MUTATIONS IN THE HUMAN ANDROGEN RECEPTOR
AND
THE ANDROGEN INSENSITIVITY SYNDROME

MUTATIES IN DE HUMANE ANDROGEENRECEPTOR
EN
HET ANDROGEEN-ONGEVOELIGHEIDS SYNDROOM

PROEFSCHRIFT

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Voor Eik, Pap, Mam, Nan en Sirbas

aa	amino acid
AIS	androgen insensitivity syndrome
AMH	anti-müllerian hormone
AR	androgen receptor
bp	base pairs
cAIS	complete androgen insensitivity
(c)DNA	(complementary) deoxyribonucleic acid
DHT	5α-dihydrotestosterone
DGGE	denaturing gradient gel electrophoresis
ER	estrogen receptor
FSH	follicle-stimulating hormone
GR	glucocorticoid receptor
GRE	glucocorticoid response element
hCG	human chorionic gonadotropin
HRE	hormone response element
kb	kilobase
kDa	kilo Dalton
Kd	dissociation constant
LH	luteinizing hormone
LNCaP	human lymph node carcinoma of the prostate (cell line)
(m)RNA	(messenger) ribonucleic acid
MR	mineralocorticoid receptor
MW	molecular weight
pAIS	partial androgen insensitivity
PR	progesterone receptor
RAR	retinoic acid receptor
RFLP	restriction fragment length polymorphism
R1881	methyltrienolone (synthetic androgen)
R5020	promegestone (synthetic progestagen)
RXR	retinoid X receptor
SBMA	spinal and bulbar muscular atrophy (Kennedy syndrome)
SSCP	single strand conformation polymorphism
T	testosterone
TDF	testis determining factor
Tfm	testicular feminization
TR	thyroid hormone receptor
UTR	untranslated region
VDR	1,25-dihydroxy-vitamin D3 receptor

CHAPTER 1

GENERAL INTRODUCTION

PREFACE

Hormone target cells contain receptors to detect incoming signals, and these receptors are responsible for the transmission of the signal. Like all other steroid hormones, the male sex hormones testosterone and 5α-dihydrotestosterone (androgens) are unable to evoke a biological response by themselves, but depend on a cellular receptor (the androgen receptor) to convey their signal. Impaired functions of the human androgen receptor are implicated in several pathophysiological situations.

In depth investigations concerning the role of the androgen receptor in the androgen insensitivity syndrome (AIS) started in the 1970ties. The cloning and characterization of the human androgen receptor gene have resulted in a major shift, from biochemical investigations of the androgen receptor protein in genital skin fibroblasts of AIS patients towards molecular studies of the androgen receptor gene. Mutations in the androgen receptor gene are considered responsible for aberrant or absent virilization in most if not all genetic male (46,XY) individuals with unimpaired testosterone production and metabolism.

Most prostate cancers are initially androgen responsive and this feature marks treatment with anti-androgens as a therapy for prostate cancer. In most patients with prostate cancer, however, an androgen-independent tumour eventually progresses. A possible role of the human androgen receptor in the switch from androgen responsive tumours to an androgen unresponsive state is a topic that could have implications for anti-androgen treatment of prostate cancer.

The androgen receptor is also being investigated in patients with the Kennedy syndrome, a motor-neuron disease. The increase in length of the polymorphic glutamine stretch in the N-terminal domain of the androgen receptor in these patients, correlates with the severity of this motor-neuron disease.

MALE SEXUAL DIFFERENTIATION AND THE ROLE OF THE ANDROGEN RECEPTOR

Human sexual development

In general there are three main events in human sexual development: the determination of genetic, gonadal and phenotypic sex.

During fertilization the **genetic** sex is determined. The ovum derived pronucleus that contains an X chromosome and 22 autosomes, fuses with a sperm derived pronucleus that contains either an X or an Y chromosome besides 22 autosomes.

The presence or absence of an Y chromosome in the embryo determines the development of the **gonadal** sex. The default pathway of human sexual differentiation is female and starts with ovarian differentiation of the embryonic bipotential gonad at 77-84 days of gestation. However, this default pathway only occurs when there has been no active initiation of testis formation. The development of the male structures requires active intervention and specific events in sexual differentiation of the male occur at earlier time points during development than the equivalent event in the female (Figure 1).

Sex steroids and anti-müllerian hormone (AMH), that are produced in the differentiating embryonic gonads, determine the **phenotypic** sex of a human embryo. The internal genitalia in males and females evolve from different embryonic structures, the wolffian and müllerian ducts respectively. The external genitalia and urethra of both sexes evolve from common structures (the genital tubercle, folds and swellings). The urogenital sinus develops into the prostate in the male, and into the vagina in the female.

Male sexual development

The first step in male sexual differentiation is the development of the testis from the embryonic bipotential gonad by 43-50 days of gestation. This event is

primarily initiated by the expression of the testis-determining gene that is located on the Y chromosome (sex-determining region of the Y chromosome; SRY). The SRY gene has been cloned by mapping of Y chromosome sequences present in XX males and absent in XY females (Palmer et al., 1990). The cDNA encodes a so-called high mobility group (HMG) protein, that contains a DNA binding domain (the HMG box). The ability of the SRY protein to bind DNA suggests that other genes 'downstream of SRY' in the sex determination pathway are subject to direct transcriptional control by SRY (Sinclair et al., 1990).

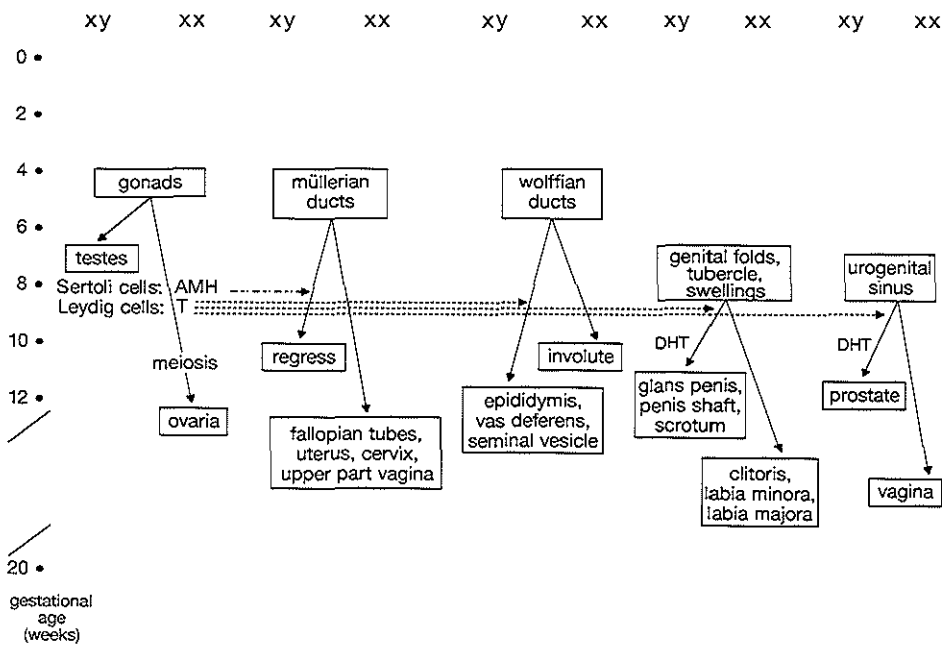

Figure 1: Flow chart of the differentiation of the human bipotential gonads, the internal and external genitalia in 46,XY males and 46,XX females from 0 to 20 weeks of gestational age. AMH: anti-müllerian hormone, T: testosterone, DHT: 5α-dihydrotestosterone.

Soon after the initial determination of the testis, the fetal Sertoli cells in the testis produce anti-müllerian hormone (AMH), a glycoprotein that is responsible for the active regression of the müllerian ducts in the male (Josso et al., 1977). Serum AMH remains detectable in human males during several years after birth, declining to very low adult levels by puberty (Hudson et al., 1990). AMH is undetectable in females before the onset of puberty when AMH, secreted by granulosa cells, is postulated to play a role in the regulation of oocyte maturation (Gustafson et al., 1993).

Leydig cells that differentiate from the interstitial cells in the testis become apparent by 60 days of gestation. The Leydig cells are responsible for the synthesis of testosterone through a multi-enzyme catalyzed process termed steroidogenesis. Testosterone is the major circulating androgen in men, and plays a critical role in the masculine differentiation of the male reproductive system. Testosterone is secreted into the bloodstream and is bound to plasma proteins (sex hormone-binding globulin and albumin) for about 98% (Westphal 1978). Initially, fetal testosterone production is autonomous and later comes under the influence of respectively human chorionic gonadotropin (hCG) from the placenta and luteinizing hormone (LH) from the fetal pituitary gland (Catt et al., 1975). LH release is regulated by the hypothalamic luteinizing hormone-releasing hormone (LHRH) and is inhibited by testosterone via a negative feedback mechanism (Schwartz and McCormack, 1972).

The local action of testosterone is responsible for the development of the wolffian ducts into epididymides, vasa deferentia, seminal vesicles and ejaculatory ducts in the male. This process is completed before 5α-reductase (see below) is expressed in these tissues (Siiteri et al., 1974).

In contrast to the development of the wolffian ducts, that is a testosterone dependent process, masculinization of the external genitalia and the development of the urogenital sinus into the urethra and the prostate occur, under the influence of the steroid hormone 5α-dihydrotestosterone (DHT), by 65-

77 days of gestation (Figure 1). DHT is actively formed from testosterone by the enzyme 5α-reductase, that is expressed in the urogenital sinus before the onset of fetal testicular testosterone secretion. A small amount of DHT is synthesized by the fetal human testis (George et al., 1987). The development of the wolffian ducts is regulated by testosterone, that is not converted to DHT due to the absence of 5α-reductase (Siiteri et al., 1974). During puberty and in later life, androgens are involved in the initiation and maintenance of spermatogenesis and the secondary sex characteristics.

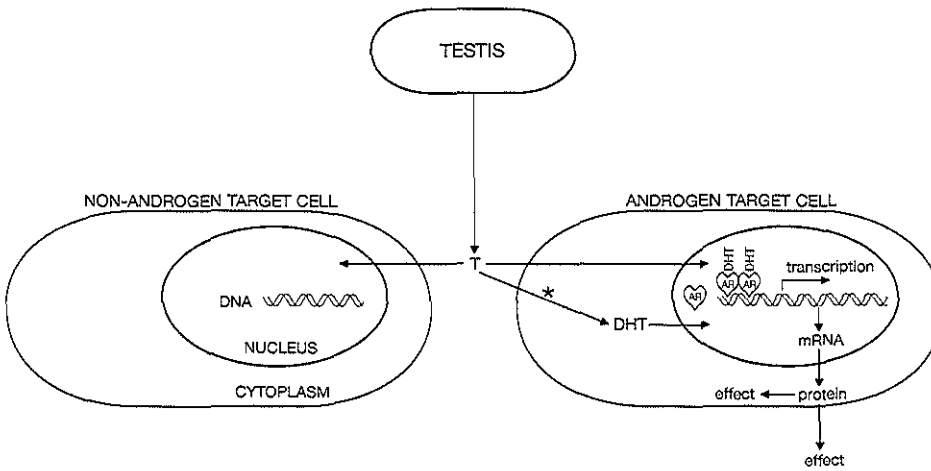

Figure 2: Schematic representation of the mechanism by which androgens regulate gene expression. Testosterone (T) enters the cell by passive diffusion and can be metabolized to 5α-dihydrotestosterone (DHT) by the androgen target cell specific enzyme 5α-reductase(*). T (in the absence of 5α-reductase) or DHT can activate the nuclear androgen receptor. The activated androgen receptor regulates the transcription of an androgen target gene.

The androgen receptor is an intracellular protein that is activated upon hormone binding and is then able to regulate the transcription of androgen target genes. The entering of testosterone into cells by passive diffusion across the cell

membrane prohibits any selectivity of androgen action at this transport level. The specificity of androgen action in androgen target cells is introduced and regulated by the availability of the enzyme 5α-reductase and by the androgen receptor (Figure 2). Both testosterone and 5α-dihydrotestosterone are able to fulfil their function only by interacting with the androgen receptor.

There is only one androgen receptor and it is an intriguing question why there are two different ligands. The mechanism by which the two ligands exert different physiological actions via the same receptor is not precisely known. A possibility might be that each ligand influences the structural constraint of the receptor-ligand complex differently, thereby permitting interaction with different transcription factors that result in the activation of different gene networks. On the other hand, the relative affinity of testosterone for the androgen receptor is approximately 38% of that of 5α-dihydrotestosterone (Veldscholte, 1993) and 5α-dihydrotestosterone is a more potent androgen than testosterone (Grino et al., 1990). This might also explain the different roles of testosterone and 5α-dihydrotestosterone during male sexual development.

Testosterone can also be aromatized to estradiol, which may result in an estrogenic response after interaction of estradiol with the estrogen receptor.

Aberrant male sexual differentiation

Male sexual differentiation is an intricate process in which many genes are involved. Consequently there are several disorders that can result in discrepancies between the genetic, gonadal and phenotypic sex of a newborn infant (McGillivray 1992).

There are three main forms of intersex conditions: disorders of gonadal differentiation, female pseudohermaphroditism and male pseudohermaphroditism.

Aberrant gonadal differentiation in a 46,XY individual can be the consequence of a mutation of the testis determining gene SRY (Swyer syndrome)(Behzadian

et al., 1991; Hawkins et al., 1992; Jager et al., 1990). The absence of a functional SRY protein will cause lack of testicular differentiation. The female internal and external genitalia remain infantile at puberty due to dysgenesis of the ovaria that are not capable to produce female sex hormones (Figure 3) (Disteche et al., 1986).

True hermaphroditism (the presence of both ovarian and testicular tissue) is a possible reason for aberrant gonadal differentiation. In Klinefelter's syndrome and Turner's syndrome the initial determination of gonadal sex is normal, but the development and function of the gonads is disturbed (for a complete review see Grumbach and Conte, 1992).

Female pseudohermaphroditism denotes the presence of ovarian tissue with partial virilization of the external genitalia. Congenital adrenal hyperplasia is the most common cause. Here the conversion of progesterone to cortisol is inhibited due to 21-hydroxylase (or other enzyme) deficiency resulting in increased androgen production by the adrenals and concomitant virilization in a female (White et al., 1987).

In **male pseudohermaphroditism** testis tissue is present in a 46,XY individual, but there is insufficient or no virilization resulting in ambiguous genitalia or a female phenotype. Male pseudohermaphroditism can be the result of a group of inherited autosomal recessive disorders caused by a deficiency of one of various enzymes involved in the conversion of cholesterol to testosterone (eg 17α-hydroxylase or 3β-hydroxysteroid dehydrogenase). Gonadotropin/Leydig cell abnormalities (failure of Leydig cells to respond to hCG or LH) are also possible causes of male pseudohermaphroditism (Rutgers 1991a). Furthermore, deficiency of the enzyme 5α-reductase impairs the conversion of testosterone into 5α-dihydrotestosterone. At birth individuals carrying a severe deficiency of this latter autosomal recessive disorder have a predominantly female phenotype, showing signs of virilization at puberty (Akgun et al., 1986; Imperato-McGinley, 1980). There is evidence for at least two different types of steroid 5α-reductase

enzymes in humans, and mutations in the steroid 5α-reductase type 2 gene are associated with 5α-reductase deficiency (Andersson and Russel, 1990; Jenkins et al., 1991; Wilson et al., 1993).

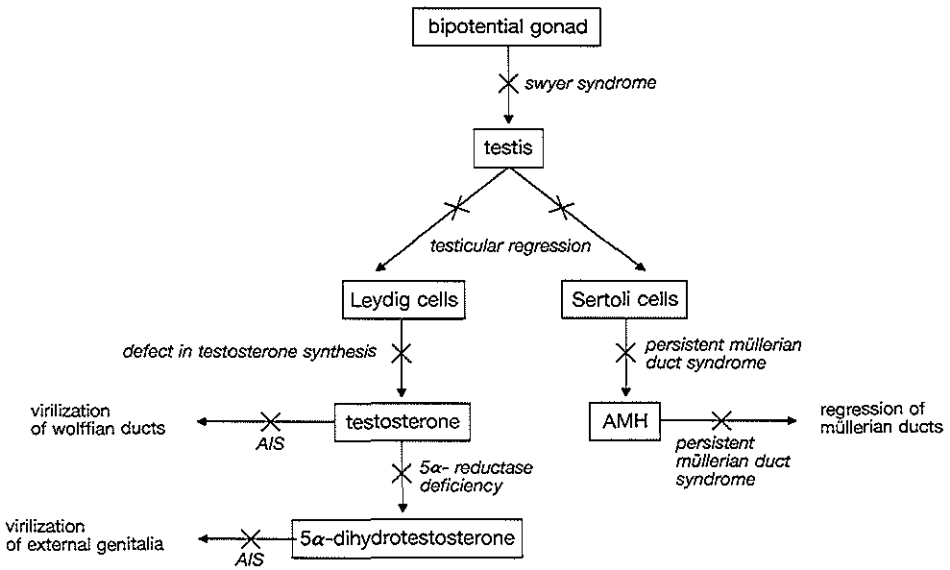

Figure 3: Male sexual differentiation of the indifferent bipotential gonad in a 46,XY embryo. Disturbances leading to intersex conditions are shown in italic.

Disturbances in the formation or action of AMH (persistent müllerian duct syndrome), and the embryonic testicular regression syndrome (vanishing testes) are also possible causes of male pseudohermaphroditism (Figure 3).

The androgen insensitivity syndrome (AIS), an X-linked genetic disorder in which both the embryonic and postnatal actions of androgens are impaired or absent due to a partly or completely non-functional androgen receptor, is the most common cause of male pseudohermaphroditism (Griffin and Wilson, 1989) (Figure 3). The incidence for complete androgen insensitivity is 1 in 60,000 male

births (Jagiello and Atwell, 1962). In the remaining part of the Introduction, the molecular characteristics of the androgen receptor and clinical consequences of mutations in the androgen receptor, will be addressed.

THE STEROID/THYROID HORMONE RECEPTOR SUPERFAMILY

The molecular structure of nuclear receptors

The human androgen receptor belongs to the still growing nuclear receptor family. This is a group of DNA binding regulatory proteins, of which the ability to modulate gene expression is controlled by the binding of a specific ligand. The ligands and their receptors are involved in the regulation of development and homeostasis in complex eukaryotes, and the receptors share a common structure (Beato, 1989; Evans, 1988). Other members of this family are the receptors for all other steroid hormones (estrogens, glucocorticoids, mineralocorticoids and progestagens), the thyroid hormone receptors, the receptors for retinoids and the vitamin D3 receptor (Figure 4).

Until late in the seventies, characterization and purification of steroid hormone receptors was attempted using radioactive ligands (Grody et al., 1982). Purification to homogeneity was unsuccessful because the receptor molecules are only present in trace amounts (10^3 to 10^4 binding sites per cell). The DNA and hormone binding properties, however, could be separated by limited proteolysis, suggesting that these functions were structurally organized in different domains (de Boer et al., 1987; Evans et al., 1988). The cDNA cloning and the deduction of the amino acid sequences of the different receptors has confirmed this prediction. Three discernible domains responsible for hormone binding, DNA binding and transactivation, respectively, have been identified. Characterization was done using mutational analysis combined with functionality studies (Dobson et al., 1989; Giguere et al., 1986; Kumar et al., 1987).

The C-terminal part of the receptor protein forms the *hormone-binding domain* that is involved in the specific binding of the receptor to its respective ligand. It is generally accepted that conserved amino acids of that region are involved in the shaping of a hydrophobic pocket, and that non-conserved residues provide the ligand selectivity. The ligand binding domain also contains regions for specific interaction with other (associated) proteins, such as the 90 kilodalton heat-shock protein (hsp90). The dissociation of the receptor complex from its associated proteins, induced by ligand binding, results in an conformational change leading to the exposure of the DNA-binding domain (Smith and Toft, 1993; Veldscholte et al., 1992).

The *DNA-binding domain* is located in the central core of the receptor protein and consists of the so-called DNA-binding zinc fingers, which are two outloopings of protein sequences each held in place by four conserved cysteine residues that coordinate with a zinc ion (Freedman et al., 1988). The C-terminal located zinc finger is assumed to be involved in protein-protein interactions, whereas the N-terminal zinc finger of the receptor is able to recognize and interact with specific DNA sequences, the hormone response elements in a target gene (Luisi et al., 1991). The high affinity binding of the nuclear receptor to a hormone response element in the promoter region of a target gene will influence the transcription of that particular gene. The DNA-binding and ligand-binding domains are able to function independently, as is demonstrated by the substitution of the DNA-binding domain of the human estrogen receptor with that of the human glucocorticoid receptor; this chimeric receptor is a functional receptor that interacts with a glucocorticoid response element, but there is a switch in ligand specificity (Green and Chambon, 1987).
Based on the high degree of homology between the DNA-binding domains of the members of the nuclear hormone receptor superfamily, a number of orphan receptors have been cloned from several vertebrates and Drosophila, for which

the ligand or the activator has not yet been identified (O'Malley, 1990; Segraves, 1991). The current theory is that the steroid receptor super-family has arisen from a common ancestor gene, a single multidomain precursor that initially mediated a simple signal transduction mechanism, and subsequently, through gene duplication and diversification, acquired increasingly complex functions (Amero et al., 1992; Evans, 1988; Picard et al., 1990).

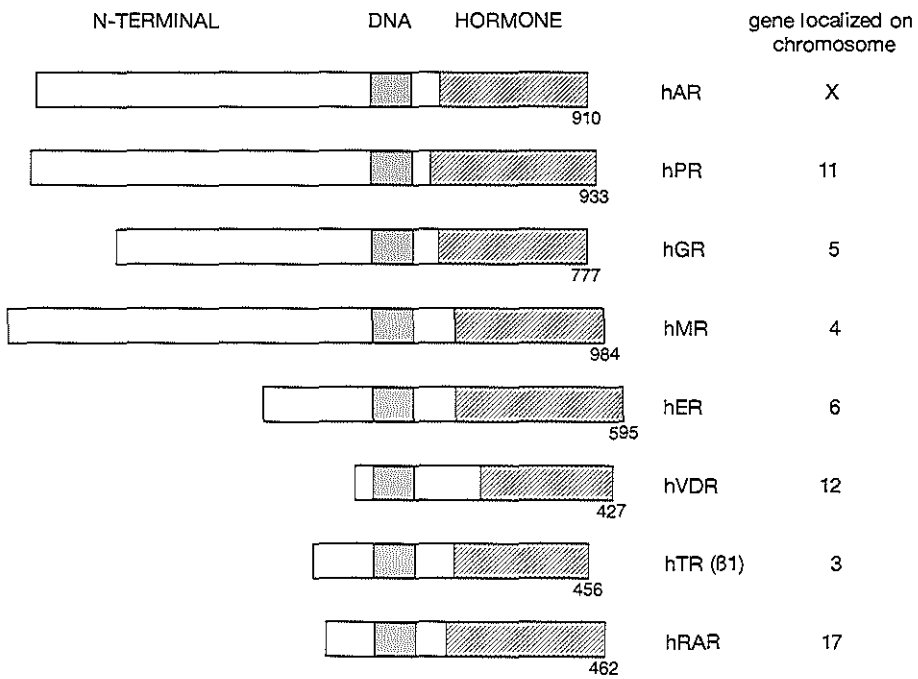

Figure 4: Members of the nuclear receptor family share a common molecular structure: an N-terminal transcription regulating domain (N-terminal), a central DNA-binding domain (DNA) and a C-terminal ligand-binding domain (hormone). The number of amino acid residues of the hAR (Faber et al., 1989; Trapman et al., 1988), hPR (Misrahi et al., 1987), hGR (Hollenberg et al., 1985), hMR (Arriza et al., 1987), hER (Green et al., 1986), hVDR (Baker et al., 1988), hRAR (Giguere et al., 1987) and hTR (Weinberger et al., 1986) are indicated.

The *N-terminal domain* of steroid/thyroid hormone receptor proteins is highly variable in size and in amino acid composition. This domain is indispensable for receptor functioning and may modulate receptor action by influencing transactivation and/or DNA binding (Kumar et al., 1987; Lees and Parker, 1989). Both the N-terminal domain and the steroid binding domain contain regions involved in transactivation.

Not only do the members of the nuclear receptor family display a high degree of homology but also the structure and organization of the regions in the promoters of the genes that are regulated by these receptors, show some similarity. Idealized hormone response elements are organized as two half sites, each 6 nucleotides with a 3 base pair gap, that exhibit an imperfect dyad symmetry axis at position 8. The response elements for the GR, PR, AR and MR resemble the idealized inverted repeat glucocorticoid response element (GRE) consensus (AGAACAnnnTGTTCT) (Freedman and Luisi, 1993). The TR and RAR are capable of activating an idealized inverted repeat with no gap (AGGTCATGACCT). The ER activates the same inverted repeat but requires a 3 bp gap (Forman et al., 1992). The promoter of the prostate-specific antigen gene contains a functional androgen response element (AGAACAgcaAGTGCT) (Riegman et al., 1991), that resembles the GRE consensus sequence.
The transcriptional activity of a nuclear receptor is also determined by protein-protein interactions. The classical steroid hormone receptors (ER and GR) interact with their HRE as homodimers. This is facilitated by the 3 bp gap in the HRE that leads to exposure of the HRE sequence half sites on the same side of the DNA helix. The half site spacing and orientation determine whether THR, RAR and VDR bind to the HRE as monomers, homodimers, or even as heterodimers with the receptor for 9-cis retinoic acid RXR. The spacing of the direct repeat determines the binding preference. A VDR:RXR heterodimer requires a 3 bp spacing, TR:RXR a 4 bp spacing, and a 5 bp spacing is required

for a RAR:RXR heterodimer (Forman et al., 1992; Green 1993). Also the interaction with other transcription factors according to the general model for transcriptional control by direct protein-protein interactions determines the nature and strength of the transcription regulating potential (Beato, 1991; Ptashne, 1988; Schüle et al., 1988; Truss et al., 1992).

Resistance syndromes

Resistance or insensitivity syndromes, due to molecular defects of a nuclear receptor, are recognized for the androgen receptor and most other members of the nuclear receptor family. The specific aspects of *androgen* insensitivity will be discussed later.

The diagnosis of an *estrogen* insensitive state is very rare, supposedly because estrogen action is essential for blastocyst implantation (George and Wilson, 1978). Recently, the disruption of the mouse estrogen receptor (ER) gene has been shown to alter reproductive function but the disruption of the estrogen receptor gene was not lethal (Lubahn et al., 1993). The absence of lethality however, might be the consequence of the remaining 5% estrogen binding activity that was detectable in animals homozygous for the estrogen receptor gene disruption (Lubahn et al., 1993). Localized estrogen resistance is seen in some breast cancers. Estrogen receptor mRNA variants have been seen in clinical breast cancer tissues that could result in aberrant receptors that prevent the action of the wild type estrogen receptor in these tissues (McGuire et al., 1991).

Vitamin D dependent rickets is a rare autosomal recessive syndrome characterized by hypocalcemia and early onset rickets despite an increase in the calcium regulating hormone 1,25-dihydroxy-vitamin D3. Mutations in the DNA-binding domain of the VDR have been identified (Hughes et al., 1988).

