Gene Expression in mouse testis during development

A thesis presented for the degree of Doctor of Philosophy in the Faculty of Veterinary Medicine, University of Glasgow.

By

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Abstract

Many genes and cellular pathways have been implicated in the initiation and regulation of testicular differentiation, development and function. The genes generally have a temporal expression profile. This study aimed to characterise several of the key genes involved in the androgen synthetic pathway, in both normal mice and also mutant strains. LH receptor and 5 α reductase were the two major genes of interest. Using molecular biology techniques it was established that the LH receptor exists in several forms throughout development, with smaller, alternate-spliced forms being the predominant transcripts expressed in testis during fetal life. It is inconclusive from this study whether these spliced forms encode functional proteins, but the consistency of their expression patterns suggests that they do. Expression levels of the two isoforms of 5 α reductase were found to be very low in mouse testis throughout development, and in addition androgen may be involved in regulation of expression of the type 1 isoform, as levels were significantly reduced in the AR-null mouse testis both at puberty and adulthood. Previously unpublished sequence from both type 1 and type2 5 α reductase transcripts was obtained using PCR primers designed from rat sequence and mouse EST sequence. The results from this study highlight the importance specific genes play in regulation and expression of others and the down stream signalling pathways which may be involved in development and regulation of a functional testis.

Real time PCR results demonstrated that not only is there a temporal differences in expression patterns with the genes of interest throughout development, but there are also differences observed between the mutant and normal strains.

Publications

O'Shaughnessy, P.J., Willerton, L. and Baker, P.J., (2002) Changes in Leydig cell gene expression during development in the mouse. *Biology of Reproduction* **66**, 966-975.

O'Shaughnessy, P.J., Johnston, H., Willerton, L. and Baker, P.J., (2002) Failure of normal adult Leydig cell development in androgen – receptor – deficient mice. *Journal of Cell Science* **15**, 3491 – 3496

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Chapter 1

Introduction

1 The Testis

The testes are central to the male reproductive system. In most mammals the testes are situated externally as they function optimally at a temperature slightly cooler than that of the rest of the body. The testes have interrelated functions, which include the production of gametes (gametogenesis) and the production of steroids (steroidogenesis). Gametogenesis results in the formation of spermatozoa, which contain the male genes and contribute to the formation of new life, and steroidogenesis results in the formation of steroid hormones, primarily testosterone which functions in the regulation of the development of spermatazoa and the differentiation, growth and functional development of accessory reproductive glands and structures.

The testis produces several different hormones including estrogen, activin and inhibin but the most important type is the androgens. Production of androgens and spermatazoa occurs in two separate compartments within the testes. The spermatazoa develop through a series of cellular differentiation stages from the precursor primordial germ cells. These are situated within the tubules and are in close association with the Sertoli cells, which at one time were also known as nurse cells, as they support and nourish the developing sperm. Spermatogenesis is the process by which the mature sperm are formed and it consists of three essential stages. Firstly, mitotic proliferation of the germ cells, in order to generate large numbers of cells. Secondly, meiotic division to create genetic diversity and halve the chromosome number and finally extensive cell modelling, to package the chromosomes for effective delivery to the oocyte. All these stages occur in the tubules of the testes aided by the Sertoli cells.

Androgen synthesis on the other hand occurs between the tubules in specialised steroidogenic cells. These are called the Leydig cells. Androgens are essential for sexual differentiation of the body during fetal life, growth and maturation of reproductive tract at puberty, and initiation and maintenance of spermatogenesis from puberty through to adult life. In addition the androgens are responsible for masculinisation of the brain and control of reproductive behaviour.

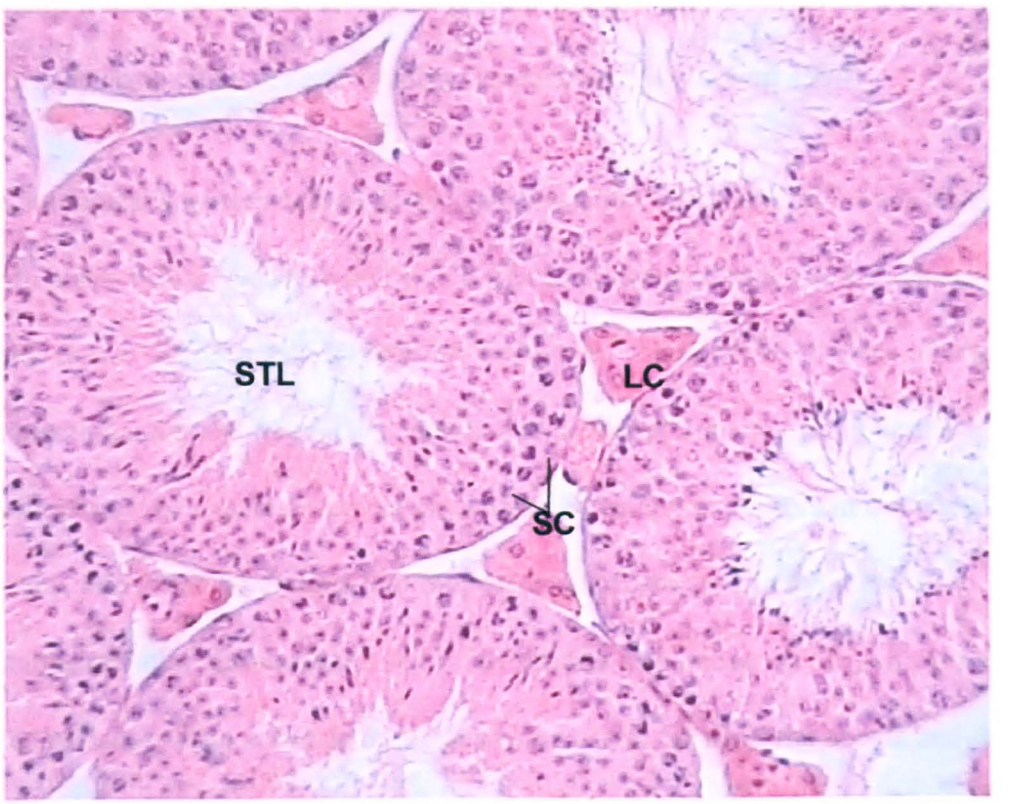

Figure 1-1 Cellular components of the testis. LC = Leydig cells, SC = Sertoli cells STL = seminiferous tubule lumen.

1.1 Sexual differentiation

The process of sexual differentiation is the commitment of a developing organism to follow either a male or female pathway. Different species have evolved different mechanisms which initiate this commitment. In invertebrates such as Drosophila melanogaster and Caenorhabdites elegans, sex determination is controlled by a gene dosage mechanism (Parkhurst and Meneely 1994). Whereas in some reptilian species sex is determined by the surrounding temperature (Crews *et al* 1994).

In mammals, a system known as male heterogamety exists within the sex chromosomes, with males being XY and females XX. The presence or absence of a Y chromosome leads to two different sex-determining pathways, which involve a series of complex interacting networks of genetic, cellular and hormonal signals leading to the development of a male or female phenotype. Development occurs in a stepwise chain of events, which can be divided into four essential processes. Firstly, there is the establishment of chromosomal sex at fertilisation also known as the genetic sex. This is accomplished when either an X or Y carrying sperm fertilises the oocyte and the nuclei of the two cells fuse leading to formation of an embryo after a series of cell divisions. Secondly, there is development of the undifferentiated gonads into either ovaries or testes (gonadal sex), This occurs due to a gene-signalling cascade which differs between the sexes, with the presence or absence of a Y chromosome determining which pathway is undertaken. Thirdly, there is differentiation of the internal duct system and external genitalia, which is in accordance with the gonadal sex. The last step in this differentiation pathway is establishment of gender. There are key areas in the brain which must be exposed to appropriate levels of testosterone during a critical time period

in development in order for male gender to be established (Gorski 1985). This is sometimes known as the psychological sex. In the absence of this occurring, the individual would adopt a female gender.

If testes develop, the male phenotype is controlled by two testicular hormones, testosterone which is produced by the fetal Leydig cells, and is required for differentiation of the internal duct system, and anti Mullerian hormone (AMH) produced by the fetal Sertoli cells, which causes regression of the Mullerian ducts. This step characterises the phenotypic sex.

A single Y-linked entity, the testis-determining factor (TDF), is required for commitment of the bipotential gonad to develop into a testis. After establishment of the gonads, all subsequent sexual characteristics arise as a result of the presence or absence of hormones produced by the testis or ovary. Pioneering experiments in this field by Alfred Jost in the 1940's showed that removal of the undifferentiated gonads from developing rabbit fetuses resulted in all rabbits developing as females (Jost 1947). Jost showed that endocrine activity in the ovaries is not essential for sexual differentiation during fetal life. In contrast the testes must be present and secrete the two essential hormones indicated above, testosterone and AMH in order for development to proceed along the male pathway of differentiation.

1.2 Development and Differentiation of the Testes

Many complex genetic, cellular and hormonal interactions must occur in order for normal development and function of the testis to be established. Specific gene expression at critical time points instructs the indifferent gonad to form a testis. Cells within will then receive various signals instructing them to differentiate in certain directions, these signals are from both hormonal and environmental influences.

Gonadal development begins with ridges appearing as mesodermal proliferations along the medial surface of the mesonephros. (O'Rahilly and Muller 1987) The outgrowth from the mesonephros is termed the gonadal ridge. Many studies since then have provided substantial evidence in support of mesonephric derived cells contributing substantially to the gonadal cell population (Beur *et al* 1993, Merchant-Larios 1998). Coelomic epithelium also has a contributory role in gonadal development and has been shown to give rise to the Sertoli and granulosa cells. (Karl and Capel 1998)

The gonadal ridges appear at around 10 days post coitum (dpc) in the mouse, and are present as a narrow band of tightly packed cells along the mesonephros. Differentiation occurs at 11.5dpc in male but not female gonads. The band of tightly packed cells is invaded by stromal cells from the mesonephros and thus histological differences between male and female gonads are established. This has been shown experimentally in mice using gonad/mesonephros grafts, and it is suggested that interactions with mesonephric cells and supporting cells in the genital ridge is important for seminiferous cord formation. (Merchant-Larios *et al.*, 1993)

1.3 Genes involved in gonadal differentiation

Many genes play a role in the initiation of sexual differentiation and subsequent development of the gonads. The importance of the role played can be established by examining the effects of absence of expression. Recent research has identified the following genes and expression of them at crucial times during development as essential for male sexual differentiation to occur.

1.3.1 SRY

The gene which triggers the testis determining cascade, *SRY* (Sex determining region, Y), is located on the Y chromosome. It consists of a single exon with a central conserved motif, which was first identified in the High Mobility Group proteins (HMG) (Sinclair *et al* 1990). This motif is termed HMG-box and has DNA-binding and bending activities, suggesting that *SRY* functions as a transcriptional regulator. Subsequent cloning and molecular studies showed that *SRY* is in fact the testes determining factor (TDF). (Goodfellow and Lovell-Badge 1993). The evidence equating *SRY* with TDF came from the following studies:

1. Demonstration that some XY females had point mutations of *SRY,* thus underlining the importance of this gene in the male pathway of differentiation. (reviewed by Goodfellow and Lovell-badge 1993)

2. Production of transgenic XX male mice expressing *SRY*. Experimental evidence showed that *SRY* is the TDF and that presence of the entire Y chromosome is not necessary for male development to occur. Only key parts of it are required (Koopman *et al* 1991).

The *SRY* gene encodes a protein comprising three regions, each one of approximately eighty amino acids. The amino terminal and carboxy terminal segments possess no obvious structural motifs and show no sequence homology to proteins in current databases; moreover, these domains appear to be evolving rapidly (Whitfield *et al* 1993) The central domain, as described above, has homology with the DNA binding HMG proteins and is thus referred to as the HMG binding region. *SRY* belongs to a subset of HMG box proteins that are expressed in a tissue-specific or developmentally regulated manner and that preferentially bind to specific DNA sequences (Laudet *et al* 1993)

Molecular studies of the *SRY* gene have revealed two areas of expression: the adult testes and the embryonic gonadal ridge during differentiation. Although biochemical studies have failed to identify genes which regulate *SRY* directly, a number of genes appear to have a crucial role in the development of the undifferentiated gonad, these include: *SF-1*, *WT1*, and *SOX 9*.

1.3.2 Steroidogenic Factor 1 (SF-1).

This gene was identified initially as a transcription factor that regulates tissue-specific expression of the cytochrome P-450 steroid hydroxylases (Ikeda *et al* 1994). These are enzymes that catalyse most of the reactions required for the synthesis of steroid hormones. *SF-1* also regulates adrenal and gonadal expression of many genes involved

in steroidogenesis, including 3ß-hydroxysteroid dehydrogenase (3βHSD), the ACTH receptor, and the steroidogenic acute regulatory (StAR) protein. Transgenic studies with knockout mice suggest that *SF-1* also regulates Sertoli cell expression of AMH (Shen *et al* 1994). Crucially however, *SF-1* knockout mice lack both gonads and adrenal glands (both arrest in development and regress) (Luo *et al* 1994) and these mice develop phenotypically as females.. Thus *SF-1* is required for differentiation and development of the gonadal ridge

SF-1 gene expression in mice begins from day 9dpc and is absent by day 12.5dpc in females, but persists in males (Ikeda *et al* 1994). With the onset of testicular differentiation, the levels of *SF-1* transcripts increase in both functional compartments of the testes: the interstitial region (i.e. androgen-producing Leydig cells) and the testicular cords (i.e. AMH producing Sertoli cells). Thus, it is possible that *SF-1* controls the production of both hormones required for male phenotypic differentiation

1.3.3 Wilms Tumour (WT-1)

The *WT-1* gene gets its name from its association with the childhood kidney cancer Wilms tumour. It is in fact a tumour suppresser gene and mutations were found to cause the Wilms tumour condition and/or gonadal and genital abnormalities (Call *et al* 1990; Caricasole *et al* 1996.) *In situ* hydbridisation and immunochemistry studies have shown *WT-1* is expressed at highest levels throughout the urogenital ridge in the developing kidney and gonad (Haber *et al* 1991; Armstrong *et al* 1992). It is believed to mediate both the outgrowth of the ureteric bud, and the response of the metanephric mesenchyme to the growth of the ureteric bud during kidney development. In *WT-1*

mutant mice, kidney development is arrested, the coelomic epithelium fails to thicken and the gonad is completely regressed by day 14.5 p.c. (Kreidberg *et al* 1993). These studies show that *WT-1* is essential for the very earliest stages of kidney and gonadal development. As far as the gonad is concerned the phenotype is very similar to that found with *SF-1* knockout mice, hence the two genes act early in gonadal development and perhaps interact directly (Lou *et al* 1994).

1.3.4 SOX9

Studies have shown that *SOX9* is expressed in the genital ridge of both male and female mouse embryos at 10.5dpc. Expression of *SOX9* is first detected in the cells of the sex cords (cord formation is the first visible indicator of testes differentiation). These cells then go on to become Sertoli cells. After 11.5dpc expression of *SOX9* is very abundant in XY embryos but is absent in XX embryos (Morais de Silva *et al* 1996). These differential patterns of expression are likely to be due to *SRY*. Studies have shown that another *SOX* gene, namely *SOX3* suppresses the expression of *SOX9*, however *SOX3* expression is itself suppressed by *SRY* (Graves, 1998). Due to the absence therefore of *SRY* in females *SOX3* is expressed and *SOX9* suppressed, thus leading to development of ovaries and related ducts and tracts. In XY individuals suppression of *SOX9* is over-ridden by expression of the *SRY*. In humans, mutations in the *SOX9* gene have been linked to a severe dwarfism syndrome known as campomelic dysplasia (Foster *et al* 1994) Patients with this syndrome have a number of congenital and skeletal abnormalities and more than 75% of XY patients show sex reversal with a gradation of genital defects.

Fibroblast growth factor 9 (*FGF9*) is also thought to have a role in *SRY* mediated events. It is thought to act upstream of *SOX9* by initiating proliferation of the mesonchymal tissue and contributes to testicular cord formation. Absence of *FGF9* expression in mice results in a female phenotype. (Colvin *et al* 2001)

1.3.5 GATA4

GATA4 is a transcription factor Which is known to play critical roles in various developmental processes, including hematopoietic and T cell differentiation, cardiac and coronary vasculature development, and liver, lung and gut morphogenesis (reviewed by Orkin, 2000, Molkentin, 2000, Ho and Glimcher, 2002) Recent studies have demonstrated that *GATA4* and its co-factor *FOG2* are required for gonadal differentiation. Mouse fetuses homozygous for a null allele of *FOG2* or homozygous for a targeted mutation in *GATA4* exhibit abnormalities in gonadogenesis. It was shown that *SRY* transcript levels were significantly reduced in XY *FOG2*(-/-) gonads at E11.5, which is the time when *SRY* expression normally reaches its peak in mice. In addition, three genes crucial for normal Sertoli cell function (*SOX9*, AMH and *Dhh*) and three Leydig cell steroid biosynthetic enzymes (p450scc, 3βHSD and p450c17) were not expressed in XY *FOG2* -/- and *GATA4* -/- gonads. (Tevosian *et al* 2002) Results from this study clearly suggest that GATA4 does play a crucial role in modulation of various genes which play a key role in the differentiation pathway.

1.3.6 Sequence of gene expression

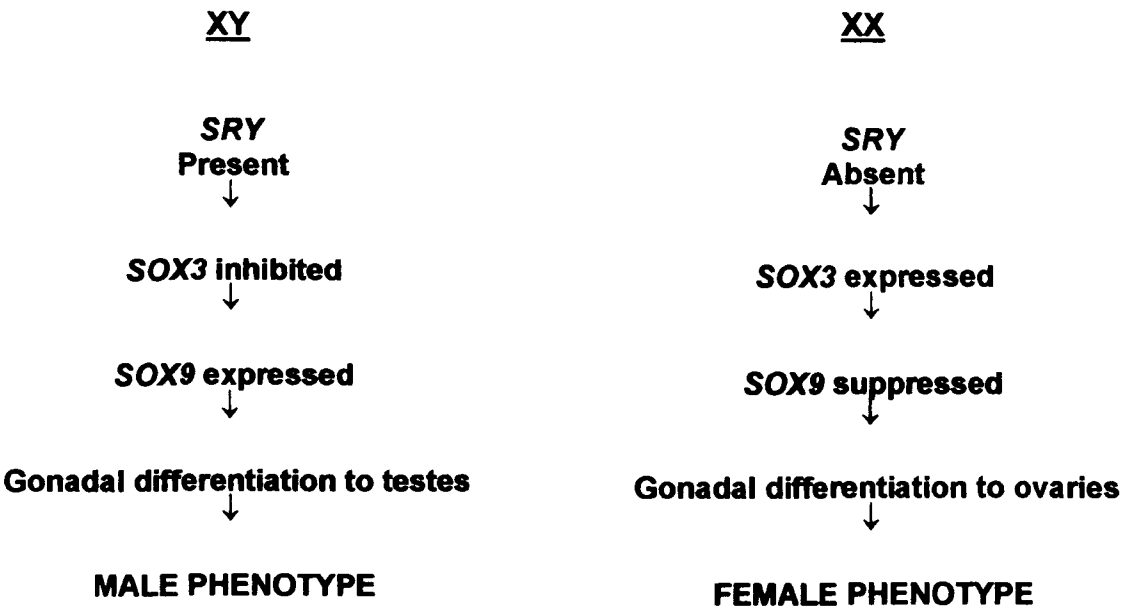

Figure 1-2 Flow diagram showing the relationship of the different key genes involved in the initiation of the sexual differentiation pathway for both males and females.

1.3.7 Desert Hedgehog

The Desert hedgehog (*Dhh*) gene encodes a signalling molecule produced by Sertoli cells, (Clarke *et al* 2000). It is expressed in the testes, but not the ovary. It has been shown to have a role in testicular development. Differentiation of peritubular myoid cells and the consequent formation of testis cords are regulated by *Dhh* (Hung-Chang yao *et al* 2002). In the mouse it is first expressed in the pre Sertoli cells at around 11.5dpc, preceded by *SRY* expression which is detectable from 10.5dpc. The *SRY* transcription factor may even be a modulator of *Dhh* expression as it is the only other known male specific gene besides AMH to be expressed at such an early stage in development. The protein encoded by *Dhh* binds to a cell surface receptor called patched (Ptc). Expression of the Ptc gene is restricted to the Leydig cells within the testes, suggesting that *Dhh*

may have a role in their regulation. Generation of a *Dhh* knockout mouse demonstrated that homozygous null females had normal reproduction, but the males were sterile. (Bitgood *et al* 1996). Besides being sterile most of the *Dhh* null male mice were also phenotypically feminised, with evidence of mammary teats and a blind vaginal opening. Internally however these animals do posses testes although they are much smaller than normal and undescended. (Clark, *et al* 2000). These findings suggest that *Dhh* is required for fetal Leydig cell differentiation and that in its absence fewer Leydig cells are present in the fetal gonad, leading to reduced androgen production and pseudohermaphroditism. (Clark *et al* 2000)

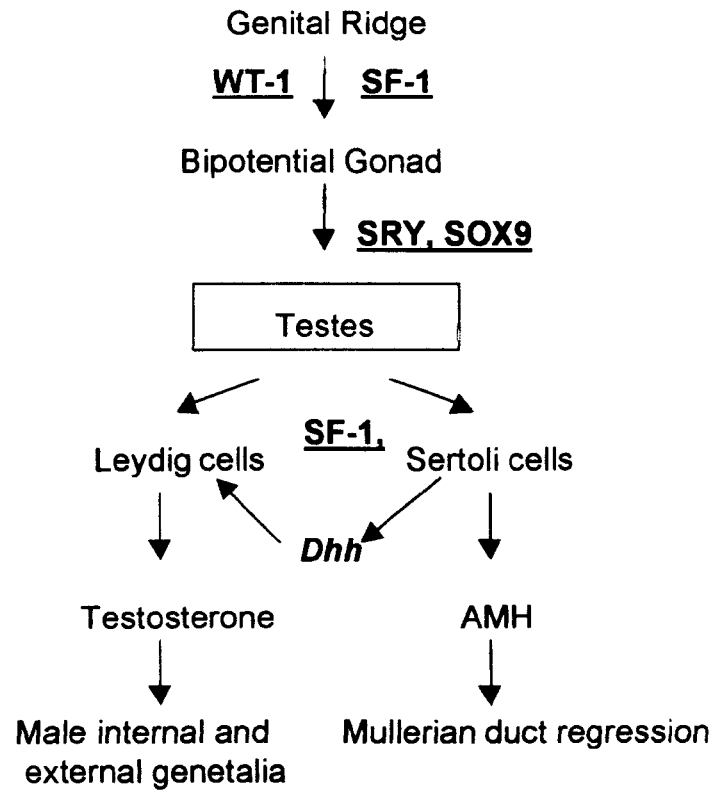

Figure 1-3 Summary diagram of key genes in gonadal development and the areas of expression

1.4 The Cells of the testis

There are several different cell types, which are present within the testes and each type plays a contributory role in maintenance of function. This can be in a structural and supporting capacity like the peritubular myoid cells which surround the seminiferous tubules. Or they may be involved in hormone production and regulate spermatogenesis like the Leydig and Sertoli cells. Alternatively, the cells themselves can give rise to mature sperm through mitotic and meiotic division of the germ cells.

1.5 Germ Cells

During gonadogenesis, precursors of the supporting and steroidogenic cell lineage are thought to be present within the gonad itself, in contrast to primordial germ cells, which originate near the stalk of the allantois and migrate by ameboid movement through the dorsal mesentery to become incorporated into the primitive gonad, proliferating as they go. Different factors control the proliferation of germ cells and their migration towards the genital ridge. Steel factor (SF) is the most important of these factors. It is a growth factor and it influences the growth and differentiation of primordial germ cells (reviewed by Merchant-Larios and Moreno-Mendoza, 2001).

The primordial germ cells arise as a small population during the earliest stages of embryogenesis and originate from different areas depending on species. In mice they are set aside from other cell lineages at around 6.5 – 7.5dpc. Once formed the primordial germ cells enter the embryonic endoderm that will give rise to the hind gut (Tam and Snow, 1981). From 9.5-11.5dpc they migrate from the hind gut to the genital ridges and

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1.6 Sertoli Cells

Testicular differentiation is marked by the appearance of fetal Sertoli cells and their organisation around the germ cells. By 12.5dpc in mice they have formed testicular cords and are surrounded by a flattened layer of peritubular myoid cells (Skinner *et al* 1985). It is accompanied by migration of cells from the mesonephros to the gonadal primordium. The Sertoli cells are believed to act as the organising centre of the male gonad as they are the first cell type to differentiate (McLaren 2000). *In situ*

hybridisation studies show that the *SRY* gene is expressed in mouse gonads at 11.5dpc (Koopman *et al* 1990). Experiments by Palmer and Burgoyne (Palmer and Burgoyne 1991) using mosaic mice generated between XX and XY cells indicate that the pre - Sertoli cell is the only one in the testis in which *SRY* exerts an effect. Therefore it is probable that *SRY* initiates testicular development through induction of the pre-Sertoli cells by instructing them to differentiate from migrating coelomic epithelium cells. *SRY* is also thought to have a proliferating effect on the Sertoli cell precursors as mitosis is enhanced in response to *SRY* (Capel 2000).

Evidence has shown the coelomic epithelium is indeed the source of pre-Sertoli cell origin, this was established through studies using a fluorescent dye which traces the route and fate of the migrating coelomic epithelium. Results showed that these injected cells differentiate to form Sertoli cells (Karl and Capel, 1998). The mesonephric cells start migrating into the gonad at 11.5dpc and these contribute to the other testis cell populations like the peritubular myoid cells which contribute to the formation of the seminiferous cords and are necessary for the maintenance of their structure.

A major role of the Sertoli cell is to define two separate and physiologically distinct compartments in the seminiferous tubule. The basal compartment, near the periphery, is sealed off from the luminal compartment by processes of adjacent Sertoli cells that "reach out" and make contact. At the point of contact these fuse to form occluding junctions. With cord and tubule formation the Sertoli cells provide an appropriate microenvironment for the development of germ cells. They form tight junctional complexes providing a protective barrier. The compartmentalisation of the seminiferous epithelium allows the Sertoli cells to establish micro environments which allow the

spermatogenic cells to proceed through meiosis and other differentiative events. The Sertoli cells also provide nutritive, regulatory and trophic factors that enhance and stimulate the survival and development of sperm.

1.7 Leydig Cells

The Leydig cells first appear in the interstitial region of the testis after cord formation, and they are derived from mesenchyme-like stem cells. Their exact embryonic origin is as yet unclear, however one hypothesis is that they are mesodermal, appearing first in the mesonephros and then migrating into the presumptive interstitial tissue. This was proposed from observations of an experiment using gonad / mesonephros grafts and co - cultures (Merchant-Larios and Moreno-Mendoza 1998), and is generally the more accepted hypothesis for origin of the Leydig cells. Another hypothesis however, is that Leydig stem cells derive from neural crest. This is based on evidence that Leydig cells express several neural specific proteins such as neural cell adhesion molecule, neurofilament protein 200 and microtubular-associated protein (Mayerhof *et al* 1992, 1996, Davidoff *et al* 1993, Middendorff *et al* 1993).

As the differentiation of the first Leydig cells occurs after that of the Sertoli cells in all species studied, it has been proposed that the trigger for differentiation may be under the paracrine action of Sertoli cells. This idea has been supported by the findings of several studies using experimental models. (Jost *et al* 1973, Byskov 1986). In further support of this is a report by Vainio *et al* (1999) who suggests that the Sertoli cells may act to suppress Wnt4 expression. Wnt4 is known to repress the early differentiation of the fetal

Leydig cells. In addition and as discussed earlier, *Dhh* secreted from the Sertoli cells is thought to initiate Leydig cell differentiation (Yao *et al* 2002).

The Leydig cells exist between the seminiferous tubules of the testes, they are present in small clusters of three to four cells and are generally located near blood vessels. This is indicative of their endocrine function, and can be observed histologically by the frothy appearance of the cytoplasm, which is a characteristic of active steroid synthesis.

In mammals ontogenesis of Leydig cell function involves at least two generations of cells. In mice the "fetal" population arises shortly after differentiation of the testis and occurs at around 12.5dpc. and 15dpc in rats. This first generation is essential for masculinisation of the fetus as they produce the androgens required to stimulate Wolffian duct development and promote male sexual differentiation. There remains some controversy as to whether this population of cells disappears, dedifferentiates or remains functionally intact during neonatal life. Current thought suggests that it is likely they persist in the adult, but are a minor component compared to the adult population of cells. (Lejeune *et al* 1998) Either way, they do appear to become functionally quiescent as androgen production decreases dramatically in the neonate testis.

A second population of Leydig cells appears from about day 5 in mice and they are functional from puberty (Habert *et al* 2001). These cells produce the androgens required for establishment of male secondary sexual characteristics. In humans this includes, deepening voice, appearance of facial and body hair, greater musculature and in other mammalian species it may be growth of horn or antlers or even a change in body colour.

The androgens are also essential for maintenance of spermatogenesis and male reproductive function.

1.7.1 Fetal Leydig cells

There is a widely accepted belief that the fetal Leydig cells originate from mesonephric cells, which invade the gonadal ridge early on in development. Studies supporting this hypothesis include grafting of gonads from E11.5 mice onto mesonephros, the donor mice were transgenic and carried markers such as β galactosidase, these markers were later traced to the peritubular myoid and interstitial cells of the testis (Buehr *et al* 1993, Merchant-Larios *et al* 1993, Nishoma *et al* 2000). Some of these migratory cells acquired ultra structural features of steroidogenic Leydig cells (Merchant-Larios and Moreno-Mendoza 1998) While it is clear that the adult Leydig cells require LH, there is now considerable evidence that differentiation and function of the fetal leydig cells does not require gonadotrophins. *In vivo* they develop in a gonadotrophin-free environment from 12.5dpc in mice and 14.4dpc in rats. LH is not detected in the pituitary until 17.5dpc in the rat by immuno histochemistry (Tougard *et al* 1977) at 16.5dpc by RT-PCR (El Gehani *et al* 1998) and 17dpc by radio immunoassay (Aubert *et al* 1985). In the plasma, LH becomes detectable on day 17.5pc (El Gehani *et al* 1998); moreover, its concentration remains very low until fetal day 19.5 and high LH levels are only observed from fetal day 20.5 onwards. (Habert and Picon, 1982). Furthermore, decapitated rat fetuses have exactly the same increase in testicular testosterone in vivo between 16.5dpc and 19.5dpc as controls (Habert and Picon, 1982).

Gonadotrophins have, nevertheless, been shown to have a positive effect on neonatal Leydig cell differentiation since a single injection of CG to neonatal rats causes a rapid increase in the number of Leydig cells, and an increase in the expression of LH receptors and steroidogenic enzymes. (Huhtaniemi *et al.*, 1982). Another study showed that testes from rat fetuses aged 20.5 or 21.5 dpc, decapitated the previous day, secreted *ex vivo* amounts of testosterone, eight times lower than did the control litter mates (Habert and Picon, 1982). This suggests that LH is required for the maintenance of physiological testicular steroidogenesis *in vivo* from fetal day 20.5 onwards. In earlier studies the requirement of LH on the differentiation of Leydig cells in late fetal life was determined by measuring the volume of Leydig cells in decapitated fetuses. An effect was found as early as 17.5 dpc, (Eguchi and Morikawa, 1968), but these results appear doubtful, as no LH is detectable in the plasma before 17.5dpc. More recently, Migrenne *et al* 2001 showed that the number of Leydig cells per testis in 21.5 dpc fetuses decapitated before onset of LH synthesis (16.5 dpc) or secretion (17.5dpc) was the same as in control litter mates. Thus the generation of new fetal Leydig cells does not depend on LH during late fetal life.

Further evidence for independent differentiation of Leydig cells exists in the hypogonadal (hpg) mouse. This is a natural mutant strain which carries a deletion mutation in the gene coding for gonadotrophin releasing hormone (GnRH), (Mason *et al* 1986). The gonads thus lack exposure to endogenous gonadotrophins during development. Studies using the *hpg* mouse have shown that fetal Leydig cell development and function in the mouse is normal in the absence of endogenous circulating gonadotrophins, and that the Leydig cells become dependent on gonadotrophins shortly after birth (O'Shaughnessy *et al.*, 1998)

A recent study has also used the hpg mouse to determine whether fetal Leydig cell differentiation and proliferation are also gonadotrophin independent. Results showed that establishment of cell number is a gonadotrophin independent event, as there was no difference between the normal and hpg embryonic testes. In contrast, the adult population of cells, which arise at day 5, are obviously greatly dependant on gonadotrophins for proliferation, as there was a marked decrease in cell number at this time in the hpg mouse when compared to the wild type (Baker and O'Shaughnessy, 2001). This study also showed that at birth Leydig cell volume in hpg mice was normal, a further indication therefore that function in these cells is normal. After birth there was a marked decrease in the amount of testosterone produced by the testes of hpg mice compared to wild type, suggesting that this is the time when gonadotrophin dependance occurs.

The fetal leydig cells are highly active, and androgen production per cell is much greater than that of the adult leydig cells (Huhtaniemi, *et al* 1984) (Tapanainen, *et al* 1984). Until fairly recently it was always assumed that the entire pathway for testosterone synthesis occurred in the fetal Leydig cells, it has since been shown however that this is not the case. The final step in the biosynthesis of testosterone is the reduction of androstendione by the enzyme 17β-hydroxysteroid dehydrogenase (17βHSD). This enzyme exists in four isoforms and it is the type three isoform which is involved in testosterone synthesis. One study in mice showed that expression of this isoform is restricted to the seminiferous tubules during development and is absent in the fetal population of Leydig cells. In the adult testis, however, expression is localised to the Leydig cells. These findings suggest that the major androgen secreted by the fetal Leydig cells is androstendione and production of testosterone up until puberty is

dependant on 17βHSD activity in the seminiferous tubules (O'Shaughnessy *et al* 2000). It has been suggested from the evidence that the fetal Leydig cells are older in evolutionary terms, than the adult cells and may have evolved to take on the endocrine, steroid producing function of the Sertoli cells (O'Shaughnessy *et al* 2000).

After initial differentiation of the fetal Leydig cells, testicular steroidogenesis increases markedly as does the number of Leydig cells. This increase is due mainly to differentiation of stem cells as differentiated fetal Leydig cells do not undergo mitosis. It was suggested that the initial induction of fetal Leydig cell differentiation was due to some Sertoli cell secreted factor (Byskov, 1986). As indicated earlier, recent evidence suggests that this is indeed the case, and it is proposed that *Dhh*, a Sertoli cell secreted signalling molecule, is responsible for induction of fetal Leydig cell differentiation as it was demonstrated that interterstitial cells ie Leydig cell precursors of 12.5dpc mouse gonads express the *Dhh* receptor patched 1 (Ptch 1) (Yao *et al* 2002). In addition, *Dhh* null mice were shown to have no p450 side chain cleavage (p450SCC) positive interstitial cells. This is a key regulatory enzyme in the steroidogenic pathway described here, and its presence is a good marker of functioning Leydig cells. Its absence in the *Dhh* null mice and absence of mature functioning Leydig cells further suggest that *Dhh* is indeed an important signalling molecule for this process (Yao *et al* 2002)

1.7.2 Adult Leydig Cells

There is strong evidence that the adult Leydig cell population also arise from undifferentiated mesenchymal cells (Lording and Krester 1972, Russell *et al* 1995, Haider *et al* 1995). These cells originate from the mesonephros and they invade they

gonadal ridge early on in development. In the rat, adult Leydig cell differentiation takes place around 10dpc (Mendis-Handagama *et al* 1987). There are several distinct stages of adult Leydig cell differentiation, the first being proliferation of the precursor cells, next the cells become committed and differentiate into Leydig cell progenitors. At this stage they simultaneously acquire 3 beta hydroxy steroid dehdrogenase (3βHSD), cytochrome P450scc and P450c17. It has been demonstrated that they acquire these steroidogenic enzymes prior to gaining LH receptors (Hardy *et al* 1989). After this they finally become mature Leydig cells, which are distinguishable histologically by their high lipid content. (Reviewed by Mendis-Handagama and Ariyaratne 2001).

In the mouse the timing of adult Leydig cell differentiation can be pinpointed to start between post natal days 7 and 10. Evidence comes from expression of the adult Leydig cell markers 3β HSD IV and relaxin like factor (Baker *et al* 1999).

In addition to regulating fetal Leydig cell differentiation , *Dhh* is thought to play a vital role in signalling initiation of adult Leydig cell differentiation. Evidence from this comes from the fact that adult Leydig cells express the *Dhh* receptor known as Ptc, and also studies using the *Dhh* knockout mouse have shown that phenotypically the males have very small and undescended testes. Histological examination shows there to be no adult Leydig cells present, only undifferentiated stem cells. (Clark, 2000). It is suggested that *Dhh* null mice lack Leydig cells precursors and that Dh is therefore required for differentiation and migration of these cells (Clark 2000).

Once the adult population of Leydig cells has differentiated and become established they do not undergo mitosis, numbers remain stable from proliferation of the stem cells.

A study by (Moore *et al* 1992) showed this to be the case. Using ^{125}I-labelled iododeoxy-uridine incorporation, they were able to identify the DNA-synthesising cells in adult rat testes. Their results showed that DNA synthesis is not associated with the adult Leydig cells. (Moore *et al.*, 1992). The presence of intermediate stages of Leydig cell differentiation in adult rat backs the proposal that stability of the population of adult Leydig cells is maintained through proliferation and differentiation of the stem cells. (Hardy *et al.*, 1989).

Although it is well established that the major regulator of adult leydig cell function is LH, there is also some evidence for a regulatory role for dihydrotestosterone (DHT). This was shown in a study whereby rat Leydig cells were isolated from testes just prior to differentiation (between days 14-28). The cells were cultured with either LH or DHT alone or coupled with other steroids or androgen receptor antagonist (to block action of DHT). The results showed that Leydig cells when cultured with LH or DHT alone did produce some testosterone. However by far the greatest effect on cell function was by culturing them with a combination of LH and DHT, this increased testosterone production dramatically, and adding an androgen receptor antagonist prevented testosterone production completely. (Hardy *et al* 1990). Some doubt surrounds these results, however, since others have reported an inability to repeat the experiments (Teerds *et al* 1989).

Another gene which may be involved in the regulation of development and function of the adult Leydig cells is platelet derived growth factor-A (PDGF-A). Mice which carry a mutation for this gene were found to show a reduction in number of morphologically recognizable Leydig cells. The differences in testis morphology between wild type and

mutant were detectable from post natal day 10 and became progressively more apparent until at post natal day 42 there were no mature Leydig cells present within the mutant testis. The conclusion drawn from this study was that PDGF-A is required for proliferation of the precursor adult Leydig cell population and in its absence this fails to occur (Gnessi *et al* 2000)

1.7.3 Differences between Fetal and Adult Leydig cells

There are several differences between the fetal and adult population of Leydig cells. One is the dependence on LH for differentiation and function. The adult Leydig cells are entirely dependent on LH for both differentiation and also maintenance of differentiated function (Payne and O'Shaughnessy 1996). Another distinction between the two is the expression of 17βHSD type 3. As previously described, this enzyme is only expressed in the adult population and not the fetal cells. 3βHSD type VI is another isoform of the hydroxy steroid enzymes which is expressed only in the adult Leydig cells (Baker *et al* 1999) These enzymes can therefore act as good markers for distinguishing between adult and fetal Leydig cells.

