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Molecular Analysis of the Androgen-Receptor Gene in a Family with Receptor-Positive Partial Androgen Insensitivity: An Unusual Type of Intronic Mutation

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Summary

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

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Introduction

Expression of a number of genes involved in male sex differentiation and development is regulated by the androgen receptor (AR). The AR belongs to the family of steroid hormone–activated transcription modulators (Evans 1988). Like the other steroid hormone receptors, the AR consists of distinct functional domains. The N-terminal part is involved in transcription activation and is encoded by exon 1 (Faber et al. 1989). Two highly conserved DNA-binding zinc clusters are encoded by exons 2 and 3. The N-terminal zinc cluster recognizes specific consensus DNA sequences, whereas the C-terminal zinc cluster is involved in dimerization (Dahlman-Wright et al. 1991; Luisi et al. 1991). Parts of exons 3 and 4 encode the hinge region, which contains a nuclear-localization signal that is involved in nuclear import, and exons 4–8 encode the ligand-binding domain (LBD).

On ligand binding, the AR undergoes conformational changes and binds to androgen-response elements (AREs) in the promoter regions of androgen-regulated target genes (Beato and Sánchez-Pacheco 1996). Recently, coactivators interacting with the LBD of steroid hormone receptors have been cloned (reviewed by Horwitz et al. 1996). One of these, ARA70, appears to be specifically involved in transcription activation by the AR (Yeh and Chang 1996).

Defects in the human AR (hAR) cause disturbed virilization in 46,XY individuals, which is called the "androgen insensitivity syndrome" (AIS) (reviewed by Quigley et al. 1995). Many qualitative and quantitative AR abnormalities, causing a broad range of AIS phenotypes, have been described (Gottlieb et al. 1997). The spectrum of phenotypes ranges from individuals with completely female external genitalia and absence of Müllerian- and Wolffian-duct derivatives (complete AIS) to patients with ambiguous genitalia (partial AIS) or with mild hypospadias (Quigley et al. 1995). The mutations that are most frequently observed are nonsense or missense point mutations. Mutations resulting in aberrant splicing are much less common; only six of them have been reported

(Gottlieb et al. 1997). In five of these, a consensus splice-donor site was mutated, resulting in complete AIS.

PCR-SSCP analysis is a screening method often used for mutation detection in the hAR. Using this method, we and others have reported that in some individuals, clinically diagnosed as having AIS, no mutation was found in the coding region and exon-flanking intronic sequences of the AR gene (Morel et al. 1994; Brüggenwirth et al. 1996; Weidemann et al. 1996). In the present study, we investigated and characterized the AR gene of a patient with partial AIS. Initially, no mutation was detected in the coding region of the AR gene in this patient, although several biochemical and cell biological assays revealed that the encoded AR of this patient was unable to bind to DNA.

Subjects and Methods

Clinical Subjects

A family with three individuals clinically suspect for AIS (II-4, III-1, and III-2; for pedigree, see fig. 1) was referred for further diagnosis, treatment, and genetic counseling. All affected individuals were 46,XY and had a female habitus with normal female external genitalia, and normal but underdeveloped testes with epididymides and vasa deferentia were present. No Müllerian remnants were found. One postpubertal patient (II-4) was Tanner P3,M5 and had axillary hair. Testosterone-synthesis disorders, which could be another cause for such a 46,XY phenotype, were excluded by analysis of the circulating levels of steroid hormones and their precursors in this patient. At the age of 15.5 years, she had a normal male level of testosterone (21 nmol/liter) (normal range in adults 10–30 nmol/liter) combined with high levels of LH (13 IU/liter) (normal range in adult males 1.5–8 IU/liter). For final proof of the diagnosis and for the purpose of genetic counseling of this family, DNA analysis of the AR gene was started. Blood cells were obtained from the three 46,XY patients, and genital skin fibroblasts (GSF) were obtained from III-1 and III-2. GSF containing wild-type AR protein and AR protein with an exon 3 deletion, derived from a patient with Reifenstein syndrome, previously described by Ris-Stalpers et al. (1994), were used for comparison. Genomic DNA from 74 unrelated individuals was used for intron 2 screening. Informed consent was obtained from all individuals.

Mutation Detection

Genomic DNA was isolated from blood cells or GSF, according to standard procedures (Sambrook et al. 1989). SSCP analysis and direct sequencing were performed as described elsewhere (Brüggenwirth et al. 1996). A total of 102 normal chromosomes from unrelated individuals were analyzed by automated sequencing. Template was made by use of intron 2 sense primer C1 and intron 3 antisense primer C2 (Lubahn et al. 1989) and was purified by use of the Boehringer High Pure PCR Product Purification Kit (Boehringer Mannheim). Sequencing was performed with antisense primer 3BB (table 1). Determination of the length of the polymorphic CAG repeat in exon 1, used as an intragenic polymorphic marker, was performed according to the method of Sleddens et al. (1992).

