are not detectable in the fetal testis before day 15.5 p.c. The factor triggering testosterone production has not yet been identified. In the primate fetus, such a factor could be hCG (Saez, 1994) but studies in the rat have so far failed to identify a similar factor (Habert and Picon, 1990; Wurzel et al., 1983) leading to speculation that either non-gonadotrophic factors may be involved or that autonomous regulation of testosterone production occurs in the fetal rat testis. The orphan nuclear receptor SF-1 has been implicated as an important transacting transcription factor essential to gonadal development (Ingraham et al., 1994; Luo et al., 1994). SF-1 regulates the expression of several cytochrome P450s enzymes (Clemens et al., 1994; Lynch et al., 1993; Morohashi et al., 1992; Zhang and Mellon, 1996) and AMH (Shen et al., 1994). The presence of SF-1 in the fetal testis from the earliest stages of gonadal development (Hatano et al., 1994; Ikeda et al., 1994) and its ability to regulate the transcription of both P450scc and P450c17 would suggest that SF-1 could be the factor that triggers and regulates early expression of some or all of the steroidogenic enzymes and thus controls early testosterone production. However, as the expression of 3ß-HSD in the fetal rat testis occurs one day before that of P450scc and P450c17, additional factors that either act independently of SF-1 or that interact with SF-1 to allow it to function differently on different genes must be involved.

Another orphan nuclear receptor, DAX-1, also appears to play important role(s) in gonadal development (Guo et al., 1995; Zanaria et al., 1994). The *DAX-1* gene is located in the DSS region of the X chromosome (Zanaria et al., 1994) and if *DAX-1* is indeed the gene which when duplicated is associated with sex reversal, it might be the first ovary determining gene identified. However, *DAX-1* is not the only gene in the DSS region and further studies will be needed to confirm whether *DAX-1* is

important in DSS (Bardoni et al., 1994). The phenotype of patients with mutations in *DAX-1* is similar to that observed in mice lacking a functional SF-1 gene and some studies have suggested that a direct interaction occurs between SF-1 and DAX-1, a hypothesis supported by the discovery of an SF-1 binding site upstream of the *DAX-1* gene (Burris et al., 1995). However, the pattern of cellular localisation described in this study does not support this theory. While these factors coimmunolocalise in many interstitial cells within the testis, some DAX-1 immunopositive cells did not show immunoexpression of SF-1. It is possible that these cells do contain SF-1 protein but at low levels, undetectable using immunocytochemistry. However, the interstitial cells that express both proteins are those that have the strongest immunoexpression of SF-1 while the cells with lower expression of SF-1 do not express DAX-1, so it seems likely that cells containing DAX-1 alone are truly SF-1 negative. In accordance with this data, an SF-1 binding element is not absolutely necessary for transcription of this gene (K.L. Parker, personal communication), and expression of DAX-1 occurs in fetuses lacking functional SF-1 (Parker, 1996) further suggesting that the interaction of these two genes/proteins is indirect rather than direct. The function of DAX-1 is not known but the timing of expression of the protein and pattern of cellular localisation described in this study as well as the phenotype of the patients with mutations in the DAX-1 gene who have a disorganised adrenal cortex (Muscatelli et al., 1994) could suggest involvement in regulation of steroidogenesis in the testis and adrenal glands. However, its role in the fetal ovary is not likely to be the same as unlike the testis the fetal ovary is steroidogenically quiescent. It is possible that DAX-1, like SF-1, acts as a transcription factor for modulation of multiple genes and therefore has additional function(s) in the development of the ovary. Alternatively, since DAX-*1* is not the only gene located in the DSS region of the X chromosome it is possible that DAX-1 is responsible for adrenal hypoplasia congenita but not for dosage sensitive XY female sex reversal and is thus not an ovary determining gene.

Another difference between adult and fetal Leydig cells, namely in their abilities to synthesise inhibin/activin subunits, has been established during this study. Inhibins and activins are well known modulators of FSH release from the pituitary (Wallach, 1996; Ying, 1988) and it is well established that the principle site of synthesis of inhibins and activins in the adult testis are the Sertoli cells (Ying, 1988). Several studies have suggested some inhibin/activin production also occurs in Leydig cells, especially in immature animals and fetuses (Maddocks and Sharpe, 1989; Roberts et al., 1989; Shaha et al., 1989). The studies described in this thesis have made use of

new, highly specific, monoclonal antibodies and have demonstrated that the major site of synthesis of inhibin α and ßB subunits in the testis during fetal life are the Leydig cells. The immunoexpression of α-subunit starts very early with positive immunostaining detectable on day 14.5 p.c. Two days later, immunoexpression of the ßB subunit was detectable, suggesting that the fetal testis has the potential to produce bioactive inhibin. The role of inhibin during fetal life is not known, however it is likely to be different from its role in the adult organism where its main role is the modulation of FSH release since FSH is not detectable in the rat fetus until day 17.5 (Aubert et al., 1985). This finding also raises the question of the regulation of inhibin expression since it has been shown that this can in turn be regulated by FSH (Ying, 1988). Several studies have demonstrated that inhibin and activin can act as local paracrine or autocrine regulators of testicular cells in immature and adult animals and it is possible that they perform a similar role(s) in the fetal testis (Boitani et al., 1995; Chen, 1993; Hsueh et al., 1987; Lin et al., 1989). In the absence of FSH stimulation regulation of expression of inhibin subunits within the fetal testis may be influenced by other local factors which are as yet unidentified. One candidate could be SF-1 as it is present in the testis at the appropriate time and as discussed has been implicated in the regulation of many genes involved in reproduction and steroidogenesis. This hypothesis is supported by the lack of inhibin expression in the fetal ovaries where SF-1 expression declines, however, no reports have been published identifying an SF-1 binding element in any of the genes encoding the inhibin subunits.

Immunoexpression of proteins examined during this study and their localisation to the different gonadal cell types during fetal and postnatal development is summarised in table 9.1.

	Fetal age in days p.c.						
Tissue/cell type	**13.5**	**14.5**	**15.5**	**16.5**	**17.5-term**	**postnatal**	**adult**
Testis: Sertoli cells	SF-1	SF-1	SF-1	SF-1	SF-1	weak SF-1	weak SF-1
						weak AR	AR
		AMH	AMH	AMH	AMH	AMH	
						Inhibin-α	Inhibin-α
						Inhibin-ßB	Inhibin-ßB
	PCNA	PCNA	PCNA	PCNA	PCNA	PCNA	
Leydig cells		3ß-HSD	3ß-HSD	3ß-HSD	3ß-HSD	3ß-HSD	3ß-HSD
			P450c17	P450c17	P450c17	P450c17	P450c17
	SF-1	SF-1	SF-1	SF-1	SF-1	weak SF-1	weak SF-1
			DAX-1	DAX-1	DAX-1	weak DAX-1	weak DAX-1
		Inhibin-α	Inhibin-α	Inhibin-α	Inhibin-α	Inhibin-α	Inhibin-α
				Inhibin-ßB	Inhibin-ßB	Inhibin-ßB	Inhibin-ßB

Table 9.1: Immunopositive staining in different gonadal cell types during fetal and postnatal development

Tissue/cell type	Fetal age in days p.c. 13.5	14.5	15.5	16.5	17.5-term	postnatal	adult
Testis: non-Leydig interstitial cells				AR	AR	AR	AR
	SF-1	SF-1	SF-1	SF-1	SF-1		
			DAX-1	DAX-1	DAX-1		
	PCNA	PCNA	PCNA	PCNA	PCNA	PCNA	
Peritubular cells					AR	AR	AR
Ovary	SF-1	SF-1	SF-1	SF-1	SF-1	?	?
			DAX-1	DAX-1	DAX-1	?	?

Table 9.1: Immunopositive staining in different gonadal cell types during fetal and postnatal development

9.2 Influence of oestrogenic chemicals on testis development

In the 1950s and 60s, several million women received treatment with DES during their pregnancy as a preventive to miscarriage (Stillman, 1982). Follow-up studies have shown that DES was not effective (Dieckmann et al., 1953) and furthermore had harmful effects on the male and female offspring from treated mothers (Bibbo et al., 1977; Gill et al., 1979; Stillman, 1982). In males, an increased incidence of cryptorchidism, hypospadias and smaller testis with lower sperm output and lower sperm quality was noted (Bibbo et al., 1977; Gill et al., 1979; Stillman, 1982). Some studies in experimental animals have also demonstrated similar deleterious effects of exposure to DES and other oestrogens on the development of the male reproductive tract in rats and mice (Boylan, 1978; McLachlan and Newbold, 1975; Nomura and Kanzaki, 1977; Vannier and Raynaud, 1980).

In recent years, several studies have reported an increasing incidence of testicular cancer, cryptorchidism and hypospadias in the normal male population in the western world (Giwercman, 1995; Giwercman and Skakkebaek, 1992; Toppari et al., 1996). In addition a number of intriguing reports recording falling sperm counts in several developed countries in the last few decades have been published (Auger et al., 1995; Carlsen et al., 1992; Irvine, 1994; van Waeleghem et al., 1994). Similarities between the problems recorded in offspring from DES treated mothers and those occurring in the normal population have lead to the suggestion of a possible connection to oestrogen exposure. Sharpe and Skakkebaek (1993) have reviewed the possible sources of exposure to oestrogenic compounds in the modern world and came to the conclusion that we are exposed to much higher amounts of oestrogens today compared to 50 years ago. One important source of oestrogenic substances are the chemicals in our environment. It has been known for several years that some PCBs and p,p-DDE (a DDT metabolite) have weak oestrogenic activity (Bittman and Cecil, 1970). In addition, for several other chemicals currently widespread in our environment oestrogenic activity has been recently established using in vitro tests (Jobling et al., 1995; White et al., 1994). Studies by Sharpe and co-workers (1995) have shown that exposure to some of these chemicals, including octylphenol (OP), also used in the studies described in this thesis, could cause the development of smaller testes and a lower sperm output in adult rats, if they were exposed whilst in utero and during lactation via their mothers. The mechanism of action of these chemicals is not yet known but the effects were noted even when the levels of treatment were low and

comparable to those to which humans may be exposed. In adult life, oestrogens influence the function of the pituitary and the testis directly via oestrogen receptors (ER) which are present in both organs (Griffin and Wilson, 1992). Similarly, ER are present in the fetal testis and pituitary (Greco et al., 1993, Majdic and Saunders, unpublished observations), suggesting that these chemicals may also act via the ER at this stage of life. Immunocytochemically, the ER were detected in the fetal Leydig cells (Majdic and Saunders, unpublished observation), suggesting possible action of oestrogenic chemicals directly on these cells. However, we were unable to detect ER in Sertoli cells, suggesting that reduced expression of SF-1 protein in these cells may be due to indirect regulation. Very recently, a second type of ER has been cloned (ER-ß; Kuiper et al., 1996), and although not much is known about its expression, it is possible that oestrogenic chemicals may act via ER-ß, expressed in Sertoli cells. In the present study, reduced expression of both the steroidogenic enzyme cytochrome P450c17 and SF-1 have been detected following exposure to DES or OP in utero. The consequences of these findings are not known but one obvious possibility is a reduction in testosterone production. Knowing the importance of testosterone for normal masculinisation (George and Wilson, 1994) one could easily imagine the deleterious effects impaired testosterone production could have. However, in this study the actual testosterone levels have not been measured and it is possible that reduced activity of P450c17 has only a limited influence on total testosterone synthesis. Similarly, it is possible that testosterone production in the fetal testis is higher than required thus enabling the fetus to compensate for any negative influences on testosterone biosynthesis during this critical period. This might explain the lack of any gross morphological changes in treated animals, however, none of the animals treated with a high dose of DES in utero, where the reduction in P450c17 activity was most prominent, were examined postnatally due to the problems with delivery in these animals. The reduced level of P450c17 mRNA in the fetal Leydig cells could either be derived from a direct influence of oestrogenic chemicals acting via the ER, or alternatively via changes in the levels of LH resulting in reduced stimulation of Leydig cells. Preliminary studies using immunocytochemistry have not demonstrated any significant reduction in the number of LHß positive cells in the pituitary from fetuses treated with either DES or OP via their mother, however, examination of mRNA expression and the measurement of LH in fetal circulation are necessary to determine whether there are changes in the concentration of this gonadotrophin. The changes in P450c17 activity in fetal Leydig cells could result from alterations in SF-1 levels as it has been shown to regulate the transcription of *CYP17* gene in vitro (Zhang and Mellon, 1996). Whether this effect of oestrogens is directly mediated via ER is not

known. There have as yet been no reports about the presence of an oestrogen response element in the SF-1 gene. The only regulatory element in the SF-1 gene so far identified is an E-box element (Nomura et al., 1995) and there are no reports about regulation of expression of SF-1 in vivo. SF-1 is expressed in several cell types in the testis and has a wide range of effects on the development of the reproductive axis. Therefore any change in SF-1 expression could have serious consequences. However, as mentioned earlier, the animals from treated mothers did not show any gross morphological abnormalities, again suggesting that compensatory mechanism(s) are in place with the expression of SF-1 being high enough to maintain normal gonadal development even after treatment. Further studies are needed to elucidate how oestrogen exposure results in reduced SF-1 expression.

In conclusion, these data have demonstrated one possible mechanism by which deleterious effects of oestrogenic chemicals could occur, but further studies will be necessary to elucidate the importance of the present findings and to further explain the mechanisms of oestrogen action on development of the reproductive tract. At present it is not yet possible to be sure that the effects attributed to oestrogenic chemicals are involved in the reported increase in incidence of male reproductive abnormalities. However, hypospadias and cryptorchidism can result from impaired testosterone action and could result from lowered testosterone production due to impaired steroidogenesis.

Appendix I: Some commonly used buffers and agars

Buffers

1x SSC
0.15M sodium chloride
0.015M sodium citrate
at pH7.0

1x TBE
0.089M Tris base
0.089M boric acid
10mM EDTA

PBS buffer, pH 7.2
0.028M NaH_2PO_4
0.072M Na_2HPO_4
0.85% NaCl

TE buffer
10mM Tris-HCl
1mM EDTA
at pH8.0

TBS buffer
0.05M Tris-Hcl, pH 7.4
0.85% NaCl

Agars

Luria Bertani (LB) broth
10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
in 1 litre water

LB-agar
LB-broth
with 1.5% bacto-agar

Appendix II: Papers related to this thesis

Majdic G, Saunders PTK: Differential patterns of expression of DAX-1 and steroidogenic factor-1 (SF-1) in the fetal rat testis. Endocrinology 1996; 137: 3586-3589.

Majdic G, Sharpe RM, O'Shaughnessy PJ and Saunders PTK: Expression of 17α-hydroxylase, 17,20 lyase in the fetal rat testis is reduced by prenatal exposure to estrogens. Endocrinology 1996; 137: 1063-1070.

Majdic G, Millar MR and Saunders PTK: Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. Journal of Endocrinology 1995; 147: 285-293.

Saunders PTK, Millar MR, **Majdic G**, Bremner WJ, McLaren TT, Grigor KM and Sharpe RM: Testicular androgen recpetor protein: distribution and control of expression. In:"Cellular and Molecular Regulation of Testicular Cells" Ed. C Desjardins. Serono Symposia, Springer Verlag: Berlin (In Press).

Sharpe RM, **Majdic G**, Fisher J, Parte P, Millar MR, O'Shaughnessy PJ, Saunders PTK: Sexual differentiation of the male and fertility in adulthood: potential interference by environmental hormonal mimics. In: "Sexual Differentiation: Clinical and biological aspects", Ed. I Hughes, Serono symposia (In press).

Majdic G, O'Shaughnessy PJ, Sharpe RM and Saunders PTK: Oestrogen effects on testicular expression of 17 alpha hydroxylase. Proceedings of 6th Zavernik memmorial meeting, Lipica, Slovenia, 8-11 November 1995 (In press).

Saunders PTK, Millar MR, Gaughan J, McKinnell C, **Majdic G** and Sharpe RM: Gene expression during spermatogenesis:regulation of protein expression by testosterone. Proceedings of 6th Zavernik memmorial meeting, Lipica, Slovenia, 8-11 November 1995 (In press).

Bibliography

Agelopoulou R., Magre S., Patsavoudi E. and Jost A. (1984) Initial phases of the rat testis differentiation in vitro. *Journal of Embryology and Experimental Morphology* **83:** 15-31.

Armstrong J. F., Pritchard-Jones K., Bickmore W. A., Hastie N. D. and Bard J. B. L. (1992) The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. *Mechanisms of Development* **40:** 85-97.

Aubert M. L., Begeot M., Winiger B. P., Morel G., Sizonenko P. C. and Dubois P. M. (1985) Ontogeny of hypothalamic luteinizing hormone-releasing hormone (GnRH) and pituitary GnRH receptors in fetal and neonatal rats. *Endocrinology* **116:** 1565-1575.

Auger J., Kunstmann J. M., Czyglik F. and Jouannet P. (1995) Decline in semen quality among fertile men in Paris during the past 20 years. *The New England Journal of Medicine* **332:** 281-285.

Avery B., Madison V. and Greve T. (1991) Sex and development in bovine in-vitro fertilized embryos. *Theriogenology* **35:** 953-963.

Baarends W. M., Van Helmond M. J. L., Post M., Van Der Schoot P. J. C. M., Carel Meijers J. H., Themmen A. P. N. and Grootegoed J. A. (1994) A novel member of the transmembrane serine/threonine kinase receptor family is specifically expressed in the gonads and in mesenchymal cells adjacent to the Mullerian duct. *Development* **120:** 189-197.

Baird P. N., Groves N., Haber D. A., Housman D. E. and Cowell J. K. (1992) Identification of mutations in the WT1 gene in tumors from patients with the WAGR syndrome. *Oncogene* **7:** 2141-2149.

Bardoni B., Zanaria E., Guioli S., Floridia G., Worley K. C., Tonini G., Ferrante E., Chiumello G., McCabe E. R. B., Fraccaro M., Zuffardi O. and Camerino G. (1994) A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. *Nature Genetics* **7:** 497-501.

Barnhart K. M. and Mellon P. L. (1994) The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone α-subunit gene in pituitary gonadotropes. *Molecular Endocrinology* **8:** 878-885.

Bartke A., Williams K. I. H. and Dalterio S. (1977) Effects of estrogens on testicular testosterone production in vivo. *Biology of Reproduction* **17:** 645-649.

Beato M., Herrlich P. and Schutz G. (1995) Steroid hormone receptors: many actors in search of a plot. *Cell* **83:** 851-857.

Behlke M. A., Bogan J. S., Beer-Romero P. and Page D. C. (1993) Evidence that the SRY protein is encoded by a single exon on the human Y chromosome. *Genomics* **17:** 736-739.

Behringer R. R., Cate R. L., Froelick G. J., Palmiter R. D. and Brinster R. L. (1990) Abnormal sexual development in transgenic mice chronically expressing Mullerian inhibiting substance. *Nature* **345:** 167-170.

Behringer R. R., Finegold M. J. and Cate R. L. (1994) Mullerian-inhibiting substance function during mammalian sexual development. *Cell* **79:** 415-425.

Bennet C. P., Docherty Z., Robb S. A., Ramani P., Hawkins J. R. and Grant D. (1993) Deletion 9p and sex reversal. *Journal of Medical Genetics* **30:** 518-520.

Bentvelsen F. M., Brinkmann A. O., van der Schoot P., van der Linden J. E. T. M., van der Kwast T. H., Boersma W. J. A., Schroeder F. H. and Nijman J. M. (1995) Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Molecular and Cellular Endocrinology* **113:** 245-253.

Bentvelsen F. M., McPhaul M. J., Wilson J. D. and George F. W. (1994) The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen. *Molecular and Cellular Endocrinology* **105:** 21-26.

Berndtson W. E., Igboeli G. and Pickett B. W. (1987) Relationship of absolute numbers of Sertoli cells to testicular size and spermatogenesis in young beef bulls. *Journal of Animal Science* **64:** 241-246.

Berndtson W. E. and Thompson T. L. (1990) Changing relationship between testis size, Sertoli cell number and spermatogenesis in Sprague-Dawley rats. *Journal of Andrology* **11:** 429-435.

Berta P., Hawkins J. R., Sinclair A. H., Taylor A., Griffiths B. L., Goodfellow P. N. and Fellous M. (1990) Genetic evidence equating SRY and the testis-determining factor. *Nature* **348:** 448-450.

Bibbo M., Gill W. B., Azizi F., Blough R., Fang V., Rosenfield R. L., Schumacher G. F. B., Sleeper K., Sonek M. G. and Weid G. L. (1977) Follow-up study of male and female offspring of DES-exposed mothers. *Journal of Obstetrics and Gynecology* **49:** 1-8.

Bittman J. and Cecil H. (1970) Estrogenic activity of DDT analogs and polychlorinated biphenyles. *Journal of Agriculture and Food Chemistry* **18:** 1108-1112.

Bloch E. (1979) Fetal gonadal endocrine activity and reproductive tract differentiation. *Contributions in Gynaecology and Obstetrics* **5:** 21-37.

Bogan J. S. and Page D. C. (1994) Ovary - Testis?-A mammalian dilemma. *Cell* **76:** 603-607.

Boitani C., Stefanini M., Fragale A. and Morena A. R. (1995) Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. *Endocrinology* **136:** 5438-5444.

Boylan E. S. (1978) Morphological and functional consequences of prenatal exposure to diethylstilbestrol in the rat. *Biology of Reproduction* **19:** 854-863.

Boyle P., Kaye S. N. and Robertson A. G. (1987) Changes in testicular cancer in Scotland. *European Journal of Cancer and Clinical Oncology* **23:** 827-830.

Bravo R., Frank R., Blundell P. A. and Macdonald-Bravo H. (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature* **326:** 515-517.

Bremner W. J., Millar M. R., Sharpe R. M. and Saunders P. T. K. (1994) Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* **135:** 1227-1234.

Bressler R. S. and Ross M. H. (1972) Differentiation of peritubular myoid cells of the testis: effects of intratesticular implantation of newborn mouse testis into normal and hypophysectomized adults. *Biology of Reproduction* **6:** 148-159.

Brink M., Humbel B. M., de Kloet E. R. and van Driel R. (1992) The unligated glucocorticoid receptor is localized in the nucleus, not in the cytoplasm. *Endocrinology* **130:** 3575-3581.

Brown L. M., Pottern L. M., Hoover R. N., Devessa S. S., Aselton P. and Flannery J. T. (1986) Testicular cancer in the United States: trends in incidence and mortality. *International Journal of Epidemiology* **15:** 164-170.

Bruening W., Bardessy N., Silverman B. L., Cohn R. A., Machin G. A., Aronson A. J., Housman D. and Pelletier J. (1992) Germline intronic and exonic mutations in the Wilms' tumor gene (WT1) affecting urogenital development. *Nature Genetics* **1:** 144-148.

Buckler A. J., Pelletier J., Haber D. A., Glasser T. and Housman D. E. (1991) Isolation, characterisation and expression of the murine Wilms' tumor gene (WT1) during kidney development. *Molecular and Cellular Biology* **11:** 1707-1712.

Buehr M., Gu S. and McLaren A. (1993) Mesonephric contribution to testis differentiation in the fetal mouse. *Development* **117:** 273-281.

Burgoyne P. S. (1993) A Y-chromosomal effect on blastocyst cell number in mice. *Development* **117:** 341-345.

Burgoyne P. S., Tam P. P. L. and Evans E. P. (1983) Retarded development of XO conceptuses during early pregnancy in the mouse. *Journal of Reproduction and Fertility* **68:** 387-393.

Burris T. P., Guo W., Le T. and McCabe E. R. B. (1995) Identification of a putative steroidogenic factor-1 response element in the DAX-1 promotor. *Biochemical and Biophysical Research Communications* **214:** 576-581.

Byskov A. G. (1986) Differentiation of mammalian embryonic gonad. *Physiological Reviews* **66:** 71-117.

Byskov A. G. and Saxen L. (1976) Induction of meiosis in foetal mouse testis in vitro. *Developmental Biology* **52:** 193-200.

Call K. M., Glaser T., Ito C. Y., Buckler A. J., Pelletier J., Haber D. A., Rose E. A., Kral A., Yeger H., Lewis W. H., Jones C. and Housman D. E. (1990) Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60:** 509-520.

Capel B. (1995) New bedfellows in the mammalian sex-determination affair. *Trends in Genetics* **11:** 161-163.

Capel B., Swain A., Nicolis S., Hacker A., Walter M., Koopman P., Goodfellow P. and Lovell-Badge R. (1993) Circular transcripts of the testis determining gene Sry in adult mouse testis. *Cell* **73:** 1019-1030.

Carlsen E., Giwercman A., Keiding N. and Skakkebaek N. E. (1992) Evidence for decreasing quality of semen during past 50 years. *British Medical Journal* **305:** 609-613.