In addition, it appears that the regulation of LH receptor gene expression in the testes is dependent on the developmental stage of the Leydig cells. LH stimulation up-regulates LHR expression in the fetal rat testis, whereas similar stimulation down-regulates receptor expression in the adult cells. (Pakarinen *et al* 1990)

1.7.4 Functional regulation of the Leydig cells

Testosterone biosynthesis in Leydig cells is dependent on two cytochrome P450 enzymes, cholesterol side-chain cleavage (P450scc) and 17α-hydroxylase/C17-20 lyase (P450c17). The expression of these two enzymes is differentially regulated by LH acting via its second messenger, cyclic adenosine 3',5'-monophosphate (cAMP), and by specific steroid hormones.

IGF may be a candidate for the initiating factor of steroidogenesis in the fetal cell population. There is evidence to suggest that IGF-1 has a regulatory role in the expression of luteinising hormone receptor (LHR). Addition of IGF-1 to cultured Leydig cells results in a dose-dependant increase in LHR gene expression and actual receptor number was increased by 300% of unstimulated control cells (Zhang *et al* 1998). Further studies have shown that when added to primary cultures, IGF-I can enhance Leydig cell steroidogenesis (Lin *et al* 1986, Lin 1995)

Several other factors have also been implicated in the involvement of modulation of testosterone synthesis by Leydig cells. Testicular macrophages are known to secrete both inhibitory and stimulatory factors. The inhibitory factors include interleukin 1 (IL 1) and TNF α. These are produced during an inflammatory reaction and their inhibitory action is directed at the steroidogenic enzymes. Once an inflammatory response is initiated Leydig cell function is depressed (Hedger, 1997). In contrast, resting resident macrophages have been shown to have a stimulatory effect on Leydig cell steroidogenesis, possibly through their trophic actions (Hedger, 1997).

Estrogen is also thought to play a regulatory role in Leydig cell steroidogenesis,. This was first proposed with the discovery that the testis concentrates estradiol (Stumpf 1969). Further studies revealed that the intersitium of the rat testis contains a specific, high affinity estrogen receptor (Kato *et al* 1974, Abney 1976) and, furthermore that Leydig cell steroidogenesis is directly inhibited by estradiol treatment (Sairam and Berman 1979, Kalla *et al* 1980). These findings suggest that the regulatory role played by estrogen in Leydig cell function is in a negative fashion. In addition, it has been demonstrated that exposure of male fetal rats to estrogen increases the incidence of cryptorchidism, (Rajfer and Walsh 1977, Yasuda *et al* 1985) a defect which is known to occur due to lack of androgens.

Most studies agree that LH is certainly a key regulator of adult Leydig cell differentiation and function whereas fetal Leydig cells can develop and function independent of its presence. Evidence for this exists in hpg mice and LHR null mice. Both strains lack cellular exposure to LH. In hpg mice this is because there is no LH, due to a mutation in the gonadotrophin releasing hormone gene and thus absence of circulating gonadotrophins. As previously mentioned, the fetal Leydig cell population differentiates and functions normally until around day 5, and only after this stage is LH required (Baker *et al* 2001)

Similarly, the LHR null mouse shows normal masculinisation at birth suggesting that it also produces normal levels of testosterone during fetal development. In addition the first transabdominal descent of the testes occurs in LHR-null mice, and this event is known to be dependant on androgen action, suggesting therefore that sufficient testosterone has been produced in the complete absence of LH action on Leydig cells. By 7 weeks there is

By 7 weeks there is a marked reduction in testosterone production in the LHR-null mice compared to normal mice suggesting that LH is required for normal functioning of the adult Leydig cells population (Zhang *et al* 2001)

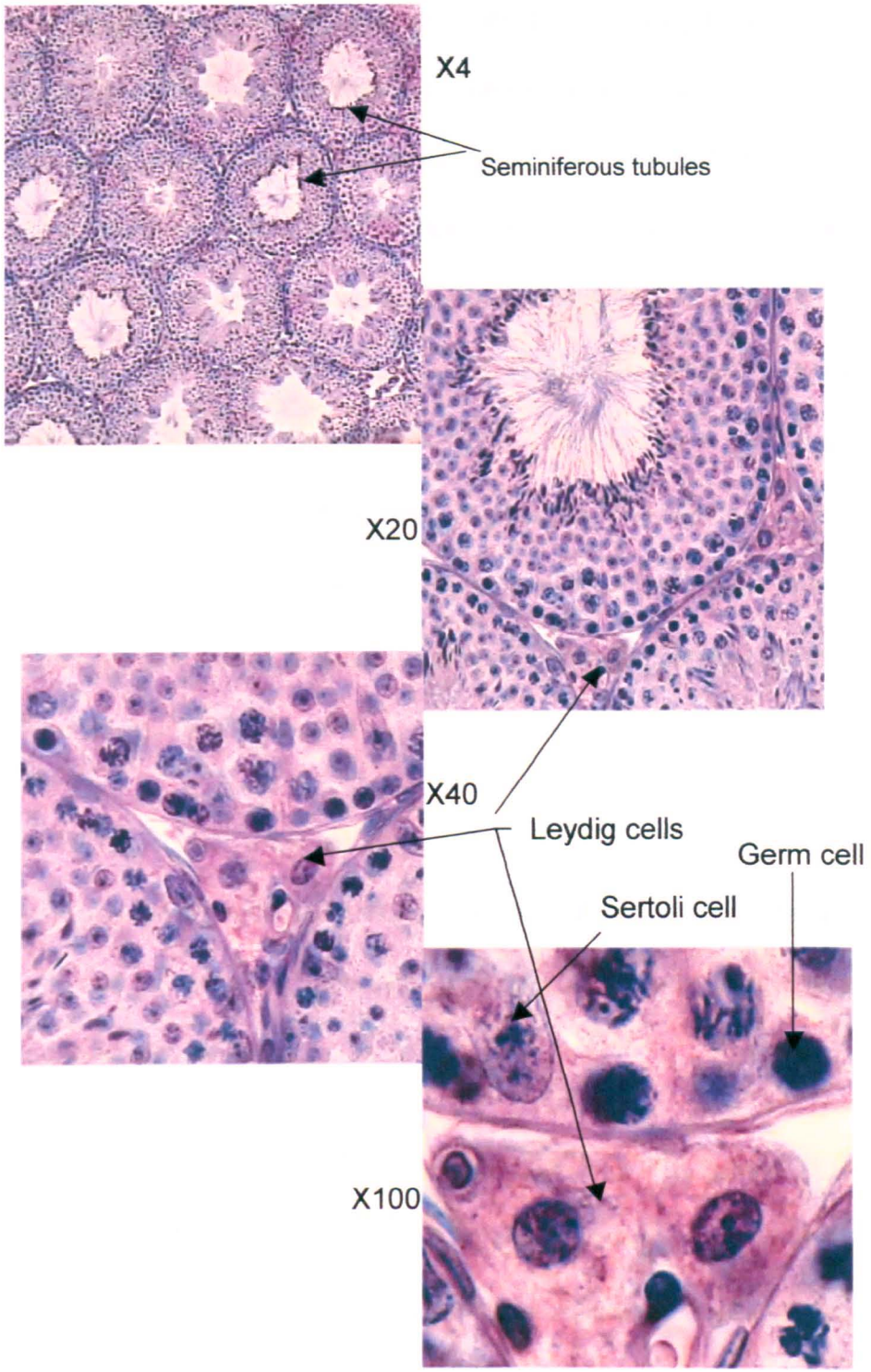


Figure 1-4 Histological sections of adult mouse testis showing different cell types and their location in relation to each other.

1.8 Gonadotrophins

The gonadotrophins are so called because their main target site is the gonads. The two main ones are follicle-stimulating hormone (FSH) and luteinising hormone (LH) Regulation of their production and release is under the control of the hypothalamus. A third gonadotrophin is produced by the placenta of some species during fetal life and is known as chorionic gonadotrophin. FSH and LH are synthesized in the same cells of the pituitary, the gonadotrophs. Both are heterodimeric glycoprotein hormones, which share a common α subunit, but have their own specific β-subunit. The hormone-specific β-subunit of each of these hormones is noncovalently linked to the α-subunit and confers receptor specificity and, hence, the biological specificity. The regulation of both α-subunit and FSH and LH β-subunit gene expression is under stringent control of the hypothalamic peptide GnRH, gonadal steroids, and gonadal peptides, including inhibins and activins (Haisenleder *et al* 1994)

In males, FSH is required for spermatogenesis and LH stimulates androgen production by the testicular Leydig cells. The cellular target site of action of LH is the Leydig cells of the testis and the theca, granulosa and luteal cells of the ovary. Target sites for FSH are the Sertoli cells and the granulosa cells.

The physiological functions of the gonadotrophins are well documented. FSH stimulates follicular maturation and granullosa cell production of estrogen in the ovary and Sertoli cell function in the testes. LH, on the other hand, stimulates theca cell production of androgen in the ovary and Leydig cell production of androgen in the testes. LH has two principle activities in the control of Leydig cell steroidogenesis: Firstly, acute stimulation

of testosterone biosynthesis, which is achieved via mobilisation and delivery of the cholesterol precursor to the inner mitochondrial membrane and secondly chronic stimulation which results in increased gene expression of the steroidogenic enzymes. The production of LH is regulated by feedback inhibition of circulating testosterone on the pituitary and hypothalamus. FSH secretion is regulated by inhibin, a peptide hormone produced by Sertoli cells, and also by circulating testosterone. This endocrine loop is known as the hypothalamic-pituitary-testicular axis.

Plasma levels of gonadotrophins fluctuate depending on the stage of development. In mice there is an increase just before and shortly after birth. Levels then decrease at around day 4 and remain low until puberty, which occurs at around day 25 – 30. Puberty is the period of development during which physical growth and sexual maturation occur. The factors responsible for triggering and timing the onset of puberty remain elusive, though it is likely that several factors are involved. Environmental factors such as, nutrition, photo-period, and population density are known to have a role. Some animals can even delay puberty if they are likely to have very poor reproductive success at that particular time. This has been clearly demonstrated in mice. (Drickamer 1990)

The high levels of gonadotrophins at puberty arise as a result of increased stimulation of the gonadotrophs of the anterior pituitary by gonadotrophic releasing hormone (GnRH). GnRH is assumed to be the most important final common mediator of all influences on reproduction conveyed through the central nervous system. Any abnormality in GnRH synthesis, storage, release or action will result in partial or complete failure of gonadal function.

After puberty, regulation of the endocrine and spermatogenic functions of the testes is maintained via hormonal interactions involving GnRH, the gonadotrophins and testosterone. They are interconnected in a feed back loop system, whereby one controls the production and release of another. The major difference between the sexes in the control of gonadal activity is that gamete and steroid production in the male occurs continuously after puberty whereas in the female these functions occur cyclically. Testosterone and LH regulate the production and release of each other, LH through steroidogenic promoting action on the Leydig cells, and testosterone by acting at target sites in the anterior pituitary and regulating production of LH. High levels of testosterone will result in a decrease in production of LH and vice versa.

1.8.1 *Hypothalamic / pituitary / gonadal axis*

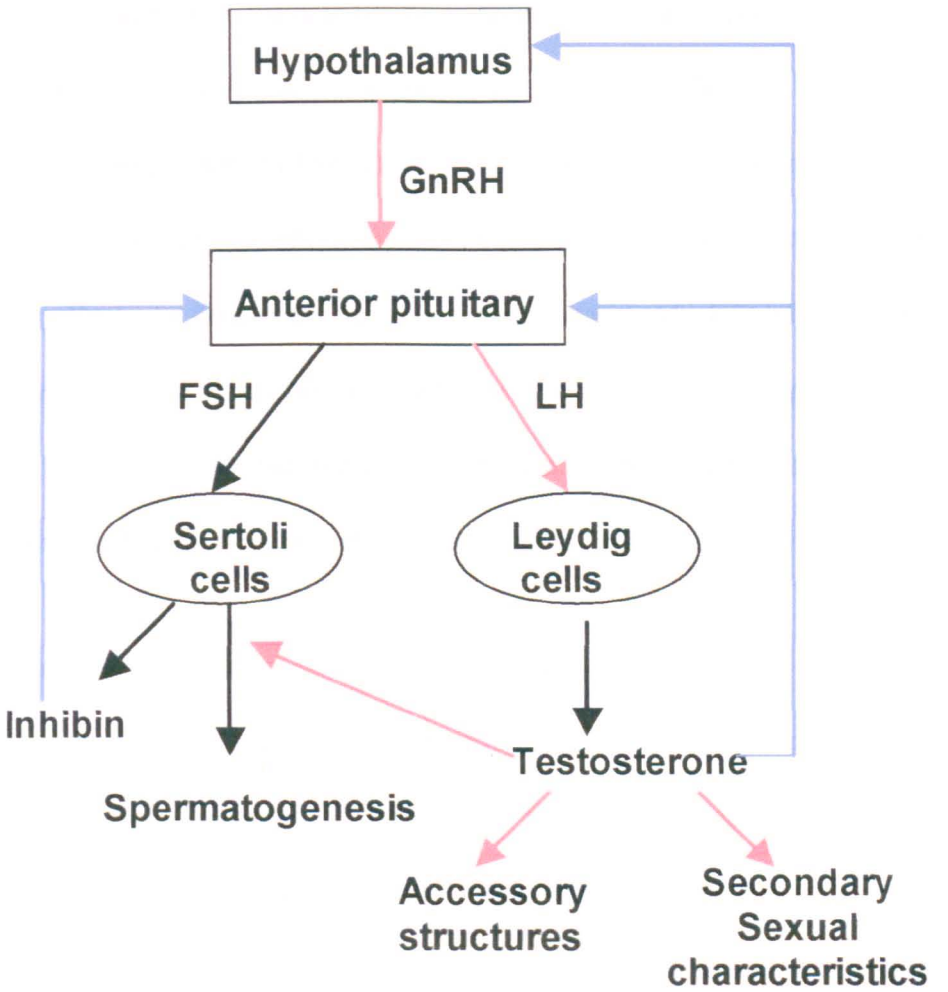


Figure 1-5 Feedback interactions between hypothalamus, anterior pituitary and testis

→ Signifies positive hormonal effect

→ Signifies negative hormonal effect

1.9 LH Receptor

The LH receptor (LHR) plays a pivotal role in the hormonal regulation of reproduction. It is a cell membrane glycoprotein hormone receptor. It is a member of a family of proteins called G-coupled protein receptors, and along with the FSH receptor and thyroid stimulating receptor (TSHR), they form a subgroup of this family. Structurally they are transmembrane receptors and have a large extra cellular hormone-binding domain attached to a seven-pass transmembrane domain, which in turn is linked to an intracellular cytoplasmic domain. The gene encoding the LH receptor consists of eleven exons, the first ten encode the extracellular domain of the receptor and the eleventh encodes the transmembrane and intracellular domain. (Dufau *et al* 1995)

The LH receptor is mainly expressed in the Leydig cells of the testes and in theca, granulosa, and luteal cells of the ovary. In both males and females the binding of LHR to its ligand LH leads to a cascade of intracellular events which result in the synthesis of steroids critical to normal development and reproductive function. The LH receptor acts by initiating a second messenger response. The primary, if not sole, second messenger mediating the actions of LH in target cells has been shown to be cyclic adenosine monophosphate (cAMP) (Hunzicker-Dunn and Birnbaumer 1985). cAMP is produced by a membrane bound enzyme called adenylate cyclase. A Gs protein mediates the LH-induced increase in adenylate cyclase activity.

1.10 G Protein coupled receptors

G protein-coupled receptors (GCRs) are a component of a modular system for the transduction of specific extracellular signals across the lipid bilayer of the cell and the conversion of those signals to an intracellular second messenger pathway. The evolutionary role of GCRs as part of a generalised cellular linkage system has been described (Kirschner 1992). The LH receptor is associated with a Gs protein which consists of three subunits; alpha (α) beta (β) and gamma (γ). In the resting state the α-subunit is bound to a GDP molecule guanine diphosphate. The protein is activated by ligand (LH) binding to the receptor. Upon activation the GDP is replaced on the α-subunit by a GTP (guanine tri phosphate), the GTP and α-subunit complex then dissociates. The G protein is said to be inactive when bound to GDP and active when bound to GTP. Upon binding to GTP this causes activation of the enzyme adenylyl cyclase. This is a membrane bound enzyme which converts adenosine tri phosphate (ATP) to the cyclic form of adenosine mono phosphate (cAMP), cAMP in turn activates protein kinase A. Meanwhile any free cAMP is quickly broken down to AMP by the enzyme phosphodiesterase.

Several types of G protein have been identified and they mediate a signalling cascade in different ways:

Gs - Activates adenylyl cyclase activity and leads to an increase in cAMP

Gi/o - Inhibits adenylyl cyclase and leads to a decrease in cAMP.

Gq/11 – Activates phospholipase C (this results in an indirect activation of adenylyl cyclase via intracellular calcium release.)

1.11 G-Protein activation

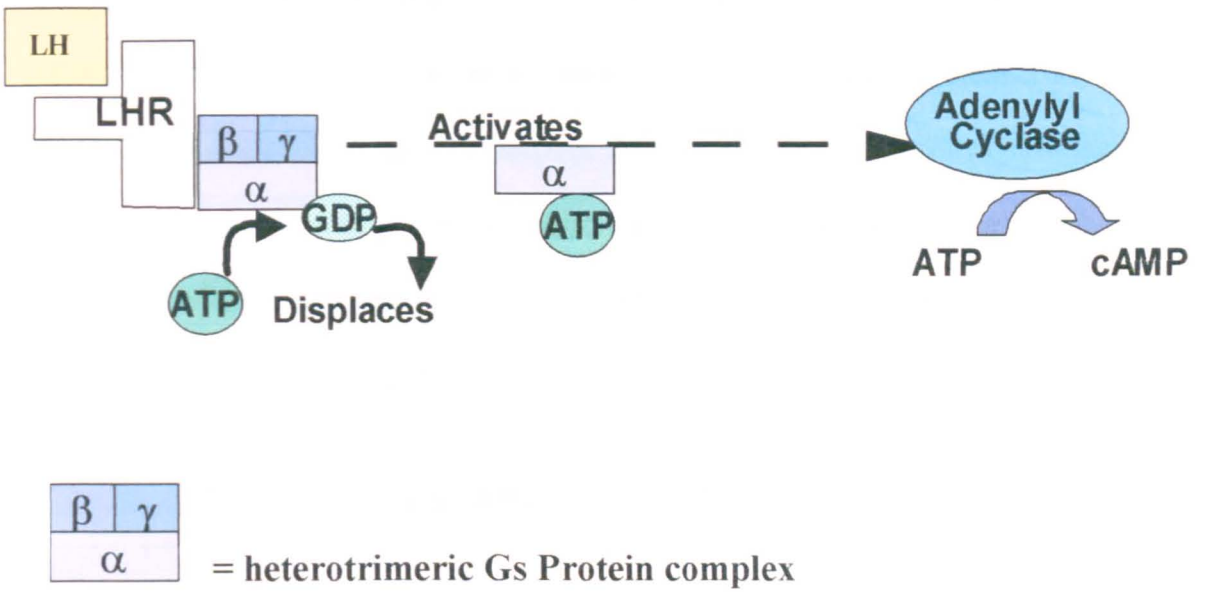


Figure 1-6 LH receptor activation of Gs protein

1. LH binds to receptor
2. This creates a binding site for Gs protein
3. This in turn changes shape of Gs protein so it releases GDP and binds GTP
4. Gs / GTP complex moves across membrane and activates adenylyl cyclase
5. Upon activation of adenylyl cyclase the Gs protein immediately releases its GTP and once again binds with GDP, thus resuming its inactive state

1.11.1 Intracellular signalling cascade initiated by cAMP

Adenylyl cyclase catalyses the formation of cAMP from adenosine triphosphate (ATP), cAMP then mediates its action by phosphorylation of the enzyme protein kinase A (PKA) that is situated in the cytoplasmic compartment of the cell. This in turn activates up regulation of expression levels of steroid acute regulatory (StAR) protein. This protein plays a key role in the initial steps of the steroidogenic pathway by delivering cholesterol to the inner mitochondrial membrane. Formation of testosterone or dihydrotestosterone is the final step in the intercellular signalling cascade initiated by the second messenger/ LHReceptor signalling pathway.

1.11.2 LH receptor signalling cascade

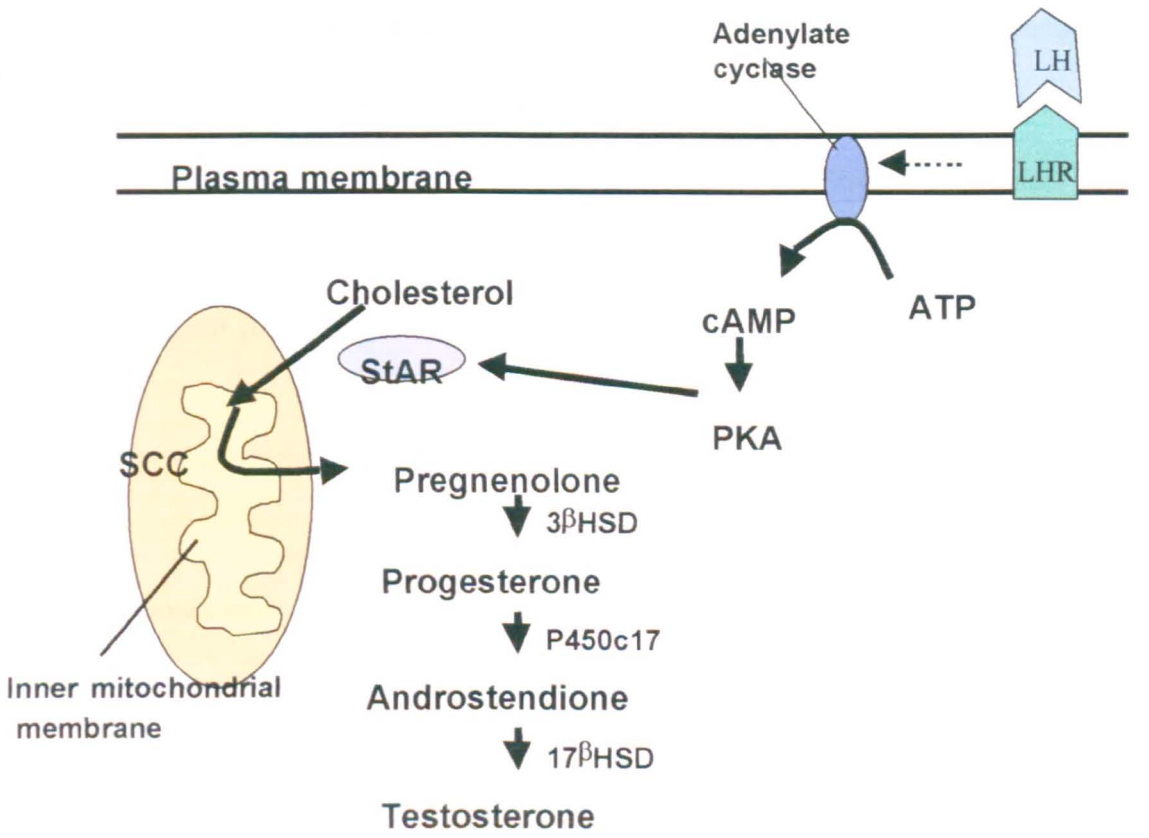


Figure 1-7 Intracellular signalling cascade initiated by LHR binding, resulting in testosterone synthesis.

1.11.3 StAR Protein

Steroidogenic acute regulatory protein (StAR) is essential for efficient gonadal steroidogenesis (Lin *et al* 1995). The role of this protein is to facilitate delivery of cholesterol to cytochrome P450scc, which is situated on the inner mitochondrial membrane. Evidence that StAR is essential for steroidogenesis is apparent from the demonstration that mutations in the StAR gene cause severe impairment of gonadal steroidogenesis at the cholesterol side chain cleavage step (Bose *et al* 1996, Nakae *et al* 1997). Targeted disruption of the mouse StAR gene results in a phenotype similar to that of congenital lipoid adrenal hyperplasia in humans (Caron *et al* 1997). cAMP influences StAR gene expression and enhances StAR activity by triggering post translational modifications (Arakane *et al* 1997). StAR is a cholesterol binding protein that appears to work on the outer mitochondrial membrane, apparently through a receptor-independent action to move cholesterol to the inner membrane, where it can become the substrate for P450scc (Stocco and Clark, 1996). Once cholesterol has been delivered to the inner mitochondrial membrane it is catalysed by the enzyme cytochrome P450scc. This is the first step in steroid synthesis.

1.12 Steroid hormones

Steroid hormones have a characteristic chemical structure consisting of 3 six-carbon rings plus one 5-carbon ring and are produced by the adrenal cortex, gonads and placenta during pregnancy. Cholesterol is the precursor of all steroid hormones. Although steroid producing cells synthesise some of their own cholesterol, most of it is delivered to the cells by plasma lipoproteins. Steroids are fat-soluble and pass easily

through cell membranes; they typically have to bind to water-soluble carrier proteins to be transported through the blood to their target tissues. There are several types of steroids and the androgens, which include testosterone, dihydrotestosterone and androstendione, belong to a group called the C19 steroids.

There are many biological steps in steroid synthesis; each one involves small changes to the molecule, mediated by specific enzymes, modifying enough for it to be deemed a separate hormone. The steroidhormones a particular cell produces, depends on the type and concentration of the enzymes present within the cell, and also its complement of peptide hormone receptors. LH receptor stimulation will result in the production of testosterone or progesterone, depending on target cell.

All steroids are highly lipid soluble and once they are synthesised they simply diffuse across the plasma membrane of the steroid producing cell and enter the interstitial fluid and then the blood. Blood vessels are always present around steroid producing cells in order for the hormones to be picked up and delivered to their target site.

1.13 Steroidogenesis.

The rate-limiting and acutely regulated step in steroid hormone biosynthesis is the translocation of cholesterol, from the mitochondrial outer membrane to the inner membrane; This is governed by the StAR protein. Here it is converted to pregnenolone by cleavage of a 6-carbon unit by the cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc). This conversion is a rate limiting and irreversible step. Pregnenolone is in turn converted to progesterone by the enzyme 3βHSD, several other pathways can

then be undertaken involving other enzymes depending on: cell type, enzymes present, and the end steroid required.

1.13.1 Steroidogenic Enzymes

There are many enzymes involved in the steroidogenic pathway and some are crucial as key rate determining regulators. The enzymes expressed in any one steroidogenic cell depend greatly on organ location of the cell. In addition one enzyme may exist as several isoforms. The gonadal synthesis of testosterone from cholesterol involves four key enzymes, namely, cytochrome p450scc, cytochrome P450 17alpha-hydroxylase/lyase (17αOH), 3β HSD and 17beta-hydroxysteroid dehydrogenase. (17β HSD).

Upon delivery of cholesterol to the inner mitochondrial membrane it is converted to pregnenolone by P450 scc. Pregnenolone itself is not a hormone, but is the immediate precursor for the synthesis of all of the steroid hormones. This conversion of cholesterol to pregnenolone is the rate limiting step in androgen biosynthesis and the rate of conversion is dependent both on the amount of P450 scc and the rate at which cholesterol is imported into mitochondria.

mRNA for all four enzymes is expressed in the mouse testes from 15dpc (Greco and Payne 1994), this is further evidence that steroidogenic activity in fetal gonads is independent of LH action as there is no full-length LH receptor transcript detected in mouse testis at this early stage in development.

1.14 Steroidogenic Pathway

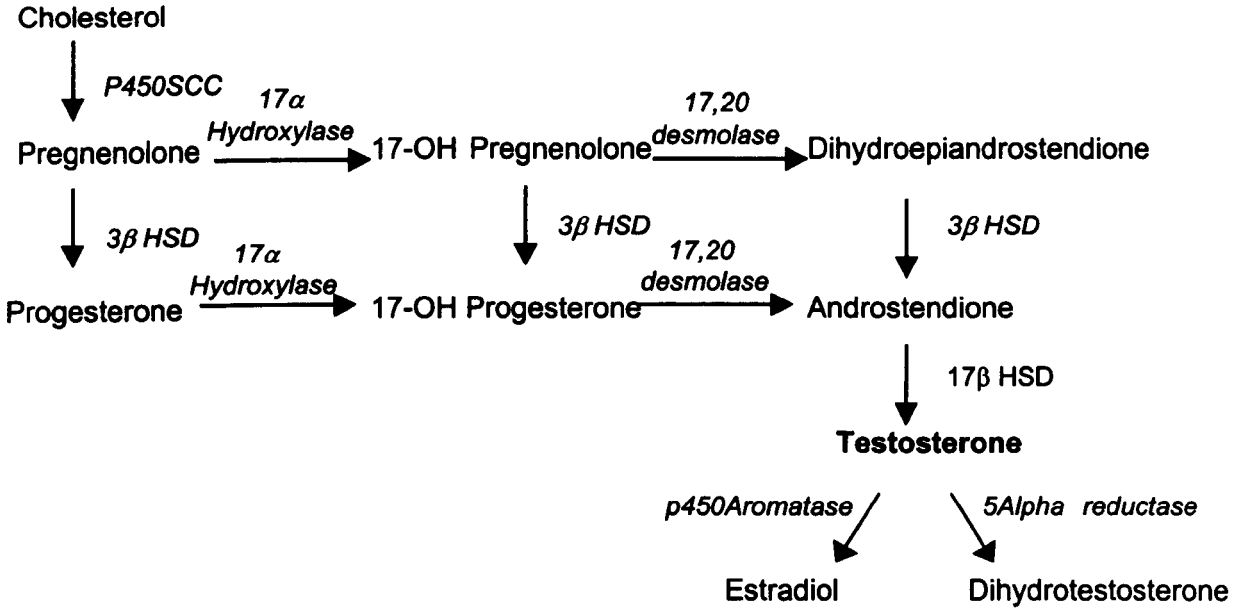


Figure 1-8 Sequence of steroidogenic pathway

Abbreviations *HSD* = Hydroxysteroid dehydrogenase, *SCC* = Side Chain Cleavage

In fetal Leydig cells the end product is androstendione whereas in adult Leydig cells testosterone is the end product of the steroid pathway (O'Shaughnessy *et al* 2000). Testosterone can also undergo further conversion to the more potent androgen dihydrotestosterone, by the enzyme 5 α reductase, or to the female hormone estrogen by the enzyme aromatase, depending on the target cell and enzymes present.

1.14.1 Steroid receptors

The receptors for steroid hormones are situated within the nucleus of target cells. The steroid / receptor complex acts as an intracellular transcription factor, and exerts positive or negative effects on the expression of target genes (Beato *et al.*, 1996; Beato and Klug, 2000). Steroid receptors consist of a ligand-binding domain, a DNA-binding domain, and several transactivation functions distributed along the molecule (Evans *et al* 1988). They have a significantly different mode of action from other hormone receptors, (eg peptide or glycoprotein) most of which affect cellular function by modulation of intracellular second messenger levels.

1.15 Masculinising effects of androgens

An early report by Berthold in 1849, showing that a testis transplanted into the abdomen of a castrated rooster prevented or reversed the effects of castration, ushered in the search for what are now known as androgens and research into how they work. However, as early as 200BC the Chinese were using processed extracts of testes to treat individuals lacking 'maleness activity'. Perhaps the two most important androgens are testosterone and dihydrotestosterone (DHT). Structurally they are very similar and they bind to the same receptor known as the androgen receptor. Administration of testosterone has such a potent effect on the body that it is now given to transsexual females desiring to become male. Hormone therapy will induce a deepening of the voice

and other desirable masculinising effects including coarsening of body hair, male pubic hair pattern, beard growth, muscle development and loss of subcutaneous fat.

1.15.1 Determinate and regulatory effects of testosterone

Testosterone has two types of effect on the body. The first is determinate and involves irreversible changes such as Wolffian duct development, establishment of external genitalia and accessory glands and masculinisation of the brain. These events occur before birth in mammals. There are also progressive changes initiated which occur from birth to puberty, causing changes such as greater musculature and bone growth and voice tone, these changes are more pronounced in sexually dimorphic species such as humans and are all examples of determinate differentiation. The determinate effects therefore are a major contributory factor of the establishment of maleness.

The second type of effect testosterone has on the body is regulatory, whereby the changes that occur are reversible, they can involve both quantitative and qualitative changes to established accessory sex organs. These actions are not involved in establishing the individual as a male or a female, but are more concerned with ensuring that the reproductive tracts and genitalia function effectively in the reproductive process. In males the regulatory actions of testosterone are more pronounced in the adult and involve initiation and maintenance of spermatogenesis, stimulation of accessory sex glands, metabolism, muscle growth, initiation of courtship behaviour and aggression towards competing males, and in some species the secondary sexual characteristics such as antler growth. The regulatory actions may be continuous or show seasonal variation.

In contrast to the determinate effects of testosterone, the regulatory effects are reversible, and involve changes to already established sex organs and glands. These actions, therefore, are not concerned with establishing the individual as male or female, but are involved in regulation of the effective functioning of the reproductive system. The regulatory effects therefore control fertility whereas the determinate effects establish maleness.

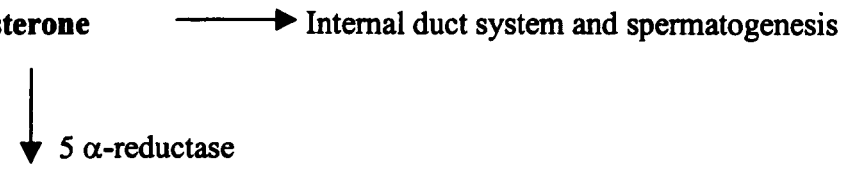
Many experiments have clearly shown that male sexual differentiation is entirely dependant on androgens, the most important one being testosterone. It initiates its effect in a determinate fashion (establishment of male structures), and has regulatory effect ie initiation and maintenance of function of structures.

Determinate	**Regulatory**
Development of Wolffian ducts	Formation of seasonal secondary sexual characteristics eg antlers
Formation of male external genitalia	Initiation and maintenance of spermatogenesis.
Gender identity and sexual preference	Regulation of sex glands and organ function

Table 1-1 Summary of determinate and regulatory effects of testosterone

1.16 Dihydrotestosterone

Although testosterone is the main steroid required by the body for masculinisation, there are certain areas that require its further conversion by the enzyme 5α-reductase to the more potent androgen, dihydrotestosterone. One area in particular is the external genitalia. Although dihydrotestosterone binds to the same receptors as testosterone it does so with much greater affinity. During development it has been found that testosterone promotes virilisation of the male urogenital and duct system in two ways



1.17 5 Alpha (α) Reductase

Testosterone is the major steroid hormone synthesized by the mature testis and consequently is the major circulating androgen, but for a short period during postnatal life in the rat (between 20-40 days), the testes produce mainly 5 α reduced androgens such as 5α-androstan-3α,17β-diol. The predominance of 5α-reduced steroids over testosterone at this time period is due to increased levels of 5 α reductase activity. The 5 α reductase enzyme exists in two forms known as type 1 and 2 and they are coded for by two separate genes. Both types are present in females and males, however the type 2 isoform is more abundant in males and the gene is expressed largely in androgen target tissue.

During fetal development, differentiation of the Wolffian ducts to epididymis and vas deferens requires testosterone, whereas differentiation of the male external genitalia appears to be dihydrotestosterone (DHT)-specific based on the expression profile of 5 α reductase type 2 enzyme, and observations in human sex reversal syndromes characterized by a deficiency of this enzyme (Thigpen *et al* 1993)

DHT is the most potent androgen and it binds selectively to the androgen receptors in genital skin and fibroblasts, making its action necessary for the development of normal male genital anatomy in the fetus. In order for normal development to proceed along the male pathway, there must be sufficient 5 α reductase activity occurring within these androgen target tissues. Mutations of the type 2 gene lead to diminished DHT formation, and hence cause masculinisation defects of varying degree (reviewed by Mahendroo and Russell 1999)

1.18 Disorders of Sexual differentiation

Disorders of sexual differentiation can occur when a bipotential gonad is incapable of developing into a testis or an ovary. The inability to develop testes may occur if a gene such as *SRY* is absent or deficient. When this is the case, a 46,XY fetus will not receive the *SRY* signal to develop testes despite the presence of a Y chromosome. At the genetic level then, disruption of *SRY* here, can result in complete absence of testes. Additionally, for example, AMH secretion accompanied by the absence of androgens or the inability to respond to androgens (due to lack of active androgen receptors) can result in a fetus lacking both male and female internal duct structures. The problems tend to originate at the genetic level. For example certain enzymes and receptors play a

crucial role in the developmental pathway. Changes in gene sequences such as point mutation or base deletion can result in many deleterious effects and completely change the anatomy and physiology of the affected individual. There are several examples of these types of developmental problems.

1.19 5 α reductase deficiency

The absence of 5 α reductase activity in certain areas of the body can have very marked effects and in extreme cases complete phenotypic feminisation can occur. If left untreated individuals remain phenotypically female until puberty. At this time there is such a surge in testosterone production that virilisation can then occur and the female genitalia then change and become male. Failure of development of the male phenotype shows that DHT is required for formation of male genetalia. At puberty, the levels of testosterone are so high that they can then exert the same effect as dihydrotestosterone at the androgen receptor (Grinno *et al* 1990), and the individuals then develop male genetalia. This particular disorder is discussed in more detail in chapter 4.

In mice there is a slight difference from humans, in that testosterone appears to play a more vital role in virilisation without the need of conversion by 5 α reductase to DHT. Evidence of this is apparent in knockout mice. Several studies have assessed the effects of the absence of one or both forms of the enzyme. Male mice lacking 5 α reductase type 1 appear phenotypically normal whereas females exhibit a parturition defect which is thought to be due to absence of cervical ripening (Mahendroo *et al* 1999). Male mice without type 2 or without types 1 and 2 had fully formed internal and external genitalia and were fertile, but had smaller prostates and seminal vesicles than normal. In

conclusion it appears testosterone is the only androgen required for differentiation and virilisation. DHT in normal male mice appears to only enhance the effect of testosterone, and the 5 α reductase enzymes have a more vital role in female reproduction. (Mahendroo *et al* 2001)

1.20 Androgenic disorders

Androgens act in their target cells via an interaction with the androgen receptor resulting in direct regulation of gene expression. The androgen receptor (AR) is comprised of 910-919 amino acids and is encoded by a gene consisting of 8 exons. The AR is a single polypeptide comprised of relatively distinct domains: an amino-terminal domain, a DNA binding domain, a hinge region, and a steroid-binding domain. The large amino-terminal domain, encoded by exon 1, is the least conserved region among the steroid receptors and is involved in transcriptional activation of target genes. The DNA binding domain, encoded by exon 2 and 3, contains two zinc finger motifs and is the most highly conserved region (Jenster *et al* 1991). It is responsible for specific binding to its cognate DNA, i.e. androgen response element of target genes. The carboxyl-terminal of the AR contains the steroid-binding domain, encoded by the 3' portion of exon 4, and exons 5-8. It is responsible for the specific high-affinity ligand binding. The carboxyl-terminal region also contains the areas involved in transcriptional regulation.

Mutations in the gene encoding the androgen receptor can result in partial to complete androgen insensitivity. Individuals have decreased dihydrotestosterone (DHT) levels, and a high ratio of testosterone/DHT, as a consequence of a secondary 5α-reductase deficiency caused by androgen resistance (Imperato-McGinley 1982).

1.20.1 Androgen Insensitivity Syndrome

Androgen Insensitivity Syndrome (AIS) is the most common cause of virilisation disorders in 46XY individuals and is the result of a defective androgen receptor which consequently prevents the normal development of both internal and external male structures in 46 XY individuals. Genetic defects of the human androgen receptor (AR) can cause a wide spectrum of androgen insensitivity syndromes (Quigley *et al* 1995) ranging from phenotypic females in those with complete AIS (CAIS) ambiguous genitalia in partial AIS (PAIS); to male infertility in minimal AIS.

The majority of these defects are due to point mutations resulting in amino acid substitutions. It is however unclear why certain mutations result in partial AIS, whereas others in the same exon cause the complete syndrome. CAIS patients have normal appearing female external genitalia due to their complete inability to respond to androgens. This is because the genital tubercle, genital swellings, and genital folds cannot masculinise in these patients despite the presence of functional testes located in the abdomen.

