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A Practical Approach to the Detection of Androgen Receptor Gene Mutations and Pedigree Analysis in Families with X-Linked Androgen Insensitivity

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ABSTRACT

Androgen insensitivity syndrome (AIS) is an X-linked disorder in which defects in the androgen receptor gene have prevented the normal development of both internal and external male structures in 46,XY individuals. This survey reports the analysis of 11 AIS subjects. The androgen receptor gene of these subjects was analyzed using polymerase chain reaction (PCR)–single-strand conformation polymorphism analysis and sequencing or sequencing of PCR-amplified androgen receptor gene fragments alone. In total, 10 single base changes and one partial gene deletion were detected. Seven single base changes resulted in an amino acid change, one resulted in the introduction of a premature stop codon, one event represented a single base insertion resulting in a frame-shift, and one single base change affected a donor splice site. The androgen receptor protein in genital skin fibroblasts from several patients was studied with respect to molecular mass after immunoprecipitation and SDS-PAGE. Two patients expressed a truncated receptor protein in agreement with the established genomic mutation. Pedigree analysis was performed to identify possible carriers for the syndrome in families of AIS patients using single-strand conformation polymorphism and restriction site analysis of PCR products. In one case, the polymorphic $(CAG)_n(CAA)$ repeat in exon 1 encoding a polyglutamine stretch was used to identify the mutant allele in a family with X-linked partial androgen insensitivity before the identification of the actual genomic mutation. PCR–single-strand conformation polymorphism analysis proved to be a fast and reliable technique to screen for androgen receptor gene mutations and to study the androgen receptor gene of family members of AIS-affected individuals. (***Pediatr Res* 36: 227–234, 1994**)

Abbreviations

AR, androgen receptor
hAR, human androgen receptor
AIS, androgen insensitivity syndrome
PCR, polymerase chain reaction
SSCP, single-strand conformation polymorphism
DHT, 5α-dihydrotestosterone

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

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secondary sex characteristics and fertility of affected children.

The AR is a member of the steroid/thyroid hormone/retinoic acid receptor zinc finger family (3), consists of 910 amino acid residues (4), and has a molecular structure homologous with that of the other family members: an N-terminal transcription-regulating domain, a DNA-binding domain, and a C-terminal ligand-binding domain (5). The number of amino acid residues can vary between individuals because of two polymorphic amino acid stretches in the N-terminal domain: a polymorphic GGN repeat encoding a glycine stretch and a polymorphic $(CAG)_n(CAA)$ repeat encoding a polyglutamine stretch. The polymorphic $(CAG)_n(CAA)$ repeat has a frequency of heterozygosity in females of 0.89 and is extremely useful for carrier diagnosis in families with AIS (6).

Although most members of the nuclear receptor family have a corresponding resistance syndrome, a mutant AR gene can cause complete androgen resistance in 46,XY individuals because of its location on the X chromosome, whereas for the other members of the nuclear receptor family the consequences of one mutant receptor allele can be compensated by the other autosomal wild-type allele.

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

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

METHODS

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

Cell culture. Fibroblasts derived from genital skin biopsies were maintained in Eagle's minimum essential medium supplemented with 10% FCS, nonessential amino acids, and antibiotics.

Specific androgen binding and Western blot analysis. Scatchard plot analysis was performed in the laboratory of origin; relevant references are included in Table 1. Genital skin fibroblasts were incubated with ^{3}H-labeled DHT (New England Nuclear, Boston, MA) in a range of 10 concentrations (0–6 nM) for 45 min at 37°C. Nonspecific DHT binding was determined using labeled DHT in the presence of 100 nM unlabeled DHT. After incubation, cells were harvested and the cell pellet resulting from a $500 \times g$ centrifugation step was washed with a Tris-HCl/EDTA buffer (0.9% NaCl, 10 mM Tris, 10 mM Na_2MoO_4, 1 mM EDTA, 0.5 mM DTT, 0.002% NaN_3, pH 7.4). The supernatant was removed and the cell pellet was stored overnight at −70°C. After thawing, 40 mL of 0.6 M KCl were added and the cells were left at 4°C for 1 h. A 0.25% charcoal suspension (150 μL) containing 0.025% dextran was added, and, after 5 min of shaking, cell debris and charcoal were removed by centrifugation at $1000 \times g$ for 10 min. One hundred mL of supernatant were used for ^{3}H counting. The protein content of the samples was measured using the BioRad protein assay kit (BioRad Laboratories, Richmond, CA). A Scatchard plot was constructed using standard statistical routines. Each assay was done in duplicate with four samples for each DHT concentration. The two maximum binding capacity and k_d values were combined, using the squared standard errors as weighing factors.

