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Molecular Basis of Androgen Insensitivity

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Mutations in the androgen receptor gene in 46,XY individuals can be associated with the androgen insensitivity syndrome, of which the phenotype can vary from a female phenotype to an undervirilized or infertile male phenotype. We have studied the androgen receptor gene of androgen insensitivity patients to get information about amino acid residues or regions involved in DNA binding and transcription activation. Genomic DNA was analysed by PCR–SSCP under two different conditions. Three new mutations were found in exon 1 of three patients with a female phenotype. A cytosine insertion at codon 42 resulted in a frameshift and consequently in the introduction of a premature stop at codon 171. Deletion of an adenine at codon 263 gave rise to a premature stop at codon 292. In both these cases, receptor protein was not detectable and hormone binding was not measurable. In a third patient, a guanine-to-adenine transition at codon 493 converted a tryptophan codon into a stop codon. Genital skin fibroblasts from this patient were not available. In exon 2 of the androgen receptor gene of a patient with receptor-positive androgen insensitivity, a cytosine-to-adenine transition, converting alanine 564 into an aspartic acid residue, resulted in defective DNA binding and transactivation. In three other receptor-positive androgen insensitivity patients no mutations were found with PCR–SSCP. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Androgens play a major role in male sexual differentiation and development. The actions of androgens are exerted through the androgen receptor (AR) which modulates transcription of androgen-responsive genes. The AR belongs to a super-family of receptors for steroid hormones, thyroid hormones and retinoids. Characteristic for the members of this family are the distinct functional domains; the N-terminal domain involved in transcription regulation, a DNA binding domain, composed of two zinc fingers, a hinge region and the C terminal ligand binding domain [1]. Mutations in the AR in 46,XY individuals are associated with the androgen insensitivity syndrome (AIS), a disorder with a wide spectrum of phenotypes. Subjects with complete AIS exhibit a female phenotype, whereas other AIS subjects show a phenotype with ambiguous genitalia, called partial AIS. The majority of the mutations reported up to now are point mutations, located in the ligand binding domain [2, 3]. AIS subjects with an AR of normal molecular mass and no abnormalities in ligand binding are an interesting group, because they may provide information about essential amino acid residues or regions, directly involved in transcription activation. Deletion mapping revealed that almost the entire N-terminal domain is necessary for full AR receptor transactivating activity [4]. Therefore, AR mutations interfering with correct receptor functioning may be expected in this domain. However, except for the expanded glutamine stretch, associated with Kennedy's disease, only six mutations in exon 1 of the AR have been reported [5–10]. Five of these resulted either directly or indirectly in the introduction of a premature stop codon.

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In this study we describe three new exon 1 mutations, all resulting in the introduction of premature stop codons. Zoppi *et al.* [10] reported a patient in which a single nucleotide substitution resulted in a premature stop at codon 60. Synthesis of AR protein was found to be initiated downstream of the termination codon. Therefore, we have investigated whether truncated AR forms were present in genital skin fibroblasts of the patients with an exon 1 mutation. A new mutation was also detected in the first zinc finger of the DNA binding domain of a patient with receptor-positive AIS. No exon 1 mutations, resulting in receptor-positive AIS, were found. In three other patients with receptor-positive AIS, no mutation was detected by polymerase chain reaction–single-strand conformation polymorphism (PCR–SSCP) analysis.

MATERIALS AND METHODS

Clinical subjects

Subject A: 46,XY index patient with complete AIS was admitted at the age of 1 yr because of a bilaterally inguinal hernia. She has a younger 46,XY sister with the same phenotype.

Subject B: 46,XY patient, diagnosed as having complete AIS. The sister of the patient's mother was known with primary amenorrhea.

Subject C: 46,XY patient, with a complete female phenotype and from a family with more affected members.

Subject D: 46,XY patient who was diagnosed as having complete AIS at birth in the absence of a positive family history. She came to medical attention because of suspected dysmorphology. After further clinical examination the diagnosis of AIS was made.