The androgen receptors on these structures fail to recognise and respond to circulating androgens. Similarly, Wolffian duct development does not occur because these duct structures also require androgens. Mullerian duct development is inhibited in CAIS individuals because MIS is secreted. by the Sertoli cells of the testes. In addition to possessing normal female external genitalia, CAIS patients also have a female duct system.

PAIS also affects 46,XY individuals. PAIS patients are born with ambiguous external genitalia due to their partial inability to respond to androgens. The genital tubercle is larger than a clitoris but smaller than a penis, a partially fused labia/scrotum may be present, the testes may be undescended, and perineal hypospadius is often present. Wolffian duct development is minimal or non-existent and the Mullerian duct system does not develop properly. PAIS patients will experience normal female breast development at puberty, along with a small amount of pubic and axillary hair.

1.21 LH Receptor mutations

The use of molecular biology techniques in the field of endocrinology has resulted in the identification of single gene mutations being the cause of many endocrine hereditary conditions. In the case of the LHR, studies have have established at the molecular level the function of these mutated genes. Both activating and inactivating mutations have been identified.

1.21.1 Activating mutations

Activating mutations in the LHR were first identified in patients with a condition known as familial male limited precocious puberty (FMPP). This occurs due to point mutations in the gene resulting in a single amino acid change in the protein. To date around seven of these mutations have been identified in humans. As a result of these mutations, onset of puberty in these boys may occur before the age of four years. The phenotype is a consequence of increased androgen production by the Leydig cells due to continuous Leydig cell stimulation through expression of the mutated LHR gene. This increase in

androgen occurs independently of stimulation from LH. Intracellular signal transduction is a result of the mutated receptor alone as after birth LH levels are generally very low. In addition to these receptors showing elevated basal activity, this will still increase upon stimulation with LH when true puberty occurs. This condition only occurs in males. There is no effect on phenotype of females with the activating mutation (Themmen *et al* 1998)

1.21.2 Inactivating mutations

Inactivating mutations are also usually a result of a single amino acid change in the protein receptor. Mutations of this type in the LHR result in a male pseudohermaphrodite characterised by a female external phenotype with a small blind ending vagina. Undescended testes are present internally but are greatly educed in size due to extreme Leydig cell hypoplasia. (Toledo *et al* 1985). Although binding with LH occurs, the mutant receptor is completely unable to initiate activation of the Gs protein and consequently adenylyl cyclase. The lack of a male phenotype in these individuals therefore is due to complete absence of androgen mediated genital development.

1.22 Mutant mouse strains

Introduction of desired mutations into ES cells and generation of knockout mice have now become a routine practice, and are a very useful tool in the field of molecular biology. Many of these mutations act as a model for certain condtitions that occur naturally in humans and provide insight and understanding about the importance of specific genes and the role they play in normal physiological function.

1.22.1 Testicular Feminising mutation (TFM)

The testicular feminised (Tfm) mouse carries a single base mutation in the gene coding for the androgen receptor. It changes the receptor structurally, which means that even in the presence of circulating androgen there can be no response, as it is not recognised by any of the target cells. Tfm is an X linked disorder and therefore only one copy of the defective gene is required for males to be affected. Females on the other hand can be carriers or mutants. In Tfm males, the testes are very small and cryptorchid. They fail to descend at the normal time (25 days) this suggests that although the testes produce testosterone it also has an auto regulatory role within the body. They do not posses mesonephric or paramesonephric derivatives and exhibit a female phenotype. Spermatogenesis is arrested and testosterone production is reduced due to a deficiency in the enzyme cytochrome P450c17, which is essential for the conversion of androstendione to testosterone. These mice display resistance to exogenous and endogenous testosterone

Due to the complete insensitivity to androgens this strain of mouse is a good model to use to examine the importance of the role of testosterone and its receptor, as it is entirely unresponsive to androgen. Testosterone clearly has a role in normal steroidogenesis in the Leydig cells, because Tfm mice have very high circulating plasma LH levels but very low testosterone levels. This is because the Leydig cells of these animals produce progesterone as the main steroid rather than testosterone.

1.22.2 Testis structure in the Tfm mouse

Histologically the testes of the Tfm mouse are much smaller (only 10% as large by weight as those of normal mice). The small size is due to the underdeveloped seminiferous tubules. There is also reduced spermatogenesis and a conspicuous quantity of interstitial tissue. (O'Shaughnessy *et al* 2002b)

1.23 LHR Knockout (LHRKO)

The LHRKO mouse was established by Zhang *et al* and reported in Jan 2001. It lacks a full-length functional LHR capable of anchoring to the plasma membrane and of signal transduction. This is due to targeted disruption of exon 11 of the gene, which encodes the transmembrane and intracellular domains. Homozygous LHRKO mice of both sexes are born phenotypically normal. Testes of the newborn males are similar in size and microscopic appearance as those of wild type (WT) littermates, and their intra-abdominal location adjacent to the urinary bladder is the same as in WT males. Postnatally, testicular growth and descent, and external genital and accessory sex organ maturation fails to occur in LHRKO males, and spermatogenesis is arrested at the round spermatid stage. The number and size of Leydig cells is dramatically reduced.

The physical differences between mutant and wild type are more obvious by day 30 as the LHRKO males have a much smaller penis, shorter anogenital distance and an underdeveloped scrotum. Serum and testicular levels of testosterone are greatly reduced compared to wild type, however these levels were still higher than those obtained from

castrated mice indicating that the testes must be able to undergo a small degree of steroidogenesis even in complete absence of LH / receptor complex activation

1.23.1 FSH receptor knockout

This mutant was generated by targeted deletion of exon one of the gene encoding the receptor (Abel *et al* 2000). FSH receptor knockout male mice have underdeveloped testis with 50% reduction in Sertoli cells, suggesting that FSHR signaling is required very early for gonadal development, maturity, and function (Wahlstrom *et al* 1983) Onset of puberty is delayed in these mice, and adult males show a reduction in serum testosterone levels compared to normal controls despite normal circulating LH concentrations. This indicates that communication between Sertoli and Leydig cells is disturbed and that this communication is vital for normal reproductive function. As a result these mice have reduced sperm production and sperm quality.

1.23.2 FSHβ Knockout

These mice have been generated by targeted deletion of exon of the FSH β subunit. Since this region confers hormonal specificity, these mice lack FSH.

Male mice deficient in FSH are fertile despite a decrease in testis size and reduced sperm number and motility (Kumar *et al* 1997) FSH-deficient female mice are infertile due to a block in the progression of ovarian folliculogenesis at the preantral stage. As expected the general phenotype of these animals is similar to that of the FSHReceptor-null mice.

1.23.3 hpg mouse (Hypogonadal)

The hpg mouse has a congenital functional gonadotrophin deficiency due to a major deletion in the gonadotrophin releasing hormone gene (GnRH) Transcription of the remaining gene does occur but there is no detection of the GnRH peptide. (Mason *et al* 1996). The lack of GnRH in the hpg mouse results in greatly reduced levels of pituitary gonadotrophins (Cattanch *et al* 1977). Homozygote hpg males are sterile although fertility can be established by administration of testosterone or dihydrotestosterone (Singh *et al* 1995). The *hpg* mouse offers an interesting model in which to study the development of Leydig cells in the testis, because it is the pituitary-gonadal axis, but not the testis, which is defective in the hpg mouse. Thus, administration of a gonadotrophin, such as LH or hCG can lead to a recovery in Leydig cell function and, hence testosterone production (Scott *et al* 1990)

1.23.4 W/W Mouse

The WW mutant mouse has a mutation affecting the gene in the White-spotting (W) locus. This disrupts the membrane-bound tyrosine kinase, c-kit. The c-kit molecule serves as a receptor for Steel factor (kit ligand, Stem cell factor), the gene product of the Sl locus. Steel factor is expressed during the migratory pathway of primordial germ cells (PGCs) and in testicular Sertoli cells. The interaction between c-kit and Steel factor is essential for the migration of PGCs to the undifferentiated gonad, and for spermatogonial proliferation within the seminiferous tubules of the established testis. In the testes of mutants homozygous for either c-kit (*W/W*) or Steel factor (*Sl/SL*), the seminiferous tubules are virtually devoid of germ cells and contain few spermatogonia;

these mice are infertile. The gonadal axis in these mice is intact, with the result that the Leydig cells seem to show a normal, wild-type phenotype and produce normal circulating levels of testosterone. (De Franca *et al* 1994)

1.24 Aims and Objectives

The overall aims of this project were to characterise expression and regulation of genes encoding the LHReceptor and 5α-reductase enzyme isoforms in the mouse testis during development. At the outset of this project it was considered that these genes were essential for the normal development and function of the Leydig cells and for masculinisation and development of the male phenotype. Despite this there were only limited studies describing the expression of these genes in the mouse and very little was known about the regulation of expression. Using molecular techniques and mutant or knockout mouse strains, studies described in this thesis set out to characterise expression patterns and their control in the mouse testis.

1.24.1 LH receptor

With repect to the LHReceptor the specific aims were

1) To establish the presence of alternatively spliced variants of the LH receptor in mouse testis during different stages of development from the embryo though to adulthood.

2) To determine the sequence of any variants present and determine whether they contain an open reading frame.

3) To determine whether there might be fetal Leydig cell-specific expression of alternate transcripts, which could be used as markers of fetal Leydig cells.

4) To establish whether there might be forms of the receptor which are different in the fetal Leydig cells and which might have constitutive activity and thus explain the gonadotrophin-independence of the fetal Leydig cells (as it subsequently turned out LHR-KO mice are normally masculinised but this was an objectives at the time).

5) To determine what endocrine factors regulate LH receptor expression during development in the mouse.

1.24.2 5 α Reductase

With respect to 5α-reductase specific aims were

1) To obtain sequence for the two isoforms of 5α reductase, type 1 and type 2 in the mouse using primers designed to conserved regions across species and, subsequently, using gene mining techniques.

2) To determine which was the predominant isoform expressed in the testis and to quantitatively assess expression of the two genes in the testes throughout mouse development and in mice lacking specific hormones or hormone receptors.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Mouse models

The mouse is a good model to use for research, and for a better understanding of mammalian embryology, development and reproduction. It is readily available, easy to feed, raise, mate and handle and it has a short gestation period (18 – 21days). In addition it produces large litters (average 8 pups per litter) and has a long period of reproductive activity (from 2 – 14 months of age). Many mutant strains exist either naturally or induced, and by studying the resulting phenotype this can allow for better understanding of the importance of function of the affected gene.

Another benefit of using mice is that they are non seasonal breeders ie they mate readily all year round with the females cycling every four days, and fertile for one day. Also with the males there is no reduction in testes size or periods of inactivity as is the case with seasonal breeders such as rams.

Normal mice were bred at the University of Glasgow Veterinary School and maintained as required under United Kingdom Home Office regulations. The mice used were derived from F1 hybrids of C3H/HeH and 101/H strains. The day of birth was designated as day 1. In order to time fetal development, males were caged with females overnight and presence of a vaginal plug the next morning was indication of a successful mating, and so was designated as embryonic day (E) 0.5.

2.2 Summary overview of lab techniques

The main practical objectives of this project were to determine presence and abundance of specific genes expressed in the mouse testes at different stages of development. Various molecular biology techniques were used to achieve this.

Testes were removed from mice at various stages of development; total RNA was then extracted from them using a sonicator and RNAzol B. The extracted RNA was then reverse transcribed in order to obtain a cDNA copy. This was amplified using PCR with primers specific to the gene of interest, and the resulting PCR product was then ran on a gel using electrophoresis. Ethidium bromide was added to the gel in order to visualise any bands present under UV light. The gel was then subjected to southern blot in order to establish if the bands present were representative of the gene of interest, or the bands were excised from the gel and processed with a mini column filter technique and sequence analysis was performed on them. The sequences obtained were then compared to known sequences in Genbank using GCG and BLAST, both of which are molecular biology computing programmes. Alternatively, levels of specific mRNA species were quantified by real-time PCR and related to an internal control (housekeeping gene) or to an external control (luciferase).

2.2.1 Messenger RNA

The central dogma of molecular biology is that DNA makes RNA makes protein. Extracting mRNA from a tissue at a specific time can give a representation of the genes being expressed. It is useful both qualitatively and quantitatively. As the DNA is copied the first type of RNA formed is known as heterogeneous nuclear RNA

(hnRNA). This contains both introns and exons. The introns which consist of non coding regions are removed by RNA splicing leaving the exons which contain the coding information. The processed intron-free mRNA is then used as a template for protein synthesis. Each set of three bases, called codons, specifies a certain amino acid in the sequence of amino acids that comprise the protein. The sequence of a strand of mRNA is based on the sequence of a complementary strand of DNA.

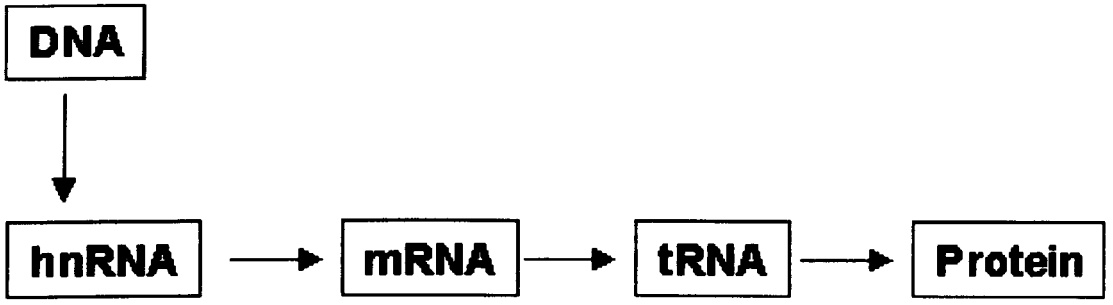


Figure 2-1 Sequence of events for gene expression

hnRNA = heteronuclear RNA

mRNA = Messenger RNA

tRNA = Transfer RNA

2.2.2 *Testes removal*

Embryonic and neonatal mice were sacrificed by decapitation and the juveniles and adults were killed by cervical dislocation. A T shaped incision was made in the lower abdomen and the testes which were situated just below the kidneys were identified

in the embryonic and neonate mice by the presence of a more profuse blood supply around them, they were also slightly more elongate and larger than the ovaries. At the ages used in these studies the sex of the fetus was confirmed by the differentiated state of the internal reproductive tract (ie, presence of Wolffian duct or Mullerian duct derivatives). Testes of the adult mice were easily identified. Once removed the testes were placed into eppendorf tubes and stored in liquid nitrogen, or they were then further processed for removal of interstitial webs, known to contain the Leydig cells. Interstitial webs were stored frozen in liquid nitrogen. It was very important that the time between killing the mice and removal of testes was kept as short as possible in order to prevent any degradation of the RNA by RNAses.

2.3 RNA Extraction

Total mRNA was extracted from the testes of individual animals using RNAzol (Biogenesis Ltd., Bournmouth, UK). RNAzol was placed on ice and 0.5ml added to tissue sample in 1ml eppendorf tube. Sample was then sonicated (see appendix) for about twenty seconds to cause disruption of the cell membranes and release cell contents.

A one-tenth volume of chloroform (50µl) was added and contents mixed using a vortex. The tube was placed on ice for 5mins, and then centrifuged for 15mins at 12500rpm. The top aqueous layer was removed with a pipette and transferred to another tube (this contained the RNA). An equal volume of isopropanol was added and tube left on ice for 30mins then centrifuged for 15mins at 12500rpm.

During centrifugation the RNA formed a pellet at the bottom of the tube and the supernatant was decanted. The pellet was then washed by addition of 500µl of 75% ethanol, vortexing for a few seconds and then re-centrifugation for 5mins. The ethanol was then removed and the tube left to air dry for a few minutes to remove all traces of ethanol. The pellet was resuspended in 30 µl H_2O (UV treated - see appendix), a one tenth volume of 3M sodium acetate pH 7 (3 µl) and 2 volumes of absolute ethanol (60 µl) were added and the tube was placed at –20°C for at least 1hour. The tube and contents were then centrifuged for 15 min at 12500 rpm. The liquid phase was removed from the tube using a pipette and the pellet was left on the bench to air dry for a few minutes, and then re-suspended in 20 µl H_2O (UV treated) by heating in a water bath at 60°C for two mins. The suspended RNA was the stored at –70oC or in liquid N_2

2.4 DNAse Treatment of RNA samples

Genomic DNA, contaminating RNA preparations, can serve as a template in PCR to produce a false positive signal from RT-PCR. The problem will only occur if primers used for subsequent PCR hybridise to a single exon. This can often be avoided during primer design but in some cases the gene structure may not be known and in many cases it is not practicable for real-time PCR (see below). False positives are easily identified by looking at the outcome of a "minus-RT" control (ie reverse transcription reaction without active enzyme), but for accurate quantification of mRNA levels or identification of some mRNA species it is important to remove contaminating DNA. Eliminating the DNA without having an effect on the total mRNA can be done using a DNAse treatment. DNAse is an enzyme, which breaks

down DNA. It efficiently hydrolyzes double and single-stranded DNA to a mixture of short oligo and mononucleotides, but leaves RNA intact.

The method in these studies used DNA-*free* (Ambion Inc, supplied by AMS biotechnology, UK). To 10µl of the RNA sample 1µl of 10x DNAse buffer was added followed by 1µl of DNAse and the reaction mix was incubated at 37oC for 30 mins. At the end of the incubation 2µl of DNAse inactivation reagent was then added to the sample and it was left on the bench at room temperature for 2mins. The mixture was then centrifuged for 1min to pellet the DNAse inactivation reagent and the supernatant was removed and transferred to another tube. The RNA prepared this way was tested for DNA contamination by PCR using primers known to amplify genomic DNA. If DNA contamination was still present a second round of DNAse treatment as above was carried out.

2.5 Reverse Transcription

Reverse transcriptase is an enzyme used to make a cDNA copy of the mRNA template. It is an RNA-dependent DNA polymerase, found in viruses, that catalyzes the synthesis of DNA from deoxyribonucleoside 5'-triphosphates, using RNA as a template.

It does so by extending an oligo dT or random hexamer primer in the 3' direction by incorporation of deoxynucleotides (dNTPs) An oligo dT primer consists of about twenty bases, all of which are thymidines It sits down at the 3' end of the RNA molecule at the poly A tail. Random hexamers are small non specific primers consisting of 6 bases and they will hybridise at multiple sites on the RNA template.

For most studies described here random hexamer primers were used. An RNAse inhibitor was usually added to the reaction as this prevents any unwanted degradation of the RNA. DTT was also added to the reaction mix to prevent oxidation of the newly formed cDNA strands

2.5.1 Procedure

In a normal reaction 2.0µl of 5 x RT buffer was added to an eppendorf along with 3.2µl .magnesium chloride ($MgCl_2$), 2.0µl of DTT and water (variable volumes to give a final volume of 10µl). These were then UV treated to eliminate any possible PCR-derived DNA contamination. Following UV-treatment, 0.5µl dNTPs, 0.5µl random hexamers, 0.3µl M-MLV reverse transcriptase, (Gibco Paisley, UK) 0.3µl of RNAse inhibitor, and 2.0µl of RNA were then added and contents mixed gently using a pipette. The mixture was then incubated in a water bath at 42°C for 1hour then placed on ice, ready to be used as a PCR template

2.6 PCR

The polymerase chain reaction was developed in the mid eighties and is a technique which is used to amplify specific target sequences of DNA, using a pair of oligonucleotide primers each complimentary to one end of the DNA target sequence. It is not essential to know the sequence in between. The primers are extended towards each other by a thermo stable DNA polymerase that is a naturally occurring enzyme, which catalyzes the formation and repair of DNA. PCR is a method for producing an extremely large number of copies of a specific DNA sequence from a DNA mixture without having to clone it. In the words of Paul Rabinow "PCR has

transformed molecular biology through vastly extending the capacity to identify, manipulate and reproduce DNA. It makes abundant what was once scarce – the genetic material required for experimentation."

The requirement of an optimal PCR reaction is to amplify a specific locus without any unspecific by-products. Therefore, annealing needs to take place at a sufficiently high temperature to allow only the perfect DNA-DNA matches to occur in the reaction. For any given primer pair, the PCR program can be selected based on the composition (GC content). The reaction cycle consists of three steps. Firstly a 95°C step to denature the double stranded DNA - they have to become single stranded in order to act as a template for the primers and new strand synthesis. Secondly an annealing step of around 56°C - this is usually an optimum temperature for a primer to anneal itself to the template strand. Lastly, there is a 72°C step for dNTPS to be incorporated into the new strand by the Taq enzyme as it extends the primers. Almost any source that contains one or more intact DNA molecule can, in theory be amplified by PCR providing appropriate primers can be designed.

2.6.1 PCR Cycles

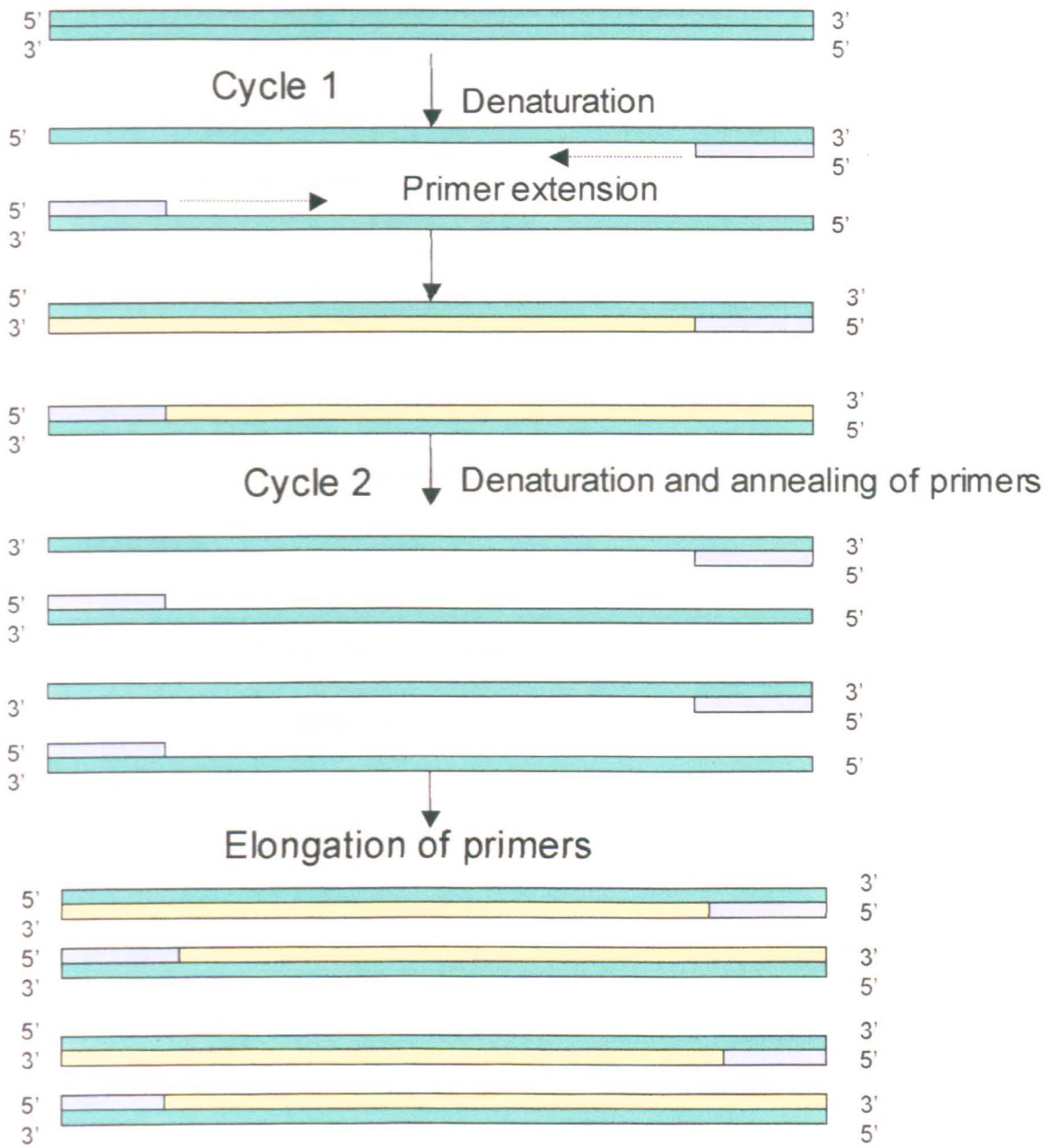


Figure 2-2 PCR cycles showing various stages of PCR amplification

- Double stranded DNA is denatured by higher temperature.
- Sequence specific primers bind to target sites.
- Elongation of primers occurs as free dNTPs' are incorporated, forming duplicate strands

2.6.2 The requirements for PCR

1. A DNA mixture containing the sequence to be amplified.

2. A pair of oligonucleotide primers about 20base pairs in length that flank the 5" and 3" regions of the DNA sequence of interest.

3. A mixture of dNTP's for new strand synthesis.

4. Thermo stable DNA polymerases eg Taq polymerase as it can survive the hot denaturation step and forms a copy of the single stranded template using the free nucleotides.

5. Buffer containing KCl, Tris Cl and MgCl, these salts provide the necessary ions reqired for primer annealing

2.6.3 Designing primers for PCR

Primers should be 17-28 bases in length, and base composition should be 45-60% guanine and cytosine (G+C). Ideally primers should end in a 3' CG or GC as this increases the efficiency of priming. Primers usually have a melting temperature (Tm) of between 55-80°C. The 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesised preferentially to any other product. Primer self-complementarity (ability to form 2° structures such as hairpins) should also be avoided, this occurs if the primer folds and anneals to itself due to high complimentarity. All primers for this study were designed by obtaining sequence of interest from Entrez then assessing which areas corresponded to the required parameters. It was also desirable to design primers spanning between two exons as

this then reduced the risk of picking up any contaminating genomic DNA during the PCR reaction.

2.6.4 PCR protocol

Each PCR reaction contained Tris/HCl buffer (75 mM, pH 9.0), $(NH_4)_2SO_4$ (20 mM), $MgCl_2$ (2 mM), dNTP's (0.2 mM each), 0.2µl Taq Polymerase (2 U per 100 µl), 1µl of the cDNA template, primers (200 nM each) and H_2O to an appropriate volume. All components, apart from dNTPs, Taq and template were treated by UV-light to remove any PCR-derived DNA contamination. Tubes were placed in the hotlid, which had been programmed to undergo the following cycles;

95°C for $1^1/_2$ minutes..... 1 cycle

94°C for 30 seconds

56°C for 30 seconds (primer annealing) } x32 cycles

72°C for 1 - 2 minute depending length of template

To ensure there was no amplification from any source other than the cDNA template, a no-template control was usually included which contained 1µl of dH_2O instead of template.

2.6.5 Nested PCR

Nested PCR means that two pairs of PCR primers were used for a single locus. The first pair amplified the locus as seen in any PCR experiment. The second pair of

primers (nested primers) bind within the first PCR product and produce a second PCR product that will be shorter than the first one. The logic behind this strategy is that if the wrong locus were amplified by mistake, the probability is very low that it would also be amplified a second time by a second pair of primers. In addition this is useful method of detection for genes which are expressed in low levels. Sometimes there are no bands present after running a gel, for this project nested PCR was then undertaken when this occurred.

2.7 Gel Electrophoresis

Gel electrophoresis is a is a very useful tool for visualising the DNA products of a PCR reaction The method is based on separation of molecules consisting of either nucleic acids or proteins on the basis of size and electric charge. Since DNA is a negatively charged molecule, it moves through an agarose gel matrix with an electric current towards the positive electrodes. The smaller molecules will travel further along the gel since they can move with much less resistance. Ethidium bromide is added to the product as it binds to DNA and can then be visualised under UV light. Molecular size markers (usually a 100 base-pair ladder) are also run on the gel along side the PCR products and can be used as a size indicator for the bands. The buffer used for loading the samples on the gel contains glycerol which makes the samples more dense and causes them to sink to the bottom of the wells so they are not lost to the surrounding buffer whilst loading. In addition the loading buffer contains the dye bromophenol blue as it allows visualisation when loading the gel and because it allows a simple estimate of sample migration through the gel during electrophoresis. After the electric current has been applied and the products have separated along the gel, the bands can be removed from the gel and either re-amplified using internal primers or processed for sequencing.

Agarose gels were prepared by adding agarose powder to a conical flask along with 0.5 x TBE buffer. The amount of agarose was usually 1% (w/v) although this could be increased to examine lower molecular size products. The agarose suspension was heated in the microwave for about 1min until all the agarose had dissolved. It was then left to cool on the bench for 5 mins and 3µl of ethidium bromide was added to the solution. This was then poured into a gel tank and a comb was inserted to provide

loading wells for the PCR products. Loading buffer was added to each PCR product and 20µl of each was added to an individual well on the gel. A voltage difference (~10V/cm) was applied to the gel and the gel was run for about 1hr

2.7.1 Southern Blotting

The Southern blot was invented by EM Southern in 1975, (Southern 1975), and it is a means of permanently immobilizing single-stranded nucleic acids to a solid support. This can be a very useful technique for identification of bands on a gel and to ensure that they do in fact represent the sequence of DNA which it was intended to amplify. It works by using a buffer and wick system, which transfers the DNA bands from a gel to a nitrocellulose membrane. During this process the DNA is made single stranded. After Southern blotting the DNA is usually detected by "probing" with a complementary DNA probe. Probe detection involves exposure of the nitrocellulose membrane to a probe of specific complementary sequence to cDNA of interest. It is labelled usually with a radioactive isotope and allowed to hybridise to the amplified products. This can then be visualised by exposure to radiographic film.

In detail the procedure involves the following steps:

1. Excess gel is trimmed and the top right hand corner cut off for orientation

2. 500ml 0.4M NaOH is prepared to act as a transfer buffer, and 4 pieces of Whatman 3MM paper are cut to the same size as the gel and one twice as long to be used as a wick. The NaOH buffer also acts to denature the DNA during the transfer process.

3. Membrane paper (positively charged nylon membrane from Boeringer Mannheim) is cut to the size of the gel, marked with a pencil in the top right hand corner and wet with transfer buffer. Gel support is placed in Pyrex dish, and transfer buffer added.

4. The paper wick is placed over the support and edges submerged in the transfer buffer. The gel is placed on top of the wick and four strips of parafilm are placed over the edges of the gel. This is to prevent the buffer from "short -circuiting" ie to ensure that the buffer flows through rather than around the gel.

5. The membrane is pre-wet in buffer and placed exactly over the gel. Air bubbles are carefully removed

6. Two pieces of 3MM paper, the same size as the gel, and wet with transfer buffer are placed over the membrane. Paper towels are cut to the same size as the membrane and stacked on top of Whatman 3MM papers to a height of about 10cm. This acts as a sink to allow flow of buffer through the membrane.

7. A glass plate is laid on top of the structure and a weight placed on top. This is left overnight to ensure transfer of DNA is complete. Membranes are then removed, washed in buffer and wrapped in saran wrap.

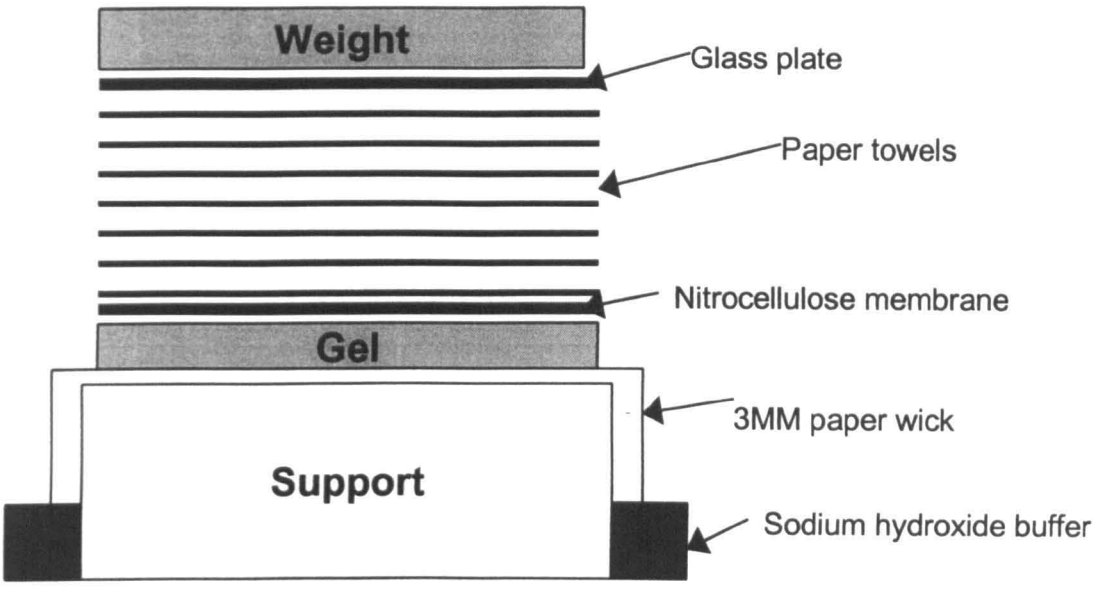


Figure 2-3 Requirements for Southern blotting

2.7.2 Southern hybridisation

In order to determine whether bands, which have been amplified by PCR, belong to the specific gene, which has been targeted, the membrane is incubated overnight at 65°C in a heat proof glass bottle along with a hybridisation solution and a radio-labelled probe. This will bind to complementary sequence on the membrane and can then be detected by exposing the membrane to Xray film. Areas where radiolabelled probe has bound will show up as dark bands on the film.

2.7.3 Prehybridisation solution

The prehybridisation solution consisted of 6 x SSC, 5 x Denhardts reagent, 0.5% SDS and 100μg/ml of denatured, fragmented salmon sperm DNA.

The nitrocellulose membrane with single stranded DNA transferred from the gel was placed in a glass bottle along with 25ml of prehybridisation solution. This was placed in oven at 65°C and left for one hour.

^{32}P labelled probe was prepared using a gel purified PCR product from amplification of exons 1-9 of LH receptor as a template and a random priming labeling kit. BioPrime labeling kit (Gibco/BRL) this contains random octamers, reaction buffer, Klenow (a DNA polymerase) and dNTP's. Probe was heated to 95°C for 5 mins to denature it. This was then added to the glass bottle containing membrane and prehybe solution. The bottle was then place in a hybridisation oven and left over night rotating at 65°C. Membrane was then washed with X2 SSC and 1% SDS for 30 min at 65°C then X1 SSC and 0.1% SDS for 5 mins.

The membrane was removed from the hybridisation bottle and wrapped in saran film, this was then placed in a cassette with film (Biomax MR). The cassette was left for an appropriate length of time (5 min to overnight) and the film developed.

2.7.4 Band Extraction

Sometimes, when PCR products have been run on a gel it may be desirable to extract the bands for further processing. The gel would be viewed under UV light box so that

desired bands were clearly visible. Bands were then removed using a scalpel blade and immediately transferred to a mini column (Chemicon). The length of time the gel was exposed to UV light was kept to a minimum as this degrades the cDNA. The mini columns containing gel slices were then centrifuged for 1 min at 13000rpm. After spinning, the gel matrix is trapped on the column and the aqueous phase containig the cDNA collects in the bottom of tube. The cDNA can then be stored at -20°C until further use.

2.8 DNA Sequencing

Sequence analysis of cDNA is the final proof of identity for a PCR product. It is also essential if the product sequences are unknown (eg due to alternate splicing). To determine the sequence of an amplified PCR product normally it would be extracted from an agarose gel, purified as above using a mini column and then sequenced. In this study all PCR products were sequenced using an ABI 310 automated sequence analyser.

2.8.1 Principle of DNA cycle sequencing

DNA sequencing is the determination of the precise sequence of nucleotides in a sample of DNA. The most popular method for doing this is called the dideoxy method. A dideoxynucleotide can be added to the growing DNA strand but when it is, chain elongation stops because there is no 3' OH for the next nucleotide to be attached to. For this reason, the dideoxy method is also called the chain termination method. Because all four normal nucleotides are also present, chain elongation proceeds normally until, by

chance, DNA polymerase inserts a dideoxy nucleotide instead of the normal deoxynucleotide. The ABI310 automated sequence analyser uses this method, and each of the dideoxynucleotides fluoresces a different colour when illuminated by a laser beam and this is detected and exact sequence of DNA template can then be determined.There are three major steps in each cycle (like in PCR), which are repeated around 30 times.

1. Denaturation at 94°C: During the denaturation, the double strand melts open to single stranded DNA

2. Annealing at 50°C: In sequencing reactions, only one primer is used, so there is only one strand copied

3. Extension at 60°C: This is the ideal working temperature for the polymerase (normally it is 72 °C, but the lower temperature is used because it has to incorporate ddNTP's, which are chemically modified with a fluorescent label. Whenever a ddNTP is incorporated into the strand the extension terminates.

4. The individual fragments are then ran on a gel within the automated sequencer, each fragment is flourescently labelled and as they migrate through the gel the flourescent molecule is excited by a laser. This then sends out light of a distinct colour. Each base has its own colour, so the sequencer can detect the order of the bases in the sequenced gene.

2.9 Hydrophobicity Analysis of translated Proteins

Hydrophobicity plots are designed to display the distribution of polar and apolar residues along a protein sequence. Hydrophilic groups are typically polar, interacting with water by hydrogen bonding. For this reason, they are called "water loving." Hydrophobic groups, on the other hand, are nonpolar, unable to interact with water, and thus are referred to as "water fearing". The hydrophobicity of the amino acids determines where the amino acid will be located in the final structure of the protein (Kyte and Doolittle 1982).

Hydrophobic amino acids tend to occur in the interior of globular proteins, while at the surface of a protein one will preferentially find hydrophilic residues. In transmembrane proteins, the regions of the chain that span the membrane tend to be strongly hydrophobic. In the case of the full-length receptor transcript, exons 1-10 encode hydrophilic amino acids, as this is the extracellular hormone-binding region of the receptor, whereas exon 11 has both hydrophobic and hydrophilic regions as the amino acid chains span the membrane in a looping fashion.

Because a hydrophobicity plot has the goal of predicting membrane-spanning segments (highly hydrophobic), or regions that are most likely exposed on the surface of proteins (hydrophilic domains). A Kyte and Doolittle Hydrophobicity plot was performed on the amino acid sequences of the three spliced transcripts and full-length form of the mouse

LH receptor. Each amino acid is given a hydrophobicity score between -4.6 and 4.6. A score of 4.6 is the most hydrophobic and a score of-4.6 is the most hydrophilic

Window size refers to the number of amino acids examined at a time to determine a point of hydrophobic character. Window size can be varied from 5 to 25 (default 7) and one should choose a window that corresponds to the expected size of the structural motif under investigation. A window size of 5-7 is good for finding hydrophilic regions. Alternatively a window size of 19-21 will make hydrophobic, membrane-spanning domains stand out rather clearly (typically > 1.6 on the Kyte and doolittle scale). A window size of 9 was used for the analysis on the LHR transcripts to accommodate both hydrophobic and hydrophyilic regions.

2.10 Real Time PCR

Real time PCR is a technological step forward from manual quantitative PCR and allows accurate quatitation of cDNA levels in a sample. It is now an important qualitative and quantitative tool in the field of molecular biology. It is qualitative in that it detects expression of a particular gene from a cDNA template, and quantitative as it shows expression levels relative to a house-keeping gene. It is similar to the manual method of semi quantitative PCR in that it assesses the amount of PCR product produced at a specific stage in the reaction, but the method of detection differs as this is achieved by a fluorescent dye. This method is much less time consuming, more accurate, and allows for larger sample numbers.