Immunoprecipitation and Western blot analysis of the AR protein obtained from approximately 5 × 106 genital skin fibroblasts were performed as described previously (8). The AR was immunoprecipitated from whole cell lysates of genital skin fibroblasts with the AR-specific MAb F39.4.1. followed by SDS-PAGE and immunoblotting using the polyclonal antibody Sp061. The AR protein on immunoblot was visualized by chemiluminescence (12).

DNA isolation and analysis. Genomic DNA was isolated from genital skin fibroblasts or from blood cells using standard methods (13). PCR reactions were done in a 100-mL reaction volume as described before (8) using the Perkin-Elmer Thermo Cycler, 2.5 U Taq polymerase (AmpliTaq), and the appropriate reaction buffer and conditions as described by the supplier (Cetus, Norwalk, CT). For PCR reactions covering the GGN repeat in exon 1, deaza deoxyguanosine triphosphate was used instead of deoxyguanosine triphosphate. Oligonucleotides used for PCR amplification of the AR gene and for direct sequencing are indicated in Table 2.

Radioactive PCR to determine the length of the $(CAG)_n(CAA)$ repeat in exon 1 or as a basis for SSCP were prepared using 22 nM ^{32}P–α-deoxyATP in a 15-μL standard PCR reaction. To determine the length of the repeat encoding the polyglutamine stretch, PCR products were size fractionated on a 6% polyacrylamide denaturing sequence gel accompanied by a standard sequence reaction. SSCP analysis was done using a nondenaturing 7% polyacrylamide gel (2% cross-linking) and either 5 or

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

	Subject										
	1†	2	3‡	4‡	5§‖	6	7	8¶	9	10	11¶
Karyotype	46,XY	ND	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY	46,XY
Evidence of defects in testosterone synthesis or metabolism	No	ND	No	No	No	ND	No	No	ND	No	No
Phenotype	M; hypospadias, small penis, infertility, no male body hair pattern, gynecomastia	F	F	F	F	F; ambiguous genitalia	F; ambiguous genitalia	F	F; cliteromegaly, labial fusion	F	F; no uterus, fallopian tubes, or vasdeferens; testis cryptorchism
Diagnosis	Reifenstein	cAIS	cAIS	cAIS	cAIS	pAIS	pAIS	cAIS	pAIS	cAIS	cAIS
Androgen-binding GSF**	Normal, Bmax 39 ± 12 fmol/mg P, k_d 0.36 ± 0.1 nM	ND	Below normal, Bmax 6-11 fmol/mg P	Normal, Bmax 29-36 fmol/mg P, k_d 1.9 nM, rapid dissociation	Not detectable	Not detectable	Not detectable	Not detectable	Below normal, Bmax 13 ± 6 fmol/mg P, k_d 62 nM, 45% thermolability	Not detectable	Not detectable
Western/immunoblot	105 kD	ND	110-kD doublet	110-kD doublet	105-kD doublet	110-kD doublet	110-kD doublet	110-kD doublet	110-kD doublet	99-kD doublet	105-kD doublet
Mutation initially defined by	Size analysis of PCR product	SSCP analysis	Sequencing of PCR product	Sequencing of PCR product	Sequencing of PCR product	Sequencing of PCR product	SSCP analysis	SSCP analysis	SSCP analysis	Sequencing of PCR product	SSCP analysis
Mutation AR gene	Deletion > 6 kb intron 2	G → T	G → C	G → A	G → T	A → G	T → C	T → C	G → A	C → T	A insertion
Location mutation	Intron 2	Exon 3	Exon 4	Exon 4	Intron 4	Exon 5	Exon 5	Exon 5	Exon 6	Exon 6	Exon 7
Protein change	Deletion of 2nd zinc finger (90%)	Arg 606 His	Asp 686 His	Asp 686 His	Δ aa 674-714	Met 733 Val	Met 736 Thr	Phe 755 Leu	Met 771 Ile	Arg 822 Stop	aa 1-837 wt sequence + 32 nonsense aa
Family analysis done using	Polymorphic $(CAG)_n(CAA)$ repeat	SSCP analysis, *Bbs*I digest	SSCP analysis, *Hin*fI digest	SSCP analysis, *Hin*fI digest	Western blot analysis, RT-PCR exon 3-5	Polymorphic $(CAG)_n(CAA)$ repeat	ND	ND	SSCP analysis, *Rsa*I digest	SSCP analysis, *Sfu*I digest	ND

