

Genotype Versus Phenotype in Families with Androgen Insensitivity Syndrome

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Androgen insensitivity syndrome encompasses a wide range of phenotypes, which are caused by numerous different mutations in the AR gene. Detailed information on the genotype/phenotype relationship in androgen insensitivity syndrome is important for sex assignment, treatment of androgen insensitivity syndrome patients, genetic counseling of their families, and insight into the functional domains of the AR. The commonly accepted concept of dependence on fetal androgens of the development of Wolffian ducts was studied in complete androgen insensitivity syndrome (CAIS) patients. In a nationwide survey in The Netherlands, all cases ($n = 49$) with the presumptive diagnosis androgen insensitivity syndrome 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. Fifty-nine percent of androgen insensitivity syndrome patients had other affected relatives. A total of 17 families were studied, seven families with CAIS (18 patients), nine families with partial androgen insensitivity (24 patients), and one family with female prepubertal phenotypes (two patients). No phenotypic variation was observed in families with CAIS. However, phenotypic variation was observed in one-third of families with partial androgen insensitivity resulting in different sex of rearing and differences in requirement of reconstructive surgery. Intrafamilial 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 the residual androgen action *in vivo* and the development of the prepubertal and adult phenotype. Patients with a functional complete defective AR had some pubic hair, Tanner stage P2, and vestigial Wolffian duct derivatives despite absence of AR expression. Vaginal length was functional in most but not all CAIS patients. The minimal incidence of androgen insensitivity syndrome in The Netherlands, based on patients with molecular proof of the diagnosis is 1:99,000. Phenotypic variation was absent in families with CAIS, but distinct phenotypic variation was observed relatively frequent in families with partial androgen insensitivity. Molecular observations suggest that phenotypic variation had different etiologies among these families. Sex assignment of patients with partial androgen insensitivity cannot be based on a specific identified AR gene mutation because distinct phenotypic variation in partial androgen insensitivity families is relatively frequent. In genetic counseling of partial androgen insensitivity families, this frequent occurrence of variable expression resulting in differences in sex of rearing and/or requirement of reconstructive surgery is important information. During puberty or normal dose androgen therapy, no or only minimal virilization may occur even in patients with significant (but still deficient) prenatal virilization. Wolffian duct remnants remain detectable but differentiation does not occur in the absence of a functional AR. In many CAIS patients, surgical elongation of the vagina is not indicated. (*J Clin Endocrinol Metab* 86: 4151-4160, 2001)

THE X-LINKED ANDROGEN insensitivity syndrome [AIS, MIM 300681/312300 (1)], encompasses a heterogeneous group of defects in the 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 undervirilized male phenotype. Intermediate phenotypes include a female phenotype with clitoromegaly and labial fusion, a phenotype with ambiguous genitalia, and 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 virilization of the external genitalia at birth. Major clinical problems are the acceptability and advisability of male *vs.* female sex assignment in patients with ambiguous genitalia. Whether virilization will increase during puberty or following androgen

Abbreviations: AIS, Androgen insensitivity syndrome; CAIS, complete androgen insensitivity syndrome; DHT, dihydrotestosterone; GSF, genital skin fibroblasts; PAIS, partial androgen insensitivity; WT, wild-type.

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 intrafamilial 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 androgen 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 a 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 amino acid 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 (2). A somatic mosaicism may be present in as many as one-third of single cases (3). To exclude modulation of the phenotype by somatic mosaicism, we studied the genotype-phenotype relationship in families with multiple affected subjects with clinical as well as molecular means.

In this study we analyzed the genotype-phenotype relationship in AIS and the occurrence and possible causes of phenotypic variation. Information on embryonic development of Wolffian ducts and development of pubic hair as well as recommendations for further treatment of the patients with a female or male phenotype was gained. Additionally, a minimal incidence of AIS could be calculated.

Subjects 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 of AIS was made when virilization in a 46,XY individual was deficient or absent, despite normal male serum levels of T and dihydrotestosterone (DHT) or a sufficient rise in T and DHT after a human CG stimulation test. In some gonadectomized patients, endocrine evaluation was lacking. In all patients the AR gene was screened for mutations. AR expression and androgen binding were studied in genital skin fibroblasts. The final diagnosis of AIS was made when an AR gene mutation was found in combination with abnormalities in androgen binding, diminished AR expression, and/or alterations of DNA binding of the hormone-AR complex. An extensive family history, covering three to four generations, was obtained. Genetic and psychological counseling was 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 genitourscopy. 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 an SHBG suppression test was done (4). SHBG serum levels are measured on days 5, 6, 7, and 8 after administration of Stanozolol 0.2 mg/kg per day orally on days 0, 1, and 2. The initial SHBG serum level (day 0) is compared with the lowest SHBG serum level obtained on either day 5 or 6 or 7 or 8 after administration of Stanozolol and expressed as a percentage of the initial value. In normal controls a decline of 35.6–62.1% is found. However, in complete androgen insensitivity syndrome (CAIS) the serum levels remains unchanged (range 92.4%–129% of the initial value), and in PAIS patients the SHBG level declines to 48.6–87% of the initial value (4). SHBG and basal or human CG stimulated serum levels of hormones were measured by RIA.