Subjects without a mutation: All patients displayed a 46,XY chromosome pattern, had no mullerian duct remnants and male hormonal levels were present in the serum. Two of them have the typical phenotype of complete AIS and one case has a phenotype with ambiguous genitalia.

The phenotype and the AR gene mutation of each subject are summarized in Table 1.

Table 1. Summary of phenotypes and AR gene mutations of index subjects

Subject	Phenotype	Mutation	Position change
A	cAIS	C insertion	codon 42
B	cAIS	A deletion	codon 263
C	cAIS	TGG→TGA	Trp493Stop
D	cAIS	GCT→GAT	Ala564Asp
E	pAIS	no mutation*	
F	cAIS	no mutation*	
G	cAIS	no mutation*	

The amino acid numbering is based on 910 residues, corresponding to a glutamine stretch of 20 residues and a glycine stretch of 16 residues.

*Only screened by PCR–SSCP.

Mutation detection

Genomic DNA, isolated from blood lymphocytes, was screened by PCR–SSCP. Seventeen primer sets for overlapping fragments were used to amplify the coding region and the exon flanking intronic regions of the hAR. A 15 μl PCR reaction mixture was used, containing 100 ng genomic DNA, 70 ng of each oligonucleotide, 40 μM of each dNTP, 10 mM Tris HCl (pH 9.0), 1.5 mM $MgCl_2$, 5 mM KCl, 10% DMSO (in case of amplifying exon 1), 1.0 μCi [α ^{32}P] dATP (Amersham, Little Chalfont, Bucks., U.K.) and 0.1 unit Supertaq DNA polymerase (HT Biotechnology Ltd, Sphaero Q, Leiden, The Netherlands). For PCR fragments covering the glycine stretch, 50% deaza dGTP was used with 50% dGTP. Reactions were denatured at 95°C and subjected to 30 cycles of denaturation at 95°C for 1 min, annealing for 2 min at different temperatures (Table 2) and elongation at 72°C for 1 min. In Table 2 oligonucleotides used for PCR amplification of the human AR gene and for direct sequencing are indicated. Samples consisting of 1 μl of PCR product and 9 μl sample buffer (95% formamide, 5% glycerol, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol FF) were denatured before loading onto a gel containing 7% acrylamide, 1× TBE buffer (Tris-borate, pH 8.2, 2.5 mM EDTA) and 5% or 10% glycerol. Gels were run for 16 h at 6 W either with 0.5× TBE buffer (5% glycerol gels) or 1× TBE (10% glycerol gels) at room temperature. Direct sequencing was performed in case an aberrant SSCP pattern was detected. Amplification took place in 100 μl reaction mixtures containing 100 ng genomic DNA, 400 ng of each primer, 200 μM of each dNTP, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 5 mM KCl, 10% DMSO (in case of amplifying exon one) and 2.5 units Amplitaq DNA polymerase (Perkin Elmer, Nieuwenkerk, a/d Yssel, The Netherlands). PCR products were purified from Seakem agarose with Spin-X columns (Costar, Badhoevedorp, The Netherlands) and about 100 ng PCR product was used as template in the cycle sequencing reaction (Sequitherm kit; Epicenter, Biozym, Landgraaf, The Netherlands). Primers, developed for PCR–SSCP, were end-labelled with T4 polynucleotide kinase in the presence of [γ ^{33}P] dATP (Amersham) and used in the cycle sequencing reaction.

Cell culture conditions

Genital skin fibroblasts were cultured in Modified Eagle's medium (MEM, containing 10% fetal calf serum, minimal essential amino acids (Gibco, Life Technologies, Breda, The Netherlands) and antibiotics at 37°C and 5% CO_2. Fibroblasts were grown to confluency, washed two times with PBS-buffer and cytosols or whole cell lysates were prepared.