The ability to monitor the real-time progress of the PCR completely revolutionizes the way one approaches PCR-based quantitation of DNA and RNA. Products of the PCR,

measured at the point in time during cycling when amplification of a PCR product is first detected (threshold cycle ct) rather than the amount of PCR product accumulated after a fixed number of cycles. The higher the starting copy number of the nucleic acid target, then the lower the ct value will be.

Real Time PCR uses threshold measurements during the exponential phase of amplicon production instead of end points. As the PCR proceeds in the TaqMan 5' nuclease assay, fluorescence is released in direct proportion to the accumulation of PCR product. Through the use of a CCD camera, fluorescence production is continually monitored in each of the reaction wells.

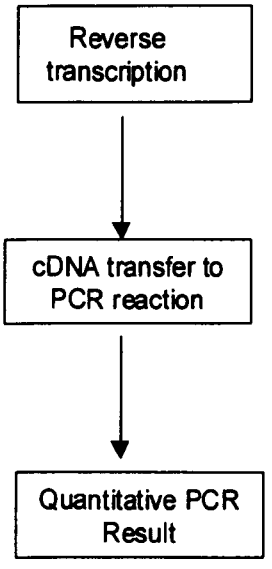


Figure 2-4 Sequence of events for semi quantitative PCR

2.10.1 Primers and Probes

The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. While the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter. Probe design and synthesis has been simplified by

PCR in that they consist of about a 20base sequence complimentary to specific areas of the gene of interest. Ideally, at least one of the primers for each gene would be designed from a region of intron/ exon boundary to avoid amplificaion of contaminating genomic DNA. In practice this is often not possible (eg lack of information of genomic structure or unsuitability of sequence across intron/exon boundaries).

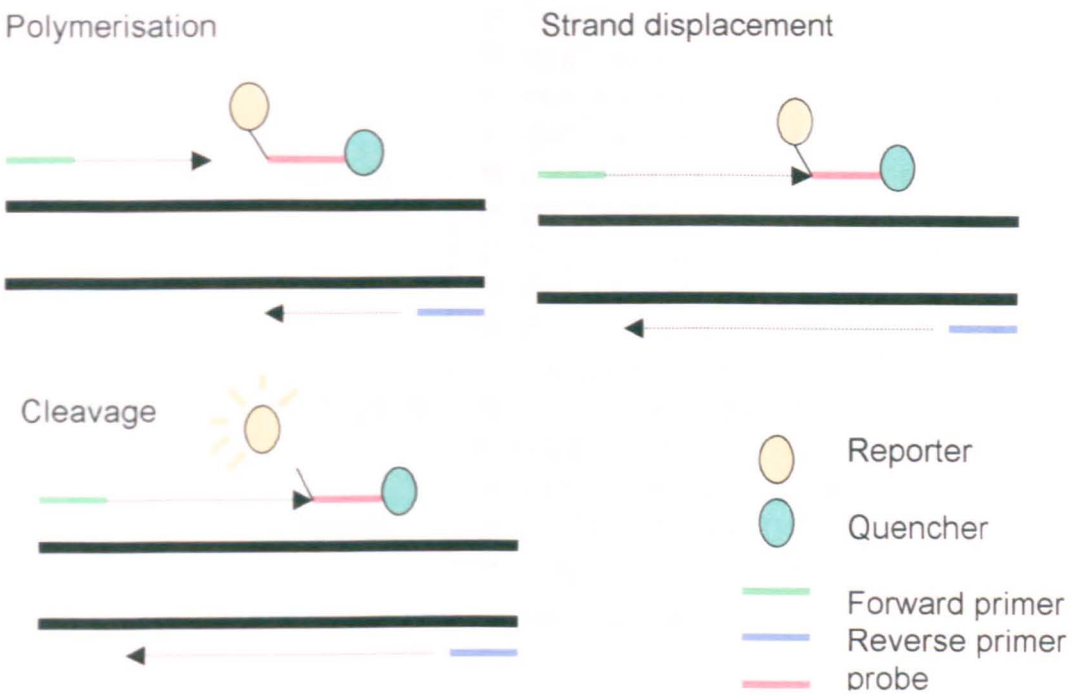


Figure 2-5 Stepwise representation of polymerisation acting on a fluorogenic probe during one extension cycle of PCR

The primers and probes to be used with the Taqman method were designed using Primer Express (Applied Biosystems, Warrington UK.) and are listed in table 2.1. Sequence information was obtained from Genbank using the Entrez search facility. Genbank contains full or partial cDNA sequences previously submitted. With 5α-reductase type1 however there was no published mouse sequence in the Genbank database at the time these studies were carried out, so sequence was obtained using EST's (expressed sequence tags), these were identified by a basic local alignment search using rat 5α-reductase type1. This process is described in detail in Chapter 4.

2.10.2 Probe sequences for genes quantified

Gene	Genbank	Sequence (5' → 3')
5αReductase Type 1	EST's	F GCGCTAGTCTACCTGGAGGGT R GAAGAGCCCACCATCTGGAG P TCCTGGCTTTCGTGGCCTTCGTG
5αReductase* Type 2		F CATCCACAGTGACTGCATGC R AGGTGGCTTGTTTACGT P AGGAAGCCCGGAGAGAGGTCATC
LH receptor	M81310	F GACCAAAAGCTGAGGCTGAGA R CAATGTGGCCATCAGGGTAGA P TGCCATCCCAATTATGCTCGGAGGA
Luciferase**		F TCGAAGTATTCCGCGTACGTG R GCCCTGGTTCCTGGAACAA P TGTTCACCTCGATATGTGCATCTGTAAAA
P450scc	AF195119	F CCAGTGTCCCCATGCTCAAAC R TGCATGGGTCCTTCCAGGTCT P TGCCTCCAGACTTCTTTCGACTCCTCAGA
StAR	L36062	F CCGGAGCAGAGTGGTGTCA R CAGTGGATGAAGCACCATGC P CAGTGGATGAAGCACCATGC

Table 2-1 Sequence of probes used for real time PCR

* Derived from new mouse sequence obtained from initial PCR studies * * Derived from sequence of mRNA provided by Promega (Madison, W1)

F = Forward primer, **R** = Reverse primer, **P** = probe

2.10.3 Standard Curves

Before performing the actual real time PCR reactions it is first necessary to construct a standard curve, which determines the appropriate dilution of cDNA template to achieve maximal amplification. A standard curve is obtained using primers and probe for gene of

interest and also an internal standard such as the house keeping gene actin or an external standard such as Luciferase. The dilution factors used in these experiments were 1, 1/5, 1/125, 1/625. These are plotted in reverse order on the graph with 625 being the most concentrate template and 1 the most dilute. The slope of the curve can be used to assess efficiency of PCR amplification. Efficiency of reaction is expressed as a percentage, with 100% being the optimal. A worked example for β actin is included below the graph. The standard curves provide useful information on amplification efficiency. In order for a relative comparison to be made between sample it is important that amplification efficiency for standard and gene of interest are the same.

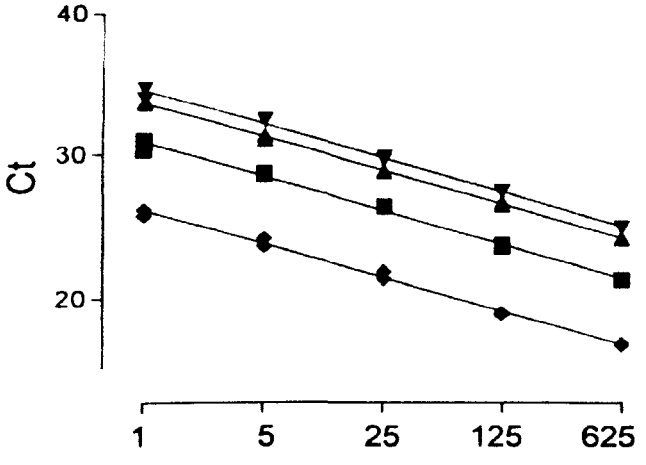


Figure 2-6 Real time PCR standard curves

These standard curves were prepared by serial dilutions of adult testis cDNA. The log of the relative template concentration was plotted against the threshold cycle (Ct) and regression analysis was used to generate the best-fit line. Results from duplicate amplifications of *luciferase* (▲), *18S* (♦), *Wbscr1* (■), and *β-actin* (▼) cDNA are shown

Calculation for efficiency of reaction for β actin

$Y = -MX + C$ $M = Y/X$ M = slope of line .

Slope of line = 1.5046 0.4343/1.5046 = 0.2886

$10^{0.2886} = 1.944 - 1 = 0.944 =$ **94% amplification efficiency**

For most of the Real time PCR experiments ß-actin was used as an internal standard. At the time this was the most commonly used house keeping gene for quantitation studies. Other experiments performed in the lab however later showed that there was not always consistency of expression levels with ß-actin and that these could vary greatly between fetal and adult tissue and it also depended on tissue type used for cDNA template. For example 1μg of cDNA from adult tissue may express much higher levels of actin than 1μg of cDNA from fetal tissue, this then introduces inconsistency and errors in measurement. For this reason an external standard was used for any subsequent reactions. This is a far more accurate method of quantitation as a known quantity of Luciferase is added to the reaction mix prior to PCR amplification.

2.10.4 Summary and order of techniques applied

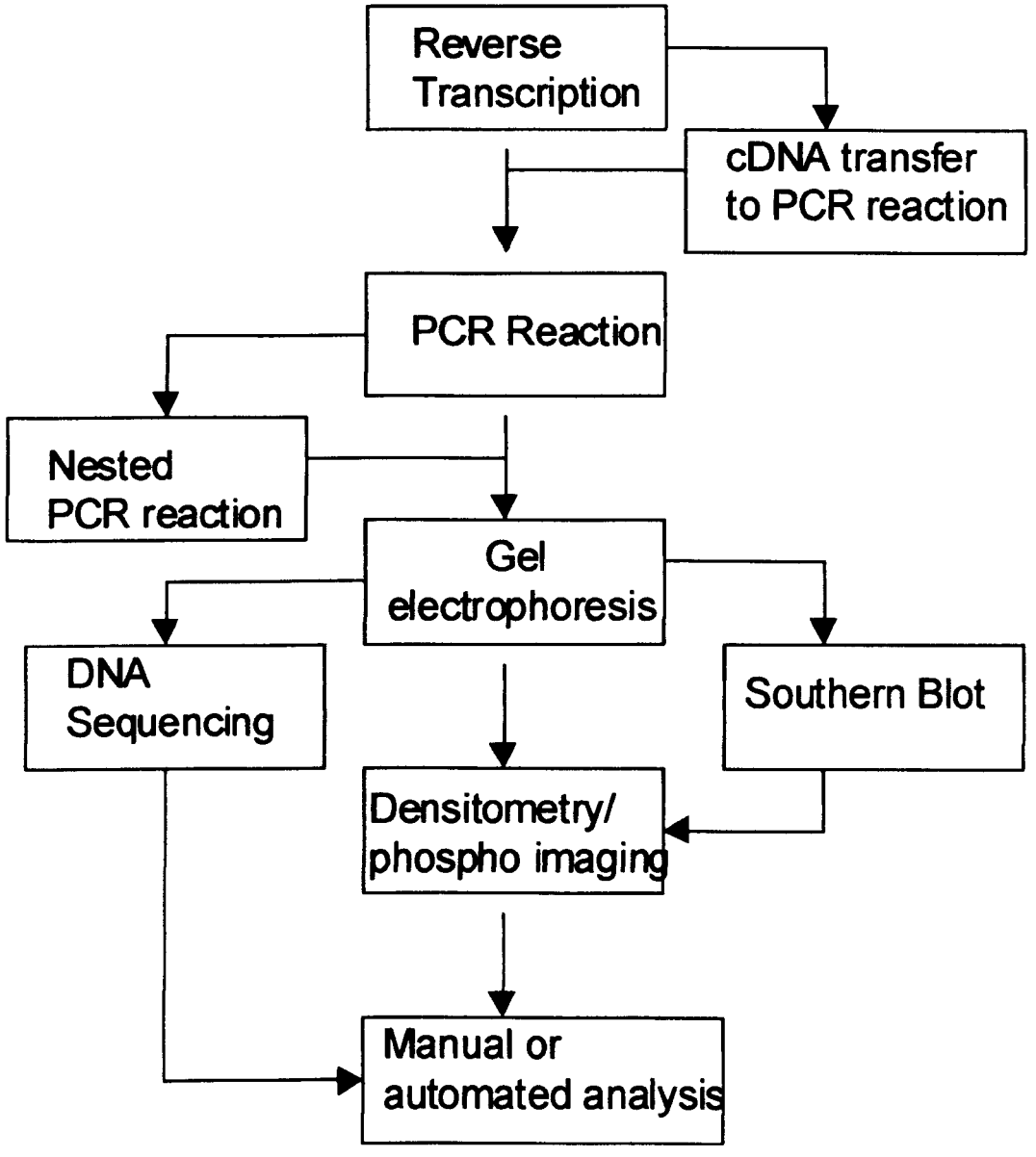


Figure 2-7 Summary flowchart of sequence of molecular biology techniques

Chapter 3

Expression of the LH Receptor in the mouse testis during development

3 LH Receptor gene

The LH receptor is encoded by a gene consisting of 11 exons and 10 introns, The processed, full length form of the mouse LH receptor gene is encoded by 2650 bases. Exons 1 – 10 each consist of about 80 bases, whereas exon 11 is much larger with 1750 bases. Variant forms of the gene encoding the LH receptor were initially identified during porcine LH / CG receptor cDNA analysis and many other studies since then have supported these findings (Misrahi 1996, Wang *et al* 1991). Cloning of the LH receptor has shown that it is a single polypeptide composed of two general domains of equivalent size (Minegish *et al* 1990). It contains an amino (N) - terminal segment, which is extracellular (Rodriguez and Ssegaloff 1990) and consists of a repeating leucine rich motif (McFarland *et al* 1989). This domain contains six potential sites for N - linked glycosylation and it has been shown that this portion of the molecule is entirely sufficient for binding hormone with high affinity (Xie *et al* 1990). The carboxy terminal contains seven regions of hydrophobic amino acids and it spans the membrane seven times, with the carboxy - terminal tail extending intracellularly. (McFarland *et al* 1989). The seven transmembrane strands, which form the transmembrane core, are linked by three exoloops, and four cyto loops.

3.1 LH Receptor Structure

The diagram of the LH receptor depicts these regions.

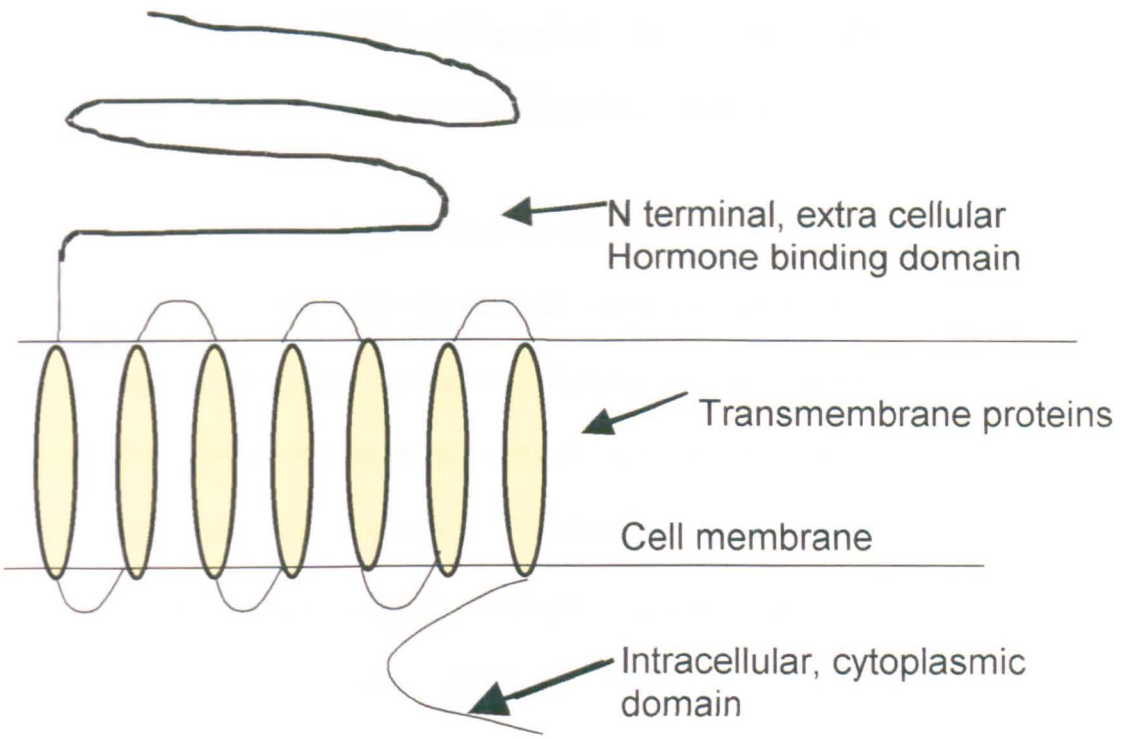


Figure 3-1 Structural diagram of LH receptor depicting functional domains

3.2 RNA Splicing

RNA splicing was discovered just over twenty five years ago, and since then many advances have been made in our understanding of the biochemical basis of the splicing reaction (Grabowski *et al* 1985, Pagdgett *et al* 1986). The general understanding of the functional significance of RNA splicing is that it has evolved as a mechanism for regulating gene expression and generating isoform diversity.

Alternative splicing usually gives rise to protein isoforms sharing regions of identity, with variation only occurring in specific domains. These different isoforms may then allow for the fine modulation of gene expression and protein function. All eukaryote RNA must first undergo a series of processing events in order for gene expression to occur. These processing events include addition of a 7-methyl guanosine to the 5' end of the RNA fragment, this is known as capping. Processing also occurs at the 3' end of the fragment with the addition of a poly adenosine (A) tail (Birnstiel *et al* 1985). At this stage the transcript still consists of exons (coding regions) and introns (non coding regions). Before translation can successfully occur the introns must be precisely excised and the exons correctly ligated to avoid disruption of the open reading frame.

Splicing involves several steps, which must occur with high precision. Most importantly, though, there must be a recognition site in order for the splicing machinery to cleave the 5' exon/intron junction and the same applies for the 3' intron / exon junction

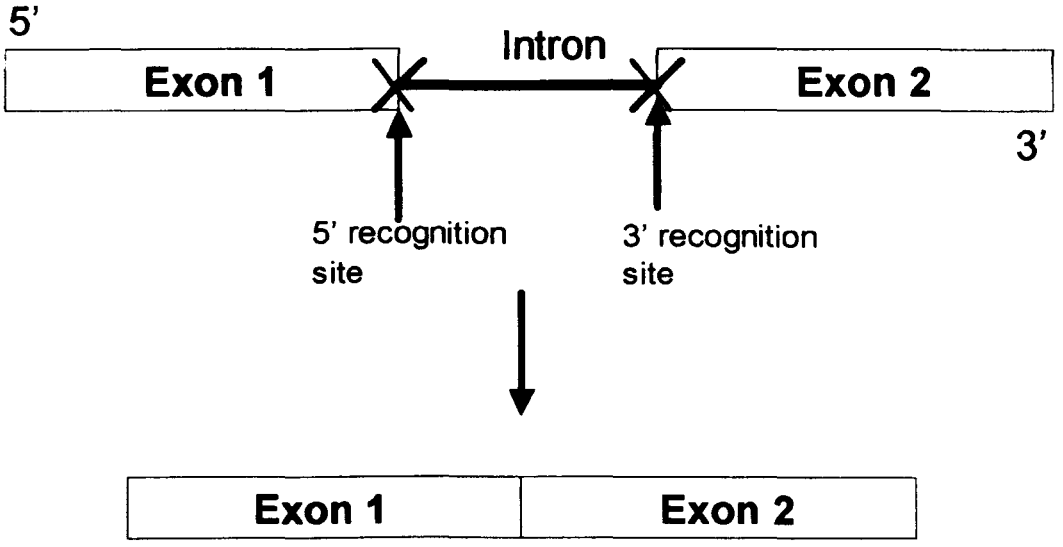


Figure 3-2 Intron exon splicing

Recognition of splicing sites tends to occur around conserved sequences of DNA. The 5' splice site consensus sequence is AG / GUR AGU, the intron exon boundary is denoted by / and the 3' splice site consensus sequence is AG / G

3.2.1 Alternative splicing in LH receptor

It is now well established that the LH receptor exists in multiple isoforms as a result of alternate splicing of the gene. Alternative isoforms have been identified and characterised in several species such as; bovine (Kawate and Okuda 1998), ovine (Bacich *et al* 1994), porcine (Loosfelt *et al* 1989) avian (You *et al* 2000) and rodent (Tena-Sempere *et al* 1994)

Several studies have identified multiple LHR isoforms common to various species suggesting a common mechanism that may be evolutionary conserved. These isoforms are present in both the ovaries and the testes indicating that it is not a sex specific phenomenon. At the time of these studies it had been shown that fetal Leydig cell function did not require LH. Since it is known that some G-proteins can show constitutive activity it was possible that some isoform of the LHR may be expressed in the fetal Leydig cells which constitutively activates the cells. The overall objectives of this part of the study were:

3.3 Objectives

1. Identification of alternate transcripts of the LH receptor gene present in mouse testes using molecular biology techniques.

2. Determination of differential expression of the truncated transcripts at various stages of testicular development, from embryonic to adult.

3. Sequence and protein analysis of the bands.

4. Quantitation of LH receptor expression using Real Time PCR

3.4 Methods and materials

Male mice, age range from embryonic day 15 through to adult were sacrificed and testes removed. Testes were identified in embryonic mice by greater vasculature than ovaries and larger size. These were then stored in liquid N_2 until required. Total RNA was extracted from whole testis using RNAzol B (described in detail in chapter 2). RNA was

reverse transcribed using random hexamers then PCR was perfomed using primer combinations spanning extracellular and transmembrane domains of mouse LH receptor. PCR products were run on a gel, which was then subjected to Southern blotting using mouse LH receptor specific probe from the extracellular region of the gene. In addition, the bands were extracted and purified then sequenced using automated sequence analysis.

The sequences obtained were then entered in a blast search to confirm if they were indeed mouse LH receptor sequence and also to assess whether alternate splicing had occurred. Alignment of bands sequences with the full-length mouse LH receptor cDNA sequence allowed for assessment of splice sites. Blast is a molecular biology computing programme available from NCBI. It allows various alignments and matches to be performed on sequences of DNA, which are entered. It searches various databases such as Genbank which contains already published DNA sequences from genes of many species and it performs a best fit alignment to DNA sequence entered. This allows you to take an unknown sequence of DNA obtained from automated sequencing and assess whether it is in fact from the gene which you were initially trying to target with PCR.

The sequence of the LH receptor bands were translated to see if an open reading frame could be applied. Protein sequences were then characterised using a Kyte and Doolittle hydrophobicity plot to determine hydrophobic or hydrophilic nature of structure.

3.4.1 Experimental Design

Primers were designed from mouse LHR cDNA sequence (Genbank M81310)

Primer Sequences

Primer number

138 – ACGGCTCGGTGCCCGACCTC

284 – TCTCACAATTAAGTCATTG

283 – TAATCAGTTAACATAGATTTATACAAGG

147 – AAGCAGTCACAGCTGCACTCT

26 – CCTCGAGCTGGCCTCGCCCGA

20 – ATAAAGCGTCTCGTTATTCGC

PCR was performed using various combinations of these primers. Originally it was intended to amplify the whole gene from 5' of exon 1 to the 3' of exon 11, using primer combination 147 / 283. Several attempts failed to produce any detectable bands on the gel, however, a nested PCR was then performed using primer combination 26 /284. This did produce some faint bands, but results were inconsistent when repeated several times. It was decided, therefore, to amplify the gene in two sections, firstly using primers complimentary to exon 1 and exon 10 in order to establish if the full-length extracellular domain was being expressed and then using primers complimentary to exons 9 and 11 in order to assess whether the full-length transmembrane/intracellular domain was

expressed. The primer combinations have been highlighted with red to represent extracellular amplification and blue to represent transmembrane amplification. PCR was perfomed on cDNA generated from mouse testis using these primer combinations for each age group. In addition to the LH receptor it was decided to examine mRNA expression of P450scc. As this is one of the key enzymes involved in the steroidogenic pathway, high expression levels can be used as an indicator of active steroid synthesis. In addition P450scc acts as a Leydig cell-specific marker and, therefore, as a positive control for sample preparation etc.

3.4.2 *Primer position*

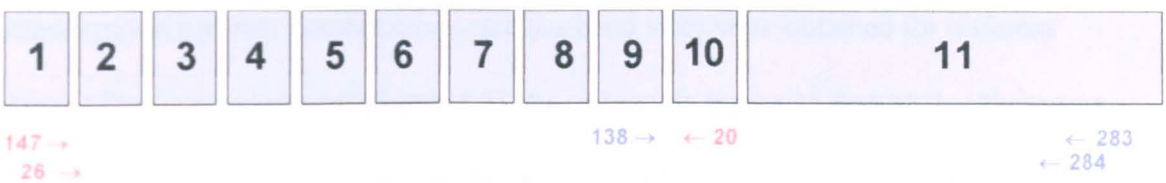


Figure 3-3 Exon structure of LHR gene showing position of primers and direction of primer extension

3.5 Hydrophobicity plots

3.6 Results

PCR with P450scc primers resulted in a band of the expected length for each of the ages looked at (Fig 3.4). The bands did vary in intensity although this was not a quantitative assay. Amplification of the extracellular region of the LHR resulted in a band of the expected size for that particular primer combination in every age group. This indicated that full-length extracellular domain was being expressed in at all ages studied (figure 3.7). Southern blot analysis (figure 3.8) confirmed that the bands amplified were indeed those belonging to the extracellular region of the mouse LH receptor. With the LHR transmembrane primer combinations, various band sizes were obtained for different stages of testis development (figure 3.5). At embryonic stages 15 and 16 dpc there was no bands of full-length receptor size, but two shorter forms were present. Bands indicative of full-length receptor were detectable from 17dpc; in addition the shorter forms were also present. Southern blot (figure 3.6) analysis confirmed that all the bands detected were indeed those of mouse LH receptor.

Over the different ages, 4 bands of varying sizes were clearly detectable after amplification of the transmembrane region. The bands varied in size from 400bp – 1400bp, the bands also varied in intensity of fluorescence which could be indicative of differences in expression levels.

The four strongest bands were selected and removed from gel (figure 3.9) Sequence analysis of the bands also confirmed that they were mouse LHR and highlighted which areas of the transmembrane region were missing due to alternate splicing.

Band 1 - 393bp

Sequence analysis showed that this transcript lacked 972bp of sequence compared to the expected ful-length sequence. The missing sequence spanned from the end of exon 10 into exon 11 (figure 3.10). This transcript did remain in frame and was therefore a truncated version of the full-length transcript. Stop codon was present after 176bp of rejoined sequence. Hydrophobicity analysis on the translated protein showed this transcript to be extremely hydrophobic in nature after the 3' splice site.

Band 2 - 488bp

Sequence analysis showed that this transcript lacked 877bp of sequence compared to the expected full-length sequence. The missing sequence spanned from the end of exon 10 into exon 11 (figure 3.11). There was a total of 877bp missing from this transcript. Translation analysis revealed that from the rejoining site, this transcipt was out of frame compared to the full-length transcript. A stop codon was present after 95bp of rejoined sequence. Hydrophobicity analysis on the translated protein showed this transcript to be hydrophobic in nature after the 3' splice site.

Band 3 - 945bp

This was the largest of the three spliced transcripts. Sequence analysis showed that this transcript lacked 420bp of sequence compared to the expected full-length sequence. The missing sequence spanned from the end of exon 10 into exon 11 (figure 3.12) There was a total of 420bp missing from this transcript. A stop codon was present after 266bp of rejoined sequence. Like band 2 this transcript was also out of frame with the full-length

transcript. Hydrophobicity analysis on the translated protein showed this transcript to be hydrophilic in nature.

3.6.1 Hydrophobicity plots

All transcripts showed identical hydrophobicity for the first 220 amino acids which span from exons 1-10. After the 3' splice site on exon 10 the amino acids differ with only transcript 1 having the same amino acid sequence as the full-length receptor. Hydrophobicity analysis revealed that the protein nature of exon 11 of this transcript was extremely hydrophobic with most of the amino acids being greater than 2 on the Kyte and Doolittle plot . Bands 2 + 3 also displayed the same extent of hydrophobicity with most of the points being between 0-2.

3.6.2 *Gel Electrophoresis*

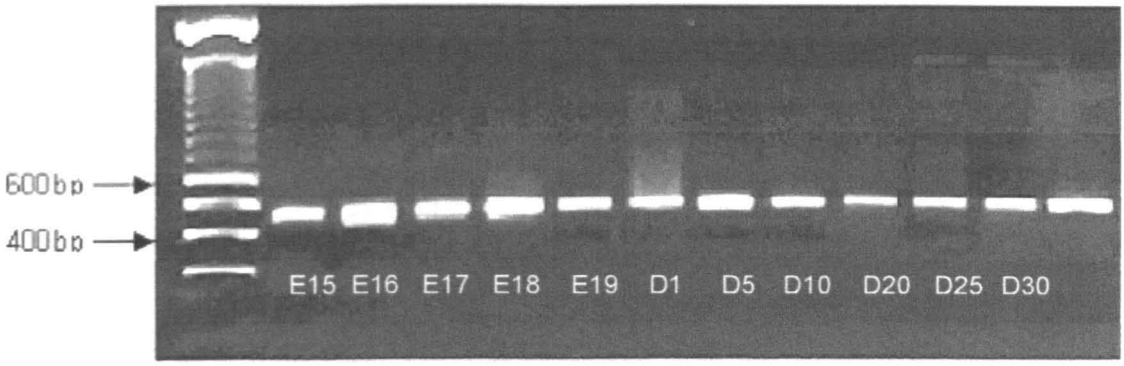


Figure 3-4 Gel image showing bands representing P450 side chain cleavage expression in mouse testes of different ages.

The enzyme p450 side chain cleavage (p450SCC) was included as a positive control for Leydig cell expression

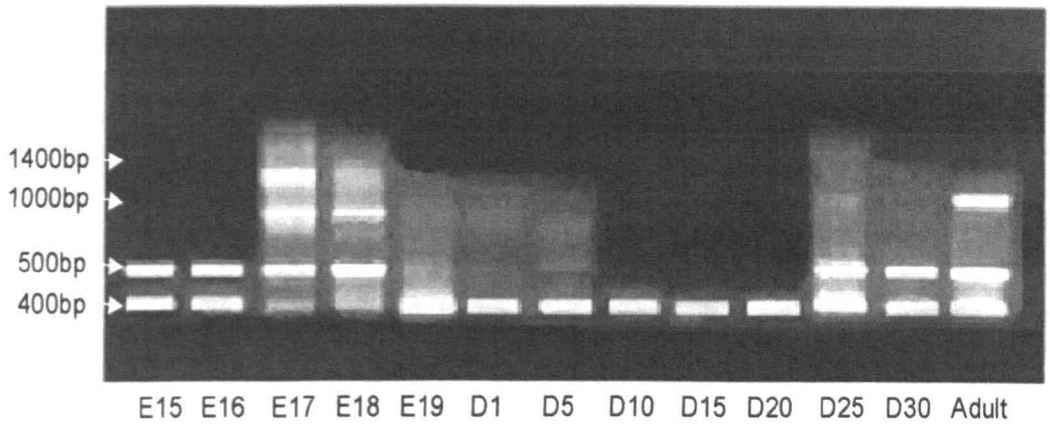


Figure 3-5 Gel picture showing LH receptor bands spanning transmembrane region

These bands were obtained from the primer combination 138 / 283 then nested with 138 / 284 from exons 9 – 11. The expected full-length size was 1365bp

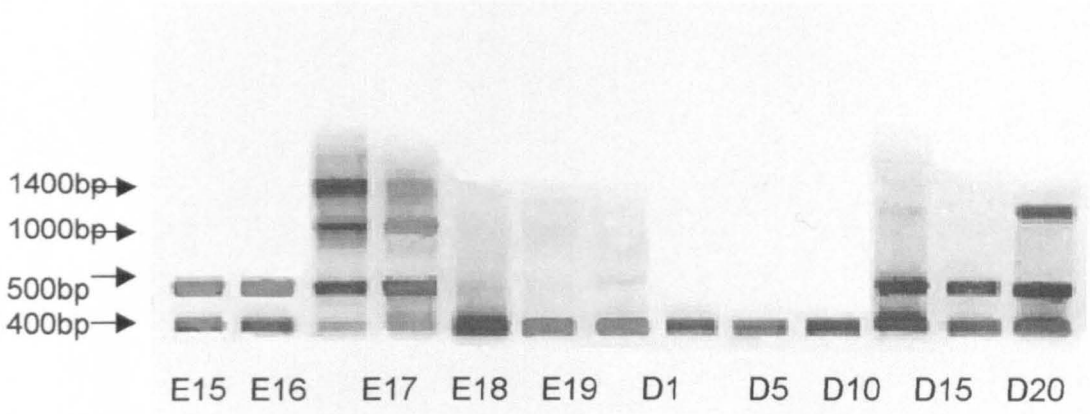


Figure 3-6 Southern blot image of gel showing bands representing transmembrane region

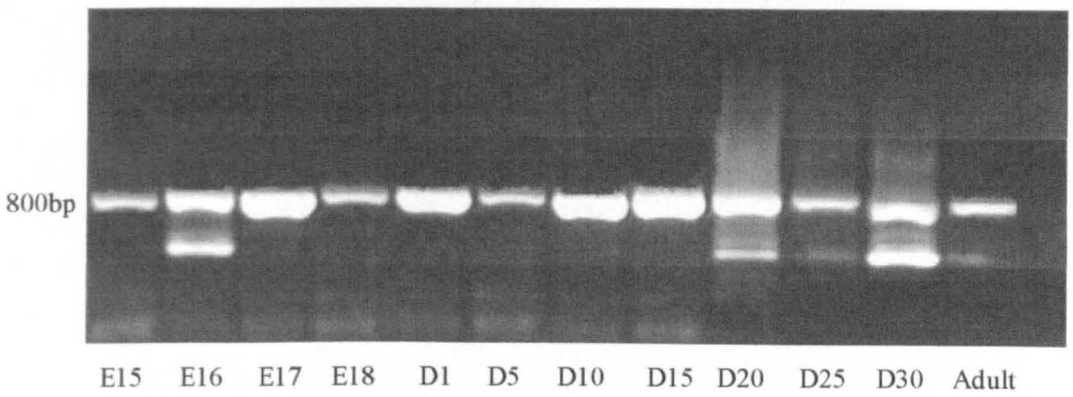


Figure 3-7 Gel 3 showing bands produced with primer combination 282/20 spanning extracellular region, exons 1 – 10.

Expected band size was 780bp

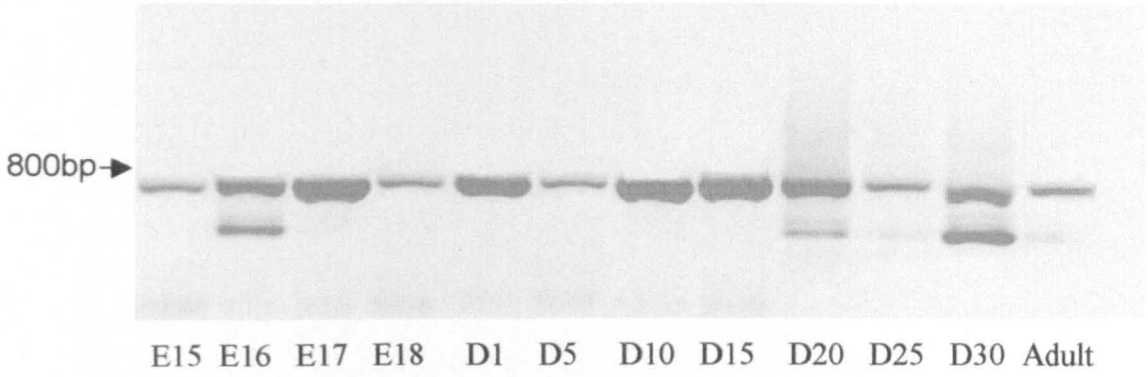


Figure 3-8 Southern blot image of bands representing extracellular region. Expected band size was 780bp

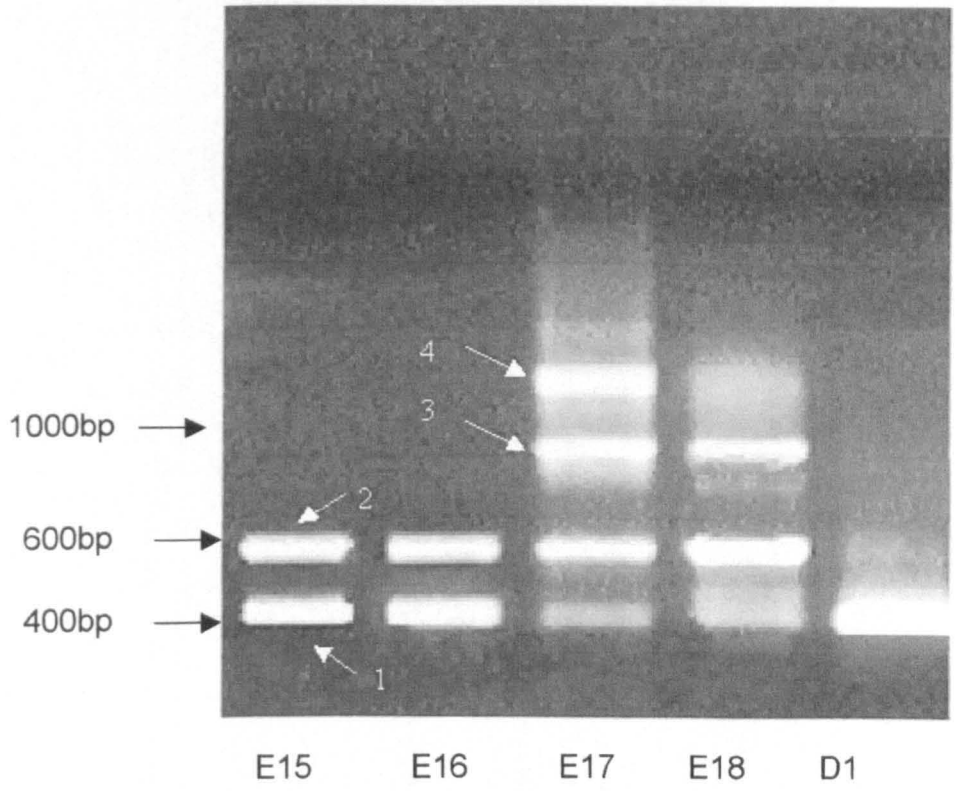


Figure 3-9. LHR bands extracted for sequencing

Band 1 393bp

Band 2 488bp

Band 3 945bp

Band 4 1365bp (full-length receptor)

All gel and southern blot images presented in this thesis are representative of the many results accumulated. PCR's were performed on each age group between 5 and 10 times. Testes from 4-8 animals per age group were used.