Classification of CAIS vs. PAIS

CAIS is differently defined. Griffin *et al.* (5) define CAIS as completely female external genitalia, paucity of axillary and pubic hair, and absent Wolffian duct derivatives. Quigley (6) defines CAIS as completely 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 of some degree of androgen responsiveness and thus classified as PAIS (7). In the classification of Sinnecker *et al.* (4), CAIS is a female phenotype with scant pubic and axillary hair (type 5a) or a female phenotype with absence of any androgen-dependent structures, such as 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 1). When this definition is applied, CAIS is used for an adult 46,XY patient with female external genitalia and absent or scant pubic hair, including Tanner stage P2. Vestigial 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.

AR gene mutation analysis

Genomic DNA was isolated from leukocytes or genital skin fibroblasts (GSF) according to standard procedures (8). The AR gene was screened for mutations with PCR-single-strand conformation polymorphism followed by direct sequencing of the PCR products suspected to carry a mutation (9). In cases of negative PCR-single-strand conformation polymorphism results, the whole AR gene was sequenced either with direct (9) or automated sequencing.

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

AR expression, molecular size, and androgen binding were studied as described (9). Either a binding assay in cytosols from GSF or a whole-cell binding assay was performed with use of the nonmetabolizable androgen R1881.

Antibodies used for Western immunostaining of the AR were 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 h, followed by 24 h in a medium containing increasing concentrations (0, 5, 30, and 100 nM) of the synthetic, nonmetabolizable androgen methyltrienolone (R1881). Whole-cell lysates were prepared from confluent cell layers in 150 cm² culture flasks, immunoprecipitated, separated on an SDS-PAGE gel, and immunostained as described (9).

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 frame shift mutation (A:II-1 and II-2) and absence of androgen binding in GSFs. Because Wolffian ducts 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 5 μ) were rehydrated, and after antigen retrieval by microwave treatment, they were immunostained with antibody F39.4, directed against the N-terminal part of the AR protein, amino acid 301–320, or with antiestrogen receptor (DAKO Corp., Glostrup, Denmark), using the avidin-biotin peroxidase complex method. Immunostaining was visualized with diaminobenzidine followed by nuclear counterstaining with Mayer's hematoxylin (10). A vas deferens of a 1-yr-old patient with 17 β 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. Nineteen of these 32 index patients had affected relatives. Of these 19 families, 17 families agreed to participate in this study. Of the two nonparticipating families, only the index patients could be studied. These two nonparticipating 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. Of the 17 patients without apparent defect in the AR, 12 patients were eventually diagnosed with 17 β -hydroxysteroid dehydrogenase 3 deficiency (as described in reference 28), two patients had 17 α -hydroxylase/17,20 lyase deficiency and two had 46, XY gonadal dysgenesis. In one patient the diagnosis remains unknown.

Incidence of AIS

An incidence rate for AIS was calculated with use of all AIS patients with proven receptor abnormalities, either isolated or familial patients, identified in this nationwide survey. Nineteen AIS patients were born in The Netherlands during the time period 1984–1993. The mean annual life birth rate in The Netherlands was 189,000 during that time period (11). Thus a minimal incidence of 1:99,000 was calculated over this 10-yr time period.

Phenotypes and families

The pedigrees of the 17 studied families are shown in Fig. 1. The subjects presumably affected as reported by family members (Fig. 1) could not be studied for reasons of family confidentiality, geographical distances, or nonparticipation in this study. Family pedigrees have been compressed for reasons of confidentiality. Thirteen of the Dutch families were of West European descent, two of Moroccan, one of Turkish descent, and one family was from Hindustan and had been living in Surinam.

The clinical and molecular studies are summarized in Table 1. Distinct phenotypic variation was observed in 3 of the 17 families with AIS, PAIS families J, K, N, or three of nine PAIS families. Relevant aspects of the phenotype not covered in Table 1 are described in more detail below.

Family C. Subject IV-2 was a girl, diagnosed at age 8 yr with a hypothalamic glioma resulting in hypogonadotrophic hypogonadism. Estrogen substitution was started at age 13. When the diagnosis of AIS was made at age 18 yr, 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 II-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 yr of age. On the finding of inguinal testes, the sex was changed to male. The genitalia consisted of a clitoris-like phallus, separate introitus vaginae and urethra, labioscrotal swellings, blind ending vagina, an utriculus prostaticus, 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 T levels of 24.5 nmol/liter and normal 5 α reductase type 2 activity as measured in genital skin fibroblasts. He started with Mesterolone 3 \times 25 mg/d p.o. during 1 yr, which resulted in the appearance of vellus facial hair. The penile size did not increase. The therapy was changed into testosterone enanthate 250 mg im once every 2 wk during 8 yr without improvement in phallic size. The patient is married and has one adopted son.