Preparation of whole cell lysates

Genital skin fibroblasts were grown to confluency and scraped in 1 ml lysis-buffer (40 mM Tris, 1 mM EDTA

Table 2. Sequence of oligonucleotides used for PCR–SSCP screening of the human AR gene

Oligo	Location	PCR fragment	Annealing temperature (°C)	Sequence
−70A	5'-UTR, exon 1	Exon 1 A*	55	GCCTGTTGAACTCTTCTGAGC
95B	Exon 1			CTTGGGGAGAACCATCCTCA
35A	Exon 1	Exon 1 B	58	TcCGCGAAGTGATCCAGAAC
95B	Exon 1			CTTGGGGAGAACCATCCTCA
80A	Exon 1	Exon 1 C	64	AGCAAGAGACTAGCCCCAGGCAGC
172B	Exon 1			CGGAGCAGCTGCTTAAGCCGGGG
160A	Exon 1	Exon 1 D	62	GCTGCCCCATCCACGTTGTCCCTGCT
250B	Exon 1			ACTCAGATGCTCCAACGCCTCCAC
240A	Exon 1	Exon 1 E	62	TGTGTAAGGCAGTGTCGGTGTCCAT
320B	Exon 1			CGCCTTCTAGaCCTTTGGTGTAAC
305A	Exon 1	Exon 1 F	64	CAGGCAAGAGCACTGAAGATACTGC
385B	Exon 1			GGTTCTCCAGCTTGATGCGAGCGTG
361A	Exon 1	Exon 1 G	58	CGCGACTACTACAACTTTCCACTGG
445B	Exon 1			CACACGGTCCATACAACTG
1A	Exon 1	Exon 1 H	55	TCCTGGCACACTCTCTTCAC
490B	Exon 1			GCCAGGGTACCACACATCAGGT
470A	Exon 1	Exon 1 I	57	GTAGCCCCCTACGGCTACA
1B	Intron 1			CAGAACACAGAGTGACTCTGC
2A	Intron 1	Exon 2	55	GTCATTTATGCCTGCAGGTT
2B	Intron 2			TCTCTCTCTGGAAGGTAAAG
3A	Intron 2	Exon 3	55	TCAGGTCTATCAACTCTTG
3B	Intron 3			GGAGAGAGGAAGGAGGAGGA
4A	Intron 3	Exon 4 A	55	ATTCAAGTCTCTCTTCCTTC
14NB	Exon 4			TGCAAAGGAGTtGGGCTGGTTG
4AA	Exon 4	Exon 4 B	55	CAGAAGCTtACAGTGTCACACA
4B	Intron 4			GCGTTCACTAAATATGATCC
5A	Intron 4	Exon 5	55	GACTCAGACTTAGCTCAACC
5B	Intron 5			ATCACCACCAACCAGGTCTG
6A	Intron 5	Exon 6	55	CAATCAGAGACATTCCTCTGG
6B	Intron 6			AGTGGTCCTCTCTGAATCTC
7A	Intron 6	Exon 7	55	TGCTCCTTCGTGGGCATGCT
7B	Intron 7			TGGCTCTATCAGGCTGTTCTC
8LA	Intron 7	Exon 8	55	AGGCCACCTCCTTGTCAAC
8B	3' UTR, exon 8			AAGGCACTGCAGAGGAGTA

*This PCR product is relatively large and was therefore digested with the restriction enzyme PstI, prior to PCR–SSCP analysis. The CAG(n)CAA repeat length was studied with primer pair 35A and 95B. Mismatches are indicated with a small letter.

pH 7.4, 10% glycerol, 10 mM dithiothreitol (DTT), 1% v/v Triton, 0.08% sodium dodecylsulfate (SDS), 0.5% sodium-deoxycholate, 600 μM phenylmethylsulfonyl fluoride, 500 μM bacitracin). After 5 min the cell extract was centrifuged for 10 min at 4000 RPM at 5°C. The supernatant was stored at −80°C.

Preparation of cytosols

Cells were scraped in 1 ml cytosol-buffer (40 mM Tris, 1 mM EDTA pH 7.4, 10% glycerol, 10 mM DTT, 10 mM molybdate, and freshly added: 600 μM phenylmethylsulfonyl fluoride, 500 μM bacitracin and 500 mM leupeptin), and homogenized with a teflon potter. The derived cell homogenates were centrifuged for 10 min at 100,000×*g*, and the supernatants stored at −80°C until use.