3.7 Sequence analysis of bands

Band 1 size 393bp - 972bp missing (highlighted in red), primer positions and intron/exon boundaries are highlighted in blue. PCR primers are highlighted in blue and Real time primers and probe are highlighted in green and under lined.

```
   1  gggccatggg gcggcgggtc ccggctctga gacagctgct ggtgctggca atgctggtgc
  61  tgaagcagtc acagctgcac tctccagagt tgtcagggtc gcgctgccct gagccctgcg
 121  actgcgcgcc ggatggtgcc ctgcgctgcc ctggccctcg agctggcctc gcccgact/at
 181  ctctcaccta tctccctgtc aaagtaatcc catcacaagc tttcagggga cttaatgagg
 241  tcgtaaaaat/ tgaaatctct cagagtgatt ccctggaaag gatagaagct aatgcctttg
 301  acaacctcct caatctgtct gaaat/actga tccagaacac caaaaacctg ctatacattg
 361  aacccggtgc ttttacaaac ctccctcggt taaaatacct /gagcatctgt aacacaggca
 421  tccggaccct cccagatgtt tcgaagatct cttcctctga atttaatttc attct/ggaaa
 481  tctgtgataa cttatacata accaccatac cagggaacgc tttccaaggg atgaatgatg
 541  agtccatcac gct/gaaactg tatggaaatg ggtttgaaga agtacaaagc catgcattca
 601  atgggacgac gctaatctcg ct/ggagttaa aagaaaacat ctacctggag aagatgcaca
 661  gtggcacctt ccaggggccc acggggccca gcatcct/gga tgtctcttcc accaaattgc
 721  aggccctgcc gagccacggg ctggagtcca ttcagacgct catcgccacg tcatcctact
 781  cactgaaaac tctgccctcc agagaaaaat tcaccagcct actggttgcc acgctgacct
 841  accctagcca ctgctgtgct ttcaggaatt tgccgaagaa aga/acagaat ttttcatttt
 901  ccatttttga aaacttttcc aaacaatgtg aaagcacagt tagagaagcg aataacgag
 961  acgct/ttattctgccatctt tgaggagaat gaactcagtg gctgggatta cgattatgact
1021  tctgttcacc caagacactc caatgtactc cagaaccaga tgctttcaat ccctgtgaag
1081  atattatggg ctatgccttc cttagggtgt tgatttggct aattaatata ctagccatct
1141  ttggcaactt gacagtcctc tttgttctcc tgaccagtcg ttataaactg acggtgcccc
1201  gcttcctcat gtgtaatctc tcctttgcag acttttgcat ggggctctac ctgctgctca
1261  ttgcctcagt ggactcccaa acaaaaggcc agtactataa ccatgccata gactggcaga
1321  cagggagtgg ctgcagtgca gctggcttct ttactgtgtt cgccagtgaa ctttctgtct
1381  atacccttac agtcatcact ctggaaaggt ggcacaccat cacctatgct gttcagctgg
1441  accaaaagct gaggctgaga catgccatcc caattatgct cggaggatgg atttttttcta
1501  ccctgatggc cacattgccc cttgtgggtg tcagcagtta catgaaagtc agcatctgcc
1561  tccccatgga tgtggaatcc actctgtcac aagtctacat attatccatc ttgctcctca
1621  atgcagtggc ctttgtcgtc atctgtgctt gctacgttag gatatacttt gcagttcaaa
1681  atccagagct gacggctcct aacaaggaca caaaaattgc taagaagatg gccatcctca
1741  tcttcacaga cttcacatgc atggcaccca tctcattctt tgccatctca gctgccttca
1801  aagtaccccc tatcactgtc accaactcaa aagttctgct ggtccttttt tatcctgtca
1861  attcttgtgc caacccattt ctgtacgcag tgttcacgaa ggcatttcag agagatttct
1921  ttctcttgct gagcagattt ggttgctgta agcaccgggc tgaactttac agaaggaagg
1981  aattttctgc atgtaccttc aactccaaaa acggctttcc aagatcaagt aagccttccc
2041  aggctgccct gaagttatcc atagtgcact gtcaacaacc tacacctcca agagtgttaa
2101  ttcagtaact gcattactga attgtatcta aatatgttcc ccccaaaaaa gtccacccaa
2161  attagtcact ttaatataat gtgttttaga aaaaatattt atctttaagc acttcaggtg
2221  aattgaaacc tgcttcaaag ggtggcccaa gacacttggt gacataaatt tcagaagggt
2281  ttagaaaatt ttttataata atttagaaag aatagttttt gtttgttgaa tctaatatta
2341  agaaaatcta agttgttgtc attttccatg tctcttgatc tttttcactt caatctgtga
2401  ttcatgttgc catctctaaa tatatattca taacagactg gaaatttaaa gtggtctttg
2461  tcctcagata gtttgataaa tacattcaag agatgcactg tgcagcgtga tagctgttag
2521  ccttacatgg taaataaaag tttcttagcc ataaaaaaaa aaaaaaaaa
```

Figure 3-10 Sequence of LHR transcript band 1

Band 2 - size 488bp - 877bp missing

```
   1 gggccatggg gcggcgggtc ccggctctga gacagctgct ggtgctggca atgctggtgc
  61 tgaagcagtc acagctgcac tctccagagt tgtcagggtc gcgctgccct gagccctgcg
 121 actgcgcgcc ggatggtgcc ctgcgctgcc ctggccctcg agctggcctc gcccgact/at
 181 ctctcaccta tctccctgtc aaagtaatcc catcacaagc tttcagggga cttaatgagg
 241 tcgtaaaaat/ tgaaatctct cagagtgatt ccctggaaag gatagaagct aatgcctttg
 301 acaacctcct caatctgtct gaaat/actga tccagaacac caaaaacctg ctatacattg
 361 aacccggtgc ttttacaaac ctccctcggt taaaatacct /gagcatctgt aacacaggca
 421 tccggaccct cccagatgtt tcgaagatct cttcctctga atttaatttc attct/ggaaa
 481 tctgtgataa cttatacata accaccatac cagggaacgc tttccaaggg atgaataatg
 541 agtccatcac gct/gaaactg tatggaaatg ggtttgaaga agtacaaagc catgcattca
 601 atgggacgac gctaatctcg ct/ggagttaa aagaaaacat ctacctggag aagatgcaca
 661 gtggcacctt ccaggggccc acggggccca gcatcct/gga tgtctcttcc accaaattgc
 721 aggccctgcc gagccacggg ctggagtcca ttcagacgct catcgccacg tcatcctact
 781 cactgaaaac tctgccctcc agagaaaaat tcaccagcct actggttgcc acgctgacct
 841 accctagcca ctgctgtgct ttcaggaatt tgccgaagaa aga/acagaat ttttcatttt
 901 ccatttttga aaacttttcc aaacaatgtg aaagcacagt tagagaagcg aataacgag
 961 acgct/ttattc tgccatcttt gaggagaatg aactcagtgg ctgggattacgattatgact
1021 tctgttcacc caagacactc caatgtactc cagaaccaga tgctttcaat ccctgtgaag
1081 atattatggg ctatgccttc cttagggtgt tgatttggct aattaatata ctagccatct
1141 ttggcaactt gacagtcctc tttgttctcc tgaccagtcg ttataaactg acggtgcccc
1201 gcttcctcat gtgtaatctc tcctttgcag acttttgcat ggggctctac ctgctgctca
1261 ttgcctcagt ggactcccaa acaaaaggcc agtactataa ccatgccata gactggcaga
1321 cagggagtgg ctgcagtgca gctggcttct ttactgtgtt cgccagtgaa ctttctgtct
1381 atacccttac agtcatcact ctggaaaggt ggcacaccat cacctatgct gttcagctgg
1441 accaaaagct gaggctgaga catgccatcc caattatgct cggaggatgg atttttctta
1501 ccctgatggc cacattgccc cttgtgggtg tcagcagtta catgaaagtc agcatctgcc
1561 tccccatgga tgtggaatcc actctgtcac aagtctacat attatccatc ttgctcctca
1621 atgcagtggc ctttgtcgtc atctgtgctt gctacgttag gatatacttt gcagttcaaa
1681 atccagagct gacggctcct aacaaggaca caaaaattgc taagaagatg gccatcctca
1741 tcttcacaga cttcacatgc atggcaccca tctcattctt tgccatctca gctgccttca
1801 aagtaccccc tatcactgtc accaactcaa aagttctgct ggtccttttt tatcctgtca
1861 attcttgtgc caacccattt ctgtacgcag tgttcacgaa ggcatttcag agagatttct
1921 ttctcttgct gagcagattt ggttgctgta agcaccgggc tgaactttac agaaggaagg
1981 aattttctgc atgtaccttc aactccaaaa acggctttcc aagatcaagt aagccttccc
2041 aggctgccct gaagttatcc atagtgcact gtcaacaacc tacacctcca agagtgttaa
2101 ttcagtaact gcattactga attgtatcta aatatgttcc ccccaaaaaa gtccacccaa
2161 attagtcact ttaatataat gtgttttaga aaaaatattt atctttaagc acttcaggtg
2221 aattgaaacc tgcttcaaag ggtggcccaa gacacttggt gacataaatt tcagaagggt
2281 ttagaaaatt ttttataata atttagaaag aatagttttt gtttgttgaa tctaatatta
2341 agaaaatcta agttgttgtc attttccatg tctcttgatc tttttcactt caatctgtga
2401 ttcatgttgc catctctaaa tatatattca taacagactg gaaatttaaa gtggtctttg
2461 tcctcagata gtttgataaa tacattcaag agatgcactg tgcagcgtga tagctgttag
2521 ccttacatgg taaataaaag tttcttagcc ataaaaaaaa aaaaaaaaa
```

Figure 3-11 Sequence of LHR transcript band 2

Band size 3 size 945bp - 420bp missing

```
   1 gggccatggg gcggcgggtc ccggctctga gacagctgct ggtgctggca atgctggtgc
  61 tgaagcagtc acagctgcac tctccagagt tgtcagggtc gcgctgccct gagccctgcg
 121 actgcgcgcc ggatggtgcc ctgcgctgcc ctggccctcg agctggcctc gcccgact/at
 181 ctctcaccta tctccctgtc aaagtaatcc catcacaagc tttcagggga cttaatgagg
 241 tcgtaaaaat/ tgaaatctct cagagtgatt ccctggaaag gatagaagct aatgcctttg
 301 acaacctcct caatctgtct gaaat/actga tccagaacac caaaaacctg ctatacattg
 361 aacccggtgc ttttacaaac ctccctcggt taaaatacct /gagcatctgt aacacaggca
 421 tccggaccct cccagatgtt tcgaagatct cttcctctga atttaatttc attct/ggaaa
 481 tctgtgataa cttatacata accaccatac cagggaacgc tttccaaggg atgaataatg
 541 agtccatcac gct/gaaactg tatggaaatg ggtttgaaga agtacaaagc catgcattca
 601 atgggacgac gctaatctcg ct/ggagttaa aagaaaacat ctacctggag aagatgcaca
 661 gtggcacctt ccaggggccc acggggccca gcatcct/gga tgtctcttcc accaaattgc
 721 aggccctgcc gagccacggg ctggagtcca ttcagacgct catcgccacg tcatcctact
 781 cactgaaaac tctgccctcc agagaaaaat tcaccagcct actggttgcc acgctgacct
 841 accctagcca ctgctgtgct ttcaggaatt tgccgaagaa aga/acagaat ttttcatttt
 901 ccatttttga aaacttttcc aaacaatgtg aaagcacagt tagagaagcg aataacgag
 961 acgct/ttattctgccatcttt gaggagaatg aactcagtgg ctgggattac gattatgact
1021 tctgttcacc caagacactc caatgtactc cagaaccaga tgctttcaat ccctgtgaag
1081 atattatggg ctatgccttc cttagggtgt tgatttggct aattaatata ctagccatct
1141 ttggcaactt gacagtcctc tttgttctcc tgaccagtcg ttataaactg acggtgcccc
1201 gcttcctcat gtgtaatctc tcctttgcag acttttgcat ggggctctac ctgctgctca
1261 ttgcctcagt ggactcccaa acaaaaggcc agtactataa ccatgccata gactggcaga
1321 cagggagtgg ctgcagtgca gctggcttct ttactgtgtt cgccagtgaa ctttctgtct
1381 atacccttac agtcatcact ctggaaaggt ggcacaccat cacctatgct gttcagctgg
1441 accaaaagct gaggctgaga catgccatcc caattatgct cggaggatgg atttttctta
1501 ccctgatggc cacattgccc cttgtgggtg tcagcagtta catgaaagtc agcatctgcc
1561 tccccatgga tgtggaatcc actctgtcac aagtctacat attatccatc ttgctcctca
1621 atgcagtggc ctttgtcgtc atctgtgctt gctacgttag gatatacttt gcagttcaaa
1681 atccagagct gacggctcct aacaaggaca caaaaattgc taagaagatg gccatcctca
1741 tcttcacaga cttcacatgc atggcaccca tctcattctt tgccatctca gctgccttca
1801 aagtacccct tatcactgtc accaactcaa aagttctgct ggtccttttt tatcctgtca
1861 attcttgtgc caacccattt ctgtacgcag tgttcacgaa ggcatttcag agagatttct
1921 ttctcttgct gagcagattt ggttgctgta agcaccgggc tgaactttac agaaggaagg
1981 aattttctgc atgtaccttc aactccaaaa acggctttcc aagatcaagt aagccttccc
2041 aggctgccct gaagttatcc atagtgcact gtcaacaacc tacacctcca agagtgttaa
2101 ttcagtaact gcattactga attgtatcta aatatgttcc ccccaaaaaa gtccacccaa
2161 attagtcact ttaatataat gtgttttaga aaaaatattt atctttaagc acttcaggtg
2221 aattgaaacc tgcttcaaag ggtggcccaa gacacttggt gacataaatt tcagaagggt
2281 ttagaaaatt ttttataata atttagaaag aatagttttt gtttgttgaa tctaatatta
2341 agaaaatcta agttgttgtc attttccatg tctcttgatc tttttcactt caatctgtga
2401 ttcatgttgc catctctaaa tatatattca taacagactg gaaatttaaa gtggtctttg
2461 tcctcagata gtttgataaa tacattcaag agatgcactg tgcagcgtga tagctgttag
2521 ccttacatgg taaataaaag tttcttagcc ataaaaaaaa aaaaaaaaa
```

Figure 3-12 Sequence of LHR transcript band 3

3.7.1 Translated transcript 1 Band size 393bp

```
      gggccatggggcggcgggtcccggctctgagacagctgctggtgctggcaatgctggtgc
    1 ---------+---------+---------+---------+---------+---------+
        A  M  G  R  R  V  P  A  L  R  Q  L  L  V  L  A  M  L  V  L
      tgaagcagtcacagctgcactctccagagttgtcagggtcgcgctgccctgagccctgcg
   61 ---------+---------+---------+---------+---------+---------+
        K  Q  S  Q  L  H  S  P  E  L  S  G  S  R  C  P  E  P  C  D
      actgcgcgccggatggtgccctgcgctgccctggccctcgagctggcctcgcccgactat
  121 ---------+---------+---------+---------+---------+---------+
        C  A  P  D  G  A  L  R  C  P  G  P  R  A  G  L  A  R  L  S
      ctctcacctatctccctgtcaaagtaatcccatcacaagctttcaggggacttaatgagg
  181 ---------+---------+---------+---------+---------+---------+
        L  T  Y  L  P  V  K  V  I  P  S  Q  A  F  R  G  L  N  E  V
      tcgtaaaaattgaaatctctcagagtgattccctggaaaggatagaagctaatgcctttg
  241 ---------+---------+---------+---------+---------+---------+
        V  K  I  E  I  S  Q  S  D  S  L  E  R  I  E  A  N  A  F  D
      acaacctcctcaatctgtctgaaatactgatccagaacaccaaaaacctgctatacattg
  301 ---------+---------+---------+---------+---------+---------+
        N  L  L  N  L  S  E  I  L  I  Q  N  T  K  N  L  L  Y  I  E
      aacccggtgcttttacaaacctccctcggttaaaatacctgagcatctgtaacacaggca
  361 ---------+---------+---------+---------+---------+---------+
        P  G  A  F  T  N  L  P  R  L  K  Y  L  S  I  C  N  T  G  I
      tccggaccctcccagatgtttcgaagatctcttcctctgaatttaatttcattctggaaa
  421 ---------+---------+---------+---------+---------+---------+
        R  T  L  P  D  V  S  K  I  S  S  S  E  F  N  F  I  L  E  I
      tctgtgataacttatacataaccaccataccagggaacgctttccaagggatgaataatg
  481 ---------+---------+---------+---------+---------+---------+
        C  D  N  L  Y  I  T  T  I  P  G  N  A  F  Q  G  M  N  N  E
      agtccatcacgctgaaactgtatggaaatgggtttgaagaagtacaaagccatgcattca
  541 ---------+---------+---------+---------+---------+---------+
        S  I  T  L  K  L  Y  G  N  G  F  E  E  V  Q  S  H  A  F  N
      atgggacgacgctaatctcgctggagttaaaagaaaacatctacctggagaagatgcaca
  601 ---------+---------+---------+---------+---------+---------+
        G  T  T  L  I  S  L  E  L  K  E  N  I  Y  L  E  K  M  H  S
      gtggcaccttccaggggccacggggcccagcatcctggatgtctcttccaccaaattgc
  661 ---------+---------+---------+---------+---------+---------+
        G  T  F  Q  G  A  T  G  P  S  I  L  D  V  S  S  T  K  L  Q
      aggccctgccgagccacgggctggagtccattcagacgctcatcgccacgtcatcctact
  721 ---------+---------+---------+---------+---------+---------+
        A  L  P  S  H  G  L  E  S  I  Q  T  L  I  A  T  S  S  Y  S
      cactgaaaactctgccctccagagaaaaattcaccagcctactggttgccacgctgacct
  781 ---------+---------+---------+---------+---------+---------+
        L  K  T  L  P  S  R  E  K  F  T  S  L  L  V  A  T  L  T  Y
      accctagccactgctgtgctttcaggaatttgccgaagaaagaacagaatttttcatttt
  841 ---------+---------+---------+---------+---------+---------+
        P  S  H  C  C  A  F  R  N  L  P  K  K  E  Q  N  F  S  F  S
      ccatttttgaaaactttccaaacaatgtgaaagcacagttagagaagcgaataacgaga
  901 ---------+---------+---------+---------+---------+---------+
        I  F  E  N  F  S  K  Q  C  E  S  T  V  R  E  A  N  N  E  T
      cgctatttggttgctgtaagcaccgggctgaactttacagaaggaaggaatttttctgcat
  961 ---------+---------+---------+---------+---------+---------+
        L  F  G  C  C  K  H  R  A  E  L  Y  R  R  K  E  F  S  A  C
      gtaccttcaactccaaaaacggctttccaagatcaagtaagccttcccaggctgccctga
 1021 ---------+---------+---------+---------+---------+---------+
        T  F  N  S  K  N  G  F  P  R  S  S  K  P  S  Q  A  A  L  K
      agttatccatagtgcactgtcaacaacctacacctccaagagtgttaattcagtaactgcatt
 1081 ---------+---------+---------+---------+---------+---------+
        L  S  I  V  H  C  Q  Q  P  T  P  P  R  V  L  I  Q  *
```

Figure 3-13 Translation of transcript 1

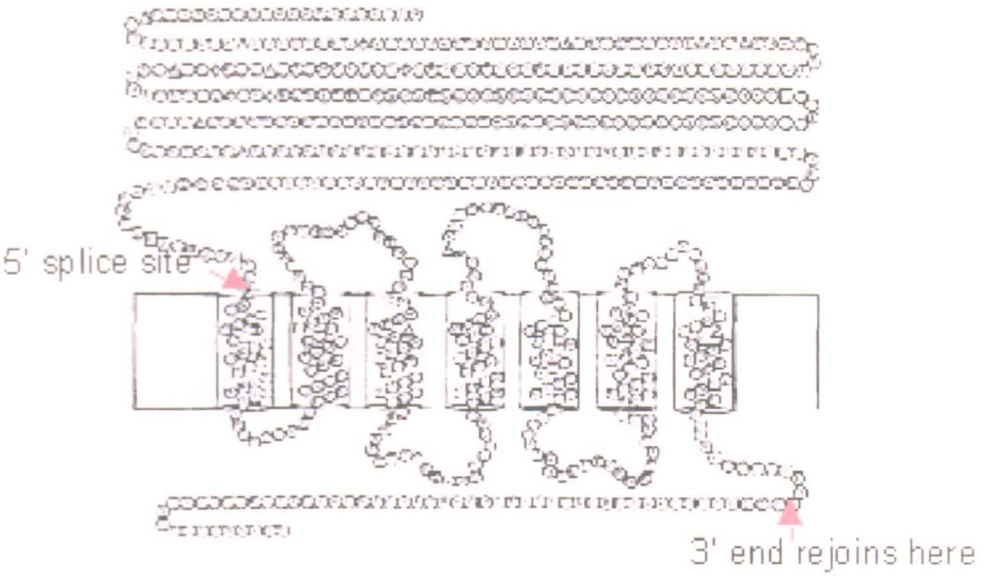


Figure 3-14 Diagram showing spice sites of transcipt 1

3.7.2 Translated Transcript 2 Band size 488bp

```
     gggccatggggcggcgggtcccggctctgagacagctgctggtgctggcaatgctggtgc
   1 ---------+---------+---------+---------+---------+---------+
       A  M  G  R  R  V  P  A  L  R  Q  L  L  V  L  A  M  L  V  L
     tgaagcagtcacagctgcactctccagagttgtcagggtcgcgctgccctgagccctgcg
  61 ---------+---------+---------+---------+---------+---------+
       K  Q  S  Q  L  H  S  P  E  L  S  G  S  R  C  P  E  P  C  D
     actgcgcgccggatggtgccctgcgctgccctggccctcgagctggcctcgcccgactat
 121 ---------+---------+---------+---------+---------+---------+
       C  A  P  D  G  A  L  R  C  P  G  P  R  A  G  L  A  R  L  S
     ctctcacctatctccctgtcaaagtaatcccatcacaagctttcaggggacttaatgagg
 181 ---------+---------+---------+---------+---------+---------+
       L  T  Y  L  P  V  K  V  I  P  S  Q  A  F  R  G  L  N  E  V
     tcgtaaaaattgaaatctctcagagtgattccctggaaaggatagaagctaatgcctttg
 241 ---------+---------+---------+---------+---------+---------+
       V  K  I  E  I  S  Q  S  D  S  L  E  R  I  E  A  N  A  F  D
     acaacctcctcaatctgtctgaaatactgatccagaacaccaaaaacctgctatacattg
 301 ---------+---------+---------+---------+---------+---------+
       N  L  L  N  L  S  E  I  L  I  Q  N  T  K  N  L  L  Y  I  E
     aacccggtgcttttacaaacctccctcggttaaaatacctgagcatctgtaacacaggca
 361 ---------+---------+---------+---------+---------+---------+
       P  G  A  F  T  N  L  P  R  L  K  Y  L  S  I  C  N  T  G  I
     tccggaccctcccagatgtttcgaagatctcttcctctgaatttaatttcattctggaaa
 421 ---------+---------+---------+---------+---------+---------+
       R  T  L  P  D  V  S  K  I  S  S  S  E  F  N  F  I  L  E  I
     tctgtgataacttatacataaccaccataccagggaacgctttccaagggatgaataatg
 481 ---------+---------+---------+---------+---------+---------+
       C  D  N  L  Y  I  T  T  I  P  G  N  A  F  Q  G  M  N  N  E
     agtccatcacgctgaaactgtatggaaatgggtttgaagaagtacaaagccatgcattca
 541 ---------+---------+---------+---------+---------+---------+
       S  I  T  L  K  L  Y  G  N  G  F  E  E  V  Q  S  H  A  F  N
     atgggacgacgctaatctcgctggagttaaaagaaaacatctacctggagaagatgcaca
 601 ---------+---------+---------+---------+---------+---------+
       M  T  T  L  I  S  L  E  L  K  E  N  I  Y  L  E  K  M  H  S
     gtggcaccttccaggggccacggggcccagcatcctggatgtctcttccaccaaattgc
 661 ---------+---------+---------+---------+---------+---------+
       G  T  F  Q  G  A  T  G  P  S  I  L  D  V  S  S  T  K  L  Q
```

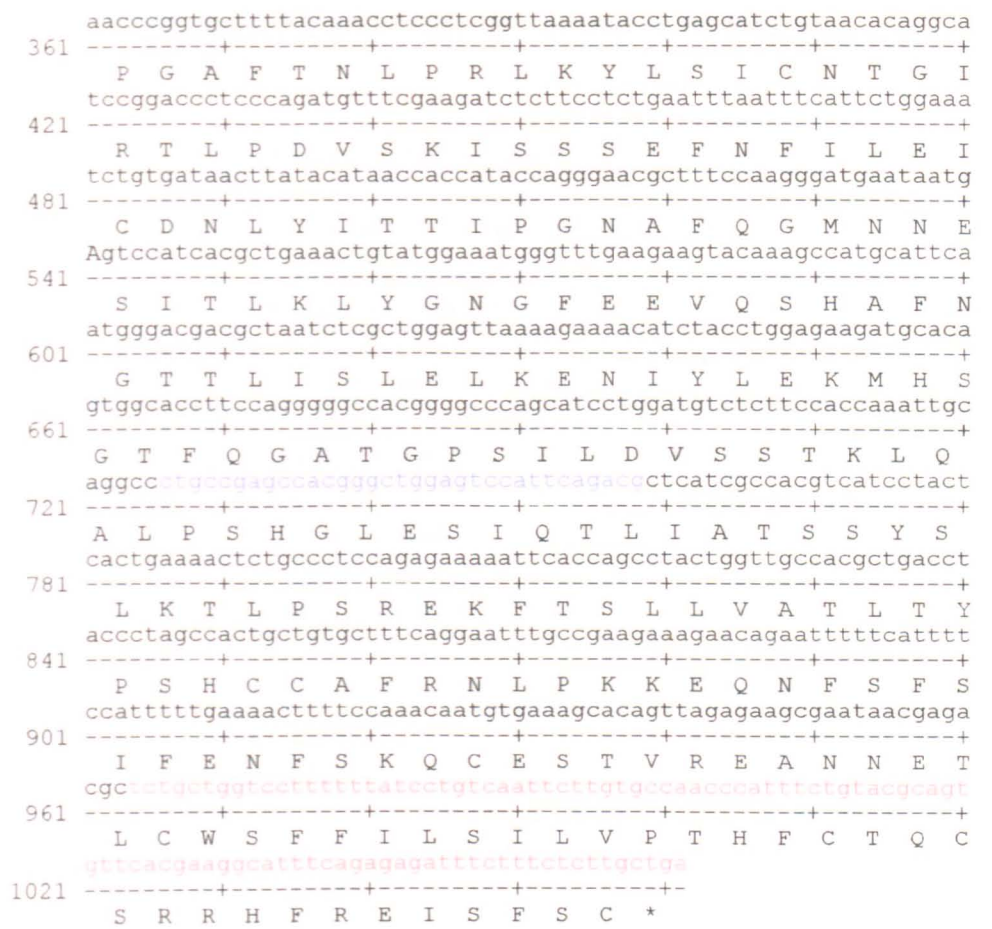


Figure 3-15 Translation of transcript 2

3.7.2 *Translated transcript 3 - Band size 945bp*

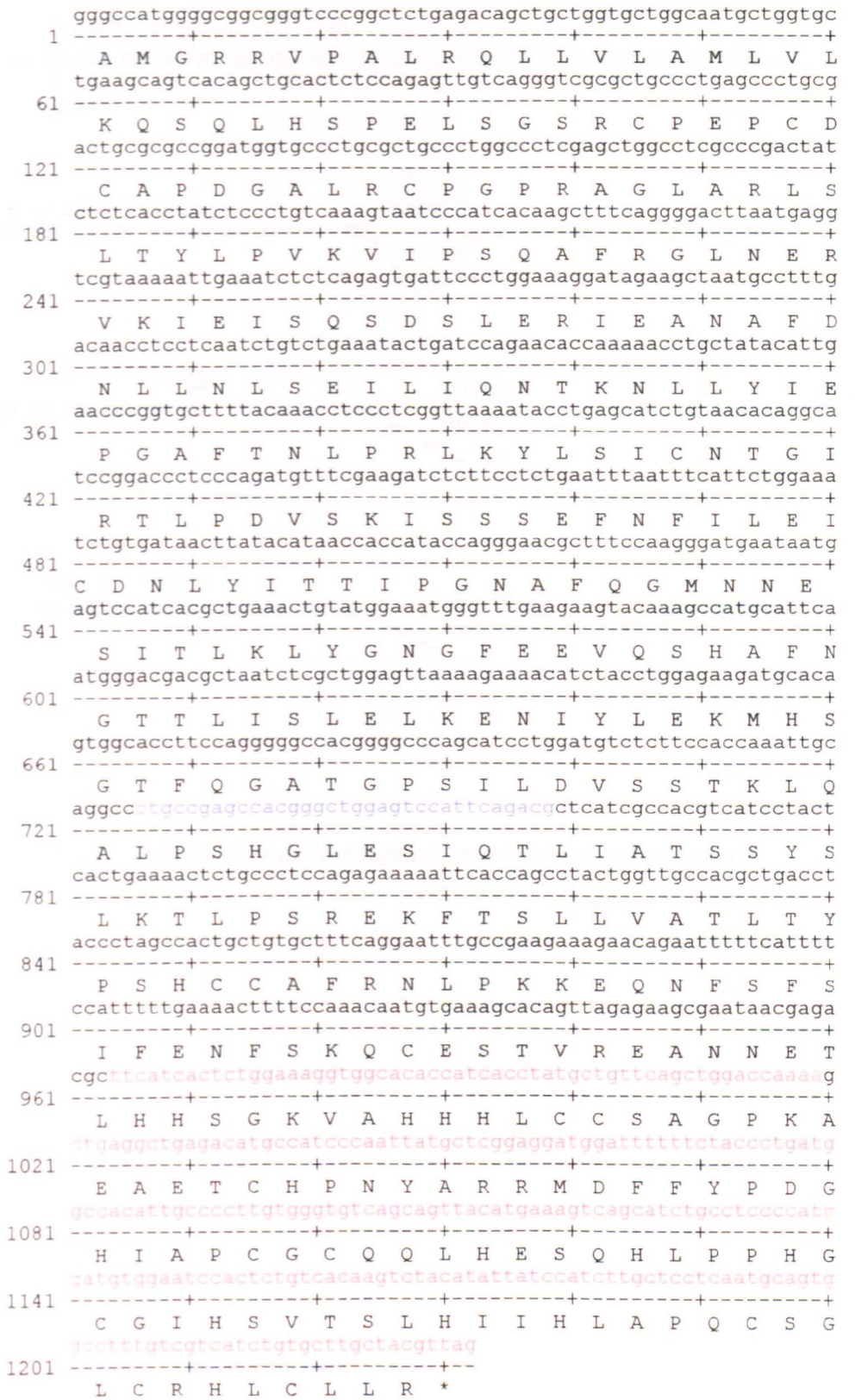


Figure 3-16 Translation of transcript 3

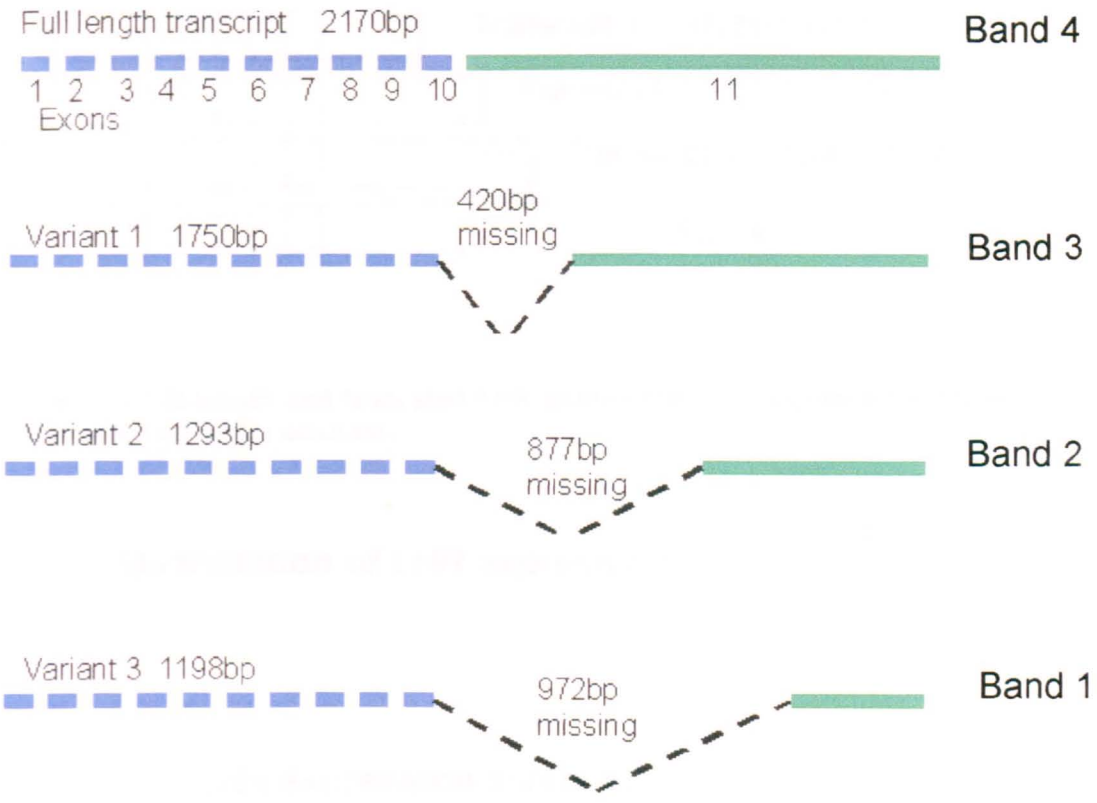


Figure 3-17 LHR transcripts highlighting spliced regions from exon 11

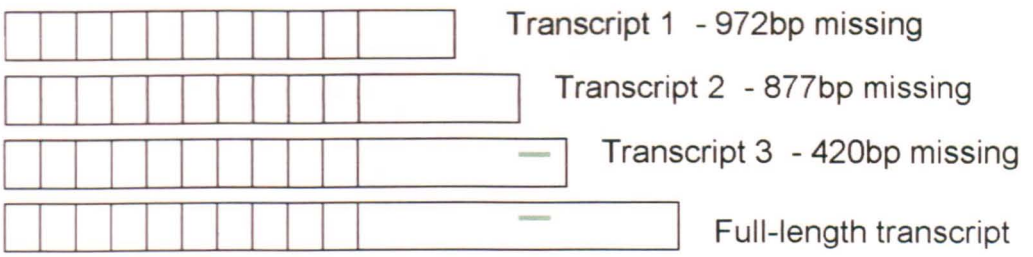


Figure 3-18 full-length and truncated LHR transcripts — signifies Real time PCR primer and probe position

3.7.3 Quantitation of LHR expression

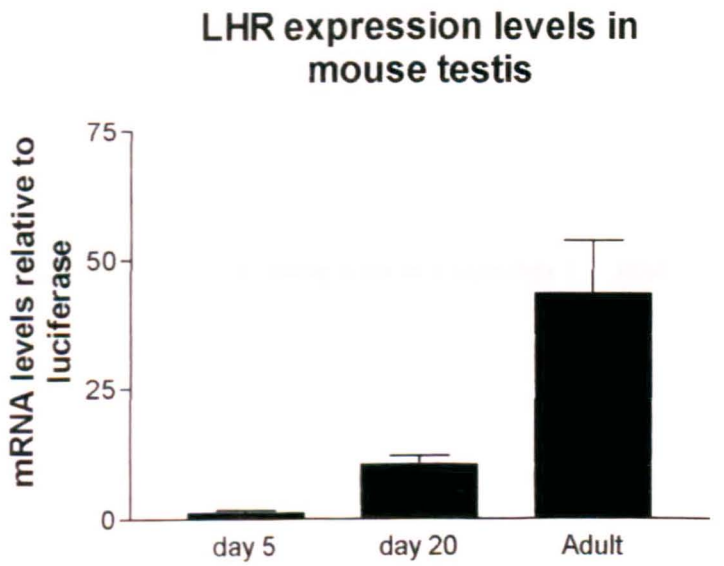


Figure 3-19 Real Time PCR Quantitation of LHR expression levels relative to luciferase

