0021-972X/01/$03.00/0
The Journal of Clinical Endocrinology & Metabolism
Copyright © 2001 by The Endocrine Society
Vol. 86, No. 3
Printed in U.S.A.

Phenotypic Variation in a Family with Partial Androgen Insensitivity Syndrome Explained by Differences in 5α Dihydrotestosterone Availability

ANNEMIE L. M. BOEHMER, ALBERT O. BRINKMANN, RIEN M. NIJMAN, MARJA C. T. VERLEUN-MOOIJMAN, PETRA DE RUITER, MARTINUS F. NIERMEIJER, AND STENVERT L. S. DROP

Division of Endocrinology, Department of Pediatrics (A.L.M.B., S.L.S.D.), and Department of Pediatric Urology (R.C.N.), Sophia Children's Hospital; Department of Endocrinology and Reproduction (A.L.M.B., A.O.B., M.C.T.V.-M., P.d.R.), Erasmus University Rotterdam; and Department of Clinical Genetics (A.L.M.B., M.F.N.), University Hospital Rotterdam, Rotterdam, The Netherlands

ABSTRACT

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

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. In both siblings serum levels of hormones were measured; a sex hormone-binding globulin (SHBG) suppression test was completed; 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 were investigated, and the 5α-reductase 2 gene was sequenced.

The decrease in SHBG serum levels in a 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 on hormone binding were identical in both siblings. However, 5α-reductase 2 activity was normal in genital skin fibroblasts from the phenotypic male patient but undetectable in genital skin fibroblasts from the phenotypic female patient. The lack of 5α-reductase 2 activity was due to absent or reduced expression of 5α-reductase 2 in genital skin fibroblasts from the phenotypic female patient. Exon and flanking intron sequences of the 5α-reductase 2 gene showed no mutations in either sibling. 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 additional to the AIS.

Distinct phenotypic variation in this family was caused by 5α-reductase 2 deficiency, additional to AIS. This 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 here is explained by differences in the availability of 5α-dihydrotestosterone during embryonic sex differentiation. (*J Clin Endocrinol Metab* **86:** 1240–1246, 2001)

ANDROGEN INSENSITIVITY SYNDROME (AIS) is an X-linked disorder of male sexual differentiation, caused by a defective or absent androgen receptor (AR; reviewed in Ref. 1). Mutations in the AR gene result in a wide range of AIS phenotypes. The phenotypic spectrum in 46,XY individuals ranges from a complete female phenotype with testes [complete AIS (CAIS)], through female phenotypes with clitoromegaly or posterior fusion of the labia minora, 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 more than 150 different AR gene mutations in over 250 AIS patients (2) illustrates the genetic heterogeneity in AIS.

The AR is a transcription factor that 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 dimerizes and migrates into the nucleus where it recruits transcription factors and binds to the promoter region of androgen-sensitive target genes (6).

With the advent of molecular analysis of the AR gene it was hoped that a correlation between a molecular defect and a particular phenotype could be established. Such a relationship would enable prediction of the response to androgen therapy in infants with PAIS, relevant for not only long-term psychosexual outcome but also for 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). Therefore, additional factors apparently may influence the effect of the mutant receptor on the development of the external genitalia.

Reduced 5α-reductase 2 activity has been described in the 70s and 80s in AIS families and has been suggested to be the cause of the observed phenotypic variation (15–17). At that time, molecular evaluation of the AR and 5α-reductase 2 genes was not yet available.

Received December 2, 1999. Revision received July 31, 2000. Rerevision received November 2, 2000. Accepted November 5, 2000.

Address correspondence and requests for reprints to: Annemie L. M. Boehmer, M.D., Ph.D., Division of Endocrinology, Department of Pediatrics, Sophia Children's Hospital, P.O. Box 2060, 3000 CB Rotterdam, The Netherlands. E-mail: a.Boehmer@JKZ-RKZ.nl.

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006

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 amino acids) is a frequently identified mutation (2). Experimental evidence is provided for a different availability of DHT in these two siblings.

Subjects and Methods

In a nationwide study on the genotype/phenotype relationship in AIS, we studied a family with eight children, of whom two were affected with AIS (Fig. 1). The parents (subjects I-1 and I-2; Fig. 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-yr-old pubertal female patient, with not yet fully developed breasts, few pubic hairs, and no axillary hair (Tanner stage M3, P2, A0), a female habitus, and female voice. She was studied after her brother was diagnosed 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 (Fig. 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, <10); estradiol, 35 pmol/L (normal males, 50–200 pmol/L); and LH, 5.7 U/L (normal males, 1.5–8 U/L). In accordance with her personal wish, she was not gonadectomized until 2 yr later, at age 15.5 yr. 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 yr, showed T/DHT ratios between 8.6 and 13.0. From age 14, T had been above 30 nmol/L.

