A Point Mutation in the Second Zinc Finger of the DNA-Binding Domain of the Androgen Receptor Gene Causes Complete Androgen Insensitivity in Two Siblings with Receptor-Positive Androgen Resistance

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We have analyzed the nucleotide sequence of complementary and genomic DNAs of the human androgen receptor (AR) gene in two siblings (patients 9006 and 9030) with receptor-positive complete androgen insensitivity (Rec$^+$-CAI). Northern analysis indicated that mRNA of the AR was normal in size. However, its expression was relatively reduced in both patients. Consistent with the normal androgen-binding capacity (496 and 552 fmol/mg DNA for patients 9006 and 9030, respectively) but decreased DNA-binding ability (168 fmol/mg DNA) measured in genital skin fibroblasts, no mutation was found in both N-terminal and ligand-binding domains of the AR. However, a single base substitution (G→A) was found in the second zinc finger of the DNA-binding domain at nucleotide 2372 of the AR cDNA in both cases. This resulted in the replacement of a highly conserved arginine residue (amino acid 614) by a histidine. When the mutated receptor plasmid was cotransfected into PC-3 cells together with the reporter chloramphenicol acetyltransferase gene, chloramphenicol acetyltransferase activity was not induced by 5α-dihydrotestosterone treatment, confirming that the mutation renders the AR nonfunctional and can, therefore, be held responsible for the clinical features in these patients. These results highlight the importance of Arginine-614 in the second zinc finger of the DNA-binding domain of the AR in the protein-DNA interaction. (Molecular Endocrinology 7: 861–869, 1993)

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INTRODUCTION

Androgens [testosterone (T) and 5α-dihydrotestosterone (DHT)] control the development of the normal male phenotype and the regulation of virilization in the adult. Their various actions are mediated by the androgen receptor (AR), a member of the steroid receptor superfamily (1). Like other members of the family, the AR acts as a *trans*-regulator of transcription; upon binding its cognate ligand, the hormone-receptor complex interacts with *cis*-acting regulatory elements to regulate the transcription of target genes. The AR gene has been cloned (2–7), and its organization as well as functional domain structure have also been determined (8–10). It comprises a variable N-terminal region involved in the modulation of gene expression, a short, well conserved DNA-binding domain characterized by the presence of two zinc fingers, and a partially con-

served C-terminal ligand-binding domain which is also involved in the receptor dimerization and *trans*-activation processes.

Subjects with complete androgen insensitivity (CAI) present with a feminine phenotype contrasting with an XY karyotype and the presence of testes secreting normal or even high level of testosterone. Measurements of androgen binding capacity in genital skin fibroblasts from these subjects have led to the distinction of a receptor-negative (Rec^--CAI) population, in whom the binding capacity was not measurable, and a receptor-positive (Rec^+-CAI) population, in whom the binding capacity was normal or slightly decreased (11). In these cases, various qualitative defects of the AR have been described to explain the insensitivity. They include increased thermal lability (12), defective stabilization by molybdate (13), defective up-regulation (14), and abnormal pHi, which suggested a structural abnormality (15). However, only the availability of molecular probes for the AR has allowed accurate delineation of the AR abnormality in these patients. In addition to a number of mutations in the ligand-binding domain in Rec^--CAI (16–18), mutations in the DNA-binding domain (19–21) or the ligand-binding domain (22, 23) have been described in patients with Rec^+-CAI. We report here the study of two siblings in whom a single base substitution in exon 3, leading to the change of a conserved arginine residue to a histidine in the second zinc finger of the DNA-binding domain of the AR, resulted in a nonfunctional AR and Rec^+-CAI.

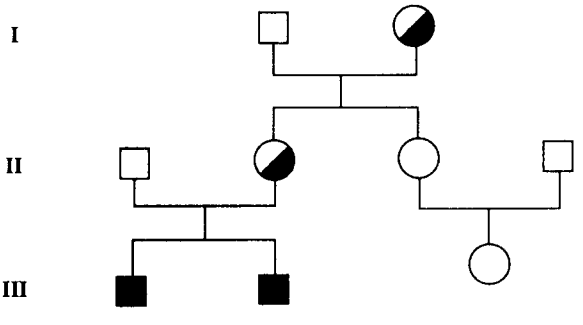

Fig. 1. Pedigree of Patients 9006 and 9030 with CAI

The *half-filled circles* represent obligate heterozygous carriers on the basis that each has the substitution mutation in exon 3 of AR in genomic DNA. The *filled squares* indicate affected hemizygous males.

RESULTS

Androgen Binding and 5α-Reductase in Genital Skin Fibroblasts of Normal and Rec^+-CAI Patients

Both patients 9006 and 9030 are affected siblings aged 15 and 13, respectively. Figure 1 represents the pedigree of this family. Both present the typically clinical phenotype of CAI. They had normal female genitalia at birth with inguinal masses suggestive of testes. Both had a karyotype at birth, which was 46,XY, but no other examination at that time.