3.7.4 *Hydrophobicity Plots*

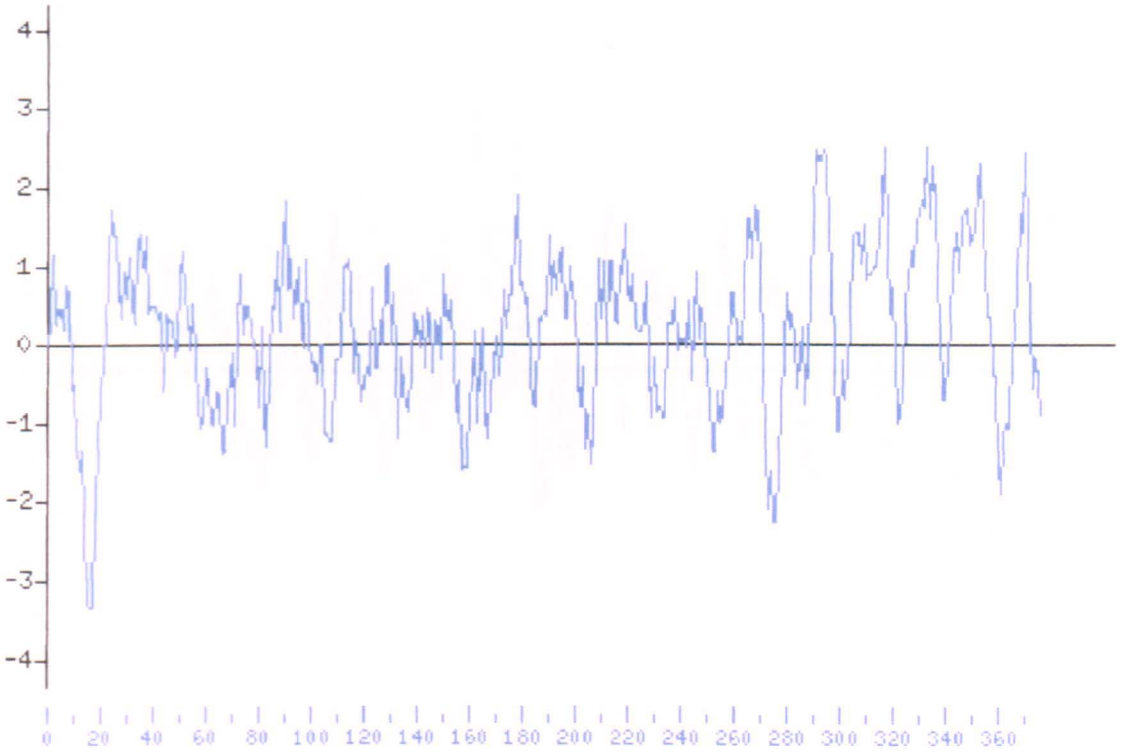


Figure 3-20 Hydrophobicity plot of transcript 1 - 393bp

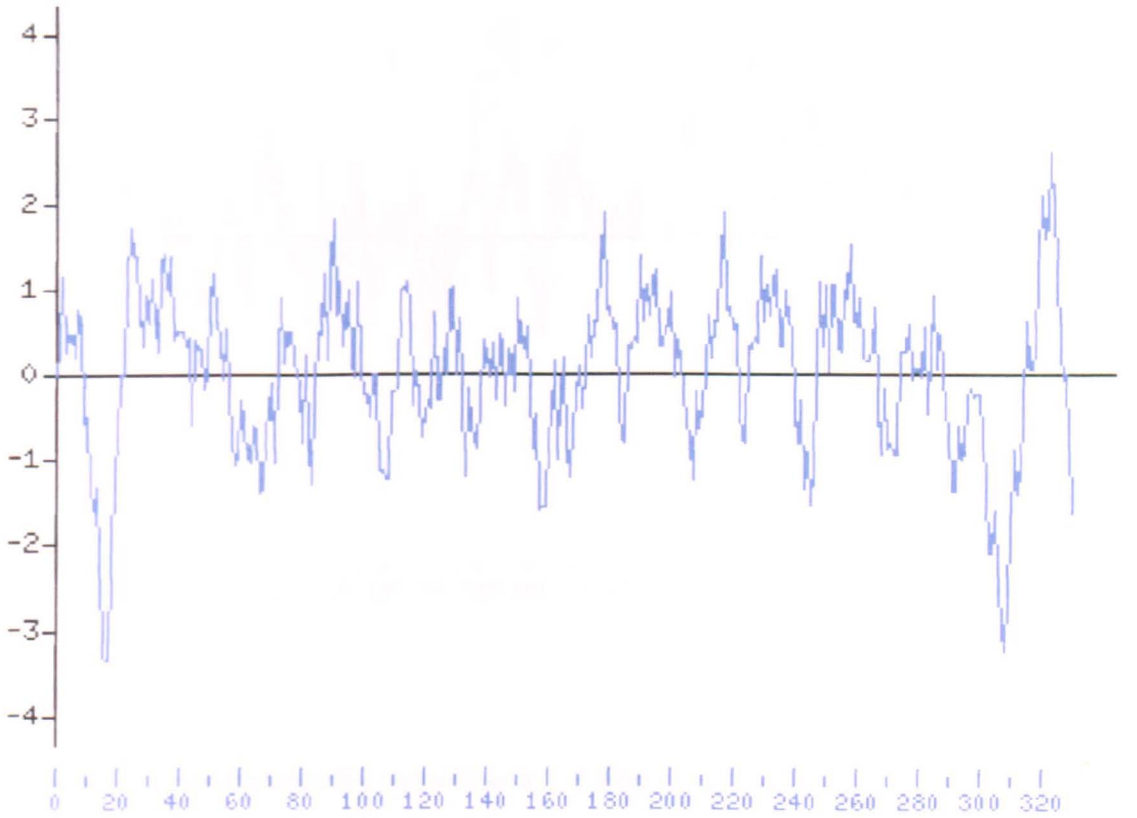


Figure 3-21 Hydrophobicity Plot of transcript 2 - 488bp

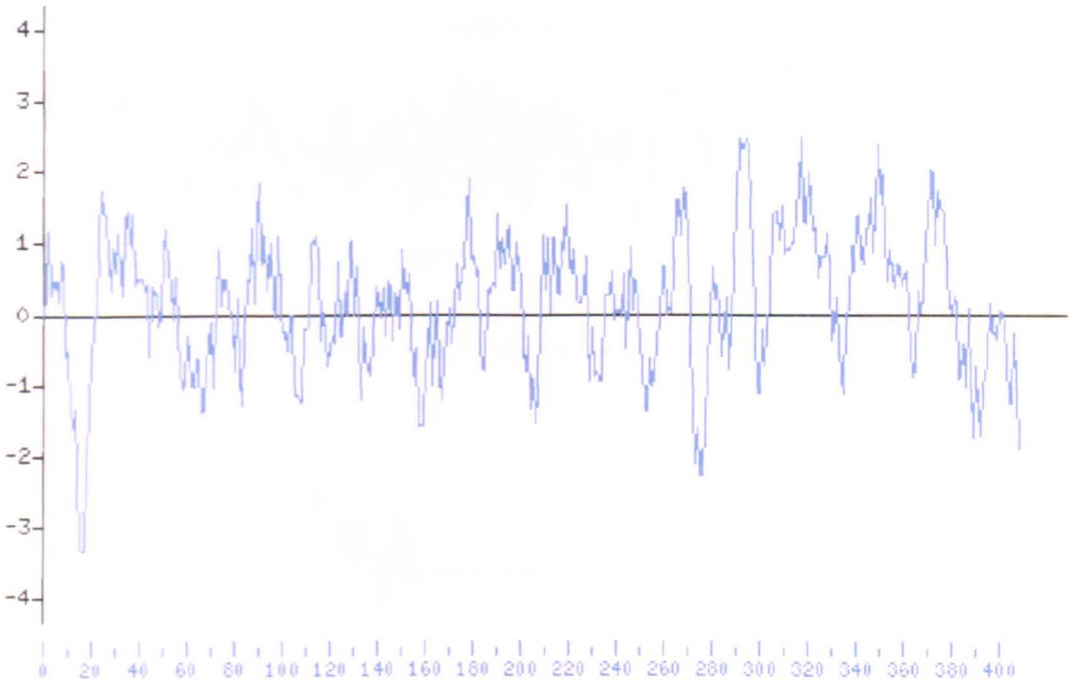


Figure 3 -22 Hydrophobicity plot of band 3 945bp

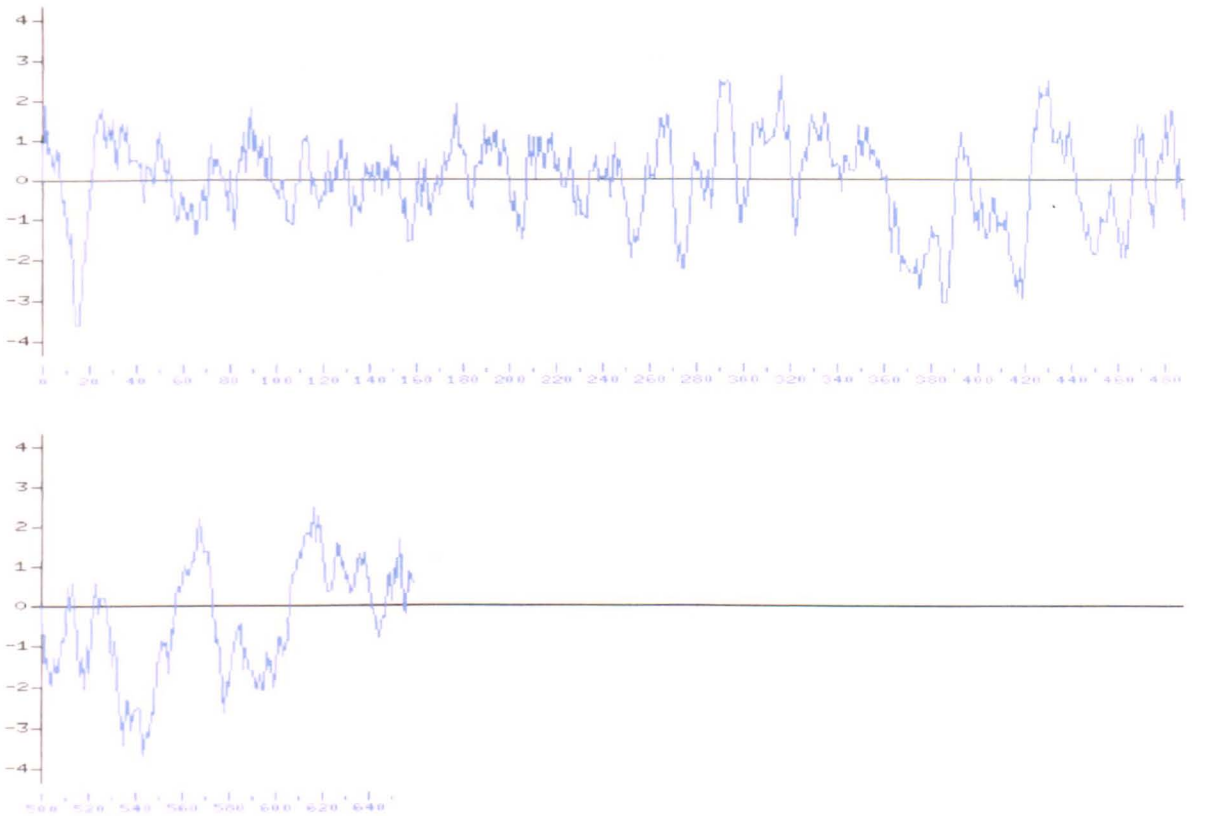


Figure 3-23Hydrophobicity Plot of full-length LH receptor

3.8 Discussion

Alternate splicing is a common phenomenon in seven transmembrane (7TM) receptors. Most of the alternate splicing occurs in the C terminus of the 7TM receptor. The extent of splice variants depends on the complexity of the gene structure, and in certain cases variant isoforms may be species specific (Reviewd by Kilpatrick *et al* 1999) There is also evidence of temporal differences in expression of 7TM receptor splice variants (Minakami *et al* 1995).

Multiple LH receptor transcripts have been detected in mammalian species such as pig (Loosfelt *et al* 1989), rat (Segaloff *et al* 1990) and avian species like turkey (You *et al* 2000). This suggests a common mechanism that may be evolutionary conserved (at least between mammalian and avian species) for the production of multiple soluble or secreted proteins from a single LHR gene due to alternate splicing to produce proteins which contain only the ligand binding domain.

Sequence analysis of the three spliced transcripts made it possible to determine exactly which region of the gene was missing. It revealed in all three cases that the 5' splice site was identical and occurred after 964bp with the consensus sequence cgct. The 3' splice region differed in all cases. Protein analysis of the transcripts revealed that only the smallest transcript, represented by band 1 continued with the same open reading frame as the full-length mouse LHR, this transcript lacked the 7 transmembrane proteins, but contained the same carboxy terminal. Sequence and protein analysis of transcripts 2 and 3 were revealed that they both contained a frame shift, whereby the amino acid sequence after the 3' splice site differed from that of the full-length receptor.

These results are in complete agreement with previous findings from work carried out on the pig LHR. This group also identified three smaller truncated forms of the receptor and in all cases the N terminal (hormone binding region) was identical and intact. Two of the variants they identified displayed a frame shift after the point of junction and thus were devoid of the putative transmembrane and intracellular domains. The third variant however conserved the same open reading frame after point of divergence and although it lacked the transmembrain domain, it still contained the putative carboxy terminal intracellular domain of the full-length receptor (Loosefelt *et al* 1989).

At the time this study commenced one of the main objectives was to determine if these truncated forms of the LH receptor played an important role in development and function of the testes. With the introduction of the LHR knock out mouse however the role of the LHR was clearly elucidated. This mouse contained a mutation in the LHR resulting in a frame shift, which completely knocked out exon 11, the transmembrane and intracellular coding region of the receptor. Complete absence of these regions suggests there would be no intracellular signalling initiation second messenger systems.

Morphologocal analysis of the gonadal and urogenital system of the LHR-null mouse at birth revealed that LHR action was not required for fetal development and function as there were no differences when compared to normal mice. It would appear that only after birth was there a requirement for expression of a functional LH receptor. This was apparent with the changes observed in the LHR-null mouse, such as reduction in testes size, androgen production and also a reduction in seminiferous tubules, accessory ducts and glands. Growth of these structures are known to be androgen dependant events so therefore it can be assumed that after birth the Leydig cells become dependant on

expression of a functional LH receptor for the production of androgen (Zhang *et al* 2001)

It is clear that in addition to the full-length LHR transcript, spliced variants of the LH receptor are expressed in the mouse testis throughout all stages of development from 15dpc. In the early embryonic stages these spliced forms appear to be the dominant form of the receptor. Their precise physiological function though cannot as yet be determined although there are several possibilities.

Splice variants of seven transmembrane proteins are often dismissed as the consequence of 'leaky transcription' and hence deemed physiologically irrelevant. This could well be the case for some variants, especially where the variant is expressed only in low amounts and perhaps does not couple to a second messenger system. However the expression patterns observed with the truncated forms of the LH receptor are remarkably consistent and of high enough levels to be deemed relevant. In addition, because they are present at all stages of development from embryo through to adult, it would seem more likely that they have a specific function to play. Although full-length receptor didn't appear to be expressed in the embryonic testes until 17dpc, this study is not conclusive enough to rule out expression in earlier stages completely. The full-length bands are very strong in the adult, but this may be more due to the fact that there is a far greater Leydig cell number in adult testes. A more sensitive method of detection would have to be deployed before ruling out the presence of full-length transcript expression in the earlier stages.

The truncated forms of the LH receptor gene may play an important role in testicular function but the extent of this is as yet unspecified. There has been a suggestion of a modulatory role in full-length receptor expression. Down-regulation of plasma

membrane receptors by homologous hormones has been found in diverse cell types. In testicular Leydig and ovarian luteal cells for example, treatment with LH/hCG decreases LH receptor content. (LaPolt *et al* 1991). Because of this the smaller truncated forms, which have a complete hormone-binding region but lack a functional transmembrane binding region can act as a sponge binding the hormone and thus preventing binding to the transmembrane located receptors. This would act to prevent down-regulation of expression of the full-length transcript. Indirectly these truncated forms, through binding to free LH may cause an up regulation in full-length receptor expression levels, as it is well documented that a decrease in hormone levels cause an increase in receptor numbers.

It is possible therefore that these truncated forms could modulate Leydig cell activity by binding to and reducing the level of available hormone for cellular signal transduction.

3.8.1 Comparison with FSH receptor spliced transcripts

Much work has been done on the FSH receptor and like the LH receptor it is known to undergo extensive alternate splicing generating multiple transcripts (Simoni *et al* 1997, Khan *et al* 1993) and several of these have been cloned and characterised from the sheep testis (Yarney *et al* 1997). Three isoforms of the FSH receptor were found and one form, which was designated FSHR3, was demonstrated to be a single transmembrane growth factor type 1 receptor (Sairam *et al* 1997). Further studies showed that the receptor is expressed on the cell surface in transfected cells exhibiting the same affinity for labelled hormone as the full-length FSH receptor transcript.

The same FSHR3 transcript has also been identified in mouse ovary. It was localised to the cell surface using a specific anti body (Babu *et al* 1999). Expression levels of this transcript were shown to be quickly up-regulated upon addition of FSH, suggesting that transcriptional activation mechanisms including splicing are initiated very quickly. This also suggests an important role for the FSHR3 receptor especially as its expression levels were consistently higher than those observed for the other two isoforms.

The FSHR3 was found to initiate a different signalling pathway by mediating rapid calcium mobilisation (Touyz, *et al* 2000) and extracellular regulated kinase activation without elevation of cAMP levels (Babu *et al* 2000). This may serve to stimulate rapid cell proliferation required for follicular growth.

These findings add strength to the possibility of an alternate role for the LHR truncated transcripts. Hydrophobicity analysis on the amino acid sequence of the proteins show that the 3' region of the truncated receptor transcripts 1 and 3 have the potential to be membrane assosciated receptors, this is highlighted by extensive hydrophobic regions indicative of transmembrane regions on the hydrophobicity plots. Transcript 2 however is more likely to have an extracellular location due to the hydrophilic nature of the entire receptor, which is shown on the hydrophobicity plot.

This work could be taken further by determining whether these alternative spliced isoforms of the LH receptor are indeed translated to functional proteins. Several approaches can be undertaken such as Western blots of protein extracted from mouse testes at different stages of development. An antibody designed to the extracellular region of the receptor should identify all transcripts if they are translated as all have an identical extracellular region.

The results suggest that the predominant forms of the receptor during embryonic stages are the two smaller truncated transcripts and that the full-length receptor is the predominant form during adulthood. It is unclear why this should be, however one suggestion is that the truncated receptor forms play an indirect role in fetal Leydig cells function by regulating hormone levels and the full-length receptor play a more functional role in the adult Leydig cells by initiating the intracellular signalling cascade which leads to androgen production.

3.8.2 Real Time PCR quantitation of LHR

The Real Time PCR results show that LHR is expressed in much higher quantities in the adult mouse testis than neonate. This is indicative of the functional aspects of the Leydig cells at these different times. The requirement for testosterone would be much greater in the adult for sperm production. This function is absent in the day 5 testis and by day 20 this is just prior to onset of puberty. The results show an increase in LHR expression around this time with highest levels in the adult. The primers and probe would detect only the full-length and largest truncated transcript as they are complimentary to regions missing in the smaller transcripts. In order to assess levels of the smaller transcripts quantitatively separate primers and probes would have to be designed around the 3' splice regions.

Chapter 4

5 α Reductase

4 Characterisation of the 5 α reductase genes, type 1 and type 2

4.1 Testosterone and dihydrotestosterone

Formation of the male phenotype is dependent upon the action of testosterone and dihydrotestosterone (DHT). Both androgens bind to the same androgen receptor, but DHT does so with much greater affinity. Upon binding, the hormone / receptor complex transclocates to the nucleus of the target cell where it acts as a transcription factor by binding to a hormone response element on the DNA.

Testosterone serves as a metabolic precursor of DHT. The conversion is catalysed by the enzyme 5α-reductase. This enzyme exists in two forms named type 1 and type 2 in the order of their discovery. The 5 α reductase conversion of testosterone to DHT is essential for many androgen actions (Russell and Wilson 1994)

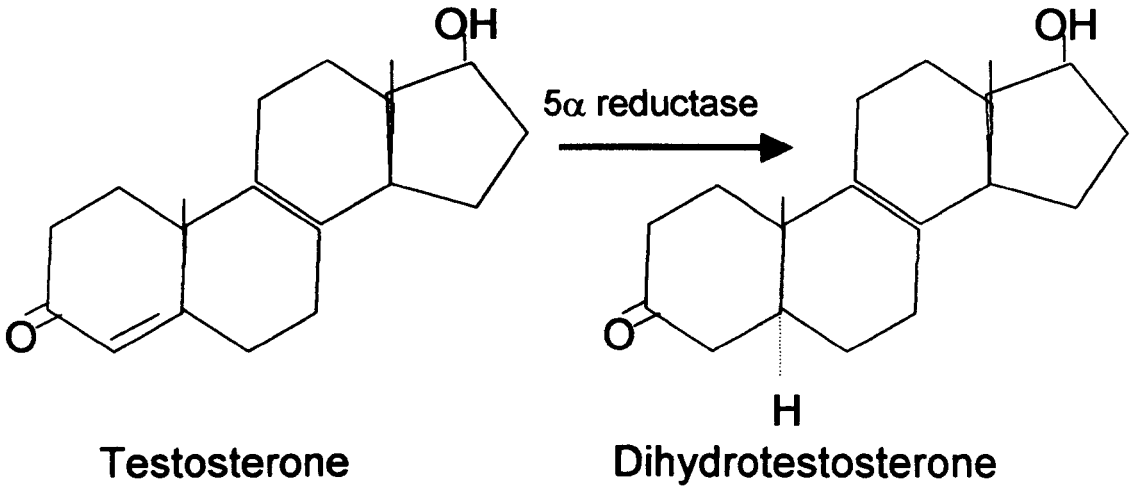


Figure 4-1 Conversion of testosterone to dihdrotestosterone by enzyme 5αreductase

Testosterone is necessary for the differentiation of Wolffian ducts into seminal vesicles, epididymis, and ejaculatory ducts whereas dihydrotestosterone is required for the differentiation of the urogenital sinus and genital tubercle into prostate, urethra, penis and scrotum. The involvement of distinct androgens in these two developmental pathways is very clear from evidence of patients with a defect in the formation of either testosterone or dihydrotestosterone.

Mutations that decrease the synthesis of testosterone affect both developmental pathways as this androgen serves as a metabolic precursor of dihydrotestosterone. (Andersson *et al* 1996). In contrast, a decrease in the synthesis of dihydrotestosterone results in a defect in the virilisation of the urogenital sinus and genital tubercal, although differentiation of Wolffian duct progresses normally. (Imperato-McGinley *et al* 1974). The two androgen model of sexual differentiation is also supported by studies in rats (Imperato-McGinley *et al* 1985)

It is not clear however whether the requirement for two androgens in the formation of the male genotype extends across the mammalian class or, indeed, why this need exists. One hypothesis to explain the need for two androgens involves the need for signal amplification in specific target tissues, this requirement for a stronger signal may arise perhaps due to poor vascularisation resulting in diminished hormone delivery, or even the presence of catabolic enzymes which specifically degrades one or the other androgen. (Penning 1997).

4.2 Isoforms of 5 α reductase

There are two isoforms of the 5α-reductase enzyme (type 1 and type 2) based on the chronological order in which their cDNA's were isolated. The enzymes are coded for by two separate, although similar, genes. The length of the coding region in both genes is just under 1000bp in length and they each contain five exons of very similar size. The intronic positions are essentially identical in both genes suggesting that the two enzymes arose by gene duplication (Russell and Wilson 1994). In humans the two genes are located on different chromosomes; type 1 is located on chromosome 5 (band p15) and type 2 is located on chromosome 2 (band 23p). The two enzymes also differ in kinetic properties. The type 1 form in the rat has a pH optima of 6 - 8 whereas the type 2 form prefers conditions slightly more acidic with a pH optima of 4 – 5. Type 1 5 α reductase is expressed predominantly in the skin and liver while type 2, on the other hand, is predominantly expressed in androgen target areas such as the prostate and genital tissue, (Imperato-McGnley *et al* 1992)

4.2.1 Type 2 Mutations

Analysis of mutations in the encoding genes elucidates why there are two forms of the 5α-reductase enzyme and also how much each type contributes to sexual differentiation and reproduction in both sexes. Distinct biological roles for each of these 5 α reductase isozymes have become apparent from studies of individuals with naturally occurring inactivating mutations or in mice with engineered knockouts of this gene.

The role of 5α-reductase in human development was assessed through studies of subjects in the Dominican Republic in the 1960's (Walsh *et al* 1974). These individuals possess a genetic mutation in the gene encoding the type 2 isoform causing a defect in its enzymatic activity. Phenotypically this results in normal development of the epididymis, vas deferens and seminal vesicle from the Wolffian ducts, due to the presence of testosterone. In the absence of its further conversion to dihydrotestosterone, however, development of the prostate and external genitalia from the urogenital sinus is limited or abnormal. These individuals form female genitalia externally, but internally they possess fully differentiated testes and a normal male duct system.

Virilisation in these individuals is eventually achieved at puberty with a massive increase in serum testosterone levels. Because of the pubertal onset of genital growth this condition has been labelled "penis at twelve" syndrome. Interestingly, individuals with type 2 deficiency have been shown to have exceptionally high levels of circulating LH (about double that found in normal males). These results suggest that a deficiency of DHT results in decreased negative feedback at the level of the hypothalamus and/or pituitary, resulting in an increase in mean plasma LH.(Canovatchel *et al* 1994)

Studies in rhesus monkeys using finisteride, a 5 α reductase type 2 inhibitor confirmed these findings, as they too developed abnormalities of the genitalia. No developmental abnormalities were observed in females. (Prahalada *et al* 1997)

Forty-five different mutations in the type two gene have been identified to date and each one results in defective enzyme activity and dihydrotestosterone production, giving rise to an intersex phenotype (Russell and Wilson 1994). These defects in virilisation due type 2 mutation suggest that this form of the enzyme plays a more crucial role in male development, particularly as areas of highest expression levels of this gene are all androgen target sites such as prostate and genitalia. (Wilson *et al* 1993.

Male mice carrying a mutation in the type 2 gene are, surprisingly, much less affected than humans. Although virilisation is incomplete, it is still distinguishably male. (Mahendroo *et al* 2001), therefore effect on phenotype is much less severe and marked only by a reduction in size of the secondary sex glands. Fertility in these individuals is unaffected. Female mice carrying this mutation show no adverse effects on the phenotype and they are indistinguishable from wild type mice with both normal development and reproduction. It would appear, therefore, that the type two enzyme plays a much more important role in males, whereas it has very little importance in females. (Mahendroo and Russell 1999)

4.2.2 Type 1 Mutations

The type 1 form of the enzyme appears to play a more important role in female mice than the type 2 form. This is evident from studies on mice carrying a mutation in the gene encoding the type 1 form. Males develop normally and reproduction is unaffected,

whereas females deficient in the type 1 isozyme of steroid 5 α reductase fail to deliver their young at term and thus manifest a parturition defect. Impaired metabolism of progesterone in the cervix of the mutant mice in late gestation leads to an accumulation of this steroid in the tissue. It would appear that a failure of cervical ripening underlies the parturition defect in mice lacking steroid 5 α reductase type1 and that this enzyme normally plays an essential role in cervical progesterone catabolism at the end of pregnancy. (Mahendroo *et al* 1999)

4.3 Objectives

At the time these studies began there was little information about the sequence of the 5α-reductase genes in the mouse, or about developmental regulation of these genes in the testis. The purpose of this part of the study therefore was:

1. To determine the sequence of mouse 5 α reductase type 1 and 2 genes using bioinformatics techniques, RT PCR and automated sequence analysis.

2. To establish expression patterns of type 1 and type 2 at different stages of development in mouse testes.

4.4 Materials and Methods

4.4.1 Strategy for determining mouse 5 α reductase sequences

Initially when the objectives of this project were proposed, the complete coding sequences of mouse 5 α reductase types 1 and 2 were unknown. Only mouse EST's of type 1 were available, and rat sequence for type 2. These were obtained from Genbank and EST databases.

Full-length sequences were not required for the proposed studies but partial sequences were necessary to design PCR primers.

To identify the sequences of mouse type 1 and 2 5 α reductase two approaches were taken. Using the molecular biology computing programme GCG, it was possible to fetch sequences of the two genes of several species from GenBank database and then perform a multi species alignment. Sequences from rat, human, monkey and pig were obtained from Genbank and lined up to identify consensus sequences; areas of highest homology between the four species which were chosen for primer design. To ensure there was no cross amplification between types 1 and 2 the primer sequences were chosen on the basis of having highest homology between species, but little homology between isoforms. The rat sequence was Blasted against mouse ESTs and matching ESTs were lined up to generate consensus sequence. Blast is a computer programme available on the NCBI web page. It allows the user to input sequences and it will then find the best-fit match for them, in addition it also allows for a pair wise comparison of two sequences.

For the type 1 isoform primers were designed from rat sequence (with highest homology to the other species compared) at the 5' end and mouse sequence obtained from EST's for the 3' region.

The same method was applied to the design of 5 α type 2 primers for both 5' and 3' regions of the sequence as there was no mouse EST's available for the type two gene, at the time this work was carried out.

4.4.2 Type 1 primer design

Alignment of rat and human type 1 sequences allowed for identification of homologous sequence regions. The 5' primers were designed this way and the initial 3' primer was designed from mouse EST sequence. After initial PCR amplification using these primers and mouse testis cDNA, it was then possible to extract and sequence the bands obtained, and then from this sequence, new mouse primers were designed. For type 1 primers only rat and human sequences were aligned, as at the time of this project these were the only full-length sequences submitted to Genbank. Sequence from four different species was aligned for designing the type 2 primers (Fig 4.2) Areas in blue indicate initial primer locations and the yellow highlighted area indicates start codon.

Type 1 alignment

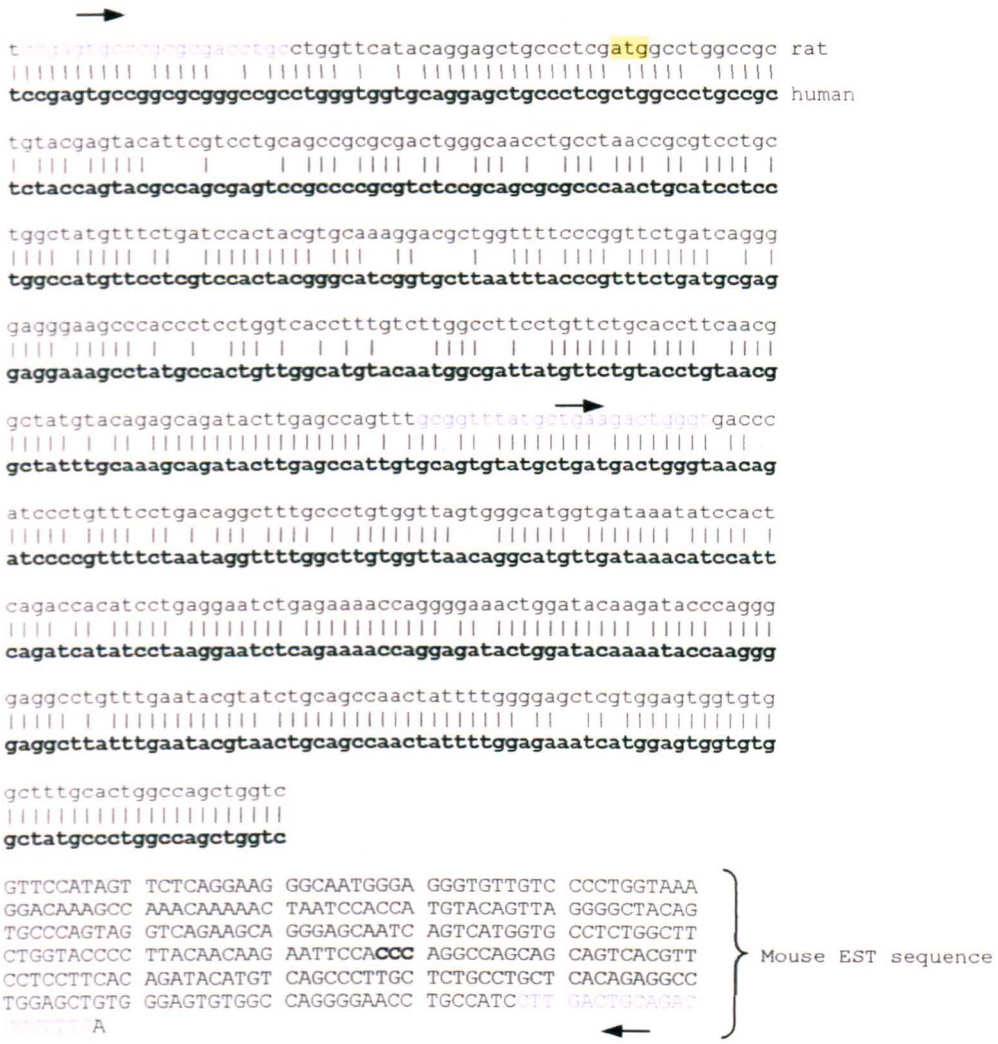


Figure 4-2 Human and rat 5 α reductase type 1 sequence alignment

Rat sequence appears in normal font and human sequence is in bold. Primer locations are highlighted in blue and start codon is in yellow

Type 2 alignment

```
human. ~~~~~~~~~~ ~~~~~~~~~~ GCGGCCACCG GCGAGGAACA CGGCGCGATG CAGGTTCAGT
monkey ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~GGCACG AGGCGCGATG CAGGTTCAGT
   pig ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~
   rat GAATTCCGGC TGAGGGGCGG CAGCTACCAA CTGTGACCAC AGGCGAGATG CAGATTGTCT

 human GCCAGCAGAG CCCAGTGCTG GCAGGCAGCG CCACTTTGGT CGCCCTTGGG GCACTGGCCT
monkey GCCAGCAGAG CCCAGTGCTG GCAGGCAGCG CCACTTTGGT CGCCCTTGGG GCACTGGTCT
   pig ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~
   rat GCCATCAGGT CCCGGTGCTG GCAGGTAGCG CCACATTGGC CACTATGGGG ACCCTGATCC

 human TGTACGTCGC GAAGCCCTCC GGCTACGGGA AGCACACGGA GAGCCTGAAG CCGGCGGCTA
monkey TGTACGTCGC GAAGCCCTCC GGCTACGGGA AGCACACGGA GAGCCTGAAG CCCGCGGCTA
   pig ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
   rat TGTGCTTAGG GAAACCCGCC AGTTACGGGA AACACACAGA GAGTGTGTCG TCGGGAGTTC

 human CCCGCCTGCC AGCCCGCGCC GCCTGGTTCC TGCAGGAGCT GCCTTCCTTC GCGGTGCCCG
monkey CCCGCCTGCC AGCCCGCGCA GCCTGGTTCC TGCAGGAGCT GCCCTCCTTC GCGGTGCCCG
   pig ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~CT GCCCTCCTTC GTGGTGCCCG
   rat CCTTCCTGCC GGCACGCATC GCCTGGTTCC TGCAGGAGTT GCCTTCCTTT GTGGTGTCGG

 human CGGGGATCCT CGCCCGGCAG CCCCTCTCCC TCTTCGGGCC ACCTGGGACG GTACTTCTGG
monkey CGGGGATCCT CGCTCGGCAG CCCCTCTCCC TCTTCGGGCC ACCTGGGACG GTGCTTCTGG
   pig CGGGGATCCT CGCCGGGCAG CCCCGTTCTC TCTTCGGCCC GCCTGCGACT GTGCTTCTGG
   rat TAGGGATGCT CGCTTGGCAG CCGCGCTCCC TCTTCGGACC GCCCGGGAAT GTCCTGCTGG

 human GCCTCTTCTG CGTACATTAC TTCCACAGGA CATTTGTGTA CTCACTGCTC AATCGAGGGA
monkey GCCTCTTCTG CGTACATTAC TTCCACAGGA CATTTGTGTA CTCACTGCTC AATCGAGGGA
   pig GCCTCTTCTC CGCACATTAC TTCCACAGGA CATTTGTTTA CTCACTGCTC ACTAGAGGGA
   rat CTCTCTTCTC TGCACATTAC TTCCACAGGA CATTTATTTA CTCGTTGCTC ACAAGAGGGA

 human GGCCTTATCC AGCTATACTC ATTCTCAGAG GCACTGCCTT CTGCACTGGA AATGGAGTCC
monkey GGCCTTATCC AGCTGTACTC ATTTTCCGAG GCATTGCCTT CTGCGCTGGA AATGGATTCC
   pig GGCCTTTTCC AGTTGTATTC CTTTTCAGAG GCTTTGTCTT CTGCATGGGA AATGGACTCC
   rat GGCCTTTCCC AGCGGTGCTG TTTTTGAGAG CCACTGCCTT CTGCATAGGG AACGGACTCC

 human TTCAAGGCTA CTATCTGATT TACTGTGCTG AATACCCTGA TGGGTGGTAC ACAGACATAC
monkey TTCAAAGCTA CTATCTGATT TACTGTGCTG AATACCCTGA TGGGTGGTAC ACAGACATAC
   pig TTCAAGGCTA CTATCTGGTT TACTGTGCCG AATACCCTGC TGAGTGGTAC ACAGACATAC
   rat TTCAAGCCTA CTACCTGGTT TACTGCGCAG AATACCCCGA GGAGTGGTAC ACAGATGTGC

 human GGTTTAGCTT GGGTGTCTTC TTATTTATTT TGGGAATGGG AATAAACATT CATAGTGACT
monkey GGTTTTGCTT GGGTGTCTTC TTATTTATTT TGGGAATGGG AGTCAACATC CATAGTGACT
   pig GGTTTAGCCT AGGTGTGTTC TTATTTATTT TGGGCATGGG AATCAACATT CACAGCGACT
   rat GGTTTAGCTT TGGTGTCTTC CTGTTTATTC TGGGGATGGG AATCAACATC CACAGTGACT

 human ATATATTGCG CCAGCTCAGG AAGCCTGGAG AAATCAGCTA CAGGATTCCA CAAGGTGGCT
monkey ATATATTGCG CCAGCTCAGG AAGCCTGGAG AAATCACCTA CAGGATTCCA AAAGGTGGCT
   pig ATATATTGCG CCAGCTCAGG AAGCCTGGAG AAGTCATCTA TAAGATTCCA CAAGGTGGCT
   rat ACACCCTGCG CCAGCTCAGG AAGCCTGGAG AAGTCATCTA TAGGATTCCT CGAGGTGGCT

 human TGTTTACGTA TGTTTCTGGA GCCAATTTCC TCGGTGAGAT CATTGAATGG ATCGGCTATG
monkey TGTTTACGTA TGTTTCTGGA GCCAATTTCC TTGGTGAGAT CATTGAATGG ATCGGCTATG
   pig TGTTCACGTA CGTTTCTGGA GCCAATTTCC TTGGTGAGAT CATTGAATGG ATCGGCTATG
   rat TGTTTACGTA TGTCTCTGGA GCCAATTTCC TGGGCGAGAT TATTGAATGG ATTGGCTACG

 human CCCTGGCCAC TTGGTCCCTC CCAGCACTTG CATTTGCATT TTTCTCACTT TGTTTCCTTG
monkey CGCTGGCCAC TTGGTCCCTC CCAGCACTTG CATTTGCATT TTTCTCAGTT TGTTTCCTTG
   pig CCTTGGCCAC TTGGTCCCTT CCAGCACTTG CATTTGCATT TTTCTCGCTT TGTTTCCTTG
   rat CCTTGGCCAC GTGGTCCGTC CCAGCCTTCG CTTTCGCCTT TTTCACACTT TGTTTCCTGG

 human GGCTGCGAGC TTTTCACCAC CATAGGTTCT ACCTCAAGAT GTTTGAGGAC TACCCCAAAT
monkey GGCTGCGAGC TTTTCACCAC CATAGGTTCT ACCTCAAGAT GTTTGAGGAC TACCCCAAAT
   pig GGCTGCGAGC TTTTCACCAT CATAGGTTCT ACGTCAAGAT GTTTGAGGAC TACCCCAAGT
   rat GGATGCAAGC CTTTTACCAC CACAGGTTCT ACCTTAAGAT GTTTAAGGAT TACCCCAAAT

 human CTCGGAAAGC CCTTATTCCA
monkey CTCGGAAAGC CCTTATTCCA
   rat C
```

Figure 4-3 multiple species alignment of 5 αreductase type 2 sequence

In addition to determining areas of species homology within the coding region, it was also important to design primers, which would not bind to 5 α reductase type 1. Once primer sequences were chosen they were entered into a blast search to ensure that they were complimentary only to the desired region of the rat 5 α reductase type 2 gene . Primer sequences have been highlighted in blue. The same technique was applied to 5 alpha reductase type 1 sequence for design of 5' primers. The 3' primers were designed from Mouse EST sequence.

4.5 PCR primers

In order to check the quality of the cDNA samples used, PCR was performed using actin primers. Actin is a house-keeping gene and generally expressed in high levels in most tissue types. Various combinations of 5 α reductase type 1 and 2 primers were tried in order to get optimal amplification.

4.5.1 Expected band sizes from primer combinations

5 alpha reductase type 1

Total size of rat sequence in GenBank = 1210bp

From start to stop codon (signified by *) = 800bp

Figure 4-4 Primer postitions for 5 α reductase type 1

323 / 326.....1176bp
323 / 335.....826
323 / 325.....800
324 / 326.....1046
324 / 325.....610
324 / 335.....650
333 / 326.....1000
333 / 335.....625
333 / 325.....585bp
334 / 326.....780
334 / 335.....400
334 / 325.....360
336 / 325.....600
336 / 335.....640

Type 1 Primer Sequences

323 - GTTGGATGAGCTCTGCCTGCT

324 - GTGCCCGCGCGACCTGCCTGG

336 - CAGGAGCTGCCCTCGATGGC

333 - ATGGCCTGGCCGCTGTACGAG

334 - GCGGTTTATGCTGAAGACTGGGT

325- ATTCCATTTGTGCTTTAGTG

335 - CTCCCATGAGCTGAGTCTGTCTGT

326- CTTGACTGCAGACCCCTTTG

All primers were designed from Rat 5 alpha reductase type 1 sequence (GenBank accession no AH00300) Except primer 326 which was designed from mouse EST sequence (accession no AA387848)

4.5.2 5 alpha reductase type 2

Total size =1800bp

Coding region = 765bp * = start and stop regions

Figure 4-5 Primer postitions for 5α reductase type 2

327 / 330.....844bp
327 / 329.....750
328 / 330.....800
328 / 329.....730
350 / 351.....570
350 / 329..... 650

Type 2 Primer Sequences

327 - ACTGTGACCACAGGCGAGATG

328 - CCGGTGCTGGCAGGTAGCGC

350 - GGTTCCTGCAGGAGCTGC

351 - CCCCAGGTTCTTACCTCCAAATG

329 - AAGCTCTCATTCATC

330 - AACTATAACAGTGTCTTGCTCAC

All primers were designed from Rat 5 alpha reductase type 2 sequence (GenBank accession no L03843) Except primers 350 and 351, which were designed from new mouse sequence

Different primer combinations gave varying results, therefore an optimal combination was chosen for both genes based on a comparison of intensity of bands obtained from same cDNA template. Essentially this consisted of two outer primers for first round amplification (x30 cycles) then two inner primers for nested PCR (x20cycles)

It was necessary to perform nested PCR as expression levels were so low that the bands were barely detectable after first round amplification.

