# Investigation of androgen receptor gene DNA sequence in patients with complete or partial androgen insensitivity syndrome or unexplained infertility

Douglas G Tincello
BSc (Edin), MB ChB (Edin)

Degree of Doctor of Medicine
University of Edinburgh
1993

# Table of contents

## Acknowledgements

During the course of my work for this thesis I have been supported and encouraged by many people, without whom the task would have been much harder. First among these must be my supervisor, Dr Philippa Saunders, whose constant encouragement, advice and undying enthusiasm renewed my spirits on many an occasion. Her ability to find a new approach to a problem and to be always cheerful whilst juggling the needs of myself, a PhD student, the rest of the department and two children is amazing. I thank her for it.

Among my colleagues, some deserve special mention for their companionship and friendship over the time of my appointment in the unit: Joe Gaughan, Sharon Maguire, Safia Qureshi, Tanya McLaren, Sarah Duthie, Chris McKinnell, Katie Turner, Gordon Coupar, Mike Millar and Tony West.

My thanks are also due to: Dr Richard Sharpe for his background presence and support and especially for reading the proof of this thesis, in the midst of all the other demands on his time; to Mr Tim Hargreave for his advice, and for teaching me a great deal about clinical andrology; and to Tom McFetters and Ted Pinner for their hard work and patience in producing the photographs. I must also thank all the staff of the MRC Reproductive Biology Unit for their friendliness and warmth in making me feel welcome, and particularly Pat Young for her assistance and Professor Dennis Lincoln for allowing me to work in his unit for much of my time.

Finally, I would like to thank Aileen and Rebecca for their support, patience and love. I am very fortunate to have such a family, and I dedicate this work to them.

## Declaration

I declare that this thesis is entirely my own composition and that the work described herein is entirely my own work. Any contribution by another individual is duly acknowledged in the text.

Douglas G Tincello

September 1993

# Chapter 1. Abstract of thesis

Androgen insensitivity is an X-linked hereditary disorder characterised by failure of virilisation during *in utero* and pubertal development. The genetic basis of the disorder is now well defined with the finding that deletions or point mutations of the androgen receptor (AR) gene are present in many affected patients. The AR is an intracellular steroid receptor and acts by binding to DNA to cause transactivation of target genes. The receptor protein possesses different structural domains which are essential to effect hormone binding, DNA binding and transactivating functions. The identification of mutations within the AR gene of affected individuals has allowed the elucidation of certain key amino acids essential for receptor function. The mutations which have been identified are located throughout the gene and cause a spectrum of functional impairment from abolition of binding to subtle effects on conformational stability of the receptor/ligand complex. In the light of this it has been suggested that androgen receptor gene mutations may be responsible for infertility in otherwise normal men.

DNA was extracted from whole blood obtained from six patients with complete androgen insensitivity and from nine patients with features of partial androgen insensitivity and the individual exons of the AR gene were amplified by polymerase chain reaction. Direct sequencing of the exons was performed to detect the presence of point mutations. Of the fifteen patients, point mutations were detected in three with complete androgen insensitivity and in four with partial insensitivity. Another of the patients showed an amplification of the glutamine homopolymeric repeat region of the receptor; this abnormality is known to be associated with spinal and bulbar muscular atrophy (Kennedy's disease) and a review of the case history suggested that he had an atypical presentation of this disease.

Blood was also obtained from a cohort of infertile men with endocrine parameters suggestive of an impairment of androgen feedback and exons of the AR gene amplified from these samples. To enable screening of the AR gene to be performed the technique of denaturing gradient gel electrophoresis was established in the laboratory using the previously identified mutations as positive controls. The samples from the infertile men were screened using this method which was able to detect 95% of mutations present in a given sample. No mutations were detected in the androgen receptor gene of any of the infertile men.

In conclusion, molecular methods have been employed to identify mutations in the AR gene of patients with clinical symptoms of androgen insensitivity but an examination of the AR gene of a selected population of infertile individuals failed to identify defects in any of the men.

# Chapter 2. Introduction and Literature Review

## 2.1. Preliminary remarks

In this chapter I will describe the current state of knowledge of the molecular basis of androgen insensitivity syndromes and outline the objectives of the research which is described in later chapters. I shall examine the mechanism of normal sexual development from conception to birth and compare this with the abnormal development seen in male pseudohermaphroditism. The central position occupied by testosterone will be emphasised and the structure and function of the androgen receptor discussed in health and disease.

## 2.2. The role of androgens in development and fertility

### 2.2.1. Gonadal control of sexual differentiation

The sex of an individual is determined by the sex chromosomes from the moment of conception when the sperm fuses with the ovum. The presence of an X and a Y chromosome within the zygote leads to differentiation as a male, while two X chromosomes will produce a female. It has been shown that the Y chromosome is the essential factor which directs male development, while female differentiation will occur in the absence of the Y chromosome. The presence of a specific female factor (i.e. the X chromosomes) is not required to initiate this process although they are essential to attain full ovarian development and normal fertility (Moore, 1988). An examination of the early embryonic development of the sexual organs reveals how the Y chromosome functions.

The Y chromosome carries several genes which are thought to control the differentiation of the primitive gonad during the seventh and eighth weeks of embryonic life. The short arm (Yp) carries the gene for testis determining factor (Vergnaud *et al.*, 1986) which stimulates the development of the indifferent gonad into an immature testis, with the formation of seminiferous cords, spermatogonia, Sertoli cells and Leydig cells. A second gene (H-Y) lies on the long arm (Yq) of the chromosome (Simpson *et al.*, 1987) and is also thought to have some role in the differentiation of the primitive gonad into a testis. It has recently been suggested that a third gene on the Y chromosome is important for normal spermatogenesis to occur. Infertile male patients have been reported with microdeletions of portions of the long arm of the Y chromosome and the term azoospermia factor (AZF) applied to the putative gene which is affected (Chandley *et al.*, 1989). These deletions have been

identified in a portion of the Yq region which is not contiguous with the H-Y antigen gene in two patients with idiopathic infertility (Ma *et al.*, 1992; Simpson *et al.* 1993) but the actual gene has not yet been identified.

By eight weeks of life the Leydig cells begin to secrete testosterone (Laycock & Wise, 1983) and the Sertoli cells of the primitive gonad produce Müllerian inhibitory factor (MIF) (Lee & Donahoe, 1993). The early (seven week) embryo has two sets of paired genital ducts: the Müllerian ducts and the Wolffian ducts. The Müllerian ducts are progenitors of the Fallopian tubes, uterus and upper two thirds of the vagina in a female, and the Wolffian ducts are progenitors of the epididymides, vasa deferentia and seminal vesicles in a male. In male embryos the presence of MIF causes regression and degeneration of the Müllerian ducts (Lee & Donahoe, 1993) while testosterone stimulates the growth and development of the Wolffian ducts. In the absence of these hormones, the Müllerian ducts will develop spontaneously and the Wolffian ducts will regress. The hormonal environment therefore determines development of the reproductive tract so that by ten weeks of life the embryo has a definable sex with either Wolffian duct structures and developing testes, or Müllerian duct tissues and ovaries. The external genitalia develop from the genital swellings and folds of the urogenital sinus. Development of these structures into penis and scrotum is driven by an active metabolite of testosterone, dihydrotestosterone (DHT), and in the absence of this stimulus, they develop into the labia majora and minora.

Thus the gonad has a central and fundamental role in the male differentiation of the early embryo. Sexual development into a female can be considered to occur by "default". The hormones secreted by the early, Y bearing, gonad drive male differentiation by inhibiting this and stimulating the growth of the male duct system.

### 2.2.2. The central role of androgens in sexual differentiation

Androgens are steroid hormones synthesized and secreted almost entirely by the Leydig cells in response to LH from the pituitary gland with about 5% of circulating testosterone synthesized by the adrenal cortex (Laycock & Wise, 1983). Testosterone circulates in the blood bound to albumin and sex hormone binding globulin. The normal plasma levels are 12-35 nmol/l in men and 0.5-2.5 nmol/l in women. In many target tissues (including seminiferous tubules, prostate and genital skin) testosterone is converted to the more potent androgen dihydrotestosterone (DHT) by the cytoplasmic enzyme 5 α-reductase. DHT is approximately 10-fold more active than testosterone and acts via the same receptor as testosterone, to which it binds with 2-fold greater affinity (Grino *et al.*, 1990).

In embryonic life testosterone is the major gonadal hormone responsible for the development and growth of the genital ducts and accessory glands (Griffin & Wilson, 1980; Moore, 1988). DHT acts mainly upon the external genitalia, causing the growth of the penis and scrotum from the genital folds and genital swellings of the embryo. At puberty and in adulthood, androgens control the growth of the genitalia (via DHT) and the development of secondary sexual characteristics: growth of muscle, facial and body hair; deepening of the voice, and increased sebum secretion (Kiraly *et al.*, 1987).

Androgens are also involved in normal spermatogenesis, acting on blood vessels, peritubular cells and the cells within the seminiferous tubules. The main sites of action appear to be the Sertoli cells and peritubular cells, since they are the only cells which possess androgen receptors (Sar *et al.*, 1990) (Fig. 2.1). Androgen action upon spermatogenesis is effective during a small window of time in the spermatogenic cycle; in the rat the available evidence indicates this to be at stage VII (Sharpe *et al.*, 1992). The precise mechanism whereby androgen stimulates and controls spermatogenesis is poorly defined, partly because the entire cell population of the tubules is engaged in complex cell-cell interactions which are extremely difficult to examine (Sharpe, 1993).

It can be seen that testosterone and dihydrotestosterone are important hormones for development and maintenance of male genital and secondary sexual development and for the maintenance of spermatogenesis. Disorders of androgen action can be predicted to have serious consequences in terms of both virilisation and fertility; such predictions are readily borne out by examining the syndromes of male pseudohermaphroditism where androgen action is impaired (see section 2.3).

## 2.3. Syndromes of androgen resistance

### 2.3.1. 5 α-reductase deficiency

5 α-reductase deficiency is an autosomal recessive hereditary condition characterised by abnormalities of sexual differentiation (Madden *et al.*, 1975; Wilson, 1992). Patients are genetically male (46 XY karyotype) with testes and Wolffian duct derivatives and androgen levels similar to normal men. The ducts terminate in a blind ending vagina and the external genitalia are female in appearance. The cause for these features is a deficiency of the 5 α-reductase enzyme which results in a failure of synthesis of DHT. Tissues which are sensitive to DHT (external genitalia and seminiferous tubules) fail to develop resulting in the mixture of male and female structures seen in the adult. One family has been found to carry a deletion of the 5 α-

reductase gene and the other cases are thought to be due to point mutations within it (Wilson, 1992). In these cases the circulating testosterone acts normally via a normal receptor on androgen-sensitive target tissues. The condition is therefore fundamentally different from androgen insensitivity.

### 2.3.2. Androgen insensitivity syndromes

Androgen insensitivity (AIS) is a term which encompasses a range of clinical entities all characterised by a tissue resistance to the effects of circulating testosterone. Levels of testosterone are elevated above the normal male range and 5 α-reductase activity is normal in these patients. The syndromes show a pattern of X-linked recessive familial inheritance (Morris, 1953; Meyer *et al.*, 1975). The incidence is estimated to be between 1 in 20,000 and 1 in 64,000 live births (Griffin & Wilson, 1987).

#### 2.3.2.i. Complete androgen insensitivity

In complete androgen insensitivity (complete AIS) total failure of virilisation of a male foetus occurs resulting in an apparently female infant at birth. The condition usually becomes apparent at the time of expected puberty when menarche and pubertal development fail to occur. Occasionally, the condition comes to medical attention in childhood when a patient presents with an inguinal hernia, which is found to contain a testis. On examination, the patient exhibits a normal female phenotype with breast development and female pattern fat deposition. The amount of pubic and axillary hair is usually scanty. Genital examination reveals a normal vulva and labia, with a short, blind-ending vagina. The gonads may be palpable within the inguinal canal but often are intra-abdominal. Investigation of these patients reveals a 46 XY karyotype and elevated blood levels of LH, FSH and testosterone.

The pathogenesis of the condition begins when the early embryo develops testes which start to synthesize and release testosterone and Müllerian inhibitory factor. MIF results in involution of the genital ducts which would give rise to the uterus and Fallopian tubes. However, in the presence of tissue resistance to testosterone and DHT, the Wolffian ducts do not develop into the accessory glands and virilisation of peripheral tissues (including the external genitalia) and the brain cannot occur Thus, the patient is phenotypically female in general body habitus and external genitalia but possesses male gonads.

Clinically, there is no treatment which can be offered. The gonads are removed because of the risk of malignant change, and oestrogens can be given to encourage breast development. These patients usually require genetic and psychological counselling to come to terms with their disease.

#### 2.3.2.ii. Partial androgen insensitivity

Partial androgen insensitivity (partial AIS) is a manifestation of the same underlying disease process, but with a less severe form of expression (Madden *et al.*, 1975). About 10% of patients with AIS show this partial form (Griffin & Wilson, 1987). Individuals are phenotypically female but show signs of virilisation with clitoromegaly and partial posterior fusion of the labioscrotal folds. Some virilisation occurs at the time of puberty. On examination, these patients are found to have testes (intra-abdominal or inguinal), a blind ending vagina, and elevated blood levels of gonadotrophins and testosterone.

#### 2.3.2.iii. Reifenstein's syndrome

Reifenstein's syndrome is another clinical variant of partial androgen insensitivity. Patients falling into this category are recognisably male, but have significant impairment of virilisation (Reifenstein, 1947; Griffin & Wilson, 1980). Common features include: severe perineoscrotal hypospadias; cryptorchidism; small testes and penis; gynaecomastia; absence of secondary facial and body hair; female pattern pubic hair; and the absence of Wolffian duct derivatives (e.g. vasa deferentia). Treatment with high dose testosterone in an attempt to induce virilisation has been performed but usually with little success (Rosenfield *et al.*, 1971; Price *et al.*, 1984).

### 2.3.3. Atypical forms of androgen resistance

In addition to the forms of androgen insensitivity detailed above there have been reported cases of patients whose clinical and biochemical features fit none of the above diagnostic categories. These cases are of interest because they reveal that the spectrum of androgen resistance is very wide and in some cases the resistance is subtle and mild. Larrea *et al.* (1978) described two brothers with gynaecomastia as the sole feature. On investigation, the patients had small prostates, a small volume ejaculate, with normal numbers of spermatozoa and elevated blood levels of gonadotrophins. Androgen binding studies were performed upon samples of breast tissue from these men, but no abnormality was detected. Two cousins (from the female line of inheritance) had similar abnormalities of virilisation, indicating that the condition appeared to follow an X-linked pattern of inheritance.

Migeon *et al.* (1984) described four male patients from three families who each presented with pubertal gynaecomastia, short penis and a small prostate. One of the men was oligospermic, while the others were azoospermic. Investigation revealed elevated blood levels of testosterone in all the men, elevated blood levels of LH in

three of them, and some reduction in androgen binding capacity in genital skin fibroblasts (GSF) from the two brothers.

Grino *et al.* (1988) reported a family in which the males were affected by gynaecomastia, sparse pubic hair growth and small genitalia but were still normally fertile. They had elevated serum levels of testosterone, but normal serum gonadotrophin levels. Androgen binding studies on genital skin fibroblasts revealed a reversible loss of binding affinity upon raising the incubation temperature and an increased dissociation rate of ligand/receptor complexes.

The presence of different phenotypes in patients with similar abnormalities of GSF receptor function suggest that there are other factors involved in virilisation of particular tissues which are important. It may be that the local activity of modifying enzymes (such as 5 α-reductase) is modulated in different tissues, leading to varying responses to the same circulating levels of testosterone. Indeed, Jukier *et al.* (1984) described a family of three brothers with Reifenstein's syndrome in which the youngest was affected much less severely. The difference in clinical severity was due to a combination of impaired androgen binding stability in the receptor which each brother had inherited and an almost total absence of 5 α-reductase activity in the older two brothers. There may be other local regulators of androgen action which are modulated in this way.

### 2.3.4. Infertile men and androgen resistance

Atypical cases of androgen resistance indicate that the range of receptor abnormalities extends beyond gross effects such as abolition of ligand binding to include less apparent defects of function such as thermolability. It has been suggested that the spectrum of abnormalities may extend to include some men whose only presenting feature is infertility. Aiman *et al.* (1979) described three men who presented with severe oligospermia; one of the men also had gynaecomastia. All three men had elevated testosterone and abnormalities of androgen binding in genital skin fibroblasts. Maximum binding was roughly 50% of normal in two and undetectable in the third. On the basis of these investigations, they suggested that a mild androgen resistance was responsible for their infertility. Aiman & Griffin (1982) determined the frequency of androgen resistance in an unselected population of infertile men. Eighteen men with idiopathic infertility were compared to ten with known abnormalities of virilisation. Maximum androgen binding sites ($B_{max}$) in the control group did not differ from normal values, whereas in all the study group the value was significantly lower, and comparable to that in patients with complete and partial AIS. They

suggested a value of $B_{max}$ of 12 fmol/mg protein as defining androgen resistance, and using this criterion applied to their subjects obtained a figure of 40% of infertile men as having significant androgen resistance. A similar picture was described by Morrow *et al.* (1987) in a sample of 21 men with sperm concentrations of less than 20 million/ml semen and infertility of more than twelve months duration. They found a wide range of androgen receptor levels in the men and 19% of these were significantly lower than in normal controls. In these men there was a mild elevation of serum LH concentrations.

However, a different result was obtained by Eil *et al.* (1985) who investigated fifteen men with 12 months of infertility, eight of whom had sperm counts of less than 20 million/ml semen. Whole cell uptake of radiolabelled androgen was used as a quantitative measure of receptor levels, and nuclear uptake to measure the function of the receptor. There was no difference in either whole cell or nuclear uptake of labelled androgen between their subjects and normal fertile men, and no correlation with sperm concentration. The apparent discrepancy between these three studies can be explained partly by the small sample sizes in each group and it seems likely that androgen insensitivity is responsible for infertility in some men, although the figure of 40% is an overestimate. It should also be borne in mind that the methods of measurement used were not sensitive enough to detect subtle abnormalities of binding affinity and none directly measured the ability of the androgen receptor to transactivate target genes.

## 2.4. The structure and function of the androgen receptor

### 2.4.1. The superfamily of steroid receptors

The androgen receptor (AR) is one of the family of steroid hormone receptors, the protein sequences of which contain regions of striking homology (Evans, 1988). The sequences of the genes for the thyroid hormone receptor, retinoic acid receptor and vitamin D receptor (Carson-Jurica *et al.*, 1990) also contain regions homologous with those of the steroid receptors. All these receptors are now generally regarded as being members of a large "superfamily" thought to have evolved from a common precursor gene (Fig. 2.2) (Evans, 1988). Included in the superfamily are several proteins which share homology with the other members but whose ligands have yet to be identified, the so-called "orphan receptors" (O'Malley & Tsai, 1992).

Androgen, progesterone and oestrogen receptors are normally located within the nucleus of the cell both in the presence and absence of ligand (Carson-Jurica *et al.*,

1990; Pratt, 1990; Jenster *et al.*, 1991), whilst the glucocorticoid receptor seems to be partially cytoplasmic in the absence of ligand, and becomes entirely intranuclear upon hormone binding. The receptors usually function as transactivating stimulators of gene transcription (Rundlett *et al.*, 1990) and exist in a complex with the heat shock proteins (section 2.4.3). Binding of the appropriate steroid ligand causes a conformational change in the receptor protein which renders it able to bind to specific recognition sites, termed hormone response elements (HREs), on the target cell DNA. Receptor/ligand complexes bound to the HRE interact with other transcription factors including RNA polymerase to promote transcription (Carson-Jurica *et al.*, 1990; Pratt, 1990). Thus, as a result of exposure to steroid hormones *de novo* synthesis of new proteins occurs within the cell which may then alter the metabolism of the cell to bring about the action of the hormone upon that tissue (Fig. 2.3).

The proteins of the steroid receptor family can each be divided into three basic functional domains which are illustrated for the androgen receptor in Fig. 2.4. The DNA binding domain has the most highly conserved sequence and possesses eight cysteine residues which are conserved throughout the entire family (Carson-Jurica *et al.*, 1990). The carboxy- terminal (C-terminal) portion of the receptor is the region responsible for binding to the specific ligand and also has several other functions which will be discussed below. The amino- terminal (N-terminal) domain varies greatly in size between the different receptors and has a number of functions which are as yet poorly defined. The androgen receptor spans a length of 918 amino acids (Chang *et al.*, 1988), encoded on eight separate exons (see section 2.5.2).

### 2.4.2. Domains of the androgen receptor

#### 2.4.2.i. The steroid binding domain

The steroid binding domain of the androgen receptor is encoded by exons 4 to 8 and spans 250 amino acids (Jenster *et al.*, 1991). Examination of the members of the superfamily reveals two regions of high homology within the domain which are hydrophobic in nature and thought to be involved directly in binding to the ligand (Carson-Jurica *et al.*, 1990). Jenster *et al.* (1991) investigated the ability of deletion mutant androgen receptors to bind steroid. They discovered that ligand binding was abolished by truncation of the C-terminal end of the domain (even if only 12 amino acids were removed) or by deletion of small portions from the N-terminal end of the steroid binding domain. Such deletions remove one of the hydrophobic regions described by Carson-Jurica *et al.* (1990). Data from the study of patients with defects of androgen and thyroid hormone action clearly indicate that the hormone binding

function of the domain is highly sensitive to individual amino acid substitutions (Sakurai *et al.*, 1989; Brown *et al.*, 1990; Wilson, 1992).

In the absence of hormone the C-terminal portion of the receptor serves to suppress the DNA binding ability of the receptor (Danielsen *et al.*, 1987). Deletion mutants lacking the hormone binding domain show constitutive transactivating function in *in vitro* systems (Jenster *et al.*, 1991). This constitutive activity is less than in a wild type receptor to which ligand is bound, consistent with the presence of a second transcriptional activating domain within the steroid binding domain which is active in the normal situation (Simental *et al.*, 1991).

Nuclear targeting of the receptor is also determined by a region within the hormone binding domain. The androgen receptor is almost totally nuclear in the absence of hormone and translocation of the remaining receptor occurs upon the addition of hormone, consistent with the presence of hormone-dependent and independent targeting signals (Jenster *et al.*, 1991). Deletion mutant studies reveal the hormone independent signal to reside in the N-terminal part of the hormone binding domain (encoded by exon 4), between amino acids 628 and 657 (Simental *et al.*, 1991). This region has homology to the nuclear localisation signal of the SV 40 large T antigen, and is also present in other members of the steroid superfamily (Guichon-Mantel *et al.*, 1989). A mutant receptor lacking this signal will still translocate to the nucleus upon binding to steroid, supporting the presence of a second, hormone dependent, nuclear localisation signal (Jenster *et al.*, 1991).

#### 2.4.2.ii. The DNA binding domain

The DNA binding domain of the receptor is the most highly conserved sequence within the receptor and contains two "zinc fingers" encoded by exons 2 and 3. Most of the research has been performed with glucocorticoid or oestrogen receptors, but the information can be extrapolated to the other members of the superfamily. Each finger contains a single zinc atom co-ordinating with four conserved cysteine residues in a tetrahedral structure (Fig. 2.5) (Freedman *et al.*, 1988). The critical role of all eight cysteine residues has been confirmed by site-directed mutagenesis experiments which systematically removed each of the cysteine residues (Hollenberg & Evans, 1988; Schena *et al.*, 1989) and abolished DNA binding and transcription.

In order to understand how the DNA binding domain of the receptor functions, one must examine the structure of the hormone response elements (HREs) which can be usefully divided into groups according to their structure (Fig. 2.6) (Berg, 1989).

The glucocorticoid response element (GRE) is a 15 base pair partial palindrome with the consensus sequence 5' GGTACA nnn TGTTCT 3' (Beato *et al.*, 1989) and can function as a gene promoter at great distances from the target gene. The spacing between consensus half sites must be three base pairs. The GRE (and experimentally designed perfect palindromes (Strahle *et al.*, 1987)) will bind to progesterone, androgen and mineralocorticoid receptors with similar affinity as to glucocorticoid receptor (Freedman, 1992). It has been shown experimentally that certain nucleotides are critical for the responsiveness of the GRE: changes to G+4, T+5, C+7, C-4 and A-5 abolish binding to glucocorticoid receptor *in vitro* and prevent induction of reporter genes *in vivo* (Nordeen *et al.*, 1990; Freedman, 1992). These nucleotides are the sites at which the DNA backbone makes specific contact with the receptor. The thymidine at position T+5 would appear to be critical for receptor discrimination as a functional oestrogen response element (ERE) differs from a GRE by only two nucleotides in each half site, including this one (Fig. 2.6) (Freedman, 1992, and references therein). The fact that HREs appear to be inverted palindromes is consistent with binding of receptor occurring as a dimer, with each molecule in opposite orientation (see section 2.4.2.iii).

The DNA binding domain is able to identify its specific response element in isolation from the rest of the receptor molecule. This ability resides within three amino acids at the C-terminal side of the base of the first zinc finger (Danielsen *et al.*, 1989; Mader *et al.*, 1989; Umesono & Evans, 1989). The two amino acids between the third and fourth cysteines, and the second amino acid after the fourth cysteine are all necessary for a glucocorticoid receptor to recognise a GRE in preference to an ERE (Fig. 2.5). Substitution of the amino acids in a glucocorticoid receptor for those in an oestrogen receptor render the receptor able to bind to an ERE but not to a GRE. In fact, steroid hormone receptors can be classed into two groups (depending on the amino acids at these sites) which correlate with the type of HRE that they recognise (Table 2.1) (Beato, 1989). The androgen receptor falls into the first group with the sequence Cysteine-*Glycine-Serine*-Cysteine and *Valine* at these sites. Additionally, the five amino acids lying between the first two cysteines of the second zinc finger are important for recognising the half site spacing of the HRE ( known as the "knuckle" of this finger) (Beato, 1989; Martinez & Wahli, 1991) which contributes to the target specificity of the receptor. Exchange of these amino acids within a glucocorticoid receptor to the oestrogen receptor sequence produced a chimaeric receptor which was able to recognise both HREs and transactivate genes through both (Danielsen *et al.*, 1989).

The three dimensional structure of the DNA binding domain has recently been elucidated by X-ray crystallography (Härd *et al.*, 1990; Luisi *et al.*, 1991). Data taken from the glucocorticoid receptor revealed the three dimensional structure of the DNA binding domain to contain two α-helices, lying C-terminal to each zinc finger, and a distorted helix within the second finger (Härd *et al.*, 1991; Freedman, 1992). The overall shape of the domain was oblong with a compact hydrophobic core containing several conserved hydrophobic amino acids, suggesting that it has an important function to perform in maintaining the tertiary structure. A virtually identical pocket has been described in the oestrogen receptor (Schwabe *et al.*, 1990).

The way in which the glucocorticoid receptor interacts with DNA has also been determined by crystallography (Luisi *et al.*, 1991; Freedman, 1992). The receptor binds to DNA as a head to head dimer. The first zinc finger of each receptor interacts with the phosphate backbone of the DNA within the HRE, with the α helix which follows it lying in the major groove and making all the specific base contacts involved in HRE binding described above. The second finger makes further specific contacts with the phosphate backbone of the DNA and also provides a dimerisation interface for the other receptor molecule (see section 2.4.2.iii) (Luisi *et al.*, 1991).

The DNA binding domain of the receptor also carries weak transcriptional activity and a ligand dependent nuclear localisation signal (Jenster *et al.*, 1991).

#### 2.4.2.iii. The dimerisation domain

As mentioned above, the glucocorticoid receptor binds to its HRE as a dimer. Kumar & Chambon (1988) showed that *in vitro* oestrogen receptor bound to its response element only in the presence of hormone, and that the binding was dimeric. Tsai *et al.* (1988) demonstrated that the two binding sites for receptor in a GRE lay on opposite strands of the DNA and that dimeric binding occurred in a co-operative fashion.

There appears to be more than one area within the receptor involved in dimerisation. The second zinc finger of the DNA binding domain interacts extensively with its fellow (Luisi *et al.*, 1991) but there is also a dimerisation signal resident within the steroid binding domain of the receptor. Lees *et al.* (1990) identified a 22 amino acid peptide which restored dimerisation ability to mutant receptors lacking it. Restoration of dimerisation also restored the DNA binding ability of the mutant receptors, indicating that dimerisation is an essential process in the normal function of the receptor. The 22 amino acid peptide sequence is highly conserved across the steroid receptor superfamily and consists of a repeating motif of hydrophobic amino

acids every seven residues (Fawell *et al.*, 1990; Parker & Bakker, 1992). In the androgen receptor this motif includes residues between 852 and 866 and overlaps one of the essential regions for ligand binding. A heptad hydrophobic repeat is a motif which has been identified in other nuclear proteins which interact with each other, including the *fos* and *jun* proteins which contain a "leucine zipper" motif (Konzarides & Ziff, 1989). Schüle *et al.* (1990) showed that *jun* was able to inhibit the transactivating ability of the glucocorticoid receptor *in vitro* in a dose dependent manner, and that this inhibition resided in the leucine zipper of *jun*. It has been proposed that steroid receptors have a dimerisation mechanism involving the binding of an α helix in the heptad repeat region of each molecule (Forman & Samuels, 1990; Lees *et al.*, 1990) and that heterodimer formation between the receptor and other nuclear proteins or steroid receptors may be a mechanism for autoregulation or transcriptional control of receptor activation (Chaucereau *et al.*, 1992; Parker & Bakker, 1992)

#### 2.4.2.iv. The N-terminal domain

The N-terminal domain of the androgen receptor is encoded by a single exon. It appears to have several functions including transcriptional activation and regulation of DNA binding specificity. Hollenberg & Evans (1988) identified a region within the N-terminal domain of the glucocorticoid receptor which was essential for normal hormone dependent activity. This region lay between residues 77 and 262 and its function was independent of site within the whole receptor. The experimental introduction of a second copy of this sequence into the receptor produced a more active molecule (Hollenberg & Evans, 1988). These data correlate with the findings of Simental *et al.* (1991) which indicated the presence of a transcriptional modulating function in the first 338 amino acids of the androgen receptor. They found that co-transfection of isolated N-terminal domains into cells bearing a reporter gene inhibited transcription by androgen receptor in a dose dependent manner, indicating that the domain interacted competitively with other transcription factors. Danielsen *et al.* (1987) found that the N-terminal region reduced non-specific DNA binding by the receptor and that this ability resided in an acidic portion (i.e. excess negative charge) of the sequence. By contrast, the C-terminal part of the receptor is basic, and Danielsen *et al.* (1987) showed that the degree of negative charge in truncated receptors correlated with the specificity of DNA binding. The transcriptional activity of the N-terminal domain appears to reside in a specific region of the domain, and it depends upon both interaction with other factors in the cell and a charge effect.

Jenster *et al.* (1991) found a similar localisation of transcriptional regulation to two regions of the domain, one between residues 51 and 211 and the other between 244 and 360. The first of these regions contains a repeating region of glutamine residues which in normal individuals can vary from 17 to 26 amino acids in length (La Spada *et al.*, 1991). There are two further repeats downstream in the exon, one of eight proline residues and the other of 27 glycine residues (Chang *et al.*, 1988). The glutamine repeat region is thought to be one of the important areas within exon 1 responsible for regulation of transcription and DNA binding since its location coincides with the functional localisations identified above. Courey & Tjian (1988) identified the presence of two glutamine rich transactivating domains in the N-terminal portion of transcription factor Sp1, which supports the suggestion that glutamine containing regions of the receptor can be involved in regulation of function.

Recently, there have been some interesting observations which illustrate the importance of the glutamine repeat, without throwing much light upon how it functions. McPhaul *et al.* (1991a) described a patient with partial androgen insensitivity in whom the genetic lesion comprised a point mutation and a truncation of the glutamine repeat to 12 residues. Both changes were necessary to reproduce the receptor abnormalities *in vitro*, which included thermolabile ligand binding (also see section 2.5.2.vi). By far the most interesting and perplexing finding has been reported by La Spada *et al.* (1991) who examined patients with a rare hereditary wasting disease, X-linked spinal and bulbar muscular atrophy (SBMA), which presents with a combination of muscular symptoms and features of androgen resistance (gynaecomastia, erectile failure and oligospermia). The genetic locus for the disease maps to the same region of the X chromosome as that of the AR gene (La Spada *et al.*, 1991). Sequencing of the AR gene in a series of thirty-five patients revealed that they all had an approximate doubling of the size of the glutamine repeat region. The absolute segregation of this size increase with the disease in these patients (and in the expected family members drawn from the pedigrees of some of the patients) indicated that this was the causative defect in the disease. These findings have been confirmed by others (Belsham *et al.*, 1992; Biancalana *et al.*, 1992; Amato *et al.*, 1993). Precisely how this mutation of the receptor affects the function of the androgen receptor within such a specific target tissue as motor neurones remains a mystery, but suggests that there may be cell specific modification of the function of the AR because of interaction with other transcription factors (see section 2.4.3).

Bocquel *et al.* (1989) provided some evidence to support the presence of cell specific modulation of function by their findings that the *in vitro* properties of different members of the steroid family varied depending on which cell type was used as expression host. Intact and deletion mutant receptors showed differences in hormone binding and transcriptional activating ability in CV-1 cells and HeLa cells, suggesting that other factors within each cell type modify the activity of the receptor.

### 2.4.3. Interaction with other transcription factors

The androgen receptor interacts with other intracellular proteins in both the bound and unbound states. Unbound AR protein exists in the nucleus complexed with the most abundant heat shock protein, HSP 90 (Pratt, 1990). The heat shock proteins are abundant, mainly cytoplasmic proteins which are found in all species (Lindquist & Craig, 1988) and are essential for normal eukaryotic cell function (Borkovich *et al.*, 1989), although their actual function is unknown. HSP 90 may be involved in "chaperon" functions related to maintaining proteins (including steroid receptors) in a particular conformation of folding; it may protect proteins against degradation within the cell, and it may be a transport protein, involved in transmembrane carriage of proteins across the nuclear envelope (Pratt, 1990).

In the case of the glucocorticoid receptor HSP 90 appears to bind to the receptor as a dimer, in association with other HSPs (HSP 70 and HSP 59). All the steroid receptors, including the androgen receptor, dissociate from the heat shock proteins in response to ligand binding, inducing a conformational change of the receptor protein (O'Malley & Tsai, 1992). This conformational change is rendered permanent by covalent modification (see below) and then DNA binding and transcriptional activation occurs.

### 2.4.4. Phosphorylation of the androgen receptor

Androgen receptor exists as a phosphoprotein in the absence of ligand. In cells from a prostate tumour cell line (LNCaP) the receptor exists as two isoforms of about 110 kDa molecular weight with most of the receptor in the heavier form (van Larr *et al.*, 1990). Golsteyn *et al.* (1990) confirmed these findings and also showed that the heavier form contained phosphotyrosine residues. The phosphorylated receptor showed higher affinity binding to cell nuclei in culture than did the unphosphorylated form and could be extracted from intact cell nuclei. Golsteyn *et al.* (1990) proposed that phosphorylation was one of the steps required to transform the receptor into its active DNA binding form. van Laar *et al.* (1991) investigated the time course of phosphorylation of the receptor in response to ligand. They showed that the receptor

has a basal level of phosphorylation which increases twofold upon exposure to ligand. This increase in phosphorylation is simultaneous with the presence of increased amounts of receptor recoverable from cell nuclei indicating that the phosphorylation is required for nuclear translocation of the cytosolic receptor.

The majority of phosphorylation sites are serine and tyrosine residues and are mainly located within the N-terminal domain of the receptor (Wilson *et al.*, 1991). Recently, Chaucereau *et al.* (1992) have demonstrated phosphorylation sites within the N-terminal domain of the progesterone receptor between residues 166 and 520. Some are sites of basal phosphorylation while other sites become phosphorylated upon binding of ligand. The precise nature of the functional changes conferred by phosphorylation of steroid receptors is not known but is the subject of intensive research.

## 2.5. The genetic basis of androgen insensitivity

Androgen insensitivity has long been recognised as an X-linked recessive disorder (Morris, 1953). In an elegant study in which GSF were cultured from two siblings with complete AIS and their mother, Meyer *et al.* (1975) demonstrated that the androgen receptor gene is located on the X chromosome. GSF from the affected siblings showed absent binding of androgen and half of the mother's clones also showed this abnormality. One of each X chromosome in every cell is inactivated to form the Barr body, so that 50% of the mother's cells expressed the product of the normal AR gene and the rest expressed the defective gene. The lack of androgen binding in the affected cells suggested that the androgen receptor gene was a likely candidate for the defective gene associated with androgen insensitivity.

### 2.5.1. Abnormalities of receptor function in androgen insensitivity

At the time that the AR gene was implicated by Meyer *et al.* (1975) it was already known that many patients with complete AIS had absent androgen binding to GSF (androgen receptor negative, AR-) (Keenan *et al.*, 1974) although not all patients with the syndrome showed demonstrable abnormalities of ligand binding (Amrhein *et al.*, 1976). This latter group were dubbed androgen receptor positive (AR+). Kaufman and colleagues showed that some patients in this AR+ group had receptors which did not upregulate their levels in response to exposure to androgens (Kaufman *et al.*, 1981a), and which also dissociated with abnormal rapidity following ligand binding (Kaufman *et al.*, 1981b). In both studies, patients with partial AIS were also studied and showed similar but less extreme abnormalities of regulation. Similarly, Brown *et*

*al.* (1982) studied three patients with AR+ complete AIS; they found the receptors of these patients to have an increased rate of dissociation from ligand, a high affinity for progesterone in competition with testosterone and increased lability at elevated temperature (42°C). A further example of qualitative abnormalities of the AR in AIS were provided by Griffin & Durrant (1982). They examined the group of patients who had grossly normal receptor binding levels and normal dissociation kinetics by employing the novel technique of centrifuging receptor/ligand complexes through a molybdenum gradient. They were able to further classify this group of patients into those with receptors which were destabilised by the gradient and those whose receptors behaved normally.

Thus, the premise was that patients with AR- insensitivity had receptors with gross structural or functional abnormalities, while the AR+ patients had receptors with subtle defects. The latter were further classified into subgroups: those with thermolabile, low affinity receptors; those with molybdenate unstable receptors, and those with apparently normal receptors. This spectrum of receptor classification was not entirely interchangeable with the clinical classification of the patients since some complete AIS patients had apparently normal receptors, but the severity of receptor function abnormality seemed to correlate broadly with the phenotypic severity. Those groups with normal or nearly normal receptors as assessed by these techniques presumably possessed receptors which were defective in some intranuclear function, such as dimerisation, DNA binding or transactivation. The technology to unravel these functions only became available following the cloning of the androgen receptor gene (see section 2.5.2).

### 2.5.2. Androgen receptor gene mutations

The androgen receptor gene lies on the long arm of the X chromosome in the Xq 11-12 region (Brown *et al.*, 1989) and its cDNA has been cloned by several groups independently (Chang *et al.*, 1988; Lubahn *et al.*, 1988; Tilley *et al.*, 1989). The gene comprises eight exons, ranging in size from 1,607 base pairs for exon 1 to 117 for exon 3 (Marcelli *et al.*, 1990a). As already mentioned, the exons of the gene share homology with the other members of the nuclear hormone receptor superfamily: exon 1 encodes the large N-terminal domain of the receptor and contains three homopolymeric repeat regions. Exons 2 and 3 each encode one of the zinc fingers of the DNA binding domain and exons 4 to 8 encode the hormone binding domain and the hinge region of the receptor between the DNA and ligand binding domains (Fig. 2.4) (Chang *et al.*, 1988). Exon 8 possesses a large 3' untranslated region following the in-frame termination codon.

Soon after the cloning of the AR gene, Brown *et al.* (1988) provided the first evidence of a genetic lesion within the AR gene of patients with AIS. They made Southern blots of genomic DNA from six patients with AR- complete AIS and probed them with DNA probes specific for the N-terminal, DNA binding and hormone binding domains. One of the patients had a deletion of a part of the steroid binding domain, and this deletion could be traced through affected siblings and the female carriers. The presence of apparently intact AR genes as assessed by Southern blotting in the other patients indicated that they might contain smaller deletions or point mutations. Shortly after this finding was published, Lubahn *et al.* (1989) identified a point mutation within the steroid binding domain of the AR gene of a patient with complete AIS. A single mutation from guanosine to adenosine in exon 7 produced a substitution of valine 866 by methionine. Subsequent *in vitro* studies using recombinant plasmids bearing mutated AR genes showed that this mutation produced a receptor protein which had a 7-fold lower binding affinity and impaired ability to activate an androgen responsive reporter plasmid at physiological concentrations of androgen (Brown *et al.*, 1990). These results supported the hypothesis that point mutations of the AR gene can be the causative lesion in the pathogenesis of androgen insensitivity. Since publication of the AR gene sequence, there has been a thorough examination of the AR gene sequence of patients with AIS across the world, resulting in the identification of many naturally occurring mutations within the gene (Fig. 2.7). Some of these mutations are of particular interest and will be discussed in more detail.

#### 2.5.2.i. Deletion mutations

Deletion mutations are known to be responsible for approximately 5% of all cases of AR gene defects (Wilson, 1992). In addition to that described above, patients with deletions of the entire gene, exon 2, exon 3, exon 5 or exons 6 & 7 (Trifiro *et al.*, 1991; Quigley *et al.*, 1992a & b; Maclean *et al.*, 1993) have been reported. These mutations serve to highlight the importance of each zinc finger to the normal function of the androgen receptor, and the importance of an intact ligand binding domain but provide little information on the detailed functions within a region.

#### 2.5.2.ii. Mutations of the hormone binding domain

Many mutations in the hormone binding domain have been described (see Fig. 2.7) and they serve to illustrate some patterns in the aetiology of mutations in the entire gene. 90% of mutations in the hormone binding domain are clustered in two regions: residues 728 to 774, and 828 to 866 (McPhaul *et al.*, 1992). The region 828 to 866 is homologous to a region of the thyroid hormone receptor where mutations are clustered in patients with hereditary thyroid hormone resistance (Refetoff *et al.*, 1993). 50% of

mutations are situated at CpG couplets within the DNA sequence (Quigley *et al.*, 1992c), which are recognised as "hot spots" for mutation by the mechanism of methylation and deamination of cytosine producing a thymidine nucleotide (Youssoufian *et al.*, 1986). This is the commonest mode of mutation, and accounts for the high frequency of premature termination codons substituting for arginine codons, because of the C to T change which converts the arginine codon (CGA) to a stop codon (TGA).

80% of mutations in the hormone binding domain occur at the site of conserved amino acids (Quigley *et al.*, 1992c). These conserved amino acids occur in all regions of the hormone binding domain, but there is a preponderance of them in exon 7, around the region of the heptad hydrophobic repeat which is involved in ligand binding and dimerisation. Mutations have been detected in several of these conserved amino acids including 855 and 866 (Lubahn *et al.*, 1989; De Bellis *et al.*, 1992). The high incidence of mutations of conserved amino acids is not really surprising in that one would expect mutations in these critical amino acids to have a marked clinical effect. More surprising is the fact that mutations of non-conserved amino acids also can have profound effects on function of the receptor, suggesting that such amino acids play an important role in maintaining the correct spatial configuration of the receptor or in causing conformational changes along the length of the receptor protein as part of the mechanics of receptor action.

It is worth noting that to date there is only one report of silent mutations detected during screening of patients (Batch *et al.*, 1992). One of these (a guanosine to adenosine mutation at 995) abolishes a restriction site and was detected in the normal population at a frequency of 8%.

#### 2.5.2.iii. Premature termination codons

Mutation of existing codons to a premature termination signal has been found to be a relatively common cause of complete AIS, partly due to the mechanism described above. Mutations of this type have been found throughout the length of the gene in exons 1, 3, 4, 6, 7 or 8 (Marcelli *et al.*, 1990a & b; Sai *et al.*, 1990; McPhaul *et al.*, 1991b; De Bellis *et al.*, 1992; Trifiro *et al.*, 1992a &b; Zoppi *et al.*, 1993). These mutations lead to the synthesis of a truncated receptor protein and it is striking how even limited truncation can render the receptor unable to bind ligand. The exon 8 mutation described by Trifiro *et al.* (1992a) removes only the last 36 amino acids of the protein but abolishes ligand binding completely. This is in agreement with the *in*

*vitro* work of Jenster *et al.* (1991) in which a deletion of the last 12 amino acids of the receptor was sufficient to abolish ligand binding.

#### 2.5.2.iv. Mutations of the DNA binding domain

Mutations within the DNA binding domain have been detected relatively infrequently when compared to those occurring within the hormone binding domain. Commonly, one of the conserved cysteine residues is mutated (Chang *et al.*, 1991; Zoppi *et al.*, 1992) and this results in disruption of the normal conformation of the finger which abolishes or severely impairs DNA binding and gene transactivation. It is worthy of note that receptors from these patients in which zinc finger mutations have been reported retain normal hormone binding characteristics (i.e. they are all AR+ patients). Mutations have also been identified in the α-helical region of the second zinc finger which is part of the dimerisation interface (De Bellis *et al.*, 1991). Marcelli *et al.* (1991a) described a patient with complete AIS having a mutation of a residue in this segment which results in loss of contact with a phosphate residue within the DNA helix and subsequent loss of receptor function.

Few mutations have been reported in the DNA binding domain in patients with partial AIS, indicating the importance of the structural integrity of this region. Mutations have been reported in the knuckle of the second finger including the substitution of alanine 596 by threonine (Klocker *et al.*, 1992). This is an amino acid conserved through all the steroid receptors except the oestrogen receptor and lies in the region responsible for recognition of half site spacing in the HRE (Beato, 1989). In co-transfection assays, this mutant androgen receptor had normal hormone binding parameters but was only able to activate a reporter gene a third as efficiently as a wild type receptor. In the glucocorticoid receptor Luisi *et al.* (1991) found that the equivalent alanine residue was one of the amino acids involved in receptor dimerisation, so a perturbation of dimerisation seems to be the likely mechanism of action in this patient. A second mutation replaces serine 597 with glycine in association with a change of arginine 617 for proline (Zoppi, *et al.*, 1992). Residue 597 also lies within the knuckle of the finger responsible for recognition of the half-site spacing of the HRE. Wooster *et al.* (1992) described a mutation of arginine 607 to glutamine in two brothers with partial AIS and breast cancer and suggested that the mutation may have rendered the AR able to activate an ERE and thus stimulate the development of the breast cancer. This residue is one of the amino acids involved in receptor HRE contact via the phosphate backbone of the DNA (Luisi *et al.*, 1991).

In view of the critical nature of the function of the DNA binding domain, it is not surprising that most of the identified mutations cause complete AIS. Examination of those mutations which cause partial AIS provide more information on the nature of the interactions of the subdomains of the zinc fingers.