In *thyroid hormone* resistance, both the products from c-erbAα (TRα) and c-erbAß (TRß) genes, encoding respectively the α and β thyroid hormone

receptor, can be involved. Most of the 347 reported subjects suffer from generalized resistance to thyroid hormone (GRTH) that shows tight linkage to the c-erbAß locus (Refetoff et al., 1993). These subjects achieve a normal metabolic state at the expense of high levels of circulating thyroid hormone maintained by non-suppressed TSH secretion. GRTH is a familial autosomal dominant disorder, and single base substitutions and insertions have been identified in the ligand binding domain of the TRß. Transcriptional inactive heterodimers consisting of wild type and mutant thyroid hormone receptors have been shown to bind to DNA in a dominant negative manner, inhibiting access to DNA of the wild type receptor homodimer (Parrilla et al., 1991; Nagaya and Jameson, 1993; Usala et al., 1991; Weiss et al., 1993).

Generalized compensated *glucocorticoid* resistance is the partial form of primary cortisol resistance characterized by increased ACTH and cortisol secretion, without clinical evidence of hypercortisolism. The clinical symptoms are the manifestations of androgen and/or mineralocorticoid excess due to high ACTH levels, causing hypertension and hyperandrogenism. In affected subjects both alleles of the glucocorticoid receptor are mutated (Hurley et al., 1991; Karl et al., 1993). For the TR or the GR the associated resistance syndrome is always partial since complete insensitivity for the action of cortisol or thyroid hormone is incompatible with life.

Although resistance syndromes as a result of mutations in nuclear receptors are not uncommon, they usually are relatively mild defects because there is nearly always a compensating effect from the other allele.

Since the AR gene is localized on the X chromosome, the clinical symptoms of androgen insensitivity in XY individuals due to a mutation in the androgen receptor gene are not compensated for by a wild type androgen receptor translation product from another allele. Mutations in the human androgen receptor causing the complete androgen insensitivity syndrome (complete AIS) are not lethal and have no detrimental effects for the patient, apart from

phenotypic sex reversal and infertility. Therefore, mutations in the androgen receptor result in a large spectrum of aberrations in androgen receptor function and provide an opportunity to investigate the structure-function relationship of the human androgen receptor.

MOLECULAR STRUCTURE OF THE HUMAN ANDROGEN RECEPTOR

The relatively low tissue concentration of the androgen receptor and the extreme susceptibility to proteolytic breakdown, compared to other steroid hormone receptors, have hampered progress in androgen receptor characterization. Consequently, the cloning of the hAR cDNA was achieved at a relatively late date. The human androgen receptor cDNA has been cloned in 1988 by several laboratories (Chang et al., 1988; Lubahn et al., 1988a, 1988b; Tilley et al., 1989; Trapman et al., 1988). The androgen receptor cDNA has also been cloned for mouse (Faber et al., 1991; He et al., 1990), rat (Chang et al., 1988; Tan et al., 1988), dog, guinea pig and clawed frog (He et al., 1990).

The full length human androgen receptor cDNA is 10.6 kb, consisting of 1.1 kb 5'-untranslated region, a 2.7 kb open reading frame coding for a 910 amino acid protein (Figure 5) and a 6.8 kb 3'-untranslated region (Faber et al., 1991).

The androgen receptor locus spans over 90 kb and has been mapped to the q11-> q12 region of the human X chromosome (Brown et al., 1989; Lubahn et al., 1988a; Trapman et al., 1988), in accordance with the fact that human androgen receptor disorders show an X-linked pattern of inheritance. Within the population, a moderate-frequency HindIII restriction fragment length polymorphism (RFLP)(Brown et al., 1989) and a StuI polymorphism in exon 1 (Batch et al., 1992) have been observed.

The human androgen receptor gene is a single copy gene comprising 8 exon sequences (Faber et al., 1991; Kuiper et al., 1989) (Figure 5).

Exons 4-8 contain the information for the hormone-binding domain that contains approximately 250 amino acid residues (Figure 5). Exon 8 also encloses the 3'-UTR. Complete deletion of the steroid binding domain results in a constitutively active protein (Jenster et al., 1991; Simental et al., 1991). Transcriptional activation of the human androgen receptor is enhanced by testosterone, 5α-dihydrotestosterone, and by the synthetic androgens R1881 and mibolerone, in a dose-dependent and cell-specific manner. Also estradiol, progesterone and several anti-androgens show a weak activity (Kemppainen et al., 1992).

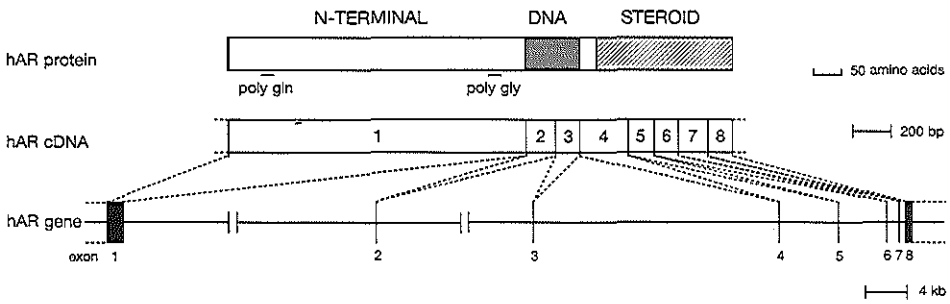

Figure 5: Relation between functional domains of the human androgen receptor protein (the N-terminal transcription regulating domain, the DNA-binding domain and the steroid-binding domain), the androgen receptor cDNA and the gene. The position of the polymorphic poly-glutamine and poly-glycine stretches are indicated.

Exons 2 and 3 encode the DNA-binding zinc fingers (Figure 5). The central part of the protein harbours the DNA-binding zinc fingers that are responsible for the tight nuclear binding to the hormone response element in an androgen target gene. A chimeric receptor protein containing the amino-terminus and DNA-binding domain of the hAR fused to the ligand-binding domain of the GR is functional after induction by glucocorticoids (Rundlett et al., 1990), demonstrating

the relative autonomy of the individual structural domains.

Exon 1 encodes the 529 amino acids N-terminal domain and includes the 5'-UTR (Faber et al., 1989, 1991) (Figure 5). One region in the N-terminal domain (amino acid residues 51-211) is essential for transcriptional activation (Jenster et al., 1991). A striking feature of the DNA sequence encoding the N-terminal domain of the androgen receptor are two polymorphic in-frame trimeric repeats, a GGN and a $(CAG)_n(CAA)$ repeat. The polymorphic GGN repeat encodes a glycine stretch, and the length of this stretch can vary from 16 to 24 glycine residues (Sleddens et al., 1993). The long GGN repeat seems to be a specific feature of the hAR; it is relatively short in the rat and mouse AR, and is not present in the human, rat and mouse GR, and in the human MR, PR and ER. This so-called Pen (GGN) repeat is also found in *Drosophila* homeotic genes, and can form a hinge region devoid of secondary structure, connecting distinct domains within a protein (Haynes et al., 1987). The function of the Pen repeat in the hAR is not clear.

The polymorphic $(CAG)_nCAA$ repeat encoding a glutamine stretch in the human androgen receptor between residues 58 and 77 (Sleddens et al., 1992), is absent at that location in the rat and mouse AR and in the hGR, hMR, hPR and hER. In the mouse and rat AR, the 'long' glutamine repeat is in the 192-196 region. The hAR also harbours a relatively short repeat of 6 glutamine residues at positions 83-88 and 192-196. The rat and mouse GR contain a glutamine stretch in this region, that varies in size between several rat strains (Gearing et al., 1993). The number of CAG repeats in the hAR is highly polymorphic and accounts together with the polymorphic GGN repeat, for the variation in amino acid number of the hAR, as reported by different laboratories. A practical application of these polymorphic tandem triplet repeats is their use in pedigree analysis in families with X-linked androgen insensitivity (Chapter 5).

The function of the polyglutamine stretch is not clear. In general there are three types of transcription activating domains: those with a net negative charge, those

with a high density of proline residues, and those with a preponderance of glutamine residues (Mitchell and Tjian, 1989). In general, GC rich sequence elements, as are present in the polymorphic repeats in the N-terminal domain of the hAR, are a target for the transcription factor Sp1 (Courey et al., 1989). The transcription factor TFIID, for example, contains 38 glutamine residues in the N-terminal region that is partly conserved between human and *Drosophila* (Peterson et al., 1990). This N-terminal region is necessary to achieve activation of transcription by the transcription factor Sp1. It is therefore likely that the polymorphic glutamine stretch in the hAR is directly involved in the regulation of transcription activation through interaction with Sp1 or other transcription factors.

The cloning of the human androgen receptor cDNA and the elucidation of the amino acid sequence has resulted in the development of polyclonal and monoclonal antibodies against the androgen receptor (van Laar et al., 1989; Zegers et al., 1991). These antibodies have greatly facilitated studies on the androgen receptor protein.

The androgen receptor in genital skin fibroblast cultures (Chapter 5) and in cell cultures derived from a human lymph node carcinoma of the prostate (LNCaP), migrates as a closely spaced 110-112 kDa doublet on SDS-PAGE (Brinkmann et al., 1988). In LNCaP cells, the upshift of the newly synthesized androgen receptor reflects receptor phosphorylation (Kuiper et al., 1991; van Laar et al., 1990). The androgen receptor in LNCaP cells is already phosphorylated in the absence of hormone, which perhaps is necessary for the acquisition of hormone binding capacity, and undergoes a hormone-induced additional phosphorylation that is accompanied by the acquisition of tight nuclear binding capacity (van Laar et al., 1991). Over 90% of the hormonally regulated phosphorylation sites are located in the N-terminal transactivating domain (Kuiper et al., 1993).

Studies on the LNCaP cell line showed that the unoccupied androgen receptor is associated with several heat-shock proteins (hsp90, hsp70, and hsp56), and

that transformation of the receptor to the tight nuclear binding form is a multistep process that involves the dissociation of heat-shock proteins from the receptor (Veldscholte et al., 1992).

The androgen receptor, which is synthesized in the cytoplasm, is too large for passive diffusion through the nuclear pores into the nucleus and the necessity for a nuclear localization signal is evident. Amino acid residues 608-625 contain a signal responsible for nuclear import of the human androgen receptor (Jenster et al., 1993). In the absence of ligand the androgen receptor is located mainly in the nucleus, and all cytoplasmic receptors translocate to the nucleus upon hormone binding (Jenster et al., 1991). Predominantly nuclear localization is also observed in glandular epithelial cells of the prostate (Husmann et al., 1990; Lubahn et al., 1988b), and in skin (Bläuer et al., 1991; Choudhry et al., 1992).

ANDROGEN INSENSITIVITY AND THE ROLE OF THE ANDROGEN RECEPTOR

Androgen insensitivity in a historical perspective

The initial recognition of the syndrome of androgen insensitivity dates back to 1937 when Pettersson and Bonnier (1937) described individuals with undescended testes in place of ovaria, who developed into externally unambiguous females.

Complete androgen insensitivity was initially termed testicular feminization (Tfm), because feminization occurred in the presence of testes that produced androgens but were also seen as the source of an estrogen-like hormone. This hormone was considered not to be estrogen because of the absence of pubic hair that was thought to represent an estrogen effect (Morris 1953). Later it was established that the presence of pubic hair is an androgen effect mediated by the androgen receptor. The first insight into the pathogenesis of Tfm comes from

Wilkins (1975), who concluded that the lack of virilization in an individual with Tfm, even after administration of androgens, resulted from resistance to the action of androgens. It is now accepted that androgen insensitivity is an X-linked genetic disorder in which defects in the androgen receptor have prevented normal sexual differentiation of internal and external genitalia.

There is some confusion in the literature with respect to the classification of 5α-reductase deficiency. Several research groups combine 5α-reductase deficiency and androgen receptor disorders under the heading androgen insensitivity (Griffin and Wilson, 1989; Pinsky et al., 1992). Others (Brown and Migeon, 1985; Verhoeven and Wilson, 1979; this thesis) keep to the original definition of Wilkins et al., (1975) where androgen insensitivity (testicular feminization) is defined based on the absence of virilization after administration of androgens. If 5α-dihydrotestosterone is administered to an individual with 5α-reductase deficiency, virilization will occur. High testosterone concentrations during puberty will also induce some virilization in these individuals. 5α-Reductase deficiency therefore is primarily an androgen biosynthesis defect. Both androgen receptor defects and 5α-reductase deficiency however, are disorders with physiological effects that are restricted to androgen target cells.

The diagnosis: complete or partial androgen insensitivity

Disorders of the androgen receptor constitute a wide clinical spectrum, where both the embryonic and postnatal actions of androgens are impaired or absent. Complete androgen insensitivity (cAIS) denotes the complete lack of virilization in a 46,XY individual with a normal synthesis and metabolism of testosterone. Often there are high plasma levels of testosterone and LH due to resistance of the negative feedback control exerted by testosterone at the hypothalamic-pituitary level. The most frequent prepubertal clinical presentation of cAIS is an inguinal hernia in an apparent female child. Overall, postpubertal primary amenorrhea combined with normal breast development and sparse pubic and

axillary hair, is the most frequent indication (Brown and Migeon, 1985; Griffin and Wilson, 1989; Pinsky and Kaufman, 1987) (Figure 6).

The exact definition of the null phenotype of the androgen insensitivity syndrome (cAIS) has been reported by Quigley et al., (1992) based on the phenotype of a 46,XY individual with a deletion on the X chromosome spanning the complete androgen receptor gene. The testis is abdominal or inguinal and wolffian duct development and masculinization of the external genitalia are completely absent. There is no true sexual terminal hair, only sparse vellus pubic and axillary hair. The presence of terminal pubic hair indicates some residual androgen action and is thus not in agreement with the diagnosis cAIS. The general physical and intellectual development is normal. In general, 46,XY individuals with cAIS are larger than normal females, but shorter than normal males (Varrela et al., 1984). This suggests the presence of genes on the Y chromosome with a general size increasing effect, and also that additive or inductive action of androgens is necessary for the completion of male body growth. The body shape of 46,XY cAIS individuals seems to be under control of estrogens and is not affected by the presence of the Y chromosome (Varrela et al., 1984). Carriers of cAIS, females with one wild type and one mutant AR allele, sometimes show a delayed menarche (Kaufman et al., 1976).

Several müllerian duct derivatives (fallopian tube, uterine smooth muscle) have been identified in surgically excised internal genital tissue of two patients with cAIS (Ulloa-Aguirre et al., 1990). This suggests that there may be some involvement of the androgen receptor with the action of anti-müllerian hormone (AMH), or that the enhanced and unopposed estrogenic milieu in the cAIS embryo might interfere with the action of AMH.

There is also a group of androgen receptor disorders that result from partial impairment of the androgen receptor (Figure 6). Clinical indications can be abnormal sexual development of individuals with a predominant male phenotype (hypospadias), or clitoromegaly in individuals with a predominantly female

phenotype or unexplained male infertility (Brown and Migeon, 1985; Griffin and Wilson, 1989; Pinsky and Kaufman, 1987). Relative or absolute excess of estrogens causes some aspects of feminization in men, particularly gynaecomastia (Wilson et al., 1980).

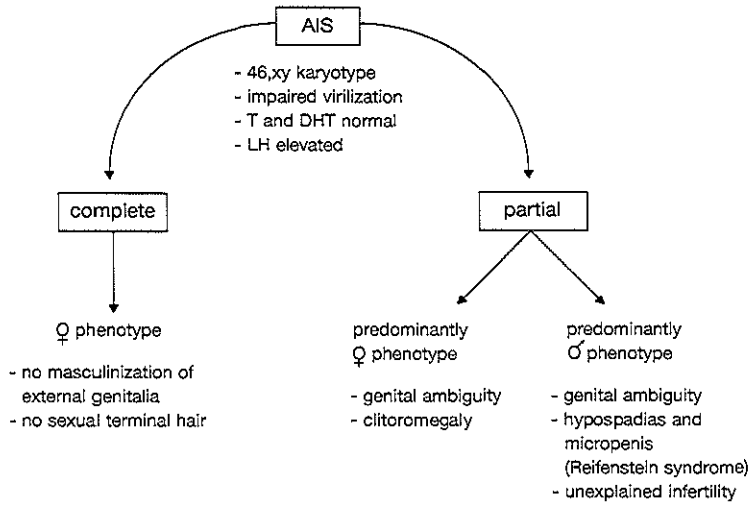

Figure 6: Clinical indications for complete or partial androgen insensitivity

The diagnosis partial androgen insensitivity can only be made by exclusion of all other possible causes of male pseudo-hermaphroditism or hypogonadism. If the clinical management of the androgen insensitivity is targeted towards further induction or maintenance of the female phenotype, the removal of the testes is indicated since they have propensity for development of tumours (Aiman and Griffin, 1982; Griffin and Wilson, 1989; Rutgers and Scully, 1991b).

The natural selection against propagation of mutations in the androgen receptor gene is high, due to the associated infertility of the affected 46,XY individuals.

Androgen insensitivity at the molecular level

Androgen insensitivity is a heterogeneous syndrome. Not only clinically but also at the biochemical and molecular level a spectrum of abnormalities has been identified. As a model for the molecular mechanism of androgen action in humans, skin fibroblasts are a useful tool. Skin is a target organ for androgens, and fibroblasts in culture retain the capacity to interact with androgens. These cells also are a source of DNA, are easily accessible, and lack receptors for progesterone and estradiol. The maximum binding capacity for androgens is about three times higher in genital skin fibroblasts (labial fold, scrotal, preputial or foreskin) than in pubic or non genital skin fibroblasts, whereas binding affinity values for androgens in the tissues are similar (Brown and Migeon, 1981).

The determination of the maximum binding capacity and dissociation constant of androgens is often measured using the synthetic androgen R1881 (methyltrienolone), that is not metabolized in genital skin and does not bind to the sex steroid binding globulin (Bonne et al., 1977). The absence of specific androgen binding sites in genital skin fibroblasts of AIS patients is termed receptor negative AIS. Absence of androgen binding sites indicates either the total absence of the androgen receptor protein or could point to a mutation in the steroid binding domain of the receptor that demolishes the binding capacity. There is also a group of AIS patients where specific androgen binding in genital skin fibroblasts is retained. This is termed receptor positive AIS. Within the latter group of receptor positive AIS, other qualitative receptor abnormalities, such as increased ligand dissociation from the receptor at physiological and/or elevated temperatures have been reported (Brown et al., 1982; Grino et al., 1989; Kaufman et al., 1981; Pinsky et al., 1981). Although the absence of receptor protein in these cases is excluded, mutations involving the ligand binding domain of the receptor can be expected. Mutations in target cell-specific and receptor associated factors are also a possible cause for AIS, but this is unlikely in case of impaired androgen binding or in case of X-linkage or an established mutation

(Pinsky et al., 1992).

In 40% of the phenotypically normal males with idiopathic infertility associated with diminished or absent sperm production, quantitative abnormalities of the androgen receptor were found in genital skin fibroblasts (Aiman and Griffin, 1982). Deficient spermatogenesis may in some cases be a mild form of androgen insensitivity. The main androgen involved in spermatogenesis is thought to be testosterone rather than 5α-dihydrotestosterone. There might be subtle defects in the androgen receptor that result in impaired sperm production while all other aspects of androgen action are unimpaired (Aiman and Griffin, 1982).

Based on ligand binding studies, a spectrum of androgen receptor defects has been described. There is no simple correlation between the level of androgen receptor binding capacity in genital skin fibroblasts and the clinical severity of the AIS, although absence of specific androgen binding is more often associated with cAIS than with pAIS (Griffin and Wilson, 1989).

ANDROGEN RECEPTOR DEFECTS IN OTHER PATHOLOGICAL SITUATIONS

Nuclear hormone receptors and cancer

Modulation of gene expression is important for intracellular signalling and plays a vital role in the control of cellular proliferation. It is therefore not surprising that abnormally active transcription factors may induce faulty gene expression (Bishop, 1991).

The first linkage between a member of the nuclear receptor family and cancer comes from the thyroid hormone receptor field. The structural relationship of the v-erbA oncogene to the human glucocorticoid receptor led to the proposal that the cellular homologue of v-erbA might encode a ligand-binding transcription

factor (Weinberger et al., 1985). This cellular homologue (c-erbA) was found to be the α-thyroid hormone receptor (Weinberger et al., 1986; Sap et al., 1986). The v-erbA oncogene is still able to bind DNA but not thyroid hormone, and represents a highly mutated version of its cellular homologue c-erbA. The thyroid hormone receptor itself is not directly implicated for an active role in the process of cancer.

The α-retinoic acid receptor, however, is implicated in the acquisition of retinoic acid resistance in patients with acute promyelocytic leukaemia who have been treated with retinoic acid (Kastner et al., 1992; Robertson et al., 1991, 1992).

The presence of estrogen receptors in human breast cancer is associated with increased survival and a longer disease-free interval, compared to patients with ER-negative tumours (Allegra et al., 1979; Bishop et al., 1979; Vollenweider-Zerargui et al., 1986). Progesterone receptor levels are upregulated by estrogens, and PR levels are generally a useful marker of hormone dependency in breast cancer. Their presence predicts an improved disease-free interval and indicates the likelihood of benefit from anti-estrogen therapy. In resistant tumours, lack of estrogen-binding capacity can be due to a total loss of receptor proteins or to a mutation that leads to either non-functional or constitutively active receptor forms. Dominant positive and dominant negative estrogen receptor variants have been found to coexist with the wild type receptor protein, in human breast cancer tissue and in the estrogen resistant human breast tumour cell line T47D (Fuqua et al., 1991; Graham et al., 1990; McGuire et al., 1991). In a human meningioma, a truncated estrogen receptor with a major deletion involving the steroid binding domain has been identified that was overexpressed. This truncated receptor protein might still be functional in stimulating estrogen responsive genes such as the PR (Koehorst et al., 1992). These data established a role for nuclear hormone receptors in the development and treatment of cancer.

The growth of most prostate cancers is androgen dependent, implying the

presence of a functional androgen receptor (Coffey and Pienta, 1987; Trapman and Brinkmann, 1991). In 80% of metastasized prostate cancers, endocrine intervention in the form of castration or the administration of anti-androgens is very effective. However, in essentially all cases an androgen independent tumour continues to grow (Coffey and Pienta, 1987). The molecular basis for this switch from androgen dependency to androgen independency of prostate cancers has not yet been resolved. Because of the central role of the androgen receptor in normal prostate development and in the growth of androgen-dependent tumours, a mutation of the androgen receptor is likely to be involved (Trapman et al., 1990, Trapman and Brinkmann, 1991).

A G to A point mutation was detected in an untreated prostate cancer, changing codon 721 in the steroid binding domain of the androgen receptor from valine to methionine (Newmark et al., 1992). A valine to methionine exchange of amino acid residue 706 in the human androgen receptor was identified in a prostatic carcinoma from a patient who failed to respond to endocrine therapy. This valine to methionine mutation at position 706 in the androgen receptor promotes transactivation, not only by testicular but also by adrenal androgens and progesterone (Culig et al., 1993). The LNCaP (Lymph Node Carcinoma of the Prostate) cell line developed by Horoszewicz et al. (1980), is a standard model cell line to study human prostate cancer. Veldscholte et al. (1990) have identified an A to G point mutation in this cell line that results in the transition of amino acid residue 868 in the steroid binding domain of the receptor from threonine into alanine. This mutation results in a mutant receptor protein that is activated not only by androgens, but also by estrogens, progestagens and anti-androgens.

The androgen receptor in the LNCaP cell line seems completely functional. Cell growth can be stimulated by the synthetic androgen R1881 and prostate-specific antigen mRNA expression can also be upregulated (Trapman et al., 1990).

The androgen receptor and motor neuron diseases

The abnormal length of the polyglutamine stretch in the human androgen receptor has been correlated with spinal and bulbar muscular atrophy (SBMA, Kennedy syndrome) (Amato et al., 1993; LaSpada et al., 1991). This very rare neuromuscular disorder becomes manifest in men between the ages of 30 and 40, and is characterized by progressive neuron degeneration and predominant proximal and bulbar muscle weakness. One frequent association is the appearance of gynaecomastia and infertility, indicative for mild androgen insensitivity (Arbizu et al., 1983).

In all SBMA patients investigated sofar the number of glutamine residues in the androgen receptor was 40 or more, whereas in individuals with no signs of SBMA or AR malfunction the number is between 12 and 32 residues. A possible consequence of an increased length of the glutamine stretch could be a change in the structural constraint of the N-terminal domain of the hAR resulting in a modified assembly of the transcription initiation complex. In cell culture experiments, the androgen receptor protein with an expanded poly-glutamine tract transactivates an androgen-responsive reporter gene subnormally, thus providing an explanation for the observed mild androgen insensitivity in some SBMA patients (Mhatre et al., 1993). However, normal transactivating properties of an androgen receptor protein with an expanded poly-glutamine tract have also been reported, indicating that such experiments should be evaluated in a cell-specific and promoter-specific context (Trapman and Brinkmann, 1993). There is a good correlation between the increased length of the CAG repeat in the human androgen receptor gene and the age of onset and severity of SBMA (Doyu et al., 1992).

An increased number (42) of CAG repeats in the human androgen receptor gene has also been identified in a patient with hypertrophic cardiomyopathy (Kaneko et al., 1993) where the underlying neurogenic disorder was diagnosed as a latent form of SBMA.

Interestingly, myotonic dystrophy (Fu et al., 1992), the fragile X syndrome and Huntington's disease have similar mutations of CG-rich tandem triplet repeats that result in changes involving gene expression, mRNA stability or other functional disorders (Nelson and Warren, 1993). In the fragile X syndrome, length variation of the CGG repeat in the FMR-1 gene is reported (Verkerk et al., 1991).

Huntington's disease is an autosomal dominant neurodegenerative disorder in which an expanded and unstable $(CAG)_n$ trinucleotide repeat in the 5' end of the IT15 gene has been identified. Normal chromosomes contain 11-34 copies of this repeat. Affected patients harbour 37-86 copies of the CAG-triplet, and the repeat length is inversely correlated with the age of onset of the disorder (Duyao et al., 1993; Huntington Disease Collaborative Research Group 1993).

SCOPE OF THE THESIS

This thesis deals with the role of the human androgen receptor in pathological situations. The major clinical consequence of impaired androgen action due to mutations in the androgen receptor gene, is androgen insensitivity (AIS).

Chapters 2, 3, and 4 describe in more detail the underlying molecular cause for the androgen insensitivity in 3 cAIS subjects and in 1 pAIS subject. The consequences of specific mutations for the ligand binding capacity and functional activity of the androgen receptor protein are addressed in detail.

Chapter 5 gives a résumé of mutations in the hAR gene identified by the Rotterdam group (patients from Canada, Germany, The Netherlands, The United Kingdom). Strategies and methods to identify carriers for the syndrome in families at risk for AIS are discussed.

Lastly, the specific role of amino acid position 868 in the human androgen receptor is evaluated. This amino acid residue came under attention when a threonine to alanine mutation was identified in the LNCaP (lymph node carcinoma of the prostate) cell line (Veldscholte et al., 1990). The substitution of threonine 868 for an alanine residue leads to an androgen receptor protein that is functional upon stimulation with androgens, progestagens, estrogens and anti-androgens. The effect on ligand binding specificity and functional activity of several different amino acid residues at position 868 is described in Chapter 6.