Patient 9006 had an LHRH stimulation test at age 2: basal LH was 38 ng/ml (normal for age, <6 ng/ml) with a peak at 135 ng/ml (normal, 25 ± 10 ng/ml). Both basal and stimulated FSH levels were also high (155 and 875 ng/ml; normal, undetectable and 75 ± 30, respectively). Statural growth was rapid (+2 SD) until the age of 6 and then slowed down. Puberty began at age 13 with an isolated mammary development but no hair growth (pubic or axillary) and no virilization. Plasma testosterone was 2.2 ng/ml (normal, 2–8 ng/ml), with a normal T/DHT ratio. LH was 15 mU/ml, and FSH was 4.7 mU/ml (normal range, 1.6–6 mU/ml and 1.5–4 mU/ml for LH and FSH, respectively). Castration was performed at age 14, and histological examination of the gonads confirmed the presence of testes. A biopsy of genital skin was obtained at the time of surgery.

Patient 9030 presented with a very similar picture. Her puberty also began at age 13 with an isolated mammary development and no hair growth (pubic or axillary) or virilization. Plasma testosterone was 1.3 ng/ml. LH was 14 mU/ml, and FSH was 1.2 mU/ml. Castration was performed at age 13, and histological examination of the gonads confirmed the presence of testes. A biopsy of genital skin was obtained at the time of surgery.

Androgen-binding capacity was 496 and 552 fmol/mg DNA in patients 9006 and 9030, respectively, which is at the lower limit of the normal values (872 ± 283 fmol/mg DNA; n = 30) (Table 1). Androgen-binding affinity was normal [dissociation constant (K_d), 0.18 nM] in patient 9006 but decreased (K_d, 0.7 nM) in patient 9030 (normal value for K_d, <0.3 nM). Prolonged incubations did not result in up-regulation of androgen-binding capacity (456 and 565 fmol/mg DNA in patients 9006 and 9030, respectively). 5α-Reductase was normal in both patients (62.3 ± 4.5 and 9.5 ± 1.2 fmol/μg DNA·h in patients 9006 and 9030, respectively; normal values, >8 fmol/μg DNA·h), confirming that this enzyme

Table 1. Ligand-Binding and 5α-Reductase Activities in Normal and Rec^+-CAI Patients

	Normal	Rec^+-CAI patients	
		9006	9030
B_{max} (fmol/mg DNA)	872 ± 283	496	552
K_d (nM)	<0.3	0.18	0.7
5α-Reductase (fmol/μg DNA·h)	>8	62.3 ± 4.5	9.5 ± 1.2

is neither androgen dependent in genital skin (24) nor involved in the mechanism of androgen insensitivity in these patients.

DNA Binding Studies

Results are shown in Fig. 2. After 20 min of incubation, total DNA binding activity (cytosol plus nucleus) was 168 ± 21 and 400 ± 80 fmol/mg DNA in patient's and control cells, respectively (Fig. 2A). However, about 40% of the specifically bound radioactivity was recovered in the cytosol of the patient's cells *vs.* 15% in control cells (Fig. 2B), which already suggests a diminished DNA binding ability in these cells. When labeled cytosol was incubated with DNA-cellulose, only 26% of the specifically bound radioactivity was recovered in the DNA-cellulose pellet using patient's cytosol *vs.* 82% with control cytosol (Fig. 2C). This binding was further reduced when the control cytosol was diluted with unlabeled activated cytosol, indicating specific binding.

