Familial disorders of sexual differentiation: a clinical and molecular genetic evaluation

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Familial disorders of sexual differentiation: a clinical and molecular genetic evaluation

Erfelijke geslachtsdifferentiatie stoornissen: klinische en moleculair genetische studies

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geboren te Rheden

Aan Hugo, Francesca en Elisabeth

Voor 'The Family'

CONTENTS

List of abbreviations

A	androstenedione
ACTH	adrenocorticotropin
AIS	androgen insensitivity syndrome
AMH	anti Müllerian hormone
AR	androgen receptor
ARE	androgen response element
Bmax	maximal number of binding sites
CAIS	complete androgen insensitivity syndrome
CAT	chloramphenicol acetyl transferase
CIS	carcinoma *in situ*
CHO	chinese hamster ovary cell line
COS-1	monkey kidney cell line
CYP17	gene encoding cytochrome P450c17
DAX-1	double dose sensitive locus-adrenal hypoplasia, critical region of X, gene 1
DBD	DNA binding domain of the androgen receptor
DHEA	dihydroepiandrosterone
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
DOC	deoxycorticosterone
E2	estradiol
FSH	follicle stimulating hormone
GSF	genital skin fibroblasts
hCG	human chorionic gonadotropin
HSD	hydroxysteroid dehydrogenase
HSD3B2	gene encoding 3β-hydroxysteroid dehydrogenase 2 enzyme
HSD17B3	gene encoding 17β-hydroxysteroid dehydrogenase 3 enzyme
hsp	heat-shock protein
kb	kilo base
kDa	kilo Dalton
Kd	equilibrium dissociation constant
LBD	ligand binding domain
LH	luteinizing hormone
Luc	luciferase
MAIS	mild androgen insensitivity syndrome
NLS	nuclear localization signal
OH	hydroxy
PAIS	partial androgen insensitivity syndrome

PCR	polymerase chain reaction
PAGE	polyacrylamide-gel electrophoresis
P-box	proximal box
preg	pregnenolone
prog	pogesterone
RT-PCR	reverse transcriptase PCR
R1881	methyltrienolone
SF-1	steroidogenic factor 1
SHBG	sex hormone-binding globulin
SRD5A2	gene encoding 5α reductase 2 enzyme
SRY	sex determining region of Y gene
SSCP	single strand conformation polymorphism
StAR	steroidogenic acute regulatory protein
T	testosterone
TAD	transcription activation domain of the androgen receptor
WT1	Wilms' tumor suppressor gene
3-D	three dimensional

Conversion factors to SI units

Testosterone ng/dl x 0.0347 = nmol/L
Androstenedione ng/dl x 0.0350 = nmol/L
DHEA ng/dl x 0.0350 = nmol/L
DHEAS μg/dl x 0.0261 = μmol/L
Estrone pg/ml x 3.69 = pmol/L
17 OH Progesterone ng/dl x 0.0303 nmol/L

CHAPTER

1 General introduction

NORMAL AND ABNORMAL HUMAN SEXUAL DETERMINATION AND DIFFERENTIATION

Sexual determination and differentiation are series of events starting with the establishment of genetic sex at fertilization, proceeding with the translation of genetic sex into gonadal sex, and culminating in the translation of gonadal sex into body sex.[1] This three-step model (Figure 1.1) is still valid, but actually (2000) much more complex. Many factors involved in normal sexual determination and differentiation became known, were cloned or defined on the molecular level during recent years. Evaluation of human disorders of sexual differentiation became crucial to demonstrate the existence of such factors and initiated the identification of genes important for sexual differentiation. Many other genes or factors are yet to follow, as in a variety of intersex syndromes the underlying defect is still unknown.

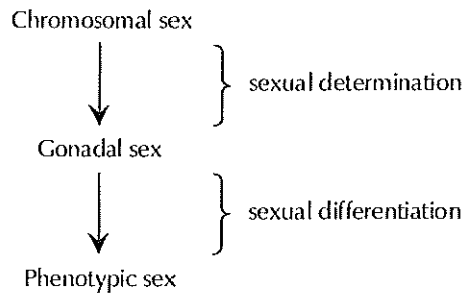

Figure 1.1
Sequential steps in sexual differentiation.

In clinical practice, the newly available knowledge on the etiology of disorders of sexual differentiation was implemented, as phenotypic/dysmorphological, cytogenetic and hormonal studies were to be followed up with protein analysis and/or gene mutation analysis. It was expected that knowledge on the evolution of a phenotype associated with a specific genotype would aid treatment of patients e.g. decisions on sex assignment. Such knowledge would also be pivotal in genetic counseling of patients family members.
The combination of clinical investigation and biochemical and molecular genetic studies will continue to improve diagnostic procedures and management options in disorders of sexual differentiation, and provide new insight into their underlying developmental processes. 'Idiopathic' disorders of sexual differentiation remain to require both clinical and fundamental research of the disease mechanisms to allow specific diagnosis, clinical management and genetic counseling.

Sexual determination

The **gonads** are formed from the intermediate mesoderm, which gives rise to the mesonephros (a primitive kidney) of which the medial part forms the indifferent genital ridges. These ridges are bipotential and differentiate at approximately the 7th week of gestation either into a testis or an ovary. Germ cells migrate to the genital ridge from the extra-embryonic mesoderm.

At conception chromosomal sex is established:

- In the presence of a Y chromosome with one X chromosome, the indifferent gonad develops into a testis
- In the absence of a Y chromosome and the presence of two X chromosomes, an ovary arises.

However, this does not cover the whole story of sex determination nowadays. Indeed the master gene that determines whether testes will arise from the indifferent gonad is *SRY* (Sex determining region of Y), localized on the Y chromosome. Studies in patients with sex determination disorders show that complete successful testicular differentiation and spermatogenesis requires the action of other genes as well. Genes upstream or downstream of *SRY* can switch on testis determination just by themselves, based on the observation that:

a) a subpopulation of 46,XX males do not have a demonstrable *SRY* gene[2],
b) 70-80% of 46,XY gonadal dysgenesis patients have no abnormalities of the SRY gene[3],
c) some forms of 46,XY gonadal dysgenesis show X-linked or autosomal inheritance[4],

Factors important for gonadal ridge formation as well as factors regulating *SRY* expression, act 'upstream' of *SRY* (Figure 1.2). Such a gene is *WT1* which ap-

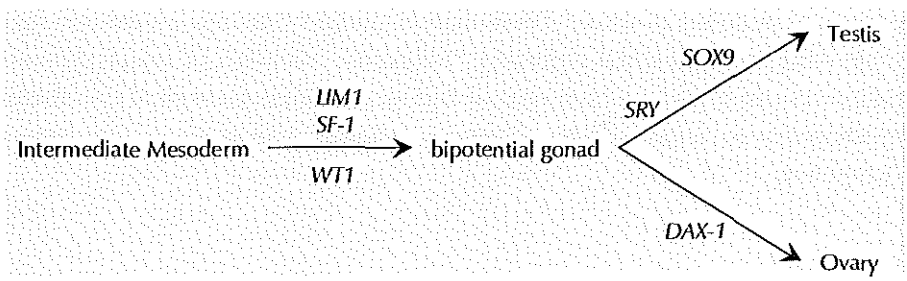

Figure 1.2
Genes in early gonadal differentiation.

pears to be essential for establishment of the bipotential gonad as well as *SRY* expression. Different mutations in the *WT1* gene in the heterozygous state, cause three syndromes: Frasier, Denys-Drash or WAGR syndrome. Frasier syndrome comprises partial or complete gonadal dysgenesis with XY sex reversal and nephropathy, Denys-Drash syndrome comprises additional Wilms' tumors. WAGR is a contiguous gene syndrome consisting of Wilms' tumors, Aniridia, Genital abnormalities and mental Retardation.[5]

Other genes that are important in the formation of the bipotential gonad are *LIM1* and *SF-1* (steroidogenic factor 1). Although human counterparts of the murine genes have been cloned, patients with mutations in these genes are yet to be identified. Mice with a homozygous deletion of *Sf-1* have an arrest in the development of gonads, adrenals and ablation of the ventromedial nucleus of the hypothalamus.[6,7] Mice with a homozygous deletion or disruption of *Lim1* have abnormalities of kidneys, gonads and brain.[8] SF-1 also plays a pivotal role in steroid hormone synthesis within the adrenal cortex and the gonads and in the expression of anti-Müllerian hormone (AMH).[9,10]

Haplo-insufficiency of another early expressed possible transcription factor, SOX9 (*SRY*-Box-related), leads to campomelic dysplasia in both sexes. Sex reversal and gonadal dysgenesis is found in 75% of 46,XY patients with *SOX9* mutations. 46,XX campomelic subjects have ovaries and a female phenotype.[11]

Another important gene for sexual determination is *DAX-1* (Double dose sensitive locus -Adrenal hypoplasia congentia, critical region of X, gene 1). *DAX-1* gene alterations result in cóngenital adrenal hypoplasia. Duplications result in sex reversal in 46,XY subjects[12] probably due to inhibition of the expression of StAR, p450scc and 3β-HSD[13] (see also section on androgen biosynthesis). *DAX-1* was suggested to be either an ovarian determinant or an inhibitor of testes determination.[14] Duplications of *DAX-1* account for only a small portion of 46,XY sex reversal cases.[15]

Thus important in the early stages of genital ridge formation are the genes *LIM1*, *WT1* and *SF-1*. *SRY* and *SOX9* are required for the subsequent differentiation of the urogenital ridge into the testes, whereas ovarian differentiation probably requires *DAX-1*[16] (Figure 1.2). All these genes are regulators of transcription.

Another candidate that may be important in genital ridge formation is the murine gene *M33*, for which a human homologue has not been cloned yet. *M33* deficient XY mice show sex reversal due to gonadal abnormalities. *M33* belongs to the polycomb genes, which are repressors of homeobox-containing and other developmentally regulated genes.[17]

Table 1.1a
Genes involved in gonadal and/or adrenal differentiation and related syndromes in 46,XY human individuals or mice

Gene	Disorder	Location	Mechanism	External Genitalia	Gonads	Associated abnormalities	Ref.
In mice:*							
LIM1	absence of kidneys and gonads	11p12-13	homozygous disruption	female	absent	absence of kidneys	(24)
SF-1	absence of adrenals, gonads and hypothalamus	9p33	homozygous disruption	female	absent		(25)
In men:							
WT1	Denys-Drash	11p13	heterozygous mutations	female → ambiguous	dysgenetic testes	renal failure; Wilms tumor	
	Frasier syndrome		defective alternative splicing of WT1 on 11p13	female	dysgenetic testes	renal failure	(26), (5)
SRY	XY gonadal dysgenesis	Yp11.3	mutations/ deletions	female	streak	none	(3)
SOX9	camptomelic dysplasia ± XY sex reversal	17q24.3-q25.1	heterozygous deletion/mutation	female → ambiguous	dysgenetic testes	skeletal abnormalities	(27)
DAX1	adrenal hypoplasia ± hypogonado-trophic hypogonadism	Xp21	heterozygous deletion/mutation DAX1	male	dysgenetic testis or ovaries	adrenal insufficiency	(28)
	XY gonadal dysgenesis		duplication of DAX1	female → ambiguous	dysgenetic testis or ovaries		(29)

* Isolation and characterization of the human homologue gene awaits further studies

Other putative gonadal differentiation genes are located on chromosomes 9p, 10q and 18p because patients with microdeletions of these loci have complete or partial gonadal dysgenesis.[18-21]

Wnt-4 was discovered as a gene that suppresses the formation of Leydig cells in the mouse ovary.[22] In the ovary of *Wnt-4* homozygous mutant female mice testosterone biosynthesis is present and Wolffian ducts continue to develop.[22] These mice do not have Müllerian ducts. Other disorders in men or mice, comprising genital malformation were found to be due to malfunction of factors regulating basic developmental processes as cell growth, tissue remodeling and epithelial-mesenchymal interactions. For example, disruption of the *TGFβ2*

Table 1.1b
Candidate genes involved in gonadal and/or adrenal differentiation and related syndromes in 46,XY human individuals or mice

Gene	Disorder	Location	Mechanism	External Genitalia	Gonads	Associated abnormalities	Ref.
In mice:*							
MTM1	XY gonadal dysgenesis	Xq28				myotubular myopathy	(30)
XH2	XY gonadal dysgenesis	Xq13.3				optic atrophy, mental retardation	(31)
In men:							
Not known	XY gonadal dysgenesis	10q	male limited autosomal dominant	female	dysgenetic testes	none	(18)
(possibly DMT1)	XY gonadal dysgenesis	9p24	male limited autosomal dominant	female → ambiguous	dysgenetic testes	none	(20) (32)

* Isolation and characterization of the human homologue gene awaits further studies

(Transforming Growth Factor β2) gene in mice leads to kidney and testicular defects among other developmental defects.[23]

A summary of the (candidate) genes or loci involved in gonadal determination and differentiation is given in Tables 1.1a and b. Because multiple new loci and genes are being identified every year, these tables may not include all known genes and loci at the time of appearance of this thesis.

Sexual differentiation[4]

Both male and female embryos possess indifferent common primordia that will feminize unless there is active interference by masculizing factors. The most important masculinizing factors are AMH, secreted by the embryonic Sertoli cells, testosterone synthesized in the Leydig cells, and DHT formed in peripheral tissues from testosterone by the enzyme 5α-reductase 2.

At the seventh week of intrauterine life, both **Müllerian** as well as **Wolffian ducts** are present in the fetus (Figure 1.3).
In the *male embryo,* Wolffian ducts differentiate into epididymides, vasa deferentia, and seminal vesicles. Their differentiation is thought to be mediated by

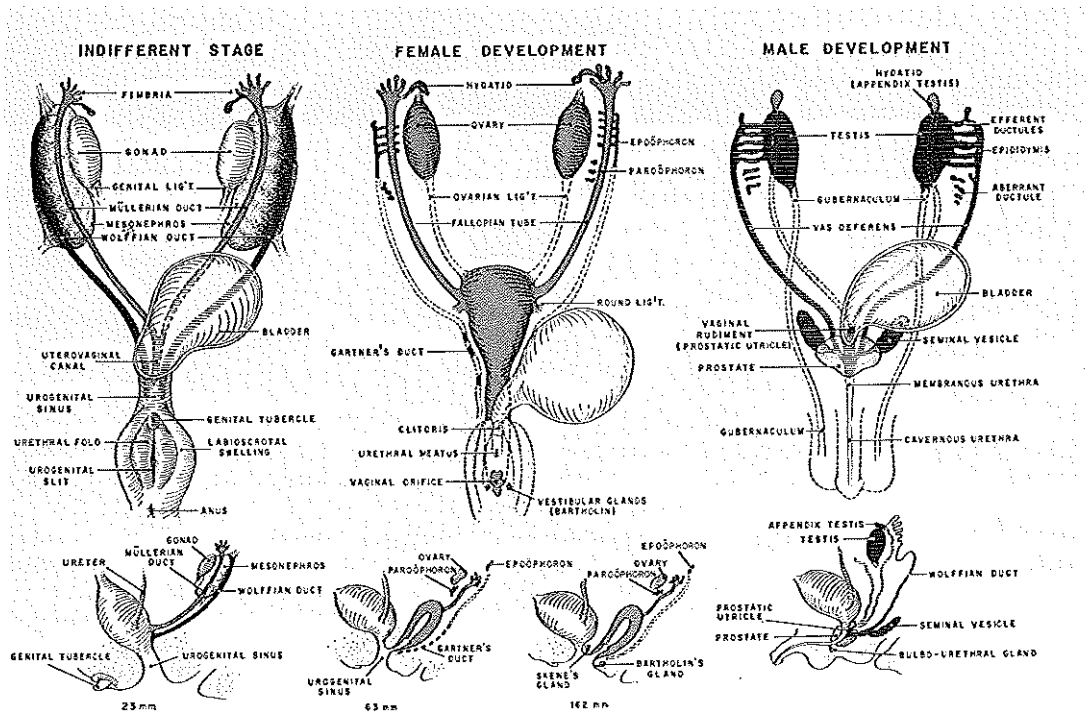

Figure 1.3
Embryonal development of the external genitalia, male versus female.
From Grumbach MM, Conte FA. 1998 Disorders of sexual differentiation. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR, editors. Williams textbook of endocrinology. 9th ed. Philadelphia: Saunders, W.B.; 1303-1425, with permission.

testosterone and not by DHT: first because 5α-reductase 2 is not expressed in Wolffian ducts until they complete their differentiation[33], and second because in 46,XY patients with 5α-reductase 2 deficiency Wolffian derivatives are normally developed.

Testicular secretion of AMH causes regression of the Müllerian ducts. Remnants of Müllerian ducts in males are the appendix testis and the utriculus prostaticus, the male equivalent of the Müllerian duct derived part of the vagina (Figure 1.3).

The prostate gland and bulbourethral glands originate from endodermal buds of the urogenital sinus and grow into the androgen-dependent urogenital mesenchyme. Their differentiation is mediated by the action of DHT.

In the *female embryo,* the ovaries do not produce AMH and the Müllerian ducts further differentiate into the upper two third of the vagina, the uterus and the fallopian tubes. In the absence of testosterone, the Wolffian ducts do

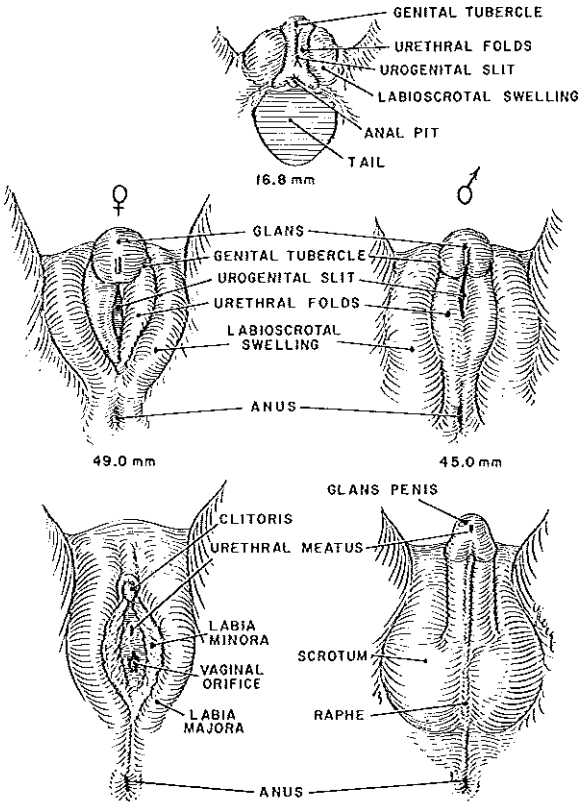

Figure 1.4
Embryonal development of the external genitalia, male versus female. From Grumbach MM, Conte FA. 1998 Disorders of sexual differentiation. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR, editors. Williams textbook of endocrinology. 9th ed. Philadelphia: Saunders, W.B.; 1303-1425, with permission.

not differentiate and remain as Gardner's ducts. During the third month of gestation, either the Müllerian or Wolffian ducts complete their development, while involution occurs simultaneously in the opposite structures (Figure 1.3).

External genitalia originate from the ectoderm-covered mesenchyme located around the anal portion of the hindgut and the primitive urogenital sinus. At the eighth week of embryonic life the external genitalia of both sexes are identical and have the capacity to differentiate in either direction (Figure 1.4).
Virilization of the external genitalia in the male fetus starts between 9-12 weeks of gestation. The urethral folds and the labioscrotal swellings fuse to form the enclosure of the urethra and the scrotum respectively, progressing from posterior to anterior. This process is completed at 12 to 14 weeks of gestation. From 10 weeks gestation to term, the penile length increases linearly.
The differentiation of the penis, scrotum and urethra is mediated by DHT. Evidence that DHT is responsible, rather than testosterone, comes from the ex-

pression of the enzyme 5α-reductase 2 in these target tissues before week 8 and the development of female external genitalia in 5α-reductase 2 deficient genetic males.
Female genitalia form in the absence of androgens. There is no fusion of the urethral fold or labioscrotal swellings and the genital tubercle grows slowly and becomes the clitoris. The urethral and the vaginal openings are separated by the anterior-posterior lengthening of the urogenital sinus. The rims of the urogenital groove turn into labia minora and the labioscrotal swellings give rise to labia majora (Figure 1.4).
In conclusion, normal testosterone/DHT production and action between 6 to 12 weeks of gestation, is needed for normal male sexual differentiation.[4] In the absence of androgens the external genetalia are female.

In males the **descent of the testes** from the abdomen into the scrotum can be divided into two stages: 1) the transabdominal movement to the internal inguinal ring, which is completed by 8-12 week of gestation, and 2) the descent through the inguinal canal into the scrotum which starts at 7 months. The mechanism of testicular descent and its possible hormonal control is still incompletely understood. Outgrowth of the gubernaculum and regression of the cranial suspensory ligament result in the transabdominal descent of the testes, in mice. In mice, gubernaculum development is induced by Insl3 while androgens cause regression of the cranial suspensory ligament.[34] This may not represent the mechanism of testis descent in men, however, as testes of CAIS patients are often located in the inguinal region, control by androgens is unlikely.

Testosterone versus DHT

Testosterone is responsible for the differentiation of the Wolffian structures. DHT controls virilization of external genitalia and the growth of a prostate during embryogenesis. In adults DHT causes the growth of a beard, the development of a male pattern pubic and axillary hair, and male pattern baldness in genetic sensitive persons.[35] Both testosterone and DHT bind to the same AR, but DHT does so with higher affinity and dissociates more slowly.[247] Furthermore, DHT is twice as potent as testosterone in bioassays.[36] High local concentrations of testosterone are necessary for differentiation of the Wolffian ducts as in hermaphrodites and in patients with incomplete gonadal dysgenesis, male duct development correlates with the degree of testicular differentiation of the adjacent gonad. Unlike the Wolffian ducts, which are adjacent to the testes and differentiate by the paracrine action of testosterone[37,38], the external genitalia and urogenital sinus receive their developmental stimuli by an-

drogen through the circulation and thus in lower concentrations. Sites of body hair growth are equally distant to the testes. It can be concluded that the differential action of the different androgens (either testosterone or DHT) in the target tissue is determined by the expression of the 5α-reductase 2 enzyme.

DISORDERS OF MALE SEXUAL DIFFERENTIATION

For normal male sexual differentiation adequate androgen secretion as well as expression of a normal androgen receptor is required at a critical time during gestation and afterwards during pubertal and adult life. In addition factors involved in spatial and timely organization of embryonic development are required. The disorders due to inadequate androgen secretion or synthesis are inherited in an autosomal recessive pattern. The disorder of inadequate androgen action is the X-linked Androgen Insensitivity Syndrome (AIS). In these disorders AMH action is normal and thus Müllerian derivatives are absent.

Leydig cell unresponsiveness to LH

The production of testosterone by the Leydig cells, which starts at 6-7 weeks of gestation[39], is a major event that initiates sexual differentiation and masculinization. Leydig cell unresponsiveness to LH/hCG because of a defective LH receptor, can result in Leydig cell agenesis or hypoplasia. In 46,XY affected subjects, external genitalia vary from completely female to a more virilized state with micropenis or even only hypospadias. A consistent correlation between receptor activity and phenotype seems to exist. In 46,XY individuals, a totally defective LH receptor leads to complete female external genitalia and residual receptor activity corresponds to residual virilization in a patient.[40-43] Furthermore, mutations in specific regions of the gene cause the LH receptor to be constituitively active while mutations in other regions are inactivating.[44]

Affected 46,XX females have amenorrhea and are infertile. However they have normal genitalia, normal breasts, pubic and axillary hair.[41,45]
At puberty some virilization may occur in patients with a male phenotype and a partially inactive LH receptor[41,43], whereas the phenotype of patients with a female phenotype remains infantile.[46] Thus in 46,XY subjects with the complete form and total receptor inactivity, breast development does not occur due to lack of estrogens (to be produced from testosterone). However, pubic

and/or axillary hair is either absent[47] or present with Tanner II[48] to IV[41] stages. The development of pubic hair is likely to be the result of adrenal androgen secretion.
There is an increased frequency of parental consanguinity. In Brazil, a disease prevalence of about 1:500,000 in the male and female population was calculated.[49] This prevalence of 1:1,000,000 in males makes Leydig cell hypoplasia a very rare cause of male pseudohermaphroditism.

Androgen biosynthesis

The synthesis of androgens from cholesterol involves several converting enzymes, which are shown in Figure 1.5. Before the actual synthesis can start, cholesterol is transferred into the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR).[50] Whereas StAR and P450scc are the rate limiting steps in hormone synthesis, P450c17 is the qualitative regulator, determining which class of steroids will be produced.[51] When P450c17 is absent, aldosterone is produced. When the 17α-hydroxylase activity of P450c17 is present cortisol is made, and when both 17α-hydroxylase and 17,20 lyase activities are present, sex steroid hormones are produced.
Both 17α-hydroxylase and 17,20 lyase activities are catalyzed by one enzyme, encoded by a single gene, *CYP17* (Figure 1.5). The ratio of 17,20 lyase/17α-hydroxylase activity of P450c17 is increased by an increased availability of electron-donating redox partners, P450 oxidoreductase or cytochrome b5, or by increased affinity of P450c17 for redox partners by Ser/Thr phosphorylation.[52] This mechanism may underlay the differential activities of this single enzyme in the adrenal versus the gonads. It might also explain the prepubertal increase of adrenal derived DHEA in children at about 8 years of age, called adrenarche.[52] Recently, *CYP17* mutations were reported causing reduced enzyme affinity for redox partners and thus leading to isolated 17,20 lyase deficiency in patients.[53,66]
In addition to its role in differentiation of the gonad, SF-1 regulates the expression of StAR, cholesterol side chain cleavage-cytochrome P450scc, 3β-hydroxysteroid dehydrogenase[13] and P450 aromatase[54], in such a way that it increases the synthesis of testosterone and decreases its conversion into estradiol. DAX-1, inhibits the expression of StAR, p450scc and 3β-HSD[13] (see also the first section of this chapter). The above examples illustrate that complex mechanisms should all operate together, in order to regulate adequately and timely steroid biosynthesis within the adrenals and within the gonads.

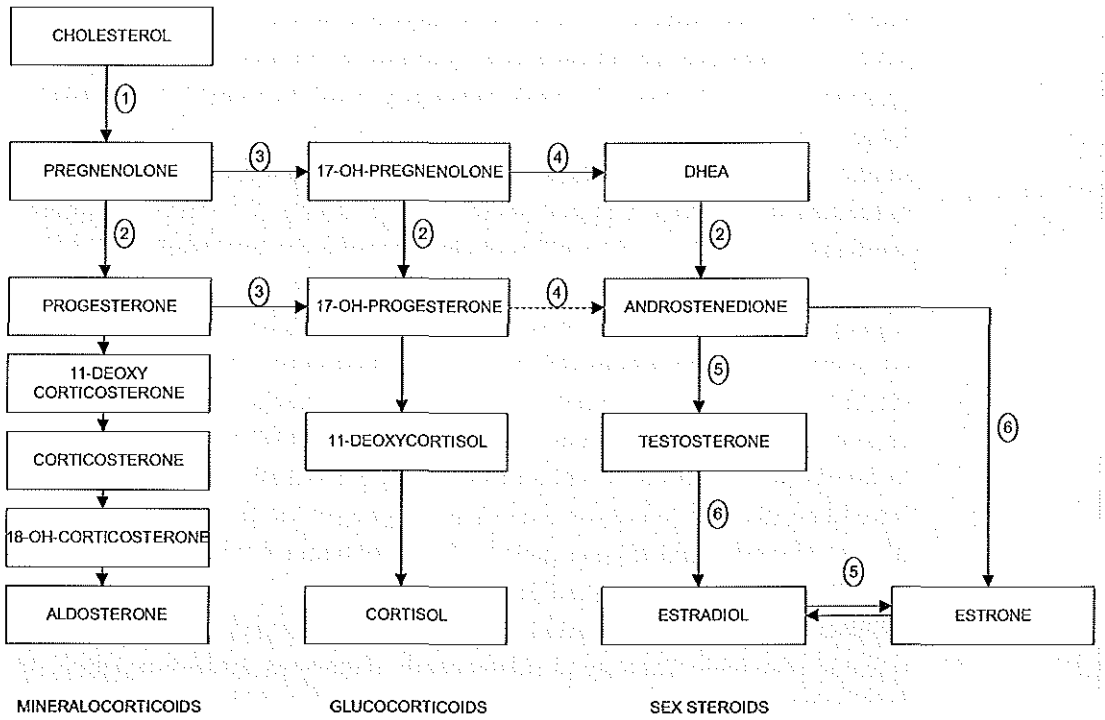

Figure 1.5
Pathway of steroid biosynthesis
Mineralocorticoid and glucocorticoid biosynthesis takes place in the adrenals, sexsteroid are made by the gonads. Enzymes involved in androgen synthesis are either short chain dehydrogenases[244] or cytochrome P450s.[245]

1: P450 side chain cleavage; 2: 3β-hydroxysteroid dehydrogenase; 3: P450c17 (17α-hydroxylase activity); 4: P450c17 (17,20 lyase activity); 5: 17β-hydroxysteroid dehydrogenase 3; 6: P450 aromatase. In humans the lyase reaction with 17-hydroxypregnenolone as a substrate is 30-fold more active than the lyase reaction with 17hydroxy-progesterone as a substrate. [51] The latter is therefore denoted with a dashed line.

Androgen biosynthesis disorders

All the disorders of steroid biosynthesis show autosomal recessive inheritance. Some enzymes involved in testosterone biosynthesis are also required for the synthesis of mineralocorticoids and glucocorticoids (Figure 1.5). Consequently disorders of testosterone synthesis can be associated with more or less severe adrenal insufficiency.

Mutations in the ***StAR*** gene result in congenital lipoid adrenal hyperplasia.[55] StAR is not required for progesterone synthesis by the placenta, and therefore pregnancies with babies with StAR deficiency come to term.[51] 46,XY affected subjects are born with female external genitalia. Through StAR-independent steroidogenesis these patients have low but detectable levels of adrenal steroid hormones and survive without treatment 1-2 months.[56,57] Trophic hormone stimulation results in accumulation of cholesterol in the fetal testis and postnatally in the adrenals leading to their destruction.[57] Little or no C18, C19, C21 steroids become detectable in serum or urine.[4] In 46,XX females, the ovaries are preserved as the ovaries only start to produce steroid hormones at the onset of puberty. The differential diagnosis with congenital adrenal hypoplasia can be made by demonstrating enlarged adrenals in StAR deficiency.[4]
In fifteen patients from 10 countries 16 different mutations in the StAR gene have been found.[57] A founder effect is likely present for one mutation found in 80% of Japanese and Korean patients and a different founder likely accounts for 78% of alleles from affected Arabs.[4]
P450scc is an enzyme, encoded by one gene, that catalyses three distinct sequential reactions. Cholesterol subsequently undergoes 20-hydroxylation, 22-hydroxylation and scission of the 20,22 carbon-carbon bond to yield pregnenolone.[4] It is thought that mutations in the P450scc gene are not compatible with life. Placental progesterone which is needed to maintain the second and third trimesters of human pregnancy, would not be synthesized.[58]

Deficiency of **3β-hydroxysteroid dehydrogenase type 2** leads to deficiency of cortisol, congenital adrenal hyperplasia, with or without signs of aldosterone deficiency, such as salt loosing, in both sexes. Affected 46,XY males may have varying degrees of undervirilization, from (rarely) entirely female external genitalia to (most often) hypospadias.[59,60] 46,XY males may also show virilization at puberty sometimes with gynaecomastia, which may be due to extra-testicular conversion by the 3β-HSD isoenzyme 1.[60] Virilization in 46,XX females can be caused by placental limitation of aromatization of androgens to estrogens early in gestation. Therefore high levels of DHEA are converted into testosterone by 3β-HSD type 1 iso-enzyme which is expressed in peripheral tissues and in the placenta. Patients with deleterious mutations have salt loss. Patients who are homozygotes or compound heterozygotes for mutations with residual 1-10% of enzyme activity, are not salt-loosing. There is no correlation between the degree in severity of the undervirilization in males and salt-loss[4] and phenotypic variation for the same mutation has been described.[61] No mutations on the

HSD3B1 or *HSD3B2* gene have been found in the late onset form of 3β-HSD deficiency.[4]
The prevalence and genetic epidemiology of 3β-HSD deficiency are not known yet.

Mutations in the *CYP17* gene which encodes an enzyme with both **17α-hydroxylase** and **17,20 lyase** activities, most frequently cause combined 17α-hydroxylase/17,20 lyase deficiency. These patients have impaired cortisol, testosterone and estrogen secretion. ACTH is elevated; consequently the adrenal cortex continuously secretes precursors including deoxycorticosterone (DOC), corticosterone and 18-hydroxy-corticosterone. An increased serum level of DOC, a mineralocorticoid, leads to hypertension, hypokalemic alkalosis, suppression of the renin-angiotensin system, and secondarily to suppression of aldosterone secretion. High levels of corticosterone, with weak glucocorticoid activity, prevents signs of cortisol deficiency and modulates secretion of ACTH.[4]
46,XY affected subjects have external genitalia ranging from female with a blind ending vaginal pouch to male with hypospadias, a small phallus and diminished sexual hair at puberty. Female 46,XX affected individuals are often diagnosed at puberty because of amennorrhea and lack of sexual hair.[4]
Cases with clinically apparently isolated 17,20 lyase deficiency were reported[62,63], but the associated mutants showed combined 17α-hydroxylase/17,20 lyase impairment when expressed *in vitro*.[64] Furthermore, such isolated 17,20 lyase deficiency in childhood changed to combined 17α-hydroxylase/17,20 lyase deficiency at young adulthood.[65] Recently, three children were reported with clinically isolated 17,20 lyase deficiency. The *in vitro* expressed mutant enzymes showed 95% reduction of 17,20 lyase activity and 35% reduction of 17α-hydroxylase activity.[53,66] The mutations had changed the electrostatic charge on the surface of the P450c17 protein, the region that normally interacts with the redoxpartner, so that binding of the enzyme to the redoxpartner was impaired.[53,66]
A prevalence of 17α-hydroxylase deficiency of 1:50,000 has been estimated, based on the number of reported patients.[4] More than 30 patients and 20 different mutations were identified. These mutations appear to be at random.[67-69]
One mutation (4 base pair duplication in exon 8) in the *CYP17* gene shows a founder effect among North American Mennonites, who have been living in isolated communities in North-America. The same mutation is found in Friesland, the north-west region in The Netherlands, where the founders of the Mennonites came from.[70]

17β-Hydroxysteroid dehydrogenase type 3 deficiency in affected 46,XY individuals causes undervirilization, ranging from completely female external genitalia (Chapter 9) to hypospadias[71] or micropenis.[72] Most patients have predominantly female genitalia. At puberty, phallus growth, rugation of labial/scrotal skin, facial hair and substantial body hair appears, sometimes resulting in gender changes.[72-74] Virilization only occurs in patients with testes *in situ* and does not occur in gonadectomized patients. This virilization may be due to extragonadal conversion of androstenedione into testosterone.[71] Gynaecomastia develops in some cases and is probably correlated with a lower testosterone/E2 ratio.[75]

46,XX females homozygous for a partially defective enzyme, showed normal sexual differentiation and pubertal development.[76,77]

A number of genes encoding 17β-HSD isoenzymes have been cloned untill now, but the isotype 3 catalyses the conversion of androstenedione into testosterone in the testes. Disruption of this gene eliminates most of the testosterone synthesis in the testes.[71,78] In pubertal patients, testicular testosterone secretion was reduced to 5% of normal, while serum concentrations were 10-40% of the normal.[71] This suggests two things:

- Peripheral conversion of androstenedione into testosterone. The 17β-HSD isoenzyme type 5 gene is expressed in muscle and liver. It is the only other enzyme known at present that catalyzes the conversion of androstenedione to testosterone although with much lower efficiency.[79] This is a candidate enzyme for peripheral conversion of androstenedione into testosterone that most likely accounts for the virilization at puberty.
- In addition, residual testicular testosterone synthesis by another isoenzyme or by partial inactivation of the 17β-HSD3 isoenzyme is present in minor amounts.

5α-Reductase 2 deficiency is often classified as a disorder of the androgen target tissues, and not as a disorder of androgen biosynthesis.[4,80] This enzyme is mainly active in the peripheral tissues and only very minimally in the testis. However it is also a disorder of androgen metabolism or even synthesis, and is therefore discussed in this section on androgen biosynthesis disorders. There are two isoenzymes 5α-reductase type 1 and type 2.

5α-Reductase type 2 is expressed in the external genitalia anlagen and urogenital sinus of both sexes from early in gestation throughout life.[81] Its impairment in 46,XY subjects, leads to external genitalia ranging from completely female to male with no overt genital defect, and absent or an underdeveloped

prostate.[35,82] Wolffian duct derivatives are normally developed[83] although small seminal vesicles were reported.[84]

At puberty all patients show some or a severe degree of virilization, often involving increased muscle mass, deepening of the voice, growth of the phallus and virilization of the genitalia[85] sometimes leading to a gender change.[35] This virilization is attributed to a combination of testosterone action, DHT formed by 5α-reductase type 1 and by residual activity of the defective 5α-reductase type 2.[85] The action of 5α-reductase type 1 which is expressed in non-genital skin, pubic skin, the liver and certain brain regions[86,87] may account for residual DHT formation. This can be concluded from the following observations:

A) ratios of T/DHT do not correlate with the severity of undervirlization[82],
B) patients homozygous for a deleterious alteration may have ambiguous genitalia at birth[88],
C) rats, prenatally exposed to high doses of the 5α-reductase type 2 inhibitor finasteride, are born with ambiguous genitalia while exposure to the androgen receptor antagonist hydroxyflutamide resulted in complete female genitalia[89],
D) development of normal or reduced amounts of pubic hair with a female distribution in adult patients with 5α-reductase type 2 deficiency.[90]

The reduced amount of facial and body hair[35] in patients with 5α-reductase type 2 deficiency suggest a role of 5α-reductase type 2 in the normal development of this hair. 5α-Reductase type 2 deficient patients are usually infertile due to underdevelopment of the prostate and seminal vesicles in addition to oligospermia or azoospermia. However, fertile patients have been reported.[35,91]

Affected 46,XX females have decreased body hair and delayed menarche, normal sebum production but no history of acne.[35] Fertility is normal.[35]

Mutations in the 5α reductase type 2 gene (SRD5A2) define the molecular genetic basis of 5α-reductase type 2 deficiency. Mutations are found in all parts of the gene, in the parts encoding substrate binding as well as in the regions for cofactor binding.[92,93] A total of 31 mutations have been identified in more than 27 ethnic groups, and identical mutations have been discovered in different ethnic groups.[35] Judged by the origin of their carriers, some are probably due to a founder effect, and some have recurred *de novo*.[83,94] About 60% of the 5α-reductase 2 deficient patients is homozygous for a mutation and around 40% of their parents report consanguinity.[83] It is a relatively rare disease, except in some geographic isolates of people with a high coefficient of inbreeding.[80] Therefore, a very low carrier frequency can be expected in the general global population. In some patients, no mutation or only one mutation has

been identified, which may be due to mutations outside the screened region of the *SRD5A2* gene.[80]

It is of special interest that 5α-reductase deficiency secondary to AIS have been described.[95,96] In contrast to patients with primary 5α-reductase deficiency, 5α-reductase activity is preserved in the liver[96,97], and is reflected by the moderately elevated ratios of serum T/DHT in AIS patients.[97] Which isoenzyme is involved was unknown.[35] In Chapter 4 we describe our further studies on this secondary 5α-reductase deficiency.

Unclassified forms of disorders of male sexual differentiation

The elucidation of gene defects in complex syndromes with incomplete sexual differentiation in 46,XY individuals shows that the steroid biosynthesis defects discussed above are only one etiologic category of incomplete sexual differentiation in 46,XY individuals. In Smith-Lemli-Opitz syndrome, a defect of delta-7-dehydrocholesterol reductase leads to diminished synthesis of cholesterol, the precursor of sex steroid hormones. Whether the virilization defect of the genitalia is due to diminished cholesterol synthesis or (in part or additionally) due to disturbance of the hedgehog system, involved in the spatial and timely organization of embryo development, is not clear.[98]
Apart from the action of the androgen-AR complex as a factor of growth and differentiation, defects in genes important in 'midline fusion' (*MID1*) can lead to hypospadias and ambiguous genitalia as in Opitz G/BBB type 1.[99,100] Mutations in some of the *HOX* genes, encoding transcription factors responsible for spatial and timely patterning of development[101-103], or other transcription factors involved in organ morphogenesis[104] were shown to be responsible for combined limb- and genital developmental disorders. These genital disorders appear to be phenotypic sexual differentiation disorders, as these patients have testes but no details on their endocrine data are yet available. Both distal limbs and the genital bud are morphogenic ends of the body and share apical growth and epithelia-mesenchymal interactions.[105] Perhaps elucidation of the pathway downstream of the HOXA or HOXD[103] or HOXD13[101] leads to the identification of genes responsible for the unsolved etiology of isolated hypospadias.

There are numerous syndromes with associated genital malformations in 46,XY subjects. For a more extensive, updated list see Smith's Recognizable Patterns of Human Malformation[106], On line Medelian Inheritance in Man (OMIM) on HTTP//:WWW3.ncbi.nlm.nih.gov/omim/ or P.O.S.S.U.M.[107] The identification

of the underlying gene defects in several of these complex syndromes will lead to a more complete picture of the genes involved in genital development.

Androgen insensitivity syndrome

Androgen Insensitivity Syndrome (AIS) is an X-linked disorder of absent or defective virilization in 46,XY individuals due to absence or deficiency of androgen action resulting from mutations in the *AR* gene. AIS displays a large phenotypic as well as mutational spectrum.

Diagnosis

The diagnosis AIS in adults is made on a 46,XY karyotype in a phenotypically female individual, or a male individual with undervirilization, despite the presence of high serum levels of testosterone and DHT. Serum LH is also elevated in these patients indicating interruption of the negative feedback mechanism. Female phenotypes have well developed breasts whereas male phenotypes usually have gynaecomastia. In neonates the diagnosis is a diagnosis *per exclusionem* of other causes of male pseudohermaphroditism. Absence of other dysmorphic features, a 46,XY karyotype with presence of testes and a normal rise of testosterone after hCG, the presence of normal hCG-stimulated serum levels of precursor hormones when compared to the hCG stimulated testosterone level and a normal hCG-stimulated T/DHT ratio. The finding of a mutation in the *AR* gene with an effect for the function of the AR protein provides definite proof of the presumptive diagnosis AIS. In prepubertal children the presumptive diagnosis is based on the same principles as described for neonates, but an SHBG-suppression test[108] may be helpful for confirmation of the diagnosis before mutation analysis is started (Chapter 12).

Clinical spectrum of AIS

The phenotypic spectrum of AIS varies from a female- to an undervirilised male phenotype or an infertile- but otherwise normal male phenotype. AIS is usually devided in complete AIS (CAIS) with complete female external genitalia, or partial AIS (PAIS) in which signs of virilization are present.
Detailed classifications were proposed by two different research groups.[108,109] Both are modifications after the Prader classification for genital development in 46,XX children with congenital adrenal hypoplasia[110] and are based on the virilization of the external genitalia only. Quigley's classification for AIS (Figure 1.6) is further used in this chapter, as it is a commonly known description of AIS. Wolffian structures are developed in AIS grade 1 to 6. Breast development

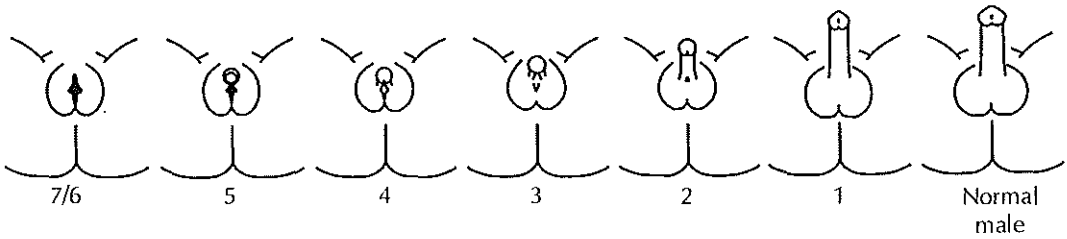

Figure 1.6
AIS classification according to Quigley et al.[109]
The different grades are defined as: **AIS grade 7** (complete AIS): Complete AIS is defined as a female genitalia with absent or sparse vulva or pubic hair, Tanner stage II. **AIS grade 6** (partial AIS with female phenotype): This PAIS phenotype shows minimal signs of androgen action. It is a female phenotype with complete female genitalia. At puberty, there is normal development of a female pattern of pubic and/or axillary hair. **AIS grade 5** (partial AIS with female phenotype): A slightly less defective in virilisation in utero leads to a female with clitoromegaly and/or posterior labial fusion. **AIS grade 4** (partial AIS with ambiguous phenotype): Neonates with scrotalized labia and a phallus of a size intermediate between a clitoris and penis, (ambiguous genitalia). **AIS grade 3** (partial AIS with male phenotype): is a phenotype with slightly more virilization and resembles a male phenotype with undervirilization. The patients have a micropenis, hypospadias, bifid-, sometimes shawl-scrotum. Cryptorchidism may be present. **AIS grade 2** (partial AIS with male phenotype): is a male phenotype with only slight undervirilization as simple hypospadias or a bifid scrotum or a micropenis and gynaecomastia. **AIS grade 1** (partial AIS with male phenotype): is a totally male phenotype with azoospermia and hormonal signs of androgen insensitivity or a fertile male with gynaecomastia.[233,246]
Wolffian structures are fully developed in AIS grade 6 to 1. Breast development is usually present and varies from fully formed female breast Tanner stage M5, in grade 7,6 and 5 to mild gynaecomastia which is more often found in grades 3 to 1. A feminine fat distribution is present in some cases with grade 3 AIS, but absent in others.[96,111-115] As in CAIS, in PAIS feminization of body contours and breast development occurs at puberty as the result of relative high estrogen concentrations in combination with androgen resistance. In general AIS is divided into complete AIS (CAIS) and partial AIS (PAIS). Virilisation of genitalia at puberty is usually absent in CAIS and absent to poor in PAIS. It seems to correlate to the degree of virilisation in utero.[111-117]

is usually present and varies from fully formed female breasts Tanner stage M5, in grade 5 till 7, to mild gynaecomastia which is more often found in grades 1 till 3. A feminine fat distribution is present in grades 4 till 7 and some cases with grade 3 AIS.[111-115] As in CAIS, in PAIS feminization of body contours and breast development occurs at puberty as the result of relative high estrogen action compared to androgen action.

Virilization of genitalia at puberty is absent in CAIS. It is absent to poor in PAIS and seems to correlate to the degree of virilization in utero.[111-117]

Complete androgen insensitivity syndrome (CAIS)[4,80,118]

- *Clinical presentation* – CAIS patients present in the neonatal period or during childhood with inguinal or labial swellings or at puberty with primary amenorrhea. A family history of AIS may lead to an early diagnosis. Occasionally, CAIS is discovered when a child is born with female genitalia, while the birth of a boy was expected because amniocentesis had shown the presence of a 46,XY fetus (personal observations) or during investigations into other abnormalities present at birth (personal observations, [119,120]). The British Pediatric Surveillance unit reported that 76% of the children with CAIS presented with uni-or bilateral hernia, whereas 14% were diagnosed because of their family history.[120] On the other hand only 1-2% of girls with inguinal hernias (reviewed by[121]) and 12% of women with primary amenorrhea may have androgen resistance.[122]
- *Prevalence* – The most accurate estimate on the prevalence of CAIS is probably that of Bangsboll et al, 1:40,800. This is based on the national registry of Denmark. Over a time period of 7 years 21 patients had the primary and secondary diagnosis of Morris syndrome and a 46,XY karyotype.[123] As only three patients were postpubertal, testosterone synthesis disorders may have been included in this group. Other estimates are 1:124,800 males, based upon a ten year study of inguinal hernias in girls in one English hospital124 and 1:200,000 by Hauser based on 'the clinical material at his disposal'.[125]
- *History*[109,125] – The earliest description of possible CAIS is in the Talmud, in the 4th century A.D.[126] At the end of the 19th, and in the early 20th century, numerous patients with CAIS were reported (reviewed in[127]). Petterson and Bonnier gave a detailed analysis of clinical and genetic features.[128] However, Morris' classical comprehensive description of the phenotype in 82 cases of CAIS[127] remains an unmatched summary of the principal features of CAIS. He gave the disorder its descriptive name: testicular feminisation. End-organ unresponsiveness to androgens as its cause was first suggested by Lawson Wilkins in 1950 (Ref.12 in[109]). However, other etiologic explanations were proposed and only by 1965-1969 the androgen insensitivity was definitively established. French et al.[129-131] showed that these patients had normal testosterone biosynthesis and metabolism but generalized tissue resistance to androgen action because they had no metabolic response to testosterone or DHT in nitrogen balance studies. The term androgen insensitivity syndrome was subsequently adopted by most clinicians; it reflects more accurately the

nature of the disorder and is psychologically more acceptable for patients and their families.

AIS animal models suggested an absent or defective AR, in the 1970s. Studies in genital skin fibroblasts of an AIS patient confirmed this diagnosis.[132,133] The heterogeneity in androgen binding defects in this syndrome was subsequently shown.[134] The molecular definition of AIS was provided by localization of the gene for the androgen receptor to Xq11-12 on the X-chromosome in 1981[135], cloning of the *AR*-gene in 1988-1989[136-139] and identification of the first mutated *AR* gene in an AIS family.[140]

- *Definition of the CAIS phenotype* – CAIS is defined as a female external phenotype with normal female breasts and female external genitalia in a 46,XY individual, despite normal male serum levels of testosterone. Sometimes the clitoris, labia minora or majora, are underdeveloped.[125] Small, juvenile nipples are also reported.[125,127] The vagina is ending blindly and of variable depth. Müllerian duct structures are either absent or vestigial.[141,142]

  Pubic and axillary hair is completely lacking in about one third of the patients, but a small amount of vulval hair is usually present.[4] Since pubic hair is androgen dependent, it has been suggested that this must be vellus hair.[109] But patients with complete *AR* gene deletions or deletions of the entire ligand binding domain of the AR do have real, although sparse pubic hair[82,140,143] (and Chapter 3). This small amount of real pubic hair may develop not until early adulthood[143] (and Chapter 3).

- *Growth and puberty* – The age of onset of puberty has not been systematically studied. The pubertal growth spurt is similar in timing and magnitude to that of normal girls. Skeletal maturation corresponds better to male standards.[144,145] The final height is in between the final height of normal women and men.[144,146,147] An eunuchoid body habitus[127] is sometimes reported but anthropometric measurements in eight CAIS patients showed normal female body proportions.[146]

  CAIS patients feminize spontaneously at puberty. Breasts develop under the influence of estrogens formed by the testes and by extra-testicular aromatization of testosterone, unopposed by the effects of androgens.[80] No or sparse amounts of pubic hair, Tanner stage P2-3 will appear.[80]

- *Wolffian / Müllerian duct derivatives* – Müllerian remnants are present in 35% of patients.[141] They consist of smooth muscle bodies that may be fused in the midline[148] or even Fallopian tubes.[141]

  In clinically defined CAIS patients Wolffian derivatives are absent or vestigial.[4,109] Data on the developmental stage of Wolffian structures is missing in

all reports of patients with a complete deletion of the *AR* gene[108,143,149] and no studies on their development in molecular well defined CAIS patients exist.

Partial androgen insensitivity syndrome (PAIS)[60,109]

- *Clinical presentation* – Patients presenting with ambiguous genitalia normally come to medical attention at or immediately after birth. Clitoromegaly, labial fusion or inguinal hernia in a phenotypic female child is sometimes first discovered during routine medical check-up or by the parents when the child is a few weeks to a few years old (personal observations). Simple hypospadias, or micropenis in boys may lead to a delayed diagnosis. Undervirilization, gynaecomastia and/or infertility are indications to seek medical attention only at/ after puberty.
- *Prevalence* – This is unknown, also due to its phenotypic variability.
- *History* – Various forms of PAIS became known during the 40ties and 50ties, without recognition of their inter-relationship. Their connection to CAIS was also not made. Reifenstein described patients with hypospadias, undescended testes and gynaecomastia, thereafter known as Reifenstein's syndrome.[150] Gilbert-Dreyfus described patients that were slightly less virilized[111] and Lubs described a predominantly female phenotype, AIS type 4.[112] Accordingly, the syndromes described respectively by Reifenstein, Lubs, Gilbert-Dreyfus and Rosewater, were identified as manifestations of the same disorder.[114]
  The link to CAIS was first made by Morris & Mahes in 1963.[151] They suggested that their cases with 'testicular feminization' and clitoral enlargement represented a heterogeneous condition closely related to 'classic testicular feminization'. A reduced response to androgens was found in a Reifenstein patients, and suggested partial AIS as the diagnosis in 1971.[152]
  Allelism of CAIS and PAIS was proposed in 1974: the same X-linked gene was thought to be involved.[114]
  Familial gynaecomastia and pubertal undervirilization in the presence of normal male genitalia, identified in 1965[153], was recognized as PAIS in 1978.[117]
  The concept that oligospermia or azoospermia in otherwise normal men could be due to AIS was introduced in 1979.[154]
- *Growth and puberty* – Females with AIS grade 5 do not develop a male phenotype, with beard growth and sex reversal.[116] Whether or not the clitoris in these patients increases in size at puberty is unclear from the available litera-

ture. Normally no prepubertal data on clitoral size are given. In one AIS grade 5 patient, the clitoris did not obviously increase in size at puberty.[116] However upon the prolonged administration of a high dose of testosterone (20 mg of methyl testosterone daily) to a patient with grade 5, clitoral enlargement appeared.[151]

Males with grade 3 AIS generally show minimal signs of virilization, minimal outgrowth of the genitalia, absent or minimal facial hair and sexual hair resembling the female pattern. Not infrequently these males have a female distribution of body fat.[111-114]

Males with grade 2 AIS may show considerable although not full virilization at puberty.[155]

The infertile male and AIS

The possible existence of isolated infertility as a phenotype of AIS was suggested based on the finding of infertility with only minimal signs of undervirilization in families including more severe cases of AIS.[154] The incidence of androgen receptor abnormalities in man with azoospermia or oligospermia was estimated to be 19% to 40%. These figures were based on diminished androgen binding by the androgen receptor in genital skin fibroblasts similar to what is found in patients with CAIS and Reifenstein syndrome.[156,157] However, mutations in the *AR* gene are found in a small number of these patients.[158-160] In the infertile men with decreased receptor capacity, some but not all had elevated testosterone and/or LH serum levels.[154,156,157] Because AR binding studies have a low specificity, AR abnormalities appear to be a very minor cause of isolated infertility (see also page 38).

Clinical variability in AIS

Morris and Mahes (1963) stated that "the complete syndrome and the syndrome with clitoral enlargement do not as a rule occur in the same family". At present there is only one reported family with coexisting CAIS and PAIS.[161] The phenotypic variation, on the other hand, in PAIS families is well documented.[114,162-164] The widest intrafamilial variations of PAIS in eight patients, ranged from clitoromegaly in the absence of labial fusion to simple hypospadias.[163] More narrow intrafamilial variation is penile hypospadias, and perineoscrotal hypospadias occurring in the same family.[165] Very mild phenotypic variation, in degree of hypospadias and amount of pubic hair, was reportedly due to secondary 5α-reductase deficiency.[96] Another family with three affected patients had the phenotypical spectrum of grade 5, grade 3 or a micropenis

and prominent scrotal raphe.[166] However, in many families no phenotypic variation was observed.

Endocrine findings in AIS[4,109]

The hormonal profile in adult patients is similar in all variants of AIS but has been most often characterized in CAIS. The hallmark is an elevated plasma **LH** and testosterone as compared to normal males, with complete or partial absence of virilization. The LH response to GnRH is normal[167] as well as the testosterone response upon hCG administration.[168,169] **FSH** levels are normal or slightly elevated as some maturation of the Sertoli cells may occur in PAIS but these structures remain immature in CAIS.[170]

Testosterone levels may be in the normal male range or elevated.

Plasma **DHT** levels are variable, either decreased or normal. The ratio T/DHT is in between the ratio in normal males and the ratio in 5α-reductase deficiency[95-97] due to a functional form of 5α-reductase type 2 deficiency.

Estradiol is increased to about twice the level in normal adult men. This increased estrogen is mainly due to LH mediated increased production in the testes and to a lesser extent due to the peripheral conversion of higher levels of androstenedione and testosterone.

Androstenedione as well as androstenedione/ testosterone ratios are also slightly higher than in normal males[97,167,171] although far from the magnitude of the increase as found in 17β-hydroxysteroid dehydrogenase 3 deficiency.

SHBG levels in CAIS patients are similar to those in normal females[167,171] and higher than in males. Androgen action lowers the SHBG concentration whereas estrogen action causes the SHBG serum concentration to rise. In PAIS patients, the SHBG concentration is in between those of males and females.

Levels of **AMH** are elevated, neonatally and at puberty, in the majority of AIS patients.[172] Presumably AMH serum levels are negatively regulated by testosterone.[172]

The **increased serum levels of both LH and testosterone** supposedly result from absence of the androgen receptor mediated negative feedback mechanism on the hypothalamus and pituitary. Studies in normal males and in one aromatase deficient male showed that the negative feedback on LH secretion in normal man is mainly mediated by testosterone, but that some testosterone must be converted to estrogen.[173-178] In CAIS patients, LH is indeed partially suppressed as LH increases even further after gonadectomy. LH does not decrease upon the administration of DHT to AIS patients whereas it does decrease after testosterone which can be aromatized to estrogen.[115] However,

estrogen levels as low as the upper normal male limit in combination with a low serum testosterone are capable of completely suppressing LH in man.[178] Therefore, the origin of elevated LH in CAIS patients is not completely clear as estrogen levels in CAIS are normally higher than in normal man and persistently elevated LH levels were seen in gonadectomized CAIS patients, despite adequate serum levels of estradiol from estrogen substitution therapy (unpublished observations). The suppression of LH has very important implications for the physician that takes care of these patients. From the report of pituitary apoplexy, due to an LH-producing adenoma in a gonadectomized CAIS patient that had not used substitution therapy for 20 years[179] it is clear that at least some suppression of LH through estradiol, should be strived for during substitution therapy.

Testicular histology

In CAIS as well as in PAIS the testes are located in the abdomen, along the course of the inguinal canal or in the labia majora. In AIS children, testicular histology is indistinguishable from age matched cryptorchid testes or testes of patients with testosterone biosynthesis disorders.[142]
In contrast to the normal situation where Leydig cells are absent in testes of prepubertal children older than one year of age, prepubertal testes of children with AIS show visible Leydig cells.[142] However, these Leydig cells lack the Leydig cell specific Reinke crystals.[142] In contrast to the report by Bale et al.[142] we have found no interstitial Leydig cells in testes of prepubertal AIS patients, but Leydig cells were frequently present in the subcapsular stroma of testes of these patients (unpublished observations).
After puberty in AIS patients, the seminiferous tubules are small, spermatogonia are sparse, and spermatogenesis is absent. A progressive reduction in the number of germ cells was suggested.[141] The Leydig cells are hyperplastic, and form adenomatous clumps. These Leydig cells resemble fetal Leydig cell, without Reinke crystals.[141] In CAIS a thick, fibrotic tunical albuginea may be present[127] and frequently cysts of either Müllerian or Wolffian duct origin are present, located on the lateral poles of the testes.[141]
Some maturation of the seminiferous tubules and the Sertoli cells may occur in PAIS. These structures remain immature in CAIS.[170]

Testicular neoplasm

Patients with AIS have a higher chance of developing germ cell and non germ cell testicular tumors, but it is unclear if this risk is higher than in isolated crypt-

Table 1.2a
Review of the literature on AIS and incidence of testicular tumors

Occurrence of neoplasm in AIS	Number of studied patients	Age of patients in years	Type of neoplasm	Ref.
0 %	23 "CAIS", from their own clinic	of total: 0-74 but predominantly between 10-29	none	(181)
9%	82 "CAIS", from literature	of total: 0-74 but predominantly between 10-29	Seminoma and various others	(181)
6-9 %	40 CAIS	16 - 83	Seminomas; CIS with early stromal invasion; malignant sex cord tumor	(141)
0 %	5 CAIS;18 PAIS	0.33-18	none	(189)
0 %	10 CAIS; 11 PAIS	18 patients <20 3 patients > 45; all CAIS	none	(123)
0 %	82 "AIS"	73 patients < 30	none	(195)
0 %	14 "AIS"		none	(196)
22 %	50 CAIS	tumors in patients > 30	Seminomas, malignant teratoma, Sertoli-Leydig cell tumor	(151)

Table 1.2b
Review of the literature on AIS and incidence of possible precancerous lesions

Occurrence of posible pre-cancerous lesions	Number of studied patients	Age of patients in years	Type of neoplasm	Ref.
0 % CAIS *38% PAIS*	4 CAIS 8 PAIS	0.1–19	CIS	(170)
0% CAIS *73% PAIS*	6 CAIS 11 PAIS	16–18.6	CIS	(188)
2.5%	40 CAIS	16–83	CIS	(141)*

* Rutger and Scully[141] reported 3 additional PAIS patients. These were not included because of incomplete diagnostic work-up for other causes of male pseudohermaphroditism.

orchidism (2.5-8.8%).[180] The cause of neoplasm in AIS is unknown. Literature data, summarized in Table 1.2a, indicate an overall risk for patients with AIS to develop malignant testicular neoplasm of 0-9%.