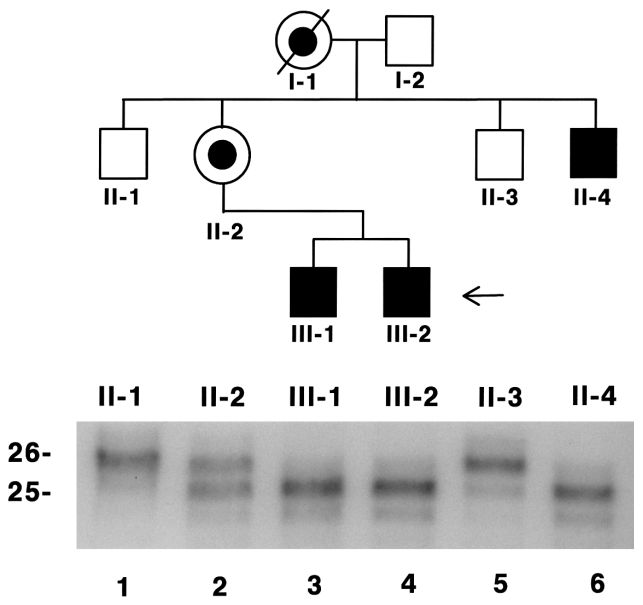

Figure 1 Pedigree and sizing of the polyglutamine stretch of the family with AIS. PCR products, obtained by amplification of genomic DNA by primers flanking the $(CAG)_nCAA$ repeat, were resolved on a 7% denaturating polyacrylamide gel. Lane 1, Normal male. Lane 2, Mother of index subject. Lane 3, 46,XY sister of index subject. Lane 4, 46,XY index subject. Lane 5, Normal male. Lane 6, 46,XY affected aunt. The index subject (III-2) is indicated by the arrow. Numbers to the left of the gel represent the number of glutamine residues encoded by the $(CAG)_nCAA$ repeat.

Reverse-Transcriptase–PCR (RT-PCR) Reaction

Total RNA was extracted from GSF by use of TRIzol reagent (Gibco BRL) and was quantified by absorption at 260 nm. Amplification of genomic DNA was prevented because the primers used in the RT-PCR reaction resulted in a PCR product spanning several introns of the AR gene. In the cDNA-synthesis reaction, the exon 5 antisense primer 5BB (table 1) was used for first-strand cDNA synthesis. cDNA amplification was performed by PCR with antisense primer 5BB combined with sense primer J3A (table 1), spanning the 3′ end of exon 1 and the 5′ end of exon 2. To obtain sufficient quantities of template DNA spanning the sequences of exons 2 and

Table 1

Oligonucleotides Used for cDNA Synthesis and Allele-Specific Hybridization

Oligonucleotide	Location	Sequence[a]
3BB	Intron 3	5′-AGAGAAAGAAAAGTATCTTAC-3′
5BB	Exon 5	5′-CGAAGTAGAGgATCCTGGAGTT-3′
J3A	Exons 1 and 2	5′-gAtGGatcCATGCGTTTGGAGACTGC-3′
14NB	Exon 4	5′-TGCAAAGGAGTtGGGCTGGTTG-3′
470A	Exon 1	5′-GTAGCCCCCTACGGCTACA-3′
Wild type	Exons 2 and 3	5′-GCTGAAGGGAAACAG-3′
69-bp insertion	Exon 2 and intron 2	5′-CTGAAGAAATACCCG-3′
Exon 3 deletion	Exons 2–4	5′-CTGAAGCCCGGAAGC-3′
2AA	Exon 2	5′-CAGAAGACCTGCCTGATCTGT0-3′

[a] Lowercase lettering indicates mismatches.

3, for direct sequencing, a nested-PCR reaction was performed by use of sense primer J3A and exon 4 antisense primer 14NB (table 1).

Ligand-Binding Study

For determination of ligand-binding characteristics of the AR of the AIS patients, a whole-cell assay was performed on GSF, as described elsewhere (Brüggenwirth et al. 1996).

Western Blot Analysis

AR protein, obtained from cultured GSF or transiently transfected Chinese-hamster-ovary (CHO) and COS-1 cells, was immunoprecipitated and analyzed by western immunoblotting according to the method of Ris-Stalpers et al. (1991).

Construction of Expression Vectors

Human wild-type AR cDNA expression plasmid pSVAR0 (Brinkmann et al. 1989) was used to construct pSVAR129, encoding an AR with 23 additional amino acid residues between the two zinc clusters. To this end, the 472-bp *Kpn*I-*Asp*I fragment from pSVAR0 was exchanged with the 541-bp *Kpn*I-*Asp*I fragment, generated by RT-PCR, from the AR mRNA of patient III-2. cDNA was synthesized as described in the Mutation Detection subsection above. In the PCR reaction, following first-strand cDNA synthesis, exon 1 sense primer 470A (table 1) was used, allowing digestion with *Kpn*I. A nested PCR was performed by use of sense primer 470A and exon 4 antisense primer 14NB (table 1). All PCR products were checked by sequencing. Expression plasmid BHEX-AR33, an expression plasmid with an in-frame deletion of exon 3, was constructed as described elsewhere (Ris-Stalpers et al. 1994). To generate pSG5AR129 and pSG5AR33, both the 541-bp *Kpn*I-*Asp*I fragment containing the additional 69 bp and the 355-bp fragment from which exon 3 had been deleted were exchanged with the 472-bp *Kpn*I-*Asp*I fragment of wild-type AR expression vector pSG5AR0 (kindly provided by Dr. A. C. B. Cato, Karlsruhe, Germany). pSG5 plasmids were used to obtain AR protein for gel-retardation assays.