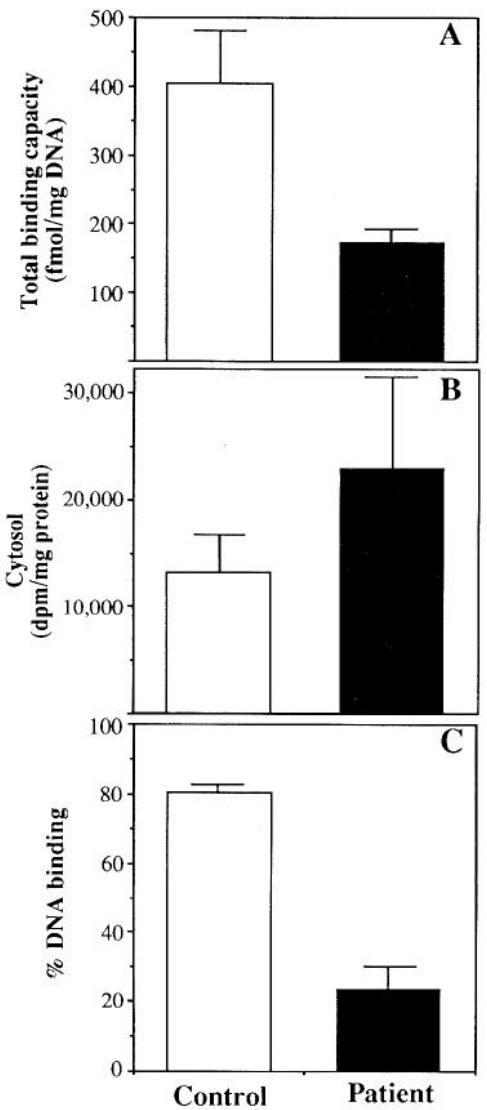

Fig. 2. DNA-Binding Studies

Cells were incubated for 20 min to yield labeled cytosol as described in *Materials and Methods*. Results are the mean ± SD of three separated experiments. Total binding and cytosol binding were calculated after subtraction of nonspecific binding. Each tube for DNA-binding studies contained 200 μl DNA-cellulose slurry including 80 μg DNA and 200 μl cytosol containing 800–1000 dpm specifically bound DHT and 70 ± 17 (control) or 33 ± 12 μg proteins.

Messenger RNA Analysis

Northern blot analysis of the expression of the AR mRNA in both patients exhibited a 10-kilobase (kb) band characteristic of the normal size of the AR mRNA (Fig. 3). This result eliminated the possibility that a gross deletion or gene rearrangement occurred. However, densitometry scanning and evaluation of the intensity of the AR-to-actin ratio revealed decreased expression of AR mRNA in both patients (24% and 8% of normal in patients 9006 and 9030, respectively).

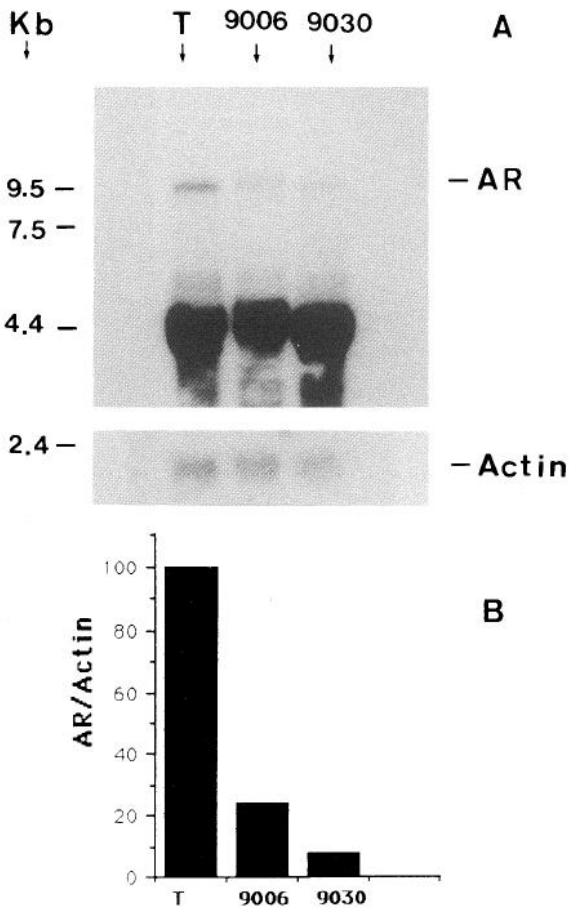

Fig. 3. Northern Blot Analysis of AR mRNA of a Normal Male Control (T) and Patients 9006 and 9030

Twenty micrograms of total RNA were electrophoresed in denaturing conditions, transferred onto a nylon membrane, and blot hybridized with a ^{32}P-labeled rat AR probe overnight at 65 C or with a cDNA probe for β-actin at 42 C for 24 h. A, Autoradiography of AR and β-actin films. B, Densitometry scanning expressed as AR-to-actin ratio.

Identification of a Molecular Defect in the AR of Patients 9006 and 9030

As the size of the AR mRNA is identical to that derived from a control RNA sample (Fig. 3), this suggested that major alterations in the gene or mRNA of AR could not be the cause of the androgen insensitivity. For the identification of any small mutations within the AR, we sequenced the entire coding region of AR cDNA. Complementary DNA was generated from total RNA isolated from genital skin fibroblasts of Rec$^+$-CAI patients and amplified by the polymerase chain reaction (PCR) using *Taq* DNA polymerase by three pairs of primers that flank the N-terminal, DNA-, or ligand-binding domain. The amplified fragments for the N-terminal, DNA-, and ligand-binding domains were then subcloned into pCR1000 or pT7Blue plasmid and sequenced. Figure 4 shows a single base substitution observed at position 2372 (G→A), resulting in the replacement of an arginine residue by a histidine at position 614 of the AR in both cases. The position numbers are based on our published sequence (3). As this mutated sequence is located in exon 3 of the AR gene, we further confirmed this mutation by isolation of genomic DNA from Rec$^+$-CAI patients and amplification of exon 3 of the AR gene for the sequence analysis. A confirmatory G→A substitution was observed in the genomic DNA of Rec$^+$-CAI patients.