#### 2.5.2.v. Commonly occurring mutations

Mutations of certain amino acids have been reported independently by several groups and many of these occur at conserved amino acid sites. The occurrence of the same mutation at the same site in unrelated individuals suggests that the affected amino acid is of particularly critical functional importance. Those amino acids falling into this group are: numbers 576, 695, 774, 831, 855 and 866 (Lubahn *et al.*, 1989; Brown *et al.*, 1990; Chang *et al.*, 1991; Marcelli *et al.*, 1991b; Ris-Stalpers *et al.*, 1991, 1992; De Bellis *et al.*, 1992; Prior *et al.*, 1992; Patterson *et al.*, 1992; Zoppi *et al.*, 1992; Kazemi-Esfarjani *et al.*, 1993). The mutations at 855 and 866 are particularly interesting because different mutations at these sites have been detected in patients with complete and partial AIS. At 855 a change from arginine to cysteine (large, positively charged to a small polar amino acid) caused complete AIS while arginine to histidine (both charged) caused partial AIS. Similarly, a change at 866 from valine to methionine (small, uncharged to large, polar amino acid) led to complete AIS while valine to leucine (both small and uncharged) produced partial AIS. Such mutations suggest that the nature of the substitution, in terms of size and charge, can have an effect upon the degree of functional impairment caused by mutations at a given site (see also Chapter 4, section 4.5).

#### 2.5.2.vi. Complex mutations

Some of the mutations which have been reported appear to cause their clinical effects by more subtle and complex mechanisms. The occurrence of a size increase of the glutamine repeat in patients with spinal and bulbar muscular atrophy and the evidence highlighting the importance of the glutamine repeat provided by McPhaul *et al.* (1991a) from a family with partial AIS has already been discussed (section 2.4.2.iv). The AR gene from these patients was found to have a point mutation at 763 (from tyrosine to cysteine) in association with a truncation of the glutamine repeat region to 12 residues. *In vitro* studies using genital skin fibroblast receptors showed the receptor to be thermolabile in binding and to have an increased rate of dissociation from ligand. Binding studies with mutated recombinant receptors *in vitro* revealed that the point mutation was responsible for the increased dissociation but that the truncation of the glutamine repeat was also necessary to reproduce all the functional abnormalities displayed by the original mutant receptor.

Marcelli *et al.* (1991b) described a mutation within exon 6 of the AR gene of a patient with AR- complete AIS. *In vitro* studies using recombinant mutant AR genes in expression vectors revealed that the point mutation caused a reduction of binding affinity and an irreversible loss of binding ability on exposure to mild temperature elevation. They also found very low levels of specific AR mRNA in genital skin fibroblasts from the patient. It was proposed that the mutation produced the AR- phenotype by a combination of the direct effect upon ligand binding and the effect which the mutation seemed to have upon the stability of the mRNA, resulting in low copy number of the receptor protein *in vivo*.

Another interesting mechanism for the generation of the AIS phenotype was reported by Ris-Stalpers *et al.* (1990) from a patient with AR- complete AIS. Analysis of the AR gene revealed a point mutation in the first nucleotide of the 5' splice site of intron 4 from the normal GT site to a TT sequence. This resulted in an mRNA 123 bases shorter than normal, leading to a protein with a deletion of 41 amino acids between 684 and 724. The mutation changed one of the two invariant nucleotides of the donor site sequence of the splice site (Green, 1986; Padgett *et al.*, 1986), which caused activation of a cryptic donor splice site within exon 4, resulting in the missing portion of exon 4 being spliced out during RNA processing. The mutant receptor lacked all androgen binding and transactivating ability in *in vitro* assays. The deleted region was part of the steroid binding domain and carries some transcriptional activation function (Jenster *et al.*, 1991).

## 2.6. Infertility as a disease

Infertility is a common problem amongst couples in Britain and around the world. Estimates put the incidence at 14% to 25% of couples having some experience of infertility (Diczfaluzy, 1986; Templeton *et al.*, 1990). The inability to conceive a child is expensive in terms of anxiety and stress for the partners, the amount of consultation time which is required and in the cost of technological investigations and treatments. Currently, infertility as a result of female factors (e.g. anovulation, tubal dysfunction) can be treated with good results. The major obstacle in improving our ability to treat couples lies in the realm of male factor infertility and unexplained infertility.

### 2.6.1. The incidence and causes of male factor infertility

Male factor infertility is the major factor in 24% of couples and is a contributory factor in up to 75% of couples presenting for treatment of infertility (Hull *et al.*, 1985; Irvine, 1992) and men with sperm dysfunction have a poor rate of fertility in follow

up of less than 27% (Hull *et al.*, 1985). It is therefore the single most prevalent cause of infertility. Male factor infertility encompasses the inability of spermatozoa to move through cervical mucus, adhere to the zona pellucida of the egg and to effect fertilisation. Most of these functions are difficult to examine and the standard semen analysis is very crude, being little more than a descriptive comment on the sample. However, there is a correlation between low sperm concentration and the inability to achieve a pregnancy (Hargreave & Elton, 1986). Low sperm counts can be caused by a variety of diseases including diabetes mellitus, bronchiectasis, epididymitis, testicular injury, mumps orchitis and drugs including sulphasalazine, alcohol, cimetidine and nitrofurantoin (Irvine, 1992). Exclusion of these known causes still leaves a subset of men with low sperm counts of unknown cause, who comprise 20% of infertile men attending infertility clinics (Hargreave, 1990).

Whilst men with oligo- and azoospermia and those with morphologically abnormal spermatozoa will be identified, many men with an apparently normal semen sample remain infertile. Hull *et al.* (1985) found 28% of men attending a large clinic in Bristol fell into this category of unexplained infertility. More specialised tests are available such as the post-coital test, sperm movement analysis and the zona-free hamster oocyte penetration test (Irvine, 1992). These tests provide useful prognostic information for each couple and can pinpoint the functional disturbance of the spermatozoa but they do not provide any information on the underlying mechanism.

### 2.6.2. Unexplained infertility

There remains in any group of infertile couples roughly 28% for whom no obvious cause can be found, and a group of men with oligospermia for which no explanation can be provided. The pregnancy rate in such a group is not good and the only effective intervention is *in vitro* fertilisation (IVF), with all the associated cost. IVF requires a reasonable concentration of spermatozoa in semen and is therefore contraindicated in those couples where the male has a low count. As we have seen, a group of these men have no recognisable reason for their low sperm count and therefore no treatment can be offered.

As discussed above (section 2.3.4), it has been suggested that a form of androgen resistance may be responsible for infertility due to oligospermia (Aiman & Griffin, 1982; Morrow *et al.*, 1987). While the figures quoted seem rather high, the rationale that mild abnormalities of androgen action can interfere with normal spermatogenesis seems plausible. Given that our understanding of the intratesticular action of testosterone is poorly understood (Sharpe, 1993), it may be that some men

have such an androgen resistance which is amenable to high dose androgen therapy as a means to improve their sperm count and fertility.

## 2.7. Aims and objectives

### 2.7.1. Characterisation of the genetic basis of androgen insensitivity

The aim of this research was to investigate the structure/function relationships of the androgen receptor gene in several ways. Firstly, I had access to patients with the complete and partial forms of androgen insensitivity drawn from all over Scotland and from northern England. Analysis of the sequence of the androgen receptor gene in these patients was undertaken to identify the genetic basis of their syndrome in terms of point mutations or deletions of the receptor.

### 2.7.2. Examination of the functional characteristics of mutant androgen receptors

Having identified mutations within the AR gene in patients with AIS, it is important to confirm that the mutation is responsible for the defects in the normal function of the receptor. I intended to investigate this by constructing recombinant mutant androgen receptor genes in plasmid DNA bearing eukaryotic promoter signals. Mutations would be introduced into the AR in a plasmid by the technique of site-directed mutagenesis (Deng and Nickoloff, 1992), and mutated plasmids transfected into mammalian cells in culture. Measurement of androgen binding ability and transactivating ability of the mutant androgen receptor would be measured *in vitro* and the results correlated with the clinical phenotype of each patient.

### 2.7.3. Screening of infertile men for the presence of androgen receptor mutations

I had access to an infertile male population numbering approximately 1,700 with all relevant information retained on computer. I selected from them a cohort with hormonal parameters suggestive of mild androgen resistance and obtained genomic DNA samples. In order to accelerate the analysis of the AR gene in these men I evaluated and established a screening method. Available methods included denaturing gradient gel electrophoresis (DGGE) (Myers *et al.*, 1987), temperature gradient gel electrophoresis (TGGE) (Riesner *et al.*, 1989) and single strand conformational polymorphism analysis (SSCP) (Orita *et al.*, 1989).

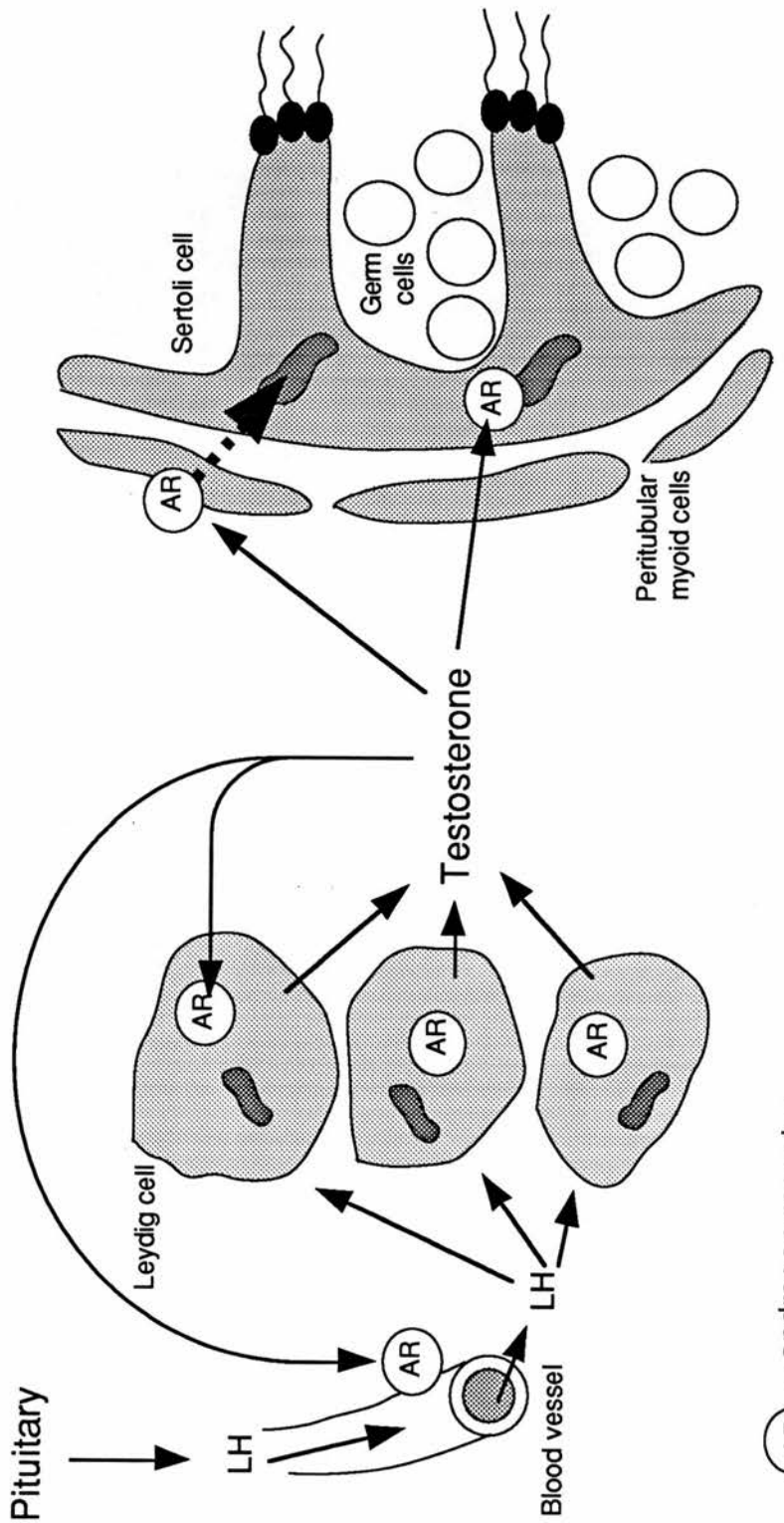
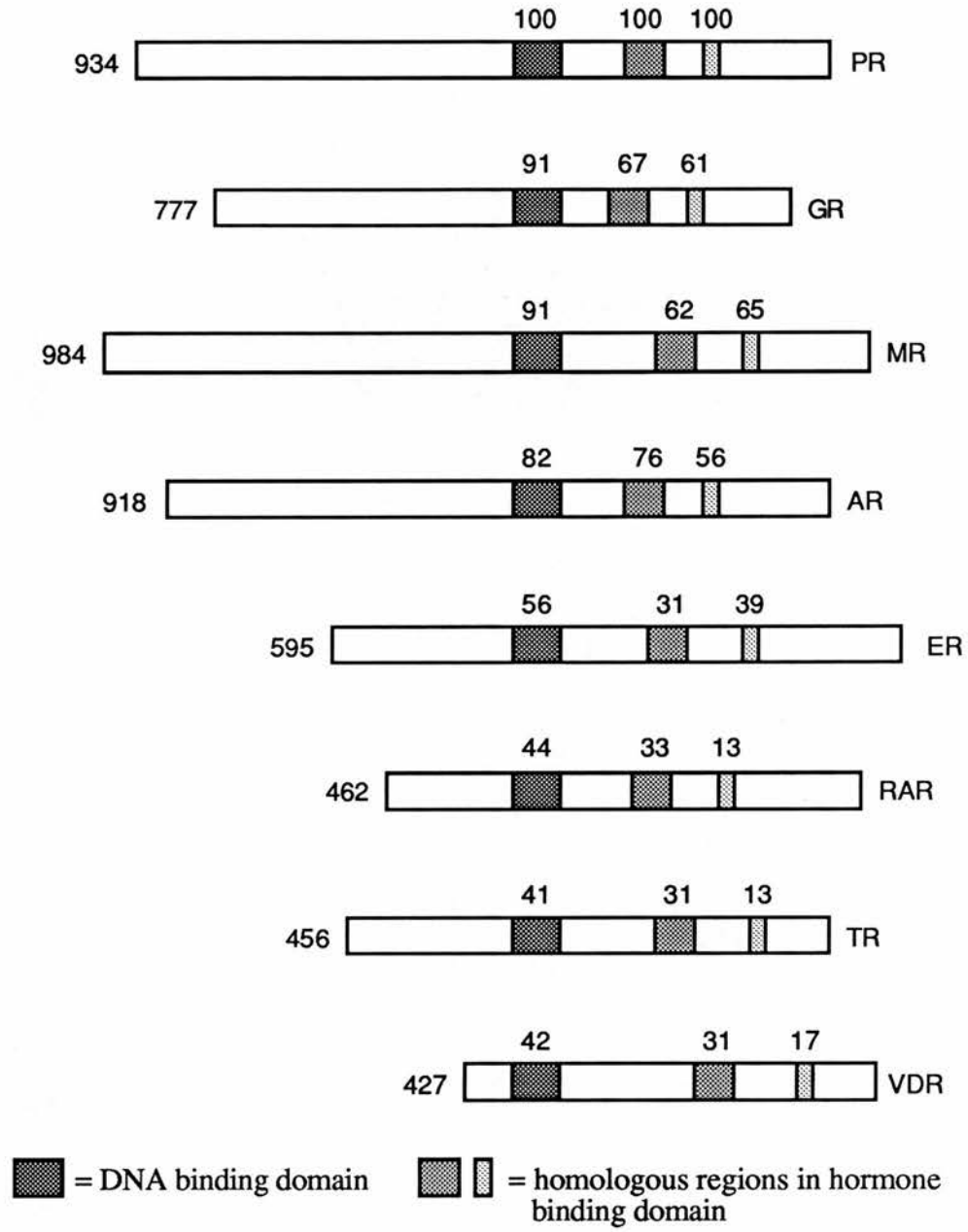



**Figure 2.1 Sites of androgen action in the testis**

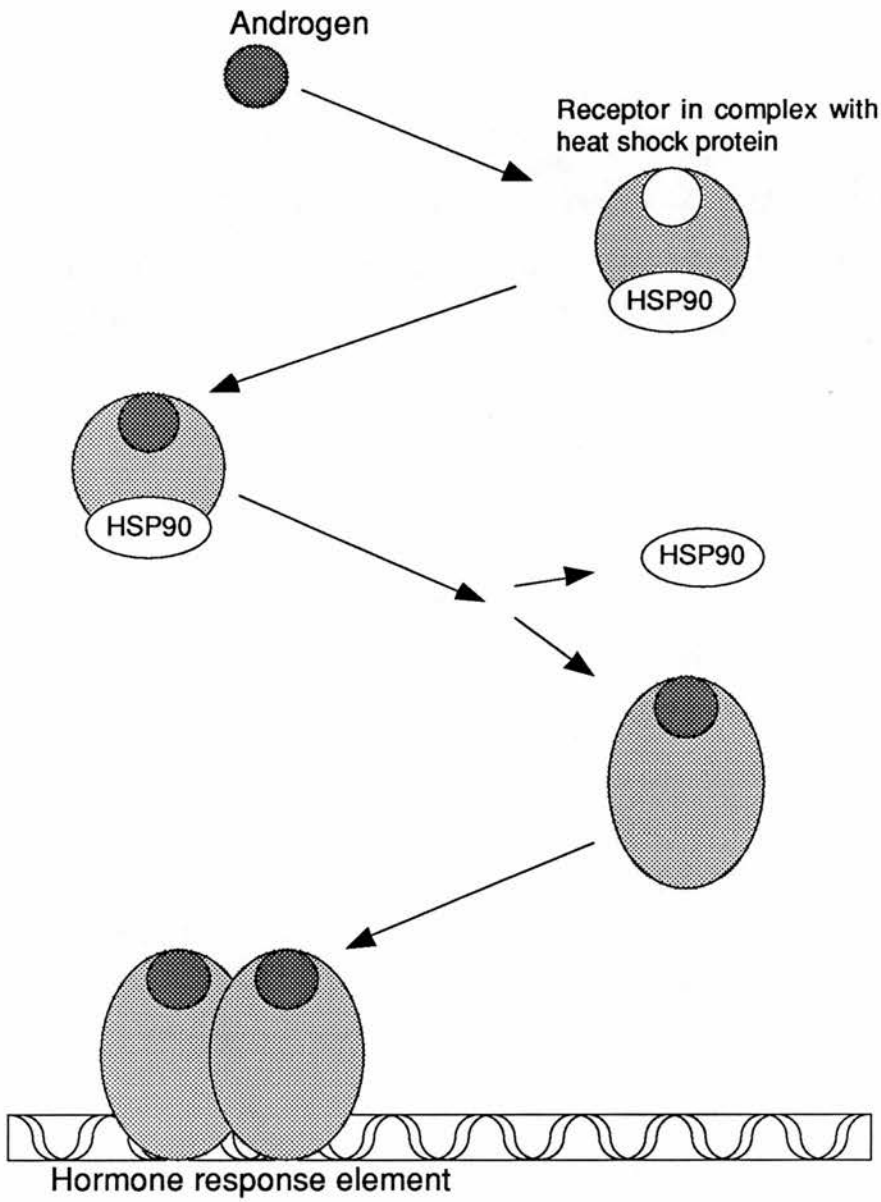
**Figure 2.2 Steroid receptor family homology**




ER = oestrogen receptor; GR = glucocorticoid; MR = mineralocorticoid
PR = progesterone; RAR = retinoic acid; TR = thyroid; VDR = vitamin D

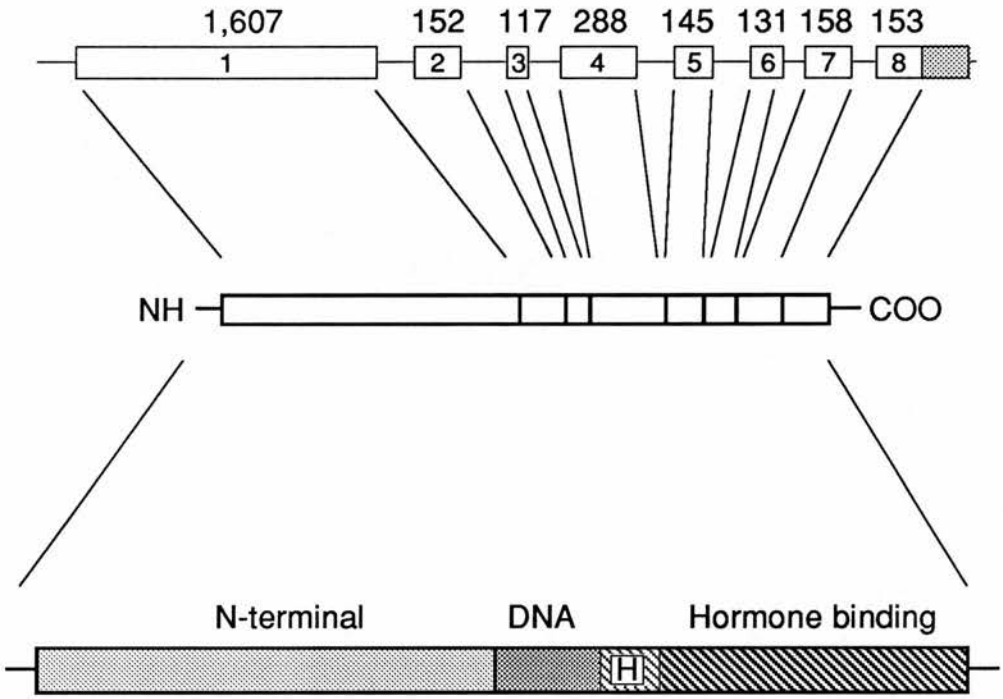
Protein sequences aligned at DNA binding domain with the amino terminus on the left. Two regions in the hormone binding domain, thought to be involved in hormone binding, are also shown. Figures above each indicate % homology of the shaded portions to the sequence of the progesterone receptor. Based on Carson-Jurica *et al.* (1990).

**Figure 2.3 Mode of action of androgen receptor**




Unliganded receptor exists in complex with heat shock protein 90. Binding of ligand induces a conformational change releasing hsp 90 and rendering the receptor able to bind to the DNA of the response element as a dimer. Binding to the response element is the first step in initiating transcription of the target gene.

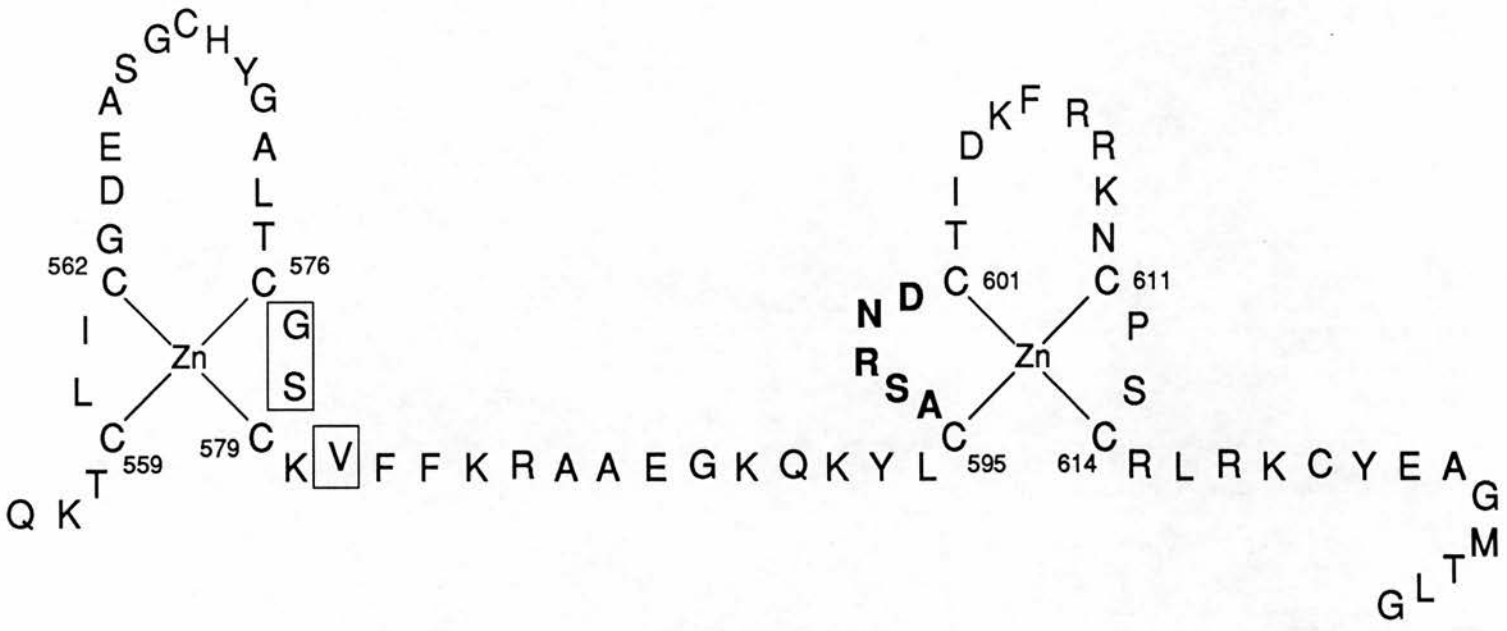
**Figure 2.4 Domains of the androgen receptor**




▒ = 3' untranslated region of exon 8

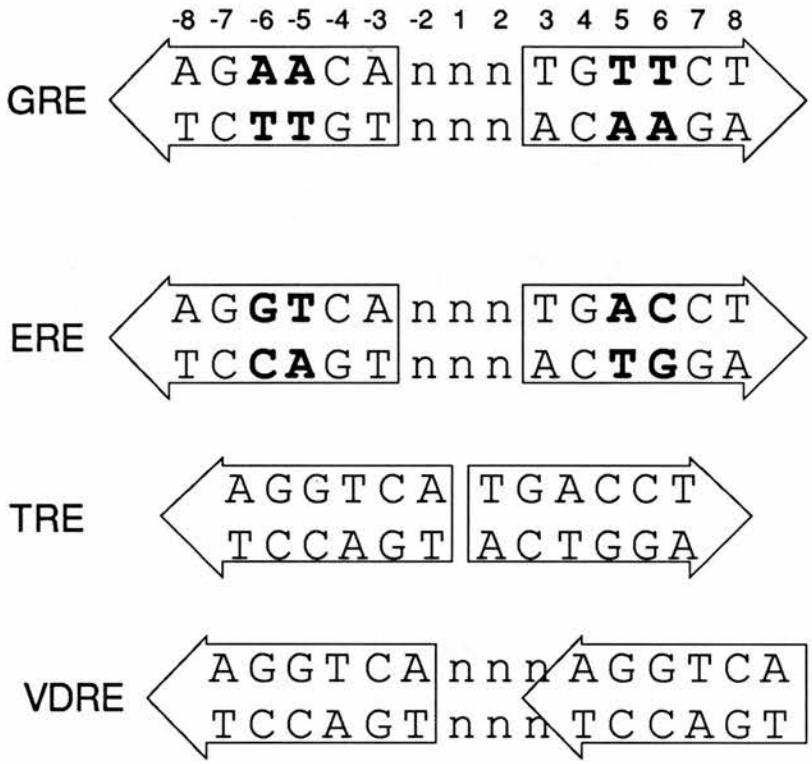
Androgen receptor coded on eight exons. The exon numbers are given within the boxes and the number of nucleotides in each indicated above. Note untranslated region at 3' end of exon 8. The receptor has three main functional domains: the amino terminal domain (exon 1), the DNA binding domain (exons 2 and 3) and the hormone binding domain (exons 4 to 8). H = hinge region of hormone binding domain.

**Figure 2.5** **Structure and sequence of the zinc finger domain of the androgen receptor**




Numbering of cysteine residues and sequence is taken from the full length receptor cDNA of Lubahn *et al.* (1988). Amino acids responsible for HRE recognition specificity are boxed, and those responsible for half site discrimination in bold type. Coding for amino acids is by IUPAC codes (see Appx. 2). Based upon Luisi *et al.* (1991).

**Figure 2.6 Idealised HRE sequences according to type**




GRE: glucocorticoid response element ERE: oestrogen response element

TRE: thyroid hormone response element VDRE: vitamin D response element

HREs shown are idealised perfect palindromes for each type of repeat. The arrows indicate the orientation of each half site. The numbering of the residues is given at the top and the DNA orientation is 5' to 3' from left to right for the upper strand. Bold type indicates the differences of sequence between the GRE and ERE. Note that the ERE, TRE and VDRE share the same half-site sequence.

Based upon Freedman, 1992.

**Figure 2.7 Map of mutations of the androgen receptor gene**

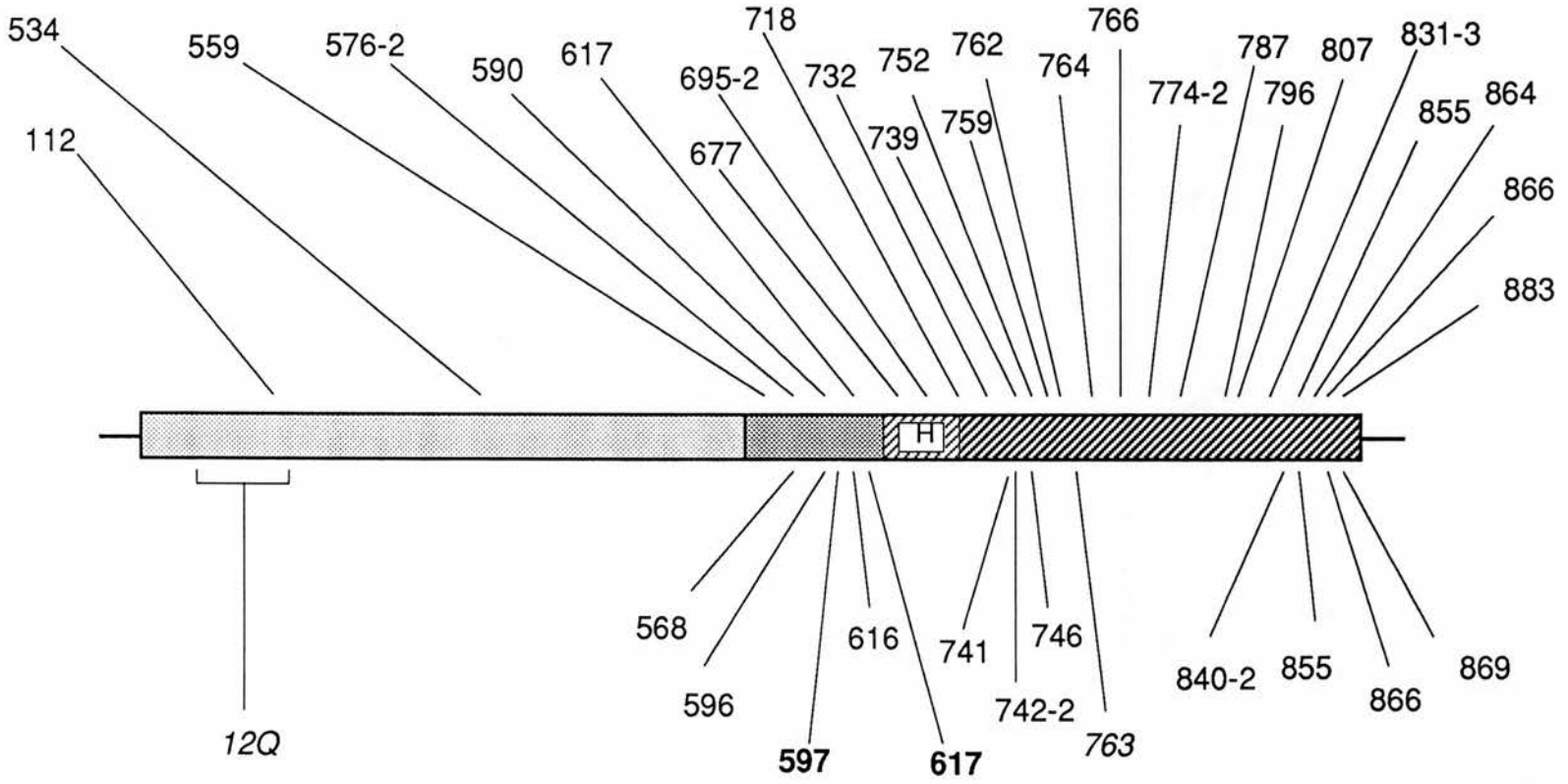



Diagram illustrates the sites of mutations detected in patients with AIS. Patients with CAIS are shown above the receptor and those with PAIS below. The two pairs of mutations shown in *italics* and in **bold** are multiple changes in two patients. References not included in the text are: Veldscholte *et al.*, 1990; Adevemo *et al.*, 1992; Brown *et al.*, 1992; Nakao *et al.*, 1992; Trifiro *et al.*, 1992b, and Wilson *et al.*, 1992. Where a residue is followed by a single figure (774-2), the indicated number of different mutations have been found.

**Table 2.1 Steroid receptor grouping by HRE type**

| Amino acids | Receptor | HRE recognised | Consensus half-site |
|---|---|---|---|
| Gly-Ser-Val | Glucocorticoid<br>Progesterone<br>Mineralocorticoid<br>Androgen | GRE | TGTTCT |
| Glu-Gly-Ala | Oestrogen<br>Thyroid hormone<br>Retinoic acid<br>Vitamin D | ERE | TGACCT |

# Chapter 3. General Methods

## 3.1. Extraction and isolation of genomic DNA

A modification of the method of Grimberg *et al.* (1989) was used for the rapid extraction of genomic DNA. Briefly, 10-20 ml of peripheral blood were sampled from each patient, mixed with 1 ml acid-citrate-dextrose buffer (ACD) (23 mM citric acid, 45 mM sodium citrate, 0.8 M glucose) and stored at -20°C before being mixed with a fourfold excess of cell lysis buffer (CLB) (0.32 M sucrose, 10 mM Tris.HCl pH 7.6, 5 mM $MgCl_2$, 1% Triton X-100). The lysate was centrifuged for 5 minutes at 2,000 revolutions/minute (rpm) at 4°C, the supernatant discarded and the pellet washed once in CLB and once in 0.5 ml of cold protein lysis buffer (PLB) (10 mM Tris.HCl pH 8.0, 10 mM NaCl, 10 mM ethylenediamine tetra-acetate (EDTA)). The pellet was centrifuged at 500 rpm for 2 minutes and resuspended in 3 ml of PLB. Proteinase K (5 units) was added and the suspension incubated at 65°C for 2 hours, with occasional agitation. Thereafter the digest was extracted twice with an equal volume of phenol saturated with TE buffer (10 mM Tris.HCL pH 8.0, 1 mM EDTA pH 8.0), and once with chloroform: isoamyl alcohol 49: 1, before 0.1 volume of 3 M sodium acetate (pH 5.5) and 2.5 volumes of 95% ethanol were added to precipitate high molecular weight DNA. Precipitated DNA was spooled out into a clean tube and 0.5 ml 95% ethanol added. The tube was centrifuged at 13,500 rpm for 5 minutes, the supernatant discarded and the pellet air dried for 10 minutes. 100 µl of sterile water were added and the sample left at 4°C overnight to dissolve.

## 3.2. Synthesis and selection of oligonucleotide primers

Oligonucleotide primers (20 to 29 base pairs) for use in polymerase chain reaction (PCR) were synthesized on a "PCR-mate" DNA synthesizer, model 391 (Applied Biosystems Ltd., Warrington, UK), recovered into 1 ml ammonia and incubated at 55°C overnight. The resulting stock solution was kept at -20°C and aliquots removed for precipitation as required. 350 µl of stock solution were mixed with 50 µl unbuffered 2 M sodium acetate and 1 ml absolute ethanol and incubated at -20°C for 30 minutes. Following centrifugation at 13,000 rpm for 15 minutes, the pellet was washed twice in 70% ethanol, dried and redissolved in 200 µl of water. The concentration of the primer was calculated from the optical density at 260 nm ($OD_{260}$) of a 1 in 200 dilution, measured in a Phillips PU 8800 UV/VIS spectrophotometer.

### 3.2.1. PCR primers

Oligonucleotide primer pairs synthesized were those published by Lubahn *et al.* (1989), chosen by them to lie within the intronic regions flanking each of exons 2 to 8 of the androgen receptor gene. Primer pairs for exon 1 were chosen to amplify overlapping portions of 200 to 500 base pairs in length to facilitate the analysis of this region (Lubahn *et al.*, 1989) (Table 3.1).

### 3.2.2. Primers for denaturing gradient gel electrophoresis

To undertake analysis by denaturing gradient gel electrophoresis (DGGE) of exons 2 to 8, primers were modified as follows: one of each primer pair was synthesized with a 40 base pair sequence ("GC-clamp") rich in guanosine and cytosine at the 5' end. The sequence of the clamp was: 5'- GCCGCCCG CCGCCGCC CGCCGCCG CCGCCCGC CGCCCGCC-3' (Brown *et al.*, 1990). The GC-clamp increases the sensitivity of DGGE in detecting sequence changes to about 95% (Sheffield *et al.*, 1989) (see Chapter 5). Exons 3 and 4 were amplified using a 5' primer lying closer to the beginning of the exon to ensure good resolution of bands in the gel by eliminating one of the higher temperature domains of each fragment (De Bellis *et al.*, 1992).

For exon 1, an identical procedure was followed except that for the glycine repeat coding region of exon 1 primers were used without the GC clamp, because the GGC repeat region functions as a clamp in this PCR fragment (De Bellis *et al*, 1992).

### 3.2.3. Primers for temperature gradient gel electrophoresis

Temperature gradient gel electrophoresis (TGGE) was performed on samples amplified with the same primers as were used for DGGE.

## 3.3. Polymerase chain reaction

Individual exons of the androgen receptor gene were amplified by the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) using pairs of oligonucleotide primers prepared as described above. The standard PCR reaction mixture was as follows: 50 mM KCl, 10 mM Tris.HCl pH 9.0, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100, 100 µM dNTPs (Pharmacia Biosystems Ltd., Milton Keynes, UK), 0.5 µM each primer, 0.5 unit *Thermus aquaticus* DNA polymerase (*Taq* polymerase, Promega Ltd., Southampton, UK), and 0.5-1.0 µg genomic DNA in a final volume of 100 µl. Each tube was layered with mineral oil to prevent evaporation. Amplification conditions typically consisted of an initial melt at 94°C for 2 minutes

followed by 30 cycles of melting for 0.5 minute at 94°C, annealing for 1 minute at 55°C and extension for 0.5-1.0 minute at 72°C. Reactions were carried out in a thermal reactor (Hybaid UK Ltd., Teddington, UK).

## 3.4. Purification and quantification of PCR products

Aliquots (10-20 µl) of each completed reaction were analysed by electrophoresis with suitable molecular weight markers in Nuseive agarose gels (0.8-3.0%) (Flowgen Instruments Ltd., Sittingbourne, UK) run in 1x TBE buffer (90 mM Tris pH 8.0, 90 mM boric acid, 2 mM EDTA) containing ethidium bromide. DNA bands were visualised by examination of the gel under ultraviolet light. Samples containing a single species of DNA of the expected size were purified using a PCR purification spin kit (Qiagen GmbH, from Hybaid UK Ltd.) or Clontech Chromaspin columns (Clontech Laboratories, from Cambridge Biosciences, Cambridge, UK) and quantified by comparison with ethidium bromide/DNA standards (Sambrook *et al.*, 1989).

Where the reaction amplified more than one species of DNA, the remainder of the sample was electrophoresed through a Nuseive agarose gel run in 1x TAE buffer (40 mM Tris pH 8.0, 40 mM sodium acetate, 1 mM EDTA). The desired band was cut from the gel and the DNA recovered using a "Geneclean kit" (USB, from Cambridge Biosciences).

## 3.5. Sequencing of PCR amplified DNA

### 3.5.1. Radioisotopic sequencing

Sequencing of purified fragments of DNA was performed using Sequenase enzyme (Cambridge Biosciences) following a modification of the standard protocol. Samples of DNA (0.5-1.0 µg) were mixed with 1 µl of dimethylsulphoxide (DMSO), 300 ng of primer, 2 µl 5x Sequenase reaction buffer (200 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and made up to a final volume of 10 µl with sterile distilled water. The double stranded DNA was thoroughly denatured by heating to 99°C for 2 minutes and then immediately placed on ice. To the mixture was added 1 µl 0.1M dithiothreitol (DTT), 2 µl labelling mix (1.5 µM of each: dGTP, dCTP, dTTP), 0.5 µl $^{35}$S-labelled dATP (10 µCi/µl) (Amersham International plc, Aylesbury, UK) and 13 units of Sequenase enzyme, diluted according to the manufacturer's instructions. The reaction was incubated at room temperature for 5-10 minutes, aliquoted to each of four tubes containing 2 µl of the appropriate

termination mix (80 μM each of dATP, dCTP, dGTP, dTTP, 50 mM NaCl and 8 μM of each di-deoxynucleotide) prewarmed to 37°C. Termination mixes were incubated at 37°C for a further 10 minutes before stopping the reactions with 4 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). Samples were stored at -20°C until analysis.

Sequencing gels were poured into a BioRad "Sequi-Gene" sequencing cell (BioRad Laboratories Ltd., Hemel Hempsted, UK). Gels were of 12 M urea, 1x TBE and 10% acrylamide (acrylamide: bisacrylamide 19: 1). Solutions were mixed immediately before use and filtered through 0.45 μm filters. Polymerisation was initiated by the addition of TEMED (N, N, N', N'-tetramethylethylenediamine) and 10% ammonium persulphate (BioRad Laboratories Ltd.). Gels were run vertically at 1,800 V and 50°C and then dried and exposed to X-ray film (Kodak X-OMAT AR) for 24 -72 hours at room temperature.

### 3.5.2. Fluorescent sequencing

Fluorescent sequencing was performed using an Applied Biosystems 373-A automatic DNA sequencer and fluorescent dye terminators according to the manufacturer's protocol (Applied Biosystems Ltd.). Reactions were carried out in 20 μl TACS buffer (80 mM Tris.HCl pH 9.0, 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$) containing 0.5-1.0 μg DNA template, 4 units of AmpliTaq™ DNA polymerase (Cetus Corporation, from Applied Biosystems Ltd.), 1 μl each Dyedeoxy™ terminator, 37.5 μM dITP, and 7.5 μM dGTP, dCTP and dTTP. Reactions were overlaid with mineral oil and sequencing carried out in a Perkin Elmer thermal cycler model 480. Typical conditions were 25 cycles of denaturing at 96°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes. For sequencing of the glutamine repeat region of exon 1, annealing and extension times were increased by 50%.

Following completion of the programme, samples were stored at 4°C until required. 80 μl of pure water were added to each sample and excess dye terminators removed by two extractions with an equal volume of phenol: water: chloroform (68: 18: 14). After the second extraction the aqueous phase was removed to a clean tube and the DNA precipitated by the addition of 15 μl 3 M sodium acetate, pH 4.5 and 300 μl absolute ethanol. Following incubation for 2 hours at 4°C, DNA was recovered by centrifugation at 13,000 rpm for 30 minutes at 4°C. The pellets were washed with 70% ethanol, left to air dry, dissolved in 4 μl of loading buffer

(formamide: 50 mM EDTA, 5:1) and heated to 95°C for 5 minutes before loading to the top of the gel.

Gels containing 6% acrylamide (acrylamide:bis-acrylamide 19:1), 8 M urea and 1x TBE in 100 ml were poured between optically clean glass plates and allowed to polymerise for 1 hour before use. Reagents were deionised using Amberlite ion exchange resin (Sigma Chemical Co. Ltd., Poole, UK) before the addition of buffer, filtered through a 0.45 μm filter and polymerisation initiated by the addition of 45 μl TEMED and 0.5 ml 10% ammonium persulphate (BioRad Laboratories Ltd.). Gels were run vertically in the Applied Biosystems 373-A sequencer at a temperature of 40°C. The gel apparatus automatically read the fluorescent signal generated and stored the data on a connected Macintosh II-Ci computer, using software supplied by Applied Biosystems.

The data generated from the automatic sequencer and the sequences manually entered from the autoradiographs of sequencing gels were analysed and compared to the androgen receptor gene sequence published by Lubahn *et al.* (1989) using the GeneJockey software package (Biosoft, Cambridge, UK).

## 3.6. Culture and storage of bacterial cells

Good microbiological practice was employed in the handling of all bacterial cultures. Stock cultures of *Escherichia coli* (*E. coli*) were stored at -70°C in 15% glycerol. Cells were streaked from stocks and cultured on LB-agarose plates (1% tryptone, 0.5% yeast extract, 1% w/v NaCl, 1.5% agar) at 37°C overnight. A single colony from each plate was picked for analysis or further manipulations. Where appropriate, ampicillin at 50 μg/ml was added to the medium. Liquid cultures of cells were prepared by inoculation of a single picked colony into a starter culture of L-broth (1% tryptone, 0.5% yeast extract, 1% w/v NaCl) and incubated at 37°C with mixing at 200 rpm. A small volume of the starter culture (typically 3 ml) was added to 1,000 ml of LB-broth and incubated at 37°C and 200 rpm until the optical density of the solution at 600 nm ($OD_{600}$) was 0.45-0.55. Cells were harvested by centrifugation at 2,000 rpm for 10 minutes. Where indicated ampicillin at 50 μg/ml was added to liquid cultures.

## 3.7. Preparation of plasmid DNA

### 3.7.1. Large scale preparation

A liquid culture was started from a single colony of bacteria containing the desired plasmid. When the $OD_{600}$ of the culture was 0.45-0.55 cells were harvested by centrifugation at 10,000 rpm and 4°C in a Beckman centrifuge. Plasmid was recovered from the cells using a Promega Magic Maxiprep kit according to the manufacturer's instructions (Promega Ltd.). Typical yields were 500-1,000 µg of plasmid DNA from 500 ml of cell culture. Purity of the DNA was calculated from the ratio of $OD_{260}$ to $OD_{280}$ and was typically greater than 1.84. DNA was taken up in sterile distilled water or TE buffer.

### 3.7.2. Small scale preparations

Small scale preparation of plasmid DNA was performed from a 3 ml culture of cells, using a Promega Magic Miniprep kit (Promega Ltd.). Typical yields were of 2.5 µg of high purity DNA.

## 3.8. Electroporation

### 3.8.1. Preparation of competent *E. coli*

A 100 ml liquid culture was prepared by inoculation with a single colony. When the $OD_{600}$ was between 0.45 and 0.50 the cell suspension was chilled on ice for 10 minutes and centrifuged at 4,000 rpm for 10 minutes at 4°C. Cells were resuspended in 100 ml ice cold 10% glycerol, incubated on ice for 1 hour and harvested by centrifugation as before. After resuspension in 10 ml cold 10% glycerol and a further 1 hour incubation on ice, cells were harvested and suspended in 400 µl cold 10% glycerol, and stored in aliquots of 40 µl at -70°C until required.