The risk for malignant germ cell tumors increases with age, from 3.6% at 25 to 33% at 50 years[181] but these risks are based on only one study. Based on the studies as summarized in Table 1.2a, the majority of patients are gonadectomized in infancy, childhood or up to their early twenties. Confirmation of these results is therefore impossible. The youngest AIS patients with malignant invasive tumors were CAIS patients between 14 and 18 years old.[181-184]

Tumors in AIS are mainly seminomas, but other identified types are: dysgerminoma, Sertoli cell tumor[185], arrhenoblastoma, teratoma, gonadoblastoma, and sex cord carcinoma.[141,186]

A very high incidence of carcinoma *in situ* (CIS) in PAIS is found by some[187,188] but not by others (Table 1.2b).[189] In infertile non-PAIS adults and in cryptorchid children, CIS can progress to invasive neoplasm after a median interval of 5 to 10 years.[170] Indeed, CIS with early stromal invasion was found in a CAIS patient.[141] Because there are no reports of malignant tumors in PAIS patients, even in their twenties or early thirties, and only two reports of CIS in CAIS patients[141,184] who have a known increased risk for neoplasia, the development of cancer from CIS in AIS remains an open question. As testes remain in situ in patients with PAIS who are raised as males, the absence of reports of PAIS patients with malignant tumors suggest that the risk of malignant tumors is low in PAIS.

Benign tumors such as hamartomas have been reported as present in 63% of patients[141] and Sertoli cell adenomas in 17-23% of the AIS patients.[127,141] The patients with a Sertoli cell adenoma were between 15 to 53 (average 27,5) years of age.[141] However, benign tumors can be precancerous. Leydig cell adenomas[190] in transition to Leydig cell neoplasia[191] and a malignant sex cord tumor that probably arose from a Sertoli cell adenomas were reported.[141] The malignant sex cord tumor was found a 71-year-old woman which suggest that only after many years Sertoli cell adenomas progress to malignancy.

Testicular biopsy as soon as the diagnosis AIS is made followed by immediate gonadectomy when CIS is found was recommended by Muller and Skakkebaek[187], but this did not become standard practice. The relatively low incidence (3.6%) of malignancies before puberty has led to the recommendation to postponement of gonadectomy until after puberty.[192,193] Seminomas, the most frequently occurring malignancy in AIS, are highly curable if discovered before extralymphatic metastases have occurred[194], which strenghtens this advice. When gonadectomy is postponed untill after puberty, breast and pubic hair develop in AIS patients type 6 and 7 by endogenously produced sex ster-

oid hormones. This is thought to be superior to puberty inducement with administered estrogens as it is a more physiological proces and strenghtens the patient in her conviction of being a female.
There are no early tumor markers available for seminomas, the most frequent tumor in AIS, as serum concentrations of hCG are elevated in only 15-20 % of patients with metastatic seminoma[194] and alpha -fetoprotein is normal in seminomas.[194]

Heterozygote phenotype

Women heterozygous for *AR* mutations causing CAIS or PAIS may show subtle symptoms of AIS. They may have a reduced amount of pubic and/or axillary hair[96,197] and may show absent androgen binding in some genital skin cell lines[198] and normal androgen binding in other GSF cell-lines derived form the same carrier.[199] These phenomena are attributed to 'Lyonisation', the random inactivation of one X chromosome at an early embryonic stage in female cells. In the adult female heterozygous for an X-linked mutation, this may lead to a mosaic state for the expression of the mutation. A delayed menarche is reported by some[149,198,200] but disputed by others.[135]

THE HUMAN ANDROGEN RECEPTOR

The AR is a ligand-depended transcription factor, and a member of the subfamily of steroid receptors. Other members of this family are the progesterone receptor, the glucocorticoid hormone receptor, the mineralocorticoid receptor and the estrogen receptor. Steroid receptors are characterized by a modular structure that is divided into 4 domains: 1) an N-terminal, transactivation domain, 2) a DNA binding domain, 3) a hinge region and 4) a C-terminal, ligand binding domain. The DNA binding domains are highly conserved between these family members and the ligand binding domains are moderately conserved. An exception are the DNA binding and ligand binding domains of the estrogen receptor with little homology to the respective domain of the other steroid receptors. The N-terminal domain has less than 15% homology between the various family members.
The *AR* gene is located on the long arm of the X-chromosome, Xq11-12, whereas the genes for the other steroid receptors are localized on the autosomes.

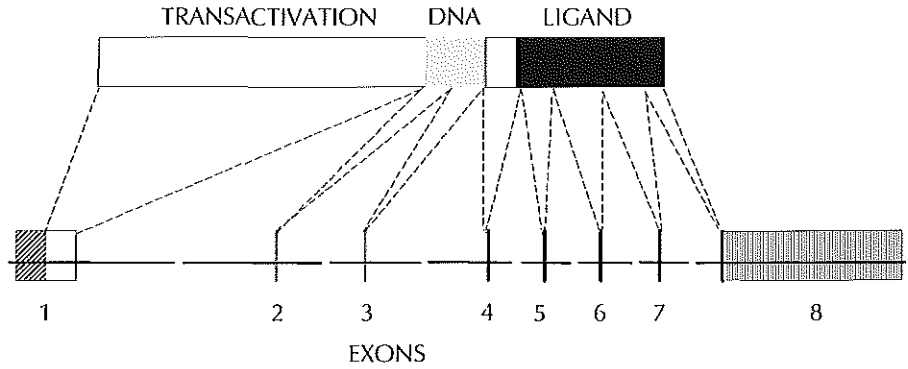

Figure 1.7
Structural organization and functional domains of the human AR. Part of exon 1 encodes the N-terminal domain, exons 2-3 the DNA binding domain, parts of exon 3 and 4 the nuclear localization domain, part of exon 4 the hinge region and exons 4-7 and part of exon 8, the ligand binding domain.

Functional domains

Figure 1.7 depicts the domain structure and gene organization of the androgen receptor. The various functional domains are encoded by eight exons. Part of exon 1 encodes the transactivation domain, exons 2 and 3 the DNA-binding domain, the first part of exon 4, the hinge region and exons 4 to 7 and part of exon 8, the ligand binding domain.

Molecular mechanism of androgen action

The model of AR function, given in Figure 1.8, shows the AR as the key protein in androgen action. The AR resides in the cytoplasm, bound to heatshock proteins. Testosterone diffuses into the cell and can be converted into DHT by the enzyme 5α-reductase in some cell types. Upon binding of androgen to the AR, which can occur either in the cytoplasm or in the nucleus, the heat shock proteins dissociate. The AR-androgen complex then binds to specific palindromic sequences (AREs) in the DNA as a dimer, becomes further phosphorylated, and activates transcription of androgen responsive genes by opening the chromatine structure, and assembly of a transcription initiation complex.

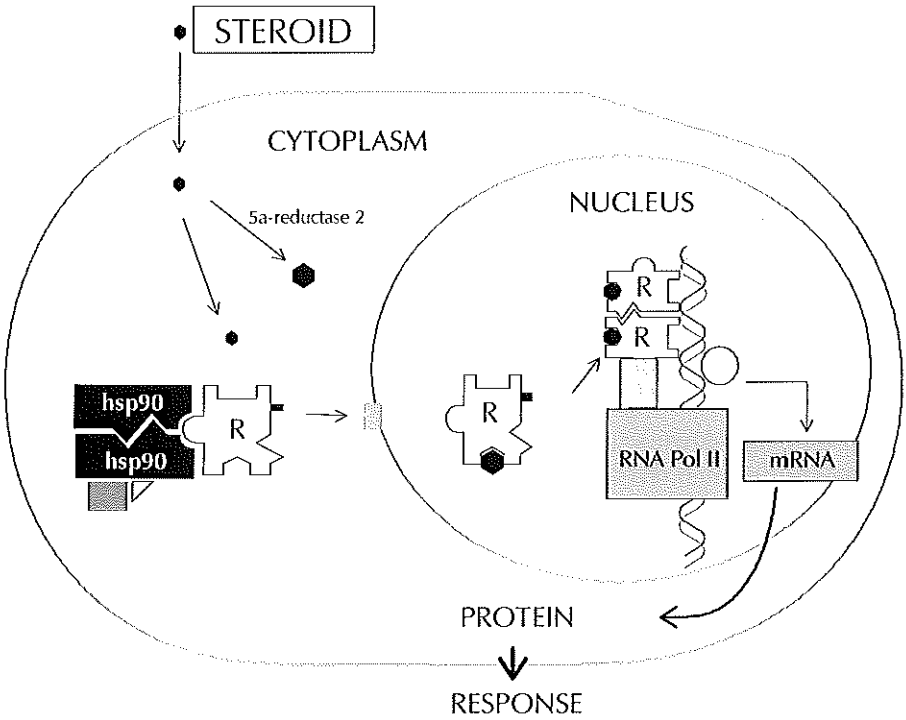

Figure 1.8
Mechanism of action of androgens. Testosterone enters the cell via passive diffusion. In the cytoplasm of target cells testosterone can be converted to the more potent androgen DHT by the enzyme 5α-reductase type 2. Both androgens can bind to the AR which resides in the cytoplasm bound to heat shock proteins (hsp90). Upon binding of androgen the AR dissociates from the heat shock proteins and binds to DNA response elements as a homodimer. Transcription co-factors are recruited and transcription of androgen responsive genes takes place.

METHODS TO STUDY AR FUNCTION

In vitro assessment of AR function

Binding characteristics

Scatchard analysis, studies on the capacity and affinity of mutant ARs for different androgens, have been in use since mid 1970. Androgen binding parameters of mutant ARs either in genital skin fibroblasts (GSF) of patients or transiently expressed in COS or CHO cell-lines are determined. Results may vary

according to the androgen used and whether an androgen can be metabolized. T can be metabolized into DHT that binds to the AR with higher affinity.[36,201] Mibolerone and R1881 are synthetic androgens that can not be metabolized in the cells. A disadvantage is that they bind with high affinity to the receptor and may thus mask very subtle differences in activity.[202] Binding capacity is expressed as Bmax and affinity of the receptor or stability of the ligand-receptor complex is expressed as Kd. In addition some authors use thermolability, the stability of the ligand-receptor complex at increased temperature as a parameter of qualitative binding.[203,204] Studies in GSF of AIS patients are useful models to demonstrate the pathogenicity of a specific mutation. They reveal whether the mutant receptor has absent, defective or normal hormone binding. However, these studies made it clear that there is no simple correlation between the severity of receptor dysfunction in GSF and a specific phenotype.
Scatchard analysis of mutant receptors expressed in COS or CHO cells has been used to test the pathogenicity of a mutation identified in a patient when GSF of that patient were not available. These assays standardize measurements on AR-ligand binding, because the mutant receptor is taken out of the genetic context of the patient, and placed in a standard context of the cell line.

Androgen receptor protein expression

In AIS patients, Western blot analysis shows whether a lowered Bmax is due to reduced expression of the AR protein.[203,205] Furthermore, it can be used to detect abnormal function of the AR. Normal AR proteins, isolated from genital fibroblast cell lines which have been cultured in the absence of androgens, migrates as a doublet of 110 and 112 kDa during SDS-PAGE. These are respectively an unphosphorylated AR isotype and a phosphorylated AR isotype. Upon binding of androgens, the AR undergoes additional phosphorylation, resulting in a third isoform of 114 kDa. AR mutants that are either partially defective in ligand binding or in DNA binding or in transcription activation, migrate with a reduced amount of the 114 kDa isoform in SDS-PAGE.[206]

In vitro *transcription activation assay*

Specific *AR* mutants can be introduced into an AR expression plasmid which is then co-transfected with a reporter plasmid into a cell-line. Upon addition of androgen, the androgen-AR complex binds to the responsive elements in the reporter plasmid and a response can be measured. Dependent on the reporter

used, this response can be luciferase of chloramphenicol acetyl transferase (CAT) activity.
Thus the effect of a mutation on the downstream part of AR function, transcription activation, can be studied. Modulating factors which may be present in the GSF cells or in the tissue of a patient are absent or different in this in vitro system, thus the receptor is tested independed from its normal genetic background. However, effects can vary with the use of different promoters in the *AR* construct, different receptor responsive elements or different cell-lines this variance hampers comparison of in vitro results with the in vivo situation. This technique has been very useful for determination of the pathogenicity of a mutation.

Adenovirus-mediated delivery of androgen-responsive reporter gene in GSF

A relatively new technique to test the AR *in* the genetic context of the patient, is the introduction of a androgen-responsive reporter gene into cultured GSFs derived from biopties of patients.[207] Thus the end point of the androgen receptor pathway is artificially measured in a system that is very close to the situation in the patient, but the androgen-response element is artificial and can be different from the AREs in the target genes. Judged by the broad range in GSF from normal males, it can only be used to test whether androgen receptor function is normal or abnormal and no gradual distinctions can be made.

Green fluorescent protein

AR-mutants fused to green fluorescent protein have been used to study the impact of mutations in the ligand binding domain.[208,209] Because the mutant receptor is made visible with a fluorescence microscope, its incorporation into the cell nucleus where it can bind to the DNA and cause transcription can be followed. Mutants with equal results in androgen binding affinity and equal results in transcriptional activity assays, revealed variance in ability to enter the nucleus which correlated with the degree in severity of the clinical phenotype.[209]

In vivo assessment of AR function

AIS patients and family studies

The study of the phenotype of a patient that carries a specific mutation provides information on the residual function of the mutant receptor during em-

bryonal life, puberty and adult life and sometimes also during androgen therapy.

SHBG suppression test

Sex hormone-binding globulin (SHBG) concentrations in serum decrease under the influence of androgens. The severity of the clinical phenotype was shown to correlate with the degree of SHBG-decline after administration of the anabolic steroid Stanozolol.[108]

AR MUTATIONS IN AIS

Nature of mutations

In over 300 patients many different *AR*-gene mutations have been identified (Figure 1.9 (page 309), [210]). Apparently, there is a wide allelic heterogeneity as well as a wide phenotypic heterogeneity in this disorder.
As predicted for X-linked diseases with absence of procreation of a 46,XY individual[211], approximately one third of the *AR* gene mutations arise *de novo*.[212,213] The mutation may have occurred either in the patient or in the germline, or in the gonads of the mother of the patient (Chapter 7).
Mutations identified in AIS patients are mostly substitution mutations. But deletions, insertions and mutations that influence splicing are also found (Table 1.3).
Some mutations are found in multiple unrelated families, indicating that the mutated residues are of importance to AR function as well as that its coding

Table 1.3
Nature of mutations in the AR gene in patients with AIS

	%
Gene or partial gene deletion	7
Non sense mutations	8
Deletion / insertion of nucleotide	5
Mutations in introns that influence splicing	4
Single codon deletions	0.5
Single base mutations resulting in amino acid substitutions	75

nucleotide sequence is mutationally prone. These mutations are located either in the DBD or the LBD.

Distribution of mutations

Figure 1.9 (page 309) shows the distribution of mutations over the *AR* gene, as deduced from the *AR* gene mutation database November 1998[210], and including multiple mutations described in this thesis. This database uses the *AR* residue numbering according to the cloned sequence of Lubahn et al.[137] In this thesis the numbering according to Brinkmann et al.[214] is used. To convert the Lubahn sequence to the Brinkmann sequence deduct 9 residues, thus R855H in Figure 1.9 is R846H in this thesis.

GENOTYPE VERSUS PHENOTYPE SUBDIVIDED BY FUNCTIONAL AR DOMAINS

Mutations in the transcription activation domain

Very few substitution mutations in exon 1 encoding the transcription activation domain have been found in AIS patients (Figure 1.9 (page 309)). There is only one substitution mutation that is without doubt causative for a PAIS phenotype because of reduced expression of the protein.[215]

As exon 1 was not always sequenced in AIS patients, we can only speculate on the low frequency of mutations in this exon:

- As the part of the AR gene encoding the transcription activation domain is the least conserved, some variation in protein structure may be more easily tolerated in this region. In our and other studies on more than 100 AIS patients, each using PCR-SSCP or DGGE, only missense and non-sense mutations were detected[204,216-218] (this thesis).
- Some substitution mutations in exon 1 may have gone undetected because of a very mild phenotype. There is only one report of systematically screening of exon 1 for mutations in normal, fertile males.[160] Only one mutation was identified in these fertile males, G213R which was also present in an infertile male and caused 15-20% reduction in transactivation capacity for physiological androgen concentrations.
- Alternatively some mutations in exon 1 are not pathogenic. Adding to the later hypothesis is the finding that mutant Pro389Ser, found in two infertile

males, showed normal transactivation[219] and did not obviously influence the clinical AIS phenotype and the phosphorylation pattern in SDS-PAGE analysis in GSF (Chapter 3). The remaining mutations, Gln194Arg[220], Leu255Pro[221], Pro389Arg[222] were found in patients with additional *AR* gene mutations with phenotypes that could well be caused by the additional mutation.

Mutations in the DNA binding domain

The DNA binding domain of the AR is encoded by exons 2 and 3. Within this region four cysteine residues, invariably present in all steroid receptors, bind a zinc ion in each of two loop structures known as 'zinc clusters'. The first zinc cluster, responsible for recognition of the target DNA sequence, is encoded by exon 2. The second zinc cluster stabilizes DNA-receptor interaction and is encoded by exon 3. CAIS is associated with absence of receptor-DNA binding while PAIS is associated with retention of some DNA binding.[223-225] Substitution of one of the four cysteine residues were found in patients with CAIS.[210] The DNA binding domain contains several residues that were found altered relatively often in AIS patients, whereas many other residues in this domain have not been found altered. Some arginine residues in the DNA binding domain are such mutational hotspots, some replacements of these arginine residues lead to PAIS others to CAIS.[210]

Mutations in the nuclear localization domain or in the hinge region

In vitro studies revealed that the nuclear localization domain, consisting of residues 608-624 and encoded by the 3' part of exon 3 and the 5' part of exon 4, is important for hormone-induced nuclear localization of the AR.[226] Such sequences are essential for the transport of large proteins, such as the AR into the nucleus. AIS patients with a missense mutation in the nuclear localization domain are infrequently found. Only one residue has been found altered, Arg608, but the ability of this mutant receptor to enter the nucleus have not been studied.[224,227]

The hinge region, located between the DBD and the LBD, encoded by the 5' region of exon 4, is a region of low sequence homology between the AR and other steroid receptors. The hinge region appears to be involved in conformational changes of the AR induced by binding of androgens and antiandrogens and is part of an interface for interacting proteins.[228-230] In addition, the hinge contains a consensus AR phosphorylation site required for optimal AR tran-

scription activity.[231] Only two residues in the hinge region have been found altered in AIS patients, residue Ile655Asn in a patient with PAIS[200] and Ala636Asp was found in PAIS[218] but also in a normal boy.[232] As in the later, the possible existence of a somatic mosaic for the *AR* gene mutation was not excluded, a conclusion on pathogenicity of this mutation can not be made.

Mutations in the ligand binding domain

The ligand binding domain is encoded by the 3' part of exon 4 and exons 5-8. Although, it is encoded by less than half of the gene the majority of mutations is found in this gene region.[210] Mutations in exons 5 and 7 are most frequently found. A mutation in almost every residue encoded by exon 5 have been found in AIS or prostate cancer patients.[210] In addition there are some amino acid residues, Arg743, Ala756, Arg765, Arg822, Arg831, Arg846, Val857 that can be considered as mutational hot-spots.

MOLECULAR BASIS OF MILD AIS PHENOTYPES

Undervirlized males and *AR* gene mutations

In two families with very subtle symptoms of AIS, very subtle alterations of the AR have been found. These AR alterations were:

- failure of receptor up regulation after prolonged exposure to androgens, increased thermolability[233] and
- increased dissociation of the AR-ligand complex (increased Kd) in GSF.[234]

The clinical abnormalities in these males were a high pitched voice, female pattern body hair with absent beard, gynaecomastia and small penis but fertility. In one family the *AR* gene was screened for mutations and was found to carry mutation Leu781Phe, a substitution of a hydrophobic amino acid for another hydrophobic aminoacid. In *in vitro* expression studies this mutant receptor showed a decreased ligand affinity and decreased transactivation.[234] This demonstrated that a subtle defective AR can cause a subtle phenotype and vise versa.

AR gene mutation Gln824Lys was found in one family with 4 males with minimal signs of AIS carrying this mutation. One of these males was proven fertile. However the mutation did not alter AR-ligand binding in *in vitro* transfection experiments[235] which might be due to insufficiency of the used technique.

Infertile males and AIS

Atypical cases of AIS indicate that the range of phenotypes may include infertility in otherwise normal men. The existence of this mild expression form of AIS was based on androgen binding characteristics in genital skin fibroblasts.[154,156] The incidence of AIS in the infertile population has been estimated to be 40%[156] but others have questioned the magnitude of this figure.[157,236] The first infertile man with a mutation in the *AR* gene was described in 1991[237] but the presented molecular evidence for a deletion of exon 4 of the *AR* gene is not convincing. Recent studies on the incidence of AIS in infertile man that used mutation analysis for detection of AIS have yielded *AR* gene mutations in only 1 out of 75 to 194 infertile males[159,160,219] even when selected for patients with elevated testosterone x LH products[219] (see also page 24).
Mutations found in infertile males are Gly213Arg, Pro 389Ser in the transcription activation domain and Asn718Lys, Glu789Gln in the LBD.
The mutation Gly213Arg showed 17-25% reduction of transcription activation in *in vitro* expression studies, not due to diminished expression.[160] However this mutation was also found in a normal fertile male.[160]
Transactivation of mutant Pro389Ser, found in two infertile males, was the same as of the wildtype receptor in *in vitro* transfection studies.[219] This mutation did not obviously influence the clinical phenotype and the phosphorylation pattern in SDS-PAGE analysis in GSF (Chapter 3). Additional transactivation assays with use of various androgen-responsive promoters may reveal whether this mutation is indeed underlying AIS in infertile males, or that Pro389Ser is an infrequent polymorphism.
The two groups that have tested mutation Glu789Gln have obtained conflicting results, normal transcription activation by one[219] and markedly decreased transcription activation by the other group.[238] This might be due to a difference in the promoter or cell lines used, and awaits publication of further details on the experimental conditions. The very mild phenotype found in the patient described by Hiort et al.[219], could also be due to somatic mosaicism, as this was not excluded in that patient.
AR gene mutation Asn718Lys was found in one male with oligospermia who had a reduced testicular volume but no signs of impairment of virilization. He became fertile upon treatment with androgen.[239] The pathogenicity of this mutation remains to be shown.

An increased risk for impaired spermatogenesis was reported to correlate with a longer but still normal polyglutamine repeat in the transcription activation

domain.[240] Elongation of the polyglutamine repeat resulting in a minimal decrease of transcription activation was reported to correlate with infertility in one study[240] but was not confirmed in another study.[219]

ANOTHER PHENOTYPE ATTRIBUTED TO *AR* GENE MUTATIONS

Another phenotype that has been attributed to *AR* gene mutations is male breast cancer. The association between male breast cancer and *AR* gene mutations became addressed because two unrelated families with Reifenstein syndrome were reported to have breast cancer.[241,242] It is of interest that these families carried alterations in adjacent amino acids in the DNA binding domain of the AR, Arg598Glu and Arg599Lys.[241,242] These alterations caused reduced transcription activation but no evidence of acting via an estrogen response element.[243] Breast cancer (*BRCA*) genes were not investigated in these patients but the occurrence of mutations at adjacent amino acids, with two affected patients in one family, suggests a more than chance association between male breast cancer and these residues or small subdomain of the AR. It might be that these mutations cause the AR regulate the expression of specific oncogenes.

SCOPE OF THE THESIS

The research presented in this thesis started with a study on phenotypes and genotypes in AIS. The aim was to investigate the phenotypic expression of *AR* gene mutations in families with multiple AIS cases and further study different mutant ARs with the use of *in vitro* studies. We wanted to know whether knowledge on the *in vivo* and *in vitro* residual activity of a mutant AR could aid sex assignment of AIS patients born with ambiguous genitalia. Furthermore, *in vivo* expression of naturally occurring AR mutants were expected to yield leads for the molecular function of specific mutated residues. In addition, the prevalence and extent of phenotypic variation, pivotal information in genetic counseling, was unknown.

During clinical evaluation of potential cases for this study on phenotypes/genotypes in AIS, patients with several other causes of male pseudohermaphroditism were encountered. The genotype/phenotype studies of these cases provided new information on these disorders with implications for clinical practice, as well as giving leads with regard to the population genetics and the molecular genetics of these disorders.

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CHAPTER

2

Molecular basis of androgen insensitivity

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SUMMARY

Mutations in the *androgen receptor* gene in 46,XY individuals can be associated with the androgen insensitivity syndrome of which the phenotype can vary from a female phenotype to an undervirilized or infertile male phenotype. To gain information about amino acid residues or regions involved in deoxyribonucleic acid binding and transcription activation, we have studied the *androgen receptor* gene of patients with androgen insensitivitysyndrome (AIS). Genomic DNA of AIS patients was analyzed by PCR-single strand conformation polymorphism analysis under two different conditions. Three new mutations in the *AR* gene were found in exon 1 of three unrelated patients with a female phenotype. A cytosine deletion at codon 42 resulted in a frameshift and consequently in the introduction of a premature stop at codon 171. Deletion of an adenine at codon 263 gave rise to a premature stop at codon 292. In both these cases, receptor protein was not detectable and hormone binding was not measurable. In a third patient, a guanine to adenine transition at codon 493 converted a tryptophan codon into a stop codon. Genital skin fibroblasts from this patient were not available. In exon 2 of the *androgen receptor* gene of a patient with receptor positive androgen insensitivity, a cytosine to adenine transition, converting alanine 564 into an aspartic acid residue, resulted in defective DNA binding and transactivation.

INTRODUCTION

Androgens play a major role in male sexual differentiation and development. The actions of androgens are exerted through the AR, which modulates transcription of androgen responsive genes. The AR belongs to a superfamily of receptors for steroid hormones, thyroid hormones and retinoids. Characteristic for the members of this family are the distinct functional domains; the NH_2-terminal domain involved in transcription regulation, a DBD, composed of two zinc-clusters, a hinge region and the C-terminal LBD.[1] Mutations in the *AR* in 46,XY individuals are associated with AIS, a disorder with a wide spectrum of phenotypes. Subjects with CAIS exhibit a female phenotype, whereas other AIS subjects show a phenotype with ambiguous genitalia, called PAIS. The majority of the mutations reported so far are point mutations, located in the LBD.[2-3] AIS subjects with an AR of normal molecular mass and no abnormalities in ligand binding are an interesting group, because they may provide information about essential amino acid residues or regions, directly involved in transcription activation. Deletion mapping revealed that almost the entire NH_2-terminal domain is necessary for full AR transactivating activity.[4] Therefore, *AR* mutations interfering with correct receptor functioning may be expected in this domain. However, except for the expanded glutamine stretch, associated with Kennedy's disease, only 6 mutations in exon 1 of the *AR* have been reported.[5-10] Five of them resulted either directly or indirectly in the introduction of a premature stop codon.

In this study we describe three new exon 1 mutations, all resulting in the introduction of premature stop codons. Zoppi et al.[9] reported a patient in whom a single nucleotide substitution introduced a premature stop at codon 60. Synthesis of AR protein was found to be initiated downstream of the termination codon. Therefore, we have investigated whether truncated AR forms were present in genital skin fibroblasts from the patients with an exon 1 mutation. A new mutation was also detected in the first zinc cluster of the DBD of a patient with receptor-positive AIS. Exon 1 mutations, resulting in receptor-positive AIS, were not found.

SUBJECTS AND METHODS

Clinical subjects

- Subject A: 46,XY index patient with CAIS was admitted at the age of 1 yr. because of a bilaterally inguinal hernias. She has a younger 46,XY sister with the same phenotype.
- Subject B: 46,XY patient, diagnosed as having CAIS. The sister of the patient's mother was known with primary amenorrhea.
- Subject C: 46,XY patient, with a complete female phenotype and from a family with more affected members.
- Subject D: 46,XY patient who was diagnosed as having CAIS at birth in the absence of a positive family history. She came to medical attention because of suspected dysmorphology. After further clinical examination the diagnosis AIS was made.

The phenotype and the *AR* gene mutation of each subject are summarized in Table 2.1.

Mutation detection

Genomic DNA, isolated from blood lymphocytes, was screened by PCR-SSCP. Seventeen primer sets for overlapping fragments were used to amplify the coding region and the exon flanking intronic regions of the human *AR (hAR)*. A 15 µl PCR reaction mixture was used, containing 100 ng genomic DNA, 70 ng of each oligonucleotide, 40 µM of each dNTP, 10 mM Tris HCl (pH 9.0), 1.5 mM $MgCl_2$, 5 mM KCl, 10% DMSO (in case of amplifying exon 1), 1.0 µCi [α^{32}P] dATP (Amersham, Little Chalfont, UK) and 0.1 unit Supertaq DNA polymerase (HT Biotechnology LTD).

Table 2.1
Summary of phenotypes and *AR* gene mutations of index subjects

Subject	Phenotype	Mutation	Position change
A	cAIS	C deletion	codon 42
B	cAIS	A deletion	codon 263
C	cAIS	TGG→TGA	Trp493Stop
D	cAIS	GCT→GAT	Ala564Asp

The amino acid numbering is based on 910 residues, corresponding with a glutamine stretch of 20 residues and a glycine stretch of 16 residues.

For PCR fragments covering the glycine stretch, 50% deaza dGTP was used with 50% dGTP. Reactions were denatured at 95°C and subjected to 30 cycles of denaturation at 95°C for 1 minute, annealing for 2 minutes at different temperatures (Table 2.2) and elongation at 72°C for 1 minute. In Table 2.2, oligo-

Table 2.2
Sequence of oligonucleotides used for PCR-SSCP screening of the hAR gene

Oligo	Location	PCR fragment	Annealing temperature (°C)	Sequence
-70A	5'-UTR, exon1	Exon 1 A*	55	GCCTGTTGAACTCTTCTGAGC
95B	Exon 1			CTTGGGGAGAACCATCCTCA
35A	Exon 1	Exon 1 B	58	TcCGCGAAGTGATCCAGAAC
95B	Exon 1			CTTGGGGAGAACCATCCTCA
80A	Exon 1	Exon 1 C	64	AGCAAGAGACTAGCCCCAGGCAGC
172B	Exon 1			CGGAGCAGCTGCTTAAGCCGGGG
160A	Exon 1	Exon 1 D	62	GCTGCCCCATCCACGTTGTCCCTGCT
250B	Exon 1			ACTCAGATGCTCCAACGCCTCCAC
240A	Exon 1	Exon 1 E	62	TGTGTAAGGCAGTGTCGGTGTCCAT
320B	Exon 1			CGCCTTCTAGaCCTTTGGTGTAAC
305A	Exon 1	Exon 1 F	64	CAGGCAAGAGCACTGAAGATACTGC
385B	Exon 1			GGTTCTCCAGCTTGATGCGAGCGTG
361A	Exon 1	Exon 1 G	58	CGCGACTACTACAACTTTCCACTGG
445B	Exon 1			CACACGGTCCATACAACTG
1A	Exon 1	Exon 1 H	55	TCCTGGCACACTCTCTTCAC
490B	Exon 1			GCCAGGGTACCACACATCAGGT
470A	Exon 1	Exon 1 I	57	GTAGCCCCCTACGGCTACA
1B	Intron 1			CAGAACACAGAGTGACTCTGC
2A	Intron 1	Exon 2	55	GTCATTTATGCCTGCAGGTT
2B	Intron 2			TCTCTCTCTGGAAGGTAAAG
3A	Intron 2	Exon 3	55	TCAGGTCTATCAACTCTTG
3B	Intron 3			GGAGAGAGGAAGGAGGAGGA
4A	Intron 3	Exon 4 A	55	ATTCAAGTCTCTCTTCCTTC
14NB	Exon 4			TGCAAAGGAGTtGGGCTGGTTG
4AA	Exon 4	Exon 4 B	55	CAGAAGCTtACAGTGTCACACA
4B	Intron 4			GCGTTCACTAAATATGATCC
5A	Intron 4	Exon 5	55	GACTCAGACTTAGCTCAACC
5B	Intron 5			ATCACCACCAACCAGGTCTG
6A	Intron 5	Exon 6	55	CAATCAGAGACATTCCTCTGG
6B	Intron 6			AGTGGTCCTCTCTGAATCTC
7A	Intron 6	Exon 7	55	TGCTCCTTCGTGGGCATGCT
7B	Intron 7			TGGCTCTATCAGGCTGTTCTC
8LA	Intron 7	Exon 8	55	AGGCCACCTCCTTGTCAAC
8B	3' UTR, exon 8			AAGGCACTGCAGAGGAGTA

*This PCR product is relatively large and was therefore digested with the restriction enzyme PstI, prior to PCR-SSCP analysis. The CAG(n)CAA repeat length was studied with primer pair 35A and 95B. Mismatches are indicated with a small letter.

nucleotides used for PCR amplification of the human *AR* gene and for direct sequencing are indicated. Samples consisting of 1 µl of PCR product and 9 µl sample buffer (95% formamide, 5% glycerol, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol FF) were denatured before loading on a gel, containing 7% acrylamide, 1 x TBE buffer (Tris-borate, pH 8.2, 2.5 mM EDTA) and 5% or 10 % glycerol. Gels were run for 16 hours at 6 Watt either with 0.5 x TBE buffer (5% glycerol gels) or 1 x TBE (10% glycerol gels) at room temperature. Direct sequencing was performed in case an aberrant SSCP pattern was detected. Amplification took place in 100 µl reaction mixtures containing 100 ng genomic DNA, 400 ng of each primer, 200 µM of each dNTP, 10 mM Tris HCl, 1.5 mM $MgCl_2$, 5 mM KCl, 10% DMSO (in case of amplifying exon 1) and 2.5 units Amplitaq DNA polymerase (Perkin Elmer, Roche Molecular Systems Inc. Branchburg, NJ USA). PCR products were purified from Seakem agarose with Spin-X columns (Costar, Badhoevedorp, the Netherlands) and about 100 ng PCR-product was used as template in the cycle sequencing reaction (Sequitherm kit, Epicenter, Biozyme, Landgraaf, the Netherlands). Primers, developed for PCR-SSCP, were end-labeled with T4 polynucleotide kinase in the presence of [γ-^{33}P] dATP (Amersham, Little Chalfont, UK) and used in the cycle sequencing reaction.

Cell culture conditions

Genital skin fibroblasts were cultured in Modified Eagle's medium (MEM, containing 10% fetal calf serum, minimal essential amino acids (Gibco, Life Technologies, Breda, the Netherlands) and antibiotics at 37°C and 5% CO_2. Fibroblasts were grown to confluence, washed two times with PBS-buffer and cytosols or whole cell lysates were prepared.

Preparation of whole cell lysates

Genital skin fibroblasts were grown to confluence and scraped in 1 ml lysis-buffer [40 mM Tris, 1 mM EDTA pH 7.4, 10 % glycerol, 10 mM dithiothreitol (DTT), 1% (vol/vol) Triton, 0.08% SDS, 0.5% sodium-deoxycholate, 600 µM phenylmethylsulfonylfluoride (PMSF), 500 µM bacitracin]. After 5 minutes the cell extract was centrifuged for 10 minutes at 4000 RPM at 5 C. The supernatant was stored at -80°C.

Preparation of cytosols

Cells were scraped in 1 ml cytosol-buffer (40 mM Tris, 1 mM EDTA pH 7.4, 10% glycerol, 10 mM DTT, 10 mM molybdate, and freshly added: 600 µM PMSF, 500 µM bacitracin and 500 mM leupeptin), and homogenized with a Teflon potter. The derived cell homogenates were centrifuged for 10 minutes at 100,000xg, and the supernatants stored at –80°C until use.

Characterization of the AR protein by SDS-PAGE and immunostaining

The AR protein was immunoprecipitated from genital skin fibroblast lysates, with monoclonal antibody F39.4.1, as described before.[11] After electrophoresis on a 7% SDS-PAGE gel, the proteins were transferred to nitrocellulose and immunostained with polyclonal antibody Sp061. After washing, the membrane was incubated with a second, peroxidase-coupled antibody, to visualize the protein.

Scatchard analysis

For studying the binding characteristics two different assays were used: either a binding assay in which genital skin fibroblast cytosols were used, or a whole cell assay. Cytosols were incubated overnight at 4 C with increasing concentrations (0 - 0.1 - 0.25 - 0.5 - 1.0 - 2.5 - 5.0 - 10.0 nM) of 17ß-hydroxy-17α-[^{3}H]-methyl-4,9,11-estratrien-3-one ([^{3}H]R1881) (NEN-DuPont de Nemours, 's-Hertogenbosch, the Netherlands), in the absence or presence of a 100 fold molar excess of non-labeled R1881 to determine non-specific binding. After protamine precipitation to remove the free steroid, specifically bound [^{3}HR1881] was measured. The total amount of protein was assayed according to the method of Bradford.[12]

Genital skin fibroblasts were incubated with increasing concentrations of [^{3}H]R1881 (0.02, 0.05, 0.3, 1.0, 3.0 nM) to obtain a saturation state. The non-specific binding was determined after incubation with a 100 fold molar excess of non-radioactive steroid. After a one-hour incubation period at 37 C, cells were washed four times with ice-cold 20 nM Tris, 0.15 mM NaCl, pH 7.4. The cells were scraped in 1 ml lysis-buffer (TEG pH 7.4: 20 mM Tris, 15 mM EDTA, 10 % (vol/vol) glycerol, 600 µM PMSF and 500 µM bacitracin). After 10 minutes centrifugation at 800xg the pellet was lysed by adding 1 ml 0.5 N NaOH and a 30 minutes incubation at 56°C. Scintillation cocktail (5 ml) Clumin (Packard) was added to 500 µl of each sample and ^{3}H-activity was measured in a scintillation counter. The amount of protein was quantified by the method of

Bradford. Scatchard analysis was carried out to determine the Kd and the Bmax.

RESULTS

The coding part of the h*AR* gene was screened by amplification of genomic DNA with a set of 17 primer pairs, followed by SSCP analysis, performed under two different conditions. In case of an aberrant PCR-SSCP profile, direct sequencing was performed. Three new exon 1 mutations, all resulting in the introduction of a premature stop codon were detected in the hAR of 3 CAIS patients (Figure 2.1). In one receptor-positive patient a mutation was found in

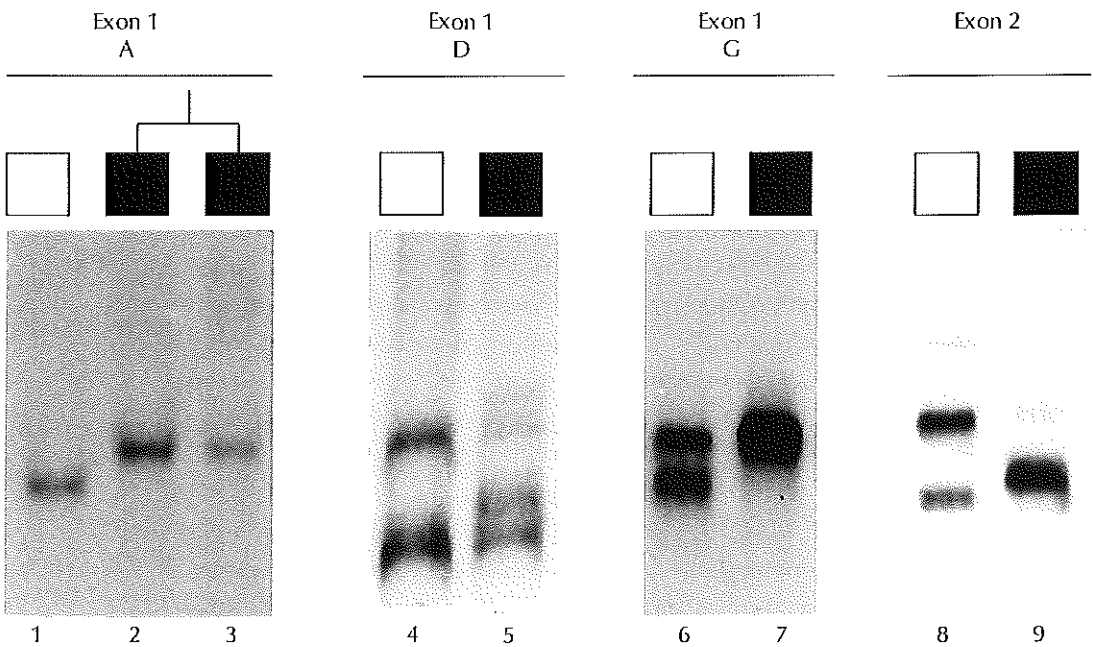

Figure 2.1
PCR-SSCP analysis of *AR* gene mutations. SSCP analysis of different parts of exon 1 and of exon 2. Lane 1, wild-type AR pattern of fragment A [nucleotides (-70)-(+286)]; lanes 2 and 3, patterns of fragment A of index patient A (2) and her 46,XY sister (3); lane 4, wild-type pattern of fragment D (nucleotides 707-958); lane 5, pattern of fragment D of patient B; lane 6, wild-type pattern of fragment G, starting at nucleotide 1402 and ending in intron 1; lane 7, SSCP pattern of fragment G of patient C; lane 8, wild-type pattern of exon 2 fragment starts in intron 1 and ends in intron 2; lane 9, exon 2 pattern of patient D. Numbers are based upon an open reading frame of 2730 nucleotides.[13]

exon 2 (Figure 2.1). Sequence analysis indicated a cytosine deletion at codon 42 of the *AR* gene of patient A resulting in a frameshift and consequently in a premature stop at codon 171 (Figure 2.2). In a second CAIS subject (B) a deletion of an adenine at codon 263 of the *AR* gene gave rise to a stop codon at position 292 (Figure 2.2). A guanine to adenine transition at codon 493 changed a tryptophan codon (TGG) into a stop codon (TGA) in the *AR* gene of patient C (Figure 2.2). The *Kpn*I restriction site, a unique site in AR cDNA was destroyed by the mutation and is informative with respect to carrier detection. Western immunoblotting was performed to investigate if a truncated AR form was expressed in genital skin fibroblasts of subjects A and B. Truncated forms might result from internal reinitiation of translation, proceeding from internal AUG codons (Figure 2.2).

In case of patient A an AR protein of 87 kDa could not be detected (Figure 2.3, lane 2). This corresponds with the observation that ligand binding was not measurable in cytosols, prepared from genital skin fibroblasts of patient A. The stop codon, introduced in the AR of patient B is also located upstream of an internal in frame AUG. However, a shortened AR protein could not be de-

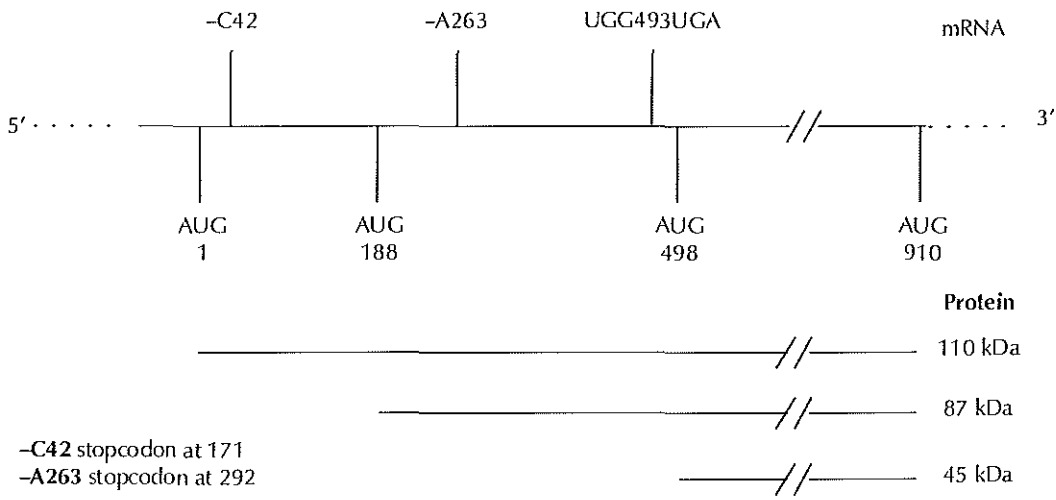

Figure 2.2
Localization of mutations and internal AUGs in exon 1 of the *AR* gene. The mutations found in exon 1 of the *AR* gene are shown; –C: deletion of a cytosine at codon 42, resulting in a premature stop at codon 171; –A: deletion of an adenine at codon 263, resulting in a premature stop at codon 292. At codon 493 a transition of a guanine to an adenine was found, directly resulting in a premature stop codon. Translation start sites and the molecular mass of proteins, resulting from translation initiation, governed by these AUGs, are depicted.

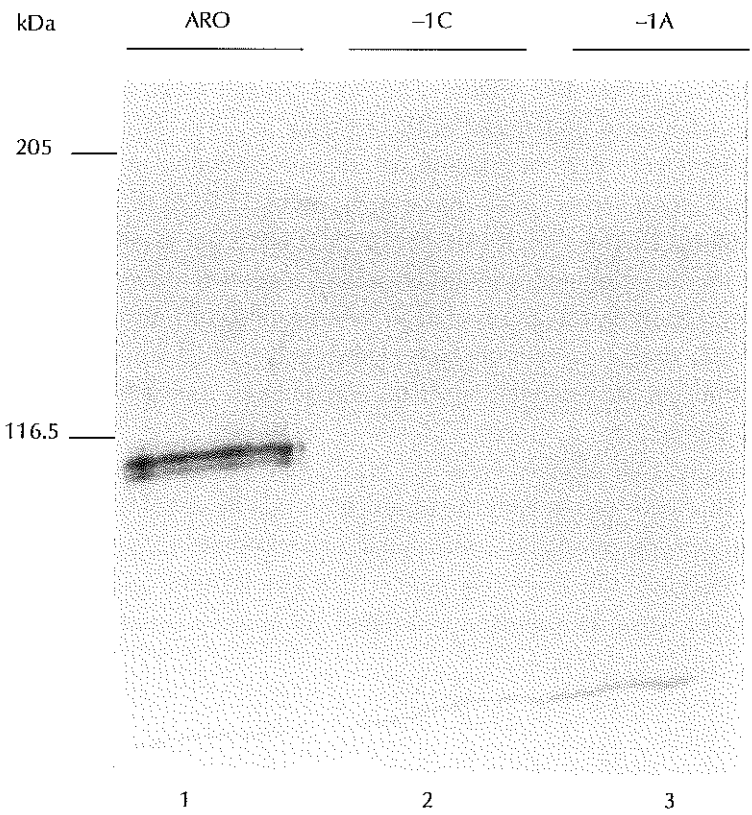

Figure 2.3
Western blot analysis of AR expression. AR protein was immunoprecipitated with monoclonal antibody F39.4.1 from genital skin fibroblast lysates, and separated on a 7% SDS-PAGE gel. After electrophoresis the proteins were transferred to nitrocellulose and immunostained with the polyclonal antibody SP061. The AR protein was visualized with a peroxidase-coupled goat anti-rabbit antibody. Lane 1, Wild-type (Wt) AR from control genital skin fibroblasts; lane 2, AR with deletion of cytosine (-1C) from genital skin fibroblasts of subject A; lane 3, AR with deletion of adenine (-1A) from genital skin fibroblasts of subject B. Molecular mass markers are indicated on the left.

tected on a Western blot (Figure 2.3, lane 3). Furthermore ligand-binding studies were negative. Genital skin fibroblasts from patient C were not available.
A new mutation was found in exon 2, which encodes the first zinc finger of the DBD of the AR. A transition of cytosine to adenine converts an alanine residue at position 564 into an aspartic acid residue. This mutation resulted in defective DNA binding and transactivation, and will be described in more detail in Chapter 6.

DISCUSSION

Genomic DNA from patients with either CAIS or PAIS was screened by PCR-SSCP analysis for mutations in the *AR* gene. Studying new mutations in receptor-positive AIS patients may reveal new information on amino acid residues in functional domains, involved in DNA binding and transcription regulation. Besides mutations in exons 2 and 3, encoding the DBD of the AR, exon 1 mutations may be expected, because the NH_2-terminal region plays a role in transcription activation.[4] In exon 1 of the *AR* gene of three CAIS patients we have found mutations resulting either directly or indirectly in the introduction of premature stop codons. Translation reinitiation is known to occur in mammalian cell mRNAs. Mammalian ribosomes can reinitiate translation at an AUG codon, after termination at an upstream site.[14-15] According to Peabody and Berg[15] it may be possible that the 40 S ribosomal subunit remains associated with the mRNA, and regains the capacity to scan along the mRNA, until it encounters an initiation codon. At this point, the loose ribosomal subunit becomes associated and synthesis of the polypeptide chain starts again. In the Tfm (testicular feminization) mouse a single nucleotide in a hexacytidine stretch (nucleotides 1107-1112) is deleted in the *AR* gene, resulting in a frame shift and the introduction of a premature stop at codon 412. Downstream of this premature stop codon three AUGs are located, in frame with the premature termination codon. A low level of high affinity binding to androgens and also to DNA could still be measured, which is indicative of reinitiation of translation.[16-18] The low protein level resulted from instability of the mRNA.[16-17] Zoppi et al.[9] described an AIS patient in which a cytosine to thymine transition, converting a glutamine (CAG) residue at position 60 in the polymorphic glutamine stretch, into a premature stop (TAG). In addition, *in vitro* experiments suggested that internal initiation occurs from the first in frame AUG codon at position 188, resulting in an 87-kDa protein. In genital skin fibroblasts a low, but detectable level of specific androgen binding with an accelerated dissociation rate was measured. In patient A, the premature stop codon was present at codon 171. A shortened protein of approximately 87 kDa, however, could not be detected in genital skin fibroblasts. These results correspond with the observation that specific androgen binding was not measurable in cytosol from the patient's genital skin fibroblasts. In the other two mutant *AR* genes we described, the stop codon occurred after the first internal AUG. No truncated receptor proteins could be detected in genital skin fibroblasts derived from patient B. It is unclear which factors dictate the efficiency with which such

translation reinitiation occurs. To some extent the efficiency of reinitiation may be governed by the position of the termination codon, relative to the subsequent downstream AUG codons.[14-15,19] In patient A, the new start is located 10 codons downstream of the premature stop codon, whereas in the patient described by Zoppi et al.[9] and in the Tfm mouse, this distance was 128 and 132-134 codons, respectively. This might be the reason why in genital skin fibroblasts from this patient A, no 87 kDa AR and ligand binding were detected.

Two isoforms of the PR have been described, a protein of normal structure (PR-B) and a shortened form (PR-A), lacking 164 amino acids at the NH_2-terminus.[20] Depending on cell type and promoter context, functional differences between the two forms have been found. PR-A can act as an activator of transcription and as a repressor of transcription by PR-B and even as an inhibitor of transcription, mediated by the glucocorticoid receptor, the mineralocorticoid receptor and the AR.[21-22] Wilson and McPhaul[23] reported that two forms of human AR, comparable to the PR isoforms, are present in human genital skin fibroblasts. An 87-kDa isoform was postulated, which constitutes 7-15% of the total AR, arising from alternative initiation of translation. We were, however, unable to detect such a 87-kDa protein in genital skin fibroblasts of a control subject.

So far, amino acid substitutions in exon 1, resulting in a receptor-positive form of AIS have not been found. However, recently a proline to leucine substitution at position 339 was found, in DNA isolated from a prostate tumor.[24] No functional studies have been performed as yet to study the significance of this mutation.

The cytosine to adenine transition at codon 564, converting an alanine residue into an aspartic acid residue in the first zinc finger of the DBD of the AR, resulted in a CAIS phenotype. The phenotype was caused by the defective DNA binding and transactivation capacities of the mutant receptor. Although the correct 3-D structure of this mutant AR is not available, the effect of the mutation can be deduced from the 3-D structure of the glucocorticoid-receptor DBD.[25] Cysteine 560, histidine 561, and tyrosine 562 are involved either in specific or a-specific contacts with the phosphate backbone of the DNA. The amino acid substitution in the mutant receptor might interfere with these contacts, resulting in defective DNA binding.

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CHAPTER

3

Genotype versus phenotype in families with androgen insensitivity syndrome

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SUMMARY

Background – Androgen insensitivity syndrome (AIS) encompasses a wide range of phenotypes that are caused by numerous different mutations in the androgen receptor *(AR)*-gene. Detailed information on the genotype / phenotype relationship in AIS is important for sex assignment, treatment of AIS patients, genetic counseling of their families and insight into functional domains of the AR. The commonly accepted concept of dependence on foetal androgens of the development of Wolffian ducts was studied in complete AIS (CAIS) patients.

Subjects and Methods – In a nationwide survey in The Netherlands, all cases (n=49) with the presumptive diagnosis AIS known to pediatric endocrinologists and clinical geneticists were studied. After studying the clinical phenotype, mutation analysis and functional analysis of mutant receptors were performed using genital skin fibroblasts and *in vitro* expression studies. Here we report the findings in families with multiple affected cases. In CAIS patients Wolffian derivatives were seen and further studied with immunostaining of the AR.

Results – 59% of AIS cases in The Netherlands had other affected relatives. A total of 17 families were studied, 7 families with CAIS (18 patients), 9 families with partial AIS (PAIS) (24 patients) and one family with female prepubertal phenotypes (2 patients). Despite a functionally completely defective AR, phenotypes with some pubic hair, Tanner stage P2, and underdeveloped Wolffian duct derivatives were observed. No phenotypic variation was observed in families with CAIS. However, phenotypic variation was observed in 1/3 of families with PAIS, resulting in different sex of rearing and differences in requirement of reconstructive surgery. Intra-familial phenotypic variation was observed for mutations R846H, M771I, and deletion of amino acid N682. Four newly identified mutations were found. Follow up in families with different AR gene mutations provided information on residual androgen action *in vivo* and development of the prepubertal- and adult phenotype. Vestigial Wolffian duct derivatives were present in CAIS patients with complete absence of AR expression.

Conclusions – Distinct phenotypic variation was observed relatively frequent in PAIS families, but was absent in families with CAIS. Molecular observations suggest that phenotypic variation had different etiologies between these families. During puberty or during androgen therapy, no or only minimal virilisation was seen even in patients with significant (but still deficient) prenatal virilisation. Sex assignment of patients with PAIS can not be based on a specific identified AR gene mutation as distinct phenotypic variation in PAIS families is relatively frequent. In genetic counseling of PAIS families, this frequent occurrence of variable expression resulting in differences in sex of rearing and/or requirement of reconstructive surger, is important information. Wolffian duct remain detectable but differentiation do not occur in the absence of a functional androgen receptor. Vaginal length was functionally in most but not all CAIS patients. The minimal incidence of AIS in The Netherlands, based on cases with molecular proof of the diagnosis is 1:99,000.

INTRODUCTION

The X-linked androgen insensitivity syndrome (AIS), encompasses a heterogeneous group of defects in the *androgen receptor (AR)* resulting in varying degrees of defective masculinization in 46,XY individuals. The phenotypic spectrum ranges from a completely female phenotype, with testes but absent Wolffian- and Müllerian duct derivatives and absent sexual hair, to an infertile or undervirilised male phenotype. Intermediate phenotypes include a female phenotype with clitoromegaly and labial fusion, a phenotype with ambiguous genitalia, a male phenotype with micropenis, or hypospadias and gynecomastia.

Sex assignment at birth of patients with partial androgen insensitivity (PAIS) is classically based upon the virilisation of the external genitalia at birth. Major clinical problems are the acceptability and advisability of male versus female sex assignment in patients with ambiguous genitalia. Whether or not virilisation will increase during puberty or following androgen therapy in a neonate with ambiguous genitalia is a crucial question. Major genetic counseling questions are the general phenotype-genotype relationship, its variability and possible intra-familial variability.

The genotype-phenotype relationship in AIS became relevant when the genetic confirmation of the diagnosis became available. A more precise prognosis was expected from the knowledge of a specific AR mutation and its residual androgenic action, which might facilitate sex assignment of a 46,XY subject with AIS and aid genetic counseling of carrier females. In addition, phenotypic expression of an AR mutation may be used for the construction of maps of functional domains of the AR. Because of the syndrome's genetic heterogeneity, every study and documentation of a mutation in an AIS patient provides important information for the function of a specific aminoacid residue.

A complicating factor for genotype/phenotype studies is the presence of a possible somatic mosaicism for the *AR* gene mutation, which can modulate the phenotype.[1] A somatic mosaicism may be present in as many as one third of single cases.[2] To exclude modulation of the phenotype by somatic mosaicism we studied with clinical as well as molecular means the genotype/phenotype relationship in families with multiple affected cases.

PATIENTS AND METHODS

Design of the study

In a nationwide study in The Netherlands, clinical data of all patients with male pseudohermaphroditism, known to pediatric endocrinologists and clinical geneticists were reviewed. All patients with the presumptive diagnosis of AIS and their families were invited to participate. The clinical diagnosis AIS was made when virilisation in a 46,XY individual was deficient or absent, despite normal male serum levels of testosterone and dihydrotestosterone (DHT) or a sufficient rise in testosterone and DHT after a human chorionic gonadotrophin (hCG) stimulation test. In some gonadectomized patients, endocrine evaluation was lacking. In all cases the androgen receptor gene was screened for mutations. Androgen receptor expression and androgen binding were studied in genital skin fibroblasts. The final diagnosis AIS was made when an androgen receptor gene mutation was found in combination with abnormalities in androgen binding and/or diminished AR expression and/or alterations of DNA-binding of the hormone-AR complex. An extensive family history, covering 3 to 4 generations, was obtained. Genetic and psychological counseling were offered to patients, parents and their relatives. Relatives at risk of being affected with AIS, or at risk of being carriers of AIS, were offered diagnosis and counseling. A written informed consent was obtained from either the patients or their parents. The study was approved by the Medical Ethical Committee of the University Hospital Rotterdam/ Medical Faculty, Erasmus University

Clinical evaluation of the patients

A complete medical history was obtained including the prenatal and neonatal history. General physical examination of patients was performed including external genital development and localization of the gonads. The presence or absence of Müllerian duct structures was determined by ultrasound or at laparotomy. Internal genitalia were further studied with genito-uroscopy. In patients with earlier reconstructive surgery, records were obtained on developmental stage of the internal and external genitalia before- and at the time of reconstruction. During gonadectomy the presence or absence of Wolffian duct structures was determined, and their development was studied by histological examination. In some patients a sex hormone binding globulin (SHBG) suppression test was done as described by Sinnecker et al.[3] SHBG and basal or

hCG stimulated serum levels of hormones were measured by radioimmunoassay.

AR gene mutation analysis

Genomic DNA was isolated from leukocytes or genital skin fibroblasts (GSF) according to standard procedures.[4] The *AR* gene was screened for mutations with PCR-SSCP followed by direct sequencing of the PCR products suspected to carry a mutation.[5] In cases of negative PCR-SSCP results, all *AR* exons and flanking intron sequences were sequenced either with direct sequencing[5] or automated sequencing.

Scatchard analysis , SDS-PAGE and immunostaining of the AR protein

AR expression, molecular size and androgen binding were studied according to previously described methods.[5] Either a binding assay in cytosols from GSF or a whole cell binding assay was performed, with use of the non-metabolizable androgen R1881.
The antibody used for Western immunostaining of the AR (F39.4.1) was directed against amino acids 301-320 in the N-terminal part of the AR.

SDS-PAGE of the AR

GSFs of subjects N IV-5 and O IV-1 were cultured in serum free medium for 24 hours, followed by 24 hours in medium containing increasing concentrations (0, 5, 30 and 100 nM) of the synthetic, non metabolizable androgen methyltrienolone (R1881). Whole cell lysates were prepared from confluent cell layers in 150 cm^2 culture flasks, immuno-precipitated, separated on a SDS-PAGE gel and immunostained as previously described.[5]

Immunohistochemistry of the AR

During gonadectomy epididymides and vasa deferentia were found in CAIS patients with a premature termination codon (B:IV-4) or a frameshift mutation (A:II-1 and II-2) and absence of androgen binding in GSFs. As Wolffian duct do not differentiate in the embryo when androgen action is absent we studied AR expression in these Wolffian ducts. Sections of formalin fixed, paraffin embedded tissue specimens (thickness of 5μ) were re-hydrated and after antigen retrieval by microwave treatment they were immunostained with antibody F39.4.1, directed against the N-terminal part of the AR-protein, amino acid

301-320, or with anti-estrogen receptor (DAKO, Denmark), using the avidin-biotin peroxidase complex method. Immunostaining was visualized with diaminobenzidine followed by nuclear counterstaining with Mayer's hematoxylin.[6] A vas deferens of a one year old patient with 17β-hydroxysteroid dehydrogenase 3 deficiency was used as a positive control.

RESULTS

Number of patients

A total of 49 index patients with possible AIS were identified. In 32 index patients an AR gene mutation was found. 19 of these 32 index patients had affected relatives. Of these 19 families, 17 families agreed to participate in this study. Of the two non-participating families only the index patients could be studied. These two non-participating families were affected with CAIS resulting from mutations that introduced a premature stop codon in exon 1 of the AR gene, rendering the AR completely defective.

Classification of CAIS versus PAIS

CAIS is differently defined. Griffin et al[7] define CAIS as complete female external genitalia, paucity of axillary and pubic hair and absent Wolffian duct derivatives. Quigley[8] defines CAIS as complete female external genitalia without pubic hair, but remnants of Wolffian duct derivatives may be found. The presence of any amount of pubic hair, is held as evidence for some degree of androgen responsiveness and thus classified as PAIS.[9] In the classification of Sinnecker et al.[3] CAIS is a female phenotype with scanty pubic and axillairy hair (type 5a), or a female phenotype with absence of any androgen dependent structures i.e. pubic and/or axillary hair (type 5b). No comment is made on the development of Wolffian duct derivatives.
Here we define CAIS as the totally abolished AR function, as in the families A-D (Table 3.1). Therefore CAIS is used for an adult 46,XY patient with female external genitalia, absent or scant pubic hair, up to Tanner stage P2. Wolffian duct derivatives may be present. PAIS is used for adults with a considerable amount of pubic hair P3-5, and with either normal female external genitalia or more virilized genitalia. To avoid confusion by semantics, the patient's phenotypes are described in detail.