To ask whether this mutation is responsible for androgen insensitivity in this family, we analyzed genomic DNA from affected and unaffected individuals within this family. Our results showed the mutation was present in the patients' mother and grandmother, who were expected to be obligately heterozygous, but not in a maternal aunt or her daughter (Fig. 1).

The arginine residue at position 614 is highly conserved within the second zinc finger of the DNA-binding domain among the steroid receptor superfamily (Fig. 5). The mutation in both patients examined resulted in the substitution of an arginine residue by a histidine at this position of the human AR gene.

Transcriptional Activity of the Mutated AR

The presence of a substituted amino acid residue within the conserved positions in the second zinc finger of the DNA-binding domain of the AR gene indicated that this AR product might not be able to interact with androgen-responsive elements of its target genes. To test this hypothesis, we reconstructed full-length AR cDNAs with or without the point mutation at nucleotide 2372 under control of the human cytomegalovirus (CMV)

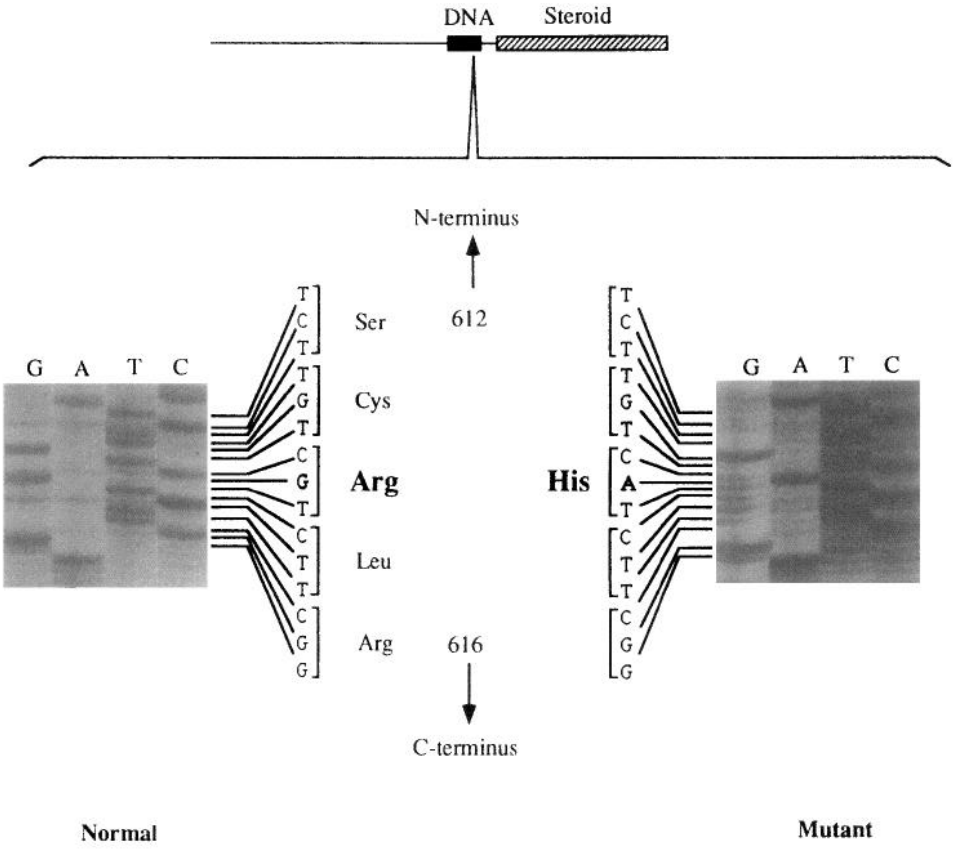

Fig. 4. Sequence Comparison of Exon 3 in Normal and Mutant ARs

The sequence shows the guanine-to-adenine substitution that results in an arginine-to-histidine substitution at amino acid residue 614.

hAR	610	Cys	Pro	Ser	Cys	**Arg**	Leu	Arg	Lys	Cys
rAR	594	-	-	-	-	-	-	-	-	-
mAR	591	-	-	-	-	-	-	-	-	-
hPR	619	-	-	Ala	-	-	-	-	-	-
hER	237	-	Gln	Ala	-	-	-	-	-	-
hGR	473	-	-	Ala	-	-	Tyr	-	-	-
hMR	655	-	-	Ala	-	-	-	Gln	-	-
hTR2	165	-	Gln	Tyr	-	-	-	Gln	Arg	-
hTR3	319	-	Gln	Phe	-	-	Phe	Gln	-	-
T3R	156	-	Gln	Glu	-	-	Phe	Lys	-	-
VDR	76	-	Gln	Ala	-	-	-	Lys	Arg	-