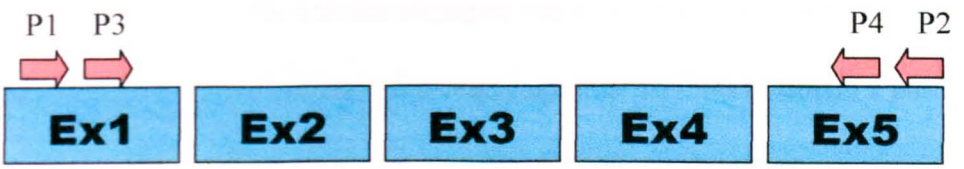


Figure 4-6 Position of primers on 5 α reductase type 1 and type 2 genes

PCR was also performed on the various templates using actin primers. Detection of expression confirmed that cDNA was of good enough quality to perform further PCR's using different primers.

4.5.3 PCR

Each PCR reaction contained Tris/HCl buffer (75 mM, pH 9.0), $(NH_4)_2SO_4$ (20 mM), $MgCl_2$ (2 mM), dNTP's (0.2 mM each), 0.2µl Taq Polymerase (2 U per 100 µl), 1µl of the cDNA template, primers (200 nM each) and H2O to an appropriate volume. All

components, apart from dNTPs, Taq and template were treated by UV-light to remove any PCR-derived DNA contamination. PCR was performed using cDNA template from mouse testes at various ages

Full details of the PCR conditions are explained in chapter 2.

Nested PCR

Because the genes of both isoforms were expressed at such low levels it was necessary to perform nested PCR on the PCR products from the first amplification. Primers located internally to the first set were used for this purpose for both 5α-reductase type 1 and 2.

The bands from gels containing nested PCR products for both type 1 and type 2 were viewed under UV light and the strongest ones were extracted using a scalpel. These were then placed into mini columns and centrifuged for 10 mins to obtain a purified PCR product. This was then used as a template for sequencing using

The sequences obtained from bands of both isoforms were entered into a blast search to ensure they were in fact specific for 5 α reductase.

4.6 Real Time PCR

Although it had been established with PCR studies that expression levels of both isoforms 5 α reductase in the testis were very low, it was decided to perform quantitative analysis to assess whether there was a temporal expression profile through out development. Much of the literature has provided evidence that type 2 is the dominant isoform expressed in androgen target tissue. With the testes being such a tissue type, it

was assumed that this isoform would be expressed in larger quantities, but it was unclear if these levels would change at all with the different age groups assessed.

Expression levels of the 5 α reductase genes were assessed relative to actin. This is a house keeping gene and known to be expressed in high levels in most tissues. At the time of carrying out these experiments this was the most widely used internal standard used for quantitation studies, however it was later revealed that this may not be the most reliable as in some tissues there can be great variability in expression levels and also depending on the role of the tissue type of interest there may be genes expressed at much higher levels. It was therefore decided that any further quantitation studies performed should be relative to an external standard such as Luciferase, this gives a more accurate indication of expression levels and this was used further work carried out to determine expression levels of the type 1 isoform. This is explained in greater detail in chapter 5.

In order to quantify expression levels of the 5 α reductase type 2 gene in mouse testes at various stages of development, the Real time PCR method of quantitation was used, which utilised the Taqman PCR method of amplification following reverse transcription of the isolated RNA (Bustin 2000). RNA was extracted from whole testis of mice at various ages using RNAzol method (Biogenesis Ltd., Bournemouth, UK) RNA was then DNAse treated (DNA-*free*, Ambion Inc, supplied by AMS biotechnology, UK) and then reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies) (method described in detail in chapter 2). The cDNA was then used as a template for Real Time PCR. The primers and probe used for Real rime PCR were designed from new mouse sequence obtained from the previous study.

Cycle number at threshold of detection calculated for both genes and results for 5 α reductase type 2 were calculated relative to actin at the same stage of amplification, these were then plotted on a graph. The cDNA template for the quantitation study was mouse testis at various stages of development

Primers and Probes for Real Time PCR

Gene	Forward primer	Probe	Reverse primer
β Actin	GCTTCTTTGCAGCTCCTTCGT	CCGGTCCACACCCGCCACC	ATATCGTCATCCATGGCGAAC
5 α reductase T1	GCGCTAGTCTACCTGGAGGGT	TCCTGGCTTTCGTGGCCTTCGTG	GAAGAGCCCACCATCTGGAG
5 α reductase T2	CATCCACAGTGACTGCATGC	AGGAAGCCCGGAGAGGTCATC	AGGTGGCTTGTTACGT

Table 4-2 Sequence or Real Time PCR primers and probes

Real Time PCR's were carried out in a 25µl volume using a 96-well plate format. Components for real time PCR were purchased from Oswel Ltd (Southampton, UK) apart from primers and probes, which were purchased from MWG Biotech (Milton Keynes, Uk) Each PCR well contained 1x reaction buffer, 5mM $MgCl_2$ 200µM dNTP's, 300nM of each primer, 200 nM probe, and 0.02U/µl enzyme (HotGoldstar). Reactions were carried out and fluorescence was detected on a GeneAmp 5700 system (Applied Biosystems, Warrington, Cheshire, UK)

4.6.1 Enzyme Activity

Included in the results section is a graph showing 5 α reductase activity. It is commonly known that gene expression levels do not always necessarily represent what is occurring at the protein level of activity. During the course of this study additional work was being undertaken in the lab assessing the enzyme activity in the mouse testis at different stages of development. It was therefore decided that it would be a useful comparison to include these results in this chapter.

Activity of 5 α reductase activity was measured by determining the conversion of a saturating concentration of titrated testosterone to DHT by homogonates of whole testis O'Shaughnessy 1991). Substrate and product were separated by thin layer chromatography and enzyme activity was expressed as pmol/min/testis.

This work was carried out by Professor O'Shaughnessy as part of a continued project, relating to this study.

4.7 Results

4.7.1 PCR

Expression levels of both genes were very low and required nested PCR in order for them to be detectable on a gel. For 5 α-reductase type 1 the most abundant levels of gene expression were detected at D20, this was also the case for the type 2 isoform. PCR amplification using βactin primers on mouse testis cDNA of various ages, gave strong bands after amplification at 30 cycles, (figure 4.7) this was a good indication that

the cDNA prepared from previously extracted RNA was of good quality. All bands were of the expected length of 380bp

5 α Reductase type 1

Initial PCR using primers 336 and 335 and amplification at 30 cycles produced only very faint bands at expected size of 640bp, suggesting low expression levels (figure 4.8). Nested PCR using primer combination 336 and 325 produced much stronger bands at expected length of 600bp (figure 4.9). These bands were extracted and purified, then sequenced.

Sequence analysis of bands showed highest homology to rat 5 α reductase type 1 when entered in a blast search. It was possible therefore to align the new mouse sequence with known rat sequence. Approximately 500bp of previously unknown mouse 5 α reductase type 1 sequence was obtained using this method.

5 α Reductase type 2

Initial PCR was performed using primers 336 and 335 and amplification at 30 cycles produced only very faint bands at expected size of 640bp, suggesting low expression levels (figure 4.10). Nested PCR using primer combination 336 and 325 produced much stronger bands at expected length of 600bp (figure 4.11). These bands were extracted and purified, then sequenced.

The sequences obtained were entered into a Blast search to see what genes they had the highest homology with. In both cases they had the highest homology with rat sequence of the same gene. By aligning the two sequences ie new mouse sequence obtained from primers targeting 5α reductase type 1 and rat 5α reductase type 1, it was possible to

assess where abouts in the gene sequence had actually been obtained from. The same was carried out for type 2. By doing several PCR's then nesting using different primer combinations for each gene, it was possible to gradually extend on the sequence obtained.

Real Time PCR

The real time PCR results demonstrated that 5 α reductase mRNA expression levels are very low throughout development in the testis. The temporal pattern of expression showed that the greatest levels of expression of both genesoccurred at around d25, (4.18A and 4.18B)Figure the peak in expression is only over a very short period of time as by d30 levels had decreased dramatically again, these low levels were maintained until adult hood and no other peaks were observed.

Enzyme activity

Results from the enzyme assay demonstrated that enzyme activity levels were very low initially from d1 and these then gradually increased at d15, the increase however was very slight until d20 – d25 when there was a seven-fold increase. These levels, however were not maintained and there was a sharp reduction in activity by d30 until barely detectable levels in the adult tissue. (Figure 4.19). These results are a measurement of total enzyme activity from both isoforms.

PCR and Sequence analysis

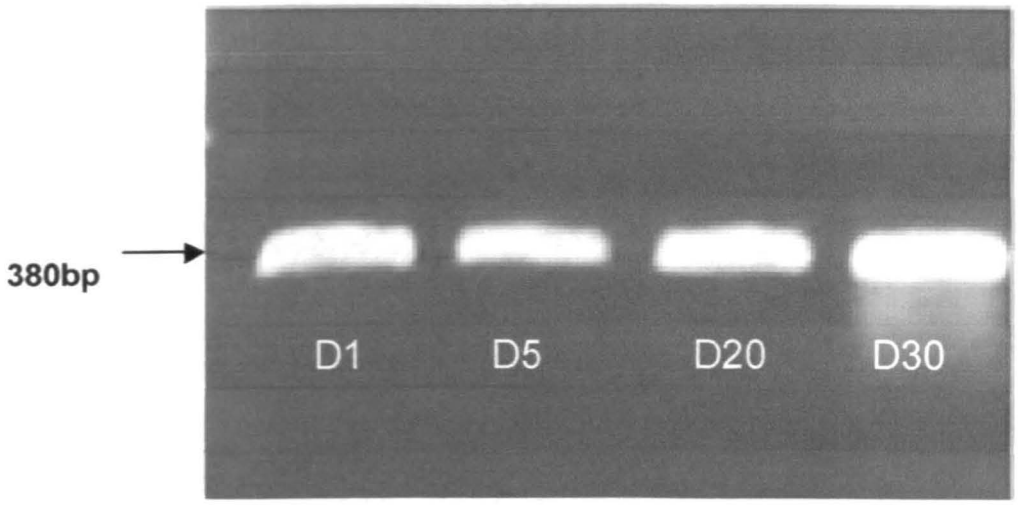


Figure 4-7 Gel image showing actin bands in selected mouse testis cDNA

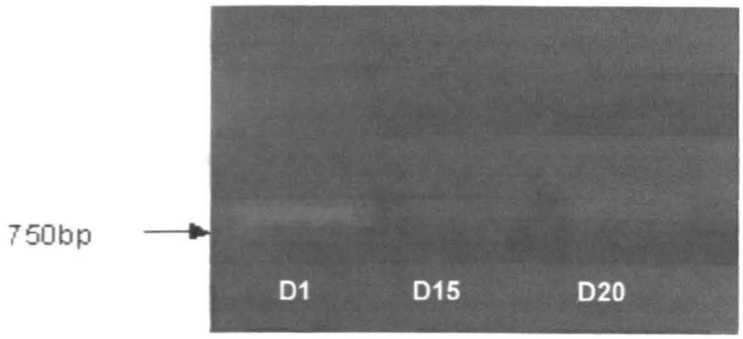


Figure 4-8 Gel image showing bands of 5alpha reductase type 1, PCR amplification was with primers 336 and 335

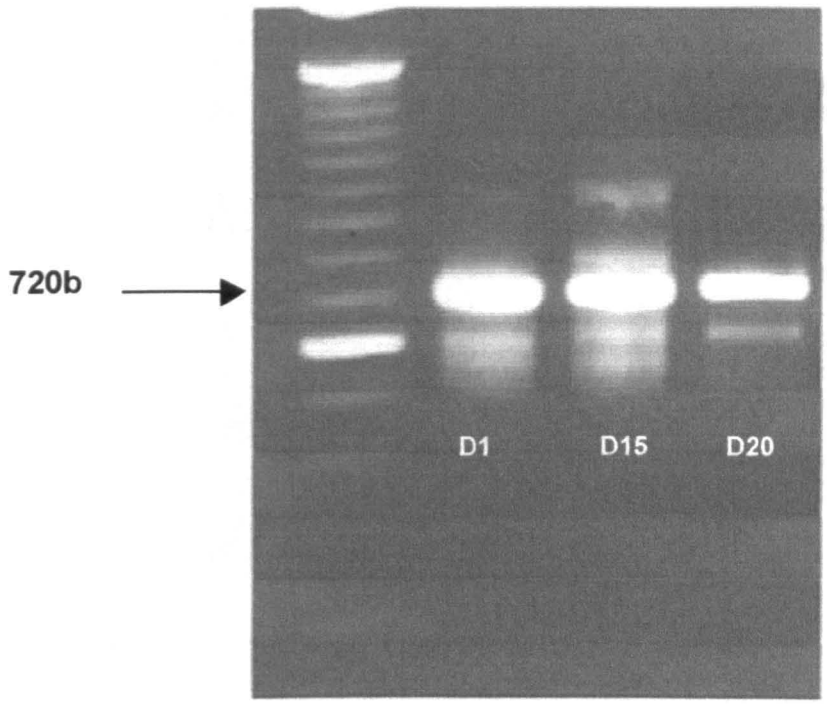


Figure 4-9 Gel image showing bands of 5alpha reductase type 1, after partial nested PCR with primers. 336 / 325

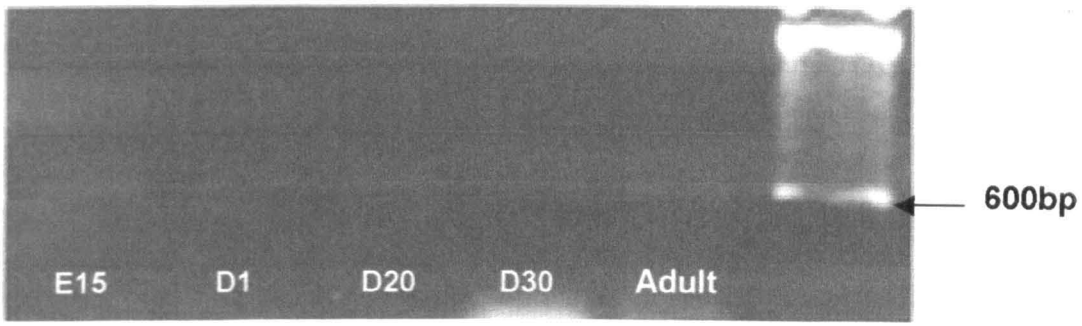


Figure 4-10 Gel image showing bands from first round PCR using 5 alpha reductase type 2 primers 350 / 329.

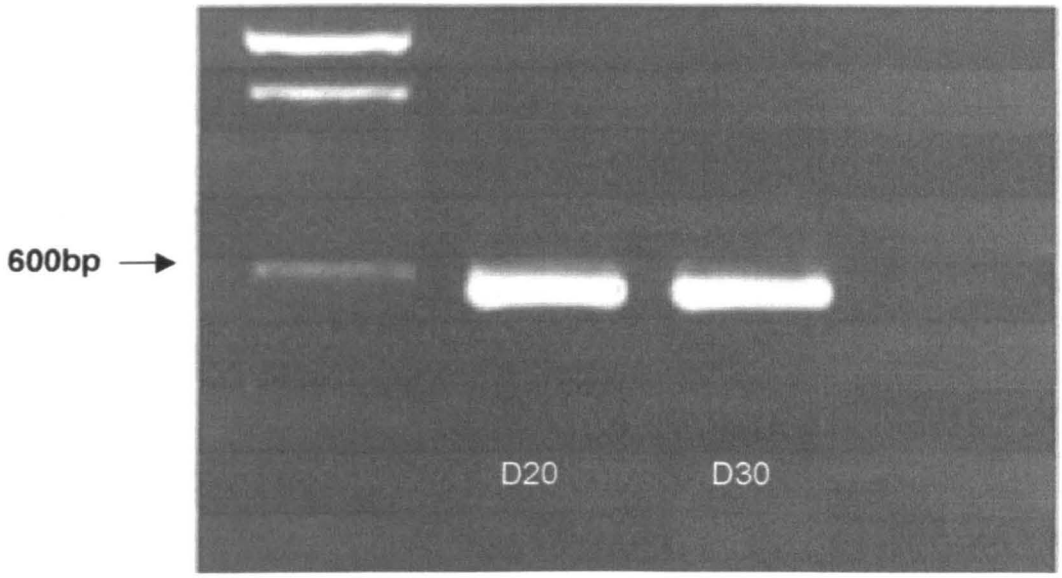


Figure 4-11 Gel image, showing bands from nested PCR using primers 350 / 351

The gel images are representative of the accumulation of experiments which were undertaken. Each PCR was performed 4-6 times for each age group and 6-8 animals were used for each age group.

These bands were extracted from gel using scalpel and purified using mini columns (Chemicon International Inc, Temecula). The cDNA obtained was then sequenced using ABI Prsim automated sequencer (protocol described in chapter 2). The sequence obtained was entered in a blast search and was found to highest sequence identity to rat 5α- reductase type 1. In addition to the mouse sequence already entered in the EST database, there were also several hundred base pairs of previously unpublished sequence. There proved to be a high level of homology between the rat and mouse sequence.

4.7.1.1 5α Reductase Type 1 mouse and rat Sequence alignment

Figure 4-12 Bestfit match of new mouse 5α-reductase type 1. and rat 5α-reductase type 1

Sequence in red and italic represents sequence obtained from PCR product of mouse testis cDNA. Sequence in black represents rat sequence. Sequence in red without complimentary black sequence represents mouse EST sequence from which the first primers were designed for type 1 isoform. Sequence in blue represents primer positions. Real time PCR primers and probe were designed from mouse EST which was a further

800bp downstream so it was not possible to highlight them. At the time of this project there was no mouse 5 α reductase sequences available in Genbank which is why the alignment was performed for both types with known rat sequence. However there is now mouse sequence available for both isoforms and a Blast search was later performed on both sequences. The best alignments produced showed around 97% homology with submitted mouse 5 α reductase sequences.

Type 1

```
ccaggggaggcctgtttgaatatgtatcttcagccaactattttggggagctcgtggagt
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
ccaggggaggcctgtttgaatatgtatcttcagccaactattttggggagctcgtggagt

gggagtggtgtggctttgcactggccagctggtccctccagggcgtggtgtttgctctgt
||   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gg---tggtgtggctttgcactggccagctggtccctccagggcgtggtgtttgctctgt

tcaccctgtgtgcactgttcaccagagcgaggcagcatcatcagtggtacctcgagaagt
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tcaccctgtgtgcactgttcaccagagcgaggcagcatcatcagtggtacctcgagaagt

ttgaagattaccccaaaacaagaaaaatactaattccattcctgctttagtgtgctgtcc
|||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
ttgaagattaccccaaaacaagaaaaatactaattccattcctgcattagtgtgctgtcc

atgctgttgtcttccataagctgagtgtctgtcttcccggtggctttgctctgagcacat
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
atgctgttgtcttccataagctgagtgtctgtcttcccggtggctttgctctgagcacat

acaagtgaattgttttccttatttctcctgc-gttccatagttctcaggaagggcaatgg
|||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
acaagtgaattgttttccttatttctcctgcagttccatagttctcaggaagggcaatgg

gagggtgttgtcccctggtaaaggacaaagccaaacaaaaactaatccaccatgtacagt
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
gagggtgttgtcccctggtaaaggacaaagccaaacaaaaactaatccaccatgtacagt

taggggctacagtgcccagtaggtcagaagcagggagcaat-agtcatggtgcctctggc
|||||||||||||||| |||||||||||||||||||||||| |||||||||||||||||
taggggctacagtgcctagtaggtcagaagcagggagcaatgagtcatggtgcctctggc

ttctggtacccccttacaacaagaattccaccc
||||||||||||||||||||||||||||||||
ttctggtacccccttacaacaagaattccaccc
```

Figure 4-13 Blast pairwise alignment of new mouse type1 sequence and submitted mouse sequence. Accession no. XM_127470

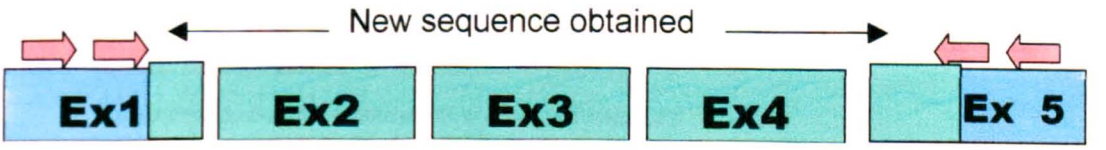


Figure 4-14 5α Reductase type 1 exon arrangement highlighting area of new sequence obtained

4.7.1.2 5α Reductase Type 2 mouse and rat Sequence alignment

Figure 4-15 Bestfit match of new mouse sequence and rat 5α-reductase type 2

= Exon/Intron boundaries

Primers are highlighted in blue, start and stop codons are highlighted in yellow and Real time PCR primers and probe are highlighted in green.

Type 2

```
ggagtaccccctcc-gcccgcacgcatagcc-ggttcctgcaggagctgccttccttgg--
ggagttcccctcctgcccgcacgcatagcctggttcctgcaggagctgccttcctttgtg

gtgacgggagggatgc-cgc--ggcagccgcgctccctctttggacctccggagaaaagt
gtgtcggtagggatgctcgcttggcagccgcgctccctctttggacctccggggaat-gt

agctagctagggtctcttctccgcacattacttccacagagacatttatttactcattag
 gct-gct-gggtctcttctccgcacattacttccacagagacatttatttactcatt-g

ctcaccagagagcagagcctttatcagcggtgatctttctgaaagccactgccttctgca
ctcaccagag-gcag-gcctttatcagcggtgatctttctgaaagccactgccttctgca

tagggaacggactcctgcaagcctattacctggtttattgcgcggaataccctgaggagt
tagggaacggactcctgcaagcctactacctggtttattgcgcggaataccctgaggagt

ggtacacagacatgcggtttagcgtcggtgtcttcttctttattctggggatggggatca
ggtacacagacatgcggtttagcgtcggtgtcttcttctttattctggggatggggatca

acatccacagtgactgcatgctgcgccagctgaggaagcccggagaggtcatctacagga
acatccacagtgactgcatgctgcgccagctcaggaagcccggagaggtcatctacagga

tcccacaaggtggcttgtttacgtatgtgtctggagccaatttcctgggcgagattattg
tcccacaaggtggcttgtttacgtatgtgtctggagccaatttcctgggcgagattattg

aatggatgggctacgctttggcc-cttggtctgttccagcctttgcttt
aatggatgggctacgctttggccacttggtctgttccagcctttgcttt
```

Figure 4-16 Blast pairwise alignment of new mouse sequence obtained and submitted mouse type 2 sequence. Accession no. AB049456

New mouse sequence is in normal font and the submitted mouse sequence is in bold

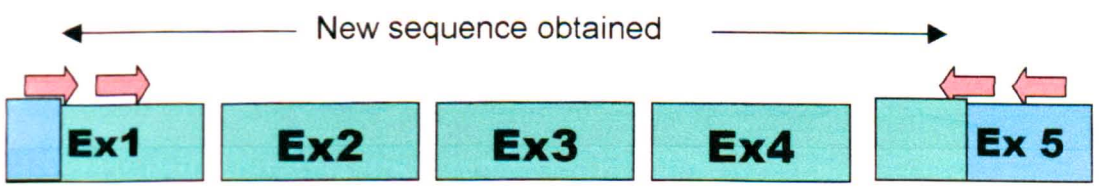


Figure 4-17 5α Reductase type 2 exon arrangement highlighting area of new sequence obtained

4.8 Real-time PCR

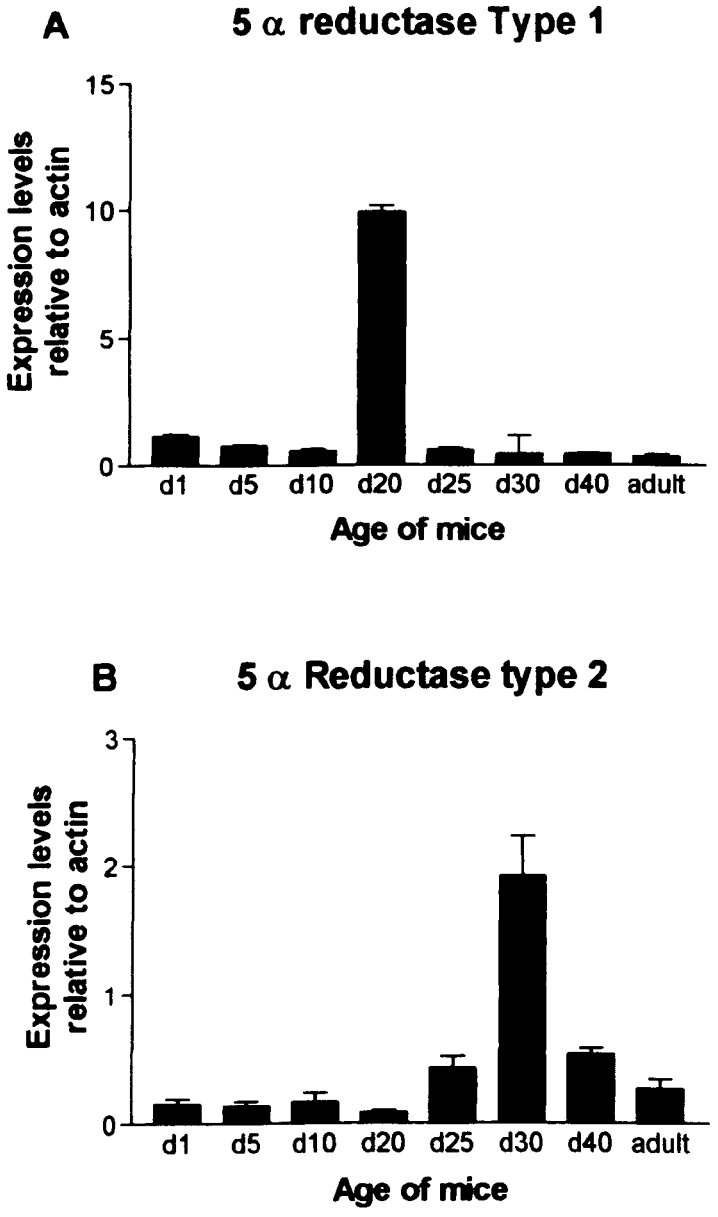


Figure 4-18 mRNA expression levels of A - 5 alpha reductase type 1 and B- type 2 in mouse whole testis at various developmental stages.

4.8.1 *Enzyme Activity Levels*

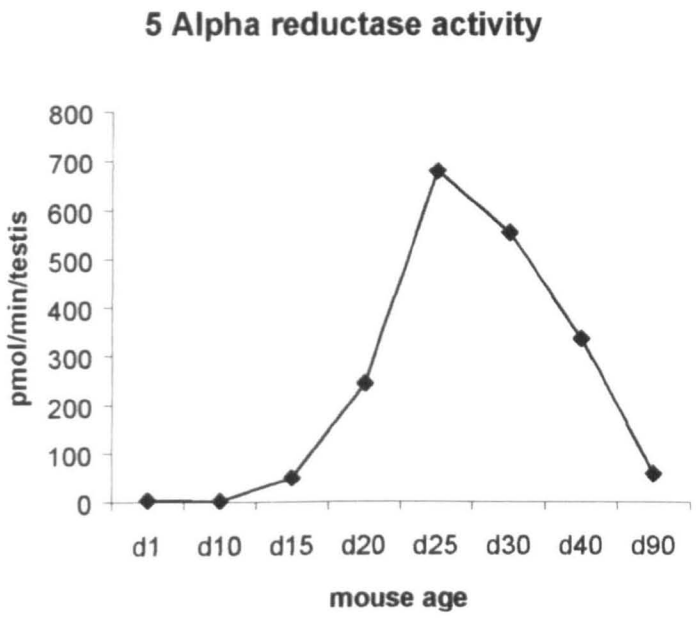


Figure 4-19 Production of DHT by mouse testis at different ages

4.9 Discussion

The results clearly show that both 5α-reductase type 1 and type 2 are expressed in the testes, although these expression levels are low and do vary depending on the stage of development. Low expression levels were confirmed by ruling out poor cDNA quality. The strong actin bands shown in figure 4.7 are indeed confirmation of the cDNA being of good quality. The bands obtained after PCR amplification suggest that there is obviously a high level of sequence homology between species as the primers designed from rat sequence were able to successful amplify the mRNA in mouse testes.

With the testis being an androgen target tissue, it was assumed that expression of type 2 isoform of 5 α reductase would be predominant over type 1. The PCR results showed that both isoforms have very low expression levels as was indicated from the very faint bands obtained on gel after primary PCR amplification. Only after a nested PCR was performed were the bands strong enough to visualise and extract from gel for purification and sequence analysis. Mouse sequence was obtained for both type 1 and 2 genes, which proved that the use of rat primers was successful for amplification. For the type 1 isoform, 80% of new sequence within the coding region of the gene was obtained and for the type 2 around 85%. These were good results as at that time there was no mouse entry of either of the 5 α reductase isoforms in GenBank. Other groups did later submit these sequences.

The PCR results showed that both isoforms appear to be equally expressed in the testis although initially it was presumed that type 1 would be much lower. Real time PCR results suggest however that the type 1 isoform is expressed in slightly higher levels than

the type 2, The highest levels for both forms being expressed around the time of puberty (d25) These results corroborate the findings of a study done in rats where the highest abundance of type 1 isoform was expressed during puberty. Immunolocalisation of the protein demonstrated the peptide is present in the interstitial tissue of the testis (Viger and Robaire 1995) Northern blot analysis revealed that the type 1 transcript was indeed present in the testis at all the stages of development. These levels however were highest between days 21 – 28. Quantitation revealed that 5 α type 1 mRNA concentrations rose by three fold between days 7 – 21, and plateaued between 21 – 28, then fell sharply between days 28 – 35. There after levels remained constant. These findings appear to be in complete agreement with the results presented.

Quantitation of the type 1 isoform was also assessed in a separate study comparing levels in normal mouse testis and AR-null. These results are presented and discussed in detail in chapter 5.

The Enzyme activity data also appears to be in agreement with the Real Time PCR quantitation study as it was demonstrated that mRNA expression are at their highest at around D25, which was also the case with enzyme activity. Mice reach puberty at D25 and these results confirm that there is a much greater need for DHT at around this time. It is largely required for prostate function and maturity of genitals. Both are requirements of successful reproductive function. In humans the conversion of testosterone to DHT in androgen target tissue is known to be largely dependant on type 2 isoform of the enzyme, whereas in mice it appears that both isoforms are involved. Expression levels were very low throughout most stages of development

One conclusion, which can be deduced from these results, is that 5 α reductase mRNA expression is developmentally regulated. This assumption can be gained from variation in mRNA expression levels at different stages.

Chapter 5

Quantitative analysis of gene expression in normal and mutant mouse testis.

5 Quantitative analysis of gene expression in normal and mutant mouse testis

It is well established that various cell types in the body are required to function in different ways at different times. This also applies to the cells of the testes. The functional aspects of any cell are entirely dependant on the genes being expressed there at any one given time. Throughout development gene expression may vary in a temporal fashion depending on the functional requirements of the cell. This also applies to levels of gene expression. This is certainly the case if a particular protein is required in large amounts at only specific times and is not essential at others. Production of testosterone by the testis is an example of this, whereby it is required in greater amounts at critical times throughout development, puberty and in adulthood. LH receptor, StAR protein, P450scc and 5α-reductase all play important roles in the androgen production pathway.

In the mouse, androgen production varies in a temporal fashion. The fetal Leydig cells begin production rapidly after Leydig cell differentiation on about embryonic day 12.5–13 (E12.5–13). Androgen levels quickly reach a peak around E17–18 before declining again at birth . Studies have shown that levels of intratesticular testosterone decline after birth, becoming very low at around days 10–20. Thereafter, testosterone levels increase more than 16-fold between days 25 and 30, and then show a further doubling up to adulthood. (O'Shaughnessy *et al* 2002) This decline in testosterone mirrors the steroidogenic activity of the testes as measured using *in vitro* studies. (O'Shaughnessy *et al* 1991).

5.1 Androgen Receptor (AR) – null mouse

The importance of the expression of specific genes can usually be established by assessing the phenotype of animals which carry a mutation in that particular gene resulting in defective or complete absence of the protein it encodes. Many mouse models now exist where specific genes have been targeted and disrupted, or the defect has arisen by a natural mutation. One example of this is the AR-null mouse (Tfm). These animals have a single base deletion causing a frame shift mutation in the gene encoding the androgen receptor (AR). The gene consists of eight exons, of which exons 2–3 code for the DNA-binding domain, and exons 4–8 code for the ligand-binding domain (Brinkmann *et al* 1989) as a result of the mutation these animals are completely insensitive to testosterone.

Studies using the AR-null mouse allow us to assess the importance of testosterone and its receptor during development and for initiation and maintenance of reproductive function. The phenotype of the AR – null mouse differs in many ways from that of wild type. The testes themselves are greatly reduced in size and they remain in the abdominal cavity in an undescended state. These testes are functional in that they do produce testosterone (in small amounts), but this is not recognised by the defective receptors and therefore cannot generate any signal. It has been shown that serum testosterone levels much lower than wild type due to a reduction in testicular androgen production (Charest et al 1991). In addition circulating LH levels are much higher. Progesterone is the major steroid produced by the AR-null testes and this has been shown to be due to a loss in 17α -hydroxylase activity (Murphy and O'Shaughnessy, 1991). This is the enzyme which converts progesterone to the 17α -hydroxy form, the precursor of androstenedione,

which itself is the precursor of testosterone. So it appears that the steroidogenic pathway in the Leydig cells is interrupted in these animals and it is arrested at progesterone production. (Murphy and O'Shaughnessy 1991)

It has now been clearly established that testosterone is the major androgen produced by the adult Leydig cells and that they are dependant on LH for this purpose, whereas the fetal population of Leydig cells produce androstendione, which they do independently of LH (O'Shaughnessy *et al* 2000). It is possible therefore that these two distinct cell populations may be affected in different ways in the AR-null mouse.

There are several possible explanations for the underlying cause of steroidogenic failure in AR-null adult mice. It could be due to: Incomplete adult Leydig cell differentiation, decreased Leydig cell precursor proliferation (resulting in greatly reduced cell numbers), or even failure of normal adult Leydig cell development.

Recent studies have characterised the normal pattern of Leydig cell proliferation and gene expression during testicular development in wild type mice (Baker and O'Shaughnessy 2001, O'Shaughnessy *et al* 2002). This allows us then to examine differences in gene expression between normal and AR-null mice to determine the role of androgens in regulating specific gene expression

5.2 FSHβ and FSH receptor knockout mice

The FSHβ and FSHR knockout mice are good experimental models to use in assessing the level of dependence that expression of a specific gene has on FSH. FSH plays an important role in the development and reproductive function in male mammals. It is a

glycoprotein hormone and is released from the anterior pituitary with the gonads as the target site.

FSH is the most important pituitary hormone regulating Sertoli cell function. The FSH receptor, like the LH receptor, is a seven transmembrane G-protein coupled receptor. In males it is unique and highly specific because the protein is selectively localized on the surface of the Sertoli cells, outside the tight junctions (Wahlstrom *et al* 1983) FSH is essential for the normal development and function of Sertoli cells. It consists of an α subunit (which is common also to LH and TSH) and a specific β subunit. In the FSHβ -/- mouse, the gene encoding the beta subunit has been disrupted resulting in FSH-deficient mice. Phenotypically females are infertile due to a block in folliculogenesis prior to antral follicle formation. Although FSH is predicted to be necessary for spermatogenesis and Sertoli cell growth in males, FSH-deficient males are fertile despite having small testes (Kumar *et al* 1997)

Another mouse model which lacks FSH signalling is the FSHReceptor (FSHR) knockout mouse. This mutant was generated by targeted deletion of exon one of the gene encoding the receptor (Abel *et al* 2000). Female mice homozygous for this mutation are infertile, they have underdeveloped uterine horns and small ovaries compared to normal . Males are fertile, although testis size is only about 50% that of normal mice.

Both models demonstrate that FSH signalling is required for establishment of normal testicular size, seminiferous tubule diameter, sperm number and motility, although certain differences are discernible in the two models with regard to circulating testosterone levels and male fertility. FSHβ mutant males are reportedly fully fertile, (Kumar *et al*

1997), while FSHR mutant males exhibit delayed sexual maturity and reduced fertility (Dierich *et al* 1998, Abel *et al* 2000, Krishnamurthy *et al* 2000)

5.3 Objectives

The aim of this study was to quantitatively analyse expression patterns of 5α-reductase and LHReceptor genes in mouse testis. And to determine the level of dependence expression of these genes have on testosterone and FSH by comparing expression levels in normal mice to those of AR-null, FSHβ-null and FSHR-null mice . In addition, for comparative purposes, the expression patterns of P450scc and steroidogenic acute regulatory (StAR) protein are reported here.

P450scc is a key enzyme involved in the synthetic pathway of testosterone, since it is the rate-limiting enzymatic step.

StAR protein is essential for normal steroidogenesis since it delivers cholesterol (a steroid substrate) to the inner mitochondrial membrane within the Leydig cells. This is also a rate determining step of the androgen production pathway.

5.4 Materials and Methods

Variations from previous chapters only are included

5.4.1 Animals

AR-null Normal and AR-null (Tfm) mice were bred on a C3h/HeH-101/H genetic background from stock animals obtained originally from the MRC Radiology Unit, (now

the MRC, Mammalian Genetics Unit, Harwell UK). Animals were maintained as required under Home Office regulations and were used for study at ages indicated in the text. The testes of normal mice undergo final descent to the scrotum at about 25 days whereas testes in AR-null mice remain intra-abdominal into adult life. To control for the failure of testicular descent in AR-null mice normal animals were surgically rendered cryptorchid at 21 days and used for experiments when adult (Murphy and O'Shaughnessy 1991). Surgical manipulations of the animals were carried out by Prof P.J. O'Shaughnessy. Animals were killed at the appropriate age and testes were frozen in liquid N_2 for subsequent study of specific mRNA levels.

FSHR-null and FSHβ-null mice were provided by Dr HM Charlton (University of Oxford). The testes were frozen in liquid N2 in Oxford and were sent to Glasgow by courier frozen in dry ice. The control groups were FHSβ+/- as these mice were phenotypically normal.

5.5 Testis Morphology

For morphological analysis, testes were incubated overnight in Bouin's fixative and then stored in 70% ethanol. Sections were cut at 2μm and were stained with Meyer's haematoxylin and eosin. Pictures of sections of FSHβ- and FSHR-null mice, prepared in the same way, were provided by Dr M. Able (University of Oxford).

5.5.1 Stereology

Testes were embedded in Technovit 7100 resin, cut into sections (20 μm thickness) and stained with Harris' haematoxylin. Total testis volume was estimated using the Cavalieri

principle (Mayhew, 1992□), and the slides used to count the number of cells were also used to measure testis volume. The optical disector technique (Wreford, 1995□) was used to count the number of Leydig cells in each testis. The numerical density of Leydig cells was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA, USA). Stereology data was provided by Dr Paul Baker.

5.5.2 Reverse Transcription and Real-time PCR

Reverse transcription and real-time PCR were carried out as described in Chapter 4 with the exception that luciferase mRNA was added to the tissue at the start of the RNA extraction process to act as an external control. This is a method developed by Prof PJ O'Shaughnessy and described in detail in Baker & O'Shaughnessy (2000). This method adds a standard known amount of luciferase mRNA to each sample which acts as a control for losses during RNA exraction and for the efficiency of cDNA synthesis. In addition, since a standard amount of luciferase RNA is added to each testis prior to RNA extraction then expression of a gene of interest relative to luciferase is a relative measure of that gene per testis. In this way levels of gene expression can be compared directly on a "per testis" basis. In studies reported here 5ng of luciferase mRNA (Promega UK, Southampton, UK) was added to each sample.