Family L. Subject IV-1 received the diagnosis AIS at age 15 yr, and a gonadectomy was performed, Tanner stage B4, 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/liter, T: 52.0 nmol/liter, DHT: 4.1 nmol/liter, E2: 235 pmol/liter, SHBG: 13.7 nmol/liter.

Subject IV-2 was 13 yr old when the diagnosis of AIS was made, Tanner stage B2, P1, A0. She had clitoromegaly, 1.5 cm in length, a urogenital sinus of 1 cm diameter, and a blindly ending vagina 1.5 cm deep. Serum LH: 2.9 IU/L, T: 11.1 nmol/liter, E2: 41 pmol/liter. Two years later she was gonadectomized. During these 2 yr, her habitus remained female and her voice and clitoral size remained unchanged. Her habitus remained female. The only sign of androgen action was the appearance of pubic hair; at age 15 yr, she had reached Tanner stage B4, P4.

Family N. This family contains both an affected female and a male individual. Subject IV-5 (subject II-5 in Boehmer *et al.* (17)), was a 13-yr-old pubertal girl, Tanner stage B3, P2, A0, with a female habitus and female voice when the diagnosis of AIS was made. External genitalia consisted of normally sized 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/liter, T 13.8 nmol/liter, DHT 1.55 nmol/liter, E2 35 pmol/liter. She was not gonadectomized until age 15.5 yr. Serum hormone levels at that time were: T 31.5 nmol/liter, DHT 2.42 nmol/liter. Her voice was still high pitched, she had no clitoromegaly, pubic hair was still P2, and axillary hair was still absent and her

TABLE 1. Phenotypes and genotypes in the studied families with AIS

| Patient | | Phenotype | | | | | Androgen receptor gene | | Androgen receptor function | | | Ref. |
|------------|-----------------------------|---------------------------|----------------|---------------------------------------|---|------------------------------|------------------------|---|----------------------------|-------------------------|---------------|------|
| AIS type | Family/patient ^a | Prader stage ^b | Sex of rearing | Tanner stage (age in yr) ^c | Wolffian/Müllerian duct structures ^d | Vagina length (at age in yr) | Exon/functional domain | Mutation | Bmax (fmol/m gP) | Kd (nM) | Protein (kDa) | |
| CAIS | A II-1 | 0 | Female | B4, P3, A1 (22) | E±/V± ^e /M+ | 6 cm (22), coitus | 1/TAD | C deletion codon 42 ^f ; frameshift → stop codon at 171 | n.m. ^g | n.m. ^g | n.m. | 6 |
| | A II-2 | 0 | Female | B5, P2, A1 (20) | E±/V± ^e /M- | 2.5 cm (1) | | | n.m. ^g | n.m. ^g | n.m. | |
| CAIS | B III-9 | 0 | Female | P2, A1 (19, 44) | E-V-/M+ | 6–7 cm (31); coitus | 1/TAD | Q478X | — | — | — | 12 |
| | 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 | B4, P2, A0 (13) | E+/V+ ^e /M- | 2 cm (13) | | | | | | |
| CAIS | C IV-1 | 0 | Female | B5, P2, A0 (14) | — | 3 cm (14) | 3/DBD | R598X | n.m. ^h | n.m. ^h | n.m. | 13 |
| CAIS | C IV-2 | 0 | Female | B5, P2 (18) | — | — | | | n.m. ^h | n.m. ^h | n.m. | |
| | D V-2 | 0 | Female | B5, P2 (25) | Not gonadectomized | 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 | B5, P2 (18) | Not gonadectomized | 4 cm (15) | | | | | | |
| CAIS | E III-1 | 0 | Female | B5, P2, A1 (55) | E+/V+/M- | 5 cm (45); surgery | 3/DBD | R606H | — | — | — | 14 |
| | E III-2 | 0 | Female | B5, P2 (40) | — | — | | | | | | |
| | E IV-1 | 0 | Female | B5, P3, A0 (26) | Not gonadectomized | — | | | | | | |
| CAIS | F II-1 | 0 | Female | B5, P2, A0 (21) | — | Shallow, coitus | 5/LBD | L735F | | | | 12 |
| | F II-2 | 0 | Female | B5, P2, A0 (21) | E+/V+/M- | surgery | | | n.m. ^h | n.m. ^h | 110 | |
| CAIS | G II-3 | 0 | Female | P2, A0 (36) | E+/V?/M- | 4.5–5 cm (36) | 5/LBD | P757A | 25 ± 3 ^h | 0.74 ± 0.8 ^h | ↓ ↓ 110 | New |
| | G III-3 | 0 | Female | B4, P2, A1 (15) | E±/V+/M+ | 7 cm (15) | | | | | | |
| | G III-4 | 0 | Female | B2, P1, A0 (11) | Not gonadectomized | — | | | | | | |
| CAIS/PAIS? | H IV-1 | 0 | Female | B2, P1, A0 (11) | E+/V+/M- | — | 5/LBD | W742R | 10 ± 3 | 0.67 ± 0.03 | — | 12 |
| | H IV-2 | 0 | Female | Prepubertal (9) | E?/V?/M- | — | | | 20 ± 4 | 0.79 ± 0.16 | | |
| PAIS | I II-4 | 0 | Female | B5, P3, A0 (15) B5, P4, A1 (18) | E+/V+/M- | 3.5 cm (0.7) 6 cm (16) | Affects splicing | Intron 2; -11 A→T | | | | 15 |
| | I III-1 | 0 | Female | Prepubertal (0.2) | E+/V+/M- | 2 cm (2) | | | 63 ^h | 0.07 ^h | 110 | |
| | I III-2 | 0 | Female | Prepubertal (2) | E+/V+/M- | 2 cm (0.2) | | | 64 ^h | 0.08 ^h | 110 | |
| PAIS | J III-1 | 0 | Female | B5, P3, A0 (27) | E+/V+/M- | 8 cm (18); coitus | 4/LBD | del AAC codon 683 | 63 ± 6 ^g | 0.38 ± 0.0 ^g | — | 16 |
| | J III-2 | I | Female | B3, P4, A0 (16) | E+/V+/M- | 8 cm (16); coitus | | | 99 ± 17 ^g | 0.60 ± 0.1 ^g | | |
| | J III-3 | 0 | Female | B5, P3, A0 (21) | E+/V+/M- | Coitus | | | | | | |
| | J III-4 | 0 | Female | ? | E?/V?/M- | Coitus | | | | | | |
| PAIS | K II-8 | II | Male | G+, P5V, A1 (23) | Not gonadectomized | Present | 6/LBD | M771I | | | — | 14 |
| | 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. ^g | n.m. ^g | | |