Fig. 5. Conservation of Arginine-614 in the DNA-Binding Domains of Other Members of the Steroid Receptor Superfamily

The amino acid residues which are identical with that of human AR (hAR) (2, 3) are shown by *hyphens*. *Numbers* indicate the amino acid positions in the receptor sequences. Sequences listed represent rat AR (rAR), mouse AR (mAR), human progesterone receptor (hPR), human estrogen receptor (hER), human glucocorticoid receptor (hGR), human mineralocorticoid receptor (hMR), human TR2 orphan receptor (hTR2), human TR3 orphan receptor (hTR3), human thyroid receptor (T3R), and human vitamin D receptor (VDR) (3, 25–27).

promoter. The pCMV-AR89 plasmid encoding the point-mutated receptor was cotransfected in PC-3 cells together with the reporter plasmid pMSG-chloramphenicol acetyltransferase (CAT). These results are shown in Fig. 6. DHT can increase the levels of CAT activity when PC-3 cells were cotransfected with the reporter and normal AR plasmids. By contrast, when PC-3 cells were cotransfected with the reporter and mutated AR plasmids, DHT stimulation of CAT activity was only slightly detected (Fig. 6).

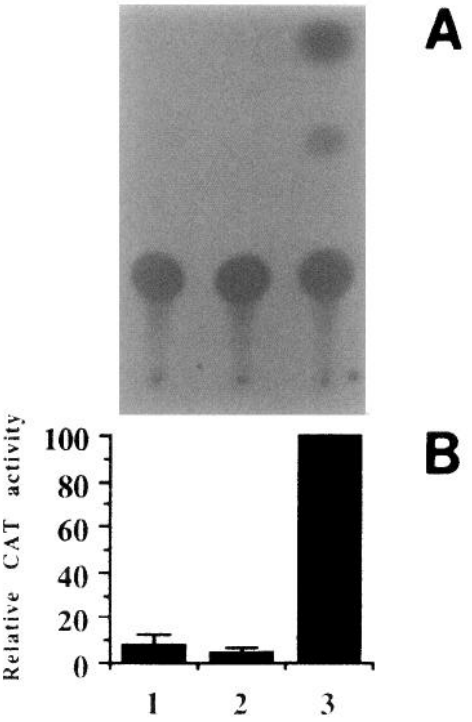

Fig. 6. Induction of CAT Activity in PC-3 Cells Cotransfected with the Reporter Plasmid pMSG-CAT and the Expression Plasmid Encoding Mutant or Normal ARs

A, Lane 1 represents the cells transfected with pMSG-CAT plasmid alone. PC-3 cells were cotransfected with pMSG-CAT and mutant (lane 2) or normal (lane 3) AR expression plasmid. After stimulation with DHT, cell extracts were prepared and assayed for CAT activity. All CAT assays were normalized for the level of β-galactosidase activity. B, The relative conversion of [^{14}C]chloramphenicol to acetylated forms in PC-3 cells was summarized from A. Each value represents the mean of three independent experiments.

DISCUSSION

We have identified a single base substitution (G⟶A) in the DNA-binding domain of the AR gene resulting in the replacement of a conserved arginine residue by a histidine at position 614 in two siblings with Rec$^+$-CAI. This mutation results in a nonfunctional receptor with decreased DNA-binding ability and could, therefore, be responsible for the CAI in both patients.

This study highlights the importance of Arginine-614 in the DNA-binding domain of the AR in the protein-DNA interaction. This arginine residue is highly conserved within the steroid receptor superfamily, suggesting that it may play a crucial role in androgen action. In addition, this residue is located next to a conserved cysteine residue involved in the formation of the second zinc finger. Both arginine and histidine have basic side chains with similar polarity and charge in the amino acid residues. However, they differ in the structure of the side chain. Histidine contains an imidazole group and a cyclic structure in the side chain of this residue. On the contrary, arginine contains a long and linear guanidinium group in its side chain. The replacement of an arginine residue containing planar group in its side chain by a histidine residue containing a cyclic group in its side chain impairs the interaction between the androgen receptor protein and androgen-responsive DNA sequences, or another androgen receptor monomer. To address this alteration of the specific residue involved in either protein-protein or protein-DNA interaction, we compare data from the known crystallographic structure of the DNA-binding domain of the glucocorticoid receptor (28) with that of the AR (Fig. 7). Arginine-614 is the only residue in the carboxy-terminal α-helix in the DNA-binding domain of the AR involved in both specific and nonspecific phosphate contacts with DNA. Thus, we hypothesize that mutation in this residue may decrease or even abolish the interaction between AR and DNA. These data indicate that the mutated residue at position 614 markedly influences protein architecture during protein-DNA interaction. Therefore, the native arginine at position 614 may be structurally and functionally important.