Primers and Probes for Real-time PCR

Sequence for LHR and 5α reductase primers and probes are in chapters 2, 3 and 4

P450scc GenBank Accsession AF195119

Forward Primer - CCAGTGTCCCCATGCTCAAAC

Probe - TGCCTCCAGACTTCTTTCGACTCCTCAGA

Reverse Primer - TGCATGGGTCCTTCCAGGTCT

StAR GenBank Accession L36062

Forward Primer - CCGGAGCAGAGTGGTGTCA

Probe - CAGAGCTGAACACGGCCCCACC

Reverse Primer - CAGTGGATGAAGCACCATGC

Luciferase (sequence provided by Promega)

Forward primer – TCGAAGTATTCCGCGTACGTG

Reverse primer – GCCCTGGTTCCTGTGGAACAA

Probe – TGTTCACCTCGATATGTGCATCTGTAAAAGCA

Primer/probe sets for LHReceptor and 5α-reductase were as in Chapter 4.

Measurement of LHReceptor and StAR mRNA levels by real-time PCR was carried out by technicians within the laboratory as part of a larger study into regulation of Leydig cell gene expression. The results are included here for comparative purposes.

5.6 Analysis of results

Results were analysed by analysis of variance. Differences between mouse groups were determined using analysis of variance followed by the Neuman-Keul test. Where heterogeneity of variance occurred values were log-transformed before analysis. Differences between AR-null animals and the appropriate control group at each age (normal animals when aged 5 and 20 days and cryptorchid animals when adult) were assessed by t-tests using the pooled variance.

5.7 Results

5.7.1 Testis morphology

Testis morphology of cryptorchid and AR-null adult mice differed greatly from that of normal mice. The diameter of seminiferous tubules was much less, with the difference being more pronounced in the AR-null testis (figure 5.1) In addition there was a marked reduction in overall testis size compared to normal (control, $112 \pm 5mm^3$, AR-null, $4.2 \pm 0.3mm^3$, cryptorchid, $21.6 \pm 2.6mm^3$). The interstitial area appeared to be significantly increased although Leydig cell morphology was not clearly different.

In FSHβ-/- and FSHR-/- mice tubule size was reduced, and germ cell number appeared less. There was no clear effect on the morphology of the Leydig cells.

5.7.2 *mRNA expression levels*

5.7.2.1 LH Receptor

The temporal pattern of expression of LH receptor mRNA was initially low at d5 with an increase at d20 and then a marked increase in adulthood (table 5.1 and figure 5.2A). Although there was a significant difference in levels between the different ages, there was no significant difference between normal, AR-null and cryptorchid mice.

5.6.2.2 5 α Reductase type 1

Expression levels of 5 α reductase type 1 were barely detectable at d5 in both normal and AR-null mice. By day 20, however, there was a marked increase in normal testes. In AR-null testes there was also an increase in expression levels, although this was relatively minor compared to normal mice. At day 20, therefore there was a significant difference in expression levels between normal mice and AR-null. In the adult, the expression levels were slightly less than those observed at day 20, although there was still a significant difference in all three groups with normal testes displaying the highest levels and cryptorchid and AR-null expressing significantly lower levels. (Table 5.2 and Figure 5.3). Expression in adult AR-null testis mice was significantly less than that in adult cryptorchid testis.

StAR

StAR expression levels showed a similar profile to that of the LH receptor in that levels were low initially at d5 increased about 5-fold at day 20 and then continued to increase significantly up to adulthood. There was no significant difference between normal, AR-null and cryptorchid animals (Table 5.3 and Figure 5.4)

P450scc

The expression pattern of P450scc was also similar to that of the LH receptor in that levels were low at day 5, increased at day 20 and then showed a marked increase up to adulthood. In contrast to the LH receptor there was a significant difference in expression levels between all three adult groups with normal having the highest levels, followed by AR-null then cryptorchid the lowest of the three. (Table 5.4 and Figure 5.5)

5.7.3 FSH mutant mouse study

To study the role of FSH in regulation of expression of LHReceptor and 5 α reductase levels of mRNA encoding these proteins were measured in normal mice and in FSHR-null and FSHβ-null mice. Levels of 5 αreductase type 1 varied between groups but there was no overall significant difference between any of the groups (Fig 5.6A) Expression of the LHReceptor was unaffected by any of the null-mutations (Fig 5.6B.) StAR mRNA expression levels were significantly higher in FSH-/- and FSHR+/- (Fig 5.6C) and P450scc levels were lower in the FSHR-/- testes (Fig 5.6D)

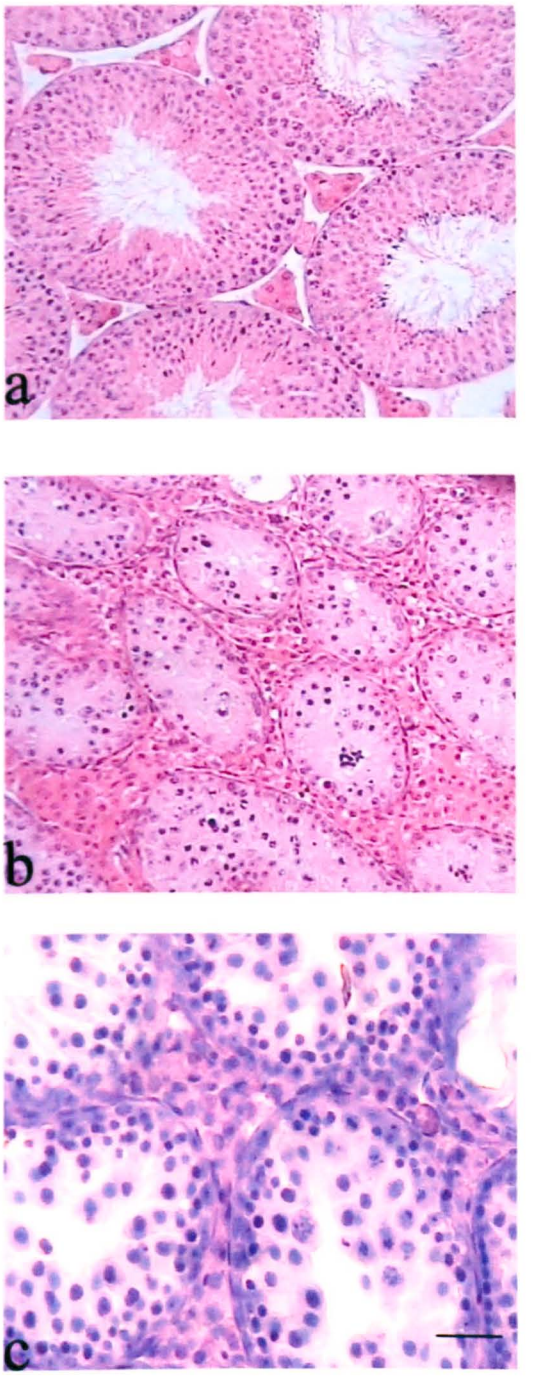


Figure 5-1 Light micrographs showing morphology of testes from A- normal, B- AR-null and C- cryptorchid adult mice. Bar = 50μm.

(Images were provided by Dr Paul Baker)

LHR mRNA expression levels relative to Luciferase			
Age of mice	**Normal**	**AR -null**	**Cryptorchid**
5 days	1.7 ± 0.3	2.3 ± 0.6	NA
20days	9.1 ± 1	9.3 ± 1.8	NA
Adult	41 ± 4	34 ± 8	32 ± 7

Table 5-1 LHR mRNA expression levels in normal, AR-null and cryptorchid mouse testis at different ages.

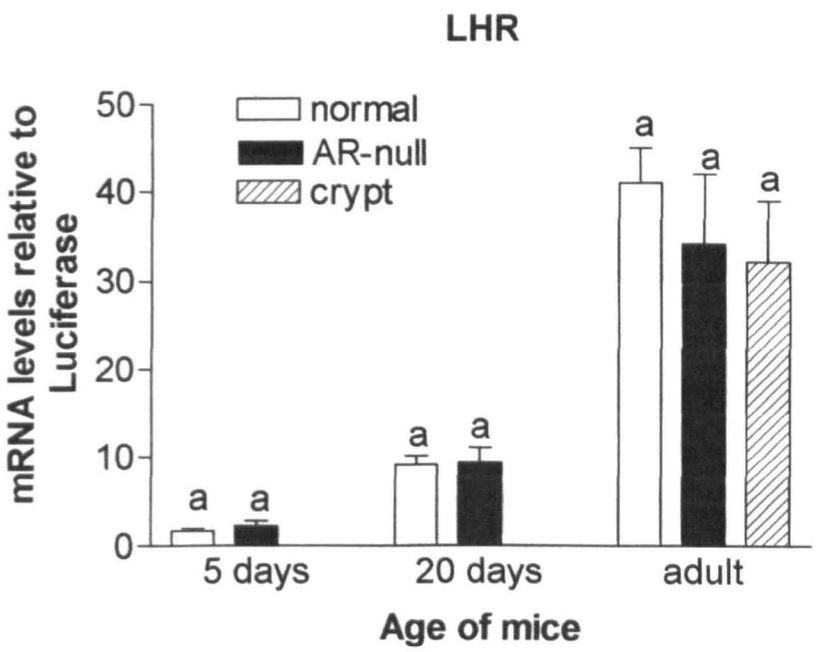


Figure 5-2 Expression of A – LHR, mRNA levels relative to luciferase in testes of normal AR-null and cryptorchid mice. Different letter superscripts indicate a significant difference in mRNA levels ($P<0.05$). data is represented as mean ± s.e.m. (n = 4-6)

5 αReductase type 1 mRNA expression levels relative to Luciferase			
Age of mice	**Normal**	**AR -null**	**Cryptorchid**
5 days	0.2 ± 0.02	0.1 ± 0.004	NA
20days	9 ± 3	1 ± 0.5	NA
Adult	5.5 ± 1	0.3 ± 0.05	1.5 ± 0.5

Table 5-2 5 α Reductase type 1 mRNA expression levels in normal, AR-null and cryptorchid mouse testis at different ages.

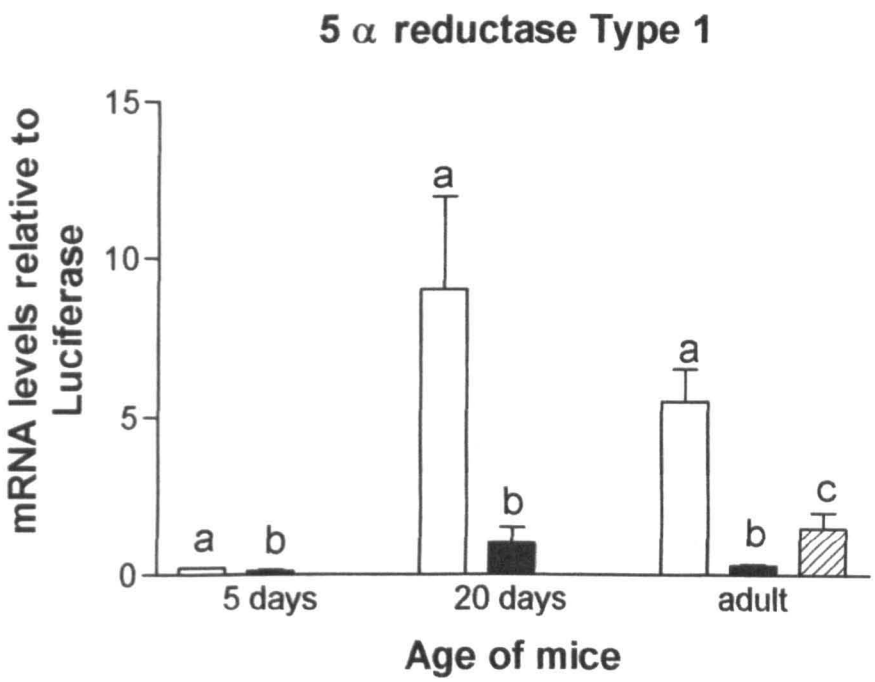


Figure 5-3 Expression of 5 α reductase type 1 mRNA levels relatine to luciferase in testes of normal AR-null and cryptorchid mice. Different letter superscripts indicate a significant difference in mRNA levels ($P<0.05$). data is represented as mean ± s.e.m. (n = 4-6)

StAR mRNA expression levels relative to Luciferase			
Age of mice	**Normal**	**AR -null**	**Cryptorchid**
5 days	4.0 ± 1.1	6.0 ± 1.3	NA
20days	22 ± 2.1	23 ± 3.2	NA
Adult	195 ± 37	189 ± 57	134 ± 18

Table 5-3 StAR mRNA expression levels in normal, AR-null and cryptorchid mouse testis at different ages.

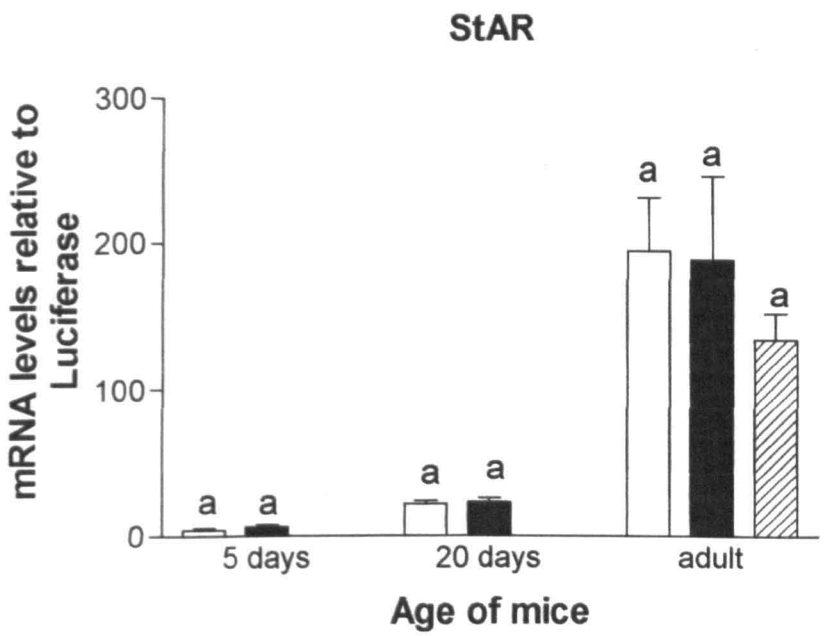


Figure 5-4 Expression of StAR mRNA levels relative to luciferase in testes of normal AR-null and cryptorchid mice. different letter superscripts indicate a significant difference in mRNA levels ($P<0.05$). data is represented as mean ± s.e.m. (n = 4-6)

P450scc mRNA expression levels relative to Luciferase			
Age of mice	**Normal**	**AR -null**	**Cryptorchid**
5 days	2.2 ± 0.5	6.1 ± 2.1	NA
20days	11.6 ± 2.2	21.6 ± 3.5	NA
Adult	242 ± 35	177 ± 38	90 ± 25

Table 5-4 P450scc mRNA expression levels in normal, AR-null and cryptorchid mouse testis at different ages. All data are expressed as mean SEM

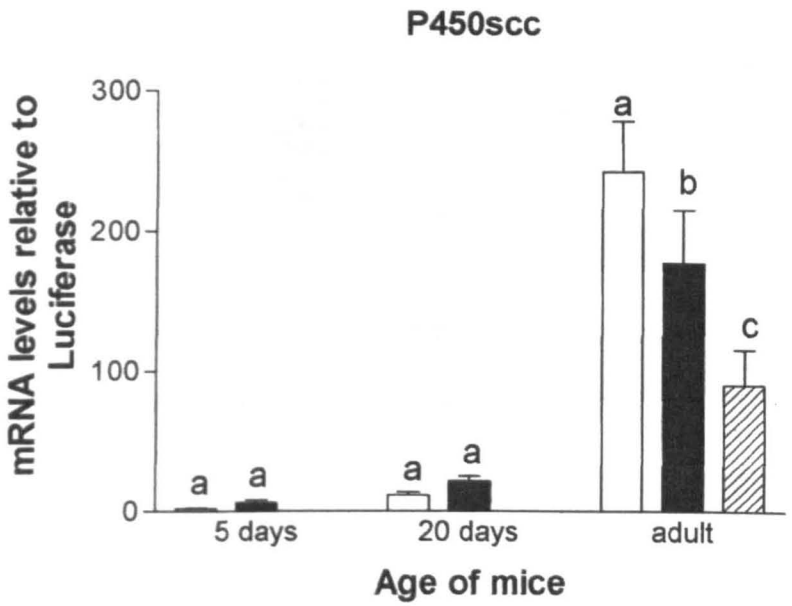


Figure 5-5 P450scc mRNA levels relative to luciferase in testes of normal, AR-null and cryptorchid mice. Different letter superscripts indicate a significant difference in mRNA levels ($P<0.05$). data is represented as mean ± s.e.m. (n = 4-6)

5.7.4 FSH mutant data

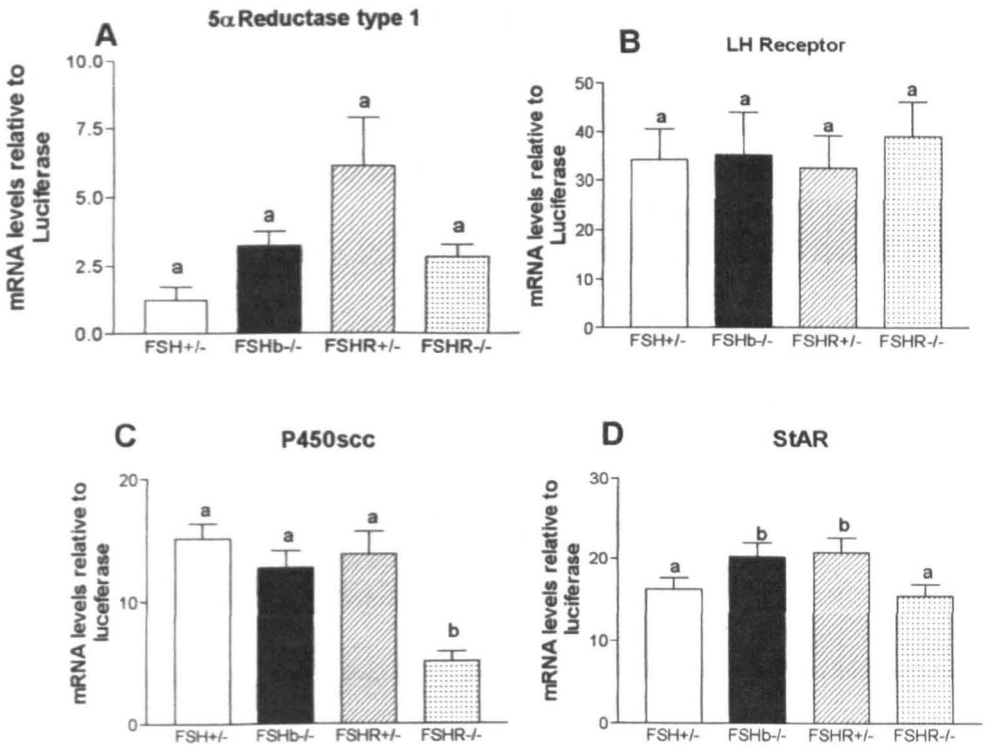


Figure 5-6 Figure 5-6 Expression of A – 5areductase type 1, B – LHR, C – P450scc and StAR mRNA levels in testes of FSHb+/-, FSHb-/-, FSHR+/- and FSH-/- mice.

The level of each mRNA species is expressed relative to an external standard (luciferase). Within each mouse group, different letter superscripts indicate a significant difference in mRNA levels ($P<0.05$). data is represented as mean ± s.e.m. (n = 4-6)

5.8 Discussion

LHR mRNA expression levels were very low in both AR-null and normal testes at 5 days, but there was an increase of about three fold in both groups by puberty, and by adulthood there was a further 4 fold increase in expression levels in all three groups with

there being no significant difference detected between any of them. These results therefore suggest that LHR mRNA expression is not dependant on androgen action. It is worth mentioning however that due to the position of the Real Time PCR primers and probe, they may have been amplifying both full-length LH receptor and the largest spliced transcript which was discussed in chapter 3. It is possible, therefore, that there could be a change in the splice pattern which would not be detected by real-time PCR. This could be examined directly by normal PCR although that is not quantitative. Alternatively, it would be possible to design the primers and probe to span the boundary of exons 10 and 11. Only full-length LH receptor would be detected in that case.

One possible factor controlling LHR mRNA expression may be LH itself. Studies in adult male rats using a single dose of the cytotoxic drug ethylene dimethane sulphonate (EDS) which is know to eliminate the mature Leydig cell population (Molenaar *et al* 1985, Morris *et al* 1986) demonstrated that repopulation of leydig cells occurs after three weeks. The cells differentiate from existing undifferentiated precursors. It has been reported that repopulation is largely dependant upon LH action (Teerds *et al* 1989). After administration of EDS there was a significant depletion of serum testosterone levels and a marked increase in serum LH levels (Tena-Sempra *et al* 1999). A key developmental event in Leydig cell differentiation is the acquisition of LH receptors. One group observed that early leydig cell precursors express a truncated form of the LH receptor (Tena-Sempre 1994 endocrinology 135 1018-1024). Full-length LH receptor expression was not detected until 15 days post recovery. (Tena-Sempre 1999) Expression of full-length LHR transcript is likely to be inversely dependant on availability of LH as levels are high immediately after EDS treatment and then they gradually return to normal. High levels of LH may prevent expression of full-length transcript.

Expression levels of 5 α reductase type 1 mRNA show a different pattern to those observed for the LHR. At all ages examined levels were very low relative to luciferase although they did vary throughout development. Levels were barely detectable in day 5 testis for both normal and AR-null testis, however by day 20 there was a marked increase in normal testis and only a slight decrease in AR-null testis. The difference in expression levels between the two groups at this stage was significant. By adulthood the levels in the normal testis had dropped slightly, but were still significantly higher than those observed for AR-null and cryptorchid testis. Interestingly, though, cryptorchid testis showed significantly greater expression levels than AR-null testes. These results strongly suggest that expression of 5αreductase type 1 mRNA in the prepubertal and the adult mouse are dependent on androgen action. In female mice 5 alpha reductase expression has been shown to be regulated by progesterone (Matsui *et al* 2002). As progesterone is a precursor of testosterone and the predominant steroid hormone in females then it would be safe to make the assumption that expression of 5 α reductase type 1 is an androgen dependant event.

In the FSH mutant mice there was a trend for increased expression levels of 5α-reductase type 1 mRNA in all three mutant forms compared to normal and in particular the FSH+/- mouse (figure 5.3A), however these levels failed to reach significance after statistical analysis. It would appear therefore that regulation of expression of 5α-reductase type 1 mRNA is independent of FSH action.

A rate limiting and acutely regulated step in LH-stimulated steroidogenesis in the gonads is the delivery of cholesterol to the inner mitochondrial membrane (Crivello and Jefcoate 1980) This was shown to be mediated by StAR protein. (Clark *et al* 1994, Stocco and

Clark 1996). This assisted transportation is a crucial step for rapid steroid synthesis as it is here the P450scc enzyme is situated. Expression of star protein in the adrenals and gonads is known to be stimulated through the cAMP signalling pathway and is closely correlated with the acute steroidogenic response of these cells to tropic hormone stimulation (Stocco and Sodeman 1991, Epstein, and Orme-Johnson1991). The results show that there was no significant difference in StAR mRNA expression levels between mouse groups at any of the ages examined. This clearly demonstrates that in absence of androgen action StAR mRNA expression is unaffected in the testis

With regard to regulation of P450scc, several groups have reported that cultured porcine Leydig cells increase synthesis of P450scc after treatment with hCG or dibutyril cAMP. This has been demonstrated at both protein level (Mason *et al* 1984) and mRNA (Orava *et al* 1989). Another study corroborated these findings using cultured mouse Leydig cells whereby cAMP added to the cutured cells resulted in a two fold increase of P450scc synthesis (Anakawe and Payne 1987). A subsequent study by the same group demonstrated that glucocorticoids inhibit both synthesis of P450scc and decrease steady state levels of mRNA in mouse Leydig cells. This inhibition of P450scc synthesis by glucocorticoids is prevented by the antiglucocorticoid RU-486. Other steroids such as progesterone and testosterone have no effect on P450scc (Hales and Payne 1989).

P450scc mRNA expression was low in day 5 and day 20 testis, levels increased dramatically in the adult testis, although a significant difference was detected between the three groups. Normal testis showed the highest levels, followed by AR-null and then cryptorchid. Our study has shown that normal adult mouse testis expressed significantly higher levels of P450scc mRNA than AR-null testis, this may suggest therefore that

testosterone has an indirect regulatory mechanism on P450 mRNA expression *in vivo*. Although these results may appear to be slightly conflicting with the previous studies which suggested that testosterone had no regulatory effect on P450scc, it is worth bearing in mind that these studies were performed *in vitro* and the mouse testis cells used for culture would have already been exposed to testosterone *in vivo*. The testis extracted from the AR-null mice in our study would have had no previous exposure to testosterone.

On comparison with these results therefore it could be suggested that prior exposure to testosterone by the testis is required for optimal expression levels of P450scc mRNA, and that further addition of testosterone has no effect.It appears therefore not to be an absolute requirement as the cells will synthesise p450scc in its absence.

Of the four genes assessed StAR and P450scc displayed the greatest levels of expression in adult testis relative to Luciferase. On comparison of results they indicate a clear difference in expression levels in two of the four genes examined in normal and AR-null testis. These differences however do not become apparent until after day 20. Because these genes are all expressed in the Leydig cells of the testis, this suggests that the fetal Leydig cell population are developing and functioning independent of androgen action. This proposal is strengthened by the fact that gene expression patterns are very similar in the normal and AR- null mice and they only start to differ after day 5. In addition Leydig cell numbers are also very similar until this stage. This was established in a different study (O'Shaughnessy *et al* 2002b).

It was also shown that the difference in Leydig cell number becomes much more apparent by day 20 where the cell count in AR-null testes was 30% less than that of

normal. The Leydig cells of the AR-null testes do proliferate at puberty through to adulthood, but to a lesser extent than normal. Leydig cell number in the adult AR-null testis is about 60% of normal. Expression levels of the LHR were in normal range on day 5 and day 20.

In normal mouse testis 5 α reductase type 1 expression levels peaked at day 20, this correlates with the onset of puberty and a greater need for the 5 α reduced androgen dihydrotestosterone. Compared to normal testis the AR-null the showed a similar peak in 5α-reductase type 1 expression levels, but overall they were significantly less than normal, they did however appear in the normal range at day 5.

Results from the FSH mutant mouse study showed there to be no difference in expression levels of LHR and 5 α reductase. This suggests therefore that mRNA expression of these genes is not dependent on FSH action in adult testis. There was however a significant decrease in the levels of P450scc in the FSHR-/- testes, but no change in the FSHβ-/-.It would have been expected that the effects of induced Null mutations in the hormone β-subunit gene or the hormone receptor gene would induce similar changes in expression levels of other genes seeing as both these mutant strains have reduced testis size and reduced spermatogenesis (Kumar et al 1997, Dierich et al 1998, Abel et al. 2000). One study however has demonstrated that there are indeed phenotypic differences between the two mutants. Leydig cell number is normal in FSHβ-/- animals whereas they are greatly reduced in the FSHR-/- in addition testosterone levels are much lower in these animal as is expression of key mRNA species associated with steroidogenesis in the affected animals (Baker *et al* 2003)

It has been proposed that the difference in Leydig cell number and androgen production may be due to the presence of constitutive activity within the FSHR in the absence of hormone ligand (Baker *et al* 2003)or that some other ligand may be acting on the receptors in the FSHβ-/- mice. If this is the case then it would suggest that P450scc expression does have some dependance on FSH receptor activation, however this cannot be the sole modulator as expression levels are merely reduced and not entirely absent.

With regard to FSH, according to the quantitation studies on LHR and 5α-reductase type 1 mRNA in the testis, it seems that both these genes are expressed in the adult mouse testis independent of FSH action. Results suggest that as all of the genes examined are known to be expressed in the Leydig cells of the testis, function and regulation of the fetal population appears to occur independently of androgen action, whereas it is necessary for optimal function in the adult population.

In summary, androgen action is only required for expression of 5 alpha reductase type 1 mRNA in both prepubertal and adult testis. The other three genes are expressed independent of androgen action, however it would appear that for optimal expression of P450scc in the adult testis androgen action is required.

Chapter 6

Discussion

6 Discussion

It is clear that there are many factors involved in the differentiation and functional regulation of the testis. This project has examined only a few of these factors, although each may play an essential role in testis development. In the wider picture, some of the genes expressed in the testis may only contribute in a temporal fashion and expression may only be required for a short, but crucial time point. This would include the genes like *SRY*, WNt4 and *SOX9*. Mutations in these genes whether in mice or in humans have shown that they do play an essential role in sexual differentiation of the testis as their absence leads to incomplete virilisation and in some cases formation of a female phenotype. Mouse models are an excellent tool for further understanding the importance of the role a particular gene may play. Absence of expression of one gene can also lead to various effects on the expression of others. Comparing the expression pattern of various genes in normal mice with the same genes in mutant animals allows us to assess in greater detail the effect of absence of protein and how this may effect several aspects of the phenotype as has been clearly shown in the AR-null and hpg mice.

6.1 LH receptor

The initial objectives of the LH receptor project were to characterise and sequence the variant forms of the receptor, these objectives were largely met. In chapter 3 the results showed clearly that there are four transcripts of the LH receptor present in mouse testis in various combinations at different stages of development. The presence and abundance of all four transcripts varied throughout the different developmental stages examined. At 15 + 16dpc there was no full-length receptor detected and only two of the shorter forms

were present. These findings would suggest either a) that androgen production is occurring independently of LH action at this stage; or b) That LH can act through the truncated receptors.

Evidence from other studies would suggest that the fetal Leydig cell population differentiates and initially functions in the absence of LH action. (O'Shaughnessy *et al* 1998). This has been demonstrated in the LHR – null mice. Androgen production occurs normally during fetal development independent of LH action. This coincides with the fact that although steroidogenesis by the testis is occurring, it is relatively low at this time period. During 17 - 18dpc there is abundant expression of the full-length receptor transcript, which may be in response to the increased requirement for androgen production. This would further amplify the intracellular mechanisms occurring in the androgenic pathway. At this stage in the mouse androgens are required for differentiation of the Wolffian ducts and external genitalia. This is a crucial time point in the mouse, and one which requires greatly increased androgen levels. This would suggest that the fetal Leydig cells are now undergoing androgen production via the intracellular signalling cascade initiated by LH receptor and ligand binding, whereas previous to this time androgen production was independent of LH intervention. (O'Shaughnessy *et al* 1998).

Results from this study (in Chapter 3) show that the full-length receptor transcript is undetectable from 19dpc - d25. This does not mean however that it is not being expressed, but merely that levels may be expressed in much lower abundance than the other transcripts, and they were too low to be detected by the molecular methods used for this study. This also coincides with a decrease in testosterone levels during this period (O'Shaughnessy *et al* 2002)

Expression of the truncated forms of the LH receptor are more abundant between the time of birth and the onset of puberty, which in the mouse occurs between d25 - d30. Form day 5 it is highly likely that the detection of any LHR transcripts is due to their presence in the adult population of Leydig cells, as it has been shown that the fetal population are no longer functionally active from this stage (Habert and Picon 1982, Tapanainen *et al* 1984, Habert and Brignaschi 1991). At day 25, this is the onset of puberty in the mouse, and is another time point in development when there is a much greater need for testosterone. The fetal Leydig cells are no longer active at this stage and detection of the LH receptor transcripts is from the adult population only.

These experiments were performed between 2-4 times on different animals for each age group examined. This was to ensure consistency and reproducibility of the results obtained. Consistency of expression of the various transcripts at specific time points was observed and it is possible therefore that these truncated forms of the receptor may have distinct biological roles. Analysis of the protein hydrophobicity plots performed on all four receptor forms revealed structural differences between them.

A study by Inhae and Tae (1991) demonstrated that both the extracellular region of the receptor and the transmembrane region can function independently in their own right. They showed this by constructing a mutant form of the receptor. One consisted of exons 1-10 only and the other consisted of exons 1+11 only. Through transfection into an expression vector they were able to produce the truncated proteins. Cells expressing these receptor forms as well as ones expressing full-length LH receptor were subjected to two different tests to determine whether either of the truncated forms were functionally viable. One test assessed binding efficiency and the other determined ability

to initiate cAMP production. Results showed that in a binding assay the truncated 1-10 form was able to bind hCG with just as high an affinity as the the full-length receptor cells. This suggests therefore that the extracellular region of the receptor can bind to ligand independently of the transmembrane region. This receptor form however was unable to initiate cAMP production in another assay

This adds further weight to the possibility of the truncated forms of the LH receptor having a functional relevance perhaps as a soluble receptor form which binds hormone but does not initiate signalling cascade.

6.2 5 α Reductase

From the results in chapter 4, we clearly see that expression of the two 5 α Reductase enzymes in the testes is very low throughout most of development. There are however two distinct peaks of increased expression and these occur coincidently at crucial time points in the mouse development. The first occurs at embryonic stages E17-E18 for a few days and the second occurs around about time of puberty.

This adds convincing evidence to the concept that the major effect of 5 α reductase is to amplify the androgenic signal at specific time points when it is required more. This is further corroborated by the fact that male mice with apparent absence of dihydrotestosterone as the result of targeted disruption of the genes for both type 1 and type 2 enzymes have fully formed internal reproductive system and only slightly affected external genitalia (Mahendroo *et al* 2001). These findings suggest that virilisation is occurring as a result of testosterone action alone and it would appear that 5 α reductase plays a less crucial role in the mouse as its absence (and therefore lack of

dihydrotestosterone) can easily be compensated by testosterone This finding is in contrast to the profound impairment in virilisation in human males with a 5 α reductase type 2 deficiency.

One explanation for difference in the two species is that in humans, prostate testosterone levels change very little with the inhibition of 5 α reductase (McConnell *et al* 1992). In mice however, disruption of the 5 α reductase genes result in prostate and testicular levels of testosterone levels of more than one hundred fold greater. (Mahendroo *et al* 2001) This is clear implication that very high levels of testosterone can perform the same role as low concentrations of dihydrotestosterone, thus although dihydrotestosterone may perform some as yet unidentified unique functions (Hsiao *et al* 2001), its major role in males is to greatly amplify a weak androgen signal.

6.3 Sequence analysis of the 5 α Reductase genes

As mentioned previously in chapter 4 at the time this study was undertaken there was very little published mouse sequence of either type 1 or 2 form of the 5 α reductase genes. The techniques applied in order to obtain new mouse sequence for both type 1 and type 2 genes proved successful and almost all of the sequence within the coding regions of both genes was obtained. This success was in part due to the high species homology enabling the design of rat primers, which were able to amplify mouse sequence. In addition molecular biology computer programmes such as BLAST allowed for identification of the sequence obtained from extracted bands of PCR products. Unfortunately by the time this project was completed, there was already submissions of

extended mouse sequence for both type 1 and 2 5 α reductase to GenBank, however initial objectives were met.

6.4 Quantitation of gene expression in normal and AR-null mouse testis

Loss of a functional androgen receptor due to a mutation in the gene encoding it clearly has detrimental effects on the phenotype of male mice. The result is pseudohermaphrodism, whereby there is a loss of internal reproductive duct systems and failure of testicular descent. This confirms that differentiation of the Wolffian duct and testicular descent are both events which rely heavily upon androgen action. In addition to the developmental defects, spermatogenesis is also disrupted with a loss of normal germ cell development although it is as yet unclear how much of this is due to the cryptorchid state of the animals and how much is due directly to loss of androgen receptors. In addition to these defects, it has been shown that adult Leydig cell numbers are greatly reduced in AR-null mice, with total cell number in AR-null testis being only 60% that of normal. (O'Shaughnessy *et al* 2002b) This is a clear indication that proliferation is also dependent, either directly or indirectly on androgen action.

With the gene expression patterns, loss of androgen action appears to have little effect on LHR mRNA levels, cryptorchidism had very little effect also with the Real time PCR quantitation results showing no significant difference between the normal, AR-null and cryptorchid adult testis. This would suggest that regulation of expression of this gene occurs independently of androgen action, either that or some compensatory mechanism

has been initiated and this then regulates expression levels, keeping them within the normal range.

Expression levels of 5 α reductase type 1 are similar in all three groups at day 5. In mice, the fetal Leydig cell population persists after birth but becomes subordinate to the adult population which begins to differentiate sometime between days 7 and 10 after birth (Vergouwen *et al.* 1991; Baker *et al.* 1999;). These expression patterns therefore are from the fetal population of Leydig cells. This suggests that the fetal cells function independant of androgen action.

Chapter 5 concentrated on gene expression in normal and mutant mouse testis. The mutant strains lacked either androgen action due to defective receptor, or FSH action due to absence of specific β unit or defective receptor. Of the four genes assessed in this chapter LHR and StAR mRNA expression occurred independently of androgen action. Evidence of this is presented by the lack of variation in expression levels between the normal, cryptorched and AR-null mouse groups assessed. In contrast, however, 5α reductase type 1 and P450scc do show variation in mRNA expression levels between groups. Normal mouse testis had the highest levels at day 20 and adult stages, with the other two groups being significantly lower. P450scc plays an important role in the androgen production pathway (discussed in detail in chapt 1) and likewise 5 α reductase in the production of the more potent androgen dihydrotestosterone. The differences in expression levels of these genes between mouse groups is therefore suggestive that certain steps in the androgen pathway may in fact require androgen action for optimisation.

All of the results in this study have centred around expression of genes in various mouse strains in a temporal fashion. For a more complete picture it would be desirable to assess the protein levels as gene expression levels dont always correlate with abundance and availability of protein.

7 Appendix

7.5 Abbreviations

AMH.....Anti mullerian hormone

AR-null.....Androgen receptor null

CAIS.....Complete androgen insensitivity syndrome

cAMP.....Cyclic adenisine monophosphate

cDNA.....Complimentary deoxy ribonucleic acid

DHT.....Dihydrotestosterone

dNTP'S.....Deoxynucleoside Triphosphates

dpc.....Days post coitum

DTT.....Dithiothreitol

EST's.....Expressed sequence tags

FSH.....Follicle stimulating hormone

Hpg.....Hypogonadal

LH.....Luteinising hormone

PCR.....Polymerase chain reaction

P450scc.....P450 side chain cleavage

PAIS.....Partial androgen insensitivity syndrome

StAR.....Steroid acute regulatory protein

TBE.....Tris-Borate EDTA.

7.6 Materials and Methods

Sonication – A method used for the disruption of cells and tissue using a metal probe which emits high frequency ultrasound vibrations. Cell membranes are ruptured as a result of this and the cell contents released.

Ultra violet (UV) light treatment – UV treatment to water or any other solution will destroy any contaminating DNA as it cross links [which?] chemical bonds to form in DNA. The exposure thus interrupts normal DNA replication.

Denhardts Reagent – this is generally made up as a x50 stock solution and is usually diluted ten fold into prehybridisation buffer

X 50 Denhardts contains;

5g Ficoll (type 400 Pharmacia), 5g polyvinylpyvrolidone, 5g bovine serum albumin and H2O to 500 mls.

X10 stock of TBE Buffer

Measure ~800ml H_2O, Add 108g Tris base. 55g Boric acid Add 9.3g EDTA Adjust volume to 1L with additional distilled dH_2O. This is diluted to a working concentration of X0.5

Bioprime Labeling kit – Gibco/BRL

DNA free – Ambion Inc, supplied by AMS Biothech, UK

Luciferase mRNA – Promega, Southampton, UK

Minicolumns – Chemicon International Inc, Temecula

M-MLV Rreverse transcriptase – Gibco, Paisley

PCR primers – MGW Biotech, Ebers-Berg, Germany

Real time PCR primers and probes – Primer Express, Aplied Biosystems, Warrington, UK

RNAzol – Biogenesis Ltd, Bournmouth, UK

8 References

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