### 3.8.2. Transformation of *E. coli*

Aliquots of electrocompetent *E. coli* were allowed to thaw on ice. Purified plasmid DNA was added to each aliquot at a concentration of 1-5 ng/vial and the suspension left on ice for 2 minutes. The cells were transferred to an ice cold 2 mm electroporation cuvette (BioRad Laboratories Ltd.) and placed in the Gene Pulser electroporation unit (BioRad Laboratories Ltd.). Electroporation was performed at 2.5 kV with a capacitance of 2.5 µFarad and resistance of 200 Ω for 15 seconds. Electroporated cells were then resuspended in 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) or LB-broth and incubated at 37°C with gentle shaking for 45 minutes. Transformed cells were harvested by centrifugation at 2,500 rpm for 5 minutes at 4°C, resuspended in 100 µl medium, spread onto LB plates with an appropriate

antibiotic for selection (see above) and incubated at 37°C overnight. For some experiments, selection was made directly in liquid culture. After the initial incubation period 4 ml medium containing the selection antibiotic was added directly to the culture bottle and incubation continued overnight.

**Table 3.1 Oligonucleotide primers for PCR amplification**

| Exon | Sequence | Strand |
|---|---|---|
| 1 | 5'-GCCTGTTGAACTCTTCTGAGC-3' | 5' |
| A1A2 | 5'-GCTGTGAAGGTTGCTGTTCCTC-3' | 3' |
| 1 | 5'-CACAGGCTACCTGGTCCTGG-3' | 5' |
| A3A4 | 5'-CTGCCTTACACAACTCCTTGGC-3' | 3' |
| 1 | 5'-GCTCCCACTTCCTCCAAGGAC-3' | 5' |
| A5A6 | 5'-CGGGTTCTCCAGCTTGATGCG-3' | 3' |
| 1 | 5'-CCAGAGTCGCGACTACTACAACTTTCC-3' | 5' |
| A7A10 | 5'-CCAGAACACAGAGTGACTCTGCC-3' | 3' |
| 2 | 5'-GCCTGCAGGTTAATGCTGAAGACC-3' | 5' |
| | 5'-CCTAAGTTATTTGATAGGGCCTTGCC-3' | 3' |
| 3 | 5'-GTTTGGTGCCATACTCTGTCCAC-3' * | 5' |
| | 5'-TTATCAGGTCTATCAACTCTTGT-3' # | 5' |
| | 5'-CTGATGGCCACGTTGCCTATGAA-3' | 3' |
| 4 | 5'-GGAGTTTAGAGTCTCTGACCAGG-3' * | 5' |
| | 5'-GATAAATTCAAGTCTCTCTTCCT-3' # | 5' |
| | 5'-GATCCCCCTTATCTCATGCTCCC-3' | 3' |
| 5 | 5'-CAACCCGTCAGTACCCAGACTGACC-3' | 5' |
| | 5'-AGCTTCACTGTCACCCCATCACCATC-3' | 3' |
| 6 | 5'-CTCTGGGCTTATTGGTAAACTTCC-3' | 5' |
| | 5'-GTCCAGGAGCTGGCTTTTCCCTA-3' | 3' |
| 7 | 5'-CTTTCAGATCGGATCCAGCTATCC-3' | 5' |
| | 5'-CTCTATCAGGCTGTTCTCCCTGAT-3' | 3' |
| 8 | 5'-GAGGCCACCTCCTTGTCAACCCTG-3' | 5' |
| | 5'-GGAACATGTTCATGACAGACTGTACACTA-3' | 3' |

All primers were used as shown to amplify exons for sequencing and to amplify exons for DGGE/TGGE analysis with the addition of the GC-clamp to the 5' primer (see text).

Exon 1 was amplified in four fragments: A1A2= nucleotides 1-427; A3A4= 384-799; A5A6= 721-1248; A7A10= 1148-1753.

For exons 3 and 4 the primers were used as follows: *= used for PCR amplification of exon for sequencing; #= used for PCR amplification of exon for DGGE/TGGE analysis.

# Chapter 4. Androgen receptor gene mutations in androgen insensitivity syndromes

## 4.1. Introduction

The occurrence of point mutations and deletions in the androgen receptor gene of patients with the various forms of androgen insensitivity (AIS) is now well documented (reviewed by Wilson, 1992). Correlation of the nature and site of such lesions with their effect upon the function of the receptor *in vitro* and the *in vivo* phenotype has provided information on the role of certain amino acids in the receptor. The elucidation of the genetic lesions in more patients with AIS will clarify further the structure/function relationships of the various regions of the receptor and also enhance our understanding of the nature of inherited disease in general.

This chapter will deal with the analysis of DNA both from patients with clinical features diagnostic of androgen insensitivity and from those patients with features suggestive of androgen resistance who could not be firmly classified. The findings will be discussed and considered in the context of the data from other researchers.

## 4.2. Patients

Blood samples were initially obtained from four patients with complete AIS and from eight with partial AIS or clinical features suggestive of partial AIS. During the course of the study samples became available from a further two patients with complete AIS and one with features suggestive of partial AIS. DNA was extracted from these samples and the AR gene analysed for the presence of mutations. Many of these patients had been seen over several years by various clinicians and the information was not complete in every case.

### 4.2.1. Complete androgen insensitivity

Patient 1 (S-AR) was referred to hospital at the age of 18 for investigation of primary amenorrhoea. On examination she had a normal female appearance with scanty pubic hair. Vaginal examination revealed a short vagina and the absence of cervix and uterus. Karyotyping confirmed the diagnosis of testicular feminisation with the finding of a normal male 46 XY pattern. She subsequently had gonadectomy performed.

Patient 2 (AC/c) was similarly referred because of primary amenorrhoea at the age of 17 years. She presented a similar picture as patient 1 and karyotyping revealed a 46 XY pattern. She underwent gonadectomy at the age of 19 and was commenced on oestrogen replacement therapy.

Patient 3 (CG) presented at age 3 years with an inguinal hernia. The diagnosis of testicular feminisation was made at the age of 6, following the birth of twin sisters who were diagnosed with the same syndrome shortly after birth. She underwent gonadectomy at age 23. She had a normal female habitus and her karyotype was 46 XY. She has a very strong family history of testicular feminisation which admirably illustrates the X-linked inheritance pattern of the condition (Fig. 4.1).

Patient 4 (JR) presented at age 4 years with bilateral inguinal herniae. She was admitted for surgical repair of these, at which time they were noted to contain testes. There appeared to be no uterine or cervical tissue present. Gonadectomy was performed and the histology reported as showing immature seminiferous tubules. Karyotyping revealed a 46 XY pattern consistent with the diagnosis of complete AIS. Plasma cortisol, ACTH and urinary steroid were measured to exclude congenital adrenal hyperplasia from the diagnosis. She is currently under annual follow up and is due to have puberty induced at a suitable age.

Patient 5 (LI) presented at the time of a hospital admission for abdominal pain at the age of 17 years. She was noted to have an absent cervix on rectal examination and a later full gynaecological examination confirmed the absence of cervix, uterus and Fallopian tubes. She was tall with long limbs, possessed a normal female habitus with adequate breast development and scanty pubic hair. Karyotyping was performed and reported as 46 XY. Laparotomy was performed at which the absence of internal genitalia was confirmed; gonadectomy was carried out and the histology reported as showing normal testicular tissue with seminiferous tubules and Leydig cell hyperplasia but with no evidence of spermatogenesis.

Patient 6 (I) also presented to hospital with primary amenorrhoea, and was found to have no internal genitalia on examination. Karyotyping confirmed the diagnosis, being reported as normal 46 XY male pattern. The patient had been referred from elsewhere and case notes were not traceable and therefore no further information was available.

### 4.2.2. Partial androgen insensitivity

A summary of serological, biochemical and *in vitro* androgen binding data available from the case notes of patients 7 to 15 is presented in Table 4.1. Patient 7 (GHg) was a 36 year old male with a 46 XY karyotype who was born with ambiguous genitalia but with palpable labial testes. He was reared as a boy and underwent numerous penile reconstruction operations. At age 13 he developed bilateral gynaecomastia requiring simple mastectomy. He had never needed to shave. His pubic hair had a female distribution, the penis was 3 cm in length and the testes measured 6 ml and 8 ml. He was married and able to have intercourse, but had never produced an ejaculate. Genital skin fibroblasts (GSF) failed to grow in culture and so 5 α-reductase activity was not measured.

Patient 8 (AC/p) was 35 years old and the youngest of three brothers. The whole family exhibited defects of virilisation and has been reported by Jukier *et al.* (1984). Patient 8 had perineoscrotal hypospadias requiring a two stage repair, gynaecomastia and a high pitched voice. Pubic hair distribution was normal but he had scanty facial hair. The GSF cell line derived from patient 8 was lost before any studies could be performed on it (Jukier *et al.*, 1984) but 5 α-reductase activity was measured and found to be normal. The two older brothers had severe abnormalities including third degree penile hypospadias, bifid scrotums and gynaecomastia. They both had sparse pubic hair and a female distribution of body fat. Specific dihydrotestosterone binding activity of genital skin fibroblasts of the patient's brothers showed a normal total number of binding sites ($B_{max}$), but the equilibrium dissociation constant (Kd) was 1.16 nmol/l, compared to 0.22 nmol/l in normal men. Thus, the brothers appear to have a combination of 5 α-reductase deficiency and impaired androgen receptor function giving rise to their undervirilisation while patient 8 would appear to have the qualitative abnormality of androgen receptor action in isolation. It was not possible to obtain DNA samples from the brothers.

Patient 9 (JS) was born with ambiguous external genitalia showing a minute phallus, non-fusion of labioscrotal folds and bilateral inguinal testes. Bilateral mastectomy was performed because of marked gynaecomastia at age 18 years. Because of repeated epididymitis the left testis was removed from the labioscrotal fold; histology showed Sertoli cell-only seminiferous tubules and abundant Leydig cells. The right testis was extremely small. In his family, abnormalities of male sexual development have been evident in three generations consistent with transmission of an X-linked recessive trait via carrier females (Fig. 4.2). Treatment with intramuscular

Sustanon 250 mg/month (30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate, 100 mg testosterone decanoate) had no effect upon secondary sexual characteristics (hair growth, sebum secretion and muscle anabolism) over two years.

Patient 10 (JM) presented at age 3 years for hypospadias repair. At 13 years he underwent right orchidopexy for an undescended right testicle. He developed gynaecomastia at 16 years and required bilateral mastectomy. Development of pubic and axillary hair began only at age 15, and he had never shaved. The patient had normal male habitus and musculature. Pubic hair was well developed but of a female pattern with no escutcheon. The penis was 3 cm long and the urethral opening found 1 cm from the tip of the glans. The right testis was 15 ml and the left 20 ml in volume, and both were of normal consistency. He had normal libido and was able to maintain erection (erect penis length of 3 cm) and achieve orgasm but could produce only a minute volume of azoospermic ejaculate. The prostate gland was small. Biopsy of the testis showed arrest of spermatogenesis at the spermatogonia stage with complete absence of spermatids. A diagnosis of Reifenstein's syndrome was made and he was commenced on long term intramuscular Sustanon treatment at 500 mg every two weeks for a period of two years. During that time there was no change in his previously normal frequency of erection and sexual activity.

### 4.2.3. Atypical androgen resistance

Patient 11 (WF) was a 44 year old man who presented with erectile impotence and failure of ejaculation. He was an apparently normal male with normal body habitus, secondary sexual characteristics and two children aged 14 and 6 years. His genitalia appeared normal with testes of 15 ml and 20 ml volume, and routine hormone screening showed a grossly elevated serum testosterone in association with an elevated steroid hormone binding globulin (SHBG) concentration and normal gonadotrophins. On direct questioning he admitted to shaving at most twice a week and his facial hair was sparse and fine. No cause for the elevation of SHBG was found and over the course of six months his sexual function appeared to deteriorate. There was no evidence of muscular or neurological deficit and no other signs of androgen insufficiency.

Patient 12 (JL) was 44 years old and had been born with perineoscrotal hypospadias and a bifid scrotum which required a three stage surgical repair. On examination he had a normally pitched voice, scanty facial hair and pubic hair of female pattern. The left testis was palpable in the scrotum, soft and 1 ml in volume.

Karyotyping revealed an apparently normal 46 XY pattern and a semen analysis showed azoospermia. He was commenced on oral fluoxymesterone 5 mg three times daily in an attempt to improve facial hair growth. Some improvement was obtained but he also developed bilateral gynaecomastia which required surgical removal. Hormone analysis revealed him to have a low serum testosterone (Table 4.1) and elevated FSH and LH. An hCG stimulation test suggested some response and he was commenced on intramuscular Sustanon at 250 mg per month. This treatment provoked some response with an increase in facial and body hair, increased libido and an increase in the girth of his penis. It was felt that the response was slightly less than optimal and on the basis of this and the nature of his previous complaints it was suggested that his androgen receptor gene should be screened to exclude any abnormality.

Patient 13 (KW) was 16 years old and had been born with bilateral cryptorchidism and perineoscrotal hypospadias. The hypospadias required several operations and at the age of 5 he was treated with topical testosterone to increase the size of his penis before a definitive repair of the defect. Some increase in size of the penis was achieved in response to this; at this time the testes were palpable at the internal inguinal ring on each side, being of very small volume. He underwent right orchidopexy at age 5, but the left side was deferred in the hope of spontaneous descent. However, this did not occur and his penis remained very small and at age 11 he had a course of intramuscular testosterone to improve the size of his genitalia before a left sided orchidopexy was performed. Good penile growth was seen in response to the injections and following the second orchidopexy he entered puberty spontaneously and seemed to develop normally, but slowly. He was discharged from follow up at age 16 with testicular volumes of 7 and 10 ml.

Patient 14 (GHd) was born in 1979 and presented at birth with severe perineoscrotal hypospadias, cryptorchidism of the left side and a firm mass of tissue in the right scrotum. He underwent examination under anaesthesia at the age of 1 week, at which time the right sided mass was excised, having the appearance of a tumour. Histology reported it to be an androblastoma, a rare tumour of gonadal stroma. Karyotyping revealed a normal 46 XY pattern. At the age of 4 years he underwent repair of the hypospadias when the left testicle was noted to be at the inguinal ring and of very small size. There was no change in the size of this over the next five years. At this time he was investigated by an endocrinologist and found to have no remaining functional testicular tissue (Table 4.1). Blood was taken at this point to exclude any

AR gene defect. At the age of 11, he was commenced on exogenous androgens to induce puberty and initial results appeared normal. However, after 4 years of androgens, although pubic and facial hair development was fairly normal, it was not fully developed and there had been only a slight increase in size of his penis and scrotum.

Patient 15 (RG) was 31 years old and presented with his wife for investigation of primary infertility. He had been born with bilateral inguinal herniae and a right hydrocoele requiring surgery. There was no history of abnormal pubertal development or gynaecomastia. On examination he appeared normally virilised with normal external genitalia. His testes were 15 ml on the left and 20 ml on the right. The right testis was very soft and could be easily pushed into the inguinal canal. A semen analysis revealed a sample of 8 ml volume with abnormal liquefaction and a sperm concentration of less than 1 million/ml semen. Serum hormone profile (Table 4.1) revealed elevated blood levels of gonadotrophins suggestive of a defect of spermatogenesis and some borderline unresponsiveness to androgens.

## 4.3. Methods

### 4.3.1. Analysis of DNA samples

DNA was extracted from the blood samples as described in Chapter 3 (section 3.1). PCR amplified exons of the AR gene from each patient were analysed by direct sequencing of the DNA (Chapter 3, section 3.5). The samples from patients obtained later in the study were analysed by the technique of denaturing gradient gel electrophoresis (DGGE) since the method had become established by that time (see Chapter 5).

### 4.3.2. Culture of genital skin fibroblasts

Genital skin fibroblasts (GSF) were cultured by Dr Malcolm Hodgins (Department of Dermatology, Robertson Building, University of Glasgow, Glasgow) and all the androgen binding, nuclear translocation and up-regulation assays were performed by him according to recognised protocols (Hodgins, 1982; Hodgins *et al.*, 1984; Ring & Hodgins, 1984).

### 4.3.3. Analysis of RNA samples

#### 4.3.3.i. Extraction of RNA

RNA was extracted from genital skin fibroblasts according to the method of Chomczynski & Sacchi (1987). GSF were grown in 75 ml culture flasks after reseeding for 48 hours before harvesting. The medium was removed and the cells

washed with 4 ml ice cold phosphate buffered saline (Flow Laboratories, Irvine, UK) before the addition of 1 ml of solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) to lyse the cells. After washing the flask with a further 1 ml of solution D, the pooled lysate was decanted into a sterile tube and mixed sequentially with 0.1 volume of 2 M sodium acetate pH 4.0, 1 volume of water saturated phenol and 0.2 volume of chloroform: isoamyl alcohol (49: 1). The tubes were shaken for 15 seconds and incubated on ice for 15 minutes before centrifugation at 10,000 rpm for 20 minutes at 4°C. The upper aqueous phase was removed to a fresh tube and the RNA precipitated by the addition of 1 volume of cold isopropanol. After incubation at -20°C for 1 hour the RNA was recovered by centrifugation at 10,000 rpm for 20 minutes at 4°C, redissolved in 0.3 ml solution D and re-precipitated by the addition of isopropanol as before. The RNA pellet was then washed in 75% ethanol, vacuum dried for 2 minutes, dissolved in 50 µl pure, RNAase-free water and the concentration and purity calculated by measuring the $OD_{260}$ and $OD_{280}$ as described in Chapter 3 (section 3.7.1). A typical value for the $OD_{260}$: $OD_{280}$ was 1.8 or greater.

#### 4.3.3.ii. Reverse transcriptase PCR

To determine whether splicing was impaired at the exon 5/6 splice site reverse transcriptase PCR (rtPCR) was performed on total RNA extracted from GSF of patient 10 using exonic primers to amplify across the whole of exons 5 and 6. These were: exon 5 (sense) : 5'- GCTTCCGCAACTTACAC -3'; exon 6 (antisense): 5'- TAATGCTGAAGAGTAGCAG -3'. Total RNA (250 ng) was added to the reaction mix which contained 0.75 µM 3' primer, 200 µM dNTPs, 10 mM $MnCl_2$, 10 mM Tris.HCl pH 8.3, 90 mM KCl and 5 units r*Tth* DNA polymerase (Cetus Corporation, from Applied Biosystems) in a final volume of 20 µl sterile water overlaid with mineral oil. r*Tth* is a DNA polymerase with reverse transcriptase activity in the presence of manganese chloride. Reverse transcription was performed by denaturing the RNA at 95°C for 5 minutes, annealing the primer at 50°C for 10 minutes followed by incubation for 15 minutes at 72°C. An additional 80 µl of PCR reaction buffer was added to each reaction to give a final 100 µl volume containing 5% glycerol, 10 mM Tris.HCl pH 8.3, 100 mM KCl, 750 µM EDTA, 0.05% Tween 20, 1.5 mM $MgCl_2$, and 0.15 µM 5' primer. Reactions were completed by 35 cycles of denaturing at 95°C for 2 minutes, annealing at 50°C for 1 minute and extension at 72°C for 2 minutes. Electrophoresis through a 2% Nuseive agarose 1x TBE gel was performed on 20 µl of sample to examine the reaction products as described in Chapter 3 (section 3.4).

## 4.4. Results

The results of sequencing and screening of the AR gene in the patients with androgen insensitivity are summarised in Tables 4.2 & 4.3. Point mutations were detected in three of the six patients with complete AIS and in four of the nine patients with partial AIS. Five of these were detected by direct sequencing (Fig. 4.3) and two by DGGE (Chapter 5, Fig. 5.4). All detected mutations were confirmed by reamplifying the affected exon from the original DNA and repeating the sequencing twice, in order to eliminate the possibility of PCR artefacts.

The AR gene sequences of patients 1, 2, 4, 6, 7-9, and 12 -13 were partly obtained by Dr. T. Padaychi; exons 2, 3, 7 and 8 were sequenced by him and the mutations in these exons in patients 1, 7, 8, and 9 were identified by him (exon 7 in patient 2 was sequenced by myself). The full sequence in these patients and the exon 1 sequences in all of them were entirely my own work (see also Tables 4.2 and 4.3)

In patient 1, a single nucleotide mutation of cytosine to thymidine which changed the codon for the conserved amino acid arginine 855 to cysteine was detected. The glutamine repeat region contained 20 residues (Fig. 4.4). Patients 2 and 3 each had a single nucleotide mutation which introduced a premature termination codon into the coding sequence of the receptor: cytosine to thymidine in the codon for arginine 831 in patient 2, and the same mutation in the codon for arginine 752 in patient 3 (Figs. 4.5 & 4.6).

Patients 7 and 8 shared the same mutation of guanosine to thymidine, changing the codon for valine 866 to leucine, although they were unrelated (Figs. 4.7 & 4.8). The length of the glutamine repeat in each patient clearly indicates this. Patient 9 possessed a point mutation within the DNA binding domain of the receptor of guanosine to adenosine which changed arginine 608 to lysine (Fig. 4.9). Patient 10 had a mutation of adenosine to guanosine, changing glutamic acid 772 to glycine (Fig. 4.10 and Fig. 5.4, Chapter 5). This mutation also altered the sequence of the 5' splice site of intron 5 but reverse transcriptase PCR of RNA isolated from GSF of this patient amplified a single band of comparable size to that generated by PCR from a plasmid bearing the AR cDNA (Fig. 4.11), consistent with the presence of a full length mRNA transcript.

In patient 11 no point mutations were detected. However, following the amplification of the glutamine repeat coding region of exon 1 a PCR product of

increased size was generated (Fig. 4.12). Sequencing of the amplified DNA revealed an approximate doubling in size of the repeat to 46 residues (Fig. 4.13).

Analysis of the entire gene in some patients was hampered by difficulties encountered in PCR amplification of exon 1 of the gene. The glutamine repeat coding region proved difficult to amplify in five of the patients and the glycine repeat coding region was only successfully amplified from two samples. These two regions have also proved refractory to PCR in the hands of other groups (Lubahn *et al.*, 1989; Marcelli *et al.*, 1990a). In a few cases, sequencing was unable to entirely resolve the identity of some bases and re-amplification of the exon was not possible. These are indicated by 'N?' in Tables 4.2 & 4.3.

Two patients (9 and 10) had had genital skin biopsies performed and androgen binding parameters had been measured on genital skin fibroblasts by Dr Malcolm Hodgins (Glasgow). The results are included in Tables 4.4 and 4.5.

## 4.5. Discussion

The androgen receptor gene sequence has been examined by a combination of direct sequencing and screening in fifteen patients with symptoms, signs and endocrine data compatible with androgen resistance. Six of these patients had complete androgen insensitivity by the criteria of female habitus, 46 XY karyotype, elevated testosterone and male gonads and mutations have been detected in the AR gene of three of them. The mutation in patient 1 changes the codon for arginine 855 into a cysteine codon. This same mutation has been detected by De Bellis *et al.* (1992) in a different patient with complete AIS and found to completely abolish androgen binding of the receptor when expressed *in vitro*. Arginine 855 has also been found to be mutated to a histidine residue in two patients with partial AIS (Chang *et al.*, 1991; Batch *et al.*, 1992) and a comparison of the change in amino acid structure in each case is revealing (Fig. 4.14). The normal amino acid is a positively charged amino acid with a bulky side chain, and in our patient with complete AIS the substituted amino acid is polar with a very small side chain group. The amino acid substituted in the patient with partial AIS is another charged amino acid with a large side chain. These features suggest that the substituted amino acid affects the function of the receptor because the charge and size of the normal residue are important. Loss of charge and size severely disrupts the function while a change to a different charged amino acid (with a larger side chain) causes a less severe effect. This residue is within the region of the heptad hydrophobic repeat thought to be involved in steroid binding and

dimerisation (Fawell *et al.*, 1990; Parker & Bakker, 1992) and the need for a charged amino acid of particular size at this position implies that arginine 855 has a role in maintaining a particular spatial configuration within the steroid binding domain which is necessary for normal function.

In patients 2 and 3, single point mutations have resulted in the introduction of premature termination codons into the reading frame of the gene. In patient 2 the codon for arginine 831 is changed by a cytosine to thymidine transition and in patient 3 the codon for arginine 752 is changed by the same nucleotide substitution (Fig. 4.5 & 4.6). The occurrence of two mutations introducing termination codons in the six patients in this study supports the theory that this is a frequent mechanism by which the complete AIS phenotype is produced (Chapter 2, section 2.5.2.iii). Both these substitutions are of the type described by Youssoufian *et al.* (1986) which occur by methylation and deamination of cytosine to thymidine at CpG couplets (see Chapter 2, section 2.5.2.ii) and which Quigley *et al.* (1992c) calculated were responsible for 50% of the mutations that have been detected in the hormone binding domain. The introduction of a termination codon will cause the synthesis of a truncated receptor protein lacking a large part of the hormone binding domain which is therefore unable to bind androgen. The introduction of a termination codon at 752 had not been described elsewhere at the time it was identified in this patient, but Pinsky *et al.* (1992) have recently reported another patient with this defect. The arginine 831 substitution has been independently identified by De Bellis *et al.* (1992) and by Ris-Stalpers *et al.* (1992) in unrelated patients. Brown *et al.* (1990) described a patient with complete AIS with a mutation of arginine 831 to glutamine as a result of substitution of the second base of the codon from guanosine to adenosine, and Pinsky *et al.* (1992) reported a substitution of leucine at the same site in another complete AIS patient.

In the three patients in this study with complete AIS, all of them had mutations at positions which have been found to be mutated in patients with complete and partial AIS by other researchers (Chang *et al.*, 1991; Batch *et al.*, 1992; De Bellis *et al.*, 1992; Evans, 1992; McPhaul *et al.*, 1992; Pinsky *et al.*, 1992; Ris-Stalpers *et al.*, 1992). That multiple defects occur at the same site and that the same defects occur in unrelated individuals suggests that the codons for these amino acids 752, 831 and 855 are particularly susceptible to mutation.

The three patients with complete AIS in whom no mutations were found present a perplexing problem. On clinical grounds there was no difference between these three and the patients described above. It may be that there are mutations lying within

the regions of exon 1 which were resistant to PCR and therefore undetected by this study. Some mutations have been described in this exon including termination codon insertions (McPhaul *et al.*, 1991; Zoppi *et al.*, 1993) and the insertion and deletion of nucleotides (Batch *et al.*, 1992). In patient 5 the entire gene was screened by DGGE and although the sensitivity of detection is around 95% (Myers *et al.*, 1985b) it is possible that this patient harbours a mutation which cannot be detected by the method. The other possibility is that these patients in fact do not have mutations within the coding sequence of the AR gene. Direct PCR of individual exons of the gene from genomic DNA will not have identified rearrangements of the gene which leave the exons intact which might be detected by Southern analysis (Brown *et al.*, 1988). However, whilst the presence of large deletions can be excluded on the basis of successful PCR reactions in each case, it is possible that a chromosomal translocation or inversion of some kind has occurred in one or more of these patients which interrupts the transcription and translation of the whole gene sequence. There may also be mutations or other abnormalities of function of the promoter and enhancer regions that modulate transcription of the AR gene which might lead to aberrant, reduced or absent synthesis of the AR protein.

Among the nine patients with partial androgen insensitivity and those with signs of impaired virilisation, defects were detected in four. Patients 7 and 8 possessed the same mutation despite being unrelated (Fig. 4.7 & 4.8). The mutation of valine 866 to leucine alters one of the key hydrophobic amino acids in the dimerisation domain of the AR. Lubahn *et al.* (1989) identified a mutation causing a substitution of methionine for the same amino acid in a patient with complete AIS and our finding of an identical mutation in two patients at the same site supports the theory put forward by them that amino acid 866 is a "hot-spot" for mutation in the same way as the amino acids discussed above. As with the mutations at position 855, an examination of the structure of the amino acids provides some clues to the mechanism by which the mutations cause their effects (Fig. 4.15). Valine and leucine are both hydrophobic with compact side chains, while methionine has a longer side chain. The change to a larger amino acid in the hydrophobic repeat of exon 7 appears to cause a severe disruption to steroid binding and dimerisation, giving rise to the complete AIS phenotype while the change in patients 7 and 8 appears to affect the normal function of the receptor less severely, judging by the less severe abnormalities in these patients.

Patient 9 was the only patient in whom a mutation was detected in the DNA binding domain of the receptor. The mutation in exon 3 of arginine 608 to lysine

changes an amino acid which lies towards the tip of the second zinc finger and is conserved throughout the entire steroid receptor superfamily (Figs. 4.9 & 4.16). The second finger is involved in orientation of the receptor for DNA binding, stabilising DNA/protein interactions between other members of the transcription complex, and providing the interface for dimerisation (Freedman, 1992). Crystallographic analysis of the second zinc finger has identified the amino acids which are in direct contact with the DNA of the hormone response element during binding of the domain to the HRE (Luisi *et al.*, 1991). Arginine 608 (and its equivalent in the other steroid receptors) makes specific contact with the phosphate backbone of the DNA within the HRE, and is thus a key residue in correctly orienting the dimerisation surface of the zinc finger. Substitution of the equivalent arginine (498) in the glucocorticoid receptor (GR) by lysine has been shown to inhibit binding to a glucocorticoid response element (GRE) *in vitro* (Schena *et al.*, 1989). This mutant GR was able to activate transcription from an MMTV-CAT (mouse mammary tumour promoter-chloramphenicol acetyl transferase) reporter gene as efficiently as a wild type GR when transfected into CV-1 cells, but showed markedly reduced activity when expressed in yeast. It is clear from the phenotype of patient 9 that arginine 608 to lysine has markedly impaired function in several target tissues, including external genitalia, testes and androgen sensitive hair follicles. Studies of nuclear uptake of receptor in GSF (Table 4.4) indicate that this mutation has some effect upon nuclear binding without abolishing it completely. This may be due to an alteration of binding affinity by the mutation directly or to failure of stabilisation of the receptor dimer. The marked tissue effects of the androgen insensitivity despite residual nuclear binding in this patient may be explained by an interaction of the AR with other steroid receptors or transcription factors. Both AR and GR can bind to the same response element (HRE) and they may compete with each other for binding to other nuclear factors such as *jun* and *fos*, and thereby regulate each other's actions (Chaucereau *et al.*, 1992; Parker & Bakker, 1992). The mutation at 608 may alter the affinity of the AR for heterodimer formation and thereby alter the normal interaction between these different molecules. Evidence for the presence of factors which modulate the action of the steroid receptors in specific cell types was provided by Bocquel *et al.* (1989) who found that the transactivating ability of the members of the steroid family varied depending on which cell type the receptors were expressed in. More recently McPhaul *et al.* (1992) described a number of patients, some of whom shared the same molecular defect of the androgen receptor but who showed markedly different severities of clinical features (e.g. complete AIS as opposed to Reifenstein's syndrome). These data indicate that other factors in addition

to the molecular lesion contribute to the physiological effect both in individual tissues and between individual patients.

Patient 10 possessed a mutation within exon 5, detected by DGGE (Fig. 5.3, Chapter 5), which altered the last nucleotide of the exon and results in a change from glutamic acid 772 to glycine. The GSF androgen receptor from this patient showed normal androgen binding capacity with a variably increased dissociation constant and a biphasic dissociation pattern indicative of loss of stability of the receptor/ligand complex. Exon 5 lies within the hormone binding domain of the androgen receptor but outside the regions involved in dimerisation and nuclear localisation (Fawell *et al.*, 1990; Jenster *et al.*, 1991). Marcelli *et al.* (1992) noted in three patients with complete AIS that the effect of mutations on receptor function in exon 5 depended on the location within the exon. Towards the N-terminal end, substitution of tyrosine 739 by arginine abolished ligand binding completely while mutations nearer the C-terminal end (phenylalanine 764 to leucine, and proline 766 to serine) altered ligand binding qualitatively yet preserved a degree of transcriptional activation in cotransfection assays. In view of the fact that patient 10 presented with defects of virilisation in conjunction with an unambiguously male phenotype and relatively normal sexual function (contrasting with the complete AIS phenotype in these other patients with exon 5 mutations (Marcelli *et al.*, 1992)) it would appear that the receptor with the mutation at amino acid 772 retains significantly more transactivating ability than those with mutations elsewhere in this exon.

The mutation of the last nucleotide of exon 5 introduces a mutation into the 5' donor splice site sequence of intron 5 at the -1 position. Although a splice site mutation has been reported in a patient with complete AIS resulting in a partial deletion of exon 4 due to activation of a cryptic site (Ris-Stalpers *et al.*, 1990), that mutation was in the invariant GT couplet at +1/+2 (Green, 1986; Padgett *et al.*, 1986) (Fig. 4.17). The sequence of the 5' site of intron 5 differs from the consensus sequence at several positions including the -1 position and so generalisations about the effect of mutations here cannot be readily made. Studies on the β-globin gene have shown that the effect of mutations in the splice site are unpredictable in any given instance (Wieringa *et al.*, 1983). Mutations in the intronic part of the sequence lead to partial inactivation of the site and result in the presence of more than one size of mRNA transcript. Activation of cryptic donor sites occurs when the mutation in the normal site alters its binding affinity for the small nuclear ribonucleoprotein particles (involved in splicing) to approach the affinity of any nearby cryptic sites (Nelson & Green,

1990). An examination of the mutations found in splice sites of several genes where cryptic site activation or exon skipping had occurred revealed that most of the mutations in the original site were at the +1 or +2 positions (where the invariant GT dinucleotide lies) while there was a relative lack of mutations at the -1 or -2 sites (Krawczak *et al.*, 1992).

On the basis of these findings one can propose that aberrant splicing of the pre-mRNA in patient 10 is unlikely to occur, but an attempt was made to verify this by amplifying exons 5 and 6 across the splice site by reverse transcriptase PCR from total RNA in order to confirm the normal size of this mRNA fragment. Samples of total RNA from patient 10 produced a single species of DNA when amplified by reverse transcriptase with exonic primers from the 5' end of exon 5 (beginning at the first base of the exon) to the 3' end of exon 6 (ending with the last base of exon). This band was the same size as those from RNA from normal male skin fibroblasts and from a plasmid bearing the human AR gene cDNA. Thus splicing at the 5' site of intron 5 appears not to be affected. However, the primers used to screen for cryptic splice site activation do not include the fourth intron (between exon 4 and 5) and it could be argued that a cryptic splice site upstream of exon 5 may have been activated. This possibility should have been explored by repeating the experiment using a 5' primer lying within or at the end of exon 4 as well.

It may be argued that the gel should have been Southern blotted and probed to exclude the presence of another species of DNA in the reaction mix which would represent a low copy number of aberrantly spliced RNA and which was not present in high enough concentration to appear on ethidium bromide staining. However, since reverse transcriptase PCR was used, even RNA species in low amounts should have been amplified many-fold in the reaction and one would expect them to be visible on the gel. The apparent weakness of the signal on the gel is compared to the PCR reaction from a highly purified DNA plasmid (lane 5, Fig. 4.11), and both lanes from RNA template have a similar intensity of staining. Therefore, I feel that a Southern blot would not have provided any extra information, but it may have been worth repeating the reverse transcriptase PCR in an attempt to improve on the amplification of the samples.

Pinsky *et al.* (1992) have described a patient with partial AIS with a mutation at amino acid 772 from glutamate to alanine. Both this change and the change in patient 10 are from an amino acid with a bulky, charged amino acid to one with a small uncharged side chain (Fig. 4.18). This situation is in contrast to that for amino acids

855 and 866 where even small changes in the size and charge of the side chain produced an effect upon the activity of the receptor (Fig. 4.15 & 4.16) and suggests that the C-terminal region of exon 5 is not subject to such severe conformational constraints as the region of exon 7 from 850 to 866.

The nature of the abnormality detected in patient 11 is particularly interesting. The size increase of the glutamine repeat region to approximately double the normal range is exactly similar to that seen in patients with spinal and bulbar muscular atrophy (SBMA) (La Spada *et al.*, 1991) where this increase in the repeat of the AR gene is thought to be the causative lesion of the disease. SBMA is an X-linked condition which presents with bulbar palsy and lower motor neurone paralysis with onset in the third decade of life (Kennedy *et al.*, 1968). Some of the reported cases show features of androgen resistance including gynaecomastia, testicular atrophy, oligospermia and failure of erection (Arbizu *et al.*, 1983). The clinical history and signs in this patient are suggestive of only a very mild abnormality of androgen action with poor facial hair growth in the presence of an elevated testosterone, associated with problems maintaining an erection. No other stigmata of undervirilisation were apparent. He showed no sign of neurological disease and was past the usual age of onset of SBMA, although a few patients have been reported where symptoms appeared beyond age 40 (Stefanis *et al.*, 1975; Barkhaus *et al.*, 1982). His testicles were of normal volume. La Spada *et al.* (1991) found that the increase in glutamine repeat was absolutely associated with the disease state, being present in 35 patients and in none of 75 control samples. It seems most likely that this man represents a late-onset variant of SBMA but there is, at present, no evidence of neurological disease. The other possibility is that the genetic lesion identified is an isolated phenomenon and not linked to any disease but in view of the size of the study of La Spada *et al.* (1991) this is very unlikely to be the case. Assuming that patient 11 goes on to develop SBMA this will be the first incidence of analysis of the AR gene providing the diagnosis, and the first description of a patient with SBMA presenting with androgen resistance rather than neurological features.

The remaining patients in whom no mutations were found are a heterogeneous group. Of these, patient 12 presented with the most undervirilised phenotype with severe genital abnormalities in association with a female type body habitus. Nevertheless he showed a considerable response to exogenous androgen both in terms of hair growth and in genital development. It is possible that a mutation exists within the glutamine or glycine repeat regions of exon 1 which would produce this pattern of

apparent androgen unresponsiveness during fetal life followed by responsiveness in later life since mutations have been reported by other workers which produce apparently isolated defects of virilisation (Larrea *et al.*, 1978; Grino *et al.*, 1988; McPhaul *et al.*, 1991a). However, since hypospadias and cryptorchidism are relatively common congenital abnormalities (Gearhart *et al.*, 1988; Evans *et al.*, 1991) and can occur in otherwise healthy infants in the presence of normal androgen action (Roberts & Lloyd, 1973), the undervirilisation and the absence of significantly elevated testosterone levels in patient 12 may be due to testicular damage as a result of his cryptorchidism.

Patient 13 appears to present exactly this picture of hypoandrogenism secondary to testicular maldescent. The aetiology of the maldescent and hypospadias is unlikely to be due to an abnormality of androgen action because there appeared to be a normal response to androgen during his treatment and following puberty. Patient 14 seems to have a primary failure of testicular development, since there was no functional tissue in the left-sided testicular remnant. The question remains whether there was any functional tissue in the right testis, because this was removed shortly after birth. Whether the genital abnormalities were due to androgen resistance is impossible to say, although the response to exogenous androgen in this patient appeared to be suboptimal (section 4.2). However, no mutation in the AR gene has been detected in this patient. Patient 15, in retrospect, is likely to be azoospermic secondary to testicular damage as a result of his cryptorchidism as a child, particularly in view of the normality of genital and general body development. The absence of defects in the AR gene is therefore not surprising.

However, the investigation of patients with atypical features of androgen resistance is still a worthwhile exercise, particularly following the report by Batch *et al.* (1993) which described the identification of a point mutation within the AR gene of two brothers with isolated perineal hypospadias. The mutation was situated in exon 8 and resulted in the replacement of isoleucine 869 by methionine. The GSF AR from these patients showed an abnormally elevated rate of dissociation, and this is the first reported case of AR gene mutations in isolated hypospadias.

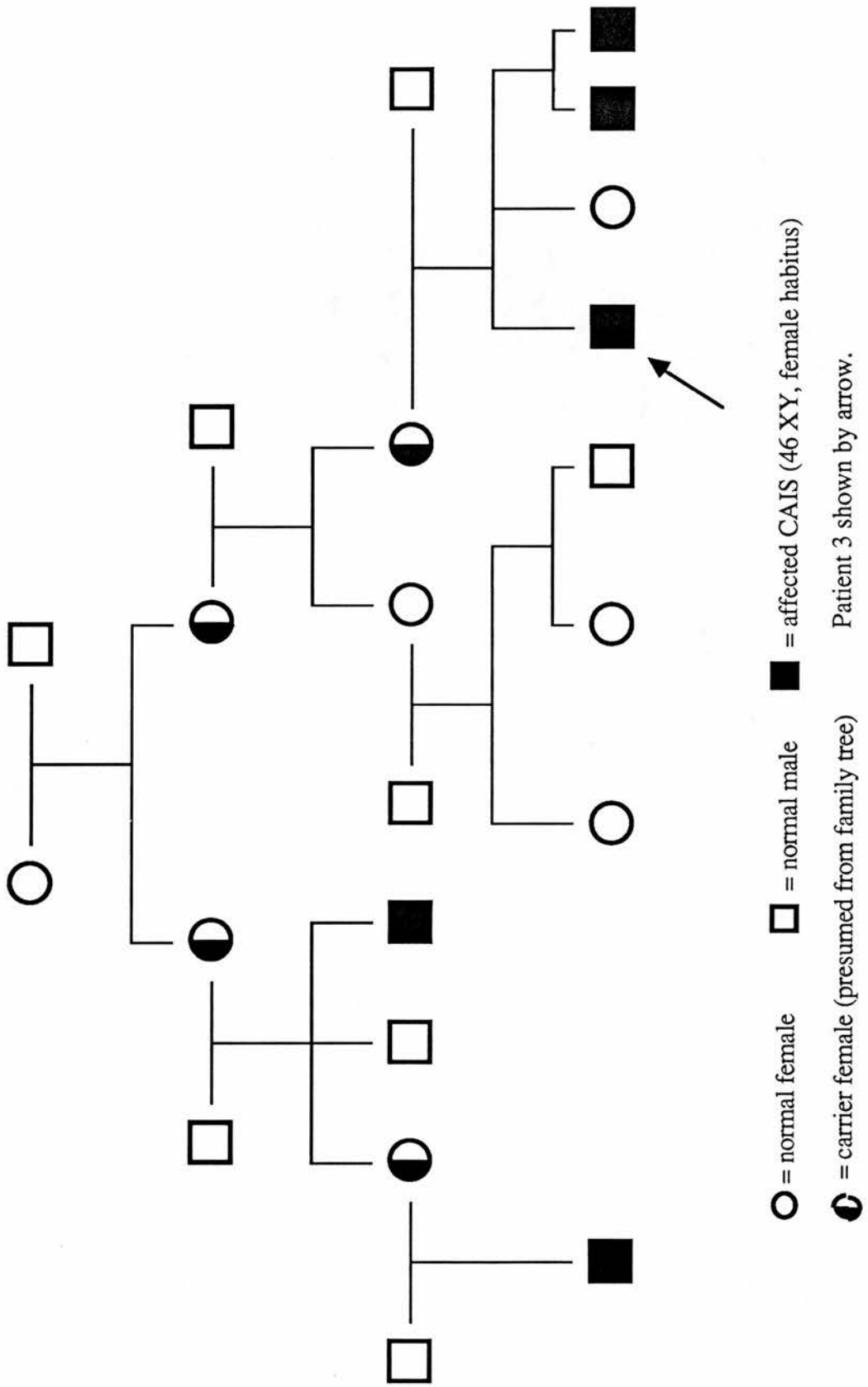



**Fig. 4.1 Family tree of patient 3 ( CG)**

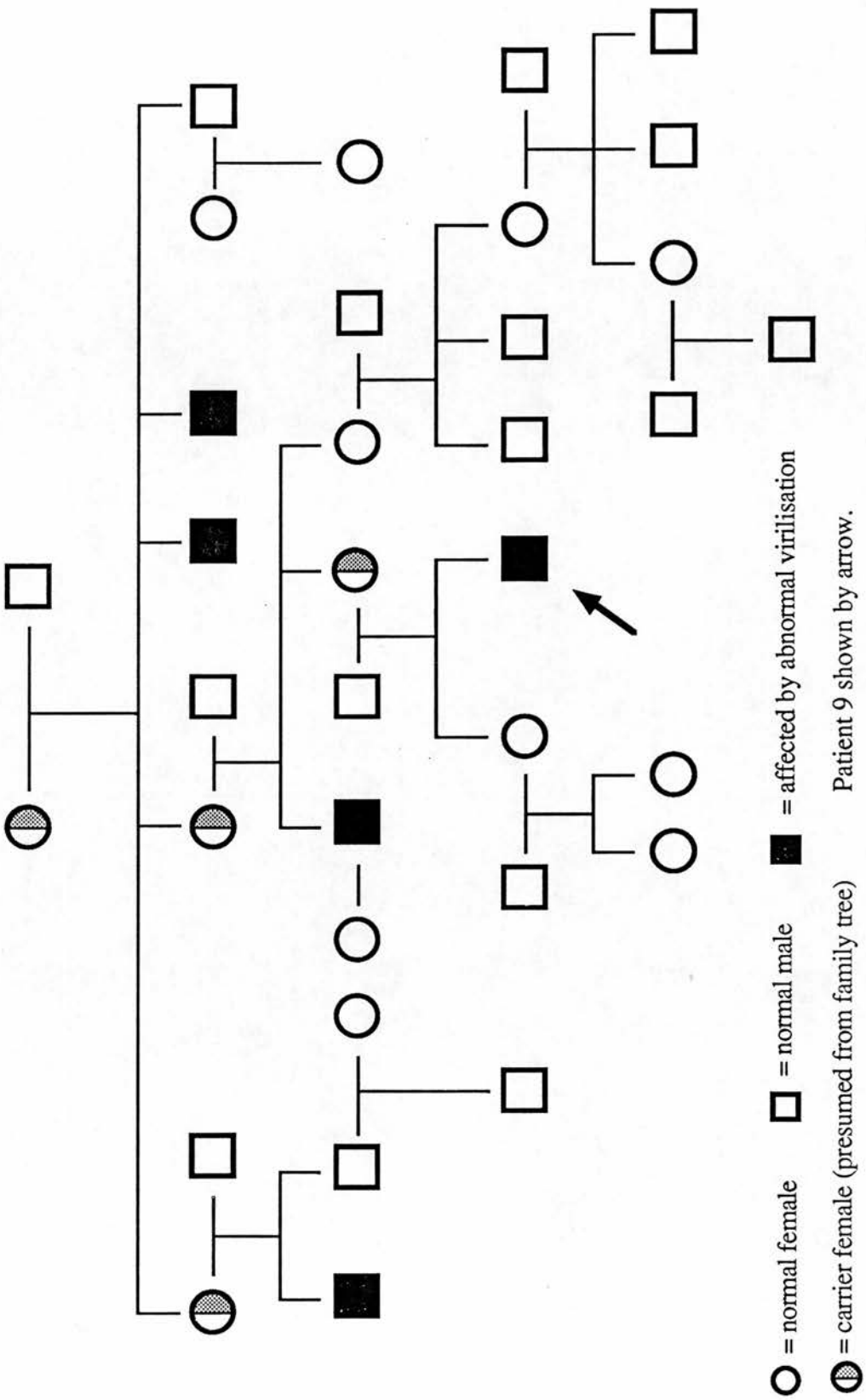
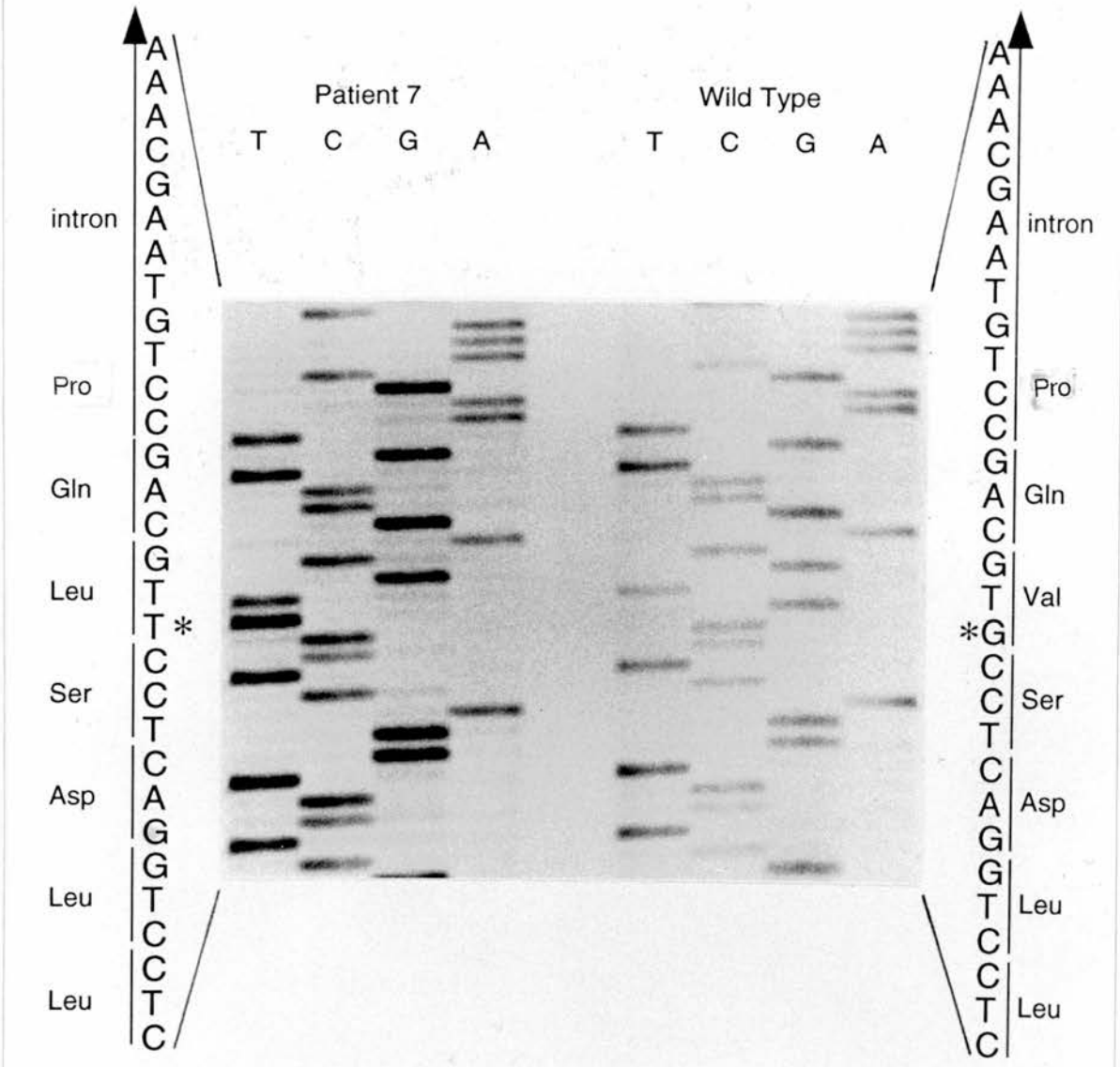