Subject II-8 (Fig. 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 (Fig. 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. Seventy-two hours after 1500 U human CG (hCG) im, T was 18 nmol/L. He was assigned the male sex. At 5 yr of age his basal serum levels were: LH, 0.1 U/L; T, 0.1 nmol/L; and DHT, <0.1 nmol/L. Seventy-two hours after 1500 U hCG im: T, 11.8 nmol/L; DHT, 2.1 nmol/L; T/DHT, 5.6. All values are within the normal range for this age.

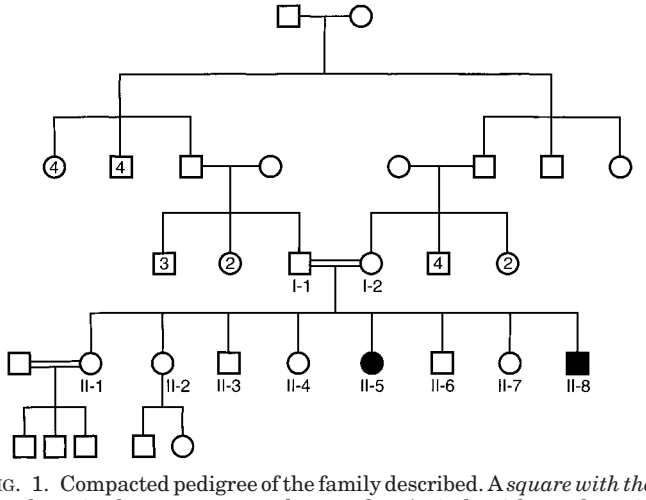

FIG. 1. Compacted pedigree of the family described. A *square with the number 3 in the center* means three males. A *circle with number 2 in the middle* means two females. The affected individuals are indicated with *black circles* or *black squares* according to their phenotypic appearance and not according to their genetic sex.

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 mosaïcism for the AR mutation as was shown by segregation analysis of the different AR alleles among affected and nonaffected family members and by allele-specific oligonucleotide analysis, respectively, as described previously (18).

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

SHBG suppression test

Androgen sensitivity in the liver can be measured *in vivo* with use of an SHBG suppression test. We have used the protocol described by Sinnecker *et al.* (19): Stanozolol (0.2 mg/kg/day, single evening dose) was administered orally at days 0, 1, and 2. Blood samples were taken before and at days 5, 6, 7, and 8 after the start of the test. The initial SHBG serum level was compared with the lowest level obtained after administration of Stanozolol (days 5, 6, 7, and 8) and expressed as a percentage of the initial value. In normal controls the SHBG serum level after Stanozolol declined to 35.6–62.1% (range) of the initial value. However, in patients with CAIS, the SHBG serum level remained unchanged, and in PAIS patients the SHBG level declined to 48.6–89.1% (range) of the initial value (19).

AR gene mutation detection

Genomic DNA was isolated from peripheral blood leukocytes and from cultured genital skin fibroblasts, following standard procedures (20).

Exon and flanking intron sequences were screened for mutations in the AR gene with the use of PCR-single-strand conformational polymorphism (21). PCR fragments suspected to harbor mutations were analyzed by direct sequencing (22). Furthermore, in DNA isolated from genital skin fibroblasts, 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 poly-glutamine and poly-glycine stretches, respectively, were determined as described previously (21, 22).

Cell culture

Skin biopsies were taken either during surgical correction of the external genitalia, or gonadectomy or circumcision. Genital skin fibroblasts were derived from biopsies of the fusion line of the labia minora of subject II-5 of control individuals, and from scrotal skin of subject II-8 and from preputium of a normal prepubertal boy obtained at circumcision. Furthermore, genital skin fibroblasts were analyzed from biopsies from preputium of a prepubertal 5α-reductase 2-deficient patient with clitoromegaly and posterior fusion of the labia minora. This patient had a T/DHT ratio after hCG of 16.5. All cell lines were cultured as described previously (22), with modifications as described with the experiments.

Androgen characteristics of the AR

Whole cell Scatchard analysis was performed on genital skin fibroblasts, as described previously (22). Genital skin fibroblast (GSF) 1 was used from 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 h, followed by 24 h in medium containing increasing concentrations (0, 5, 30, and 100 nM) of the synthetic, nonmetabolizable androgen methyltrienolone (R1881). GSF 1 was used from subject II-5. Whole cell lysates were prepared, immunoprecipitated, separated on a SDS-PAGE gel, and immunostained as described previously (22).