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Aberrant splicing of androgen receptor mRNA results in synthesis of a nonfunctional receptor protein in a patient with androgen insensitivity

(testicular feminization/steroid receptor/point mutation/splice donor site/male sexual differentiation)

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ABSTRACT **Androgen insensitivity is a disorder in which the correct androgen response in an androgen target cell is impaired. The clinical symptoms of this X chromosome-linked syndrome are presumed to be caused by mutations in the androgen receptor gene. We report a G → T mutation in the splice donor site of intron 4 of the androgen receptor gene of a 46,XY subject lacking detectable androgen binding to the receptor and with the complete form of androgen insensitivity. This point mutation completely abolishes normal RNA splicing at the exon 4/intron 4 boundary and results in the activation of a cryptic splice donor site in exon 4, which leads to the deletion of 123 nucleotides from the mRNA. Translation of the mutant mRNA results in an androgen receptor protein ≈5 kDa smaller than the wild type. This mutated androgen receptor protein was unable to bind androgens and unable to activate transcription of an androgen-regulated reporter gene construct. This mutation in the human androgen receptor gene demonstrates the importance of an intact steroid-binding domain for proper androgen receptor functioning *in vivo*.**

Androgens play an essential role in the control of male sexual differentiation and development and in the maintenance of normal male reproductive function (1). Androgen action is mediated by the low-abundance intracellular androgen receptor protein, a member of the superfamily of ligand-responsive transcription regulators that includes the retinoic acid receptors, the thyroid hormone receptors, and the other steroid hormone receptors (2–4).

The human androgen receptor is composed of 910 amino acids, as deduced from the cDNA sequence (5–9). The corresponding gene is located on the X chromosome and has a length of >90 kilobases (kb) (5, 10, 11). The information for the protein-coding region is separated over eight exons. The sequence encoding the N-terminal domain is present in one large exon (exon 1) (8). The DNA-binding domain is encoded by exons 2 and 3, and the information for the steroid-binding domain is distributed over five exons (exons 4–8) (10). The positions of the exon/intron boundaries are conserved among progesterone, estrogen, and androgen receptor genes (10, 12, 13).

A number of aberrations of male sexual differentiation and development are associated with defects in the androgen receptor protein (1, 14). These defects can vary from a complete female phenotype in a 46,XY individual [complete androgen insensitivity syndrome (AIS)] to partial disorders of male sexual differentiation (partial AIS). Both the complete and the partial form of AIS can manifest at the protein level in either the absence or the presence of androgen binding. In the latter case, qualitative defects in androgen binding have been reported (1, 14). Therefore, naturally occurring mutations in the androgen receptor are a potentially interesting source for the investigation of receptor structure–function relationships. In addition, the variation in clinical syndromes provides the opportunity to correlate a mutation in the androgen receptor structure with the impairment of a specific physiological function of the androgen receptor.

Here we report a point mutation in the splice donor site of intron 4 of the androgen receptor gene of a patient with complete AIS and describe the consequences for androgen receptor properties.

MATERIALS AND METHODS

Index Patient. Clinical and biochemical data concerning patient 20.1 (age 17) showed a 46,XY karyotype but a female habitus with unambiguously female external genitalia. Serum concentrations of testosterone, dihydrotestosterone, and follitropin were within the normal range for men. Serum lutropin levels were 5 times higher than normal. Androgen receptor binding was assessed in genital skin fibroblast monolayers, cultured from a skin biopsy of the labia majora (15). Androgen binding could not be detected (maximal binding in genital skin fibroblasts of controls, >18 fmol/mg of protein). These data led to the diagnosis of complete AIS with no detectable androgen binding to receptors.

Cell Culture. Genital skin fibroblasts were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, and antibiotics. COS-1 cells (simian virus 40-transformed monkey kidney fibroblasts) were grown in Dulbecco's modified Eagle's medium supplemented with 5% dextran/charcoal-treated fetal bovine serum and antibiotics.

RNA Preparation. Total cellular RNA was isolated by the guanidinium isothiocyanate method (16). cDNA was synthesized using 4 μg of total RNA, 100 ng of oligodeoxynucleotide primer (E8: 5'-AAGGCACTGCAGAGGAGTA-3'), 10 units of avian myeloblastosis virus reverse transcriptase (Promega), and 10 units of RNase inhibitor (RNasin; Promega). Synthesis was done according to the standard protocol (Promega).

DNA Amplification and Sequencing. Amplification by the polymerase chain reaction (PCR; ref. 17) took place in 100-μl reaction mixtures containing 1 μg of genomic DNA or 2% of

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Abbreviations: AIS, androgen insensitivity syndrome; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; LTR, long terminal repeat.

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the cDNA-synthesis reaction mixture. PCR mixtures contained 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 μmol of each dNTP, 17 μg of bovine serum albumin, 2 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Amersham), and 600 ng of each oligonucleotide. Amplification was performed during 24 cycles; each cycle included denaturation for 1 min at 92°C, primer annealing for 2 min at 60°C, and primer extension for 1–5 min at 70°C. For Southern blotting, samples were electrophoresed in 2% agarose, transferred to nitrocellulose, and hybridized as described previously (probe C in ref. 10). For sequence analysis, amplified fragments were made blunt-ended and inserted into the *Sma* I site of M13mp18 (18) prior to sequencing by the dideoxy chain-termination method (19). The following oligonucleotides were used (mismatches are indicated by lowercase letters): I3, ATTCAAGTCTCTCTTCCTTC; I4, GCGTTCACTAAATATGATCC; E1, ggatCCACATGCGTTTGGAGACTGC; E4, CAGAAGCTtACAGTGTCACACA; E5, CGAAGTAGAGgATCCTGGAGTT; E8, AAGGCACTGCAGAGGAGTA.

Construction of the Expression Vectors. A human androgen receptor cDNA expression vector (pAR_0) was constructed using the simian virus 40 early promoter and the rabbit β-globin polyadenylylation signal (20). The $pAR_{\Delta 674-714}$ expression vector (pAR_Δ) was generated by exchanging the 898-base-pair *Kpn* I–*Eco*RI fragment of pAR_0 with the mutant 775-bp *Kpn* I–*Eco*RI fragment obtained by amplification of cDNA with oligonucleotides flanking the *Kpn* I site in exon 1 and the *Eco*RI site in exon 6 (5′-GACTTCACCGCACCTGATG-3′ and 5′-TGCTGAAGAGTAGCAGTGCT-3′).

Transfection. COS cells were transfected by the calcium phosphate precipitation method (21). For immunoblotting studies, 5×10^6 COS cells were transfected with 40 μg of either pAR_0 or pAR_Δ and 40 μg of pTZ (Pharmacia) carrier plasmid. For binding studies, 10^7 COS cells were transfected with 80 μg of either pAR_0 or pAR_Δ and 80 μg carrier plasmid. For transcription studies, 5×10^5 cells were transfected with 2.5 μg of either pAR_0 or pAR_Δ, 2.5 μg of MMTV–CAT reporter gene [bacterial chloramphenicol acetyltransferase (CAT) gene under control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR)], and 2.5 μg of pCH110 (β-galactosidase reporter plasmid; Pharmacia). Carrier DNA (pTZ) was added to give a total of 10 μg of DNA per dish. pSV2cat (2.5 μg) was used in a control experiment. Each experiment was carried out in duplicate.

Western Blot Analysis. COS cells were lysed in 40 mM Tris·HCl, pH 7.0/1 mM EDTA/4% (vol/vol) glycerol/10 mM dithiothreitol/2% SDS, and 1 volume of the protein fraction of the whole cell lysate was precipitated with 5 volumes of methanol. SDS/PAGE (0.1 mg of protein per lane), Western blotting, and immunostaining with antibody Sp061 (diluted 1:1000) were done as described (22).

Hormone Binding Assay. Transfected COS cells were cultured for 3 days in steroid-depleted medium and then incubated for 1 hr at 37°C with 0.1–10 nM [^{3}H]R1881 (17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one) in the presence of a 500-fold molar excess of triamcinolone acetonide. Nonspecific binding was determined in parallel incubations with an additional 100-fold molar excess of nonradioactive R1881. Separation of bound and unbound steroid was achieved by the oil microassay method (23).

β-Galactosidase and CAT Assays. β-Galactosidase was assayed (24) by incubation of 5 μl of cell extract with 10 μl of 1 nM 4-methylumbelliferyl β-D-galactopyranoside (Koch Light) for 30 min at 37°C. The reaction was terminated by adding 200 μl of 1 M $NaHCO_3$ and fluorescence was determined at 365 and 448 nm. The CAT assay was essentially as described (25). After correction for transfection efficiency (β-galactosidase assay), CAT activity was quantitated (26).

RESULTS

Southern blotting with specific androgen receptor cDNA probes showed that genomic DNA from genital skin fibroblasts of patient 20.1 contained the complete coding region of the gene (data not shown). To investigate whether a point mutation or a small gene deletion might have caused the absence of hormone binding, exons 4–8, which encode the steroid-binding domain, and exons 2 and 3, which encode the DNA-binding domain, were amplified from genomic DNA and sequenced (17). Sequences were found to be identical with the previously published wild-type structure with only one exception: a G → T mutation at position 1 in the splice donor site of intron 4 (Fig. 1). This mutation was detected in each of four independent clones produced by two separate PCR amplifications. RNA was isolated from genital skin fibroblasts of patient 20.1 and first-strand cDNA was prepared using an oligonucleotide primer corresponding to an exon 8 sequence. The resulting cDNA was amplified using exon 4- and 5-specific primers. Amplified fragments were analyzed by size fractionation in a 2% agarose gel and hybridization with a cDNA probe specific for the steroid-binding domain of the human androgen receptor. This resulted in the detection of only one fragment, which, however, was shorter than the corresponding fragment from the wild-type receptor. Amplification of a cDNA fragment spanning exons 2–8 also resulted in one amplification product with a similar length difference, implying total abolishment of normal RNA splicing and the effective use of only one alternative splice site (Fig. 2*a*).

Sequence analysis of the mutant fragment revealed the use of a cryptic splice donor site, CAG/GTGTAG at position 2020/2021 (10) in exon 4 of the human androgen receptor gene. The use of this cryptic splice site results in the deletion of 123 nucleotides from the mRNA (Fig. 2*b*).

Translation of the deleted mRNA would result in an in-frame deletion of 41 amino acids (residues 674–714; ref. 10) in the steroid-binding domain of the androgen receptor protein. To investigate whether the deleted mRNA could be translated, androgen receptor expression vectors were constructed. Expression vectors containing either the wild-type sequence (pAR_0) or the mutated sequence ($pAR_{\Delta 674-714}$) were transiently expressed in COS-1 cells. Western immunoblotting using a polyclonal antibody (SP061; ref. 22) directed against the human androgen receptor showed the presence of

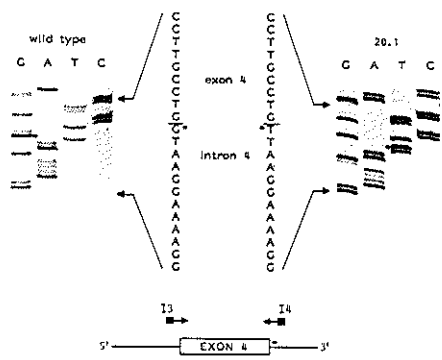

FIG. 1. Sequence comparison of the exon 4/intron 4 boundaries of the wild-type (*Left*) and mutant 20.1 (*Right*) androgen receptor genes. Asterisks indicate the single base substitution (G → T) in the splice donor site. Genomic DNA was amplified using oligonucleotide primers I3 and I4 as indicated.

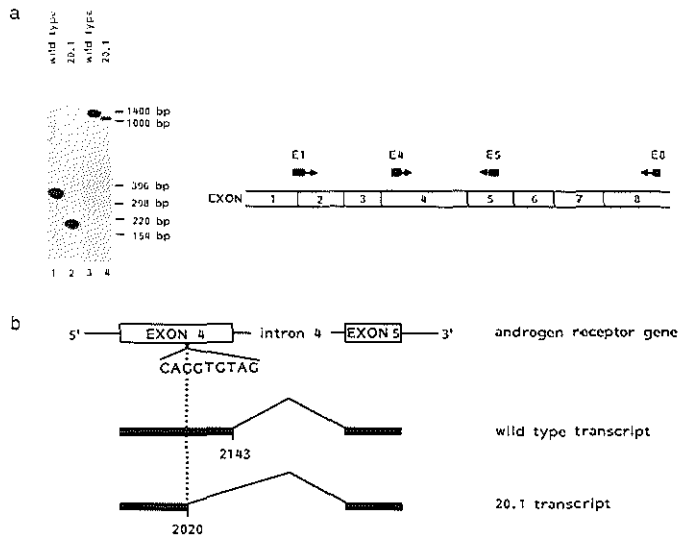

FIG. 2. (*a*) Size comparison of amplified androgen receptor cDNA. Oligonucleotides E4 and E5 (lanes 1 and 2) or E1 and E8 (lanes 3 and 4) were used after cDNA synthesis with oligonucleotide E8. RNA was isolated from genital skin fibroblasts of patient 20.1 (lanes 2 and 4) and from control cells (lanes 1 and 3). Marker sizes and the relative positions of the oligonucleotide primers are indicated. (*b*) Sequencing of the E4–E5 amplification product elucidated the position of the cryptic splice donor site in exon 4 of the androgen receptor gene, resulting in the deletion of 123 nucleotides from the mRNA of patient 20.1.

comparable amounts of the pAR_0 protein (calculated molecular mass, 98,845 Da) and the $pAR_{\Delta 674-714}$ protein (94,334 Da) (Fig. 3). The protein bands around 50 kDa and 70 kDa are probably due to proteolytic breakdown. A protein product of 50 kDa originating from an alternative initiation site of translation lacks ≈400 amino acid residues from the N-terminal domain and would in that case have lost the epitope for Sp061 recognition.

No androgen receptor expression was found after mock transfection (Fig. 3). Immunoblot detection of the normal or mutant androgen receptor in genital skin fibroblasts was not possible, probably due to the low concentration of androgen receptor protein in these cells.

In genital skin fibroblasts of the patient, no binding of androgens to androgen receptors was detected, and the clinical syndrome of this patient indicates the inability of the receptor to regulate transcription of androgen target genes. To investigate whether the mutation described above could

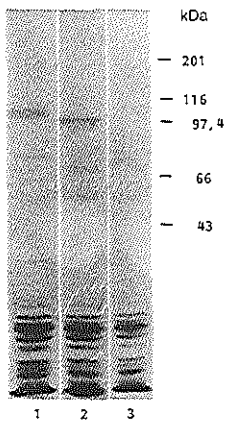

FIG. 3. Western blot analysis of protein products of pAR_0 (wild-type sequence; lane 1), $pAR_{\Delta 674-714}$ (mutant sequence; lane 2) and pSV2cat (lane 3) expressed in COS cells and analyzed by SDS/7.5% PAGE. The androgen receptor was visualized by immunostaining with the polyclonal antibody Sp061.

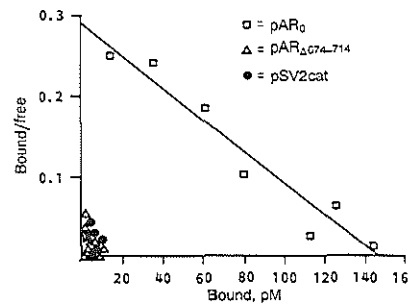

FIG. 4. Scatchard plot analysis of androgen ([^{3}H]R1881) binding in COS cells transfected with pAR_0, $pAR_{\Delta 674-714}$, or pSV2cat.

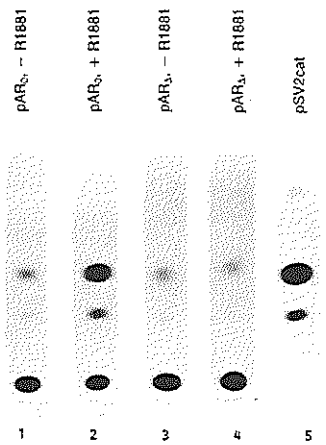

FIG. 5. Regulation of MMTV–CAT expression in COS cells cotransfected with pAR_0 (lanes 1 and 2) or $pAR_{\Delta 674-714}$ (lanes 3 and 4). Lane 5 shows the pSV2cat control. The cells were cultured in the absence (lanes 1, 3, and 5) or presence (lanes 2 and 4) of 0.1 nM R1881. Autoradiograms display the conversion of [^{14}C]chloramphenicol to acetylated products.

be the cause of absence of hormone binding and transcription activation, androgen binding and MMTV-LTR-driven CAT expression were determined in COS cells transfected with either pAR_0 or $pAR_{\Delta 674-714}$. pSV2cat was used as a control. Specific binding of the synthetic androgen [^{3}H]R1881 could not be demonstrated for the $pAR_{\Delta 674-714}$ protein, whereas the pAR_0 protein showed a maximum binding capacity of 730 fmol/mg of protein and a dissociation constant of 0.5 nM (Fig. 4).

In the presence of the synthetic androgen R1881, the wild-type androgen receptor protein expressed in COS cells activated the expression of the MMTV–CAT reporter gene, but the $pAR_{\Delta 674-714}$ deletion mutant did not (Fig. 5). The deletion mutant was also unable to activate transcription in the absence of R1881, indicating that the mutant receptor protein is not constitutively active. When the CAT activity induced by pAR_0 in the presence of R1881 was set to 100%, the relative CAT activity in the absence of R1881 was 19%. The relative CAT activity induced by $pAR_{\Delta 674-714}$ in either the presence or the absence of R1881 was 14%. The low CAT activity observed in the case of transfection with $pAR_{\Delta 674-714}$ and in the absence of hormone in the case of pAR_0 was considered background activity stemming from the MMTV–CAT construct. COS cells transfected with MMTV–CAT alone also show this low basal CAT activity (20).

DISCUSSION

In this study a point mutation in a splice donor site of the androgen receptor gene was characterized in detail. It is well documented (27) that an effective splice donor site resembles the consensus sequence $\begin{matrix} C \\ A \end{matrix}AG/GU\begin{matrix} A \\ G \end{matrix}AGU$ (Fig. 6*a*). Within this consensus splice sequence, the G at intron position 1 is obligatory. Mutation of this G leads to abolishment of normal splicing and to aberrant splicing products due to the activation of one or more cryptic splice sites (30–32). A G → T mutation at intron position 1 can, in an *in vitro* model, lead to an upstream shift of the cleavage site of 1 nucleotide (33). The G → T mutation reported here also generates a possible splice site 1 nucleotide upstream from the original junction, but this site is not activated in the mutant *in vivo*.

Recognition by means of hybrid formation of the splice donor site with nucleotides 4–11 of the U1 small nuclear RNA is one of the key steps in the splicing mechanism (28, 29). The most frequently formed hybrids comprise only 5–7 bp. For the human androgen receptor gene, the splice donor sequence normally used at the exon 4/intron 4 boundary (CTG/GTAAGG) is able to form 7 bp with U1 RNA. Obviously, in the wild-type situation this splice site is preferred to the cryptic splice site (CAG/GTGTAG) although the latter has six possible base-pairing positions with U1 RNA (including G·U pairing) and conforms to the splice consensus rule (Fig. 6).

Only a few naturally occurring mutations involving human steroid hormone receptors have been described. A recently published mutation involves a single nucleotide change leading to an amino acid change in the steroid-binding domain of the androgen receptor of a complete AIS patient with evidence of X chromosome linkage. The mutated protein has a decreased affinity for ligand but the effect on androgen target-gene activation has not been investigated (34). A deletion of part of the androgen receptor gene of a complete AIS patient also has been published (35). The effect of this deletion on receptor synthesis or function has not been established.

A steroid receptor that lacks the steroid-binding domain may show constitutive transcriptional activity; this has been demonstrated for progesterone, glucocorticoid, and estrogen receptors (2–4). The same holds for the androgen receptor. When the complete steroid-binding domain is deleted the receptor has a constitutive transcriptional activity that is about 30% of the activity induced by the wild-type androgen receptor (unpublished data).

The lack of androgen binding found in genital skin fibroblasts of patient 20.1 is the result of the deletion of 41 amino acids (residues 674–714) in the steroid-binding domain of the androgen receptor. Residues 674–714 of the receptor are located in a region that displays a high degree of sequence conservation in the family of steroid hormone receptors (5, 7). The importance for steroid binding and transcription regulation of a region similar to the one deleted in the

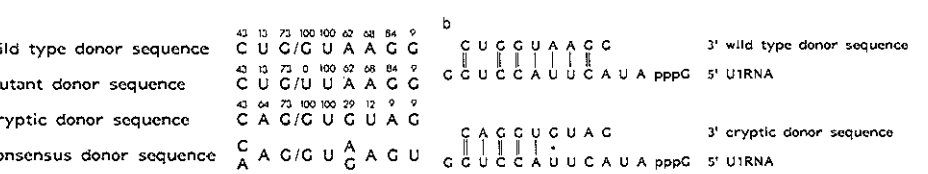

FIG. 6. (*a*) Comparison of the wild-type splice donor site of intron 4, the mutant splice site, and the cryptic splice donor site with the consensus donor sequence according to Mount (27). The percentage of occurrence is indicated. (*b*) Possible base-pairing of nucleotides 4–11 of U1 small nuclear RNA with the wild-type or the cryptic splice donor site according to previous models (28, 29). G·C pairing is indicated by double bars, A·U pairing by single bars, and G·U pairing by dots.

androgen receptor of AIS patient 20.1 has been established for other steroid hormone receptors (2–4), where *in vitro* generated deletions in this region abolish hormone binding and decrease the ability of the protein to activate transcription. For the glucocorticoid receptor, it has been postulated that the 90-kDa heat shock protein is able to associate with this part of the steroid-binding domain (36). This region of the glucocorticoid receptor also harbors a nuclear localization signal (37) and a domain that has a potent effect on transcription (38).

Whether there is a parallel between the glucocorticoid receptor and the androgen receptor regarding the function of this region is still unresolved. The deletion mutant described here does not enable us to answer this question, because it probably changes the folding of the receptor in such a way that the structure of the ligand-binding pocket is destroyed. Further research on the effect of deletions and single amino acid mutations of the androgen receptor on hormone binding and the regulation of androgen target-gene expression will provide more insight into the mechanism of androgen receptor action.

We thank Dr. Anton Grootegoed for helpful discussions and for reading the manuscript. This investigation was supported by the Netherlands Organization for Scientific Research and by the Netherlands Cancer Foundation.

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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

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We have identified two different single nucleotide alterations in codon 686 (GAC; aspartic acid) in exon 4 of the human androgen receptor gene in three unrelated families with the complete form of androgen insensitivity. One mutation (G → C) results in an aspartic acid → histidine substitution (with 15–20% of wild-type androgen-binding capacity), whereas the other mutation (G → A) leads to an aspartic acid → asparagine substitution (with normal androgen-binding capacity, but a rapidly dissociating ligand-receptor complex). The mutations eliminate a *Hinf*I restriction site. Screening for the loss of the *Hinf*I site in both families with the Asp → Asn mutation resulted in the recognition of heterozygous carriers in successive generations of each. Both mutant androgen receptors were generated *in vitro* and transiently expressed in COS and HeLa cells. The receptor proteins produced had the same altered binding characteristics as those measured in fibroblasts from the affected subjects. R1881-activated transcription of a GRE-tk-CAT reporter gene construct was strongly diminished by both mutant receptors and was only partially restored using a 100-fold higher concentration of ligand compared with wild-type receptor. Thus, aspartic acid-686 appears essential for normal androgen receptor function. Substitution of this amino acid residue, by either histidine or asparagine, results in androgen insensitivity and lack of androgen-dependent male sexual differentiation. (Molecular Endocrinology 5: 1562–1569, 1991)

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Molecular Endocrinology

INTRODUCTION

The actions of both testosterone and dihydrotestosterone during male sexual differentiation are mediated through the androgen receptor, a member of the steroid/thyroid hormone/retinoic acid receptor family (1–5). The molecular mechanism of transactivation by these receptor proteins involves a structural modification of the receptor upon ligand binding. This enables

the ligand-receptor complex to interact with *cis*-acting regulatory elements in such a way that the expression of a target gene is modulated (6). All members of the steroid/thyroid hormone/retinoic acid receptor family have a similar functional domain structure: a variable N-terminal region, which is involved in modulation of gene expression; a short well conserved DNA-binding domain, which is characterized by the presence of two so-called zinc finger motifs; and a partially conserved C-terminal ligand-binding domain, which is important for receptor dimerization and transactivation (5–7).

The androgen insensitivity syndrome is due to genetic defects in the X-linked androgen receptor gene that prevent normal male differentiation of the internal and external genitalia in affected 46,XY individuals. In contrast to androgen-insensitive individuals, in whom hormone binding to the receptor or binding of the receptor-hormone complex to DNA is impaired, some androgen-insensitive individuals have normal levels of androgen receptors that do not show any abnormality in hormone or DNA binding (1, 8). The molecular cause of the dysfunctioning of these androgen receptors could reside in androgen receptor mutations that inactivate transcription regulation without affecting hormone binding or tight nuclear binding.

The cloning and characterization of cDNA encoding the human androgen receptor and the elucidation of the structural organization of the human androgen receptor gene have increased our knowledge of the properties of the human androgen receptor and have facilitated the study of molecular defects associated with androgen insensitivity (2–4, 9–12). In addition, the development of rapid and sensitive amplification methods using the polymerase chain reaction (PCR) have improved the simplicity and speed with which single base changes in human DNA can be identified (13). A limited number of different point mutations and one partial and one complete deletion of the androgen receptor gene have been described in patients with the complete form of androgen insensitivity (11, 12, 14–19).

Here we present the detailed characterization of two point mutations affecting the same nucleotide in the androgen receptor gene of three unrelated individuals with the complete form of androgen insensitivity.

RESULTS

Androgen Receptor Binding Kinetics

Assay of intact genital skin fibroblasts with up to 5 nM [^{3}H]mibolerone revealed a concentration of specific androgen-binding activity in subjects 882718, 51198, and RDL within the normal range for genital skin fibroblasts, but a subnormal level in subject 21.1 (Table 1). At 37 C, the mibolerone-androgen receptor complexes in the cells of 882718, 51198, and RDL dissociated about 8-fold faster than wild-type complexes (Fig. 1). Because of the very low number of binding sites in the fibroblasts of subject 21.1, no reliable dissociation data could be obtained. The K_d values of androgen binding are abnormal for both 882718 cells (1.9 nM) and 51198 cells (0.7 nM); the normal upper limit is 0.25 nM. The K_d of androgen binding for 21.1 cells could not be determined due to the very low number of binding sites. Up-regulation of specific androgen-binding sites was determined during prolonged incubation with 5 nM mibolerone. After 1 and 20 h of incubation, 882718 cells had, respectively, 29 and 31 fmol/mg protein; 21.1 cells had, respectively, 11 and 5 fmol/mg protein. Normal cells double their androgen receptor levels within 20 h. The lack of measurable up-regulation in the presence of ligand is most likely due to the increased dissociation rate of the receptor-ligand complex.

Table 1. Number of Specific Binding Sites for [^{3}H]Mibolerone in Genital Skin Fibroblasts of Affected and Normal Subjects

Sample	No. of Binding Sites (fmol/mg Protein)
Normal	15–40 (30)
882718	29–36 (4)
51198	29–31 (3)
RDL	19–20 (2)
21.1	6–11 (3)

The number of determinations is in parentheses.

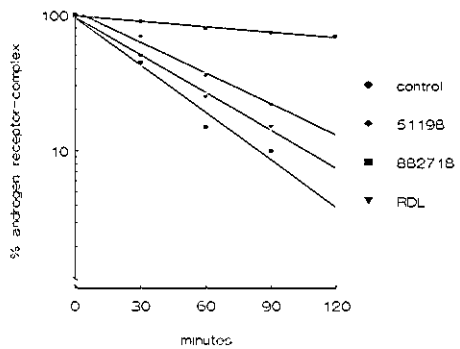

Fig. 1. Dissociation Rates of Mibolerone-Androgen Receptor Complexes at 37 C in Genital Skin Fibroblasts of Subjects 882718, 51198, and RDL and a Normal Individual

Identification of the Molecular Defects

To study whether a major deletion in the androgen receptor gene was the cause of the androgen insensitivity in subjects 21.1 and 882718, Southern blot analysis of genomic DNA was performed after digestion with *Bam*HI, *Eco*RI, *Hin*dIII, and *Sst*I. No large androgen receptor gene deletions were found using a set of androgen receptor cDNA hybridization probes (data not shown). The receptor defects in both 21.1 and 882718 pointed to an abnormality in the steroid-binding domain.