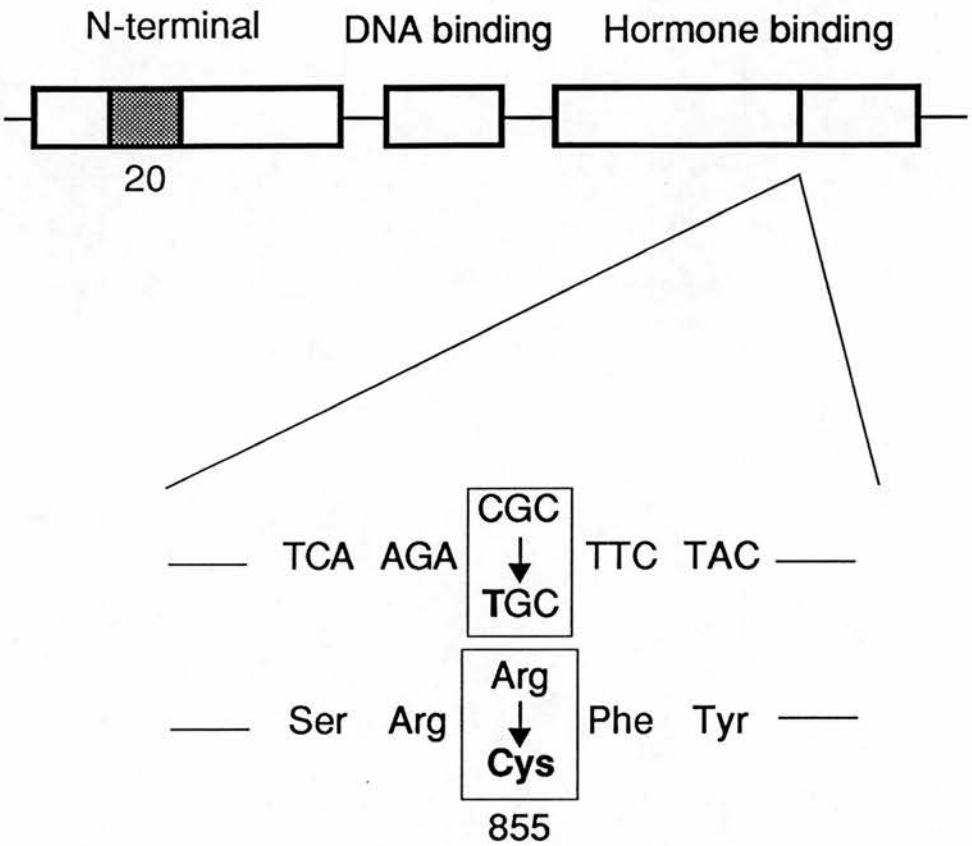

**Figure 4.2 Family tree of patient 9 ( JS)**

**Figure 4.3 Autoradiograph of a mutation in exon 7**



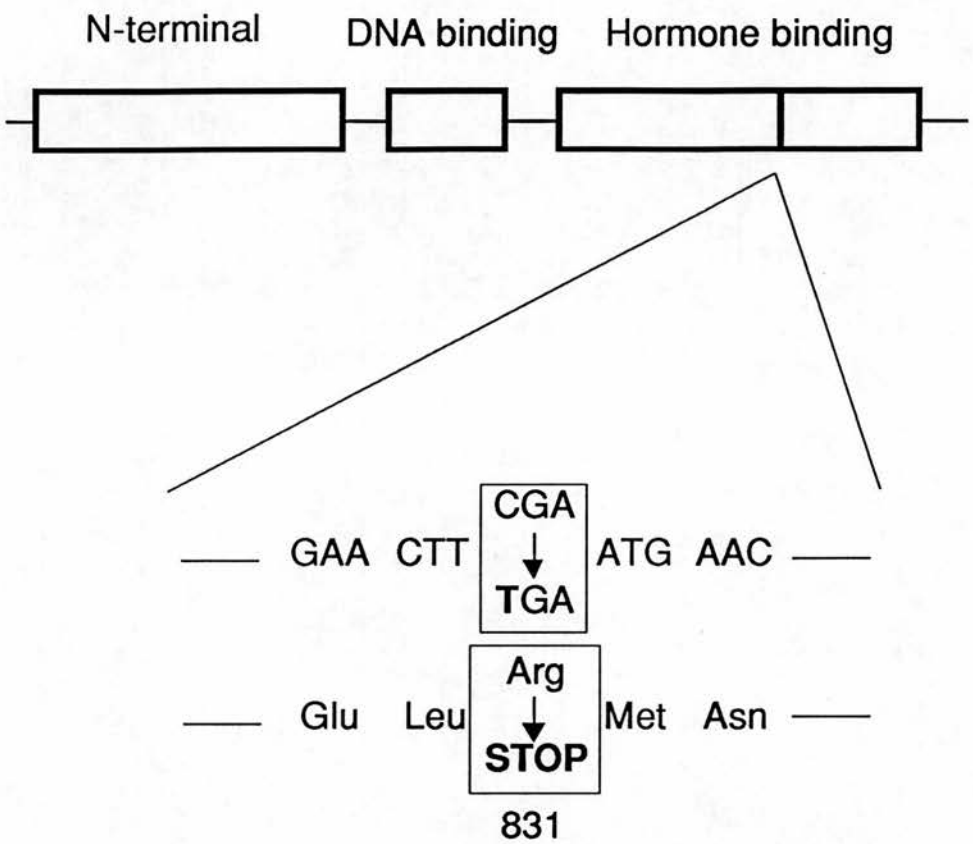

Portion of autoradiograph of sequencing of exon 7 from patient 7. Sense runs from the bottom of the photograph and the coded amino acids are indicated at the sides. The mutation is indicated by an asterisk (*) at the point in the photograph and in the schematic figure to the side.

**Figure 4.4 Mutation detected in patient 1**



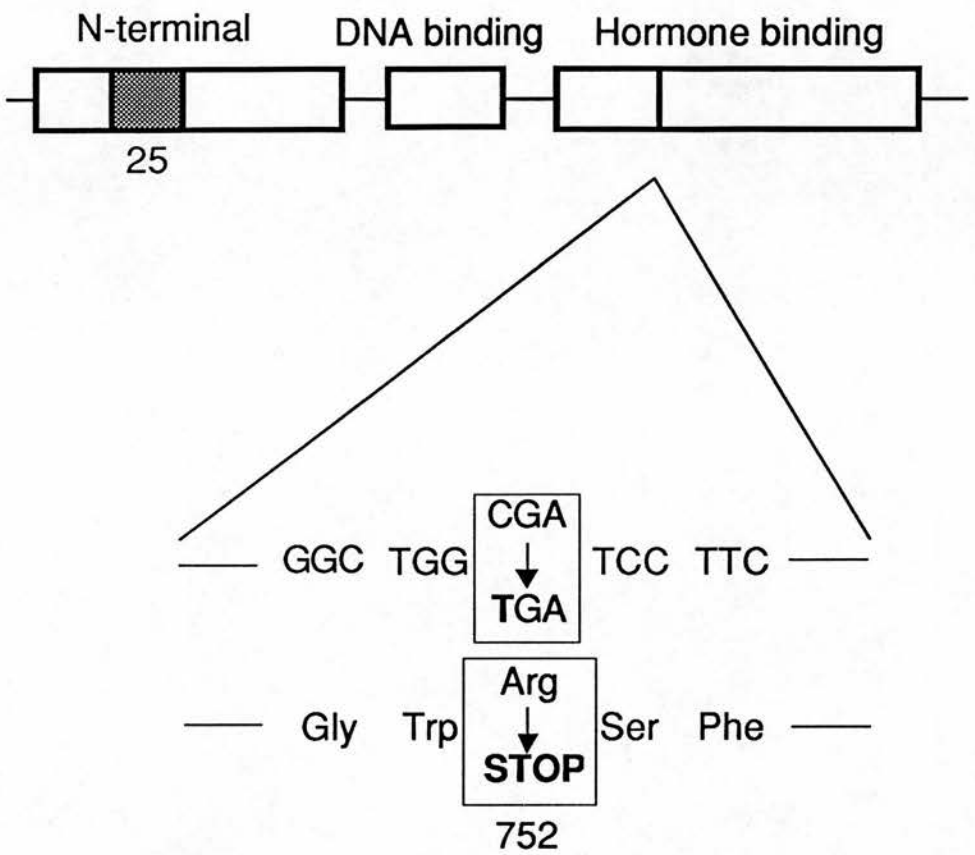

Schematic representation of the mutation detected in patient 1. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.5 Mutation detected in patient 2**



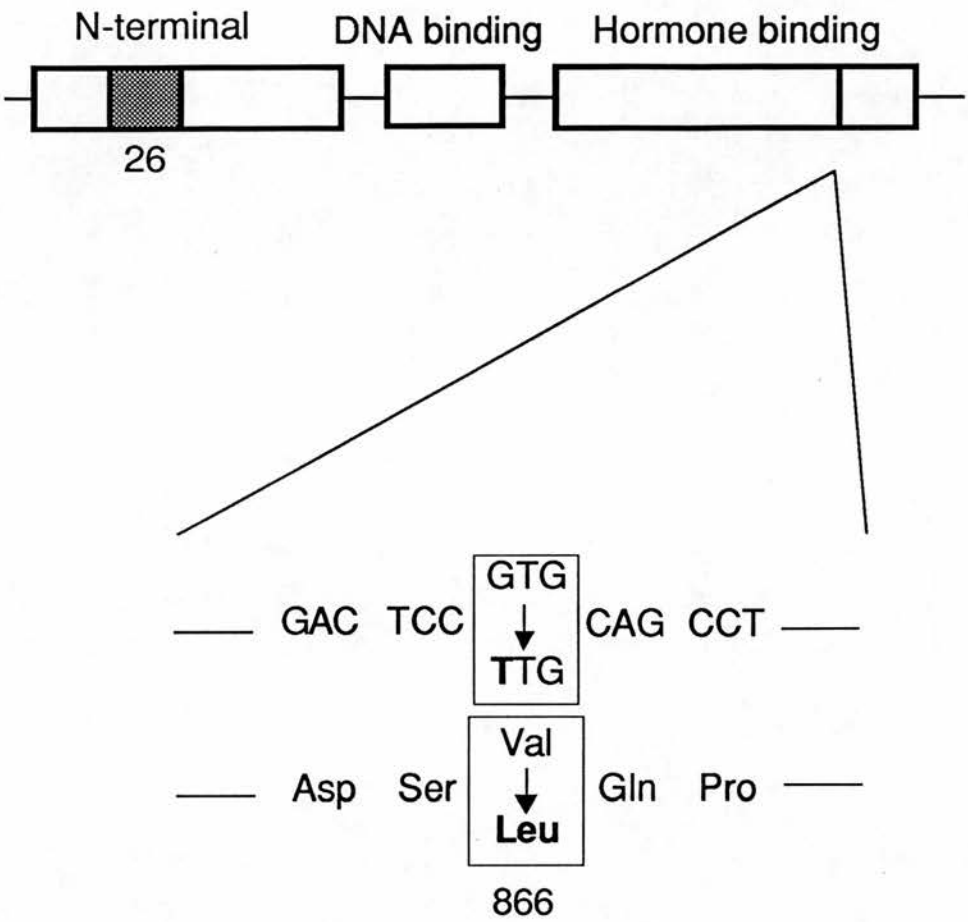

Schematic representation of the mutation detected in patient 2. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.6 Mutation detected in patient 3**



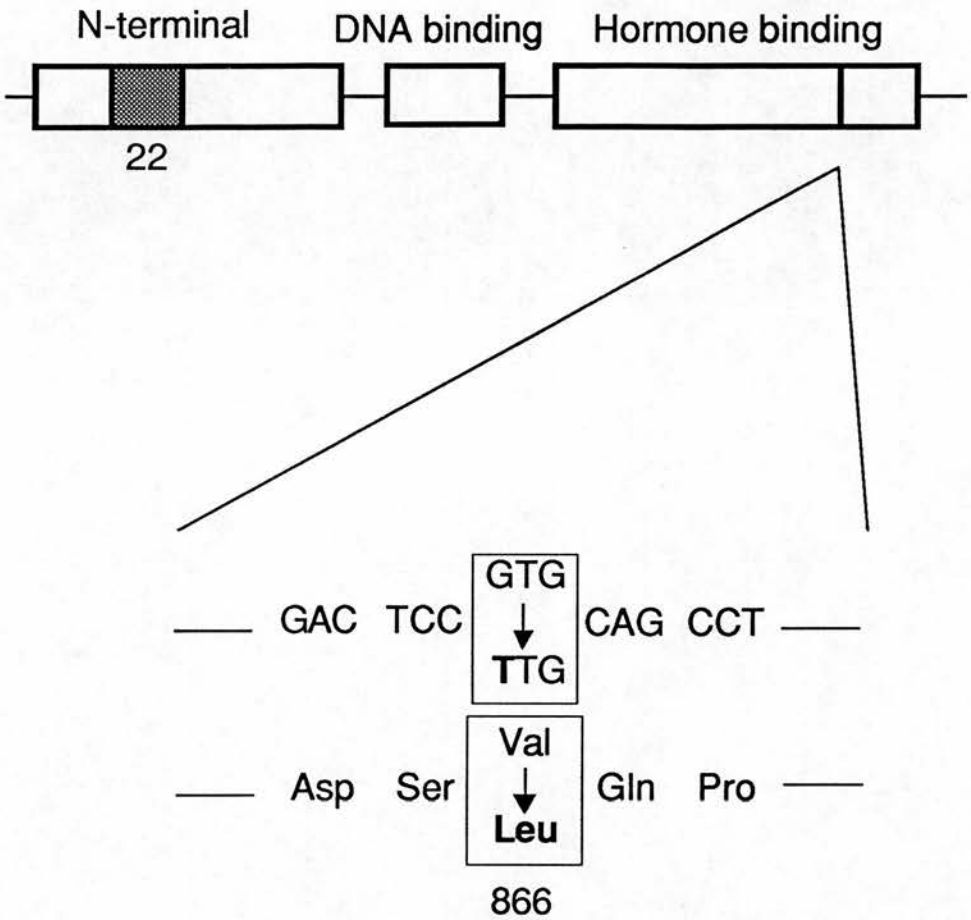

Schematic representation of the mutation detected in patient 3. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.7 Mutation detected in patient 7**



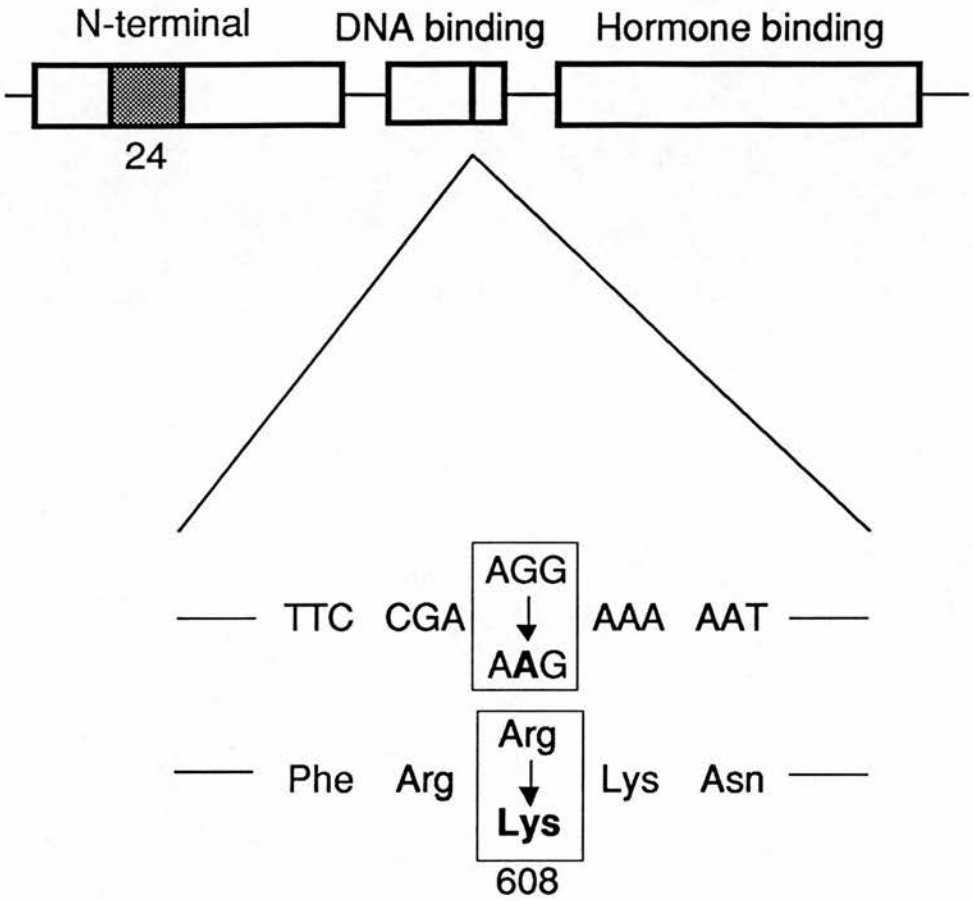

Schematic representation of the mutation detected in patient 7. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.8 Mutation detected in patient 8**



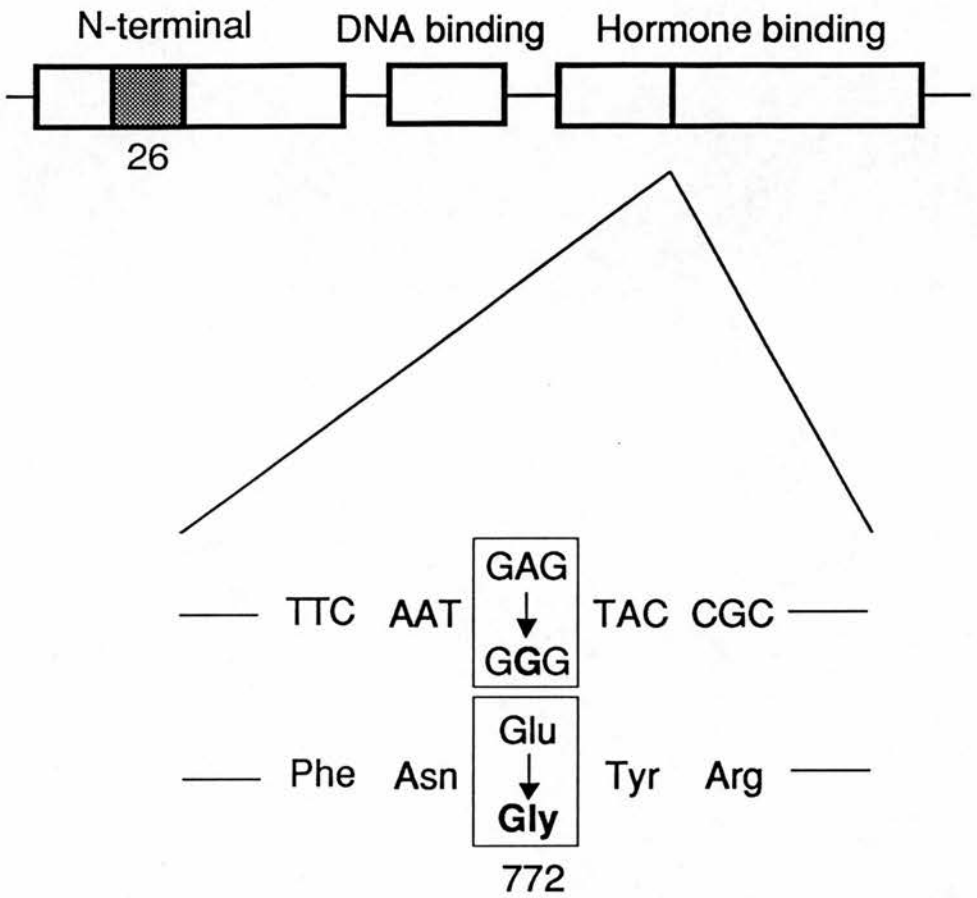

Schematic representation of the mutation detected in patient 8. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.9 Mutation detected in patient 9**



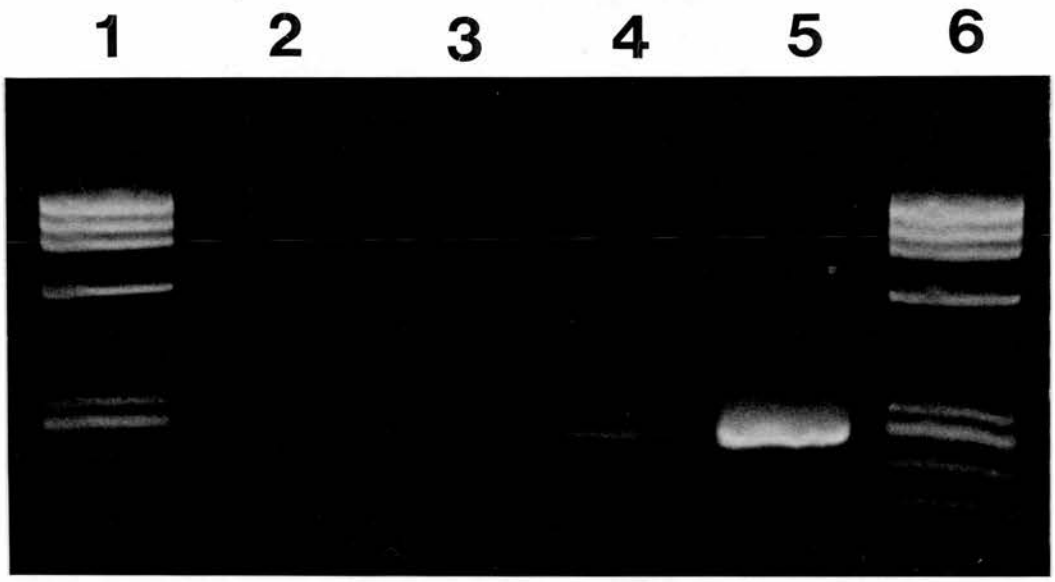

Schematic representation of the mutation detected in patient 9. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.10 Mutation detected in patient 10**



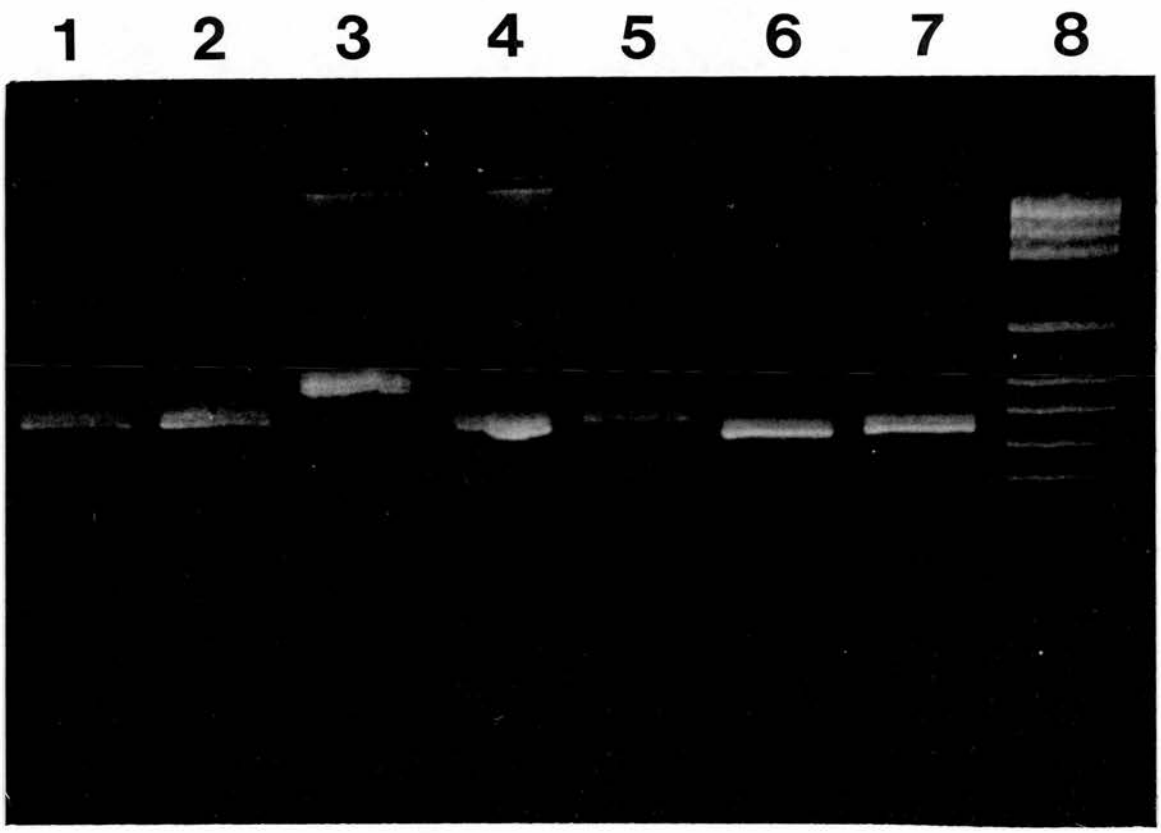

Schematic representation of the mutation detected in patient 10. The substituted nucleotide and the presumed coding change are indicated in **bold** type.

**Figure 4.11 Agarose gel of rtPCR of exon 5/6**



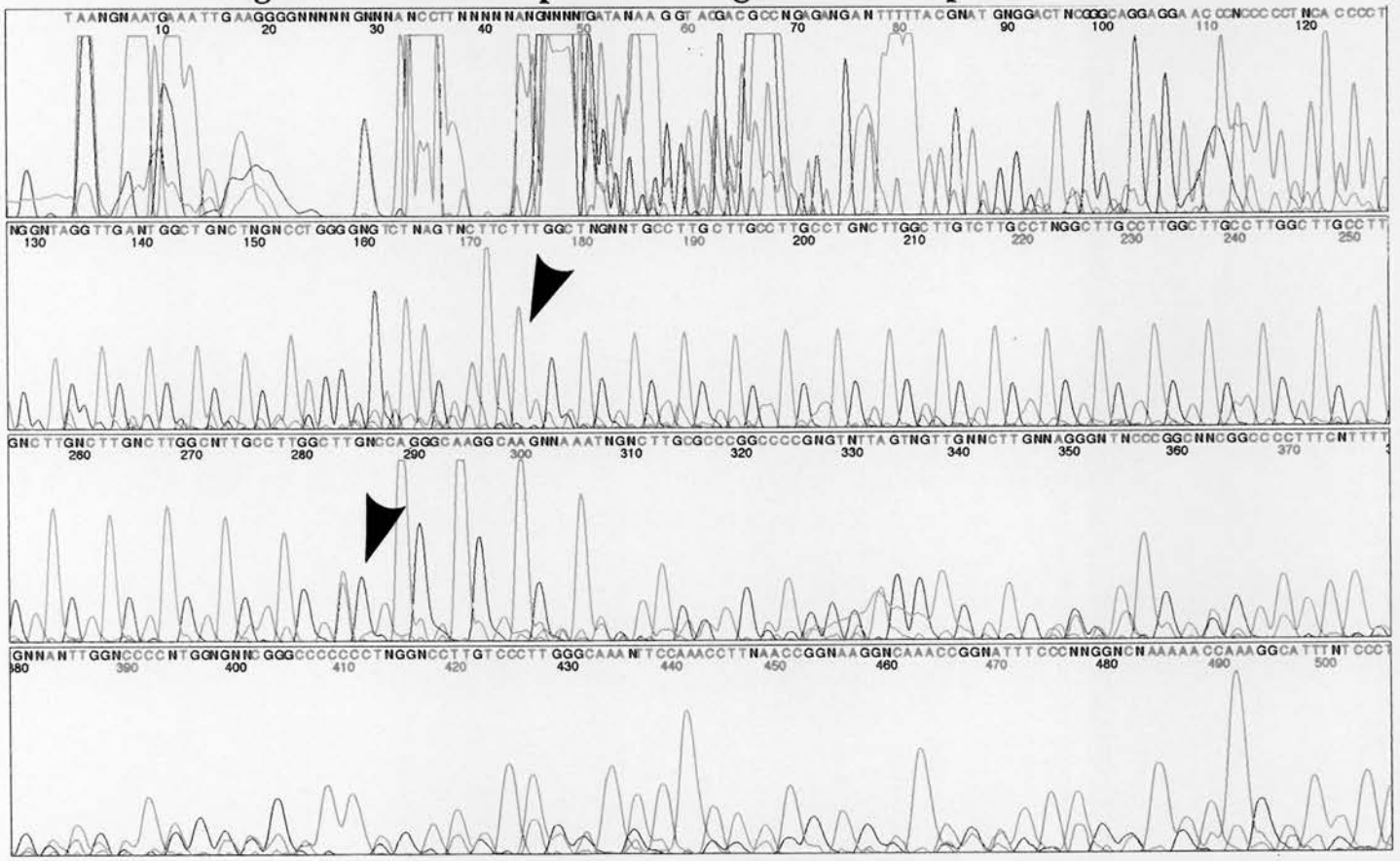

Agarose gel of reaction products from rtPCR across exons 5 & 6. The expected size of PCR product was 276 bp. Lanes 1 & 6: ϕX134/Hae III DNA markers: ϕX134 digested to completion with Hae III. Sizes of the visible bands are: 1,353, 1078, 872, 603, 310, 281, 271, 234, 194. lane 2: no DNA control; lanes 3: whole RNA from patient 10; lane 4: whole RNA from normal male GSF; lane 5: pSV.ARo. 10µl of completed reaction added to each lane.

**Figure 4.12 Gel of PCR fragment A1A2 of exon 1**



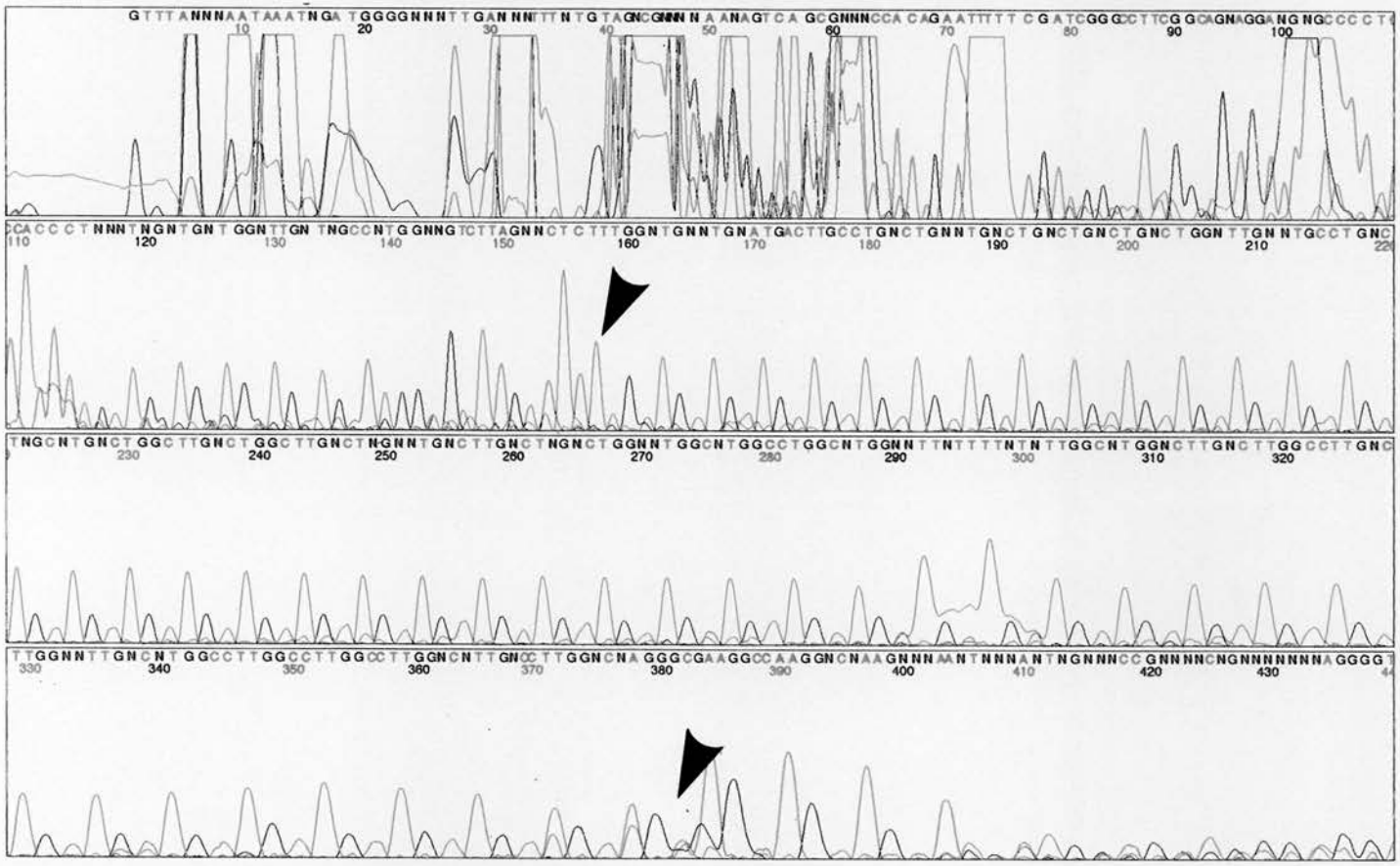

Agarose gel of exon 1 PCR product containing the glutamine repeat coding region. Lane 1: 22 repeats; lane 2: 22 repeats (patient 5); lane 3: 46 repeats (patient 11); lane 4: 20 repeats; lane 5: 25 repeats; lane 6: 18 repeats; lane 7: 20 repeats; lane 8: pGEM DNA markers: pGEM digested separately with *Sin I*, *Hinf I* & *Rsa* I. Sizes of the visible bands are: 2645, 1605, 1198, 676, 517, 460, 396, 350 bp. The no DNA control contained no bands (not shown in photograph). 10 µl completed reaction mix added to each lane.

**Figure 4.13a Expansion of glutamine repeat of exon 1**



Chromatogram data files from patient 5 (this page) and patient 11 (Fig 4.13b overleaf). Patient 5 has 23 repeats, patient 11 has 46 (indicated by arrows). Sequencing performed on antisense strand; note that the base calls are inaccurate due to the nature of the repeat. The signal of the chromatogram is easily read and the CAG triplets can be easily seen.

**Figure 4.13b Expansion of glutamine repeat of exon 1**



Chromatogram data files from patient 5 (Fig. 4.13a previous page) and patient 11 (this page). Patient 5 has 23 repeats, patient 11 has 46 (indicated by arrows). Sequencing performed on antisense strand; note that the base calls are inaccurate due to the nature of the repeat. The signal of the chromatogram is easily read and the CAG triplets can be easily seen.

**Figure 4.14 Comparison of amino acid substitution and phenotype at position 855**

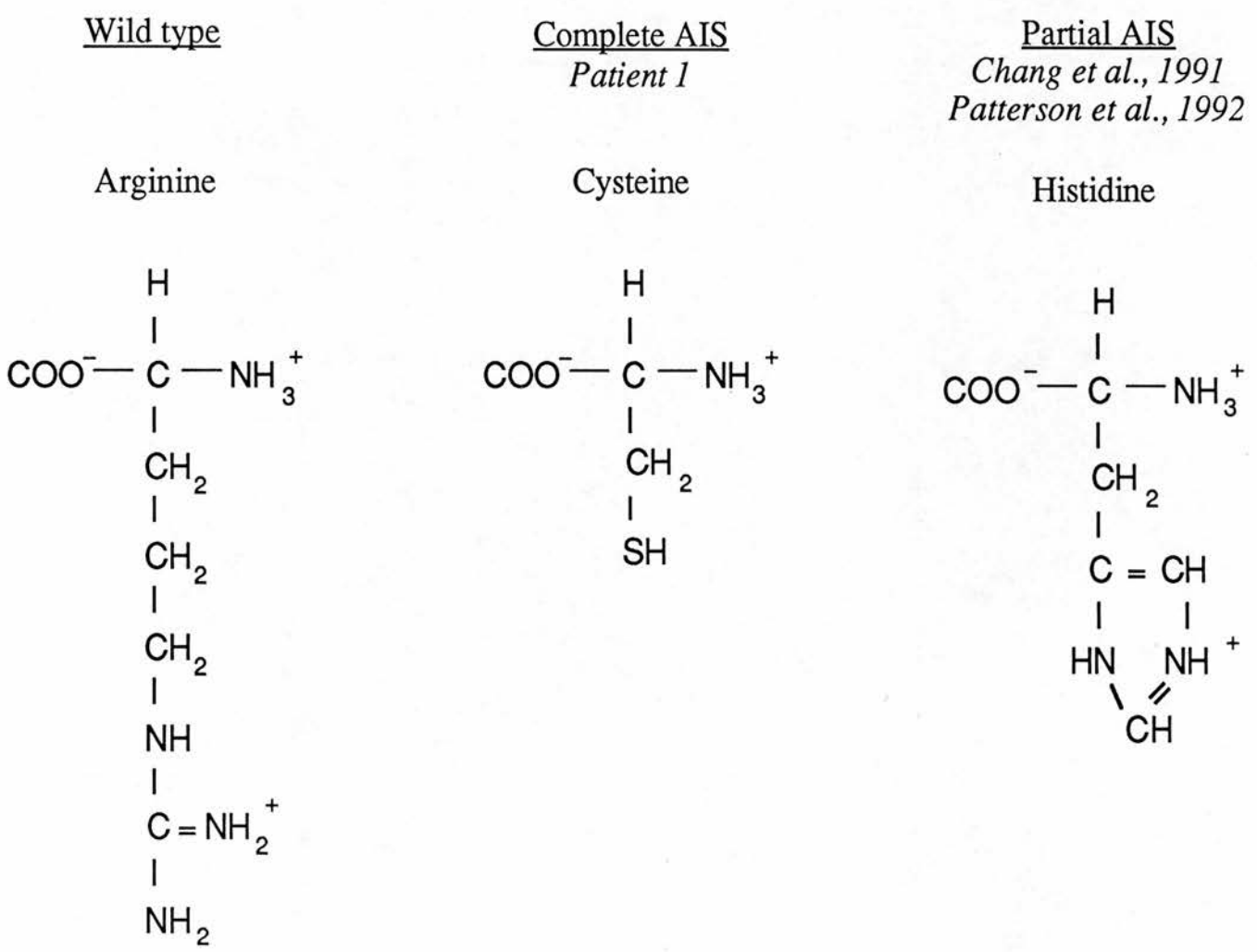
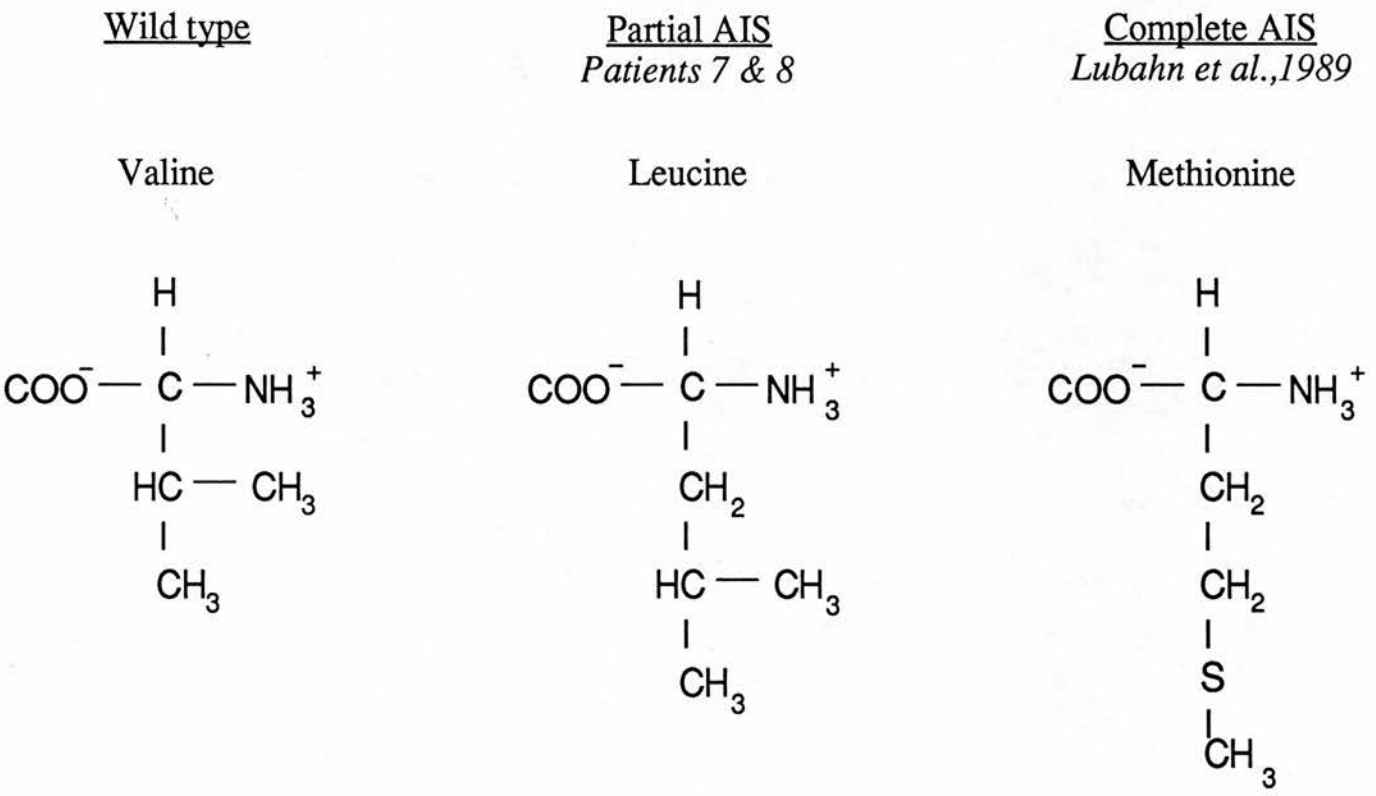
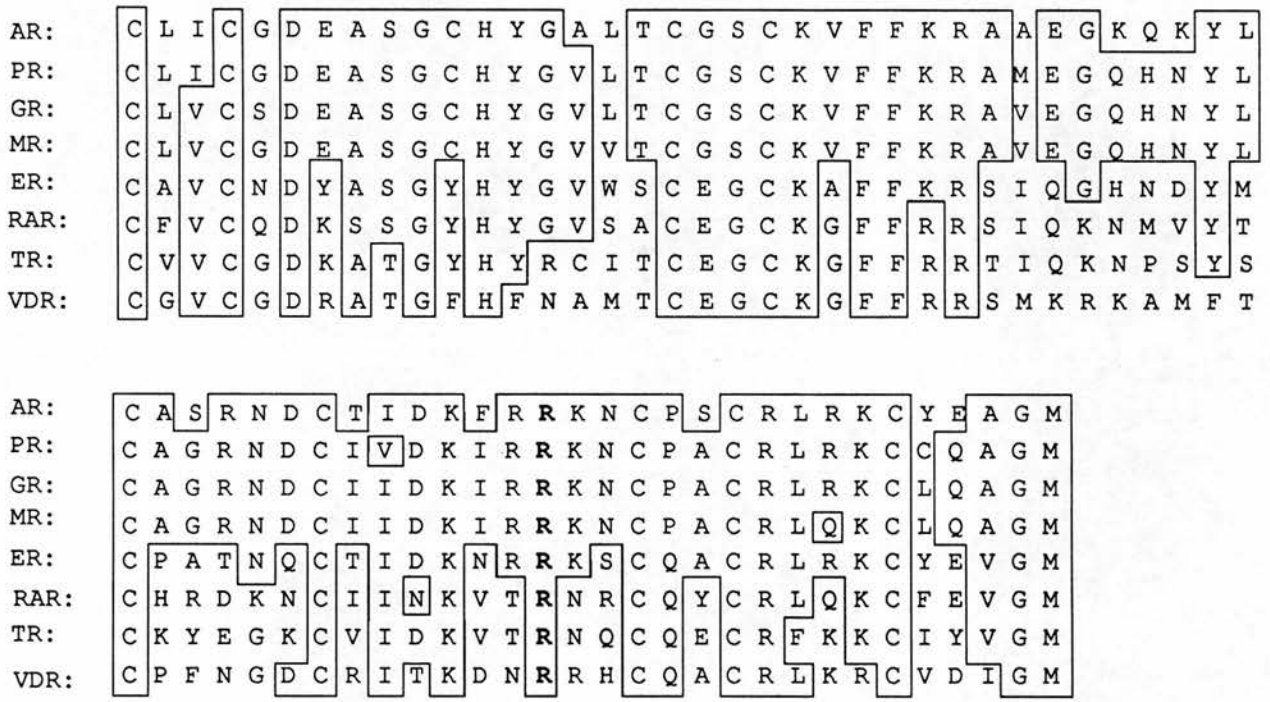


**Figure 4.15 Comparison of amino acid substitution and phenotype at position 866**



## Figure 4.16 Sequence homology of the zinc fingers of the steroid receptor family




Zinc finger sequences aligned by homology. Homologous amino acids are boxed. The first zinc finger is in the upper panel, the second in the lower. Arginine 608 and equivalents are shown in bold type. Amino acids are represented by IUPAC codes (Appx. 2). AR = androgen receptor; PR = progesterone; GR = glucocorticoid; MR = mineralocorticoid; ER = oestrogen; RAR = retinoic acid; TR = thyroid hormone; VDR= vitamin D. Based on Freedman, 1992.