TABLE 1. Continued

| Patient | Phenotype | | | | | | Androgen receptor gene | | Androgen receptor function | | | Ref. | |
|---------|-----------|-----------------------------|---------------------------|------------------------------------|---------------------------------------|---|------------------------------|---|----------------------------|-------------------------|-------------------|------|---------------|
| | AIS type | Family/patient ^a | Prader stage ^b | Sex of rearing | Tanner stage (age in yr) ^c | Wolffian/Müllerian duct structures ^d | Vagina length (at age in yr) | Exon/functional domain | Mutation | Bmax (fmol/mg P) | Kd (nM) | | Protein (kDa) |
| PAIS | K IV-10 | II | Female | Prepubertal | E+/V+/M- | Present | | | | n.m. ^g | n.m. ^g | | |
| | L IV-1 | II | Female | B5, P4, A0 (15) | E+/V+/M- | 2 cm (15) | 5/LBD | L759M | 90 ^h | 0.47 ^h | 110 | New | |
| | L IV-2 | II | Female | B2, P1, A0 (13) M4, P4, A0 (15) | E+/V+/M- | 1.5 cm (13) | | | 40 ^h | 0.47 ^h | 110 | | |
| PAIS | M II-2 | II | Female | B5, P5 (adult) | — | Shallow, surgery | 5/LBD | M733V | | | | 14 | |
| PAIS | M III-1 | II | Female | Prepubertal | — | Utricle | | | n.m. | n.m. | 110 | | |
| | N IV-5 | II | Female | B4, P2, A0 (15) | E+/V+/M- | 2.5 cm (15) | 7/LBD | R846H | 87 | 0.9 ^h | 110 | 17 | |
| PAIS | N IV-8 | III | Male | Prepubertal | E+/V+/M- | Absent (5) | | | 61 | 0.5 ^h | 110 | | |
| | O III-20 | III | Male | Prepubertal | Not gonadectomized | — | | | | | | 19 | |
| PAIS | O IV-1 | II | Female | Prepubertal | E+/V+/M- | Shallow (birth) | 1 & 7/TAD & LBD | P389S/R846H | 86 ^h | 0.99 ^h | 110 ^e | | |
| | P II-2 | III | Male | — | Not gonadectomized | Absent | 2/DBD | Tyr562H | | | | New | |
| | P II-3 | III | Male | — | Not gonadectomized | Absent | | | | | | | |
| | P III-1 | III | Male | Prepubertal | Not gonadectomized | Absent | | | 41 ^h | 0.07 ^h | 110 | | |
| PAIS | Q III-6 | III | Male | G+, P5V, A1 (21) | — | Absent | Affects splicing | Deletion >6 kb of intron 2; including branch-point sequence | 39 ± 12 ^g | 0.36 ± 0.1 ^g | 105 | 19 | |
| | Q IV-1 | III | Male | Prepubertal | Not gonadectomized | Absent | | | | | | | |

P5V is female distribution of pubic hair, inverted triangle. Coitus means coitus regularly without major difficulties; surgery means neovagina was constructed. Age at evaluation is shown between brackets. — means not investigated; n.m. means not measurable; ↓ ↓ means diminished amount present.

^a Patients are denoted with roman-arabic numbers corresponding to their position in the pedigree, Fig. 1 (for example III-13, V-3, etc.).