* M, predominant phenotype male; F, predominant phenotype female; cAIS, complete androgen insensitivity syndrome; pAIS, partial androgen insensitivity syndrome; P, protein; GSF, genital skin fibroblasts; wt, wild type; Bmax, maximum binding capacity; aa, amino acids; nd, not done.

† Identification and characterization of mutation including Western blot analysis of genital skin fibroblasts (unpublished observations).

‡ Identification and characterization of mutation published in ref 8.

§ Identification and characterization of mutation published in ref 9.

‖ Western blot analysis of family published in ref 10.

¶ Binding data and clinical description published in ref 11.

** The normal value for specific androgen binding in all laboratories is >15 fmol/mg P.

Table 2. *Oligonucleotides used for PCR and sequencing**

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

* Nucleotide (nt) numbering based on an open reading frame of 2730 nucleotides. All sequences are displayed in 5′ → 3′ orientation; mismatches are indicated by lower case.

† Nucleotide position relative to the ATG start codon. This PCR product is relatively large and was therefore digested with the restriction enzyme *PstI* before SSCP analysis. This procedure also makes it possible to disregard the abberant SSCP profile due to the polymorphic $(CAG)_n(CAA)$ repeat.

10% glycerol. Samples consisted of 1 μL of PCR product and 9 μL of loading dye (95% formamide, 5% glycerol, 10 mM EDTA). Before loading on gel, the samples were denatured for 5 min at 100°C, followed by a quick chill on ice. Electrophoresis was performed at room temperature for 16 h at 7 W constant power.

The yield of five PCR reactions was pooled for one direct sequencing reaction after purification from a 2% agarose gel using Qiaex (Qiagen Inc., Chatsworth, CA). Sequencing was done using the dideoxy-chain termination method (14).

RESULTS

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

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

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

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

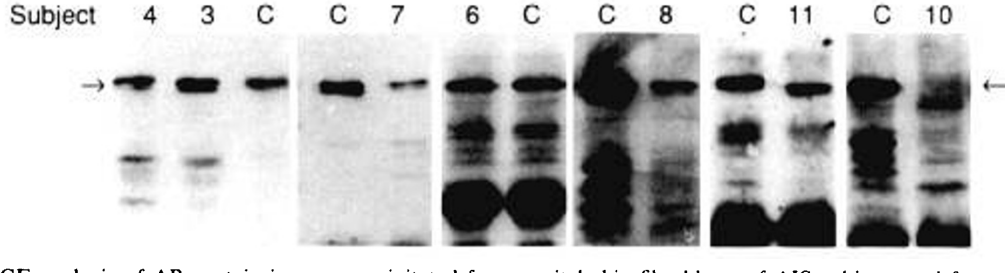

Figure 1. SDS-PAGE analysis of AR protein immunoprecipitated from genital skin fibroblasts of AIS subjects and from control genital skin fibroblasts. Patients are identified at the top of each lane; numbers correspond to those in Table 1 (*C*, control fibroblast strains). The position of the 110-kD AR doublet is indicated by *arrows*.

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

Subject 1 has a deletion in intron 2 of the AR gene, which leads to an alternative splicing event resulting in a deletion of exon 3 sequence in 92% of the transcripts. Upon translation, this will give rise to a receptor protein with an in-frame deletion of the second DNA-binding zinc finger. This 105-kD protein was present in genital skin fibroblasts of the patient, the 8% wild-type protein was not detectable. Because the 105-kD protein is completely nonfunctional with respect to transactivation, the partial virilization of the AIS subject must be the consequence of the limited amount of wild-type AR protein (15).

Western blot analysis of the AR of subject 5, where amino acids 674–714 from the AR are deleted in frame, showed an AR protein of approximately 105 kD; her mother and affected sibling also showed the expected smaller AR protein (10).

In none of the receptor-negative AIS subjects was the absence of specific androgen binding caused by the absence of AR protein.