**Figure 4.17 Consensus splice site sequence compared to the 5' splice site of intron 5 of the AR gene**

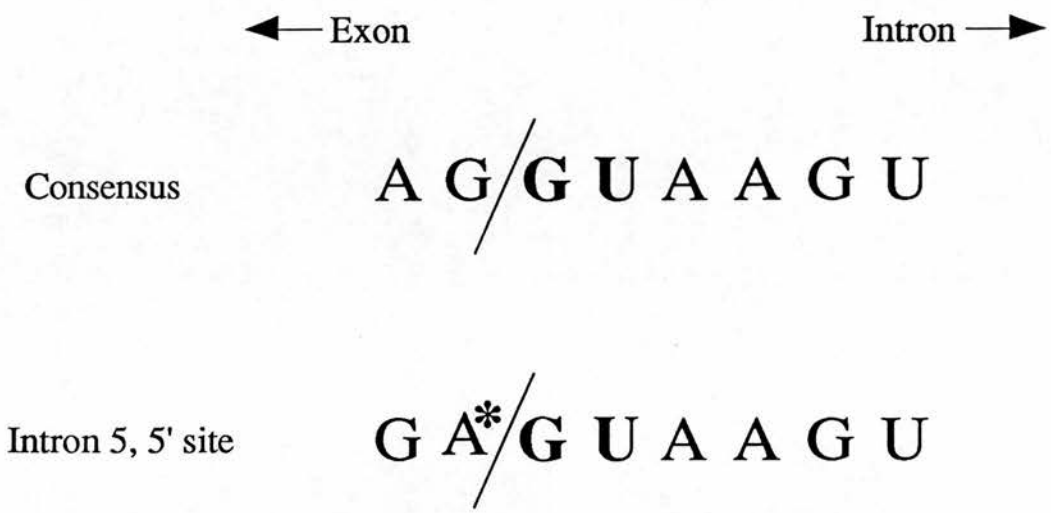
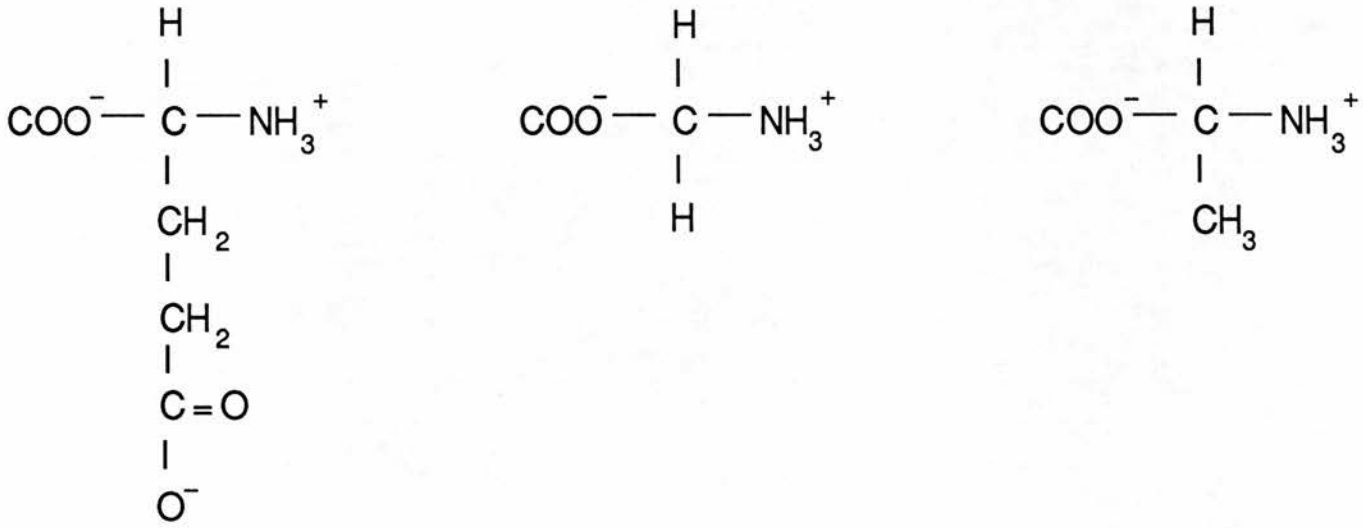



Figure comparing the consensus splice site sequence with the sequence of the 5' splice site of intron 5. The GU dinucleotide in **bold** type is the invariant donor site; the base marked with an asterisk (*) is the mutated base in patient 10 (A to G).

**Figure 4.18 Comparison of amino acid substitution and phenotype at position 772**

| Wild type | Partial AIS<br>*Patient 10* | Partial AIS<br>*Pinsky et al.,1992* |
|---|---|---|
| Glutamate | Glycine | Alanine |



## Table 4.1 Hormonal and other parameters from patients 7 to 15

| Patient | Testosterone | LH | FSH | SHBG | Oestradiol | Sperm concentration | Prolactin | Bmax | Kd |
|---|---|---|---|---|---|---|---|---|---|
| | *nmol/l* | *IU/l* | *IU/l* | *nmol/l* | *pmol/l* | *million/ml* | *mU/ml* | *mg/fmol protein* | *nmol/l* |
| Normal range | 12 to 35 | <6.0 | <6.0 | 25 - 30 | < 200 (male) | > 20 | 60-390 | 34 ± 2.6 | 0.27 ± 0.056 |
| 7 (GHg) | 90.0 | >32.0 | >32.0 | N/A | 211.0 | No ejaculate | N/A | Fibroblasts failed to grow | |
| 8 (AC) | 62.3 | 24.1 | 16.1 | N/A | 223.0 | 0.0 | N/A | Fibroblasts failed to grow | |
| 9 (JS) | 9.7 | 48.5 | 63.4 | 17.0 | 15.7 | No ejaculate | 167.0 | 52.8 | 0.2 |
| 10 (JM) | 80.0 | 14.7 | 1.3 | 68.0 | 152.5 | 0.0 | 86.0 | 26.8-55.0 * | 0.23-0.85* |
| 11 (WF) | 58.5 | 8.5 | 5.8 | 127.0 | 148.0 | N/A | 276.0 | N/A | N/A |
| 12 (JL) | 3.8 | 9.2 | 43.5 | 47.5 | 137.0 | 0.0 | 252.0 | N/A | N/A |
| 13 (KW) | 6.6 | 1.7 | 3.2 | N/A | N/A | N/A | N/A | N/A | N/A |
| 14 (GHd) | 0.9 | 1.7 | 27.2 | N/A | <67.0 | No ejaculate | N/A | N/A | N/A |
| 15 (RG) | 24.8 | 10.9 | 17.4 | N/A | N/A | 0.6 | N/A | N/A | N/A |

N/A = not available or not measured

* Bmax and Kd for this patient variable. Half life of the receptor/ligand complex was measured (Table 4.5).

**Table 4.2 Analysis of samples from patients with complete AIS**

| Patient | Exon 1 | | | | Exon | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A1A2* | *A3A4* | *A5A6* | *A7A10* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| 1 (S-AR) | 20 | N | | | n | n | N | N | N | ***d*** | n |
| 2 (AC) | | N | | | n | n | N | N | N | ***d*** | n |
| 3 (CG) | 25 | N | N | | N | N | N | ***D*** | N | N | N |
| 4 (JR) | | N | | N | n | n | N | N? | N | n | n |
| 5 (LI) | 23 | N | N | | N | N | N | N | N | N | N |
| 6 (I) | | N | | | n | n? | N? | N | N | n | n |

N=normal sequence; N?=one or more uncertain base calls in exon.; ***D*** or ***d***=defect detected (Table 4.6). Exon 1 segments defined by primers used (Chapter 3, Table 3.1). Column A1A2 gives the number of CAG (glutamine) repeats in the homopolymeric region of that segment.

The mutation in patient 1 was identified by Dr T. Padaychi, although the sequence work was completed by myself.

Exons recorded in lower case letters were sequenced manually; the other exons were sequenced fluorescently. All the regions of exon 1 apart from A1A2 were confirmed as having normal sequence by DGGE analysis (see Chapter 5).

**Table 4.3 Analysis of samples from patients with partial AIS and features of androgen resistance**

| Patient | Exon 1 | | | | Exon | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A1A2* | *A3A4* | *A5A6* | *A7A10* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| 7 (GH) | 26 | | | | n | n | N | N | N | ***d*** | n |
| 8 (AC) | 22 | | | | n | n | N | N | N | ***d*** | n |
| 9 (JS) | 24 | N | | | n | ***d*** | N | N | N | n | n |
| 10 (JM) | 26 | N | N | N | N | N | N | ***D*** | N | N | N |
| 11 (WF) | ***46*** | N | N | | N | N | N | N | N | N | N |
| 12 (JL) | | N | N | | n | n | N | N | N | n | n |
| 13 (KW) | | | | | n | n | N? | N | N | n | n |
| 14 (SH) | 24 | | | | N | N | N? | N | N | N | N |
| 15 (RG) | 21 | N | N | | N | N | N | N | N | N | N |

N=normal sequence; N?=one or more uncertain base calls in exon.; ***D*** or ***d***=defect detected (Table 4.6). Exon 1 segments defined by primers used (Chapter 3, Table 3.1). Column A1A2 gives the number of CAG (glutamine) repeats in the homopolymeric region of that segment.

Patients 7 to 10 were diagnosed as partial AIS or Reifenstein's syndrome. The remainder had some features of androgen resistance (see section 4.2).

The mutations in patients 7, 8 and 9 were identified by Dr T. Padaychi, but the complete sequence was obtained by myself.

Exons recorded in lower case letters were sequenced manually; the other exons were sequenced fluorescently. All the regions of exon 1 apart from A1A2 were confirmed as having normal sequence by DGGE analysis (see Chapter 5).

**Table 4.4 Nuclear translocation assay from patient 9**

| | Specific binding of radioactivity (%) | | | | |
|---|---|---|---|---|---|
| | Patient 2 | | Control | | p |
| Nuclei | 27.13 | (1.75) | 36.48 | (4.05) | <0.001 |
| Cytosol | 47.40 | (4.25) | 35.78 | (6.67) | <0.05 |

Numbers are the mean (SD) of four experiments. p values, calculated using Student's t test, are of the significance of difference between patient and control in each line of the table.

Note: non-specific binding did not exceed 10% of total binding in whole cells and was negligible in isolated nuclei. Recovery of nuclei was 40-50%.

Data from M.B. Hodgins, University of Glasgow.

**Table 4.5 Half-life determination for GSF of patient 10**

| | | | Half-life (min) |
|---|---|---|---|
| Patient 10 | Exp. 1 | i: | 18 |
| | | ii: | 144 |
| | Exp. 2 | | 100 |
| Control | | | 160 |

Note biphasic response in patient. 70% of complexes dissociated at 60 min.

Data from M.B. Hodgins, University of Glasgow.

# Chapter 5. Screening for androgen receptor gene mutations

## 5.1. Introduction

### 5.1.1. Aims of screening

The spectrum of androgen insensitivity has been reported to include patients with isolated abnormalities of virilisation and genital development in addition to those with testicular feminisation (complete AIS) and partial insensitivity syndrome (partial AIS). Larrea *et al.* (1978) identified a family with gynaecomastia in isolation which appeared to follow an X-linked pattern of inheritance and more recently a family with gynaecomastia, small genitalia yet with normal fertility were described (Grino *et al.*, 1988). When the androgen receptor (AR) expressed in genital skin fibroblasts (GSF) from these patients was analysed, subtle abnormalities of androgen binding were detected. It has been suggested on the basis of several similar GSF receptor studies that a mild form of androgen resistance may be responsible for idiopathic infertility (Aiman *et al.*, 1979) and that the incidence in the infertile population could be as high as 40% (Aiman & Griffin, 1982) although other groups have doubted the magnitude of this figure (Eil *et al.*, 1985; Morrow *et al.*, 1987). The data from individual patients in these studies are convincing even if the conclusions regarding incidence are less so, and it therefore seems possible that mild abnormalities of androgen action could be responsible for infertility in a subset of patients.

In the light of these data, the findings of Akin *et al.*, (1991) were unexpected. They analysed the AR gene in seven men with azoospermia who all had serum levels of testosterone and gonadotrophins within the normal range and found that it was not possible to amplify exon 4 of the AR gene of one of them by PCR using the primers described by Lubahn *et al.* (1989). The presence of a deletion of exon 4 was confirmed by Southern analysis of digested DNA using AR specific probes (Akin *et al.*, 1991). It is surprising that a deletion of this exon apparently has no effect upon the action of androgen in all other tissues of this patient since he had normal endocrine parameters of androgen feedback. The finding of one in seven azoospermic men carrying a deletion of the AR gene is almost certainly not a true estimate of the frequency of such lesions.

It was decided to investigate the frequency of AR gene mutations in the infertile population by obtaining blood samples from a selected group of men from the patients attending a busy infertility clinic. The frequency of mutations of the AR gene in this

group was expected to be low and in order to facilitate and accelerate the search for mutations in a number of patient samples I established a screening method.

### 5.1.2. Available techniques for screening

The sequencing of the AR gene from a large number of patients is time consuming and laborious. Since in male patients there is only one copy of the gene, screening techniques for gene mutations should be readily applicable to search for changes in the AR gene. There are three methods of screening currently in use: single stranded conformational polymorphism analysis, denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis.

#### 5.1.2.i Single stranded conformational polymorphism

Single strand conformational polymorphism analysis (SSCP) exploits the presence of a unique sequence in a fragment of single stranded DNA (Orita *et al.*, 1989). Briefly, PCR reactions are performed in the presence of $^{32}$P-dATP to radiolabel the amplified DNA, samples are denatured by heating and immediately loaded onto a non-denaturing polyacrylamide gel and subjected to electrophoresis. The position of the bands is visualised by autoradiography. The sensitivity of this method depends upon the formation of bonds between nucleotides within the single strand of DNA, which produces a characteristic conformation of that strand and thus a specific mobility through the gel. A change in sequence will alter the configuration of the double stranded regions of each strand and hence alter the mobility of it through the gel. The presence of a mutation is indicated by radiolabelled bands appearing on the autoradiograph at positions different to that of control samples. The ability of the method to detect mutations in any given sequence is quoted as being 80-90% (Hayashi, 1992), and to achieve this two gel runs have to be performed in different conditions. SCCP can be performed equally well with unlabelled DNA samples, when silver or ethidium bromide staining is used to visualise the sample bands.

#### 5.1.2.ii Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a method of screening which requires no radio-isotopes and has a sensitivity of 95% or greater (Myers *et al.*, 1987). Double stranded DNA undergoes denaturation in the presence of an elevated temperature or a high concentration of denaturant (usually urea and formamide). With increasing concentration of denaturant, strand separation initially occurs in a reversible fashion producing a partially single stranded molecule, termed a "Y-form". The concentration of denaturant at which the Y-form is generated ($D_m$) differs for each DNA molecule or fragment and is a function of its sequence. As denaturant

concentration is raised beyond the $D_m$ of a molecule irreversible strand separation will occur.

DGGE exploits this characteristic of DNA. DNA samples are run through a polyacrylamide gel which contains a linearly increasing gradient of denaturants (urea and formamide) aligned parallel to the direction of electrophoresis. As each sample reaches the point in the gel where denaturant concentration is equal to its $D_m$ it separates into the Y-form. The Y-form has an extremely low mobility through the gel and the sample is retarded at the position corresponding to the $D_m$. Thus, samples with different sequences (and therefore a different $D_m$) will reach different positions in the gel. Samples showing differential mobility from controls can be sequenced to determine the exact mutation.

The appearance of the Y-form depends upon the presence of subdomains within the DNA which separate (or "melt") at different concentrations. The retention of some double stranded DNA is essential to produce the Y-form with its low mobility. If there are any mutations within the region which remains double stranded they will not be detected. In order to improve the detection of such mutations, the addition of a GC-clamp has been recommended (Myers *et al.*, 1985a & b; Sheffield *et al.*, 1989). The GC-clamp is a 40 base pair sequence of guanosine and cytosine nucleotides which can be added to the 5' end of one oligonucleotide used to amplify the samples by PCR. The clamp remains double stranded throughout the range of denaturant concentration used in the gels and so the whole length of the sample of interest becomes single stranded, revealing any sequence changes within it. By the use of the GC-clamp over 95% of possible mutations present in a length of DNA can be detected (Sheffield *et al.*, 1989).

#### 5.1.2.iii Temperature gradient gel electrophoresis

Temperature gradient gel electrophoresis (TGGE) is a method essentially similar to DGGE (Riesner *et al.*, 1989) which also exploits the characteristic melting properties of DNA. The denaturing environment is provided by means of exposing the gel (of a fixed concentration of urea and formamide) to a linearly increasing temperature gradient. The effect of temperature and denaturant gradients are entirely equivalent (Myers *et al.*, 1987) and the two techniques provide exactly similar information. The temperature at which strand separation occurs is termed $T_m$. TGGE should be methodologically easier to perform since there is no requirement for pouring gradient gels and the gel run time can be reduced.

In both TGGE and DGGE samples can be mixed with an equal amount of control DNA in order to enhance the visibility of the altered mobility of the mutant samples (De Bellis *et al.*, 1992). After denaturing and reannealing, if a sample contained a mutation there would be two homodimers with matched sequences (the normal control and the mutated strand) and two heterodimers with a mismatched base due to annealing of non-complementary strands (Fig. 5.1). Such a mixture produces four bands on the gel because each has a different melting temperature and the heterodimers denature much sooner than the homodimers.

On consideration of the three methods available it was decided to establish a TGGE system for screening of AR gene samples because it did not involve the use of radio-isotopes or the pouring of gradient gels. However, due to various technical problems (see below) the alternative DGGE system was also attempted and became the method of choice.

## 5.2. Patients

Patients were selected from a busy infertility/andrology clinic. The records from each couple have been stored on computer since 1978 (Hargreave & Elton, 1986) and data were available from 1,700 couples. Follow up information was available on all couples. Criteria for selection, in an effort to identify those men with features suggestive of mild androgen resistance, comprised testosterone > 30 nmol/l, FSH > 6 IU/l, LH > 6 IU/l and a sperm concentration < 20 million/ml semen. When applied to the clinic population, these criteria selected a group of 50 men, 11 of whom wished no further follow up and 32 of whom failed to respond to three standard letters inviting them to attend. A final group of 7 men was successfully recruited and blood was sampled from them. Thus, less than 1% of the total clinic population were studied. Relevant details of the patients involved in the study are shown in Table 5.1. Patient 7 was particularly interesting because he had a brother who was infertile with azoospermia, and this suggested some inherited problem which may have been responsible for their infertility.

## 5.3. Methods

### 5.3.1. Temperature gradient gel electrophoresis

The equipment for TGGE was obtained from Diagen GmbH, Hamburg, Germany (via Hybaid UK Ltd., Teddington, Middlesex, UK) and comprised an aluminium plate with circulation tubes at each end to which waterbaths set to the limit temperatures

were connected. The plate could be arranged such that the temperature gradient was either parallel or perpendicular to the direction of electrophoresis. The gel was laid horizontally on the plate and temperature equilibration allowed to occur over 30 minutes. The plate, the buffer tanks and the gel were supported within a perspex cabinet.

Gels were of 8% acrylamide (acrylamide: bis-acrylamide 37.5:1), 20 mM MOPS (4-morpholinopropanesulphonic acid), 8 M urea, 1 mM EDTA and 2% glycerol. Polymerisation was initiated by the addition of TEMED and 10% ammonium persulphate and the gels allowed to set for 1 hour. GC clamped exons from patients were mixed 1:1 with clamped exons from DNA of normal fertile men and added to 1 volume of denaturing buffer (400 mM MOPS, 8 M urea, 10 mM EDTA), denatured by boiling for 5 minutes and allowed to re-anneal at 50°C for 15 minutes before loading to the gel. After electrophoresis gels were stained by silver staining as follows: gels were washed twice in 10% ethanol/0.5% acetic acid (v/v) for 3 minutes, stained in 0.1% $AgNO_3$, (w/v in distilled water) for 10 minutes and then rinsed twice in distilled water. The stain was developed by washing in 1.5% NaOH, 0.01% $NaBH_3$ (w/v), 0.15% formaldehyde (v/v) for 20 minutes and then fixed in 0.75% $NaCO_3$ (w/v) for 10 minutes. All manipulations were carried out in a large glass dish on a rocker plate.

For each exon of the AR gene PCR amplification was carried out with the addition of the 40 base pair GC clamp to the 5' primer, and amplified DNA purified as described in Chapter 3 (sections 3.2 to 3.4). A preliminary gel was run with the temperature gradient (typically 20°C - 60°C) applied perpendicularly to the direction of electrophoresis, with the DNA added in a long slot across the top of the gel, and electrophoresed for 2.5 hours at 300 V. The melting properties of each sample were shown as a curve of melting behaviour against temperature after staining (Fig. 5.2). A temperature bracket embracing the melting range of the fragment (the steep part of the curve) was calculated from this curve and used as the limits of the temperature gradient for subsequent gels. These gels were run with the temperature gradient and electric field in the same orientation. In this parallel orientation, samples containing a mismatch or mutation compared to the control DNA migrated as three or four distinct bands on staining (Fig. 5.3). Exons from these patients were then re-amplified by PCR without the GC clamp and sequenced (Chapter 3, sections 3.3 & 3.5).

### 5.3.2. Denaturing gradient gel electrophoresis

Denaturing gradient gels were run on the same apparatus used for TGGE but at a constant temperature of 60°C at 75 V for 16 to 20 hours, and stained as above. 100% denaturant was 7 M urea with 40% formamide and gradient limits for the different exons were as described by De Bellis *et al.* (1992). Typical limits were 35% and 60%. Gels were poured using a 40 ml capacity gradient maker by the addition of the lower concentration mix to the higher with constant stirring after the addition of 20 µl TEMED and 50 µl 10% ammonium persulphate to each mixture. Samples were run on the gels without mixing and annealing with control samples.

## 5.4. Results

Initial attempts to establish the TGGE system were performed using samples known to contain mutations from those patients described in Chapter 4. Perpendicular gels to establish the temperature gradient conditions for each exon were performed without difficulty (Fig. 5.2). Parallel gels were run with exon 7 samples to confirm that the method was sensitive and reproducible (Fig. 5.3). Samples from the patients with mutations in exon 7 showed the expected pattern of four bands produced by mixing control and mutant samples before loading to the gel.

However, repeat runs of the same samples and gels of other exons proved very troublesome with a smear of DNA rather than defined bands being visualised. Modification of the electrophoresis voltage or duration of annealing time made no significant difference to the reproducibility of the gel runs. A switch to DGGE was therefore made with an immediate improvement in resolution and reproducibility of the gel data (Fig. 5.4). In view of the greater reliability of DGGE, all of the data from the infertile patients in this chapter were obtained using this method rather than TGGE.

The results of screening of AR gene exons from the infertile patients by DGGE are shown in Table 5.2. All exons of the gene from 2 to 8 were amplified in all patients and found to be normal. Exon 1 proved difficult to amplify in many of the patients, especially across the glutamine and glycine repeat coding regions. The glycine repeat region of exon 1 did not amplify from the DNA of any patient, and the glutamine repeat region failed to amplify in patient 2. Of the exons of the gene examined by DGGE no samples with altered mobility were detected. The glutamine repeat region of exon 1 (bases 250-312) showed variation in size between patients from 20 to 25 CAG codons which is within the normal range for this region (La Spada *et al.*, 1991).

## 5.5. Discussion

In this chapter I have described the establishment of a screening technique for the androgen receptor gene. Initially TGGE was used because it appeared to be the most straightforward to set up, and there was no need to pour gradient gels. The technique was claimed to be rapid and sensitive. However, after early success with control samples known to contain mutations, the method became unreliable and inconsistent to use. The difficulties stemmed from two sources: the annealing procedure and also from the method of controlling the temperature.

The annealing procedure for samples was intended to enhance the ease of detection of mutations by creating four hybrid molecules in each sample. However, this step proved troublesome, producing partially annealed or single stranded samples resulting in smears of DNA on the gel, obscuring any defined bands and rendering interpretation of the data impossible; single stranded DNA provides no information on TGGE. Adjustments to the length and temperature of the annealing step were made in an effort to encourage complete reannealing but with no improvement in the results of the gels.

The maintenance of the stable linear temperature gradient is of critical importance to the success of TGGE. Even minor fluctuations in the temperature at either end of the gel plate will disturb the gradient and thereby cause the position of $T_m$ of any sample to move slightly in the gel. If this occurs it will result in a broad, smeared band in the region of the $T_m$ rather than a sharp one. The relative positions of the bands cannot then be interpreted with any confidence. The temperature control of the cold end of the apparatus was troublesome in particular since it tended to rise with increased run times. With the water baths available this situation could not be overcome and the decision was taken to switch to denaturing gradient gels.

The use of denaturing gradient gels and DGGE was very successful. Because the methods are equivalent the same primers and samples could be used for DGGE as were used for TGGE. De Bellis *et al.* (1992) have used this system successfully for examining the AR gene of patients with androgen insensitivity. We adopted the same gradient parameters as these investigators but did not routinely anneal samples with control DNA in the light of previous difficulties with this step. With the use of samples with a GC clamp, DGGE is sensitive enough to detect at least 95% of mutations present in a given strand without the need to undertake annealing (Sheffield *et al.*, 1989) and this was considered sufficient for the study. The use of gradient gels

completely eliminated the problems associated with fluctuations in gradient limits seen with the TGGE and the technique was easily and reproducibly performed.

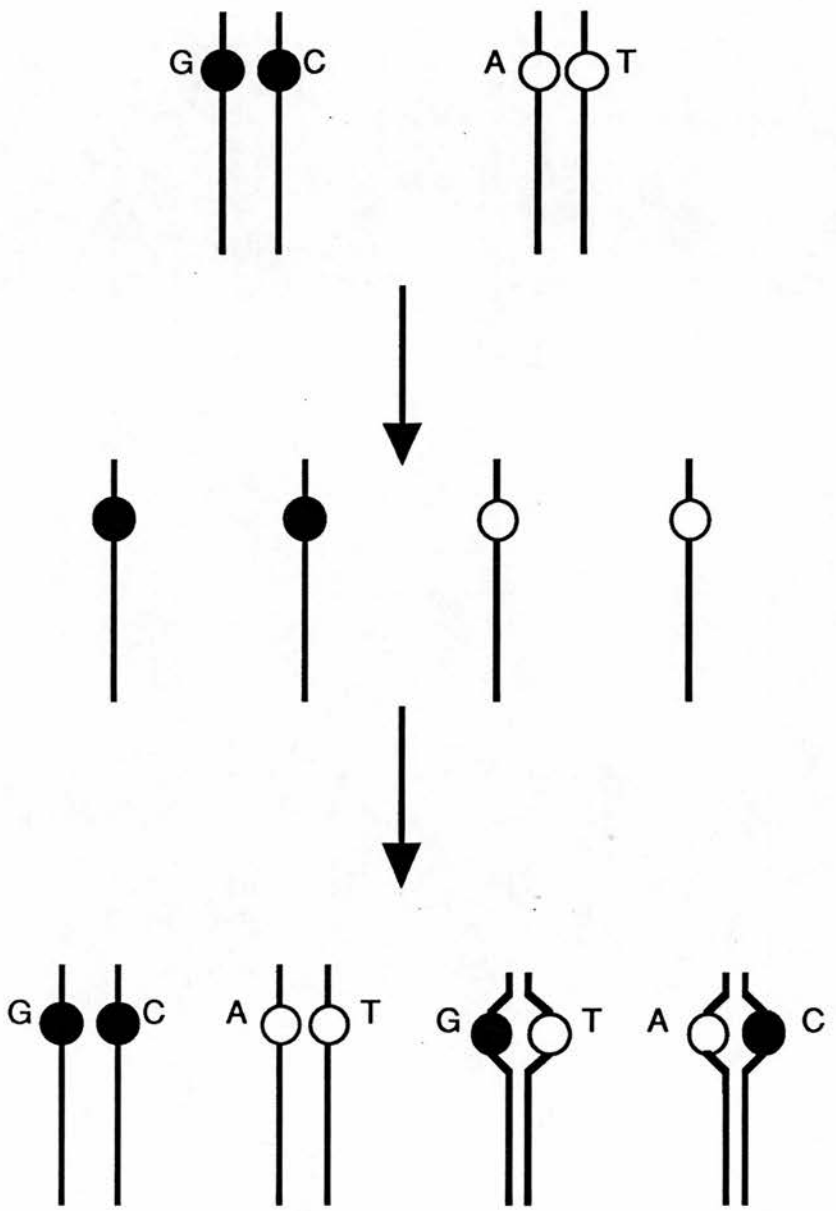
The results from the analysis of the 7 patients with infertility are shown in Table 5.2. No mutations were detected in any of these patients and the glutamine repeat region was of normal size in each. This cohort of men is small, but has been selected on the basis of there being an identifiable disturbance of androgen feedback. The criteria of elevated testosterone and LH with low sperm density are comparable to the biochemical findings of patients with recognised androgen insensitivity associated with impaired receptor function. The LH-testosterone product (LH-T) is used as an index of androgen resistance (Morrow *et al.*, 1987) and in all of our patients was elevated well above the normal range (26-97) (Table 5.1). This is similar to the results of Morrow *et al.* (1987) who found the LH-T elevated in all their cohort of infertile men.

Using these criteria we have selected the 3% of 1,700 men attending an infertility clinic most at risk of an abnormality of androgen receptor function on the basis of their endocrine profile. From the seven responders in this cohort, none possessed a mutation and that they all presumably carry a normal, functional androgen receptor. This finding appears to contradict the results of Aiman & Griffin (1982) and Morrow *et al.* (1987) who found in independent studies that between 20% and 40% of a sample of infertile men had levels of androgen receptors in GSF lower than normal controls. However, the presence of a normal androgen receptor gene does not necessarily lead to the expression of normal levels of receptor. Aiman & Griffin (1982) found that the $B_{max}$ of the infertile men with severe oligospermia (less than 1 million/ml semen) was significantly lower than in controls while in less severe oligospermia there was no difference, but they only reported the mean result for each group. Morrow *et al.* (1987) found low levels of receptor in only 5 men from a sample of 21 with idiopathic infertility, but reported the results on each patient which indicated that the range of $B_{max}$ was somewhat wider in the infertile group, although the mean values were not significantly different. It is difficult to interpret the results of Aiman & Griffin in the absence of the individual results from each patient, particularly in the light of the findings of Eil *et al.* (1985) who examined the GSF androgen binding sites and nuclear uptake of androgen in a group of 15 infertile and oligospermic men. They found no difference in the mean values or the range between patients and controls, although the infertile group had more men with results at the low end of the range. Thus, one can say that infertile, oligospermic men fall into a group

who tend to express lower levels of normal androgen receptor than other men, but it is not possible to say whether this is the cause of their low sperm count and fertility. Oligospermic men may represent merely the bottom end of the normal range of fertility for which there is no definable, correctable cause.

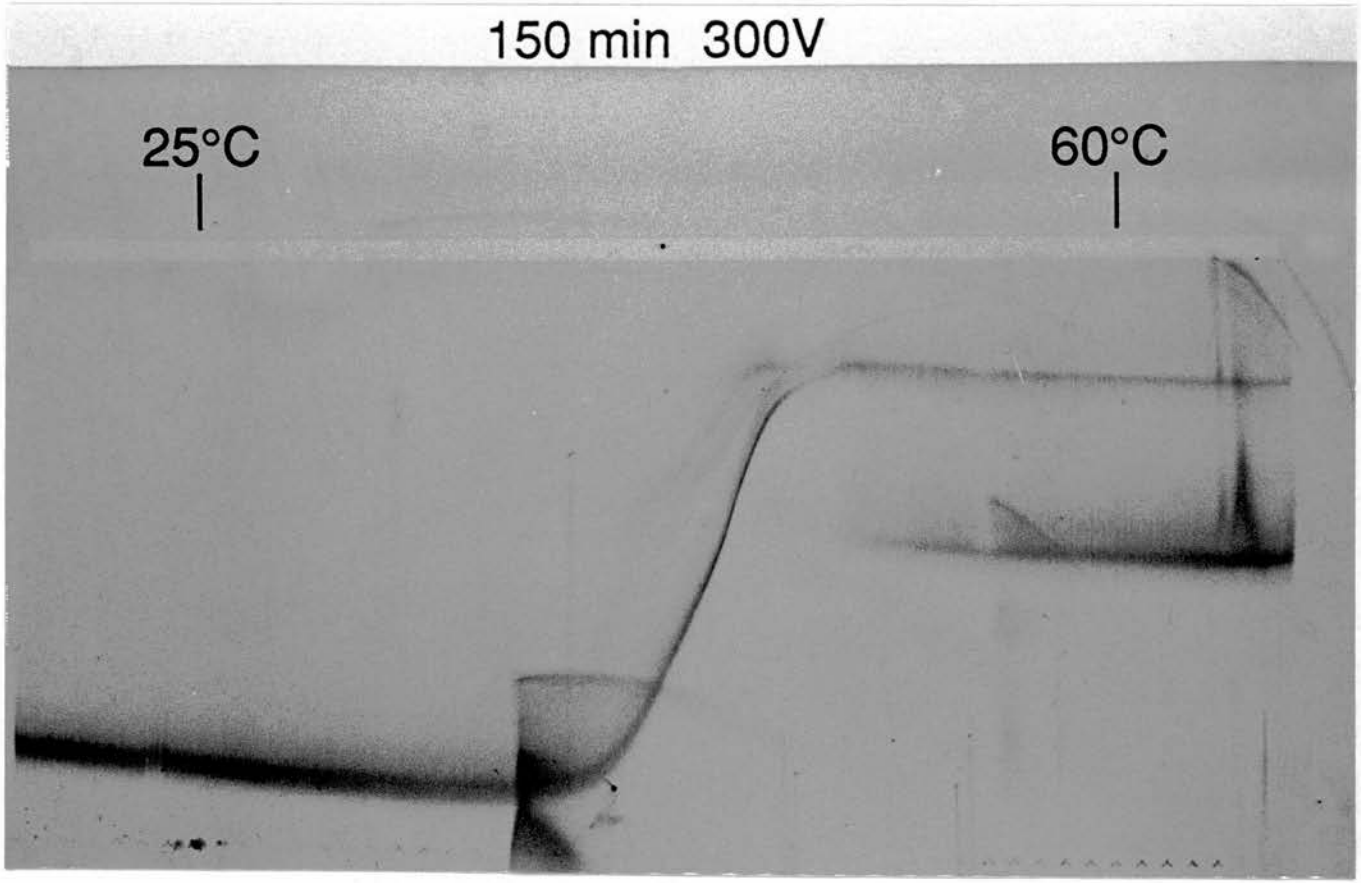
However, the findings of Akin *et al.* (1991) appear to contradict the assumption that AR mutations in infertile men will have any endocrine markers at all. Of seven azoospermic men with normal endocrine profiles, they found a deletion of exon 4 in one of them. It is highly surprising that such a gross deletion appears to have no effect upon the endocrine feedback of the hypothalamic pituitary axis (particularly when exon 4 carries the hormone independent nuclear localisation signal (Simental *et al.*, 1991)) and implies that the selection of patients with endocrine features of such perturbation may be somewhat misguided. None of the patients in this study were azoospermic and perhaps a screening programme aimed at a large number of azoospermic men would be more fruitful.

**Figure 5.1 Diagram illustrating heterodimer formation**



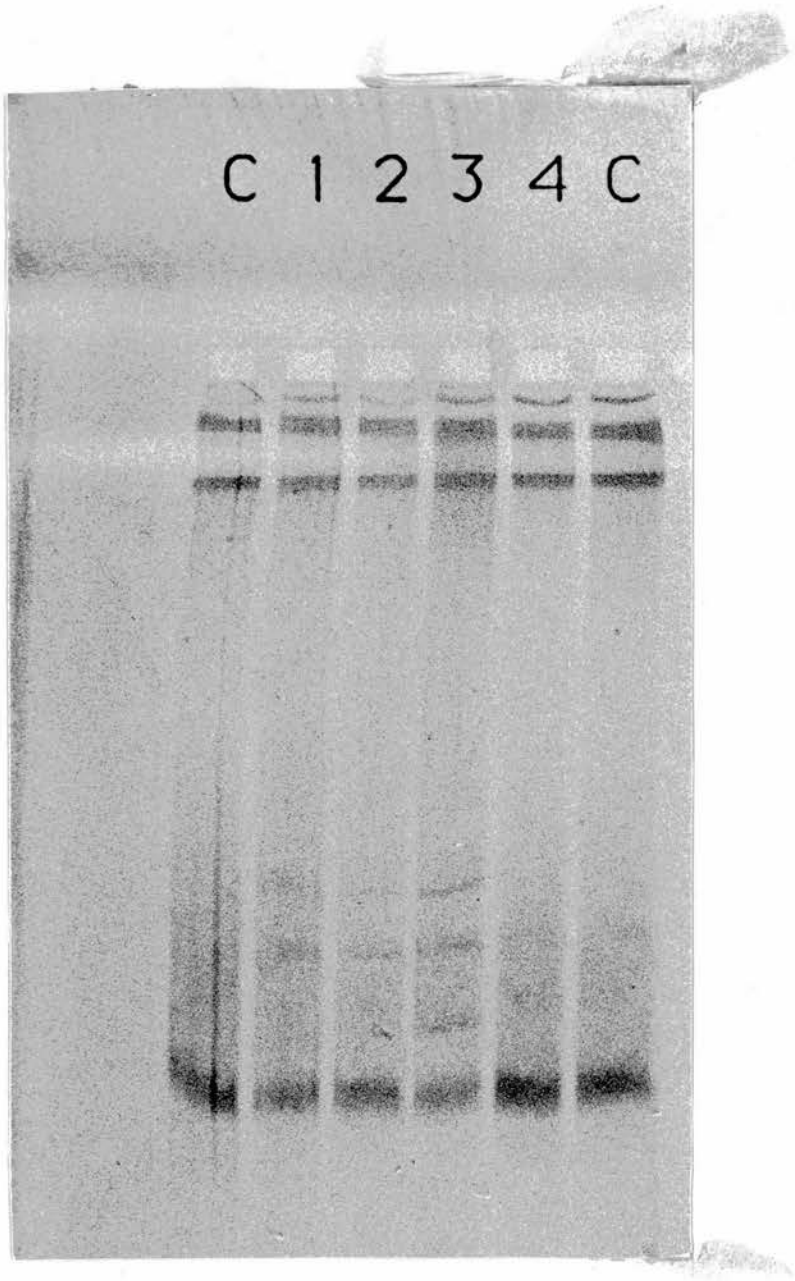

Annealing of separated strands from two samples with a single base substitution produces two homodimers and two heterodimers. Pairing bases are shown in the same shading.

**Figure 5.2 Perpendicular TGGE gel**



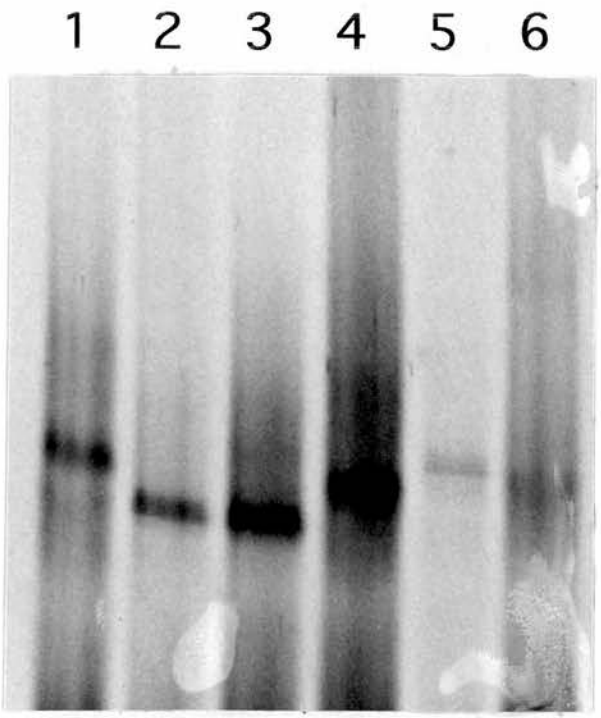

Photograph of perpendicular gel of exon 2. The steep part of the curve represents the partially denatured "Y-form of the DNA, and the limits of the curve correspond to the temperature limits to be used in a parallel gel.

**Figure 5.3 Parallel TGGE of exon 7**




Photograph of parallel gel of exon 7. C: control, normal DNA from a fertile man; lane 1: patient 1 (C to T mutation); lane 2: patient 7 (G to T mutation); lane 3: patient 8 (G to T mutation); lane 4: patient 4 (no mutation). Electrophoresis was performed for 4.5 hours at 300 V.

**Figure 5.4 Parallel DGGE of exon 5**




Photograph of DGGE gel of exon 5. Lanes 1 and 6: control DNA; lanes 2 & 3: patient 10 (A to G mutation); lane 4: control DNA; lane 5: patient 3 (C to T mutation). Electrophoresis was performed for 16 hours at 75 V. The C to T mutation results in a lower $D_m$ and a sooner retardation in the gel than control, while the A to G mutation results in a higher $D_m$ and a later retardation.

**Table 5.1 Hormonal profile of infertile patients**

| Patient | Testosterone (nmol/l) | LH (U/l) | FSH (U/l) | LHxT | Sperm (Million/ml) | Comment |
|---|---|---|---|---|---|---|
| | 12 to 35 | <6.0 | <6.0 | 26-97* | >20 | |
| 1 | 32.60 | 14.20 | 24.70 | 462.92 | 0.50 | |
| 2 | 33.00 | 12.60 | 24.80 | 415.80 | 33.00 | Testicular maldescent |
| 3 | 49.40 | 8.10 | 2.80 | 400.14 | 9.00 | Diabetes mellitus |
| 4 | 31.10 | 9.10 | 10.80 | 283.01 | 0.15 | |
| 5 | 41.70 | 8.40 | N/A | 350.28 | 8.02 | |
| 6 | 36.30 | 11.00 | N/A | 399.30 | 4.00 | |
| 7 | 43.20 | 6.90 | 17.50 | 298.08 | 0.00 | Azoospermic brother |
| Mean | 38.19 | 10.04 | 16.12 | 326.19 | 6.83 | |

LHxT = LH testosterone product; N/A = not available.

*Morrow *et al.*, 1987

**Table 5.2 Screening of AR gene from infertile men**

| Patient | Exon 1 | | | | Exon | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A1A2* | *A3A4* | *A5A6* | *A7A10* | *2* | *3* | *4* | *5* | *6* | *7* | *8* |
| 1 | 25 | N | N | | N | N | N | N | N | N | N |
| 2 | | N | N | | N | N | N | N | N | N | N |
| 3 | 27 | N | N | | N | N | N | N | N | N | N |
| 4 | 24 | N | N | | N | N | N | N | N | N | N |
| 5 | 26 | N | N | | N | N | N | N | N | N | N |
| 6 | 24 | N | N | | N | N | N | N | N | N | N |
| 7 | 25 | N | N | | N | N | N | N | N | N | N |

N=normal sequence. Exon 1 segments defined by primers used (Chapter 3, Table 3.1). Column A1A2 gives the number of CAG repeats (coding for glutamine) in the homopolymeric region of that segment.

# Chapter 6. Site-directed mutagenesis

## 6.1. Introduction

Since the initial discovery of mutations within the androgen receptor (AR) gene of patients with clinical features of androgen resistance (Lubahn *et al.*, 1989; Wilson, 1992), confirmation that these changes alone are responsible for the *in vivo* features of the syndrome has been sought by a variety of *in vitro* investigations. A number of workers have reproduced point mutations within cloned AR cDNAs by site-directed mutagenesis and have examined the ability of the proteins synthesized from the modified cDNA to bind androgen (Marcelli *et al.*, 1990a, Ris-Stalpers *et al.*, 1991) and to activate gene transcription (Brown *et al.*, 1990) after introducing the cDNA into a suitable cell line host.