^b External genitalia are denoted according to the Prader classification (20), the age at assessment is in between brackets.

^c Pubertal stage is denoted according to the Tanner classification (19).

^d E: epididymides, V: vas deferens, M: müllerian remnants. E±: underdeveloped epididymis; E+: normally developed epididymis; E?: no data available on the developmental stage of epididymides.

^e See Fig. 2.

^f Previously published C insertion codon 42 is incorrect (9, 12); this should be C deletion codon 42.

^g Binding assay with use of cytosols; normal range Bmax: 39–169 fmol/mg P, Kd: 0.03–0.13 nM.

^h Binding assay with use of whole cells; normal range Bmax: >10 fmol/mg P, Kd: 0.1–0.4 nM.

TAD, Transcription activation domain of the AR; DBD, DNA-binding domain of the AR; LBD, ligand-binding domain of the AR.

breasts had grown to B4. Subject IV-8 (subject II-8 in Boehmer *et al.* [17]) 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; he 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 yr.

Subject III-21 was born with a micropenis, labioscrotal swellings, and a urogenital sinus and was raised as a boy. He died after 1 yr from a congenital heart malformation.

Subject IV-1 was born with a micropenis, labioscrotal swellings, separate vaginal and urethral openings, and a 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 a micropenis and severe hypospadias at the age of 19 yr and 13 yr, respectively. At present they are both married, reportedly with satisfactory sexual lives but without children.

Subject III-1 was born with a phallus 0.75 cm in length without palpable corpora cavernosa, a urogenital sinus, and a hypoplastic scrotum containing testes. The male sex was assigned according to the parents' wish, which was based on the families' experience with two uncles. Phallus length increased to 1.8 cm after 4 months of treatment with 0.25 mg testosterone propionate 100 im/3 wk. At age 6 yr, the phallus had grown to become 2.5 cm in the absence of androgen treatment.