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

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

We established that the female relatives III-6 and III-7 of subject 9 (Fig. 3*A*) do not carry the mutant AR allele.

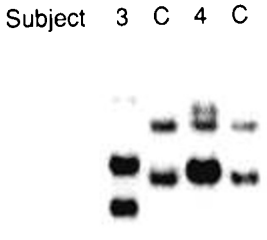

Figure 2. SSCP analysis showing exon 4 of the hAR gene amplified from genomic DNA. Exon 4 SSCP profile of patient 3 (G → C mutation of nucleotide 2056) (*lane 1*), control (*lane 2*), patient 4 (G → A mutation of nucleotide 2056) (*lane 3*), and control (*lane 4*).

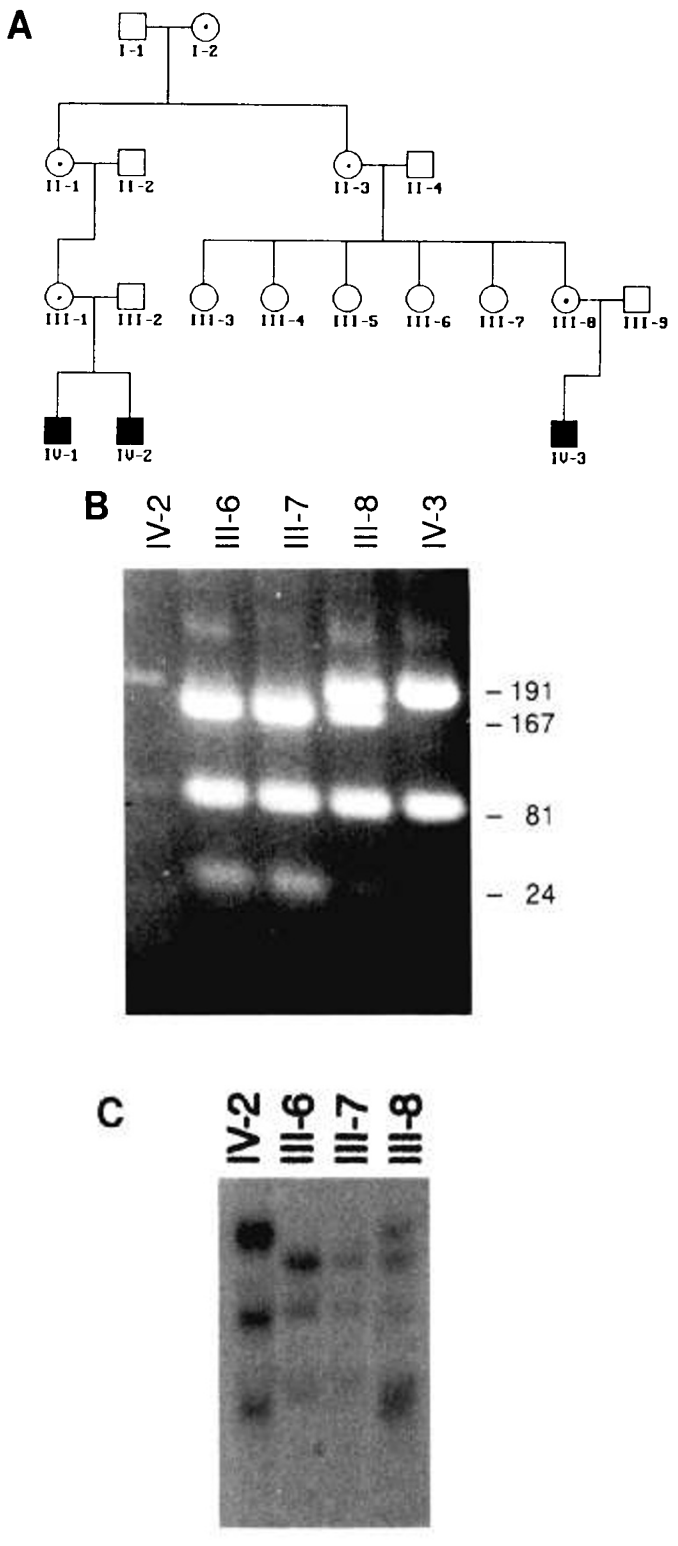

Figure 3. Partial pedigree analysis of the family of AIS subject 9 (IV-2) by *Rsa*I digestion and SSCP analysis of PCR amplified exon 6. *A*, pedigree of subject 9 (IV-2). *B*, PCR-amplified exon 6 digested with *Rsa*I size fractionated on a 2% ethidium bromide-stained agarose gel. The affected members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