Recombinant plasmids bearing the androgen receptor gene in a highly expressed form have been used in *in vitro* transfection assays of normal receptor function within CV-1 cells (Quarmby *et al.*, 1990) and COS cells (Jenster *et al.*, 1991). The plasmid AR can be mutated by a variety of means and the androgen binding parameters compared to controls after transfection into these cell lines (Marcelli *et al.*, 1990b; Ris-Stalpers *et al.*, 1990). However, measurement of normal hormone binding does not indicate a normally functioning receptor, since mutations of the DNA binding domain would not be expected to show abnormal hormone binding. Co-transfection of an androgen responsive reporter plasmid, such as one carrying the chloramphenicol acetyl transferase gene (CAT), under the control of the mouse mammary tumour virus (MMTV) promoter region to act as an androgen responsive HRE, allows the transactivating ability of mutant receptors to be measured directly (Brown *et al.*, 1990).

In order to examine the effect of the mutations which were identified in the patients with androgen insensitivity (Chapter 4), and to confirm that they altered receptor function, an attempt was made to mutate the AR cDNA supplied in a eukaryotic expression vector. The technique described by Deng & Nickoloff (1992) was employed as this allows the mutation of cDNA to be performed within any plasmid which possesses a unique restriction endonuclease site. Two oligonucleotides are required in the mutagenesis step: one to introduce the desired mutation and the second to eliminate a unique restriction site in the vector (Fig. 6.1). The plasmid is denatured and the two oligonucleotides are simultaneously allowed to anneal to one

strand. The annealed primers are used for elongation to synthesize a pool of "mutant" plasmid using the original as template which is then transformed into *E. coli* defective in mismatch repair. Transformed bacteria are pooled, cultured and the plasmid DNA extracted and digested with the restriction enzyme whose site should have been eliminated from the plasmid by the specific oligonucleotide. The digested plasmid DNA is retransformed into bacteria and should result in a high selection of the mutant plasmid since the original unmutated plasmid is linearised and will therefore be transformed with approximately 100-fold less efficiency than the circular (mutated) plasmid DNA.

## 6.2. Methods

### 6.2.1. Selection of plasmid and oligonucleotide primers

The AR cDNA containing plasmid, pSV.ARo, was a gift from Dr Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands) and contained a full length cDNA of the human androgen receptor gene subcloned into a eukaryotic expression vector where its transcription was under the control of the SV 40 early promoter region and origin of replication, and terminated by the polyadenylation signal of the rabbit β-globin gene. Selection in prokaryotes was achieved using the β-lactamase gene for ampicillin resistance (Fig. 6.2). The sequence of the plasmid was not available but a unique *Sfi I* site was identified within the SV40 origin of replication. The sequences of this selection primer and of three mutagenic primers (designed to mutate specific single bases within the AR cDNA, as identified from the patients in Chapter 4) are given in Table 6.1. Primers were synthesized on an Applied Biosystems "PCR-mate" DNA synthesizer, model 391 (Applied Biosystems Ltd., Warrington, UK) as described in Chapter 3 (section 3.2).

### 6.2.2. Phosphorylation of primers

Before use, the primers were phosphorylated at their 5' ends so as to be suitable templates for the action of DNA ligase. Primer DNA (1 μg) was mixed with 2 μl 10x kinase buffer (500 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$, 50 mM dithiothreitol (DTT), 10 mM ATP), 10 units of T4 polynucleotide kinase (Promega Ltd., Southampton, UK) and made up to 20 μl with sterile distilled water. The mixture was incubated at 37°C for 1 hour before terminating the reaction by heating to 65°C for 10 minutes to inactivate the enzyme. Phosphorylated primers were stored at -20°C until required.

### 6.2.3. Mutagenesis of plasmid

The protocol provided with the Transformer™ site-directed mutagenesis kit (Clontech Laboratories Inc., from Cambridge Bioscience) was followed. Briefly, 0.1 µg purified plasmid DNA was mixed with 0.1 µg phosphorylated selection primer, 0.1 µg phosphorylated mutagenic primer, 2 µl 10x annealing buffer (200 mM Tris.HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl) and made up to 20 µl with sterile distilled water. The DNA was denatured at 100°C for 5 minutes and then plunged onto ice and incubated for 5 minutes at 0°C to allow the primers to anneal. To each tube was added 2 units of T4 DNA polymerase (an enzyme lacking strand displacement activity (Masumune & Richardson, 1971; Nossal, 1974)), 2 units of T4 DNA ligase, 3 µl 10x synthesis buffer ("optimised concentrations" of nucleotides in 200 mM Tris.HCl pH 7.5; Clontech Laboratories Inc.) and sterile distilled water to a final volume of 30 µl and the mixture incubated at 37°C for 4 hours. Following incubation, the samples were heated to 65°C for 5 minutes to inactivate the enzymes.

### 6.2.4. Transformation of bacteria

A specialised strain of *E. coli*, BMH 71-18 *mut S*, was used for transformation. This strain is defective in mismatch repair (Wallace *et al.*, 1981) and is used to prevent repair of the mutant strand. For electroporation, the entire purified DNA mixture from each mutagenesis reaction was diluted fivefold and 1 µl (1 ng) used per 40 µl aliquot of cells. Transformation and selection of transformed cells was performed in liquid culture (Chapter 3, sections 3.6 to 3.8).

### 6.2.5. Digestion of plasmid DNA

The overnight culture of transformed bacteria was harvested and the plasmid DNA recovered using a Promega Magic Miniprep kit (Promega Ltd., Southampton) (Chapter 3, section 3.7.2). Purified plasmid DNA (500 ng) was mixed with 10 units of *Sfi I* and 2 µl of 10x restriction buffer (60 mM Tris.HCl pH 7.5, 60 mM $MgCl_2$, 500 mM NaCl, 10 mM DTT) (Promega Ltd.) in a final volume of 30 µl and incubated at 50°C for 3 hours, before the addition of another 10 units of enzyme and additional incubation for 3 hours. The enzyme was inactivated by heating for 5 minutes to 70°C, before removing the enzyme and reaction buffer by filtration through a Chromaspin TE-100 column (Clontech Laboratories Inc., via Cambridge Bioscience, Cambridge). This purified, digested DNA (3 ng) was used in the second transformation step; following electroporation and recovery (Chapter 3, section 3.8), the cells were centrifuged at 3,000 rpm for 5 minutes, resuspended in 100 µl of LB-broth and spread over an LB-agarose plate containing ampicillin for selection. The plate was incubated at 37°C overnight.

### 6.2.6. Selection and screening of transformants

The plasmid containing the AR cDNA possessed no colour selection system and therefore, in each experiment, transformation of a control plasmid, pUC19M DNA (provided in kit), was performed in parallel. This plasmid, derived from the wild type pUC19, contains a mutation within the coding sequence of the *lac Z* gene which introduces a termination codon. Mutagenesis of this plasmid using the provided primers restores the functional *lac Z* gene to allow positive colonies of bacteria to be identified by their blue colour when grown on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-thiogalactopyranoside (IPTG). Following mutagenesis and digestion of the control plasmid, bacteria transformed with pUC19M were mixed with 40 µl of X-gal (20 mg/ml) and 10 µl of IPTG (20 mM) (Promega Ltd.) immediately before plating out. The ratio of blue colonies to the total number of colonies on each plate was used to calculate the efficiency of the mutagenesis procedure.

Colonies of bacteria from the mutated pSV.ARo plates were inoculated into liquid cultures and plasmid DNA recovered the following day by Promega Magic Minipreps (Chapter 3, section 3.7.2). Following agarose gel electrophoresis of the DNA to confirm the presence of intact circular plasmid DNA, PCR amplification of the mutated region of the plasmid was performed for each sample and the PCR product purified and sequenced (Chapter 3, section 3.5). The primers used to amplify the relevant portions of the AR cDNA are given in Table 6.2.

## 6.3. Results

Initially mutagenesis was attempted at three sites in the AR cDNA: guanosine to adenosine at nucleotide 1843 (arginine 608 to lysine); cytosine to thymidine at nucleotide 2265 (arginine 752 to STOP), and cytosine to thymidine at nucleotide 2574 (arginine 855 to cysteine). The protocol provided with the Transformer™ kit was followed for the first two experiments and mutagenesis was performed with the control experiment in parallel in every case. In each experiment the control rate of mutagenesis appeared satisfactory but in every case the selected colonies from the mutated pSV.ARo plates yielded wild type plasmid after sequencing (Table 6.3, Exp. 1 & 2).

In an effort to improve the success rate of the mutagenesis, modifications of the basic method were introduced. Initially, the restriction digestion incubation was increased to 8 hours to ensure complete digestion of the unmutated parent strand and

thus avoid a high rate of transformation with wild type plasmid. There was no improvement in the yield of mutant DNA following this step (Table 6.3, Exp. 3).

An additional Chromaspin column purification step was introduced following synthesis of the mutant strand and before the initial transformation to minimise the transfer of partially synthesized DNA, enzymes and possible contaminants which may have interfered with the transformation efficiency. Once again, there was no improvement in the outcome (Table 6.3, Exp. 4)

Finally, the mutagenesis was attempted with the modification that the DNA polymerase and DNA ligase were added sequentially rather than simultaneously with incubation at 37 °C between each addition. This was done because of the large size of the plasmid used (7.2 kilobases) in an effort to encourage strand synthesis to be completed before ligation occurred and prevent the possible synthesis of truncated plasmids which were confounding the results of transformation. Following the addition of 2 units of T4 DNA polymerase and incubation at 37°C for 2 hours, a further 2 units of T4 DNA polymerase were added together with 2 units of T4 DNA ligase and incubation continued for a further 2 hours. Finally, 2 units of T4 DNA ligase were added and a final 2 hour incubation at 37°C carried out before heating to 65°C for 5 minutes to inactivate the enzymes. Unfortunately, this modified protocol was no more successful than the others (Table 6.3, Exp.5).

A summary of the results of the mutagenesis experiments is presented in Table 6.3.

## 6.4. Discussion

Methods for site-directed mutagenesis of plasmid DNA which have been developed during the past 15 years utilise a variety of techniques to achieve the desired goal of the insertion of mutant bases into the DNA. All of the available methods make use of single stranded plasmid DNA as a template, either for synthesis of a mutant strand from a specifically designed oligonucleotide primer annealed to the strand (Zoller & Smith, 1983), or as a template for nick-translation in the presence of modified nucleotides (Müller *et al.*, 1978). These methods are hampered by the continued presence of the unmutated strand in the final DNA sample and the presence of *in vivo* repair mechanisms which result in low yields of the desired mutant DNA (Kramer *et al.*, 1984; Dohet *et al.*, 1985). Subsequently, modifications have been introduced in

an effort to improve the yield of mutant DNA from these procedures and thereby reduce the need to screen large numbers of samples to find mutated plasmid.

Kunkel (1985) published a method in which he claimed that the yield of mutated DNA was increased to the order of 60-80%. Single stranded DNA was prepared using a bacteriophage grown in an *E. coli dut⁻ ung⁻* strain. This strain lacks the gene for the enzyme dUTPase (*dut*) and therefore contains an increased intracellular pool of dUTP (uracil triphosphate) which is incorporated into newly synthesized DNA in competition with dTTP (thymidine triphosphate). This DNA is biologically active in this strain which also lacks the gene for the enzyme uracil glycolase (*ung*). In cells containing this enzyme, the uracil residues are hydrolysed leaving abasic sites which are lethal in single stranded DNA. The uracil containing plasmid is used as a template in combination with an oligonucleotide mutagenic primer and the newly synthesized DNA transfected into an *ung⁺* host bacteria. The template strand is hydrolysed resulting in selection of mutant DNA which is then amplified. The ability of this method to select the mutant strand and lyse the template allows the selection of mutant DNA without having to resort to phenotypic selection since the percentage of mutant DNA in the surviving bacteria is high (Kunkel, 1985).

An alternative method where phosphorothioate analogues (i.e. containing a sulphur species) of nucleotides were incorporated during synthesis of the mutant strand was described by Taylor *et al.* (1985a & b). The phosphorothioate bonds between nucleotides are resistant to digestion by many restriction enzymes (Taylor *et al.*, 1985a) which therefore can be used to produce a "nicked" form of the double stranded plasmid with the digested bonds all present in the template strand. Digestion with exonuclease III removes the nicked native strand, allowing resynthesis of the complementary mutant strand (Taylor *et al.*, 1985b). A third approach involves synthesizing the mutant strand from the template in the presence of 5-methyl-dCTP (Vandeyar *et al.*, 1988). The presence of methylated cytosine within the restriction sites of certain restriction enzymes results in the site being nicked in the non-methylated strand rather than being completely cleaved. Subsequent exonuclease III digestion removes the native strand in a manner similar to the technique of Taylor *et al.*, (1985b).

All of the methods described above rely upon the synthesis of single stranded DNA for use as a template before the start of the procedure and are therefore time consuming to perform. The method chosen for use during the current study was that of Deng & Nickoloff (1992) which had the advantage of being flexible and rapid to

perform, requiring only two overnight bacterial cultures and could be performed on any double stranded plasmid, with no need for helper phage rescue of single stranded DNA. It was therefore disappointing that we failed to detect any mutants in five experiments in spite of modifications to the method to take account of the large size of of the pSV.ARo plasmid and to ensure completion of each step of the procedure.

The fact that good numbers of colonies contained mutant plasmid in the positive control group indicates that there was no problem with the fundamentals of the method and good numbers of transformed colonies were obtained from the cells transformed with pSV.ARo. The problem appeared to be one of failure to incorporate the mutations into the vector. The possible reasons for the failure of the method include: incomplete linearisation of the parental plasmid before transformation, leading to high numbers of wild type transformants; synthesis of truncated plasmid DNA lacking a portion of the sequence, and some problem with the mutagenic primers themselves. In all experiments recovered plasmids were subjected to electrophoresis in agarose gels to confirm the presence of circular DNA of the appropriate size, and full length copies were present in all samples (data not shown). The adjustments made to the incubation time with the enzyme *Sfi I* were designed to overcome any problems with incomplete digestion of the parental DNA, as was the incorporation of an additional purification step. No improvement in the yield of mutants was observed with either of these steps.

The nature of the oligonucleotides chosen remains a likely cause for the failure of the method. The elimination oligonucleotide changes the *Sfi I* site from GGCCnnnnnGGCC to GACCnnnnnGGTC which is not recognised and both it and all the mutagenic primers were designed according to the guidelines suggested by Deng & Nickoloff (1992). The oligonucleotides were subjected to electrophoresis through a non-denaturant polyacrylamide gel which confirmed the presence of a species of the expected length in each case (data not shown). It may be possible that the phosphorylation step to prepare the oligonucleotides was not efficient, rendering them unable to provide substrate sites for the action of DNA polymerase once annealed to the template, but the experiments were performed with freshly phosphorylated primers once the potential problem had been recognised. It would be advisable to repeat the mutagenesis experiments using freshly made oligonucleotides containing different sequences and modifications of the duration of the reaction steps in order to optimise the procedure but unfortunately the constraints of time rendered this option impossible.

The plasmid pSV.ARo was a gift from Dr Albert Brinkmann and has been used in i*n vitro* investigation of mutations of the AR gene (Ris-Stalpers *et al.*, 1991, 1992). In these studies, mutagenesis was performed by ligating restriction endonuclease digestion fragments into the wild type cDNA. The fragments were obtained by digesting cDNA synthesized by reverse transcriptase PCR using RNA from genital skin fibroblasts from affected patients. This method is another time consuming approach, but it has generated useful data using the pSV.ARo as a template and vector.

In retrospect, the design of the mutagenesis strategy was probably seriously flawed. The only unique site which could be identified was the *Sfi I* site within the SV40 origin of replication. The mutagenesis changed two nucleotides within this region, and it is impossible to predict whether these mutations would affect the normal function of the region. An untested mutation within the region could have abolished two critical functions: the control of replication of the plasmid and the promoter function of the region on the expression of the AR gene in the host cell.

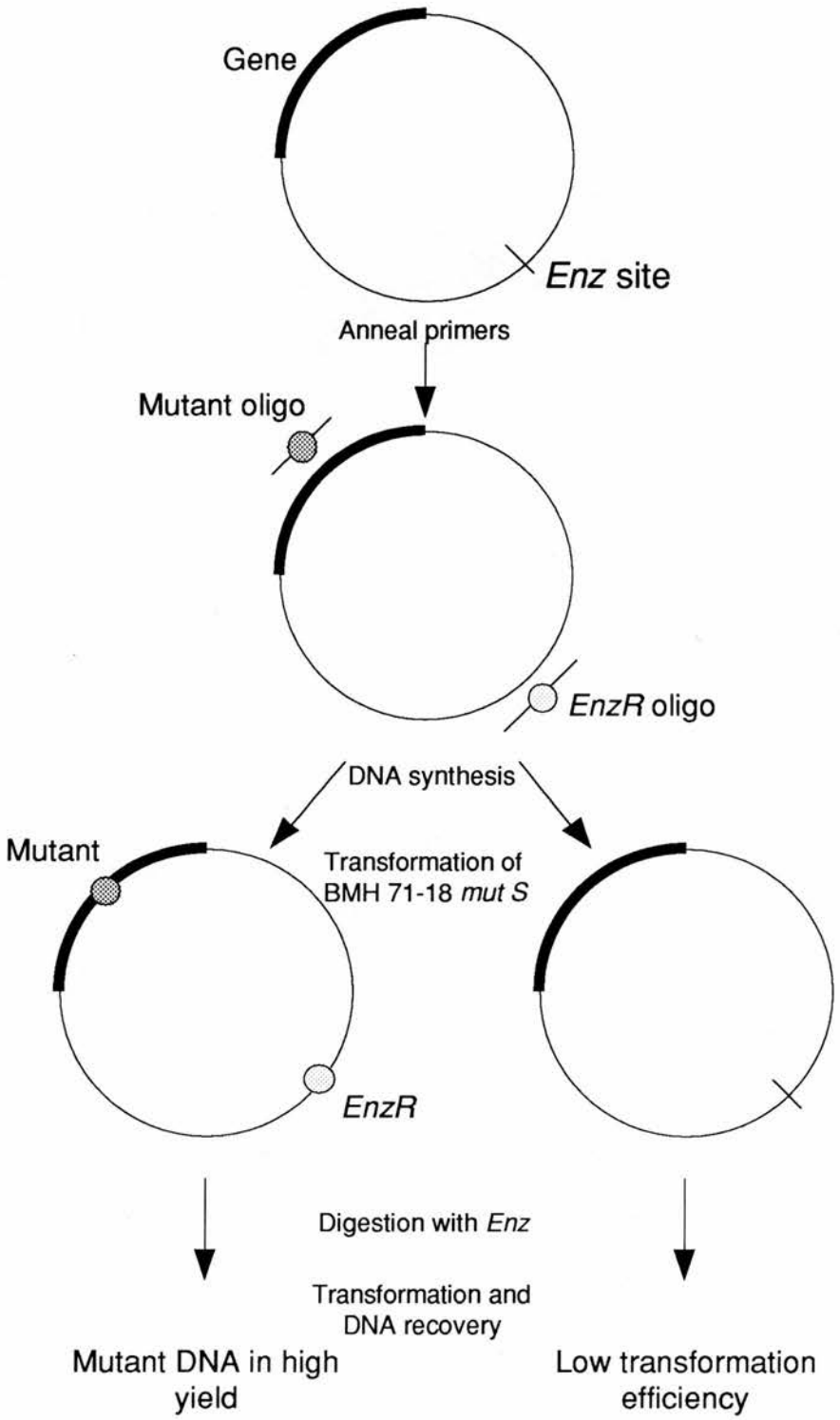
The aim of the experiments was to produce a plasmid capable of expressing mutant AR in a cell line *in vitro* (COS cells were to be used). If the mutant plasmid was defective in replication to high copy number in the host cell, or incapable of controlling expression of the mutant gene, the results of any transfection studies and measurements of mutant AR function would have been meaningless.

To overcome this problem, ideally another restriction site should have been chosen; this would have been facilitated if the sequence map of the whole plasmid had been available. An alternative would have been to have performed mutagenesis on the AR plasmid with the *Sfi I* mutation primer and a dummy primer within the AR gene sequence. Then the effect of mutation within the origin of replication could have been measured directly *in vitro* ; the control experiment would also have excluded any other effect of the mutagenesis procedure upon the remainder of the plasmid sequence.

The mutagenesis method chosen has been declared to be accurate and specific (Deng & Nickoloff, 1992). However, exposing a vector of such large size to these enzymes may introduce extraneous mutations elsewhere in the coding region of the AR gene. Had the necessary time been available, it would have been better to subclone the region of the AR to be mutated into another smaller vector for the actual mutagenesis. This would have allowed confirmation of the mutation by sequencing

more easily, and would probably have made the mutagenesis more successful because a smaller plasmid vector would have been used.

**Figure 6.1 Schematic representation of mutagenesis**




*EnzR* oligo eliminates restriction site for the enzyme *Enz*. Gene is any target gene. Mutant sites are indicated by bullets.

**Figure 6.2 Androgen receptor gene plasmid pSV.ARo**

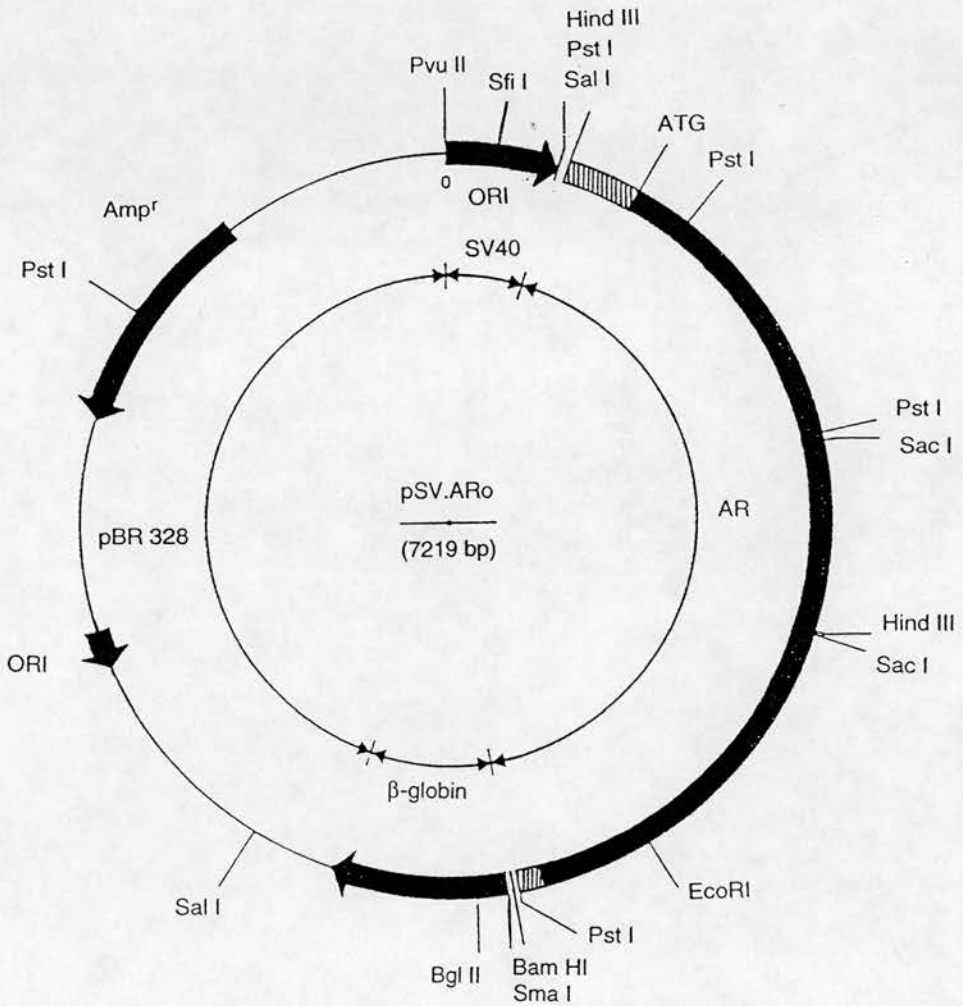



Diagram of pSV.ARo showing orientation of cDNA to the origin of replication and the polyadenylation signal. Some of the restriction sites are shown. Courtesy of Dr A.O. Brinkmann.

## Table 6.1 Mutagenic and selection primers

| Primer sequence | Mutation |
|---|---|
| 5'-GAGGCCGAG***A***CCGCCTCGG***T***CTCTGAGCTA-3' | Elimination of *SfiI* site |
| 5'-GATAAATTCCGAA***A***GAAAAATTGTCCATC-3' | Arg608 → Lys |
| 5'-GTTTGCCATGGGCTGG***T***GATCCTTCACCAATGTC-3' | Arg752 → STOP |
| 5'-CATCCTGCTCAAGA***T***GCTTCTACCAGCTC-3' | Arg855 → Cys |

The first oligonucleotide is the selection oligo. All others mutate bases within the coding sequence of the receptor. Bases mutated from wild type sequence shown in ***bold italic*** type.

## Table 6.2 Primers used to amplify androgen receptor cDNA

| Exon | Sequence | Strand |
|---|---|---|
| 3 | 5'- GGAAACAGAAGTACCTGTG -3' | 5' |
| | 5'- CTCCCAGAGTCATCCCTG -3' | 3' |
| 5 | 5'- GCTTCCGCAACTTACAC -3' | 5' |
| | 5'- TCATTGAAAACCAGATC -3' | 3' |
| 7 & 8* | 5'- TCTTTGATGAACTTCGAATGA -3' | 5' |
| | 5'- TCACTGGGTGTGGAAATA -3' | 3' |

All primers are exonic. *The 5' primer for exons 7 & 8 lies within the first third of exon 7. All other primers lie at the ends of the relevant exon.

## Table 6.3 Results of mutagenesis experiments

| Experiment | | | pUC19M controls | | | pSV.ARo | |
|---|---|---|---|---|---|---|---|
| | | | No. colonies | | Efficiency | | |
| No. | Mutation | Modification | Blue | Total | (%) | Colonies selected | Number mutated |
| 1 | Arg608/Lys | – | 13 | 19 | 68 | 1 of 1 | 0 |
| | Arg752/STOP | | | | | 7 of 18 | 0 |
| | Arg855/Cys | | | | | 6 of 10 | 0 |
| 2 | Arg608/Lys | – | 18 | 23 | 78 | 6 of 16 | 0 |
| | Arg752/STOP | | | | | 6 of 14 | 0 |
| | Arg855/Cys | | | | | 6 of 17 | 0 |
| 3 | Arg608/Lys | Increase digest time | 19 | 21 | 90 | 8 of 8 | 0 |
| | Arg752/STOP | | | | | 9 of 9 | 0 |
| | Arg855/Cys | | | | | 10 of 10 | 0 |
| 4 | Arg608/Lys | Additional purification | 24 | 29 | 83 | 4 of 4 | 0 |
| | Arg752/STOP | | | | | 6 of 6 | 0 |
| | Arg855/Cys | | | | | 2 of 2 | 0 |
| 5 | Arg608/Lys | Increased synthesis time | 15 | 17 | 88 | 6 of 6 | 0 |
| | Arg752/STOP | | | | | 7 of 9 | 0 |
| | Arg855/Cys | | | | | 7 of 10 | 0 |

Figures in the "colonies selected" column indicate the number of transformed colonies selected from the total number appearing on the agarose plate after final antibiotic selection.

# Chapter 7. General Discussion

## 7.1. Introductory remarks

In this thesis I have described the analysis of the androgen receptor (AR) gene sequence of patients with androgen insensitivity syndromes and from a cohort of infertile men. Two methods of screening the AR gene for mutations have been established and used successfully in the detection of point mutations and an effort has been made to examine the functional parameters of mutant androgen receptors. I shall discuss the relevance of these findings in the light of the data from other researchers and also indicate the future direction which the work might take.

## 7.2. Mutations in patients with AIS

The analysis of the AR gene has been carried out for six patients with complete AIS, four with partial AIS and five with features suggestive of androgen resistance. Following PCR amplification, mutations were detected in eight of the patients tested (Table 7.1). Point mutations were detected in three patients with complete AIS, all four with partial AIS who were tested and in one patient who appears to have Kennedy's disease. Since the cloning of the AR gene (Chang *et al.*, 1988), examination of the gene for the presence of mutations in patients with AIS has been undertaken by several groups of investigators and has resulted in a rapid increase in the available information about the effects of defects in this gene. Indeed, whilst the current study has been under way, many publications have appeared describing AR gene mutations. Some of the mutations detected in this study have also been described in patients investigated by other groups: arginine 752 to a termination signal; arginine 831 to a termination signal; arginine 855 to cysteine, and valine 866 to leucine (De Bellis *et al.*, 1992; McPhaul *et al.*, 1992; Pinsky *et al.*, 1992; Ris-Stalpers *et al.*, 1992).

The location of the hormone binding domain mutations identified in the patients in this study reinforces the observation of McPhaul *et al.* (1992) that 90% of mutations in this domain are clustered in two regions (residues 728 to 774 and 828 to 866 (Chapter 2, section 2.5.2.ii)), one of which is homologous to a region of the thyroid hormone receptor where most of the identified mutations lie (Refetoff *et al.*, 1993). This similarity indicates that these regions are probably critically important in the function of the receptor. Within these regions, the occurrence of the same mutations at

the same amino acid position in unrelated patients may indicate that the nucleotides of these codons are particularly susceptible to mutation. Three of the four mutations listed above are due to a cytosine to thymidine transition, believed to result from methylation and deamination of the pyrimidine as described by Youssoufian *et al.* (1986). Other mutations have been identified in more than one patient at positions throughout the gene: namely amino acids 695, 732, 749, 752, 762, 764, 774, 780, 831, 840, 855, 864 and 866 (Lubahn *et al.*, 1989; Brown *et al.*, 1990, 1992; Mebarki *et al.*, 1990; Chang *et al.*, 1991; De Bellis *et al.*, 1991, 1992; Marcelli *et al.*,1991b, 1992; Batch *et al.*, 1992; Evans, 1992; Jakubiczka *et al.*, 1992; McPhaul *et al.*, 1992; Pinsky *et al.*, 1992; Prior *et al.*, 1992; Ris-Stalpers *et al.*, 1991; Trifiro *et al.*, 1992b). Patients sharing the same mutation generally appear to express the same phenotype and GSF from them show the same functional abnormalities (but see below). All of the duplicate sites lie within the steroid binding domain of the receptor and this tendency for multiple mutations in the domain to manifest with clinical symptoms further supports the notion that this portion of the receptor is critical in normal androgen action. However, whilst mutations associated with symptoms of AIS have been found in other regions of the receptor (notably within the DNA binding domain, exons 2 and 3) there have been no reports to date of duplicate mutations in this region, a fact which is difficult to explain.

In addition to the presence of duplicate mutations, some amino acids have been found to be changed to different residues in different patients. Clinical information regarding the severity of AIS in these individuals and, where possible, examination of the function of the AR protein has added considerably to our understanding of the spatial and charge restraints and requirements of each critical amino acid (Chapter 4, section 4.5). For example, substitution of the normal arginine at position 855 for cysteine replaces a residue with a positively charged side chain by one with a small polar side chain (patient 1, Chapter 4; De Bellis *et al.*, 1992) and has been found to result in the expression of a complete AIS phenotype because the receptor appears unable to bind androgen (De Bellis *et al.*, 1992; McPhaul *et al.*, 1992). Alternatively, substitution of histidine at this site (with a charged side chain) results in a partial AIS phenotype (Chang *et al.*, 1991; Batch *et al.*, 1992) and a receptor with disturbed binding properties (Batch *et al.*, 1992; McPhaul *et al.*, 1992). Taken together, these findings would be consistent with a charge dependent configuration or interaction at this site which is critical for normal receptor function. Correlation of these data with the crystallographic analysis of the steroid receptors (Luisi *et al.*, 1991), together with the information on the structure/function relationships of the various subdomains of

the receptor, and their interaction with ligand and hormone response elements will add to our understanding of normal receptor function and provide a mechanism by which the mutations cause disease.

However, it would appear that the nature of the amino acid substitution is not the only factor which affects the function of the AR. For example, a mutation of arginine 855 to histidine was identified in a patient with partial AIS by several groups (Chang *et al.*, 1991; Batch *et al.*, 1992) but McPhaul *et al.* (1992) identified two patients with this mutation, one of whom had partial AIS and the other of whom had complete AIS. The GSF AR binding characteristics in each patient were the same. Similarly, McPhaul *et al.* (1992) found the mutation of valine 866 to methionine in a patient with partial AIS, while the same mutation in patients with complete AIS has been identified by Lubahn *et al.* (1989) and Pinsky *et al.* (1992). The conclusion from these findings must be that other factors act to modulate the action of the receptor.

Evidence for such factors has been provided by Bocquel *et al.* (1989) who found that *in vitro* assays of receptor function produced different results depending upon the type of host cell used. Some clinical studies have also provided clues to the presence of other cellular factors; Jukier *et al.* (1984) described a family of brothers with variable severity of undervirilisation where the most severely affected had 5 $\alpha$-reductase deficiency in association with an abnormality of the androgen receptor, while the less severely affected brother had normal 5 $\alpha$-reductase activity, but the same abnormal AR (patient 8, Chapter 4). The activity of the enzyme appeared to synergise with the receptor defect in this family. The presence of an AR gene mutation in spinal and bulbar muscular atrophy (Kennedy's disease) which appears to cause a specific effect upon the viability of spinal motor neurones provides circumstantial evidence for the presence of a cell specific interaction between the AR and some other intracellular factor (Chapter 2, section 2.4.2.iv). Whilst there is, therefore, evidence for interaction between the AR and other intracellular factors having an effect upon the action of the receptor in different target tissues which can modify the clinically apparent changes in this patient, elucidation of the precise nature of these interactions will require further study.

The three patients in the study with complete AIS where no mutations were detected are a perplexing group. Whilst the failure to detect mutations may be due in part to methodological difficulties, the possibility remains of alternative mechanisms for the causation of the complete AIS phenotype. As discussed (Chapter 4, section 4.5), rearrangements of the gene or mutations within the promoter regions, or

message translation abnormalities are all possibilities and deserve to be considered and examined experimentally. Such work was outside the scope of this study but would require an examination of the mechanics of the initiation of transcription and translation of the gene. The transcription initiation sites of the AR gene have only recently been identified (Faber *et al.*, 1991) in the region upstream of the gene. The promoter region lacks TATA/CCAAT boxes and has two separate sites for initiation (AR-TIS I and AR-TIS II). Preliminary data indicate that utilisation of the two sites is regulated differently: AR-TIS I is dependent upon sequences close to itself for activity, while AR-TIS II depends upon the presence of an upstream GC-box which contains an Sp1 binding site (Faber *et al.*, 1993). Mutations introduced into the site abolish transcription *in vitro*. These early findings indicate that mutations and other structural defects of the transcription sites and their regulators can affect AR gene transcription, and thus activity.

## 7.3. Infertile patients

The screening of a small cohort of infertile men for mutations of the AR gene was also undertaken during the course of this study. The study was hampered by difficulties in recruiting patients and in some degree by methodological difficulties. Patients were selected on the assumption that there would be endocrine markers of receptor dysfunction in terms of gonadotrophin profile and serum testosterone.

The seven men in this study were a subset of the 3% with oligospermia or azoospermia who showed elevated testosterone and gonadotrophins selected from a population of 1,700 men attending a local clinic. In this highly selected group no mutations were detected in exons 2 to 8 of the AR gene. Problems in amplification of GC-rich domains meant that the glycine repeat coding region of exon 1 was not completely sequenced but no evidence of the presence of defects was found in the remainder of exon 1. The current data would be consistent with the argument that mutations of the androgen receptor gene are not common causes of infertility. However, additional data from a population of azoospermic men with no endocrine abnormality would be useful in order to expand the study of Akin *et al.* (1991) who have published a paper in which they describe the detection of a deletion of exon 4 of the AR gene in one of seven azoospermic men with normal endocrine parameters. Their finding of a mutation in 1 of 7 men is almost certain to be partly due to fortunate sampling, otherwise a comparable size of sample in this study would have been expected to produce a positive result. This defect in association with normal hormonal parameters is in agreement with Aiman & Griffin (1982) who proposed that infertility

could be due to androgen resistance without endocrine markers. It is difficult to see how androgen resistance can be present with no perturbation of the pituitary-testicular axis unless the androgen receptor is defective in some testis specific function.

In considering the pathogenesis of infertility, the recent results of Ma *et al.* (1992) should also be borne in mind. They detected microdeletions in the Y chromosome of two azoospermic men, in the region of the azoospermia factor (AZF) (Simpson *et al.*, 1993). There are likely to be multiple aetiologies of non-obstructive azoospermia, and it may be that interaction between the androgen receptor, testis-specific factors and possibly the product of the AZF are all important for normal spermatogenesis.

## 7.4. Trinucleotide repeats

The finding of an amplification of the glutamine repeat region of exon 1 of the AR in patient 11 who was referred following routine endocrine screening is exciting. This patient appears to have a late onset form of X-linked spinal and bulbar muscular atrophy (SBMA) (Kennedy *et al.*, 1968; Arbizu *et al.*, 1983). The amplification of the CAG trinucleotide repeat has been shown to be associated with this disease in a large study (La Spada *et al.*, 1991). It has recently been shown that this repeat is variable and unstable in transmission during meiosis, and that the larger repeats tended to be associated with more severe disease of earlier onset (Biancalana *et al.*, 1992; La Spada *et al.*, 1992). Trinucleotide repeat amplifications appear to be responsible for a number of inherited diseases and provide a mechanism to explain some previously perplexing observations on the transmission of genetic disease, examples of which are given below.

Fragile X syndrome is an X-linked dominant disorder with reduced penetrance characterised by mental retardation (Fryns, 1989). Either sex may exhibit mental retardation when carrying the affected chromosome. It has recently been shown that a trinucleotide CGG repeat of thirty codons lies in the region of the fragile site of the X chromosome (Verkerk *et al.*, 1991). This repeat shows a variable increase in size in DNA from affected patients relative to control samples and appears to be within the coding region of a gene (FMR-1) which lies in this vicinity also.

Myotonic dystrophy is an autosomal dominant condition characterised by muscle wasting and myotonia in association with cardiac problems, cataracts, testicular atrophy and diabetes (Harper, 1989). The disease occurs in three forms of increasing

severity; often the age of onset falls and the severity of symptoms increases in successive generations of affected families (termed "anticipation") (Howeler *et al.*, 1989). A CTG trinucleotide repeat which is expanded in affected patients has been identified at the genetic locus for myotonic dystrophy (Brook *et al.*, 1992). In addition, the repeat sequence also increases in size through successive generations (Harley *et al.*, 1992) and the size of the repeat sequence correlates with disease severity and young age of onset (Redman *et al.*, 1992; Tsilfidis, *et al.*, 1992), giving a molecular basis for the phenomenon of anticipation.

The third disease recently attributed to trinucleotide repeat expansion is Huntington's disease (Huntington's Disease Collaborative Research Group, 1993). A gene has been identified which carries a CAG trinucleotide repeat which was expanded in all of 75 disease families studied. The size of the repeat was variable in normal patients. Thus far, the identity of the encoded protein is unknown, and the repeat lies within the coding region.

All these different diseases share the same underlying mechanism of an amplification of a trinucleotide repeat region but the mechanism of how they exert their effects is still unknown. In SBMA, the repeat lies within the coding region of the gene while in myotonic dystrophy, the repeat is not translated (Brook *et al.*, 1992). The repeat in fragile X syndrome lies within the 5' untranslated region of the gene FMR-1 (Verkerk *et al.*, 1991), but the site in the Huntington's disease gene may be in either situation (Huntington's Disease Collaborative Research Group, 1993). There is little evidence to indicate how the mutation is effective. It may be a spatial effect which causes disruption of the conformation of the three dimensional structure of the DNA, interfering with the activity of the gene or an adjacent gene, but the presence of a truncation of the repeat in the AR gene of a patient with partial AIS (McPhaul *et al.*, 1991) suggests that in this case the mechanism may be more subtle and involve some perturbation of function of the protein. The finding of an elargement of the CAG repeat in SMBA also suggests the possibility of some dominant negative action of the mutant protein, inhibiting a normal protein-protein interaction.

## 7.5. Future direction

This study has examined the AR gene of patients with androgen insensitivity and of a cohort of infertile men. The mutations identified in the patients with AIS have added to the database of mutations responsible for the syndrome and have contributed to the understanding of the mechanism of action of the steroid receptor. The examination of

the entire coding region of the gene has been slightly hampered by difficulties with PCR amplification of certain regions and it would be worth a concerted effort to modify the protocol in order to improve the efficiency of the PCR and allow completion of the analysis of the patients (in particular the infertile cohort). The inclusion of 7-deaza-guanosine as a nucleotide in the PCR reaction (Innis, 1990) was adopted briefly in an attempt to improve the specific amplification of the GC-rich glycine repeat coding region of exon 1, but there was no immediate improvement. Additional effort attempting to optimise the use of this nucleotide in PCR amplification of exon 1 would probably be of benefit since this approach has resulted in successful amplification of GC-rich domains of other genes, notably the CGG repeat in the FMR-1 gene in fragile X syndrome (Kremer *et al.*, 1991).

The continued analysis of the AR gene of patients with AIS as they come to clinical attention will be useful to complement the available data on the location of AR gene mutations and how they affect the function of the receptor protein. The number of patients is likely to be low in view of the low incidence of the disease and the small size of the Scottish population. Additional information about regions upstream of the start site of transcription of the AR gene is now required and this would be worthy of an entirely new study initially focussing upon patients with no demonstrable defect within the coding region of the AR gene but symptoms and signs of AIS, such as patients 4 to 6 in this study.

Modification and optimisation of the method for site-directed mutagenesis should be undertaken in order to reproduce the sequence of the AR detected in the AIS patients in this study, and to utilise *in vitro* assays to determine whether the function of the resultant AR protein is affected. This will be particularly useful in the patients where data from genital skin fibroblasts are not available and there is no information about receptor status and binding capacity of GSF. The mutations introducing premature termination codons are more predictable in their effect, but it is nevertheless worth confirming the translation of a truncated protein.

The analysis of infertile men for the presence of AR gene mutations should be continued to complete the analysis of exon 1, as discussed above, and to provide a larger sample size. In view of the findings of Akin *et al.* (1991) a sample of azoospermic men should be studied. The likelihood of a low rate of mutation detection will make such a study labour intensive even using the DGGE technique established in this study, but even negative findings such as were obtained in this study are of interest in resolving the debate about the prevalence of AR gene mutations

in infertility.

In conclusion, this study has identified mutations in the AR gene of patients with androgen resistance which have contributed to a greater understanding of the mechanism of action of these mutations in the pathogenesis of the disease. They have also, in context with the data of other workers, provided insights into the mechanics of the steroid receptor function in the normal situation. A preliminary study of a cohort of infertile men has been undertaken without finding any evidence of AR gene mutations.

**Table 7.1. Summary of all detected AR gene mutations**

| Patient | Nucleotide | Change | Amino acid | Change |
|---|---|---|---|---|
| 1 (complete AIS) | 2574 | CGC → TGC | 855 | Arg → Cys |
| 2 (complete AIS) | 2502 | CGA → TGA | 831 | Arg → STOP |
| 3 (complete AIS) | 2265 | CGA → TGA | 752 | Arg → STOP |
| 7 (partial AIS) | 2607 | GTG → TTG | 866 | Val → Leu |
| 8 (partial AIS) | 2607 | GTG → TTG | 866 | Val → Leu |
| 9 (partial AIS) | 1834 | AGG → AAG | 608 | Arg → Lys |
| 10 (Reifenstein's) | 2326 | GAG → GGG | 772 | Glu → Gly |
| 11 (?Kennedy's) | Exon 1 | Glutamine repeat | No. of residues | Increased to 46 Gln |

Note: numbering of amino acids in this table and throughout the thesis is taken to be that of the androgen receptor gene sequence published by Lubahn *et al.*, 1989.

# Bibliography


Adevemo, O., Kontula, K. & Jänne, O.A. (1992) Androgen insensitivity syndrome resulting from a point mutation in exon 6 of androgen receptor gene. *74th Meeting US Endocrine Soc.* Abs. 225.

Aiman, J., Griffin, J.E., Gazak, J.M., Wilson, J.D. & MacDonald, P.C. (1979) Androgen insensitivity as a cause for infertility in otherwise normal men. *New Engl. J. Med.* 300, 223-227.

Aiman, J. & Griffin, J.E. (1982) The frequency of androgen receptor deficiency in infertile men. *J. Clin. Endocrinol. Metab.* 54, 725-732.

Akin, J.W., Behzadian, A., Tho, S.P.T. & McDonough, P.G. (1991) Evidence for a partial deletion in the androgen receptor gene in a phenotypic male with azoospermia. *Am. J. Obstet. Gynaecol.* 165, 1891-1894.

Amato, A.A., Prior, T.W., Barohn, R.J., Snyder, P., Papp, A. & Mendell, J.R. (1993) Kennedy's disease: a clinicopathologic correlation with mutations in the androgen receptor gene. *Neurology* 43, 791-794.

Amrhein, J., Meyer, W., Jones, H. & Migeon, C. (1976) Androgen insensitivity in man: evidence for genetic heterogeneity. *Proc. Natl. Acad. Sci. USA* 73, 891-894.

Arbizu, T., Santamaría, J., Gomez, J.M., Quílez, A. & Senra, J.P. (1983) A family with adult spinal and bulbar muscular atrophy, X-linked inheritance and associated testicular failure. *J. Neurol. Sci.* 59, 371-382.

Barkhaus, P.E., Kennedy, W.R., Stern, L.Z. & Harrington, R.B. (1982) Hereditary proximal and bulbar motor neuron disease of late onset. *Arch. Neurol. (Chic.)* 39, 112-116.

Batch, J.A., Williams, D.M., Davies, H.R., Brown, B.D., Evans, B.A.J., Hughes, I.A. & Patterson, M.N. (1992) Androgen receptor gene mutations identified by SSCP in fourteen subjects with androgen insensitivity syndrome. *Hum. Mol. Genet.* 1, 497-503.

Batch, J.A., Evans, B.A., Hughes, I.A., Patterson, M.J. (1993) Mutations of the androgen receptor gene identified in perineal hypospadias. *J. Med. Genet.* 30, 198-201.

Beato, M. (1989) Gene regulation by steroid hormones. *Cell* 56, 335-344.

Beato, M., Chalepakis, G., Schauer, M. & Slater, E.P. (1989) DNA regulatory elements for steroid hormones. *J. Steroid Biochem.* 32, 737-748.

Belsham, D.D., Greenberg, C.R., Faiman, C., Yee, W-C. & Wrogemann, K. (1992) Clinical applications of mutational analysis in families with androgen receptor defects: complete and partial androgen insensitivity syndrome and spinal and bulbar muscular atrophy. *74th Meeting of US Endocrine Soc.* Abs. 361.

Berg, J. (1989) DNA binding specificity of steroid receptors. *Cell* 57, 1065-1068.