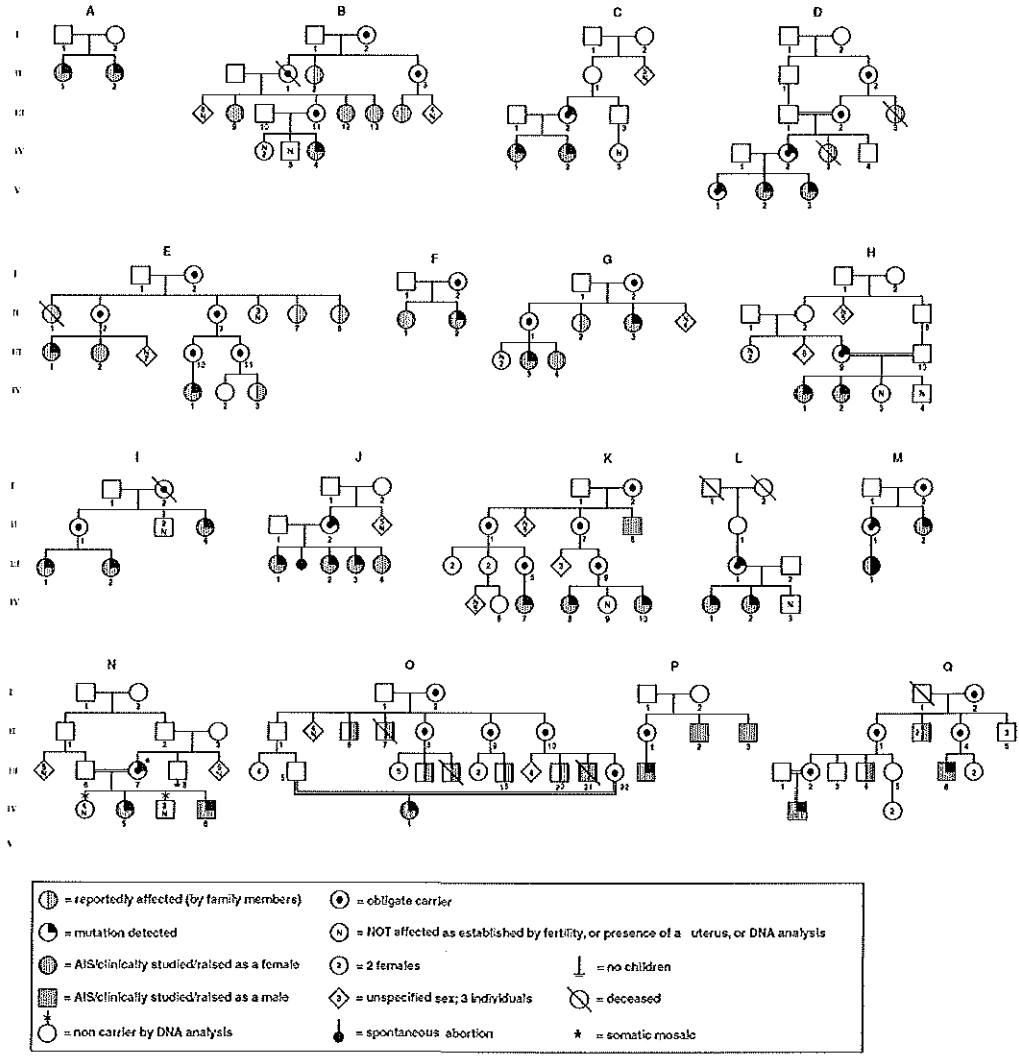

Figure 3.1
Pedigrees of the studied families.

Phenotypes and families

The pedigrees of the 17 studied families are shown in Figure 3.1. The subjects presumably affected as reported by family members (Figure 3.1) could not be studied for reasons of family confidentiality, geographical distances or non par-

ticipation in this study. Family pedigrees have been compressed for reasons of confidentiality. Thirteen families were of West-European descent, two of Moroccan, one of Turkish descent and one originated from Surinam (Hindustan).

The clinical and molecular studies are summarized in Table 3.1. Distinct phenotypic variation was observed in three of the 17 families with AIS, PAIS families J,K,N or three out of nine PAIS families. Relevant aspects of the phenotype not covered in Table 3.1 are described in more detail below.

- *Family C* – Subject IV-2 was a girl, diagnosed at age 8 years with a hypothalamic glioma resulting in hypogonadotrophic hypogonadism. Estrogen substitution was started at age 13. When the diagnosis AIS was made at age 18, she had developed pubic hair Tanner stage 2. Both testes appeared to be atrophic upon gonadectomy. No information on Wolffian duct derivatives was available.
- *Family D* – Subjects III-3 and IV-3 were reported to have amenorrhea and scanty pubic hair but no medical records were available.
- *Family J* – During gestation of subject III-2 the mother received injections to prevent a miscarriage. Details of this treatment were not available. Subject II-2 had female genitalia with clitoral hypertrophy and posterior labial fusion.
- *Family K* – Subject II-8 was raised as a girl until 2 years of age. Upon the finding of inguinal testes the sex was changed to male. The genitalia consisted of a clitoris-like phallus, separate introitus vaginae and urethra, labio-scrotal swellings, blind ending vagina, an utriculus prostatus and inguinal testes. He underwent several corrective surgical procedures for his genitalia and developed gynecomastia at age 16. At age 23 he had a micropenis, female distribution of pubic hair, very little axillary hair and no facial hair, despite serum testosterone levels of 24.5 nmol/L and normal 5α-reductase type 2 activity as measured in genital skin fibroblasts. He started with Mesterolon 3 x 25 mg /day p.o. during one year which resulted in the appearance of vellus facial hair. The penile size did not increase. The therapy was changed into testosterone enanthate 250 mg i.m. once every 2 weeks during 8 years without improvement in phallic size. The patient is married and has one adopted son.
- *Family L* – Subject IV-1: when the diagnosis AIS was made at age 15, gonadectomy was performed, Tanner stage M4, P4, A0. She had a minimally enlarged clitoris, 1.0 cm in length, a urogenital sinus of 3-4 cm diameter, a blindly ending vagina 2.0 cm deep and inguinal testes. Serum LH: 27.2 IU/L,

testosterone: 52.0 nmol/L, DHT: 4.1 nmol/L, estradiol: 235 pmol/L, SHBG: 13.7 nmol/L.

Subject IV-2 was 13 years old when the diagnosis AIS was made, Tanner stage M2, P1, A0. She had clitoromegaly, 1.5 cm in length, an urogenital sinus of 1 cm diameter, a blindly ending vagina 1.5 cm deep. Serum LH: 2.9 IU/L, testosterone: 11.1 nmol/L, estradiol: 41 pmol/L. Two years later she was gonadectomized. During these two years she neither had lowering of the voice nor increase of clitoral enlargement. Her habitus remained female. The only sign of androgen action was the appearance of pubic hair; at age 15 years she had reached Tanner stage M4,P4.

- *Family N* – This family contains both an affected female and a male individual. Subject IV-5 (subject II-5 in Boehmer et al.[16]) was a 13 year old pubertal girl, Tanner stage M3, P2, A0 , with a female habitus and female voice when the diagnosis AIS was made. External genitalia consisted of normal size clitoris, normal labia majora, posterior fusion of the labia minora leading to an urogenital sinus and a 2.5 cm deep, blindly ending vagina that was connected with the urogenital sinus. Serum levels of: LH 5.7 U/L, testosterone 13.8 nmol/L, DHT 1.55 nmol/L, estradiol 35 pmol/L. She was not gonadectomized until two years later, at age 15.5 years. Serum hormone levels at that time were: testosterone 31.5 nmol/L, DHT 2.42 nmol/L. Her voice was still high pitched, she had no clitoromegaly, pubic hair was still P2 and axillary hair was still absent while her breast had grown to M4.

  Subject IV-8 (is subject II-8 in Boehmer et al.[16]) was born with perineoscrotal hypospadias, micropenis with well developed corpora cavernosa, a bifid scrotum that contained testes and transposition of the scrotum. The male sex was assigned because of these anatomical findings.
- *Family O* – Subject III-20 was raised as a male but could not be studied. His relatives reported that he had a small phallus, hypospadias, inguinal testes, gynecomastia, and absent beard at the age of 19 years.

  Subject III-21 was born with micropenis, labioscrotal swellings, urogenital sinus and was raised as a boy. He died after one year from a congenital heart abnormality.

  Subject IV-1 was born with a micropenis, labioscrotal swellings, separate vaginal and urethral openings, shallow blindly ending vagina. On the basis of the external genitalia the infant was assigned the female sex and gonadectomized.
- *Family P* – Subjects II-2 and II-3: were noted by the urologist to have micropenis and severe hypospadias at the age of 19 and 13 years respectively. At

Table 3.1
Phenotypes and genotypes in the studied families with AIS

Patient		Phenotype					Androgen Receptor Gene		Androgen Receptor Function			Ref.
AIS Type	Family/ Patient #	Prader stage ∅	Sex of rearing:	Tanner stage (age in years)	Wolffian/ Müllerian duct Structures ⊗	Vagina length (at age in years)	Exon/ functional domain	Mutation	Bmax (fmol/ mgP)	Kd (nM)	Protein (kDa)	
CAIS	A II-1	0	Female	M4,P3,A1 (22)	E±/V±*/M+	6 cm (22), coitus	1/ TAD	C deletion codon 42 (♦); frameshift→ stop codon at 171	n.m.[a]	n.m.[a]	n.m.	(5)
	A II-2	0	Female	M5,P2,A1 (20)	E±/V±*/M-	2.5 cm (1)			n.m.[a]	n.m.[a]	n.m.	
CAIS	B III-9	0	Female	P2,A1 (19, 44)	E-/V-/M+	6-7cm(31); coitus	1/ TAD	Q478X	-	-	-	(10)
	B III-12	0	Female	P2,A1 (28)	E-/V-/M+	6-7 (28), coitus						
	B III-13	0	Female	P2,A1 (32)	-	coitus						
	B IV-4	0	Female	M4,P2,A0 (13)	E+/V+*/M-	2 cm (13)						
CAIS	C IV-1	0	Female	M5,P2,A0 (14)	-	3 cm (14)	3/ DBD	R598X	n.m.[b]	n.m.[b]	n.m.	(11)
	C IV-2	0	Female	M5,P2 (18)	-	-			n.m.[b]	n.m.[b]	n.m.	
CAIS	D V-2	0	Female	M5,P2 (25)	n.g.	2.5 cm (0) 4 cm (19)	6 / LBD	deleted C in codon 791 frameshift →stop codon at 798	-	-	-	new
	D V-3	0	Female	M5,P2 (18)	n.g.	4 cm (15)						
CAIS	E III-1	0	Female	M5,P2,A1 (55)	E+/V+/M-	5 cm (45); surgery	3/ DBD	R606H	-	-	-	(12)
	E III-2	0	Female	M5, P2 (40)	-	-						
	E IV-1	0	Female	M5,P3,A0 (26)	n.g.	-						
CAIS	F II-1	0	Female	M5,P2,A0 (21)	-	shallow, coitus	5/ LBD	L735F				(13)
	F II-2	0	Female	M5,P2,A0 (21)	E+/V+/M-	surgery			n.m.[b]	n.m.[b]	110	

CAIS	G II-3	0	Female	P2, A0 (36)	E+/V?/M-	4.5-5 cm (36)	5/ LBD	P757A	25±3 [b]	0.74±0.8 [b]	↓↓ 110	new
	G III-3	0	Female	M4,P2,A1 (15)	E±/V+/M+	7 cm(15)						
	G III-4	0	Female	M2,P1,A0 (11)	n.g.	-						
CAIS/ PAIS ?	H IV-1	0	Female	M2,P1,A0 (11)	E+/V+/M-	-	5/ LBD	W742R	10±3	0.67±0.03	-	(13)
	H IV-2	0	Female	prepubertal (9)	E?/V?/M-	-			20±4	0.79±0.16		
PAIS	I II-4	0	Female	M5,P3,A0 (15) M5,P4,A1 (18)	E+/V+/M-	3.5 cm (0.7) 6 cm (16)	affects splicing	intron 2; -11 A→T (♣)				(14)
	I III-1	0	Female	prepubertal (0.2)	E+/V+/M-	2 cm (2)			63 [b]	0.07 [b]	110	
	I III-2	0	Female	prepubertal (2)	E+/V+/M-	2 cm (0.2)			64 [b]	0.08 [b]	110	
PAIS	J III-1	0	Female	M5,P3,A0 (27)	E+/V+/M-	8 cm (18); coitus	4/ LBD	del AAC codon 683	63±6 [a]	0.38 ±0.0 [a]	-	(15)
	J III-2	I	Female	M3,P4,Ao (16)	E+/V+/M-	8 cm (16); coitus			99±17 [a]	0.60 ±0.1 [a]		
	J III-3	0	Female	M5,P3,A0 (21)	E+/V+/M-	coitus						
	J III-4	0	Female	?	E?/V?/M-	coitus						
PAIS	K II-8	II	Male	G+,P5∇,A1 (23)	n.g.	present	6/ LBD	M771I			-	(12)
	K IV-7	I	Female	prepubertal	E+/V+/M-	present						
	K IV-8	II	Female	prepubertal	E+/V+/M-	3.5 cm (0.2)			n.m. [a]	n.m. [a]		
	K IV-10	II	Female	prepubertal	E+/V+/M-	present			n.m. [a]	n.m. [a]		
PAIS	L IV-1	II	Female	M5,P4,A0 (15)	E+/V+/M-	2 cm (15)	5/ LBD	L759M	90 [b]	0.47 [b]	110	new
	L IV-2	II	Female	M2,P1,A0 (13) M4,P4,A0 (15)	E+/V+/M-	1.5 cm (13)			40 [b]	0.47 [b]	110	
PAIS	M II-2	II	Female	M5,P5 (adult)	-	shallow, surgery	5/ LBD	M733V				(12)
	M III-1	II	Female	prepubertal	-	utriculus			n.m.	n.m.	110	
PAIS	N IV-5	II	Female	M4,P2,A0 (15)	E+/V+/M-	2.5 cm (15)	7/ LBD	R846H	87	0.9 [b]	110	(16)
	N IV-8	III	Male	prepubertal	E+/V+/M-	absent (5)			61	0.5 [b]	110	

Table 3.1 – continued

Patient		Phenotype					Androgen Receptor Gene		Androgen Receptor Function			Ref.
AIS Type	Family/ Patient #	Prader stage ∅	Sex of rearing:	Tanner stage (age in years)	Wolffian/ Müllerian duct Structures ⊗	Vagina length (at age in years)	Exon/ functional domain	Mutation	Bmax (fmol/ /mgP)	Kd (nM)	Protein (kDa)	
PAIS	O III-20	III	Male	prepubertal	n.g.	-						(17)
	O IV-1	II	Female	prepubertal	E+/V+/M-	shallow (birth)	1 & 7/ TAD & LBD	P389S/ R846H	86 [b]	0.99 [b]	110 *	
PAIS	P II-2	III	Male	-	n.g.	absent	2/ DBD	Tyr562H				new
	P II-3	III	Male	-	n.g.	absent						
	P III-1	III	Male	prepubertal		absent			41 [b]	0.07 [b]	110	
PAIS	Q III-6	III	Male	C+,P5∇,A1 (21)	-	absent	affects splicing	deletion > 6 kb of intron 2; including branch-point sequence	39±12 [a]	0.36±0.1 [a]	105	(18)
	Q IV-1	III	Male	prepubertal	n.g.	absent						

#: Patients are denoted with roman-arabic numbers corresponding to their position in the pedigree, Figure 3.1 (for example III-13; V-3 etc.).
∅: External genitalia are denoted according the Prader classification (19), the age at assesment is in between brackets .
P5∇ female distribution of pubic hair, inverted triangle.
⊗: E: epididymides, V: vas deferens, M: müllerian remnants. E±: underdeveloped epididymis; E+ normal developed epididymis; E ?: no data available on the developmental stage of epididymides
n.g. = not gonadectomized
"Coitus" : coitus regularly without major difficulties, "Surgery" : neovagina was constructed. Age at evaluation is shown between brackets.
TAD = Transcription activation domain of the AR
DBD = DNA Binding domain of the AR
LBD = Ligand binding domain of the AR
- = not investigated
n.m. = not measurable
♦ Previouly published C insertion codon 42 is incorrect (5, 13), this should be: C deletion codon 42
[a] Binding assay with use of cytosols; normal range Bmax: 39-169 fmol/mg P, Kd: 0.03-0.13 nM
[b] Binding assay with use of whole cells; normal range Bmax: >10 fmol /mg P, Kd: 0.1-0.4 nM
* see Figure 3.3 (page 83)
↓↓ : diminished amount present

present they are both married, reportedly with satisfactory sexual lives but without children.
Subject: III-1 was born with a phallus of 0.75 cm length with no palpable corpora, an urogenital sinus, and a hypoplastic scrotum that contained testes. The male sex was assigned following the parents wish which was based on the families experience with the two uncles. Phallus length increased to 1.8 cm after a 4 months treatment with 0.25 mg testosterone propionaat 100 i.m. / 3 weeks. At age 6 years the phallus had grown to become 2.5 cm in the absence of androgen treatment.

- *Family Q* – Subject III-6: Was raised as a girl. At 2 years of age, inguinal hernias containing testes were found, prompting male sex re-assignment. He had a phallus of 2.5 cm in length with hypospadias, bifid scrotum, testes bilaterally in the inguinal region. He developed gynecomastia at age 14. At age 21 the penis had increased in circumference but it still measured 2.5 cm in length despite serum testosterone levels between 30 and 40 nmol/L during at least 5 years. He had reached Tanner stage P4, A1 but facial hair was absent. No prostate was palpable. Unilateral gonadectomy was done at age 26 because of a palpable, slowly growing tumor. A benign Sertoli cell proliferation was found. Subject III-4 was raised as a male and was not studied. His wife reported that he had a small but functional penis and had ejaculations. The marriage remained infertile.
  Subject IV-1 was born with ambiguous genitalia consisting of a phallus 2.3 cm, bifid scrotum, inguinal testes and scrotal hypospadias. He was raised as a boy.

Adult patients with a female phenotype and Prader stage 0 or I reported that intercourse was satisfactory. Some applied vaginal dilator therapy when they started to be sexually active.

SHBG-suppression test

Two sisters with CAIS in family H, H:IV-1 and IV-2, showed no decline in SHBG serum levels (both 100% of the initial SHBG serum levels) as is expected for CAIS patients (normal males < 63.4 %; PAIS: 63.4-93%; CAIS: >92%. CAIS is here defined as a female phenotype without any signs of virilization and scanty or absent pubic and/or axillary hair.[20] In CAIS patient B:IV-4, a maximal decrease of 82% of the initial SHBG serum level was obtained as is observed for PAIS. In two siblings N:IV-5 and IV-8 with PAIS the SHBG suppression was 73.5% and 92% respectively, the latter value is in the normal

range for CAIS.[20] In the studied patients we found an overlap in values in CAIS and PAIS patients.

Genotype and receptor phenotype

The identified mutations are shown in Table 3.1. Four of the 18 identified AR gene mutations have not been reported before. All patients had polymorphic Glutamine and Glycine repeats in Exon 1 between 14-30 and 19-24 respectively, which are within the normal range.[9,21] The results of Scatchard analysis and SDS-PAGE are also shown in Table 3.1.

AR protein expression in vestigeal Wolffian duct derivatives

Structures that macroscopically resembled vasa deferentia and epididymides were found in patients with a frameshift mutation (A:II-1 and II-2) or a premature termination codon (B:IV-4) in the AR gene. As it is generally accepted that Wolffian duct differentiation is dependend on fetal androgens[22], these patients were an ideal model to challenge this idea. The structures that were macroscopically identified as Wolffian duct derived, histologically resembled Wolffian ducts that had remained vestigeal.[23] These structures did not express AR protein, as was shown by immunohistochemistry of tissue form AIS patients A:II-1 and B:IV-4 (Figure 3.2 (page 313)). In a well differentiated vas deferens of a one year old patient with 17β-hydroxysteroid dehydrogenase type 3 deficiency abundant AR-protein was present (Figure 3.2 (page 313)).

Hormone dependent AR phosphorylation

AR protein isolated from wild type GSFs cultured in the absence of androgens migrates as a doublet of 110 and 112 kDa during SDS-PAGE, reflecting dephosphorylated AR isotype and a phosphorylated AR isotype respectively. Upon binding of androgens, the AR undergoes additional phosphorylation, resulting in a third isoform of 114 kDa (Figure 3.3, lower lane). AR mutants that are either partially defective in ligand binding or in DNA binding or in transcription activation, migrate with a reduced amount of the 114 kDa isoform in SDS-PAGE[24] (Figure 3.3, upper lane). At relatively low androgen concentrations of 5 nM of R1881, GSFs with mutant R846H or P389S+R486H have equally reduced amounts of the third isoform of 114 kDa as compared to the wild type. Increased androgen levels did not induce the 114 kDa band as in the wild-type cells (Figure 3.3). Moreover, both, the single and the double mutant AR have an equally deficient hormone induced upshift of the 114kDa

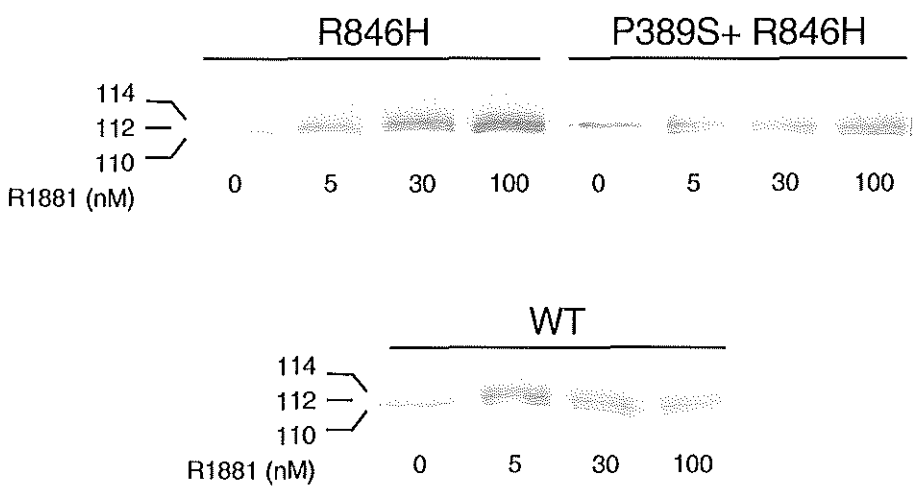

Figure 3.3
SDS-PAGE of mutant R486H (family N, PAIS) versus P389S+R468H (family O, PAIS). GSFs of patients (N: IV-8 and O: IV-1, respectively) were cultured in the presence of increasing concentrations (0-100 nM) of the synthetic, nonmetabolizable androgen R1881. The molecular sizes of the AR-isoforms are indicated at the left side (110-112-114 kDa).

AR isoform. A deficient hormone induced upshift is in concordance with the increased dissociation of the AR hormone complex in GSF of the mutant R846H.

Incidence of AIS

An incidence rate for AIS can be calculated with use of all AIS cases, either isolated or familial cases, identified in this nationwide survey with proven receptor abnormalities. Nineteen AIS cases were born in The Netherlands in the time period 1984-1993. The mean annual live birth rate in The Netherlands was 189.000 in that time period.[25] Thus a minimal incidence of 1:99.000 was calculated over this ten year time period.

DISCUSSION

59% of AIS cases had other affected relatives which is in accordance with Haldanes rule that 1/3 of cases with diseases that prevent procreation of 46,XY

patients are the result of de novo occurrence of mutations.[26,27] The study on phenotypic variation was done on patients with familial AIS, having received the X-linked AR mutation from their carrier mother, and having it in all their cells. The possible influence of somatic mosaicism resulting from postzygotic mutations was so excluded. Also, comparison of multiple cases from one family enabled analysis of intra- and interfamilial variability of the disorder.

Phenotypic variation in AIS

No phenotypic variation was observed in families with CAIS, confirming that phenotypic variation in families with CAIS is very rare. Only one family with coexistence of CAIS and PAIS has been described to date.[28]
However, distinct phenotypic variation was observed in one third of the families with PAIS and included families with a truly female phenotype. In one family two affected individuals were raised as girl and boy respectively, because of a wide phenotypic difference. In two other families the variation had implications for the indication of reconstructive surgery. Therefore sex assignment at birth of patients with PAIS can not be based on a specific identified AR gene mutation.

Molecular mechanisms of variability

The cause of phenotypic variation in PAIS might have different molecular backgrounds. For instance, in family N with mutation R846H we found 5α-reductase 2 deficiency in genital skin fibroblasts of subject N:IV-5 as the cause of the more severely impaired virilisation (manuscript submitted). This 5α-reductase deficien-cy is secondary to the primary AR defect and caused by the absent or severely reduced expression of 5α-reductase 2. This explains the observed phenotypic variation, as the action of mutant R846H can be influenced dramatically by differences in availability of DHT.[29,30]
In mutation M771I, also as described before[28], very diverse phenotypes are found, which are less clearly explained.[28] Affected members of that family had either an external female or a Reifenstein phenotype. The M771I mutation rendered the AR qualitatively defective and caused decreased expression of the AR-protein in Scatchard analysis of GSF[28] and in in vitro expression studies.[29] However, M771I mutant receptor, showed hardly any activity in a transactivation assay in HeLa cells, even in the presence of high levels of androgen.[29] The in vivo observed residual virilisation (family K; Table 3.1) is not

explained by these in vitro results. This may be due to differences in genetic background of genital target cells versus HeLa cells.
The deletion of Asn 683 in the ligand binding domain of the AR, in family J results in an increased dissociation of the androgen/receptor complex in GSF (Table 3.1). It is unfortunate that unidentified injections were administered during gestation of patient J:III-2; they might have been (most likely) either progesterone or multivitamins. Some AR gene mutations found in prostate cancer have been shown to widen the binding specificity of the AR to progestagens and estrogens.[31,32] These androgen receptors still cause androgen responsive gene expression, however this effect is mediated also by progestagens or estrogens in addition to androgens. Whether this mutation widens the ligand specificity remains to be tested in in vitro expression studies.

Two families, one with mutation R846H and one with the double mutation P389S & R846H, enabled the study of the impact of mutation P389S on the phenotype. Mutation R846H resulted in external genitalia Prader stage II and III in family N. The additional mutation P389S, present in family O, resulted also in Prader stage III, a less severe impairment of virilisation than Prader stage II. Therefore mutation P389S was not of major influence on the clinical phenotype in family O.
The stability of the hormone-R846H mutant receptor complex nor the hormone induced upshift of the 110-112 kDa isotype, was influenced by the additional mutation P389S as was shown by Scatchard analysis (Table 3.1) and SDS-PAGE (Figure 3.3). Mutation P389S has also been found in an infertile, but otherwise normal male[17] but a causative relationship between genotype and phenotype is not clear as in vitro experiments showed almost identical transcription activation for mutant P389S and the wild-type receptor.[17] Therefore P389S might alternatively be an infrequent polymorphism in the AR gene.

Residual capacity for virilisation

Follow up into puberty and adulthood provided information on the residual capacity for virilisation. Patients born with complete female genitalia, Prader stage 0, did not show virilisation. Some developed pubic hair, Tanner stage P3 or P4 as the only sign of residual androgen action. Patients born with Prader stage II had been raised as females and one was raised as a male. In adulthood the latter patient, K:II-1, had a female distribution of body fat, gynecomastia, a female distribution of pubic hair and a microphallus despite prolonged androgen therapy. Patients born with Prader stage III in families P, and Q also

showed absent to minimal virilisation at puberty. The adult patients, raised as males, had severely undervirilised genitalia and absence of a beard. In conclusion AIS patients in this study showed no to minimal virilisation at puberty, even the patients that were born with ambiguous genitalia.

Wolffian derivatives and pubic hair

Development of Wolffian duct derivatives such as epididymis and vas deferens and the appearance of pubic hair are androgen dependent.[7,9] However, scant pubic hair Tanner stage 2 and/or scant axillary hair was found in all (post) pubertal patients, which must have developed under the influence of other factors than AR action. Structures resembling vestigeal Wolffian duct derived structures were repeatedly found in CAIS patients (in families A-E). The nature of the AR gene mutations in these families predicted they would lead to truncated, non functional.[33] AR proteins. Complete absence of the AR in these structures was confirmed by Scatchard analysis and SDS-PAGE analysis in GSFs (families A & C) and immunohistochemistry of the underdeveloped vas deferens and epididymis (families A & B, Figure 3.3). Thus in the absence of a functional AR, pubic hair develops minimally and Wolffian ducts remain macroscopically detectable but undifferentiated. In the 17β-hydroxysteroid 3-deficient patient Wolffian ducts were normally developed which may be due to the action of androstenedione or minimal amounts of testosterone formed during embryonic development.[34]

Indication for vagina construction

In many CAIS patients surgical vaginal elongation is not indicated. Although the vagina is less deep than in normal women, the vaginal depth was sufficient for intercourse, albeit that vaginal dilator therapy had to be applied. However, in PAIS patients, vaginal neoplasty is often required to allow sexual activity.

Genetic epidemiology

Through the design of this study we could calculate a minimal incidence for AIS of 1:99,000. This incidence is an underestimation because AIS patients that come to medical attention with primary amenorrhea, usually consult gynecologists and are therefore not included. However, it is the first incidence figure based on cases with a confirmed diagnosis of AIS. The previously published prevalence rates vary between 1:4,000 to 1:128,400.[9] The estimation from a Danish patients registry[35] of 1:40,800 is probably the most accurate to

date. However it is based on 46,XY-female phenotypes with testes and the diagnosis AIS was not confirmed either by mutation analysis nor with an SHBG-suppression test. That group may have included cases of 17β-hydroxysteroid dehydrogenase type 3 deficiency (incidence 1:147,000[36]) and of 5α-reductase 2 deficiency. Therefore this figure may be an overestimation. The true incidence of AIS is probably between 1:40,800 and 1:99,000.

CONCLUSIONS

Phenotypic variation was observed in 1/3 of the PAIS families and resulted in differences in indication for of corrective surgery and in differences in sex of rearing in one family. AIS displays a large genotypic heterogeneity, molecular analysis does not always explain the variance in phenotype. Accordingly, this impredictability in PAIS families is important to consider in genetic counseling of PAIS, as it may lead to differences in sex assignment and indications for reconstructive surgery. Wolffian ducts remain vestigial in the absence of a functional AR protein as was shown by immunhistochemistry in molecular defined patients with CAIS. Vaginal dept is functionally in many adult CAIS cases.

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CHAPTER

4

Phenotypic variation in a family with partial androgen insensitivity syndrome caused by differences in 5α-dihydrotestosterone availability

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SUMMARY

Background – Mutations in the androgen receptor *(AR)* gene result in a wide range of phenotypes of the androgen insensitivity syndrome (AIS). Inter- and intra familial differences in the phenotypic expression of identical AR mutations are known, suggesting modifying factors in establishing the phenotype.

Methods – Two 46,XY siblings with partial AIS sharing the same AR gene mutation, R846H, but showing very different phenotypes, are studied. Their parents are first cousins. One sibling with grade 5 AIS was raised as a girl; the other sibling with grade 3 AIS was raised as a boy (grading according to Quigley et al (1)). In both siblings serum levels of hormones were measured, an SHBG suppression test was done and mutation analysis of the *AR* gene, Scatchard, and SDS-PAGE analysis of the AR protein was performed. Furthermore, 5α-reductase 2 expression and activity in genital skin fibroblasts (GSF) were investigated and the 5α-reductase 2 gene was sequenced.

Results – SHBG decrease in an SHBG-suppression test did not suggest differences in androgen sensitivity as the cause of the phenotypic variation. Also androgen binding characteristics of the AR, AR expression levels and the phosphorylation pattern of the AR upon hormone binding were identical in both sibs. However, 5α-reductase 2 activity was normal in GSF from the boy but undetectable in GSF from the affected girl. The lack of 5α-reductase 2 activity was due to absent or reduced expression of 5α-reductase 2 in GSF from the girl. Exon and flanking intron sequences of the 5α-reductase 2 gene, showed no mutations in either sib. Additional intragenic polymorphic marker analysis gave no evidence for different inherited alleles for the 5α-reductase 2 gene in the two siblings. Therefore the absent or reduced expression of 5α-reductase 2 is likely to be secondary to the AIS.

Conclusions – Distinct phenotypic variation in this family was caused by 5α-reductase 2 deficiency, secondary to AIS. This secondary 5α-reductase deficiency is due to absence of expression of the 5α-reductase iso-enzyme 2 as shown by molecular studies. The distinct phenotypic variation in AIS is here explained by differences in the availability of androgens.

INTRODUCTION

Androgen insensitivity syndrome (AIS) is an X-linked disorder of male sexual differentiation, caused by a defective or absent androgen receptor (AR) (reviewed in[1]). Mutations in the androgen receptor gene result in a wide range of AIS phenotypes. The phenotypic spectrum in 46,XY individuals ranges from a complete female phenotype with testes (CAIS), through female phenotypes with clitoromegaly or posterior fusion of the labia majora, to a male phenotype with hypospadias and/or micropenis and gynaecomastia, or even a normal male phenotype with infertility, all defined as partial AIS (PAIS).[1] The finding of mostly different *AR* gene mutations in over 250 AIS patients[2] illustrates the genetic heterogeneity in AIS.

The AR is a transcription factor, which binds either testosterone (T) or 5α dihydrotestosterone (DHT); However, DHT is bound with higher affinity and has a slower dissociation rate from the receptor than T.[3-5] When androgen is bound to the AR, the complex dimerises and migrates into the nucleus where it recruits transcription factors and binds to the promotor region of androgen sensitive target genes.[6]

With the advent of molecular analysis of the *AR* gene it was hoped that correlations between a molecular defect and any phenotype might become established. Such a relationship would enable prediction of the response to androgen therapy in infants with PAIS, therefore aid sex assignment and improve long term psychosexual outcome. That information would become also pivotal in genetic counseling of parents and other identified female carrier relatives. Ten years after cloning of the *AR* gene[7-10], it is obvious that there is no simple genotype-phenotype relationship in this phenotypically and genotypically heterogeneous syndrome. Identified mutations are associated with different phenotypes in the same kindred[11-14] or rarely with CAIS in one kindred and with PAIS in another.[2] Additional factors apparently may influence the effect of the mutant receptor upon the development of the external genitalia. Understanding of these modulating factors is essential in genetic counseling and clinical management of patients and families with AIS.

Here, a family with distinct phenotypes in two siblings with the same AR-gene mutation is reported. The identified mutation in the *AR* gene, R846H (amino acid numbering based on 20 glutamine residues and 16 glycine residues, thus a total of 910 aminoacids) is a frequently occurring recurrent mutation.[2] A different availability of DHT in these two siblings is detected and further

investigated with molecular techniques. Reduced 5α-reductase 2 activity was found before molecular evaluation was available and may explain the observed phenotypic variation.[15-17]

SUBJECTS AND METHODS

In nationwide study on the genotype/phenotype relationship in AIS, we studied a family with eight children of whom two were affected with AIS (Figure 4.1). The parents (subjects I-1 and I-2; Figure 4.1) were of Moroccan descent and first cousins: their fathers were brothers. There was no family history of ambiguous genitalia.

Subject II-5 was a 13-year old pubertal girl, Tanner stage M3, P2, A0, with a female habitus and female voice. She was studied after her brother was diag-

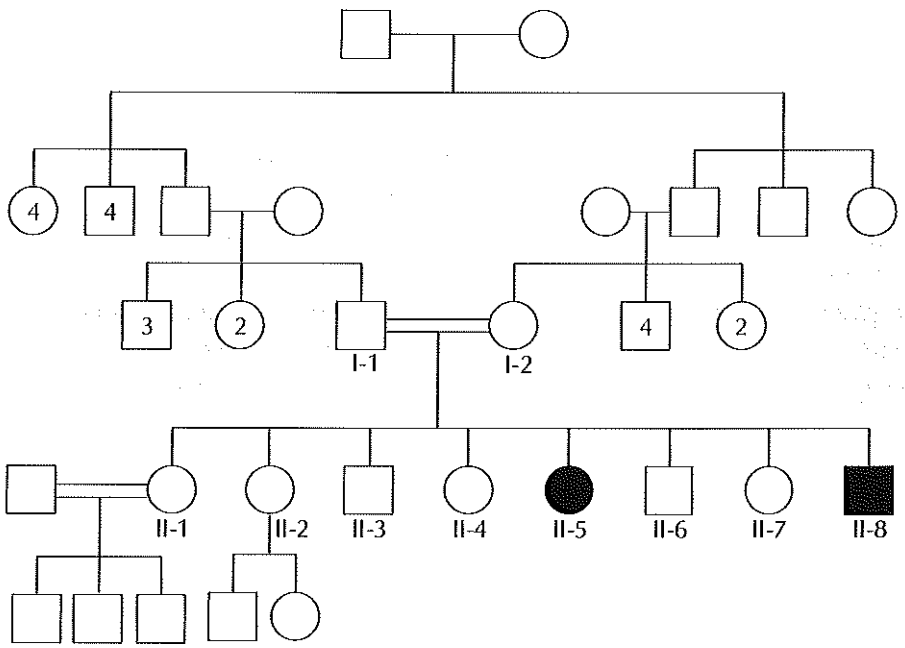

Figure 4.1
Compacted pedigree of the family described. A square with the figure 3 in the middle means three males. A circle with a figure 2 in the middle means 2 females. The affected individuals are indicated with filled circles or filled squares according to their sex of rearing.

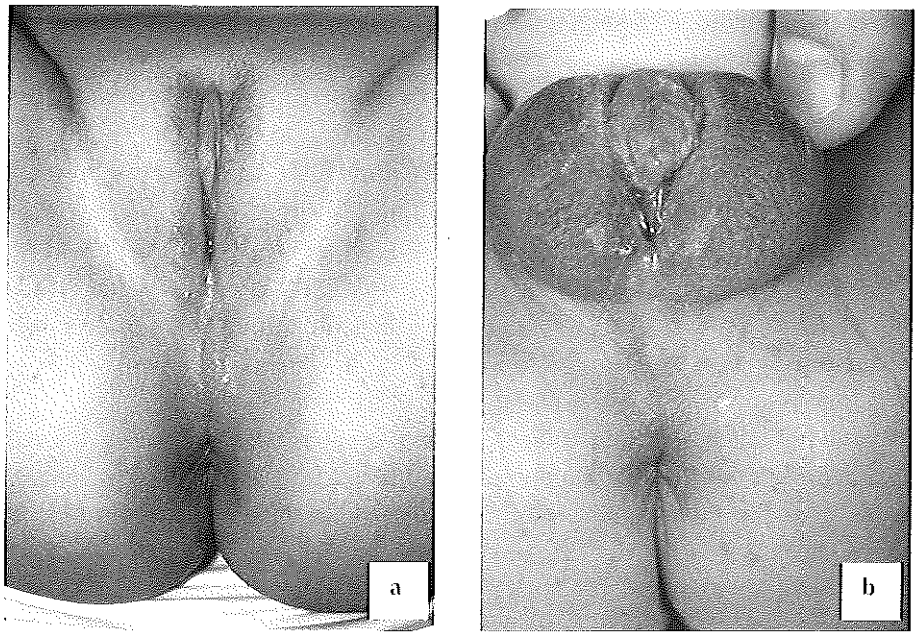

Figure 4.2
External genitalia of subject II-5 at age 15.5 years (a) and subject II-8 at 1 month of age (b).

nosed with AIS. The karyotype was 46,XY. External genitalia were a normally sized clitoris, normal labia majora, posterior fusion of the labia minora leading to an urogenital sinus (Figure 4.2a) and a shallow (2.5 cm in length), blindly ending vagina that was connected with the urogenital sinus. Testes were localized bilaterally in the inguinal region. The following serum levels of hormones were determined: T 13.5 nmol/L (range in normal males 10-30 nmol/L), DHT 1.55 nmol/L, T/DHT 8.7 (normal males T/DHT <10), estradiol 35 pmol/L (normal males: 50-200 pmol/L), LH 5.7 U/L (normal males 1.5 - 8 U/L). Following her personal wish, she was not gonadectomized until two years later, at age 15.5 years. Normally developed epididymides and vasa deferentia were found. Müllerian duct derivatives were absent. Serum hormone levels at that time were: T 31.5 nmol/L, DHT 2.42 nmol/L, T/DHT 13, estradiol 156 pmol/L and LH 8.4 U/L. Her voice had remained high-pitched, the clitoral size remained normal, pubic hair had remained Tanner stage P2, axillary hair was still absent, and her breasts had grown to M4. T and DHT, measured every 6 months from age 13.5 years, showed T/DHT ratios between 8.6 and 13.0. From age 14, T had been above 30 nmol/L.

Subject II-8 (Figure 4.1), karyotype 46,XY, was born with perineoscrotal hypospadias, a micropenis with well developed corpora cavernosa, a bifid scrotum containing testes and transposition of the scrotum (Figure 4.2b). Müllerian duct remnants were absent, as was established by ultrasound. Bilaterally epididymides were palpable. Serum hormone levels measured at age 4 days: T 0.64 nmol/L. 72 Hours after 1500 units of hCG i.m,. T was 18 nmol/L. He was assigned the male sex. At 5 years of age his basal serum levels were: LH 0.1 U/L , T 0.1 nmol/L, DHT < 0.1 nmol/L. 72 hours after 1500 U of hCG i.m.: T 11.8 nmol/L, DHT 2.1 nmol/L, T/DHT 5.6. All values are within the normal range for this age.

I-1 and II-3 were normally virilized healthy adult males. II-6 was a normal healthy prepubertal boy with a normal penis length and a normal testis volume for his age. The mother, subject I-2, had a gonadal/somatic mosaicism for the AR mutation.[18]

The protocol of this study was approved by the medical ethical committee of the University Hospital Rotterdam.

AR gene mutation detection

Genomic DNA was isolated from peripheral blood leukocytes and from cultured genital skin fibroblasts (GSF), following standard procedures.[19]
Exon and flanking intron sequences were screened for mutations in the AR gene with the use of PCR-SSCP.[20] PCR fragments suspected to harbor mutations were analyzed by direct sequencing.[20] Furthermore in DNA isolated from GSF, the entire AR gene of subject II-5 was sequenced.

AR gene CAGn(CAA) / GGN repeat length

CAGn(CAA) and GGN repeat lengths in exon 1 of the AR-gene, encoding polyglutamine and poly-glycine streches respectively, were determined as described.[20,21]

Cell culture

Skin biopsies were taken either during surgical correction of the external genitalia, or gonadectomy or circumcision. GSF were derived from biopsies of (the fusion line of) the labia minora of subject II-5 and controls, and from scrotal skin of subject II-8 and from preputium of a normal prepubertal boy

obtained at circumcision. All cell-lines were cultured as described previously[20] with modifications as described with the experiments.

Androgen binding by the AR

Whole cell Scatchard analysis was performed on GSF as described previously.[20] GSF#1 was used for subject II-5.

SDS-PAGE of the AR

Confluent cell layers in 150 cm^2 culture flasks, were cultured in serum free medium for 24 hours, followed by 24 hours in medium containing increasing concentrations (0, 5, 30 and 100 nM) of the synthetic, non metabolizable androgen methyltrienolone (R1881). GSF#1 was used for subject II-5. Whole cell lysates were prepared, immuno-precipitated, separated on a SDS-PAGE gel and immunostained as previously described.[20]

5α-Reductase assay

To reduce bias on 5 α-reductase 2 activity caused by clonal origin of a cell-line[22] or by site of origin of the biopsies[23], two GSF cell-lines (GSF#1 and GSF#2) derived from separate biopsies taken 1.5 years apart and one PSF cell-line of subject II-5 were used for studies.
GSF cell-lines derived from biopsies from subject II-8, from a normal male, from a normal female, and from a 5α-reductase 2 deficient patient homozygous for mutations H231R in the 5α-reductase 2 gene, were used as controls.
As 5α-reductase 2 activity increases with serial subcultures[24,25], all cell-lines used were the 7th subculture.
In order to reduce possible bias by confluency rate, all cell-lines were grown in 75 cm^2 culture flasks with medium containing 10% fetal calf serum. They were harvested 7 days after subculture. At that time the cell-lines were confluent and the flasks contained ~$1,2x10^6$ cells.

Harvesting of cells

Cells were washed multiple times with PBS and with 20 mM Tris Saline pH 7.4, then scraped in Tris Saline and pelleted at 800 x g. Pellets were washed twice in Tris Saline. Cell-free extracts were prepared by 4 cycles of freezing in liquid nitrogen and thawing.

Enzyme assay

40 µl of cellfree extracts were incubated with 10 µl of 30 mM NADPH and 50 µl reaction mixture (reaction mixture consisted of 500 µl 10 mMTris citrate, pH 5.5, 2.4 pmol of 1,2,6,7 ^{3}H-testosterone (Amersham) and 7.6 pmol testosterone (Steraloids)) at 37° C for 1 hour. The reaction was stopped on ice. Each incubation was done in duplo. Assays were done in triplicate.

To all samples 10 µl of a steroid mixture containing androstanedione, androstenedione, DHT, testosterone, 3α-androstanediol, each 1mg/ml ethanol, was added, before extraction of radioactivity with a total of 3 x 500 µl ethylacetate. Extracts were evaporated to dryness and the residues were dissolved in 50µl ethanol and chromatographed in dichloromethane: ethylacetate: methanol (85:15:3) on a 0.25 mm layer silica gel plates, 20 x 20 cm (Merck). Steroids were visualized in a control lane by spraying with 20% H_2SO_4 in methanol and developing at 100°C for 15 minutes. Fractions were collected in separate vials, resuspended in 500µl ethanol and ^{3}H concentrations were counted. 5α-reductase activity was calculated from the sum of ^{3}H-radioactivity in the androstanediol, DHT, 3α-androstanediol fractions divided through the sum of ^{3}H-radioactivity in androstanediol, androstenediol, DHT, T, 3α androstanediol fractions. The amount of protein in each cellfree extract was determined with Bradford reagens.[26]
5α-Reductase activity was expressed as femtomoles of 5α reduced steroids formed per mg protein/hour.

Analysis of the *5α-reductase 2* gene and polymorphic marker analysis

Exon and flanking intron sequences of the *5α-reductase type 2 (SRD5A2)* gene from subject II-5 were analyzed with direct sequencing after amplification of fragments with primers described by Hiort et al.[27] As an intragenic polymorphic marker, codon 89 in exon 1 of the *SRD5A2* gene was used. This codon is either CTA or GTA[28,29] with unknown allele frequencies. Genomic DNA isolated from GSF#1 (subject II-5) and GSF (subject II-8) was used.

Reverse transcriptase PCR (RT-PCR) of 5α-reductase 2 mRNA

Cell lines were cultured and harvested as for the 5α-reductase assays.
Total RNA was extracted from GSF and PSF cell-lines using Trizol reagent (GibcoBRL) and quantified by absorption at 260 nm. cDNA was synthesized from 2.5 µg RNA with the use of an oligo dT primer (Promega). Of each inves-

tigated cell line, cell pellets from different cell culture flasks were pooled and divided in aliquots. RNA was isolated from these aliquots in separate experiments. The various aliquots of RNA were subjected to several independent RT-PCR experiments.

As a control for cDNA synthesis, β actin was used. β Actin was amplified with antisense primer GAGGTAGCAGGTGGCGTTTACGAAGAT and sense primer AAGGATTCCTATGTGGGCGACGAG. Primers used for amplification of the *5α-reductase 2* gene were: antisense primer 5B: 5' TGACAGTTTTCATCAGCATTG 3' specific for 3' untranslated sequences in exon 5 and sense primer 120A: 5' CACTGGAAATGGAGTCCTTC 3', starting at codon 120 in exon 2. These primers were used in a PCR reaction as described below.

3 µl of the obtained cDNA reaction product was used in a 50 µl PCR amplification reaction. 50 µl PCR reaction mix contained 1.5 mM $MgCl_2$. Conditions for the PCR in a Biometra cycle sequencer were as follows: hot start at 94°C for 5 minutes, then 35 cycles at 94°C for 1 minute, at 55°C for 30 seconds, at 72°C for 1 minute, and final extension for 10 minutes at 72°C. The PCR product was visualized after electrophoresis on a 2% agarose gel that contained ethidium bromide. Amplification of genomic DNA was prevented because intervening introns were in total ~ 7.3 kb in size, and the Amplitaq polymerase (Perkin Elmer) can not amplify DNA of this size under the used conditions. The resulting PCR product was subcloned into a plasmid using the TOPO TA cloning kit (Invitrogen) and subjected to automated sequencing.

RESULTS

SHBG suppression test

An SHBG suppression test[30] showed a maximal decrease in SHBG of 73.5 % on days 4, 5, 6 and 7 in the girl, subject II-5 (normal males < 63.4 %; PAIS: 63.4-93%; CAIS: >92%[30]), while a maximal decrease of 92 % on day 7 was seen in the boy, subject II-8.

Mutation detection and identification in AR-gene

With PCR-SSCP followed by direct sequencing of the ^{AR} gene, mutation R846H in the ligand binding domain of the AR was identified in both individuals II-5 and II-8. No other mutations were identified upon sequencing of the exon,- and flanking intron sequences of the *AR* gene of subject II-5. Therefore, the

Table 4.1
Scatchard analysis of the AR in GSF of both affected siblings

	II-5; girl	II-8; boy	Normal values
Bmax (fmoles/mg protein)	87	61	>20
Kd (nM)	0.9	0.5	<0.1

presence of additional mutations in the *AR* gene are not the cause of this phenotypic variation.

Length of (CAG)n CAA and GGN repeats

In both siblings the (CAG)nCAA and GGN repeats in exon 1 of the AR gene mutant-allele carried 14 (CAG)nCAA (published in[18]) and 24 GGN.

Androgen binding characteristics

Androgen binding characteristics of the androgen receptor in GSF of subjects II-5 and II-8 (Table 4.1) show an increased equilibrium dissociation constant

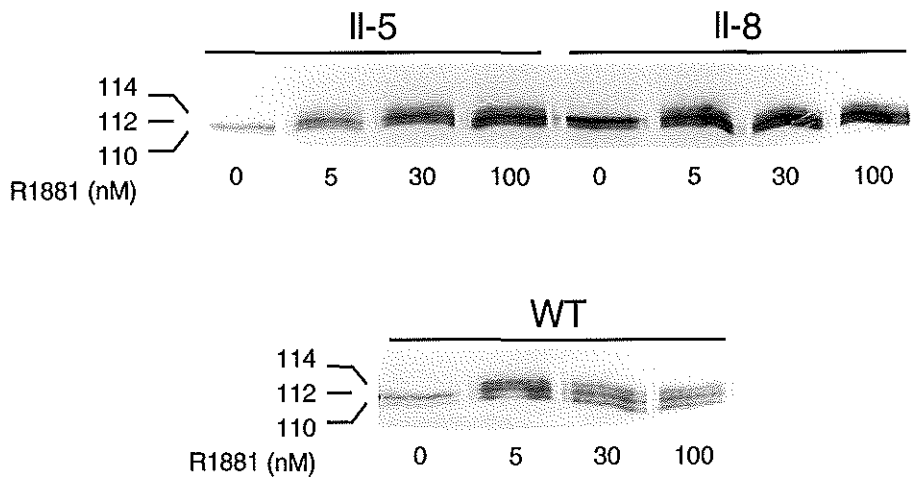

Figure 4.3
Hormone dependent AR phosphorylation in GSF.
GSF of both siblings (II-5; II-8) and a normal prepubertal boy (WT) were cultured in the absence or presence of increasing concentrations (0-100nM) of the non-metabolizable androgen R1881. The molecular sizes of the AR-isoforms are indicated at the left side (110-112-114 kDa).

(Kd) but a normal number of binding sites (Bmax). The difference in Bmax and Kd values between the two siblings should be interpreted as a variance of Scatchard analysis.

Hormone dependent AR phosphorylation

AR protein isolated from wild type cells cultured in the absence of androgens migrates as a doublet of 110 and 112 kDa during SDS-PAGE. These represent an unphosphorylated AR isotype and a phosphorylated AR isotype, respectively. Upon binding of androgens, the AR undergoes additional phosphorylation, resulting in a third isoform of 114 kDa. AR mutants that are either partially defective in ligand binding or in DNA binding or in transcription activation, migrate with a reduced amount of the 114 kDa isoform in SDS-PAGE[31] (Figure 4.3). At a relative low androgen concentration of 5 nM of R1881, GSFs of II-5 and II-8 have equally reduced amounts of the third isoform of 114 kDa as compared to the wild-type. Increased androgen levels did not induce the 114 kDa band as in the wild-type cells (Figure 4.3). Moreover, both siblings have an equally deficient hormone induced upshift of the 114kDa AR isoform. A deficient hormone induced upshift is in agreement with the increased dissociation of the AR hormone complex in GSF of both patients.

5α-Reductase 2 activity

Of subject II-5, two different GSF cell lines were deficient in 5α-reductase 2, similar as in a 5α-reductase 2 deficient patient homozygous for SRD5A2 mutation H231R. However, the sibling II-8 had normal 5α-reductase 2 activity in GSF (Table 4.2).

Analysis of the *5α-reductase 2* gene and polymorphic marker analysis

No mutations were found in genomic DNA of subject II-5 and subject II-8 upon sequencing exon and flanking intron sequences of the *5α-reductase type 2* gene.
Both siblings were heterozygous for a known polymorphism in exon 1 of the *5α-reductase 2* gene, CTA/GTA, codon 89 (data not shown). A homozygous defect in the *5α-reductase 2* gene, inherited from the consanguineous parents and present in other parts of the gene than the sequenced parts such as introns or in a gene promotor, was thus excluded.

Table 4.2
5α-Reductase 2 activities in GSF from control persons and 46,XY patients with various forms of male pseudohermaphroditism

Subject/Disorder	Age at biopsy (years)	5α-reductase 2 activity pmoles/mg protein/h♦ mean	(range)
normal male	1	73	(44-139)
normal female	16	41*	
II-5 GSF#1; PAIS	13	0	(0-0)
II-5 GSF#2; PAIS	15	0	(0-0)
II-8; PAIS	5	88	(42-154)
5α-reductase 2 deficiency	4	0	(0-0)

In all assays the recovered radioactivity represented at least 84% of the added ^{3}H-testosterone. Variation between duplo experiments was < 11 %.
♦Mean and range (in brackets) are given. Ranges in different experiments are shown in brackets.
* One assay, in duplicate

5α-Reductase 2 mRNA expression

After RT-PCR of 5α-reductase 2 mRNA using a primer combination as outlined in subjects and methods, a 460 bp fragment can be expected. No 5α-reductase 2 cDNA was detectable after RT-PCR of total RNA preparations from GSF#1 and GSF#2 of subject II-5 whereas in total RNA preparations from GSF of subject II-8, a band of 460 bp was detected (Figure 4.4). This band was subcloned and sequenced and exhibited the wild-type *5α-reductase 2* gene

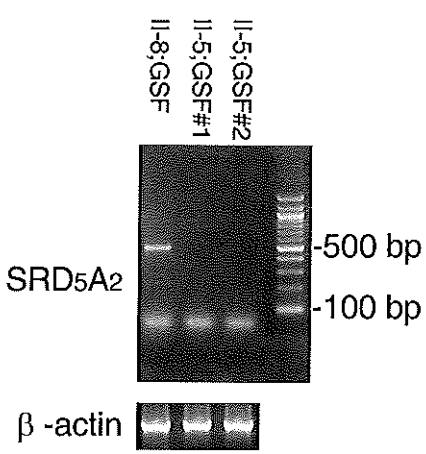

Figure 4.4.
RT-PCR of 5α-reductase 2 mRNA. RNA extracted from GSF#1; GSF#2 of subject II-5 and RNA from GSF of subject II-8 was used. β Actin expression was used as a control for cDNA synthesis.

sequence. Therefore, the absence of 5α-reductase type 2 activity in GSFs of subject II-5 is due to lack of or reduced expression of 5α-reductase type 2.

DISCUSSION

In this family a very different phenotypic expression of AIS is observed in sibs carrying the same mutation in the *AR* gene. Additional mutations in the *AR* gene or differences in length of the glutamine or glycine repeat were excluded as of influence on the phenotype in this family. Furthermore, phosphorylation of the AR upon hormone binding, was equally reduced in GSF of both siblings, irrespective whether low or high concentrations of the potent, non metabolizable androgen R1881, were used. Phosphorylation of the AR occurs after DNA binding of the ligand-AR complex on hormone responsive elements and during or following transcription of androgen regulated genes.[31] The equally reduced phosphorylation of ARs in both siblings and comparable clinical androgen responsiveness in both siblings as determined by SHBG-suppression tests, provides additional evidence that the AR itself is not responsible for the distinct variation in phenotype and suggests an important role for factors other than the AR in determining the phenotype.

The identified mutant AR, due to mutation R846H, is normally expressed in GSF but has an decreased affinity for androgens (Table 4.1).[32,33] When the R846H mutant AR is stimulated with DHT instead of testosterone, the transcriptional deficit becomes less[32,33] and the functional defect can be partially corrected by the repeated addition of DHT.[32]

5α-Reductase activity was totally absent in GSFs of subject II-5 (Table 4.2). As DHT, formed in the embryonal urogenital tissues by 5α-reductase 2[34], causes elongation and enlargement of the urogenital tubercle and fusion of the urogenital swellings and folds during the development of the embryo[35], the phenotypic difference between the siblings might well be due to this difference in DHT. A 5α-reductase 2 deficiency secondary to the primary defect AIS was found to cause phenotypic differences in other families with AIS and was established by 5α-reductase 2 assays in GSFs and hormonal analysis in serum.[15-17] These observations were made before the cloning of the *AR*[7,10,36] and *5α-reductase 2* genes.[28] The nature of the decreased 5α-reductase 2 activity remained unidentified. The repeated observation of this 5α-reductase 2 deficiency secondary to AIS and identification of a mutant AR which is

especially dependent on DHT for residual androgen action, provides a basis for further studies on secondary 5α-reductase 2 deficiency.
RT-PCR experiments in this family show that the 5α-reductase 2 deficiency in subject II-5 is due to reduced expression of the *5α-reductase 2* gene *(SRD5A2)*. A homozygous defect in the *SRD5A2* gene inherited from the consanguineous parents was excluded as no mutations in the *SRD5A2* gene were found in both siblings and the presence of mutations in the remaining intronic sequences or the promotor of the *SRD5A2* gene was made very unlikely as both siblings are heterozygotes for the CTA/GTA polymorphism in exon 1.
With molecular means we show that secondary 5α-reductase 2 deficiency in the presented family is due to absent or reduced expression of the 5α-reductase 2 enzyme.
The etiology of this secondary 5α-reductase 2 deficiency is not clear. Disruption of a feed forward control mechanism by which formation of trace amounts of DHT induces 5α-reductase 2 activity thereby increasing DHT synthesis and triggering a positive developmental cascade, is one possible explanation. Such a feed forward mechanism exists in the rat embryonic urogenital tract where 5α-reductase type 1 and 2 expression is increased by either testosterone or DHT.[37] A feed forward mechanism is also present in adult rat prostate[38] but is absent in embryonic rat prostate.[37] In humans, the presence of such a positive regulation by androgens is suggested by the presence of reduced 5α-reductase 2 expression in urogenital swellings and tubercles in female fetuses, 1/3 of that in males who have higher levels of androgens.[39] Arguments against positive regulation of 5α-reductase 2 enzyme activity in humans are the observations that in many CAIS patients 5α-reductase 2 activity is normal[23,40] and during in vitro culture of GSF no increase of 5α-reductase activity is observed after stimulation with androgen.[41] In GSF, 5α-reductase 2 is the predominantly expressed and active iso-enzyme.[42]
Others have suggested a disbalance between estrogen and androgen action as the cause for secondary 5α-reductase deficiency.[16,23]

These observations support the hypothesis that differences in the availability of various androgens in different target tissues could lead to phenotypic variation between AIS patients that carry the same *AR* gene mutation. Minimal phenotypic variation was shown to be the result of secondary 5α-reductase 2 deficiency.[17] We show that distinct phenotypic variation can also be caused by secondary 5α-reductase 2 deficiency. Furthermore, biochemical and molecular genetic evidence is provided that absence or reduced expression of the 5α-re-

ductase 2 iso-enzyme is the underlying cause of secondary 5α-reductase deficiency. It is an example of how gene products of many genes interact in providing a phenotype and provides information to be implemented in genetic counseling of families with androgen insensitivity.

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CHAPTER

5

Molecular analysis of the androgen receptor gene in a family with receptor-positive partial androgen insensitivity: an unusual type of intronic mutation

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Summary

In the coding part and the intron-exon boundaries of the *androgen receptor* gene of a patient with partial androgen insensitivity, no mutation was found. The androgen receptor of this patient displayed normal ligand-binding parameters, and migrated as a 110-112 kDa doublet on SDS-PAGE in the absence of hormone. However, after culturing of the patient's genital skin fibroblasts in the presence of hormone, the slower migrating 114-kDa protein, which reflects hormone-dependent phosphorylation, was hardly detectable. Furthermore, receptor protein was undetectable in the nuclear fraction of the fibroblasts, after treatment with hormone, which is indicative of defective DNA binding. By sequencing part of intron 2, a T→A mutation was found 11 base pairs upstream of exon 3. In our screening of 102 chromosomes from unrelated individuals this base pair substitution was not found, indicating that it was not a polymorphism. mRNA analysis revealed that splicing involved a cryptic splice site located 71/70 base pairs upstream of exon 3, resulting in generation of mRNA with an insert of 69 nucleotides. In addition, a small amount of a transcript with a deleted exon 3 and a very low level of wild-type transcript were detected. Translation of the extended transcript resulted in an androgen receptor protein with 23 amino acid residues inserted in between the two zinc clusters, displaying defective DNA binding and defective transcription activation.

INTRODUCTION

Expression of a number of genes involved in male sex differentiation and development is regulated by the AR. The AR belongs to the family of steroid hormone-activated transcription modulators.[1] Like the other steroid hormone receptors, the AR consists of distinct functional domains. The NH_2-terminal part is involved in transcription activation and is encoded by exon 1.[2] Two highly conserved DNA-binding zinc clusters are encoded by exons 2 and 3. The NH_2-terminal zinc cluster recognizes specific consensus DNA sequences, whereas the C-terminal zinc cluster is involved in dimerization.[3-4] Parts of exons 3 and 4 encode the hinge region, which contains a NLS that is involved in nuclear import, and exons 4-8 encode the LBD.