In this particular case, the G → A mutation in exon 6 destroys a site for the restriction enzyme *Rsa*I, resulting in a 191-bp fragment and a 81-bp fragment when the mutant exon 6 PCR fragment is digested with *Rsa*I. When exon 6 is PCR amplified from the wild-type allele, the 191-bp fragment is digested by *Rsa*I, and digestion in this case results in three fragments of 167, 81, and 24 bp (Fig. 3*B*). SSCP analysis is also informative. In the case of subject 9 and several of her family members, the SSCP profile of exon 6 confirmed the results obtained by the restriction site analysis of amplified exon 6 (Fig. 3*C*).

Sometimes there is an urgent demand to investigate whether carriers for the syndrome are present in the family before the identification and characterization of the actual AR gene mutation, or the nature of the mutation prevents the reliable detection of carriers with the presently used methods. In such cases, SSCP analysis and restriction site analysis are not suitable for investigations of the pedigree. To investigate the family members of subject 1 (pedigree in Fig. 4*A*), before the actual identification of the underlying genomic mutation, the polymorphic $(CAG)_n(CAA)$ repeat in exon 1 of the hAR gene was used. The mutant AR gene was found to contain a repeat length of 20, and this cosegregated with the mutant phenotype in this family. The obligate carriers II-2, II-5, and III-2 also had the 20-repeat allele. Subject III-5 inherited the other maternal allele (Fig. 4*B*). Hence she was proven not to be a carrier for the Reifenstein syndrome. After identification of the genomic mutation in the AR gene of patient 1, confirmation of the pedigree analysis by PCR-SSCP or restriction enzyme analysis proved to be impossible. No amplification product of exon 3 can be obtained using our standard primers because of the deletion of the annealing site of the upstream PCR primer (15). Affected individuals can be identified by the absence of a specific exon 3 amplification product. Carriers still show the amplification product from the other (normal) X chromosome.

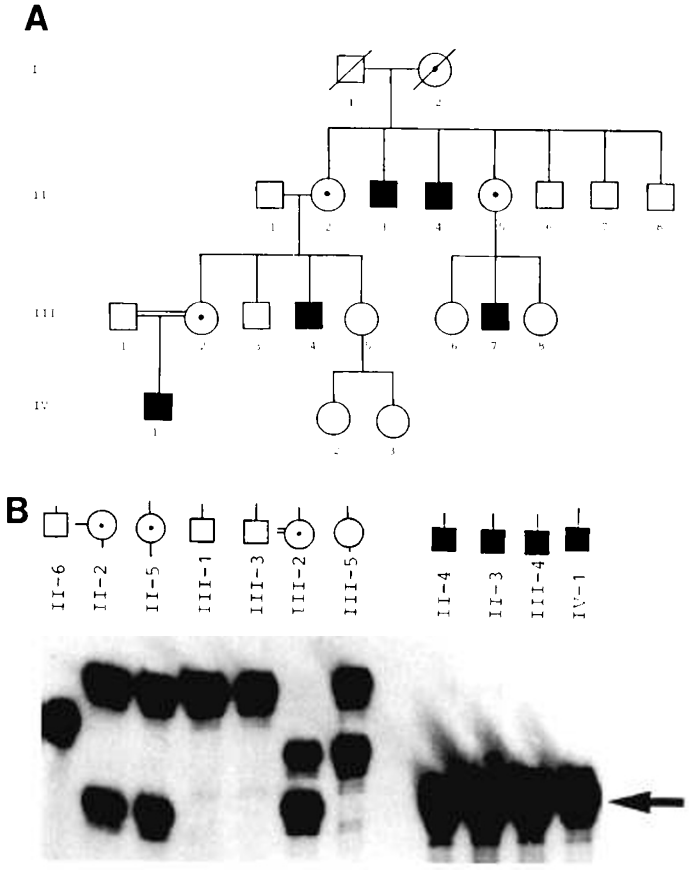

Figure 4. Family analysis of AIS subject 1 (III-7). *A*, pedigree of family with familial X-linked hypospadias (Reifenstein syndrome). *B*, PCR-amplified polymorphic $(CAG)_n(CAA)$ repeat (poly-Gln-stretch) of the hAR in a family with X-linked hypospadias due to a deletion of more than 6 kb in intron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

DISCUSSION

This paper describes mutations in the hAR gene of subjects with either complete or partial androgen insensitivity. The majority of mutations in the ligand-binding domain of the hAR mainly cluster in two regions of the gene (16). One of these regions is homologous with a region in the thyroid hormone receptor gene that is a known cluster site for mutations that cause generalized thyroid hormone resistance (17).