Carr B. R. (1992). Disorders of the ovary and female reproductive tract. In Williams Textbook of Endocrinology, J. D. Wilson and D. W. Foster, eds. Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: Harcourt Brace Jovanovich, Inc., pp. 733-798.

Carson-Jurnica M. A., Schrader W. T. and O'Malley B. W. (1990) Steroid receptor superfamily: structure and functions. *Endocrine Reviews* **11:** 209-220.

Cattanach B. M., Iddon C. A., Charlton H. M., Chiappa S. A. and Fink G. (1977) Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* **269:** 338-340.

Chen C. C. (1993) Editorial: Inhibin and activin as paracrine/autocrine factors. *Endocrinology* **132:** 4-5.

Chiefetz S., Weatherbee J. A., Tsang M. L., Anderson J. K., Mole J. E., Lucas R. and Massague J. (1987) The transforming growth factor-ß system, a complex pattern of cross-reactive ligands and receptors. *Cell* **48:** 409-415.

Chiquoine A. D. (1954) The identification, origin and migration of primordial germ cells in the mouse embryo. *Anatomical Record* **118:** 135-146.

Clemens J. W., Lala D. S., Parker K. L. and Richards J. S. (1994) Steroidogenic factor-1 binding and transcriptional activity of the cholesterol side-chain cleavage promoter in rat granulosa cells. *Endocrinology* **134:** 1499-1508.

Clermont Y. and Perey B. (1957) Quantitative study of the cell population of the seminiferous tubules in immature rats. *American Journal of Anatomy* **100:** 241-267.

Codesal J., Regadera J., Nistal M., Regadera Rejas J. and Paniagua R. (1990) Involution of human fetal Leydig cells. An immunohistochemical, ultrastructural and quantitative study. *Journal of Anatomy* **172:** 103-114.

Cooke P. S. and Meisami E. (1991) Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. *Endocrinology* **129:** 237-243.

Cooke P. S., Young P. and Cunha G. R. (1991a) Androgen receptor expression in developing male reproductive organs. *Endocrinology* **128:** 2867-2873.

Cooke P. S., Young P., Hess R. A. and Cunha G. R. (1991b) Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs. *Endocrinology* **128:** 2874-2879.

Cooke P. S., Zhao Y. D. and Bunick D. (1994) Triiodothyronine inhibits proliferation and stimulates differentiation of cultured neonatal Sertoli cells: possible mechanism for increased adult testis weight and sperm production induced by neonatal goitrogen treatment. *Biology of Reproduction* **51:** 1-6.

Cortes D., Muller J. and Skakkebaek N. E. (1987) Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. *International Journal of Andrology* **10:** 589-596.

Darney K. J. and Ewing L. (1981) Autoregulation of testosterone secretion in perfused rat testes. *Endocrinology* **109:** 993-995.

Darney K. J., Zirkin B. R. and Ewing L. L. (1996) Testosterone autoregulation of its biosynthesis in the rat testis: inhibition of 17α-hydroxylase activity. *Journal of Andrology* **17:** 137-142.

Dauvois S., White R. and Parker M. (1993) The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *Journal of Cell Science* **106:** 1377-1388.

De Riviers M., Hocherau De Riviers M. T. and Blanc M. R. (1980) Control of Sertoli and germ cell populations in the cock and sheep testes. *Reproduction, Nutrition and Development* **20:** 241-249.

de Winter J. P., Vanderstichele H. M. J., Verhoeven G. and Timmerman M. A. (1994) Peritubular myoid cells from immature rat testes secrete activin A and express activin receptor type II in vitro. *Endocrinology* **135:** 759-767.

Dechelotte P., Chassagne J., Labbe A., Afane M., Scheye T., de Laguillaumie B. and Boucher D. (1989) Ultrastructural and immunohistochemical evidence of in situ differentiation of mononuclear phagocyte system cells in the interstitium of human fetal testis. *Early Human Development* **20:** 25-36.

Di Clemente N., Wilson C., Faure E., Boussin L., Carmillo P., Tizard R., Picard J. Y., Vigier B., Josso N. and Cate R. (1994) Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. *Molecular Endocrinology* **8:** 1006-1020.

Dieckmann W. J., Davis M. E. and Rynkiewicz L. M. (1953) Does administration of diethylstilbestrol during pregnancy have therapeutic value? *American Journal of Obstetrics and Gynecology* **66:** 1062-1081.

Dorrington J. H. and Armstrong D. T. (1975) Follicle-stimulating hormone stimulates estradiol-17ß synthesis in cultured Sertoli cells. *Proceedings of National Academy of Sciences USA* **72:** 2677-2681.

Dorrington J. H. and Khan S. A. (1993) Steroid production, metabolism, and release by Sertoli cells. In The Sertoli Cell, L. Russell and M. Griswold, eds. Clearwater: Cache River Press, pp. 538-549.

Dressler G. R., Deutsch U., Chowdhury K., Nornes K. O. and Gruss P. (1990) Pax-2, a new paired-box-containing gene and its expression in the developing excretory system. *Development* **109:** 787-795.

Dubin R. A. and Ostrer H. (1994) Sry is a transcriptional activator. *Molecular Endocrinology* **8:** 1182-1192.

Fechner P. Y., Marcantonio S. M., Jaswaney V., Stetten G., Goodfellow P. N., Migeon C. J., Smith K. D. and Berkowitz G. D. (1993a) The role of the Sex-Determining region Y gene in the etiology of 46,XX maleness. *Journal of Clinical Endocrinology and Metabolism* **76:** 690-695.

Fechner P. Y., Marcantonio S. M., Ogata T., Rosales T. O., Smith K. D., Goodfellow P. N., Migeon C. J. and Berkowitz G. D. (1993b) Report of a kindred with X-linked (or autosomal dominant sex-limited) 46,XY partial gonadal dysgenesis. *Journal of Clinical Endocrinology and Metabolism* **76:** 1248-1253.

Feldman S. C. and Bloch E. (1978) Developmental pattern of testosterone synthesis by fetal rat testes in response to luteinizing hormone. *Endocrinology* **102:** 999-1007.

Fisch H. and Goluboff E. T. (1996) Geographical variations in sperm counts: a potential cause of bias in studies of semen quality. *Fertility and Sterility* **65:** 1044-1046.

Forest M. G., Cathiard A. M. and Bertrand J. A. (1973) Evidence of testicular activity in early infancy. *Journal of Clinical Endocrinology and Metabolism* **37:** 148-151.

Foster J. W., Dominguez-Steglich M. A., Guioli S., Kwok C., Weller P. A., Stevanovic M., Weissenbach J., Mansour S., Young I. D., Goodfellow P. N., David Brook J. and Schafer A. J. (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* **372:** 525-530.

Gangnerau M. N., Funkenstein B. and Picon R. (1982) LH/hCG receptors and stimulation of testosterone biosynthesis in the rat testis: changes during fetal development in vivo and in vitro. *Molecular and Cellular Endocrinology* **28:** 499-512.

Gangnerau M. N. and Picon R. (1987) Onset of steroidogenesis and differentiation of functional LH receptors in rat fetal testicular cultures. *Archives of Andrology* **18:** 215-224.

George F. W. and Wilson J. D. (1994) Sex determination and differentiation. In The Physiology of Reproduction, E. Knobil and J. D. Neill, eds. New York: Raven Press, pp. 3-28.

Gilbert S. F. (1994) Developmental Biology. Subderland, Massachusetts: Sinauer Associates, Inc.

Gill W. B., Schumacher G. F. B., Bibbo M., Straus F. H. and Schoenberg H. W. (1979) Association of diethylstilbestrol exposure in utero with cryptorchidism, testicular hypoplasia and semen abnormalities. *Journal of Urology* **122:** 36-39.

Giwercman A. (1995) Declining semen quality and increasing incidence of abnormalities in male reproductive organs - fact or fiction? *Human Reproduction* **10:** 158-164.

Giwercman A. and Skakkebaek N. E. (1992) The Human Testis – an organ at risk? *International Journal of Andrology* **15:** 373-375.

Goodfellow P. N. and Lovell-Badge R. (1993) SRY and sex determination in mammals. *Annual Review of Genetics* **27:** 71-92.

Greco T. L., Duello T. M. and Gorski J. (1993) Estrogen receptors, estradiol, and diethylstilbestrol in early development: the mouse as a model for the study of estrogen receptors and estrogen sensitivity in embryonic development of male and female reproductive tracts. *Endocrine Reviews* **14:** 59-71.

Greco T. L., Furlow J. D., Duello T. M. and Gorski J. (1992) Immunodetection of estrogen receptors in fetal and neonatal male mouse reproductive tracts. *Endocrinology* **130:** 421-429.

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

Green R., Burril M. and Ivy A. (1939) Experimental intersexuality: modification of sexual development of the white rat with a synthetic estrogen. *Proceedings of Society for Experimental Biology and Medicine* **41:** 169-170.

Griffin J. E. and Wilson J. D. (1989) The androgen resistance syndromes: 5α-reductase deficiency, testicular feminization and related syndromes. In The Metabolic Basis of Inherited Disease, C. R. Scriver, W. S. Beaudet and S. D. Valle, eds. New York: McGraw-Hill, pp. 1919-1944.

Griffin J. E. and Wilson J. D. (1992) Disorders of the testes and the male reproductive tract. In Williams Textbook of Endocrinology, J. D. Wilson and D. W. Foster, eds. Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: Harcourt Brace Jovanovich, Inc., pp. 799-852.

Grosschedl R., Giese K. and Pagel J. (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends in Genetics* **10:** 94-100.

Grumbach M. M. and Styne D. M. (1992) Puberty: Ontogeny, Neuroendocrinology, Physiology and Disorders. In Williams Textbook of Endocrinology, J. D. Wilson and D. W. Foster, eds. Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: Harcourt Brace Jovanovich, Inc., pp. 1139-1121.

Gruss P. and Walther C. (1992) Pax in development. *Cell* **69:** 719-722.

Guerrier D., Tran D., VanderWinden J. M., Hideux S., Van Outryve L., Legeai L., Bouchard M., Van Vliet G., De Laet M. H., Picard J. Y., Kahn A. and Josso N. (1989) The persistent Mullerian duct syndrome: a molecular approach. *Journal of Clinical Endocrinology and Metabolism* **68:** 46-52.

Guo W., Mason J. S., Stone C. G., Morgan S. A., Madu S. I., Baldini A., Lindsay E. A., Biesecker L. G., Copeland K. C., Horlick M. N., Pettigrew A. L., Zanaria E. and McCabe E. R. B. (1995) Diagnosis of X-linked adrenal hypoplasia congenita by mutation analysis of the *DAX1* gene. *Journal of the American Medical Association* **274:** 324-330.

Gustafson M. L. and Donahoe P. K. (1994) Male sex determination: current concepts of male sexual differentiation. *Annual Review of Medicine* **45:** 505-524.

Haber D. A., Sohn R., Buckler A. J., Pelletier J., Call K. M. and Housman D. E. (1991) Alternative splicing and genomic structure of the Wilms' tumor gene WT1. *Proceedings of the National Academy of Sciences USA* **88:** 9618-9622.

Habert R. (1993) In vivo acute testicular testosterone response to injection of luteinizing hormone in the rat fetus. *Acta Endocrinologica* **128:** 268-73.

Habert R. and Picon R. (1982) Control of testicular steroidogenesis in foetal rat: effect of decapitation on testosterone and plasma luteinizing hormone-like activity. *Acta Endocrinologica* **99:** 466-473.

Habert R. and Picon R. (1990) Attempts for identification of a chorionic gonadotrophin-like bioactivity in the rat placenta which stimulates the testosterone secretion of the fetal testis in vitro. *Biology of Neonate* **58:** 24-31.

Hacker A., Capel B., Goodfellow P. and Lovell-Badge R. (1995) Expression of Sry, the mouse sex determining gene. *Development* **121:** 1603-1614.

Haisenleder D. J., Dalkin A. C. and Marshall J. C. (1994) Regulation of gonadotropin gene expression. In The Physiology of Reproduction, E. Knobil and J. D. Neill, eds. New York: Raven Press, pp. 1793-1813.

Hakulinen T., Andersen A. A., Malker B., Pukkala E., Schou G. and Tulinius H. (1986) Trends in cancer incidence in the Nordic countries. *Acta Pathologica Microbiologica et Immunologica Scandinavica* **94:** 151.

Hales D. B., Sha L. and Payne A. H. (1987) Testosterone inhibits cAMP-induced de novo synthesis of Leydig cell cytochrome P-45017α by an androgen receptor-mediated mechanism. *Journal of Biological Chemistry* **262:** 11200-11206.

Hall P. F. (1994) Testicular steroid synthesis: Organization and regulation. In The Physiology of Reproduction, E. Knobil and J. D. Neill, eds. New York: Raven Press, pp. 1335-1361.

Haqq C., Lee M. M., Tizard R., Wysk M., DeMarinis J., Donahoe P. K. and Cate R. L. (1992) Isolation of the rat gene for Mullerian inhibiting substance. *Genomics* **12:** 665-669.

Haqq C. M., King C.-Y., Donahoe P. K. and Weiss M. A. (1993) SRY recognises conserved DNA sites in sex-specific promotors. *Proceedings of the National Academy of Sciences USA* **90:** 1097-1101.

Haqq C. M., King C.-Y., Ukiyama E., Falsafi S., Haqq T. N., Donahoe P. K. and Weiss M. A. (1994) Molecular basis of mammalian sexual determination: activation of Mullerian inhibiting substance gene expression by SRY. *Science* **266:** 1494-1500.

Hardy M. P., Zirkin B. R. and Ewing L. L. (1989) Kinetic studies on the development of the adult population of Leydig cells in the testes of the pubertal rat. *Endocrinology* **124:** 762-770.

Harley V. R. (1993) Genetic control of testis determination. In Molecular Biology of the Male Reproductive System, D. de Kretser, ed. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press Inc., pp. 1-19.

Harley V. R. and Goodfellow P. N. (1994) The biochemical role of SRY in sex determination. *Molecular Reproduction and Development* **39:** 184-193.

Harley V. R., Jackson D. I., Hextall P. J., Hawkins J. R., Berkowitz G. D., Sockanathan S., Lovell-Badge R. and Goodfellow P. N. (1992) DNA binding activity of recombinant SRY from normal males and XY females. *Science* **255:** 453-456.

Hatano O., Takayama K., Imai T., Waterman M. R., Takakusu A., Omura T. and Morohashi K. (1994) Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. *Development* **120:** 2787-2797.

Herbison A. E. and Theodosis D. T. (1992) Localisation of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. *Neuroscience* **50:** 283-298.

Herbst A. L., Robboy S. J. and Scully R. E. (1974) Clear cell adenocarcinoma of the vagina and cervix in young females: an analysis of 170 registry cases. *American Journal of Obstetrics and Gynecology* **119:** 713-720.

Hess R. A., Cooke P. S., Bunick D. and Kirby J. D. (1993) Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. *Endocrinology* **132:** 2607-2613.

Honda S., Morohashi K., Nomura M., Takeya H., Kitajima M. and Omura T. (1993) Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *Journal of Biological Chemistry* **268:** 7494-7502.

Houston C. S., Opitz J. M., Spranger J. W., MacPherson R. I., Reed M. H., Gilbert E. F., Herrmann J. and Schinzel A. (1983) The campomelic syndrome: review, report of 17 cases, and follow-up on the currently 17-year old boy first reported by Maroteaux et al. in 1971. *American Journal of Medical Genetics* **15:** 3-28.

Hsueh A. J. W., Dahl K. D., Vaughan J., Tucker E., Rivier J., Bardin C. W. and Vale W. (1987) Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone stimulated androgen biosynthesis. *Proceedings of the National Academy of Sciences USA* **84:** 5082-5086.

Huggins C. and Moulder P. V. (1945) Estrogen production by Sertoli cell tumors of the testes. *Cancer Research* **5:** 510-514.

Huhtaniemi I. (1994) Fetal testis - a very special endocrine organ. *European Journal of Endocrinology* **130:** 25-31.

Huhtaniemi I. and Pelliniemi L. J. (1992) Fetal Leydig cells: Cellular origin, morphology, life span, and special functional features. *Proceedings of the Society for Experimental Biology and Medicine* **201:** 125-140.

Huhtaniemi I. T., Nozu K., Warren D. W., Dufau M. L. and Catt K. J. (1982) Acquisition of regulatory mechanisms for gonadotropin receptors and steroidogenesis in the maturing rat testis. *Endocrinology* **111:** 1711-1720.

Hutson J. C. (1990) Changes in the concentration and size of testicular macrophages during development. *Biology of Reproduction* **43:** 885-890.

Ikeda Y., Lala D. S., Luo X., Kim E., Moisan M. P. and Parker K. L. (1993) Characterization of the mouse *FTZ-F1* gene, which encodes a key regulator of steroid hydroxylase gene expression. *Molecular Endocrinology* **7:** 852-860.

Ikeda Y., Shen W., Ingraham H. A. and Parker K. L. (1994) Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Molecular Endocrinology* **8:** 654-662.

Imai T., Globerman H., Gertner J., Kagawa N. and Waterman M. R. (1993) Expression and purification of functional human 17α-hydroxylase/17,20-lyase (P450c17) in *Escherichia coli*. *Journal of Biological Chemistry* **268:** 19681-19689.

Ingraham H. A., Lala D. S., Ikeda Y., Luo X., Shen W., Nachtigal M. W., Abbud R., Nilson J. H. and Parker K. L. (1994) The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes and Development* **8:** 2302-2312.

Irvine D. S. (1994) Falling sperm quality. *British Medical Journal* **309**.

Jenster G., van der Korput J. A. G. M., van Vroonhoven C., van der Kwast T. H., Trapman J. and Brinkman A. O. (1991) Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Molecular Endocrinology* **5:** 1396-1404.

Jeske Y. W. A., Bowles J., Greenfield A. and Koopman P. (1995) Expression of a linear Sry transcript in the mouse genital ridge. *Nature Genetics* **10:** 480-482.

Jobling S., Reynolds T., White R., Parker M. G. and Sumpter J. P. (1995) A variety of environmentaly persistent chemicals, including some phthalate plasticizers, are weakly estrogenic. *Environmental Health Perspectives* **103:** 582-586.

Jobling S., Sheahan D., Osborne J. A., Matthiessen P. and Sumpter J. P. (1996) Inhibition of testicular growth in trout exposed to environmental estrogens. *Environmental Toxicology and Chemistry* **15:** 194-202.

Johnson A., Varner D. D., Tatum M. E. and Scrutchfield W. (1991) Season but not age affects Sertoli cell number in adult stallions. *Biology of Reproduction* **45:** 404-410.

Johnson L. and Nguyen H. B. (1986) Annual cycle of the Sertoli cell population in adult stallions. *Journal of Reproduction and Fertility* **76:** 311-316.

Josso N., Boussin L., Knebelmann B., Nihoul-Fekete C. and Picard C. Y. (1991) Anti-Mullerian hormone and intersex states. *Trends in Endocrinology and Metabolism* **2:** 227-233.

Josso N., Cate R., Picard J. Y., Vigier B., DiClemente N., Wilson C., Imbeaud S., Pepinsky R. B., Guerrier D., Boussin L., Legsai L. and Carre-Eusebe D. (1993) Anti-Mullerian hormone: the Jost factor. *Recent Progress in Hormone Research* **48:** 1-59.

Josso N. and Picard J. Y. (1986) Anti-Mullerian hormone. *Physiology Review* **66:** 1038-1090.

Jost A. (1953) Problems in fetal endocrinology: the gonadal and hypophyseal hormones. *Recent Progress in Hormone Research* **8:** 379-418.

Jost A., Vigier B. and Prepin J. (1972) Free-martins in the cattle: the first steps of sexual organogenesis.*Journal of Reproduction and Fertility* **29:** 349-379.

Jost A., Vigier M., Prepin J. and Perchellet J. P. (1973) Studies on sex differentiation in mammals. *Recent Progress in Hormone Research* **29:** 1-33.

Juneau C., Dupont E., Luu-The V., Labrie F. and Pelletier G. (1993) Ontogenesis of 3ß-hydroxysteroid dehydrogenase Δ^5-Δ^4 isomerase in the rat ovary as studied by immunocytochemistry and in situ hybridization. *Biology of Reproduction* **48:** 226-234.

Kaipia A., Toppari J., Huhtaniemi I. and Paranko J. (1994) Sex difference in the action of activin-A on cell proliferation of differentiating rat gonad. *Endocrinology* **134:** 2165-2170.

Kalla N. R., Nisula B. C., Menard R. and Loriaux D. L. (1980) The effect of estradiol on testicular testosterone biosynthesis. *Endocrinology* **106:** 35-39.

Kalloo N. B., Gearhart J. P. and Barrack E. R. (1993) Sexually dimorphic expression of estrogen receptors but not androgen receptors in human fetal external genitalia. *Journal of Clinical Endocrinology and Metabolism* **77:** 692-698.

Kaufman M. (1992) Atlas of Mouse Development. London: Academic Press.

Keeney D. S., MendisHandagama S. M. L. C., Zirkin B. R. and Ewing L. L. (1988) Effect of long term deprivation of luteinizing hormone on Leydig cell volume, Leydig cell number, and steroidogenic capacity of the rat. *Endocrinology* **123:** 2906-2915.

Kelce W., Stone C., Laws S., Gray L., Kemppianen J. and Wilson E. (1995) Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. *Nature* **375:** 581-585.

King W. J. and Greene G. L. (1984) Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* **307:** 745-747.

Kluin P. M., Kramer M. R. and De Rooij D. G. (1984) Proliferation of spermatogonia and Sertoli cells in maturing mice. *Anatomia, Embryologia* **169:** 73-78.

Knebelmann B., Boussin L., Guerrier D., Legeai L., Kahn A., Josso N. and Picard N. Y. (1991) Anti-Mullerian hormone Bruxelles: a nonsense mutation associated with persistent Mullerian duct syndrome. *Proceedings of the National Academy of Sciences USA* **88:** 3767-3771.

Kogawa K., Nakamura T., Sugino K., Takio K., Titani K. and Sugino H. (1991) Activin-binding protein is present in pituitary. *Endocrinology* **128:** 1434-1440.

Koopman P., Gubbay J., Vivian N., Goodfellow P. N. and Lovell-Badge R. (1991) Male development of chromosomally female mice transgenic for Sry. *Nature* **351:** 117-121.

Koopman P., Munsterberg A., Capel B., Vivian N. and Lovell-Badge R. (1990) Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature* **348:** 450-453.

Korach K. S. (1994) Insights from the study of animals lacking functional estrogen receptor. *Science* **266:** 1524-1527.

Kosco M. S., Loseth K. J. and Crabo B. G. (1989) Development of the seminiferous tubules after neonatal hemicastration in the boar. *Journal of Reproduction and Fertility* **87:** 1-11.

Kreidberg J. A., Sariola H., Loring J. M., Maeda M., Pelletier J., Housman D. and Jaenisch R. (1993) WT-1 is required for early kidney development. *Cell* **74:** 679-691.

Krishnan A. V., Stathis P., Permuth S. F., Tokes L. and Feldman D. (1993) Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* **132:** 2279-2286.

Ku C.-Y., Loose-Mitchell D. S. and Sanborn B. M. (1994) Both Sertoli and peritubular cells respond to androgens with increased expression of an androgen response element reporter. *Biology of Reproduction* **51:** 319-326.

Kuiper G. G. J. M., Enmark E., Pelto-Huikko M., Nilsson S. and Gustafsson J. (1996) Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Science USA* **93:** 5925-5930.

Kurki P., Vanderlaan M., Dolbeare F., Gray J. and Tan E. M. (1986) Expression of proliferating cell nuclear antigen (PCNA/cyclin) during the cell cycle. *Experimental Cell Research* **166:** 209-219.

Kuroda M., Lee M. M., Ragin R. C., Hirobe S. and Donahoe P. K. (1991) Mullerian inhibiting substance production and cleavage is modulated by gonadotropins and steroids. *Endocrinology* **129:** 2985-2992.

Kuroda T., Lee M. M., Haqq C. M., Powell D. M., Manganaro T. F. and Donahoe P. K. (1990) Mullerian inhibiting substance ontogeny and its modulation by FSH in the rat testis. *Endocrinology* **127:** 1825-1832.

Lacy D., Vinson G. P., Collins P., Bell J., Fyson P., Pudney J. and Pettit A. J. (1968) Isolation of estradiol from testes enriched in Sertoli cells. *Excerpta Medica* **184:** 1019-1029.

Lala D. S., Rice D. A. and Parker K. L. (1992) Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of *fushi tarazu*-factor I. *Molecular Endocrinology* **6:** 1249-1258.

Laudet V., Stehelin D. and Clevers H. (1993) Ancestry and diversity of the HMG box superfamily. *Nucleic Acids Research* **21:** 2493-2501.