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006

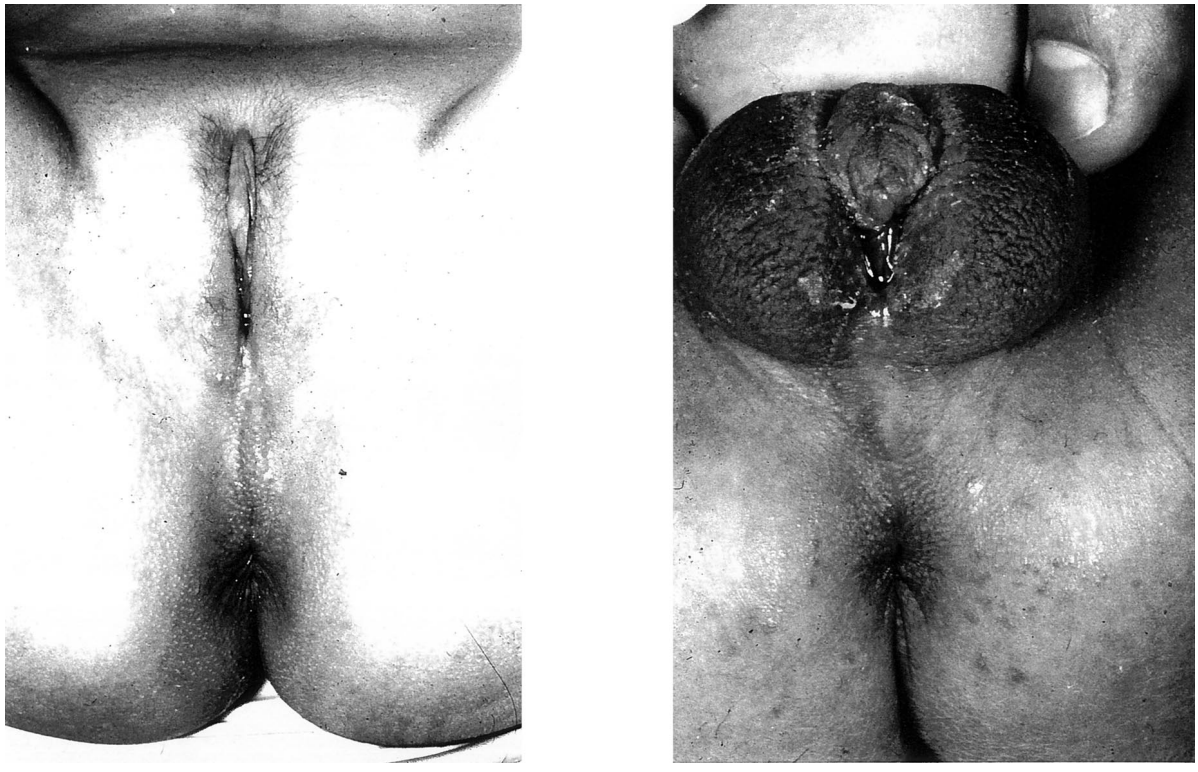

FIG. 2. External genitalia of subject II-5 at age 15.5 yr (A) and subject II-8 at 1 month of age (B).

5α-Reductase 2 assay

5α-Reductase 2 activity can be influenced by either clonal origin of the cell line (23) or by the site of origin of the biopsies (24). Therefore, two different GSF cell lines from subject II-5 (GSF 1 and 2) derived from separate biopsies taken 1.5 yr apart were used for the 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 a known pathogenic mutation in the 5α-reductase 2 gene (H231R) were used as controls.

Because 5α-reductase 2 activity increases with serial subcultures (25, 26), all cell lines used were the seventh subculture.

To reduce possible bias by confluency rate, all cell lines were grown in 75-cm^2 culture flasks with medium containing 10% FCS. They were harvested 7 days after subculture. At that time, the cell lines were confluent and the flasks contained $\sim 1.2 \times 10^6$ 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 $800 \times g$. Pellets were washed twice in Tris saline. Cell-free extracts were prepared by four cycles of freezing in liquid nitrogen and thawing.

Enzyme assay. Forty microliters of cell-free extracts were incubated with 10 μl of 30 mM NADPH and 50 μl reaction mixture [reaction mixture consisted of 500 μl of 10 mM Tris citrate (pH 5.5), 2.4 pmol 1,2,6,7 ^{3}H-testosterone (Amersham Pharmacia Biotech, Little Chalfont, UK), and 7.6 pmol testosterone (Steraloids)] at 37 C for 1 h. The reaction was stopped on ice. Each incubation was done in duplicate. Assays were done in triplicate.