Six different abnormalities have been described so far in patients with Rec$^+$-CAI. First, a deletion of exon 3 leading to the synthesis of a receptor protein that

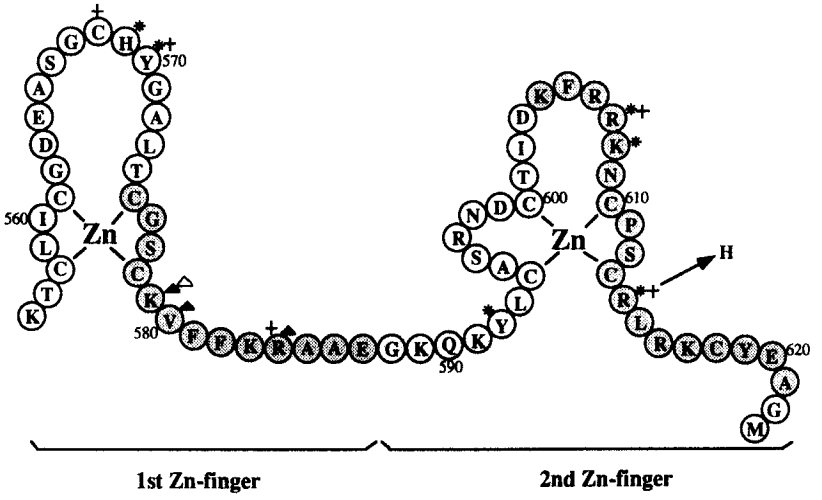

Fig. 7. Schematic Structure of the Human AR DNA-Binding Domain

Numbering corresponds to that of the full-length AR cDNA. Residues involved in phosphate contacts with DNA at the specific (569, 570, 592, 607, 608, and 614) and nonspecific (568, 570, 584, 607, and 614) sites are indicated by *asterisks* and *crosses*, respectively, compared with data of the known crystallographic structure of the GR (28). Residues making the amino- (575–587), carboxy-terminal (610–621), and middle (604–609) α-helical segments are *shaded*. The *solid and open triangles* show base contacts at the specific (579, 580, and 584) and nonspecific (579) sites, respectively. The mutant derivative (Arg^{614}→His) is indicated by the *arrow*.

fuses exons 2 and 4 maintains the reading frame of the AR coding segment. This receptor has retained a normal binding capacity but is inactive in assays of receptor functions (21).

Second, a mutation in the second zinc finger of the DNA-binding domain of the AR has been reported which differs from our finding in position (19, 20). This base mutation was a G→C conversion resulting in an Arg→Pro substitution at position 615 (corresponding to position 616 based on our sequence). Although this arginine residue was not conserved in the steroid receptor superfamily, the replacement of this arginine by another residue (proline, lysine, or glutamine) impaired receptor activity.

Third, two mutations have been detected in exon 2 comprising the first zinc finger of the DNA-binding domain of the AR (20). Both mutations resulted in the replacement of the cysteine residue into a tryptophan and arginine at positions 557 and 574, respectively. These substitution mutations abolished receptor function and the capacity of the receptor to bind to DNA.

Finally, two point mutations have been reported in the ligand-binding domain of the AR. The mutated AR exhibited a normal level of ligand-binding capacity in genital skin fibroblasts but presented qualitative abnormalities. In the first paper, the replacement of a valine residue by a methionine at position 866 resulted in a modified specificity of the AR (22). In the second one, the replacement of an aspartic acid by either a histidine or an asparagine at position 686 gave rise to a receptor protein with increased dissociation kinetics (23). In both cases, patients presented with the Rec^+-CAI phenotype.

It is relatively easy to imagine a mechanism through which a defective DNA-binding domain could prevent the receptor from normally *trans*-activating the transcription of androgen-regulated genes in spite of normal hormone binding. A mutation in the ligand-binding domain resulting in an inactive AR may further indicate that the ligand-binding domain contains a transcriptional activation function in addition to its ligand-binding function (9, 10). Whereas our patients had almost normal and similar binding capacity as measured in genital skin fibroblasts, AR mRNA expression was decreased in both patients but lower in patient 9030. However, the assessment of AR mRNA in cultured genital skin fibroblasts is difficult and not accurate enough that conclusions can be drawn from the difference between the patients. Nevertheless, variable and most often decreased levels of mRNA have been reported in patients with androgen insensitivity (29). The possibility that additional mutations in the promoter region of the AR gene resulting in the alteration of AR mRNA levels cannot be excluded. However, variation in AR mRNA levels could in itself, whatever its causes, modulate the degree of androgen resistance. It is of interest to note that a marked decrease of steady state level of vitamin D receptor mRNA has been reported in patients with mutations within the vitamin D receptor coding segment (30). It is, therefore, possible that mutations of the AR alter the turnover of the AR mRNA or protein, thus modulating the degree of androgen resistance.