Lawson, K. A. and Hage, W. J. (1994) Clonal analysis of the origin of primordial germ-cells in the mouse. *Ciba foundation symposia* **182**: 68-89.

Lee M. M., Cate R. L., Donahoe P. K. and Waneck G. L. (1992) Developmentally regulated polyadenilation of two discrete mRNAs for Mullerian inhibiting substance. *Endocrinology* **130:** 847-853.

Lee M. M. and Donahoe P. (1993) Mullerian inhibiting substance: a gonadal hormone with multiple functions. *Endocrine Reviews* **14:** 152-164.

Lillie F. R. (1916) The theory of free-martin. *Science* **43:** 611-613.

Lillie F. R. (1917) The free-martin, a study of the action of sex hormones in fetal life of cattle. *Journal of Experimental Zoology* **23:** 271-451.

Lin D., Sugawara T., Strauss J. F., Clark B. J., Stocco D. M., Saenger P., Rogol A. and Miller W. L. (1995) Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267:** 1828-1831.

Lin T., Calkins H., Morris P. L., Vale W. W. and Bardin C. W. (1989) Regulation of Leydig cell function in primary culture by inhibin and activin. *Endocrinology* **125:** 2134-2150.

Lopez-Casillas F., Wrana J. L. and Massague J. (1993) Betaglycan presents ligand to the TGF signalling receptor. *Cell* **73:** 1435-1444.

Lording D. W. and De Kretser D. M. (1972) Comparative ultrastructural and histochemical studies of the interstitial cells of the rat testis during fetal and postnatal development. *Journal of Reproduction and Fertility* **298:** 261-269.

Lorence M., Murray B., Trant J. and Mason J. I. (1990) Human 3ß-hydroxysteroid dehydrogenase/Δ5-4 isomerase from placenta: expression in non steroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology* **126:** 2493-2498.

Lubahn D. B., Brown T. R., Simental J. A., Higgs H. N., Migeon C. J., Wilson E. M. and French F. S. (1989) Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proceedings of the National Academy of Science USA* **86:** 9534-9538.

Lubahn D. B., Joseph D. R., Sullivan P. M., Willard H. F., French F. S. and Wilson E. M. (1988) Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* **240:** 327-330.

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

Lynch J. P., Lala D. S., Peluso J. J., Luo W., Parker K. L. and White B. A. (1993) Steroidogenic factor-1, an orphan nuclear receptor, regulates the expression of the rat aromatase gene in gonadal tissues. *Molecular Endocrinology* **7:** 776-786.

Maddocks S. and Sharpe R. M. (1989) Assessment of the contribution of Leydig cells to the secretion of inhibin by the rat testis. *Molecular and Cellular Endocrinology* **67:** 113-118.

Magre S. and Jost A. (1980) The initial phases of testicular organogenesis in the rat. *Archives of Anatomy and Microscopy* **69:** 297-318.

Majdic G., Millar M. R. and Saunders P. T. K. (1995) Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. *Journal of Endocrinology* **147:** 285-293.

Majdic G., Sharpe R. M., O'Shaughnessy P. J. and Saunders P. T. K. (1996) Expression of cytochrome P450 17α-hydroxylase/C17-20 lyase (P450c17) in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology* **137:** 1063-1070.

Manova K., Huang E. J., Angeles M., DeLeon V., Sanchez S., Pronovost S. M., Besmer P. and Bachvarova R. F. (1993) The expression of the c-kit ligand in gonads of mice supports a role for the c-kit receptor in the oocyte growth and in proliferation of spermatogonia. *Developmental Biology* **157:** 85-99.

Manova K., Nocka K., Besmer P. and Bachvarova R. F. (1990) Gonadal expression of c-kit encoded at the W locus of the mouse. *Development* **10:** 1057-1069.

Massague J. (1990) The transforming growth factor-ß family. *Annual Review of Cell Biology* **6:** 597-641.

Matsunaga E. (1981) Genetics of Wilms' tumor. *Human Genetics* **57:** 231-246.

Matzuk M. M., Finegold M. J., Su J. J., Hsueh J. W. and Bradley A. (1992) α-Inhibin is a tumour suppressor gene with gonadal specificity in mice. *Nature* **360:** 313-319.

Matzuk M. M., Kumar T. R., Vassalli A., Bickenbach J. R., Roop D. R., Jaenisch R. and Bradley A. (1995) Functional analysis of activins during mammalian development. *Nature* **374:** 354-356.

McElreavey K., Vilian E., Abbas N., Herskowitz I. and Fellous M. (1993) A regulatory cascade hypothesis for mammalian sex determination: SRY represses a negative regulator of male development. *Proceedings of the National Academy of Sciences USA* **90:** 3368-3372.

McElreavy K., Vilain E., Abbas N., Costa J. M., Souleyreau N., Kucheria K., Boucekkine C., Thibaud E., Brauner R., Flamant F. and Fellous M. (1992) XY sex reversal associated with a deletion 5' to the SRY "HMG box" in the testis determining region. *Proceedings of the National Academy of Sciences USA* **89:** 11016-11020.

McLachlan J. A. and Newbold R. R. (1975) Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. *Science* **190:** 991-992.

McLaren A. (1991) Development of the mammalian gonad: The fate of the supporting cell lineage. *BioEssays* **13:** 151-156.

McLaren, A. (1994) Germline and soma: interactions during early mouse development. *Seminars in Developmental Biology* **5**: 43-49.

Michel U., Farnworth P. and Findlay J. K. (1993) Follistatins: more than follicle-stimulating hormone suppressing proteins. *Molecular and Cellular Endocrinology* **91:** 1-11.

Miller W. L. (1988) Molecular biology of steroid hormone synthesis. *Endocrine Reviews* **9:** 295-318.

Mittwoch U. (1993) Blastocysts prepare for the race to be male. *Human Reproduction* **8:** 1550-1555.

Mittwoch U., Delhanty J. D. A. and Beck F. (1969) Growth of differentiating testes and ovaries. *Nature* **224:** 1323-1325.

Moger W. H. (1980) *In vitro* inhibitory effect of estradiol on testosterone production. *Journal of Steroid Biochemistry* **13:** 61-66.

Moore A., Krummen L. A. and Mather J. P. (1994) Inhibins, activins, their binding proteins and receptors: interactions underlying paracrine activity in the testis. *Molecular and Cellular Endocrinology* **100:** 81-86.

Morohashi K., Honda S., Inomata Y., Handa H. and Omura T. (1992) A common *trans*-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. *Journal of Biological Chemistry* **267:** 17913-17919.

Morohashi K., Iida H., Nomura M., Hatano O., Honda S., Tsukiyama T., Niwa O., Hara T., Takakusu A., Shibata Y. and Omura T. (1994) Functional difference between Ad4BP and ELP, and their distributions in steroidogenic tissues. *Molecular Endocrinology* **8:** 643-653.

Morohashi K., Zanger U. M., Honda S., Hara M., Waterman M. R. and Omura T. (1993) Activation of CYP11A and CYP11B gene promotors by the steroidogenic cell-specific transcription factor, Ad4BP. *Molecular Endocrinology* **7:** 1196-1204.

Morris J. F., Madden S. L., Tournay O. E., Cook D. M., Sukhatme V. P. and Rauscher F. (1991) Characterization of the zinc finger protein encoded by the WT1 Wilms' tumor locus. *Oncogene* **6:** 2339-2348.

Munsterberg A. and Lovell-Badge R. (1991) Expression of the mouse anti-Mullerian hormone gene suggests a role in both male and female sexual differentiation. *Development* **113:** 613-624.

Muscatelli F., Strom T. M., Walker A. P., Zanaria E., Recan D., Meindl A., Bardoni B., Guioli S., Zehetner G., Rabl W., Schwarz H. P., Kaplan J., Camerino G., Meitinger T. and Monaco A. P. (1994) Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature* **372:** 672-676.

Nagy F. (1972) Cell division kinetics and DNA synthesis in the immature Sertoli cells of the rat testis. *Journal of Reproduction and Fertility* **28:** 389-395.

Nakamura T., Takio K., Eto Y., Shibai H., Titani K. and Sugino H. (1990) Activin-binding protein from rat ovary is follistatin. *Science* **247:** 836-838.

Naylor G. C., Mierure J. P., Weeks J. A., Castaldi F. J. and Romano R. R. (1992) Alkylphenol ethoxylates in the environment. *Journal of American Oil Chemists Society* **69:** 695-703.

Nistal M. and Paniagua R. (1979) Leydig cell differentiation induced by stimulation with HCG and HMG in two patients with hypogonadotropic hypogonadism. *Andrologia* **11:** 211-222.

Nomura M., Bartsch S., Nawata H., Omura T. and Morohashi K. (1995) An E box element is required for the expression of the *ad4bp* gene, a mammalian homologue of *ftz-f1* gene, which is essential for adrenal and gonadal development. *Journal of Biological Chemistry* **270:** 7453-7461.

Nomura T. and Kanzaki T. (1977) Induction of urogenital abnormalities and some tumors in the progeny of mice receiving diethylstilbestrol during pregnancy. *Cancer Research* **37:** 1099-1104.

Nonneman D., Ganjam V., Welshons W. and Vom Saal F. (1992) Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. *Biology of Reproduction* **47:** 723-729.

Nozu K., Dufau M. L. and Catt K. J. (1981a) Estradiol receptor-mediated regulation of steroidogenesis in gonadotropin-desensitized Leydig cells. *Journal of Biological Chemistry* **256:** 1915-1922.

Nozu k., Matsuura S., Catt K. J. and Dufau M. L. (1981b) Modulation of Leydig cell androgen biosynthesis and cytochrome P-450 levels during estrogen treatment and human chorionic gonadotropin-induced desensitization. *Journal of Biological Chemistry* **256:** 10012-10017.

O'Riordan J. L. H., Malan P. G. and Gould R. P. (1988) Essentials of Endocrinology. Oxford, London, Edinburgh: Blackwell Scientific Publications.

O'Shaughnessy P. J. and Murphy L. (1991) Steroidogenic enzyme activity in the rat testis following Leydig cells destruction by ethylene-1,2-dimethansulphonate and during subsequent Leydig cell regeneration. *Journal of Endocrinology* **131:** 451-457.

Oesterlind A. (1986) Diverging trends in incidence and mortality of testicular cancer in Denmark, 1943-1982. *British Journal of Cancer* **53:** 501-505.

Orth D. N., Kovacs W. J. and DeBold C. R. (1992) The Adrenal Cortex. In Williams Textbook of Endocrinology, J. D. Wilson and D. W. Foster, eds. Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: Harcourt Brace Jovanovich, Inc., pp. 489-620.

Orth J. M. (1982) Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anatomical Record* **203:** 385-492.

Orth J. M. (1984) The role of FSH in controlling Sertoli cell proliferation in testes of fetal rats. *Endocrinology* **115:** 1248-1255.

Orth J. M., Gunsalus G. M. and Lamperti A. A. (1988) Evidence from Sertoli cell-depleted rats indicates that spermatid numbers in adult testis depends on numbers of Sertoli cells produced during perinatal development. *Endocrinology* **122:** 787-794.

Packer A. I., Besmer P. and Bachvarova R. F. (1995) Kit ligand mediates survival of type A spermatogonia and dividing spermatocytes in postnatal mouse testis. *Molecular Reproduction and Development* **42:** 303-310.

Pakarinen P., Proshlyakova E. and Huhtaniemi I. (1994) Pituitary-gonadal interactions in perinatal rats: relationships of plasma luteinizing hormone and testosterone concentrations, and pituitary levels of LH subunit mRNAs. *Neuroendocrinology* **60:** 42-49.

Pao C. C., Kao S. M., Hor J. J. and Chang S. Y. (1993) Lack of mutational alteration in the conserved regions of ZFY and SRY genes of 46,XY females with gonadal dysgenesis. *Human Reproduction* **8:** 224-228.

Parker K. L. (1996) The nuclear receptor SF-1 acts at multiple levels of the reproductive axis. In 10th International Congress of Endocrinology, San Francisco, USA, pp. 36.

Parker M. G. (1991) Nuclear Hormone Receptors. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press Inc.

Patek C. E., Kerr J. B., Gosden R. G., Jones K. W., Hardy K., Muggleton-Harris A. L., Handyside A. H., Whitingham D. G. and Hooper M. L. (1991) Sex chimaerism, fertility and sex determination in the mouse. *Development* **113:** 311-325.

Payne A. H. (1990) Hormonal regulation of cytochrome P450 enzymes, cholesterol side-chain cleavage and 17α-hydroxylase/C17-20 Lyase in Leydig cells. *Biology of Reproduction* **42:** 399-404.

Payne A. H. and Youngblood G. (1995) Regulation of expression of steroidogenic enzymes in Leydig cells. *Biology of Reproduction* **52:** 217-225.

Pelletier J., Bruening W., Kashtan C. E., Mauer S. M., Manivel J. C., Striegel J. E., Houghton D. C., Junien C., Habib R., Rouser L., Fine R. N., Silverman B. L., Haber D. A. and Housman D. (1991a) Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development. *Cell* **67:** 437-447.

Pelletier J., Bruening W., Li F. P., Haber D. A., Glaser T. and Housman D. E. (1991b) WT1 mutations contribute to abnormal genital system development and hereditary Wilms' tumor. *Nature* **353:** 431-434.

Pelletier J., Schalling M., Buckler A. J., Rogers A., Haber D. A. and Housman D. E. (1991c) Expression of the Wilms' tumor gene WT1 in the murine urogenital system. *Genes and Development* **5:** 1345-1356.

Pelliniemi L. J., Frojdman K. and Paranko J. (1993) Cell biology of testicular development. In Molecular Biology of the Male Reporductive System, D. de Kretser, ed. San Diego, New York, Boston, London, Sydney, Tokyo, Toronto: Academic Press Inc., pp. 20-65.

Pepinsky R. B., Sinclair L. K., Chow E. P., Mattaliano R. J., Manganaro T. F., Donahoe P. K. and Cate R. L. (1988) Proteolytic processing of Mullerian inhibiting substance produces a transforming growth factor-ß-like fragment. *Journal of Biological Chemistry* **263:** 18961-18964.

Peschon J., Behringer R. R., Cate R. L., Harwood K. A., Idzerida R. L., Brinster R. L. and Palmiter R. D. (1992) Directed expression of an oncogene to Sertoli cells in transgenic mice using Mullerian inhibiting substance. *Molecular Endocrinology* **6:** 1403-1411.

Picon R. (1976) Testosterone secretion by foetal rat testes in vitro. *Journal of Endocrinology* **71:** 231-238.

Pieau C. (1996) Temperature variation and sex determination in reptiles. *BioEssays* **18:** 19-26.

Pointis G., Latreille M. T. and Cedard L. (1984) Regulation of testosterone production in fetal testicular cells: effect of androgens. *Experientia* **40:** 756-757.

Prince F. P. (1990) Ultrastructural evidence of mature Leydig cell and Leydig cell regression in the neonatal human testis. *Anatomical Record* **228:** 405-413.

Qiu P. C., Gaudette M. F., Robinson D. H. and Crain W. R. (1995) Expression of the mouse testis-determining gene Sry in male preimplantation embryos. *Molecular Reproduction and Development* **40:** 196-204.

Quigley C. A., De Bellis A., Marschke K. B., El-Awady M., Wilson E. M. and French F. S. (1995) Androgen receptor defects: historical, clinical and molecular perspectives. *Endocrine Reviews* **16:** 271-321.

Rabinovici J., Goldsmith P. C., Roberts V. J., Vaughan J., Vale W. and Jaffe R. B. (1991) Localization and secretion of inhibin/activin subunits in the human and subhuman primate fetal gonads. *Journal of Clinical Endocrinology and Metabolism* **73:** 1141-1149.

Ranniki A. S., Zhang F. P. and Huhtaniemi I. T. (1995) Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. *Molecular and Cellular Endocrinology* **107:** 199-208.

Rey R. A., Campo S. M., Bedecarras P., Nagle C. A. and Chemes H. E. (1993) Is infancy a quiscent period of testicular development? Histological, morphometric, and functional study of the seminiferous tubules of the Cebus monkey from birth to the end of puberty. *Journal of Clinical Endocrinology and Metabolism* **76:** 1325-1331.

Roberts V., Meunier H., Sawchenko P. and Vale W. (1989) Differential production and regulation of inhibin subunits in rat testicular cell types. *Endocrinology* **125:** 2350-2359.

Roberts V., Sawchenko P. E. and Vale W. (1991) Expression of inhibin/activin subunit messenger ribonucleic acids during rat embryogenesis. *Endocrinology* **128:** 3122-3129.

Roberts V. J. and Barth S. L. (1994) Expression of messenger ribonucleic acids encoding the inhibin/activin system during mid-and late-gestation rat embryogenesis. *Endocrinology* **134:** 914-923.

Robertson D. M., Klein R., de Vos F. L., McLachlan R. I., Wettenhall R. E. H., Hearn M. T. W., Burger H. G. and de Kretser D. M. (1987) The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochemical and Biophysical Research Communications* **149:** 744-749.

Rommerts F. F. G., Krugar-Sawnarain B. C., Van Woerkom-Blik A., Grootegoed J. A. and Van der Molen J. H. (1978) Secretion of proteins by Sertoli cell enriched cultures: effects of follicle stimulating hormone, dibutyryl cAMP and testosterone and correlation with secretion of oestradiol and androgen-binding protein. *Molecular and Cellular Endocrinology* **10:** 39-55.

Roosen-Runge E. C. and Anderson D. (1959) The development of the interstitial cells in the testis of the albino rat. *Acta anatomica* **37:** 125-137.

Roosen-Runge E. C. and Leik J. (1968) Gonocyte degeneration in the postnatal male rat. *American Journal of Anatomy* **122:** 275-300.

Rossi P., Dolci S., Albanesi C., Grimaldi P., Ricca R. and Geremia R. (1993) Follicle-stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. *Developmental Biology* **155:** 68-74.

Rouiller V., Gangnerau M. N., Vayssiere J. L. and Picon R. (1990) Cholesterol side-chain cleavage activity in rat fetal gonads: a limiting step for ovarian steroidogenesis. *Molecular Cellular Endocrinology* **72:** 111-120.

Rundlett S. E., Wu S.-P. and Miesfeld R. L. (1990) Functional characterizations of the androgen receptor confirm that the molecular basis of androgen action is transcriptional regulation. *Molecular Endocrinology* **4:** 708-714.

Russel D. L. and Findlay J. K. (1995) The N-terminal peptide of the inhibin α subunit: What are its endocrine and paracrine roles? *Trends in Endocrinology and Metabolism* **6:** 305-311.

Russell L. D., Bartke A. and Gosh J. C. (1989) Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion and flow. *American Journal of Anatomy* **184:** 179-189.

Russell L. D. and Peterson R. N. (1984) Determination of the elongate spermatid-Sertoli cell ratio in various mammals. *Journal of Reproduction and Fertility* **70:** 635-641.

Ryan G., Steele-Perkins V., Morris J. F., Rauscher F. J. and Dressler G. R. (1995) Repression of Pax-2 by WT1 during normal kidney development. *Development* **121:** 867-875.

Ryner L. C. and Swain A. (1995) Sex in the 90's. *Cell* **81:** 483-493.

Sachs A. (1990) The role of poly(A)in the translation and stability of mRNA. *Current Opinions in Cell Biology* **2:** 1092-1098.

Saez J. M. (1994) Leydig cells: endocrine, paracrine and autocrine regulation. *Endocrine Reviews* **15:** 574-624.

Sar M., Sahu A., Crowley W. R. and Kalra S. P. (1990) Localisation of neuropeptide-Y immunoreactivity in estradiol-concentrating cells in the hypothalamus. *Endocrinology* **127:** 2752-2756.

Satoh M. (1985) The histogenesis of the gonad in rat embryos. *Journal of Anatomy* **143:** 17-37.

Saunders P. T. K., Millar M. R., Majdic G., McLaren T. T., Bremner W. J., Grigor K. M., Chandley A. C. and Sharpe R. M. (1996) Testicular androgen receptor protein: distribution and control of expression. In Molecluar and Cellular Endocrinology of the Testis, E. S. Desjardins, ed.: Serono, In press.

Schlatt S. and Weinbauer G. F. (1994) Immunohistochemical localization of proliferating cell nuclear antigen as a tool to study cell proliferation in rodent and primate testis. *International Journal of Andrology* **17:** 214-222.

Schwall R., Schmelzer C. H., Matsuyama E. and Mason A. J. (1989) Multiple actions of recombinant activin-A *in vivo*. *Endocrinology* **125:** 1420-1423.

Seller M. J. and Perkins-Cole K. H. (1987) Sex difference in mouse embryonic development at neurulation. *Journal of Reproduction and Fertility* **79:** 159-161.

Shaha C., Morris P. L., Chen C. C., Vale W. and Bardin C. W. (1989) Immunostainable inhibin subunits are in multiple cell types of testicular cells. *Endocrinology* **125:** 1941-1950.

Sharpe R. M. (1994) Regulation of Spermatogenesis. In The Physiology of Reproduction, 2nd Edn, E. Knobil and J. D. Neill, eds. New York: Raven Press, pp. 1363-1434.

Sharpe R. M., Fisher J., Millar M. R., Jobling S. and Sumpter J. S. (1995) Gestational and/or neonatal exposure of rats to environmental estrogenic chemicals results in reduced testis size and daily sperm production in adulthood. *Environmental Health Perspectives* **103:** 1136-1143.

Sharpe R. M., Maddocks S., Millar M., Saunders P. T. K., Kerr J. B. and McKinnell C. (1992) Testosterone and spermatogenesis: identification of stage-dependent, androgen-regulated proteins secreted by adult rat seminiferous tubules. *Journal of Andrology* **13:** 172-184.

Sharpe R. M. and Skakkebaek N. E. (1993) Are oestrogens involved in falling sperm counts and disorders of the reproductive tract? *Lancet* **341:** 125-126.

Shen W., Moore C. C. D., Ikeda Y., Parker K. L. and Ingraham H. A. (1994) Nuclear receptor steroidogenic factor-1 regulates the Mullerian inhibiting substance gene: a link to the sex determination cascade. *Cell* **77:** 651-661.

Shi S.-R., Chaiwun B., Young L., Cote R. and Taylor C. (1993) Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *Journal of Histochemistry and Cytochemistry* **41:** 1599-1604.

Shimasaki S., Koga M., Buscaglia M. L., Simmons D. M., Bicsak T. A. and Ling N. (1989) Follistatin gene expression in the ovary and extragonadal tissues. *Molecular Endocrinology* **3:** 651-659.

Shimonaka M., Inouye S., Shimasaki S. and Ling N. (1991) Follistatin binds to both activin and inhibin through common beta-subunit. *Endocrinology* **128:** 3313-3315.

Siiteri P. K. and Wilson J. D. (1974) Testosterone formation and metabolism during male sexual differentiation in the human embryo. *Journal of Clinical Endocrinology and Metabolism* **38:** 113-125.

Sinclair A. H., Berta P., Palmer M. S., Hawkins J. R., Griffiths B. L., Smith M. J., Foster J. W., Fishchauf A.-M., Lovell-Badge R. and Goodfellow P. N. (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature* **346:** 240-250.

Skinner M. K. (1991) Cell-cell interactions in the testis. *Endocrine Reviews* **12:** 45-77.

Smith O. W. (1948) Diethylstilbestrol in the prevention and treatment of complications of pregnancy. *American Journal of Obstetrics and Gynecology* **56:** 821-830.

Soto A. M., Justicia H., Wray J. W. and Sonnenschein C. (1991) p-Nonylphenol: an estrogenic xenobiotic released from "modified" polystyrene. *Environmental Health Perspectives* **92:** 167-173.

Spencer S. J., Rabinovici J. and Jaffe B. (1990) Human recombinant activin-A inhibits proliferation of human fetal adrenal cells *in vitro*. *Journal of Clinical Endocrinology and Metabolism* **1990:** 1678-80.

Stillman R. J. (1982) In utero exposure to diethylstilbestrol: adverse effects on the reproductive tract and reproductive performance in male and female offspring. *American Journal of Obstetrics and Gynecology* **142:** 905-921.

Sun E. L. and Gondos B. (1981) Proliferative activity in the rabbit testis during postnatal development. In Development and Function of Reproductive Organs, A. Byskov and H. Peters, eds. Amsterdam: Excerpta Medica, pp. 140-148.

Suominen J. and Vierula M. (1993) Semen quality of Finnish men. *British Medical Journal* **306:** 1579-1581.

Swain A., Zanaria E., Hacker A., Lovell-Badge R. and Camerino G. (1996) Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nature Genetics* **12:** 404-409.

Taketo M., Parker K. L., Howard T., Tsukiyama T., Wong M., Niwa O., Morton C. C., Miron P. M. and Seldin M. F. (1995) Homologs of drosophila Fushi-Tarazu Factor-1 map to mouse chromosome 2 and human chromosome 9q33. *Genomics* **25:** 565-567.