To all samples 10 μl of a steroid mixture containing androstanedione, androstenedione, DHT, testosterone, 3α-androstanediol, each 1 mg/mL ethanol, were added, before extraction of radioactivity with a total of 3×500 μL ethylacetate. Extracts were evaporated to dryness, and the residues were dissolved in 50 μl ethanol and chromatographed in dichloromethane:ethylacetate:methanol (85:15:3) on a 0.25-mm layer silica gel plate, 20×20 cm (Merck, Darmstadt, Germany). Steroids were visualized in a control lane by spraying with 20% H_2SO_4 in methanol and developing at 100 C for 15 min. Fractions were collected in separate vials, resuspended in 500 μl ethanol, and were counted in a liquid scintillator counter. 5α-Reductase activity was calculated from the sum of ^{3}H-radioactivity in the androstanediol, DHT, and 3α androstanediol fractions divided through the sum of ^{3}H radioactivity in the androstanediol, androstenediol, DHT, T, and 3α androstanediol fractions. The amount of protein in each cell-free extract was determined according to Bradford (27).

5α-Reductase activity was expressed as femtomoles of 5α reduced steroids formed per milligram of protein per hour.

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

Exon and flanking intron sequences of the 5α-reductase type 2 gene (SRD5A2) 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 with unknown allele frequencies (29, 30). Genomic DNA isolated from GSF 1 (subject II-5) and GSF (subject II-8) was used.

RT-PCR of 5α-reductase 2 messenger RNA (mRNA)

Total RNA was extracted from GSF cell lines using Trizol reagent (Life Technologies, Inc., Breda, The Netherlands) and quantified by absorption at 260 nm. Complementary DNA (cDNA) was synthesized from 2.5 μg RNA with the use of an oligo dT primer (Promega Corp., Madison, WI). Of each investigated cell line, cell pellets from different cell culture flasks were pooled and divided in equal 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 10 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.

Three microliters of the obtained cDNA reaction product was used in a 50-μl PCR amplification reaction. The 50-μL PCR reaction mix contained 1.5 mM $MgCl_2$. Conditions for the PCR reactions in a Biometra

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006

cycle sequencer were: hot start at 94 C for 5 min, then 35 cycles at 94 C for 1 min, at 55 C for 30 sec, at 72 C for 1 min, and final extension for 10 min 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 Corp., Norwalk, CT) cannot 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, San Diego, CA) and subjected to automated sequencing.

Results

SHBG suppression test

An SHBG suppression test (19) showed a maximal decrease in SHBG of 73.5% on days 5, 6, 7, and 8 in the female patient, subject II-5 [normal males, <63.4%; PAIS, 63.4–93%; CAIS, >92% (31)], whereas a maximal decrease of 92% on day 7 was seen in subject II-8. It can be concluded that both siblings display a suppression in the PAIS range.

Mutation detection and identification in the AR gene

With PCR-single-strand conformational polymorphism, 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 on sequencing of the exon and flanking intron sequences of the AR gene of subject II-5.

Length of (CAG)nCAA and GGN repeats

In both siblings the (CAG)nCAA and GGN repeats in exon 1 of the AR gene mutant-allele carried 14 glutamine and 24 glycine residues (18), respectively.

AR binding characteristics

AR binding characteristics in GSF of subjects II-5 and II-8 (Table 1) show an increased equilibrium dissociation constant (K_d) but a normal number of binding sites (B_{max}). The difference in B_{max} and K_d 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. On 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 during SDS-PAGE (Ref. 31; Fig. 3). At a relatively low androgen concentration of 5 nM R1881, GSFs of II-5 and II-8 express equally reduced amounts of the third isoform of 114 kDa compared with the wild type. Increased androgen levels did not induce the 114-kDa band as in the wild-type cells (Fig. 3). Moreover, both siblings have an equally deficient hormone induced upshift of the 114 kDa 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, sibling II-8 had normal 5α-reductase 2 activity in GSF (Table 2).

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

No mutations were found in the 5α-reductase type 2 gene of subjects II-5 and II-8 on sequencing exon and flanking intron sequences.

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 promoter, is, therefore, very unlikely.

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 GSFs 1 and 2 of subject II-5, whereas in total RNA preparations from the GSF of subject II-8 a band of 460-bp was detected (Fig. 4). This band was subcloned and sequenced and exhibited the wild-type 5α-reductase 2 gene sequence. Therefore, the absence of 5α-reductase type 2 activity in GSFs of subject II-5 is most likely 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 siblings carrying the same mutation in the AR gene. Additional mutations in the AR gene or differences in the length of the polyglutamine or polyglycine repeats were not found. AR gene promoter mutations are very unlikely because AR protein expression is similar and normal in both siblings. Furthermore, phosphorylation of the AR on hormone binding, was equally reduced in genital skin fibro-

TABLE 1. Scratchard analysis of the AR in GSFs of both affected siblings

	Subject II-5, female phenotype	Subject II-8, male phenotype	Normal values
B_{max} (fmol/mg protein)	87	61	>20
K_d (nM)	0.9	0.5	<0.1