Biancalana, V., Serville, F., Pommier, J., Julien, J., Hanauer, A. & Mandel, J.L. (1992) Moderate instability of the trinucleotide repeat in spinobulbar muscular atrophy. *Hum. Mol. Genet.* 1, 255-258.

Bocquel, M.T., Kumar, V., Stricker, C., Chambon, P. & Gronmeyer, H. (1989) The contribution of the N- and C-terminal regions of steroid receptors to activation of transcription is both receptor and cell specific. *Nucleic Acid Res.* 17, 2581-2595.

Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J. & Lindquist, S. (1989) hsp 82 is an essential protein that is required in higher concentration for growth of cells at higher temperatures. *Mol. Cell. Biol.* 9, 3919-3930.

Brook, J.D., McCurragh, M.E., Harley, H.G., Buckler, A.J., Church, D., Aburatani, H., Hunter, K., Stanton, V.P., Thirion, J.P., Hudson, T., Sohn, R., Zemelman, B., Snell, R.B., Rundle, S.A., Crow, S., Davies, J., Shelbourne, P., Buxton, J., Jones, C., Juvonen, V., Johnson, K., Harper, P.S., Shaw, D.J. & Housman, D.E. (1992) Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3' end of a transcript encoding a protein kinase family member. *Cell* 68, 799-808.

Brown, T.R., Maes, M., Rothwell, S.R. & Migeon, C.J. (1982) Human complete androgen insensitivity with normal dihydrotestosterone receptor binding capacity in cultured genital skin fibroblasts: evidence for a qualitative abnormality of the receptor. *J. Clin. Endocrinol. Metab.* 55, 61-69.

Brown, T.R., Lubahn, D.B., Wilson, E.M., Joseph, D.R., French, F.S. & Migeon, C.J. (1988) Deletion of the steroid binding domain of the human androgen receptor gene in one family with complete androgen insensitivity syndrome: evidence for further genetic heterogeneity in this syndrome. *Proc. Natl. Acad. Sci. USA* 85, 8151-8155.

Brown, C.J., Goss, S.J., Lubahn, D.B., Joseph, D.R., Wilson, E.M., French, F.S. & Willard, H.F. (1989) Androgen receptor locus on the human X chromosome—regional localisation to Xq 11-12 and description of a DNA polymorphism. *Am. J. Hum. Genet.* 44, 264-269.

Brown, T.R., Lubahn, D.B., Wilson, E.M., French, F.S., Migeon, C.J. & Corden, J.L. (1990) Functional characterisation of naturally occurring mutant androgen receptors from subjects with complete androgen insensitivity. *Mol. Endocrinol.* 4, 1759-1772.

Brown, T.R., Newmark, J. & Ghirri, P. (1992) Six naturally occurring mutations in the 144-base pairs of exon E of the human androgen receptor gene. *74th Meeting US Endocrine Soc.* Abs. 1506.

Carson-Jurica, M.A., Schrader, W.T. & O'Malley, B.W. (1990) Steroid receptor family: structure and functions. *Endocrine Reviews* 11, 201- 220.

Chandley, A.C., Gosden, J.R., Hargreave, T.B., Spowart, G., Speed, R.M. & McBeath, S. (1989) Deleted Yq in the sterile son of a man with a satellited Y chromosome (Yqs). *J. Med. Genet.* 26, 145-153.

Chang, C., Kokontis, J. & Liao, S. (1988) Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc. Natl. Acad. Sci. USA* 85, 7211-7215.

Chang, Y.T., Migeon, C.J. & Brown, T.R. (1991) Human androgen insensitivity syndrome due to androgen receptor gene point mutations in subjects with normal androgen receptor levels but impaired biological activity. *73rd Meeting US Endocrine Soc.* Abs. 28.

Chaucereau, A., Savouret, J-F. & Milgrom, E. (1992) Control of biosynthesis and post-translational modification of the progesterone receptor. *Biol. Reprod.* 46, 174-177.

Chomczynski, P. & Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* 162, 156-159.

Courey, A.J. & Tjian, R. (1988) Analysis of Sp1 in vivo reveals multiple transcriptional domains including a novel glutamine rich activation motif. *Cell* 55, 887-898.

Danielsen, M., Northrop, J.P., Jonklaas, J. & Ringold, G.M. (1987) Domains of the glucocorticoid receptor involved in specific and nonspecific DNA binding, hormone activation and transcriptional enhancement. *Mol. Endocrinol.* 1, 816-822.

Danielsen, M., Hinck, L. & Ringold, G.M. (1989) Two amino acids within the knuckle of the first zinc finger specify DNA response element activation by the glucocorticoid receptor. *Cell* 57, 1131-1138.

De Bellis, A., Quigley, C.A., Cariello, N.F., Ho, K.C. & French, F.S. (1991) DNA and steroid binding domain mutations of the androgen receptor cause partial or complete androgen insensitivity. *73rd Meeting US Endocrine Soc.* Abs. 1141.

De Bellis, A., Quigley, C.A., Cariello, N.F., El-Awady, M.K., Sar, M., Lane, M.V., Wilson, E.M. & French, F.S. (1992) Single base mutations in the human androgen receptor gene causing complete androgen insensitivity: rapid detection by a modified denaturing gradient gel electrophoresis technique. *Mol. Endocrinol.* 6, 1909-1920.

Deng, W.P. & Nickoloff, J.A. (1992) Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Analyt. Biochem.* 200, 81-88.

Diczfaluzy, E. (1986) World Health Organisation: Special Programme of Research Development and Research Training in Human Reproduction: The first fifteen years: a review. *Contraception* 34, 1-97.

Dohet, C., Wagner, R. & Radman, M. (1985) Repair of defined single base-pair mismatches in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 82, 503-505.

Eil, C., Gamblin, G.T., Hodge, J.W., Clarke, R.V. & Sherin, R.S. (1985) Whole cell and nuclear androgen uptake in skin fibroblasts from infertile men. *J. Andrology* 6, 365-371.

Evans, B., Williams,D. & Hughes, I. (1991) Normal postnatal androgen production and action in isolated micropenis and isolated hypospadias. *Arch. Dis. Child.* 66, 1033-1036.

Evans, B.A.J. (1992) Detection of a point mutation within the androgen receptor gene in a family with complete androgen insensitivity and subsequent prenatal diagnosis. *J. Endocrinol.* 135 *Supp.*, Abs. P26.

Evans, R.M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* 240, 889-895.

Faber, P.W., van Rooij, H.C.J., van der Korput, H.A.G.M., Baarends, W.M., Brinkmann, A.O., Grootegoed, J.A. & Trapman, J.A. (1991) Characterisation of the human androgen receptor transcription unit. *J. Biol. Chem.* 266, 10743-10749.

Faber, P.W., van Rooij, H.C.J., Schippert, H.J., Brinkmann, A.O. & Trapman, J. (1993) Two different, overlapping pathways of transcription initiation are active on the TATA-less human androgen receptor promoter. J. Biol. Chem. 268, 9296-9301.

Fawell, S.E., Lees, J.A., White, R. & Parker, M.G. (1990) Characterisation and co-localisation of steroid binding and dimerisation activities in the mouse oestrogen receptor. *Cell* 60, 953-962.

Forman, B.M. & Samuels, H.H. (1990) Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol.* 4, 1293-1301.

Freedman, L.P., Luisi, B.F., Korsun, Z.R., Basavappa, R., Sigler, P.J. & Yamamoto, K.R. (1988) The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* 334, 543-546.

Freedman, L.P. (1992) Anatomy of the steroid receptor zinc finger region. *Endocrine Reviews* 13, 129-145.

Fryns, J-P. (1989) X-linked mental retardation and the fragile X syndrome: a clinical approach. In: *The fragile X syndrome*. Davies, K.E., ed. Oxford University Press, Oxford.

Gearhart, J., Linhard, H., Berkovitz, G., Jeffs, R. & Brown, T. (1988) Androgen receptor levels and 5 α-reductase activities in preputial skin and chordee tissue of boys with isolated hypospadias. *J. Urol.* 140, 1243-1246.

Golsteyn, E.J., Goren, H.J., Lehoux, J.G. & Lefebvre, Y.A. (1990) Phosphorylation and nuclear processing of the androgen receptor. *Biochem. Biophys. Res. Commun.* 171, 336-341.

Green, M.R. (1986) Pre-mRNA splicing. *Ann. Rev. Genet.* 20, 671-708.

Griffin, J.E. & Durrant, J.L. (1982) Qualitative receptor defects in families with androgen resistance: failure of stabilisation of the fibroblast cytosol androgen receptor. *J. Clin. Endocrinol. Metab.* 55, 465-474.

Griffin, J.E. & Wilson, J.D. (1980) The syndromes of androgen resistance. *New Engl. J. Med.* 302, 198-209.

Griffin, J.E. & Wilson, J.D. (1987) Syndromes of androgen resistance. *Hospital Practice* 22, 159-176.

Grimberg, J., Nawoschik, S., Belluscio, L., McKee, R., Turck, A. & Eisenberg, A. (1989) A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. *Nucleic Acid Res.* 17, 8390.

Grino, P.B., Griffin, J.E., Cushard, W.G. & Wilson, J.D. (1988) A mutation of the androgen receptor associated with partial androgen resistance, familial gynaecomastia and fertility. *J. Clin. Endocrinol. Metab.* 66, 754-761.

Grino, P.B., Griffin, J.E. & Wilson, J.D. (1990) Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* 126, 1165-1172.

Guichon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M. & Milgrom, E. (1989) Mechanisms of nuclear localisation of the progesterone receptor: evidence for interaction between monomers. *Cell* 57, 1147-1154.

Härd, T., Kellenbach, E., Boelens, R., Maler, B.A., Dahlman, K., Freedman, L.P., Carlstedt-Duke, J., Yamamoto, K.R., Gustafsson, J-Å. & Kaptein, R. (1990) Solution structure of the glucocorticoid receptor DNA-binding domain. *Science* 249, 157-160.

Hargreave, T.B. (1990) Introduction. In: *The management of male infertility.* Hargreave, T.B. & Soon, T.E., eds. P.G. Publishing. Singapore.

Hargreave, T.B. & Elton, R.A. (1986) Fecundability rates from an infertile male population. *Br. J. Urology* 58, 194-197.

Harley, H.G., Brook, J.D., Rundle, S.A., Crow, S., Reardon, W., Buckler, A.J., Harper, P.S., Housman, D.E. & Shaw, D.J. (1992) Expansion of an unstable DNA region and phenotypic variation in myotonic dystrophy. *Nature* 355, 545-546.

Harper, P.S. (1989) Myotonic dystrophy. Second Edition. W.B. Saunders Co., London.

Hayashi, K. (1992) PCR-SSCP: A method for detection of mutations. *GATA*. 9, 73-79.

Hodgins, M.B. (1982) Binding of androgens in 5 α-reductase-deficient human genital skin fibroblasts: inhibition by progesterone and its metabolites. *J. Endocrinol.* 94, 415-427.

Hodgins, M.B., Duke, E.M.C. & Ring, D. (1984) Carrier detection in the testicular feminization syndrome: deficient binding of 5 α-DHT in cultured skin fibroblasts from mothers of patients with complete androgen insensitivity. *J. Med. Genet.* 21, 178-181.

Hollenberg, S.M. & Evans, R.M. (1988) Multiple and cooperative transactivation domains of the human glucocorticoid receptor. *Cell* 55, 899-906.

Howeler, C.J., Busch, H.F.M., Geraedts, J.P.M., Niermeijer, M.F. & Staal, A. (1989) Anticipation in myotonic dystrophy: fact or fiction? *Brain* 112, 779-797.

Hull, M.G.R., Glazener, C.M.A., Kelly, N.J., Conway, D.I., Foster, P.A., Hinton, R.A., Coulson, C., Lambert, P.A., Watt, E.M. & Desai, K.M. (1985) Population study of causes, treatment and outcome of infertility. *Br. Med. J.* 291, 1693-1697.

Huntington's Disease Collaborative Research Group (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72, 971-983.

Innis, M.A. (1990) PCR with 7-deaza-2'-triphosphate. In: *PCR protocols.* Innis, M.A., Gelfand, D.H., Sninsky, J.J. & White, T.J., eds. Academic Press Inc., Harcourt Brace Jovanovich, London.

Irvine, D.S. (1992) Assessment of spermatogenesis. *Current Obstet. & Gynaecol.* 2, 20-26.

Jakubiczka, S., Werder, E.A. & Wieacker, P. (1992) Point mutation in the steroid binding domain of the androgen receptor gene in a family with complete androgen insensitivity syndrome (CAIS). Hum. Gen. 90, 311-312.

Jenster, G., van der Korput, H.A.G.M., van Vroonhoven, C., van der Kwast, T.H., Trapman, J. & Brinkmann, A.O. (1991) Domains of the human androgen receptor involved in steroid binding, transcriptional activation and subcellular localisation. *Mol. Endocrinol.* 5, 1396-1404.

Jukier, L., Kaufman, M., Pinsky, L. & Peterson, R.E. (1984) Partial androgen resistance associated with secondary 5 α-reductase deficiency: Identification of a novel qualitative androgen receptor defect and clinical implications. *J. Clin. Endocrinol. Metab.* 59, 679-689.

Kaufman, M., Pinsky, L. & Feder-Hollander, R. (1981a) Defective up-regulation of the androgen receptor in human androgen insensitivity. *Nature* 293, 735-737.

Kaufman, M., Pinsky, L., Simard, L. & Wong, S.C. (1981b) Defective activation of androgen receptor complexes. A marker of androgen insensitivity. *Mol. Cell. Endocrinol.* 25, 151-162.

Kazemi-Esfarjani, P., Beitel, L.K., Trifiro, M., Kaufman, M., Rennie, P., Sheppard, P., Matusik, R. & Pinsky, L. (1993) Substitution of valine 865 by methionine or leucine in the human androgen receptor causes complete or partial androgen insensitivity, respectively with distinct androgen receptor phenotypes. *Mol. Endocrinol.* 7, 37-46.

Keenan, B., Meyer, W., Hadjian, A., Jones, H. & Migeon, C. (1974) Syndrome of androgen insensitivity in man; absence of 5 α-DHT binding protein in skin fibroblasts. *J. Clin. Endocrinol. Metab.* 38, 1143-1146.

Kennedy, W.R., Alter, M. & Sung, J.H. (1968) Progressive proximal spinal and bulbar muscular atrophy of late onset— a sex-linked recessive trait. *Neurology* 18, 671-680.

Kiraly, C.L., Collan, Y. & Alen, M. (1987) Effect of testosterone and anabolic steroids on the size of the sebaceous glands in power athletes. *Am. J. Dermatopathol.* 9, 515-519.

Klocker, H., Kaspar, F., Eberle, J., Uberreiter, S., Radmayr, C. & Bartsch, G. (1992) Point mutation in the DNA binding domain of the androgen receptor in two families with Reifenstein's syndrome. *Am. J. Hum. Genet.* 50, 1318-1327.

Konzarides, T. & Ziff, E. (1989) The rôle of the leucine zipper in the fos-jun interaction. *Nature* 336, 646-651.

Kramer, B., Kramer, W. & Fritz, H-J. (1984) Different base/base mismatches are corrected with different efficiencies by the methyl-directed mismatch-repair system of *E. coli*. *Cell* 38, 879-887.

Krawczek, M., Reiss, J. & Cooper, D.N. (1992) The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: causes and consequences. *Hum. Genet.* 90, 41-54.

Kremer, E.J., Pritchard, M., Lynch, M., Yu, S., Holman, K., Baker, E., Warren, S.T., Schlessinger, D., Sutherland, G.R. & Richards, R.I. (1991) Mapping of DNA instability at the fragile X to a trinucleotide repeat sequence p(CCG)n. *Science* 252, 1711-1714.

Kumar, V. & Chambon, P. (1988) The oestrogen receptor binds tightly to its responsive element as a ligand induced homodimer. *Cell* 55, 145-156.

Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82, 488-492.

Larrea, F., Benavides, G., Scaglia, H., Kofman-Alfaro, S., Ferrusca, E., Medina, M. & Perez-Palacios, G. (1978) Gynaecomastia as a familial incomplete male pseudohermaphroditism type I; A limited androgen resistance syndrome. *J. Clin. Endocrinol. Metab.* 46, 961-970.

La Spada, A.R., Wilson, E.M., Lubahn, D.B., Hareling, A.E. & Fischbeck, K.H. (1991) Androgen receptor gene mutations in X linked spinal and bulbar muscular atrophy. *Nature* 352, 77-79.

La Spada, A.R., Roling, D.B., Harding, A.E., Warner, C.L., Speigel, R., Hausmanowa-Patrusewicz, I., Yee, W.C. & Fischbeck, K.H. (1992) Meiotic stability and genotype-phenotype correlation of the trinucleotide repeat in X linked spinal and bulbar muscular atrophy. *Nat. Genet.* 2, 301-314.

Laycock, J.F. & Wise, P.H. (1983) The Gonads. In: *Essential Endocrinology*. Second Edition. Oxford University Press, Oxford.

Lee, M.M. & Donahoe, P.K. (1993) Müllerian Inhibiting Substance: a gonadal hormone with multiple functions. *Endocrine Rev.* 14, 152-164.

Lees, J.A., Fawell, S.E., White, R. & Parker, M.G. (1990) A 22 amino acid peptide restores DNA binding activity to dimerisation defective mutants of the oestrogen receptor. *Mol. Cell. Biol.* 10, 5529-5531.

Lindquist, S. & Craig, E.A. (1988) The heat shock proteins. *Ann. Rev. Genet.* 22, 631-677.

Lubahn, D.B., Joseph, D.R., Sar, M., Tan, J., Higgs, H.N., Larson, R., French, F.S. & Wilson, E.M. (1988) The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. *Mol. Endocrinol.* 2, 1265-1275.

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

Luisi, B.F., Xu, W.X., Otwinowski, Z., Freedman, L.P., Yamamoto, K.R. & Sigler, P.B. (1991) Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352, 497-505.

Ma, K., Sharkey, A., Kirsch, S., Vogt, P., Keil, R., Hargreave, T.B., McBeath, S. & Chandley, A.C. (1992) Towards the molecular localisation of the AZF locus: mapping of microdeletions in azoospermic men within 14 subintervals of interval 6 of the human Y chromosome. *Hum. Mol. Genet.* 1, 29-33.

Maclean, H.E., Chu, S., Warne, G.L. & Zajac, J.D. (1993) Related individuals with different androgen receptor gene deletions. *J. Clin. Invest.* 91, 1123-1128.

Madden, J.D., Walsh, P.C., MacDonald, P.C. & Wilson, J.D. (1975) Clinical and endocrinologic characterisation of a patient with the syndrome of incomplete testicular feminisation. *J. Clin. Endocrinol. Metab.* 41, 751-760.

Mader, S., Kumar, V., de Verneuil, H. & Chambon, P. (1989) Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid responsive element. *Nature* 338, 271-273.

Marcelli, M., Tilley, W.D., Wilson, C.M., Griffin, J.E., Wilson, J.D. & McPhaul, M.J. (1990a) Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid residue 588 causes complete androgen resistance. *Mol. Endocrinol.* 4, 1105-1116.

Marcelli, M., Tilley, W.D., Wilson, C.M., Wilson, J.D., Griffin, J.D. & McPhaul, M.J. (1990b) A single nucleotide substitution introduces a premature termination codon into the androgen receptor gene of a patient with receptor negative androgen resistance. *J. Clin. Invest.* 85, 1522-1528.

Marcelli, M., Zoppi, S., Grino, P.B., Griffin, J.E., Wilson, J.D. & McPhaul, M.J. (1991a) A mutation in the DNA binding domain of the androgen receptor gene causes complete testicular feminisation in a patient with receptor positive androgen resistance. *J. Clin. Invest.* 87, 1123-1126.

Marcelli, M., Tilley, W.D., Zoppi, S., Griffin, J.E., Wilson, J.D. & McPhaul, M.J. (1991b) Androgen resistance associated with a mutation of the androgen receptor at amino acid 772 (Arg - Cys) results from a combination of decreased messenger ribonucleic acid levels and impairment of receptor function. *J. Clin. Endocrinol. Metab.* 73, 318-325.

Marcelli, M.; Zoppi, S.; Wilson, C.M.; Griffin, J.E.; Wilson, J.D. & McPhaul, M.J. (1992) Amino acid substitutions in a small segment of exon 5 of the human androgen receptor gene cause complete testicular feminisation (CTF) by different mechanisms. *74th Meeting US Endocrine Soc.* Abs. 224.

Martinez, E. & Wahli, W. (1992) Characterisation of hormone response elements. In: *Nuclear Hormone Receptors: Molecular Mechanisms, Cellular Functions, Clinical Abnormalities.* First Edition. Parker, M.G., ed. Academic Press. London.

Masumune, Y. & Richardson, C.C. (1971) Strand displacement during deoxyribonucleic acid synthesis at single strand breaks. *J. Biol. Chem.* 246, 2692-2701.

McPhaul, M.J., Marcelli, M., Tilley, W.D., Griffin, J.E., Isidro-Gutierrez, R.F. & Wilson, J.D. (1991a) Molecular basis of androgen resistance in a family with a qualitative abnormality of the androgen receptor and responsive to high dose androgen therapy. *J. Clin. Invest.* 87, 1413-1421.

McPhaul, M.J., Marcelli, M., Tilley, W.D., Griffin, J.E. & Wilson, J.D. (1991b) Androgen resistance caused by mutations in the androgen receptor gene.. *FASEB J.* 5, 2910-2915.

McPhaul, M.J., Marcelli, M., Zoppi, S., Wilson, C.M., Griffin, J.E. & Wilson, J.D. (1992) Mutations in the ligand binding domain of the androgen receptor gene cluster in two regions of the gene. *J. Clin. Invest.* 90, 2097-2101.

Mebarki, F., Gasmi, M., Forest, M.G., David, M., Chatelain, P. & Morel, Y. (1990) Analysis of polymerase chain reaction (PCR) amplified exons of the androgen receptor gene in 14 patients with genetic androgen resistance. *72nd Meeting US Endocrine Soc.* Abs. 791.

Mebarki, F., Forest, M.G., Lauras, B., Bertrand, A.M., Chatelain, P., David, M. & Morel, Y.(1993) De novo mutation of the androgen receptor gene is not a rare event in androgen insensitivity syndrome as suggested by segregation of the trinucleotide repeat in exon 1 and sequencing. *75th Meeting US Endocrine Soc.* Abs. 602.

Meyer, W.J., Migeon, B.R. & Migeon, C.J. (1975) Locus on the human X chromosome for dihydrotestosterone receptor and androgen insensitivity. *Proc. Natl. Acad. Sci. USA* 72, 1469-1472.

Migeon, C.J., Brown, T.R., Lanes, R., Palacios, A, Amrhein, J.A. & Schoen, E.J. (1984) A clinical syndrome of mild androgen insensitivity. *J. Clin. Endocrinol. Metab.* 59, 672-680.

Moore, K.L. (1988) The Urogenital System. In: *The Developing Human. Clinically Oriented Embryology*. Fourth Edition. Wonsiewicz, M., ed. W.B. Saunders Co. Philadelphia.

Morris, J.M. (1953) The syndrome of testicular feminisation in male pseudohermaphrodites. *Am. J. Obstet. Gynaecol.* 65, 1191-1211.

Morrow, A.F., Gyorki, S., Warne, G.L., Burger, H.G., Bangah, M.L., Outch, K.H., Mirovics, A. & Baker, H.W.G. (1987) Variable androgen receptor levels in infertile men. *J. Clin. Endocrinol. Metab.* 64, 1115-1121.

Müller, W., Weber, H., Meyer, F. & Weissman, C. (1978) Site directed mutagenesis in DNA: generation of point mutations in cloned β globin complementary DNA at the positions corresponding to amino acids 121 to 123. *J. Mol. Biol.* 124, 343-358.

Myers, R.M., Fischer, S.G., Lerman, L.S. & Maniatis, T. (1985a) Modification of the melting properties of duplex DNA by attachment of a GC-rich DNA sequence as determined by denaturing gradient gel electrophoresis. *Nucleic Acid Res.* 13, 3131-3129.

Myers, R.M., Fischer, S.G., Lerman, L.S. & Maniatis, T. (1985b) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acid Res.* 13, 3131-3145.

Myers, R.M., Maniatis, T., Lerman, L.S. (1987) Detection and localisation of single base changes by denaturing gradient gel electrophoresis. *Meth. Enzymol.* 155, 501-527.

Nakao, R., Haji, M., Yanase, T., Ogo, A., Takayanagi, R., Katsube, T., Fukumaki, Y. & Nawata, H. (1992) A single amino acid substitution ($Met^{786}$-Val) in the steroid binding domain of human androgen receptor leads to complete androgen insensitivity. *J. Clin. Endocrinol. Metab.* 74, 1152-1157.

Nelson, K.K. & Green, M.R. (1990) Mechanism for cryptic splice site activation during pre-mRNA splicing. *Proc. Natl. Acad. Sci. USA* 87, 6253-6257.

Nordeen, S.K., Suh, B.J., Kuhnel, B. & Hutchison, C.A. (1990) Structural determinants of a glucocorticoid receptor recognition element. *Mol. Endocrinol.* 4, 1866-1873.

Nossal, N.G. (1974) DNA synthesis on a double-stranded DNA template by the T4 bacteriophage DNA polymerase and the T4 gene 32 DNA unwinding protein. *J. Biol. Chem.* 249, 5668-5676.

O'Malley, B.W. & Tsai, M-J. (1992) Molecular pathways of steroid receptor action. *Biol. Reprod.* 46, 163-167.

Orita, M., Suzuki, Y., Sekiya, T. & Hayashi, K. (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* 5, 874-879.

Padgett, R.A., Grabowski, P.J., Konarska, M.M., Seiler, S & Sharp, P.A. (1986) Splicing of mRNA precursors. *Ann. Rev. Biochem.* 55, 1119-1150.

Parker, M.G. & Bakker, O. (1992) Concluding Remarks. In: *Nuclear Hormone Receptors: Molecular Mechanisms, Cellular Functions, Clinical Abnormalities*. First Edition. Parker, M.G., ed. Academic Press. London.

Pinsky, L., Trifiro, M., Kaufman, M., Beitel, L.K., Mhatre, A., Kazemi-Esfarjani, P., Sabbaghian, N., Lumbroso, R., Alvarado, M., Vasiliou, M. & Gottlieb, B. (1992) Androgen resistance due to mutation of the androgen receptor. *Clin. Invest. Med.* 15, 456-472.

Pratt, W.B. (1990) Interaction of hsp90 with steroid receptors: organising some diverse observations and presenting the newest concepts. *Mol. Cell. Endocrinol.* 74, C69-C76.

Price, P., Wass, J.A.H., Griffin, J.E., Leshin, M., Savage, M.O., Large, D.M., Bu'Lock, D.E., Anderson, D.C., Wilson, J.D. & Besser, G.M. (1984) High dose androgen therapy in male pseudohermaphroditism due to 5 α-reductase deficiency and disorders of the androgen receptor. *J. Clin. Endocrinol. Metab.* 74, 1496-1508.

Prior, L., Bordet, S., Trifiro, M.A., Mhatre, A., Kaufman, M., Pinsky, L., Wrogemann, K., Belsham, D.D., Pereira, F., Greenberg, C., Trapman, J., Brinkmann, A.O., Chang, C. & Liao, S. (1992) Replacement of arginine 773 by cysteine or histidine in the human androgen receptor causes complete androgen insensitivity with different receptor phenotypes. *Am. J. Hum. Genet.* 51, 143-155.

Quarmby, V.E., Kemppainen, J.A., Sar, M., Lubahn, D.B., French, F.S. & Wilson, E.M. (1990) Expression of recombinant androgen receptor in cultured mammalian cells. *Mol. Endocrinol.* 4, 1399-1407.

Quigley, C.A., Simental, J.A., Marschke, K.B., Lubahn, D.B., Wilson, E.M. & French, F.S. (1992a) *J. Cell. Biochem. Supp.* 16C Abs. L323.

Quigley, C.A., Evans, B.A.J., Simental, J.A., Marschke, K.B., Sar, M., Lubahn, D.B., Davies, P., Hughes, I.A., Wilson, E.M. & French, F.S. (1992b) Complete androgen insensitivity due to deletion of exon C of the androgen receptor gene highlights the importance of the second zinc finger of the androgen receptor in vivo. *Mol. Endocrinol.* 6, 1103-1112.

Quigley, C.A., De Bellis, A. & Lubahn, D.B. (1992c) The nature and distribution of amino acid alterations in the androgen receptor in the androgen insensitivity syndrome. *74th Meeting US Endocrine Soc.* Abs. 223.

Redman, J.B., Fenwick, R.G. Jr., Fu, Y-H., Pizzuti, A., Caskey, C.T. (1993) Relationship between parental trinucleotide GCT repeat length and severity of myotonic dystrophy in offspring. *JAMA* 269, 1960-1965.

Refetoff, S., Weiss, R.E. & Usala, S.J. (1993) The syndromes of resistance to thyroid hormone. Endocrine Rev. 14, 348-399.

Reifenstein, E.C. (1947) Hereditary familial hypogonadism. *Proc. Am. Fed. Clin. Res.* 3, 86.

Riesner, D., Steger, G., Zimmat, R., Owens, R.A., Wagenhofer, M., Hillen, W., Vollbach, S. & Henco, K. (1989) Temperature-gradient gel electrophoresis of nucleic acids: analysis of conformational transitions, sequence variations, and protein-nucleic acid interactions. *Electrophoresis* 10, 377-389.

Ring, D. & Hodgins, M.B. (1984) The regulation of androgen receptor turnover in genital skin fibroblasts by 5 α-DHT. *Biochem. Soc. Transact.* 12, 658-659.

Ris-Stalpers, C., Kuiper, G.G.J.M., Faber, P.W., Schweikert, H.U., van Rooij, H.C.J., Zegers, N.D., Hodgins, M.B., Degenhart, H.J., Trapman, J. & Brinkmann, A.O. (1990) Aberrant splicing of androgen receptor mRNA results in synthesis of a non-functional receptor protein in a patient with androgen insensitivity. *Proc. Natl. Acad. Sci. USA* 87, 7866-7870.

Ris-Stalpers, C., Trifiro, M.A., Kuiper, G.G.J.M., Romali, G., Sai, T., van Rooij, H.C.J., Kaufman, M., Rosenfield, R.L., Liao, S., Schweikert, H-U., Trapman, J., Pinsky, L. & Brinkmann, A.O. (1991) Substitution of aspartic acid 686 by histidine or asparagine in the human androgen receptor leads to a functionally inactive protein with altered hormone binding characteristics. *Mol. Endocrinol.* 5, 1562-1569.

Ris-Stalpers, C., Blaeij, T.J.P., Sleddens, H.F.B.M., Trapman, J. & Brinkmann, A.O. (1992) Androgen receptor mutants and functional activity. *74th Meeting of US Endocrine Soc.* Abs. 1008.

Roberts, C. & Lloyd, S. (1973) Observations on the epidemiology of simple hypospadias. *Br. Med. J.* 1, 768-770.

Rosenfield, R.L., Lawrence, A.M., Liao, S. & Landau, R.L. (1971) Androgens and androgen responsiveness in the feminising testis syndrome. Comparison of complete and "incomplete" forms. *J. Clin. Endocrinol.* 32, 625-632.

Rundlett, S.E., Wu, X-P. & Miesfeld, R.L. (1990) Functional characterisation of the androgen receptor confirms that the molecular basis of androgen action is transcriptional activation. *Mol. Endocrinol.* 4, 708-714.

Sai, T.J., Seino, S., Chang, C.S., Trifiro, M., Pinsky, L., Mhatre, A., Kaufman, M., Lambert, B., Trapman, J., Brinkmann, A.O. Rosenfield, R.L. & Liao, S. (1990) An exonic point mutation of the

androgen receptor gene in a family with complete androgen insensitivity. *Am. J. Hum. Genet.* 46, 1095-1100.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf S.J., Higuci, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487-494.

Sakurai, A., Takeda, K., Ceccarelli, P., Nakai, A., Seino, S.,Bell, G.I., Refetoff, S. & DeGroot, L.J. (1989) Generalised resistance to thyroid hormone associated with a mutation in the ligand binding domain of the human thyroid hormone receptor β. *Proc. Natl. Acad. Sci. USA.* 86, 8977-8981.

Sar, M., Lubahn, D.B., French, F.S. & Wilson, E.M. (1990) Immunohistochemical localisation of the androgen receptor in rat and human tissues. *Endocrinology* 127, 3180-3186.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A laboratory manual.* Second Edition. Cold Spring Harbor Laboratory Press.

Saunders, P.T.K., Padayachi, T., Tincello, D.G., Shalet, S.M. & Wu, F.C.W. (1992) Point mutations detected in the androgen receptor gene of three men with partial androgen insensitivity syndrome. *Clin. Endocrinol.* 37, 214-220.

Schena, M., Freedman, L.P. & Yamamoto, K.R. (1989) Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. *Genes Dev.* 3, 1590-1601.

Schüle, R., Rangarajan, P., Klieuer, S., Ransome, L.J., Bolado, J., Yang, N., Verma, I.M. & Evans, R.M. (1990) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* 62, 1217-1226.

Schwabe, J.W.R., Neuhaus, D. & Rhodes, D. (1990) Solution structure of the DNA binding domain of the oestrogen receptor. *Nature* 348, 458-461.

Sharpe, R.M., Maddocks, S., Millar, M., Kerr, J.B., Saunders, P.T.K. & McKinnel, C. (1992) Identification of stage-specific, androgen-regulated proteins secreted by adult rat seminiferous tubules. *J. Androl.* 13, 172- 184.

Sharpe, R.M. (1993) Experimental evidence for Sertoli-germ cell and Sertoli-Leydig cell interactions. In: *The Sertoli Cell.* First Edition. Russell, L.D. & Griswold, M.D., eds. Cache River Press.

Sheffield, V.C., Cox, D.R., Lerman, L.S. & Myers, R.M. (1989) Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl. Acad. Sci. USA.* 86, 232-236.

Simental, J.A., Sar, M., Lane, M.V., French, F.S. & Wilson, E.M. (1991) Transcriptional activation and nuclear targetting signals of the human androgen receptor. *J. Biol. Chem.* 266, 510-518.

Simpson, E., Chandler, P., Goulmy, E., Disteche, C.M., Ferguson-Smith, M.A. & Page, D.A. (1987) Separation of the genetic loci for the H-Y antigen and for testis determination on human Y chromosome. *Nature* 326, 876-878.

Simpson, E., Chandler, P., Goulmy, E., Ma, K., Hargreave, T.B. & Chandley, A.C. (1993) Loss of the 'azoospermia factor' (AZF) on Yq in man is not associated with loss of HYA. *Hum. Mol. Genet.* 2, 469-471.

Stefanis, C., Papapetropoulos, T., Scarpalezos, S., Lygidakis, G. & Panayiotopoulos, C.P. (1975) X-linked spinal and bulbar muscular atrophy of late onset— a separate type of motor neurone disease? *J. Neurol. Sci.* 24, 493-503.

Strahle, U., Klock, G. & Schutz, G. (1987) A DNA sequence of 15 base pairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression. *Proc. Natl. Acad. Sci. USA* 84, 7871-7875.

Taylor, J.W., Schmidt, W., Cosstick, R., Okruszek, A. & Eckstein, F. (1985a) The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. *Nucleic Acid Res.* 13, 8749-8764.

Taylor, J.W., Ott, J. & Eckstein, F. (1985b) The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acid Res.* 13, 8765-8785.

Templeton, A., Fraser, C. & Thompson, B. (1990) The epidemiology of infertility in Aberdeen. *Br. Med. J.* 301, 148-152.

Tilley,W.D., Marcelli, M., Wilson, J.D. & McPhaul, M.J. (1989) Characterisation and expression of a cDNA encoding the human androgen receptor. *Proc. Natl. Acad. Sci. USA* 86, 327-331.

Tincello, D.G., Hargreave, T.B., Wu, F.C., Padayachi, T. & Saunders, P.T. (1992a) Mutations in the androgen receptor gene of patients with androgen insensitivity. *J. Endocrinology* 132 *Supp.*, Abs. 87.

Tincello, D.G., Wu, F.C.W., Hargreave, T.B. & Saunders, P.T.K. (1992b) Temperature gradient gel electrophoresis can detect point mutations in the androgen receptor gene in patients with androgen insensitivity. *J. Endocrinol.* 135 *Supp.*, Abs. P27.

Tincello, D.G., Hargreave, T.B., Saunders, P. T. & Wu, F.C. (1993) Clinical, biochemical and molecular aspects of and response to high dose testosterone in male pseudohermaphroditism (Reifenstein syndrome). *J. Endocrinol.* 137 *Supp.*, Abs. P63.

Trifiro, M., Gottlieb, B., Pinsky, L., Kaufman, M., Prior, L., Belsham, D.D., Wrogemann, K., Brown, C.J., Willard, H.F., Trapman, J., Brinkmann, A.O., Chang, C., Liao, S., Segovich, F. & Jung, J. (1991) The 56/58 kDa androgen-binding protein in male genital skin fibroblasts with a deleted androgen receptor gene. *Mol. Cell. Endocrinol.* 75, 37-47.

Trifiro, M., Prior, R.L., Sabbaghian, N., Pinsky, L., Kaufman, M., Nylen, E.G., Belsham, D.D., Greenberg, C.R. & Wrogemann, K. (1992a) Amber mutation creates a diagnostic Mae I site in the androgen receptor gene of a family with complete androgen insensitivity. *Am. J. Med. Genet.* 40, 493-499.

Trifiro, M., Pinsky, L., Bordet, S., Alvarado, C., Sabbaghian, N., Lumbroso, R., Mhatre, A., Beitel, L. & Kaufman, M. (1992b) Transregulatory competence of mutant androgen receptors (AR) in the presence of androgen-receptor complex instability. *74th Meeting US Endocrine Soc. Abs.* 1007.

Tsai, S.Y., Carlstedt-Duke, J., Weigel, N.L., Dahlman, K., Gustafsson, J-Å., Tsai, M-J. & O'Malley, B.W. (1988) Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. *Cell* 55, 361-369.

Tsilfidis, C., MacKenzie, A.E., Mettler, G., Barcelo, J., Korneluk, R.G. (1992) Correlation between CTG trinucleotide repeat length and frequency of severe congenital myotonic dystrophy. *Nat. Genet.* 1, 192-195.

Umesono, K. & Evans, R.M. (1989) Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* 57, 1139-1146.

Vandeyar, M.A., Weiner, M.P., Hutton, C.J. & Batt, C.A. (1988) A simple and rapid method for the selection of oligodeoxynucleotide-directed mutants. *Gene* 65, 129-133.

van Laar, J.H., Bolt-de-Vries, J., Zegers, N.D., Trapman, J. & Brinkmann, A.O. (1990) Androgen receptor heterogeneity and phosphorylation in human LNCaP cells. *Biochem. Biophys. Res. Commun.* 166, 193-200.

van Laar, J.H., Berrevoets, C.A., Trapman, J., Zegers, N.D. & Brinkmann, A.O. (1991) Hormone dependent androgen receptor phosphorylation is accompanied by receptor transformation in human lymph node carcinoma of the prostate cells. *J. Biol. Chem.* 266, 3734-3738.

Veldscholte, J., Ris-Stalpers, C., Kuiper, G.G., Jenster, G., Berrevoets, C., Claasen, E., van Rooij, H.C., Trapman, J., Brinkmann, A.O. & Mulder, E. (1990) A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem. Biophys. Res. Commun.* 172, 534-540.

Vergnaud, G., Page, D.C., Simmler, M-C., Brown, L., Rouyer, F., Noel, B., Botstein, D., de la Chapelle, A. & Weissenbach, J. (1986) A deletion map of the human Y chromosome based on DNA hybridisation. *Am. J. Hum. Genet.* 38, 109-124.

Verkerk, A.J.M.H., Pieretti, M., Sutcliffe, J.S., Fu, Y-H., Kuhl, D.P.A., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F., Eussen, B.E., van Ommen, G.J.B., Blonden, L.A.J., Riggins, G.J., Chastain, J.L., Kunst, C.B., Galjaard, H., Caskey, C.T., Nelson, D.L., Oostra, B.A. & Warren, S.T. (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell* 65 905-914.

Wallace, R.B., Johnson, M.J., Suggs, S.V., Miyoshi, K-I., Bhatt, R. & Itakura, K. (1981) A set of synthetic oligodeoxyribonucleotide primers for DNA sequencing in the plasmid vector. *Gene* 16, 21-26.

Wieringa, B., Meyer, F., Reisner, J. & Weissman, C. (1983) Unusual splice sites revealed by mutagenic inactivation of an authentic splice site of the rabbit β globin gene *Nature* 301, 38-43.

Wilson, E.M., Simental, J.A., French, F.S. & Sar, M. (1991) Molecular analysis of the androgen receptor. *Ann. N.Y. Acad. Sci.* 637, 56-63.

Wilson, J.D. (1992) Syndromes of androgen resistance. *Biol. Reprod.* 46, 168-173.

Wilson, C.M., Griffin, J.E., Wilson, J.D., Marcelli, M., Zoppi, S. & McPhaul, M.J. (1992) Immunoreactive androgen receptor expression in subjects with androgen resistance. *J. Clin. Endocrinol. Metab.* 75, 1474-1478.

Wooster, R., Mangion, J., Eeles, R., Smith, S., Dowsett, M., Averill, D., Barrett-Lee, P., Easton, D.F., Ponder, B.J. & Stratton, M.R. (1992) A germline mutation in the androgen receptor gene in two brothers with breast cancer and Reifenstein syndrome. *Nat. Gen.* 2, 132-134.

Youssoufian, H., Kazazian, H.H., Phillips, D.G., Arouis, S., Tsiftis, G. & Brown, W.A. (1986) Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. *Nature* 324, 380-382.

Zoller, M.J. & Smith, M. (1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. *Meth.. Enzymol.* 100, 469-500.

Zoppi, S., Marcelli, M., Deslypere, J-P., Griffin, J.E., Wilson, J.D. & McPhaul, M.J. (1992) Amino acid substitutions in the DNA binding domain of the human androgen receptor are a frequent cause of receptor-positive androgen resistance. *Mol. Endocrinol.* 6, 409-415.

Zoppi, S., Wilson, C.M., Harbison, M.D., Griffin, J.E., Wilson, J.D. & McPhaul, M.J. (1993) Complete testicular feminization caused by amino-terminal truncation of the androgen receptor with downstream initiation. *J. Clin. Invest.* 91, 1105-1112.

# Appendix 1. Abbreviations

| | |
|---|---|
| ACD: | Acid citrate dextrose buffer |
| AR+: | Androgen receptor positive (androgen insensitivity) |
| AR-: | Androgen receptor negative (androgen insensitivity) |
| ATP: | Adenosine triphosphate |
| AZF: | Azoospermia factor |
| $B_{max}$: | Maximum binding sites |
| Complete AIS: | Complete androgen insensitivity syndrome |
| CAT: | Chloramphenicol acetyl transferase |
| cDNA: | Complementary DNA |
| CLB: | Cell lysis buffer |
| C-terminal: | Carboxy-terminal end of peptide chain |
| CTP: | Cytosine triphosphate |
| DGGE: | Denaturing gradient gel electrophoresis |
| DHT: | Dihydrotestosterone |
| $D_m$: | Concentration of denaturants at which DNA begins to separate into single strands |
| DMSO: | Dimethylsulphoxide |
| DNA: | deoxyribonucleic acid |
| DTT: | dithiothreitol |
| EDTA: | Ethylenediamine tetra-acetate |
| ERE: | Oestrogen response element |
| Exp.: | Experiment |
| FSH: | Follicle stimulating hormone |
| GRE: | Glucocorticoid response element |
| GSF: | Genital skin fibroblast |
| GTP: | Guanosine triphosphate |
| hCG: | human chorionic gonadotrophin |
| HRE: | Hormone response element |
| HSP: | Heat shock protein |
| IPTG: | isopropyl-β-thiogalactopyranoside |
| ITP: | Inosine triphosphate |
| Kd: | Dissociation constant |
| LH: | Luteinising hormone |
| LH-T: | LH x testosterone product |
| MIF: | Müllerian inhibitory factor |

| | |
|---|---|
| MMTV: | Mouse mammary tumour virus |
| MOPS: | 4-morpholinopropanesulphonic acid |
| mRNA: | Messenger ribonucleic acid |
| N-terminal: | Amino-terminal end of peptide chain |
| Partial AIS: | Partial androgen insensitivity syndrome |
| PCR: | Polymerase chain reaction |
| PLB: | Protein lysis buffer |
| rpm: | Revolutions per minute |
| rtPCR: | Reverse transcriptase PCR |
| SBMA: | Spinal and bulbar muscular atrophy |
| SHBG: | Steroid hormone binding globulin |
| SOC: | 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose |
| SSCP: | Single strand conformational polymorphism |
| Sustanon: | 30 mg testosterone propionate, 60 mg testosterone phenylpropionate, 60 mg testosterone isocaproate, 100 mg testosterone decanoate |
| TAE: | Tris/acetate/EDTA buffer: 40 mM Tris pH 8.0, 40 mM sodium acetate, 1 mM EDTA |
| TBE: | Tris/Borate/EDTA buffer: 90 mM Tris pH 8.0, 90 mM boric acid, 2 mM EDTA |
| TE: | Tris/EDTA buffer: 10 mM Tris.HCL pH 8.0, 1 mM EDTA |
| TEMED: | N, N, N', N'-tetramethylethylenediamine |
| TGGE: | Temperature gradient gel electrophoresis |
| $T_m$: | Temperature at which DNA begins to separate into single strands |
| TTP: | Thymidine triphosphate |
| UTP: | Uridine triphosphate |
| X-gal: | 5-bromo-4-chloro-3-indolyl-β-D-galactoside |
| Yp: | Short arm of Y chromosome |
| Yq: | Long arm of Y chromosome |

# Appendix 2. IUPAC codes for amino acids

| Amino acid | IUPAC code | Three letter code |
|---|---|---|
| Alanine | A | Ala |
| Arginine | R | Arg |
| Asparagine | N | Asn |
| Aspartic acid | D | Asp |
| Cysteine | C | Cys |
| Glutamic acid | E | Glu |
| Glutamine | Q | Gln |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Leucine | L | Leu |
| Lysine | K | Lys |
| Methionine | M | Met |
| Phenylalanine | F | Phe |
| Proline | P | Pro |
| Serine | S | Ser |
| Threonine | T | Thr |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Valine | V | Val |

# Appendix 3. Addresses of suppliers

Applied Biosystems Ltd.
Kelvin Close
Birchwood Science Park
Warrington
Cheshire WA3 7PB

Amersham International plc
Lincoln Place
Green End
Aylesbury
Buckinghamshire HP20 2TP

BioRad Laboratories Ltd.
BioRad House
Maylands Avenue
Hemel Hempsted
Hertfordshire HP2 7TD

Biosoft
22 Hills Road
Cambridge
Cambridgeshire
CB2 1JP

Cambridge Bioscience
25 Signet Court
Newmarket Road
Cambridge
Cambridgeshire CB5 8LA

Flow Laboratories
Irvine
Scotland
KA12 8NB

Flowgen Instruments Ltd.
Broad Oak Enterprise Village
Broad Oak Road
Sittingbourne
Kent ME9 8AQ

Hybaid UK Ltd.
111-113 Waldegrave Road
Teddington
Middlesex TW11 8LL

Pharmacia Biosystems Ltd.
Biotechnology Division
Davy Avenue
Knowlhill
Milton Keynes MK5 8PH

Promega Ltd.
Delta House
Enterprise Road
Chilworth Research Centre
Southampton SO1 7NS

# Appendix 4. Published abstracts & papers

## British Andrology Society: Anglo-Scandinavian Conference on Andrology, London, 6th - 7th December 1991

### Point mutations in the androgen receptor gene of patients suffering from androgen insensitivity syndrome.

D.G. Tincello, P.T.K. Saunders*, F.C.W. Wu* and T.B. Hargreave.
Department of Surgery, Western General Hospital, Crewe Road, Edinburgh and * MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh.