Because the steroid-binding domain is encoded by exons 4, 5, 6, 7, and part of exon 8 of the androgen receptor gene, these regions were amplified from genomic DNA isolated from cultured genital skin fibroblasts of subjects 21.1 and 882718 and subsequently sequenced. As illustrated in Fig. 2, we identified two different point mutations at nucleotide 2056 of exon 4, converting a G to a C in subject 21.1 and a G to an A in subject 882718. The mutation in subject 21.1 causes the substitution of an aspartic acid residue by a histidine residue at position 686. The aspartic acid residue at position 686 is highly conserved within the steroid-binding domain of the progesterone, glucocorticoid, and mineralocorticoid receptor (Figs. 3 and 4). In subject 882718 the aspartic acid residue 686 is substituted by asparagine. The sequence of exons 5–8 and the sequence of exons 2 and 3 (coding for the DNA-binding domain) were normal for subjects 21.1 and 882718.

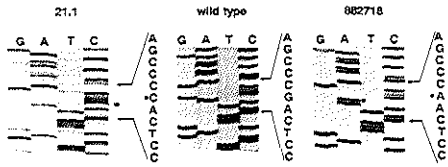

Fig. 2. Partial Sequence of Androgen Receptor Exon 4

Left, The guanine to cytosine mutation that changes the sense of codon 686 from Asp (GAC) to His (CAC) in subject 21.1. *Middle*, The partial sequence of wild-type androgen receptor exon 4. *Right*, The guanine to adenine transition that changes the sense of codon 686 from Asp (GAC) to Asn (AAC) in subject 882718.

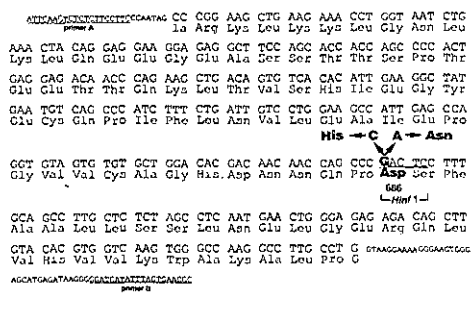

Fig. 3. Location of the Point Mutations in Exon 4 of the Androgen Receptor Gene

Nucleotide and amino acid sequences of exon 4 of the androgen receptor gene with portions of its flanking introns (*smaller lettering*). The intronic primers (*underlined*) used for PCR amplification are shown. The guanine to adenine and guanine to cytosine mutations at codon 686 and the consequent aspartic acid → asparagine and aspartic acid → histidine substitutions are *highlighted in bold*. The *Hin*fI recognition sequence (GANTC) abolished by the mutation is *underlined*.

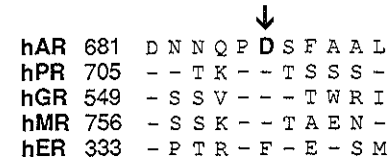

Fig. 4. Conservation of Aspartic Acid-686 in the Steroid-Binding Domains of Other Members of the Superfamily of Steroid Receptors

An aspartic acid residue is located at position 710 in the human progesterone receptor (hPR), at position 554 in the human glucocorticoid receptor (hGR), and at position 756 in the human mineralocorticoid receptor (hMR). For comparison, part of the steroid-binding domain of the human estrogen receptor (hER) is shown.

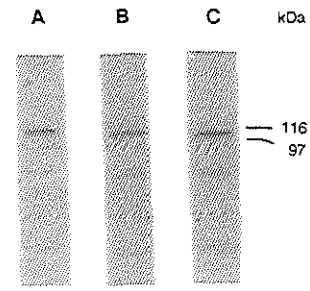

Fig. 5. Western Blot Analysis of Wild-Type and Mutant Androgen Receptors Transiently Expressed in COS Cells

Total cellular lysates of COS cells transfected with wild-type (A), mutant 882718 (B), or mutant 21.1 (C) androgen receptor cDNA were analyzed by SDS-PAGE after immunoprecipitation with a monoclonal human androgen receptor antibody. Immunostaining was performed using a polyclonal human androgen receptor antibody.

Functional Properties of Mutant Receptors

To determine whether both mutations found in the androgen receptor gene affected the functional properties of the androgen receptor, mutant cDNAs were transiently expressed in COS cells. Western blot analysis of total cellular lysates revealed the expression of a 110-kDa protein for both mutant receptors 21.1 and 882718 in concentrations almost equivalent to those of the wild-type receptor (Fig. 5). In lysates from mock-transfected cells, no androgen receptor protein could be detected. A 4-fold reduction in binding capacity was observed for mutant receptor 21.1 compared with the wild-type receptor, whereas the binding capacity of mutant receptor 882718 was slightly diminished (Table 2). The K_d of androgen binding of mutant receptor 882718 is 0.42 nM; the range for the wild-type receptor expressed in COS cells varied from 0.03–0.11 nM. The K_d for mutant receptor 21.1 could not be measured

Table 2. Number of Specific Binding Sites for [^{3}H] Methyltrienolone (R1881) in COS Cells Transfected with the Wild-Type and Mutant Androgen Receptors

Receptor	No. of Binding Sites (fmol/mg Protein)
Wild-Type	1568 ± 777 (10)
Mutant 882718 (Asp→Asn)	421 ± 131 (6)
Mutant 21.1 (Asp→His)	132 ± 20 (6)

Values are the mean ± SD; the number of determinations is in parentheses.

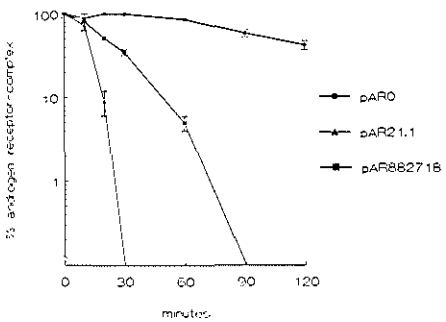

Fig. 6. Dissociation Rates of Methyltrienolone-Androgen Receptor Complexes at 37 C in COS Cells Transfected with Mutant (882718 and 21.1) or Wild-Type Androgen Receptors

COS cells were incubated with 5 nM [^{3}H]R1881 for 2 h, subsequently washed twice with saline, and incubated for 0–120 min in the presence of 1 μM R1881. After the chase with unlabeled R1881, specific binding was measured. The results were plotted as the percentage of [^{3}H]R1881-androgen receptor complex remaining after the chase (*bars* represent the range of duplicates). The percentage of [^{3}H]R1881-androgen receptor complex remaining was 0 at 60, 90, and 120 min for pAR21.1 and at 90 and 120 min for pAR882718.

accurately due to the relatively low number of binding sites.

The high dissociation rate originally observed in 882718 cells was also investigated in the reconstructed mutant receptors after transfection of COS cells. The results shown in Fig. 6 illustrate that substitution of asparagine for aspartic acid results in an approximately 5-fold increase in the dissociation rate of the ligand-receptor complex compared with the wild-type receptor. For mutant 21.1, an 8-fold increased dissociation rate was found. The decrease in bound radioactive ligand that could be detected is not due to receptor breakdown, because during an incubation period of 4 h with 5 nM [^{3}H]R1881, the amount of labeled mutant androgen receptor remained constant.

Transcriptional Activity of the Mutant Receptors

The functional activity of the mutant receptors was tested in HeLa cells after cotransfection of androgen receptor expression vectors containing the wild-type receptor (pARo), mutant 21.1, or mutant 882718 receptors together with the GRE-tk-CAT reporter gene. In Fig. 7, the results of this experiment are shown. The wild-type receptor caused a hormone-dependent induction of transcription that was maximal at 0.1 nM R1881. In contrast, both mutant receptors needed a 50- to 100-fold higher ligand concentration to attain only 10–30% maximal chloramphenicol acetyltransferase (CAT) activity.

Analysis of Exon 4 in Affected Families

Both point mutations in exon 4 abolished a *Hin*fI restriction site which is unique in this exon. To confirm the mutation in other affected siblings, genomic DNA from exon 4 was amplified by PCR and subsequently digested with *Hin*fI. The size of the intact amplified fragment is 370 nucleotides, while *Hin*fI digestion results in two fragments of 227 and 143 nucleotides, respectively. In Fig. 8A, the results for the analysis of the 882718 pedigree (family 1) are shown. Exon 4 of the affected siblings in this family yielded only the intact 370-nucleotide fragment. Exon 4 from the father has the normal *Hin*fI digestion pattern, whereas the mother's DNA yielded the mutant large fragment and the two normal fragments. The *Hin*fI site was used to screen for the occurrence of mutations at the same position in other androgen-insensitive patients and their relatives. In this way, of 39 investigated cases a second family (family 2) was found with the Asp → Asn mutation. Sequence analysis revealed the presence of the same G → A transition as that found in family 1. In this

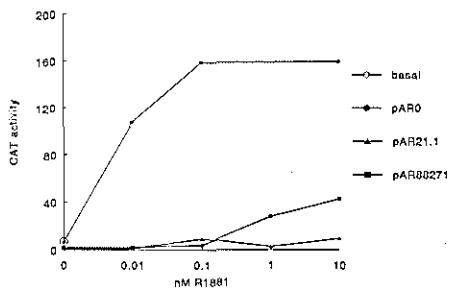

Fig. 7. R1881-Dependent Induction of CAT Activity in HeLa Cells Cotransfected with Wild-Type and Mutant Androgen Receptor Expression Plasmids and the GRE-tkCAT Reporter Gene

HeLa cells were cotransfected with expression plasmids for, respectively, wild-type, mutant 882718, or mutant 21.1 androgen receptors and with GRE-tkCAT at equivalent DNA concentrations, and exposed to 10^{-11}-10^{-6} M concentrations of methyltrienolone (R1881). CAT activity is plotted as disintegrations per min (thousands) of [^{14}C]butyryl-chloramphenicol recovered in the xylene phase *vs.* the concentration of R1881. In addition, the basal activity of the GRE-tkCAT construct is indicated.

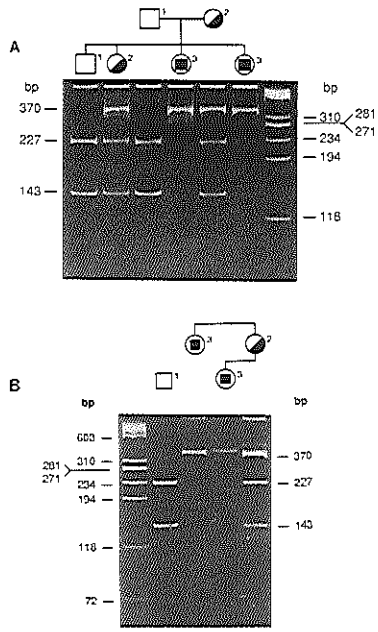

Fig. 8. Partial Pedigrees of the Canadian Families 1 and 2 and PAGE Analysis of PCR-Amplified Androgen Receptor Exon 4 DNA Digested with *Hinf*I

A, Family 1. The two affected siblings (3) only have the undigested PCR product containing exon 4. Their father and brother (1) only have the two expected wild-type digestion products. Their mother and sister (2) have both the undigested and digested products indicative of heterozygosity. B, Family 2. The affected members (3) only have the undigested PCR product. The unrelated normal male (1) has the two expected digestion products. The obligate heterozygote (2) has both the undigested and digested products. The size markers were generated by *Hae*III digestion of ϕX174RF DNA.

second Canadian family, the affected child and a maternal aunt were hemizygous for the mutant allele, and the mother was heterozygous (Fig. 8B).

DISCUSSION

The application of the PCR for analysis of point mutations has enabled us to discover two such mutations in the same codon of the androgen receptor gene from members of three unrelated families with the complete androgen insensitivity syndrome. The mutations are located in the N-terminal part of the steroid-binding domain of the human androgen receptor (exon 4) that is involved in both androgen binding and transcriptional regulation. The homologs of codon 686 are conserved as aspartic acid in the steroid-binding domains of the receptors for progesterone, glucocorticoid, and mineralocorticoid (4, 20–22), and the mutations change a negatively charged aspartic acid to either a neutral asparagine or a neutral/positively charged histidine residue.

Upon ligand binding, steroid hormone receptors transform to a form with enhanced binding affinity to hormone response elements. The transformation of the receptor molecule involves the unmasking of the DNA-binding domain by either a direct change in the receptor protein structure or dissociation of a nonsteroid-binding protein. It is not clear whether the abnormal binding characteristics of mutants 21.1 and 882718 are due to charge differences alone or whether the domain conformation in this region of the androgen receptor is also altered. Both possibilities could explain the altered stability of the androgen receptor-hormone complexes observed in genital skin fibroblasts and in transfected COS cells producing the mutant androgen receptors.

The most likely explanation for the lack of transcriptional regulation by both mutants at physiological ligand concentrations (0.5–1 nM) is that the relatively short half-life of the ligand-receptor complex leads to an insufficient amount of transformed receptor molecules for transcription activation. Impaired transformation or a fully transformed but short-lived mutant receptor molecule (rapid dissociation of ligand) could cause complete loss of androgen receptor functional activity. In this particular situation, an androgen receptor with an increased dissociation rate may not be able to elicit androgen receptor-mediated responses at physiological hormone concentrations. A high androgen concentration could stabilize nuclear interactions of the defective androgen receptor and thereby result in transcription activation, as was observed at pharmacological levels of R1881. The androgen receptors in patients 882718 and 21.1 with their increased ligand dissociation rate, however, are not functionally active in the presence of physiological levels of androgen.

The *trans*-regulating domains of the human androgen receptor have not been defined in detail as yet, so it is possible that codon 686 is located in such a domain. Constitutive activity with respect to transcription regulation has been assigned to androgen receptor protein products of cDNA constructs spanning exon 1–4 sequences (12, 23). Transcriptional activation studies after substitution of aspartic acid-686 (by histidine or asparagine, respectively) in these constitutively active receptor mutants could be indicative for a direct role of aspartic acid-686 in transcription activation.

Reports to date on androgen receptor gene structure in patients with the complete form of androgen insensitivity indicate that gross deletions within the androgen receptor gene are infrequent (11, 12, 14–18). The locations of the different mutations reported cannot be assigned to a single site, but are spread throughout the ligand-binding and DNA-binding domains. The present investigation, however, indicates that nucleotide 2056 in codon 686 is sensitive to mutational alterations. This mutation can be hereditary, as was found in the two

unrelated Canadian families. Extensive investigations for the abolishment of the *HinfI* site in individuals with androgen insensitivity and rapidly dissociating androgen-receptor complexes could substantiate this point further.

MATERIALS AND METHODS

Description of Affected Subjects and Families

Family 1 Subjects 882718 and 51198 are affected childhood siblings with one normal brother and sister. Each has the typical clinical phenotype of complete androgen insensitivity, with ultrasound documentation of absent uterus and histologically verified testes. Their mother is Canadian of Italian origin. She has three normal brothers and seven normal sisters. None of her sisters had delayed menarche, an occasional sign of heterozygosity, and none has had an affected child. Specific androgen binding to genital skin fibroblasts has been evaluated (Table 1 and Fig. 1).

Family 2 RDL is one of four adult affected sisters with the typical phenotype of complete androgen insensitivity. Their mother, a Canadian of French origin, developed very sparse axillary and pubic hair, an occasional sign of heterozygosity. There are five clinically normal daughters. One of them has had an affected child. The genital skin fibroblasts of RDL were classified as having deficient, but near-normal, specific androgen-binding activity in a previous report (24).

Subject 21.1 was thought to be a normal female until the age of 18 yr, when she sought medical advice because of primary amenorrhea. The parents are not related. Physical examination revealed a female habitus with well developed breasts and a female distribution of body fat. Axillary and pubic hair was scanty. The vagina was 6 cm in depth and blind ending. During gonadectomy bilaterally small testes were found situated in the inguinal canal. On histological examination, the gonads appeared similar to those of undescended testes (with no signs of spermatogenesis). The Leydig cells were well developed, and some tubular adenomas were also present. Laboratory findings revealed the following: serum testosterone, 10 ng/ml (normal range for men, 3–12); dehydroepiandrosterone sulfate, 4990 ng/ml (normal, 800–5600); estradiol, 40 pg/ml (normal, 6–44); LH, 20.2 mIU/ml (normal, 1.5–9.2) and FSH, 4.4 mIU/ml (normal, 1–14). 5α-Reductase activity was determined in cell homogenates of genital skin fibroblasts and was found to be 0.8 pmol/mg protein·h; the normal range for men is above 1 pmol/mg protein·h. It is assumed that the subnormal 5α-reductase activity is secondary to the patient's androgen receptor defect (25). The karyotype was 46,XY.

Cell Culture

Genital skin fibroblasts and COS cells were cultured in Eagle's Minimum Essential Medium supplemented with nonessential amino acids and antibiotics. Fibroblasts were maintained in medium containing 10% fetal calf serum, COS cells were maintained in medium supplemented with 5% dextran-charcoal-treated fetal calf serum (100 ml serum were treated twice for 30 min at 58 C with 0.1 g Dextran T300 and 1 g charcoal). HeLa cells were cultured in Dulbecco's Minimum Essential Medium supplemented with nonessential amino acids, antibiotics, and 5% fetal calf serum. Transcription-regulating studies were performed in HeLa cells and hormone-binding studies in COS cells. Both cell types were cultured in medium supplemented with 5% fetal calf serum treated with dextran-coated charcoal.

DNA and RNA Isolation

Genomic DNA was isolated from genital skin fibroblast monolayers using standard methods (26). Total cellular RNA was isolated by the guanidinium isothiocyanate method (27).

Complementary DNA Preparation

Complementary DNA was synthesized using 4 μg total cellular RNA, 100 ng primer (5′-T GCT GAA GAG TAG CAG TGC T-3′) corresponding to nucleotides 2397–2416 of the antisense strand of the androgen receptor gene, 10 U AMV reverse transcriptase (Promega, Madison, WI), and 10 U RNasin according to the protocol of the manufacturer.

DNA Amplification and Sequencing

The androgen receptor nucleotide sequence was determined after PCR amplification of genomic DNA or cDNA fragments. Oligonucleotide primers were synthesized using an Applied Biosystems 381A synthesizer (Foster City, CA). The following primers were designed according to the cDNA sequence and the intron sequence of the human androgen receptor gene: exon 4 primer A, 5′-ATTCAAGTCTCTCTTTCCTTC-3′; primer B, 5′-GCGTTCACTAAATATGATCC-3′; exon 5 primer A, 5′-GACTCAGACTTAGCTCAACC-3′; primer B, 5′-ATCACCACCAACCAGGTCTG-3′; exon 6 primer A, 5′-CAATCAGAGACATTCCTCTGG-3′; primer B, 5′-AGTGGTC-CTCTCTGAATCTC-3′; exon 7 primer A, 5′-TGCTCCTTCG-TGGGCATGCT-3′; primer B, 5′-TGGCTCTATCAGGCTGTT-CTC-3′; exon 8 primer A, 5′-ACCTCCTTGTCACCCTGT-3′; and primer B, 5′-AAGGCACTGCAGAGGAGTA-3′.

Amplification by PCR (13, 18) took place in 100-μl reaction mixtures containing 1 μg genomic DNA or 2% of the cDNA synthesis reaction mixture. Amplification conditions were: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 2 mM of each dNTP, 17 μg BSA, and 600 ng of each oligonucleotide. Samples were covered with 70 μl mineral oil. After an initial denaturation step for 5 min at 92 C, 2 U *Taq* polymerase (Amersham, Arlington Heights, IL) were added. Amplification was performed using a Techné (Cambridge, UK) DNA incubator during 24 cycles; each cycle included denaturation for 1 min at 92 C, primer annealing for 2 min at 60 C, and primer extension for 1–5 min at 70 C. After the last cycle, samples were incubated at 70 C for 5–9 min. The amplification product was recovered after chloroform-iso-amyl alcohol extraction and ethanol precipitation. Amplified fragments were blunt end ligated in the *SmaI* site of M13 mp18–19 (28) and sequenced using the dideoxy chain termination method (29). Complementary DNA expression vectors were sequenced after denaturation of 2 μg plasmid with 2 μl 2 N NaOH for 10 min at room temperature and subsequent precipitation.

Construction of Expression Vectors

All molecular cloning experiments were carried out using standard procedures. A human androgen receptor cDNA expression vector (pAR_o) was constructed using the simian virus-40 early promoter and the rabbit β-globin poly(A) signal (30). Mutant androgen receptor expression vectors ($pAR_{21.1}$ and pAR_{882718}) were generated by exchanging the 898-base-pair *KpnI-EcoRI* fragment of pAR_o with the mutant *KpnI-EcoRI* fragments that were obtained from PCR-amplified cDNA after reverse transcription of RNA isolated from the subjects' genital skin fibroblasts using oligonucleotides flanking the *KpnI* and *EcoRI* sites (5′-GACTTCACCGCACCTGATG-3′ and 5′-TGCTGAAGAGTAGCAGTGCT-3′). The correct nucleotide sequence of the expression plasmid has been assessed by sequencing.

Transfection

COS and HeLa cells grown to 50–60% confluency were transfected using the calcium phosphate precipitation method (31). For binding and immunoblotting studies, 5×10^6 COS cells were incubated with 10 μg pAR_o, 5 μg pTZ19 (carrier plasmid), and 5 μg pCH 110 (Pharmacia, Piscataway, NJ). For transcription-regulating studies, 5×10^5 HeLa cells in 3.5-cm dishes were transfected with 1.5 μg pAR_o, 0.75 μg pG29GtkCAT, and 0.75 μg pCH 110. The pG29GtkCAT contains two mouse mammary tumor virus glucocorticoid response element/progesterone response element/androgen response element (GRE/PRE/ARE) sequences up-stream of the tkCAT fusion gene (32).

Immunoprecipitation and Western Blot Analysis

Approximately 5×10^6 cells were lysed in 800 μl buffer [40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10% glycerol (vol/vol), 10 mM dithiothreitol, 10 mM sodium molybdate, 50 mM sodium fluoride, 0.6 mM phenylmethylsulfonylfluoride, 0.5-mM bacitracin, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, and 0.08% (wt/vol) sodium dodecyl sulfate (SDS)] at 4 C for 5 min. The cells were scraped from the surface, and the lysate was centrifuged (10 min; $1700 \times g$). The supernatant was stored at −80 C.

A mouse monoclonal antibody raised against a synthetic peptide corresponding to amino acid residues 301–320 of the human androgen receptor (33) was coupled to goat antimouse agarose (Sigma, St. Louis, MO) in a 1:4 dilution in PBS. Portions of 320 μl cell lysate were added, and the mixture was incubated for 2 h at 4 C. The mixtures were pelleted, washed three times with PBS, boiled for 3 min with 60 μl sample buffer [40 mM Tris-HCl (pH 6.8), 5% (vol/vol) glycerol, 2% (wt/vol) SDS, 10 mM dithiothreitol, and 0.2% (wt/vol) bromophenol blue] and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE, Western blotting, and immunostaining using the polyclonal human androgen antibody SP061 (diluted 1:1000) were performed as described previously (34).

Hormone Binding and Dissociation Assay

To determine the binding characteristics of genital skin fibroblasts or COS cells transfected with the wild-type and mutant androgen receptors, triplicate dishes were incubated for 2 h at 37 C with increasing concentrations (0.1–5 nM) of [^{3}H] mibolerone ([^{3}H]7α,17α-dimethyl-19-nortestosterone) or [^{3}H] R1881 ([^{3}H]17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one) in serum-free medium buffered with 15 mM HEPES, pH 7.4. Nonspecific binding was measured in parallel incubations containing an additional 200-fold molar excess of radioinert ligand. To determine the dissociation rate of the ligand-receptor complex in genital skin fibroblasts or in COS cells containing either wild-type or mutant receptors, cells were incubated for 2 h at 37 C with 5 nM tritiated ligand, followed by incubation for 0–120 min at 37 C with a 200-fold molar excess of radioinert ligand. After incubation, the dishes were placed on ice and washed twice with ice-cold 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 0.2% BSA and twice in the same Tris buffer without BSA. The dishes were placed at room temperature and incubated for 5 min with 0.1% trypsin. The cells were scraped with a rubber policeman and pelleted. The cell pellet was washed once in Tris buffer without BSA and subsequently solubilized in 0.5 N NaOH and sampled for protein measurement and radioactivity content.

CAT Assay

Twenty-four hours after transfection, HeLa cells were incubated for another 24 h with or without 10 nM R1881 before measuring CAT activity in the cell lysates. CAT activity was measured as described previously (35).

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Differential Splicing of Human Androgen Receptor Pre-mRNA in X-linked Reifenstein Syndrome Due to a Deletion Involving a Putative Branch Site

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Summary

The analysis of the androgen receptor (AR) gene, mRNA, and protein in a subject with X-linked Reifenstein syndrome (partial androgen insensitivity) is reported. The presence of two mature AR transcripts in genital skin fibroblasts of the patient is established, and, by reverse transcriptase-PCR and RNase transcription analysis, the wild-type transcript and a transcript in which exon 3 sequences are absent without disruption of the translational reading frame are identified. Sequencing and hybridization analysis show a deletion of >6 kb in intron 2 of the human AR gene, starting 18 bp upstream of exon 3. The deletion includes the putative branch-point sequence (BPS) but not the acceptor splice site on the intron 2/exon 3 boundary. The deletion of the putative intron 2 BPS results in 90% inhibition of wild-type splicing. The mutant transcript encodes an AR protein lacking the second zinc finger of the DNA-binding domain. Western immunoblotting is used to show that the mutant AR protein is expressed in genital skin fibroblasts of the patient. The residual 10% wild-type transcript can be the result of the use of a cryptic BPS located 63 bp upstream of the intron 2/exon 3 boundary of the mutant AR gene. The mutated AR protein has no transcription-activating potential and does not influence the transactivating properties of the wild-type AR, as tested in cotransfection studies. It is concluded that the partial androgen-insensitivity syndrome of this patient is the consequence of the limited amount of wild-type AR protein expressed in androgen target cells, resulting from the deletion of the intron 2 putative BPS.

Introduction

The human androgen receptor (hAR) is a ligand-dependent transacting transcription factor belonging to the steroid hormone/thyroid hormone/retinoic acid receptor zinc-finger family (O'Malley 1990). The androgen receptor (AR) mediates the actions of testosterone and 5α-dihydrotestosterone in male sexual development and in the maintenance of normal male reproductive functions during adult life (Griffin and Wilson 1989).

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It is generally accepted that mutations in the AR gene are the cause of the X chromosome–linked androgen insensitivity syndrome (AIS). The nature of these mutations can range from complete or partial gene deletions to single base changes and can result in a range of defects in virilization of a 46,XY individual, defects ranging from male infertility or aberrant virilization (partial AIS [pAIS]) to a genetic male with a complete female habitus (complete AIS [cAIS]) (Brinkmann and Trapman 1992; Griffin 1992; Pinsky et al. 1992). The human AR (hAR) is a phosphoprotein with 910 amino acid residues (Van Laar et al. 1991). The number of amino acid residues, however, can vary, because of polymorphic glutamine and glycine stretches located in the N-terminal domain of the AR (Chang et al. 1988; Lubahn et al. 1988; Trapman et al. 1988; Tilley et al. 1989; McPhaul et al. 1991; Sleddens et al. 1991).