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

The Arg606His mutation identified in this study in patient 2 has also been reported for the homologous amino acid residue of the human vitamin D receptor (18). In this case, the vitamin D receptor was inactive in promoting transcription in a cotransfection assay. This suggests that the Arg606His mutation in the AR is responsible for the complete AIS in patient 2. A mutation at methionine residue 733 in the AR has been reported before, although the transition in this case was not to valine, as in subject 6, but to isoleucine (19). This amino acid mutation also concerned a patient with partial AIS but with detectable androgen binding in genital skin fibroblasts. Both the Met771Ile mutation of patient 9 and the Arg822Stop mutation of patient 10 were reported previously in unrelated subjects (19, 20).

In the case of subjects 6, 7, 8, and 9, it is likely that the amino acid change in the steroid-binding domain results in a receptor protein in which the steroid-binding capacity itself is (partly) unimpaired but the dissociation of the ligand from the receptor complex is enhanced. The receptor will be totally nonfunctional in patient 8 and partly unable to regulate transcription of AR target genes in the partial AIS subjects 6, 7, and 9. Genital skin fibroblast

cultures of subjects 10 and 11 express a truncated receptor protein due to the introduction of a premature stop codon and a frame-shift resulting in a premature stop codon, respectively. Based on experiments of *in vitro* expressed truncated AR proteins (5), the receptor proteins of subjects 10 and 11 are not expected to bind ligand and consequently are not expected to acquire transactivating potential. These aspects fully explain the complete AIS of these two patients.

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

If all this evidence points to androgen insensitivity, a biopsy to obtain genital skin fibroblasts for the determination of the number of AR and the quality of the binding can be very informative. In addition, immunoblot analysis of the AR protein from genital skin fibroblasts can provide important information with regard to the most likely location or nature of a possible mutation, especially if immunoblots can be probed with specific MAb raised against different epitopes of the receptor. If there is impaired androgen binding but a 110-kD AR protein can be immunoprecipitated, all evidence points to an amino acid substitution in the steroid-binding domain of the receptor. When a truncated receptor protein is immunoprecipitated, a mutation in the AR gene resulted in the introduction of a premature stop codon, possibly the consequence of a frame-shift. In the case of receptor-negative AIS, in which no AR protein can be immunoprecipitated from genital skin fibroblasts, a mutation on the level of the promoter of the AR gene leading to diminished transcription or the expression of a truncated receptor protein that lacks the epitope for the antibody is likely.

The localization of the genomic mutation responsible for the androgen insensitivity is best established by the PCR-based SSCP analysis or a similar screening method such as denaturing gradient gel electrophoresis (20). The SSCP analysis is a quick and reliable method to screen for mutations in the AR gene. Subsequent to DNA isolation, a radioactive PCR and a sequencing gel procedure have to be done, making it possible to obtain results within 48 h. In our hands, the detection rate of mutations in the hAR gene using SSCP analysis is approximately 90%.

With respect to pedigree analysis, the most appropriate technique largely depends on the nature of the mutation. When a mutation has created or destroyed a recognition site for a restriction enzyme, restriction enzyme analysis of a PCR-amplified DNA fragment containing the mutation will be informative. Sometimes, however, it is difficult to digest a PCR product completely, which can lead to false interpretations. Therefore, we usually confirm the restriction site analysis with an SSCP profile of the family members. In cases in which SSCP analysis is not possible or in which the mutation itself has not been identified, the use of the polymorphic $(CAG)_n(CAA)$ repeat in exon 1 of the hAR can be very informative. The size of the polyglutamine repeat in the normal population varies from 11 to 31 with a high frequency of 21–23 residues (6, 21, 22). However, the use of this polymorphic repeat is only possible when a certain diagnosis of X-linked androgen insensitivity within a family has been established.

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

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