On ligand binding, the AR undergoes conformational changes and binds to AREs in the promoter regions of androgen-regulated target genes.[5] Recently, coactivators, interacting with the LBD of steroid hormone receptors have been cloned (reviewed by Horwitz et al.[6]). One of these, ARA70, appears to be specifically involved in transcription activation by the AR.[7]

Defects in the hAR cause disturbed virilization in 46,XY individuals, which is called AIS (reviewed by Quigley et al.[8]). Many qualitative and quantitative AR abnormalities, causing a broad range of AIS phenotypes, have been described.[9] The spectrum of phenotypes ranges from individuals with completely female external genitalia and absence of müllerian and wolffian duct derivatives (complete AIS) to patients with ambiguous genitalia (partial AIS) or with mild hypospadias.[8] The mutations that are most frequently observed, are nonsense or missense point mutations. Mutations resulting in aberrant splicing are much less common, only six of them have been reported.[9] In five of these, a consensus splice donor site was mutated, resulting in complete AIS.

PCR-SSCP analysis is a screening method, often used for mutation detection in the hAR. Using this method, we and others have reported that in some individuals, clinically diagnosed as having AIS, no mutation was found in the coding region and exon flanking intronic sequences of the *AR* gene.[10-12] In the present study, we investigated and characterized the *AR* gene of a patient with partial AIS. Initially no mutation was detected in the coding region of the *AR* gene in this patient, although several biochemical and cell biological assays revealed that the encoded AR of this patient was unable to bind to DNA.

SUBJECTS AND METHODS

Clinical subjects

A family with three individuals clinically suspect for AIS (II-4, III-1 and III-2; for pedigree, see Figure 5.1) was referred for further diagnosis, treatment and genetic counseling. All affected individuals were 46,XY and had a female habitus with normal female external genitalia, and normal but underdeveloped testes with epididymides and vasa deferentia were present. No müllerian remnants

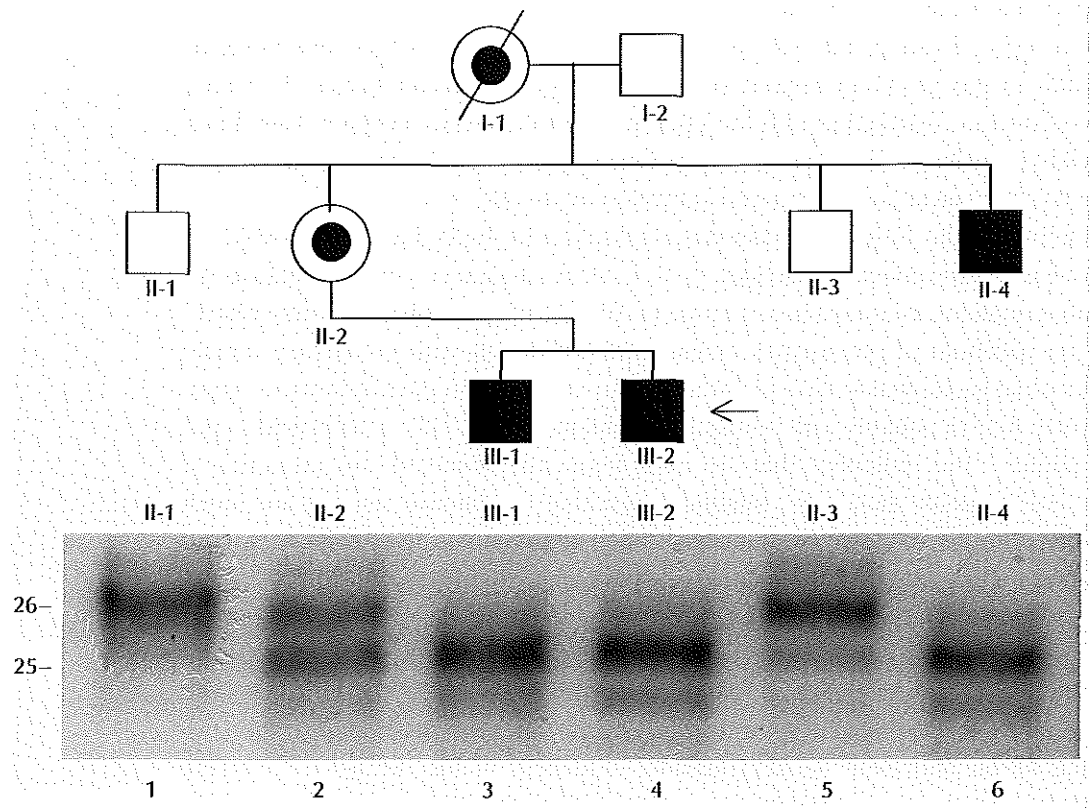

Figure 5.1
Pedigree and sizing of the poly-glutamine stretch of the present family with AIS. PCR products, obtained by amplification of genomic DNA by primers flanking the (CAG)nCAA repeat, were resolved on a 7% denaturating polyacrylamide gel. Lane 1, normal male; lane 2, mother of the index subject; lane 3, 46,XY sister of the index subject; lane 4, 46,XY index subject; lane 5, normal male; lane 6, 46,XY affected aunt. The index subject (III-2) is indicated with an arrow. Numbers in the left margin of the lower part of the figure represent the numbers of glutamine residues encoded by the (CAG)nCAA repeat.

were found. One postpubertal patient (II-4) was Tanner P3, M5 and had axillary hair. Testosterone synthesis disorders, which could be another cause for such a 46,XY phenotype, were excluded by analysis of the circulating levels of steroid hormones and their precursors in this patient. At the age of 15.5 yrs., she had a normal male level of testosterone (21 nmol/liter) (normal range in adults: 10-30 nmol/liter) combined with high levels of LH (13 IU/liter) (normal range in adult males: 1.5-8 IU/liter). For final proof of the diagnosis and for the purpose of genetic counseling of this family, DNA analysis of the *AR* gene was started. Blood cells were obtained from the three 46,XY patients, and genital skin fibroblasts from III-1 and III-2. Genital skin fibroblasts containing wild-type AR protein and AR protein with an exon 3 deletion, derived from a patient with Reifenstein syndrome, previously described by Ris-Stalpers et al.[13], were used for comparison. Genomic DNA from 74 unrelated individuals was used for intron 2 screening. Informed consent was obtained from all individuals.

Mutation detection

Genomic DNA was isolated from blood cells or genital skin fibroblasts, according to standard procedures.[14] Single strand conformation polymorphism analysis and direct sequencing were performed as described previously.[11] A total of 102 normal chromosomes from unrelated individuals were analyzed by automated sequencing. Template was made using intron 2 sense primer C1 and intron 3 antisense primer C2[15], and purified by use of the Boehringer High

Table 5.1
Oligonucleotides used for cDNA synthesis and allele specific hybridization

Oligonucleotide	Location	Sequence
3BB	intron 3	5' AGAGAAAGAAAAGTATCTTAC 3'
5BB	exon 5	5' CGAAGTAGAGgATCCTGGAGTT 3'
J3A	exon 1 - exon 2	5' gAtGGatcCATGCGTTTGGAGACTGC 3'
14NB	exon 4	5' TGCAAAGGAGTtGGGCTGGTTG 3'
470A	exon 1	5' GTAGCCCCCTACGGCTACA 3'
wild-type	exon 2 - exon 3	5' GCTGAAGGGAAACAG 3'
69 bp insertion	exon 2 - intron 2	5' CTGAAGAAATACCCG 3'
exon 3 deletion	exon 2 - exon 4	5' CTGAAGCCCGGAAGC 3'
2AA	exon 2	5' CAGAAGACCTGCCTGATCTGT 3'

Lower case lettering indicates mismatches

Pure PCR Product Purification Kit (Boehringer Mannheim). Sequencing was performed with antisense primer 3BB (Table 5.1). Determination of the length of the polymorphic CAG repeat in exon 1, used as an intragenic polymorphic marker, was performed according to Sleddens et al.[16]

Reverse-transcriptase-PCR (RT-PCR) reaction

Total RNA was extracted from genital skin fibroblasts using TRIzol reagent (Gibco BRL) and quantified by absorption at 260 nm. Amplification of genomic DNA was prevented because the primers used in the RT-PCR reaction resulted in a PCR product spanning several introns of the *AR* gene. In the cDNA synthesis reaction, the exon 5 antisense primer 5BB (Table 5.1) was used for first strand cDNA synthesis. cDNA amplification was performed by PCR with antisense primer 5BB combined with sense primer J3A (Table 5.1), spanning the 3' end of exon 1 and the 5' end of exon 2. To obtain sufficient quantities of template DNA spanning exon 2 and 3 sequences, for direct sequencing, a nested-PCR reaction was performed, by use of sense primer J3A and exon 4 antisense primer 14NB (Table 5.1).

Ligand-binding study

For determination of ligand-binding characteristics of the AR of the AIS patients, a whole cell assay was performed on genital skin fibroblasts as described elsewhere.[11]

Western blot analysis

AR protein, obtained from cultured genital skin fibroblasts or transiently transfected CHO and COS-1 cells, was immunoprecipitated and analyzed by Western immunoblotting according to Ris-Stalpers et al.[17]

Construction of expression vectors

Human wild-type AR cDNA expression plasmid pSVAR0[18] was used to construct pSVAR129, encoding an AR with 23 additional amino acid residues between the two zinc clusters. To this end, the 472-bp *Kpn*I-*Asp*I fragment from pSVAR0 was exchanged with the 541 bp *Kpn*I-*Asp*I fragment, generated by RT-PCR from the AR mRNA of patient III-2. cDNA was synthesized as described in *Mutation detection* above. In the PCR reaction, following first strand cDNA synthesis, exon 1 sense primer 470A (Table 5.1) was used, allowing digestion

with *Kpn*I. A nested PCR was performed by use of sense primer 470A and exon 4 antisense primer 14NB (Table 5.1). All PCR products were checked by sequencing. Expression plasmid BHEX-AR33, an expression plasmid with an in-frame deletion of exon 3, was constructed as described elsewhere.[13] To generate pSG5AR129 and pSG5AR33, the 541 bp *Kpn*I-*Asp*I fragment containing the 69 additional bp, and the 355 bp fragment from which exon 3 had been deleted were exchanged with the 472 bp *Kpn*I-*Asp*I fragment of wild-type AR expression vector pSG5AR0 (provided by Dr. A.C.B. Cato, Karlsruhe, Germany). pSG5 plasmids were used to obtain AR protein for gel-shift assays.

Cell culture and transfections

Genital skin fibroblasts and COS-1 cells were cultured as described by Ris-Stalpers et al.[19] CHO cells were treated like COS-1 cells. CHO cells were plated at 7 cm^2 wells and grown for 24 hours before they were transiently transfected, by the use of the calcium phosphate method[20], with AR expression plasmid (10 ng DNA/ml precipitate suspension) and the MMTV-Luc reporter plasmid (2 µg DNA/ml precipitate suspension).[21] Carrier DNA (pTZ19) was added to 20 µg DNA/ml precipitate suspension, and 90 µl precipitate suspension was added per well. Twenty-four hours after transfection, cells were incubated with increasing concentrations of R1881. Luciferase assays were performed as described before, after 24 hours incubation.[22] Each receptor mutant was assayed three times in triplicate, by use of three independently isolated expression-plasmid preparations. Luciferase activity was related to basal activity, measured in the absence of hormone. CHO cells, used for expression studies, were plated in 175 cm^2 culture flasks and transfected with 200 µl DNA precipitate, as described above, by use of the calcium phosphate method. COS-1 cells were transiently transfected in 80 cm^2 culture flasks with 9.4 µg expression plasmid, by use of the diethylaminoethyldextran method.[23] Cells were shocked with 80 µM chloroquine for 2 hours. Transfected CHO and COS-1 cells were washed after 24 hours and cultured for another 24 hours in either the presence or absence of 10 nM R1881.

Preparation of cytosolic fractions and nuclear extracts

Genital skin fibroblasts were grown until confluence in 175 cm^2 culture flasks, incubated for 24 h in culture medium[19] containing 10% hormone-depleted fetal calf serum, and were cultured for another 24 h with medium either with or without 10 nM R1881. Next, cells were washed twice in PBS, were col-

lected in 1 ml lysis buffer A [40 mM Tris, 1 mM EDTA, 10% (vol/vol) glycerol, 10 mM DTT, 10 mM Na_2MoO_4, 0.5 mM bacitracin, 0.5 leupeptin, 0.6 mM PMSF], and subjected to four freeze-thaw cycles, followed by 10-min centrifugation at 800xg in a Biofuge (Heraeus) at 4 C. The supernatant was centrifuged for 10 min. at 400,000xg at 4 C (TLA120.2 rotor; Beckman, Fullerton, CA). The cytosol fraction (supernatant) was stored at -80 C until use. The pellet, remaining after the first 800xg centrifugation step was resuspended in buffer B (buffer A with 0.2 % Triton X-100) and incubated for 5 min at 4 C, followed by 10 min centrifugation at 800xg in a Biofuge 13. The resulting pellet (nuclear fraction) was washed with buffer C (buffer A without leupeptin), and was resuspended in equal volumes of 1 M NaCl and 0.5 M NaCl and incubated for 1 hour at 0 C. Nuclear extract (supernatant) was obtained by centrifugation for 10 min at 400,000xg at 4 C (TLA120.2 rotor). The total nuclear extract and 250 µl cytosol fraction were taken separately for immunoprecipitation.

Gel-shift assay

After transfection with pSG5AR0, pSG5AR33 or pSG5AR129, COS-1 cells were collected in 5 ml PBS. The pellet was resuspended in extraction buffer [10 mM NaH_2PO_4 (pH 7.4), 0.4 M KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM bacitracin, 0.5 mM leupeptin and 0.6 mM PMSF, 10 mM DTT] and was frozen and thawed four times, followed by 10 min centrifugation at 400,000xg (TLA120.2 rotor) at 4 C. The double-stranded DNA probe, containing the ARE from the TAT promoter (5'-TCGA*CTGTACAGGATGTTCT*AGCTACT 3') (half-sites in italics), was obtained from Promega (Woerden, the Netherlands). Labeling and purification of the DNA probe and the gel-shift assay were performed as previously described by Brüggenwirth et al.[24] (see also Chapter 6).

Allele-specific oligonucleotide hybridization

RT-PCR and nested-PCR reactions were performed as described above (*see the Construction of expression vectors subsection above*). The resulting PCR product was amplified once more in a PCR reaction of 30 cycles, by use of exon 1 sense primer 470A and exon 4 antisense primer 14NB. Plasmid (pSVAR0, BHEX-AR33, and pSVAR129) fragments were amplified once under identical conditions, by use of the same primers. Dot blots were prepared in a Schleicher & Schuell apparatus according to the manufacturer's protocol. In the case of PCR product obtained by plasmid amplification, an equivalent amount of DNA was spotted. Membranes were preincubated for 10 min with hybridi-

zation mix [50 mM NaH_2PO_4, 0.75 M NaCl, 5 mM EDTA (= 5xSSPE); 1% SDS; 0.05 mg/ml herring sperm DNA]. For each of the splice variants, specific oligonucleotides were designed: a wild-type probe, an exon 3 deletion probe, and a 69-bp insertion probe (Table 5.1) (Pharmacia Biotech Benelux). The filters were subsequently hybridized and rinsed as described by Boehmer et al.[25], but at a temperature of 35 C instead of 37°C. The membranes probed with wild-type probe or exon 3 deletion probe were washed for an additional 10 and 15 min, respectively, in 0.1 x SSC/0.1 % SDS at 38°C, before exposure. After autoradiography, the membranes were stripped and the procedure was repeated with probe 2AA (Table 5.1) as a control.

RESULTS

Screening for mutations

PCR-SSCP analysis was performed under two conditions to screen for mutations in the *AR* gene of an index subject with partial AIS. In the coding part and the exon-flanking intronic sequences, no mutation was found. To study segregation of a presumed AR defect, the length of the *AR* gene CAG repeat was determined in DNA from several family members. Results are presented in Figure 5.1. The mother (II-2) of the two 46,XY sisters (III-1 and III-2) had two different alleles, one with a (CAG)24CAA unit coding for 25 glutamine residues and one with a (CAG)25CAA unit coding for 26 glutamine residues. The three affected 46,XY individuals (II-4, III-1 and III-2) had the (CAG)24CAA allele. Therefore, X-linked inheritance could not be excluded (Figure 5.1). The marker was informative, since two brothers of the 46,XX carrier (II-1 and II-3) had the (CAG)25CAA allele.

Receptor characteristics

AR protein isolated from genital skin fibroblasts from the index patient (III-2) appeared as a normal 110-112 kDa doublet after SDS-PAGE and immunoblotting. Hormone-binding parameters were determined in a whole cell-binding assay of genital skin fibroblasts. The receptor displayed a Kd of 0.08 nM and a Bmax of 64 fmol/mg protein, both within the normal range (Kd: 0.03-0.13 nM; Bmax: 39-169 fmol/mg protein). The AR of patient III-1 displayed a Kd of 0.07 nM and a Bmax of 63 fmol/mg protein. Therefore, the 46,XY individuals (III-1 and III-2) in this AIS family were classified as having receptor-positive AIS.

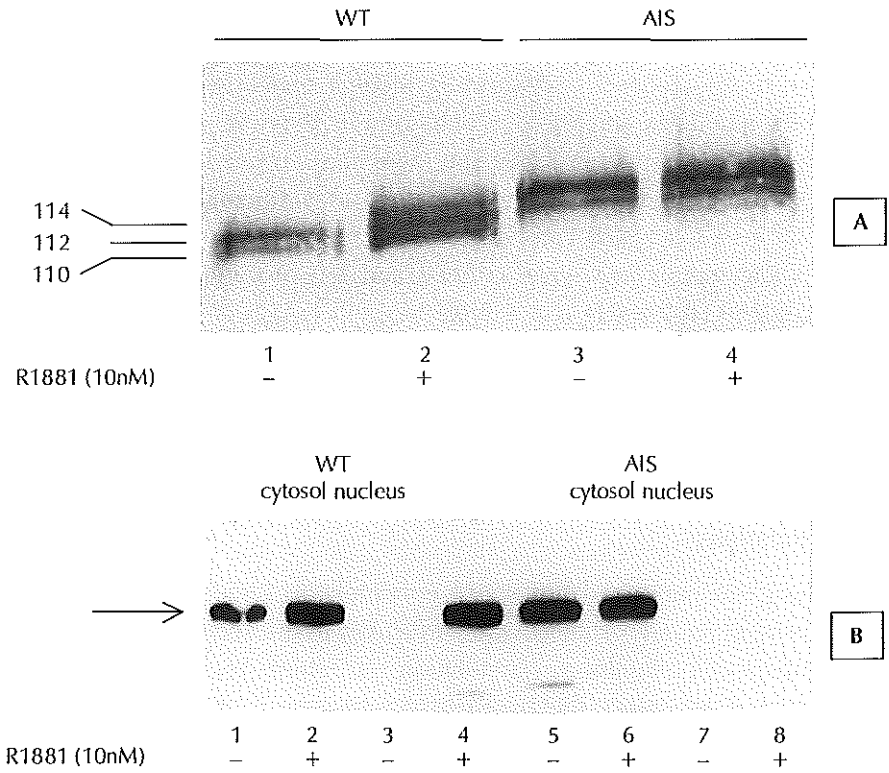

Figure 5.2
Western blot analysis of wild-type and mutant androgen receptor proteins.
A – Western blot, showing receptor isotypes of the wild-type AR and the mutant AR. AR protein was prepared from genital skin fibroblasts, that had been cultured in the absence (-) or presence (+) of 10 nM R1881. After immunoprecipitation with monoclonal anti-AR antibody F39.4.1, receptor protein was separated on a 7% SDS-PAGE gel. After immunoblotting the blot was incubated with Sp061, a polyclonal anti-AR antibody. The blot was washed and then incubated with an alkaline phosphatase-coupled goat-anti-rabbit antibody to visualize the AR. Lanes 1 and 2, wild-type AR; lanes 3 and 4, mutant AR.
B – Subcellular localization of the wild-type AR and the mutant receptor in genital skin fibroblasts of patient III-2, after culturing of genital skin fibroblasts in the absence or presence of R1881. Genital skin fibroblasts were cultured for 24 hours either in the absence (-) or presence (+) of 10 nM R1881. Nuclear and cytosol fractions were prepared, and the AR was immunoprecipitated with monoclonal antibody F39.4.1 from both the total nuclear extract and one-fourth of the cytosol fraction, Western blotting and immunostaining were performed as described in the Legend to Figure VI.2A. AR was visualized with a peroxidase-coupled goat-anti-rabbit antibody. Lanes 1 and 2, cytosol fraction from wild-type genital skin fibroblasts; lanes 3 and 4, nuclear extract from wild-type genital skin fibroblasts; lanes 5 and 6, cytosol fraction from patient III-2 genital skin fibroblasts; lanes 7 and 8, nuclear extract from patient III-2 genital skin fibroblasts.

Hormone-dependent receptor phosphorylation

The AR is phosphorylated and many potential phospho-sites are located in the part encoded by exon 1. In preparations from cells cultured in the absence of hormone, usually two receptor isotypes are present, which are visible as a 110-112 kDa doublet on a SDS-PAGE immunoblot.[26]
AR protein was isolated from control genital skin fibroblasts and from genital skin fibroblasts of the index patient (III-2). In both genital skin fibroblast preparations, cultured in the absence of androgens, the 110 and the 112 kDa AR isotypes were present (Figure 5.2A, lanes 1 and 2). The AR from patient III-2 contains a relatively long glutamine stretch (25 glutamines compared with 19 in the control AR), resulting in a slower migration pattern. On hormone binding, the AR undergoes additional phosphorylation, reflected by a 114-kDa isoform. The appearance of this isoform is dependent on DNA binding and/or transcription activation.[27] This hormone-induced phosphorylation was used as a marker for proper receptor functioning. Control cells cultured in the presence of hormone displayed the expected, slower migrating, third isoform (114 kDa) (Figure 5.2A, lane 2). However, the 114-kDa isoform was hardly detectable in preparations derived from patient III-2 (Figure 5.2A, lane 4).

Subcellular localization studies in genital skin fibroblasts of the index subject

Because the appearance of the 114-kDa isoform depends on DNA binding[27], we investigated whether AR was detectable in the tightly nuclear bound AR fraction in genital skin fibroblasts from the patient. Control genital skin fibroblasts and genital skin fibroblasts from the index patient were cultured in either the absence or presence of 10 nM R1881, and cytosol and nuclear fractions were prepared. When cells were cultured in the absence of hormone, AR was found in the cytosol fraction (Figure 5.2B, lanes 1 and 5), but not in the nuclear fraction (lanes 3 and 7). After culture in the presence of hormone, although AR was still detectable in the cytosol fractions (lanes 2 and 6), wild-type AR was clearly present in the nuclear extract (lane 4), whereas AR protein was not observed in the nuclear extract of the patient's genital skin fibroblasts (lane 8). This suggests a defect in DNA binding of the AR in the index patient (III-2).

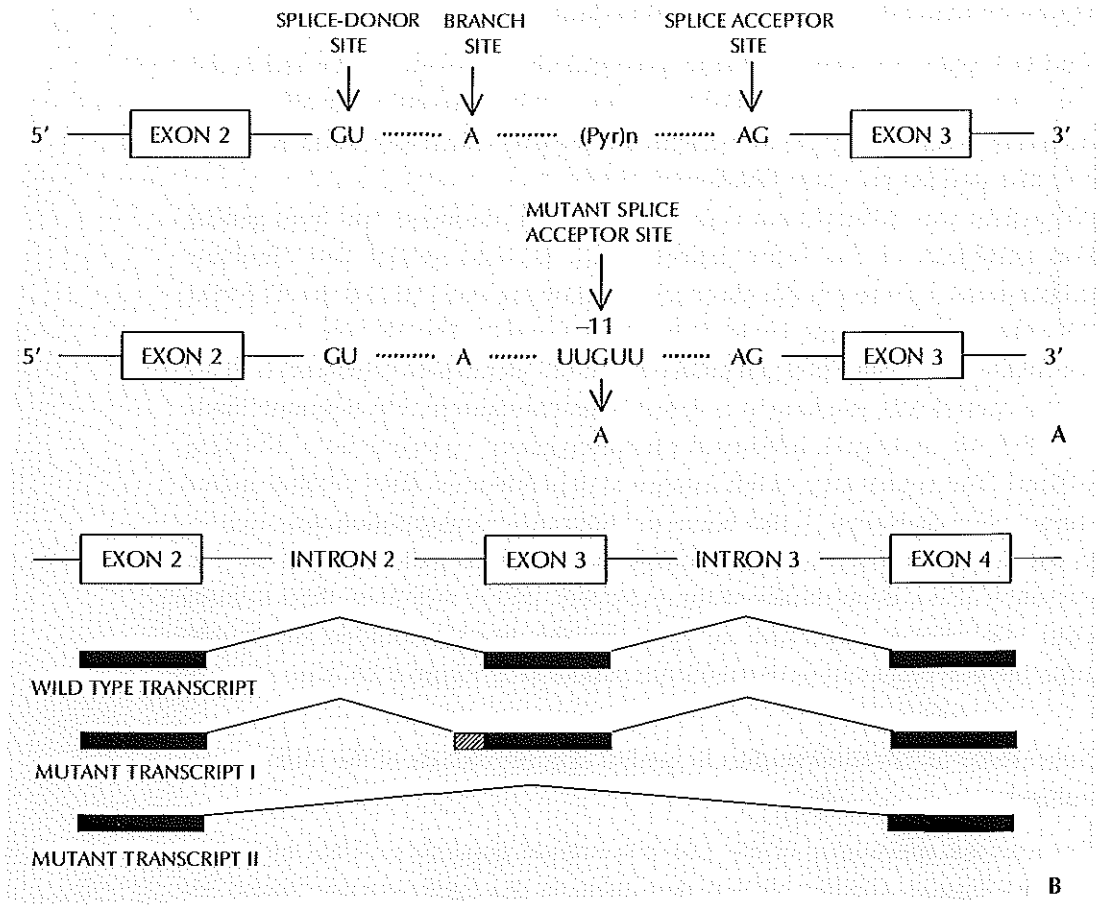

Figure 5.3
Characterization of the point mutation.
A – Position of the point mutation, found in intron 2. Represented are exons 2 and 3 and significant flanking intron 2 sequences of both wild-type and the mutant AR pre-mRNAs. The positions of the splice donor site, the branch site, and the splice acceptor site are indicated. The mutation is located at position -11 in intron 2 in the conserved pyrimidine-rich region 5' upstream of the splice acceptor site of intron 2.
B – Illustration of the wild-type and the aberrant splicing process, resulting from the intron 2 mutation. Black bars represent exons 2, 3, and 4; gray bar represents 69 additional nucleotides. Mutant transcript I and mutant transcript II were found by RT-PCR studies on genital skin fibroblast mRNA from the index subject.

Mutation detection and the effect of the mutation on the splicing process

Although PCR-SSCP analysis was not informative about an *AR* gene mutation in patient III-2, the *AR* gene was studied in more detail. Sequencing of the flanking intronic regions of exons 2 and 3 of the *AR* gene of patient III-2 demonstrated a mutation (T→A) 11 bp upstream of exon 3 (Figure 5.3A). The same

mutation was found in the *AR* gene of patients II-4 and III-1. Because the mutation is located in the region where forward primer (primer 3A) anneals, the mutation remained undetected in PCR-SSCP analysis. In RT-PCR experiments, two different receptor variants were found. Predominantly, a transcript containing 69 additional nucleotides between the sequences of exons 2 and 3 (mutant transcript I), and a smaller amount of a transcript from which exon 3 was deleted (mutant transcript II) (Figure 5.3B) were detected. Translation of transcript I will result in an AR with a 23 amino acid insertion between the two zinc clusters, and transcript II encodes a protein that lacks the second zinc cluster. Wild-type transcript was not detected by RT-PCR.
SDS-PAGE and immunoblot analysis revealed only a protein with an increased molecular mass in genital skin fibroblasts from patient III-2 (Figure 5.2A, lanes 3 and 4). The 23 additional amino acid residues, as well as the relatively long glutamine stretch, contributed to the slower migration pattern seen with SDS-PAGE analysis, as compared with the control AR.
To prove that the mutation at position -11 is not a common polymorphism, 102 normal chromosomes from unrelated individuals were screened for the presence of this mutation in intron 2 of the *AR* gene. The mutation was not detected in these control individuals. In addition, a larger part of intron 2 of the *AR* gene of the index patient was sequenced to exclude the presence of an additional mutation that could have induced the preferential use of the cryptic splice site. No other alterations were found in intron 2 up to position -137.

Immunoblot analysis of the AR protein

The expression plasmids pSG5AR0, pSG5AR129, and pSG5AR33, encoding the wild-type AR, AR TI, and AR TII, respectively, were transiently expressed in COS-1 cells. In the cells transfected with pSG5AR0, the 114-kDa isoform was detected after culturing in the presence of hormone. However cells, transiently transfected with either one of the receptor mutants almost lacked this third isoform (Figure 5.4A, lanes 4 and 6).

Gel-retardation assay

In order to establish whether DNA binding by the mutant AR was affected, *in vitro* binding to a consensus ARE was studied. In the presence of polyclonal antibody Sp197, which stabilizes the protein-DNA complex[28], the wild-type receptor was able to bind to a consensus ARE, resulting in a shifted probe (Figure 5.4B, lane 5). No shifted probe was detected for the mutant AR TI and TII (Fig-

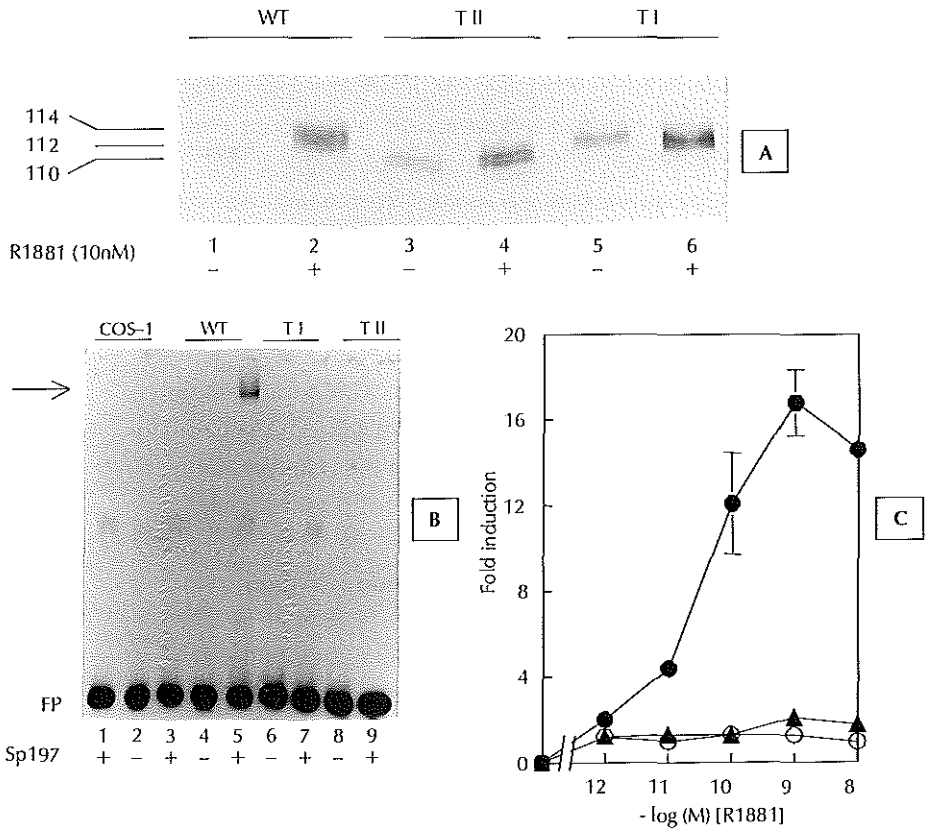

Figure 5.4
Functional analysis of wild-type and mutant androgen receptors.
A – Hormone-induced upshift of the AR proteins. AR protein variants were expressed in COS-1 cells after transfection with pSG5AR0, pSG5AR33, or pSG5AR129. The cells were cultured for 24 hours in the absence (-) or presence (+) of 10 nM R1881. After immunoprecipitation using monoclonal antibody F39.4.1, SDS-PAGE was performed followed by immunoblotting. The AR was detected by immunostaining using polyclonal antibody Sp061 and an alkaline phosphatase-coupled anti-rabbit-antibody. Lanes 1 and 2, wild-type AR; lanes 3 and 4, AR deletion mutant; lanes 5 and 6, AR insertion mutant.
B – Gel-shift assay. Cell extracts, prepared from COS-1 cells were incubated for 10 min. on ice in binding buffer with poly[dI-dC]-poly[dI-dC], either in the presence (+) or absence (-) of polyclonal anti-AR antibody Sp197. Labeled ARE probe (50,000 cpm) was added and the samples were incubated for 20 min. at room temperature. Complexes were analyzed by polyacrylamide gel electrophoresis, as described in *Subjects and Methods*. The position of the shifted complexes is indicated by an arrow, and FP indicates the position of the free probe. Lane 1, free probe; lanes 2 and 3, COS-1 extract; lanes 4 and 5, wild-type AR (WT); lanes 6 and 7, mutant AR, encoded by transcript I (TI); lanes 8 and 9, mutant AR, encoded by transcript II (TII).
C – Transcriptional activity. CHO cells were cotransfected with MMTV-Luc reporter plasmid and with either wild-type or mutant AR expression plasmid. Twenty-four hours before a luciferase assay was performed, cells were incubated with increasing concentrations of R1881. Each datapoint was tested in triplicate. ●: mean-fold induction ± SEM of wild-type AR, for three different experiments; ○: mean-fold induction ± SEM calculated for AR mutant with the insertion of 23 amino acids; ▲: mean-fold induction ± SEM of the AR mutant with the exon 3 deletion.

ure 5.4B, lanes 7 and 9) or in the control lane (Figure 5.4B, lane 3). The amount of receptor protein was checked by Western blotting and immunostaining. Comparable amounts of AR protein were incubated.

Transcription-activation assay

The wild-type AR showed transcription activation on the MMTV promoter in CHO cells (Figure 5.4C). However, AR TI and TII were unable to activate transcription on an MMTV-Luc promoter (Figure 5.4C). SDS-PAGE, followed by immunoblotting, showed that AR protein expression was identical for the three different receptor molecules (results not shown).

Allele-specific oligonucleotide hybridization

To investigate whether a wild-type transcript was present in genital skin fibroblasts of the two affected siblings in the partial-AIS family, an allele specific oligonucleotide-hybridization assay was developed. First-strand cDNA was synthesized from total mRNA, derived from genital skin fibroblasts, and was amplified in three consecutive PCR reactions. mRNA was isolated from wild-type genital skin fibroblasts, from genital skin fibroblasts from patients III-1 and III-2, and as a control from genital skin fibroblasts derived from a patient with partial AIS who has been described previously by Ris-Stalpers et al.[13] The mutation, found in this latter patient causes differential splicing, resulting in 10% wild-type mRNA and 90% mRNA of a splice variant with a deletion of exon 3. The PCR products were spotted on membranes and subsequently hybridized with oligonucleotides specific for the splice variants and the wild-type AR (Table 5.1). Hybridization with the wild-type probe revealed wild-type mRNA in genital skin fibroblasts from a normal control male, in genital skin fibroblasts from the partial- AIS patient (positive control), and in genital skin fibroblasts from one of the 46,XY patients (III-1) (Figure 5.5, WT-1b, 1c, and 1d, respectively). In genital skin fibroblasts from patient III-2, wild-type transcript was either not present or below the detection limit of the assay (Figure 5.5, WT-1e). TI was only present in genital skin fibroblasts from patients III-1 and III-2 and not in wild-type genital skin fibroblasts (Figure 5.5, TI-3d and 3e, respectively). TII was detected in genital skin fibroblasts of the positive control and patient III-1 (Figure 5.5, TII-5c and 5d, respectively). The signal at position e in Figure 5.5 (patient III-2), which was comparable to the a-specific signal at position b in Figure 5.5, resulted from cross-hybridization of the probe with wild-type DNA and was considered as background. DNA, amplified from expression

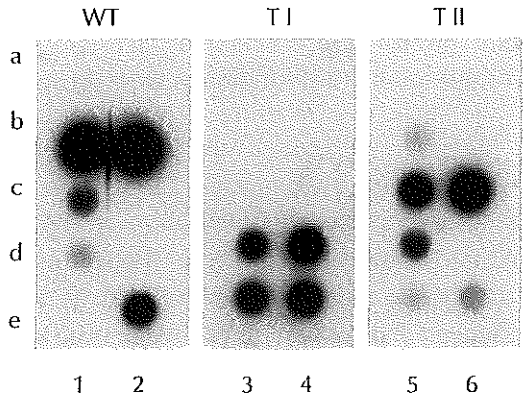

Figure 5.5
Allele specific oligonucleotide hybridization. DNAs were amplified using exon 1 sense primer 470A and exon 4 antisense primer 14 NB in two consecutive PCR reactions. The expression plasmids (pSVAR0, BHEX-AR33, and pSVAR129), used as controls, were amplified in one PCR reaction. The resulting PCR products were spotted on three separate membranes, which were subsequently hybridized with allele specific oligonucleotides as described in *Subjects and Methods*. Membrane WT: wild-type probe; membrane TI: 69 bp insertion mutant; membrane TII: exon 3 deletion mutant. For lanes 1 (WT), 3 (TI), and 5 (TII), a = blank; b = amplified cDNA of normal control; c = amplified cDNA of a patient with Reifenstein syndrome; d = amplified cDNA of patient III-1; e = amplified cDNA of patient III-2. For lanes 2 (WT), 4 (TI), and 6 (TII), a = denaturation buffer; b = amplified wild-type expression plasmid; c = amplified expression plasmid (BHEX-AR33); d = amplified expression plasmid (pSVAR129); e = mixture of amplified expression plasmids pSVAR0, BHEX-AR33, and pSVAR129 (1:1:8).

plasmids, which was used as a positive control, is visible in rows 2, 4 and 6 of Figure 5.5. Comparable amounts of DNA were spotted, as was assessed by hybridization with an exon 2 probe (results not shown).

DISCUSSION

It is well established that AIS is caused by mutations in the *AR* gene. However, reports have appeared about patients with an AIS phenotype in which no mutation was detected by use of PCR-SSCP analysis[11-12] and DNA sequencing[10], in spite of clear phenotypic, endocrinological, and biochemical evidence for AIS. Mutations might be missed because PCR-SSCP is not 100% sensitive. Often, only the exonic sequences and their flanking intronic regions are screened, leaving mutations in intronic and promoter regions undetected. For the AR, if

cells from patients are available, RT-PCR studies and Western immunoblotting can be very informative, in particular when mutations are present in intronic regions.[13,19]

In the present report, a family presenting with partial AIS is described. No mutation was detected in the *AR* gene of the index patient after PCR-SSCP analysis or sequencing of the exons. However, biochemical studies revealed that the AR of the affected family members was unable to bind to DNA. In the absence of hormone, wild-type receptor derived from transiently transfected COS-1 cells migrates as a 110-112 kDa doublet.[28] AR preparations derived from cells cultured in the presence of hormone contain, in addition to the 110-112 kDa doublet, a slower-migrating, 114-kDa isoform, representing hormone-dependent phosphorylation.[27] It has been shown that the appearance of the 114-kDa isoform is correlated with DNA binding and/or transcription activation.[27] In the present report, hormone-dependent phosphorylation of the AR protein was used as an indicator of receptor dysfunctioning. Since the 114-kDa isoform was hardly detectable in genital skin fibroblasts derived from the index subject (III-2) and her sister (III-1), cellular distribution of the AR from the index subject was studied. AR was undetectable in the tight nuclear-bound fraction of hormone-exposed genital skin fibroblasts from the index subject, which points to disturbed AR-DNA binding and, consequently, to a defect in transcription activation. Sequencing analysis revealed a mutation at position -11 in intron 2, in the *AR* gene of both siblings, their 46,XY aunt, and the index patient's mother (carrier of the mutation). Since this mutation was not found in 102 chromosomes derived from unrelated controls, we have concluded that it is not a common polymorphism.

The effect of the mutation on the splicing process was studied. Splicing of pre-mRNA begins with the cleavage at the 5' splice donor site, which is attacked by the 2-OH group of the branch-site adenosine. The 3' splice site is attacked by the newly formed 3'-OH of the upstream exon, the intron is released in the form of a lariat, and the exons are joined.[29] Around the splice acceptor site a consensus sequence, (T/C, T/C, T, T, T/C, T/C, T/C, T/C, T/C, T/C, N, C, A, G, G) important for proper splicing, is present.[30] The present AR mutation was located in this pyrimidine-rich consensus sequence preceding the splice acceptor site. Aberrant splicing took place, resulting in different transcripts encoding defective ARs, which might explain the observed phenotype. The cryptic splice site, that was used is located at position -71/-70 in intron 2, thus resulting in a mRNA variant containing an insertion of 69 nucleotides. We de-

termined that sequences in and around this cryptic splice site did not contain any mutation, thereby not enhancing preferable usage of this site.
According to Nakai and Sakamoto[31], almost all major cryptic splice sites, which are activated by mutations, are located within 100 nucleotides from the original splice site. On the basis of comparison with a consensus sequence, the cryptic splice site located at position -71/-70 in the AR shows more homology with the consensus sequence than does the wild-type splice acceptor site (the cryptic splice site is T, C, T, T, T, T, C, T, G, T, T, C, T, A, G, A; the wild-type intron 2 splice site: T, A, T, T, T, G, T, T, C, T, C, C, C, A, G, C). However, RT-PCR studies performed on wild-type mRNA demonstrated that this cryptic splice site is normally not used. Watakabe et al.[32] proposed that suboptimal 3' splice site sequences require stimulation by downstream sequences. Thus, exon 3 sequences of the AR may play a role in splice acceptor site selection. The newly created splice acceptor site at position -11/-10 was not used, probably because, compared with the novel cryptic site at position -71/-70, the sequence was less favorable for splicing. A small amount of a transcript, from which exon 3 was deleted, was detected. Wild-type transcript was not observed, although this could be expected on the basis of the partial-AIS phenotype of the affected individuals. We concluded that the wild-type splice acceptor site has become weaker because of the base pair substitution at position -11. Alternatively, closely spaced splice sites can inactivate each other because of sterical hindrance of bound splicing factors.[33] Nelson and Green[34] showed with *in vitro* splicing experiments that, in a β-globin intron, insertion of a new splice donor site close to the wild-type splice donor site prevented splicing at both sites. Therefore, aberrant splicing in our patients could also be the result of interference by two closely spaced splice acceptor sequences.

Quigley et al.[35] have previously described the so-called null phenotype of AIS. A deletion of the X chromosome spanning the complete *AR* gene caused complete AIS. The patient showed inguinal or abdominal testes, no wolffian-duct development, and absence of masculinization of the external genitalia. Sparse pubic and axillary hair was detected. Also, a complete external female phenotype and absence of secondary hair were seen in a 46,XY individual with a complete deletion of the *AR* gene, as reported by Hiort et al.[36] All AIS subjects in the family reported in this paper showed remnants of vasa deferentia and epididymides. The aunt (II-4), clinically investigated postpubertally, has pubic and axillary hair. These phenotypic characteristics suggest that some residual AR activity is present. Therefore, DNA binding and transcription-activation capacities of the splice variants were investigated. The AR, expressed in genital

skin fibroblasts from the index subject has 23 additional amino acids between the first zinc cluster and the second zinc cluster. Ducouret et al.[37] cloned a teleost-fish glucocorticoid receptor (GR) with 9 additional amino acid residues between the two zinc clusters. This fish GR, however, was still capable of activating a reporter gene, from which it was concluded that the folding of the GR could compensate for separation of the two zinc clusters by an extra 9-amino-acid stretch. The AR mutant with the insertion of 23 amino acid residues did not bind to a consensus ARE (on the basis of a gel-retardation assay). Consequently, there was absence of transcription activation. The splice variant with a deletion of the second zinc cluster was also unable to bind specifically to DNA, corresponding to the results reported by Quigley et al.[38] and Ris-Stalpers et al.[13] On the basis of these results and in view of the phenotype of the affected family members, we concluded that splicing was not completely aberrant. Indeed, a very small amount of wild-type transcript was detected in genital skin fibroblasts of patient III-1, by use of the allele-specific oligonucleotide-hybridization method. The transcript with the deletion of exon 3 (i.e., AR TII) was detected in genital skin fibroblasts of patient III-1 and not in genital skin fibroblasts of patient III-2. However, this was not reproduced in all experiments, since AR TII had previously been detected by RT-PCR studies using RNA from patient III-2.

The intronic mutation discussed in this paper was missed by PCR-SSCP analysis. There are several other explanations for the apparent absence of AR mutations in several cases of AIS. Neutral mutations, for example, have to be interpreted with caution. Richard and Beckmann[39] found a synonymous-codon mutation (GGC→GGT) in the cDNA of the *calpain (CANP3)* gene, which turned out to be pathogenic, because a splice donor site was created. Kallio et al.[40] suggested that in 46,XY subjects without a mutation in the *AR* gene and a typical AIS phenotype, post-receptor defects might be the cause of the disease. This may involve receptor-specific cofactors or corepressors. Recently, a family with dominant inheritance of thyroid hormone resistance was reported, which could not be linked to defects in the *thyroid hormone-receptor* α or β genes.[41] It was postulated that an abnormal cofactor, playing a role in regulation of thyroid-hormone action, might be involved. Most cofactors, reported to date are not AR specific, so mutations in these factors will probably be lethal or give rise to complex phenotypes. One coactivator, ARA70, which binds specifically to the ligand-bound AR, has been reported by Yeh and Chang.[7] It is not known whether mutations in the gene, encoding *ARA70* correlate with certain forms of AIS.

In conclusion, the mutation presented in this paper remained undetected with PCR-SSCP analysis. AR genes from AIS subjects for whom the clinical diagnosis is well established, and in whom no mutation can be detected after PCR-SSCP screening and additional sequencing of the coding parts of the gene should be analyzed for intronic mutations. RT-PCR studies and Western immunoblotting, using cultured genital skin fibroblasts, can provide important information.

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CHAPTER 6

Substitution of Ala-564 in the first zinc cluster of the DNA-binding domain of the androgen receptor by Asp, Asn, or Leu exerts differential effects on DNA binding

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Summary

In the androgen receptor of a patient with androgen insensitivity the alanine residue at position 564 in the first zinc cluster of the DNA-binding domain was substituted by aspartic acid. In other members of the steroid-receptor family, either valine or alanine is present at the corresponding position, suggesting the importance of a neutral amino acid residue at this site. The mutant receptor was transcriptionally inactive, which corresponded to the absence of specific DNA binding in gel-retardation assays and its inactivity in a promoter-interference assay. Two other receptor mutants with a mutation at this same position were created to study the role of position 564 in the human androgen receptor on DNA binding in more detail. Introduction of asparagine at position 564 resulted in transcription activation of a mouse mammary tumor virus promoter, although at a lower level compared to the wild-type receptor. Transcription activation of an $(ARE)_2$-TATA promoter was low, and binding to different hormone response elements could not be visualized. The receptor with a leucine residue at position 564 was as active as the wild-type receptor on a mouse mammary tumor virus promoter and an $(ARE)_2$-TATA promoter, but interacted differentially with several hormone response elements in a gel-retardation assay. The results of the transcription activation and DNA-binding studies could partially be predicted from three-dimensional modeling data. The phenotype of the patient was explained by the negative charge, introduced at position 564.

INTRODUCTION

The *AR* gene is composed of eight exons and encodes a protein of 910 amino acids with an apparent molecular mass of 110 kDa.[1] The AR belongs to a superfamily of nuclear receptors for steroid hormones, thyroid hormones, vitamin D, and retinoids. These receptors are characterized by distinct functional domains: an NH_2-terminal part, involved in transcription activation, a DBD, a hinge region, and a C-terminal part involved in ligand binding, dimerization and transcription activation.[2-3] The DBD of steroid receptors is encoded by two exons and consists of two functionally different DNA-binding zinc clusters.[4] Steroid receptors bind to HREs as homodimers, in contrast to several other nuclear receptors that can heterodimerize with the retinoid X receptor.[4] Although the structure of the DBD is well conserved between nuclear receptors, several groups of receptors bind to specific DNA sequences.[5] The GR and ER DBDs interact with distinct, although related HREs.[4,6] Three amino acid residues located in the so-called P box (proximal box) are essential for specific interaction with base pairs from the HRE, located in the major groove of DNA.[7] The GR, the AR, the MR, and the PR recognize the same HRE (AGAACAnnnTGTTCT).[5] Specificity with respect to transcription activation is probably introduced by auxiliary factors, which can change the affinity and specificity of binding sites.[4] However, recently Claessens *et al.*[8] reported an ARE in the probasin promoter that is AR specific. The consensus HRE for steroid receptors is an imperfect palindromic sequence, consisting of two half-sites, spaced by three nucleotides.[9] Binding of the first receptor molecule enhances binding of the second molecule. Important determinants for this so-called cooperativity of binding are the spacing between the two half-sites of the HRE and protein-protein contacts.[9]

Male sex differentiation and development proceed under direct control of the male sex hormones testosterone and 5α-dihydrotestosterone, and actions of both androgens are mediated by the AR. Mutations in the *AR* gene of 46,XY individuals are associated with AIS, a disorder of sex differentiation. Many abnormalities have been described, causing a wide spectrum of phenotypes, ranging from subjects with an external female phenotype and the absence of müllerian and wolffian duct derivatives, which is the CAIS, to a phenotype with ambiguous genitalia, called PAIS.[10] The most frequently reported defects are point mutations in the ligand- and DNA-binding domains of the AR.[10-11]

In the present study a mutation in exon 2 of a subject with CAIS is reported. The alanine residue at position 564 in the DBD was substituted into aspartic acid (mutant A564D). The effect of the A564D mutation on AR function was investigated, as was the effect of an asparagine substitution (mutant A564N) and a leucine substitution (mutant A564L) at this same position. These studies were completed with molecular modeling.

SUBJECTS AND METHODS

Materials

Primers were obtained from Pharmacia Biotech Benelux (Roosendaal, the Netherlands). [γ-^{32}P]ATP (specific activity: 3000 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). 17ß-Hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881; specific activity 85 Ci/mmol) and unlabeled R1881 were purchased from New England Nuclear-DuPont de Nemours ('s-Hertogenbosch, The Netherlands). The double stranded probe, containing an ARE, derived from the tyrosine aminotransferase (TAT) promoter (half-sites in *italics*) was obtained from Promega (Woerden, The Netherlands). The 27 bp oligonucleotides, used to produce two other double stranded probes, (half-sites in *italics*), containing, respectively, the strongest ARE from the MMTV promoter[12] and a consensus ARE[13], were obtained from Pharmacia Biotech Benelux.

ARE TAT	:	5' TCGA*CTGTACA*GGA*TGTTCT*AGCTACT 3'
ARE MMTV	:	5' TCGAC*GTTACA*AAC*TGTTCT*AGCTACT 3'
ARE consensus	:	5' TCGAC*GGTACA*GTT*TGTTCT*AGCTACT 3'

Clinical data

The patient exhibiting female external genitalia, atrophic epididymides and vasa deferentia, was diagnosed as having AIS at the age of 1 yr. in the absence of a positive family history. A blind ending vagina was present, the uterus was absent and testes with a normal histology for a boy of this age were present in the inguinal canal. Testosterone synthesis disorders were excluded as a cause of the 46,XY sex reversal. Genital skin fibroblasts were obtained from the index patient for Scatchard analysis and structural analysis of the *AR* gene. AR sequence analysis of relatives was performed on white blood cell genomic DNA.

Mutation detection

PCR-SSCP analysis and direct sequencing were performed as described previously.[14]

Ligand-binding study

For determination of ligand-binding characteristics of the AR of the AIS subject, Genital skin fibroblasts were incubated with serial dilutions [^{3}H]R1881 (0.02, 0.05, 0.3, 1.0, 3.0 nM, respectively) in serum free medium. The binding assay was performed as described previously.[14]

Western blot analysis

AR protein, derived from genital skin fibroblasts or transiently transfected CHO cells was immunoprecipitated and analyzed by Western immunoblotting according to the method of Ris-Stalpers et al.[15]

Construction of AR expression vectors

pAR(0), a human wild-type AR complementary DNA expression plasmid was described previously.[1] Expression plasmids encoding the various mutants, pAR(A564D), pAR(A564N) and pAR(A564L) respectively, were constructed by site directed mutagenesis. The *Kpn*I-*Asp*I digested fragment of pAR(0) was exchanged with mutated *Kpn*I-*Asp*I fragments generated in two separate PCR amplifications.[16] Sense primer 470A[14], located upstream of the *Kpn*I site in exon 1, was combined with an antisense primer, containing the mutation (antisense primers: construct A564D, 5'CATGTGAGAtCTCCATAGTGACAC 3';
construct A564N, 5'CATGTGAGAttTCCATAGTGACACCC 3';
construct A564L, 5' CATGTGAGAagTCCATAGTGACACCC 3').

A sense primer, introducing the mutation (sense primers:
construct A564D, 5' GTGTCACTATGGAGaTCTCACATG 3';
construct A564N, 5' GGTGTCACTATGGAaaTCTCACATGTGG 3';
construct A564L, 5' GGTGTCACTATGGActTCTCACATGTGG 3'),
was used in combination with an antisense primer 14NB[14], located downstream of a unique *Asp*I site in exon 4. One microliter of both PCR products was used as template in a second PCR reaction using primers 470A and 14NB. The resulting PCR fragment was digested with *Kpn*I and *Asp*I, and exchanged for the corresponding wild-type fragment in pAR0.

pSG5AR(0), a human wild-type AR cDNA expression vector (provided by Dr. A.C.B. Cato, Karlsruhe, Germany) was used for transient transfection of COS-1 cells. pSG5AR(A564D), pSG5AR(A564N) and pSG5AR(A564L) were constructed by exchanging the 472 bp *Kpn*I-*Asp*I insert from pSG5AR(0) and the *Kpn*I-*Asp*I fragment from the pSVAR plasmids, encoding the variant ARs.
MMTV-Luc reporter plasmid, cytomegalovirus (CMV)-Luc and the CMV-$(ARE)_3$-Luc reporter plasmids and pJH4-$(ARE)_2$-TATA-Luc, containing the TATA-box and an Sp1-site derived from the *Oct* 6-gene promoter have been described previously.[17-19]

Cell culture conditions and transfections

Genital skin fibroblasts and COS-1 cells were cultured as previously described.[20] CHO cells were cultured according to the COS-1 cell culture protocol. The CHO cells, used for transcription activation studies and promoter-interference assays were plated in 7 or 11 cm^2 (promoter-interference assay) wells and grown for 24 hours. Cells were cotransfected, using the calcium-phosphate method[21], with AR expression plasmid (10 ng/ml precipitate) and reporter plasmid (2 µg/ml precipitate). Carrier DNA (pTZ19) was added to a total of 20 µg DNA/ml precipitate, and 90 µl precipitate was added per well. In the promoter-interference assay, 300 ng AR expression plasmid and 30 ng reporter plasmid (CMV-Luc or CMV-$(ARE)_3$-Luc), respectively, were added per ml precipitate. pTZ19 was added to a total of 20 µg DNA/ml precipitate, and 250 µl precipitate were added to 11 cm^2 wells. The transfection and luciferase assay were performed as described before.[22] Both transcription activation studies and the promoter-interference assay were performed at least three independent times in triplicate, using three independent isolates of expression plasmid. In case of transcription activation studies, luciferase activity was expressed, relative to basal activity measured after culturing in the absence of hormone. For promoter interference studies, luciferase activity in cells, transfected with CMV-$(ARE)_3$-Luc and AR expression plasmid and cultured in the absence of hormone was set at 100%. Inhibition of promoter activity in the presence of hormone was expressed relative to this 100% activity. CHO cells used for expression studies by Western blotting were also transiently transfected by the calcium-phosphate method. To this end, cells were plated in 175 cm^2 culture flasks and transiently transfected with 20 µg expression plasmid. COS-1 cells were plated in 80 cm^2 culture flasks and transfected with 9.4 µg expression plasmid, using the diethylaminoethyl-dextran method.[23] Twenty-

four hours before harvesting, CHO and COS-1 cells were washed and incubated with medium containing 1 nM R1881.

Gel-retardation assays

Transfected COS-1 cells were collected in 5 ml PBS and the pellet was resuspended in extraction buffer [10 mM NaH_2PO_4 (pH 7.4), 0.4 M KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM bacitracin, 0.5 mM leupeptin and 0.6 mM PMSF, 10 mM DTT] and subjected to four freeze- thaw cycles, followed by 10 minutes centrifugation at 400,000xg at 4 C in a TLA120.2 rotor (Beckman, Fullerton, CA) in a Beckman Optima TLX ultracentrifuge. The TAT ARE containing probe (5'-TCGAC*TGTACAGGATGTTCT*AGCTACT-3') (half-sites in *italics*) was obtained from Promega. Two other probes were produced by annealing a 27-bp oligonucleotide with an oligonucleotide of complementary sequence. One of them (5'-TCGAC*GTTACAAACTGTTCT*AGCTACT-3') (half-sites in *italics*) contains the strongest ARE from the MMTV promoter[12], and the other probe (5'-TCGAC*GGTACAGTTTGTTCT*AGCTACT-3') (half-sites in *italics*) contains a consensus ARE.[13] The ARE containing probes were end-labeled using T4 polynucleotide kinase and double stranded probe was purified from a 4% acrylamide gel, in 0.5 x TBE (1 x TBE= 50 mM Tris base, 50 mM boric acid, 1 mM EDTA, pH 8.6). Cellular extracts were incubated in binding buffer [10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 4% ficoll], 1 µg polydeoxyinosinic-deoxycitidylic acid (poly[dI-dC]-poly[dI-dC]) in the absence or presence of the polyclonal AR antibody Sp197 (10-fold diluted).[24] After an incubation period of 10 minutes on ice, 2 µl purified DNA probe (50,000 cpm/µl) were added and incubation was continued for 20 minutes at room temperature. The 20-µl sample was separated on a 4% polyacrylamide gel in 0.5 x TBE. Gels were fixed for 10 minutes in 10% acetic acid-10 % methanol, and subsequently dried and exposed.

Molecular modeling

The crystal structure of the rat GR DBD, bound to a GRE was used as a basis upon which the 3-D AR models were built. The 3-D model of AR bound to a GRE has previously been described.[25] The A564D, A564N and A564L mutants were built according to the same strategy as that used to build the wild-type model. Briefly, the side-chains of the AR mutants that were substituted in the GR model were placed in energetically favorable conformations, using the SMD program.[26] The whole system was then energy minimized with the

AMBER program (Pearlman et al. 1991, University of California, San Francisco, CA). During the optimization process, the oligonucleotide was kept frozen to prevent unrealistic deviation from the initial crystal structure. Moreover, positional restraints on the backbone and on side-chains of conserved residues were applied and gradually released during the optimization. Figure 6.5A was generated with the Insight II viewer (Biosym Technologies, San Diego, CA) and Figure 6.5B was generated using the program MOLSCRIPT.[27]

RESULTS

Mutation detection

Genomic DNA of the index patient was used to amplify the coding part and intronic sequences flanking the exons of the *AR* gene, followed by SSCP analy-

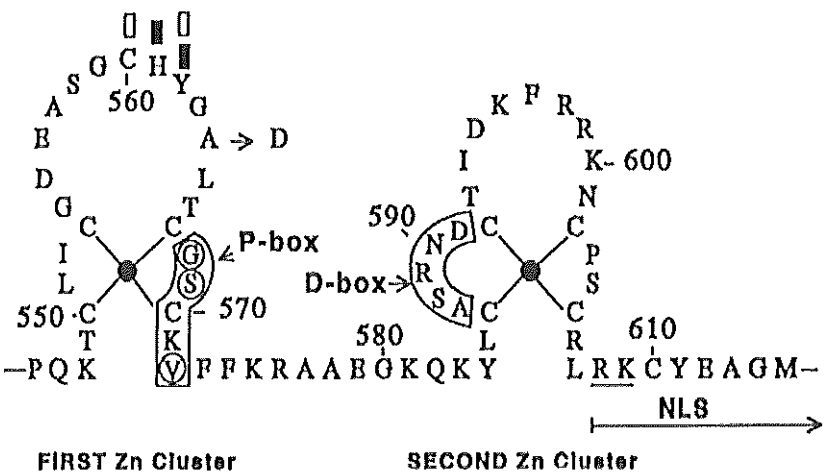

Figure 6.1
Sequence of the AR DBD with the A→D mutation, located in the first zinc cluster. The mutation found in the index patient's AR is present at position 564 of the AR DBD, located near the P-box of which the circled amino acid residues are involved in ARE recognition. The boxes indicate amino acid residues that interact with the phosphate backbone of DNA, either at specific (black boxes) or at nonspecific sites (open boxes).[25,28] The second zinc cluster contains the D-box, which is involved in dimerization with the other AR. The first part of the nuclear localization signal (NLS) is also shown *(underlined)*. The numbering of the various codons is based on a total of 910 amino acid residues in the human AR.[1]

sis performed under two different conditions. An aberrant banding pattern was found for exon 2, which encodes the first zinc cluster of the DBD. Direct sequencing showed a single nucleotide substitution at codon 564 (C to A) that resulted in substitution of alanine to aspartic acid (Figure 6.1). The numbering of amino acid residues throughout the text is based on a total number of 910 amino acid residues in the human AR.[1] The mutation created a *Bgl*II site, which was used to investigate the segregation of this mutation in the family of the index patient. The mother and grandmother of the index patient were heterozygous carriers of this AR mutation (results not shown).