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006

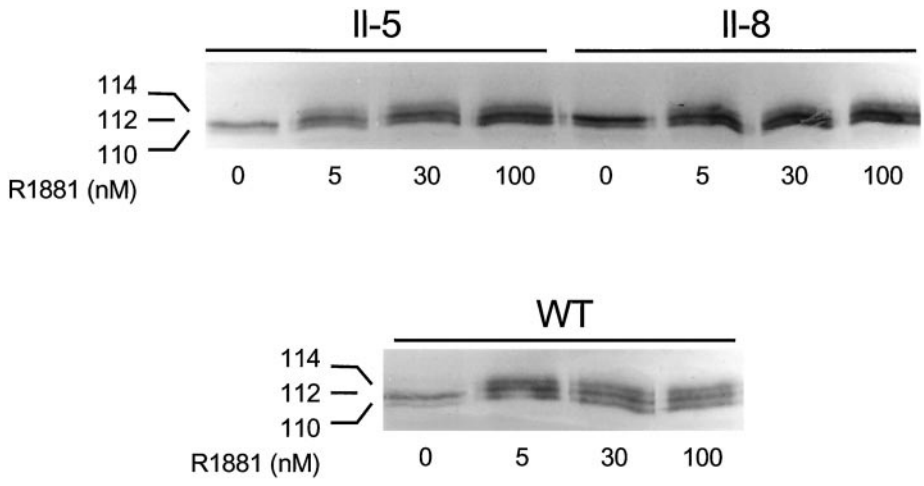

FIG. 3. Hormone-dependent AR phosphorylation in GSFs. GSFs of both siblings (II-5 and II-8) and a normal prepubertal boy (WT) were cultured in the absence or presence of increasing concentrations (0–100 nM) of the nonmetabolizable androgen R1881. The molecular sizes of the AR isoforms are indicated at the *left* (110, 112, and 114 kDa). AR protein isolated from wild-type cells cultured in the absence of androgens migrates as a doublet of 110 and 112 kDa, representing an unphosphorylated 110 kDa AR isotype and a phosphorylated 112 kDa AR isotype, respectively. On binding of androgens, the AR undergoes additional phosphorylation, resulting in a third isoform of 114 kDa. At a relative low androgen concentration of 5 nM R1881, GSFs of siblings II-5 and II-8 have equally reduced amounts of the third isoform of 114 kDa compared with the wild type. Increased androgen levels did not induce the 114-kDa band as in the wild-type cells, and both siblings have an equally deficient hormone-induced upshift of the 114-kDa AR isoform.

TABLE 2. 5α-Reductase 2 activities in GSFs from control persons and 46,XY patients with AIS or 5α-reductase 2 deficiency

Subject/disorder	Age at biopsy (yr)	5α-reductase 2 activity (pmol/mg protein/h)[a]		No. of experiments
		Mean	Range	
Normal male	1	73	44–139	6
Normal female	16	41		1
II-5 GSF 1; PAIS	13	0.2	0–0.3	2
II-5 GSF 2; PAIS	15	0	0–0	2
II-8; PAIS	5	88	42–154	5
5α-Reductase 2 deficiency	4	0.4	0–1.3	3

In all assays the recovered radioactivity represented at least 84% of the added ^{3}H-testosterone. All assays were done in duplicate, variation between duplicate measurements was <11%.

[a] Mean and range are given. Ranges in different experiments are shown.

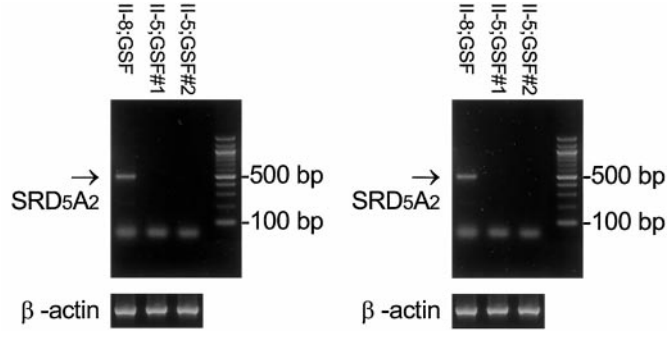

FIG. 4. RT-PCR of 5α-reductase 2 mRNA. RNA extracted from GSFs 1 and 2 of subject II-5 and RNA from the GSF of subject II-8 was used. β Actin expression was used as a control for cDNA synthesis. The amplified 5α-reductase 2 cDNA fragment, 460 bp in size, is indicated with an *arrow*.

blasts of both siblings, irrespective whether low or high concentrations of the nonmetabolizable androgen R1881 were used. Phosphorylation of the AR occurs during 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 and comparable clinical androgen responsiveness in both siblings, as determined by SHBG suppression tests, provide evidence that the AR itself is not responsible for the distinct variation in phenotype and suggest an important role for factors other than the AR in determining the phenotype.