The N-terminal domain of the AR harbors transcript-

tion-activating functions, the central DNA-binding domain consists of two zinc fingers, and the ligand-binding domain is located in the C-terminal part of the protein (Jenster et al. 1991). On ligand binding, the C-terminal zinc finger is assumed to be involved in dimerization, whereas the N-terminal zinc finger of the AR is able to recognize and interact with the hormone-responsive elements in a target gene, thus regulating the transcription of that gene, as has been shown for the glucocorticoid receptor (Luisi et al. 1991).

hAR, mRNA presents itself as a 10.6-kb transcript, containing relatively large 5'- and 3'-UTRs (1.1 and 6.8 kb, respectively). In human prostate tissues and human foreskin fibroblasts, an additional mRNA species of 8 kb is present, generated by alternative splicing in the 3'-UTR (Lubahn et al. 1988; Faber et al. 1991).

The diversity of the AIS, both in the molecular cause and in the resulting clinical characteristics of the syndrome, provides detailed information about the structure-function relationship of the hAR protein. In the present studies the nature of the AR protein in a patient with a severe form of X-linked pAIS is presented. In this particular case, differential pre-mRNA splicing resulting from a genomic deletion involving the intron 2 branchpoint sequence is the molecular cause of the syndrome.

Subject, Material and Methods

The Propositus

The propositus is a member of a family in which four of nine males are affected by X-linked Reifenstein syndrome. The 46,XY index patient was diagnosed as having pAIS, with infertility, hypospadias, small penis, absence of typical male body hair pattern, and gynecomastia. Genital skin fibroblasts of the patient showed a normal specific binding capacity of 39 ± 12 fmol/mg protein, with a K_d of 0.36 ± 0.1 nM. These values are within the normal range. There was no indication of defects in the testosterone or 5α-dihydrotestosterone synthesis.

DNA and RNA Isolation and Analysis

Genomic DNA was isolated from genital skin fibroblast monolayers or from white blood cells by using standard methods (Sambrook et al. 1989). Total cellular RNA was extracted using 3 M LiCl and 6 M urea (Auffray and Rougeon 1980), followed by phenol and chloroform extractions. RNase protection analyses (Sambrook et al. 1989) were performed using 20 μg total RNA and a ^{32}P probe complementary to the cDNA fragment *SacI/StuI* ranging from the 3' part of exon 2 to the 5' part of exon 4 (fragment B in Kuiper et al. 1989). First-strand cDNA synthesis was performed using 4 μg total RNA, 100 ng oligonucleotide (oligo) complementary to part of exon 6 (5'-TGCTGAAGAGTAGCAGTGCT-3'), 10 units AMV reverse transcriptase (Promega), and 10 units RNasin, according to the protocol of the manufacturer. These cDNAs were amplified by PCR using the above oligo as primer, along with an oligo situated in exon 1 (5'-GACTTCACCGCACCTGATG-3'). All PCRs were done in a 100-μl reaction volume, using the Perkin Elmer thermocycler and 2.5 units *Taq* polymerase (AmpliTaq) and the appropriate reaction buffer and conditions as described by the supplier (Cetus). A typical cycling protocol was denaturation for 1 min at 94°C, primer annealing for 2 min at 55°C, and primer extension for 1–3 min at 72°C, for 25–30 cycles. The template was either 1 μg genomic DNA or 2% of the cDNA synthesis reaction mixture. The fragments were size fractionated on 2% agarose gels, ethidium bromide stained, and directly sequenced using the dideoxy chain termination method (Sanger et al. 1977) after purification with Qiaex (Qiagen). The yields of five PCR reactions were pooled for one direct sequencing reaction with 500 ng of sequencing primer. Oligos used for PCR and sequencing were as follows (s = sense; as = antisense; and mismatches in the sequences are indicated by lowercase letters): 1s, 5'-GTTTGGTGCCATACTCTGTCCAC-3'; 2s, 5'-TCAGGTCTATCAACTCTTG-3'; 3s, 5'-TGTCCATCTTGTCGTCTTgGcgccTGTTATGAAGCAGGGATG-3'; 4s, 5'-TCCTCCTCCTTCCTCTCTCC-3'; 1as, 5'-GACGAgAAGATGGAgAATTcTTCCTTCGG-3'; 2as, 5'-CATCCCTGCTTCATAACAggcgCcAAGACGACAAGATGGACA-3'; 3as, 5'-CTGATGGCCACGTTGCCTATGAA-3'; and 5s, 5'-AGAAGTGTCTGTTCATGTCC-3'.

Cell Culture Conditions and Transfections

Genital skin fibroblasts and HeLa cells were cultured in Eagle's minimum essential medium supplemented with nonessential amino acids and antibiotics. Fibroblasts were maintained in medium containing 10% FCS. HeLa cells in medium containing 5% full or 5% dextran charcoal-treated (DCC) FCS. HeLa cells grown to 50%–60% confluency were transfected using the calcium phosphate precipitation method (Chen and Okayama 1987) essentially as described elsewhere (Ris-Stalpers et al. 1991).

AR Expression Vectors

The original hAR cDNA expression vector pSV.AR_0 (Brinkmann et al. 1989) was slightly modified with re-

spect to the restriction-enzyme sites, rendering the *Eco*RI site in the open reading frame unique (BHEX-ARO; a gift from Dr. L. Pinsky). The unique *Kpn*I site and the unique *Eco*RI site were used to generate an expression plasmid with an in-frame deletion of exon 3 (BHEX-ARΔ3), by exchanging the 898-bp *Kpn*I-*Eco*RI fragment of the wild-type expression plasmid with the 781-bp *Kpn*I-*Eco*RI fragment lacking the exon 3 sequence obtained by amplification of cDNA of the patient. The deletion mutant was sequenced to verify the correct reading frame and to exclude misincorporation of nucleotides by *Taq* polymerase.

CAT Assays

HeLa cells were cotransfected with AR expression plasmids and the pG29GtkCAT reporter plasmid (Schule et al. 1988). Twenty-four hours after transfection, cells were incubated for another 24 h, with or without 1 nM of the synthetic androgen methyltrienolone (R1881) or 10 nM dexamethasone, before the CAT activity in the cell lysates was measured essentially as described by Seed and Sheen (1988). Data were corrected for the amount of protein in the cell lysates, and the transfection efficiency was determined by using the CAT activity induced by dexamethasone via the endogenous glucocorticoid receptor.

Immunoblot Analysis

Immunoprecipitation and western blot analysis of the AR protein obtained either from $\sim 5 \times 10^6$ genital skin fibroblasts or from transiently transfected HeLa cells were performed as described elsewhere (Ris-Stalpers et al. 1991). The AR protein on immunoblot was visualized by chemiluminescence (Veldscholte et al. 1992).

Results

Identification of the Genomic Deletion Involving the Intron 2 Branch Site of the hAR Gene

The initial amplification of all hAR coding sequences from genomic DNA of the patient was successful for all coding sequences of the gene except for exon 3, which encodes the second DNA-binding zinc finger. By using several primer combinations corresponding to intron sequences upstream and downstream of exon 3 and exon 3 sequence itself, the presence of a deletion of part of intron 2 and possibly part of exon 3 was established (fig 1).

To narrow the boundaries of the deletion, the intron sequences upstream of exon 3 from genomic DNA of

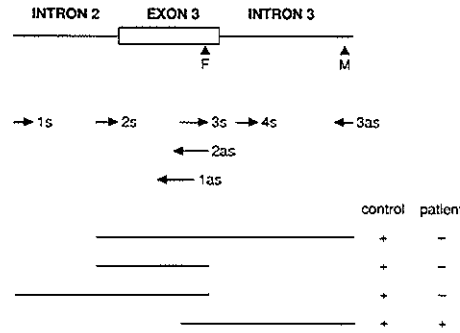

Figure 1 Amplification of exon 3 of the hAR gene and flanking intron sequences: schematic illustration of exon 3 and flanking sequences. Oligos for PCR (s and as), as well as location of restriction sites, are indicated. F = *Fok*I; and M = *Msc*I. The (dis)ability of specific fragments, to be amplified by PCR, from genomic DNA of either the patient or a control sample is shown.

the index patient were amplified by inverse PCR (Ochman et al. 1988). Genomic DNA of the pAIS subject was digested with *Msc*I, ligated, and redigested with *Fok*I (fig 1). The linearized DNA was amplified using oligos 1as and 4s, and the resulting 500-bp fragment was sequenced using oligo 4s. Subsequent sequence analysis identified intron 3 sequence, the *Msc*I site, followed by an unknown sequence, 18-bp intron 2 sequence including the splice acceptor site, and part of exon 3. Comparison with the wild-type genomic sequence revealed the presence of the complete exon 3 coding sequence and 18-bp wild-type intron 2 sequence upstream of exon 3 (including the splice acceptor site at the intron 2/exon 3 junction) and an additional 262 bp of unknown intron 2 sequence (fig 2). This was confirmed by PCR directly on genomic DNA by using primer 2as situated in exon 3 and a primer (5s) situated in the unknown intron 2 sequence, located ~200 bp upstream of the intron 2/exon 3 boundary, followed by direct sequencing of this amplification product. We also used this amplification product to probe digests of two genomic phages, in order to determine the size of the deletion.

In an earlier study, both the structural organization of the hAR gene and the intron sizes were determined (Kuiper et al. 1989). No genomic clones spanning the complete intron 2 region could be identified, and the size of intron 2 was determined to be ≥15 kb. One of the phages described in that study (designated "8.2")

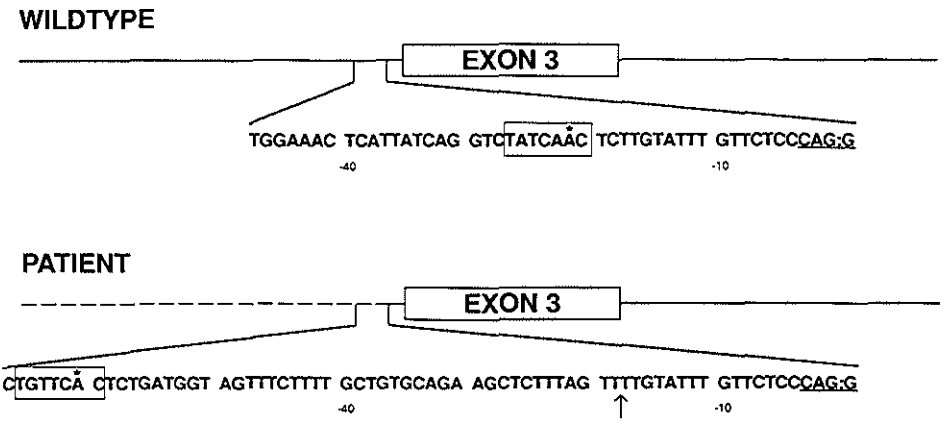

Figure 2 Partial mapping of the deletion in intron 2, using inverse PCR: direct sequencing results of the PCR product generated using oligos 1as and 4s after digesting genomic DNA of the Reifenstein patient with *MscI*, ligation, and subsequent recutting of the ligated DNA by *FokI*. The position where the sequence deviates from the wild-type sequence is indicated by an arrow, and the splice acceptor site is underlined. The wild-type putative branch site and the putative cryptic branch site, in the wild-type and mutant AR gene, respectively, are boxed, and the branch nucleotide is indicated by an asterisk.

contains exon 2 and 8-kb downstream intron 2 sequence. Hybridization of digested 8.2 phage DNA by the probe described above resulted in a positive hybridizing signal, indicating that the deletion originates in this area. A phage (designated "9.2"; Kuiper et al. 1989) containing exon 3 and 6-kb upstream intron 2 sequence was also digested. Hybridization in this case resulted in a positive band, corresponding to a genomic fragment containing exon 3 itself. No hybridization was seen with the 6-kb upstream sequence of exon 3. Since these two phages do not overlap, it was concluded that the part deleted from intron 2 is ≥6 kb. When a sense oligo in exon 2 and an antisense oligo in exon 3 were used, no positive PCR signal could be obtained from the mutated AR gene, indicating that the size of the remaining intron 2 sequence is >1,500 bp (data not shown).

Effect of the Intron Deletion on RNA Splicing

RNA was isolated from genital skin fibroblasts of the patient and from control genital skin fibroblasts. First-strand cDNA synthesis was performed using an AR-specific oligo located in exon 6 of the hAR gene. Subsequent PCR amplification using this oligo and a sense oligo situated in exon 1 resulted in two amplification products—contrary to control RNA, which showed only one amplification product (fig. 3). Sequencing of both amplification products showed the minor product to be the wild-type transcript and the major product to be a transcript from which the complete exon 3 sequence was deleted.

An RNase protection assay using a ^{32}P-labeled *SacI*(exon 2)-*StuI*(exon 4) fragment was performed to establish the relative amounts of both transcripts. Densitometric analysis of the autoradiograms showed a ratio of 8% wild-type transcript to 92% exon 3 deleted transcript (in three separate experiments the ratios of wild-type transcript:exon 3 deleted transcript were 8:92, 13:87, and 4:96) (figs. 4 and 5).

Binding Characteristics and Immunoblot Analysis of the Mutant AR Protein

The complete deletion of exon 3 from the hAR mRNA transcript is in-frame and will, on translation, result in a normal ligand-binding protein with a deletion of 39 amino acids from the DNA-binding domain of the hAR. Both the wild-type and the mutant AR protein are expected to bind ligand normally. Genital skin fibroblasts of the patient showed a specific binding capacity for androgens that was within the normal range (see Subject, Material, and Methods).

Immunoprecipitation of the AR from genital skin fibroblasts cultured from a biopsy of the pAIS patient

with the AR-specific monoclonal antibody F39.4.1 was followed by SDS-PAGE and immunoblotting using the polyclonal antibody Sp061. Visualization was done by chemiluminescence. This procedure showed only a mutant protein with an apparent molecular mass of 100 kD in genital skin fibroblasts of the patient. The translation of the 10% wild-type transcript was below the detection limit. Similar to the wild-type AR protein, the mutant AR protein migrated as a doublet, with the majority of protein in the band with the highest apparent molecular mass (Jenster et al. 1991) (fig. 6). The mutant AR doublet, however, was much less spaced, compared with the wild-type AR doublet.

Functional Activity of the Mutant AR Protein in HeLa Cells

In order to analyze the functional activity of the mutant AR, an hAR expression plasmid was generated with an in-frame deletion of exon 3 (BHEX-ARΔ3). The BHEX-AR0 (wild-type expression vector) and BHEX-ARΔ3 constructs were transiently transfected in HeLa cells with the p29GREtk-CAT reporter gene. Two ratios (1:1 and 1:8) of the wild-type and the Δ3 construct were transfected. Only the wild-type construct showed transactivation potential in het presence of the synthetic androgen R1881. The ARΔ3 construct had

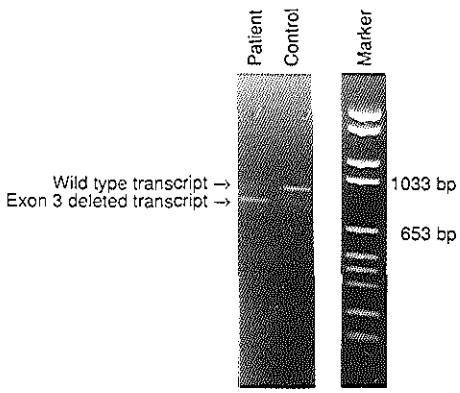

Figure 3 Alternative splicing of the AR pre-mRNA, as a result of a partial intron 2 deletion of the hAR gene: RT-PCR on RNA isolated from genital skin fibroblasts of the patient and from a control genital skin fibroblast strain. First-strand synthesis and PCR amplification using an exon 6-exon 1 primer combination shows a majority of mutant transcript and a minority of wild-type transcript in RNA from the patient.

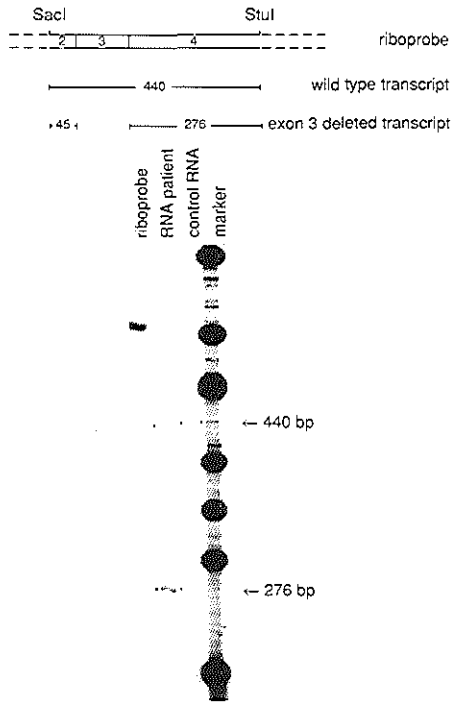

Figure 4 RNase protection assay: Expression of both the wild-type AR mRNA and a transcript lacking exon 3 sequences in genital skin fibroblasts of the index patient. A schematic representation of the RNase protection analysis, including the used riboprobe, is given. The marker used is pBR322 *HinfIxEco*RI.

no transactivating properties with or without ligand and did not influence the transactivating properties of the wild-type expression vector when cotransfected with equal amounts of AR0 or with eight times in excess of it (table 1). The lack of transactivating potential of the mutant AR was not due to underexpression of the construct in HeLa cells, as was assessed by western/immunoblotting analysis (data not shown).

Discussion

AR disorders in the AIS are well documented. There is no indication for a hot spot for mutations; rather, there is a whole range of mutations, varying from (par-

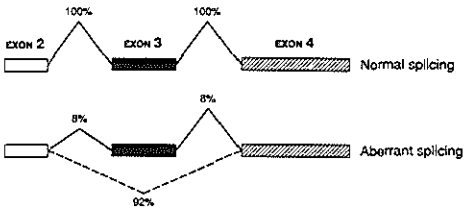

Figure 5 Schematic illustration of exons 2–4 of the hAR gene and of the alternative splicing event resulting from the partial intron 2 deletion. The solid lines represent wild-type splicing events, and the dashed line represents the aberrant splicing event. The ratio of the two splicing events, as determined by densitometric determination of the intensity of the bands shown in fig. 4, is indicated.

tial) gene deletions to single base changes, which result in the introduction of either a premature stop codon or an amino acid change.

In the present study a unique, >6-kb deletion in intron 2 of the AR gene, which does not involve any protein-coding sequences or splice acceptor/donor sequences, is reported. The deletion leaves 18 bp upstream of exon 3 intact. The intronic deletion has serious consequences for the splicing of the AR pre-mRNA, because of the deletion of the putative branch-point sequence (BPS).

The first event in pre-mRNA splicing is the cleavage at the 5′ splice site. Subsequently a loop or lariat structure is formed where an adenosine residue (the branch

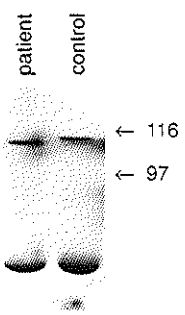

Figure 6 SDS-PAGE analysis of hAR protein immunoprecipitated from genital skin fibroblasts of the patient and of control fibroblasts. The position of marker proteins is indicated.

Table 1

Transcriptional Activity of the ARΔ3 Mutant

BHEX-AR0:BHEX-ARΔ3[a]	CAT Activity[b] (%) −R1881	+R1881
1:0	<1	100 (n=7)
0:1	<1	<1 (n=7)
1:1	<1	142 ± 37 (n=5)
1:8	<1	118 ± 8 (n=2)

NOTE.—HeLa cells were transiently cotransfected with different amounts (0.5–5 μg/ml precipitate) of the expression plasmids BHEX-AR0 and BHEX-ARΔ3 and the reporter plasmid pG29GtkCAT. CAT activity was measured in cell lysates after culturing for 24 h in the absence (−R1881) or presence (+R1881) of 1 nM R1881.

[a] Ratio of the amounts of expression plasmids used.

[b] Presented as a percentage relative to that of the wild-type AR expression plasmid, which was set at 100% for each individual experiment. The number of experiments and the SD of the mean are indicated.

nucleotide) is linked to the 5′ end of the intervening sequence. Following the lariat formation, cleavage at the 3′ splice site takes place, resulting in the excision of the intron as a lariat and in the concomitant ligation of both exons.

The site of branch formation is typically located 20–50 nt upstream of the 3′ splice site, which is always the first AG sequence downstream of the BPS. The consensus sequence for the mammalian BPS and acceptor splice site is T/C,T/C,T/C,G/A,A,T/C. . .(T/C)$_{11}$. . .N,C/T,A,G:G (Reed and Maniatis 1985; Padgett et al. 1986; also see references therein). In eukaryotes, base pairing occurs between the BPS and the small nuclear ribonucleoprotein particle U2snRNP, indicating the direct involvement of the BPS in the assembly of the spliceosome (Wu and Manley 1991). The BPS in intron 2 of the AR gene fits the consensus sequence closely, and the branch nucleotide is on position −22, within the range of 20–50 nt upstream of the acceptor splice site. The AG:G used as splice acceptor site is the first AG:G downstream of the putative BPS. The intron 2 deletion described in the present study ends 18 bp proximal to the 3′ end of the intron and completely abolishes the putative BPS.

The mammalian BPS shares sequence homology with the yeast "TACTAAC" BPS, which is critical in yeast; only small deviations from the consensus sequence in yeast are tolerated (Langford et al. 1984). It has been

reported that the deletion of mammalian BPS either only slows down the splicing reaction or does not influence the pre-mRNA splicing efficiency at all, as was shown for the rabbit β-globin IVS 2, where no specific internal intron sequences are required for correct and efficient splicing, but only a minimal intron length of 80 nucleotides (Wieringa et al. 1984). However, in this case it cannot be totally excluded that a cryptic branch site has been utilized.

The results presented in this study strongly indicate that the deletion of the BPS is very deleterious for pre-mRNA splicing. The deletion of the putative BPS in intron 2 of the hAR gene inhibits 90% of wild-type splicing, by skipping the acceptor splice site on the intron 2/exon 3 boundary, which results in a transcript with an in-frame deletion of exon 3 sequence. That the BPS in this case is not indispensable for proper pre-mRNA splicing is evident, since ~10% wild-type transcript is still present, as shown by reverse transcriptase-PCR (RT-PCR) and RNAse protection. Whether the wild-type splicing event is the result of the use of a cryptic BPS is not clear. A possible BPS at position −63 in the mutant AR intron 2 sequence, which partly fits the consensus sequence, could function as such. There are, however, four other AG dinucleotides between the putative cryptic BPS and the regular splice acceptor site. RT-PCR demonstrated that they are not used as such. If this cryptic BPS is involved in the 10% wild-type splicing, then it is clearly a weak BPS probably due to the deviation from the consensus sequence on the first base upstream of the branch nucleotide.

Aberrant splicing events are a relatively rare phenomenon in AIS. Elsewhere we have reported a G→T mutation on position 1 in the splice donor site of intron 4 in the AR gene of a completely androgen-insensitive individual, which completely abolished wild-type splicing (Ris-Stalpers et al. 1990).

Mutations in the second DNA-binding zinc finger of members of the steroid hormone receptor family have been described before elsewhere (Hughes et al. 1988; Sone et al. 1990; Chang et al. 1991; Marcelli et al. 1991; Klocker et al. 1992; Zoppi et al. 1992). They result in either partial or complete hormone insensitivity. It is therefore not unexpected that the in-frame deletion of the second DNA-binding zinc finger of the hAR gene, due to a genomic deletion inclusing exon 3 sequences, results in cAIS. The protein in vitro is unable to activate transcription of an androgen-reporter gene, as has been shown both by Quigley et al. (1992) and in the present study. Specific androgen binding in genital skin fibroblasts of the Reifenstein patient is within the normal range and does not corroborate the supranormal concentration found by Quigley et al. (1992) in a cAIS patient with an exon 3 deletion because of failure of autologous down-regulation.

The occurrence of a mutant AR protein in combination with the wild-type protein in an androgen-insensitive patient has not been described before. The partial syndrome of the patient described here must be the result of an insufficient amount of functional AR protein, since the mutant protein itself is inactive. Cotransfection studies showed that the mutant protein has no dominant negative effect on the wild-type protein, as has been described for a mutant estrogen receptor. Mutant estrogen receptors lacking the second DNA-binding zinc finger inhibit estrogen-dependent transcription activation in a dominant negative fashion when cotransfected with the wild-type estrogen receptor and a reporter plasmid. This inhibitory effect probably occurs through protein-protein interactions (Wang and Miksicek 1991).

A synergistic effect on transcription-activating potential has been described for a transcription factor SP1 mutant lacking all of the DNA-binding zinc fingers in combination with the wild-type transcription factor (Courey et al. 1989; Pascal and Tjian 1991). The glutamine-rich activation domains involved in this superactivation are also present in the hAR, but we have found no evidence of superactivation of the mutant AR protein lacking the second DNA-binding zinc finger.

Acknowledgments

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A PRACTICAL APPROACH TO THE DETECTION OF ANDROGEN RECEPTOR GENE MUTATIONS AND PEDIGREE ANALYSIS IN FAMILIES WITH X-LINKED ANDROGEN INSENSITIVITY (AIS)

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ABSTRACT

Androgen insensitivity (AIS) is an X-linked disorder where defects in the androgen receptor gene have prevented the normal development of both internal and external male structures in 46,XY individuals. This survey reports the analysis of 11 AIS subjects. The androgen receptor (AR) gene of these subjects was analyzed using PCR-single-strand conformation polymorphism (SSCP) analysis and sequencing, or sequencing of PCR amplified AR gene fragments alone.

In total ten single base changes and one partial gene deletion were detected. Seven single base changes resulted in an amino acid change, one in the introduction of a premature stopcodon, one event represented a single base insertion resulting in a frameshift and one single base change affected a donor splice site. The androgen receptor protein in genital skin fibroblasts from several patients was studied with respect to molecular mass after immunoprecipitation and SDS-PAGE. Two patients expressed a truncated receptor protein in agreement with the established genomic mutation. Pedigree analysis was performed to identify possible carriers for the syndrome in families of AIS patients using SSCP and restriction site analysis of PCR products. In one case the polymorphic $(CAG)_n(CAA)$ repeat in exon 1 encoding a polyglutamine stretch was used to identify the mutant allele in a family with X-linked partial androgen

insensitivity, prior to the identification of the actual genomic mutation.
PCR-SSCP analysis proved to be a fast and reliable technique to screen for androgen receptor gene mutations and to study the AR gene of family members of AIS affected individuals.

INTRODUCTION

The most common cause of male pseudohermaphroditism is the androgen insensi-tivity syndrome (AIS) (1). Androgen insensitivity is a heterogenous syndrome where the masculine development of both internal and external structures of a 46,XY individual may be affected. The clinical phenotype of affected individuals consists of a spectrum of defects in male sexual differentiation. In the most severe cases a 46,XY individual is presented with a female phenotype (complete AIS). In other cases it may concern a 46,XY phenotypic female with clitoromegaly or ambiguous genitalia (partial AIS), or a phenotypic male with severe hypospadias (Reifenstein syndrome) or unexplained infertility (2). In all these cases the clinical syndrome results from diminished or absent androgen action due to a (partly) non-functioning androgen receptor, although testosterone synthesis is unimpaired (1). Defining all the possible mutations in the androgen receptor in patients with partial androgen insensitivity, may eventually lead to a better understanding and may be even a better way of predicting the further development of the secondary sex characteristics and fertility of affected children.