Functional properties of the mutant receptor

The AR protein was isolated from genital skin fibroblasts, obtained from the index subject. Molecular mass was checked by immunoblotting after immunoprecipitation. The AR protein migrated as a normal 110-112 kDa doublet on SDS-PAGE (data not shown). For Scatchard analysis, cultured genital skin fibroblasts were incubated for 1 hour with increasing concentrations of [^{3}H]R1881 either in the presence or absence of a 200-fold molar excess of non-radioactive R1881. Both the Kd (0.07 nM) and Bmax (58 fmol/mg protein) values were within the normal range (Kd: 0.03-0.13 nM, Bmax: 39-169 fmol/mg protein), indicating that ligand binding was not affected by the mutation.

Transcriptional activity of AR A564D

AR A564D was inactive in CHO cells cotransfected with $(ARE)_2$-TATA-Luc, in contrast to the wild-type AR (Figure 6.2A). The promoter of this reporter construct contains a TATA-box and Sp1 site derived from the *Oct-6* gene promoter, and two AREs.[17] In general, comparable levels of wild-type and mutant receptor protein were expressed in transiently transfected CHO cells, as verified by SDS-PAGE and immunoblotting (for example, see Figure 6.3C). AR A564D was also unable to activate transcription from the complex MMTV promoter in CHO cells (Figure 6.2B).

In vivo DNA binding of AR A564D

In vivo DNA binding was studied by a promoter-interference assay. CHO cells were cotransfected with CMV-$(ARE)_3$-Luc. Three consensus AREs are inserted between the TATA-box of the constitutively active CMV promoter and the transcription start site of the *luciferase* gene.[13] Binding of the AR hinders the assembly of a transcription initiation complex and, therefore, also interferes

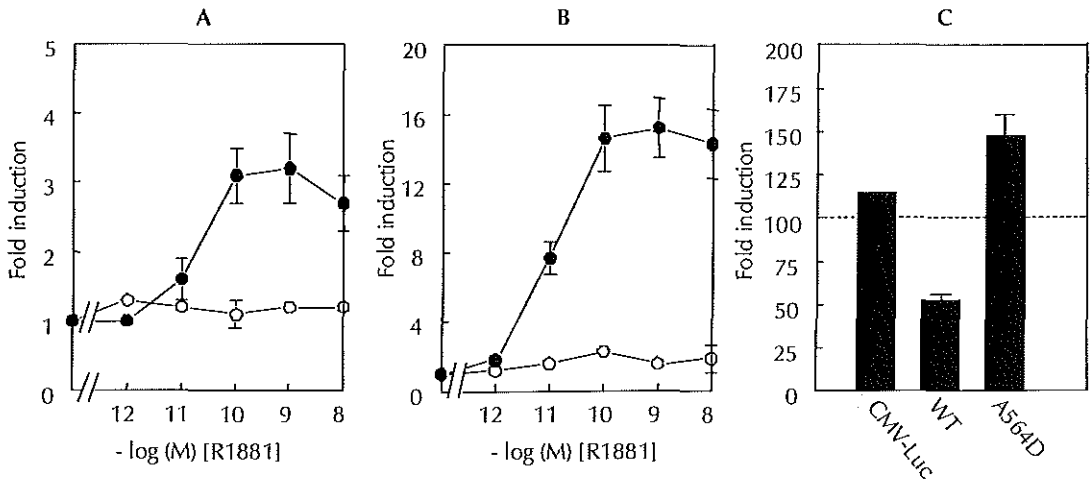

Figure 6.2

– **A and B:** Transcriptional activity of AR A564D at different promoters. CHO cells were cotransfected with a reporter plasmid [$(ARE)_2$-TATA-Luc or MMTV-Luc] and the wild-type or mutant expression plasmid. After 24 hours cells were cultured with medium containing increasing concentrations of R1881 for another 24 hours before a luciferase assay was performed. Each data point was tested in triplicate. **A,** Induction of luciferase activity was calculated from five different experiments, in which $(ARE)_2$-TATA-Luc was used as a reporter gene. Symbols represent the mean ± SEM (●: wild type, O: A564D). Transcription activation by AR A564D was significantly different from that by the wild-type AR (by Student's *t* test, $p = 0.05$). **B,** Induction of luciferase activity was calculated from three different experiments, in which MMTV-Luc was used as a reporter gene. Symbols represent the mean ± SEM (●, wild type; O, A564D). Transcription activation by AR A564D was significantly different from that by wild-type AR (by Student's *t* test, $p = 0.05$).

– **C:** Promoter interference of wild-type AR and AR A564D. CHO cells were transiently transfected with expression plasmid and CMV-$(ARE)_2$-Luc reporter plasmid. Twenty-four hours after transfection, cells were cultured in the absence or presence of 1 nM R1881 and incubated for another 24 hours before performing a luciferase assay. Each data point was tested in triplicate. CMV-Luc reporter construct cotransfected with pAR0 was taken as a control (n=2). The luciferase signal, measured in the absence of R1881 was arbitrarily set at 100%, and activities, measured in the presence of 1 nM R1881 were related to these values. The mean promoter activity ± SEM in the presence of hormone is represented.

with the expression of the *luciferase* gene.[19] The level of inhibition is taken as a measure of specific DNA binding. In the presence of 1 nM R1881, the wild-type AR showed a 48% reduction of luciferase activity whereas no reduction was seen in cells cotransfected with AR A564D (Figure 6.2C). The AR could sequester factors that are essential for transcriptional activity of the CMV promoter (squelching). However, no reduction of luciferase expression was seen in cells cotransfected with CMV-Luc (Figure 6.2C).

Transcriptional activities of AR A564N and AR A564L

To investigate whether the inactivity of AR A564D was caused by steric hindrance or by a conformational change due to the introduction of a negative charged amino acid residue, the alanine residue was replaced by either a leucine residue (A564L) or an asparagine residue (A564N). Leucine has a larger side chain, like the aspartic acid residue. However, leucine is a neutral amino acid as is the alanine residue present in the wild-type receptor. Asparagine has also a larger side chain, but is a polar amino acid residue. CHO cells were transiently cotransfected with AR expression plasmids and $(ARE)_2$-TATA-Luc. Wild-type AR and AR A564L showed comparable activation of the minimal promoter at increasing amounts of R1881, whereas AR A564N showed strongly reduced transcription activation compared to the wild-type AR (Figure 6.3A). On the more complex MMTV promoter AR A564L showed activity comparable to that of the wild-type receptor and AR A564N displayed a low level of hormone induced transcription activation (Figure 6.3B). All proteins were expressed, and in general, expression levels were comparable (Figure 6.3C).

In vivo DNA binding of AR A564N and AR A564L

DNA binding was studied in CHO cells, cotransfected with CMV-$(ARE)_3$-Luc (Figure 6.3D). In contrast to the wild-type AR, luciferase expression was not lowered after cotransfection of cells with AR A564N and culture in the presence of hormone. AR A564L showed 38% inhibition, which was significantly different ($p<0.05$) from the 48% inhibition observed for the wild-type receptor. Protein expression levels were identical for all mutant receptors (see also Figure 6.3C).

In vitro DNA binding, comparing different AREs

The DNA-binding capacities of the wild-type and the different AR mutants were tested *in vitro* in gel-retardation assays, using probes containing various AREs. AR was produced in transfected COS-1 cells. The amount of receptor protein was checked by Western blotting, followed by immunostaining. Comparable amounts of AR in COS-1 cellular extracts were incubated with a ^{32}P-labeled probe in either the absence or presence of polyclonal antibody Sp197, which stabilizes AR-dimers bound to the DNA.[24] Wild-type AR and AR A564L did bind to the ARE, derived from the TAT promoter (Figure 6.4A: lanes 3 and 9), whereas binding of AR A564D and AR A564N could not be detected (Figure 6.4A: lanes 5 and 7). Gel-retardation assays were also performed with two

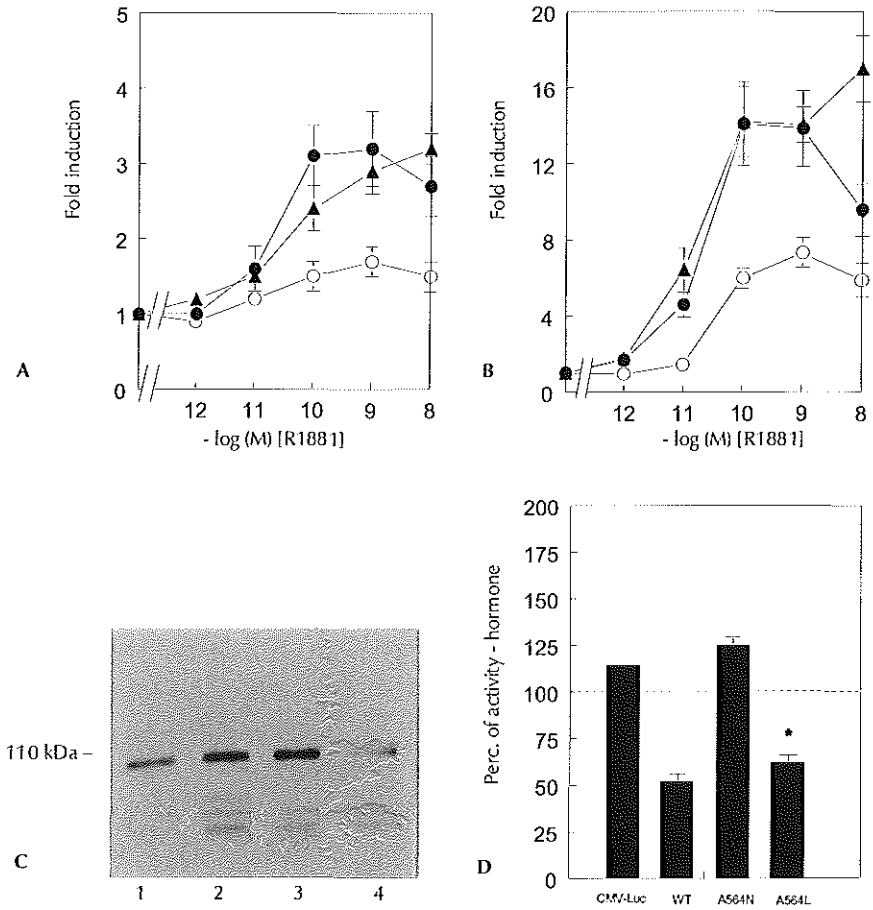

Figure 6.3

– A and B: Transcription activation by the wild-type receptor and A564N and A564L using different reporter genes. CHO cells were cotransfected with a reporter plasmid [$(ARE)_2$-TATA-Luc or MMTV-Luc] and either the wild-type or one of the mutant expression plasmids. Twenty-four hours after transfection, cells were cultured in medium containing increasing concentrations of R1881 for another 24 hours. Each data point was tested in triplicate. A, Induction of luciferase activity was calculated from five different experiments in which $(ARE)_2$-TATA-Luc was used as a reporter gene. Symbols represent the mean ± SEM (●: wild type, O: A564N, ▲: A564L). Transcription activation by AR A564N was significantly different from that displayed by the wild-type AR (by Student's *t* test, $p = 0.05$). B, Induction of luciferase activity was calculated from five different experiments in which MMTV-Luc was used as a reporter gene. Symbols represent the mean ± SEM (●: wild type, O: A564N, ▲: A564L). Transcription activation by AR A564N was significantly different from activation, displayed by the wild-type receptor (Student's *t*-test, $p = 0.05$).

– C: Western blot analysis of wild-type and mutated ARs after transient transfection in CHO cells. Culture flasks with CHO cells were transfected with expression plasmid and after 48 hours cell lysates were prepared. From these lysates, the receptor was immunoprecipitated with monoclonal antibody F39.4.1 and separated on a SDS-PAGE gel. After blotting, receptor protein was detected with polyclonal antibody Sp061 and an alkaline phosphatase-coupled goat anti-rabbit antibody. Lane 1, wild-type AR; lane 2, AR A564D; lane 3, AR A564N; lane 4, AR A564L.

other probes. One of the probes contained the strongest ARE from the MMTV promoter[12], which was also present in $(ARE)_2$-TATA-Luc. The other probe contained a consensus ARE[13], which was also cloned behind the constitutively active CMV promoter that was used for promoter interference studies. Wild-type AR was able to shift the probes in the presence of antibody, indicative of specific DNA binding (Figure 6.4B: lane 3, and Figure 6.4C: lane 3). Neither probe could be shifted with AR A564D (Figure 6.4B, lane 5, and Figure 6.4C, lane 5) or AR A564N (Figure 6.4B, lane 7, and Figure 6.4C, lane 7), although A564N showed transcription activation on a complex MMTV promoter and even on a minimal ARE promoter. AR A564L interacted with both probes, although less efficiently than the wild-type AR, which is in agreement with the results of the promoter-interference assay (Figure 6.4B, lane 9, and Figure 6.4C, lane 9).

Molecular modeling

The alanine residue at position 564 is buried, as it is involved in a hydrophobic cluster that is mainly formed by leucine 551, isoleucine 552, cysteine 610, alanine 613, and methionine 615 (Figures 6.1 and 6.5, A and B). The Cα-Cß bond of the alanine residue at position 564 is directed towards the cysteine residue at position 610 in the protein core. The backbone of residue 564 is hydrogen-bonded with the backbone of histidine residue 561, as both residues belong to a ß-hairpin (Figure 6.5B). They are located at the same side of the hairpin at facing positions. The histidine residue at position 561 is involved in direct contacts with DNA and participates in ARE recognition (Figure 6.5B). Molecular modeling showed that in the A564D mutant the aspartic acid residue is still buried. However, burying of charged residues is unfavorable, unless a compensatory charge forms a salt bridge and is also buried at the same site. The only way AR A564D can take a more favorable conformation, is by breaking of the ß-hairpin and the hydrogen bonding with the histidine residue

– **D:** Promoter-interference assay of AR A564N and AR A564L. The DNA-binding properties of the mutated receptors were also tested in intact cells. CHO cells were transiently transfected with expression plasmid and CMV-$(ARE)_2$-Luc reporter plasmid. Twenty-four hours after transfection, cells were cultured in the absence or presence of 1 nM R1881 and incubated for another 24 hours before performing a luciferase assay. Each data point was tested in triplicate. The CMV-Luc reporter construct, cotransfected with pAR0 was taken as a control (n=2). The luciferase signal, measured in the absence of R1881 was arbitrarily set at 100%, and activities, measured in the presence of 1 nM R1881 were calculated relative to these values. The mean promoter activity ± SEM in the presence of hormone is represented (n=4). *, Significantly different from inhibition shown by the wild-type receptor (by Student's *t* test, $p < 0.05$).

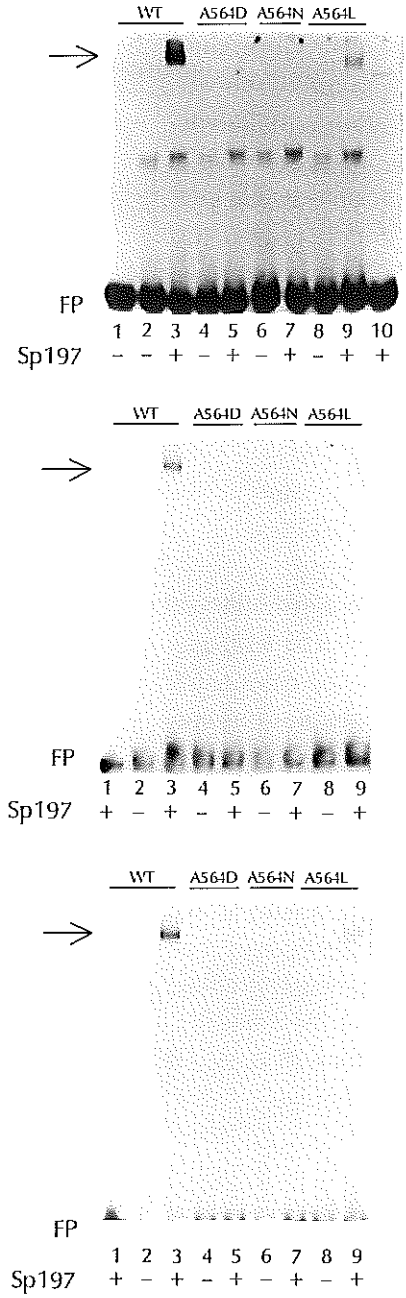

Figure 6.4
Gel-shift assay with three different ARE-probes.
A labeled ARE-probe (50,000 cpm) was incubated with nuclear extracts, prepared from transiently transfected COS-1 cells. Incubations were performed in the absence (-) or presence (+) of the polyclonal antibody Sp197. The complexes were analyzed by polyacrylamide gel electrophoresis as described in *Experimentals*. The position of the shifted complex is indicated by an arrow, FP indicates the position of the free ^{32}P-probe.
– **A:** The probe contained an ARE, derived from the TAT promoter. Lane 1, No receptor protein; lanes 2 and 3, wild-type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L; lane 10, free probe. After incubation of the probe with the antibody, no specific shifted band could be seen.
– **B:** The probe contained the strongest ARE, derived from the MMTV promoter (Ham et al. 1988). Lane 1, No receptor protein; lanes 2 and 3, wild-type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L.
– **C:** The probe contained an consensus ARE.[13] Lane 1: No receptor protein; lanes 2 and 3, wild-type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L.

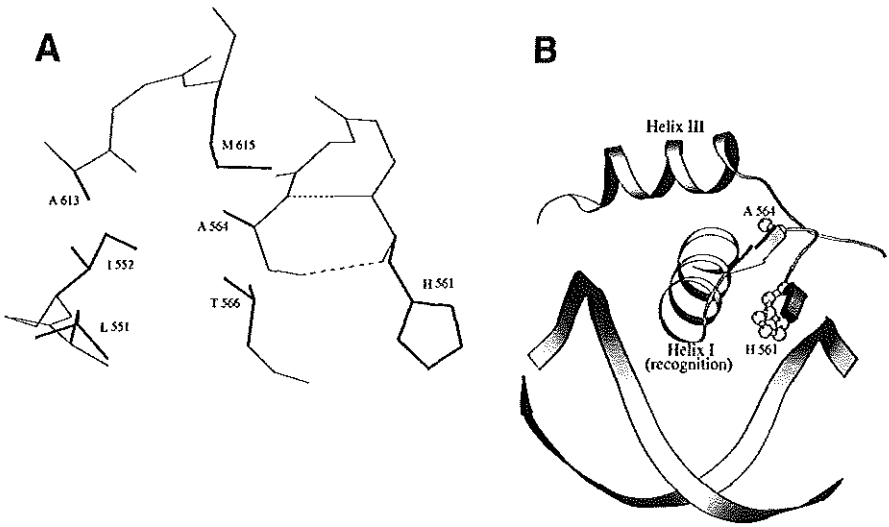

Figure 6.5
– **A:** View of the hydrogen bonding between the alanine residue at position 564 and the histidine residue at position 561. The hydrogen bonding is shown by the dashed lines. Also shown are residues constituting the hydrophobic pocket in which the alanine residue at position 564 is buried (i.e. leucine 551, isoleucine 552, threonine 566, alanine 613, and methionine 615). The backbone is shown by thin lines, and the side chains are shown as thick lines.
– **B:** Schematic view of part of the ARE and the wild-type AR. Helix I, helix III, and the side chains of the histidine residue at position 561 and the alanine residue at position 564 (ball and stick) are shown. The histidine residue and the alanine residue belong to a small ß-hairpin (strands shown as thick arrows) and the histidine residue makes direct contacts with DNA. Part of the ARE is displayed as long thin antiparallel arrows.

at position 561. This most likely affects the conformation of the histidine residue 561, resulting in disturbed ARE recognition. The asparagine residue in A564N should lead to smaller perturbations because it will remain buried, although asparagine is a polar residue. The modeling showed that hydrogen bonding of asparagine with threonine 566 may roughly compensate for the unfavorable burying of polar atoms. Modeling of the A564L mutant showed that the larger leucine side-chain could be accommodated with out difficulties in the hydrophobic pocket. Burying of the larger hydrophobic surface may even provide additional stability to the AR. Therefore, the leucine mutant was not expected to significantly perturb DNA recognition.

DISCUSSION

The DBD is the most conserved region within the nuclear-receptor family. Characteristic are the eight cysteines in this domain, forming the two zinc clusters. Zinc ion-coordinated binding is essential for proper folding and DNA binding. The structure of the DBDs of the GR and ER respectively, in complex with their response element on the DNA, was solved by X-ray crystallography. Almost similar conformations were displayed.[28-29] The AR DBD is more closely related to the GR DBD sequence than to the ER DBD sequence. Fifteen amino acid residues in the AR DBD, which is defined as a 65 amino acid domain starting at lysine 548, are different from the GR DBD.[30-31] Only 5 of them are located in the first zinc cluster, and 1 of these should be considered as a conservative change according to the chemical properties of its side-chain.[31] Therefore, it seems reasonable to deduce the structural consequences of mutations in the AR DBD from the 3-D structure of the GR DBD.

In the present paper the characterization of a mutation at position 564 in the first zinc cluster of the DBD of the human AR is reported. Mutations in the first zinc cluster of the AR have been described previously.[11] Some of them disrupt the zinc cluster structure because one of the cysteines is substituted, resulting in a CAIS phenotype.[11] Alanine 564 is partially conserved in other members of the receptor family. At the corresponding position in the vitamin D receptor (VDR) an alanine residue is present as well.[32] In the human ER, human GR, human PR, and human MR a valine residue is located at the corresponding position, implying that the presence of a neutral amino acid residue at this position is critical for proper interaction of the receptor with DNA.[6,30,33-34] In the AR of the patient, described in this report, alanine was substituted by the negatively charged aspartic acid residue. The aspartic acid residue is located upstream of an α-helical region that is exposed to the major groove of DNA, and downstream of cysteine 560, histidine 561 and tyrosine 562, which are involved in specific as well as non-specific contacts with the phosphate backbone of the DNA.[28] Substitution of the latter residues by non-conservative amino acids in the GR resulted in loss of function *(in vivo)* and *in vitro* reduced DNA-binding affinity was seen.[35] Warriar et al.[36] substituted the cysteine residue at position 560 in the human AR by a serine residue. Although this is a relatively conservative change, decreased DNA binding and transcription activation were observed, which was attributed to the instability of the AR mutant-DNA complex.

We showed that AR A564D displayed defective transcription activation. Specific binding to DNA, which was studied *in vitro* by gel-shift assays with oligonucleotide probes containing different AREs, and *in vivo* with a promoter-interference assay, could not be detected. From 3-D modeling studies it became clear that the alanine residue at position 564 is buried in a hydrophobic cluster (Figures 6.1 and 6.5A). A hydrogen bond is formed between the backbone of alanine 564 and the backbone of histidine 561. This latter residue is involved in direct interaction with the phosphate backbone of the DNA and is conserved in other nuclear receptors. Yagi et al.[37] reported a patient with hereditary 1,25-dihydroxyvitamin D-resistant rickets caused by substitution of the conserved histidine at position 35 of the VDR, comparable to histidine 561 in the AR. The phenotype of the patient was caused by perturbation of the conserved site that contacts the phosphate backbone of DNA. For AR A564D, the computer model displayed almost the exact conformation as that seen for the wild type, showing that there is no steric hindrance due to the mutation. However, buried charged residues are only observed in proteins when they can form salt bridges with residues of opposite charge. Therefore, the modeled conformation appears unlikely. Probably, aspartic acid 564 adopts a more favorable conformation, and as a result, the main chain hydrogen bond between aspartic acid 564 and histidine 561 will be broken, which has consequences with respect to DNA binding.

To investigate the role of the alanine residue at position 564 in more detail, the residue was also replaced by an asparagine or a leucine residue. 3-D modeling predicted that introduction of an asparagine residue should have intermediate effects with respect to transcription activation. Hydrogen bonding between asparagine 564 and threonine 566 might compensate for unfavorable burying of the polar amino acid residue. Substitution by an asparagine residue resulted in a less stable AR-DNA complex which had clear consequences for transcription activation on a minimal $(ARE)_2$-TATA promoter and to a lesser extent on a complex MMTV promoter. DNA binding was not observed *in vitro* by gel-shift analysis. DNA-binding capacity remained undetectable, even when studied in whole cells. However, the functionality of AREs is determined by additional transcription factor binding sites in the vicinity of AREs. Interaction with other proteins might stabilize the AR-DNA complex. This might explain as well the stronger activation of AR A564N on the MMTV promoter, compared to its activity on the minimal $(ARE)_2$-TATA promoter.[8,38-39]
Modeling showed that no particular constraint resulted from the larger size of the leucine residue. It was predicted that the leucine residue, because of its

larger hydrophobic surface, provides even more favorable stability to the AR. However, reduced DNA-binding affinity, *in vitro* as well as *in vivo*, was observed for AR A564L. Transcription activation was comparable with activation displayed by the wild-type AR. Apparently, molecular modeling has some limitations, with respect to the prediction of complex interactions which might be explained by the fact that the model was based upon the crystal structure, which was solved for the GR DBD. In addition, functional studies were performed with the intact receptor and not only the DBD.

In conclusion, the negative charge, introduced by the aspartic acid residue, destabilizes the normal conformation of the AR DBD, resulting in disturbed ARE recognition, in agreement with the phenotype of the patient expressing this mutant receptor. Results from the functional assays were partially supported by predictions, made by 3-D modeling. Although not predicted by molecular modeling, steric hindrance might have an impact on the DNA-binding capacities of AR A564N and AR A564L.

Acknowledgements

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CHAPTER

7

Germline and somatic mosaicism in the androgen insensitivity syndrome; implications for genetic counseling

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The androgen insensitivity syndrome (AIS)(MIM 300681/312300) is a disorder of male sexual differentiation caused by a defective, deficient, or absent androgen receptor (AR). The AR gene is located on the X-chromosome, therefore AIS is an X-linked disease of which 46,XX individuals can be carriers.
Mutation detection in the AR gene is increasingly used for diagnosis, carrier detection for genetic counseling and prenatal diagnosis.[1-7]
In numerous X-linked or autosomal dominant diseases as Duchenne muscular dystrophy, hemophilia A, lethal osteogenesis imperfecta, neurofibromatosis type I, and tuberous sclerosis somatic or germline mosaicism for "de novo" mutations has been found. In those cases the mutation was present in some of the cells in one of the clinically unaffected parents.[8-14]
Here, we provide the molecular and genetic evidence for the occurrence of germline and somatic mosaicism in a carrier of AIS, a mosaicism that was undetectable with PCR-SSCP and direct sequencing.

In a consanguineous family of Moroccan descent (pedigree in Figure 7.1A) studied as part of a study on genotype versus phenotype relationship in AIS, an AR gene mutation was found in two affected siblings (exon 7; codon 846 change of nucleotide G to A; arginine to histidine). In vitro studies have proved this R846H mutation to be pathogenic.[15] With the use of PCR-SSCP, no other mutations were found in the coding sequences and flanking intron sequences of the AR gene. Both patients (II-5 and II-8, Figure 7.1A) had the partial form of AIS with a dramatic difference in severity of the phenotype (described in chapter 4). However with PCR-SSCP and direct sequencing after separate PCR reactions of the appropriate fragment, we could not detect this mutation in DNA derived from peripheral lymphocytes of the mother (I-2, Figure 7.1B). Several polymorphisms in the AR gene[16-18] are known and are sometimes used in prenatal diagnosis when a causative mutation is yet unidentified.[2,19] We used the $(CAG)_nCAA$ repeat in exon 1 (indicated hereafter as CAGs) to study the segregation of the mutant allele in this family (Figure 7.1C). The mutant AR allele contained 14 trinucleotide repeats in both affected siblings (II-5 and II-8). The mother (I-2) showed, in addition to the allele with 14 CAGs, an AR allele with 21 CAGs on the other X-chromosome. Therefore, this marker seemed informative in this segregation study. It was surprising that one unaffected brother (II-7) had also inherited the AR allele with 14 CAGs, but without the mutation (Figure 7.1B). This segregation pattern proved that a germline mosaicism was present in the mother. By use of an allele-specific oligonucleotide slot-blot assay of PCR products from the same DNA samples of the various family members, it was shown that the mother's (I-2) DNA hybrid-

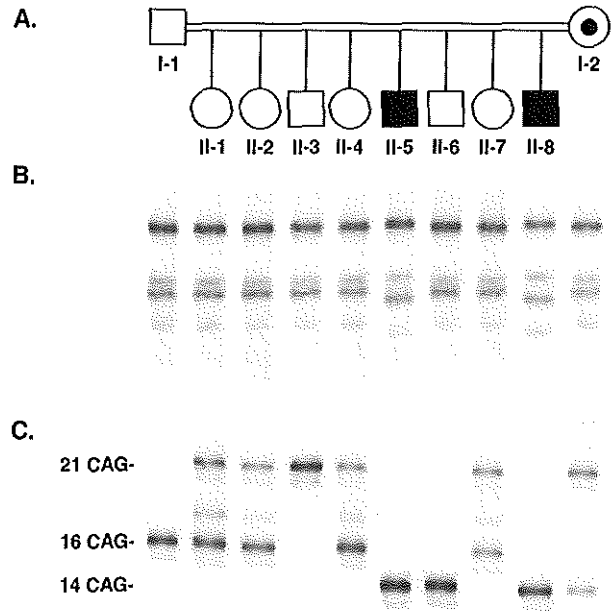

Figure 7.1
A – Pedigree of the family with two siblings with partial AIS and the R846H mutation, nucleotide change G→A in exon 7 of the AR-gene. The parents (I-1, I-2) were consanguineous (their grandfathers were sibs) and from Moroccan ancestry. ■ 46,XY individuals with androgen insensitivity. II-5 was raised as a girl and had predominantly female genitalia with labial fusion. II-8 had a micropenis, bifid-, shawlscrotum and cryptorchidism and was raised as a boy. □ = normal male phenotype; O = normal female phenotype as established after clinical examination.
B – Segregation analysis of the R846H mutation using PCR-SSCP for exon 7 of the AR-gene (as described by Ris-Stalpers et al.[23] DNA was derived from peripheral lymphocytes. The SSCP was repeated with separate PCR reactions giving identical results.
C – CAG-trinucleotide-repeat analysis in exon 1 of the AR-gene (as described by Ris-Stalpers et al.[23]).

ized with the normal as well as with the R846H probe (Figure 7.2A and 7.2B). This demonstrated, in addition to a germline mosaicism, the presence of a somatic mosaicism for R846H and the wild-type sequence in the mother (I-2). The intensity of the hybridization signal of the R846H versus the normal allele suggested that the amount of R846H was less than 10% of the normal allele in peripheral lymphocytes (Figure 7.2).

This observation of somatic and germline mosaicism points to a methodological, as well as a counseling, problem in AIS. PCR-SSCP and direct sequencing gave false negative results in carrier testing of the mother. The presented observation of somatic/germline mosaicism, also in AIS, necessitates careful interpre-

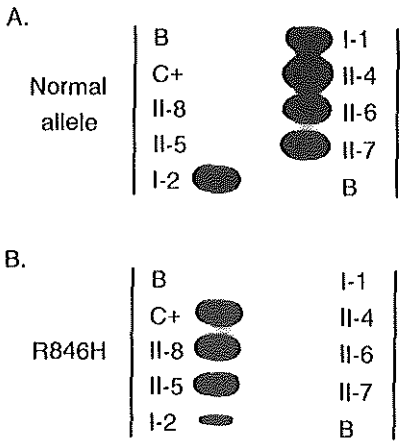

Figure 7.2
Slot-blot hybridization of the family's DNA with normal and R846H allele-specific oligonucleotides.
Hybridization with allele specific oligonucleotides of a slot blot containing PCR products of exon 7 of the AR. Hybridization was carried out with oligonucleotides containing either the wildtype sequence (Figure 7.1A) or the R846H mutant sequence (Figure 7.1B). DNA derived from peripheral lymphocytes of the various family members was used. PCR of exon 7, was done in a 50 µl reaction using 5' TGC TCC TTC GTG GGC ATG CT 3' as forward primer and 5' TGG CTC TAT CAG GCT GTT CTC 3' as backward primer. Slot blots were prepared in a Schleicher & Schuell apparatus according to the manufacturer's protocol. Allele specific oligonucleotides: WT 5'TCA AGA CGC TTC TAC 3' and R846H 5' TCA AGA CAC TTC TAC 3' were labelled with γ^{32}P-ATP.[24] Hybond filters N-plus were prehybridized at 37°C with hybridizationmix (5% SSPE {200mM $NaH_2PO_4H_2O$, 3M NaCl, 20 mM EDTA pH 7.4}. Subsequently one filter was incubated with the wildtype and one with the mutant oligonucleotide at 37°C for one hour. Filters were rinsed in 3 x SSC + 0.1% SDS, followed by 3 consecutive washsteps each for 5 minutes in 1x SSC + 0.1% SDS, 0.3x SSC + 0.1% SDS, 0.1x SSC + 0.1% SDS at 37°C, then rinsed in 3 x SSC and exposed to an X-rayfilm. Numbers (I-1, I-2, e.g) correspond to the numbers of family members in figure 7.1. B= blanc control, C+= positive control of an unrelated 46,XY AIS-patient with the R846H mutation.

tation of data in 'single' cases of AIS. This especially applies when the HindIII-, or trinucleotide repeat- polymorphisms in exon 1 of the AR gene are being used as a marker for the affected gene when a mutation is yet unidentified. Carrier detection should preferably be based on mutation-specific testing.
Allele-specific slot-blot analysis was fully informative in this case. This finding urges the use of a second unrelated mutation detection method for confirmation and carrier detection. However, mutation detection in mosaic cases will never be 100% sensitive because the ratio of the mutant to the wild-type allele can be small or mutant DNA is absent in somatic cells as in germline mosaicism.
In families with a single affected child one must consider the possibility of maternal somatic/germ-line mosaicism. The precise recurrence risk for AIS is difficult to calculate, in the absence of larger data sets. However, the ubiquitous occurrence of DNA replication errors, justifies general consideration of this factor in *de novo* AIS cases. Sisters of affected 'single cases' certainly have an indication for carrier testing. Mothers of 'single cases' may wish prenatal diagnosis in a future pregnancy. This will be fully reliable if based upon mutation detection.

As in other disorders caused by *de novo* mutations, in AIS, mutations occurring at different stages of development have been observed:

- germline mosaicism with or without somatic mosaicism in a mother, as suggested once before[20] , and documented in this study;
- *de novo* mutations in children of unaffected parents[6, 21];
- somatic mosaicism in an AIS patient.[22]

This knowledge is helpful in remaining cautious in genetic counseling: "never say, there is no recurrence risk for a sibling of a new case in the family."

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CHAPTER

8

Androgen Insensitivity Syndrome: transmission of information and emotional reactions of parents and adult patients to the clinical diagnosis of AIS and its confirmation by androgen receptor gene mutation analysis

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SUMMARY

The emotional reaction of parents and adult patients on disclosure of the clinical diagnosis of androgen insensitivity syndrome (AIS) and its later confirmation by gene mutation analysis were assessed, as well as their decision to inform their relatives, friends and others. A semi-structured interview and three questionnaires were used. Parents came from eighteen different families with a total of 20 children with AIS (15 Complete AIS, 5 Partial AIS), 19 raised as girls, 1 as a boy. Ten adult CAIS women came from 6 families.
The emotional reaction of both groups on AIS was strong, long lasting and appeared to have a negative effect on openness towards dissemination of information. Both groups inform only persons they can trust because of fear of stigmatization and out of feelings of shame. The availability of DNA-testing does not convert the reticent attitude about AIS into a more open one towards potential carriers.

INTRODUCTION

Androgen Insensitivity Syndrome (AIS) is an X-linked disorder of male sex differentiation with a incidence of 1:99.000.[1] AIS is due to an absent or defective androgen receptor (AR) function and comprises a spectrum of phenotypic abnormalities.[2] Subjects with complete androgen insensitivity (CAIS) have normal female external appearance at birth. They may manifest before puberty with inguinal hernia containing testes, or after puberty because of primary amenorrhoea and lack of pubic hair. Internally, they have a short, blind ending vagina and absence of uterus and ovaries.

Patients with CAIS are always raised as girls, their gender role behavior and gender identity is female[3], their sexual orientation is heterosexual.[4-6] Children with partial androgen insensitivity (PAIS) usually present directly at birth because of ambiguous external genitalia. The wide spectrum of genital development ranges from severe hypospadias, bifid scrotum, and bilateral cryptorchidism to partial fusion of labia and/or clitoromegaly. In all cases there is absence of female internal sex organs. Sex assignment in PAIS is complicated, because these patients virilize insufficiently at puberty. Children with PAIS raised as girls are at risk to develop cross-gender role behavior and cross-gender identification.[3]

Confrontation with an intersex condition is a traumatic experience for parents and also for patients themselves at an age when they comprehend their condition.[3] Parents of patients with either CAIS or PAIS experience problems of accepting the discordance between genotype and phenotype of their child. They often find it very difficult to inform their child about AIS and to offer adequate support. Parents of girls with PAIS find it hard to cope with the cross-gender behavior of their daughter. Female young adult patients have problems in the acceptance of sparse pubic hair, absence of menses, infertility and lifelong need of hormone replacement therapy and vaginoplasty. Male patients may feel inadequate with an extreme small penis (looking more like a clitoris). Also they have to cope with corrective surgery for hypospadias at an early age, inability to urinate while standing, insufficient virilization at puberty and, incidentally, lifelong hormone replacement therapy and implantation of artificial testes.

Since cloning of the AR gene in 1988, mutation analysis of the androgen receptor enables confirmation of the clinical diagnosis of AIS. If the AIS gene

mutation in a family is found, precise carrier testing for female relatives of AIS patients is possible.
Female carriers have a 25% chance of an affected 46,XY child.
Before DNA-testing became available, most parents of children with AIS kept the disorder secret to the majority of their relatives and friends. They were only inclined to inform close relatives or friends for emotional support. The knowledge that female relatives may be carriers and that the carrier state is now detectable confronts them with the dilemma whether the privacy of their child has more priority than the moral obligation to inform potential carriers.

This study analyses the emotional reactions of parents and adult patients on the clinical diagnosis of AIS, its confirmation by AR gene mutation analysis and their handling of information about AIS. For the latter we analyzed how and to what extent information would be transmitted towards potential female carriers of AIS, other relatives and non-relatives before and after DNA testing. Moreover, we studied the motivations (not) to inform relatives and non-relatives about AIS. Insight into these decisions and motivations will give indications for a future support strategy by professional workers for parents of AIS children, adolescents and adult patients.

METHODS

In a nationwide study in the Netherlands all AIS patients known to (pediatric) endocrinologists, clinical geneticists, pediatric surgeons and pediatric urologists were asked to participate in a study on the genotype and phenotype in AIS. Between 1993 and 1998 19 families with 21 children and 18 adult patients who had been clinically diagnosed with AIS took part in a clinical study assessing the phenotype/genotype relationship in AIS at its various gene mutations. All participants in the clinical study were asked to take part in the psychological study by their physician.
The psychological study involved a number of standardized questionnaires and an extensive semi-structured interview with open and precoded questions. The interviews were held twice: after the clinical diagnosis AIS but before the DNA test result and within 3 months after the DNA test result was disclosed. Since the majority of the parents and their children received psychological counseling by the first author as part of the information program, the interviews of the parents were carried out by a research assistant to avoid bias. The adult

patients were interviewed by the first author. The interviews were rated by 3 independent clinical psychologists: interjudge agreement was achieved by consensus.[7]

The interviews assessed the psychological reactions to the diagnosis of AIS by exploring the presence of feelings of shock, sadness, anger, guilt and shame immediately after the diagnosis (as remembered) and remaining until the time of the interview. The acceptance of various aspects of AIS (discrepancy between geno/phenotype, appearance of external genitalia, infertility and carrier state of mother) was evaluated by rating the interview material on a 5-point scale for these items from 'not at all' to 'completely'. Support from the partner was also rated on a 5-point scale. The answers of mothers and fathers were evaluated separately. The transmission of information about AIS was assessed by the experiences of parents and adult patients about being informed themselves and their own dissemination of information on AIS to relatives, family, friends and others. We also monitored the timing of informing others: immediately after the clinical diagnosis, 4-6 weeks later and after the DNA confirmation of the clinical diagnosis. The content of the information (complete, incomplete and evasive or misleading) was recorded as well as the reaction perceived from the informed persons. As the total of relatives who might be informed about AIS and its hereditary nature were taken the first- and second-degree relatives and from the third degree relatives the children of aunts and uncles of the parents or adult patients (> 12 years of age).

Psychometric data were obtained through psychological questionnaires: the Impact of Event Scale[8], the Beck Hopelessness Scale[9] and the Social Support Scale.[10] The Impact of Event Scale (IES) is a self-report scale measuring the current degree of subjective impact of a specific event (in this case AIS). It estimates two dimensions: (1) intrusion of unwanted ideas and thoughts into consciousness and (2) conscious denial-avoidance. The response categories are: never, seldom, often and continuously. The IES has a 7 item intrusion subscale (score range 0-35) and an 8 item denial-avoidance subscale (score range 0-40). The Beck Hopelessness Scale (BHS) measures hopelessness or pessimistic expectations on the future. A score of 9 or higher on a scale of 0-20 indicates depression and possible suicidal behavior. The social support scale measures the availability of and satisfaction with social support.

Statistical analysis

Differences between groups were assessed with the chi-square test, within groups with the paired sample test and considered significant for $p < 0.05$.

RESULTS

Parents of AIS children

Demographic and medical data

Eighteen families (18 mothers and 15 fathers) having 20 children with AIS consented to participate in the psychological study. Fifteen parent-couples lived together (in one couple the mother had died, the father had remarried), 2 parents were divorced, one mother was a single parent. In 9 families the AIS patient was the only child. The average age of the fathers was 39 years (SD=10.4) and those of the mothers 36 years of age (SD=8.7). All parents were of Caucasian origin and almost all had the Dutch nationality (2 fathers and 1 mother had another European nationality). Nearly half the parents (n=8) were Roman Catholic, 4 were Protestant, the others had no religious affiliation. Twelve fathers and 9 mothers received at least high school, 5 fathers and 2 mothers had at least a polytechnic degree.

An AIS gene mutation was found in 19/20 children (2/19 *de novo* mutations). Carrier detection was positive in 14/17 of the mothers. All parents received genetic counseling before DNA analysis and all but one after disclosure of the DNA test results.

The mean age of the 20 children with AIS was 8.7 years (SD=6.6). Twelve girls and one boy were prepubertal (9 months-11 years), the others were adolescents. Fifteen girls had the complete, four girls and one boy the partial form of AIS. Although there was some doubt at birth about sex-assignment in three children (two PAIS patients with virilization of the external genital and one CAIS patient with gonads in the labiae) none of them were sex-reassigned. Nineteen of the twenty children were at birth assigned to the female sex and one to the male sex. The external genitalia were completely female (Prader 1) in 15 girls, mildly virilized (Prader 2) in 3 girls, more severely virilized (Prader 3) in 1 girl and incompletely male (Prader 4) in the boy.

The mean age at diagnosis was 3.7 years (range: 1 day-16 years).

In the neonatal period the diagnosis of CAIS was made in 3 children: because of labial gonads (1 girl), a suspicion of a chromosomal disorder (1 girl) and family history (1 girl, with an affected sister). In the neonatal period the diagnosis PAIS was made in two children: 1 boy and 1 girl because of ambiguous external genitalia. Before one year of age the diagnosis CAIS was made in six girls and between 1 and 10 years of age in four girls because of inguinal hernia or gonads in the inguinal canal. One girl with PAIS showed clitoral hypertrophy at age 2.3 years. Two girls with CAIS presented with primary

amenorrhoea at age 17. The diagnosis PAIS was made in two sisters at the age of 12 and 14.6 due to an accident of the youngest which resulted in an abdominal operation, and subsequent family study in the older sister.
Surgery of the external genitalia was performed in one PAIS girl at age 2.3 years (clitoris reduction and construction of the labia minora), in one PAIS girl at age 5.4 years (clitoris reduction and vaginoplasty) and in the PAIS boy at age 1.6 and 2.0 years (correction of the hypospadias reconstruction of the bifid scrotum and orchidopexy and corrections of fistula). Of the 19 girls 13 had had a gonadectomy: at ages <1.6 in 8 girls, at ages 2-7 years in 3 girls and at ages 14-16 years in 2 girls.

Information process to parents.

All parents except one couple were informed upon all aspects of AIS when the clinical diagnosis was made. The other couple received only evasive information on the internal genitalia and fertility until their PAIS-daughter was 5 years old when they eventually were fully informed. All parents received extensive psychological counseling during the period of diagnostic procedure and several years thereafter. Parents of 16 children received information about the AIS diagnosis in several interviews (2 or more) by either the pediatric endocrinologist alone (n=4) or in cooperation with the psychologist (n=12). In all these cases the children were not present since they were too young. Four pairs of parents were informed about the AIS diagnosis in the presence of their daughters who were between 10 and 17 years of age. These girls also received extensive counseling alone or in combination with their parents from the psychologist.
In the interview seventy percent of the parents reported adequate knowledge about the recurrence risk in subsequent children and all parents appeared to be well informed about the pattern of hereditary transmission.

Parents' emotional reaction to the AIS diagnosis

The parents' recollection about their first reaction and their actual feelings about the diagnosis are given in Table 8.1. The average time between the moment the diagnosis was disclosed to the parents and the time of interviewing was 5 years (SD= 4.7 years). Shortly after the diagnosis the majority of both parents reacted with shock, grief, anger and shame, and mothers with guilt. Feelings of grief and shame remained actual in both parents, feelings of shock, anger and guilt also in mothers. Mothers reacted

Table 8.1
Past and present emotional reactions of 19 mothers (M) and 17 fathers (F) of AIS children (n=20); at diagnosis (mean 5 years earlier) and in the present time

	% experienced									
experienced	shock		grief		anger		guilt		shame	
	M	F	M	F	M	F	M	F	M	F
never	10	12	0	29	32	71	37	94	47	53
at diagnosis, not in the present time	53	65	58	29	42	18	16	6	10	6
at diagnosis and in the present time	37	4	42	41	26	12	47	0*	42	41

* significant $p<0.001$

significantly more often than fathers with feelings of guilt (chi-square=13.5 df=2 p=0.001).

There was no difference between the emotional reaction on the AIS diagnosis of parents with a PAIS child (n parents=4) and parents with a CAIS child (n mothers=13 n, fathers=11). No significant difference was found between parents who were informed about the AIS diagnosis less than 3 years earlier (n=9) or since more than 3 years (n=9). In only three girls AIS was diagnosed after the onset of puberty. The emotional reaction of their parents appeared to be different concerning feelings of shock (more shock), grief (more grief) and shame (less shame) from the other parents.

On the identification of the mother as a carrier 54.5% of the parents reacted with disappointment. Forty-five percent of these parents reported that this information had had a positive effect on their relationship and 20% a negative one. Compared to the emotional reaction of parents to the clinical diagnosis of AIS, their emotional reaction to the DNA test result (n mothers=11, n fathers=9) appeared to be similar, with the exception of feelings of shame, which were absent. Guilt feelings towards potential female carriers were reported by seventy five percent of the parents, the others indicated that they felt indifferent.

On the Impact of Event Scale the mean scores on 'intrusion of unwanted ideas and thoughts' of fathers (mean=12.08 sd=3.65) and mothers (mean=13.58 sd=3.92) were not significantly different, nor their mean scores on 'denial-avoidance' (mean fathers=13.67 sd=8.66; mean mothers =10.75 SD=3.44). Since normative data for the IES are lacking, the results of the parents on this

scale will be described. Father's scores on 'intrusion' ranged from 7 to 17, those of the mothers from 7 to 19. Fathers' scores on 'denial-avoidance' ranged from 8 to 18 (one score was 39) and those of mothers from 8 to 20. This means that parents reported that they felt sometimes (between seldom and often) overwhelmed by unwanted thoughts about AIS and sometimes avoided thoughts about AIS.

Parents' acceptance of AIS

No significant differences could be found between fathers and mothers in accepting the various aspects of AIS.
Infertility was the most difficult aspect of AIS for the majority of the parents (84% of the mothers, n=19 and 75% of the fathers, n=16)

Social support and coping with AIS of parents.

Sufficient social support was perceived by the majority of the parents and loss of friends because of AIS did not occur. The influence of AIS on their relationship was reported as positive by 34% of the parents, as negative or equivocal by 28% and 39%. Lack of support from the partner in coping with AIS was indicated by 40% of the mothers.

Expectancy of future

Hopelessness on the Beck Hopelessness Scale was equal in fathers (mean=3.08 SD=1.44 n=12) and mothers (mean=4.14 SD=2.35 n=14). Only one mother had a score in the clinical range (score 10). Pessimistic feelings for the future were absent in most parents. Reduced chances for their child to find a partner were expressed by about half of the parents, and 25% were doubtful about this.

Informing of AIS-children by parents.

Most parents planned to inform their child about the AIS in three steps: education about the biological and psychological aspects of normal sexual development (step 1), information at the ages of 11 or 12 about AIS with the exception of the XY-karyotype (step 2) and disclosure of the chromosome pattern after the age of sixteen (step 3).
Parents of six adolescents had effectuated this process. One adolescent girl was informed by her gynecologist. The ages at which the girls were informed ranged from eleven to sixteen. All children who were not informed were prepubertal (mean age 3.2, range 9 months - 11 years of age, n=13).

Grief was the principal reaction in 5/6 girls on the AIS information, but all were able to cope reasonably well. Infertility, absence of pubic hair, lack of menstruation and underdevelopment of the vagina appeared to be hard to accept. One girl reacted with a depression, requiring therapy with anti-depressants. One girl receiving a diagnosis of PAIS at the age of ten experienced this as an acknowledgment of her male identification and male gender role. She was confused for some time about her gender identity and played with the option of sex-reassignment, but eventually she decided to remain a girl.

Dissemination of AIS information to relatives and others by parents before and after DNA testing.

A total of 186 persons were informed about AIS by the parents; 64% relatives (n=119) and 36% non-relatives (n=67). From the mothers' female relatives 42% (57/137) were informed (Table 8.2); 97% of the 57 were potential carriers. Nearly one third of the male relatives of the mother were told about AIS. In the family of the father a similar proportion of females and males were informed. There was no significant difference between the proportion of informed relatives in the families of the father and the mother of the AIS patient. The proportion of informed relatives was not influenced by incompleteness of the family (divorce, single mother).
From the informed non-relatives (n=67) one third were friends and two thirds were colleagues, employers, employees, teachers of the child and acquaintances. Of all the people who were informed about AIS, 66% (127/194) were told so shortly after the child was diagnosed, 30% (59/194) 4-6 weeks after the diagnosis was made and 4% (8/194) after disclosure of the DNA test result.
The motives of the parents to inform people about AIS was in 66% (n=193) that they wanted to share emotions with people they felt close to and in 11%

Table 8.2
Proportion of parents' relatives informed about AIS

		Total informed	
		n	%
Family of the mother:	females (n=130)	57	42
	males (n=109)	29	27
Family of the father:	females (n=45)	16	36
	males (n=51)	14	27
Total (n=342)		114	33

Table 8.3
Proportion of informed relatives and non-relatives of parents and the degree of completeness of transmitted information about AIS

	Mothers' family		Fathers' family		Non-rel.
	Females n=57 %	Males n=29 %	Females n=18 %	Males n=15 %	n=67 %
Complete information	81	62	72	27	21
Partial information	17	24	28	53	39
Misinformation	2	14	6	20	40

that they felt morally obliged. Twenty three percent of the parents who disclosed information to employers, employees of the parents and teachers of the child regretted their decision.

All parents reported that they explicitly did not inform at least one first or second degree relative who might be a carrier. Motives for not informing were for 64% of the parents that they did not feel close to this/these person(s). Other reasons were that the potential carrier was childless or beyond childbearing age or that the parents of this potential carrier woman had to transmit the information about AIS to their offspring or that the DNA test result was not yet known.

Half the people who were informed about AIS were informed about all the aspects of AIS (the male karyotype, hereditary nature, absence of female internal genitalia and infertility). One third were partially informed (infertility and/or absence of female internal genitalia) and 19% were informed in evasive, masking terms. Table 8.3 shows that more than two thirds of all informed female relatives and male relatives of mothers were completely informed about AIS. The majority of the males in fathers' family and the non-relatives were partially or misinformed about AIS.

Half the parents had received a positive reaction of those whom they informed, the other half had reacted neutral.

Parents of children with PAIS (n=4) tended to inform twice as many relatives in mothers' family than parents of children with CAIS 17/37=55%; 20/101=20%). No differences were found between these groups of parents in respect to time of transmitting the information, the motives to inform nor the reaction of those who were informed.

Adult AIS patients

Demographic and medical data

Of the adult AIS patients 10/18 participated in the psychological study. The non-participant a.o. included multiple cases from one family and a male PAIS patient, whose physician advised against participation. The 10 patients belonged to 6 different families, one adult patient was a sister of a mother in the parent study. All patients had the Dutch nationality. Seven patients were Roman Catholic, 2 were reformed and one had no religious affiliation. Nine patients received at least high school, 5 had at least a polytechnic degree.
The AR gene mutation was detected in all patients. The carrier state of the mothers was established in 1/10 by mutation analysis and in 3/10 by family history, in the others these studies were unavailable. All adult patients were well informed about the hereditary aspects of AIS.
The mean age of the adult women with AIS was 40.5 years (range 24.11-70 years; SD=15.4). There were 4 young adult women (24.11 and 31.1 years), five adults (38.8 and 56.1 years) and one elderly (70.3 years) woman.
The mean age at diagnosis was 14.2 years (SD 4.6). All had CAIS, none were sex-reassigned. All had normal female external genitalia (Prader 1). Eight women had a permanent relationship, one was widowed, two had adopted children.
Diagnosis was before 7 years in 4/10 due to inguinal hernia and in 6/10 at ages 16-19 years because of primary amenorrhoea. Gonadectomy was done in 9/10 (in 1 at age 7, in 8 at ages 15-53) and vaginoplasty in one.

Information process to adult patients.

Of the four young adult women, three were completely informed about AIS by their parents in early adolescence and one by her gynecologist upon diagnosis when she was 18 years. The six older women received only incomplete, evasive or misleading information from their family doctor or gynecologist. In adolescence they heard about being infertile and unable to menstruate. They had no name for their condition, no information about their genetic sex and their internal genitalia (some were only told that their ovaries had to be removed). They learned the real facts about their condition only years later (26-53 years).
Only one set of parents of the adult patients received extensive psychological counseling during the period of diagnostic procedures and several years thereafter. Their daughter received counseling for emotional problems in latency and psychotherapy for problems with acceptation of the diagnosis in

Table 8.4
Past and present emotional reactions of 10 adult women with AIS; at diagnosis (*mean 26 years earlier*) and in the present time

period of experience	shock %	grief %	anger %	shame %
never	20	10	30	0
at diagnosis, not in the present time	20	50	20	10
at diagnosis and in the present time	60	40	50	90

adolescence. Five more women had been in psychotherapy for acceptation problems including sexual problems.
Eight of the nine partners were fully informed about AIS by their wives. All women were relieved by their partners' positive reaction and supportive attitude. Only one woman had kept her chromosome pattern secret for her partner.

Emotional reaction on AIS diagnosis by adult patients

The average interval between the moment the diagnosis was disclosed to the adult patients and the interview was 26.5 years (SD=13.5 years). The major impact remembered of the diagnosis (table 8.4) was shock, grief, anger and shame. These feelings remained prominent into present time in 40% or more of the adult patients: especially shame was a frequent actual feeling (90 %).
Adult patients reported on the Impact of Event Scale that they felt sometimes (between seldom and often) overwhelmed by unwanted thoughts about AIS (mean score: 15.5, range 7-24, SD=5.8 n=8) and sometimes avoided thoughts about AIS (mean score 16.3, range 11-22, SD=4.5).

Acceptance of AIS in adult patients

Acceptance of infertility and discrepancy between genotype and phenotype was poor in respectively 90% (mean score = 1.5) and 60% (mean score = 2) of the adult patients. The external genitalia of all 10 women were female but only 3/10 were satisfied with their secondary sexual characteristics. Patients were dissatisfied with underdevelopment of pubic hair and breasts and with their stature (too masculine).

Social support of adult patients

Social support was rated as sufficient on the Social Support Scale by the majority of the adult patients. However in their childhood or adolescence 7/10 adult patients did not receive any support at all form their parents and 3/10 received only support from their mothers. Six women had lost friend(s) because of their problems with AIS. The support by their partner was rated as good or excellent. This included that 50% discussed their feelings about AIS frequently with their partner.

Expectancy of future

The majority of the adult patients reported on the Beck Hopelessness Scale that they did not suffer from pessimistic feelings for the future (mean hopelessness score = 5.34, SD = 4.92, n=9). However two were feeling so deeply depressed that they needed psychological treatment (hopelessness score >10). Reduced chances to find a partner were expressed by 60% of the adult patients.

Dissemination of AIS information by adult patients before and after DNA testing

Information was transmitted to 229 persons, 189 relatives (82.5%) and 40 non-relatives (17.5%). One third was informed by the adult patients and two third by their parents. The majority of the female relatives on mother's side 83/115 (72%) and all siblings (32 sisters and 21 brothers) were informed. From the potential female carriers 64% (78/122) were informed.

Adult patients did not know the total number of their male relatives in their mothers' and fathers' family. From the male relatives in fathers' family who were known to the adult patient only 8/10 fathers were informed and in mothers' family only 11 males. All partners were informed and 4 patients also informed some of their in-laws (n=20). The majority of the non-relatives (25/40) were friends.

Data about the timing of dissemination of information (shortly after the diagnosis and 4-6 weeks later) could not be assembled from adult patients because they did not have reliable information. The 3/10 adult patients who had received information about DNA-testing did not inform additional persons. The motive of adult patients to inform was the desire to share emotions to people they felt close to (97%), only 3% felt morally obliged to inform relatives. From the persons who were informed (n=229), none were misinformed: 73% knew all aspects of AIS (96% relatives and 4% non-relatives), and 27% knew some

aspects (45% relatives and 55% non-relatives). The majority of the informed persons reacted positively (89%).

Adult patients and parents compared

Feelings of shame were significantly more often reported by adult patients than mothers of children (chi-square=7.3 df=2 p=0.03 n mothers=19, n adult pat.=10). Adult patients and mothers did not differ in their feelings of shock, grief and anger. Acceptance of the discordancy between genotype and phenotype was significantly lower in adult patients than mothers of children, but they did not differ in the other aspects of AIS. In coping with AIS adult patients and mothers did not differ in the scores on intrusion and avoidance of AIS, nor in their pessimistic feelings (mean intrusion score adult pat.=15.5 SD=5.8 n=8, mean intrusion score mothers=13.6 SD=3.9 n=12; mean denial score adult pat.=16.3 SD=4.5 n=8, mean denial score mothers=10.8 SD=3.6 n=12; mean hopelessness score adult pat.= 5.3 SD=4.9 n=9, mean hopelessness score mothers=4.1 SD=2.4 n=14).

DISCUSSION

Comparison of emotional reactions in parents and adult patients

Reactions of shock, grief and anger on the AIS diagnosis were strong and long lasting in mothers of AIS children and adult patients. Shame was more frequently reported by adult patients. Acceptation of infertility appeared to be as difficult for mothers as adult patients, but acceptation of the discordancy between genotype and phenotype and acceptation of secondary sexual characteristics were more difficult for adult patients. In adult patients the male chromosome pattern appears to lead to feelings of shame which have a negative influence on the acceptation of the female body. It is possible that adult patients have more feelings of shame than parents because of the taboo on issues of sex when they grew up. Mothers may have less feelings of shame than adult patients because their daughters are still too young to have sexual feelings and experiences. Moreover their daughters may not be inclined to discuss their sexual feelings/ problems with their mothers. A third explanation can be the timing of diagnosis. The majority of the adult patients were diagnosed in late adolescence because of primary amenorrea, the majority of the children were diagnosed at an early age. Adolescents with primary

amenorrea worry about the functioning of their female body: they feel often different from their female peers and may have feelings of shame. The confirmation of these worries with the AIS diagnosis is often a very big disappointment, which can enhance their feelings of shame.
Although both parents and adult patients report strong emotional reactions on the AIS diagnosis and a lot of acceptation problems, they both indicate that they cope rather well and feel sufficiently supported by family and friends. Social support is received from a careful selection of people they trust, with whom they share emotions. The result is that they both report that positive reactions are much more frequent than negative ones.
Since the emotional reactions to AIS of parents and adult patients are rather similar the question arises whether these reactions are specific for AIS or a more common emotional reaction on a chronic condition. Therefore, we compared the emotional reactions of parents with a daughter with Turner Syndrome (TS)[11] with those of AIS parents. Feelings of shock, anger and shame were similar, but guilt more frequently reported by AIS mothers (chi-square=14.3 df=2 p=0.001 n ais=19 n TS=64) and grief more frequently by AIS fathers (chi-square=14.3 df=2 p=0.00 n ais=17, n TS=55). Apparently being a carrier or potential carrier of AIS appears to result in guilt feelings in AIS-mothers.

In contrast to many reports that CAIS presents typically in late adolescence with primary amenorrhea[12], CAIS was diagnosed in our study in 13 children (87%) before the onset of puberty. The symptoms were in the majority an inguinal hernia or gonads in the inguinal canal. In two girls primary amenorrea led to the CAIS diagnosis. The adult (CAIS) patients in this study were diagnosed during childhood in 40% (inguinal hernia or gonads in the inguinal canal) and in late adolescence in 60% because of primary amenorrhoea.
The question is whether parents who are informed about AIS before the onset of puberty of their AIS child are better able to support this child in the acceptance of AIS because they have a longer period of preparation than parents who are informed later. The three parents who were informed about AIS after the onset of puberty of their daughter reported more feelings of shock and grief but less shame than mothers who were informed before puberty. Definite conclusions are not possible. However, parents (and patients) becoming informed after puberty may have the difficult task to deal with their own and their AIS daughter's feelings, which complicates adaptation.