5α-Reductase activity was found to be totally absent in genital skin fibroblasts of subject II-5 (Table 2). DHT, formed in the embryonal urogenital tissues by 5α-reductase 2, causes elongation and enlargement of the urogenital tubercle and fusion of the urogenital swellings and folds during the development of the embryo (32, 33). We hypothezised that the phenotypic differences between the siblings might be due to the difference in availability of DHT, especially because both siblings carry a particular mutant AR (R846H). When this R846H mutant AR is stimulated with DHT instead of testosterone, the transcriptional deficit becomes less (34, 35) and the functional defect can even be partially corrected by the repeated addition of DHT (34). Therefore, a difference in availability of DHT between the two siblings could have been of particular influence on the phenotype.

Although, serum dihydrotestosterone levels at puberty in subject II-5 are low but detectable and once fell within the low normal range, similar DHT serum levels are found in pubertal/adult 5α-reductase 2-deficient patients with deficient 5α-reductase 2 activity as established in GSFs (16, 36). When 5α-reductase 2-deficient patients enter puberty they start to synthesize some DHT. The source of this DHT may be either peripheral conversion by 5α-reductase type 1 in the liver and skin or, in some cases, residual activity of the mutant enzyme.

A 5α-reductase 2 deficiency secondary to the primary de-

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006

fect AIS has been reported to cause phenotypic differences in other families with AIS and was established by 5α-reductase 2 assays in GSFs and by hormonal analysis in serum (15–17). It has been suggested that in AIS patients 5α-reductase 2 enzyme activity is preserved in the liver but deficient in the periphery, in contrast to the autosomal recessive inherited syndrome of 5α-reductase 2 deficiency wherein a generalized severe defect of both hepatic and peripheral 5α-reductase is found (16, 37). These observations were made before cloning of the AR and 5α-reductase 2 genes (7, 10, 29, 38). The nature of the decreased 5α-reductase 2 activity remained unidentified. The repeated observation of this 5α-reductase 2 deficiency secondary to AIS and the 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 10 reductase 2 deficiency in GSFs of 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. The presence of mutations in the remaining intronic sequences or the promoter region of the SRD5A2 gene is very unlikely because both siblings are heterozygotes for the *C*TA/*G*TA polymorphism in exon 1.

With molecular means we show that the additional 5α-reductase 2 deficiency in the presented subject II-5 with AIS is due to absent or reduced expression of the 5α-reductase 2 enzyme.

The etiology of this additional 5α-reductase 2 deficiency is not clear. One possible explanation is disruption of a feed forward control mechanism: formation of trace amounts of DHT that are bound to the AR induces 5α-reductase 2 activity thereby increasing DHT synthesis and triggering a positive developmental cascade. Such a positive feedback mechanism exists in the rat embryonic urogenital tract where 5α-reductase type 2 expression is increased by either T or DHT (39). A positive feedback mechanism is also present in adult rat prostate but is absent in embryonic rat prostate (39, 40). 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. The expression of 5α-reductase 2 in female fetuses is one third of the expression of 5α-reductase 2 found in males, who have higher levels of androgens (36). 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 and during *in vitro* culture of GSFs no increase of 5α-reductase activity is observed after stimulation with androgen (24, 41, 42). In GSFs, 5α-reductase 2 is the predominantly expressed and active iso-enzyme (43).

Others have suggested a unbalance between estrogen and androgen action as the cause for secondary 5α-reductase deficiency (16, 24).

An interesting possibility is an additional autosomal recessively inherited defect in a factor regulating 5α-reductase 2 expression, inherited from the consanguineous parents by subject II-5. Because 5α-reductase 2 expression appears in fetal rats before testicular androgen synthesis starts, it was suggested that the early regulation and most likely the initial induction events are androgen independent in rat (38). Which factors control the temporal and cell type-specific pattern of 5α-reductase enzymes in the rat or in man is presently unknown.

These observations support the hypothesis that differences in the availability of DHT in different target tissues could lead to phenotypic variation between AIS patients who carry the same AR gene mutation. This study shows that distinct intrafamilial phenotypic variation can be associated with additional 5α-reductase 2 deficiency, information to be implemented in genetic counseling of families with androgen insensitivity.

Additional 5α-reductase 2 deficiency was shown to be associated with undetectable 5α-reductase 2 mRNA levels in GSFs. The total lack of enzyme activity is unlikely to be due to mutations in the 5α-reductase 2 gene itself. Possible causes for this additional 5α-reductase 2 deficiency are a defective autocatalyic regulation or an autosomal recessively inherited defect in a regulatory protein that controls the cell type-specific and temporal expression pattern of the 5α-reductase 2 gene.