Tapanainen J., Kellokumpu-Lehtinen P., Pelliniemi L. and Huhtaniemi I. (1981) Age-related changes in endogenous steroids of human fetal testis during early and midpregnancy. *Journal of Clinical Endocrinology and Metabolism* **52:** 98-102.

Tapanainen J., Kuopio T., Pelliniemi L. J. and Huhtaniemi I. (1984) Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biology of Reproduction* **31:** 1027-1035.

Toppari J., Larsen J. C., Christiansen P., Giwercman A., Grandjean P., Guillette L. J., Keiding N., Leffers H., McLachlan J. A., Meyer O., Muller J., Rajpert-De Meyts E., Scheike T., Sharpe R. M., Sumpter J. and Skakkebaek N. E. (1996) Male reproductive health and environmental chemicals with estrogenic effects. *Environmental Health Perspectives* : In press.

Torres M., Gomez-Pardo E., Dressler G. R. and Gruss P. (1995) Pax-2 controls multiple steps of urogenital development. *Development* **121:** 4057-4065.

Tsai-Morris C., Knox G., Luna S. and Dufau M. L. (1986) Acquisition of Estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat Leydig cells. *Journal of Biological Chemistry* **261:** 3471-3474.

Tsukiyama T., Ueda H., Hirose S. and Niwa O. (1992) Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. *Molecular and Cellular Biology* **12:** 1286-1291.

Tsunoda Y., Tokunaga T. and Sugie T. (1985) Altered sex ration of live young after transfer of fast- and slow-developing mouse embryos. *Gamete Research* **12:** 301-304.

Tucker P. K. and Lundrigan B. L. (1993) Rapid evolution of the sex determining locus in old world mice and rats. *Nature* **364:** 715-717.

Ueno N., Ling N., Ying S.-Y., Esch F., Shimasaki S. and Guillemin R. (1987) Isolation and partial characterization of follistatin: a single chain Mr 35,000 monomeric protein that inhibits the release of follicle stimulating hormone. *Proceedings of the National Academy of Sciences USA* **84:** 8282-8286.

Vale W., Rivier C. and Hsueh A. (1988) Chemical and biological characterization of the inhibin family of protein hormones. *Recent Progress in Hormone Research* **44:** 1-34.

Vale W., Rivier J., Vaughan J., McClintock R., Corrigan A., Woo W., Karr D. and Spiess J. (1986) Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* **321:** 776-779.

Van Haster L. H., de Jong F. H., Docter R. and de Rooij D. G. (1993) High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. *Endocrinology* **133:** 755-760.

van Steensel B., Jenster G., Damm K., Brinkmann A. O. and van Driel R. (1995) Domains of the human androgen receptor and glucocorticoid receptor involved in binding to the nuclear matrix. *Journal of Cellular Biochemistry* **57:** 465-478.

van Waeleghem K., de Clercq N., Vermuelen L., Schoonjans F. and Comhaire F. (1994) Detoriation of sperm quality in young Belgian men during recent decades. *Human Reproduction* **9:** Abstract 73.

Vannier B. and Raynaud J. P. (1980) Long-term effects of prenatal oestrogen treatment on genital morphology and reproductive function in the rat. *Journal of Reproduction and Fertility* **59:** 43-49.

Vergouwen R. P. F. A., Jacobs S. G. P. M., Huiskamp R., Davids J. A. G. and de Rooij D. G. (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. *Journal of Reproduction and Fertility* **93:** 233-243.

Verhoeven G. (1980) Androgen receptor in cultured interstitial cells derived from immature testis. *Journal of Steroid Biochemistry* **13:** 469-474.

Verhoeven G., Dierickx P. and de Moor P. (1979) Stimulation effect of neurotransmitters on the aromatization of testosterone by Sertoli cell-enriched cultures. *Molecular and Cellular Endocrinology* **13:** 241-253.

Vierula M., Niemi M., Keiski A., Saatanen M., Saarikoski S. and Suominen J. (1996) High and unchanged sperm counts of Finnish men. *International Journal of Andrology* **19:** 11-17.

Visser J. A., McLuskey A., van Beers T., Weghuis D. O., van Kessel A. G., Grootegoed J. A. and Themmen A. P. N. (1995) Structure and chromosomal localization of the antimullerian hormone type II receptor gene. *Biochemical and Biophysical Research Communications* **215:** 1029-1036.

Vornberger W., Prins G., Musto N. A. and Suarez-Quian C. A. (1994) Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* **134:** 2307-2316.

Voutilainen R. and Miller W. L. (1986) Developmental expression of genes for the steroidogenic enzymes P450scc (20,22-Desmolase), P450c17 (17α-hydroxylase/17,20-Lyase), and P450c21 (21-hydroxylase) in the human fetus. *Journal of Clinical Endocrinology and Metabolism* **63:** 1145-1150.

Voutilainen R. and Miller W. L. (1987) Human Mullerian inhibitory factor messenger ribonucleic acid is hormonally regulated in the fetal testis and in adult granulosa cells. *Molecular Endocrinology* **1:** 604-608.

Wagner T., Wirth J., Meyer J., Zabel B., Held M., Zimmer J., Pasantes J., Bricarelli F. D., Keutel J., Hustert E., Wolf U., Tommerup N., Schemmp W. and Scherer G. (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* **79:** 1111-1120.

Wallach E. E. (1996) Inhibin, activin and follistatin in reproductive medicine. *Fertility and Sterility* **65:** 459-469.

Warren D. W., Haltmeyer G. C. and Eik-Nes K. B. (1973) Testosterone in the fetal rat testis. *Biology of Reproduction* **8:** 560-565.

Warren D. W., Huhtaniemi I. T., Dufau M. L. and Catt K. J. (1987) Regulation of LH receptors and steroidogenesis in the foetal rat testis in vivo. *Acta Endocrinologica* **115:** 189-195.

Warren D. W., Huhtaniemi I. T., Tapanainen J., Dufau M. L. and Catt K. J. (1984) Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. *Endocrinology* **114:** 470-475.

Watanabe Y. G. and Daikoku S. (1979) An immunohistochemical study on the cytogenesis of adenohypophysial cells in fetal rats. *Developmental Biology* **68:** 557-567.

White R., Jobling S., Hoare S. A., Sumpter J. P. and Parker M. G. (1994) Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135:** 175-182.

Whitfield L. S., Lovell-Badge R. and Goodfellow P. (1993) Rapid sequence evolution of the mammalian sex-determining gene SRY. *Nature* **364:** 713-715.

Wilcox A. J., Baird D. D., Weinberg C. R., Hornsby P. P. and Herbst A. L. (1995) Fertility in men exposed prenatally to diethylstilbestrol. *New England Journal of Medicine* **332:** 1411-1415.

Wilson J. D. and Lasnitzki I. (1971) Dihydrotestosterone formation in fetal tissues of the rabbit and rat. *Endocrinology* **89:** 659-668.

Winslow S. G. and Henkart P. A. (1991) Polyinosinic acid as a carrier in the microscale purification of total RNA. *Nucleic Acids Research* **19:** 3251-3253.

Witschi E. (1948) Migration of the germ cells of the human embryos from the yolk sac to the primitive gonadal folds. *Contributions in Embryology* **32:** 67-82.

Woodruf T. K., Meunier H., Jones P. B. C., Hsueh A. J. W. and Mayo K. E. (1987) Rat inhibin: molecular cloning of α- and ß-subunit complementary deoxyribonucleic acids and expression in the ovary. *Molecular Endocrinology* **1:** 561-568.

Wrana J. L., Attisano L., Carcamo J., Zentella A., Doody J., Laiho M., Wang X. F. and Massague J. (1992) TGF-ß signals through a heteromeric protein kinase receptor complex. *Cell* **71:** 1003-1114.

Wright E., Hargrave M. R., Christiansen J., Cooper L., Kun J., Evans T., Gangadharan U., Greenfield A. and Koopman P. (1995) The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nature Genetics* **9:** 15-20.

Wurzel J. M., Curatola L. M., Gurr J. A., Goldsmidt A. M. and Kourides I. A. (1983) The luteotropic activity of rat placenta is not due to chorionic gonadotropin. *Endocrinology* **113:** 1854-1857.

Xu K. P., Yadav B. R. and King W. A. (1992) Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. *Molecular Reproduction and Development* **31:** 249-252.

Ying S. Y. (1988) Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocrine Reviews* **9:** 267-293.

Yoshinaga K., Nishikawa S., Ogawa M., Hayashi S.-I., Kunisada T., Fujomoto T. and Nishikawa S.-I. (1991) Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development* **113:** 689-699.

Zanaria E., Muscatelli F., Bardoni B., Strom T. M., Guioli S., Guo W., Laill E., Moser C., Walker A. P., McCabe E. R. B., Meitinger T., Monaco A. P., Sassone-Corsi P. and Camerino G. (1994) An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature* **372:** 635-641.

Zhang F., Hamalainen T., Kaipia A., Pakarinen P. and Huhtaniemi I. (1994) Ontogeny of luteinizing hormone receptor gene expression in the rat testis. *Endocrinology* **134:** 2206-2213.

Zhang P. and Mellon S. H. (1996) The orphan nuclear receptor steroidogenic factor-1 regulates the cyclic adenosine 3',5'-monophosphate-mediated transcriptional activation of rat cytochrome P450c17 (17α-hydroxylase/c17-20 lyase). *Molecular Endocrinology* **10:** 147-158.

Zhou Z.-X., Lane M. V., Kemppainen J. A., French F. S. and Wilson E. M. (1995) Specificity of ligand-dependent androgen stabilization: receptor domain interactions influence dissociation and receptor stability. *Molecular Endocrinology* **9:** 208-218.

Zondek B. (1934) Mass excretion of oestrogenic hormone in the urine of the stallion. *Nature* **133:** 209-210.

Zwingman T., Erickson R. P., Boyer T. and Ao A. (1993) Transcription of the sex-determining region genes Sry and Zfy in the mouse preimplantation embryo. *Proceedings of the National Academy of Sciences USA* **90:** 814-817.

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Expression of Cytochrome P450 17α-Hydroxylase/C17–20 Lyase in the Fetal Rat Testis Is Reduced by Maternal Exposure to Exogenous Estrogens

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ABSTRACT

Testosterone is required for normal development of the male reproductive tract. Synthesis of testosterone occurs in the Leydig cells and is dependent upon the expression of several enzymes, including cytochrome P450 17α-hydroxylase/C17–20-lyase (P450c17), which is highly regulated within the testis. The aim of the present study was to investigate whether maternal exposure to estrogenic chemicals was able to affect Leydig cell function in the developing male fetus at the time of masculinization. Pregnant rats were injected sc with diethylstilbestrol (DES; 100 or 500 μg/kg), 4-octylphenol (OP; 100 or 600 mg/kg), or vehicle (oil, control) on days 11.5 and 15.5 postcoitum. Doses were chosen to reflect the reported estrogenic potency of the chemicals *in vitro*. On day 17.5, fetal testes were fixed before performing *in situ* hybridization and immunocytochemistry, used for extraction of RNA, or homogenized in phosphate buffer for determination of 17α-hydroxylase enzyme activity.

There was no difference between fetuses from control and treated mothers in either the overall histology of the testes or the apparent number of Leydig cells, as determined by immunocytochemistry with an antibody directed against 3β-hydroxysteroid dehydrogenase. However, there was a consistent and striking reduction in the amount of P450c17 detected by immunocytochemistry in testes from the groups given the higher dose of DES or OP. These observations were supported by measurement of 17α-hydroxylase activity, which was significantly reduced compared with that in controls (6.25 ± 0.65 pmol/testis·min) in fetuses from animals treated with 100 μg/kg DES (4.27 ± 0.39; $P < 0.05$), 500 μg/kg DES (1.4 ± 0.47; $P < 0.001$), or 600 mg/kg OP (4.25 ± 0.33; $P < 0.05$). RT-PCR and *in situ* hybridization revealed that these changes were mirrored by reductions in P450c17 messenger RNA in testes from fetuses from treated mothers compared with control levels.

In conclusion, maternal treatment with either a potent synthetic estrogen (DES) or a putative environmental estrogen (OP) results in reduced expression of the messenger RNA and protein for P450c17 in fetal Leydig cells. These results, therefore, provide a mechanism by which inappropriate exposure of the fetus to estrogenic chemicals might have an adverse affect on fetal steroid synthesis and masculinization. (*Endocrinology* **137:** 1063–1070, 1996)

NORMAL DEVELOPMENT of the testis and male reproductive tract is a tightly regulated process. The earliest step in formation of the testis from the indifferent gonad is the differentiation of Sertoli cells, which occurs after expression of Sry (the testis-determining factor) (1) on day 10.5 in the mouse. The Sertoli cells then orchestrate the formation of the testis cords and synthesize and secrete anti-Mullerian hormone (2). In the rat on or about day 15 of gestation, Leydig cells differentiate from mesenchymal cells in the interstitial region of the testis between the seminiferous cords (3). Fetal Leydig cells are the site of synthesis of testosterone, which is essential for masculinization of the reproductive tract and external genitalia and the development of a normal male phenotype (4).

During the 1950s to 1970s, some women were given diethylstilbestrol (DES), a potent estrogen, as a preventative for miscarriage. Studies have shown that the offspring of these women had an increased incidence of abnormalities of their reproductive tracts. In the adult males, an increased incidence of hypospadias, cryptorchidism, as well as smaller testes and semen abnormalities have been reported (5). However, a recent follow-up survey of the same patients did not find their fertility to have been reduced (6). Prenatal exposure of male mice to DES has been associated with epididymal cysts, cryptorchidism, and, in some cases, sterility (7). In the past 50 yr, the incidence of testicular cancer has increased progressively in many developed countries, whereas average sperm counts have fallen by 40–50% during the same time period, and other developmental male reproductive disorders also have increased in incidence (8, 9). Similarities between the reproductive problems of males born to DES-treated mothers and those occurring in the rest of the population has led to the hypothesis that one potential cause of male reproductive abnormalities may be inappropriate exposure to estrogens during fetal life (8, 9).

Synthesis of testosterone is dependent upon the expression of four steroidogenic enzymes (10), including P450 17α-hydroxylase/C17–20-lyase (P450c17), which is highly regulated in the Leydig cells, and 3β-hydroxysteroid dehydrogenase (3βHSD), which appears to be largely constitutive (11, 12). The messenger RNAs (mRNAs) encoding these enzymes are present in fetal mouse testis on day 13.5 postcoitum (pc) (approximately equivalent to day 15.5 in the rat) (13). In the fetal rat testis, testosterone synthesis is detectable on day 15.5 of pregnancy (14), with testosterone production reaching its peak around day 18.5 pc and then declining (15).

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In the adult testis, testosterone production by Leydig cells can be inhibited by exposure to estrogens (16, 17), and it has been shown that this inhibition is associated with lower activity of the 17α-hydroxylase enzyme (18, 19). Estrogen receptors (ER) have been detected using immunocytochemistry in fetal mouse testes (including the interstitial cells) and associated ducts on days 13, 15, and 17 of gestation (20). Incubation of fetal rat Leydig cells recovered on day 21 of pregnancy with estradiol is reported to result in decreased androgen production and an increase in ER detectable by binding assay (21).

In the present study we examined the effects of maternal treatment with DES or a putative environmental estrogen, 4-octylphenol (OP), at two different times during pregnancy, corresponding to the development of the fetal testis (day 11.5) and the onset of Leydig cell steroidogenesis (day 15.5). Our results show that administration of these estrogenic chemicals to the mother results in a specific reduction in steroidogenic enzyme activity within the fetal testis associated with reduced levels of P450c17 mRNA and protein.

Materials and Methods

Animals, treatments, and tissue recovery

Adult female rats were placed in individual cages with male rats and checked for the presence of copulatory plugs each morning. The day when the plug was found was taken as day 0.5 pc. Pregnant females were injected sc on days 11.5 and 15.5 pc with DES (Sigma Chemical Co., St. Louis, MO; 100 or 500 μg/kg) or OP (Aldrich Chemical Co., Rouses Point, IL; 100 or 600 mg/kg) in corn oil (1 ml/kg) or with the vehicle alone (controls). Pregnant females were killed on day 17.5 pc by inhalation of carbon dioxide followed by cervical dislocation. Fetuses were examined under a dissecting microscope, and the testes were recovered. Testes were either immersion fixed in Bouin's solution for 5–6 h before processing for immunocytochemistry and *in situ* hybridization (22) or used for extraction of RNA or determination of enzyme activity (see below). Fetal age was confirmed by morphological examination of the fetuses (23).

RNA extraction

Dissected testes from two to five fetuses from the same litter were pooled in 1 ml TRI reagent (Sigma) and homogenized by passing through a 27-gauge needle. Homogenate was mixed thoroughly with chloroform (0.2 ml; IBI, Cambridge, UK) and kept at room temperature for 15 min. Samples were then centrifuged at 12,000 × *g* for 15 min before the upper aqueous phase was transferred to fresh tubes. RNA was precipitated in the presence of 3 μg polyinosine (Pharmacia, Piscataway, NJ) (24) using 0.5 ml isopropanol (Sigma) for 15 min at −20 C. Samples were centrifuged at 12,000 × *g* for 15 min to pellet the RNA, which was washed with 75% ethanol, air-dried, and dissolved in 20 or 50 μl distilled water. The concentration of RNA was determined by measuring OD at 260 nm, and its quality was checked by electrophoresis of 5 μg/lane in a denaturing agarose gel (25).

RT-PCR

Primers based on the sequence of the rat CYP17 gene (26) upstream 5′-CTTGTCGGACCAAGGAAAAGGCGT (bases 344–367, sense strand) and downstream 5′-AGCCAGCTGATCGTGCAGC (bases 669–692, antisense strand) were synthesized on an Applied Biosystems PCR mate (ABI, Warrington, Cheshire, UK). Reverse transcription was undertaken using 250–500 ng total RNA in a 20-μl volume containing 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 1 mM $MnCl_2$, 200 μM of each deoxy-NTP, 5 U Tth polymerase (ABI) (27), and 0.75 μM antisense primer. The conditions used were 65 C for 5 min, 53 C for 5 min, and 70 C for 15 min, and the samples were then placed on ice. The reaction volume was increased to 100 μl, and PCR was undertaken in buffer containing 1.5 mM $MgCl_2$, 40% glycerol, 10 mM Tris-HCl (pH 8.3), 890 mM KCl, 6 mM EGTA, 0.4% Tween 20 ($MnCl_2$ in the first reaction solution was chelated by the EGTA), and 0.15 μM of the upstream primer. PCR conditions were denaturation at 95 C for 3 min, followed by 30 cycles of amplification at 94 C for 0.5 min, 55 C for 1 min, and 70 C for 2 min, with a final elongation at 70 C for 10 min. The expected size of the amplified product was 348 bp. To provide an internal control, cyclophilin complementary DNA (cDNA) was amplified simultaneously in every reaction, using upstream primer 5′-GTGGCAAGTCCATCTACG (bases 263–280 sense strand) and downstream primer 5′-CAGTGAGAGCAGAGATTACAG (bases 626–646 antisense strand) based on the sequence of rat cyclophilin (28). The expected size of the amplified product was 380 bp. Amplified product(s) were analyzed by electrophoresis on a 2% agarose gel run in Tris-borate-EDTA buffer using standard methods. The cDNAs were subcloned into pCRII vector according to the manufacturer's instructions (Invitrogen Corp., San Diego, CA). The specificity of amplification was confirmed by sequencing the cloned fragments of P450c17 and cyclophilin cDNAs using an ABI automated sequencer and fluorescent primers, as previously described (29).

In situ hybridization

To synthesize sense and antisense riboprobes, p450c17-pCRII was linearized with *Xho*I (antisense) and *Bam*HI (sense), and complementary RNA (cRNA) was synthesized with SP6 or T7 RNA polymerase, respectively (Boehringer Mannheim). Probes were labeled with [^{35}S]UTP (400 Ci/mmol; Amersham, Aylesbury, UK), as described previously (22), and purified using RNA spin columns (IBI). Paraffin sections (5 μm) were mounted on slides coated with 3-aminopropyl triethoxy-silane (Sigma), dried overnight at 50 C, dewaxed in xylene, and rehydrated in graded ethanols. Sections were hybridized with 10^6 cpm [^{35}S]cRNA in 40 μl hybridization buffer at 50 C overnight. Next day, sections were washed in 4 × SSC (1 × SSC contains 0.15 M NaCl and 15 mM sodium citrate, pH 7) for 10 min, incubated in ribonuclease A (Sigma) solution (2 mg/ml in 0.01 M Tris-HCl, 0.05 M NaCl, and 5 mM EDTA, pH 8.0) for 30 min at 37 C, washed twice for 5 min each time in 2 × SSC, and finally washed in 0.1 × SSC-30% formamide for 10 min at 40 C. Sections were dehydrated in graded ethanols, air-dried, dipped in undiluted photographic emulsion (NTB-2, Eastman Kodak, Rochester, NY), exposed at 4 C in a light-tight box for 2 weeks, and processed as previously described (22). To ensure that all of the tissue sections were hybridized and washed under the same conditions, sections from control and treated animals were mounted on the same slide. Sections were observed under brightfield illumination using an Olympus BH2 microscope (Olympus Corp., New Hyde Park, NY) and a ×40 objective. Images were captured using a data translation quick capture card and a Macintosh IIcx computer, and quantified using NIH image 1.57 program. To determine the abundance of P450c17 mRNA, the number of silver grains in 10 random fields in the interstitial region of the testes was counted. Background counts were determined by counting 10 random fields in regions of the same sections that were outside the interstitium, *e.g.* mesonephros, and the average of the grain counts in these regions was deducted from the grain densities determined for the interstitial regions. The silver grains were counted in 2–3 testes from 3 different litters for each treatment group, and the group mean ± SEM were calculated. Sections from the same testes were also probed with sense strand riboprobes, and no differences between grain densities in the different regions of the section were observed.

Immunocytochemistry

Rabbit IgGs directed against recombinant human P450c17 (30) were a gift from Prof. Michael Waterman (Vanderbilt University, Nashville, TN), and rabbit antihuman 3βHSD (31) was a gift from Prof. Ian Mason (Department of Clinical Biochemistry, University of Edinburgh, Edinburgh, UK). The staining procedure employed was similar for both antibodies. Sections (5 μm) were mounted on slides coated with 3-aminopropyl triethoxy-silane (Sigma) and dried overnight at 50 C. Before incubation with primary antibodies, sections were dewaxed, rehydrated in graded ethanols, and washed in water and TBS (0.05 M Tris-HCl, pH

7.4, and 0.85% NaCl). Sections to be incubated with antiserum to P450c17 were subjected to antigen retrieval (32) by microwaving in 0.01 M citrate buffer (pH 6.0) on full power for 20 min and thereafter standing for 20 min without disturbance. All sections were blocked using normal swine serum (Dako, High Wycombe, UK) diluted 1:5 in TBS. Both antibodies were diluted 1:1000 in normal swine serum in TBS before incubation on sections under plastic coverslips overnight at 4 C. On the following day, coverslips were removed, and sections were washed twice in TBS (5 min each wash), incubated for 30 min with biotinylated swine antirabbit Ig (Dako) diluted 1:500 in TBS, then washed again in TBS (twice for 5 min each time). For detection of bound antibodies, sections were first incubated with avidin-biotin complex conjugated with horseradish peroxidase for 30 min and washed twice in TBS (5 min each time). Color reaction product was developed by incubating sections in a mixture of 0.05% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris-HCl, pH 7.4, and 0.01% hydrogen peroxide. After 5–15 min, sections were washed in water, counterstained with hematoxylin, dehydrated in graded ethanols, cleared in xylene, and coverslipped using Pertex mounting medium (Cell Path, Hemel Hempstead, UK). The specificity of the antibodies was confirmed using normal rabbit serum instead of the primary antibodies; no staining was observed in these controls.