This study shows a discrepancy between information about AIS received by parents of children and parents of adult patients. The first were nearly all completely informed about the diagnosis, the second were either not, or insufficiently or evasively informed. An authoritative medical attitude towards parents of AIS children that are adults at present may have precluded their parents from receiving or asking full explanations from physicians that had the opinion that the parents and patients were unable to cope with the knowledge of a 'male karyotype'. The result was that their children were badly informed and confused for years, even after hearing the whole story about their condition. It appeared for some of them impossible to incorporate the correct information into their distorted self-image. Although Money was one of the first to promote complete enlightenment for parents and patients, he recommended information about the genotype for the patient which can nowadays be seen as too evasive ("the Y is in a sense functioning as an X with two arms broken off"[13]). The purpose of his advice was to spare the patient "a false concept of her cells as male, for her cells belong to her self image". Nowadays most pediatricians are convinced that parents need to be informed about the development of sex differentiation in their child of which the karyotype is an essential but not the single component, to obtain a clear picture of the condition and to be able to explain it to their child. As for the children, we are living in a time where all information is accessible for even young children with the help of the Internet. The effect of this development is already visible in our results about informing adolescents in the parents' group. Although the policy was to explain all aspects of AIS without the karyotype, all parents informed their children completely. They did not intend to do this, but their children asked for more explanation.

Parents and adult patients show a similar behavior on transferring information about the AIS diagnosis. They both inform twice as many or more relatives, compared to non-relatives and they both inform their relatives more completely than their non-relatives. They do not inform non-relatives about the male karyotype out of fear of stigmatization.
Parents and adult patients are reticent in the dissemination of information towards potential carriers: parents inform 42% and adult patients 68%. The availability of DNA-testing and its genetic nature do not alter their cautious strategy since the motives for informing is mostly based on trust and not on moral obligation. Parents seem to be more open about AIS than adult patients. Adult patients inform female relatives and friends, parents inform also relatives of the father, the male relatives of the mother and colleagues, employers, employees,

teacher of the child. The motive for parents to inform this last category is that they felt forced to explain absence from work or school because of hospital visits with their child. They sometimes regretted their decision. The reason why adult patients are more reserved than parents may be their feelings of shame. They inform potential carriers or AIS patients who are both companions in misfortune. The high percentage of positive reactions parents and adult patients received indicates that they did not misjudge the persons they informed.
Parents feel that informing potential carriers is often not in the interest of their child because disclosure of the genetic nature of AIS implies also disclosure of the total condition of which the potential carrier is up to then unaware. It is possible that parents and adult patients fear the anger of potential carriers (who after DNA testing may appear not to be carriers) by bringing them threatening news. The guilt feelings of the mothers who appeared to be specific for AIS might also be a barrier. The low percentage of persons informed about their carrier risk especially in parents (42%) seems not to be specific for AIS. In the fragile-X syndrome De Vries[14] found that in 19 families 34% of the relatives were informed about their carrier risk. Studies on informing relatives about a genetic disorder have been published (autosomal recessive: cystic fibrosis, hereditary breast/ovarian cancer). Comparison with AIS families is useful although the mode of inheritance is different. Julian-Reynier[15] reports that 34% of those who received genetic counseling for hereditary breast/ovarian cancer distributed the letter summarizing the genetic consultation to the relatives. Reasons not to inform at least one first or second degree relative about cystic fibrosis[16] or hereditary breast/ovarian cancer[15] were not feeling close to these persons or not feeling responsible.

Since AIS is a rare condition large groups are seldomly collected. Compared to other studies this study collected a large group of pediatric patients (n=20) and a comparable group of adult patients (n=10).[4-6,17] However, in such small groups the power of statistical analysis is small and results are largely descriptive, but meaningful in the clinical management and genetic counseling.

Recommendations

The emotional reaction of parents and adult patients to the AIS diagnosis was strong and long lasting. Long-term psychological counseling may be needed for

both groups. Mothers and other female relatives need extra care because the identification as a carrier may strengthen their already strong guilt feelings.
Although parents nowadays are better supported and informed to inform their AIS children during childhood, adolescence brings again problems needing support. Feelings of shame and guilt in the mothers may be barriers to help their daughters to cope with infertility and sexual relationships. Adolescent AIS girls need adequate sexuological help to overcome their shyness to enter into a sexual relationship. Support groups such as the AIS Support Group* can also be helpful. Our impression is that AIS adolescent girls are well able to cope with the AIS information given by their parents, but it remains difficult for them to incorporate the information about their genotype into their self-image.

Our study shows that the strategy of parents and adult patients in informing relatives, friends and others worked well, due to the control they had on the choice of persons they informed and the content of the information they disseminated. However, access to AIS information will become easier by means of electronic devices such as the Internet, which means that control may become more difficult. Disclosure of the name of the condition means full disclosure. In those cases where the intention is to give incomplete information, we recommend not to disclose the name of the condition. Since it is very likely that in the future the age of AIS children and adolescents for informing will decline, the information on Internet must be presented in a comprehensive way and adapted to their age level.
Parents and adolescent patients for whom confirmation of the clinical diagnosis of AIS with DNA-testing will nowadays be immediately available need extensive genetic counseling and psychological support in order to help them to make their own decision about informing potential carriers. Pressing them to inform these potential carriers out of moral obligation seems to be bad advice.

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* Androgen Insensitivity Syndrome support group: http://www.medhelp.org.ww/ais

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CHAPTER

17β-Hydroxysteroid dehydrogenase 3 deficiency: diagnosis, phenotypic variability, population genetics and world-wide distribution of ancient and *de novo* mutations

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Abstract

Background – 17β-Hydroxysteroid dehydrogenase-3 (17βHSD3) deficiency is an autosomal recessive form of male pseudohermaphroditism caused by mutations in the *HSD17B3* gene.

Methods – In a nationwide study on male pseudohermaphroditism among all pediatric endocrinologists and clinical geneticists in The Netherlands, eighteen 17βHSD3-deficient index cases were identified, 12 of whom initially had received the tentative diagnosis Androgen Insensitivity Syndrome (AIS). The phenotypes and genotypes of these patients were studied. Endocrine diagnostic methods were evaluated in comparison to mutation analysis of the *HSD17B3* gene. RT-PCR studies were performed on testicular RNA of patients homozygous for two different splice site mutations. The minimal incidence of 17βHSD3 deficiency in The Netherlands and the corresponding carrier frequency were calculated. Haplotype analysis of the chromosomal region of the *HSD17B3* gene in Europeans, North Americans, Latin Americans, Australians and Arabs was used to establish whether recurrent identical mutations were ancient or had repeatedly occurred *de novo*.

Results – In genotypically identical cases, phenotypic variation for external sexual development was observed. Gonadotrophin-stimulated serum testosterone/androstenedione ratios in 17βHSD3-deficient patients were discriminative in all cases and did not overlap with ratios in normal controls or with ratios in AIS patients. In all investigated patients both *HSD17B3* alleles were mutated. The intronic mutations 325+4;A→T and 655-1;G→A disrupted normal splicing, but a small amount of wild type mRNA was still made in patients homozygous for 655-1;G→A. The minimal incidence of 17βHSD3 deficiency in The Netherlands was shown to be 1:147,000, with a heterozygote frequency of 1:135. At least four mutations, 325+4;A→T, N74T, 655-1;G→A and R80Q, found worldwide, appeared to be ancient and originating from genetic founders. Their dispersion could be reconstructed through historical analysis. The *HSD17B3* gene mutations 326-1;G→C and P282L were *de novo* mutations.

Conclusions – 17βHSD3 deficiency can be reliably diagnosed by endocrine evaluation and mutation analysis. Phenotypic variation can occur between families with the same homozygous mutations. The incidence of 17βHSD3 deficiency is 0.65-times the incidence of AIS which is thought to be the most frequent known cause of male pseudohermaphroditism without dysgenic gonads. A global inventory of affected cases demonstrated the ancient origin of at least four mutations. The mutational history of this genetic locus offers views into human diversity and disease, provided by national and international collaboration.

INTRODUCTION

17β-hydroxysteroid dehydrogenase-3 (17βHSD3) deficiency is an autosomal recessive form of male pseudohermaphroditism due to impaired testicular conversion of androstenedione to testosterone[1]. 46,XY homozygotes or compound heterozygotes for mutations in the *HSD17B3* gene have testes and normally developed Wolffian duct derivatives. However they show undervirilisation of the external genitalia which are often female with or without clitoromegaly and/or labial fusion and a blind-ending vagina. Therefore, patients are most often raised as females.[2] Less often ambiguous external genitalia[3-5], male genitalia with micropenis[6], or hypospadias[4,7] are reported. Virilisation occurs at puberty and is probably due to extra testicular conversion of androstenedione into testosterone.[4] Thus, the diagnosis 17βHSD3 deficiency should be made before the onset of puberty and followed by gonadectomy, in cases with complete female genitalia. In the less frequently seen, partially virilized, 17βHSD3-deficient patients, the diagnosis should be made directly after birth, because androgen treatment may result in a nearly normal male phenotype in adulthood[8,9], and male sex assignment can be considered. 46,XX 17βHSD3-deficient cases are normal, asymptomatic females.[10,11]

17βHSD3 deficiency is clinically indistinguishable from androgen insensitivity syndrome (AIS) in prepubertal patients but the diagnosis can be made from elevated serum androstenedione and decreased serum testosterone/androstenedione ratios after hCG stimulation.[12,13] Unfortunately, the diagnostic power of endocrine diagnostics is not optimal because of the lack of normal ranges in strictly age-matched controls. An improved diagnostic procedure became available after cloning of the *HSD17B3* gene and detection of 16 different mutations in 21 index patients.[5,10,11,14-16] Eleven of these mutations, resulting in amino acid substitutions, were proven to be pathogenic. For the identified splice site mutations this proof is still lacking.

Except for a high prevalence in an isolated Arabic population[17,18], 17βHSD3 deficiency is thought to be a rare disease.[4,19]

In a nationwide study on male pseudohermaphroditism in The Netherlands (population 15.5 x 10^6 in 1998) we found 18 index cases with 17βHSD3 deficiency. Of those, 12 initially received the tentative diagnosis AIS. Here, we evaluate the phenotype/ genotype relationship for several mutations. The diagnostic value of testosterone/androstenedione ratios is compared to that of mutation analysis. Molecular genetic proof for the pathogeny of frequently identi-

fied splice site mutations is provided. The incidence and carrier frequency of 17βHSD3 deficiency in the Dutch population is investigated. The finding of identical mutations in unrelated families from diverse ethnic background is further investigated. Evidence is provided that some mutations in this gene may be quite ancient.

SUBJECTS AND METHODS

Design of the study

A nationwide survey on male pseudohermaphroditism, among all 9 major pediatric and 7 clinical genetic centers in The Netherlands, resulted in the identification of 18 index patients and 2 siblings with a tentative diagnosis of 17βHSD3 deficiency. Some had been diagnosed previously; others were identified during this 5 yr study. In addition, three affected siblings from Turkey were analyzed.

A diagnosis of 17βHSD3 deficiency was established by review of medical history including prenatal exposures, a 46,XY karyotype, physical examination of the patients, cysto-uroscopy, histological examination of the gonads and Wolffian duct derivatives if possible, additional hCG-tests if possible and mutation analysis of the *HSD17B3* gene. Furthermore four generation pedigrees were constructed.

Here we report this total of 19 index patients and 4 affected siblings. The study was approved by the medical ethical committee of the University Hospital Rotterdam. Written informed consent was obtained from either the patients or their parents.

DNA samples from patients with 17βHSD3 deficiency from all over the world with similar *HSD17B3* gene mutations as those found in these 19 index patients, were used in a study on the origin of these mutations.

Endocrine evaluation

Testosterone/androstenedione (T/A) ratios of patients with 17βHSD3 deficiency were compared to those in age-matched normal males and AIS patients.

Androstenedione and testosterone serum levels in 17βHSD3-deficient cases were measured by RIA, in different laboratories in The Netherlands; the inter-laboratory variation coefficient was maximally 15% for androstenedione and 6% for testosterone (Dutch council for clinical chemistry). The following served

as normal controls: 9 normal boys, 1-3 months old; 25 normal prepubertal boys, aged 4 months to 12 yr[20] and 20 normal adult males. For comparison, T/A ratios were determined in AIS patients with proven androgen receptor mutations: 1- to 3- month-old infants before (n=2) and after hCG stimulation (n=6); in prepubertal cases 4 months to 12 yr after hCG stimulation (n=3), and in (post)pubertal cases (basal T/A ratios, n=17; hCG stimulated T/A ratios, n=5).

T/A ratios in the above controls and AIS patients were determined according to the method described by Verjans et al.[21] without chromatography for testosterone with a coated tube RIA (Diagnostic Products, Los Angeles, CA) for androstenedione.

Genomic DNA isolation and mutation detection

Genomic DNA was extracted from peripheral blood leukocytes or from cultured genital skin fibroblasts following standard procedures.[22] In the *HSD17B3* gene and androgen receptor gene, exons and flanking intron sequences were screened for mutations using PCR and single strand conformation polymorphism (PCR-SSCP).[15,23] PCR fragments of the introns/exons suspected of harboring mutations were analyzed by automated sequencing.

Ribonucleic acid (RNA) extraction, complementary DNA (cDNA) synthesis and PCR amplification of cDNA

RNA was extracted as previously described[24] from testes obtained at gonadectomy of patients homozygous for the 325+4;A→T mutation (patient 1-I) or the 655-1;G→A mutation (patient 9-III) and from a normal 46,XY male (tissue donor bank). cDNA synthesis was performed with an oligo (deoxythymidine) dT primer as described[24] and further amplification was performed with primers 1AA-11B and 1AA-6BB, 1AA-3BB or 9AA-11B (for localization of the primers, see Figure 9.2).

Primer sequences are:

1AA, ACACAGAGAGCCACGGCCAG;
3BB, ATCTCTGTGGCAATGGCCTCTA;
6BB, ACGGAGGTGATGTTACAATG;
9AA, GCCCTGCAAGAGGAATATAAAGCA and
11B, GAGGAAAAGGTTGTGCTGGACTCCT.

Fifty microliters of reaction mix for PCR contained 1.5 mmol/L $MgCl_2$. Conditions for the PCR in a Biometra cycle sequencer were as follows: hot start at 94°C for 5 min, then 35 cycles at 94°C for 1 min, at annealing temperature for 1 min, at 72°C for 1 min, and final extension for 10 min at 72°C. Annealing temperatures were as follows: primer pairs 1AA-11B and 1AA-6BB: 55°C, 1AA-3BB: 62°C and 9AA-11B: 65°C.
The resulting PCR products were subcloned into a plasmid using the TOPO TA cloning kit (Invitrogen, San Diego, CA) and subjected to automated sequencing.

Carrier frequency of the 325+4;A→T mutation in the *HSD17B3* gene

The carrier frequency of 17βHSD3 deficiency and of the 325+4;A→T mutation was calculated under the assumption of a Hardy-Weinberg equilibrium and on basis of the fact that 46,XX homozygous/compound heterozygous cases are asymptomatic.[25] Therefore, the carrier frequency is 2pq, with $q=\sqrt{2z/N}$ (z is the number of diseased newborns or the number of 325+4;A→T alleles and N is the total number of newborns during a time period; p=1-q). To test the calculated carrier frequency of 325+4;A→T, exons 3 of 200 Dutch normal control individuals were screened with PCR-single strand conformation polymorphism.[15] As a positive control a carrier of the 325+4;A→T mutation was used.

Haplotyping alleles

Polymorphic extragenic markers on chromosome 9p22.3, AFM023XH8, D9S1786 and D9S1851 (Genethon Resource Center, 91000 Evry, France)[26] were used to genotype the 17βHSD3-deficient patients described in Table 9.1 (except for patient 19) and their parents. Consequently their haplotypes could be derived. Thirty AIS patients that were identified during this same nationwide survey on male pseudohermaphroditism and 20 of their relatives were used as controls, providing a total of 74 independent alleles. Possible associations of a specific haplotype with a specific mutation were investigated by statistical analysis using the Student's *t*-test. *P* values were calculated exact according to a multiple hypergeometric distribution.

Study of genetic origin of recurrent mutations worldwide

The haplotypes of 18 index cases were compared with the genotypes (FM023XH8, D9S1786, D9S1851) of 12 unrelated patients from all over the world. These patients carried the same mutations as the 18 index cases. Some

had previously been described as denoted in Table 9.3[15]; others have not been reported before.

RESULTS

Patients

All 17βHSD3-deficient patients had a 46,XY karyotype and were initially raised as girls. Table 9.1 summarizes data on genotype, phenotype, age of and reason for referral, endocrine evaluation, year of birth, ethnic background and parental consanguinity. Female-like external genitalia were present in all but three patients (patients 6, 9-I, 9-II). Most cases were referred because of inguinal masses or abnormal external genitalia in infancy or childhood. In a few cases virilisation at puberty prompted referral. Virilisation consisted of rugged, pigmented skin of labia majora, enlarged clitoris (> 3 cm), and male pattern body hair in patients 6, 9-I, 9-II and 11-I and lowering of the voice in patients 6 and 11-I. Patient 7 was gonadectomized early in puberty, at age 13 yr, Tanner M2, and had an enlarged clitoris at time of gonadectomy.

Interfamilial phenotypic variability was found in homozygotes for the 325+4;A→T mutation. Two sisters (no. 1-I, 1-II) and two unrelated patients (2, 3) had complete female genitalia at birth. Another unrelated patient (no. 4) had virilised genitalia at birth, that allowed a gender reversal from female to male when the diagnosis 17βHSD3 deficiency was made at age 2 yr.

Patients 1-I, 1-II, 2, 5, 6, 7, 8, 10, 12, 13, 14, 16 and 17 had initially received the tentative diagnosis AIS. No interfamilial relationships were found. Clinical data and *in vivo* and *in vitro* testosterone synthesis studies of patient 18 were described previously.[28]

Endocrine evaluation

T/A ratios in 17βHSD3-deficient patients, controls, and AIS patients are summarized in Figure 9.1. No overlap between the gonadotrophin-stimulated T/A ratios in 17βHSD3-deficient patients and controls or AIS patients was observed. Depending on the presence or absence of the physiological LH surge at the time of serum sampling, T/A ratios did show some overlap in the age group of 1-3 months. T/A ratios in 17βHSD3 deficient-patients initially diagnosed with AIS did not differ from ratios in the other 17βHSD3 patients. The highest T/A ratios in 17βHSD3-deficient patients of 0.84 before hCG and 0.94 after

Table 9.1
Clinical Data of 17β-hydroxysteroid dehydrogenase 3 Deficient Patients and Mutations in *HSD17B3* Gene: 23 patients in 19 families.

family/ patient #	*mutations*	*prepubertal external genitalia: Prader stage 27*	*age at referral (in years)*	*reason for referral*	*endocrine evaluation at age (in years)*	*T/A basal level*	*T/A after hCG*	*year of birth*	*ethnic origin*	*parental consanguinity*
Homozygotes										
1-I	325+4;A→T	female	0.8	inguinal mass	12	0.22	0.19	1982	Dutch	no
1-II	325+4;A→T	female	0.3	family history		ND	ND	1989		
2	325+4;A→T	female	0.1	inguinal mass		ND	ND	1993	Dutch	no
3	325+4;A→T	female	birth	inguinal mass	0.25	0.07	0.25	1998	Dutch	yes
4	325+4;A→T	2	birth	abnormal genitalia	2	ND	0.45	1995	Dutch	no
5	N74T	1	4	abnormal genitalia	4	0.62	0.50	1986	Dutch	no
6	N74T	not known	16	virilisation at puberty	16	0.19	ND	1972	Dutch	yes
7	R80Q	1	13	virilisation at puberty	13	0.84	0.94	1983	Dutch	no
8	326-1;G→C	female	8	inguinal mass		ND	ND	1970	Dutch	no
9-I	655-1;G→A	not known	16	virilisation at puberty	17	0.08	0.09	1976	Turkish	yes
9-II	655-1;G→A	not known	15	family history	16	0.1	0.09	1977		
9-III	655-1;G→A	1	10	family history	10	0.05	0.15	1985		
10	A188V	female	2	inguinal mass	10	1.08	0.56	1973	Turkish	no
Compound heterozygotes										
11-I	325+4;A→T/N74T	1	14	virilisation at puberty	14	0.30	ND	1982	Dutch	no
11-II	325+4;A→T/N74T	1	10	family history	10	0.4	0.6	1986		
12	325+4;A→T/N74T	1	0.1	inguinal mass	1.3	ND	0.28	1985	Dutch	no
13	325+4;A→T/R80Q	1	0.1	inguinal mass	0.1	0.16	ND	1986	Dutch	no
14	325+4;A→T/R80Q	1	birth	abnormal genitalia	0.1	0.3	0.38	1991	Dutch	no
15	325+4;A→T/326-1;G→C	1	2	inguinal mass	2	ND	0.35	1986	Dutch	no
16	325+4;A->T/326-1;G→C	1	0.1	inguinal mass		ND	ND	1988	Dutch	no
17	325+4;A→T/P282L	1	3.5	abnormal genitalia	6	1.0	0.27	1989	Dutch	no
Heterozygotes										
18	N130S/ -- (G289S)	1	birth	abnormal genitalia	0.6	0.4	0.29	1982	W-Indian	no
19	no DNA available	2	2	inguinal mass	15	ND	0.34	1972	Dutch	not known

ND= not determined; Patient #19 is included for disease incidence calculations; no DNA was available.

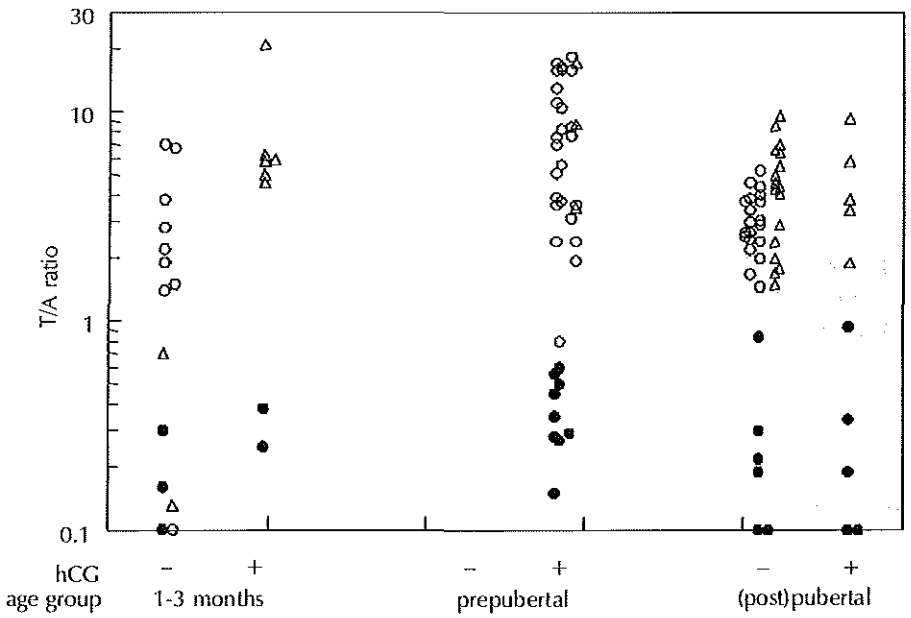

Figure 9.1
T/A ratios in serum of 17βHSD3-deficient patients (•), in serum of age-matched controls (o) or age-matched androgen insensitivity patients (Δ), before (-) or after (+) hCG stimulation. Ratios in individual 17βHSD3-deficient patients from this study are also shown in Table 1.

hCG, was found in one pubertal patient (no. 7) homozygous for a known partially inactivating mutation.[14]

Mutations

In 18 index patients, 9 different splice site or amino acid substitution mutations were identified (Table 9.1; Figure 9.2). No DNA could be obtained from patient #19. Recurrent mutations found among unrelated Dutch index patients were 325+4;A→T (15 alleles), N74T (6 alleles), R80Q (4 alleles) and 326-1;G→C (4 alleles). One patient (no. 18; Table 9.1) was heterozygote for a proven pathogenic mutation, N130S.[16] The second identified mutation, G289S, is supposed to be a neutral polymorphism[16]; we assume that the other allele contained another mutation outside the coding region, because males heterozygous for mutations in the HSD17B gene are normal, as was established in the fathers of these patients.

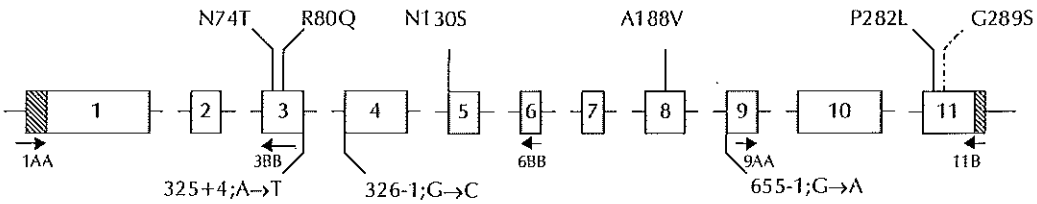

Figure 9.2
Mutations in the HSD17B3 gene identified in this study. The numbered *gray boxes* indicate exons; *lines* indicate intron sequences. 5' and 3' untranslated regions are denoted by *hatched bars*. Mutations leading to substitution of an amino acid are indicated *above* the gene, mutations in splice sites are indicated *below*. The neutral polymorphism G289S is denoted with a *dashed line*. Localization of the primers used for PCR amplification of cDNA has been indicated with *arrows*.

RNA splicing in homozygotes for 325+4;A→T or 655-1;G→A

PCR amplification of cDNA with primer pair 1AA-11B resulted in a 1016 bp product in the control (Figure 9.3A+B). In patient 1-I, mutation 325+4;A→T, no wildtype transcript was detected. Instead a transcript with deletion of exon 3 (941 bp or 454 bp when using primer pair 1AA-11B or 1AA-6BB respectively) and in minor amounts a transcript with deletion of exon 3 and 4 (833

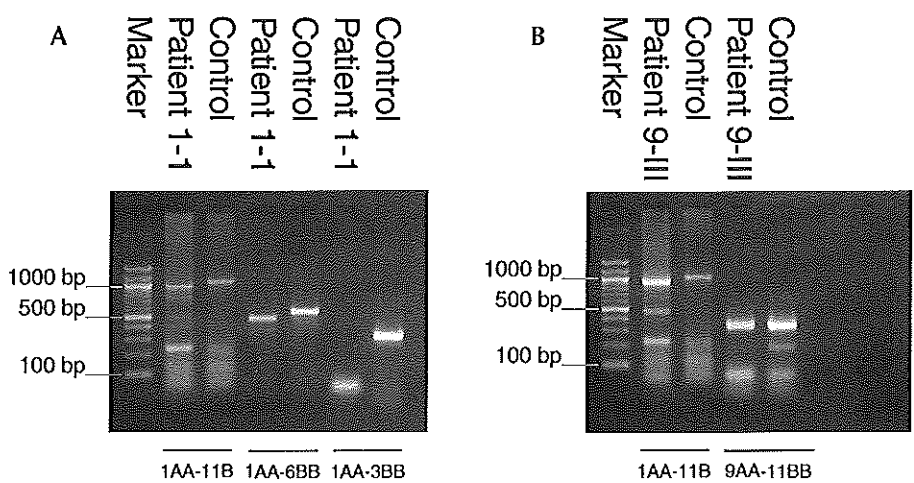

Figure 9.3
RT-PCR of testicular RNA from patient 1-I homozygous for mutation 325+4;A→T (A) and of patient 9-III homozygous for mutation 655-1;G→A (B) versus testicular RNA from a normal healthy male (control).

bp and 346 bp with the respective primer combinations) were present (Figure 9.3A). Both *HSD17B3* gene transcripts render the message out of frame.
In the patient with mutation 655-1;G→A, a transcript with a in-frame deletion of exon 9 was present (with primer pair 1AA-11B, 950 bp in weight). In minor amounts a transcript with an inframe deletion of exon 9 and 10 of 800 bp was present. A wildtype transcript was found only with use of primer pair 9AA-11B (Figure 9.3B). Therefore, only minute amounts of wildtype transcript are present as compared to the mutant transcripts.
The 175 bp band is found in cDNA of patient 9-III as well as in cDNA of the control and is therefore not specific for the patient (Figure 9.3B). Additional bands of 123 bp, 230 bp, 492 bp and 738 bp were unknown sequences and were present in cDNA of patient 1-I, 9-III as well as in cDNA of the control (Figure 9.3A+B). The transcript with a deletion of exon 3 till 11 of 396bp (primer pair 1AA-11B), present in cDNA of patient 1-I, patient 9-III as well as in cDNA of the control (Figure 9.3A+B), can also be regarded as aspecific for the mutants.

Incidence of 17β-hydroxysteroid dehydrogenase deficiency in The Netherlands

Of the total of 23 patients (19 families), 20 were born in The Netherlands between 1969-1999 including patients 10 and 18 (Table 9.1). The mean annual birth rate in that period was 190,000.[29] Thus a minimal incidence at birth of 28/20 x 190.000 = 1:266,000 can be calculated. However, the first 17βHSD3-deficient patient was described in 1971.[30] Very likely many earlier cases of 17βHSD3 deficiency will have received other diagnoses. Affected cases that manifest only with virilisation at puberty, born after 1987 will not be diagnosed until 1999 or later. The number of patients born in the 1980s is probably the most representative group for calculation of incidence data; most cases born in that period will be symptomatic by 1998. For the 1980s (mean birth rate 176,000) the calculated minimal incidence is 1:147,000.

Carrier frequency

The heterozygote frequency for the Dutch population, as calculated from the incidence of 17βHSD3 deficiency of 1:147,000 in the 1980s, is 1:135. For the 325+4;A→T mutation it is 1:210, based on eight 325+4;A→T alleles found in index patients born in the 80s. In a study of 200 random controls (400 chromosomes) no 325+4;A→T mutations were found. This finding does not con-

Table 9.2
Haplotype frequencies of chromosomes that carry either the 325+4;A→T or the N74T mutation in Dutch 17βHSD3 deficient patients versus a Dutch control group.

Haplotype	325+4;A→T	N74T	control	325+4;A→T	N74T	control	325+4;A→T	N74T	control
	AFM023XH8			D9S1786			D9S1851		
1	0	5	0	0	0	4	0	0	3
2	0	0	33	0	0	8	0	0	1
3	12	0	40	0	0	1	1	0	8
4	0	0	1	12	0	17	7	2	34
5				0	0	9	2	3	13
6				0	0	29	0	0	10
7				0	0	4	0	0	1
8				0	5	2			
Total alleles tested	12	5	74	12	5	74	10	5	70

The frequency of a specific haplotype for each marker is given in patients and controls and for each mutation. The frequency figures give the numbers of the observed haplotypes. The different haplotypes are numbered (1,2,3, etc) according to increasing gel mobility. No PCR product could be obtained with the primer combination for marker D9S1851 in 4/74 control alleles and two out of twelve 325+4;A→T mutant alleles.

tradict the calculated carrier rate, as there is a $(209/210)^{200}=38\%$ chance for this outcome.

Founders of *HSD17B3* mutations in the Dutch population

The haplotypes for chromosomes in the Dutch patients carrying either mutation 325+4;A→T or N74T, versus controls are shown in Table 9.2. The 325+4;A→T mutations were observed on the same 3/4 haplotype for the flanking markers AFM023XH8/ D9S1786 (~100 kb on the centromeric and telomeric sides, respectively). For the more distant marker (~1500 kb) D9S1851, recombination (allele 3, 4 or 5) had occurred. This confirmed that there was no close genetic relationship between families, as was established by pedigree analysis.

Table 9.3
Polymorphic marker haplotypes of mutations or polymorphic marker genotypes of patients of different geographic origin with 17βHSD3 deficiency.

Patient's origin	Genotype/Haplotype	Mutations
The Netherlands	3/4/3,4,5	325+4;A→T
Munich[15]	33/44/44	325+4;A→T/325+4;A→T
Australia	33/44/44	325+4;A→T/325+4;A→T
Pittsburg[15]	23/45/24	325+4;A→T/Q176P
S. Francisco 3[15]	3/4/4	325+4;A→T
The Netherlands	1/7/5	P282L
S.Francisco 3[15]	3/4/4	P282L
The Netherlands	2/7/5,6 or 3/4/5	326-1;G→C
Brazil 1	33/22/11	326-1;G→C/326-1;G→C
Brazil 2	33/22/11	326-1;G→C/326-1;G→C
S.Paulo 1[15]	33/24/46	R80Q/326-1;G→C
The Netherlands	3/4/4	R80Q
S.Paulo 1[15]	33/24/46	R80Q/326-1;G→C
Gaza[15]	33/44/66	R80Q/R80Q
S.Paulo 3[15]	33/44/66	R80Q/R80Q
Portugal	33/44/36	R80Q/E215D
Turkey	2/6/6	655-1;G→A
Syria[15]	22/66/44	655-1;G→A/655-1;G→A
Greece[15]	22/66/66	655-1;G→A/655-1;G→A

Haplotypes of mutations were derived by segregation analysis, thus by comparison of the polymorphic marker genotype of the patients that carried specific mutations and the polymorphic marker genotype of their parents who were heterozygotes for a specific mutation. Not of all patients parents DNA was available. In these cases the polymorphic marker genotype is given. The order of genotype/haplotype numbers is: AFM023XH8, D9S1786, D9S1851 respectively. Some of these patients were reported in Andersson et al.[15] and are denoted with[15]. The other patients are first described in this study.

Likewise, mutation N74T was associated with haplotype 1/8/4,5 (AFM023XH8/ D9S1786/ D9S1851) .

The association between mutation 325+4;A→T and haplotype 3/4 is significant {$P<0.05$ (AFM023XH8) and $P<0.01$ (D9S1786)} and also between N74T and haplotype 1/8 {$P<0.00000001$ (AFM023XH8) and $P<0.00001$ (D9S1786)}. Thus it is likely that both mutations were introduced by two genetic founders for all Dutch patients. Mutation 326-1;G→C on the other hand occurred on different haplotypes (2/7/5,6 and 3/4/5; Table 9.3) which suggests a recurrent *de novo* mutation.

Haplotypes of disease chromosomes found in patients worldwide

The geographic distribution of mutations reported in this study as being found worldwide is shown in Figure 9.4 (page 317); haplotypes and marker genotypes are shown in Table 9.3. The 325+4;A→T mutations in Dutch, Germans, white Australians and white Americans share the same marker genotype and are likely to be identical by descent. Likewise, the mutation R80Q in Dutch, in Arabs in Gaza, in white Brazilians and in white Portuguese patients and the mutation 655-1;G→C in Turkish, Syrian and Greek patients (Table 9.3, Figure 9.3) are due to common founders. The mutations 326-1;G-C and P282L have different intra and/or inter-ethnic haplotypes; therefore, these mutations must have recurrently occurred *de novo*.

DISCUSSION

Pathogeny of splice site mutations

We obtained evidence for the pathogeny of the frequently found 325+4;A→T and 655-1;G→A splice site mutations. Both mutations disrupt normal splicing. The transcripts found in the patient homozygous for 325+4;A→T are out of frame and therefore non functional (Figure 9.3A). No wildtype transcript was identified in the patient homozygous for mutation 325+4;A→T (Figure 9.3A). As all tested substitution mutations in exon 9 completely abolish enzyme activity[14,31], the transcripts with a deletion of exon 9 or with a deletion of exon 9 and 10, found in the patient homozygous for 655-1;G→A, are likely to be nonfunctional. A wildtype transcript was found in lesser amounts than the mutant transcripts in a patient homozygous for mutation 655-1;G→A (Figure 9.3B).

Genotype, phenotype relationship

Recurrence of several mutations in multiple patients offered the opportunity for genotype/ phenotype comparison. Prepubertal compound heterozygotes had clitoromegaly and labial fusion regardless of whether the mutations had been shown to cause truly female genitalia in homozygote form (325+4;A→T or 326-1;G→C; patient no. 15 and 16) or to render the enzyme completely defective in in vitro studies (P282L, patient no.17).[15]

During childhood, homozygotes for mutation 325+4;A→T had either truly female genitalia or ambiguous genitalia. Thus distinct phenotypic variation can occur between homozygotes for the same mutation. Possibilities for the residual-, prenatal source of androgen in patient no. 4 are 1) testosterone formation by another 17βHSD iso-enzyme, 2) the variable formation of a small amount of wildtype transcript or 3) somatic mosaicism for one wildtype allele, sometimes caused by reverse mutations.[32] However, somatic mosaicism for the mutation and a normal allele was excluded by allele-specific oligonucleotide hybridization analysis (data not shown). Therefore, the activity of another prenatally expressed iso-enzyme or the possible presence of a wildtype transcript are more plausible explanations. As the outcome of aberrant splicing is variable, the absence of a wildtype transcript in one homozygous patient for 325+4;A→T, does not exclude the possible presence of a wildtype transcript in another patient. This could not be tested in patient 4 because this patient was raised as a boy and consequently was not gonadectomized.
The presence of a wild-type transcript in testicular RNA of the 655-1;G→C homozygous patient (no. 9-III), predicts that phenotypic variation between homozygotes for this mutation could occur, depending on the amount of wildtype transcript formed. Indeed phenotypic variation between families is observed as the affected children in family #9 were thought to be normal girls during childhood which is distinctly different from the ambiguous genitalia with which two other unrelated 655-1;G→C homozygous patients had been born.[5,33] Again, androgen formation in peripheral tissues or even in the testes by another 17β-hydroxysteroid dehydrogenase iso-enzyme could also be the cause of this phenotypic difference.
It seems clear that no specific phenotype is associated with a specific mutation.

Diagnostics

Gonadotropin-stimulated T/A ratios allowed accurate selection of 17βHSD3-deficient cases. However, low T/A ratios are not specific for 17βHSD3 deficiency but are sometimes also found in patients with other defects in testosterone synthesis or Leydigcell hypoplasia. Therefore T/A ratios should only be used when a hCG-stimulated response of serum testosterone or/and serum androstenedione is observed. With additional mutation analysis the diagnosis can hardly be missed. All but 1 of the 18 tested patients were identified as homozygous or compound heterozygous for *HSD17B3* mutations. The remaining case, a 46,XY female with testes had unmistakable endocrine evidence

of 17βHSD3 deficiency: an abnormally low T/A ratio and the absence of androstenedione to testosterone conversion in testicular tissue.[28]
Based on the ethnic descent of a patient, a prediction on the expected mutations can be made (Figure 9.4 (page 317)). This greatly facilitates mutation analysis. Furthermore, the West-Europeans in this study all had mutations in exon 3, in both splice sites of intron 3 or in exon 11. Mutation analysis can therefore initially be focussed on these particular, relatively small parts of the gene.
In conclusion, endocrine evaluation is an important tool for the selection and diagnosis of patients suspected of 17βHSD3 deficiency. Mutation analysis, facilitated by knowledge of the ethnic distribution of mutations, provides additional proof.

Incidence and carrier frequency

17βHSD3 deficiency is a relatively common cause of male pseudohermaphroditism in The Netherlands, minimally in 1:147,000 newborns. In comparison, the minimal incidence of AIS in the Netherlands is 1:99,000 (unpublished data, based on this same nationwide survey). Previous incidence data for AIS vary between 1:40,800 and 1:128,000 births[34-37] and are based on antiquated diagnostic criteria such as inguinal hernia in girls or X-chromatin-negative bodies in buccal smear of affected girls. Quite likely these series include unidentified 17βHSD3-deficient patients and give a biased, too high incidence rate for AIS.
Like other autosomal recessive diseases, 17βHSD3 deficiency may show increased frequencies among populations with a high intermarriage rate. In Arabs in Gaza among whom intermarriage is frequent[38], the incidence is 1:200-300, most likely all homozygotes for the R80Q mutation.[10] In contrast, the Caucasian Dutch population is heterogeneous, the intermarriage rate is low and the disease is caused by several different mutations. The carrier frequency for 17βHSD3 deficiency in The Netherlands was calculated to be 1:135.

Founders

Recurrence of mutations N74T and 325+4;A→T in the Dutch is very likely due to common founders. Unfortunately, founder analysis of the other described patients with N74T[39] was not possible. 325+3;A→T is also carried by other Caucasians living worldwide. All patients with mutation 325+4;A→T have the same haplotype for 17HSDB3 gene flanking markers. Thus the com-

mon founders may have lived in Europe, and European immigrants brought the mutation to the U.S. and Australia.

An interesting founder effect may be present in the R80Q mutation, common among Arabs in various parts of Israel, some with Druze ancestors from Lebanon and Syria.[40] Their relationship with the same founder of the mutation in Dutch, Portuguese, and white Brazilians prompts the speculation that this mutation became introduced by the Phoenicians who migrated from an area in present day Syria, Lebanon and Israel around 750 BC towards Portugal and Spain to search for metals and timber.[41-43] From there, the mutation was brought to Brazil by the Portuguese colonists and to The Netherlands during the Spanish rule in the 16th-17th centuries. Alternatively, the mutation may have been introduced in Portugal and Spain by the Moors who had empires on the Iberian peninsula from AD 711 untill 1492 and came from, for example Lebanon and Syria. The Lebanese and Druze are genetically descendants of the Phoenicians[43], and large numbers of Arabs from Lebanon and Syria, among which Druze, immigrated in the 19th century in South America.[44]

The 655-1;G→A mutation found in Turks, Greeks and Syrians might have spread over these populations during the Ottoman empire which included these three countries between AD 1359 and 1565.[45] The Ottoman empire is known to have contributed to the racial admixture of that area.[43,46]

The recurrent *de novo* occurrence of other mutations such as 326-1;G→C and P282L supports the conclusion that the genetic basis of 17βHSD deficiency is determined by multiple founders as well as recurrent *de novo* mutations.

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CHAPTER

10

Patients with mutations in the putative redox partner binding site of the 17,20 lyase enzyme

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SUMMARY

Background – Combined 17α-hydroxylase/17,20 lyase deficiency and isolated 17,20 lyase deficiency are rare autosomal recessive diseases associated with mutations in the *CYP17* gene. The *CYP17* gene encodes the bifunctional enzyme which is responsible for both the 17α-hydroxylase and the 17,20 lyase reactions. It is expressed in the adrenals and the gonads, and these enzyme activities are crucial for the synthesis of cortisol, and sex steroid hormones (17α-hydroxylase), or sex steroid hormones alone (17,20 lyase). Understanding the regulation of 17,20 lyase activity is important for unraveling the mechanisms for adrenarche and, in women, hyperandrogenism. Furthermore, analysis of patients and their mutations will permit more insight into the structure-function relationship in 17α-hydroxylase / 17,20 lyase enzyme.

Methods – Phenotypes and genotypes of four patients, including two siblings, were studied with either combined 17α-hydroxylase / 17,20 lyase or 17,20 lyase deficiency.

Results – Two 46,XY siblings with 17,20 lyase deficiency but nearly normal 17α-hydroxylase activity were homozygous for the mutation R347H. Residue 347 lies in the putative redox partner binding site of P450c17 which is involved in the differential regulation of the 17α-hydroxylase and 17,20 lyase activities of P450c17. In these two patients, homozygosity for R347H in the *CYP17* gene leads to 17,20 lyase deficiency and only minimal 17α-hydroxylase impairment as cortisol levels are low, but could still be stimulated by ACTH.

Two unrelated 46,XY (post)pubertal women with combined deficiency, were compound heterozygous for the mutation R347C, with as the second mutation either a newly identified deletion of 25 bp in exon 1 or a duplication of 4 bp in exon 8. Compound heterozygosity for mutation R347C and the deletion in exon 1 or duplication in exon 8, was found to be associated with a more severe impairment of 17α-hydroxylase activity. Cortisol was present at low normal levels, but its concentration did not increase upon ACTH-stimulation.

Conclusions – Therefore, the R347C mutant is a candidate for further unraveling the differential regulation of 17α-hydroxylase/ 17,20 lyase activity, when tested in *in vitro* expression studies. Alternatively, the observed differences in impairment of 17α-hydroxylase enzyme activity is age-related in these patients.

INTRODUCTION

Combined 17α-hydroxylase/17,20 lyase deficiency and isolated 17,20 lyase deficiency are rare autosomal recessive diseases associated with mutations in the *CYP17* gene.[1,2] This gene, located on chromosome 10q24.3[3] encodes the bifunctional enzyme 17α-hydroxylase/17,20 lyase (cytochrome P450c17) and is expressed in the adrenals and the gonads. The enzyme activities are crucial for the synthesis of both cortisol and sex steroid hormones (17α-hydroxylase; 3 in Figure 5, Chapter 1) or sex steroid hormones alone (17,20 lyase; 4 in Figure 5, Chapter 1). Understanding the regulation of 17,20 lyase activity is important for unraveling the mechanisms for adrenarche in both sexes and hyperandrogenism in women. Furthermore, analysis of patients and their mutations will permit more insight into the structure-function relationship of P450c17.
In 46,XY subjects, the deficiency or decreased activity of the 17,20 lyase enzyme causes defective virilisation of the external genitalia during prenatal development. These patients do have testes, and Müllerian derivatives are absent. At puberty, breast development and pubic hair will not develop. 17α-Hydroxylase catalyses the reaction preceding the 17,20 lyase reaction. In 17α-hydroxylase, deficiency low renin hypertension, hypokalemia and metabolic alkalosis are seen besides male pseudohermaphroditism and hypogonadism.

Twenty-six different mutations in the *CYP17* gene have been described untill now, which either lead to complete or partial deficiency and can be devided in two groups: 1) mutations that abolish 17α-hydroxylase and 17,20 lyase activities, and 2) mutations that affect lyase activity but leave sufficient 17α-hydroxylase activity, resulting in isolated 17,20 lyase deficiency.[2,4]
However, patients with isolated 17,20 lyase activity carry *CYP17* mutations that impair both 17α-hydroxylase/17,20 lyase activities when tested *in vitro*.[5] Furthermore, in some cases 17,20 lyase deficiency during childhood changed to 17α-hydroxylase/17,20 lyase deficiency at adulthood.[6]
Despite these difficulties, *in vivo* as well as *in vitro* studies indicate that the mutations R347H, R358Q[2], F417C[4] are associated with isolated 17,20 lyase deficiency. These mutations all lie in or near a part of the gene that encodes the putative redox-partner binding site, where P450 oxidoreductase interacts with the P450c17 enzyme to donate the electrons used for catalysis. In vitro experiments showed 50%, 50% and 26% of residual 17α-hydroxylase activity, respectively, for these mutations, and undetectable 17,20 lyase activity in all three. The lyase reaction increased to 5% of wild type activity in the presence

of high levels of the redox partner or an electron donor that is not dependent on binding to the enzyme.[2,4]

We describe 4 patients with different mutations in one of the putative redox partner binding site, R347. The patients homozygous for R347H have isolated 17,20 lyase deficiency as described before. However, the patients who are compound heterozygous for mutation R347C and a deleterious mutation, have combined partial 17α-hydroxylase/complete 17,20 lyase deficiency.

PATIENTS AND METHODS

Patients I-1 and I-2 are siblings from consanguineous Moroccan parents. Both 46XY siblings were born with ambiguous external genitalia, consisting of a small phallus, penoscrotal hypospadias and labio-scrotal folds which had a pigmented, rugated skin in patient I-1 (Figure 10.1a and b). Patient I-1 was assigned the male sex and I-2 the female sex on the basis of the designated sex at birth by the parents. There were no clinical signs of insufficient cortisol secretion. Both siblings underwent surgery several times without complications (in the absence of a hydrocortisone stress scheme). Results of endocrine evaluation is shown in Table 10.1A. In the hCG test, serum levels of several hormones were determined in blood samples taken 0 and 72 hours after the intramuscular administration of 1500 U hCG.
In the ACTH test, venous catheters were inserted and 30' afterwards serum levels of several hormones were determined in blood samples taken 0', 30' and 60' after the intravenous administration of ACTH.

Patient II: 46XY, was born with complete female external genitalia and raised as a girl. She was the first child of consanguineous Dutch parents. The family history did not reveal sexual differentiation disorders. At age 2 months, she had bilateral inguinal hernias that contained testes. The presumptive diagnosis androgen insensitivity (AIS) was made and a gonadectomy was performed. She was re-evaluated at age 10. AIS was excluded by molecular studies in genital skin fibroblasts, mutation analysis of the androgen receptor gene as well as by a normal decline of serum SHBG levels in a SHBG-suppression test.[7] The results of an ACTH stimulation test are shown in Table 10.1B. Her blood pressure was 140/80 mmHg at age 10. She underwent surgery uneventfully twice.

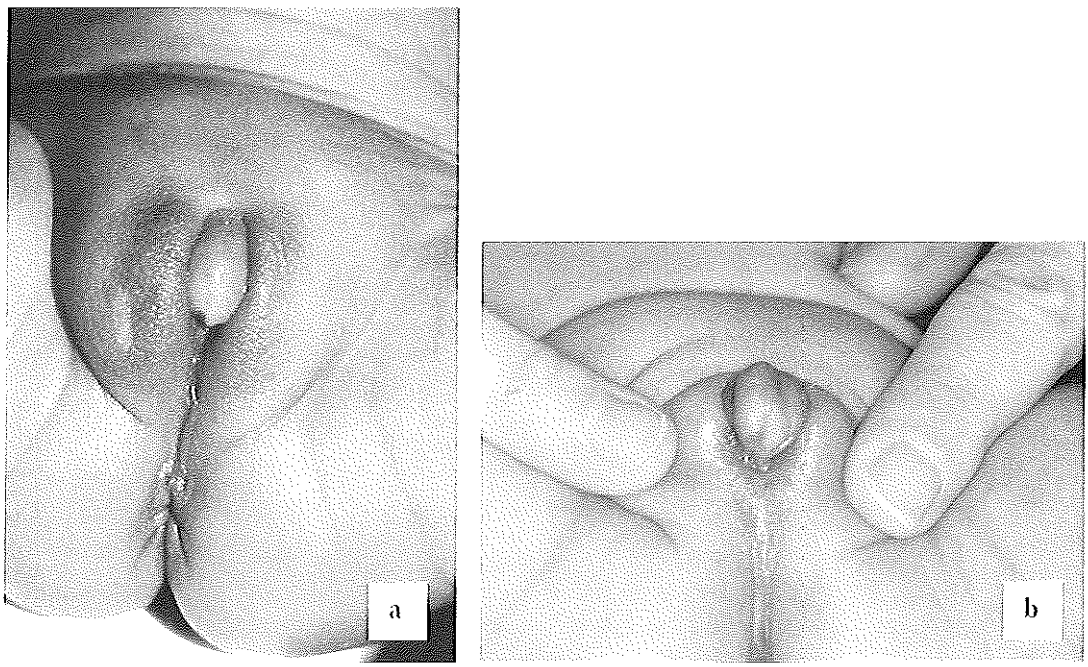

Figure 10.1
External genitalia of patients I-1 (a) and I-2 (b).

Patient III, raised as a girl, was evaluated at age 14, because of delayed puberty. She had a 46XY karyotype and complete female external genitalia, with absent uterus and fallopian tubes. The diagnosis AIS was made and she underwent gonadectomy. At age 28 she was reevaluated. No mutations in the androgen receptor gene were found and SHBG serum levels declined normally in an SHBG-suppression test, excluding AIS. Results of an ACTH test are shown in Table 10.1B. Her blood pressure was 170/90 mmHg. She underwent surgery several times without complications. She had developed breasts on estrogen substitution. Her main complaint was the complete absence of pubic hair. After the diagnosis 17α-hydroxylase/17,20 lyase deficiency was made, testosterone propionate cream therapy was started and pubic hair developed.

Mutation analysis of the *CYP17* gene

Genomic DNA was isolated from either leukocytes or genital skin fibroblasts according to standard procedures.[8]
Exons 1-8 of the *CYP17* gene were individually amplified by PCR[9] followed by SSCP analysis and direct sequencing.[10] In order to determine whether two mu-

Table 10.1A
Hormonal evaluation of patients I-1 and I-2.

	Patient I-1							Patient I-2			
Age	2 weeks		1 month	2 months		7 years		2 weeks		2.5 years (gonadectomized)	
Test/Time	basal	72 h after hCG	basal	basal	72 h after hCG	basal (0′)	after ACTH (60′)	basal	72 h after hCG	basal (0′)	after ACTH (60′)
LH (IU/L)			20 (2-26)					6.0 (2-26)			
FSH (IU/L)		3	1.8					4.7			
Progesterone (nmol/L)	8.6 (0.5-1.3)					2.7	6.1			2.6	11.0
17OHprogesterone (nmol/L)				4.5 (0.03-5.2)	14.9	1.3 (0.4-2.1)	1.5 (3.5-6)	13.4 (0.33-5.2)	19.6	1.3 (0.1-3.5)	2.1 (2.0-8.1)
DHEA (nmol/L)				0.25 (0.9-8.2)	0.35	0.7 (0.4-4.9)	0.44 (1.2-9.4)	2.0 (0.9-8.2)	1.4	0.1 (0.3-1.5)	0.3 (0.7-3.3)
DHEAS (µmol/L)				0.4 (0.03-0.6)	0.3 (0.1-0.5)	<0.4 (0.03-0.6)	<0.4 (0.03-0.8)	<0.4 (0.05-1.0)	<0.4	<0.4 (0.03-0.5)	<0.4 (0.03-0.6)
Androstenedione (nmolL)	0.73 (0.9-15)			0.14 (0.9-1.8)	0.15 (1.0-4.0)	0.10 (0.7-3.8)	0.09 (0.4-1.6)	0.3 (0.9-15)	0.4	0.07 (0.2-1.7)	0.05 (0.4-2.4)
Testosterone (nmol/L)	4.0 (0.03-26)	4.1 (6.8-15)	6.2 (0.03-26)	0.7 (0.03-26)	4.1 (6.8-15)	0.02 (<0.5)	0.05	1.2 (0.03-26)	2.6 (6.8-15)	0.01 (<0.5)	0.09 (0.07-0.21)
DHT (nmol/L) ♣				0.2	0.7	<0.02	<0.02	0.5	0.8		
ACTH (ng/L) (pmol/L)*	78 (20-80)		11.2* (1.3-9.2)*	4.4* (1.3-9.2)*		9.3* (1.3-9.2)*		9.7* (1.3-9.2)*		6.6* (1.3-9.2)*	
Compound-S				1.9 (0.3-5.8)	3.3			17.7 (0.3-5.8)	14.3		
Cortisol (nmol/L)	99 (80-579)		350 (80-579)	194 (80-579)	187	190 (80-579)	385 (>500)	270 (83-579)	150	243 (80-579)	427 (>500)
Plasma Renin Activity (nmol/L/H)								4.12 (4)			
Sodium (mmol/L)	133 (135-140)										
Potasium (mmol/L)	5.8 (3.5-6.0)										

♣: values in normal males; T/DHT <10
(.....) values in age matched normal males

Table 10.1B
Hormonal evaluation of patients II and III.

	Patient II (gonadectomized; no estrogen substitution)			Patient III (gonadectomized; estrogen substitution)		
Age	10 years		11 years	28 years		
Test/Time	basal	after ACTH (60')	basal	basal 10.00	afterACTH(60')	basal 19.30
LH (IU/L)	6.4	5.4		10.6 (1.5-8)		11.6 (1.5-8)
FSH (IU/L)	41.5	46.5		37.3 (2-7)		30.9 (2-7)
Progesterone (nmol/L)	7.4	8.4		6.1 (0.5-2)	12.2	5.2 (0.5-2)
17OHProgesterone (nmol/L)	2.4 (0.4-2.1)	2.6 (3.5-6.0)		1.9 (<10)	2.4	0.6 (<10)
DHEA (nmol/L)	0.3 (0.4-4.9)	0.5 (1.2-9.4)		0.0 (3.5-25)	0.0	0.5 (3.5-25)
DHEAS (µmol/L)	0.28	0.19		0.25 (7-17)	0.16	0.26 (7-17)
Androstenedione (nmol/L)	0.29 (0.24-0.84)	0.13 (0.4-1.6)		0.57 (2-10)	0.51	0.3 (2-10)
Testosterone (nmol/L)	0.1	0.1		0.1		0.1
ACTH (ng/L)			159.0 (<60)	43.0 (<60)		49.2 (<60)
DOC (nmol/L)				96 (<50)	73	20 (<50)
Cortisol (nmol/L)	216 (200-800)	228 (>500)	237 (200-800)	232 (200-800)	285 (>500)	178 (<75% of 10.00h)
Renin (pg/ml)			12.5 (60-300)			10.2 (60-300)
Aldosterone (pg/ml)			435 (50-200)			187 (50-200)
Sodium (mmol/L)			141(137-145)			137 (137-145)
Potassium (mmol/L)			3.7 (3.5-5.0)			3.8 (3.5-5.0)

tations identified in a patient were localized on separate alleles, allele-specific amplification was carried out (patient 2), or DNA of the parents was sequenced (patients I-1, I-2 and III).

RESULTS

Clinical diagnosis

In Patients I-1 and I-2, the diagnosis of isolated 17,20 lyase deficiency was made, based on the low basal serum levels of DHEA, DHEAS, androstenedione and testosterone, the insufficient rise of these levels after hCG stimulation and normal basal serum levels of ACTH, cortisol and renin,. However, a slight impairment of 17α-hydroxylase was suggested by an abnormal rise in progesterone serum levels after ACTH stimulation while 17OH progesterone levels remained normal. Furthermore, an insufficient rise of cortisol serum levels after the intravenous adminitration of 0.25 mg of ACTH, was observed in both siblings.

Patient II had elevated levels of ACTH combined with low-normal serum levels of cortisol and absence of the normal response of cortisol to ACTH. Her blood pressure was elevated and she had a low plasma renin and elevated aldosterone levels as in 17α-hydroxylase deficiency. Despite the presence of normally developed testes her phenotype was completely female. Therefore, the diagnosis combined partial 17α-hydroxylase/complete 17,20 lyase deficiency was made.

Although Patient III had normal basal levels of ACTH, and a low normal cortisol, the cortisol levels did not rise after ACTH stimulation. She had a low plasma renin activity (PRA) combined with normal aldosterone values, thus an abnormally low PRA/aldosterone ratio and hypertension as in 17α-hydroxylase deficiency. Furthermore, she had completely female genitalia and total absence of pubic hair and breast development at the age of puberty. Therefore the diagnosis combined partial 17α-hydroxylase/complete 17,20 lyase deficiency was made.

Mutations

The mutations identified in the *CYP17* gene, are shown in Table 10.2. Patient I-1 and I-2 are homozygous for the R347H mutation. The parents of patient I-1 and I-2 were proven to be heterozygous carriers of the R347H mutation.

Table 10.2
Mutations in the *CYP17* gene

Mutation	Effect of mutation	Patients I-1 and I-2	Patient II	Patient III
R347H	amino acid substitution	++		
R347C	amino acid substitution		+	+
deletion bp 204-228 in exon 1	frameshift; stopcodon at codon 94		+	
CATC duplication on position 1867 in exon 8	frameshift of C-terminal 26 aminoacids			+

++ homozygous
+ heterozygous

Patient II was a compound heterozygote for two newly identified mutations: R347C and a 25 basepair deletion in exon 1 that renders the message out of frame.
Patient III was a compound heterozygote for mutation R347C and a 4 base duplication near codon 480.
Localization of two different mutations identified in the *CYP17* gene in a single patient on separate alleles was confirmed by allele-specific amplification (patient II) and by finding the parents to be heterozygous for the respective mutations (patient III).

DISCUSSION

Isolated 17,20 lyase deficiency was associated with homozygosity for the mutation R347H in two siblings as was found in a previously described case.[2] The partially virilized genitalia of these 46XY children and the ability to synthesize some testosterone are in accordance with the findings *in vitro* that in the presence of high concentrations of redox partner there was an approximately 5% residual activity of the lyase.[2] ACTH stimulation in these patients resulted in an increase of the serum progesterone concentrations. This suggests some expression of the *in vitro* observed 50% impairment of 17α-hydroxylase activity[2] in homozygous patients for mutation R347H.

The combined deficiency in patients II and III was caused by compound heterozygosity, for a novel mutation R347C combined with two different mutations on the other allele. In patient III this was a 4 bp duplication in exon 8 previously observed in several families of Dutch and German Mennonite descent.[11] Its homozygous presence leads to complete combined 17α-hydroxylase/17,20 lyase deficiency.[12] In patient II the second mutation was a 25 bp deletion in exon 1, leading to a frameshift with a premature stopcodon at position 94, a newly identified mutation. We expect that this mutation does not lead to the production of any functional protein.

Based of these results, we conclude that heterozygosity for mutation R347C in the redox partner binding site, combined with a mutation in the other allele which completely abolishes 17α-hydroxylyase and 17,20 lyase activities, leads to complete lyase deficiency and partial hydroxylase deficiency. In contrast to patients homozygous for mutation R347H (patients I-1, I-2 and ref. 2), who retain a relatively adequate capacity to synthesize cortisol, these two compound heterozygous patients were just able to maintain minimal basal cortisol levels. Therefore, the R347C mutant is a candidate for further unraveling the differential regulation of 17α-hydroxylase/ 17,20 lyase activity, when tested in *in vitro* expression studies. Alternatively, the observed differences in impairment of 17α-hydroxylase enzyme activity is age-related in these patients as patients II and III had already passed adrenarche, whereas patients I-1 and I-2 had not.

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CHAPTER

11

Etiologic studies in severe or familial hypospadias

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SUMMARY

Background – Hypospadias is a congenital anomaly occurring in 1:250 to 1:830 live male births, of which 20% are cases with a severe form of hypospadias. The recurrence risk in families is high. In the majority of cases the underlying etiology remains unknown, which hampers further management and treatment based on the specific requirements associated with a specific etiology.

Patients and Methods – In a single-center study 63 unselected cases with severe hypospadias were studied for all presently known causes of hypospadias, with use of clinical as well as molecular biological techniques. Also 16 families with hypospadias were analyzed for possible presence of androgen receptor gene mutations.