References

1. **Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS.** 1995 Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev. 16:271–321.
2. **Gottlieb B, Beitel LK, Lumbroso R, Pinsky L, Trifiro M.** 1999 Update of the Androgen Receptor Gene Mutations Database. Hum Mutat. 14:103–114.
3. **Zhou ZX, Lane MV, Kemppainen JA, French FS, Wilson EM.** 1995 Specificity of ligand-dependent androgen receptor stabilization: receptor domain interactions influence ligand dissociation and receptor stability. Mol Endocrinol. 9:208–218.
4. **Grino PB, Griffin JE, Wilson JD.** 1990 Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. Endocrinology. 126:1165–1172.
5. **Maes M, Sultan C, Zerhouni N, Rothwell SW, Migeon CJ.** 1979 Role of testosterone binding to the androgen receptor in male sexual differentiation of patients with 5 α-reductase deficiency. J Steroid Biochem. 11:1385–1392.
6. **Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO.** 1991 Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. Mol Endocrinol. 5:1396–1404.
7. **Trapman J, Klaassen P, Kuiper GG, et al.** 1988 Cloning, structure and expression of a cDNA encoding the human androgen receptor. Biochem Biophys Res Commun. 153:241–248.
8. **Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM.** 1988 Cloning of human androgen receptor complementary DNA and localization to the X chromosome. Science. 240:327–330.
9. **Tilley WD, Marcelli M, Wilson JD, McPhaul MJ.** 1989 Characterization and expression of a cDNA encoding the human androgen receptor. Proc Natl Acad Sci USA. 86:327–331.
10. **Chang C, Kokontis J, Liao S.** 1988 Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science. 240:324–326.
11. **Batch JA, Davies HR, Evans BA, Hughes IA, Patterson MN.** 1993 Phenotypic variation and detection of carrier status in the partial androgen insensitivity syndrome. Arch Dis Child. 68:453–457.
12. **Imasaki K, Hasegawa T, Okabe T, et al.** 1994 Single amino acid substitution (840Arg→His) in the hormone-binding domain of the androgen receptor leads to incomplete androgen insensitivity syndrome associated with a thermolabile androgen receptor. Eur J Endocrinol. 130:569–574.
13. **Rodien P, Mebarki F, Mowszowicz I, et al.** 1996 Different phenotypes in a family with androgen insensitivity caused by the same M780I point mutation in the androgen receptor gene. J Clin Endocrinol Metab. 81:2994–2998.
14. **Evans BA, Hughes IA, Bevan CL, Patterson MN, Gregory JW.** 1997 Phenotypic diversity in siblings with partial androgen insensitivity syndrome. Arch Dis Child. 76:529–531.
15. **Kuttenn F, Mowszowicz I, Wright F, et al.** 1979 Male pseudohermaphroditism: a comparative study of one patient with 5 α-reductase deficiency and three patients with the complete form of testicular feminization. J Clin Endocrinol Metab. 49:861–865.