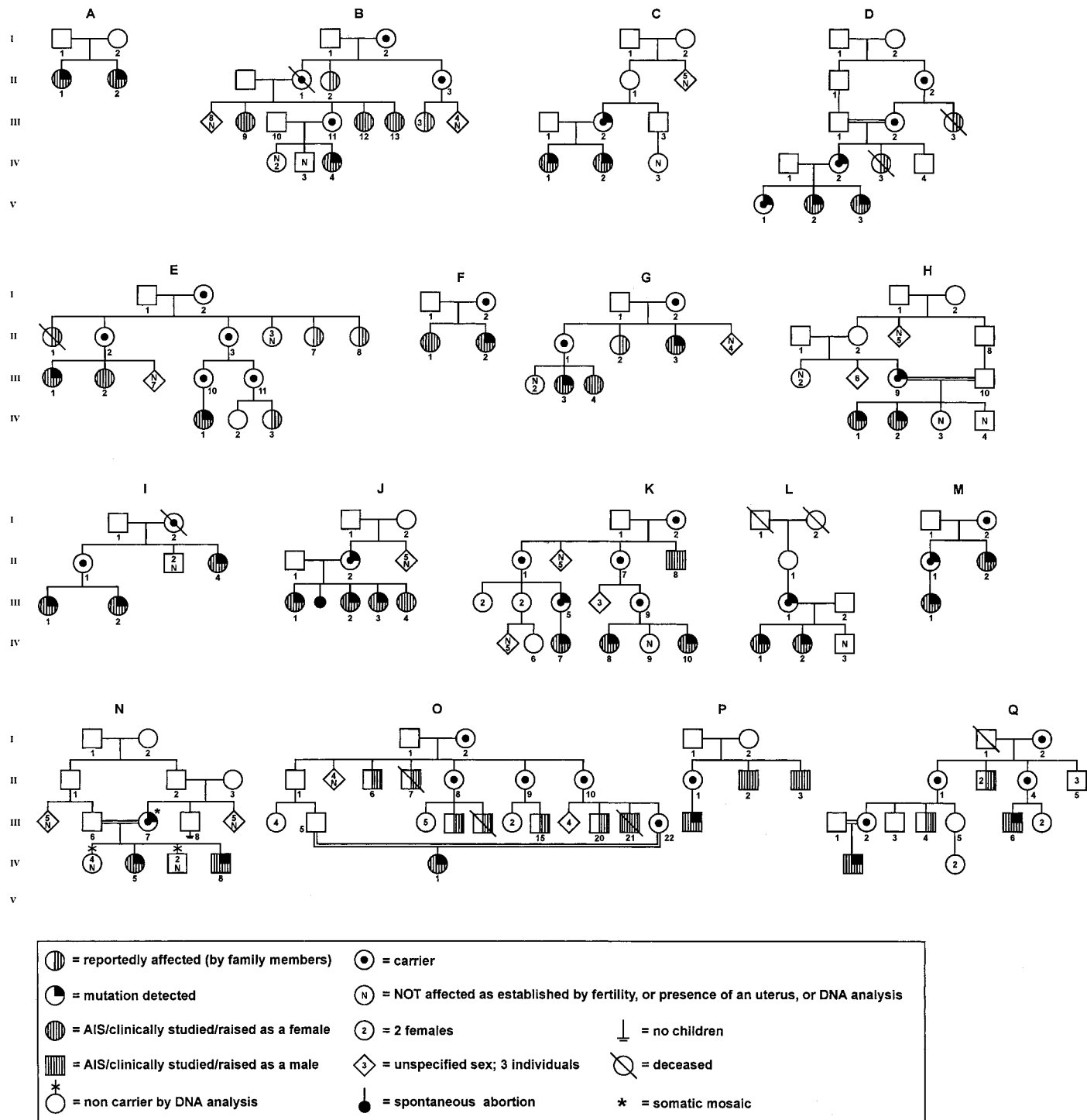


FIG. 1. Pedigrees of the studied families.

Family Q. Subject III-6 was raised as a girl. At 2 yr of age, inguinal hernias containing testes were found, prompting male sex reassignment. He had a phallus of 2.5 cm in length with hypospadias, bifid scrotum, and bilateral testes in the inguinal region. He developed gynecomastia at age 14 yr. At age 21 yr, the penis had increased in circumference, but it still measured 2.5 cm in length despite serum T levels between 30 and 40 nmol/liter during at

least 5 yr. He had reached Tanner stage P4, A1, but facial hair was absent. No prostate was palpable. Unilateral gonadectomy was done at age 26 yr 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 became sexually active.

SHBG suppression test

Two sisters with CAIS in family H, H:IV-1, and H: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 < 62.1%; PAIS: 48.6–87.0%; CAIS: > 92%). CAIS is here defined as a female phenotype without any signs of virilization and scanty or absent pubic and/or axillary hair. 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 (4). In the patients studied, we found an overlap of values between CAIS and PAIS patients.

Genotype and receptor phenotype

The identified mutations are shown in Table 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 and 30 and 19 and 24, respectively, which are within the normal range (11, 21). The results of Scatchard analysis and SDS-PAGE are also shown in Table 1.

AR protein expression in vestigial Wolffian duct derivatives

Structures macroscopically resembling vasa deferentia and epididymides were found in patients with a frame shift mutation (A:II-1 and A:II-2) or a premature termination codon (B:IV-4) in the AR gene. Because it is generally accepted that Wolffian duct differentiation is dependent on fetal androgens (22), these patients were an ideal model to substantiate this idea. The structures that were macroscopically identified as Wolffian duct derivatives histologically resembled vestigial Wolffian ducts (23). These structures did not express AR protein, as was shown by immunohistochemistry of tissue from AIS patients A:II-1 and B:IV-4 (Fig. 2). In a well-differentiated vas deferens of a 1-yr-old patient with 17 β -hydroxysteroid dehydrogenase type 3 deficiency, studied as a control, abundant AR protein was present (Fig. 2).

Hormone dependent AR phosphorylation

AR protein isolated from wild-type (WT) GSFs cultured in the absence of androgens migrates as a doublet of 110 and 112 kDa during SDS-PAGE, reflecting a dephosphorylated and a phosphorylated AR isotype. On binding of androgens, the AR undergoes additional phosphorylation, resulting in a third isoform of 114 kDa (Fig. 3, lower lane). AR mutants that are either partially defective in ligand or DNA binding or in transcription activation migrate with a reduced amount of the 114-kDa isoform in SDS-PAGE (24) (Fig. 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, compared with the WT. Increased androgen levels did not induce the 114-kDa band as in the WT cells (Fig. 3). Moreover, both, the single and the double mutant AR have an equally deficient hormone induced upshift of the 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.

Discussion

Fifty-nine percent of AIS patients had other affected relatives, which is in accordance with Haldanes rule that one-third of patients with X-linked diseases that prevent procreation of 46,XY patients is the result of *de novo* mutations (25, 26).

Genetic epidemiology

The design of this study enabled us to 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 patients with a confirmed diagnosis of AIS. The previously published prevalence rates vary between 1:4,000 and 1:128,400 (7). Until now the estimate from the Danish patient registry (27) of 1:40,800 was probably the most accurate. However, it is based on 46,XY female phenotypes with testes, and the diagnosis of AIS was not confirmed either by mutation analysis or with an SHBG suppression test. Therefore, that study group may have included cases of 17 β hydroxysteroid dehydrogenase type 3 deficiency (incidence 1:147,000 [28]), 17 α -hydroxylase/17,20 lyase deficiency, 46,XY gonadal dysgenesis, and 5 α reductase 2 deficiency. Accordingly, 1:40,800 may be an overestimate. The true incidence of AIS is probably between 1:40,800 and 1:99,000.

Phenotypic variation in AIS

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 thus excluded. Also, comparison of multiple subjects from one family enabled analysis of intra- and interfamilial variability of the disorder.

No phenotypic variation was observed in families with CAIS, confirming that phenotypic variation in families with CAIS is very rare because only one family with coexistence of CAIS and PAIS has been described to date (29).