Results – In 31% of cases with severe hypospadias the underlying etiology was resolved. Of these 32%, 17% was due to complex genetic syndromes, 9,5% was due to chromosomal anomalies, one case had vanishing testes syndrome, one had androgen insensitivity syndrome, and one had 5α-reductase type 2 deficiency. Based on hormone stimulation tests, Leydig cell hypoplasia and disorders of testosterone biosynthesis were suspected in some patients but were not confirmed by mutation analysis of the respective genes. Familiar hypospadias was due to androgen insensitivity in only one family, but no other etiologies were identified in this group.

Conclusions – With use of patient's history, physical examination, karyotyping, hormonal evaluation including hCG testing in prepubertal cases and additional biochemical and molecular genetic evaluation, an etiologic diagnosis could be made in 31% of cases with severe hypospadias. This diagnosis has implications for further management and treatment of the patients. In addition, familial hypospadias is rarely due to androgen insensitivity syndrome.

INTRODUCTION

Hypospadias is a congenital anomaly occurring in 1:250 to 1:830 live male births[1], of which 20% are cases with a severe form of hypospadias (i.e. penoscrotal or perineal hypospadias).[2] The recurrence risk in families is high[3], 14% for a sibling and 8% when the father is affected.[4]

Severe hypospadias are often associated with other genital abnormalities such as micropenis, bifid scrotum, penoscrotal transposition, and cryptorchidism and may represent an intersex phenotype.[2] Known etiologic factors equal those for intersex disorders and include complex genetic syndromes[5], chromosomal abnormalities[3,6-8], WT1 mutations[9], Leydig cell hypoplasia[10], testosterone synthesis disorders[11-15], 5α-reductase type 2 deficiency[16], and androgen insensitivity.[17-20] In addition maternal ingestion of estrogens or progestagens during pregnancy may cause hypospadias.[1] An etiologic role for genetic and environmental factors was postulated since ethnic as well as geographic differences in incidence exist and the incidence is increasing in many countries.[1] However in the majority of cases, the etiology of hypospadias remains unresolved.[21]

Among single etiologic factors, chromosomal abnormalities are found in 5-12% of cases.[3,6-8] Androgen receptor mutations are infrequently found in patients with hypospadias.[19-21] Reduced testosterone responses to hCG in prepubertal boys with hypospadias was found in some[22-24] but not in other studies.[25] A 30% incidence of 3β-hydroxysteroid dehydrogenase type II deficiency and 17,20-lyase deficiency was recently described in 30 patients with severe forms of hypospadias[26] whereas others, also using hormonal evaluation found no such cases.[21,25] Using a variety of diagnostic techniques for established causes of hypospadias one third of 33 cases of severe hypospadias, could be clearly classified.[21]

We present the results of a single-center study of 63 patients with severe hypospadias using a variety of diagnostic techniques. In addition the prevalence of androgen receptor (AR) gene mutations was studied in 27 familial hypospadias patients with possible X-linked inheritance.

PATIENTS AND METHODS

Recruitment of patients

The databases available in the Departments of Plastic and Reconstructive Surgery and Pediatric Urology as well as the database of the University Hospital

Rotterdam, (functioning as a regional specialized center for hypospadias), were searched for patients with hypospadias. All cases referred between 1966 and 1995 with severe hypospadias as well as familial cases with any degree of hypospadias, were included. As severe hypospadias were classified: cases with penoscrotal, scrotal or perineal hypospadias with or without cryptorchidism, micropenis and/or bifid- or transposed scrotum, (i.e. Prader stage III-IV[27]). Patients with intersex phenotypes (Prader stage II), severe undervirilisation such as labioscrotal folds, or a pseudovaginal pouch, were not included. Androgen insensitivity syndrome (AIS) was specifically studied in familial cases. Familial cases were further selected for a possible X-linked inheritance pattern; patients with a paternal transmission were excluded.
The patient or the parents of the underaged cases received written information about the purpose and methods of this study and were asked to give consent for participation. The Medical Ethical Committee of the University Hospital Rotterdam approved this study.
From a total of 88 patients with a severe form of hypospadias, 63 patients aged between 2 weeks to 33 years could be studied. 16 patients refused to participate, 8 patients were lost to follow up and one patient died in an accident.
Also, 27 patients with familial hypospadias from 16 different families aged 2 to 42 years were selected. Two other families were excluded because of paternal inheritance. Twelve patients from seven different families refrained from participation.

Methods

A complete medical history was obtained from all patients and their parents. Maternal medication/intoxications, especially during the first trimester of gestation of the patient were addressed. Family history and pedigrees were obtained, focussing on hypospadias/genital ambiguity, infertility, neonatal deaths and consanguinity. All patients received a general physical examination, including dysmorphological evaluation to detect possible complex syndromes. The preoperative analysis and description of the genitalia and the localization of the testes were obtained from the medical records. In adults, we checked for undervirilisation such as a high pitched voice, a female distribution and/or reduced amount of sexual hair, female body habitus, or gynecomastia. Testicular volumes were measured using of the Prader-orchidometer and stretched penile length was measured between symphysis and glandular tip.[26]
Ultrasonographic investigation for urinary tract abnormalities and Müllerian remnants was performed in cases with severe hypospadias and other genital

abnormalities such as bifid scrotum or abnormalities in other organ systems. Blood samples were obtained for hormonal evaluation, DNA analysis of the AR and cytogenetic studies.

Hormonal evaluation

Serum levels of LH, FSH, 17-hydroxyprogesterone, testosterone (T), 5α dihydro-testosterone (DHT) and sex hormone binding globulin (SHBG) were measured by radio immuno assay. In prepubertal patients a human chorionic gonadotrophin (hCG) test was performed. Blood samples were taken before, and 72 hours after the intramuscular administration of 1500 IU hCG. Results were compared with those obtained in normal healthy boys aged 1-2 years (n=7), 2-6 years (n=10) and 6-12 years (n=10).[28] Depending on age, increases of serum T less than 6.8 nmol/L, less than 7.1 nmol/L and less than 3.3 nmol/L respectively, were considered abnormal. Furthermore, in patients with postpubertal or hCG stimulated 17-hydroxyprogesterone serum levels exceeding 25% of the T level, additional androstenedione (A) serum levels were measured in order to differentiate between 17,20 lyase deficiency and 17β-hydroxysteroid dehydrogenase 3 (17βHSD3) deficiency. In patients with an insufficient T rise and low 17-hydroxyprogesterone, progesterone and dehydro-epi-androsterone (DHEA) were measured in order to detect a 3β-hydroxysteroid dehydrogenase type II deficiency, 17α-hydroxylase/17,20 lyase deficiency or Leydig cell hypoplasia.

Cytogenetic studies

Standard GTG-banding and chromosome analysis were performed on metha-phase spreads from peripheral blood leukocytes and from cultured fibroblasts grown from skin biopsies (only patients 1, 5 and 6, Table 11.4A). FISH studies used the centromeric probes pBam X5 (X-centromere) and pDP97 (Y-centro-mere). Hybridization and signal detection were performed as described by De Vries et al.[29]

Androgen receptor assessment

DNA was extracted from peripheral leukocytes using standard procedures.[30] *Androgen receptor* genes of all patients with severe hypospadias as well as *an-drogen receptor* genes of all index patients with familial hypospadias, were screened for mutations with the use of PCR-single strand conformation poly-morphism (SSCP) as previously described.[31] Genital skin fibroblast cell-lines of

patients with *AR* gene mutations were assayed for androgen receptor binding according to the previously described method.[31]

5α-Reductase type 2 assessment

5α-reductase type 2 activity was measured in genital skin fibroblasts of one patient suspected of 5α-reductase type 2 deficiency because of an elevated serum T/DHT ratio.[32]

LH receptor gene or *HSD17B3* gene analysis

In cases with insufficient serum T response to hCG and low serum levels of progesterone, DHEA, 17-hydroxyprogesterone and androstenedione after hCG, all eleven exons of the *LH receptor* gene was sequenced using methods previously described by Martens et al.[33] and Atger et al.[34]
In cases with an insufficient rise in T after hCG and a decreased T/A ratio in serum compared to normal controls[35], the gene encoding 17β-hydroxysteroid dehydrogenase 3 (17βHSD3), *HSD17B3*, was screened for mutations according to the previously described method.[35]

RESULTS

Hypospadias: grade and associated genital abnormalities

The severities of the hypospadias and the associated abnormalities have been summarized in Tables 11.1 and 11.2, respectively.
Associated genital abnormalities in familial cases were micropenis in one family and transposition in 3 families. Intra-familial phenotypic variation was observed in all but one family.

Family history

4 patients with severe hypospadias and 2 index patients with familial hypospadias were offspring of a consanguineous marriage. One patient with a severe hypospadias had a 46,XY sibling with undervirilized genitalia that died in utero, at the gestational age of 28 weeks. No further diagnosis was made.

Intrauterine exposures

The mothers of two boys continued oral anticonception during the first 3 months of gestation. The mother of a third boy used isoxsuprine 3x20 mg/ day from start till the end of pregnancy in addition to hydroxyprogesterone 250 mg /10 days in the first 3 months of gestation and allylestrenol in the 3rd till 6th month of gestation.

Complex genetic syndromes

Complex syndromes were established in 11 patients with severe hypospadias (Table 11.3); in 8/11 these autosomal recessive, X-linked or sporadic syndromes could be associated with hypospadias. In 3/11 the complex syndrome could not be matched to any known syndrome.

Associated non-genital abnormalities

In the group with severe hypospadias a unilateral Wilms tumor (Drash syndrome) was present in one patient, hydronephrosis in another. Small for gestational age and prematurity were observed in these two cases. Two digestive tract abnormalities and one cardiac abnormality were found.
In the group of familial non-syndromic hypospadias, one patient had reflux uropathy, one patient had a hypoplastic arm and hand, and one had pyloric hypertrophy.

Cytogenetic studies

Among the 63 karyotyped patients with severe hypospadias abnormal findings were seen in 8 cases (#1-8; Table 11.4A). Patient

Table 11.1
Hypospadias-types among severe and familial cases

Type of hypospadias	Severe n	Familial n
Perineal	7	
Scrotal	17	
Peno-scrotal	39	5
Penile		10
Coronal		6
Glandular		6
Total	63	27

Table 11.2
Associated genital anomalies among 63 patients with severe hypospadias

	n
Micropenis +cryptorchidism+shawl scrotum	6
Other anomalies, single or combined:	
• Micropenis	11
• Buried penis	4
• (Partially) bifid scrotum	26
• (Slight) scrotal transposition	16
• Unilateral/bilateral cryptorchidism	11
• Unilateral testis atrophy / agenesis	4
• Absence of associated anomalies	19

Table 11.3
***Complex genetic syndromes* as established in patients with severe hypospadias**

syndrome	n
Smith-Lemli-Opitz	2
Opitz-Frias	1
Opitz G / BBB	1
Silver Russel	1
Cornelia de Lange	1
Joubert	1
Drash	1
Other unidentified complex syndrome	3
Total	11

Table 11.4A
Chromosomal findings and phenotypes in patients with severe hypospadias

#	karyotype	type of hypospadias	findings by urethro-cystography/ ultrasonography or inspection	Serum hormones	gonads at gonadectomy
1	L mos 45,X [36] / 46,XY [60] F mos 45,X [21] / 46,XY [54]	scrotal, micropenis	urogenital sinus with proximal vagina, rudimentary tubae + uterus	-	dysgenetic testes
2	L chi 46,XX [21] / 46,XY [9]	perineal, unilateral cryptorchism	utricule	3 years old: LH normal (< 0.1 IU/L) normal T after hCG (12 nmol/L)	-
3	L 46,XY (n=32) In gonadal cells: X and Y sequences	penoscrotal, bifid scrotum, unilateral cryptorchidism	no Müllerian remnants.	1 year old: LH normal (<0.1 IU/L) FSH normal (1.3 IU/L) normal T after hCG (8.4 nmol/L)	one dysgenetic ovary, one testis
4	L mos 45,X [38] / 46,X,i(Y)(P11.1) [12]	penoscrotal, bifid scrotum	no Müllerian remnants	2 years old: LH normal (< 0.1 IU/L) FSH normal (0.8 IU/L) normal T after hCG (7.6 nmol/L)	-
5	L mos 45,X [2] / 46,XY [48] F mos 45,X [16] / 46,XY [16]	scrotal, unilateral cryptorchism	utricule	-	one testis, one dysgenetic ovary + rudimentairy uterus, tubae
6	L 46,XX (n=50) F 46,XX (n=12)	perineal, scrotal transposition	no Müllerian remnants normal sized adult testes	1 year old: LH normal (<0.1 IU/L) FSH normal (1.4 IU/L) normal T after hCG (7.1 nmol/L)	-
7	L 47,XYY (n=100)	penoscrotal, bifid scrotum	no Müllerian remnants normal sized adult testes	1 year old: LH normal (<0.1 IU/L) FSH normal (0.4 IU/L) normal T after hCG (16 nmol/L)	-
8	L mos 47,XY, +mar [27] / 48,XY,+2 mar [1] / 46,XY [100]	perineal, bifid scrotum.	maternal progesterone use. no Müllerian remnants., normal sized adult testes	Adult: LH normal (6.6 IU/L) FSH normal (5.6 IU/L) T normal (18.8 nmol/L)	-

Table 11.4B
Chromosomal findings and phenotypes in patients with familial hypospadias

#	karyotype	type of hypospadias	findings by urethro-cystography/ ultrasonography or inspection	Serum hormones	gonads at gonadectomy
9	L mos 46, del(X)(q21) [1] / 46,XY [99]	familial, penile	normal sized adult testes	Adult: LH normal (5.6 IU/L) FSH normal (4.0 IU/L) T normal (19.6 nmol/L)	affected brother 46,XY and similar endocrine data
10	L mos 46,XY, fra(2)(q13) [5] / 46,XY, del(2)(q13) [4] / 46,XY [41]	familial, coronal	normal sized adult testes	Adult: LH normal (2.7 IU/L) FSH normal (2.3 IU/L) T normal (9.9 nmol/L)	affected brother 46,XY and similar endocrine data
11	L 46,XY, inv(11)(q21q23.3) mat	familial, coronal	normal sized adult testes	Adult: LH 2.1, FSH <0.1 IU/L, T 15.9 nmol/L	mother has same inversion, affected brother could not be tested

L = karyotype in leukocytes
F = karyotype in fibroblasts
mos = mosaic
chi = chimera
[ ] = nr of cells in each clone
n= number of cells investigated
T = testosterone
- = not investigated

3, Table 11.4A, with one dysgenic ovary and one testis showed a normal 46,XY karyotype in peripheral leukocytes but FISH studies on the dysgenic ovary showed the presence of only X-chromosomal DNA sequences in a high number of cells, while other cells showed X as well as Y chromosome sequences. This suggested the presence of chimeric karyotypes, being either XX/XY or X/XY. In the case #1, 5, and 6 the chromosomal findings in fibroblast confirmed those seen in leukocytes. The abnormal karyotypes in cases #1- 6 are known to be associated with ambiguous genitalia/ hypospadias.[36,37] However, an XYY karyotype ,as in case #7, is not known to be associated with hypospadias. In case #7 the extra Y chromosome might cause a tall stature as its most conspicuous feature. With its known population frequency of 1.5/1000 live births, this finding can be interpreted as an accidental one. In case #8 the extra chromosomal material in 27% of the cells might have contributed to the hypospadias. However, till now this material could not be identified, moreover also maternal progesterone ingestion during gestation could have caused the hypospadias.

In the group with familiar hypospadias a low grade mosaicism for structural chromosomal anomalies was found in 2 cases (# 9 and 10, Table 11.4B) while affected brothers had normal karyotypes. Therefore it is unlikely that these chromosomal abnormalities are associated with the hypospadias. In the last familial case (# 11, Table 11.4B) the normal mother had a similar paracentric inversion as the index patient. We could not study his affected brother. Chromosomal aberrations are apparently not a frequent cause of familial hypospadias. All these patients were adults with LH, FSH and testosterone serum levels in the normal range, adequate LH/T ratios and adult sized testes.

Androgen receptor analysis

Among 63 severe hypospadias patients, one pathogenic *AR* gene mutation, R846H[38] (numbering based on 20 glutamine and 18 glycine residues in the N-terminal part of the protein), was found. Androgen binding was abnormal in this AIS patient (Bmax 29 fmol/mg protein; normal > 20; Kd 0.6 nM; normal <0.3). An SHBG-suppression test also indicated AIS, for a maximum SHBG decrease to 92% of the initial SHBG serum concentration was observed.[39] This patient had a penoscrotal hypospadias, micropenis, bifid scrotum and cryptorchidism.

In one family with 5 males with hypospadias and a Reifenstein syndrome phenotype (Partial AIS) a deletion of 6 kb resulting in a deletion of exon 2 was found.[40,41]

In none of the other patients was an *AR* gene mutation identified, using PCR-SSCP of the entire *AR* gene.
The highly polymorphic CAGn(CAA) stretch in exon 1 of the *AR* gene contained between 14 and 34 codons in all index cases studied, which is in the normal range.[42]

Endocrine evaluation

In cases with severe hypospadias, unilateral vanishing testes syndrome was found in one patient with an insufficient rise of serum T after hCG, compared to age matched controls.
No abnormal responses of serum testosterone in combination with abnormal responses of progesterone/17-hydroxyprogesterone or DHEA were observed. Therefore, neither 3β-hydroxysteroid dehydrogenase II deficiency nor 17α-hydroxylase/17,20 lyase deficiency were underlying the hypospadias in severe hypospadias. Decreased T/A ratios were found in 2 patients with severe hypospadias (A & B in Table 11.5), suggesting 17βHSD3 deficiency. In one patient elevated T/DHT ratios suggested 5α-reductase 2 deficiency (Patient C in Table 11.5).
In familiar hypospadias, two patients (D, E in Table 11.5) showed an abnormal low rise of serum T and its precursors after hCG and therefore Leydig cell hypoplasia was suspected.
Patients suspected for 17βHSD3 deficiency, 5α-reductase 2 deficiency and Leydig cell hypoplasia were further studied with biochemical or molecular genetic methods.

Mutation or biochemical analyses of testosterone biosynthesis defects or LH-receptor defects (Table 11.5)

The clinical and hormonal diagnosis of 17βHSD3 deficiency in patients A and B (Table 11.5) could not yet be confirmed at mutation analysis of the *HSD17B3* gene, using PCR-SSCP analysis.
5α-reductase type 2 activity in genital skin fibroblasts of patient C was totally absent, confirming the diagnosis 5α-reductase type 2 deficiency. The LH-receptor genes of patients D (Table 11.5; familial, distal penile hypospadias) and E (Table 11.5; familial, penoscrotal hypospadias) were investigated for mutations. Patients D was heterozygous for mutation R124Q, which rendered the LH-receptor partially inactive in *in vitro* experiments.[43] However this mutation was not present in an affected brother of patient D, which made a

Table 11.5
Patients with hypospadias and abnormal hormonal serum values.

Patient #	Age at evaluation	T	Progesterone	17OH Progesterone	DHEA	A	T/A	DHT	T/DHT	Suspected diagnosis
Severe hypospadias										
A	12	3.9				5.5	0.7			17βHSD3 def.
B	5	3.3				5.5	0.6			17βHSD3 def.
C	6	8.5		6.6		0.22		0.2	43	5α-reductase 2 def.
Familial hypospadias										
D	7	1.7	2.5	0.4	1.6	1.0		0.1	0.5	Leydig cell hypoplasia
E	6	2.9	1.5	0.4	0.6	0.1		0.3	0.5	Leydig cell hypoplasia
Ranges in age-matched controls	6-12	>3.3	1.2-2.5	<5.3		0.9-4.1	> 1.0 ref.(35)		5.2-18.6	

T = testosterone
A = androstenedione
DHT = 5α-dihydrotestosterone
DHEA = dehydroepiandrosterone
All values in mmol/L, all values after hCG stimulation

causative role of a mutant LH receptor for this familial hypospadias unlikely. No mutations were identified in the LH-receptor gene of patient E.

DISCUSSION

In a single-center study, we have investigated currently known etiologic factors for severe hypospadias in a large cohort of patients, which resulted in detection of the underlying defects in 31 % of cases.

Maternal hormone ingestion

In one patient (Table 11.4, case 8) maternal ingestion of progestagens during the first three months of pregnancy could have caused his hypospadias but whether there is a relationship of intra-uterine exposure to progestins and hypospadias is not clear.[1] A genetic origin of his hypospadias can not be ruled out as his karyotype showed a low grade mosaicism with unidentified marker chromosome material.
The mothers of two boys, one with glandular hypospadias and one with severe hypospadias, had used hormonal contraception during the first trimester of pregnancy. It is not known whether the continued use of hormonal contraception during the first trimester of pregnancy is associated with an increased risk for hypospadias in the fetus.

Complex syndromes

In 17% of the patients with severe hypospadias, the hypospadias was part of a complex genetic syndrome. This emphasizes the value of dysmorphological evaluation when examining a neonate with severe hypospadias. A family history and examination of the parents may also be helpful to establish a diagnosis. In cases with suspected Smith-Lemli-Opitz syndrome, the diagnosis can be confirmed by biochemical methods.

Abnormal karyotypes

9,5% (6/63) of the cases with severe hypospadias are associated with sex chromosome aberrations.[37] The observed 9,5% of chromosomal aberrations is comparable with the 9.6% found by Yabumoto[7] and 12% found by Albers et al.[21]. FSH levels in the patients with gonadal dysgenesis were in the normal

range for age matched controls (prepubertal cases), and were therefore not indicative for the diagnosis.
Patients with chromosomal abnormalities are at risk of developing malignancies. For patients with gonadal dysgenesis the risk for malignancies is 20%.[37] This high risk of malignancy necessitates karyotyping in the diagnostic work-up of all patients with severe hypospadias, especially because in these patients normal serum hormone levels were found and urethro-cystography or ultrasonography did detect the presence of Müllerian remnants only in one case. An utricle is observed in many other hypospadias cases[44] and is therefore not pathognomonic.

Androgen receptor abnormalities

In the group with severe hypospadias only in one patient a known pathogenic mutation R846H[45] was detected, confirming former findings that *AR* gene mutations are infrequent in patients with severe hypospadias.[19,20,46] Familial cases of hypospadias without other genital abnormalities due to *AR* gene mutations, have been reported.[18] In one familial case with severe hypospadias, a deletion in the *AR* gene, disrupting normal mRNA splicing, was identified.[40] In the family of this patient multiple affected cases over several generations were present.[41] The adult cases in this family were typical examples of cases of Reifenstein syndrome and the family history was nearly conclusive for AIS. These results suggest that *AR* gene mutations are not a frequent cause of familial hypospadias.

Insufficient androgen biosynthesis

In one patient the hypospadia was associated with vanishing testis syndrome as was established upon laparotomy. Leydig cell hypoplasia was excluded as a cause in two cases by *LH receptor* gene mutation analysis. Whether these patients have a delayed maturation of the hypothalamic-pituitary-testicular axis as was suggested by Allen et al.[24] was not further studied. Based on endocrine studies, no cases of 3β-hydroxysteroid dehydrogenase II deficiency or 17α-hydroxylase/17,20 lyase deficiency were identified in our population. These results are very different form their prevalence of 50% reported by Aaronson et al.[26] It is therefore of interest to know whether these patients harbored mutations in the respective genes. Although T/A ratios suggested the diagnosis 17βHSD3 deficiency in two patients with severe hypospadias (cases A and B in Table 11.5) no mutations in their *HSD17B3* gene were found upon PCR-SSCP

analysis. The questions remains unanswered whether the hypospadias is due to a mild form of 17βHSD3 enzyme deficiency and undetected *HSD17B3* gene mutations. One patient had 5α-reductase 2 deficiency, which was established by T/DHT ratios as well as 5α-reductase 2 assays in genital skin fibroblasts. Although a rare disease, 5α-reductase 2 deficiency is important to consider in children with severe hypospadias. Likewise, although not found in this study, androgen synthesis disorders must be excluded by means of hormonal evaluation including hCG testing in prepubertal boys, as hormonal treatment in these disorders if often indicated.

Idiopathic hypospadias

The etiology in the remaining cases (69%) is the challenge for further research. These patients form a well defined group in whom several causes have been excluded. They seem to have normally functioning testes. Delayed onset of androgen secretion during the crucial time of gestation, remains one possible cause in these cases. Moreover, there are first indications that hypospadias might be due to a defect in androgen action that is not due to mutations in the coding region of the AR.[47] Disruption of unidentified genes is likely to underlay the hypospadias in some of the familiar hypospadias. In addition more and more evidence is gathered that widely used industrial or agricultural chemicals have hormone mimicking effects.[1] Whether such chemicals are responsible for the increasing incidence of hypospadias in many countries is a question, which has evoked many ongoing studies.

Diagnostic work-up

The aim of this study was to determine the incidence of various causes for severe hypospadias and to implement this information in recommendations for a diagnostic work-up for this disorder. The diagnostic work-up used in this study (schematically represented in Figure 11.1) is based on Grumbach and Conte[37], Ritzén and Hintz[48] and Albers et al.[21], each with a few modifications. We favor an hCG test in every prepubertal boy as testosterone synthesis is normally low at that age and defects of testosterone biosynthesis cannot be excluded without such a test. In addition, the peak of the neonatal LH-surge drops somewhere during the first three to six months of life and is therefore difficult to use. Furthermore, we have not performed an SHBG test in all 46,XY cases with a normal hCG induced response of testosterone. Although an abnormally small decrease in SHBG was found in our patient with severe hypospadias due

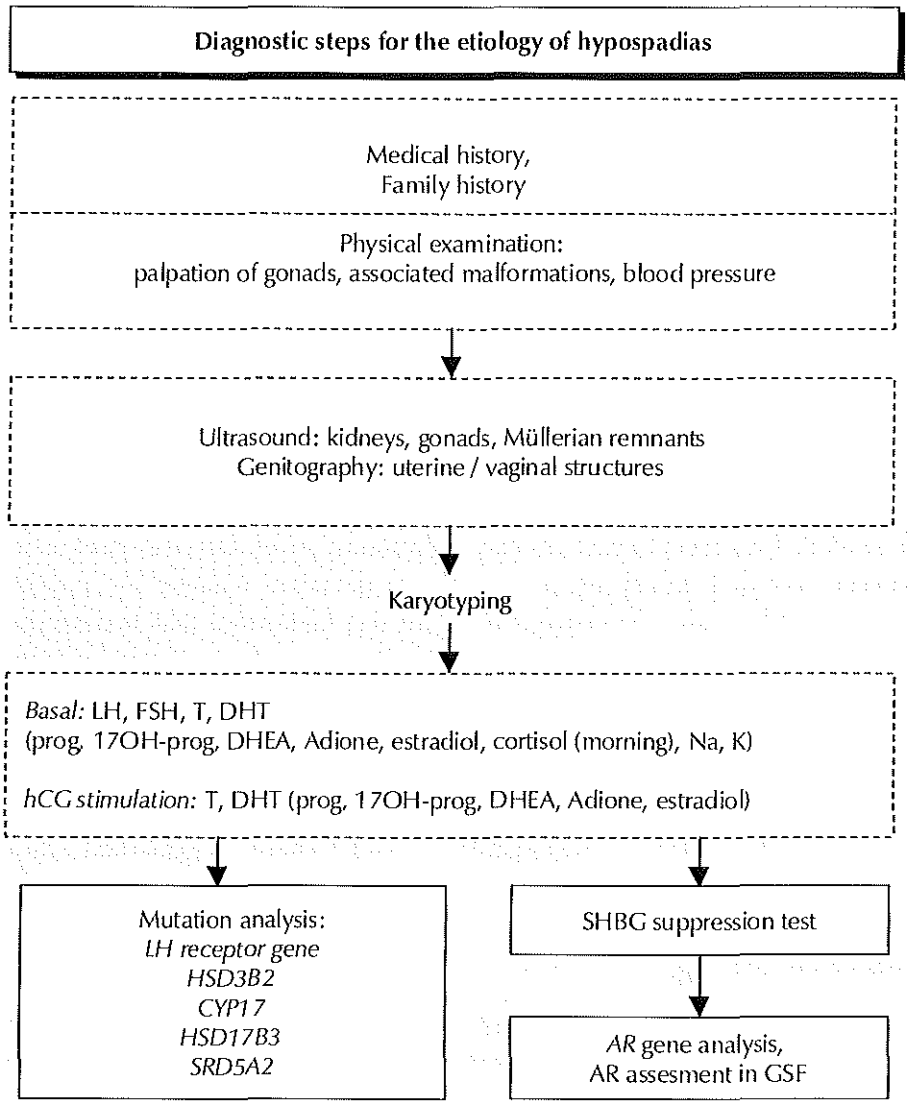

Figure 11.1
Schematic representation of the diagnostic steps used in this study.
Arrows indicate sequential steps followed in every patient. Boxed (uninterrupted line) tests were only performed after a presumptive diagnosis was made on the basis of the previous steps.

to AIS, false negative results in hypospadias patients have been described in two others.[21] Somatic mosaicism which may be not uncommon for this mild phenotype of a disease known to be frequently due to *de novo* mutations.[49-51] is a hypothetical cause of false negative results of the SHBG suppression test. However, as mutation analysis of the *AR* gene is an elaborate procedure which is not commonly available, the SHBG-suppression test might provide the diagnostic tool for this diagnosis per exclusionem.

CONCLUSIONS

In a total of 31% of severe hypospadias cases its etiology became clear. Based on hormone stimulation tests, Leydig cell hypoplasia and disorders of testosterone biosynthesis were suspected in some patients but were not confirmed by mutation analysis of the respective genes.

The identification of the etiology has implications for further treatment, prognosis, and genetic counseling. Syndromal cases may be associated with other disabling complications. Prognosis based on the syndromal diagnosis and counseling for recurrence risks are important for these patients and their parents. Patients with sex chromosomal aberrations are at high risk of developing malignancies.[36,37] In patients with 17βHSD3 deficiency, corrective surery with additional priming with testosterone in early childhood and the naturally occurring virilisation during puberty can give rise to functionally and cosmetically acceptable male genitalia.[52,53] The naturally occurring virilisation at puberty in 5α-reductase 2 deficient patients and treatment with DHT might result in an increase of virilisation. In contrast, in the long term follow up of hypospadias, often complex sexual ambiguity was observed in patients with AIS.[54] Patients with AIS sometimes respond well to high doses of androgens during a prolonged period of time during puberty.[55] Furthermore all of the above described disorders have their specific recurrence risks. An early diagnosis with adequate treatment will lead to better cosmetic and functional results and yields important information for reproductive decisions.
The unidentified causes of hypospadias, which play a role in 69% of the patients, remain a challenge for further research.

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CHAPTER

12

Disorders of sexual differentiation in 46,XY individuals: implementation of gene mutation analysis in clinical practice

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INTRODUCTION

Many genes encoding proteins involved in normal male sex differentiation have been cloned during recent years. Mutation analysis became possible for confirmation of an etiologic diagnosis in 46,XY individuals with undervirilisation. Earlier, the diagnosis in disorders of sex differentiation was based on phenotypic/dysmorphological, cytogenetic and hormonal studies which is usually sufficient in adolescents and adults with male pseudohermaphroditism, since at that age there is a full expression of clinical and hormonal phenotypes. However, in neonates and prepubertal children the diagnosis is often more difficult as the anatomical phenotypes are not yet fully expressed and sex steroid hormone synthesis is normally low. An early diagnosis for sex assignment in the neonatal period of patients with ambiguous genitalia is often essential; in the disorders of testosterone synthesis virilisation may occur by endogenous biosynthesized hormones or androgen substitution therapy, whereas in the androgen insensitivity syndrome (AIS) virilisation is generally limited.

hCG- and ACTH stimulation tests are essential to establish the type of endocrine dysfunction in neonates and prepubertal children. The interpretation of hCG tests in these children is hampered by the absence of a standard protocol for an hCG test, resulting in paucity of data on age matched ranges of androgen- and precursor hormone serum concentrations in controls and patients with the various disorders. The advent of DNA diagnosis in the analysis of disorders of androgen synthesis circumvents part of these problems; however, functional analysis of the effects of a mutation remains an essential part of a diagnostic evaluation.

AIS was a diagnosis *per exclusionem* in neonates and prepubertal children until androgen receptor binding tests in genital skin fibroblasts became available. However, androgen receptor binding is normal in a considerable number of AIS patients.[1] Since the cloning of the androgen receptor (*AR*) gene in 1988/1990[2-5], mutation analysis of the *AR* gene provides the key diagnostic tool for AIS. Even more, carrier detection and prenatal diagnosis became available.

The cloning of the human genes for 17α-hydroxylase/17,20 lyase[6], 17β-hydroxysteroid dehydrogenase 3[7], and 5α-reductase 2[8], in 1987, 1994 and 1991 respectively, enabled mutation detection and prenatal diagnosis for patients and their families. The additional use of gene mutation analysis for confirmation of a clinical diagnosis of a particular disorder of testosterone synthesis or AIS and their use in genetic counseling is summarized in this chapter.

DIAGNOSTIC PROCEDURES

Gene mutation analysis is always preceded by stepwise conventional diagnostic procedures, which usually yield a presumptive diagnosis. Our initial diagnostic procedures in neonates presenting with ambiguous genitalia or otherwise suspected of having a disorder of sex differentiation, follows a modification of a scheme proposed by Grumbach and Conte[9], outlined in Figure 12.1. As the evaluation, treatment and counseling of disorders of sex differentiation requires a multidisciplinary approach, referral to a specialized center is highly recommended. A team including a pediatric endocrinologist, pediatric surgeon, pediatric urologist and a child psychologist is primarily involved. In addition a pediatric radiologist/ultrasonographist, clinical geneticist and gynecologist should be consulted on a regular basis.

Initial diagnostic procedures

In Figure 12.1 the stepwise diagnostic procedures are summarized that are initially required in neonates/prepubertal children with ambiguous genitalia or with a female phenotype with inguinal gonads.

Specific attention is directed to:

Family history

Parental consanguinity, presence of similar cases among maternal siblings, premature and small for gestational age births, virilisation of a girl at puberty, infertility, hypospadias, gynaecomastia, congenital malformations, dysmorphology.

Pregnancy

Premature and small for gestational age births, maternal medication for example use of progestins during pregnancy, ovarian / adrenal tumors.

Physical examination

General physical examination, blood pressure.
Special attention for:
- Dysmorphology, congenital defects.

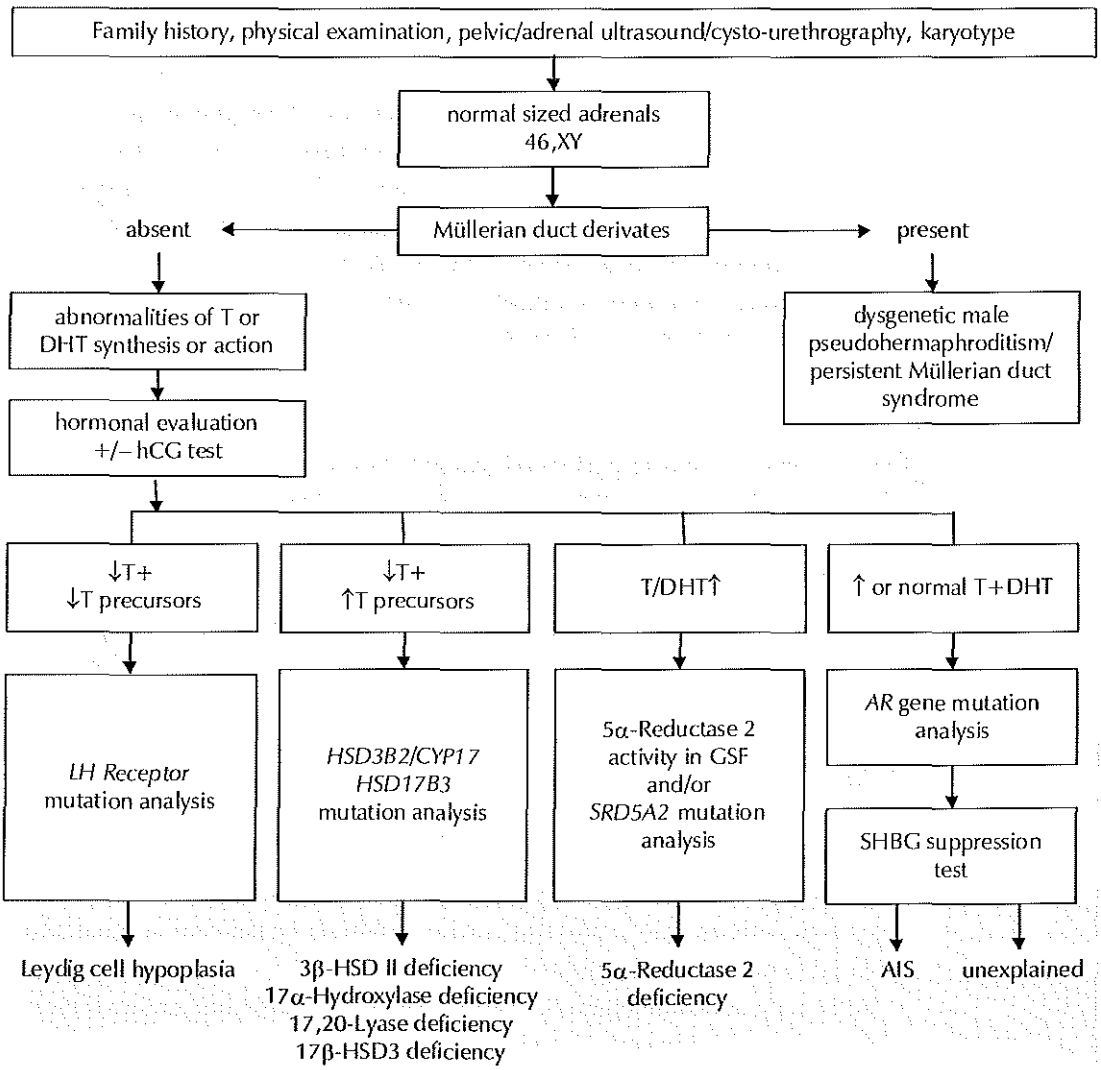

Figure 12.1
Sequential diagnostic steps in 46,XY individuals with disorders of sexual differentiation.

- Genitalia: Localization of the gonads, aspect and size of the phallus, presence of chordae; localization of the meatus urethrae, introitus vaginae and possible posterior fusion of labia, shape of the labia/scrotum, hyperpigmentation. The typing of the degree of virilisation of external genitalia has been proposed by Prader.[10]

Ultrasonography

Ultrasonography of the internal genital organs is of critical importance, but may be inconclusive especially in very small children. Genitography or vagino-cystoscopy may be required for more precise information on the anatomy of the pelvic structures. However, laparotomy, performed for example for gonadectomy, sometimes reveals what has remained undetectable by other visualization techniques. Adrenal normo-, hypo-, or hyperplasia differentiates between sex steroid hormone synthesis, or action defects, combined sex steroid/ glucocorticoid deficiency, or StAR deficiency, respectively.

Karyotype

Chromosome analysis in peripheral lymphocytes; if chromosomal mosaicism is suspected: skin fibroblast culture for cytogenetic analysis

Storage of a DNA-sample

As only small volumes of blood can be taken from neonates, the leukocytes for DNA isolation and plasma for endocrine evaluation can be obtained from the same sample.

On the basis of the obtained information, in most cases a distinction can be made between patients with:

- complex genetic syndromes,
- patients with chromosomal disorders (gonadal dysgenesis),
- patients with disorders affecting cortisol synthesis (congenital adrenal hypoplasia/ lipoid adrenal hyperplasia),
- 46,XY patients with relatively normal cortisol synthesis but undervirilisation due to lack of androgens or androgen insensitivity.

Initial differential diagnostic considerations

The most common causes of male pseudohermaphroditism are XY gonadal dysgenesis, AIS (incidence in The Netherlands 1:99,000; Chapter 3), and 17β-hydroxysteroid dehydrogenase deficiency (incidence in The Netherlands, 1:147,000; Chapter 9). There are reports on a very high incidence of 3β-hydroxysteroid dehydrogenase in hypospadias[11], which however is not confirmed in our studies (chapter 11). The incidence or prevalence of 17α-hydroxylase/ 17,20 lyase deficiency is not known. Judged by the number of reported patients, combined 17α-hydroxylase/ 17,20 lyase deficiency seems far more

common than isolated 17,20 lyase deficiency.[9] The prevalence of association of undervirilisation in 46,XY individuals or hypospadias and complex genetic syndromes is not known.

Neonates and prepubertal children

The phenotypes of various disorders giving rise to male pseudohermaphroditism in neonates and prepubertal children are very similar. External genitalia range from completely female through a micropenis with severe hypospadias and bifid scrotum to simple hypospadias.[9,12-17] Discriminating diagnostic anatomical features may be the presence of other dysmorphologies or absence or presence of Müllerian duct structures. Müllerian duct structures are present in most cases of gonadal dysgenesis, but absent in the other causes of male pseudohermaphroditism.

Hypertension and hypokalemia have been described in 46,XY infants with combined 17α-hydroxylase / 17,20 lyase deficiency.[16] However, although cortisol synthesis is diminished, these patients may not show signs of cortisol deficiency because of high DOC serum concentrations with mineralocorticoid activity.[9] For this reason, the phenotype resembles that of other causes of male pseudohermaphroditism. 3β-Hydroxysteroid dehydrogenase deficiency can also present either with or without salt wasting.

Pubertal children and adults

Patients with Leydig cell hypoplasia, 17α-hydroxylase/ 17,20 lyase deficiency, or 3β-hydroxysteroid deficiency do not enter puberty. Breast development and pubic hair are absent or sparse. In contrast, patients with 17β-hydroxysteriod dehydrogenase 3 deficiency or 5α-reductase 2 deficiency show variable degrees of virilisation at puberty: phallic size increases, the voice may lower and sexual hair growth follows a male distribution pattern (Chapter 9).[18-20] Even gender changes from female to male sex have been documented.[18-20]

In AIS patients virilisation during puberty correlates with the prenatal virilisation grade.[21] Thus, no virilisation is seen in AIS children with completely female external genitalia whereas some virilisation may occur in AIS patients with severe hypospadias and micropenis (Chapter 1).

In pubertal children and adults, the salient features of the different causes of male pseudohermaphroditism become apparent during hormonal studies (for details, see Table 12.1).

In conclusion: in pubertal children and adults a diagnosis can usually be made based on anatomical, chromosomal and endocrine features. However, ana-

Table 12.1
Diagnostic findings in endocrine evaluation of the disorders of androgen synthesis

Defect	Diagnostic parameters
Leydig cell hypoplasia	LH ↑, Testosterone ↓, Testosterone precursors ↓
3β-hydroxysteroid dehydrogenase 2 deficiency	Preg/Prog ↑, 17OH Preg/17OH Prog ↑, DHEA/Adione ↑
17α-hydroxylase deficiency/17,20 lyase deficiency	Preg/17OH Preg ↑, Prog/17OH Prog ↑, DOC ↑, 18OH DOC ↑, Corticosterone ↑, Renin ↓
17,20 lyase deficiency	17OH Preg/DHEA ↑, 17OH Prog/Adione ↑, Cortisol =
17β-hydroxsteroid dehydrogenase 3 deficiency	Androstenedione ↑, Testosterone/Androstenedione ↓
androgen insensitivity	LH ↑, Testosterone ↑
5α-reductase 2 deficiency	Testosterone/DHT ↑

↑ : elevated as compared with normal controls
↓ : decreased as compared with normal controls
= : comparable with normal controls

Preg: pregnendone
Prog:: progesterone
Adione: androstenedione

tomical features in neonates and children are not diagnostic. Endocrine evaluation requires an hCG test, of which the interpretation is difficult because of lack of age-matched control values.

Second order investigations

hCG stimulation test
ACTH stimulation test
SHBG suppression test
AMH and inhibin determination in serum
Karyotype in (genital) skin fibroblast in case of suspected mosaicism
Functional studies of the AR in genital skin fibroblasts
FISH-analysis on interphase nuclei in cases of suspected XO/XY mosaics
Laparoscopy

Mutation analysis of a gene indicated by the previous diagnostic procedures as likely associated with the disease.

Differential diagnostic considerations, endocrine evaluation

In neonates and prepubertal children endocrine evaluation by hCG and sometimes ACTH testing is essential (see below).

hCG test

There is no uniform protocol for an hCG test. Androgen responses vary according to the dose and frequency of injected hCG[22] and according to the age of the infant / child. We used doses of 1500 U i.m. hCG (standard dose for every age group and body size; blood sampling was done prior to and 72 hours after this injection; modified after Smals et al.[23]). As in testosterone synthesis disorders either the ratio between the various precursors of testosterone synthesis or the ratio between these precursors and testosterone is more indicative for a specific diagnosis than merely the rise of testosterone, serum concentrations of both testosterone and the precursors should be determined (Table 12.1).
A correct interpretation of basal and stimulated serum concentrations of steroid hormones and their precursors in patients, requires observations of serum concentrations in age matched controls studied with the same hCG protocol. However, only a few laboratories established such normal values for every hormone in age matched normal boys. Unfortunately antibodies and protocols used for radio-immunoassays tend to be changed after which the normative data are corrected for the new method. This may lead to slight inaccuracies of the corrected normative data. Despite these problems samples from diagnostic cases should preferably be analyzed in a laboratory that does have these data. The normal values for the used hCG test protocol (serum sampling at 0 and at 72 hours after 1500 IU hCG) are given in Table 12.2 (derived from de Muinck Keizer-Schrama and Hazebroek[46]).

While interpreting hCG test results one should keep in mind that a normal (rise in) testosterone for age does not exclude testosterone biosynthesis disorders nor gonadal dysgenesis. A normal rise of T can be observed in patients with gonadal dysgenesis on the basis of mosaicism such as XO/XY or XX/XXY (Chapter 11) or in (pre)pubertal cases with 17βHSD3 deficiency (Chapter 9).[9] In the latter, testosterone/androstenedione ratios are more reliable parameters than testosterone serum concentrations (Chapter 9).

Table 12.2A
Normal serum levels of steroid hormones and their precursors in prepubertal boys and adult men: Basal (-) and hCG stimulated (+) serum concentrations.

age in years	prog (nmol/L)	prog (nmol/L)	17OHprog (nmol/L)	17OHprog (nmol/L)	DHEA (nmol/L)	DHEA (nmol/L)	A (nmol/L)	A (nmol/L)	T (nmol/L)	T (nmol/L)
hCG	-	+	-	+	-	+	-	+	-	+
<1			0.33-5.23♠		0.9-8.18♠		0.21-1.89♠		0-25	
1-2 (n=5)	1.0-2.3	1.1-2.7	<1.2-1.6	<1.2-2.3			<0.35-0.8	0.5-1.0	<0.2	6.8-15
2-5 (n=10)	<0.5-2.0	<0.5-3.0	<1.2-3.1	<1.2-3.5	-	-	<0.35-1.0	0.6-1.5	<0.1	>5.7
6-12 (n=10)	1.2-3.6	1.2-2.5	<2.4-6.0	<2.4-5.3	-	-	0.4-3.9	0.9-4.1	0.1-0.4	3.3-6.5
Adult (n=10)	0.5-2		0.7-4.9		1-25		2-10		10-30	

Table 12.2B
Normal values for ratios between steroid hormones and their direct precursors in serum of prepubertal boys and adult men: Basal (-) and after hCG stimulation (+) (serum concentrations in nmol/L).

age in years	T/A	17OH Preg/ DHEA	17OH Prog/ A	17OH Prog/ A	Preg/ 17OH Preg	Prog/ 17OH Prog	Prog/ 17OH Prog	T/DHT
HCG	+	-	-	+	-	-	+	+
<1								
1-2 (n=5)	8.5-17		<3.0	<3.0		<2.0	<1.7	<16.7
2-6 (n=10)	3.6-18.4		<11	<8.0		<0.9	<0.9	<17.3
6-12 (n=10)	0.8-7.0	<0.5♦	<8.5	<5.0	<1♦	<0.7	<0.5	<18.6
Adult (n=10)	1.5-4.6							

De Muinck Keizer-Schrama and Hazebroek[46]
♦ After Forest MG et al[24] and Kaufman FR et al.[25]
♠ After Lashansky G. et al.[26]

ACTH test

The commonly used protocol for the ACTH test is blood sampling before, at 30 and at 60 minutes after the intravenous administration of 0.25 mg ACTH. Normal values for steroid hormones and their precursors in neonates, prepubertal and pubertal children are documented by Lashansky G et al.[26]

SHBG-suppression test

Androgen sensitivity in the liver can be measured in vivo with use of an SHBG-suppression test. A decline in serum SHBG levels is seen in androgen normosensitive individuals upon administration of androgen. When AIS is suspected this a useful and relatively quick test. In our experience this test can also be used in gonadectomized patients, and has been useful to establish a diagnosis in cases that had been gonadectomized before a proper hormonal evaluation was done Unfortunately it is not informative in children below the age of 1 year because of the physiological SHBG rise during the first year of life[17], nor in patients with mosaics for a mutant *AR* and a normal *AR*.
The protocol has been described by Sinnecker et al.[17]: Stanozolol (Stromba ®) (0.2 mg/kg/day, single evening dose) is administered orally at day 0, 1 and 2. Blood samples are taken before and at days 5, 6, 7, 8 after the start of the test. The initial SHBG serum level is compared to the lowest level obtained after administration of Stanozolol (days 5, 6, 7, 8) and expressed as a percentage of the initial value. In normal controls the SHBG serum level after Stanozolol declines to 35.6-62.1% (range) of the initial value. However, in patients with CAIS, the SHBG serum level remains unchanged, and in PAIS patients the SHBG level declines to 48.6-89.1% (range) of the initial value.[17] (Table 12.3).

AMH / inhibin determination

In children with intersex conditions Sertoli cell function can be assessed by measuring AMH and inhibin serum levels, in addition to testing Leydig cell function by testosterone secretion. AMH is low in XY patients with abnormal testicular development (pure or gonadal dysgenesis) but is normal or elevated in patients with impaired testosterone secretion. AMH is elevated in AIS patients during the first year of life and at puberty.[27,28] Thus AMH and inhibin serum levels can help to differentiate between defects of male sexual differentiation caused by abnormal testicular development and defects resulting from impaired secretion of androgens or resulting from androgen insensitivity.
Inhibin B is a marker of Sertoli cell function in boys and granulosa cell function in girls. During the first 2 years of life, particularly in the first few months, in-

Table 12.3
Influence of gonadectomy on the outcome of SHBG supression tests in patients with AIS, 17α hydroxylase/17,20 lyase deficiency and 17β hydroxysteroid dehydrogenase 3 deficiency.

AIS grade	age in years	Gonad-ectomized / estrogen substitution	SHBG; % of initial SHBG serum con-centration	*AR* gene mutation
6/7	11	yes / no	100	W742R
6/7	9	yes / no	108	W742R
6	12	no	100	Q478X
6	30	yes / no	97	Gln59X
5	13	no	73	R846H
3	5	no	92	R846H
Controls				
17α-hydroxylase/17,20 lyase deficiency	28	yes / yes	33	compound heterozygous *CYP17* mutations
17α-hydroxylase/17,20 lyase deficiency	10	yes / no	35	compound heterozygous *CYP17* mutations
17β-hydroxysteroid dehydrogenase 3 def.	8	yes /no	45	homozygous *HSD17B3* mutation
17β-hydroxysteroid dehydrogenase 3 def.	11	yes/no	49	compound heterozygous for *HSD17B3* mutations
17β-hydroxysteroid dehydrogenase 3 def.	6	no/no	46	compound heterozygous for *HSD17B3* mutations

(Data are from patients described in this thesis; SHBG supression test according to the method described by Sinnecker et al.[16])

hibin B serum levels are high in normal boys, even exceeding serum levels in adult men. In normal girls inhibin B levels are lower than in boys albeit more variable during the first year of life, and in the second year very low or even undetectable.[28]

IMPLEMENTATION OF MUTATION ANALYSIS

In general

In some prepubertal patients endocrine parameters may be inconclusive. In the absence of enzyme or protein assays for the respective key enzymes of steroid biosynthesis in non gonadectomized patients, mutation analysis of the respective genes suspected to be involved may be used for a definite diagnosis. Mutation analysis is facilitated in 3β-hydroxysteroid dehydrogenase 2-, 17α-hydroxylase / 17,20 lyase-, 17β-hydroxysteroid 3-, 5α-reductase 2-, deficiency because of the limited size of the genes, clustering of mutations and/or founder effects (Chapter 9).[29-33] In AIS and Leydig cell hypoplasia mutation analysis is more time consuming as the androgen receptor and LH receptor genes are relatively large and mutations are scattered throughout these genes.[12,34]
PCR-SSCP followed by sequencing will reveal the mutations in many cases. However, when no mutation is found and the clinical diagnosis is suggestive, sequencing of the entire gene will be needed. Most reliable would be RT-PCR followed by sequencing of the cDNA or even quantitation of expression of the RNA by RNA'se protection. However, in many cases the tissue required for RNA extraction is not available.

Mutation analysis in Leydig cell hypoplasia

Mutations in either of the two functional domains of the LH-receptor are found in patients with Leydig cell hypoplasia.[12] Mutation analysis is time consuming, as most families will show novel mutations.[12] In infants and children with a low response of androgens to hCG and no exaggerated response of androgen precursors, the finding of *LH receptor* gene mutations yields a definite diagnosis of Leydig cell hypoplasia. However, many patients with a clinically diagnosis of Leydig cell hypoplasia have no detectable *LH receptor* gene mutation even after extensive sequencing of all exons and flanking intron sequences of the gene.[12] Whether mutations in non sequenced parts of the *LH receptor* gene or genetic heterogeneity are explanations, remains to be solved.

Mutation analysis in 3β-hydroxysteroid dehydrogenase 2 deficiency

Patients with the classical salt wasting form of 3β-hydroxysteroid dehydrogenase deficiency are homozygotes or compound heterozygotes for point mutations including nonsense and missense mutations in the *HSD3B2* gene that es-

sentially abolish 3β-hydroxysteroid dehydrogenase activity in the adrenals and gonads.[9] Patients with the non-salt losing form are homozygotes or compound heterozygotes for mutations that result in <10 % of normal enzyme activity in vitro.[9] In the late onset form or non classical 3β-hydroxysteroid dehydrogenase deficiency no mutations are found in the *HSD3B2* gene, hence the molecular basis remains to be defined.

Mutation analysis in 17α-hydroxylase / 17,20 lyase deficiency

The *CYP17* gene encodes both the 17α-hydroxylase and 17,20 lyase enzyme activities. The gene consists of 8 exons and is located on chromosome 10 at 10q24-q25. Over 30 patients with 20 different mutations have been identified. These mutations occur throughout the gene without predilection.[16,31,35,36] A founder effect for one deleterious mutation (4 base pair duplication in exon 8) in the *CYP17* gene is present among North-American Mennonites, a small religious sect of Dutch-Friesian descent.[37] This mutation was spread also within The Netherlands, as in two unrelated Dutch individuals not from Friesian origin, the same mutation was identified (Chapter 10).

Mutation analysis in 17β-hydroxysteroid dehydrogenase 3 deficiency

In neonates and in prepubertal children T/A ratios after hCG differ with ratios in normal boys or with ratios in age matched AIS patients and are conclusive in all studied 17β-hydroxysteroid dehydrogenase 3 deficient cases (Chapter 9). The *HSD17B3* gene consists of 11 exons and is ~1.3 kb in size.[7] Mutation analysis should prove the presence of 17β-hydroxysteroid dehydrogenase 3 deficiency in cases with marginally abnormal T/A ratios. In 17/18 affected patients two mutated alleles and only one mutated allele in 1/18 were found (Chapter 9). Founder effects for some mutations were observed. Due to founder effects some mutations are more frequently observed in specific geographic regions (Chapter 9). Therefore, mutation analysis may start with searching for those founder mutations of the geographic area of the patient's origin. Furthermore, most mutations reported as found in individuals from West-European origin are located either in the 3' part of intron 3 or in exon 3. Therefore mutation detection is an efficient diagnostic tool for 17β-hydroxysteroid dehydrogenase 3 deficiency despite the relatively large number of exons.

Mutation analysis of the *AR* gene in AIS

The androgen receptor gene consists of 8 exons, ~2.8 kb of coding region. Mutations identified in AIS patients are spread over the entire coding region of the gene, with some mutational hot-spots in exon 5 and exon 7 (Chapter 1).[34] Over 75 % of mutations are substitution mutations.[34] But partial gene deletions, gene deletions, insertion mutations and mutations that influence splicing are also found (see Chapter 1, Table 1.3).[34]
The large heterogeneity in mutations hampers quick results of mutation analysis. Furthermore, sequencing of exon 1 with a high CG content, may be cumbersome.
The presumptive diagnosis AIS is strengthened by the results of an SHBG test, Scatchard analysis or Western blotting of the AR isolated form genital skin fibroblasts, if available. Although mutation analysis may be impeded by mutational heterogeneity or technical reasons, it is essential for the diagnosis and genetic counseling of the family (and the mother's relatives) in this X-linked disorder as it is the only available method for carrier detection.

Mutation analysis in 5α-reductase 2 deficiency

Overlap in T/DHT ratios between normal controls, AIS patients and patients with 5α-reductase 2 deficiency exists.[38] Biochemical analysis of enzyme activity in cultured genital skin fibroblasts may not provide a definite diagnosis in these cases (Chapter 4). Mutation analysis of the *5α-reductase 2* gene (*SRD5A2*) and sometimes additionally mutation analysis of the *AR* gene, yield a diagnosis in those cases.
The *5α-reductase 2* gene, *SRD5A2*, consists of 5 exons.[39] In a few well documented cases only one allele was found mutated and no mutation was found on the other allele of the two mutations were found. Some mutations may apparently be located outside the sequenced regions of the gene.[39] Diagnostic problems may be prevented, if limitations of mutation analysis are considered.

GENETIC COUNSELING USING GENE MUTATION DETECTTION

3β-hydroxysteroid dehydrogenase-, 17α-hydroxylase/ 17,20 lyase-, 17β-hydroxysteroid 3 dehydrogenase-, 5α-reductase 2 deficiency

These disorders of testosterone or DHT biosynthesis show autosomal recessive inheritance. As their incidence is low, their carrier frequency is equally low. Therefore, mutation analysis will especially be used for confirmation of the diagnosis and for prenatal diagnosis for parents who previously had an affected child. Carrier detection among relatives of the parents is especially useful in consanguineous relationships.

AIS

Mutation analysis of the *AR* gene not only facilitates a precise diagnosis of AIS, but also enables carrier detection in this X-linked disorder. This is especially important as heterozygous 46,XX women show either no or variable characteristics, such as patchy distribution of pubic and axillary hair.[40] Moreover, only mutation analysis allows exclusion of heterozygosity at least in women with a 50% risk of having received the gene from their mother.

In properly defined AIS patients, an *AR* mutation is actually detected in 100% of CAIS patients (unpublished observations). For PAIS patients the detection rate of mutations is unknown. The group of patients with PAIS phenotypes tested by an SHBG-suppression test in our center was too small for such analysis.

In families with multiple AIS patients where no *AR* gene mutation has yet been identified, intragenic polymorphic markers were used for carrier detection and prenatal diagnosis.[41-45] However this should only been applied when the diagnosis AIS is without doubt, for example as shown by an SHBG-suppression test. In isolated AIS cases (when a mutation found in the index case but not in the mother) a somatic or gonadal mosaicism in the mother is important to consider as a cause of risk of recurrence for the mother, and the risk of being a carrier in her healthy daughter (Chapter 7). A mother of an AIS child who does not show the mutation of her child in her somatic DNA, still has a risk of having another affected child because of possible gonadal mosaicism. Such a mother has an indication for prenatal diagnosis, and her daughter for carrier testing (Chapter 7).

Some mutations that render the AR protein totally defective are expected to lead to a complete (CAIS) phenotype in every affected family member (Chapters 2 and 3). However, when the mutation does not cause a truncated protein or is other than a complete or partial gene deletion, the phenotype can not be predicted (Chapter 3). This alters the argument by some authors that prenatal diagnosis for complete AIS should not be practiced, as persons with complete AIS can live lives as normal but infertile females.[42,45] In partial AIS families, phenotypic variation was observed in approximately one third of the families, sometimes even leading to differences in sex of rearing (Chapter 3). In others the need for reconstructive surgery varied (Chapter 3).
It is important to consider the causes for phenotypic variability among cases with the same mutation. Carriers and their partners should face the difficult consideration about the possibility of phenotypic variation and therefore sometimes differences in sex of rearing of affected children.

Other problems in genetic counseling are the acceptation of the parents and family of the disorder in their affected child, on the one hand, and choices about future reproduction in the view of the risk of recurrence, the implications of the disorder, and the option of prenatal diagnosis.
Dissemination of information of the clinical diagnosis AIS and the possibility of carrier detection by gene mutation analysis among potential carrier relatives, was found to be restricted to relatives felt to be trustworthy, because of fear of stigmatization and feelings of shame (Chapter 8).

IN CONCLUSION

Mutation analysis is a diagnostic test with a high sensitivity and specificity for male pseudohermaphroditism. Therefore its should be applied when anatomical and endocrinological investigations have led to a presumptive diagnosis. Furthermore, in families with AIS and in consanguineous families with testosterone synthesis disorders, genetic counseling and prenatal diagnosis should ideally be available using mutation analysis.

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CHAPTER

13

General discussion and future directions

THE RATIONALE FOR GENOTYPE/PHENOTYPE STUDIES

The application of molecular genetic methodologies in many human diseases, enabled genotype / phenotype analysis. Critics of such studies expect phenotypic expression of a mutant gene would always be quite unpredictable, as the phenotype is the result of ontogenetic development. During ontogenesis, genetic and non-genetic factors interact, producing successive states, each of which is the prerequisite and also determines the conditions for the next to follow. Despite this complexity, genotype/phenotype studies have contributed to our understanding of the etiology of many syndromes and diseases. Furthermore, information on structure-function relationships would have required much longer to become established if developmental and molecular biologists had to rely on experimental model systems only. In this thesis presents an example has been presented of how genotype versus phenotype studies can answer clinical as well as molecular questions.