Cell Culture and Transfections

GSF and COS-1 cells were cultured as described by Ris-Stalpers et al. (1990). CHO cells were treated like COS-1 cells. CHO cells were plated in 7-cm^2 wells and were grown for 24 h before they were transiently transfected, by use of the calcium phosphate method (Chen and Okayama 1987), with AR expression plasmid (10 ng DNA/ml precipitate suspension) and the mouse mammary tumor virus (MMTV)–Luc reporter plasmid (2 μg DNA/ml precipitate suspension) (de Ruiter et al. 1995). Carrier DNA (pTZ19) was added to an end concentration of 20 μg DNA/ml precipitate suspension, and 90 μl precipitate suspension was added per well. Twenty-four hours after transfection, cells were incubated with increasing concentrations of R1881. Luciferase assays were performed as described above, after 24-h incubation (Kuil et al. 1995). Each receptor mutant was assayed three times in triplicate, by use of three independently isolated expression-plasmid preparations. Luciferase activity was related to basal activity, measured in the absence of hormone. CHO cells, used for expression studies, were plated in 175-cm^2 culture flasks and were transfected with 200 μl DNA precipitate, as described above, by use of the calcium phosphate method. COS-1 cells were transiently transfected in 80-cm^2 culture flasks with 9.4 μg expression plasmid, by use of the DEAE-dextran method (Gerster et al. 1987). Cells were shocked with 80 μM chloroquine for 2 h. Transfected CHO and COS-1 cells were washed after 24 h and were cultured for another 24 h in either the presence or absence of 10 nM R1881.

Preparation of Cytosolic Fractions and Nuclear Extracts

GSF were grown until confluence in 175-cm^2 culture flasks, incubated for 24 h in culture medium (Ris-Stal-

pers et al. 1990) containing 10% hormone-depleted FCS, and were cultured for another 24 h with medium either with or without 10 nM R1881. Next, cells were washed twice in PBS, were collected in 1 ml lysis buffer A (40 mM Tris, 1 mM EDTA, 10% [v/v] glycerol, 10 mM DTT, 10 mM Na_2MoO_4, 0.5 mM bacitracin, 0.5 leupeptin, and 0.6 mM phenylmethylsulfonylfluoride [PMSF]), and were homogenized by freeze-thawing four times, followed by 10 min centrifugation at 800 *g* in a Biofuge (Heraeus) at 4°C. The supernatant was centrifuged for 10 min at 400,000 *g* at 4°C (TLA120.2 rotor; Beckman). The cytosol fraction (supernatant) was stored at −80°C until used. The pellet remaining after the first 800-*g* centrifugation step was resuspended in buffer B (buffer A with 0.2% Triton X-100) and incubated for 5 min at 4°C, followed by 10 min centrifugation at 800 *g* in a Biofuge 13. The resulting pellet (nuclear fraction) was washed with buffer C (buffer A without leupeptin) and was resuspended in equal volumes of 1 M NaCl and 0.5 M NaCl and incubated for 1 h at 0°C. Nuclear extract (supernatant) was obtained by centrifugation for 10 min at 400,000 *g* (TLA120.2 rotor) at 4°C. The total nuclear extract and 250 μl cytosol fraction were taken separately for immunoprecipitation.

Gel-Retardation Assay

After transfection with pSG5AR0, pSG5AR33, or pSG5AR129, COS-1 cells were collected in 5 ml PBS. The pellet was resuspended in extraction buffer (10 mM NaH_2PO_4, pH 7.4, 0.4 M KCl, 1 mM EDTA, 10% [v/v] glycerol, 0.5 mM bacitracin, 0.5 mM leupeptin, 0.6 mM PMSF, and 10 mM DTT) and was frozen and thawed four times, followed by 10 min centrifugation at 400,000 *g* (TLA120.2 rotor) at 4°C. The double-stranded DNA probe containing the ARE from the tyrosine aminotransferase (TAT) promoter (5′-TGAC*TGTACA*GGA*TGTTCT*AGCTACT-3′) (half sites are in italics) was obtained from Promega. The ARE probe was end-labeled by use of T4 polynucleotide kinase and γ-^{32}P-ATP (specific activity 3,000 Ci/mmol; Amersham). The double-stranded probe was purified from a 4% acrylamide gel, in 0.5 × TBE (1 × TBE = 50 mM Tris base, 50 mM boric acid, and 1 mM EDTA, pH 8.6). Cellular extracts were incubated in binding buffer (10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM DTT, 1 mM EDTA, and 4% Ficoll) containing 1 μg poly-dIdC, in the absence or presence of AR antibody (Ab) Sp197 (the epitope is amino acid residues 1–20 from the AR) (Kuiper et al. 1993). After 10 min incubation on ice, 2 μl purified DNA probe (50,000 cpm/μl) was added, and incubation was continued for 20 min at room temperature. The 20-μl sample was separated on a 4% polyacrylamide gel in 0.5 × TBE. Gels were fixed for 10 min in 10% acetic acid and 10% methanol and subsequently were dried and exposed.