Androgens are responsible for development of the male phenotype. Syndromes of androgen insensitivity ranging in severity from slight undervirilisation to complete testicular feminisation have been described in patients with an XY karyotype (Grino *et al.,* 1988). These latter patients are phenotypically female but have a blind-ending vagina and lack a uterus and Fallopian tubes. They also have testes either intra-abdominally or within the inguinal canal. Typically they present with primary amenorrhoea or an inguinal hernia.

The first step in androgen action is binding to a specific cellular receptor (Carson-Jurica *et al.*, 1990). The cloning and sequencing of the androgen receptor (AR) cDNA and the mapping of a single gene to the X-chromosome has facilitated examination of its structure in patients with androgen insensitivity (Chang *et al.*, 1988; Lubahn *et al.*, 1988). We have examined the sequence of the androgen receptor gene in patients who are insensitive to androgen (testicular feminisation).

Whole blood was mixed with acid-citrate buffer and DNA extracted from the nuclei of leucocytes. Portions of DNA encoding the exons of the human androgen receptor gene were amplified by polymerase chain reaction using specific pairs of oligonucleotide primers (Lubahn *et al.*, 1989). The amplified DNA was purified and the sequence determined. To date we have detected single point mutations in two of four patients under investigation. In patient 1, a single base pair change (C to T) within an area of the receptor gene known to encode the steroid binding portion of the receptor, results in an arginine residue being replaced by cysteine. In patient 2 the codon for amino acid 831 (CGA= arginine) has been changed to a 'stop' codon(TGA). This patient would therefore appear to be unable to translate a full length receptor protein from her mRNA.

In conclusion, techniques of DNA amplification and sequencing have enabled us to pin point the genetic defects responsible for the abnormal phenotype of two patients suffering from complete androgen insensitivity syndrome.

## 11th Joint meeting of British Endocrine Societies, Harrogate, 23rd - 26th March 1992

### Mutations in the androgen receptor gene of patients with androgen insensitivity

(Tincello *et al.*, 1992a)

D.G. Tincello*, T.B. Hargreave*, F.C. Wu,T. Padayachi, and P T. Saunders. MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, &*University Department of Surgery, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, UK

Androgens are responsible for development of the male phenotype. Syndromes of androgen insensitivity range in severity from complete failure of virilisation (complete AIS) to partial androgen insensitivity (partial AIS) with hypospadias, gynaecomastia and small testes. The first step in androgen action is binding to a specific cellular receptor. The cloning and sequencing of the androgen receptor (AR) cDNA has facilitated examination of its structure. We have examined the sequence of the AR gene in patients with both complete AIS and partial AIS in order to determine the genetic basis of their androgen resistance.

Genomic DNA was extracted from whole blood. DNA encoding the exons of the AR gene was amplified by polymerase chain reaction (Lubahn *et al*, 1989), purified and sequenced. To date we have detected single point mutations in five of sixteen patients under investigation. In patient 1, (complete AIS), the codon for amino acid 831 (CGA= arginine) has been changed to a 'STOP' codon (TGA). This would produce a truncated receptor protein lacking a portion of the steroid binding domain. In patient 2, also with complete AIS, a single base pair change (C to T) within an area of the receptor gene coding for the steroid binding portion of the receptor, results in an arginine residue (855) being replaced by cysteine. Two unrelated patients with partial AIS have the same mutation substituting valine for leucine at position 866, again in the steroid binding region of the receptor. Patient 5, with partial AIS, has a mutation within the DNA binding domain of the receptor, changing a lysine to arginine at residue 607 of the second "zinc finger " of the domain, closely involved in DNA binding. This amino acid is conserved through the entire steroid receptor family. We plan to examine the androgen receptor gene in the remaining patients by the technique of temperature gradient gel electrophoresis. This method allows the analysis of DNA fragments on the basis of sequence composition (Myers *et al.*, 1985a).

In conclusion, techniques of DNA amplification and sequencing have enabled us to pin-point the genetic defects responsible for the abnormal phenotype of five patients suffering from varieties of androgen insensitivity. We find that both the nature of the amino acid substitution caused and its location can have a profound effect upon the function of the AR and the consequent phenotype of the individual.

**5th Simpson Symposium, 8th - 11th September, Edinburgh 1992**

## Detection of point mutations in the androgen receptor gene of patients with androgen insensitivity by the use of temperature gradient gel electrophoresis

D.G. Tincello†, F.C.W. Wu#, T.B. Hargreave† & P.T.K. Saunders*

*MRC Centre for Reproductive Biology, 37 Chalmers St., Edinburgh, UK;†University Department of Surgery, Western General Hospital, Edinburgh, UK;#Department of Medicine, University of Manchester Medical School, Salford, Greater Manchester, UK

Androgen insensitivity (AIS) is a rare X-linked inherited disorder characterised by a failure of androgen action. Patients either show an unambiguously female phenotype (complete AIS) in association with a total failure of androgen action; or a phenotype within a spectrum from ambiguous genitalia with labioscrotal fusion through hypospadias to isolated gynaecomastia, in conjunction with a partial defect in androgen action (partial AIS) (Wilson, 1992). Defects in the androgen receptor (AR) gene have been found in these patients ranging from deletions of whole or part of the gene (Brown *et al.*, 1988) to single mutations scattered throughout the eight exons of the gene, causing single amino acid substitutions, premature termination codons (Marcelli *et al.*, 1990a) and aberrant splicing of messenger RNA (Ris-Stalpers *et al.*, 1990).

The AR gene is coded for on eight exons and is over 3000 base pairs long. Screening of a large number of patients is very laborious. We have used the technique of temperature gradient gel electrophoresis (TGGE) to screen individual exons of the AR gene for mutations. TGGE involves the electrophoresis of DNA fragments in a polyacrylamide gel over a linearly increasing temperature gradient. At a certain temperature (Tm, governed by the sequence composition of the fragment) the DNA strands begin to separate and the fragment is retarded in the gel by these single stranded regions. A single base mutation is sufficient to alter the temperature (Tm) at which this separation occurs and fragments with different Tms will migrate different distances in the gel (Riesner *et al.*, 1989). Thus, samples which are mutated relative to control DNA can be easily identified by their final position in the gel. Only these identified samples need to be sequenced to determine the nature of the mutation.

We have established a TGGE system in our laboratory using samples from patients previously identified as having mutations as positive controls (Saunders *et al.*, 1992). In addition, using TGGE we have identified two novel point mutations in two more patients: a C to T mutation creating a premature termination codon in exon 5, in a patient with complete AIS; an A to G mutation causing the substitution of glycine for the normal glutamine at amino acid 772, in a patient with partial AIS. Thus TGGE is an efficient method for the detection of point mutations in the AR gene.

## Society for Endocrinology 183rd Meeting, London, 25th - 27th November 1992

### Temperature gradient gel electrophoresis can detect point mutations in the androgen receptor gene in patients with androgen insensitivity

(Tincello *et al.*, 1992b)

D.G. Tincello, F.C.W. Wu#, T.B. Hargreave & P.T.K. Saunders* University Department of Surgery, Western General Hospital, Edinburgh, UK; MRC Centre for Reproductive Biology, 37 Chalmers St., Edinburgh, UK; #Department of Medicine, University of Manchester Medical School, Salford, Greater Manchester, UK

TGGE is a fairly new technique for the analysis of DNA fragments on the basis of their sequence in addition to their size (Riesner *et al.*, 1989). Samples of DNA purified from a polymerase chain reaction are electrophoresed through a polyacrylamide gel subject to a linearly increasing temperature gradient. At a certain temperature (Tm, governed by the sequence composition of the fragment) the DNA duplex will partially separate and the fragment will be retarded in the gel. A single base mutation is sufficient to alter the temperature (Tm) at which this separation occurs and fragments with different Tms will therefore migrate different distances in the same gel. Thus, samples which are mutated relative to control DNA can be easily identified by their final position in the gel. Only these identified samples need to be sequenced to determine the nature of the mutation. This technique of denaturing gradient gel electrophoresis is able to detect up to 95% of mutations within a DNA fragment (Myers *et al.*, 1987). The method can be rendered more sensitive by PCR amplification of the fragment with a GC clamp attached to one end (Sheffield *et al.*, 1989).

Androgen insensitivity (AIS) is a rare X-linked inherited disorder characterised by a failure of androgen action. Affected patients are either phenotypically female, with absent internal genitalia and male gonads (complete insensitivity or complete AIS) or partially virilised, with ambiguous genitalia, hypospadias and gynaecomastia (Wilson, 1992). Causative defects in the androgen receptor (AR) gene have been found in these patients ranging from deletions of whole or part of the gene (Brown *et al.*, 1988) to single mutations scattered throughout the eight exons of the gene (Marcelli *et al.*, 1990b).

Detection of AR gene mutations in these patients can be laborious by standard techniques of gene sequencing and so we have established a TGGE system in our laboratory. Samples from patients previously identified as having mutations were used as positive controls (Saunders *et al.*; 1992). Subsequently, we have identified novel point mutations in two patients: a C to T mutation creating a premature termination codon in exon 5, in a patient with complete AIS; an A to G mutation causing the substitution of glycine for the normal glutamic acid at amino acid 772, in a patient with partial AIS.

## 12th Joint Meeting of British Endocrine Societies, Liverpool, 29th March - 1st April 1993

### Clinical, biochemical & molecular aspects of and response to high dose testosterone in male pseudohermaphroditism (Reifenstein syndrome)

(Tincello *et al.*, 1993)

D.G. Tincello, T.B. Hargreave, P T. Saunders* and F.C. Wu. University Department of Surgery, Western General Hospital, Edinburgh EH4 2XU; *MRC Reproductive Biology Unit, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9EW, & Dept. Medicine, Hope Hospital, Salford, Manchester.

Androgen insensitivity (AIS) is an X-linked inherited disorder characterised by a failure of androgen action. Reifenstein's syndrome is one of the clinical manifestations of the partial form of the syndrome, where the individual presents with an obviously male phenotype, but is poorly virilised (Wilson, 1992). There is usually marked hypospadias, gynaecomastia and the testes are small and often undescended. Secondary sexual development is impaired. Defects in the androgen receptor (AR) gene have been found in patients with AIS ranging from deletions of whole or part of the gene to point mutations scattered throughout the gene, mainly in the exons encoding the hormone binding or DNA binding domains.

We present two men with Reifenstein's syndrome. Patient 1 had hypospadias, gynaecomastia, testicular maldescent, and a small penis. Patient 2 had ambiguous genitalia at birth, was reared as a boy and given androgen therapy with little response. His genealogy shows an X linked recessive trait of impaired virilisation. Genital skin fibroblasts (GSF) from patient 1 had a normal Bmax & elevated Kd for androgen; dissociation was shown to be biphasic. GSF from patient 2 showed normal Bmax & Kd. Nuclear localisation of liganded receptor was impaired. Both men were subjected to high dose testosterone therapy and serum testosterone and gonadotrophins measured. Patient 1 showed some suppression of gonadotrophins during administration of 5 mg/kg/day testosterone propionate; patient 2 had little change during the entire period. Patient 1 developed positive nitrogen balance, but no change in sebum secretion. Patient 2 also developed positive nitrogen balance, but showed a normal response in sebum secretion.

The AR gene of both men has been sequenced. Patient 1 has a mutation of arginine 608 to lysine in exon 3 (Saunders *et al.*; 1992) and patient 2 a mutation in exon 5 from glutamic acid 772 to glycine (Tincello *et al.*, 1992b). The site of these mutations correlates with the *in vivo* and *in vitro* data above. A mutation in exon 5 (hormone binding domain) results in a mild reduction in binding affinity for androgen, which can be overcome by high dose therapy; a mutation in exon 3 (DNA binding domain) impairs the ability of the receptor to bind to DNA and activate target gene, and this effect is not altered by high dose androgens.

Clinical Endocrinology (1992) 37, 214–220

# Point mutations detected in the androgen receptor gene of three men with partial androgen insensitivity syndrome

**P. T. K. Saunders, T. Padayachi, D. G. Tincello, S. M. Shalet* and F. C. W. Wu**

*MRC Reproductive Biology Unit, Edinburgh, and *Department of Medicine and Endocrinology, Christie Hospital & Holt Radium Institute, Wilmslow Road, Manchester M20 9BX, UK*

*(Received 14 January 1992; returned for revision 6 February 1992; finally revised 6 March 1992; accepted 8 April 1992)*

**Summary**

**OBJECTIVE Determine the sequence of the androgen receptor gene in men with impaired responsiveness to androgens in order to identify the molecular basis of their under-virilization.**

**DESIGN Blood samples were used as the source of genomic DNA. Portions of the androgen receptor gene were amplified by polymerase chain reaction and sequenced.**

**PATIENTS Samples were obtained from three patients and five normal fertile controls. Patients were all 46 XY and were undervirilized with ambiguous external genitalia, gynaecomastia and infertility.**

**MEASUREMENTS Total cellular DNA was purified from peripheral blood leucocytes. Pairs of oligonucleotide primers designed to flank the individual exons of the androgen receptor gene were synthesized. The specific regions of the androgen receptor were amplified from the samples of cellular DNA by polymerase chain reaction. Amplified DNA was purified, sequenced and compared to the published sequence.**

**RESULTS In all three patients point mutations in the androgen receptor gene were detected but no defects were detected in samples from normal controls. In two of the patients, an identical single nucleotide change from G to T was detected. This nucleotide was within the codon for amino acid 866 and would change it from valine to leucine. Amino acid 866 is found within an area of the steroid binding domain thought to be involved in receptor dimerization. Within the repetitive sequence of exon I patient 1 had 21 glutamine residues and patient 2 had 25. In the third patient a single change of G to A would result in incorporation of lysine in place of a conserved arginine residue at position 607 within the second zinc finger of the DNA binding domain. The sequence of the androgen receptor gene of the mother of the third patient revealed her to be heterozygous for the same defect.**

**CONCLUSION Patients 1 and 2 are unrelated although they have an identical point mutation in their androgen receptor gene. A patient with complete androgen insensitivity syndrome has been reported to have a defect at the same position causing the amino acid substitution of methionine for valine. Therefore we confirm that the nature of the amino acid change in the peptide sequence of the androgen receptor as well as its location within the protein, can have a profound effect on the phenotypic severity of androgen resistance. Studies on mutated receptors from individuals with a wide range of degrees of androgen resistance may enable us to construct a map of the key amino acids in the different domains of the protein.**

Correspondence: Dr Philippa T. K. Saunders, MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh EH3 9EW, UK.

Androgens are essential for the determination, maintenance and expression of the male phenotype. They are responsible for masculinization of the brain, the reproductive tract and the external genitalia during development *in utero* (Bardin, 1986). Subsequently, androgens are responsible for the development and maintenance of secondary sexual characteristics, libido and fertility. A spectrum of androgen resistance syndromes has been described ranging in severity from complete feminization to slight under-virilization with normal fertility (Griffin & Wilson, 1989; Grino *et al.*, 1988).

Normal androgen action is initiated by the interaction of the steroid with a specific receptor expressed in target tissues. The cDNA for the human androgen receptor (AR) has been cloned, sequenced and its amino acid structure determined (Chang *et al.*, 1988; Lubahn *et al.*, 1988). The AR is a member of the steroid receptor family in which the basic structural organization of the receptor cDNA and protein is highly conserved (Carson-Jurnica *et al.*, 1990) and can be considered as a series of amino acid domains which act separately, and cooperatively, to confer the full function of the receptor. The AR is encoded on eight exons (Lubahn *et al.*, 1989; Marcelli *et al.*, 1990). The hormone-binding domain which confers steroid specificity on the AR is found towards the C-terminus of the protein encoded on exons 4 to 8 (Carson-Jurnica *et al.*, 1990; Ris-Stalpers *et al.*, 1990). The DNA binding domain is characterized by the presence of two zinc fingers, which are encoded on exons two and three and are in the mid region of the receptor. The zinc fingers interact

with specific recognition sequences of androgen regulated genes, the hormone-response elements (HRE), to cause alterations in the rate of gene transcription (Carson-Jurnica *et al.*, 1990). The N-terminal domain has recently been shown to interact with other domains to promote full transcriptional activity (Simental *et al.*, 1991).

Patients with female phenotype diagnosed as suffering from complete androgen insensitivity (testicular feminization or CAIS) have been investigated for genetic defects. There is a clear pattern of familial inheritance of the syndrome via heterozygous carrier females consistent with the location of a single AR gene on the X chromosome (Lubahn *et al.*, 1988). Defects in the AR gene of individuals with CAIS have been detected which appear to be responsible for their inability to respond to endogenous androgens. The alterations documented range from deletions and premature stop codons (Brown *et al.*, 1988; Marcelli *et al.*, 1990; Ris-Stalpers *et al.*, 1990; Quigley *et al.*, 1991) to point mutations in the AR coding sequence leading to changes in key amino acids in the steroid-binding or DNA-binding domains (Lubahn *et al.*, 1989; Zoppi *et al.*, 1991). Limited information has been available for patients with the milder forms of androgen resistance. However, recently McPhaul and co-workers (1991) have described a family with undervirilization where two defects in the receptor protein appeared to act cooperatively to impair the ability of the receptor protein to activate genes. In addition, at a recent meeting, single point mutations have been recorded for patients described as suffering from partial AIS (De Bellis *et al.*, 1991; Chang *et al.*, 1991).

In this paper we present data from three patients with clinical features of partial AIS, in whom we have detected point mutations in their AR gene. Each of the changes found would cause an alteration in an amino acid incorporated into a key domain of the AR protein consistent with impaired functioning of the receptor *in vivo*.

## Materials and methods

### *Patients*

Peripheral blood was obtained from three patients with incomplete androgen insensitivity and from five normal men of proven fertility. Informed consent was obtained from each individual. Patient 1 is a 36-year-old man (46 XY) who had several operations for hypospadias as a child (the severity was unrecorded). At age 13 he developed bilateral gynaecomastia requiring simple mastectomy. He has never needed to shave. His pubic hair has a female distribution, the penis is 3 cm in length and the testes measure 6 ml and 8 ml. He is married and is able to have intercourse, but has never produced an ejaculate (i.e. he is azoospermic). Serum testosterone is elevated at 90 nmol/l, FSH > 32 U/l, LH > 32U/l, with no response to a gonadotrophin releasing hormone test. Genital skin fibroblasts failed to grow in culture and so 5α-reductase activity was not measured.

Patient 2 is 35 years old and the youngest of three brothers. The whole family have under-virilization and have been reported by Jukier *et al.* (1984). Patient 2 had perineo-scrotal hypospadias requiring a two stage repair, gynaecomastia and a high pitched voice. Pubic hair distribution was reasonable but he had scanty facial hair. He is azoospermic with a serum testosterone of 62·3 nmol/l, FSH of 16·1 U/l and LH of 24·1 U/l. His two brothers also had third degree penile hypospadias, a bifid scrotum and developed gynaecomastia. They both have sparse pubic hair and a female distribution of body fat. Specific dihydrotestosterone binding activity of genital skin fibroblasts of the patient's brothers showed a normal $B_{max}$, but the equilibrium dissociation constant was 1·16 nmol/l, compared to 0·22 nmol/l in normal men. Unfortunately, the cell line derived from patient 2 was lost before any studied could be performed (Jukier *et al.*, 1984). The patient showed normal 5α-reductase activity, although his brother had virtually undetectable levels. Thus, the clinical picture in the brothers appears to be a combination of 5α-reductase deficiency and impaired androgen receptor function. By inference, patient 2 would appear to have the qualitative abnormality of androgen receptor action in isolation.

Patient 3 is 48 years old (46 XY) and was diagnosed as suffering from Reifensteins's syndrome; he was born with ambiguous external genitalia (minute phallus, bilateral inguinal testes) and received weekly androgen injections to age 5. At 18 years old bilateral mastectomy was performed because of marked gynaecomastia. The right testis has been biopsied and the left testis removed following repeated epididymitis; both contained Sertoli cell-only seminiferous tubules and abundant Leydig cells. In cells from a genital skin biopsy specific high affinity binding sites for 5α-dihydrotestosterone was 50 fmol/mg protein (normal range 16·5–55·0) with a *K*d of 0·2 nmol/l (normal 0·05–0·53) at 37°C; 5α-reductase activity was normal (M. B. Hodgkins, University of Glasgow, unpublished results). This patient has not virilized despite repeated courses of androgen therapy. In his family, abnormalities of male sexual development have been evident in three generations consistent with transmission of an X-linked recessive trait via carrier females (Fig. 1). Blood samples were obtained from his mother, his sister and her two daughters.

### *Isolation of genomic DNA and amplification of AR*

Blood (10 ml) was mixed with acid–citrate–dextrose buffer (1 ml/10 ml whole blood) and DNA extracted as detailed by

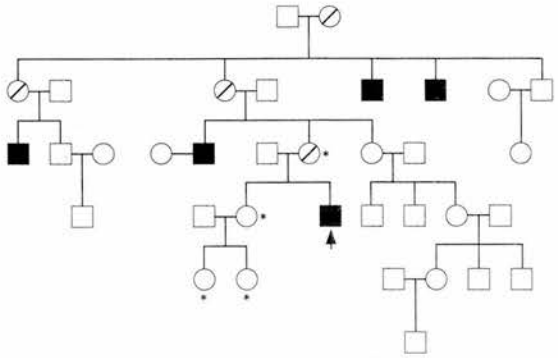


**Fig. 1** Family history of patient 3 showing the incidence of partial androgen insensitivity in males from three generations. The affected individuals (closed boxes; patient 3 arrowed) had high pitched voices, gynaecomastia, hypospadias, and were hypogonadal. * Individuals from whom blood was obtained; ○, carrier females. The carrier status of individuals not directly examined was inferred from the appearance of the defect in their offspring. The patient was examined by one of us (FCWW) and the family tree prepared from information supplied by him.

Sambrook *et al.* (1989). Polymerase chain reaction (PCR; Saiki *et al.*, 1988) was performed either using pairs of oligonucleotide primers (18–20 mers) detailed below or those published by Lubhan *et al.* (1989). Oligonucleotide primers (written 5′–3′): exon 6–7 = 5′ (2845-) TATCGCATGCA-CAAGTCCCG, 3′ (3097-rev) AAGCGTCTTGAGCAG-GATGT; exon 7–8 = 5′ (3005-) TCTTTGATGAACTTCG-AATGA, 3′ (3288-rev) TCACAGGGTGTGGAAATAGA. The standard PCR reaction mixture was as follows: 100 μl/tube containing 50 mM KCl, 10 mM Tris-HCl, pH9 at 25°C, 1·5 mM $MgCl_2$, 0·01% gelatin, 0·1% Triton X100, 100 μM dNTPs (Pharmacia Ltd, Milton Keynes, UK), 0·5 μM each primer, 2·5U *Thermus aquaticus* DNA polymerase (Taq polymerase, Promega Ltd, Southampton, UK) and 0·5–1 μg genomic DNA. Amplification conditions typically consisted of an initial melt at 94°C for 2 min followed by 30 cycles of melting for 0·5 min at 94°C, annealing for 1 min at 55°C and extension for 0·5–1 min at 72°C. Amplified double stranded AR DNA was purified by gel electrophoresis in NuSieve agarose (FMC, Flowgen), recovered using a Geneclean kit (USB, Cambridge BioSciences, UK) and quantified. DNA was denatured by boiling and sequenced using Sequenase (Cambridge BioSciences) according to the standard protocol and GTP or ITP nucleotide mixes but with the following minor modifications. Dimethyl sulphoxide (DMSO) was added to DNA during the annealing reaction (6:1, DNA:DMSO) and to termination mixes at a ratio of 9:1 (mix:DMSO).

## Results

Amplified exons were of the expected size in all controls and patients. Direct sequencing of PCR amplified DNA from normal fertile controls revealed no differences from the published sequence (Chang *et al.*, 1988) consistent with the PCR amplification producing a faithful copy of the original DNA. In contrast, in the patients with partial AIS, the following point mutations were detected. In exon 7 of patients 1 and 2 there was a single base pair change (G to T) in the codon for amino acid 866 (Fig. 2). The codon TTG replacing the normal GTG at this position results in the inclusion of leucine in place of valine in this part of the AR which has been identified as part of the steroid-binding domain. DNA amplified from exon 1 to include the hyper-variable domain (which begins at amino acid 62; Chang *et al.*, 1988) of patients 1 and 2 was found to be of different lengths on agarose gels. Direct sequencing confirmed that patient 1 had 21 glutamine residues within this region whilst patient 2 had 25.

Patient 3 had a single nucleotide substitution in exon 3 where a change from G to A (Fig. 3) results in a change in the amino acid in position 607 from arginine to lysine; he had a normal complement of 23 glutamine residues in exon 1. This arginine is within the 'tip' of the second zinc finger of the DNA binding domain (Fig. 4) and is conserved in other members of the steroid receptor family. His mother was found to be heterozygous for the defective AR whilst his sister and her daughters had normal DNA sequence for the AR on both of their X-chromosomes (data not shown). Amplification and sequencing of exons in which base pair changes were detected was repeated three times. Except for the changes detailed above no other changes have been detected in these patients.

## Discussion

Three male patients with partial AIS, characterized by under-virilization with ambiguous external genitalia, gynaecomastia and infertility, were investigated to determine whether they had mutations in the gene encoding their androgen receptor (AR). In all cases point mutations in the nucleotide sequence were detected which would give rise to a change in one amino acid in the AR protein. In patients 1 and 2 an identical mutation was detected in the codon for amino acid at position 866. This would result in a change from valine to leucine in the AR protein. Sequence of the polymorphic homopolymeric (CAG) repeats in exon 1 revealed 21 codons in patient 1 and 25 codons in patient 2 in this position. This therefore confirms family investigations which indicated that they are not related.

It is notable that Lubahn and co-workers (1989) have

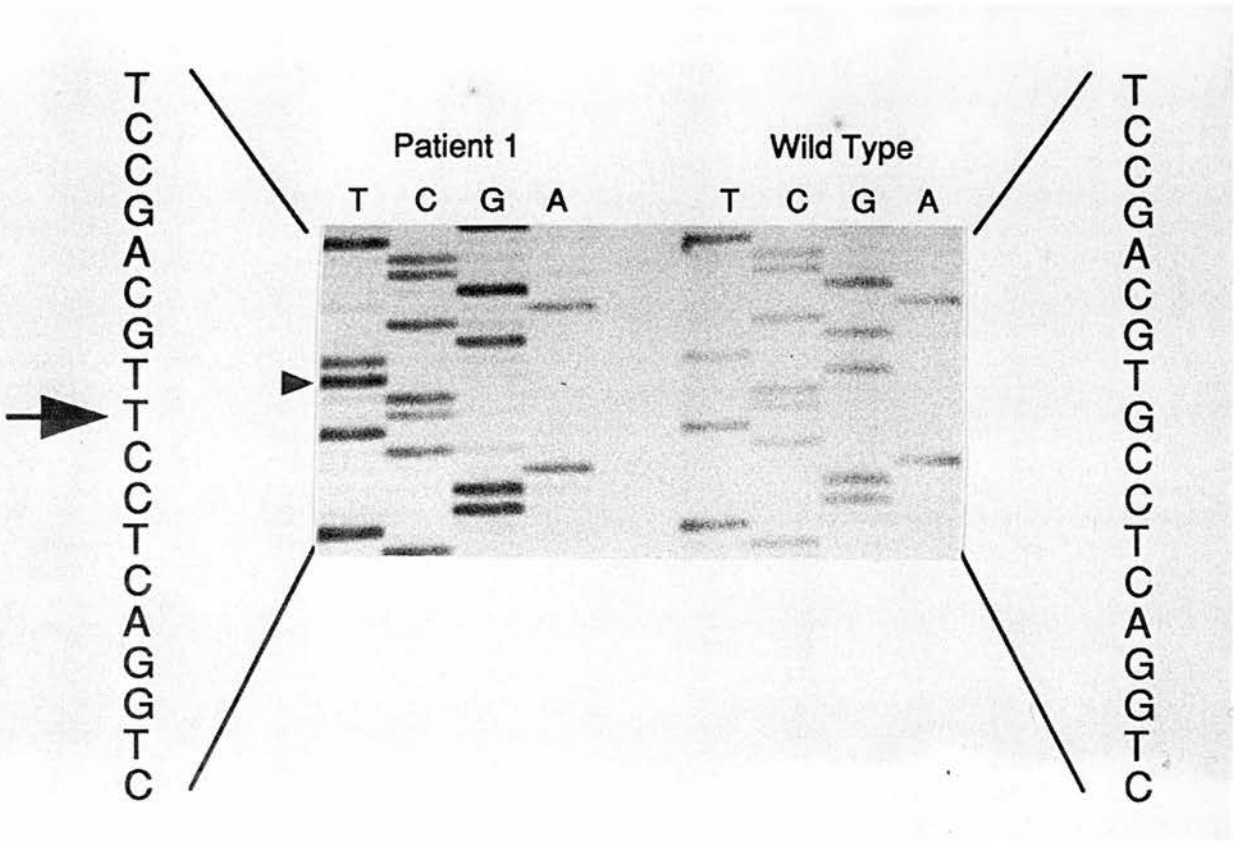



**Fig. 2** Sequence data from exon 7 of patient 1 compared to that of a control. A single base pair change (G to T) in the codon for amino acid 866 was observed (arrowed).

described a patient with complete AIS in which a point mutation at the same nucleotide resulted in incorporation of methionine in place of valine. Examination of the amino acid structure of valine and leucine reveals the slight nature of the change in patients 1 and 2, with both being non-polar and only differing by a single methyl group (Fig. 3). In the patient with complete AIS (Lubahn *et al.*, 1989; Fig. 3) the amino acid substitution (methionine in place of valine) results in a more substantial change in the size and structure of the amino acid than that seen in our patients with partial AIS. Brown *et al.* (1990) used transfection of COS cells with mutant AR to recreate this defect *in vitro* and found that an AR with methionine in this position was able to activate a reporter gene but only at concentrations of steroid unlikely to occur naturally.

Work on steroid receptors has indicated that amino acid 866 falls within a portion of the receptor protein involved in dimerization after ligand binding (Fawell *et al.*, 1990). One of the key hydrophobic amino acids in the dimerization domain of the AR is believed to be valine 866, a finding consistent with the amino acid at this position having a profound effect on function of the receptor *in vivo*. The finding of an identical base change in these two patients would support the idea advanced in a previous paper (Lubahn *et al.*, 1989), that this site is a 'hot-spot' for mutation.

In favour of this hot-spot theory, Ris-Stalpers and co-workers (1991) have similarly identified two different point mutations in three unrelated families at codon 686, a conserved aspartic acid in exon 4. In these families changes of this amino acid to histidine or asparagine resulted in impaired receptor function and a CAIS phenotype.

In patient 3 a nucleotide substitution in exon 3 results in a codon for lysine in place of arginine. His mother was heterozygous for the same defect. The amino acid so changed (607) is found within the second zinc finger and is conserved as arginine in all members of the steroid receptor family (Carson-Jurnica *et al.*, 1990). Previous clinical investigation of this man had indicated that the binding of androgen to receptor protein from this individual was in the normal range, consistent with the finding of normal nucleotide sequence in the steroid binding domain. Deletion of the entire second zinc finger of the AR has been detected in a

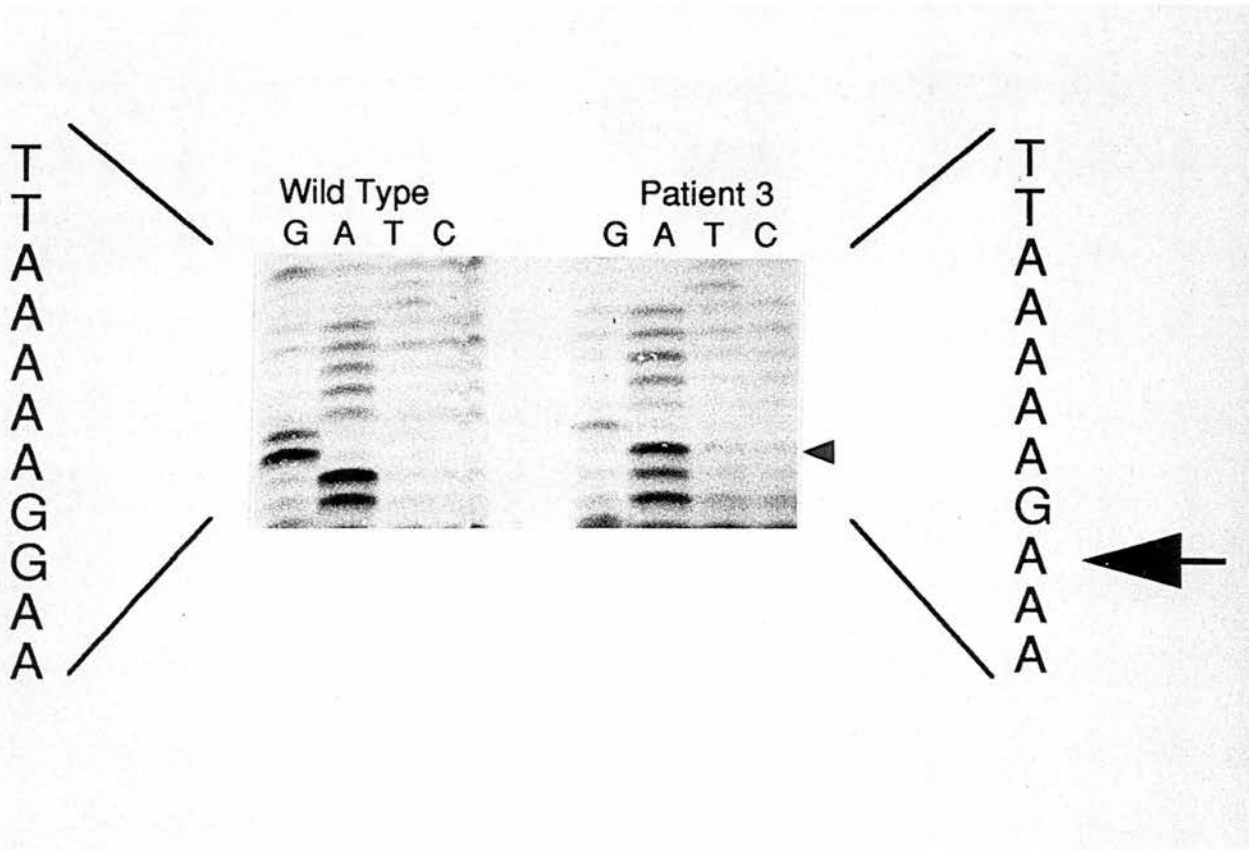



**Fig. 3** Sequence data from exon 3 of patient 3 compared to a normal control. A single base pair change (G to A) was detected.

patient with complete AIS (Quigley *et al.*, 1990). In addition, De Bellis *et al.* (1991) report a point mutation at amino acid 616 (leucine to arginine) in a partial AIS subject and Marcelli *et al.* (1991) a change from arginine to proline at position 615 in a patient with complete AIS.

The role(s) of the two zinc fingers in determination of the specificity of interaction with hormone response elements and subsequent modification of gene transcription has been the subject of several studies. Work on oestrogen receptors has demonstrated that the identity of the three amino acids at the base of the first zinc finger plays a key part in the recognition specificity of the receptor for the androgen receptor hormone response element (Mader *et al.*, 1989). Studies in which mutations were introduced into the second zinc finger of the glucocorticoid receptor have suggested that this region extends away from the DNA and interacts with proteins of the transcription machinery (Hard *et al.*, 1990). In patient 3, binding of the occupied AR to the hormone response element may occur but the rate of activation of transcription of androgen-regulated genes may be reduced compared to normal. All patients had a complement of CAG repeats in exon 1 within the normal range of 17 to 26. McPhaul and co-workers (1991) found this region to be truncated in a partial AIS subject and La Spada *et al.* (1991) found it to be much increased in size in individuals with X-linked spinal and bulbar muscular atrophy. Confirmation of the effect of the mutations described in this paper on the biological function of the AR will be explored further by transfection and dimerization studies.

In patients with *complete* AIS substitutions tend to involve a change in both the size *and* charge of the amino acid, e.g. arginine to cysteine; arginine to glutamine (Brown *et al.* 1990), an exception being the substitution of methionine for valine at position 866 where only the size of the amino acid is changed (Lubahn *et al.*, 1989). Data for two unrelated individuals in which the codon for amino acid 855 (arginine) was changed support this hypothesis. An arginine to cysteine (basic to neutral) change was detected in a complete AIS patient (De Bellis *et al.*, 1991) whilst substitution of histidine (basic to basic) resulted in partial AIS (Chang *et al.*, 1991).

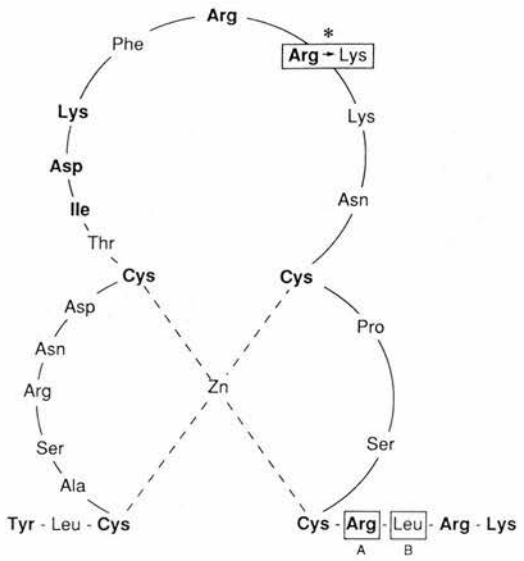



**Fig. 4** Amino acid sequence of the second zinc finger of the AR drawn to be homologous with the structure of that of the glucocorticoid receptor (Severne *et al.*, 1988), showing the location of the amino acid substitution in patient 3 (arginine to lysine, *). Amino acids conserved between the different members of the steroid receptor family are shown in bold type. Other mutations reported for patients with AIS are boxed: A, Arg 615 to Pro (Zoppi *et al.*, 1991); B, Leu 616 to Arg (De Bellis *et al.*, 1991).

Our data, in which partial AIS resulted from 'conservative' amino acid substitutions; arginine to lysine (basic to basic) and valine to leucine (neutral to neutral) are consistent with these findings.

In conclusion, examination of previous reports suggest to us that the nature of the amino acid substitution as well as its location can have a profound influence on the function of the receptor protein and consequent phenotype of the individual. Studies on mutated AR from individuals with a wide range of degrees of androgen resistance has contributed to identification of some of the key amino acids within the different domains of the AR.

**Acknowledgements**

We thank Jillian Charlton, Sharon Maguire, Sr E. Kacser, Dr Richard Sharpe, Edward Pinner and Professor C. R. W. Edwards and the staff of the Department of Medicine, Western General Hospital, Edinburgh for their assistance. Drs Chang and Liao (University of Chicago) kindly made available plasmid containing AR cDNA used in setting up of the methods. T. Padayachi was supported by ICI (South Africa), and D. Tincello by the Scottish Home and Health Department.

**References**

Bardin, C.W. (1986) Pituitary-testicular axis. In *Reproductive Endocrinology. Physiology, Pathophysiology and Clinical Management* Second edition. (ed. S. S. C. Yen & R. B. Jaffe), pp. 177–199. Saunders Co.

Brown, T.R., Lubahn, D.B., Wilson, E.M., Joseph, D.R., French, F.W. & Migeon, C.J. (1988) Deletion of the human androgen receptor gene in one family with complete androgen insensitivity syndrome: evidence for further genetic heterogeneity in this syndrome. *Proceedings of the National Academy of Sciences, USA*, **85,** 8151–8155.

Brown, T.R., Lubahn, D.B., Wilson, E.M., French, F.S., Migeon, C.J. & Corden, J.L. (1990) Functional characterisation of naturally occurring mutant androgen receptors from subjects with complete androgen insensitivity. *Molecular Endocrinology*, **4,** 1759–1772.

Carson-Jurnica, M.A., Schrader, W.T. & O'Malley, B.W. (1990) Steroid receptor family: structure and functions. *Endocrine Reviews*, **11,** 201–220.

Chang, C., Kokontis, J. & Liao, S. (1988) Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science*, **240,** 324–326.

Chang, Y.T., Migeon, C.J. & Brown, T.R. (1991) Human androgen insensitivity syndrome due to androgen receptor gene point mutations in subjects with normal androgen receptor levels but impaired biological activity. *73rd Annual Meeting of The Endocrine Society, USA*, Abstract 28.

De Bellis, A., Quigley, C.A., Cariello, N.F., Ho, K.C. & French, F.S. (1991) DNA and steroid binding domain mutations of the androgen receptor cause partial or complete androgen insensitivity. *73rd Annual Meeting of The Endocrine Society, USA*, Abstract 1141.

Fawell, S.E., Lees, J.A., White, R. & Parker, M.G. (1990) Characterisation and co-localisation of dimerisation activities in mouse oestrogen receptor. *Cell*, **60,** 956–62.

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

Grino, P.B., Griffin, J.E., Cushard, W.G. & Wilson, J.D. (1988) A mutation of the androgen receptor associated with partial androgen resistance, familial gynaecomastia and fertility. *Journal of Clinical Endocrinology and Metabolism*, **66,** 754–761.

Hard, T., Kellenbach, E., Boelens, R., Maler, B.A., Dahlman, K., Freedman, L.P., Carstedt-Duke, J., Yamamoto, K.R., Gustafsson, J-A. & Kaptein, R. (1990) Solution structure of the glucocorticoid receptor DNA-binding domain. *Science*, **249,** 157–159.

Jukier, L., Kaufman, M., Pinsky, L. & Peterson, R.E. (1984) Partial androgen resistance associated with secondary 5α-reductase deficiency: Identification of a novel qualitative androgen receptor defect and clinical implications. *Journal of Clinical Endocrinology and Metabolism*, **59,** 679–689.

La Spada, A.R., Wilson, E.M., Lubhan, D.B., Harding, A.E. & Fishbeck, K.H. (1991) Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature*, **352,** 77–79.

Lubahn, D.B., Brown, T.R., Simental, J.A., Higgs, H.N., Migeon, C.J., Wilson, E.M. & French, F.S. (1989) Sequence of the intron/exon junctions of the coding region of the human androgen

receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proceedings of the National Academy of Sciences, USA*, **86,** 9534–9538.

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

Mader, S., Kumar, V., de Verneuil, H. & Chambon, P. (1989) Three amino acids of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-responsive element. *Nature*, **388,** 271–274.

Marcelli, M., Tilley, W.D., Wilson, C.M., Griffin, J.E., Wilson, J.D. & McPaul, M.J. (1990) Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at amino acid residue 588 causes complete androgen resistance. *Molecular Endocrinology*, **4,** 1105–1116.

Marcelli, M., Zoppi, S., Grino, P.B., Griffin, J.E., Wilson, J.D. & McPhaul, M.J. (1991) A mutation in the DNA-binding domain of the androgen receptor gene causes complete testicular feminisation in a patient with receptor-positive androgen resistance. *Journal of Clinical Investigation*, **87,** 1123–1126.

McPhaul, M.J., Marcelli, M., Tilley, W., Griffin, J.E., Isidro-Gutierrez, R.F. and Wilson, J.D. (1991) Molecular basis of androgen resistance in a family with a qualitative abnormality of the androgen receptor and responsiveness to high dose androgen therapy. *Journal of Clinical Investigation*, **87,** 1413–1421.

Quigley, C.A., Friedman, K.J., Lafreniere, R.G., Silverman, L.M., Willard, H.F. & Lubhan, D.B. (1991) Androgen insensitivity due to total deletion of the androgen receptor gene: analysis of a family and of carrier status. *73rd Annual Meeting of The Endocrine Society, USA*. Abstract 1144.

Quigley, C.A., Simental, J.A., Evans, B.A., Lubahn, D.R., Hughes, I.A. & French, F.S. (1990) Androgen insensitivity due to deletion of the second zinc finger of the androgen receptor. *72nd Meeting of the Endocrine Society. USA*. Abstract 793.

Ris-Stalpers, C., Kuiper, G.G.J.M., Faber, P.W., Schweikert, H.U., van Rooij, H.C.J., Zegers, N.D., Hodgkins, M.B., Degenhart, H.J., Trapman, J. & Brinkman, A.O.. (1990) Aberrant splicing of androgen receptor mRNA results in synthesis of a nonfunctional receptor protein in a patient with androgen insensitivity. *Proceedings of the National Academy of Sciences, USA*, **87,** 7866–7870.

Ris-Stalpers, C., Trifiro, M.A., Kuiper, G.G.J.M., Jenster, G., Romalo, G., Sai, T., van Rooij, H.C.J., Kaufman, M., Rosenfeild, R.L., Liao, S., Schweikert, H-U., Trapman, J., Pinsky, L. & Brinkman, A.O. (1991) Substitution of aspartic acid 686 by histidine or asparagine in the human androgen receptor leads to a functionally inactive protein with altered hormone-binding characteristics. *Molecular Endocrinology*, **5,** 1562–1569.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, **239,** 487–494.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*. Second edition. New York: Cold Spring Harbour Laboratory Press.

Severne, Y., Wieland, S., Schaffner, W. & Rusconi, S. (1988) Metal-binding finger structures in the glucocorticoid receptor defined by site-directed mutagenesis. *EMBO Journal*, **7,** 2503–2508.

Simental, J.A., Sar, M., Lane, M.V., French, F.S. & Wilson, E.M. (1991) Transcriptional activation and nuclear targeting signals of the human androgen receptor. *Journal of Biological Chemistry*, **266,** 510–518.

Zoppi, S., Marcelli, M., Griffin, J.E. & Wilson, J.D. (1991) Point mutations in the DNA-binding domain are a frequent cause of receptor-positive androgen resistance. *73rd Annual Meeting of The Endocrine Society, USA*, Abstract 254.