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 a girl and a boy, respectively, because of wide phenotypic differences. In two other families, the variation had implications for the indication of reconstructive surgery. Molecular analysis of the AR does not always explain the variance in phenotype. We must conclude

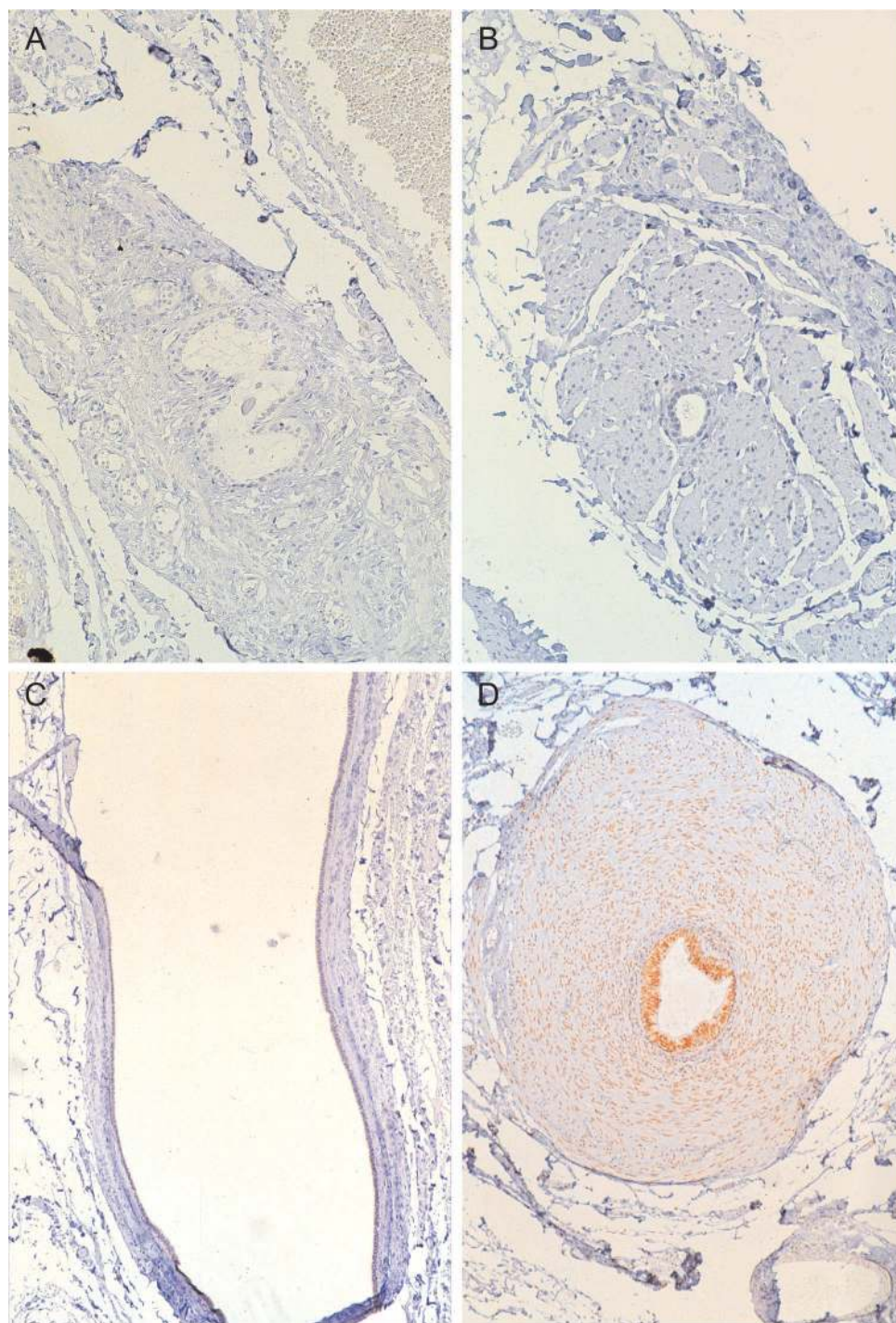


FIG. 2. Immunohistochemistry of gonadal tissue sections of patients. Staining with a monoclonal antibody against the N-terminal part of the AR. A and B, Structures resembling Wolffian ducts in patient B: IV-4. C, Longitudinal section through a structure resembling the vas deferens of subject A: II-1. D, Vas deferens of a patient with 17β -hydroxysteroid dehydrogenase type 3 deficiency

that sex assignment at birth of patients with PAIS cannot be based on a specific AR gene mutation. This unpredictability in PAIS families is also important to consider in genetic counseling of PAIS because it may lead to differences in sex assignment and differences in indications for reconstructive surgery.