Allele-Specific Oligonucleotide Hybridization

RT-PCR and nested-PCR reactions were performed as described above (see the Construction of Expression Vectors subsection above). The resulting PCR product was amplified once more in a PCR reaction of 30 cycles, by use of exon 1 sense primer 470A and exon 4 antisense primer 14NB. Plasmid (pSVAR0, BHEX-AR33, and pSVAR129) fragments were amplified once under identical conditions, by use of the same primers. Dot blots were prepared in a Schleicher & Schuell apparatus according to the manufacturer's protocol. In the case of PCR product obtained by plasmid amplification, an equivalent amount of DNA was spotted. Membranes were preincubated for 10 min with hybridization mix (50 mM NaH_2PO_4, 0.75 M NaCl, 5 mM EDTA [5 × SSPE], 1% SDS, and 0.05 mg herring sperm DNA/ml). For each of the splice variants, specific oligonucleotides were designed: a wild-type probe, an exon 3–deletion probe, and a 69-bp-insertion probe (table 1) (Pharmacia Biotech Benelux). The filters were subsequently hybridized and rinsed as described by Boehmer et al. (1997), but at a temperature of 35°C instead of 37°C. The membranes probed with wild-type probe or exon 3–deletion probe were washed for an additional 10 or 15 min, respectively, in 0.1 × SSC and 0.1% SDS at 38°C, before exposure. After autoradiography, the membranes were stripped and the procedure was repeated with probe 2AA (table 1) as a control.

Results

Screening for Mutations

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

Receptor Characteristics

AR protein isolated from GSF from the index patient (III-2) appeared as a normal 110–112-kD doublet after SDS-PAGE and immunoblotting. Hormone-binding parameters were determined in a whole cell–binding assay of GSF. The receptor displayed an equilibrium dissociation constant (K_d of 0.08 nM and a maximal number of binding sites (B_{max}) of 64 fmol/mg protein, both within the normal range (K_d 0.03–0.13 nM; B_{max} 39–169 fmol/mg protein). The AR of patient III-1 displayed a K_d of 0.07 nM and a B_{max} of 63 fmol/mg protein. Therefore, the 46,XY individuals (III-1 and III-2) in this AIS family were classified as having receptor-positive AIS.

Hormone-Dependent Receptor Phosphorylation

The AR is phosphorylated, and many potential phosphorylation sites are located in the part encoded by exon 1. In preparations from cells cultured in the absence of hormone, usually two receptor isotypes are present, which are visible as a 110–112-kD doublet on an SDS-PAGE immunoblot (Kuiper et al. 1991). AR protein was isolated from control GSF and from GSF of the index patient (III-2). In both GSF preparations, cultured in the absence of androgens, both the 110- and the 112-kD AR isotypes were present (fig. 2*A,* lanes 1 and 2). The AR from patient III-2 contains a relatively long glutamine stretch (25 glutamines, compared with 19 in the control AR), resulting in a slower migration pattern. On hormone binding, the AR undergoes additional phosphorylation, reflected by a 114-kD isoform. The appearance of this isoform is dependent on DNA binding and/or transcription activation (Jenster et al. 1994). This hormone-induced phosphorylation was used as a marker for proper receptor functioning. Control cells cultured in the presence of hormone displayed the expected, slower-migrating, third isoform (114 kD) (fig. 2*A,* lane 2). However, the 114-kD isoform was hardly detectable in preparations derived from patient III-2 (fig. 2*A,* lane 4).

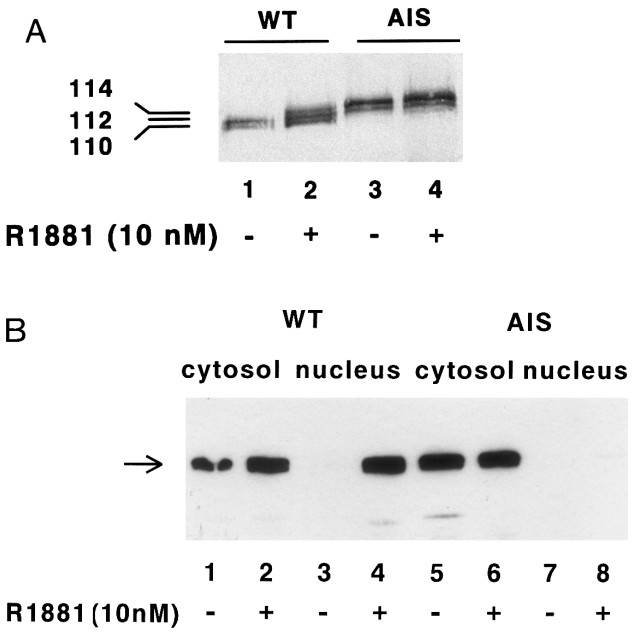

Figure 2 Western blot analysis of wild-type and mutant AR proteins. *A,* Western blot, showing receptor isotypes of wild-type AR and mutant AR. AR protein was prepared from GSF that had been cultured in the absence (−) or presence (+) of 10 nM R1881. After immunoprecipitation with monoclonal anti-AR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