Measurement of 17α-hydroxylase activity

The activity of 17α-hydroxylase was measured as described previously (33). In the current experiments, fetal testes from two animals (four testes) were pooled and homogenized in 0.4 ml PBS (0.05 M PBS, pH 7.6). As substrate for the enzyme, 10^6 cpm [^{3}H]progesterone (88 Ci/mmol; Amersham) were dissolved in 35 μl dimethylsulfoxide and mixed with 0.87 ml phosphate buffer (0.05 M; pH 7.2) containing NADPH (final concentration, 1 mmol/liter; Sigma). Homogenized testes (100 μl) were mixed with [^{3}H]progesterone in phosphate buffer and incubated at 37 C for 20 min. Nonradioactive progesterone (Sigma) was added to the incubation mixture to give a final substrate concentration of 2 μM, which previous studies have shown to be saturating (33). Reactions were stopped by adding 100 μl 1 M NaOH to each tube in ice. To allow determination of steroid recovery, ^{14}C-labeled testosterone (50 Ci/mmol) and androstenedione (50 Ci/mmol; Amersham) were added (2000 cpm of each to each reaction), and the steroids were extracted from the solution using 5 ml toluene (AnalaR, BDH, Poole, UK). The organic phase was recovered, dried by heating to 45 C under a stream of nitrogen, dissolved in a few drops of methanol, and applied to a plastic-backed silica gel plate (Whatman, Clifton, NJ). Steroids were separated by TLC in chloroform-ether (7:1, vol/vol). Fluorescent bands (detected under UV light) corresponding to 17-hydroxyprogesterone, androstenedione, and testosterone were cut from the plates, placed in vials (Packard Instrument Co., Caversham, Berkshire, UK) and mixed with 6 ml scintillation fluid (Ecoscint A, National Diagnostic, Atlanta, GA). The amount of each radiolabeled steroid was determined in a β-counter using a program to detect both ^{3}H (counting range, 5–304 kiloelectron volts) and ^{14}C (counting range, 325–765 kiloelectron volts) simultaneously. The activity of 17α-hydroxylase was expressed as picomoles of radiolabeled progesterone converted by one testis in 1 min. Controls were run in each experiment using PBS without added tissue. The 17α-hydroxylase activity was measured in eight separate pools of four testes from five different litters for the control group and in six separate pools of four testes from three different litters for each of the treatment groups.

Statistical analysis

Data from each experiment were subjected to ANOVA to determine whether there were significant effects of treatment. Where these were indicated, subgroup comparisons between means for the control and individual treatment groups were then made using the variance from the experiment as a whole as the measure of error.

Results

Expression of P450c17 mRNA

Multiple reactions were performed to determine the expression of P450c17 mRNA in individual samples of testes from control and treated animals; primers directed against cyclophilin (28) were added at the same time as those for the enzyme to allow an internal check on the efficiency of the amplification reaction. The comparisons shown in Fig. 1 revealed an apparent reduction in the amount of P450c17 cDNA amplified from the testes of fetuses exposed to high dose DES (lanes 3 and 5). The method was not considered sufficiently quantitative to determine whether changes had occurred after OP treatment (lane 2). The amounts of cyclophilin cDNA amplified from all samples were approximately similar.

In situ hybridization using a specific radiolabeled cRNA directed against rat P450c17 resulted in the detection of abundant silver grains, as expected, in the interstitial region of the day 17.5 fetal testis after exposure to photographic emulsion for 2 weeks (Fig. 2). Fewer silver grains were detected in fetal testes from the animals treated with DES (Fig. 2c) than in those from controls (Fig. 2a). Sense controls did not show any signal above background (not shown). The results were confirmed and extended by image analysis (Fig. 3), which revealed a slight reduction in grain counts in the interstitial region after low dose OP treatment (Fig. 2b).

Detection of P450c17 and 3βHSD protein in fetal Leydig cells

The gross histologies of the testes recovered on day 17.5 of gestation from treated and control animals were similar.

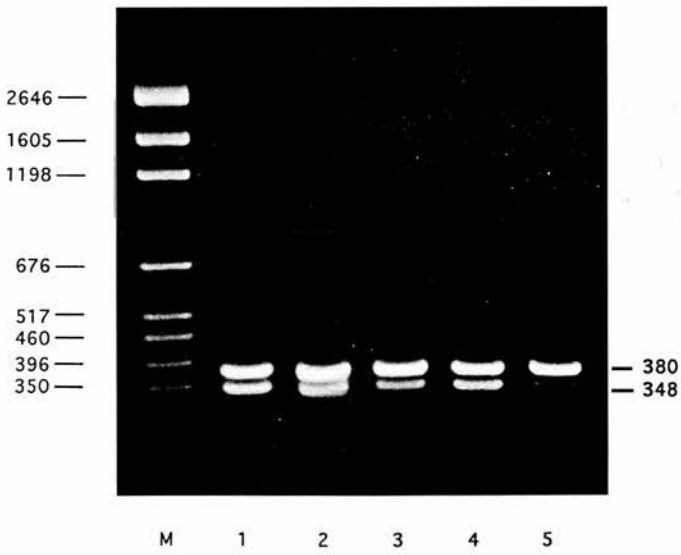

FIG. 1. Coamplification of mRNAs for P450c17 and cyclophilin using RT-PCR. The amplified products were 348 bp for P450c17 (*lower band*) and 380 bp for cyclophilin (*upper band*); all samples contained 500 ng total RNA isolated from fetal rat testes isolated on day 17.5 of gestation from mothers treated as follows: lane 1, control; lane 2, OP (100 mg/kg); lane 3, DES (500 μg/kg); lane 4, control; lane 5, DES (500 μg/kg). Note that although all fetal samples contain roughly the same amount of cyclophilin mRNA, maternal treatment with DES resulted in an apparent reduction in P450c17 mRNA.

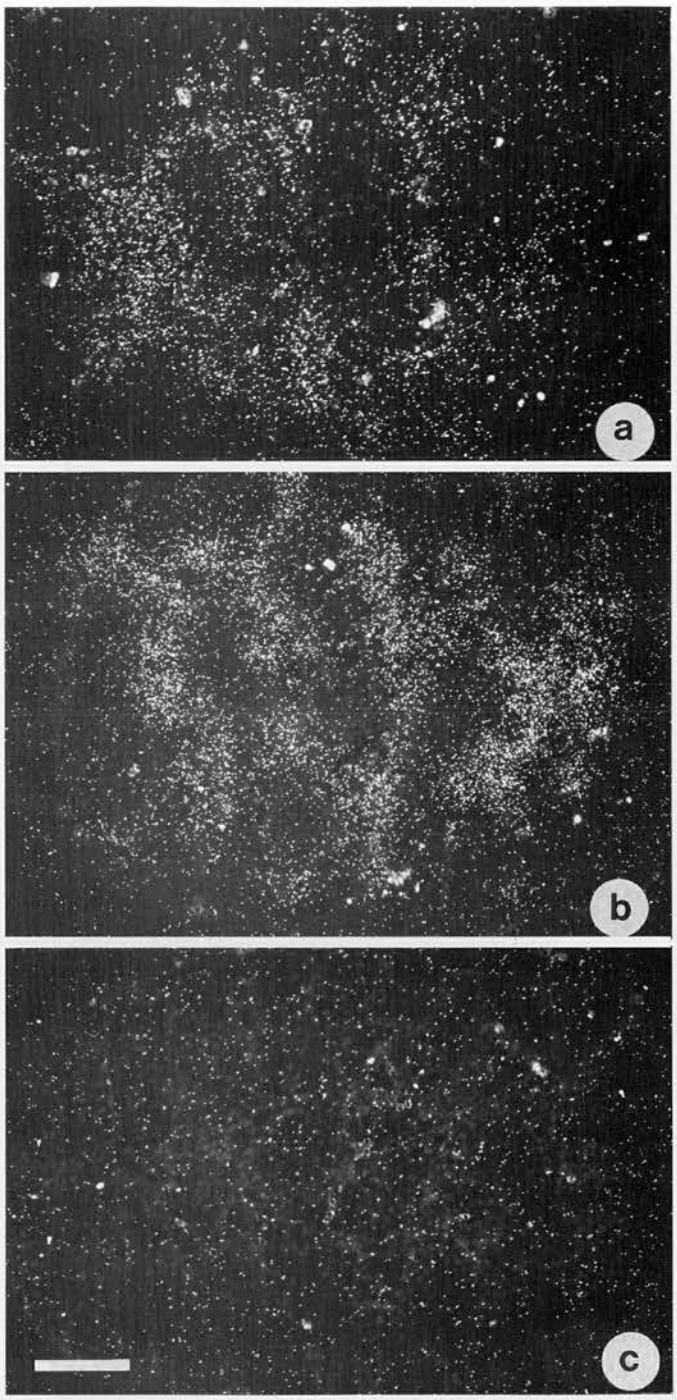

FIG. 2. *In situ* hybridization using ^{35}S-labeled cRNA to rat P450c17 mRNA on sections of fetal rat testis. a, Control; b, OP treatment (100 mg/kg) of mother; c, DES treatment (500 μg/kg) of mother. Exposure to emulsion was for 2 weeks; all sections were exposed on the same slide. Note that a strong concentration of grains is present in the control rat testis, and brightfield illumination revealed that they were concentrated mainly within the interstitial region of the testis. The specific hybridization signal appeared reduced in the testes of males deriving from mothers treated with 500 μg/kg DES. Magnification, ×100; *bar* = 100 μm.

Testes from fetal males recovered from untreated mothers contained numerous Leydig cells that were stained strongly after immunohistochemistry with primary antibodies directed against the steroidogenic enzymes P450c17 and 3βHSD (Figs. 4 and 5). Specific immunostaining of P450c17 within fetal Leydig cells appeared the same in testes of untreated control mothers (Fig. 4a) and those from mothers treated with 100 mg/kg OP (Fig. 4b) or 100 μg/kg DES (Fig. 4d). However, there was a marked reduction in immunostaining when testes were recovered from fetuses of mothers given 600 mg/kg OP (Fig. 4c) or 500 μg/kg DES (Fig. 4e). In contrast, when fetal testes were immunostained for 3βHSD, there was no apparent reduction in the number of interstitial cells with positive immunostaining in testes from treated mothers (Fig. 5, b and c) compared with the control value (Fig. 5a).

Enzymatic activity of 17α-hydroxylase in fetal testes from control and treated mothers

17α-Hydroxylase activity was measured in four testes pooled from two fetuses. Eight such pools from five litters were analyzed for the control group, and six pools from three litters were analyzed for each treatment group. Enzyme activity in animals treated with the high (500 μg/kg) or low (100 μg/kg) dose of DES was reduced by 78% ($P < 0.001$) or 30% ($P < 0.05$), respectively, compared to enzyme activity in control animals. In animals treated with the high (600 mg/kg) dose of OP, enzyme activity was reduced by 30% ($P < 0.05$), whereas the lower dose (100 mg/kg) of OP resulted in an 18% reduction ($0.07 > P < 0.05$) in enzyme activity compared to that in control animals (Fig. 6).

Discussion

Testosterone is essential for the maintenance of fertility in adult life (34) and development of the male reproductive tract during fetal life (4). In the rat, synthesis of testosterone in the Leydig cells of the fetal testis starts on day 15 pc and then increases rapidly over the next few days to reach a peak on day 18.5 (14). The results obtained in the present study in which estrogenic chemicals were administered to mothers around this time demonstrate that estrogens could interfere with steroid production in the testis during fetal life by inhibiting the expression and activity of P450c17. The long term consequences of this reduction in enzyme activity are not known, but deficits in various aspects of masculinization are an obvious possibility.

The reduction in 17α-hydroxylase enzyme activity in fetal testes resulting from estrogen administration to the mothers was accompanied by reduced expression of P450c17 mRNA, as suggested by RT-PCR analysis and confirmed by *in situ* hybridization, consistent with the effect of the estrogenic chemicals being mediated at the level of transcription of the CPY17 gene. In the same tissue sections in which reduced immunostaining for 17α-hydroxylase was observed, the number of Leydig cells expressing 3βHSD did not change. Studies on steroidogenesis by adult Leydig cells have demonstrated that expression of P450c17 is acutely regulated by LH stimulation and that levels of enzyme fall rapidly in the

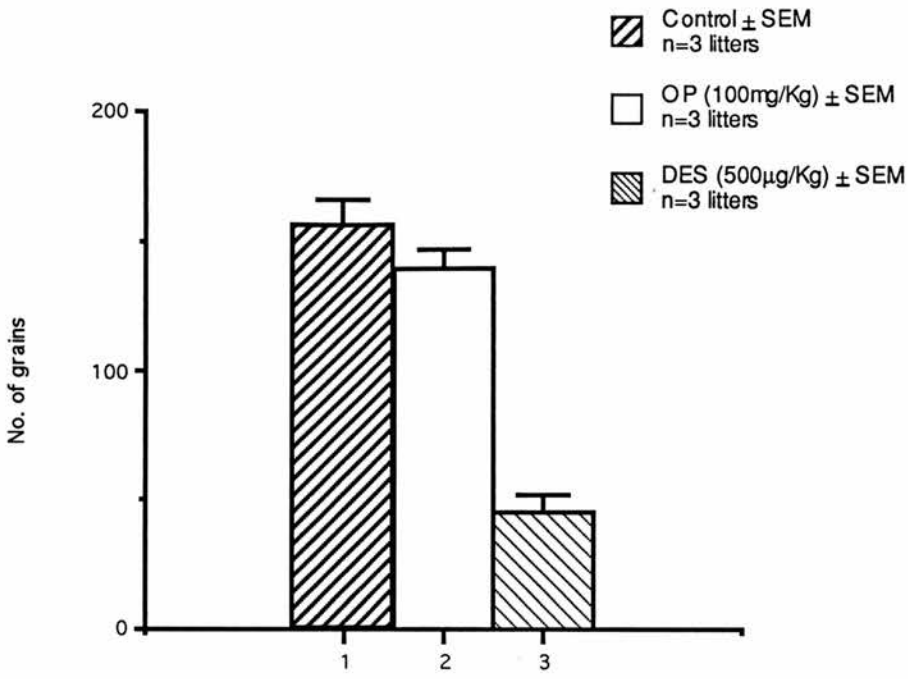

FIG. 3. Quantification of the results obtained by *in situ* hybridization for P450c17 in fetal rat testis. The number of silver grains detected in the interstitial region of the testis was reduced significantly in animals from mothers exposed to 500 μg DES (column 3; $P < 0.001$) compared with controls (lane 1). Maternal treatment with 100 mg/kg OP resulted in a slight reduction in grain counts in the interstitium when corrected for background, but this did not reach significance.

absence of cAMP, whereas expression of 3βHSD remains unchanged under the same circumstances (11, 35). In rats, plasma LH levels remain low until fetal day 19.5, and it has been concluded that although LH receptors are present in the fetal rat testis on or about day 15.5 (36), activation of expression of steroidogenic enzymes and fetal testicular testosterone production involves factors other than LH, presumably originating within the testes (37). Recently, a factor termed SF-1/Ad4BP present in fetal gonads and adrenals during and after sexual differentiation has been shown to regulate the expression of steroidogenic enzymes, and this may, therefore, be the missing regulator of fetal Leydig cell steroidogenesis (38, 39).

Classical estrogen action is mediated via an intracellular receptor that, after steroid binding, interacts with specific regions of DNA and acts as a transcriptional regulator for selected genes (40). ER have been detected in the genital tract of the male mouse using binding assays (41) and immunocytochemistry (20). Within the testis, ER immunostaining was present in interstitial cells, including Leydig cells, on fetal days 15–19 (42). Tsai-Morris and co-workers (21) reported that exposure of fetal rat Leydig cells (day 21) *in vitro* to estradiol resulted not only in a decrease in the synthesis of testosterone, but also in an increase in ER detected by binding assays. They concluded that the action of estrogens was mediated at the level of the nucleus, consistent with the action of estrogen being mediated via its receptor, because incubation of isolated microsomes with estrogen failed to affect enzyme activities (43). In the mouse, P450 aromatase mRNA was detected in testes on and after day 17 (13), suggesting that fetal and neonatal testis have the capacity to produce estrogens locally. Recent studies of mice in which expression of the ER gene was disrupted selectively found that the mature adult males were infertile because of impaired spermatogenesis, but appeared to have anatomically normal accessory organs (44).

DES is a known potent estrogen, and previous observations in man (5) and animals (7) have indicated that it is likely to exert adverse effects during the time of masculinization and reproductive tract development. In contrast, there are no published studies of the effects of OP at this time, although it has been shown to be weekly estrogenic *in vitro* (45). OP is an alkylphenolic compound formed as a metabolite of some nonionic surfactants that are used widely in industrial and some household detergents as plastic and petrol additives, spermicides, dispensers for insecticides, *etc.* (46). However, the level of human exposure is largely unknown. The present data are the first to show that maternal exposure to high levels of such compounds can suppress 17α-hydroxylase activity. OP has been shown previously to bind to the ER and stimulate estrogen-dependent growth of breast cancer cells and gene expression *in vitro* to a similar extent as 17β-estradiol, albeit with approximately 1000-fold less potency (45). Male trout exposed to low levels of OP (38.5 μg/liter) in the surrounding water synthesize vitellogenin, an estrogen-inducible protein normally only expressed in the female, and as a result have reduced testis size (47). Our own studies have shown that exposure of male rats to OP during gestation and neonatal life, whether administered by injection or via the drinking water (48), results in small, but significant, reductions in testicular size and sperm production in adulthood. These changes are thought to reflect alterations in Sertoli cell number rather than altered Leydig cell function, but it is possible that suppression of 17α-hydroxylase activity could have contributed to these observations.

It is presumed that the present effects of DES and OP on P450c17 are the result of effects mediated via the ER, although it has been reported recently that the major and persistent metabolite of DDT (1,1,1-trichloro2,2(p-chlorophenyl)ethane, p,p'-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene), which had previously been implicated in the induction of abnormalities in male sex development in wild-

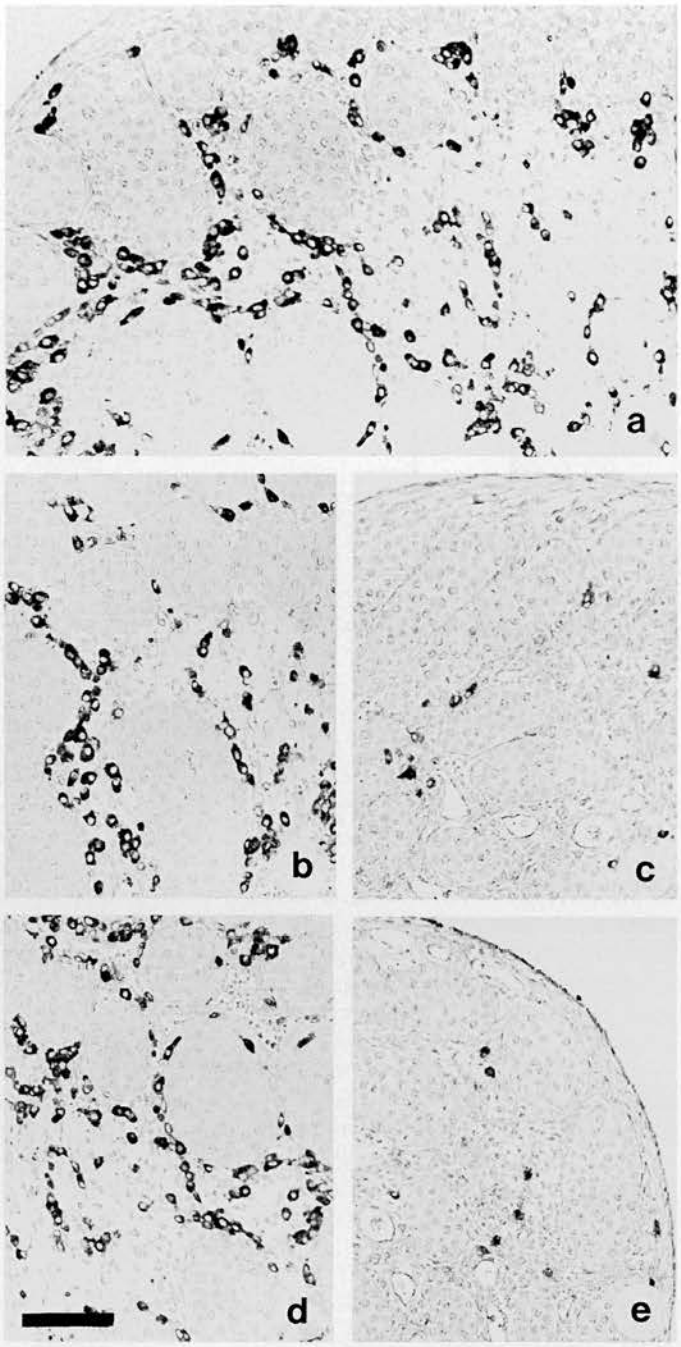

FIG. 4. Immunocytochemical staining of Leydig cells in fetal rat testis using antibodies directed against P450c17. a, Testis from control group; b and c, testes from fetuses of mothers treated with 100 and 600 mg/kg OP, respectively; d and e, testes from fetuses of mothers treated with 100 and 500 μg/kg DES, respectively. Note that specific immunostaining of P450c17 is reduced in fetuses from the higher dose treatment groups (c and e) compared with controls (a). Magnification, ×100; *bar* = 100 μm.

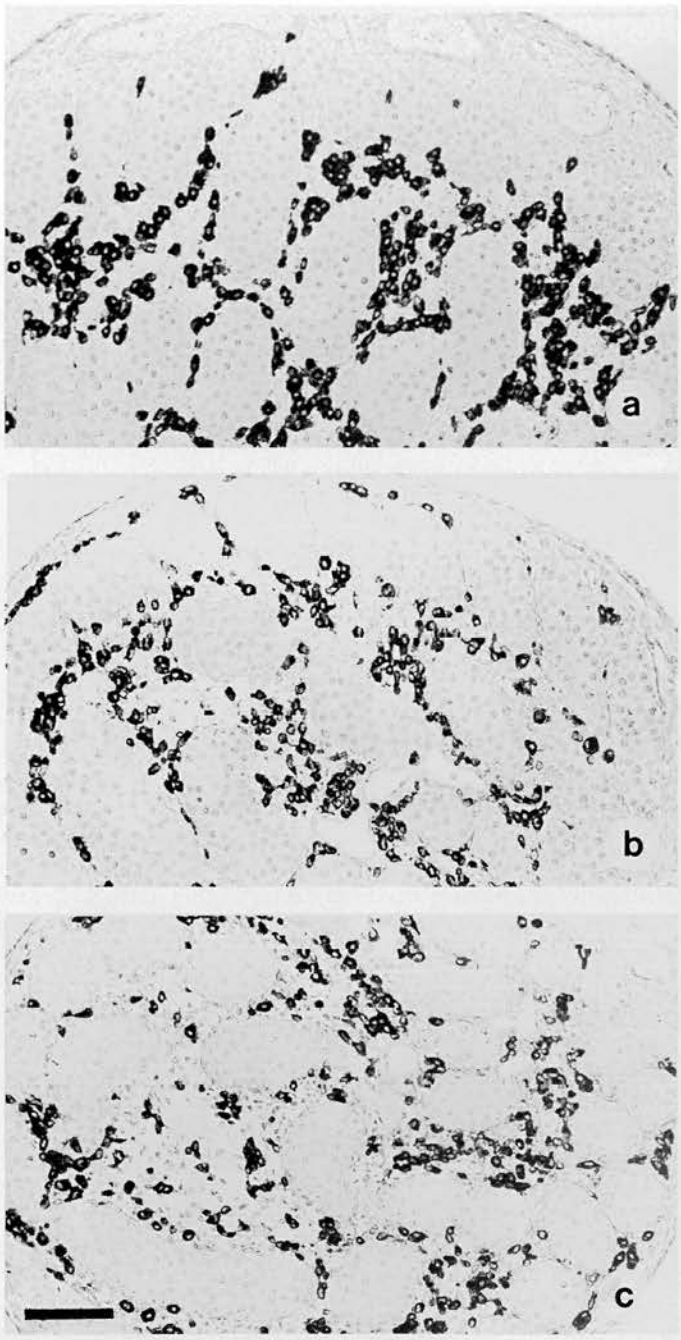

FIG. 5. Immunocytochemical staining of Leydig cells in fetal rat testis using antibodies directed against 3βHSD. a, Testis from control; b, testis from 600 mg/kg OP treatment group; c, testis from 500 μg/kg DES treatment group. Note that the number of Leydig cells appears the same in all groups. Magnification, ×100; *bar* = 100 μm.

life, bound more efficiently to the androgen receptor than it did to the ER (49) and that DES could also bind to the androgen receptor. In the present study we do not believe that the effects on Leydig cell 17α-hydroxylase activity observed after treatment with DES or OP reflect interaction at the level of the AR, as our earlier studies have shown that the few AR present within the testis at this time are not present in Leydig cells (50).