The androgen receptor is a member of the steroid/thyroid hormone/retinoic acid receptor zinc finger family (3), consists of 910 amino acid residues (4) and has a molecular structure homologous to the other family members: an N-terminal transcription regulating domain, a DNA-binding domain, and a C-terminal ligand binding domain (5). The number of amino acid residues can vary between individuals due to two polymorphic amino acid stretches in the N-terminal domain: a polymorphic GGN repeat encoding a glycine stretch and a

polymorphic $(CAG)_n(CAA)$ repeat encoding a polyglutamine stretch. The polymorphic $(CAG)_n(CAA)$ repeat has a frequency of heterozygosity in females of 0.89 and is extremely useful for carrier diagnosis in families with AIS (6).

Although most members of the nuclear receptor family have a corresponding resistance syndrome, the androgen receptor due to its genomic location on the X-chromosome shows the full effect of the syndrome in 46,XY individuals, not compensated by a translation product from an other autosomal allele as do the other members of the receptor family.

In this study the localization and characterization of mutations in the human androgen receptor gene from 11 unrelated subjects suffering from different degrees of androgen insensitivity is reported. Several mutations have been identified by single-strand conformation polymorphism (SSCP) analysis (7), other mutations could be confirmed by PCR-SSCP analysis. The SSCP analysis technique, in most cases combined with restriction enzyme analysis of PCR products, was also successfully employed to screen family members of AIS patients for heterozygosity of the syndrome. The polymorphic $(CAG)_n(CAA)$ repeat in exon 1 of the human androgen receptor gene was employed to study family members of an AIS patient, who at a later date was diagnosed as having a genomic deletion of over 6 kb from intron 2 of the hAR gene.

Immunoprecipitation of the androgen receptor from genital skin fibroblast lysates, obtained from either receptor binding negative or receptor binding positive AIS patients was performed to investigate if a receptor protein was present.

MATERIALS AND METHODS

Clinical subjects. Subjects were initially diagnosed in different clinics in the Netherlands, Germany, Canada and the United Kingdom. The diagnosis of partial or complete AIS was made taking into account the karyotype, the clinical phenotype, laboratory data including the male hormone levels in plasma when available, and relevant family history (summarized in Table 1). Informed consent was obtained from all subjects or their parents.

Cell culture. Fibroblasts derived from genital skin biopsies were maintained in Eagle's Minimum Essential Medium supplemented with 10 % fetal calf serum, nonessential amino acids and antibiotics.

Table 1: Identification of mutations in the hAR gene and analysis of the AR protein from genital skin fibroblasts of AIS patients analyzed in the Rotterdam laboratory.

subject	karyotype	evidence of defects in testosterone synthesis or metabolism	phenotype	diagnosis	androgen binding GSF	western/ immunoblot	mutation initially identified by	mutation AR gene	location mutation	protein change	family analysis done using
1 a)	46, XY	no	M; hypospadias, small penis, infertility, no male body hair pattern, gynecomastia	Reifenstein	normal, Bmax 39± 12 fmol/mg P, Kd 0.36 ± 0.1 nM	105 kDa	size analysis of PCR product	del > 6kb	intron 2	deletion of 2nd zinc finger (90%)	polymorphic (CAG)nCAA repeat
2	not done	not done	F	cAIS	not done	not done	SSCP analysis	G → T	exon 3	Arg 606 His	SSCP analysis, Bbsl digest
3 b)	46, XY	no	F	cAIS	below normal, Bmax 6-11 fmol/mg P	110 kDa doublet	sequencing of PCR product	G → C	exon 4	Asp 686 His	SSCP analysis, HinfI digest
4 b)	46, XY	no	F	cAIS	normal, rapid dissociation, Bmax 29-36 fmol/mg P, Kd 1.9 nM	110 kDa doublet	sequencing of PCR product	G → A	exon 4	Asp 686 Asn	SSCP analysis, HinfI digest
5 c,d)	46, XY	no	F	cAIS	not detectable	105 kDa doublet	sequencing of PCR product	G → T	intron 4	Δ aa 674-714	western blot analysis, RT-PCR exon 3-5
6	46, XY	not done	F; ambiguous genitalia	pAIS	not detectable	110 kDa doublet	sequencing of PCR product	A → G	exon 5	Met 733 Val	polymorphic (CAG)nCAA repeat
7	46, XY	no	F; ambiguous genitalia	pAIS	not detectable	110 kDa doublet	SSCP analysis	T → C	exon 5	Met 736 Thr	not done
8 e)	46, XY	no	F	cAIS	not detectable	110 kDa doublet	SSCP analysis	T → C	exon 5	Phe 755 Leu	not done
9	46, XY	not done	F; clitoromegaly, labial fusion	pAIS	below normal, 13± 6 fmol/mg P, Kd 62 nM, 45% thermolability	110 kDa doublet	SSCP analysis	G → A	exon 6	Met 771 Ile	SSCP analysis, RsaI digest
10	46, XY	no	F	cAIS	not detectable	99 kDa doublet	sequencing of PCR product	C → T	exon 6	Arg 822 Stop	SSCP analysis, SfuI digest
11 e)	46, XY	no	F; no uterus, fallopian tubes or vas deferens, testis cryptorch	cAIS	not detectable	105 kDa doublet	SSCP analysis	A insertion	exon 7	aa 1-837 wild type sequence + 32 nonsense aa	not done

a) Identification and characterization of mutation including western blot analysis of genital skin fibroblasts: manuscript submitted
b) Identification and characterization of mutation published in ref 8
c) Identification and characterization of mutation published in ref 20
d) Western blot analysis of family published in ref 12
e) Binding data and clinical description published in ref 21
M: predominant phenotype male; F: predominant phenotype female
cAIS: complete androgen insensitivity syndrome; pAIS: partial androgen insensitivity syndrome
aa: amino acid residues

Specific androgen binding and western blot analysis. Scatchard plot analysis was performed in the laboratory of origin, relevant references are included in table 1. In general: Genital skin fibroblasts were incubated with ^{3}H-labelled 5α-dihydrotestosterone (DHT)(NEN) in a range of 10 concentrations (0-6nM) for 45 minutes at 37 °C. Nonspecific DHT binding was determined using labelled DHT in the presence of 100 nM unlabelled DHT. After incubation, cells were harvested and the cell pellet resulting from a 500xg centrifugation step was washed with a TrisHCl/EDTA buffer (0.9% NaCl, 10 mM Tris, 10 mM Na_2MoO_4, 1 mM EDTA, 0.5 mM DTT, 0.002% NaN_3 pH:7.4). The supernatant was removed and the cellpellet was stored overnight at -70 °C. After thawing, 40 ml 0.6 M KCl was added and the cells were left at 4 °C for one hour. A 0.25% charcoal suspension (150 µl), containing 0.025% dextran was added and after 5 minutes shaking, cell debris and charcoal were removed by centrifugation at 1000xg for 10 minutes. 100 ml supernatant was used for ^{3}H counting. The protein content of the samples was measured using the Biorad protein assay kit. A Scatchard plot was constructed using standard statistical routines. Each assay was done in duplicate with 4 samples for each DHT concentration. The two B_{max} and Kd values were combined, using the squared standard errors as weighing factors.

Immunoprecipitation and western blot analysis of the androgen receptor protein obtained from approximately 5 x 10^{6} genital skin fibroblasts were performed as described previously (8). Briefly: the AR was immunoprecipitated from whole cell lysates of genital skin fibroblasts using the AR specific monoclonal antibody F39.4.1. followed by SDS-PAGE and immunoblotting using the polyclonal antibody Sp061. The androgen receptor protein on immunoblot was visualized by chemiluminescence (9).

TABLE 2: Oligonucleotides used for PCR and sequencing

amplified fragment	sense oligonucleotide	antisense oligonucleotide
(-) 77[1,2] - exon 1: nucleotides 286	GCC TGT TGA ACT CTT CTG AGC	CTT GGG GAG AAC CAT CCT CA
exon 1: nucleotides 266 - 526	AGC AAG AGA CTA GCC CCA GGC AGC	CGG AGC AGC TGC TTA AGC CGG GG
exon 1: nucleotides 466 - 766	CTG CCC CAT CCA CGT TGT CCC TGC T	GAC TCA GAT GCT CCA ACG CCT CCA C
exon 1: nucleotides 707 - 958	TGT GTA AGG CAG TGT CGG TGT CCA T	CGC CTT CTA GaC CTT TGG TGT AAC
exon 1: nucleotides 886 - 1165	CAG GCA AGA GCA CTG AAG ATA CTG C	GGT TCT CCA GCT TGA TGC GAG CGT G
exon 1: nucleotides 1075 - 1313	CGC GAC TAC TAC AAC TTT CCA CTG G	GCT GTc tAG AGA GTG TGC CAG GAT GAG
exon 1: nucleotides 1291 - 1488	TCC TGG CAC ACT CTC TTC AC	GCA AGG GTA CCA CAC ATC AGG T
exon 1: nucleotides 1411 - intron 1	TAG CCC CCT ACG GCT ACA	CAG AAC ACA GAG TGA CTC TGC
exon 1: $(CAG)_nCAA$ repeat	TCC GCG AAG TGA TCC AGA AC	CTT GGG GAG AAC CAT CCT CA
exon 2 and flanking sequences	GTC ATT TAT GCC TGC AGG TT	TC TCT CTC TGG AAG GTA AAG
exon 3 and flanking sequences	TCA GGT CTA TCA ACT CTT G	CTG ATG GCC ACG TTG CCT ATG AA
exon 4 and flanking sequences	ATT CAA GTC TCT CTT CTT TC	GCG TTC ACT AAA TAT GAT CC
	CAG AAG CTt ACA GTG TCA CAC A	GCG TTC ACT AAA TAT GAT CC
	ATT CAA GTC TCT CTT CCT TC	TGC AAA GGA GTt GGG CTG GTT G
exon 5 and flanking sequences	GAC TCA GAC TTA GCT CAA CC	ATC ACC ACC AAC CAG GTC TG
exon 6 and flanking sequences	CAA TCA GAG ACA TTC CTC TGG	AGT GGT CCT CTC TGA ATC TC
exon 7 and flanking sequences	TGC TCC TTC GTG GGC ATG CT	TGG CTC TAT CAG GCT GTT CTC
exon 8 and flanking sequences	AG GCC ACC TCC TTG TCA AC	AA GGC ACT GCA GAG GAG TA

nucleotide numbering based on an open reading frame of 2730 nucleotides
all sequences are displayed in 5' -> 3' orientation
mismatches are indicated by lower case
[1]nucleotide position relative to the ATG start codon
[2]This PCR product is relatively large and was therefore digested with the restriction enzyme PstI prior to SSCP analysis. This procedure also makes it possible to disregard the abberant SSCP profile due to the polymorphic $(CAG)_n(CAA)$ repeat.

DNA isolation and analysis. Genomic DNA was isolated from genital skin fibroblasts or from blood cells using standard methods (10). PCR reactions were done in a 100 ml reaction volume as described before (8) using the Perkin Elmer Thermo Cycler, 2.5 units Taq polymerase (AmpliTaq) and the appropriate reaction buffer and conditions as described by the supplier (Cetus). For PCR reactions covering the GGN repeat in exon 1, deaza dGTP was used instead of dGTP. Oligonucleotides used for PCR amplification of the AR gene and for direct sequencing are indicated in Table 2.

Radioactive PCRs to determine the length of the $(CAG)_n(CAA)$ repeat in exon 1 or as a basis for SSCP were prepared using 22 nM ^{32}PdATP in a 15 µl standard PCR reaction. To determine the length of the repeat encoding the polyglutamine stretch PCR products were size fractioned on a 6% polyacrylamide denaturing sequence gel accompanied by a standard sequence reaction. SSCP analysis was done using a nondenaturing 7% polyacrylamide gel (2% crosslinking) and either 5 or 10% glycerol. Samples consisted of 1 µl PCR product and 9 µl loading dye (95% formamide, 5% glycerol, 10 mM EDTA). Before loading on gel the samples were denatured for 5 minutes at 100 °C, followed by a quick chill on ice. Electrophoresis was performed at room temperature , for 16 hours at 7 Watt constant power.

The yield of five PCR reactions was pooled for one direct sequencing reaction after purification from a 2% agarose gel using Qiaex (Qiagen Inc. USA). Sequencing was done using the dideoxy-chain termination method (11).

RESULTS

Specific androgen binding in genital skin fibroblasts

Binding studies on genital skin fibroblasts were performed in the laboratories of origin and are included in Table 1. From all patient material available initially those with aberrant androgen binding characteristics were selected for further characterization of the underlying genomic defect. In genital skin fibroblasts of the subjects 6 and 7 no specific androgen binding was detectable. These patients have been diagnosed as partial AIS and therefore the androgen receptor should have some residual ligand binding activity. Either a partially functioning constitutive receptor protein or an increased dissociation of the ligand-receptor complex due to a mutation in the steroid binding domain of the receptor could be responsible for the pAIS in these patients.

In genital skin fibroblasts of subject 1 (Reifenstein syndrome) a normal binding capacity was established and the dissociation constant for androgens was within the normal range. Patient 4 also displayed a normal binding capacity for androgens but an increased dissociation rate of the ligand-receptor complex was observed (8).

All other complete AIS subjects had low or undetectable levels of specific androgen binding in their genital skin fibroblasts (Table 1).

Western blot analysis of the androgen receptor protein

Immunoprecipitation of the androgen receptor protein was done for all subjects whose genital skin fibroblasts were available (Figure 1, Table 1). The androgen receptor of AIS subjects 3, 4, 6, 7, 8 and 9 displayed the expected normal pattern of a 110 kDa doublet. In the case of subjects 6 and 7 this decreased the likelihood of the presence of a truncated androgen receptor protein with constitutive activity.

The AR immunoprecipitated from genital skin fibroblasts of subjects 10 and 11 showed androgen receptor specific protein bands of approximately 100 and 105 kDa respectively, in agreement with the later established mutations in the hAR gene.

Subject 1 has a deletion in intron 2 of the androgen receptor gene which leads to an alternative splicing event resulting in a deletion of exon 3 sequence in 92% of the transcripts. Upon translation this will give rise to a receptor protein with an inframe deletion of the second DNA binding zinc-finger. This 105 kDa protein was present in genital skin fibroblasts of the patient, the 8% wild type protein was not detectable . Since the 105 kDa protein is completely non-functional with respect to transactivation, the partial virilization of the AIS subject must be the consequence of the limited amount of wild type androgen receptor protein (Ris-Stalpers et al., submitted).

Western blot analysis of the androgen receptor of subject 5, where amino acids 674-714 from the androgen receptor are deleted in frame showed an androgen receptor protein of approximately 105 kDa, her mother and affected sibling also showed the expected smaller androgen receptor protein (12).

In none of the receptor negative AIS subjects was the absence of specific androgen binding due to the absence of androgen receptor protein.

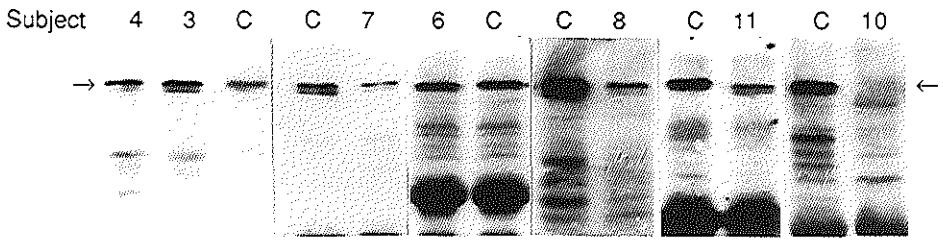

Figure 1: SDS-PAGE analysis of AR protein immunoprecipitated from genital skin fibroblasts of AIS subjects and from control genital skin fibroblasts. Patients are identified on top of each lane, numbers correspond to Table 1 (c= control fibroblast strains). The position of the 110 kDa androgen receptor doublet is indicated by arrows.

Mutations in the androgen receptor gene

Using PCR amplification of genomic DNA followed by size analysis, SSCP analysis and sequencing of the amplified products, the whole coding region of the AR gene of 11 subjects suffering from androgen insensitivity was screened for mutations. In total 11 mutations were identified and characterized (Table 1). Ten single base changes and one partial gene deletion were identified. Only the G -> T mutation in intron 4 of the hAR gene (patient 5) was undetectable by SSCP analysis under the different conditions as mentioned in the materials and methods section. All other mutations were either firstly identified using SSCP analysis or could be assigned a deviant SSCP profile on hindsight. Subjects 3 and 4 where the same G nucleotide is mutated in a C or an A respectively, showed clearly distinct SSCP profiles (Figure 2).

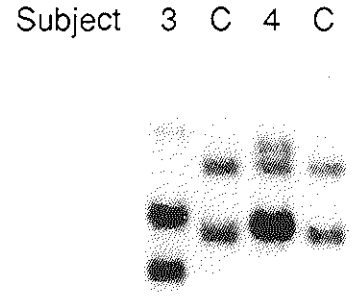

Figure 2: SSCP analysis showing exon 4 of the hAR gene amplified from genomic DNA. Exon 4 SSCP profile of patient 3 (G -> C mutation of nucleotide 2056)(lane 1), control (lane 2), patient 4 (G -> A mutation of nucleotide 2056)(lane 3) and control (lane 4).

Pedigree analysis

Several of the AIS subjects belong to families with additionally affected members. In order to identify possible carriers we used different methods to analyze their pedigrees (Table 1).

We established that the female relatives III-6 and III-7 of subject 9, (Figure 3A), do not carry the mutant androgen receptor allele. In this particular case the G -> A mutation in exon 6 destroys a site for the restriction enzyme RsaI, resulting in a 191 bp and a 81 bp fragment when the mutant exon 6 PCR fragment is digested with RsaI. When exon 6 is PCR amplified from the wild type allele, the 191 bp fragment is digested by RsaI and digestion in this case results in three fragments of 167, 81 and 24 bp (Figure 3B). SSCP analysis is also informative. In the case of subject 9 and several of her family members the SSCP profile of exon 6 confirms the results obtained by the restriction site analysis of amplified exon 6 (Figure 3C).

Sometimes there is an urgent demand to investigate if carriers for the syndrome are present in the family prior to the identification and characterization of the actual AR gene mutation, or the nature of the mutation prevents the reliable

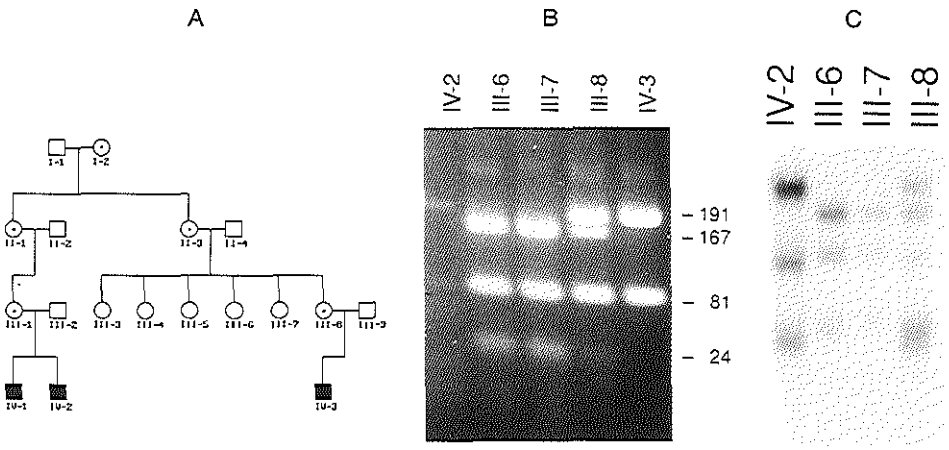

Figure 3: Partial pedigree analysis of family of AIS subject 9 (IV-2) by RsaI digestion and SSCP analysis of PCR amplified exon 6.
***A**: pedigree of subject 9.(IV-2) **B**: PCR amplified exon 6 digested with RsaI size fractionated on a 2% ethidium bromide stained agarose gel. The affected members IV-2 and IV-3 have the 191 and 81 bp fragments, family members III-6 and III-7 show the wild type 167, 81 and 24 bp fragments and the obligate heterozygote III-8 has both the wild type and the mutant fragments. **C**: SSCP analysis of radioactive PCR amplified exon 6 of patient IV-2, unaffected individuals III-6 and III-7, and obligate carrier III-8.*

detection of carriers with the presently used methods. In such cases SSCP analysis and restriction site analysis are not suitable for investigations of the pedigree. To investigate the family members of subject 1 (pedigree in figure 4A), prior to the actual identification of the underlying genomic mutation, the polymorphic $(CAG)_n(CAA)$ repeat in exon 1 of the human androgen receptor gene was used. The mutant androgen receptor gene was found to contain a repeat length of 20 and this co-segregated with the mutant phenotype in this family. The obligate carriers II-2, II-5 and III-2 also had the 20 repeat allele. Subject III-5 inherited the other maternal allele (Figure 4B). Hence she was proven not to be a carrier for the Reifenstein syndrome. After identification of the genomic mutation in the androgen receptor gene of patient 1, confirmation of the pedigree analysis by PCR-SSCP or restriction enzyme analysis proved to be impossible. No amplification product of exon 3 can be obtained using our

standard primers, due to the deletion of the annealing site of the upstream PCR primer (Ris-Stalpers et al, submitted). Affected individuals can be identified by the absence of a specific exon 3 amplification product. Carriers still show the amplification product from the other (normal) X-chromosome.

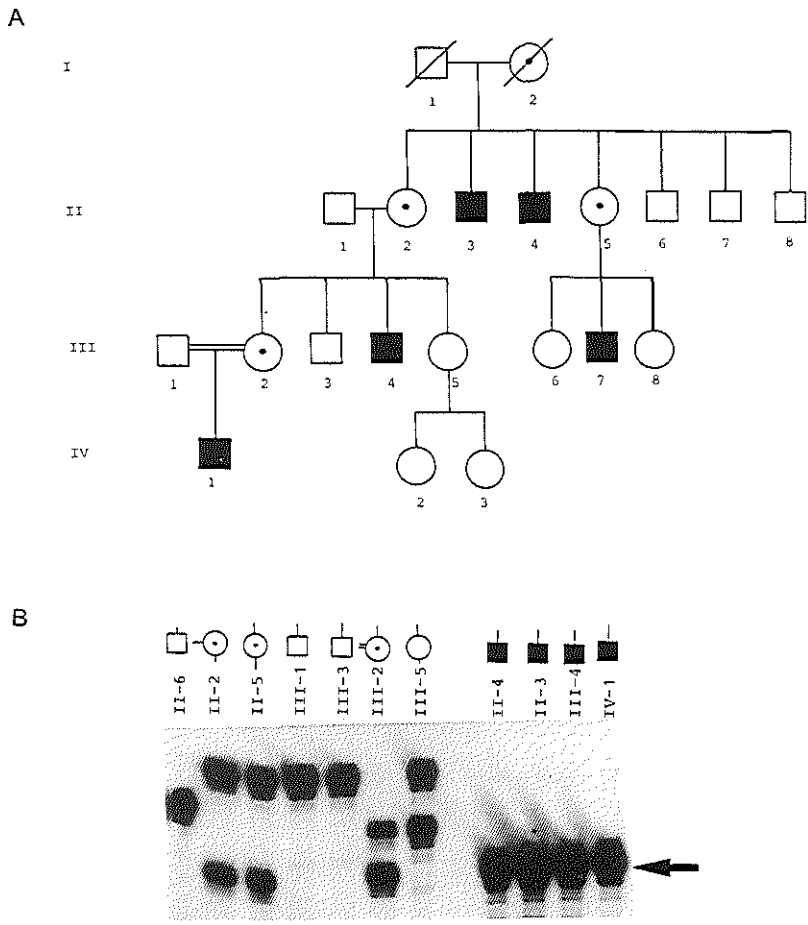

Figure 4: Family analysis of AIS subject 1 (III-7). A: pedigree of family with familial X-linked hypospadias (Reifenstein syndrome). B: PCR amplified polymorphic $(CAG)_n(CAA)$ repeat (poly-Gln-stretch) of the hAR in a family with X-linked hypospadias due to a deletion of over 6 kb in intron 2. The mutant allele is indicated by an arrow.

DISCUSSION

This paper describes mutations in the human androgen receptor gene of subjects with either complete or partial androgen insensitivity. The majority of mutations in the ligand binding domain of the human androgen receptor mainly cluster in two regions of the gene (13). One of these regions is homologous to a region in the thyroid hormone receptor gene which is a known cluster site for mutations that cause generalized thyroid hormone resistance (14).

It is generally accepted that a single mutation does not correlate with a specific AIS phenotype and there is also no apparent clustering of mutations found responsible for either the partial or the complete form of the androgen insensitivity syndrome. There are however a number of the same codons in the androgen receptor gene that are mutated in several unrelated AIS patients so it seems that certain positions in the gene are mutated more frequently. The total number of different mutations however is too high to be able to speak of hot spots for mutations in the hAR gene.

The Arg606His mutation identified in this study in patient 2 has also been reported for the homologous amino acid residue of the human vitamin D receptor (15). In this case the vitamin D receptor was inactive in promoting transcription in a cotransfection assay. This suggests that the Arg606His mutation in the androgen receptor is responsible for the cAIS in patient 2. A mutation at methionine residue 733 in the AR has been reported before, although the transition in this case was not to valine as in subject 6, but to isoleucine (16). This amino acid mutation also concerned a patient with pAIS but with detectable androgen binding in genital skin fibroblasts. Both the Met771Ile mutation of patient 9 and the Arg822Stop mutation of patient 10 were reported previously in unrelated subjects (16,17).

In the case of subjects 6, 7, 8 and 9 it is likely that the amino acid change in the steroid binding domain results in a receptor protein where the steroid binding capacity itself is (partly) unimpaired but where the dissociation of the ligand from

the receptor complex is enhanced. The receptor will be totally non-functional in case of patient 8 and partly unable to regulate transcription of androgen receptor target genes, in case of the pAIS subjects 6, 7 and 9. Genital skin fibroblast cultures of subjects 10 and 11 express a truncated receptor protein due to the introduction of respectively a premature stopcodon or a frameshift resulting in a premature stopcodon. Based on experiments of in vitro expressed truncated androgen receptor proteins (5) the receptor proteins of subjects 10 and 11 are not expected to bind ligand and consequently not to acquire transactivating potential. These aspects fully explain the complete androgen insensitivity syndrome of these two patients.

Possible clinical indications of AIS can be sexual ambiguity at birth, an inguinal hernia or primary amenorrhea. In all these cases the possible diagnosis of androgen insensitivity has to be made based on a 46,XY karyotype, the presence of testes, the absence of indications of defects in the synthesis or metabolism of androgens, if possible sustained by a positive (X-linked) family history.

If all this evidence points to androgen insensitivity, a biopsy to obtain genital skin fibroblasts for the determination of the number of androgen receptors and the quality of the binding can be very informative. In addition, immunoblot analysis of the androgen receptor protein from genital skin fibroblasts can provide important information with regard to the most likely location or nature of a possible mutation. Especially if immunoblots can be probed with specific monoclonal antibodies raised against different epitopes of the receptor. If there is impaired androgen binding while a 110 kDa androgen receptor protein can be immunoprecipitated, all evidence points to an amino acid substitution in the steroid binding domain of the receptor. When a truncated receptor protein is immunoprecipitated, a mutation in the AR gene has resulted in the introduction of a premature stopcodon, possibly the consequence of a frameshift. In case of

receptor negative AIS where no androgen receptor protein can be immunoprecipitated from genital skin fibroblasts, a mutation on the level of the promoter of the androgen receptor gene leading to diminished transcription, or the expression of a truncated receptor protein that lacks the epitope for the antibody is likely.