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006

16. **Imperato-McGinley J, Peterson RE, Gautier T, et al.** 1982 Hormonal evaluation of a large kindred with complete androgen insensitivity: evidence for secondary 5 α-reductase deficiency. J Clin Endocrinol Metab. 54:931–941.
17. **Jukier L, Kaufman M, Pinsky L, Peterson RE.** 1984 Partial androgen resistance associated with secondary 5 α-reductase deficiency: identification of a novel qualitative androgen receptor defect and clinical implications. J Clin Endocrinol Metab. 59:679–688.
18. **Boehmer AL, Brinkmann AO, Niermeijer MF, Bakker L, Halley DJ, Drop SL.** 1997 Germ-line and somatic mosaicism in the androgen insensitivity syndrome: implications for genetic counseling (Letter). Am J Hum Genet. 60:1003–1006.
19. **Sinnecker GH, Hiort O, Nitsche EM, Holterhus PM, Kruse K.** 1997 Functional assessment and clinical classification of androgen sensitivity in patients with mutations of the androgen receptor gene. German Collaborative Intersex Study Group. Eur J Pediatr. 156:7–14.
20. **Miller SA, Dykes DD, Polesky HF.** 1988 A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16:1215.
21. **Ris-Stalpers C, Hoogenboezem T, Sleddens HF, et al.** 1994 A practical approach to the detection of androgen receptor gene mutations and pedigree analysis in families with x-linked androgen insensitivity. Pediatr Res. 36:227–234.
22. **Bruggenwirth HT, Boehmer AL, Verleun-Mooijman MC, et al.** 1996 Molecular basis of androgen insensitivity. J Steroid Biochem Mol Biol. 58:569–575.
23. **Griffin JE, Allman DR, Durrant JL, Wilson JD.** 1981 Variation in steroid 5 α-reductase activity in cloned human skin fibroblasts. Shift in phenotypic expression from high to low activity upon subcloning. J Biol Chem. 256:3662–3666.
24. **Pinsky L, Kaufman M, Straisfeld C, Zilahi B, Hall CS.** 1978 5α-reductase activity of genital and nongenital skin fibroblasts from patients with 5α-reductase deficiency, androgen insensitivity, or unknown forms of male pseudohermaphroditism. Am J Med Genet. 1:407–416.
25. **Lamberigts G, Dierickx P, De Moor P, Verhoeven G.** 1979 Comparison of the metabolism and receptor binding of testosterone and 17 β-hydroxy-5 α-androstan-3-one in normal skin fibroblast cultures: influence of origin and passage number. J Clin Endocrinol Metab. 48:924–930.
26. **Mowszowicz I, Kirchhoffer MO, Kuttenn F, Mauvais-Jarvis P.** 1980 Testosterone 5 α reductase activity of skin fibroblasts. Increase with serial subcultures. Mol Cell Endocrinol. 17:41–50.
27. **Bradford MM.** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72:248–254.
28. **Hiort O, Sinnecker GH, Willenbring H, Lehners A, Zollner A, Struve D.** 1996 Nonisotopic single-strand conformation analysis of the 5 α-reductase type 2 gene for the diagnosis of 5 α-reductase deficiency. J Clin Endocrinol Metab. 81:3415–3418.
29. **Andersson S, Berman DM, Jenkins EP, Russell DW.** 1991 Deletion of steroid 5 α-reductase 2 gene in male pseudohermaphroditism. Nature. 354:159–161.
30. **Labrie F, Sugimoto Y, Luu-The V, et al.** 1992 Structure of human type II 5 α-reductase gene. Endocrinology. 131:1571–1573.
31. **Jenster G, de Ruiter PE, van der Korput HA, Kuiper GG, Trapman J, Brinkmann AO.** 1994 Changes in the abundance of androgen receptor isotypes: effects of ligand treatment, glutamine-stretch variation, and mutation of putative phosphorylation sites. Biochemistry. 33:14064–14072.
32. **Grumbach MM, Conte FA.** 1998 Disorders of sexual differentiation. In: Wilson JD, Foster DW, Kronenberg HM, Larsen PR, eds. Williams' textbook of endocrinology, ed 9. Philadelphia: W.B. Saunders; 1303–1425.
33. **Thigpen AE, Silver RI, Guileyardo JM, Casey ML, McConnell JD, Russell DW.** 1993 Tissue distribution and ontogeny of steroid 5 α-reductase isozyme expression. J Clin Invest. 92:903–910.
34. **Marcelli M, Zoppi S, Wilson CM, Griffin JE, McPhaul MJ.** 1994 Amino acid substitutions in the hormone-binding domain of the human androgen receptor alter the stability of the hormone receptor complex. J Clin Invest. 94:1642–1650.
35. **Bevan CL, Brown BB, Davies HR, Evans BA, Hughes IA, Patterson MN.** 1996 Functional analysis of six androgen receptor mutations identified in patients with partial androgen insensitivity syndrome. Hum Mol Genet. 5:265–273.
36. **Wilson JD, Griffin JE, Russell DW.** 1993 Steroid 5 α-reductase 2 deficiency. Endocr Rev. 14:577–593.
37. **Zhu Y-S, Katz MD, Imperato-McGinley J.** 1998 Natural potent androgens: lessons from human genetic models. In: Hughes IA, ed. Bailliere's clinical endocrinology and metabolism. Sexual differentiation. London: Bailliere Tindal; 83–114.
38. **Lubahn DB, Joseph DR, Sar M, et al.** 1988 The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. Mol Endocrinol. 2:1265–1275.
39. **George FW, Russell DW, Wilson JD.** 1991 Feed-forward control of prostate growth: dihydrotestosterone induces expression of its own biosynthetic enzyme, steroid 5 α-reductase. Proc Natl Acad Sci USA. 88:8044–8047.
40. **Berman DM, Tian H, Russell DW.** 1995 Expression and regulation of steroid 5 α-reductase in the urogenital tract of the fetal rat. Mol Endocrinol. 9:1561–1570.
41. **Jenkins JS, Ash S.** 1971 The metabolism of testosterone by skin in normal subjects and in testicular feminization. J Endocrinol. 49:515–520.
42. **Mowszowicz I, Melanitou E, Kirchhoffer MO, Mauvais-Jarvis P.** 1983 Dihydrotestosterone stimulates 5 α-reductase activity in pubic skin fibroblasts. J Clin Endocrinol Metab. 56:320–325.
43. **Mestayer C, Berthaut I, Portois MC, et al.** 1996 Predominant expression of 5 α-reductase type 1 in pubic skin from normal subjects and hirsute patients. J Clin Endocrinol Metab. 81:1989–1993.

Downloaded from jcem.endojournals.org at Medical Library Erasmus MC on November 28, 2006