The importance and effect(s) of reduced 17α-hydroxylase enzyme activity induced by exogenous estrogens during fetal life and the consequent reduction in testosterone production are not clear. The formation of a testis with normal appearance despite estrogen administration on day 11.5 (a time before gonadal differentiation) suggests that expression of the genes involved in gonadal differentiation, including

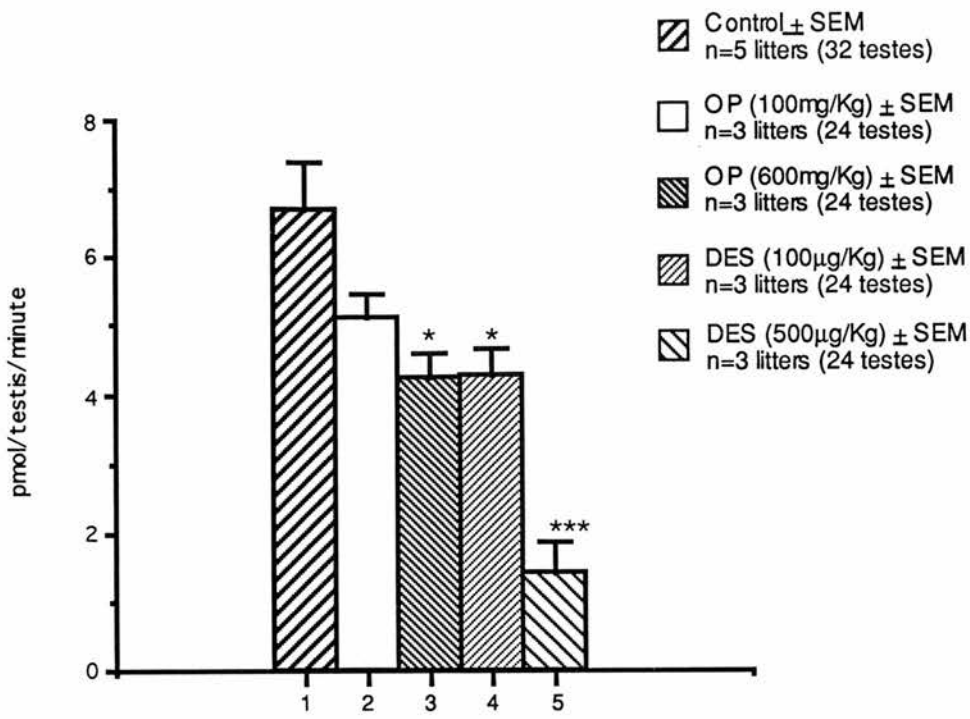

FIG. 6. Level of 17α-hydroxylase enzyme activity in fetal testes isolated on day 17.5 of gestation from mothers treated with vehicle (control; lane 1), OP (600 or 100 mg/kg; lanes 2 and 3), or DES (500 or 100 μg/kg; lanes 4 and 5). Each *bar* is the mean ± SEM for eight (control group) or six (all treated groups) determinations using tissue pooled from two male fetuses per determination. *, $P < 0.05$; ***, $P < 0.001$ (compared with control group).

SRY and SOX-9 (51), was unaffected, although we have not as yet addressed this question directly. Long term effects on male reproductive function have been reported after maternal treatment with DES in both humans and rodents (5, 42), although a recent survey did not find any evidence that long term male fertility had been affected (6). However, the question remains as to whether extended exposure to low doses of estrogens throughout life could have a long term and deleterious effect on Leydig cell steroidogenesis. Evidence that the effects of intrauterine exposure to low doses of estrogen might be reflected in altered Leydig cell function in adulthood is provided by an intriguing report from Nonneman and co-workers (52), in which they found that mice differed in their adult reproductive characteristics according to whether they developed *in utero* between two male fetuses, which have higher testosterone levels, or between two female fetuses, which have higher estradiol levels. They recorded lower levels of 17α-hydroxylase activity in adult male mice that had been adjacent to female fetuses than those next to males, and higher androgen receptor in the prostates of these same mice, leading to the conclusion that low level estrogen exposure exerts a significant influence on androgen metabolism and responsiveness.

In conclusion, our data show that maternal exposure to estrogens such as OP and DES inhibits 17α-hydroxylase activity in the fetal rat testis. The mechanism(s) that bring about this reduction and its consequences remain to be elucidated. We cannot yet conclude that the reduction in enzymatic activity and consequent reduction in androgen synthesis necessarily explain the incidence of reproductive problems in male offspring from DES-treated mothers or the rise in the reported incidence of similar problems in the general population. However, the present observations suggest that the effect of long term exposure to estrogenic chemicals, especially environmental estrogens, on Leydig cell function needs to be more fully explored.

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References

1. **Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R** 1990 Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 348:450–454
2. **Lee M, Donahoe P** 1993 Mullerian inhibiting substance: a gonadal hormone with multiple functions. Endocr Rev 14:152–164
3. **Magre S, Jost A** 1980 The initial phases of testicular organogenesis in the rat. Arch Anat Microsc 69:297–318
4. **George FW, Wilson JD** 1994 Sex determination and differentiation. In: Knobil E, Neill JD (eds) The Physiology of Reproduction. Raven Press, New York, pp 3–28
5. **Gill W, Schumacher G, Bibbo M, Straus F, Schoenberg H** 1979 Association of diethylstilbestrol exposure *in utero* with cryptorchidism, testicular hypoplasia and semen abnormalities. J Urol 122:36–39
6. **Wilcox A, Baird D, Weinberg C, Hornsby P, Herbst A** 1995 Fertility in men exposed prenatally to diethylstilbestrol. N Engl J Med 332: 1411–1415
7. **McLachlan J, Newbold R, Bullock B** 1975 Reproductive tract lesions in male mice exposed prenatally to diethylstilbestrol. Science 190: 991–992
8. **Sharpe RM, Skakkebaek NE** 1993 Are oestrogens involved in falling sperm counts and disorders of the reproductive tract? Lancet 341:125–126
9. **Toppari JJ, Larsen J, Christiansen P, Giwercman A, Grandjean P, Guillette L, Keiding N, Leffers H, McLachlan J, Meyer O, Muller J, Rajpert-De Meyts E, Scheike T, Sharpe RM, Sumpter JS, Skakkebaek NE** 1995 Male Reproductive Health and Environmental Chemicals with Estrogenic Effects. Ministry of the Environment and

Energy, Danish Environmental Protection Agency, vol Miljoprojekt, Copenhagen, p 290
10. **Miller WR** 1988 Molecular biology of steroid hormone synthesis. Endocr Rev 9:295–318
11. **Keeney DS, MendisHandagama SMLC, Zirkin BR, Ewing LL** 1988 Effect of long term deprivation of luteinizing hormone on Leydig cell volume, Leydig cell number, and steroidogenic capacity of the rat. Endocrinology 123:2906–2915
12. **O'Shaughnessy PJ** 1991 Steroidogenic enzyme activity in the hypogonadal (hpg) mouse testis and effect of treatment with luteinizing hormone. J Steroid Biochem Mol Biol 39:921–928
13. **Greco TL, Payne AH** 1994 Ontogeny of expression of the genes for steroidogenic enzymes P450 side chain cleavage, 3β-hydroxysteroid dehydrogenase, P450 17α-hydroxylase/C17–20 lyase, and P450 aromatase in fetal mouse gonads. Endocrinology 135:262–268
14. **Warren D, Haltmeyer G, Eik-Nes K** 1973 Testosterone in the fetal rat testis. Biol Reprod 8:560–565
15. **Tapanainen J, Kuopio T, Pelliniemi L, Huhtaniemi I** 1984 Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. Biol Reprod 31:1027–1035
16. **Verjans HL, de Jong FH, Cooke BA, Van der Molen H, Eik-Nes KB** 1974 Effects of estradiol benzoate on pituitary and testis function in normal adult male rats. Acta Endocrinol (Copenh) 77:636–672
17. **Moger W** 1980 In vitro inhibitory effect of estradiol on testosterone production. J Steroid Biochem 13:61–66
18. **Kremers P, Tixhon C, Gielen J** 1977 17α-Hydroxylase and testosterone biosynthesis in rat testis. J Steroid Biochem 8:873–877
19. **Wang C, Erickson G, Hopper B, Hsueh A** 1980 Direct inhibitory effect of enclomiphene citrate and estradiol on testis functions in hypophysectomized rats. Biol Reprod 22:645–653
20. **Greco T, Furlow J, Duello T, Gorski J** 1992 Immunodetection of estrogen receptors in fetal and neonatal male mouse reproductive tracts. Endocrinology 130:421–429
21. **Tsai-Morris C, Knox G, Luna S, Dufau ML** 1986 Acquisition of Estradiol-mediated regulatory mechanism of steroidogenesis in cultured fetal rat Leydig cells. J Biol Chem 261:3471–3474
22. **Millar MR, Sharpe RM, Maguire SM, Saunders PTK** 1993 Cellular localisation of messenger RNAs in rat testis; application of digoxigenin-labelled probes to embedded tissue. Cell Tissue Res 273:269–277
23. **Kaufman MH** 1992 Atlas of Mouse Development. Academic Press, London, pp 453–468
24. **Winslow SG, Henkart PA** 1991 Polyinosinic acid as a carrier in the microscale purification of total RNA. Nucleic Acids Res 19:3251–3253
25. **Davis LG, Dibner MD, Battey JF** 1968 Basic Methods in Molecular Biology. Elsevier, New York, pp 1–388
26. **Fevold H, Lorence M, McCarthy J, Trant J, Kagimoto M, Waterman MR, Mason JI** 1989 P450–17-alpha from testis: characterization of a full length cDNA encoding a unique steroid hydroxylase capable of catalyzing both delta-4- and delta-5-steroid-17,20 lyase reactions. Mol Endocrinol 3:968–975
27. **Myers TW, Gelfand DH** 1991 Reverse transcription and DNA amplification by a *Thermus thermophilius* DNA polymerase. Biochemistry 30:7661–7666
28. **Danielsson P, Forss-Petter S, Brow M, Calavetta L, Douglass J, Milner R, Sutcliffe J** 1988 p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA 7:261–267
29. **Saunders PTK, McKinnell C, Millar MR, Gaughan J, Turner KJ, Jegou B, Syed V, Sharpe RM** 1995 Phosphatidylethanolamine binding protein is an abundant secretory product of haploid testicular germ cells in the rat. Mol Cell Endocrinol 107:221–230
30. **Imai T, Globerman H, Gertner J, Kagawa N, Waterman MR** 1993 Expression and purification of functional human 17α-hydroxylase/17,20-lyase (P450c17) in *Escherichia coli*. J Biol Chem 268:19681–19689
31. **Lorence M, Murry B, Trant J, Mason JI** 1990 Human 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase from placenta: expression in non steroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C_{21} and C_{19} steroids. Endocrinology 126: 2493–2498
32. **Shi S-R, Chaiwun B, Young L, Cote R, Taylor C** 1993 Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. J Histochem Cytochem 41:1599–1604
33. **O'Shaughnessy PJ, Murphy L** 1991 Steroidogenic enzyme activity in the rat testis following Leydig cells destruction by ethylene-1,2-dimethansulphonate and during subsequent Leydig cell regeneration. J Endocrinol 131:451–457
34. **Sharpe RM** 1994 Regulation of Spermatogenesis. In: Knobil E, Neill JD (eds) The Physiology of Reproduction, ed 2. Raven Press, New York, pp 1363–1424
35. **Payne AH, Youngblood G** 1995 Regulation of expression of steroidogenic enzymes in Leydig cells. Biol Reprod 52:217–225
36. **Warren D, Huhtaniemi I, Tapanainen J, Dufau ML, Catt KJ** 1984 Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. Endocrinology 114:470–475
37. **Pakarinen P, Proshlyakova E, Huhtaniemi I** 1994 Pituitary-gonadal interactions in perinatal rats: relationships of plasma luteinizing hormone and testosterone concentrations, and pituitary levels of LH subunit mRNAs. Neuroendocrinology 60:42–49
38. **Lala D, Rice D, Parker KJ** 1992 Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression is the mouse homolog of fushi tarazu-factor 1. Mol Endocrinol 6:1249–1258
39. **Hatano O, Takayama K, Imai T, Waterman MR, Takakusu A, Omura T, Morohashi K** 1994 Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 120:2787–2797
40. **Carson-Jurnica MA, Schrader WT, O'Malley BW** 1990 Steroid receptor superfamily: structure and functions. Endocr Rev 11:201–219
41. **Cooke P, Young P, Hess R, Cunha G** 1991 Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs. Endocrinology 128:2874–2879
42. **Greco T, Duello T, Gorski J** 1993 Estrogen receptors, estradiol, and diethylstilbestrol in early development: the mouse as a model for the study of estrogen receptors and estrogen sensitivity in embryonic development of male and female reproductive tracts. Endocr Rev 14:59–71
43. **Nozu K, Matsuura S, Catt KJ, Dufau ML** 1981 Modulation of Leydig cell androgen biosynthesis and cytochrome P-450 levels during estrogen treatment and human chorionic gonadotropin-induced desensitization. J Biol Chem 256:10012–10017
44. **Korach K** 1994 Insights from the study of animals lacking functional estrogen receptor. Science 266:1524–1527
45. **White R, Jobling S, Hoare S, Sumpter JS, Parker M** 1994 Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135:175–182
46. **Naylor G, Mierure J, Weeks J, Castaldi F, Romano R** 1992 Alkylphenol ethoxylates in the environment. J Am Oil Chemists Soc 69:695–703
47. **Jobling S, Sheahan D, Osborne J, Matthiessen P, Sumpter JS** 1995 Inhibition of testicular growth in trout exposed to environmental estrogens. Environ Toxicol Chem 103:1136–1143
48. **Sharpe RM, Fisher J, Millar MR, Jobling S, Sumpter JS** 1995 Gestational and/or neonatal exposure of rats to environmental estrogenic chemicals results in reduced testis size and daily sperm production in adulthood. Environ Health Perspect, in press
49. **Kelce W, Stone C, Laws S, Gray L, Kemppainen J, Wilson E** 1995 Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375:581–585
50. **Majdic G, Millar MR, Saunders PTK** 1995 Immunolocalisation of androgen receptors to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. J Endocrinol 147: 285–293
51. **Capel B** 1995 New bedfellows in the mammalian sex-determination affair. Trends Genet 11:161–163
52. **Nonneman D, Ganjam V, Welshons W, Vom Saal F** 1992 Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. Biol Reprod 47:723–729

0013-7227/96/$03.00/0
Endocrinology

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Differential patterns of expression of DAX-1 and steroidogenic factor-1 (SF-1) in the fetal rat testis.

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ABSTRACT Gonadal differentiation is dependent upon a cascade of molecular and morphological events. Steroidogenic factor 1 (SF-1) and DAX-1 have been implicated in this process. A SF-1 binding site has been reported to be present on the *DAX-1* gene. We therefore used immunocytochemistry to determine whether these two transcription factors are co-expressed in the fetal testis. DAX-1 was immunolocalised to the cytoplasm of interstitial cells from fetal testis of rat and human from day 15.5 and 16 weeks of gestation respectively. SF-1 was detected in nuclei of fetal Sertoli and interstitial cells. In the fetal rat expression of SF-1 in interstitial cells was not uniform; cells with abundant SF-1 all contained 3-βHSD and were classified as Leydig cells. DAX-1 expression was not exclusive to cells which contained SF-1 and SF-1/DAX-1 co-expressing cells were not exclusively fetal Leydig cells. We conclude that in the fetal testis expression of the DAX-1 protein is not dependent upon the presence of SF-1 although SF-1 is present at an earlier stage of gestation in the gonad. DAX-1 may therefore play a separate or complementary role to that of SF-1 in the modulation of testicular gene expression and differentiation.

In the past few years a number of new genes expressed during the period of fetal gonadal development have been identified (1, 2) although the role(s) of most have yet to be elucidated. One gene product shown to play an essential role in gonadal development and steroidogenesis is the transcription factor steroidogenic factor-1 (SF-1) (3, 4). Another gene, *DAX-1*, has been localised to the interval on the X-chromosome associated with dosage-dependent sex reversal (5). The DAX-1 protein has been shown to bind to DNA (6) and in common with SF-1, DAX-1 appears to be a member of the nuclear receptor hormone superfamily (7, 8) the ligand(s) of which are as yet unidentified. Notably a putative SF-1 binding element has been identified in the 5' flanking region of human *DAX-1* and binding has been demonstrated in vitro (9). However, the question of whether SF-1 may determine DAX-1 expression has not been explored in vivo.

We have investigated the patterns of expression of SF-1 and DAX-1 in the fetal testis and present the first photographic evidence of the pattern of immunoexpression of DAX-1 in the fetal testis of human and rat. Notably, SF-1 is synthesised within fetal testicular cells at an earlier stage of development and in a wider range of cells than the DAX-1 protein. Within the testicular interstitium expression of DAX-1 protein, as determined by immunocytochemistry, occurs in SF-1 positive as well as SF-1 negative cells.

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Materials and methods

Animals and Tissues Fetal tissues were obtained from timed mated rats on days 13.5 to 20.5 of gestation and fixed in Bouin's as described previously (10). Human fetal testis fixed in Bouin's were from a collection at the MRC Human Genetics Unit, Edinburgh accumulated from routine autopsy examinations between 1991 and 1993.

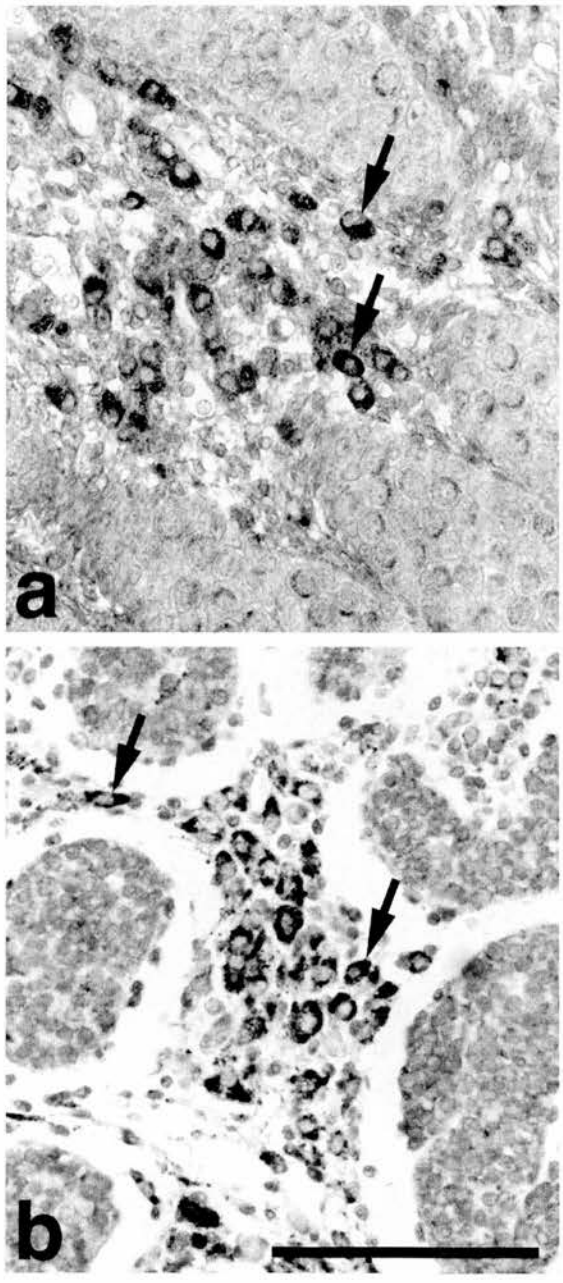
Immunohistochemistry Immunolocalisation was carried out on 5μ sections mounted on coated slides using our standard methods (10) except that human tissues were not microwaved Rabbit anti-human DAX-1 was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and used at 1:100. The antibody was reported to bind to DAX-1 of human, mouse and rat origin on Western blots. Rabbit anti-bovine AdBP4 (SF-1) was a kind gift from Dr Ken-ichirou Morohashi (Kyushu University, Japan) and was used at 1:200. Rabbit anti-human 3-beta hydroxysteroid dehydrogenase (3-βHSD) was a kind gift from Professor Ian Mason (University of Edinburgh, UK) and was used at 1:1000. Sections were counter stained with hematoxylin. Specificity of the antibodies was checked by either replacing the primary, antigen-specific antiserum with normal rabbit serum or by preabsorption with peptide.

Results

DAX-1 immunostaining in fetal testis

In the fetal testis of the rat (Fig 1a) and human (Fig 1b) DAX-1 was immunolocalised to the cytoplasm of interstitial cells. In the rat weak immunostaining was first detectable on day 15.5 p.c.

Fig. 1 Immunolocalisation of DAX-1 protein to interstitial cells of the fetal testis. Panel a, fetal rat testis day 18.5 of gestation; panel b, fetal human testis 24 weeks gestation. Note the absence of expression of DAX-1 in Sertoli cells within the seminiferous cords and the cytoplasmic localization of the protein in these fixed tissue sections (arrows). Bar = 100μM



(not shown). The intensity of immunostaining remained low on day 16.5 but increased thereafter (Fig 1a, day 18.5 p.c.) and remained strong throughout fetal life. Staining was also observed in a perinuclear region of some fetal gonocytes but this was non-specific. In the human weak immunostaining for DAX-1 was present at 16 weeks gestation (earliest age examined) and was increased in intensity at 18 (not shown) and 24 weeks (Fig 1b) of pregnancy. No immunostaining of interstitial cells was observed when the antiserum was preabsorbed with the cognate peptide or when the primary specific antiserum was replaced by non-immune serum (not shown). In the rat DAX-1 was also detected in the cytoplasm of interstitial cells in adult testis as well as in the fetal ovary and in the cortex of the fetal adrenal gland (not shown).

Relationship between DAX-1 positive cells and those expressing SF-1 and 3-βHSD in fetal rat testis

Consistent with previous reports (3, 4) SF-1 was immunolocalised to interstitial and Sertoli cell nuclei on day 14.5 p.c. The intensity of immunostaining remained higher in interstitial than Sertoli cell nuclei throughout gestation in the rat. On day 17.5 (Fig 2) intensity of immunostaining within nuclei of interstitial cells was variable however those containing abundant nuclear SF-1 appeared to be steroidogenically active Leydig cells as they also contained cytoplasmic 3β-HSD (Fig. 2a intense blue). In contrast to SF-1 the population of interstitial cells containing DAX-1 was more restricted (Fig 2b blue color) Not all DAX-1 positive cells contained SF-1 in their nuclei (Fig 2b arrows) and many interstital cells containing SF-1 were DAX-1 negative (Fig 2b, arrowheads). Co-localization of SF-1 and DAX-1 did not appear to be confined to presumptive fetal Leydig cells.

Discussion

DAX-1 and SF-1 are both believed to act as transcriptional regulators and have been proposed as modulators of gene expression during gonadal and adrenal development. The demonstration that gonadal development fails to occur normally in SF-1 knockout mice has convincingly shown that expression of this protein is essential during fetal life (3). The precise role(s) of DAX-1 in the gonad have yet to be elucidated although it has recently been reported that DAX-1 mRNA is expressed in the gonadal ridge and in the adult testis of the

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mouse (6, 11). The presence of an SF-1 binding site on *DAX-1* (9) led us to anticipate that SF-1 might co-localise with DAX-1 in the fetal testis and/or be expressed in a less mature cell type. These results demonstrate for the first time immunolocalization of DAX-1 to fetal testis of rat and human. In contrast to SF-1 which was detected in the nuclei of both interstitial and Sertoli cells, specific DAX-1 immunostaining was present only in cells within the interstitium. DAX-1 protein was first detectable by immunocytochemistry on day 15.5 of gestation in the rat and at 16 weeks of gestation in the human. Studies using a homologous cDNA probe to determine the pattern of expression of DAX-1 mRNA by in situ hybridisation are planned to address the issue of whether a wider population of cells within the fetal testis express DAX-1 mRNA than has been detected by immunocytochemistry in the present study.

DAX-1 was localised to the cytoplasm of interstitial cells in fixed tissue sections from both rat and human during fetal life. This finding is somewhat surprising as the presence of putative DNA and ligand binding domains in DAX-1 suggest this protein, like SF-1, may function as a transcription factor (6). In the same sections SF-1 was found exclusively in cellular nuclei consistent with previous data using frozen tissue sections (4). We have as yet no explanation for the localization of DAX-1 but have seen a similar pattern of staining using antisera directed against peptides of the estrogen receptor (unpublished observations) but not the androgen receptor (10) on fixed sections of fetal testis processed in an identical way to those in the current paper. Estrogen receptors have been reported to undergo nucleocytoplasmic shuttling which is energy dependent (12). Previous investigations using frozen tissue sections have reported that monoclonal antibodies localize to nuclei of target cells (13) and we speculate that in the case of ER, and possibly DAX-1, the fixation of the tissue may have influenced the subcellular localization of the protein in keeping with results reported for the glucocorticoid receptor (14).