The localization of the genomic mutation responsible for the androgen insensitivity is best established by the PCR based SSCP analysis or a similar screening method such as denaturing gradient gel electroforesis (DGGE)(17). The SSCP analysis is a quick and reliable method to screen for mutations in the androgen receptor gene. Subsequent to DNA isolation, a radioactive PCR and a sequencing gel procedure have to be done, making it possible to obtain results within 48 hours. In our hands the detection rate of mutations in the hAR gene using SSCP analysis is approximately 90%.

With respect to pedigree analysis the most appropriate technique largely depends on the nature of the mutation. When a mutation has created or destroyed a recognition site for a restriction enzyme, restriction enzyme analysis of a PCR amplified DNA fragment containing the mutation will be informative. Sometimes however, it is difficult to digest a PCR product completely, what can lead to false interpretations. Therefore we usually confirm the restriction site analysis with an SSCP profile of the family members. In cases where SSCP analysis is not possible or where the mutation itself has not been identified, the use of the polymorphic $(CAG)_n(CAA)$ repeat in exon 1 of the human androgen receptor can be very informative. The size of the polyglutamine repeat in the normal population varies from 11 to 31 with a high frequency of 21-23 residues (6, 18, 19). However, the use of this polymorphic repeat is only possible when a certain diagnosis of X-linked androgen insensitivity within a family has been established.

Mutations in the androgen receptor gene are a relatively common cause of male pseudohermaphroditism and clinical ascertainment of cases is good. The defining of all possible mutations and their effect upon receptor function correlated with the clinical phenotype provides the opportunity to gain information about the structure function relationship of the human androgen receptor and its various aspects in male sexual differentiation. It will also lead to a better understanding of the prognosis for AIS affected children and might even help in assessing the role and effect of high androgen levels in inducing virilization, during puberty or as a result of medication.

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THREONINE ON AMINO ACID POSITION 868 IN THE HUMAN ANDROGEN RECEPTOR IS ESSENTIAL FOR ANDROGEN BINDING SPECIFICITY AND FUNCTIONAL ACTIVITY

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The human androgen receptor gene in the androgen sensitive prostate tumor cell line (LNCaP) contains a point mutation in codon 868 resulting in the substitution of threonine by alanine. This amino acid change is responsible for the increased affinity of the mutant receptor protein for progestagens and estrogens.
To further elucidate the role of threonine 868 on androgen binding capacity, specificity and functional activity, threonine 868 was substituted by six different amino acid residues. Substitution by aspartic acid, lysine or tyrosine totally eliminated androgen binding and the mutated androgen receptors did not have any transcriptional activating potential with either R1881, R5020 or estradiol. Introduction of a serine or an alanine broadened the steroid specificity, as did the introduction of a cysteine to a lesser degree. It is concluded that threonine on position 868 of the human androgen receptor limits the ligand specificity of the receptor to androgens.

Androgen regulated gene expression is mediated by the androgen receptor (AR), a protein belonging to the superfamily of receptors for steroid hormones, thyroid hormones and retinoids (1). Several single base changes in the androgen receptor gene resulting in amino acid substitutions have been identified in subjects with the androgen insensitivity syndrome, indicating the importance of the androgen receptor in normal male sexual differentiation (2). We have reported a point mutation in the androgen receptor gene at codon 868 of an androgen-sensitive tumor cell line (LNCaP) derived from a metastatic lesion of a human prostatic carcinoma (3). This mutation resulted in a threonine to alanine substitution in the steroid binding domain rendering the receptor protein responsive to androgens as well as to estrogens, progestagens and anti-androgens (3). Similar mutations are implicated in prostate tumors which do not respond to hormonal therapy. Only recently a point mutation resulting in a valine to methionine substitution in the steroid binding domain of the human androgen receptor gene has been reported in prostate tumor tissue (4).

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To study the role of threonine 868 in the human androgen receptor, mutant androgen receptor expression constructs were generated in which the codon for threonine 868 was substituted for triplets encoding either alanine, aspartic acid, cysteine, lysine, serine or tyrosine. These receptor constructs were transiently expressed in COS and HeLa cells and their steroid binding specificity and their ability to activate transcription of an androgen receptor responsive reporter gene was studied using androgens, progestagens and estrogens as ligands.

MATERIALS AND METHODS

Construction of androgen receptor expression vectors: The wild type human androgen receptor cDNA expression vector pAR0 (5) was modified on amino acid position 868 by specific mutagenesis using the polymerase chain reaction (PCR) as described by Higuchi et al (6). Briefly: for each construct a sense and an antisense oligonucleotide primer were constructed where the mismatches in the primer sequence upon translation result in the incorporation of the desired mutant amino acid (Table 1). In the primary PCR reactions, an oligonucleotide located upstream of the EcoRI site in exon 6 (5' - TGAGGCACCTCTCTCAAGA - 3'), and the antisense mutant primer were used on pAR0 template, and an oligonucleotide primer located downstream of the EcoRI site in exon 8 (5'- CAAAGTCTGAAGGTGCCATG - 3') combined with the sense mutant primer were used on a genomic DNA fragment containing exon 8 sequences (phage 18.1 in ref 7). 1% of both products were mixed and used in a second PCR with the oligonucleotiodes flanking both EcoRI sites as described above. The 642 bp product was digested with EcoRI and the resulting 500 bp fragment was exchanged with the 500 bp wild type EcoRI-EcoRI fragment from pAR0. The T868A mutant expression vector used was the pARL expression vector as described in ref. 3. All mutant expression vectors were sequenced to verify the mutation and the correct reading frame. A typical PCR protocol was: 1 minute 94 °C, 2 minutes 55 °C, 1 minute 72 °C for 25 cycles using 2,5 units AmpliTaq according to specifications of the manufacturer (Cetus).

Cell culture and transfection conditions: COS-1 and HeLa cells were cultured in Eagles minimal essential medium (Gibco) supplemented with either 5% full or 5% dextran charcoal treated (DCC) fetal calf serum, antibiotics and non-essential amino acids. Cells grown to 50-60% confluency were transfected using the calcium-phosphate precipitation method (8) essentially as described before (9).

Hormone binding assays: COS cells were harvested 48 hours after transfection by scraping in buffer (10) and the cytosol fraction was prepared by 3 freeze (20 seconds

TABLE 1: Oligonucleotides used to create specific amino acid substitutions on position 868 in the human androgen receptor

construct	amino acid introduced	senso oligonucleotide used to introduce specific amino acid substitution
T868C	cysteine	5' - CTG CAT CAG TTC TGT TTT GAC CTG CT - 3'
T868D	aspartic acid	5' - CTG CAT CAG TTC GAT TTT GAC CTG CT - 3'
T868K	lysine	5' - CTG CAT CAG TTC AAG TTT GAC CTG CT - 3'
T868S	serine	5' - CTG CAT CAG TTC TCC TTT GAC CTG CT - 3'
T868Y	tyrosine	5' - CTG CAT CAG TTC TAT TTT GAC CTG CT - 3'

Mismatches are underlined.
Corresponding antisense oligonucleotides are complementary.
In the T868A (LNCaP) mutant threonine 868 is replaced by alanine.

liquid nitrogen) thaw (10 minutes 10 °C) cycles followed by centrifugation at 12,000xg for 10 minutes. Cytosol (supernatant) was incubated for 16 hours at 4 °C, with 10 nM of the radiolabeled synthetic androgen methyltrienolone (^{3}H)R1881 (NEN, Boston) in the presence of either unlabeled R1881, R5020 or estradiol in 1 or 100-fold molar excess. Alternatively after whole cell incubations at 37 °C for 2 hours, cells were harvested in 0.1% trypsin, the supernatant was discarded and cell pellets were solubilized in 0.5N NaOH. Non specific binding was determined using a 200-fold molar excess unlabeled R1881. Separation of bound and unbound steroid was done by protamine-HCl precipitation (10). Radioactivity was determined by liquid scintillation counting.
Cat assays: HeLa cells co-transfected with androgen receptor expression plasmids and the pG29GtkCAT reporter plasmid (11) were 24 hours after transfection incubated for an additional 24 hours with either 1, 10 or 100 nM R1881, R5020 or estradiol, or 10 nM Dexamethasone before CAT activity in the cell lysates was measured as described (12). Data were corrected for the protein content of the cell lysates and the CAT activity induced by dexamethasone via the endogenous glucocorticoid receptors was used as an internal control.
Western blot analysis: The androgen receptor was immunoprecipitated from COS cells transfected as described above using the AR specific monoclonal antibody F39.4.1 and analyzed by SDS-PAGE and immunoblotting using the polyclonal antibody Sp061. Staining was done using alkaline phosphatase (9).

RESULTS

The wild type androgen receptor and six different androgen receptor proteins specifically mutated at amino acid position 868 were transiently expressed in COS-1 cells to study their steroid binding capacity and specificity. The wild type human androgen receptor protein on SDS-PAGE has an apparant molecular mass of 110 kDa and migrated as a tightly spaced doublet which is the result of post-translational phosphorylation, in the N-terminal domain of the receptor (13,14). Immunoblots demonstrated that all the constructs were expressed in COS cells showing the expected pattern after SDS-PAGE and in virtually equal amounts (Figure 1).
Substitution of threonine 868 by either alanine, serine, or cysteïne did not markedly influence the R1881 binding capacity as determined in a whole cell binding assay at 37 °C. The introduction of tyrosine, aspartic acid or lysine eliminated specific R1881 binding

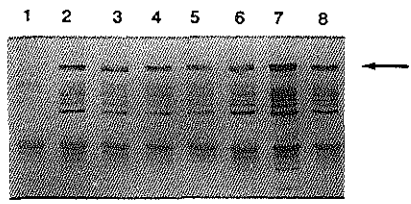

FIGURE 1.
SDS-PAGE analysis of immunoprecipitated androgen receptor from transiently transfected COS cells. Lane 1: mock transfected COS cells, lane 2: pARo, lane 3: T868A, lane 4: T868K, lane 5: T868C, lane 6: T868S, lane 7: T868Y, lane 8: T868D. The position of the 110 kDa androgen receptor protein specific doublet is indicated by an arrow.

TABLE 2: Specific R1881 binding capacity of androgen receptor protein mutated on amino acid position 868

construct	specific R1881 binding sites (fmoles/mg protein)
pAR0	165 ± 58 (3)
T868A	205 ± 79 (3)
T868C	131 ± 63 (3)
T868S	169 ± 66 (3)
T868K	nd (3)
T868Y	nd (3)
T868D	nd (2)

Hormone binding was determined in whole cell fractions of transfected COS cells at 37 °C using 10 nM (^{3}H) R1881. Non specific binding was determined using a 200-fold molar excess unlabeled ligand. Values are the mean ± SD. The number of determinations is in parenthesis.
nd = not detectable.

completely (Table 2). Similar results were obtained in a cytosol binding assay at 4 °C (data not shown).

To investigate the ligand binding specificity of the wild type and the mutant androgen receptors expressed in COS cells, competition experiments of (^{3}H)R1881 labeled receptor with the synthetic progestagen R5020 and with estradiol were performed in cytosol fractions. At 1-fold molar excess, both R5020 and estradiol as compared to R1881, did not markedly influence specific R1881 binding in the case of the wild type receptor protein, and only partly in case of the alanine, cysteine and serine mutants (Table 3). At 100-fold molar excess of competitor, the cysteine mutant resembled the wild type androgen receptor protein while the serine mutant displayed a pattern comparable with the alanine (LNCaP) androgen receptor mutant (Table 3).

The transactivating potential of the wild type androgen receptor and the receptors mutated at position 868 were tested in HeLa cells in a co-transfection assay with the pG29GtkCAT reporter gene which can function as an androgen responsive gene. The mutant receptors that did not demonstrate specific R1881 binding were also unable to

TABLE 3: Competition for R1881 binding by androgen receptor proteins mutated on amino acid position 868 by different ligands

	(^{3}H)R1881 bound, % of control					
	R1881		R5020		E_2	
	1-fold	200-fold	1-fold	100-fold	1-fold	100-fold
pAR0	49	0	99	12	103	35
T868A	32	4	67	0	74	13
T868C	51	3	89	8	94	47
T868S	47	0	77	0	90	9

Androgen binding was determined in cytosol fractions of transfected COS cells at 4 °C using 10 nM (^{3}H)R1881.
Competition of R5020 and E_2 for specific R1881 binding was determined using different molar excesses of cold competitor. One of three typical experiments is presented. Percentages are the average of duplicate determinations. (^{3}H)R1881 bound in the absence of competitor was set at 100% for each construct (=control).

TABLE 4: Functional activity, induced by R1881, of androgen receptor proteins mutated at amino acid residue 868

	% CAT activity induced by R1881			
	0 nM	1 nM	10 nM	100 nM
pAR0	-	100	80	91
T868A	-	61	43	41
T868C	-	45	60	25
T868S	-	45	45	35
T868K	-	-	-	-
T868Y	-	-	-	-
T868D	-	-	-	-

Androgen receptor mediated hormone induction of CAT activity determined in HeLa cells after co-transfection with androgen receptor expression constructs and the reporter gene pG29GtkCAT.
Presented data are one of three typical experiments.
Background activity (<1%) is indicated by -.

acquire activation potential under the influence of R1881 (Table 2 and Table 4). The alanine, serine and cysteïne mutants did acquire functional activity in the presence of R1881, but not to the same extent as the wild type receptor (Table 4), despite equal binding capacities (Table 2).

The wild type androgen receptor gains functional activity in the presence of 10-100 nM estradiol, but not at similar high R5020 concentrations (Table 5). This difference however is not a reflection of the relative binding affinities of estradiol and R5020 according to Table 3, indicating that a decreased binding affinity for the receptor (as measured in a competition assay) does not prohibit the generation of a functional receptor-ligand complex. The T868A (LNCaP) mutant responded equally well to R5020 and to estradiol, as has been shown before (3). The T868S mutant receptor was more responsive to both R5020 and estradiol as compared with the T868C mutant receptor protein. The T868C mutant responded better to R5020 compared with the wild type receptor protein (Table 5).

TABLE 5: Functional activity, induced by either R1881, R5020 or estradiol (E_2) of androgen receptor proteins mutated at amino acid residue 868

	% CAT activity induced by					
	nM R1881		nM R5020		nM E_2	
	0	1	10	100	10	100
pAR0	-	100	2	1	20	32
T868A	-	100	64	63	62	62
T868C	-	100	6	18	9	22
T868S	-	100	35	50	58	58
T868K	-	-	-	-	-	-
T868Y	-	-	-	-	-	-
T868D	-	-	-	-	-	-

Functional activity of wild type and mutant androgen receptors co-transfected in HeLa cells with the pG29GtkCAT reporter gene. The CAT activity resulting from incubation with 1nM R1881 was arbitrarily set at 100%.
Presented data are those of one of two representative experiments. Individual determinations were performed in triplicate.
Background activity (<1%) is indicated by -.

DISCUSSION

The ligand binding domain of steroid receptors in general is an independent functioning entity (15). The high degree of homology between the ligand binding domains of the steroid hormone receptors suggests that conserved amino acids are involved in shaping of the hydrophobic pocket and that ligand specificity is determined largely by the non-homologous amino acid residues. The integrety of a domain in the mouse estrogen receptor, corresponding to hAR residues 844-878, may be involved in dimerization. This region consists of hydrophobic residues arranged in a heptad repeat which is conserved in all nuclear receptors (16).

The markedly changed steroid specificity of the human androgen receptor in LNCaP cells, where threonine 868 was substituted for alanine, led to several hypotheses concerning the underlying function of threonine 868. The threonine residue at position 868 is unique for the human, rat and mouse androgen receptor. The human progesterone and glucocorticoid receptor carry a cysteïne at this position and the human estrogen receptor a serine residue (Figure 2). In the human estrogen receptor receptor cysteïne 530 can be covalently labelled by aziridine analogs of estrogens and amino acid residues in this area of the receptor may be involved in discrimination between estrogens and anti-estrogens (17). The equivalent of hAR residue 868 in the mouse and rat glucocorticoid receptor is a cysteïne which can be crosslinked by triamcinolone acetonide (18,19). Although the residues corresponding to hAR amino acid 868 are not totally conserved for a particular receptor within the different species it could be that a cysteïne or serine residue at position 868 would shift the steroid specificity towards a higher binding affinity for progestagens, estrogens or glucocorticoids respectively. In general this does not seem to be the case. Only the relative functional activity of the T868C mutant for R5020 is slightly increased. The effect of glucocorticoids was not analyzed since HeLa cells contain a substantial amount of glucocorticoïd receptors.

All steroid hormone receptors are phosphoproteins (20-24) and it has been shown that basal phosphorylation is indispensable for ligand binding activity of the estradiol

							868						
hAR	Arg	Glu	Leu	His	Gln	Phe	Thr	Phe	Asp	Leu	Leu	Ile	Lys	(862-874)
hPR	Lys	Gln	*	*	Leu	Tyr	Cys	Leu	Asn	Thr	Phe	*	Gln	(885-897)
hGR	Glu	Asn	*	Leu	Asn	Tyr	Cys	*	Gln	Thr	Phe	Leu	Asp	(730-742)
hMR	Ser	Asp	*	Leu	Glu	*	Cys	*	Tyr	Thr	Phe	Arg	Glu	(936-948)
hER	Gly	Met	Glu	*	Leu	Tyr	Ser	Met	Lys	Cys	Lys	Asn	Val	(521-533)

FIGURE 2.
Amino acid homology of part of the hormone binding domain of the steroid hormone receptors. The amino acid sequence of the human androgen receptor residues 862-874 (hAR, ref 27) was aligned with the sequences of the human progesterone receptor (hPR, ref 28), the human glucocorticoid receptor (hGR, ref 29), the human mineralocorticoid receptor (hMR, ref 30) and the human estrogen receptor (hER, ref 31). Identical amino acids are indicated by asterisks.

receptor (23). The phosphorylation on tyrosine in the steroid binding domain is essential for the activation of hormone binding of in vitro synthesized hER (25). For the LNCaP androgen receptor it has been established that over 90 percent of the phosphorylation sites are localized in the N-terminal part of the androgen-receptor protein (14) but this does not rule out the possibility that in the wild type androgen receptor basal phosphorylation of threonine 868 could be involved in ligand binding and/or specificity. Introduction of the aromatic side chain containing tyrosine, which can also be phosphorylated, or the acidic side chain containing aspartic acid, which mimics the negative charge of a phosphorylated residue, totally inhibited specific androgen binding and these mutated androgen receptors were unable to activate transcription of a CAT reporter gene under the influence of either R1881, R5020 or estradiol. This is also the case when a basic side chain containing lysine was substituted at this position. These data strongly suggest that basal phosphorylation on threonine 868 is not essential for androgen specificity and that the introduction of a charged or relative bulky amino acid at this position is detrimental for androgen binding and receptor functional activity.

The substitution of cysteïne 656 in the rat glucocorticoid receptor by either glycine or serine resulted in mutant receptors with a higher affinity for glucocorticoids compared to the wild type receptor accompanied by a decreased relative affinity for cross-reacting steroids (26). The results in this study indicate that a threonine residue at position 868 limits the ligand specificity to androgens and that the introduction of either an alanine, a serine or a cysteïne at this position leaves the androgen binding capacity intact, but broadens the binding specificity for other ligands, while the introduction of either a lysine, a tyrosine or an aspartic acid destroys the binding capacity for androgens progestagens and estrogens.

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CHAPTER 7

GENERAL DISCUSSION

PREFACE

The cloning of the human androgen receptor cDNA and the elucidation of the structure of the gene have greatly facilitated investigations concerning the androgen receptor at the molecular level. These investigations are indicated in patients with possible X-linked androgen insensitivity, or when there is an indication for the Kennedy syndrome. Also a substantial number of prostate cancers have been screened for mutations in the androgen receptor gene, and this has resulted in the discovery of three point mutations (Culig et al., 1993; Newmark et al., 1992; Veldscholte et al., 1990).

MUTATIONS IN THE HUMAN ANDROGEN RECEPTOR GENE

Specific amplification and sequencing of parts of the coding region of the androgen receptor gene of AIS-affected individuals, has resulted in the discovery of mutations in the androgen receptor gene. The further development of rapid methods to screen for mutations in amplified gene products, such as single strand conformation polymorphism (SSCP) analysis (Orita et al., 1989) and denaturing gradient gel electrophoresis (DGGE) analysis (Myers et al., 1987), has markedly enhanced the capacity to investigate AIS patients and their families for the underlying genomic cause for the AIS syndrome.

In total 91 mutations have been reported in the human androgen receptor, excluding variations in the polyglutamine stretch associated with Kennedy's syndrome. A list of the 91 mutations in the human androgen receptor can be found in the appendix to the General Discussion (page 117).

Most of the mutations in the human androgen receptor gene concern patients with established androgen insensitivity. Two mutations were identified in prostate cancers and one in a human prostate tumour cell line (LNCaP). Two mutations

were seen in subjects with pAIS in combination with breast cancer.
Of the 91 mutations, 79 are single-base pair changes leading to either amino acid substitutions, the introduction of premature stopcodons, or a defective splice donor site. Chapter 2 describes the mutation of the intron 4 donor splice site; the other single-base change in a splice consensus site entails the splice donor site on the exon 6 boundary.
In 2 instances a single base insertion has been identified, 10 mutations involve a deletion (either a single base deletion, or a partial or a complete gene deletion). One gene deletion consists of intron sequences but involves the branch site, thereby affecting pre-mRNA splicing (described in Chapter 4).

THE DISTRIBUTION AND NATURE OF MUTATIONS IN THE ANDROGEN RECEPTOR GENE

The human androgen receptor gene

There are no outstanding major mutational hot spots for mutations in the hAR gene. There is also no correlation between the location of a specific mutation and the severity of the associated clinical syndrome of androgen insensitivity (Figure 1).
Mutations in the human thyroid hormone receptor beta gene, that are responsible for generalized resistance to thyroid hormone, seem to cluster in two CG-rich areas of the gene (Weiss et al., 1993). One of these regions corresponding to amino acid residues 822-857 in the human androgen receptor also seems to be an analogous cluster region for mutations causing androgen insensitivity (McPhaul et al., 1992) (Figure 1).
There are a number of situations where the same amino acid is involved, but is being mutated differently: aspartic acid 686 (Chapter 3) and 855, methionine 733, arginines 765, 822 and 846, and valine 857. Most of these mutations

involve CpG dinucleotides and perhaps could be considered minor hot spots. The majority of the identified mutations is located in the steroid binding domain. This is a direct consequence of the initial selection of the patients for analysis of the androgen receptor gene. Most complete AIS patients were clinically well defined, studied by analysis of the AR protein in genital skin fibroblasts, and these patients were used in the initial studies for gene mutation analysis. The majority of cAIS patients is receptor negative. In retrospect, this is due to either a premature stop codon or an amino acid change in the steroid binding domain that affects ligand binding. The complete absence of the androgen receptor protein as a result of a complete deletion of the gene has been reported only twice (see Appendix).

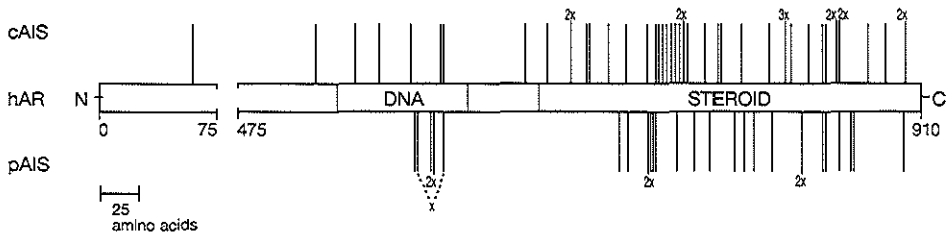

Figure 1: Single nucleotide mutations in the human androgen receptor gene. The bar depicts the hAR protein and the numbers refer to the amino acid positions. Marks above reflect the positions of mutations resulting in cAIS. Marks below those resulting in pAIS.

Generally there is a correlation between the severity of the androgen insensitivity and the chemical nature of the amino acid change. An amino acid substitution in the ligand binding domain of the human androgen receptor can result in very subtle changes in steroid binding specificity and ligand binding dissociation kinetics, depending on the nature of the amino acids involved. Conservative mutations are generally associated with pAIS, non-conservative amino acid changes with cAIS. When the aliphatic side chain containing valine residue at

position 857 (see Appendix) is mutated to the structural similar leucine, the result is partial androgen insensitivity. When on the other hand a methionine (sulphur containing side chain) or a glutamic acid is incorporated at this position, the mutated androgen receptor is associated with cAIS. In one case this mutation is associated with pAIS; the cause for this discrepancy is not clear.

In the case of aspartic acid 686, the mutation to the structurally related asparagine results in an androgen receptor protein with a normal binding capacity but increased dissociation of the ligand receptor complex. When aspartic acid 686 is mutated to histidine, the resulting receptor protein displays a drastically reduced binding capacity for androgens combined with an increased dissociation rate. The physiological end result in both cases however is identical. The patients in which these mutations were identified are both complete androgen insensitive (Chapter 3).

To investigate whether the amino acid substitutions Asp686Asn and Asp686His were involved not only in the increased ligand dissociation, but were also directly influencing the transactivation potential of the androgen receptor, these specific amino acid substitutions were incorporated into a constitutive androgen receptor mutant (pAR14 in Jenster et al., 1991). This constitutive mutant is truncated after amino acid 713 and displays a 40% wild type activity, either in the presence or absence of ligand, when cotransfected in COS cells with the GREtkCAT reporter gene. The additional mutations on amino acid position 686 did not markedly influence the constitutive transactivating potential of pAR14. The constitutively active truncated androgen receptor protein with either the additional Asp686Asn or Asp686His mutation displayed 90% of constitutive transcriptional activity compared to the unmutated truncated androgen receptor protein (unpublished data). These data suggest that position 686 is not directly involved in the transactivation potential of the androgen receptor, and that the cAIS in the subjects with a mutation of amino acid residue 686 results from increased ligand dissociation.

The growth of prostate cancers in general is androgen dependent, and initially prostate cancers respond well to endocrine therapy. At a later stage, however, in most cases an androgen independent tumour continues to grow, rendering the anti-androgen therapy superfluous. In two, out of 67, prostate cancers a mutation in the androgen receptor gene has been identified (Culig et al., 1993; Newmark et al., 1992). This relatively low frequency of mutations in the androgen receptor gene leads to the conclusion that structural changes in the androgen receptor do not seem to play a role in early stages of prostatic carcinoma. However, it is also possible that at these early stages only a small percentage of prostatic tumour cells, that is not detected by the methods currently used, contain a mutated androgen receptor.

A mutation in the androgen receptor gene of the partially androgen-dependent prostatic tumour cell line LNCaP was also reported (Veldscholte et al., 1990). In this case, the Thr868Ala mutation in the human androgen receptor was originally detected in the LNCaP cell line at passage 70, but is also present in passage number 20 of the same cell strain. The LNCaP cell line is aneuploid, probably as a result from prolonged *in vitro* culturing of the cell line. No evidence for the presence of the wild type androgen receptor allele in cultured LNCaP cells was found. These facts suggest that this mutation was also present in the prostate cancer of the original patient. The mutation in the human androgen receptor of threonine 868 into alanine gives rise to a receptor protein that is not only functional upon activation with androgens, but also to a lesser extent with estrogens, progestagens and anti-androgens (Veldscholte et al., 1990).

The mutation of valine residue 706 to methionine (Culig et al., 1993), also results in an androgen receptor protein with aberrant ligand specificity. The mutant receptor protein is functional with 5α-dihydrotestosterone and synthetic androgens, but also adrenal androgens and progesterone mediate a higher trans-activation through the mutant than through the wild type receptor (Culig et al., 1993).