Subcellular Localization Studies in GSF of the Index Subject

Because the appearance of the 114-kD isoform depends on DNA binding (Jenster et al. 1994), we investigated whether AR was detectable in the tightly nuclear-bound AR fraction in GSF from the patient. Control GSF and GSF from the index patient were cultured in either the absence or presence of 10 nM R1881, and cytosol and nuclear fractions were prepared. When cells were cultured in the absence of hormone, AR was found in the cytosol fraction (fig. 2*B,* lanes 1 and 5) but not in the nuclear fraction (fig. 2*B,* lanes 3 and 7). After culture in the presence of hormone, although AR was still detectable in the cytosol fraction (fig. 2*B,* lanes 2 and 6), wild-type AR was clearly present in the nuclear extract (fig. 2*B,* lane 4), whereas AR protein was not observed in the nuclear extract of the patient's GSF (fig. 2*B,* lane 8). This suggests a defect in DNA binding of the AR in the index patient (III-2).

Mutation Detection and the Mutation's Effect on the Splicing Process

Although PCR-SSCP analysis was not informative about an AR-gene mutation in patient III-2, the AR gene was studied in more detail. Sequencing of the flanking intronic regions of exons 2 and 3 of the AR gene of

patient III-2 demonstrated a mutation (T→A) 11 bp upstream of exon 3 (fig. 3*A*). The same mutation was found in the AR gene of patients II-4 and III-1. Because the mutation is located in the region where the forward primer (fig. 3*A*) anneals, the mutation remained undetected in PCR-SSCP analysis. In RT-PCR experiments, two different receptor variants were found. Predominantly, a transcript containing 69 additional nucleotides between the sequences of exons 2 and 3 (mutant transcript I) and a smaller amount of a transcript from which exon 3 was deleted (mutant transcript II) (fig. 3*B*) were detected. Translation of transcript I will result in an AR with a 23-amino-acid insertion between the two zinc clusters, and transcript II encodes a protein that lacks the second zinc cluster. Wild-type transcript was not detected by RT-PCR. SDS-PAGE and immunoblot analysis revealed only a protein with an increased molecular mass in GSF from patient III-2 (fig. 2*A*, lanes 3 and 4). The 23 additional amino acid residues, as well as the relatively long glutamine stretch, contributed to the slower migration pattern seen with SDS-PAGE analysis, as compared with the control AR.

To prove that the mutation at position −11 is not a common polymorphism, 102 normal chromosomes from unrelated individuals were screened for the presence of this mutation in intron 2 of the AR gene. The mutation was not detected in these control individuals. In addition, a larger part of intron 2 of the AR gene of the index patient was sequenced to exclude the presence of an additional mutation that could have induced the preferential use of the cryptic splice site. No other alterations were found in intron 2, up to position −137.

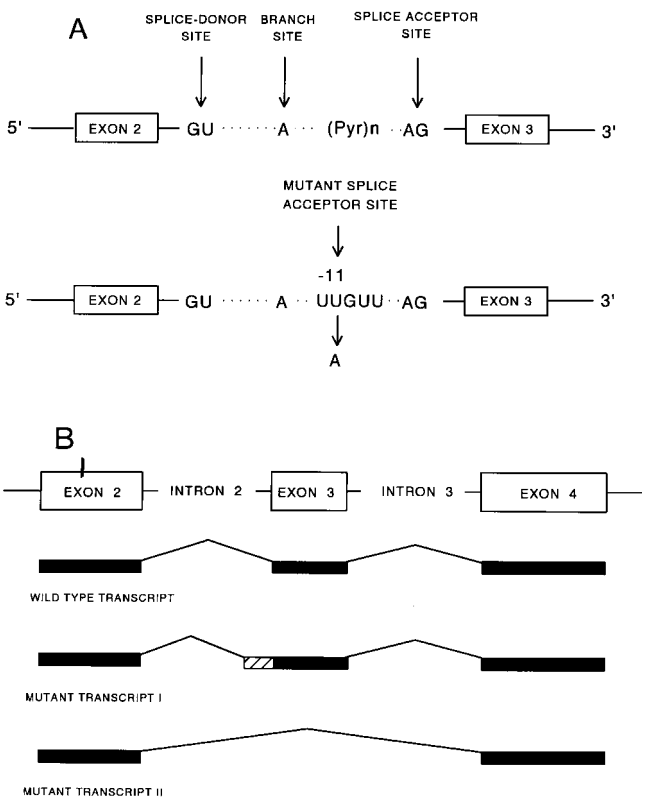

Figure 3 Characterization of point mutation. *A*, Position of point mutation found in intron 2. Represented are exons 2 and 3 and significant sequences flanking intron 2, of both wild-type and mutant AR pre-mRNAs. The positions of the splice-donor site, the branch site, and the splice-acceptor site are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

Immunoblot Analysis of the AR Protein

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

Gel-Retardation Assay

In order to establish whether DNA binding by the mutant AR was affected, in vitro binding to a consensus ARE was studied. In the presence of polyclonal Ab Sp197, which stabilizes the protein-DNA complex (Kuiper et al. 1993), the wild-type receptor was able to bind to a consensus ARE, resulting in a shifted probe (fig. 4*B*, lane 5). No shifted probe was detected for the mutant AR TI and AR TII (fig. 4*B*, lanes 7 and 9) or in the control lane (fig. 4*B*, lane 3). The amount of receptor protein was checked by western blotting and immunostaining. Comparable amounts of AR protein were incubated.