Characterization of the AR protein by SDS–PAGE and immunostaining

The AR protein was immunoprecipitated from genital skin fibroblast lysates, with monoclonal antibody F39.4.1., as described before [11]. After electrophoresis on a 7% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel, the proteins were transferred to nitrocellulose and immunostained with polyclonal antibody, Sp061. After washing, the membrane was incubated with a second, peroxidase coupled antibody, to visualize the protein.

Scatchard analysis

For studying the binding characteristics two different assays were used: either a binding assay in which genital skin fibroblast cytosols were used, or a whole cell assay. Cytosols were incubated overnight at 4°C with increasing concentrations (0–0.1–0.23–0.5–1.0–2.5–5.0–10.0 nM) of 17β-hydroxy 17α-methyl-^{3}H (^{3}H-R1881) (Dupont, Dordrecht, The Netherlands), in the absence or presence of a 100-fold molar excess of non-labelled R1881 to determine non-specific binding. After protamine precipitation to remove the free steroid, specifically bound ^{3}H-R1881 was measured. The total amount of protein was assayed according to the method of Bradford [12].

Genital skin fibroblasts were incubated with increasing concentrations of ^{3}H-R1881 (0.02, 0.05, 0.3, 1.0, 3.0 nM) to obtain a saturation state. The non-specific

binding was determined after incubation with a 100-fold molar excess of non-radioactive steroid. After a 1-h incubation period at 37°C, cells were washed four times with ice-cold 20 nM Tris, 0.15 mM NaCl, pH 7.4. The cells were scraped in 1 ml lysis-buffer (TEG pH 7.4: 20 mM Tris, 15 mM EDTA, 10% v/v glycerol, 600 μM phenylmethylsulfonyl fluoride and 500 μM bacitracine). After 10 min centrifugation at 800×*g* the pellet was lysed by adding 1 ml 0.5 N NaOH and a 30 min incubation at 56°C. Scintillation cocktail (5 ml Clumin (Packard, Tilburg, The Netherlands)) was added to 500 μl of each sample and ^{3}H-activity was measured in a scintillation counter. The amount of protein was quantified by the method of Bradford [12]. Scatchard analysis was carried out to determine the dissociation constant (K_d) and the number of binding sites (B_{max}).

RESULTS

The coding part of the hAR gene was screened by amplification of genomic DNA with a set of 18 primer pairs, followed by SSCP analysis, performed under two different conditions. In case of an aberrant PCR–SSCP profile, direct sequencing was performed. Three new exon 1 mutations, all resulting in the introduction of a premature stop codon were detected in the hAR of three complete AIS patients (Fig. 1). In one receptor-positive patient a mutation was found in exon 2 (Fig. 1). In three other patients, diagnosed as also having a receptor-positive form of AIS, no aberrant PCR–SSCP pattern was detected.

Sequence analysis indicated a cytosine insertion at codon 42 of the AR gene of patient A, resulting in a frameshift and consequently in a premature stop at codon 171 (Fig. 2). In a second complete AIS subject (B) a deletion of an adenine at codon 263 of the AR gene gave rise to a stop codon at position 292 (Fig. 2). A guanine-to-adenine transition at codon 493 changed a tryptophan codon (TGG) into a stop codon (TGA) in the AR gene of patient C (Fig. 2). A Kpn I restriction site, which is a unique site in AR cDNA, was destroyed by the mutation and is informative with respect to carrier detection.