For AIS, our understanding of the phenotype-genotype heterogeneity evolved since identification of the *AR* gene. Mutations leading to a truncated protein certainly cause CAIS, but CAIS can also be cause by single missense mutations. PAIS is caused by mutations disrupting normal splicing, small deletions or point mutations. Phenotypic variation is rarely observed in CAIS families, whereas it is present in about one third of PAIS families. Molecular mechanisms underlying phenotypic variation are diverse. Here, an additional mechanism of influence of the expression of a mutant AR phenotype was detected in a consanguineous family with PAIS, in which a reduced expression of 5α-reductase 2 leads to a more severely impaired virilisation in one of the affected siblings (Chapter 4).

Genotype-phenotype studies contribute substantially to our understanding of human biology. The reciprocal benefits for both medicine and biology are exemplified in the 17βHSD3 study. The potential of mutation analysis for confirmation of a clinical diagnosis is shown. Mutation analysis was also used for the establishment of the ranges of T/A ratios in 17βHSD3 deficient versus non-17βHSD3 deficient cases. Observed interfamilial phenotypic variation for *HSD17B3* gene mutations affecting RNA splicing, was studied with molecular methods. The opportunity to study 17βHSD3 deficient patients and their mutations in an international, world-wide collaboration resulted in a global map of distribution of different *HSD17B3* mutations and offered views into the history of the human population. The study on etiology of hypospadias, with

use of clinical as well as with molecular means provides guidelines for diagnostic procedures in patients with hypospadias.

In the next paragraph, the genotype/phenotype relationship in AIS is summarized with the use of all published AIS cases and their *AR* gene mutations.[1]

GENOTYPE VERSUS PHENOTYPE IN AIS

The genotype/ phenotype relationship in AIS is not a straightforward one. There is no direct correlation between mutations in a specific functional domain and resulting phenotype. However some general principles are found:

Mutations leading to CAIS versus mutations leading to PAIS

Deleterious *AR* gene mutations cause the most severe degree of androgen insensitivity, CAIS. Thus complete gene deletions, partial gene deletions and mutations resulting in premature termination codons are solely found in CAIS and not in PAIS (Chapters 2 & 3, [1]). Truncated androgen receptors have been shown *in vitro* to be unable to cause transactivation and /or ligand binding.[2] Even truncated ARs missing a small part of the NH2-terminal domain of the AR protein which are sometimes detectable with immunoblotting[3], and the AR proteins resulting from alternative translation starting after a premature termination codon in exon 1[4], impede transactivation.[2]
Deletions of single codons are found in CAIS as well as in PAIS patients. The resulting phenotype depends on the localization and importance of the residue. Deletions in the DNA binding domain have led to CAIS[1] , in the ligand binding domain to either CAIS or PAIS.[1]
Mutations that affect splicing are found in CAIS as well as in PAIS patients. The resulting phenotype depends on the residual function of the mutant AR and on the amount of functional or wild type transcript produced by alternative splicing (Chapters 3 & 5, [5,6]).
Missense mutations leading to amino acid substitution comprise the vast majority of mutations in AIS. In general, PAIS patients carry more conservative amino acid substitutions compared to CAIS patients but there are many exceptions.[1] Some amino acid residues have been found substituted with different residues that result either in CAIS or PAIS.[1] On the other hand, alteration of some residues consistently results in identical phenotypes.[1] The severity of the resulting

Table 13.1
Binding characteristics of mutant ARs in GSF of AIS patients or transfected cell lines. Only ARs with mutations in the LBD are depicted.

	binding absent	capacity and affinity reduced	capacity reduced	affinity reduced	increased thermo-lability	normal binding
CAIS (n=70)	61%	9%	21%	9%	0%	0%
PAIS (n=57)	9%	14%	21%	33%	5%	18%

After ref.[1] with some corrections based on the original reports, studied cases only.

phenotype, might indicate the degree of importance of this residue for the secondary structure of the AR or AR function.
Missense mutations leading to amino acid substitution in the DNA binding domain, result in PAIS if some degree of DNA binding capacity is retained, and in CAIS when DNA binding is totally abolished.[7-10]
Missense mutations leading to amino acid substitution in the ligand binding domain cause either absent binding, decreased ligand affinity, reduced expression of the mutant AR or a combination. Absence of androgen binding is more often observed in CAIS, whereas qualitatively defective binding is observed for mutants in PAIS (Table 13.1). AR mutants of some PAIS patients did not show impaired binding but transactivation was impaired indicating that other, still unknown functions of the receptor were impaired.[11,12]

Possible causes of phenotypic variation in AIS

Theoretically, phenotypic variation in AIS can be caused by:

- ***Somatic mosaicism***

About 30 % of the cases with AIS result from *de novo* mutations. A part of these patients may have a somatic mosaicism for the mutation and the normal gene.[13] Depending on the percentage of cells in which the mutation is present versus 'normal' androgen sensitive cells, a more virilised phenotype can be observed than is expected when all cells carry the AR gene mutation.[14]

- ***Mutations that influence splicing***

Some mutations that lead to defective splicing of the AR can lead to the production of some wild type transcript in addition to the production of a mutant

transcript by alternate splicing (Chapter 6). The amount of wild type transcript produced may vary, resulting in variance of phenotypes.

• ***Qualitatively defective AR***

Candidates for phenotypic variation due to variance in availability of androgens, are mutations that render the AR qualitatively defective. Sometimes, these mutant ARs show partial- or complete restoration of *in vitro* transcription activity when stimulated with increased or repeated amounts of different androgens. Such mutations are: S694G[15], P746L[16], Y754C[17], Y754C[18], R765H[7], M798V[17], R831C[12], I833T[16], R846H[12,19], D855N[20], I860M.[12]

Indeed phenotypic variation within and between AIS families for some of the aforementioned mutations (e.g. Y754C+ 12 GLN[21], R831H[1,22,23], R846H (Chapter 3, [1]), I833T[16,24]) have been observed. Furthermore, androgen therapy resulted in phallus elongation in patients with AIS grade 3 (mutations S694G and T754C in combination with a shortened glutamine repeat)[18,25,26] illustrating that the *in vitro* residual responsiveness of this mutation is also present *in vivo*.

• ***Mutations that influence interactions with coactivators***

Mutations that cause a disturbed interaction with coactivators might theoretically lead to differential phenotypes, as the expression level of such factors might vary in different patients. Mutation Q789E is a candidate for disturbed interaction with cofactors, as it showed impaired transcription activation without impairment of ligand binding in *in vitro* transfection studies.[12] Mutation Q789E apparently causes very different phenotypes, AIS grade 5[12,27] or infertility in an otherwise normal male.[27] In addition, it was detected as a somatic mutation in prostate cancer.[28] Alternatively, Q789E is located within a functional subdomain of the LBD, which is involved in the NH2-terminal/COOH-terminal domain interaction of the androgen receptor.[29]

AIS phenotypes without mutations in the *AR* gene

There are several reports of patients with AIS without a detectable mutation in the AR gene (Chapter 6, [16,30]). Possible explanations are:

• ***Incorrect diagnosis***

Patients with 17βHSD3 deficiency or 17,20 lyase deficiency (Chapters 8 and 9) and gonadal dysgenesis are sometimes initially diagnosed as AIS.

• ***Sensitivity of the PCR-SSCP technique***

Detection of a mutation with PCR-SSCP depends on whether the mutation causes a conformational change when a PCR product is electrophoresed. Examples of mutations that were not detected by PCR-SSCP are the mutation described in Chapter 3, intron 2, at nucleotide position -11 which is located at the 5' end of the PCR product. Another mutation which involves a C deletion at position 2372 in exon 6 which did not result in a conformational change, likely because the sequence consists of six Cs in a row (Chapter 3). Furthermore, mutations either in the polymorphic CAG (Chapter 3) or CGG repeats can not be detected by PCR-SSCP because of the variable nature of these repeats. In addition, some patients may be somatic mosaics for an AR mutation which is undetectable by PCR-SSCP in leukocyte-DNA (Chapter 7). Direct sequencing of the AR gene is therefore indicated in clinically well classified AIS patients without apparent mutation in the AR gene as detectable by PCR-SSCP.

• ***Mutations in introns***

As was shown in Chapter 5, mutations not located in the exon flanking sequences but further into an intron can be missed even when the AR gene is sequenced.

• ***Mutations in the 5' or 3' UTR***

There is only one report of a patient with a mutation in the 5' UTR. However this mutation was detected because the sequence was within the amplified region in the direct sequencing procedure.[31] In our studies we have used primers covering a considerable part of the 5'UTR, -70 bp for the codon encoding the first amino acid (Chapter 2), but no mutations were identified in this region.

• ***Mutations in the promoter***

There are no reports of AIS patients with mutations in the known promoter sequences of the AR. We analyzed a patient with Reifenstein syndrome without detectable mutation in the AR gene at direct sequencing. However this patient's genital skin fibroblast showed reduced expression of the AR protein. This suggested a possible mutation in the AR gene promoter. However, no abnormalities were found in the sequenced-, known promoter sequences (as published by Faber et al.[32]; unpublished observations A.L.M. Boehmer / H.T. Brüggenwirth).

• ***Defect in another factor necessary for normal AR function***

No patients with defects in another factor necessary for normal AR function have been reported to date. Most known coactivators are not specific for AR function, including ARA70[33], which was formerly suggested to act as a specific coactivator.[33-35] However the number of coactivators cloned is rapidly increasing, awaiting analysis of their specificity.[36,37]

PHENOTYPE VERSUS GENOTYPE IN SEX DIFFERENTIATION DISORDERS

Several genes have recently been identified, playing additional roles in sex determination and differentiation. These genes include some of the HOX genes (responsible for spatial and timely patterning of development)[38,39], RING finger genes (body axis patterning and control of cell proliferation)[40], regulators of HOX- and other developmentally regulated genes[41], genes encoding transcription factors involved in morphogenesis of several organs, or in general cell proliferation and differentiation processes.[41-44]

A combination of several techniques will enable rapid expansion of our understanding of factors important for sex determination and differentiation. These include: cloning homologous human genes of animal genes that are found to be important for sex determination and differentiation[39,41], analysis of knockout mice phenotypes[41,43,45], positional cloning using chromosomal break points in humans[40,46-48], linkage analysis in families with (complex) syndromes including disturbed sexual differentiation[49,50], and further phenotypic studies revealing the etiology of the disorder in families with sexual differentiation disorders.[51,52] Some of these factors may influence the timing in development of important structures or organs[49,50] others may prove to be important for the hormonal control of sexual differentiation.

FUTURE DIRECTIONS

Molecular studies in AIS

Quantitation of the residual activity of a partially defective androgen receptor that is transiently expressed and tested by luciferase assays, is difficult. This hampers genotype / phenotype studies in AIS. The use of adenovirus mediated delivery of an androgen-responsive reporter gene into cultured genital fibroblast of a patient, to measure the level of AR function in situ, is one way to minimize the artifacts inherent to such assays.[53,54] In addition genital skin fibroblasts of the patient are likely a better model for the actual situation in the androgen target cell, of the patient, than COS or HeLa cells as used in the transient expression assays.

Phenotypes of patients and *in vitro* AR function are presently used to test and fine tune 3D models of the AR. These models will be useful in explaining a phenotype of a newly identified mutation in the AR in some, but not all cases.

Mechanism of secondary 5α-reductase 2 deficiency

We have shown that a 5α-reductase 2 deficiency secondary to AIS, is due to absence of expression of the 5α-reductase 2 mRNA (Chapter 4). The underlying mechanism of this reduced expression leading to secondary 5α-reductase deficiency is still unknown. More tissues of AIS patients with a secondary 5α-reductase deficiency need testing for the presence of *SRD5A2* mRNA, to develop further understanding.

Further studies in 17β-hydroxysteroid dehydrogenase 3 deficiency

The residual activity of several mutant 17βHSD3 enzymes identified in patients with 17βHSD3 deficiency remain to be tested *in vitro*. The pathogenicity of mutation N74T in the *HSD17B3* gene became very likely as mutation N74T was not found in any of the 74 tested alleles from AIS patients and their family members. Based on the clinical phenotype the N74T *HSD17B3* mutant is expected to have some residual activity (Chapter 9).

Towards the etiology of 'idiopathic' hypospadia

As hypospadias is one of the most common congenital malformations, the elucidation of the etiology of idiopathic hypospadias is a real challenge. A

relevant, well defined group for further analysis may be the cases with hypospadias and Prader stage III or IV, in whom other known causes of hypospadias have been excluded (Chapter 11).
When in knockout mice the disruption of a gene under study leads coincidentally to hypospadias or sex reversal, human homologous genes may be screened for defects in hypospadias patients. A candidate mouse gene has been identified for patients with cryptorchidism in such a 'coincidental way'.[45]

Apart from genetic causes, hypospadias may be caused by environmental (both synthetic and naturally derived) chemicals with anti-androgenic activity.[55] These environmental chemicals give no full explanation for the ethnic and geographic differences in the prevalence of hypospadias, but might be partially related to the increasing incidence of hypospadias in many countries.[56]
Chemicals with hormone mimicking effects are under full investigation now. An example is the agricultural fungicide Vinclozolin, which was shown to cause hypospadias in prenatally exposed male rats.[55] If there is an effect of Vinclozolin on human embryos and at what level of exposure is unknown, and needs further testing. Further toxicological studies are needed to determine the extent of danger of these chemicals.

Differential regulation of 17,20 lyase and 17α-hydroxylase enzyme activity by residue R347

We have identified a new mutation R347C in the *CYP17* gene of two unrelated children with partial 17α-hydroxylase/complete 17,20 lyase deficiency. In two other patients residue R347 had been altered to histidine which resulted in isolated 17,20 lyase deficiency (Chapter 9). Both mutations are in the same residue in the putative redox partner binding site, important in 'awakening' 17,20 lyase enzyme activity instead of 17α-hydroxylase activity.[57]
Mutation R347H completely abolishes 17,20 lyase activity whereas 50% of wild type 17α-hydroxylase activity is retained.[57]. Based on the clinical phenotype of our patients we expect the less conservative mutation R347C to lead to more severe impairment of 17α-hydroxylase activity than the R347H mutation (Chapter 10).
In vitro expression of this R347C mutant *CYP17* and determination of the 17α-hydroxylase enzyme activity versus 17,20 lyase enzyme activity, in the presence or absence of an electron donor, should yield more information on the importance of this residue in regulating the 17,20 lyase versus 17α-hydroxylase enzyme activity of $P450_c17$.

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CHAPTER

14

Summary

This thesis describes clinical and molecular genetic studies on familial disorders of sexual differentiation in 46,XY individuals (male pseudohermaphroditism). Studies on the androgen insensitivity syndrome, 17β-hydroxysteroid dehydrogenase 3 deficiency, 17αhydroxylase/17,20 lyase deficiency and on severe and familial hypospadias are described.

The normal sexual determination and differentiation and the disorders of male sexual differentiation are reviewed in **Chapter 1**.
Many genes, involved in the complex developmental process of gonadal determination and sexual differentiation, became identified in the past 10 years. Normal male sexual differentiation depends on adequate androgen secretion and expression of a normal androgen receptor, at a critical time during gestation and afterwards during puberty and adult life.
Since the cloning of genes involved in sexual differentiation, mutation analysis has become available to establish a definitive diagnosis. Moreover mutation analysis opened the way to carrier detection and to mutation based genetic counseling.
The X-linked androgen insensitivity syndrome (AIS) is a disorder of undervirilization in 46,XY individuals due to an absent or defective androgen receptor (AR). After cloning of the AR gene multiple different mutations were identified in AIS patients. It became apparent that AIS, with a broad phenotypic spectrum, also displayed a broad genotypic spectrum.
This study addressed the genotype-phenotype relationship in AIS. Detailed knowledge of such a relation might be valuable in establishing the sex of rearing in patients born with ambiguous genitalia due to androgen insensitivity and in genetic counseling. The long term psychological reactions of the parents and adult patients to hearing the diagnosis AIS and the possibility of carrier detection were studied simultaneously.

In **Chapter 2** several families with AIS, their AR gene mutations, and the effect of the mutations on the AR protein function are described. Molecular techniques used throughout this study are described in detail. Three new mutations were found in exon 1 of the AR gene in three patients with a female phenotype. A mutation in exon 2 of the AR gene of an additional patient, resulted in defective DNA binding and transactivation.

Chapter 3 describes the genotype/phenotype relationship in 17 families with 43 patients with AIS. Their phenotypes, genotypes and molecular consequences of these genotypes are analyzed. AR gene mutations leading to a

completely defective AR protein lead invariably to complete AIS. However, *AR* gene mutations leading to a partially defective AR protein may result in phenotypic variation even within a family. In as many as 1/3 of the families with partial AIS such intra familial phenotypic variation was observed. Molecular observations suggest that phenotypic variation had different etiologies between these families.
In patients with a completely defective AR protein, Wolffian duct remnants remained detectable, but immunohistochemical evaluation showed no expression of AR protein. Thus, in patients with molecular proof of total absence of a functional AR, no differentiation occurs of Wolffian ducts but they can be macroscopically identified. During puberty or during androgen therapy, no or only minimal virilization was seen even in patients with significant (but still deficient) prenatal virilization. Vaginal length was functional in most but not all patients with complete AIS.
The minimal incidence of AIS in the Netherlands is 1:99.000 based on cases with molecular genetic evidence of the diagnosis.

The mechanism of phenotypic variation in one family with AIS is studied in detail in **Chapter 4**. In the studied family with distinct phenotypic variation, one *AR* gene mutation that rendered the AR partially defective was identified. A 5α-reductase 2 deficiency was detected in genital skin fibroblasts of the most severely undervirilized sibling. Althoug, mutations in the gene encoding 5α-reductase 2 were not found, RT-PCR studies showed no expression of 5α-reductase 2 in genital skin fibroblasts. Intragenic polymorphic marker analysis showed that presence of mutations in the regions outside the sequenced parts of the gene encoding 5α-Reductase 2 were unlikely. 5α-Reductase 2 deficiency, secondary to the primary defect AIS, seems to be the underlying cause of phenotypic variation in this family.

In some patients with AIS no mutations in the *AR* gene are detected upon initial investigations. In such a family (**Chapter 5**), all 3 affected individuals had the same X-chromosome as demonstrated with intragenic polymorphic markers. The AR protein displayed an abnormal phosphorylation pattern suggesting that the AR was indeed defective. An AR gene mutation was identified in intron 2, which abolished the splice acceptor site in intron 2. However a cryptic splice acceptor site at position -70/-71 in intron 2 was activated. Two non-functional transcripts were produced in the patient's genital skin fibroblasts. Only minimal amounts of a normal, functional AR gene transcript were present which explains the partial AIS phenotype, with pubic hair as the only sign of

the action of androgen in a female phenotype with fully female external genitalia.

Chapter 6 describes the in vitro and additional three dimensional modeling studies into the effect on DNA binding when residue Ala 564, located in the part encoding the first zinc cluster of the AR is altered. In one patient with the complete form of AIS this residue was found substituted by Asp. In vitro studies showed that this resulted in the introduction of an extra negative charge, which destabilizes the normal conformation of the AR DNA binding domain. This explains the phenotype of this patient.

In **Chapter 7** the phenomenon of somatic / gonadal mosaicism is described in a family with AIS and two affected children. The AR gene mutation, identified in both children, was not found in DNA derived from leukocytes of the mother. Somatic mosaicism was proven using intragenic polymorphic marker and allele specific oligonucleotide hybridization. Somatic or gonadal mosaicism must always be considered in a mother of an affected AIS child but with apparent absence of an *AR* mutation in maternal leukocytes. Such a mother, has a risk of recurrence and her daughter has a chance of being a carrier. When carrier detection is done, this knowledge helps to remain cautious in genetic counseling: "Never say, there is no recurrence risk for a sibling of a new case in the family".

In **Chapter 8** a study into the emotional reactions of patients and their parents on disclosure of the clinical diagnosis of AIS and its later confirmation by gene mutation analysis is described. Eighteen families with a total of 20 children with AIS as well as ten adult women with complete AIS participated in a semi-structured interview and in completing three standardized psychological questionnaires.
The emotional reaction of parents and adult patients is strong, long-lasting and appears to have a negative effect on openness toward dissemination of information. Parents and patients inform a limited number of persons. Confirmation by mutation analysis did not alter the feelings of AIS patients and their parents, nor did it alter the reticent attitude about AIS into a more open one towards potential carriers.

Chapter 9 describes 18 patients with 17β-hydroxysteroid dehydrogenase 3 (17βHSD3) deficiency of which the majority had received the tentative diagnosis AIS. Hormonal tests were evaluated together with mutation analysis for di-

agnostic purposes. The diagnosis 17βHSD3 deficiency can be reliably diagnosed with use of both, endocrine evaluation and mutation analysis.
Identified putative splice site mutations in the gene encoding 17βHSD3 (*HSD3B2*), were proven to disrupt normal splicing with use of RT-PCR. Furthermore, the identified phenotypic variation in patients homozygous for these splice site mutations could be explained by alternative splicing.
Founder effects were identified with use of haplotype analysis and a control group. A global inventory by haplotype analysis of *HSD3B2* mutations in affected cases from Europe, Near Asia, the USA, Brazil and Australia, demonstrated the ancient origin of at least 4 mutations. The minimal incidence of 17βHSD3 deficiency in The Netherlands was shown to be 1:147.000 with a heterozygote frequency of minimal 1:135.

Chapter 10 describes the phenotype and genotype of 4 patients from 3 families with either isolated 17,20 lyase deficiency or combined 17αhydroxylase/17,20 lyase deficiency. Two siblings were homozygous for mutation R346H, occurring in the putative redox binding site of the CYP17 enzyme, which resulted in isolated 17,20 lyase deficiency. Two unrelated other patients with combined 17αhydroxylase/17,20 lyase deficiency were compound heterozygotes for mutation R346C and either a newly identified deletion of 25bp in exon 1 or a duplication of 4bp in exon 8. In vitro studies of the identified mutations R346C and R346H are expected to yield clues on the role of this residue in the two different enzyme activities, both hydroxylase and lyase, which are encoded by the same *CYP17* gene.

In **Chapter 11** a study on the etiology of severe- or familial hypospadias is described. Patients with severe (n=63) or familial (n=27) hypospadias all referred to a single center were investigated. With use of a diagnostic scheme including clinical evaluation, chromosome analysis, hormonal evaluation and mutation analysis of several genes involved in male sexual differentiation, the etiology was identified in 31% of the cases. Of these 31%, 17% was associated with complex genetic syndromes (such as Smith-Lemli-Opitz, Opitz Frias and Opitz-G/BBB). In 9.5% an abnormal karyotype was found such as 45X/46XY or 46XX/46XY. Only one patient had the vanishing testes syndrome and in just 3% either AIS or 5α-reductase 2 deficiency was established. The results of this study imply that a diagnostic protocol of a child with severe hypospadias may include at least dysmorphological evaluation, chromosome analysis and hormonal evaluation to substantiate evidence of androgen synthesis disorders or AIS. In specific cases, gene mutation analysis is indicated. The identification of

a definitive etiology has important implications on further treatment, prognosis and genetic counseling.

The evaluation of a patient with a disorder of sexual differentiation requires a multidisciplinary approach. A practical clinical protocol with sequential diagnostic steps is advocated in **Chapter 12**. When careful clinical, radio-diagnostic and sono-graphic evaluation, chromosome analysis and endocrinological studies have led to a presumptive diagnosis, gene mutation analysis is a diagnostic test with a high sensitivity and specificity for the etiology in the male undervirilization syndrome. Furthermore, for families with AIS and for (consanguineous) families with androgen synthesis disorders gene mutation analysis is essential for genetic counseling and prenatal diagnosis.

In **Chapter 13** general correlations between genotype and phenotype in AIS and possible origins of phenotypic variation are being discussed. Furthermore, several causes of undetectable *AR* gene mutations in patients with the presumptive diagnosis of AIS are reviewed.
Topics of interest for further research are proposed, including: further studies on secondary 5α-reductase 2 deficiency, differential regulation of 17,20 lyase and 17α-hydroxyase enzyme activity and studies on the etiology of idiopathic hypospadias.

HOOFDSTUK

15 Samenvatting

Onderwerp van dit proefschrift zijn een aantal oorzaken van erfelijke stoornissen in de geslachts differentiatie, met name stoornissen in de gevoeligheid van weefsel van mannelijk geslachtshormoon (androgeenongevoeligheidssyndroom) en een aantal stoornissen die leiden tot verminderde aanmaak van mannelijk geslachtshormoon (17β-hydroxysteroid dehydrogenase 3 deficiëntie, en 17α-hydroxylase/17,20 lyase deficiëntie). Ook worden aanlegstoornissen van het uitwendige mannelijk genitaal (hypospadie) onderzocht.

De normale ontwikkeling van het geslacht van mensen met een 46XY chromosomenpatroon en stoornissen in die geslachtsontwikkeling, worden beschreven in **Hoofdstuk 1**.
De ontwikkeling van het geslacht bij de mens bestaat uit geslachtsdeterminatie en geslachtsdifferentiatie. Tijdens de geslachtsdeterminatie wordt door de geslachtschromosomen, (XY of XX) en zoals recent is gebleken ook door een aantal autosomale genen bepaald, of de indifferente- gonade (=geslachtsklier) in het embryo, zal uitgroeien tot een testis of een ovarium. Bij de geslachtsdifferentiatie wordt het in- en uitwendig geslacht verder gevormd onder invloed van hormonen die geproduceerd worden door de gedifferentieerde gonaden. Wanneer de geslachtsdeterminatie of -differentiatie niet goed verloopt, ontstaat een stoornis in de geslachtelijke ontwikkeling.
Bij geslachtsdifferentiatie in mannelijke richting, spelen androgenen (mannelijke geslachtshormonen) en de androgeenreceptor, een onmisbare rol. Wanneer tijdens de kritieke fase van de embryonale ontwikkeling in een 46XY embryo er een stoornis van de aanmaak van mannelijk hormoon bestaat, of de receptor daarvoor op de cellen niet aanwezig is of niet functioneert, zullen uitwendige en inwendige geslachtsorganen niet of onvolledig in mannelijke richting uitgroeien. Het uitwendige geslachtsorgaan kan vrouwelijk zijn, ofwel niet duidelijk of onvolledig mannelijk. Een voorbeeld van dat laatste is dan een mannelijk geslachtsorgaan met een kleine penis (micropenis) en/of een afwijkende uitmonding van de urineleider (hypospadie). Bij deze personen met een 46XY chromosomenpatroon is er een wisselende mate van aanlegstoornis, gaande van uitwendig volledig vrouwelijk, tot een enigszins onderontwikkeld mannelijk uitwendig geslacht.

De diagnostiek, behandeling en begeleiding van patiënten met een geslachtsdifferentiatiestoornis bestaan uit:
- diagnosestelling
- chirurgische correctie in gevallen met een afwijkend genitaal
- verwijdering van geslachtsklieren wanneer de patiënt opgroeit als vrouw

- hormoontherapie (wanneer zinvol)
- psychologische begeleiding
- erfelijkheidsadvies, draagsterschapsonderzoek, nagaan keuze mogelijkheden bij verhoogd risico

Bij geslachtstoewijzing spelen een rol:
- de aanleg van de verschillende in-/uitwendige geslachtsorganen,
- de mogelijke reactie op mannelijk hormoon (uit het lichaam of toegediend) en
- de verwachting van het resultaat van correctieve chirurgie in de mannelijke richting.

Androgeenongevoeligheid (AOS) is een stoornis van de mannelijke geslachtelijke ontwikkeling, veroorzaakt door een defecte of dysfunctionerende androgeenreceptor (AR). Door de klonering van het *AR* gen in 1988-1989 werd mutatieanalyse in patiënten met AOS mogelijk. Het aantonen van een afwijking in het gen voor de AR receptor gaf een grote precisie aan de diagnose. Tot die tijd was AOS een diagnose die alleen door uitsluiten van andere oorzaken gesteld kon worden in pasgeborenen en kinderen voor de puberteit. Stimulatietesten met hormonen tonen een normale oploop van testosteron en 5α-dehydrotestosteron (DHT), en daaruit blijkt dat de aanmaak van mannelijk hormoon normaal is. In gekweekte huidcellen van een stukje genitale huid (genitale huidfibroblasten) kan men de binding van hormoon aan de AR testen. Soms ziet men daarbij geen afwijkingen. Dit wordt "AR positieve AOS" genoemd. Deze testen waren daardoor niet erg gevoelig voor het aantonen van AOS.
Door het beschikbaarkomen van mutatieanalyse kwam er een nieuwe, nauwkeurige diagnostische test en een methode voor draagsterschapsbepaling voor deze geslachtsgebonden (X-gebonden) aandoening beschikbaar. Hierdoor werd de relatie tussen genmutatie, moleculair fenotype en klinisch fenotype in toenemende mate belangrijk. Bij erfelijkheidsadvies is het te verwachten tekort in vermannelijking bij een toekomstig kind belangrijke informatie voor de ouders. Ook voor geslachtstoewijzing en voorspellen van de mate van vermannelijking tijdens de puberteit of tijdens behandeling met hormonen is het van belang om (rest)activiteit van de AR te kunnen voorspellen.

De studies in dit proefschrift gaan over stoornissen in de mannelijke geslachtsontwikkeling. Daarbij was de relatie van belang tussen klinische kenmerken van androgeen ongevoeligheid, de *AR* gen mutatie en het AR eiwit. Deze

kennis heeft belangrijke consequenties bij geslachtstoewijzing, chirurgische correctie en begeleiding van genderontwikkeling vooral bij gedeeltelijk vermannelijkte patiënten. De psychologische gevolgen van de diagnose en omgang met de diagnose AOS werden afzonderlijk bestudeerd door in dit onderwerp gespecialiseerde psychologen. Daarnaast is kennis van prognose, draagsterschap onderzoek, en risicobepaling van belang bij het erfelijkheidsadvies aan ouders en andere familieleden van patiënten.

In **Hoofdstuk 2** worden enkele patiënten met androgeen ongevoeligheid, hun mutaties in het *AR* gen en het effect van deze mutaties voor het AR eiwit beschreven. Enkele van de gebruikte moleculair biologische technieken worden hier beschreven. Drie nieuwe mutaties in exon 1 van het *AR* gen werden gevonden in 3 patiënten met een vrouwelijk fenotype. Een mutatie in exon 2 resulteerde in verminderde DNA binding en transactivatie.

In **Hoofdstuk 3** wordt een grootschalig Nederlands onderzoek beschreven van alle patiënten met mannelijk pseudohermaphroditisme (=46XY personen met onvoldoende virilisatie) en de waarschijnlijkheidsdiagnose androgeen ongevoeligheid, die bekend waren bij de kinderartsen-endocrinologen, kinderchirurgen, urologen, klinische genetici en sommige internisten en gynaecologen in alle academische centra en enkele grote perifere ziekenhuizen in Nederland. Patiënten en ouders werden benaderd via de behandelend arts, met de vraag om deel te nemen aan het onderzoek. In dezelfde periode werden nieuwe patiënten met mogelijk AOS bestudeerd. De fenotypes van patiënten werden onderzocht, mutaties in het AR gen opgespoord en het effect van de mutatie op de expressie en werking van de AR in genitale huidfibroblasten onderzocht. Sommige mutaties werden in vitro tot expressie gebracht na overbrengen in gekweekte cellijnen en getest. Stambomen werden opgesteld en mogelijke draagsters en aangedane familieleden werden benaderd, gevolgd door genotype/fenotype studies van deze familieleden. De fenotypes van patiënten met dezelfde mutatie binnen families en tussen families werden bestudeerd.
Op deze wijze werden in totaal 17 families met 43 patiënten en 8 afzonderlijke gevallen bestudeerd.
Door de landelijke opzet en nauwkeurige diagnostiek door middel van DNA onderzoek kon een nauwkeurige bepaling worden gedaan van de incidentie van AOS. De incidentie cijfers liepen eerder uiteen van 1:4.000 tot 1:128.400. In onze studie wordt een incidentie van minimaal 1:99.000 gevonden.
De genotype/fenotype relatie in AOS blijkt bijzonder complex te zijn. Er zijn internationaal meer dan 200 verschillende mutaties gevonden verspreid over

bijna het gehele coderende gedeelte van het *AR* gen. Genotype/ fenotype relatie binnen families, waarbij het fenotype van een specifieke mutatie in een aantal patiënten tegelijk bestudeerd kan worden, toont het volgende: *AR* gen mutaties die leiden tot een compleet defect eiwit geven altijd een compleet AOS fenotype. Echter *AR* gen mutaties die resulteren in een gedeeltelijk defect AR eiwit kunnen leiden tot fenotypische variatie ook binnen één familie. In 1/3 van de families met partiële AOS wordt een duidelijke fenotypische variatie gevonden. Deze bevinding is van belang voor het erfelijkheidsadvies, daar in dezelfde familie fenotypische meisjes met enkel verkleving van de schaamlippen én kinderen met een onduidelijk uitwendig geslacht kunnen voorkomen. Bij de meeste, maar niet alle vrouwen met een compleet defecte of afwezige AR bleek de vagina een functionele lengte te hebben.

Hoofdstuk 4 beschrijft een oorzaak voor fenotypische variatie. Twee kinderen uit een gezin hebben dezelfde AR mutatie. De jongen heeft een micropenis en een ernstige hypospadie en een meisje (ook met een 46XY chromosomen patroon) heeft een gedeeltelijke fusie van de labia. Er was geen verschil tussen beide kinderen in AR eiwit expressie, of in androgeen binding door de AR in genitale huidfibroblasten. Bij het meisje bleek 5α-reductase 2 enzym activiteit in genitale huidfibroblasten totaal afwezig, terwijl deze bij de jongen normaal was. Mutatieanalyse en intragene polymorfe marker analyse van het 5α-reductase 2 gen toonde geen mutatie in een exon of intron. Met reverse transcriptase (RT) PCR werd daarop een verminderde expressie van 5α reductase 2 mRNA aangetoond bij het zusje. Deze verminderde expressie van dit enzym is secundair aan het primaire defect, de androgeen ongevoeligheid. Het 5α-reductase 2 enzym zorgt voor de omzetting van testosteron in het sterkere androgeen DHT. Bij dit meisje met AOS veroorzaakte het secundaire 5α-reductase 2 enzym defect een verminderde prenatale en pubertaire virilisatie.
Een dergelijk secundair 5α-reductase 2 defect is al eerder gesuggereerd als bijdragende factor aan de klinische expressie van AOS. In ons onderzoek werd met RT-PCR genexpressieonderzoek en bepaling van enzymactiviteit aangetoond, dat er sprake kan zijn van verminderde expressie van het 5α-reductase 2 enzym, in personen met dezelfde mutatie in het AR gen.

Een duidelijk klinisch voorbeeld van AOS bij een patiënt waarbij geen mutatie in het coderende gedeelte van her AR gen gevonden wordt, is beschreven in **Hoofdstuk 5.** In deze familie waren drie patiënten met de klinische diagnose partiële AOS. Met behulp van intragene polymorfe markers werd aangetoond dat deze drie hetzelfde X-chromosoom hadden, hetgeen X-gebonden over-

erving ondersteunde. Het AR eiwit liet een afwijkend fosforyleringspatroon zien, wat suggereert dat het AR eiwit defect was. Uiteindelijk werd een mutatie gevonden in een intron, op aanzienlijke afstand van het begin van een exon. Deze mutatie verhindert echter de normale splitsing van pre-mRNA. Dit toont het belang van nucleotiden voorafgaand aan de eigenlijke splitsingsplaats van een exon zijn voor het normale verloop van mRNA splitsing. Door de gevonden mutatie ontstaat een nieuwe ongebruikte acceptatie splitsingsplaats op positie -10/-11 in intron 2 en worden een cryptische acceptatie splitsingsplaats op positie -70/-71 in intron 2 en de acceptatie splitsingsplaats op positie -1/-2 in intron 3 geactiveerd. Zo ontstaan twee verschillende, niet-functionele eiwitten en een afwezige AR functie bij in vitro testen. Met een gevoelige techniek werd een kleine hoeveelheid normaal AR eiwit aangetoond in genitale huidfibroblasten. De aanwezigheid van een zeer kleine hoeveelheid normaal AR eiwit, verklaart het vrouwelijke fenotype in deze familie, mèt pubis beharing als een miniem effect van het mannelijk hormoon.
Dus bij patiënten 'zonder' mutatie in het *AR* gen kan een intronmutatie het fenotype verklaren. en mutaties buiten consensus splitsingsplaatsen kunnen mRNA splitsing beïnvloeden, doordat ze de juiste base sequentie scheppen voor ontstaan van nieuwe splitsingsplaatsen.

De *in vitro* expressie van een mutatie in het *AR* gen, gevonden bij een patiënt met de complete vorm van AOS, wordt beschreven in **Hoofdstuk 6**. Het effect van deze mutatie op de binding van de AR aan DNA, voorafgaand aan transcriptieactivatie, werd onderzocht. Door deze mutatie wordt een neutraal aminozuur vervangen door een negatief geladen aminozuur. Onderzocht werd of de verminderde binding van de mutante AR aan het DNA veroorzaakt werd door ladingsveranderingen of door veranderingen in de vorm van het AR- androgeen-DNA complex. Het fenotype van de patiënt lijkt verklaard door de ladingsverandering in deze specifiek plaats van de AR.

Hoofdstuk 7 beschrijft een familie waarbij 2 van de 8 kinderen AOS hadden. In het DNA van de moeder, verkregen uit haar leukocyten, werd echter de mutatie die bij de kinderen aanwezig was, niet gevonden. Door de overerving van een intragene polymorfe marker te volgen en via allel specifieke oligonucleotide hybridisatie is aangetoond dat bij de moeder een somatisch mosaicisme bestond voor de AR gen mutatie. De mogelijkheid van een somatisch of gonadaal mosaicisme moet worden overwogen bij iedere moeder van een schijnbaar sporadische patiënt met AOS. Een somatisch/gonadaal mozaïek bij moeder geeft een duidelijk risico op herhaling voor een volgend kind en is een

reden voor draagsterschapsonderzoek bij alle dochters van de betrokken vrouwen. In volgende zwangerschappen van moeders die op basis van mutatie analyse geen draagster lijken te zijn maar wel een eerder kind met AOS hebben gekregen, is er desgewenst een indicatie voor prenatale diagnostiek.

In **Hoofdstuk 8** wordt een studie beschreven naar de emotionele reacties op lagere termijn in 18 families met 20 kinderen met AIS alsmede bij 10 volwassen vrouwen met AOS, op het stellen van de diagnose AOS en de latere bevestiging van die diagnose met de recente DNA diagnostiek. Emotionele reacties van patiënten en hun ouders zijn hevig en blijven langdurig bestaan. Deze reacties lijken een negatief effect te hebben op openheid over de aandoening. Ouders van patiënten lichten een beperkt aantal mensen in over de aandoening. Bevestiging van de klinische diagnose AOS met behulp van DNA diagnostiek veranderde de gevoelens over de diagnose van patiënten en hun ouders niet. Teven leidt de mogelijkheid van DNA diagnostiek niet tot meer openheid of het inlichten van mogelijke draagsters in de familie.

Door de klonering van het verantwoordelijke gen voor 17βHSD3 deficiëntie, een testosteron synthese stoornis en andere oorzaak van mannelijk pseudohermaphroditisme, kwam mutatie analyse beschikbaar voor diagnostiek. **Hoofdstuk 9** beschrijft een aantal patiënten met de aanvankelijke waarschijnlijkheidsdiagnose AOS waarbij geen *AR* gen mutatie gevonden werd. Aanvullende endocrinologische-, fenotypische en moleculaire tests toonde dat het veelal ging om 17β-hydroxysteroid-dehydrogenase 3 (17βHSD3) deficiëntie. Deze diagnose werd vroeger vooral op basis van verhoogde serum androsteendion en verlaagde testosteron/androsteendion ratio in serum gesteld. Men beschouwde het als een relatief zeldzaam ziektebeeld. De klassieke endocrinologische diagnostiek werd vergeleken met mutatie analyse als diagnostisch middel. De conclusie is dat bij gebruik van beide methoden, de diagnose 17βHSD3 deficiëntie onomstotelijk is te stellen. Draagsterschap onderzoek van de autosomaal recessief erfelijke 17βHSD3 deficiëntie, is alleen mogelijk met mutatie analyse.
Veel Nederlands patiënten van niet-consanguïne ouderparen bleken homozygoot te zijn. Er werd een klein aantal specifieke mutaties bij herhaling gevonden in verschillende- niet verwante- families. Tevens waren de mutaties per etnische afkomst verschillend. Door een bijzondere internationale samenwerking kon de verspreiding van deze mutaties in een aantal bevolkingsgroepen worden bestudeerd. Met extra-gene polymorfe marker analyse in de 17βHSD3 deficiënte patiënten en één controle groep, werd op basis van

statistische analyse een founder effect gevonden voor enkele van deze mutaties. Andere mutaties zijn waarschijnlijk bij herhaling opnieuw ontstaan. Door vergelijking van Nederlandse patiënten met patiënten uit verschillende delen van de wereld kon voor een aantal van deze gen mutaties, worden herleid, uit welke populatie de founder (de eerste mutatie drager, die tot verspreiding heeft geleid) afkomstig was, mede gebaseerd op historische gegevens. Zo blijkt een *HSD17B3* genmutatie door West-Europese immigranten verspreid te zijn onder blanke Noord-Amerikanen en blanke Australiërs. Een andere mutatie is mogelijk erg oud en is verspreid door de Phoeniciers, komend vanuit de regio die tegenwoordig Syrië, Israël en Libanon beslaat. Weer een andere mutatie is mogelijk ten tijde van het Ottomaanse Rijk verspreid, in welke periode een vermenging ontstond van de verschillende volkeren in klein Azië, en aangrenzende gebieden.
Voor 17βHSD3 deficiëntie kon voor het eerst de minimale incidentie in Nederland worden vastgesteld op 1:146.000. In vergelijking, de incidentie van AOS is 1:99.000. Terwijl vroeger werd aangenomen dat AOS de meest frequente oorzaak van mannelijk pseudohermafroditisme is, dan is 17βHSD3 deficiëntie dus belangrijk in de differentiaaldiagnose.
Van twee van de geïdentificeerde intron mutaties werd door RT-PCR studies duidelijk dat ze pathogeen zijn. Bij patiënten homozygoot voor deze splitsingmutaties kan fenotypische variatie optreden, hetgeen veroorzaakt kan worden door alternerend splitsen met een verschillende opbrengst in afwijkende en normale enzym eiwitten.

Hoofdstuk 10 behandelt een andere testosteron synthesestoornis waarvoor mutatieanalyse inmiddels mogelijk is, is gecombineerde 17α-hydroxylase/17,20 lyase deficiëntie dan wel geïsoleerde 17,20 lyase deficiëntie. Het *CYP17* gen codeert voor zowel de 17α-hydroxylase als de 17,20 lyase enzym activiteit. Het is niet precies bekend waarom in de bijnier zowel pre- als post pubertair en voornamelijk 17α-hydroxylase activiteit aanwezig is, terwijl in de testes beide 17α-hydroxylase en 17,20 lyase enzym activiteiten tot expressie komen. Posttranslatie modificaties van het eiwit zijn voor deze differentiële expressie waarschijnlijk verantwoordelijk.
Onder kinderen met mannelijk pseudohermaphroditisme werden drie patiënten uit 2 families gediagnostiseerd met een geïsoleerde 17,20 lyase deficiëntie dan wel gecombineerde 17α-hydroxylase/17,20 lyase deficiëntie. De patiënten met geïsoleerde 17,20 lyase deficiëntie waren homozygoot voor mutatie R346H in het *CYP17* gen. In 2 andere patiënten resulteerde een compound heterozygotie van mutatie R346C en een totaal defect allel, in een ge-

combineerde 17α hydroxylase/17,20 lyase deficiëntie. Residu R346 van het *CYP17* gen speelt mogelijk een belangrijke rol in differentiële expressie van ofwel 17α-hydroxylase enzym activiteit ofwel 17,20 lyase enzym activiteit. Bestudering van het in vitro fenotype kan aanwijzingen opleveren over het mechanisme van differentiële expressie van beide functies van hetzelfde *CYP17* gen.

Hoofdstuk 11: hypospadie is een van de meest voorkomende aangeboren afwijkingen bij jongens waarvan in de meeste gevallen de etiologie onbekend blijft. Hypospadie kan echter ook een van de verschijnselen zijn van AOS, androgeen synthese stoornissen, gonadale dysfunctie en complexe genetische syndromen. De waarde van mutatieanalyse en nauwkeurige genetische diagnostiek werd onderzocht in een cohort van patiënten dat tussen 1966 en 1995 was verwezen naar het Academische Ziekenhuis Rotterdam, met een ernstige (n=63) of familiair voorkomende (n=27) hypospadie. Toegepast werden: lichamelijk onderzoek, endocrinologische evaluatie, karyotypering en mutatie analyse van verschillende genen betrokken bij de normale mannelijke ontwikkeling. De oorzaak kon vastgesteld worden bij 31% van de index patiënten met een ernstige hypospadie. 17% werd veroorzaakt door complexe genetische syndromen, 9.5% door chromosomale afwijkingen, 3% door AOS en androgeen synthese stoornissen en een patiënt had het vanishing testes syndroom.
Deze resultaten tonen de wenselijkheid aan, om bij jongetjes met een ernstige hypospadie onderzoek te doen naar chromosoomafwijkingen en complexe genetische syndromen, evenals endocrinologisch onderzoek. Een hCG test kan testosteronsynthese stoornissen en 5α-reductase 2 deficiëntie aantonen. Op grond van de endocrinologische evaluatie kan mutatie analyse van het betrokken gen bijvoorbeeld een testosteronsynthese stoornis aantonen. Het tijdrovende karakter van AR gen analyse maakt routinematig toepassing bij jongetjes met een ernstige hypospadie onmogelijk.

In hoofdstuk 12 wordt de plaats van de DNA-diagnostiek bij de diagnostiek van geslachtsdifferentiatiestoornissen ontwikkeld tijdens deze studie verder onderzocht. Het sluitstuk van het proefschrift is een aangepast protocol voor diagnostiek van patiënten met geslachtsdifferentiatiestoornissen.

In **Hoofdstuk 13** wordt een samenvatting gegeven van de genotype/fenotype relatie in AOS voorzover deze kan worden afgeleid. Over de mogelijke oorza-

ken van fenotypische variatie in AOS en het niet vinden van een *AR* gen mutatie bij AOS wordt gespeculeerd.
Toekomstige gebieden van onderzoek, voortvloeiend uit de in dit proefschrift beschreven studies, worden voorgesteld zoals: aanvullende studies naar secundaire 5α-reductase 2 deficiëntie, *in vitro* studies van mutaties gevonden bij 17βHSD3 deficiëntie, verder onderzoek naar de oorzaken van hypospadie bij patiënten waarbij na uitgebreide aanvullende diagnostiek geen oorzaak gevonden werd en in vitro studies naar het effect van mutatie R347C in het *CYP17* gen op de 17α-hydroxylase en 17,20 lyase enzym activiteit.

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June 1996
36th Meeting of the European Society for Pediatric Endocrinology, Montpellier, France.
Title: An intron mutation in the androgen receptor (AR) gene as a cause of receptor positive androgen insensitivity syndrome (AIS).

September 1996
Najaars vergadering voor Fertiliteits studies, Leuven, Belgium.
Title: Androgeen ongevoeligheid: klinische en moleculair genetische aspecten.

June 1997
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Title: 17β-hydroxysteroid dehydrogenase 3 (17βHSD3) deficiency: mutational and phenotypic spectrum, diagnostic pitfalls.

September 1997
Androgens '97. Clermont-Ferrant, France.
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November 1997
Jaarlijks congres van de Nederlandse Vereniging voor Kindergeneeskunde, Veldhoven.
Titel: Genotype versus fenotype van erfelijke geslachtsdifferentiatie stoornissen.

November 1998
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Titel: Geslachtsdifferentiatie stoornissen: diagnostische uitdagingen.

November 1999
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Titel: Erfelijke geslachts differentiatie stoornissen: klinische en moleculair genetische studies.

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"Androgen insensitivity syndrome, somatic mosaicism and extreme phenotypic variation within one family; implications for genetic counseling. Sex differentiation: Clinical and biological aspects." Cambridge UK.

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CURRICULUM VITAE

Annemie Boehmer was born in 1966 in Velp where she grew up and attended secondary school at the Rhedens Lyceum. She passed her exam (vwo-B) in 1984. From 1984 until 1985 she did a propedeutic exam in Biomedical Sciences at Leiden University. She attended Medical School at the same university from 1985 to 1993. While in Medical School, she worked as a research assistant on tumor angiogenesis in the Department of Pathology of Leiden University, under the direction of Prof. D.J. Ruiter. Her medical school thesis work, on the role of γδ T lymphocytes in the immune system, was performed from 1990 to 1991 in the laboratory of Prof. J.G. Seidman in the Department of Genetics at Harvard Medical School, Boston, MA, USA. During her Medical School internship, she worked on the genetic basis of polysyndactyly with Prof. C.E. Seidman, Department of Genetics, Harvard Medical School, Boston, MA, USA. After receiving her medical degree in June 1993, the worked as an intern in the department of Pediatric Oncology, Amsterdam Medical Center / Emma Children's Hospital. From October 1993 until December 1998, the research described in this doctoral thesis was performed under the direction of Prof. S.L.S. Drop, Prof. M.F. Niermeijer, and Dr. A.O. Brinkmann. Annemie Boehmer started her clinical pediatric residency in January 1999, at the Sophia Children's Hospital/University Hospital Rotterdam (heads: Prof.Dr. H.A. Büller and Prof.Dr. H.J. Neijens). She is married to Hugo van Berckel with whom she has two children: Francesca, born in 1995, and Elisabeth, born in 1997.

Color illustrations

CHAPTER 1

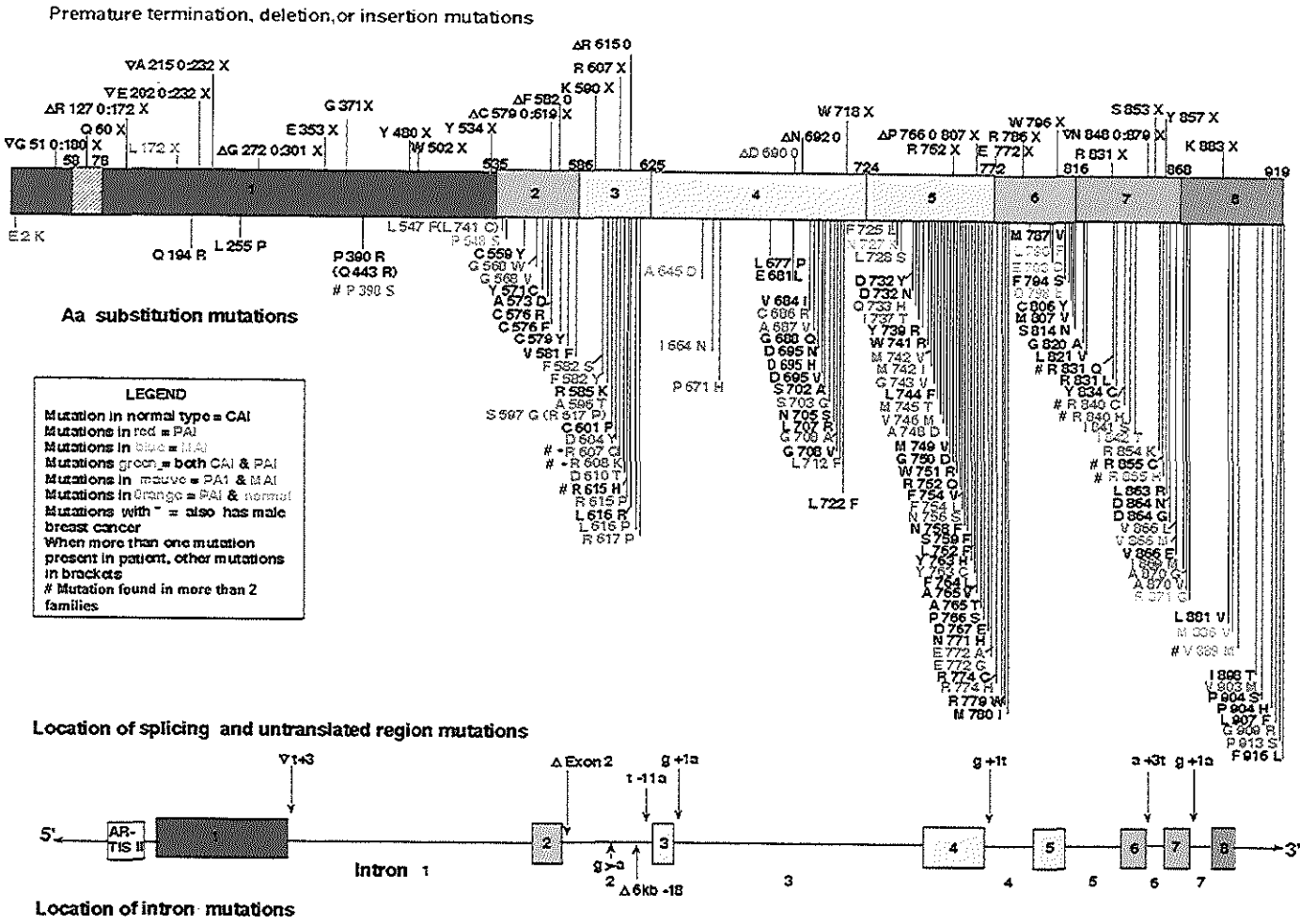

Figure 1.9
Distribution of mutations over the AR gene, as deduced from the AR-gene mutation database Http://WWW.McGill.CA/androgendb, November 1998 and includes the mutations as described in this thesis. Reproduced with permission. This data base uses the AR residue numbering according to the cloned sequence of Lubahn et al.[137]

CHAPTER 3

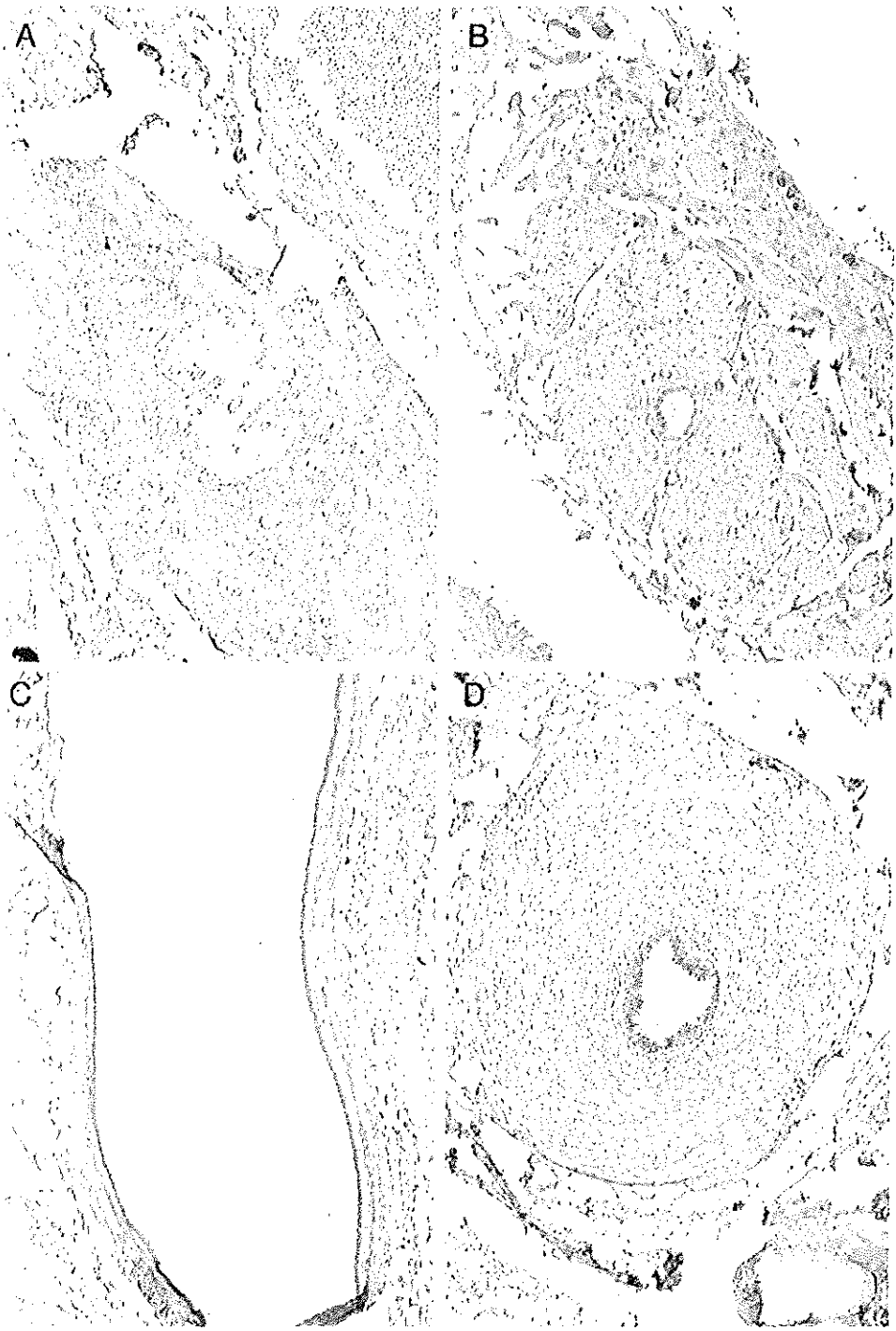

Figure 3.2
Immunohistochemistry of tissue sections of patients. Staining with a monoclonal anti-body against the N-terminal part of the AR.
A+B Structures resembling Wollfian ducts in patient B:IV-4.
C Longitudinal section through a structure resembling the vas deferens of subject A:II-1.
D Vas deferens of a patient with 17β-hydroxysteroid dehydrogenase type 3 deficiency.

CHAPTER 9

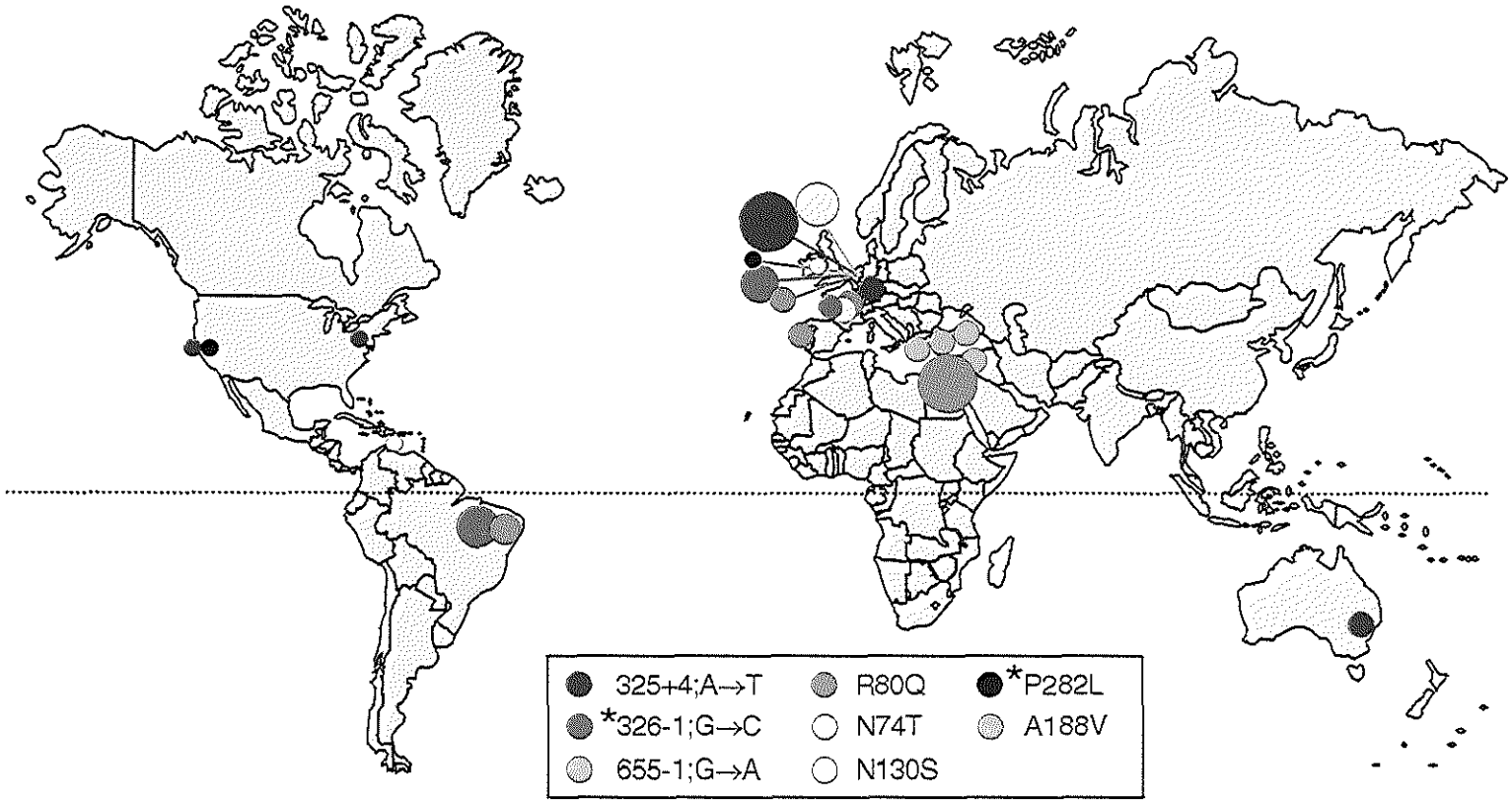

Figure 9.4
Global distribution of identical HSD17B3 gene mutations as found in this study.[5,15,16,39] The size of the circles corresponds to the number of alleles that have been found for a specific mutation in a specific area. However the number of R80Q alleles in Israel is much larger than indicated.[10] The different mutations are color coded. *, Mutations with proven *de novo* recurrence.