Transcription-Activation Assay

The wild-type AR showed transcription activation on the MMTV promoter in CHO cells (fig. 4*C*). However, AR TI and AR TII were unable to activate transcription on an MMTV-Luc promoter (fig. 4*C*). SDS-PAGE followed by immunoblotting showed that AR-protein expression was identical for the three different receptor molecules (results not shown).

Allele-Specific Oligonucleotide Hybridization

To investigate whether a wild-type transcript was present in GSF of the two affected siblings in the partial-AIS family, an allele-specific oligonucleotide–hybridization

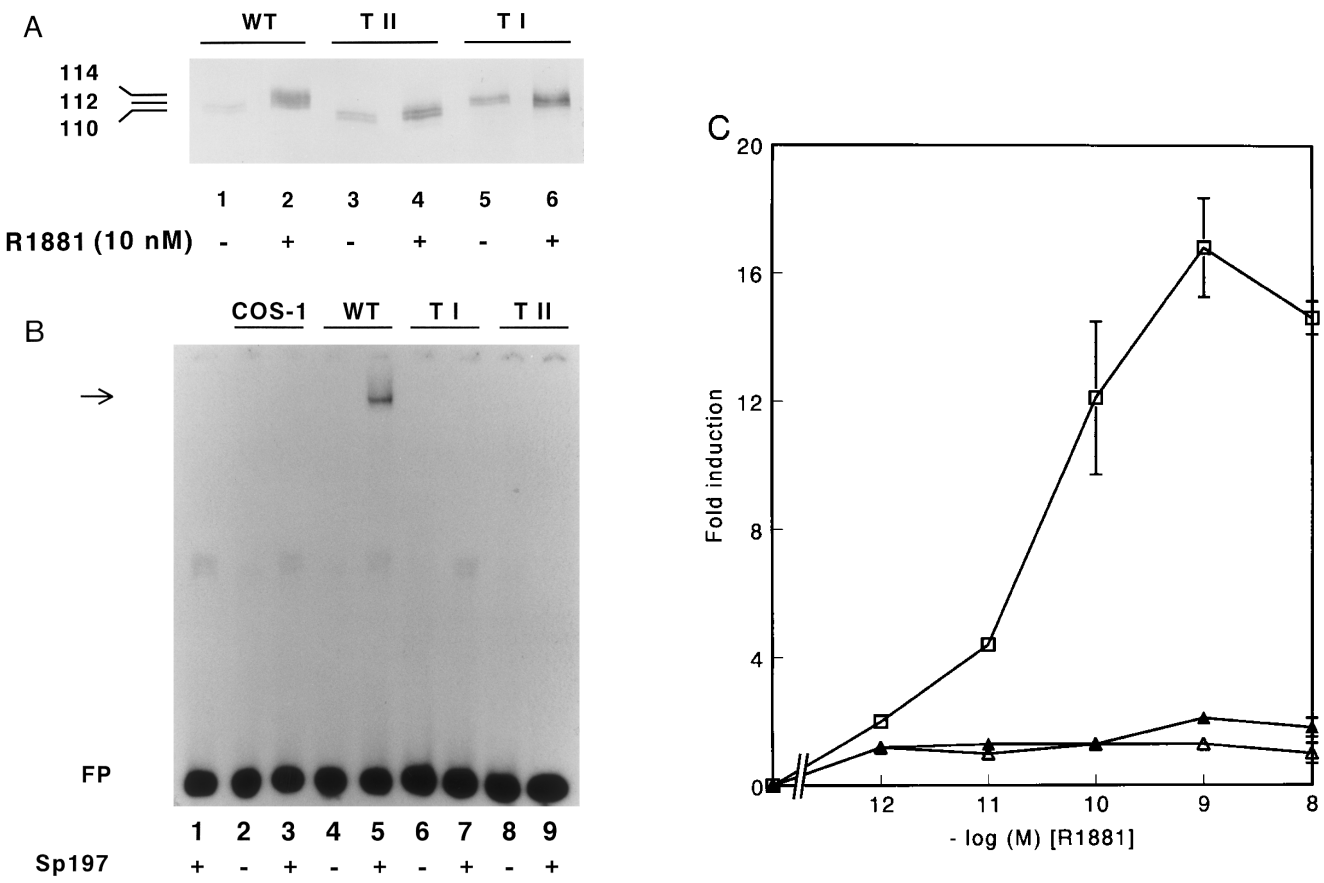

Figure 4 Functional analysis of wild-type and mutant AR. *A,* Hormone-induced upshift of AR proteins. AR protein variants were expressed in COS-1 cells after transfection with pSG5AR0, pSG5AR33, or pSG5AR129. The cells were cultured for 24 h in the absence (−) or presence (+) of 10 nM R1881. After immunoprecipitation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