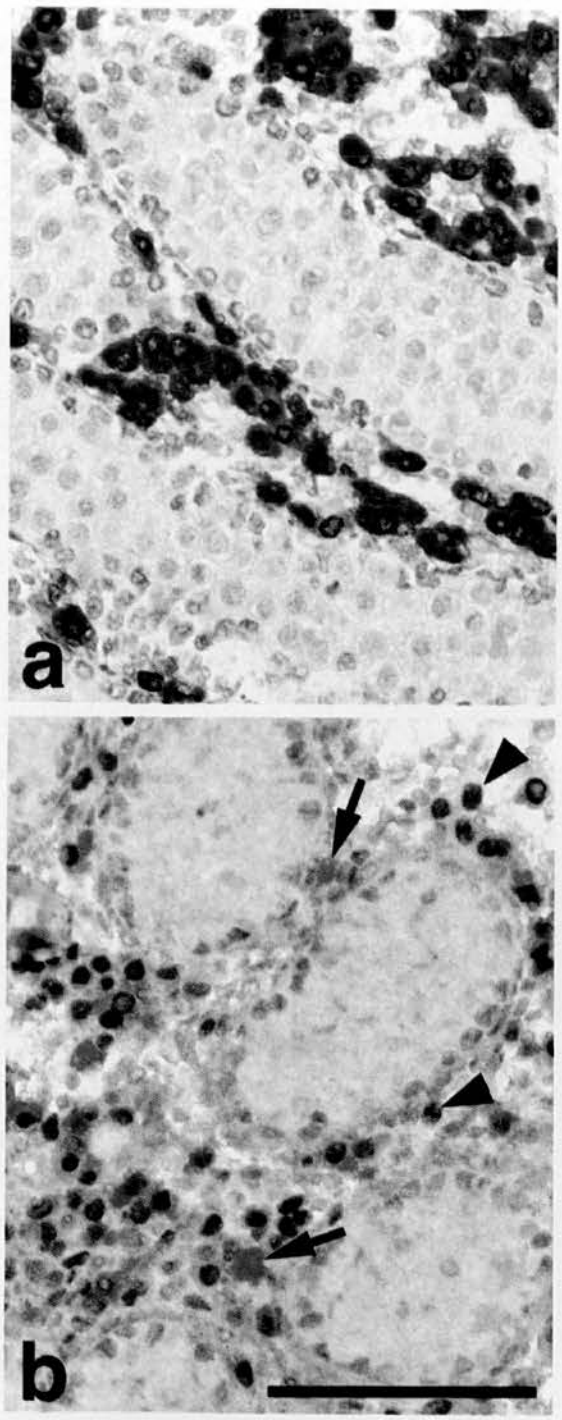

Fig. 2 Co-localisation of SF-1, 3β-HSD and/or DAX-1 to interstital cell populations in the fetal rat testis on day 17.5 of gestation, Panel a, double immunolocalisation of SF-1 and 3β-HSD. SF-1(brown color) is present in the nuclei of all cells expressing 3β-HSD (intense blue color) in their cytoplasm (steroidogenic Leydig cells). SF-1 is also present in the nuclei of other interstitial cells and Sertoli cells within the seminiferous cords; panel b, double immunolocalisation of SF-1(brown color) and DAX-1(blue color). Some interstital cells contain SF-1 but no DAX-1 (arrowheads), others contain DAX-1 but no SF-1 (arrows) whilst co-localisation is also observed. Bar = 100μM

A recent study (11) detected DAX-1 mRNA in mice using RNase protection and whole mount in situ hybridization. DAX-1 mRNA was present in the interstitial cell compartment in adult mice and was presumed to be in the adult Leydig cells. However in the fetal mouse DAX-1 mRNA was present on day 11.5 p.c. (approximately equivalent to day 13 in the rat) and declined after day 12.5 a finding apparently at odds with the present study although no protein data are included in that study (11) and a direct comparison between species is therefore not possible. In a preliminary experiment we have been able to detect significant amounts of DAX-1 mRNA in fetal rat testis on day 17.5 by Northern blotting (unpublished observations).

Immunolocalization of SF-1 in interstitial cells in the fetal rat testis was not uniform and double immunostaining revealed that those cells containing abundant nuclear SF-1 also contained the steroidogenic enzyme 3β-HSD expressed in fetal Leydig cells (15). It has been reported that fetal Leydig cells have no mitotic activity and therefore the increase in their numbers occurring between days 16.5 and 18.5 of gestation in the rat, at a time when DAX-1 is expressed, may be due to recruitment and differentiation of mesenchymal-type interstitial cells (15). In a previous study, we identified a population of fetal interstitial cells that were immunopositive for androgen receptor (AR) but not for 3β-HSD (10), and which we proposed could be progenitors of Leydig cells. The DAX-1 positive cell population identified in the current study may overlap partially with those containing AR but it has not yet been possible to evaluate this. Interestingly, no DAX-1 immunostaining was detected in Sertoli cells although these contained SF-1. We conclude that whereas SF-1 may regulate *DAX-1* expression in other tissues such as the adrenal, in the testis the situation is complex and SF-1 and/or DAX-1 acting individually or together may modulate gene transcription and functional differentiation of fetal interstitial and Leydig cells.

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References

1. **Capel B** 1995 New bedfellows in the mammalian sex-determination affair. Trends in Genetics 11: 161-163.
2. **Ryner L, Swain A** 1995 Sex in the 90's. Cell 81: 483-493.
3. **Luo X, Ikeda Y, Parker KL** 1994 A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77: 481-490.
4. **Hatano O, Takayama K, Imai T, Waterman M, Takakusu A, Omura T, Morohashi K** 1994 Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. Development 120: 2787-2797.
5. **Bardoni B, Zanaria E, Guioli S, Floridia G, Worley K, Tonini G, Ferrante E, Chiumello G, McCabe E, Fraccaro M, Zuffardi O, Camerino G** 1994 A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal. Nature Genetics 7: 497-501.
6. **Zanaria E, Muscatelli F, Bardoni B, Strom T, Guioli S, Guo W, Laill E, Moser C, Walker A, McCabe E, Meitinger T, Monaco A, Sassone-Corsi P, Camerino G** 1994 An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. Nature 372: 635-641.
7. **Carson-Jurnica MA, Schrader WT, O'Malley BW** 1990 Steroid receptor superfamily: structure and functions. Endocrine Reviews 11: .
8. **O'Malley BW, Conneely O** 1992 Orphan receptors: in search of a unifying hypothesis for activation. Molecular Endocrinology 6: 1359-1361.
9. **Burris T, Guo W, Le T, McCabe E** 1995 Identification of a putative steroidogenic facotr-1 response element in the DAX-1 promotor. Biochemical and Biophysical Research Communications 214: 576-581.
10. **Majdic G, Millar MR, Saunders PTK** 1995 Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. Journal of Endocrinology 147: 285-293.
11. **Swain A, Zanaria E, Hacker A, Lovell-Badge R, Camerino G** 1996 Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. Nature Genetics 12: 404-409.
12. **Dauvois S, White R, Parker MG** 1993 The antiestorgen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. Journal of Cell Science 106: 1377-1388.
13. **King WJ, Greene GL** 1984 Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. Nature 307: 745-747.
14. **Brink M, Humbel B, de Kloet E, van Driel R** 1992 The unligated glucocorticoid receptor is localized in the nucleus, not in the cytoplasm. Endocrinology 130: 3575-3581.
15. **Saez JM** 1994 Leydig cells: endocrine, paracrine and autocrine regulation. Endocrine Reviews 15: 574-624.

Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts

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Abstract

Androgens are required for the development of male internal and external genitalia. Androgen action is mediated by an intracellular receptor which acts as a transcription factor following activation by ligand binding. The aim of the present study was to define the time of appearance of androgen receptor (AR) in the male fetal rat gonad using immunohistochemistry. Intact fetuses (days 13·5–16·5) or testicular tissue (days 16·5–20·5 and days 3–7 postnatal) were fixed in Bouins' solution and processed into paraffin wax. On day 16·5 nuclear AR were present in mesenchymal cells surrounding the Wolffian duct but those around the Mullerian duct were receptor negative. During the following day (17–18) the abundance of nuclear staining increased, becoming detectable in the epithelial cells of the Wolffian ducts. Within the testis some nuclear staining was apparent at day 17 but was confined to interstitial cells surrounding the seminiferous cords. As development of the testis proceeded the abundance of nuclear AR in peritubular and elongated mesenchymal cells increased. AR were not detected in fetal Leydig cells expressing 3β-hydroxysteroid dehydrogenase nor in the ovaries or associated ducts of female fetuses at the same ages.

In conclusion, in the rat we have found AR expression detectable by immunohistochemistry in mesonephric mesenchyme to be confined to that underlying the Wolffian ducts and to be absent from the area around the degenerating Mullerian duct. On and after day 17 of gestation AR is present in Wolffian duct epithelial cell nuclei and within the testis it is confined to peritubular and interstitial cells which may have migrated from the mesonephros. Fetal Leydig cells were receptor negative. Within the seminiferous cords AR in Sertoli cells remained low until after birth and some perinuclear staining was detected in cells thought to be gonocytes. We believe this to be the first report of immunolocalisation of AR to fetal testicular interstitial cells.

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Introduction

Androgens are essential for masculinisation of the reproductive tract and the external genitalia during development *in utero* (see George & Wilson 1994). Androgen action is mediated by a specific intracellular receptor expressed in target tissues which binds to testosterone and dihydrotestosterone with high affinity (Carson-Jurnica *et al.* 1990). Ligand binding is essential for activation of the receptor which then binds to specific targets on DNA resulting in modulation of the level of gene expression (Rundlett *et al.* 1990). In the absence of a functional androgen receptor protein genetic males (XY karyotype) develop a female phenotype (androgen insensitivity syndrome, Griffin & Wilson 1989).

In the fetus the gonads develop along the inner surface of the mesonephros. On day 13 post coitum (p.c.) in the rat, germ cells can be recognised among the differentiating Sertoli cells and on day 14 cells within the testis can be seen to be organised into distinct cords with germ cells surrounded by Sertoli cells (Pelliniemi *et al.* 1993). Synthesis of anti-Mullerian hormone mRNA by the Sertoli cells can be detected on day 13 in the rat (Lee & Donahoe 1993) and production of testosterone by fetal Leydig cells is reported to start on day 15·5 p.c. and to be maximal on days 18·5–19·5 (Warren *et al.* 1972).

Cooke *et al.* (1991) examined expression of androgen receptor protein (AR) in male reproductive organs (excluding the testis) of the mouse from day 13 onwards using a ligand binding method. They reported the presence of binding in mesenchymal cells at all times examined but found epithelial expression of AR to be delayed, with only the epithelium of the efferent ductules positive on day 16 (approx. rat day 18). Androgen receptors have been detected, using immunocytochemistry, in external genitalia of both male and female human fetuses at 18–22 weeks of gestation after the time of androgen-dependent differentiation of the external genitalia (Kalloo *et al.* 1993).

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Recently we have successfully used an antigen retrieval method (Shi *et al.* 1993) in combination with a specific anti-androgen receptor antibody to visualise stage-dependent expression of AR in nuclei of Sertoli cells of the rat during the spermatogenic cycle (Bremner *et al.* 1994). In view of the important role of androgen in the development of normal male phenotype (Griffin & Wilson 1989) and testicular function in the adult (Sharpe 1994) we decided to use immunohistochemistry to determine the ontogeny of AR expression in the rat testis and associated ducts. We have found that the first expression of AR within the fetal rat testis occurs in interstitial cells on or about the end of day 17 of gestation. Significant nuclear staining is not seen in Leydig cells or in Sertoli cells during fetal life. Mesenchymal cells surrounding ducts destined to form the epididymis and seminal vesicles contain abundant nuclear AR on day 16·5 whilst the cells surrounding the degenerating Mullerian duct are AR negative.

Materials and Methods

Animals and tissues

Adult female Wistar rats (aged 90–100 days) obtained from our colony were maintained under standard animal house conditions. Timed matings were carried out by the introduction of an adult male and the presence of a vaginal plug in the morning was taken as evidence of mating during the previous 12 h (day 0·5). Animals were killed by inhalation of carbon dioxide and subsequent cervical dislocation. Whole fetuses (days 11·5–17·5) and testes and kidneys dissected from fetuses on days 16·5–20·5 (delivery on day 21–23 in our colony) and from neonates (days 3, 5, 7) were immersion-fixed in Bouins' solution for 6–7 h prior to processing into paraffin wax (see Millar *et al.* 1993). The age of the fetuses was confirmed by morphological examination (see Kaufman 1992).

Histology and immunohistochemistry

For examination of testicular histology during fetal life, serial sections (5 microns) were cut through fetuses which had been oriented in paraffin blocks using heated forceps (see Kaufman 1992), mounted on slides and stained with haematoxylin and eosin. To perform immunocytochemistry, sections (5 microns) were cut and mounted on slides coated with 3-aminopropyl triethoxy-silane (Sigma Chemical Co., Poole, Dorset, UK), dried overnight (50 °C), dewaxed, rehydrated in graded ethanols, washed in water, submerged in 0·01 M citrate buffer (pH 6) and microwaved on full power (650 W, 4 times 5 min). After standing for 20 min without disturbance in the microwave oven, sections were washed once (5 min) in TBS (0·05 M Tris–HCl pH 7·4, 0·85% NaCl) and blocked using normal swine serum diluted in TBS (1:5, NSS-TBS). Anti-human androgen receptor antibody raised in rabbits against the first 17 amino acids of the AR which are conserved between rat and human, was purchased from Novocastra (Newcastle, UK). This polyclonal antibody was diluted 1:20 in NSS-TBS (see above) and incubated on the sections overnight at 4 °C under plastic coverslips. Control sections were incubated either with non-immune rabbit serum (diluted 1:20) or antiserum which had been incubated with a 20-fold excess of free peptide (hAR 1–17). After removal of coverslips, sections were washed twice in TBS (5 min), incubated for 30 min with biotinylated swine anti-rabbit immunoglobulin (Dako, High Wycombe, Bucks, UK) diluted 1:500 in NSS-TBS, then washed again in TBS (2 times 5 min). For detection of bound antibodies, sections were first incubated with alkaline phosphatase conjugated to avidin-biotin complex (Dako) for 30 min, washed in TBS (2 times 5 min), followed by a final wash in Tris–Mg buffer (100 mM Tris–HCl pH 9·5, 100 mM NaCl, 50 mM $MgCl_2$). Colour reaction product was developed by incubation in a solution containing 337·5 μg/ml nitro-blue tetrazolium, 175 μg/ml 5-bromo-4-chloro-3-indolyphosphate and 1 mM levamisol in Tris–Mg buffer for 30–120 min (de Jong *et al.* 1985). Sections were counterstained with haematoxylin, dehydrated in absolute alcohol, cleared in xylene and coverslipped using Pertex mounting medium (Cell Path plc, Hemel Hempstead, Herts, UK).

To determine which cells within the interstitial region of the fetal testis belonged to the Leydig cell population, immunostaining using antibodies against androgen receptor (as described above) was followed by that using rabbit anti-human 3β-hydroxysteroid dehydrogenase (3β-HSD; Lorence *et al.* 1990) kindly donated by Professor Ian Mason, Department of Clinical Biochemistry, University of Edinburgh. Immunostaining with anti-AR was followed by a quick wash in double distilled water and 2 washes in TBS (5 min each). Sections were blocked with normal swine serum (diluted 1:5 in TBS) for 30 min, followed by incubation with antibodies against 3β-HSD (1:500 dilution) overnight at 4 °C. Sections were washed twice in TBS (5 min each wash), incubated with swine anti-rabbit immunoglobulins (Dako) for 30 min at room temperature, washed again 2 times 5 min in TBS and incubated with rabbit peroxidase-antiperoxidase complex (Dako) for 30 min at room temperature. After two washes in TBS, sections were equilibrated for 5 min in 0·02 M sodium acetate buffer (pH 5·2). Colour reaction was developed by incubation in a mixture of 0·02% 3-amino-9-ethylcarbazole (Sigma) in 10 ml 0·02 M sodium acetate buffer (pH 5·2) containing 1·2 ml dimethylsulphoxide (DMSO; BDH, Merck Ltd, Lutterworth, Leics, UK) and 0·01% hydrogen peroxide. After 5–10 min, sections were washed in double distilled water and coverslipped using aquamount (BDH) mounting medium. Specificity of the 3β-HSD antibody was checked by using normal rabbit serum; no colour reaction was obtained.

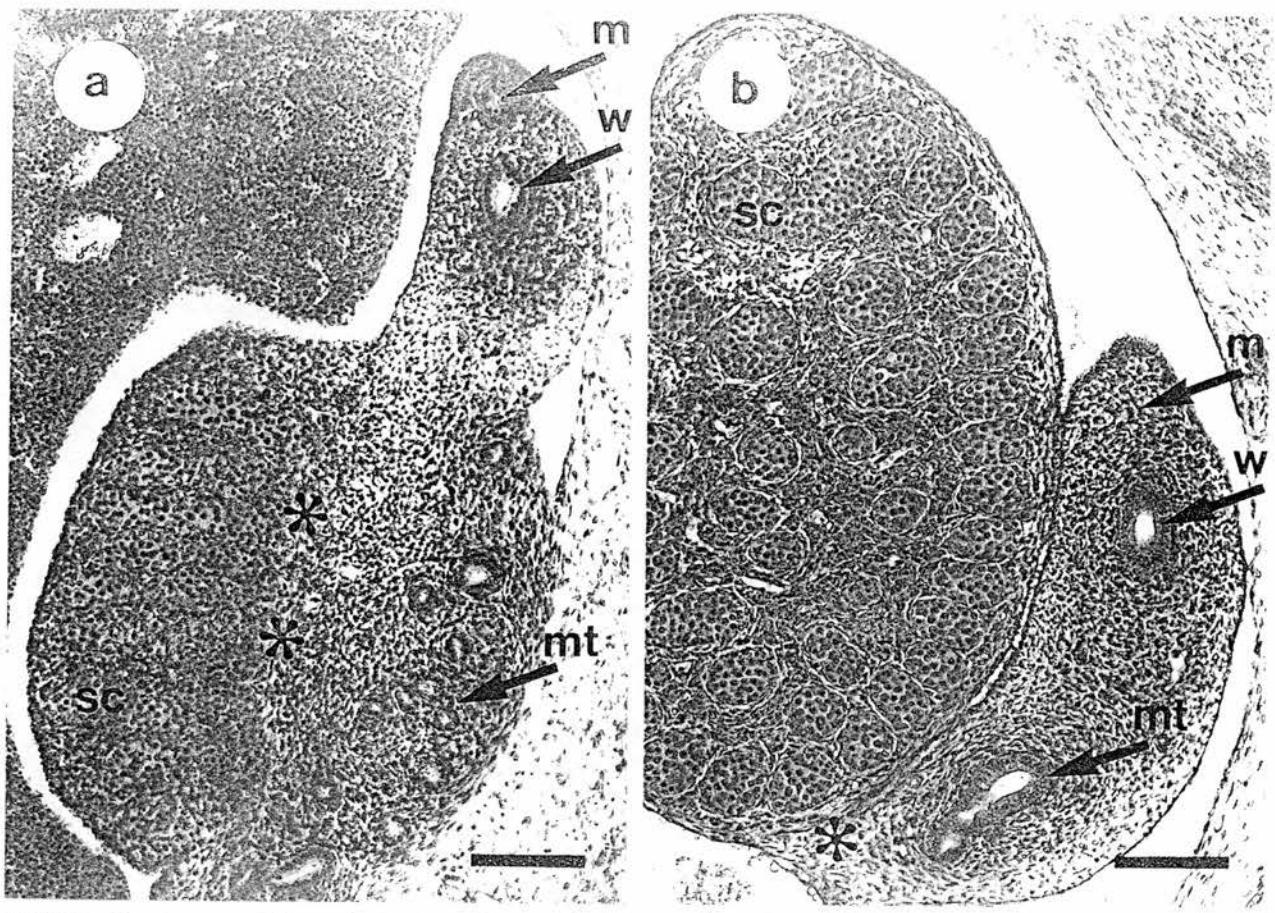

FIGURE 1. Development of the rat fetal testis; sections are from fetuses of timed mated females. Fetuses were fixed in Bouins' solution and stained with haematoxylin and eosin. (a) Testis and associated structures on day 14·5 of gestation; testis containing identifiable seminiferous cords (sc). m=Mullerian duct, w=Wolffian duct, mt=mesonephric tubules, *=mesorchium. (b) Day 17·5 of gestation. Labelling as in (a). Note increased testis size, distinct solid seminiferous cords, and absence of a Mullerian duct. Bar=100 μm.

Results

Fetal development of the testis

In the fetuses recovered from timed mated females in our colony male and female gonads were first easily distinguishable from each other on day 14·5 at which time the testis appeared larger than the corresponding ovary and there was some organisation into seminiferous cords giving it a characteristic 'striped' appearance (Fig. 1*a*). The appearance of the rat testis on day 15·5 appeared comparable to that of a day 13·5 mouse embryo illustrated in Kaufman's Atlas of Mouse Development (Kaufman 1992). On day 17·5, within the testis distinct seminiferous cords containing germ cells could be distinguished easily (Fig. 1*b*, labelled SC) surrounded by the cells of the interstitium. Outside the testis the Mullerian duct was no longer distinct on day 17 whilst, in contrast, the Wolffian duct and mesonephric tubules were enlarged (Fig. 1*b*).

Detection of androgen receptor by immunohistochemistry

In the testis and associated structures there was no clear immunolocalisation of nuclear AR protein on or before day 15·5. On day 16·5 (Fig. 2*a*) nuclear AR was clearly present in the nuclei of mesenchymal cells surrounding the Wolffian duct and within the epithelium of the mesonephric tubules and their underlying mesenchyme, but was absent from the cells around the Mullerian duct. The central region of the degenerating mesonephros was only weakly positive. At this time the adrenal gland, thymus and mammary gland rudiment were all stained positively (not shown). As day 16 progressed and on day 17 (Fig. 2*b*,*c*) nuclear staining of mesenchymal cells surrounding the ducts increased and staining of nuclear AR in the cells of the epithelium of the Wolffian duct became more [illegible] day AR positive cells were present within the testis itself and these were confined to the interstitial region (open arrow). In female fetuses

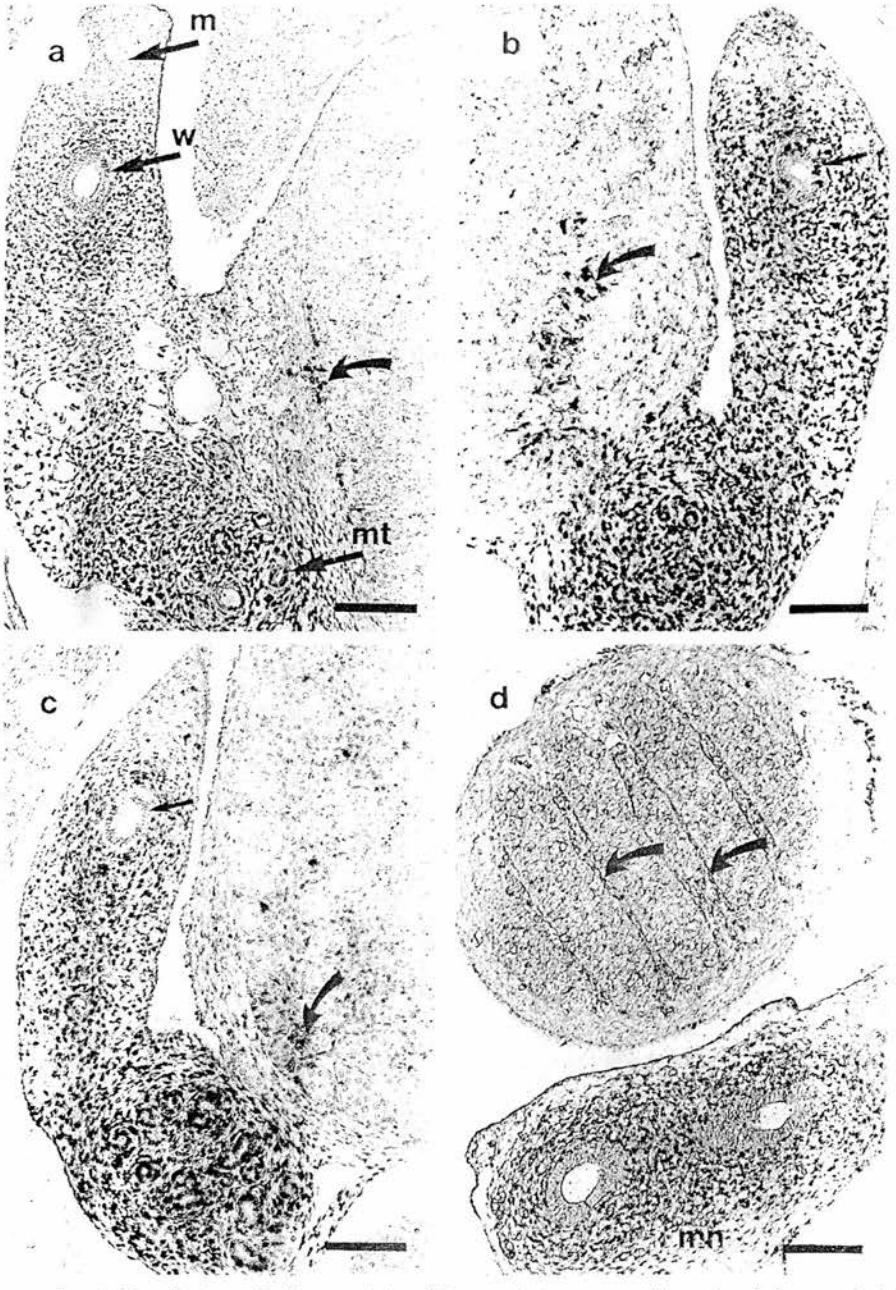

FIGURE 2. Immunocytochemical localisation of cells containing AR protein in testes and associated ducts and their underlying mesenchyme on days 16·5–18·5 of gestation. (a) On day 16·5 AR protein is clearly present in the nuclei of cells within the mesenchymal cells surrounding the Wolffian duct (arrowed, w) and within the epithelium of the mesonephric tubules (arrowed, mt) and its underlying mesenchyme, but is absent from the cells around the degenerating Mullerian duct (arrowed, m). The central region of the degenerating mesonephros is only weakly positive. A few cells of the interstitial population between the seminiferous cords within the testis are positive (curved arrow). During day 17 ((b) 17·0, (c) 17·5) nuclear staining of mesenchymal cells surrounding the developing ducts is strongly positive and nuclear AR in the epithelium of the Wolffian duct is very prominent (arrow). Even late on day 17 few AR positive cells are present within the testis (curved arrow). (d) Isolated testis and mesonephric tubules from day 18·5. The testis has been cut so that the seminiferous cords are seen in longitudinal section. Positive AR staining within the testis is confined to interstitial cells (curved arrows). Sections were counterstained with haematoxylin. mn = mesonephros. Bar = 100 μm.

examined at the same ages no specific AR nuclear staining was ever observed in the ovary or Mullerian ducts (not shown).