MATERIALS AND METHODS

Cell Culture

Fibroblast strains were obtained from skin biopsies and propagated in a 4/1 mixture of medium 199/Dulbecco's modified

Eagle's medium supplemented with antibiotics and 10% fetal calf serum as previously described (24).

Androgen-Binding Capacity and Metabolism

Androgen-binding capacity and metabolism were measured as previously described (31). The maximum binding capacity and the apparent K_d of the AR were derived from Scatchard plot analysis. Binding capacity was expressed as femtomoles of DHT bound per mg DNA. For each patient, binding capacity was measured at least twice in separate experiments. In some experiments, incubations were continued for 18 h to assess up-regulation of the AR. These conditions usually resulted in a 2- to 3-fold increase of the normal AR (11). DNA assay was performed after perchloric acid extraction as described by Burton (32).

5α-Reductase Enzyme Assay

5α-Reductase activity was measured as previously described (31) and calculated as the amount of DHT and diols formed in 1 h and expressed as femtomoles per μg DNA/h.

DNA-Binding Studies

The ability of patient 9006's AR to bind DNA was examined in comparison with that of a normal control subject, using a DNA-cellulose slurry technique adapted from Sato *et al.* (33). Briefly, confluent cell monolayers were put in serum-free medium. Twenty-four hours later, the medium was changed for serum-free medium containing 5 nM [^{3}H]DHT (SA, 125 Ci/mmol) alone (group A) or with a 200-fold excess of unlabeled DHT (group B). Cells were incubated for 20 min in normal culture conditions. Our experience showed that 15–20% of the labeled AR was recovered in the cytosol in low-salt buffer. In addition, another group of plates (patient and control) were incubated in the presence of 1 μM unlabeled DHT to provide unlabeled activated AR (group C). At the end of incubation, cells were collected in cold PBS. Plates from each group were pooled to produce three cell pellets: A, B, and C. Each was homogenized in buffer A (50 mM Tris, 1 mM EDTA, 10% glycerol, and 0.25 mM dithiothreitol, pH 7.4) in a glass-glass homogenizer with 10 strokes of a hand-driven pestle. The resulting homogenates were centrifuged at 12,000 x *g* for 30 min to yield cytosol fractions A (total binding), B (nonspecific binding), and C (unlabeled activated AR). Two hundred microliters of cytosol (50–70 μg protein) were incubated in the presence of 200 μl DNA-cellulose slurry in buffer A, containing approximately 80 μg DNA according to the manufacturer's indications. Incubations were for 1 h at 0 C with intermittent shaking in a total vol of 1.2 ml. Tubes, in duplicate, contained either 200 μl cytosol A or B or 200 μl cytosol A or B and a 3-fold excess of cytosol C to assess DNA binding specificity. After incubations, 2 ml ice-cold buffer A were added to all tubes, quickly followed by vortexing and centrifuging at 1,200 x *g* for 5 min. The packed pellets were washed three more times with 2 ml buffer A. The radioactivity associated with the washed pellets was counted.

RNA Extraction

Cells (usually 6 × 10^6) grown to confluency and placed in serum-free medium 24 h before extraction were harvested using trypsin-EDTA, collected in serum-free medium, and centrifuged at 200 x *g* for 15 min. The cell pellet was washed three times with sterile saline and transferred into two eppendorf tubes (1.5 ml). The cell pellet was either extracted immediately or stored at −80 C. Extraction was performed using the one-step RNAzol extraction procedure described by Chomczynski and Sacchi (34). The cell pellet was carefully suspended in 600 μl RNAzol (Bioprobe Systems, France), and 60 μl chloroform/isoamylic alcohol (24/1) were added. Tubes were vigorously agitated, allowed to stand 15 min in ice, and then centrifuged at 12,000 rpm, 2 C, for 15 min. The aqueous phase was transferred into a clean eppendorf tube, and RNA was precipitated in isopropanol for 2 h at −80 C.

Northern Blot Analysis

Twenty micrograms of total RNA extracts from genital skin fibroblasts dissolved in 0.5% sodium dodecyl sulfate were size-fractionated on 1% agarose gel containing 2.2 M formaldehyde (35) and the gel blotted in 20× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) onto nylon membrane (Hybond N$^+$, Amersham, Arlington Heights, IL) and hybridized overnight at 65 C with a rat AR probe (36) linearized by *Bam*HI, cut, and ^{32}P labeled by *in vitro* transcription using the Riboprobe Gemini II system and T7 RNA polymerase (Promega, Madison, WI). Membranes were washed five times for 30 min with 0.1× SSC, 0.1 sodium dodecyl sulfate, and revealed by autoradiography. After exposition, membranes were stripped and rehybridized to a human β-actin cDNA probe (37). RNAs from each patient have been run four times against different controls, and the quantitative analysis was reproducible (± 15%).