assay was developed. First-strand cDNA was synthesized from total mRNA, was derived from GSF, and was amplified in three consecutive PCR reactions. mRNA was isolated from wild-type GSF, from GSF from patients III-1 and III-2, and as a control from GSF derived from a patient with partial AIS who has been described previously by Ris-Stalpers et al. (1994). The mutation found in this latter patient causes differential splicing, resulting in 10% wild-type mRNA and 90% mRNA of a splice variant with a deletion of exon 3. The PCR products were spotted on membranes and subsequently hybridized with oligonucleotides specific for the splice variants and the wild-type AR (table 1). Hybridization with the wild-type probe revealed wild-type mRNA in GSF from a normal control male, in GSF from the partial-AIS patient (positive control), and in GSF from one of the 46,XY patients (III-1) (fig. 5, WT-1b, WT-1c, and WT-1d, respectively). In GSF from patient III-2, wild-type transcript was either not present or below the detection limit of the assay (fig. 5, WT-1e). TI was present only in GSF from patients III-1 and III-2 and not in wild-type GSF (fig. 5, TI-3d and TI-3e, respectively). TII was detected in GSF of the positive control and patient III-1 (fig. 5, TII-5c and TII-5d, respectively). The signal at position e in figure 5 (patient III-2), which was comparable to the a-specific signal at position b in figure 5,

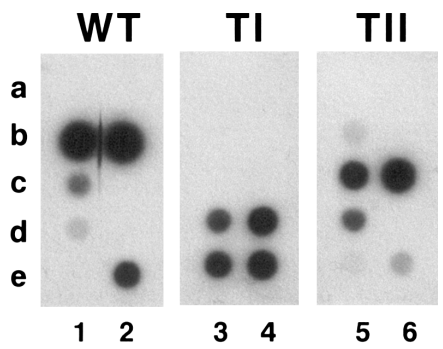

Figure 5 Allele-specific oligonucleotide hybridization. DNAs were amplified by use of exon 1 sense primer 470A and exon 4 antisense primer 14 NB, in two consecutive PCR reactions. The expression plasmids used as controls (pSVAR0, BHEX-AR33, and pSVAR129) were amplified in one PCR reaction. The resulting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

resulted from cross-hybridization of the probe with wild-type DNA and was considered as background. DNA amplified from expression plasmids, which was used as a positive control, is visible in rows 2, 4, and 6 of figure 5. Comparable amounts of DNA were spotted, as was assessed by hybridization with an exon 2 probe (results not shown).

Discussion

It is well established that AIS is caused by mutations in the AR gene. However, reports have appeared about patients with an AIS phenotype in which no mutation was detected by use of PCR-SSCP analysis (Brüggenwirth et al. 1996; Weidemann et al. 1996) and DNA sequencing (Morel et al. 1994), in spite of clear phenotypic, endocrinological, and biochemical evidence for AIS. Mutations might be missed because PCR-SSCP is not 100% sensitive. Often, only the exonic sequences and their flanking intronic regions are screened, leaving mutations in intronic and promoter regions undetected. For the AR, if cells from patients are available, RT-PCR studies and western immunoblotting can be very informative, in particular when mutations are present in intronic regions (Ris-Stalpers et al. 1990, 1994).

In the present report, a family presenting with partial AIS has been described. No mutation was detected in the AR gene of the index patient after PCR-SSCP analysis or sequencing of the exons. However, biochemical studies revealed that the AR of the affected family members was unable to bind to DNA. In the absence of hormone, wild-type receptor derived from transiently transfected COS-1 cells migrates as a 110–112-kD doublet (Kuiper et al. 1991). AR preparations derived from cells cultured in the presence of hormone contain, in addition to the 110–112-kD doublet, a slower-migrating, 114-kD isoform, representing hormone-dependent phosphorylation (Jenster et al. 1994). It has been shown that the appearance of the 114-kD isoform is correlated with DNA binding and/or transcription activation (Jenster et al. 1994). In the present report, hormone-dependent phosphorylation of the AR protein has been used as an indicator of receptor dysfunctioning. Since the 114-kD isoform was hardly detectable in GSF derived from the index subject (III-2) and her sister (III-1), cellular distribution of the AR from the index subject was studied. AR was undetectable in the tightly nuclear-bound fraction of hormone-exposed GSF from the index subject, which points to disturbed AR-DNA binding and, consequently, to a defect in transcription activation. Sequencing analysis revealed a mutation at position −11 in intron 2, in the AR gene of both siblings, their 46,XY aunt, and the index patient's mother (carrier of the mutation). Since this mutation was not found in 102 chromosomes derived from unrelated controls, we have concluded that it is not a common polymorphism.

The effect of the mutation on the splicing process has been studied. Splicing of pre-mRNA begins with cleavage at the *5* splice-donor site, which is attacked by the 2-OH group of the branch-site adenosine. The 3′ splice site is attacked by the newly formed 3′-OH of the upstream exon, the intron is released in the form of a lariat, and the exons are joined (Sharp 1985). Around the splice-acceptor site a consensus sequence (T/C, T/C, T, T, T/C, T/C, T/C, T/C, T/C, T/C, N, C, A, G, G) important for proper splicing is present (Shapiro and Senapathy 1987). The present AR mutation was located in this pyrimidine-rich consensus sequence preceding the splice-acceptor site. Aberrant splicing took place, resulting in different transcripts encoding defective ARs, which might explain the observed phenotype. The cryptic splice site that was used is located at position −71/−70 in intron 2, thus resulting in an mRNA variant containing an insertion of 69 nucleotides. We determined that sequences in and around this cryptic splice site did not contain any mutation, thereby not enhancing preferable usage of this site.