To investigate if the interesting effect on the steroid binding capacity of alanine on position 868 in the human androgen receptor was a unique phenomenon, site directed mutagenesis was employed to substitute the wild type threonine for different amino acid residues. These studies, described in Chapter 6, show that the amino acid residue 868 (threonine) in the wild type receptor is responsible for the restriction of ligand specificity and functional activity of the hAR in response to androgens. It is therefore unlikely that the mutations on amino acid position 868 in the human androgen receptor directly influence the DNA binding and transactivating properties of the receptor.

Breast cancer in the human male is thought to develop in response to androgen deficiency and/or under conditions associated with estrogen excess. Adequate testicular androgen production is reported to have a protective role against breast cancer in men (Thomas et al., 1992). Interestingly, there are now two reports of subjects where a mutation in the human androgen receptor is associated with both pAIS and breast cancer (Lobaccaro et al., 1993b; Wooster et al., 1992). Both mutations are located in the second DNA binding zinc finger and are located on adjacent amino acid residues. Whether the possible ability of the mutated androgen receptor to activate estrogen responsive genes, or the loss of the protective effect of androgens is related to the development of breast cancer, is not yet clear. The mutation of amino acid residue 599 from arginine to lysine, that according to Lobaccaro et al. (1993b) is associated with breast cancer, has also been reported in a pAIS individual without breast cancer (Saunders et al., 1992). However, this patient had a bilateral mastectomy after puberty because of gynaecomastia.

Mutations in the human androgen receptor gene affecting pre-mRNA splicing are uncommon. In Chapter 3 a mutation in the exon 4/intron 4 splice consensus site is described, that totally destroys splicing at that boundary and results in

alternative splicing because of the recognition of a cryptic splice donor site in exon 4. This splicing event results in the in-frame deletion of 41 amino acids from the steroid binding domain in the androgen receptor of a complete AIS patient. The deletion of 41 amino acids from the steroid binding domain of the human androgen receptor results in a receptor protein that is unable to bind ligand and does not have any transcriptional activating potential (Chapter 2). It is unknown, whether this is a consequence of the deletion of amino acids directly involved in interaction with the ligand, or whether the internal deletion of the steroid binding domain affects protein folding resulting in the destruction of the hydrophobic ligand binding pocket is not known.

A partial intron 2 deletion exceeding 6 kb in the androgen receptor gene of patients suffering from partial androgen insensitivity (Reifenstein Syndrome) involves the putative branch site and leads to the skipping of exon 3 splicing in 92% of the pre-mRNA (Chapter 4). The translation product is an androgen receptor protein with an in-frame deletion of the second DNA-binding zinc finger. We (Chapter 4) and others (Quigley et al., 1992b) have shown that the deletion of the second DNA-binding zinc finger totally abolishes the transactivating potential of the receptor protein. In this patient, the remaining 8% wild type androgen receptor protein was not sufficient to achieve complete virilization. This, to our knowledge, is the first report on a human branch site mutation.

Most screening assays for androgen receptor gene mutations employ primers relatively close to the intron/exon borders to amplify a specific gene fragment. Since branch point sequences are typically located 20 to 50 nucleotides upstream of the intron/exon boundary (Reed et al., 1985), a mutation in a branch point sequence might well go undetected in routine androgen receptor gene screening.

Most studies on androgen receptor gene mutations do not involve any (co-)transfection studies, in which the binding activity and the transactivating potential of the mutant androgen receptor protein is compared to the wild type

protein. This approach, however, is essential for establishing the structure/function relationship of the human androgen receptor in detail, and to make a definite statement regarding the molecular cause of the clinical symptoms of androgen insensitivity.

Mutations in the rat and mouse androgen receptor gene

Androgen insensitivity is also recognized in other, non-human species such as rat and mouse.

The rat androgen receptor cDNA encodes a protein of 902 amino acid residues (Chang et al., 1988) with 5 repeated amino acid motives in the N-terminal domain (Tan et al., 1988). Stanley and Gumbreck (1973) discovered AIS in a strain of King/Holtzman rats. A single base alteration in the AR gene of that Tfm rat leads to the replacement of arginine (CGG) 734 by glutamine (CAG) (Yarbrough et al., 1990). The reduced binding capacity of this mutated androgen receptor in transfected cells, is comparable to that of the AR in tissue extracts of the Tfm rat (Yarbrough et al., 1990).

The mouse androgen receptor cDNA contains an open reading frame of 2697 base pairs, encoding an 899 amino acid protein (Charest et al., 1991; Faber et al., 1991; Gaspar et al., 1990). The coding sequence is divided over 8 exons (Faber et al., 1991). The overall homology on the amino acid level with the rat AR is 96% and with the hAR 85%. The amino acid sequence for the DNA- and steroid-binding domains is totally conserved between these species. The homopolymeric stretches of glutamine and glycine residues are at approximately the same position as in the rat AR, but the composition is different (Faber et al., 1991).

In the Tfm mouse a C nucleotide, in a stretch of six cytosine residues between positions 1106 and 1113 in the N-terminal domain encoding part of the mRNA is deleted (He et al., 1991). The single nucleotide deletion results in a frame shift and a premature stopcodon at nucleotides 1235-1237. Internal initiation of

transcription proceeds from the inframe ATG codon 1507-1509. This smaller androgen receptor protein was identified by gel filtration and sucrose density gradients (Young et al., 1989). This mutation may result in an unstable mRNA (Charest et al., 1991; Gaspar et al., 1990). A similar instability of mRNAs containing a premature termination codon has been described for human androgen receptor mutants (Marcelli et al., 1990). The low amount of N-terminal truncated androgen receptor protein is responsible for the 10% residual androgen binding activity of the mAR in the Tfm mouse (Gaspar et al., 1991).

CpG DINUCLEOTIDES AS MUTATIONAL HOT SPOTS IN THE ANDROGEN RECEPTOR GENE?

Errors in the eukaryotic DNA replication process occur at a frequency between 10^{-9} to 10^{-11} mis-incorporated nucleotides per base (Nalbantoglu et al., 1983). Nucleotide substitutions arise either by chemical (deamination of 5-methylcytosine or depurination), physical (DNA slippage), or enzymatic (postreplicative mismatch repair or exonucleolytic proofreading) mechanisms (Cooper and Krawczak, 1990). Point mutations in vertebrate genes are known to be non-random. This is partly due to the hypermutability of the methylated dinucleotide CpG. Deamination of 5-methylcytosine to thymidine in this doublet results in a C → T or G → A substitution depending upon the strand in which the cytosine is methylated. CpG is a hot spot for mutations in vertebrate genomes, and the redundancy of the genetic code provides the ability to avoid the detrimental effects of cytosine methylation by evading codons containing a CpG dinucleotide. Complete avoidance, however, is not seen, not even for the CGA (arginine) codons, where a C → T transition would result in a termination codon. For the mutations described in Chapter 5, CpG dinucleotides are involved in 4 out of 9 point mutations; in 2 cases methylation-mediated deamination is

involved. The number of mutations, however, is relatively low compared to other studies. For all the human androgen receptor point mutations published, those occurring in CpG nucleotides account for 32% of the total (Tables 1 and 2). If only those mutations consistent with methylation-mediated deamination are considered, this percentage falls to 23% (Table 1, Table 2), which is slightly lower compared to the 31% noted by Cooper and Youssoufian (1988) and Cooper and Krawczak (1990). The fact that for the human androgen receptor 50% of all C -> T mutations involve a CpG, while for the 12 human genetic diseases studied by Cooper and Krawczak this value is 75%, is the main contribution to this discrepancy. Whether this is a specific property of the AR gene is not known.

Table 1: Point mutations in the human androgen receptor gene.

Initial nt	Nucleotide resulting from single base-pair change				Total
	G	A	T	C	
G	·	26	5	5	36
A	10	·	2	1	13
T	3	2	·	6	11
C	3	2	12	·	17
	16	30	19	12	77

Table 2: Point mutations in CpG dinucleotides in the human androgen receptor gene.

Mutation	Number	Percentage
CG → TG	6	24
CG → CA	13	52
CG → other	6	24
total	25	100

GENERAL CONCLUSION

The cloning of the human androgen receptor cDNA and the elucidation of the organization of the gene have made it possible to search for mutations in the androgen receptor gene of androgen insensitive patients. The development of the Polymerase Chain Reaction (PCR) and related fast screening techniques such as the Single Strand Conformation Polymorphism (SSCP) analysis, have greatly increased the potential and feasibility to screen for gene mutations. There is no single hot spot that can be held responsible for either most cases of the complete or the partial form of androgen insensitivity. Compared to other genetic disorders, mutations in the human androgen receptor gene consistent with CpG methylation and deamination, are relatively underrepresented. However, the triplets encoding amino acids 608, 686, 822, 831, 846 and 857 can be considered minor hot spots. Mutation of a specific nucleotide in the triplet encoding these amino acid residues has been associated with AIS in more than one family. In all these mutations a CpG dinucleotide is involved.

The absence of major mutational hot spots obligates the screening of the complete coding region of the human androgen receptor gene when investigating the hAR gene in relation with AIS. When a mutation in the androgen receptor is found in a patient with cAIS it is important to establish, using *in vitro* analysis of the expressed mutant androgen receptor protein, that indeed the receptor is completely non-functional with respect to its transactivation potential. Furthermore, the ligand binding potential of the *in vitro* expressed androgen receptor mutant, should reflect the androgen binding characteristics of the receptor, as determined in genital skin fibroblasts of the cAIS patient. The mutation in the androgen receptor can be considered causally related to the clinical syndrome of cAIS, if these data match.
In most cases when there is a patient with pAIS carrying a mutation in the androgen receptor gene, the causal relationship between mutation and clinical syndrome is not unequivocal. The *in vitro* analysis of a partly functional receptor protein overexpressed in experimental cell systems, makes if difficult to distinguish between normal and partially aberrant receptor functions. In these cases, the analysis of the hAR gene in family members of the index patient should establish a positive linkage between the X-linked androgen insensitivity and the mutant allele.

The identification of a mutation in an index case of AIS allows family studies for heterozygote detection and prenatal diagnosis. Moreover, naturally occurring androgen receptor mutant proteins provide detailed information on the relationship between the structure and the function of the human androgen receptor.
Further *in vitro* studies on mutant androgen receptors might even help in assessing the benefit of treatment with high amounts of androgens during male sexual differentiation and development. These studies could aid the clinician in predicting the *in vivo* effect of androgen therapy on androgen receptor

functioning in patients with pAIS due to a mutant androgen receptor.

The involvement of mutant androgen receptor proteins in prostate cancer, male breast cancer and the Kennedy syndrome, is a new area for research. These investigations might provide insight into the role of androgens and the androgen receptor, not only in male sexual development, but also in cancer and other diseases.

APPENDIX

PUBLISHED MUTATIONS IN THE HUMAN ANDROGEN RECEPTOR GENE

(Partial) Gene deletions involving multiple exons

Exons involved	*Patient phenotype*	*Reference*
6 and 7	cAIS	MacLean et al., 1993
3-8	cAIS	Brown et al., 1988 French et al., 1990
5-8	cAIS	Brown et al., 1988 French et al., 1990
1-8	cAIS	Quigley et al., 1992 Trifiro et al., 1992

Mutations in exon 1

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
CAG -> TAG	Gln60stop, and downstream initiation	cAIS	Zoppi et al., 1993
ΔA	frame shift 127	cAIS	Batch et al., 1992
ATCC insertion	frame shift at position 200	cAIS	Batch et al., 1992
TAC -> ?	Tyr525stop	cAIS	McPhaul et al., 1991b

Mutations in exon 2

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
TGC -> TAC	Cys550Tyr	cAIS	McPhaul et al., 1993, Zoppi et al., 1992
TGT -> CGT	Cys567Arg	cAIS	McPhaul et al., 1993, Zoppi et al., 1992

Mutations in exon 3

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
complete Δexon 3	Δ 580-618	cAIS	Quigley et al., 1992b
complete _ exon 3 in 92% of the mRNA, due to an intron 2 deletion	Δ 580-618 in 92% of the protein	pAIS	Ris-Stalpers et al., 1994 (Chapter 4)
AAA -> TAA	Lys581Stop	cAIS	Marcelli et al., 1990
CGCC -> CACC	Ala587Thr	pAIS	Klocker et al., 1992
AGC -> GGC and CGG -> CCG	Ser588Gly and Arg608Pro	pAIS	Zoppi et al., 1992
CGA -> CAA	Arg598Gln	pAIS + breast cancer	Wooster et al., 1992
AGG -> AAG	Arg599Lys	pAIS + breast cancer	Lobaccaro et al., 1993b
AGG -> AAG	Arg599Lys	pAIS	Saunders et al., 1992
CGT -> CAT	Arg606His	cAIS	Ris-Stalpers et al., in press (Chapter 5)
CGG -> CCG	Arg608Pro	cAIS	Marcelli et al., 1991

Mutations in exon 4

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
ATT -> ATA	Ile655Asn	cAIS	Pinsky et al., 1992
GAG -> AAG	Glu672Lys	cAIS	Hiort et al., 1993
Ggt -> Gtt	Δ 674-714	cAIS	Ris-Stalpers et al., 1990 (Chapter 2)
Δ AAC	Δ 683	cAIS	Batch et al., 1992
CGAC -> CCAC	Asp686His	cAIS	Ris-Stalpers et al., 1991 (Chapter 3)
CGAC -> CAAC	Asp686Asn	cAIS	Ris-Stalpers et al., 1991 (Chapter 3)
TCT -> GCT	Ser693Ala	cAIS	Pinsky et al., 1992
AAT -> AGT	Asn696Ser	cAIS	DeBellis et al., 1992
CGTG -> CATG	Val706Met	prostate cancer	Culig et al., 1993
TGG -> TGA	Trp709stop	cAIS	Sai et al., 1990

Mutations in exon 5

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
TTA -> TCA	Leu719Ser	pAIS	McPhaul et al., 1992
CGTG ->CATG	Val721Met	prostate cancer	Newmark et al., 1992
GAC -> TAC	Asp723Tyr	cAIS	Pinsky et al., 1992
CAG-> CAG/T	Gln724Gln/His	pAIS	Hiort et al., 1993
TGG -> CGG	Trp732Arg	cAIS	McPhaul et al., 1992, Wilson et al., 1992
ATG -> GTG	Met733Val	pAIS	Ris-Stalpers et al., in press (Chapter 5)
ATG -> ATA	Met733Ile	pAIS	Batch et al., 1992
GGG -> GTG	Gly734Val	pAIS	Nakao et al., 1993
ATG -> ACG	Met736Thr	pAIS	Ris-Stalpers et al., in press (Chapter 5)
GCC -> GAC	Ala739Asp	pAIS	McPhaul et al., 1992
ATG -> GTG	Met740Val	cAIS	De Bellis et al., 1992, Jakubiczka et al., 1992
GGC -> GAC	Gly741Asp	cAIS	Batch et al., 1992
CGA -> TGA	Arg743stop	cAIS	Pinsky et al., 1992
TTC -> GTC	Phe745Val	cAIS	Lobaccaro et al., 1993a
TCC -> TTC	Ser750Phe	cAIS	De Bellis et al., 1992
CTC -> TTC	Leu753Phe	cAIS	Batch et al., 1992

TAC -> TGC and shortened CAG repeat in exon 1	Tyr754Cys Δ 12 Gln	pAIS	McPhaul et al., 1991a
TTC -> CTC	Phe755Leu	cAIS	McPhaul et al., 1992, Ris-Stalpers et al., in press (Chapter 5)
CGCC -> CACC	Ala756Thr	cAIS	Batch et al., 1992, Sweet et al., 1992
GCC -> GTC	Ala756Val	cAIS	Pinsky et al., 1992
CCT -> TCT	Pro757Ser	cAIS	McPhaul et al., 1992
complete Δ exon 5	Δ 715-910	cAIS	MacLean et al., 1993

Mutations in exon 6

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
GAG -> GCG	Glu763Ala	pAIS	Pinsky et al., 1992
CGC -> CAC	Arg765His	cAIS	Batch et al., 1992, Prior et al., 1992
CGC -> TGC	Arg765Cys	cAIS	Brown et al., 1990, Marcelli et al., 1991, McPhaul et al., 1992, Prior et al., 1992
ATG -> ATA	Met771Ile	pAIS	Batch et al., 1992, Ris-Stalpers et al., in press (Chapter 5)
CGA -> TGA	Arg777stop	cAIS	Pinsky et al., 1992
ATG -> GTG	Met778Val	cAIS	Nakao et al., 1992
GAG -> ?	Glu784Asp	pAIS	Pinsky et al., 1992
TGG -> TGA	Trp787stop	cAIS	Marcelli et al., 1990
CAA -> GAA	Gln789Glu	pAIS	Batch et al., 1992
AGC -> AAC	Ser805Asn	pAIS	Pinsky et al., 1992

Mutations in exon 7

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
GGG -> GCG	Gly811Ala	cAIS	Kasumi et al., 1993
CTG -> GTG	Leu 812Val	pAIS	Pinsky et al., 1992
CGA -> TGA	Arg822stop	cAIS	Ris-Stalpers et al., in press (Chapter 5), Brown et al., 1990, De Bellis et al., 1992
CGA -> CTA	Arg822Leu	cAIS	Pinsky et al., 1992
CGA -> CAA	Arg822Gln	cAIS	McPhaul et al., 1992
TAC -> TGC	Tyr825Cys	cAIS	McPhaul et al., 1992, Wilson et al., 1992
CGT -> TGT	Arg831Cys	pAIS	McPhaul et al., 1992
CGT -> CAT	Arg831His	pAIS	Hiort et al., 1993, McPhaul et al., 1992
ATT -> ACT	Ile833Thr	cAIS	Hiort et al., 1993
A insertion	wild type aa 1-837	cAIS	Ris-Stalpers et al., in press (Chapter 5)
TCA -> TGA	Ser844stop	cAIS	Wilson et al., 1992
AGA -> AAA	Arg845Lys	pAIS	McPhaul et al., 1992
CGC -> TGC	Arg846Cys	cAIS	De Bellis et al., 1992, McPhaul et al., 1992
CGC -> CAC	Arg846His	pAIS	Batch et al., 1992, McPhaul et al., 1992
GAC->GGC	Asp855Gly	cAIS	De Bellis et al., 1992
GAC -> AAC	Asp855Asn	cAIS	Batch et al., 1992

CGTG -> CTTG	Val857Leu	pAIS	Hiort et al., 1993, Kazemi-Esfarjani et al., 1993
CGTG -> CATG	Val857Met	cAIS pAIS	Brown et al., 1990, Kazemi-Esfarjani et al., 1993, Lubahn et al., 1989 McPhaul et al., 1992
CGTG -> CGAG	Val857Glu	cAIS	McPhaul et al., 1992

Mutations in exon 8

Mutation[1]	*Position amino acid change*[2]	*Patient phenotype*	*Reference*
ATT -> ATG	Ile860Met	pAIS	Batch et al., 1992
AGA -> GGA	Arg862Gly	pAIS	Pinsky et al., 1992
CACT -> CGCT	Thr868Ala	LNCaP	Veldscholte et al., 1990
CAAG -> CTAG	Lys874stop	cAIS	Trifiro et al., 1991b
CGTG -> CATG	Val880Met	cAIS	Pinsky et al., 1992
GTG -> ATG	Val894Met	pAIS	McPhaul et al., 1992
CCC -> TCC	Pro895Ser	cAIS	Pinsky et al., 1992
CCC -> CAC	Pro895His	cAIS	McPhaul et al., 1992

[1] In some cases an extra nucleotide is represented to show the presence of a CG dinucleotide involved in the mutation.
[2] The amino acid residue numbering is based on 910 amino acid residues, corresponding with a glutamine stretch of 20 residues and a glycine stretch of 16 residues.

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In the androgen insensitivity syndrome (AIS) male sexual development is disturbed, even although adequate amounts of testosterone and 5α-dihydrotestosterone are available. The underlying molecular basis of mutations in the androgen receptor, that result in androgen insensitivity, is the subject of this thesis.

Chapter 1 is a general introduction to this thesis. The major events in human sexual differentiation, and the specific role of the androgen receptor in male sexual development are addressed. Possible causes of aberrant male sexual differentiation, such as AIS due to androgen receptor dysfunctioning, are discussed. The molecular structure of nuclear hormone receptors in general and the androgen receptor, a member of the nuclear hormone receptor family, in particular, are presented.

Chapter 2 shows the mutation of the first nucleotide in intron 4 of the human androgen receptor gene of a complete androgen insensitive syndrome (cAIS) subject. This mutation abolishes pre-mRNA splicing at the exon 4/intron 4 border. In this case, activation of a cryptic donor splice site in exon 4 results in a pre-mRNA transcript with a deletion of 123 nucleotides of exon 4 sequence. The mutant translation product has an in-frame deletion of 41 amino acids in the steroid binding domain, and is completely non-functional with respect to ligand binding and transcription activation.

In Chapter 3 it is demonstrated that the mutation of amino acid residue 686 (aspartic acid) in the human androgen receptor to either asparagine or histidine results in a receptor protein that is non-functional with respect to the transactivation of an androgen responsive reporter gene, explaining the complete androgen insensitivity of the affected subjects. However, in a transient transfection system, both mutant receptor proteins display different ligand binding characteristics that correlate with the ligand binding properties of the receptors in genital skin fibroblast cultures of both patients.

Chapter 4 addresses the deletion of over 6 kb from intron 2 of the androgen

receptor gene of a partial androgen insensitive patient. The partial intron deletion leads to skipping of exon 3 sequences in 92% of the pre-mRNA. The limited amount of wild type androgen receptor mRNA and protein was insufficient to induce proper virilization in this particular patient.

While Chapters 2,3 and 4 discuss in detail the mutations established in the androgen receptor gene of several AIS subjects and the consequences for androgen receptor functionality, Chapter 5 gives an overview of the mutations identified in the Rotterdam laboratory (patients from different countries). Evaluations concerning approaches to analyze families with X-linked androgen insensitivity for possible carriers of the mutant allele are presented.

Chapter 6 describes *in vitro* studies on the human androgen receptor where specific amino acid substitutions were introduced at position 868. These studies show that the wild type amino acid (threonine) is important for the specificity of the receptor to androgens.

Finally, Chapter 7 constitutes a general discussion. All known mutations in the androgen receptor gene resulting in either partial or complete androgen insensitivity, and those mutations implicated in prostate cancer, are listed. Most mutations (77 out of 89) are single nucleotide substitutions, and result in either an amino acid change (65), the introduction of a premature stop codon (10), or affect pre-mRNA slicing (2). There is no correlation between the location of a specific mutation and its clinical consequences, and there are no major mutational hot spots. Multiple substitutions of six amino acid residues are associated with androgen insensitivity and each triplet encoding these amino acids contains a CpG dinucleotide. These positions in the human androgen receptor gene may be considered minor hot spots.

In het androgeen-ongevoeligheids-syndroom (AIS), is de mannelijke sexuele ontwikkeling gestoord, ondanks het feit dat de mannelijke geslachtshormonen testosteron en 5α-dihydrotestosteron in voldoende mate aanwezig zijn. De onderliggende moleculaire basis van mutaties in de androgeenreceptor, die resulteren in AIS, zijn het onderwerp van dit proefschrift.

Hoofdstuk 1 is de algemene introductie van dit proefschrift. De belangrijkste gebeurtenissen tijdens de menselijke geslachtsdifferentiatie, en de specifieke rol van de androgeenreceptor in mannelijke sexuele ontwikkeling, komen aan bod. Mogelijke oorzaken van afwijkende mannelijke sexuele differentiatie, zoals AIS worden besproken. De moleculaire structuur van kernreceptoren in het algemeen, en die van de androgeenreceptor (die ook tot deze groep receptoren behoort) in het bijzonder, worden gepresenteerd.

Hoofdstuk 2 behandelt de mutatie van het eerste nucleotide in intron 4 van het humane androgeenreceptor-gen van een patiënt met de complete vorm van het androgeen-ongevoeligheids-syndroom (cAIS). Deze mutatie verhindert de splitsing van het pre-mRNA op de exon 4/intron 4 overgang. De mutatie veroorzaakt de activering van een cryptische donor splitsingsplaats in exon 4, hetgeen resulteert in een mRNA met een deletie van 123 nucleotiden in de exon 4 sequentie. Het gemuteerde translatieprodukt vertoont een interne deletie van 41 aminozuren in het steroïd-bindende domein van de androgeenreceptor, waardoor het leesraam niet wordt verstoord. De gemuteerde receptor is niet-functioneel met betrekking tot hormoon-binding en transcriptie-activatie.

Hoofdstuk 3 laat zien dat de mutatie van aminozuurresidu 686 (asparaginezuur) van de humane androgeenreceptor naar asparagine of histidine resulteert in een receptoreiwit dat niet meer functioneel is. Dit verklaart de complete androgeen-ongevoeligheid van de patiënten met deze mutaties. In een celsysteem waar deze gemuteerde eiwitten tijdelijk tot expressie worden gebracht, vertonen de gemuteerde receptoreiwitten veranderde hormoon-bindende karakteristieken, en deze correleren met de hormoon bindende eigenschappen zoals ze aangetoond

zijn in genitale-huidfibroblastculturen van deze patiënten.

Hoofdstuk 4 behandelt de deletie van meer dan 6 kb van intron 2 van het androgeenreceptorgen van een patiënt met een partiële vorm van AIS. De partiële intron deletie resulteert in het overslaan van exon 3 sequenties in 92% van het mRNA. De gelimiteerde hoeveelheid wild-type receptor mRNA en eiwit was niet voldoende om complete virilisatie te induceren bij deze patiënt.

Terwijl de Hoofdstukken 2, 3 en 4 mutaties in het androgeenreceptor gen van verschillende AIS patiënten in detail bespreken, geeft Hoofdstuk 5 een overzicht van alle mutaties die in het Rotterdamse laboratorium geïdentificeerd zijn (patiënten uit verschillende landen). Dit hoofdstuk bevat tevens een evaluatie met betrekking tot de mogelijke manieren om families met X-gebonden androgeen-ongevoeligheid te onderzoeken op dragerschap van het gemuteerde allel.

Hoofdstuk 6 beschrijft de *in vitro* experimenten betreffende de humane androgeenreceptor, waarbij specifiek het aminozuurresidu 868 gemuteerd werd. Deze studies laten zien dat het aminozuur op deze positie in de wild type receptor bijdraagt aan de hormoon specificiteit van de receptor voor androgenen.

Tenslotte betreft Hoofdstuk 7 de algemene discussie. Alle gepubliceerde mutaties van het humane androgeenreceptor-gen die in complete of partiële androgeen-ongevoeligheid resulteren, en de mutaties die geïmpliceerd zijn in prostaatkanker, worden gepresenteerd. De meeste mutaties (77 van de 89) zijn nucleotide-substituties, die resulteren in een aminozuurverandering (65), de introductie van een vroegtijdig stopkodon (10), of die gevolgen hebben voor de splitsing van het pre-mRNA (2). Er is geen correlatie tussen de positie van een mutatie en het fenotype van de patiënt. Ook is er geen duidelijke 'hot spot' voor mutaties die de oorzaak zijn van AIS. Er zijn 6 aminozuurposities waar mutaties geassocieerd met AIS meer dan eens voorkomen. De tripletten die voor deze aminozuren coderen, bevatten een CpG dinucleotide. Deze posities in het humane androgeenreceptor-gen kunnen gezien worden als 'minor hot spots'.

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Eiko, chantage werkt!

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