Complementary DNA Preparation

The following primers were used for separate analysis of the N-terminal domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD), which cover the entire AR cDNA: primer 5 of NTD, 5′-GTGCAGTTAGGGCTGGGAAGG-3′; primer G of NTD, 5′-CGGGTTCTCCAGCTTGATGCG-3′; primer 2 of DBD, 5′-TCGCGACTACTACAACTTTC-3′; primer 4 of DBD, 5′- TGGCTCAATGGCTTCCAGGA-3′; primer A of LBD, 5′- GTGGAAATAGATGGGCTTGA-3′; and primer B of LBD, 5′-TCACACATTGAAGGCTATGA-3′. Complementary DNAs were synthesized using 1 μg total cellular RNA by the GeneAmp RNA PCR Kit (Perkin Elmer-Cetus, Norwalk, CT) according to the manufacturer's specifications. Amplification by PCR was performed using two-thirds of the cDNA synthesis reaction mixture and the above pairs of primers (100 ng each) in a total vol of 100 μl by Perkin Elmer-Cetus apparatus during 30 cycles. Each cycle included denaturation for 1 min at 95 C, annealing for 2 min at 60 C, and extension for 3 min at 72 C. After the last cycle, samples were incubated at 72 C for 7 min. The amplified products were recovered after phenol/chloroform extraction and ethanol precipitation.

Genomic DNA Extraction and Amplification

Genomic DNA was isolated from fibroblasts (3–5 × 10^6) of patients 9006 and 9030 or from lymphocytes of the family members as described by Davis *et al.* (38). Two oligonucleotide primers (exon 3 up, 5′-TGGTGCCATACTCTGTCCAC-3′; exon 3 down, 5′-ATGGCCACGTTGCCTATGAA-3′) were selected to generate a DNA fragment corresponding to the exon 3 region in the AR gene (22) by PCR. Amplification conditions consisted of denaturation for 1 min at 94 C, annealing for 2 min at 60 C, and extension for 3 min at 72 C through 30 cycles.

DNA Cloning and Sequencing

Amplified cDNAs and genomic DNA fragments were cloned by either the TA (InVitrogen, San Diego, CA) or pT7Blue cloning system (Novagen, Madison, WI) as described in the manufacturer's instructions. Sequence was analyzed by the dideoxynucleotide chain termination method (39) directly on plasmid DNA (40).

Plasmid Construction

The full-length coding sequence of the human AR (2, 3) pSK-AR3 digested with *Bam* HI was cloned into the pCMV-neo

vector at the *Bam*HI site under the human CMV promoter, termed pCMV-AR. To construct the full-length mutant AR gene, pSK-AR3 was cut with *Bam*HI and *Hind*III, and a 2.3-kb DNA fragment was cloned into the *Bam*HI- and *Hind*-III digested pBluescript SK$^+$ (Stratagene, La Jolla, CA). A 260-base pair DNA fragment was generated from the plasmid containing the DNA-binding domain of the AR gene with a point mutation digested with *Hind*III and *Dde*I. This fragment was cloned together with a 1.1-kb fragment derived from pSK-AR3 cut with *Bam*HI and *Dde*I into the *Bam*HI- and *Hind*III-digested pBluescript vector. Then, the plasmid containing the 2.3-kb fragment was cut with *Bam*HI/blunt and *Hind*III and cloned into the *Xho*I/blunt and *Hind*III-digested plasmid containing the 1.4-kb fragment to generate a full-length AR gene with mutation. Finally, the mutant AR plasmid digested with *Bgl*II and *Bam*HI was cloned into the pCMV-neo vector at the compatible *Bam*HI site under the CMV promoter, named pCMV-AR89.

Cell Transfection and Enzyme Assays

Five micrograms of pCMV-neo vector containing normal (pCMV-AR) or mutant androgen receptor coding regions (pCMV-AR89) were cotransfected by the calcium phosphate precipitate method (41) into PC-3 human prostatic carcinoma cells (1×10^6 cells per plate) (42) with 5 μg pMSG-CAT, a plasmid containing the full-length mouse mammary tumor virus long terminal repeat sequence linked with the CAT gene (Pharmacia, Piscataway, NJ). In transfections of PC-3 cells, 2 μg of the β-galactosidase expression vector pCH110 (Clontech, Palo Alto, CA) were cotransfected to normalize the transfection efficiency. After incubation at 37 C for 6 h, cells were treated with 7.5% glycerol in HBS buffer (25 mM HEPES, pH 7.0, 0.75 mM Na_2HPO_4, and 140 mM NaCl) for 2 min. Twenty four hours after transfection, cells were stimulated by DHT (10 nM). Cell extracts were prepared and assayed for CAT and β-galactosidase activities at 48 h after transfection (41).

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