In testes isolated from rat fetuses towards the end of pregnancy (days 18·5–20·5) nuclear AR was immunolocalised to the cells forming a narrow layer around the individual tubules (Fig. 2*d* curved arrows, Fig. 3*a,b*). The location of these cells would be consistent with identification as peritubular myoid cells which form a distinct unicellular layer around the seminiferous tubules in the adult rat testis (Skinner 1991). Other cells within the interstitial area including the central mesenchyme of the testis (Fig. 3*b*) were also AR positive. Outside the testis mesenchymal cells underlying the Wolffian duct and mesonephric tubules and the respective epithelial cells contained abundant nuclear AR (Fig. 3*a,b*). Although Sertoli cell AR nuclear immunostaining was clearly distinguishable on postnatal days 3, 5 (Fig. 3*c*) and 7 it remained significantly lower than that detected in the adjacent peritubular cells and other interstitial cells. Preabsorption of antiserum with AR peptide abolished specific immunostaining (Fig. 3*d*).

In order to address whether cells within the interstitial population were members of the Leydig cell population, double immunostaining with antibodies directed against AR and 3β-HSD, a constitutively expressed, steroidogenic enzyme essential to production of testosterone by Leydig cells (Payne & Youngblood 1995), was undertaken. The results obtained (Fig. 4) clearly demonstrated that nuclear AR immunostaining was confined to cells which did not express 3β-HSD and that on the days examined (days 17·5–19·5 p.c.) the fetal Leydig cell population did not appear to be responsive to androgens via the AR.

Discussion

In the fetal rat testis synthesis and secretion of testosterone is detectable on day 15·5 following almost immediately after differentiation of the fetal Leydig cells from mesenchymal cells surrounding the primitive sex cords. Maximal output of testosterone by the fetal Leydig cells occurs on days 18·5–19·5 at the time when numbers of cells are at their highest (reviewed by Huhtaniemi 1994). Androgens act via a specific intracellular receptor that interacts with nuclear DNA to bring about changes in the expression of androgen responsive genes (reviewed in Parker 1991). Androgens, and a functional androgen receptor, are essential for normal development of male genitalia and a number of studies have addressed the timing and distribution of androgen receptors in the developing urogenital system using binding assays (Takeda *et al.* 1985, Cooke *et al.* 1987, 1991). These studies and those using *in vitro* approaches have implicated the cells of the mesenchyme as the primary targets for androgen action during fetal life. This concept is supported by the results obtained when mesenchymal or epithelial samples from Tfm mice (which have a defect in their AR) were used in combination with those from normal animals; the presence of a normal AR in the mesenchymal cells was found to be essential for development (Lasnitzi & Mizuno 1980). In the current study we have identified clear and abundant nuclear AR in mesenchymal cells surrounding the Wolffian duct on day 16 of pregnancy. However, AR were not distributed uniformly throughout the mesenchymal cells within the mesonephric area being absent from the cells surrounding the Mullerian duct, which is destined to regress under the influence of anti-Mullerian hormone (AMH/MIS; Lee & Donahoe 1993), and sparse in the central region directly adjoining the testis. Some cells within the epithelial layer of the Wolffian duct, especially in the area which will develop into the epididymis, contained nuclear AR on day 16·5 and the nuclei of nearly all the epithelial cells of the ducts were positively stained for AR on and after day 17. The present results are consistent with the location of AR described in earlier reports using ligand binding but seem to place expression of AR in the epithelial cells of the ducts one to two days earlier than might have been expected from reports in the mouse (Cooke *et al.* 1991) reflecting the greater sensitivity of the immunohistochemical technique. The results also show that expression of AR within the mesenchymal cells of the mesonephric region is not uniform.

In the adult testis the primary target for androgen action is believed to be the Sertoli cells within the seminiferous epithelium (reviewed by Sharpe 1994) although both peritubular myoid cells and Leydig cells also express AR. In the rat only the Sertoli cells were found to exhibit stage-dependent expression of nuclear AR (Bremner *et al.* 1994) which correlates with the reported stage-dependent effects of testosterone (Sharpe *et al.* 1992). In the fetal rat we failed to find convincing immunolocalisation of nuclear AR in the testis before day 17 p.c., at which time a few cells of the interstitial cell population did appear positive. As testicular development progressed the number of cells in the interstitial cell population with AR nuclear immunostaining increased. Based on their locations within the testis and lack of staining with antibody against 3β-HSD we would identify these AR positive cells as being peritubular and interstitial mesenchymal cells, both of which have a fibroblastic appearance. In the adult testis peritubular myoid cells around the tubules have been shown to contain AR (Verhoeven 1980, Bremner *et al.* 1994). In an interesting series of experiments *in vitro*, Buehr *et al.* (1993) found that cells from the mesonephric region migrated into the testis on or after day 11·5 p.c. in the mouse and contributed to the interstitial cell population including those in the peritubular layer. In the absence of an influx of cells from the mesonephros normal differentiation of testis cords did not occur, consistent with the synthesis of components of the extracellular matrix by peritubular cells contributing to organisation of the testis.

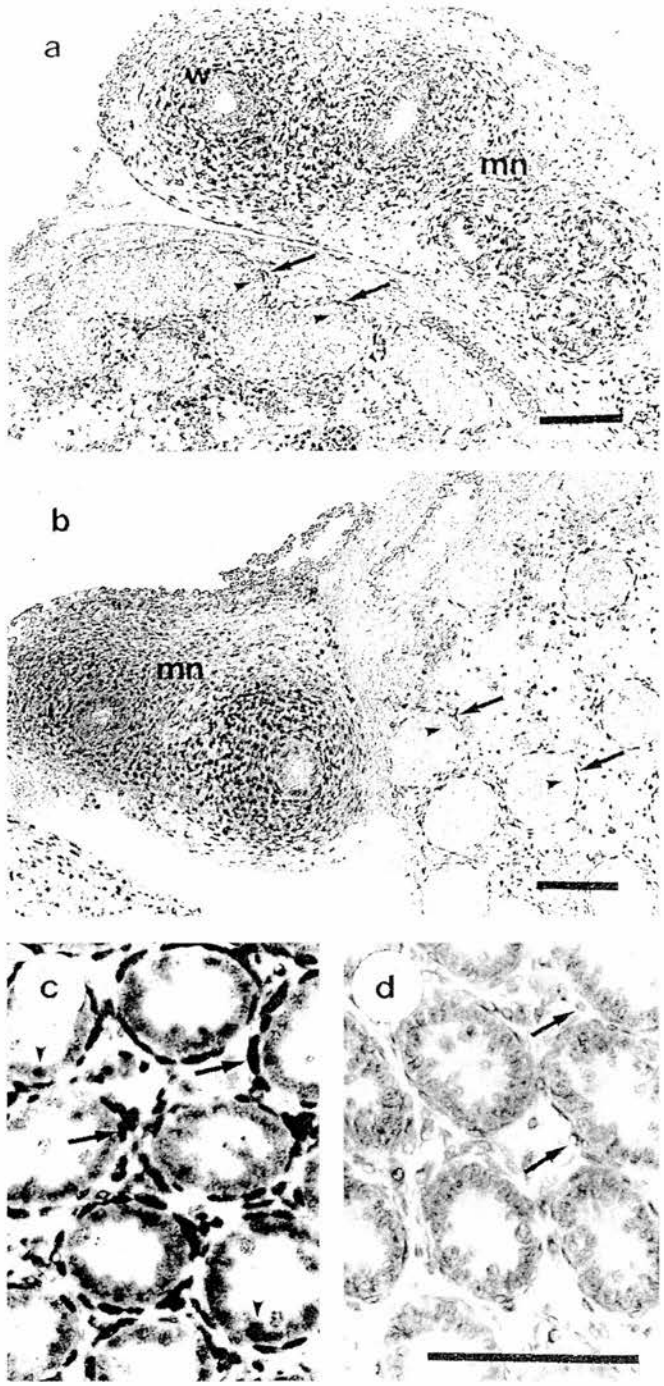

FIGURE 3. Immunocytochemical detection of nuclear androgen receptor in presumptive peritubular cells surrounding seminiferous tubules from day 19·5 of gestation and in Sertoli cells after birth. As pregnancy progresses and the testis continues to enlarge the amount of nuclear AR distinguishable within the testis increases but remains confined to the interstitial cell population especially the presumptive peritubular cells surrounding the solid seminiferous cords (arrows). (a) Day 19·5. (b) day 20·5. Staining of Sertoli cells (arrowheads) was not convincing until after birth (see (c)) although on day 20·5 sometimes cells within the seminiferous cords did appear to have faint perinuclear staining (arrowheads) and these were provisionally identified as gonocytes. After birth (c) (day 5) AR immunostaining in peritubular cells was strong and some AR could be detected within the seminiferous cords. The specificity of staining was checked by preabsorption of the antibody with excess peptide (d). All sections were counterstained with haematoxylin. Bar=100 μm.

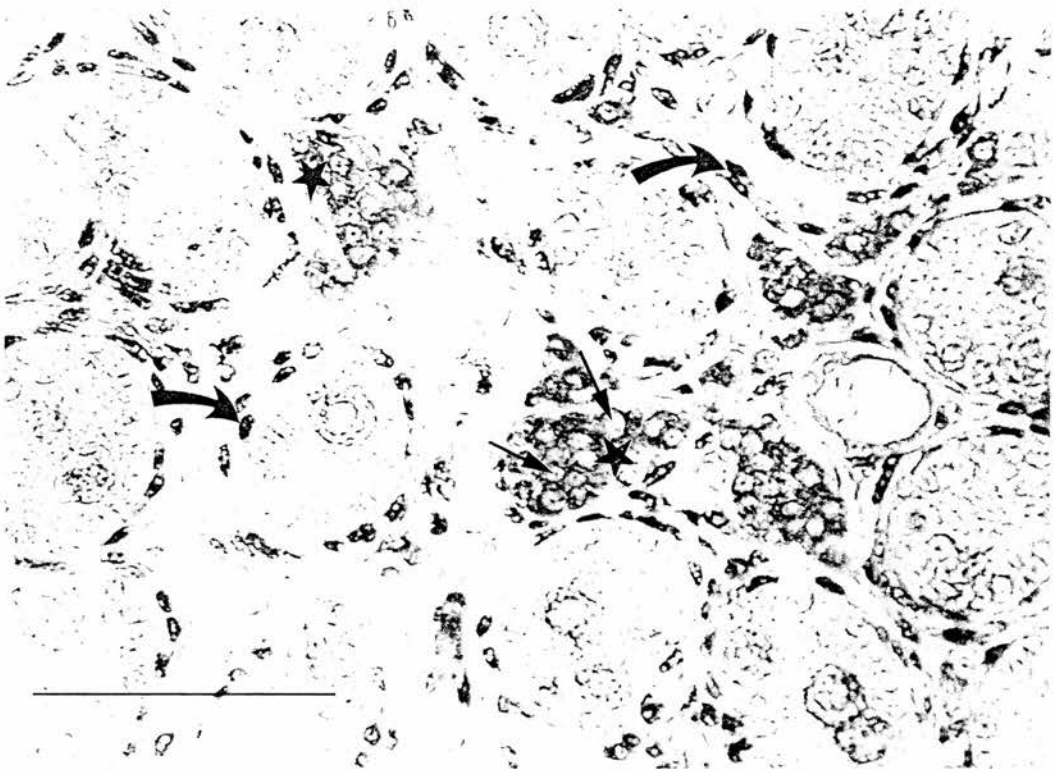

FIGURE 4. Immunostaining of 19·5-day-old fetal rat testis with antibodies directed against AR and 3β-HSD, a steroidogenic enzyme expressed only in Leydig cells. Cytoplasm of the Leydig cells stained strongly positive with antibodies against the steroidogenic enzyme 3β-HSD (light brown staining, asterisks). However, the Leydig cells nuclei do not show any staining with antibodies against AR (thin arrows). In contrast, peritubular cells and interstitial mesenchymal cells have nuclei immunostained using anti-AR (curved arrows). Bar=100 μm.

In the context of the current findings it is interesting to speculate that the cells in the fetal testis containing AR have developed from the same cell population which expresses AR in the mesonephros and which we were able to immunostain on day 16·5 p.c. Previous studies have suggested that a peritubular sheath of cells is present at birth but these do not become 'myoid' cells until puberty and they do so under the influence of testosterone (Bressler & Ross 1972, Skinner 1991). Our results demonstrating abundant immunostaining of AR would indicate that the peritubular cells have the potential to be responsive to androgens during fetal life and this may have implications for the development of the fetal testis. Peritubular cells from immature rats (postnatal days 15–25) have recently been shown to secrete activin-A (de Winter *et al.* 1994) and to respond to androgens with increased expression of an androgen response element reporter (Ku *et al.* 1994). Previous studies have shown that, at this time, they can secrete a range of growth factors and regulatory agents (reviewed by Skinner 1991) the production of some of which may be testosterone dependent and influence function of the fetal testis.

In the results of double immunostaining experiments we found evidence of three clear populations of interstitial cells; the peritubular cells discussed above, AR positive cells with elongated nuclei and clumps of fetal Leydig cells which contained no AR. We speculate that the AR positive mesenchymal-like interstitial cells might be progenitors of the fetal Leydig cells and that androgen action could play a role in their differentiation. In immature rats, differentiation of adult type Leydig cells from mesenchymal progenitor cells has been reported to be advanced by androgen and luteinizing hormone (LH) (Hardy *et al.* 1990, Shan *et al.* 1995) and it is notable that expression of LH receptors is reported to occur in the fetal rat testis on and after day 15·5 (Warren *et al.* 1984). We found positive staining of AR protein within Sertoli cells of the seminiferous cords was generally unconvincing prior to birth although in some sections we did observe perinuclear staining in large round cells which we believe to be gonocytes (Clermont & Perey 1957). Previous reports have demonstrated an increase in the expression of AR in immature Sertoli cells of the rat over the period when spermatogenesis is becoming established between days

14 and 28 of postnatal life and establishment of stage-dependent immunostaining of Sertoli cells in the rat (Bremner *et al.* 1994, Ku *et al.* 1994).

To detect the AR protein in our sections of fixed tissue it was necessary to undertake antigen retrieval of the samples by microwaving in citrate buffer (Shi *et al.* 1993). In the absence of this pretreatment, which was recommended by the supplier of the antiserum, no immunostaining was detected (M R Millar, unpublished observations). In a separate study using adult rat testes AR immunostaining of Sertoli cell nuclei at stages IV-VII has been reported both following antigen retrieval (Bremner *et al.* 1994) and in samples embedded in polyester wax and not subjected to antigen retrieval (Vornberger *et al.* 1994). The immunogen used to raise the antiserum used in this study is located at the extreme N terminal end of the receptor protein in the region associated with ligand independent-transcriptional activation (Jenster *et al.* 1991). Antigen retrieval, due to the high temperatures used, is believed to cause 'unmasking' of antigenic sites and has been particularly applicable to proteins with a nuclear location, and can sometimes be mimicked by treatment of sections with proteolytic enzymes (Shi *et al.* 1993). AR are known to associate with other proteins both before and after ligand binding (Carson-Jurnica *et al.* 1990) and a recent study has shown retention of receptor-bound androgen is enhanced by an interaction between the AR N terminal and steroid binding domains (Zhou *et al.* 1995). We have not determined whether the detection of AR by the antibody directed against the N terminal epitope is dependent upon the conformation of the AR protein but in previous investigations have assumed that immunohistochemical localisation of AR reflects the presence of an active ligand receptor because of the correlation with known sites of androgen action (Bremner *et al.* 1994).

In conclusion, we have shown that some cells within the mesonephric mesenchyme contain AR as early as day 16 of gestation, consistent with these cells being a primary target of androgen action, but that cells around the Mullerian duct remain receptor negative. In the fetal rat testis AR detected by immunohistochemistry are confined to cells of the interstitial population which may have migrated from the mesonephros, but are absent from Leydig cells. In the rat, androgen action within the testis during fetal life seems likely to be confined to the peritubular and interstitial mesenchymal cell population.

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References

Bremner WJ, Millar MR, Sharpe RM & Saunders PTK 1994 Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* **135** 1227–1234.

Bressler RS & Ross MH 1972 Differentiation of peritubular myoid cells of the testis: effects of intratesticular implantation of newborn mouse testis into normal and hypophysectomized adults. *Biology of Reproduction* **6** 148–159.

Buehr M, Gu S & McLaren A 1993 Mesonephric contribution to testis differentiation in the fetal mouse. *Development* **117** 273–281.

Carson-Jurnica MA, Schrader WT & O'Malley BW 1990 Steroid receptor superfamily: structure and functions. *Endocrine Reviews* **11** 201–219.

Clermont Y & Perey B 1957 Quantitative study of the cell population of the seminiferous tubules in immature rats. *American Journal of Anatomy* **100** 241–267.

Cooke PS, Young PF & Cunha GR 1987 Androgen dependence of growth and epithelial morphogenesis in neonatal mouse bulbourethral glands. *Endocrinology* **121** 2153–2160.

Cooke PS, Young P & Cunha GR 1991 Androgen receptor expression in developing male reproductive organs. *Endocrinology* **128** 2867–2873.

George FW & Wilson JD 1994 Sex determination and differentiation. In *The Physiology of Reproduction*, pp 3–28. Eds E Knobil & JD Neill. New York: Raven Press.

Griffin JE & Wilson JD 1989 The androgen resistance syndromes: 5α-reductase deficiency, testicular feminisation and related syndromes. In *The Metabolic Basis of Inherited Disease*, pp 1919–1944. Eds CR Scriver, WS Beaudet & SD Valle. New York: McGraw-Hill.

Hardy MP, Kelce WR, Klinefelter GR & Ewing LL 1990 Differentiation of Leydig cell precursors *in vitro*: a role for androgens. *Endocrinology* **127** 488–490.

Huhtaniemi I 1994 Fetal testis – a very special endocrine organ. *European Journal of Endocrinology* **130** 25–31.

Jenster G, van der Korput J, van Vroonhoven C, van der Kwast T, Trapman J & Brinkman A 1991 Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Molecular Endocrinology* **5** 1396–1404.

de Jong ASH, van Kessel-van Vark M & Raap AK 1985 Sensitivity of various visualization methods for peroxidase and alkaline phosphatase activity in immunoenzyme histochemistry. *Histochemistry* **17** 1119–1130.

Kalloo NB, Gearhart JP & Barrack ER 1993 Sexually dimorphic expression of estrogen receptors but not androgen receptors in human fetal external genitalia. *Journal of Clinical Endocrinology and Metabolism* **77** 692–698.

Kaufman M 1992. *Atlas of Mouse Development*. London: Academic Press.

Ku C-Y, Loose-Mitchell DS & Sanborn BM 1994 Both Sertoli and peritubular cells respond to androgens with increased expression of an androgen response element reporter. *Biology of Reproduction* **51** 319–326.

Lasnitzi I & Mizuno T 1980 Prostatic induction: interaction of epithelium and mesenchyme from normal wild-type mice and androgen insensitive mice with testicular feminization. *Journal of Endocrinology* **85** 423–428.

Lee MM & Donahoe P 1993 Mullerian inhibiting substance: a gonadal hormone with multiple functions. *Endocrine Reviews* **14** 152–164.

Lorence MC, Murry BA, Trant JM & Mason JI 1990 Human 3β-hydroxysteroid dehydrogenase/delta(5)->(4) isomerase from

placenta: expression in non steroidogenic cells of a protein that catalyses the dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology* **126** 2493–2498.

Millar M, Sharpe RM, Maguire SM & Saunders PTK 1993 Cellular localisation of messenger RNAs in rat testis; application of digoxigenin-labelled probes to embedded tissue. *Cell and Tissue Research* **273** 269–277.

Parker MG 1991. *Nuclear Hormone Receptors*. London: Academic Press.

Payne AH & Youngblood GL 1995 Regulation of expression of steroidogenic enzymes in Leydig cells. *Biology of Reproduction* **52** 217–225.

Pelliniemi LJ, Frojdman K & Patanko J 1993 Embryological and prenatal development and function of Sertoli cells. In *The Sertoli Cell*, pp 87–113. Eds LD Russell & MD Griswold. Clearwater, Florida: Cache River Press.

Rundlett SE, Wu S-P & Miesfeld, RL 1990 Functional characterizations of the androgen receptor confirm that the molecular basis of androgen action is transcriptional regulation. *Molecular Endocrinology* **4** 708–714.

Shan L-X, Hardy DO, Catterall JF & Hardy MP 1995 Effects of luteinizing hormone (LH) and androgen on steady state levels of messenger ribonucleic acid for LH receptors, androgen receptors, and steroidogenic enzymes in rat Leydig cell progenitors *in vivo*. *Endocrinology* **136** 1686–1693.

Sharpe RM 1994 Regulation of spermatogenesis. In *The Physiology of Reproduction*, edn 2, pp 1363–1434. Eds E Knobil & JD Neill. New York: Raven Press.

Sharpe RM, Maddocks S, Millar M, Kerr JB, Saunders PTK & McKinnell C 1992 Testosterone and spermatogenesis: identification of stage-specific, androgen-related proteins secreted by adult rat seminiferous tubules. *Journal of Andrology* **13** 172–184.

Shi S-R, Chaiwun B, Young L, Cote R & Taylor C 1993 Antigen retrieval technique utilizing citrate buffer or urea solution for immunohistochemical demonstration of androgen receptor in formalin-fixed paraffin sections. *Journal of Histochemistry and Cytochemistry* **41** 1599–1604.

Skinner MK 1991 Cell-cell interactions in the testis. *Endocrine Reviews* **12** 45–77.

Takeda H, Mizuno T & Lasnitzki I 1985 Autoradiographic studies of androgen binding sites in the rat urogenital sinus and postnatal prostate. *Journal of Endocrinology* **104** 87–92.

Verhoeven G 1980 Androgen receptor in cultured interstitial cells derived from immature testis. *Journal of Steroid Biochemistry* **13** 469–474.

Vornberger W, Prins G, Musto N & Suarez-Quian C 1994 Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* **134** 2307–2316.

Warren DW, Haltmeyer GC & Eik-New KB 1972 Synthesis and metabolism of testosterone in the fetal rat testis. *Biology of Reproduction* **7** 94–99.

Warren DW, Huhtaniemi IT, Tapanainen J, Dufau ML & Catt KJ 1984 Ontogeny of gonadotrophin receptors in the fetal and neonatal rat testis. *Endocrinology* **96** 1226–1229.

de Winter JP, Vanderstichele HMJ, Verhoeven G & Timmerman MA 1994 Peritubular myoid cells from immature rat testes secrete activin A and express activin receptor type II *in vitro*. *Endocrinology* **135** 759–767.

Zhou Z-X, Lane M, Kemppainen J, French F & Wilson E 1995 Specificity of ligand-dependent androgen stabilization: receptor domain interactions influence dissociation and receptor stability. *Molecular Endocrinology* **9** 208–218.

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