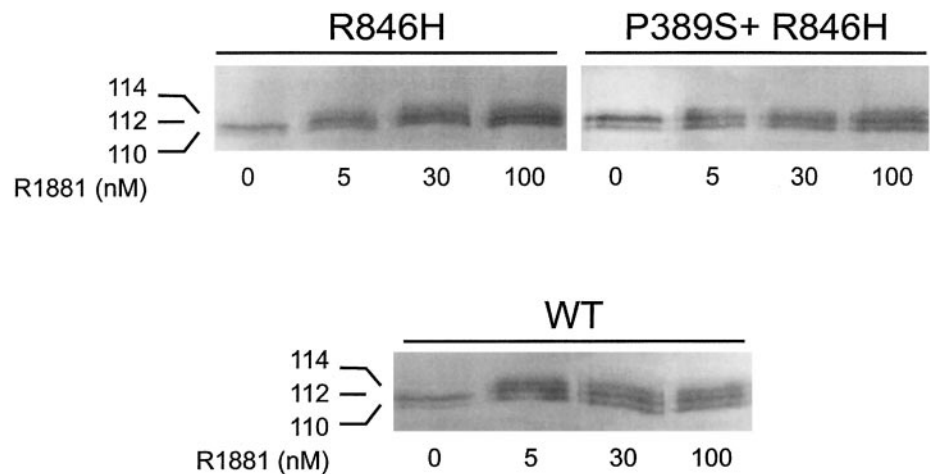
Molecular mechanisms of variability

The phenotypic variation in PAIS might have different molecular backgrounds. For instance, in family N with mu-

tation R846H, we found a 5α reductase 2 deficiency in genital skin fibroblasts of subject N:IV-5 as the cause of the more severely impaired virilization (30). This 5α reductase deficiency was shown to be caused by the absent or severely reduced expression of 5α reductase 2. Because the action of mutant R846H can be influenced dramatically by differences in availability of DHT (31, 32), this explains the observed phenotypic variation.

In family K no differences in 5α reductase type 2 activity were found in GSFs of subjects K: II-8 and K: IV-10. In this

FIG. 3. SDS-PAGE of mutant R486H (family N, PAIS) vs. 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).



family, with AR-gene mutation M771I, diverse phenotypes are found for which no clear explanation can be given. Affected members of another family with AR gene mutation M771I (29) also showed phenotypic variation, ranging from a female to a Reifenstein phenotype. The M771I mutation rendered the AR qualitative defective and caused decreased expression of the AR protein in Scatchard analysis of GSF (29) and in *in vitro* expression studies (31). However, M771I mutant receptor showed hardly any activity in a transactivation assay in HeLa cells, even in the presence of high levels of androgen (31). The *in vivo* observed residual virilization (family K; Table 1) is not explained by these *in vitro* results. This may be owing to differences in genetic background of genital target cells vs. 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 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 (33, 34). These ARs 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 and 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 virilization 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 and the hormone-induced upshift of the 110- to 112-kDa isotype was influenced by the additional mutation P389S as was shown by Scatchard analysis (Table 1) and SDS-PAGE (Fig. 3). Mutation P389S has also been found in an infertile but otherwise normal male (35), but a causative relationship between genotype and phenotype is not clear because *in vitro* experiments showed almost identical transcription activation for mu-

tant P389S and the WT receptor (35). Therefore, P389S might alternatively be an infrequent polymorphism in the AR gene.

Residual capacity for virilization

Follow-up into puberty and adulthood provided information on the residual capacity for virilization. Patients born with complete female genitalia, Prader stage 0, did not show virilization even if they had not been gonadectomized until their early 20s (<27 yr of age). The development of pubic hair is presumably dependent on adrenal androgens as this was also observed in gonadectomized patients. 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 a prolonged but not high dose of androgen therapy (for details, see phenotypes and families in *Results*). Patients born with Prader stage III in families P and Q also showed absent to minimal virilization at puberty. They did not receive additional androgen therapy. The adult patients, raised as males, had severely undervirilized genitalia and absence of a beard. In conclusion, AIS patients in this study showed no to minimal virilization 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 (9, 11). 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 vestigial 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, nonfunctional AR proteins (36). Complete absence of the AR in these structures was confirmed by Scatchard analysis and SDS-PAGE Western immunoblotting of GSFs in families A and C. Immunohistochemistry of the underdeveloped vas deferens and epididymis was done in families A and B (Fig. 3).

Thus, in the absence of a functional AR, pubic hair develops minimally and Wolffian ducts remain macroscopically detectable but undifferentiated. In the 17 β hydroxysteroid 3-deficient patient Wolffian ducts were normally developed, which may be owing to the action of androstenedione or minimal amounts of testosterone formed during embryonic development (37).

Indication for vaginal reconstruction

In many CAIS patients, surgical vaginal elongation was 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.

Acknowledgments

We are indebted to the families that participated in this study and grateful for the expert graphical assistance of Ruud Koppenol, technical assistance of Theo Hoogenboezem and Patrick Jansen, and the help of Jan Maarten Wit, Monique de Vroede, and Guy Massa in providing clinical data of some of the patients.

Received January 24, 2001. Accepted May 7, 2001.

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This work was supported by a grant from The Netherlands Organization for Scientific Research GB-MW 900-716-606 (A.L.M.B.).

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