According to Nakai and Sakamoto (1994), almost all major cryptic splice sites that are activated by mutations are located <100 nucleotides from the original splice site.

On the basis of comparison with a consensus sequence, the cryptic splice site located at position −71/−70 in the AR appears to be closer to the consensus sequence than does the wild-type splice-acceptor site (the cryptic splice site is T, C, T, T, T, T, C, T, G, T, T, C, T, A, G, A; the wild-type intron 2 splice site is T, A, T, T, T, G, T, T, C, T, C, C, C, A, G, C). However, RT-PCR studies performed on wild-type mRNA demonstrated that this cryptic splice site is normally not used. Watakabe et al. (1993) proposed that suboptimal 3′ splice-site sequences require stimulation by downstream sequences. Thus, exon 3 sequences of the AR may play a role in splice-acceptor-site selection. The newly created splice-acceptor site at position −11/−10 was not used, probably because, compared with the novel cryptic site at position −71/−70, the sequence was less favorable for splicing. A small amount of a transcript from which exon 3 was deleted was detected. Wild-type transcript was not observed, although this could be expected on the basis of the partial-AIS phenotype of the affected individuals. We concluded that the wild-type splice-acceptor site has become weaker because of the base-pair substitution at position −11. Alternatively, closely spaced splice sites can inactivate each other because of sterical hindrance of bound splicing factors (Black 1991). Nelson and Green (1988) showed with in vitro splicing experiments that, in a β-globin intron, insertion of a new splice-donor site close to the wild-type splice-donor site prevented splicing at both sites. Therefore, aberrant splicing in our patients could also be the result of interference by two closely spaced splice-acceptor sequences.

Quigley et al. (1992) have described the so-called null phenotype of AIS. A deletion of the X chromosome spanning the complete AR gene caused complete AIS. The patient showed inguinal or abdominal testes, no Wolffian-duct development, and absence of masculinization of the external genitalia. Sparse pubic and axillary hair were detected. Also, a complete external female phenotype and absence of secondary hair were seen in a 46,XY individual with a complete deletion of the AR gene, as reported by Hiort et al. (1996). All AIS subjects in the family reported in the present paper showed remnants of vasa deferentia and epididymides. The aunt (II-4), clinically investigated postpubertally, has pubic and axillary hair. These phenotypic characteristics suggest that some residual AR activity is present. Therefore, DNA binding and transcription-activation capacities of the splice variants were investigated. The AR expressed in GSF from the index subject has 23 additional amino acids between the first zinc cluster and the second zinc cluster. Ducouret et al. (1995) cloned a teleost-fish glucocorticoid receptor (GR) with nine additional amino acid residues between the two zinc clusters. This fish GR, however, was still capable of activating a reporter gene, from which it was concluded that the folding of the GR can compensate for separation of the two zinc clusters by an extra 9-amino-acid stretch. The AR mutant with the insertion of 23 amino acid residues did not bind to a consensus ARE (on the basis of a gel-retardation assay). Consequently, there was absence of transcription activation. The splice variant with a deletion of the second zinc cluster was also unable to bind specifically to DNA, corresponding to the results reported by Quigley et al. (1992) and Ris-Stalpers et al. (1994). On the basis of these results and in view of the phenotype of the affected family members, we concluded that splicing was not completely aberrant. Indeed, a very small amount of wild-type transcript was detected in GSF of patient III-1, by use of the allele-specific oligonucleotide–hybridization method. The transcript with the deletion of exon 3 (i.e., AR TII) was detected in GSF of patient III-1 and not in GSF of patient III-2. However, this was not reproduced in all experiments, since AR TII previously had been detected by RT-PCR studies using RNA from patient III-2.

The intronic mutation discussed in this paper was missed by PCR-SSCP analysis. There are several other explanations for the apparent absence of AR mutations in several cases of AIS. Neutral mutations, for example, have to be interpreted with caution. Richard and Beckmann (1995) found a synonymous-codon mutation (GGC→GGT) in the cDNA of the calpain (CANP3) gene, which turned out to be pathogenic, because a splice-donor site was created. Kallio et al. (1996) suggested that, in 46,XY subjects without a mutation in the AR gene and with a typical AIS phenotype, postreceptor defects might be the cause of the disease. This may involve receptor-specific cofactors or corepressors. Recently, a family with dominant inheritance of thyroid-hormone resistance was reported, which could not be linked to defects in the thyroid hormone–receptor α or β genes (Weiss et al. 1996). It was postulated that an abnormal cofactor, playing a role in regulation of thyroid-hormone action, might be involved. Most cofactors reported to date are not AR specific, so mutations in these factors will probably be lethal or give rise to complex phenotypes. One coactivator, ARA70, which binds specifically to the ligand-bound AR, has been reported by Yeh and Chang (1996). It is not known whether mutations in the gene encoding ARA70 correlate with certain forms of AIS.

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

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