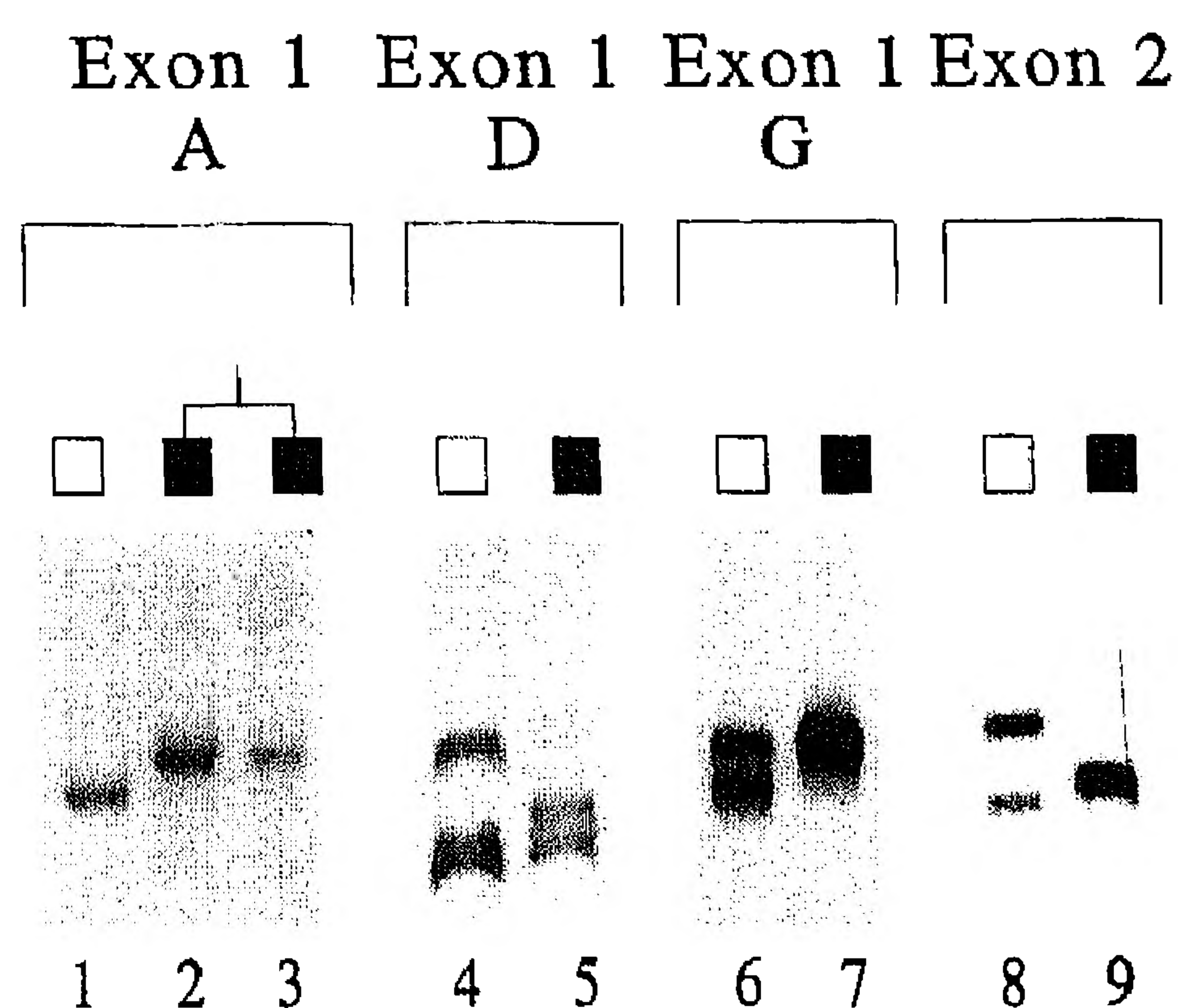

Fig. 1. PCR–SSCP analysis of androgen receptor gene mutations. SSCP analysis of different parts of exon 1 and of exon 2. Lane 1: wild-type AR pattern of fragment A [nucleotides (−70)–(+286)]; lanes 2 and 3: patterns of fragment A of index patient A [2] and her 46,XY sister [3]; lane 4: wild-type pattern of fragment D (nucleotides 707–958); lane 5: pattern of fragment D of patient B; lane 6: wild-type pattern of fragment G, starting at nucleotide 1402 and ending in intron 1; lane 7: SSCP pattern of fragment G of patient C; lane 8: wild-type pattern of exon 2 fragment starts in intron 1 and ends in intron 2; lane 9: exon 2 pattern of patient D. Numbers are based upon an open reading frame of 2730 nucleotides [13].

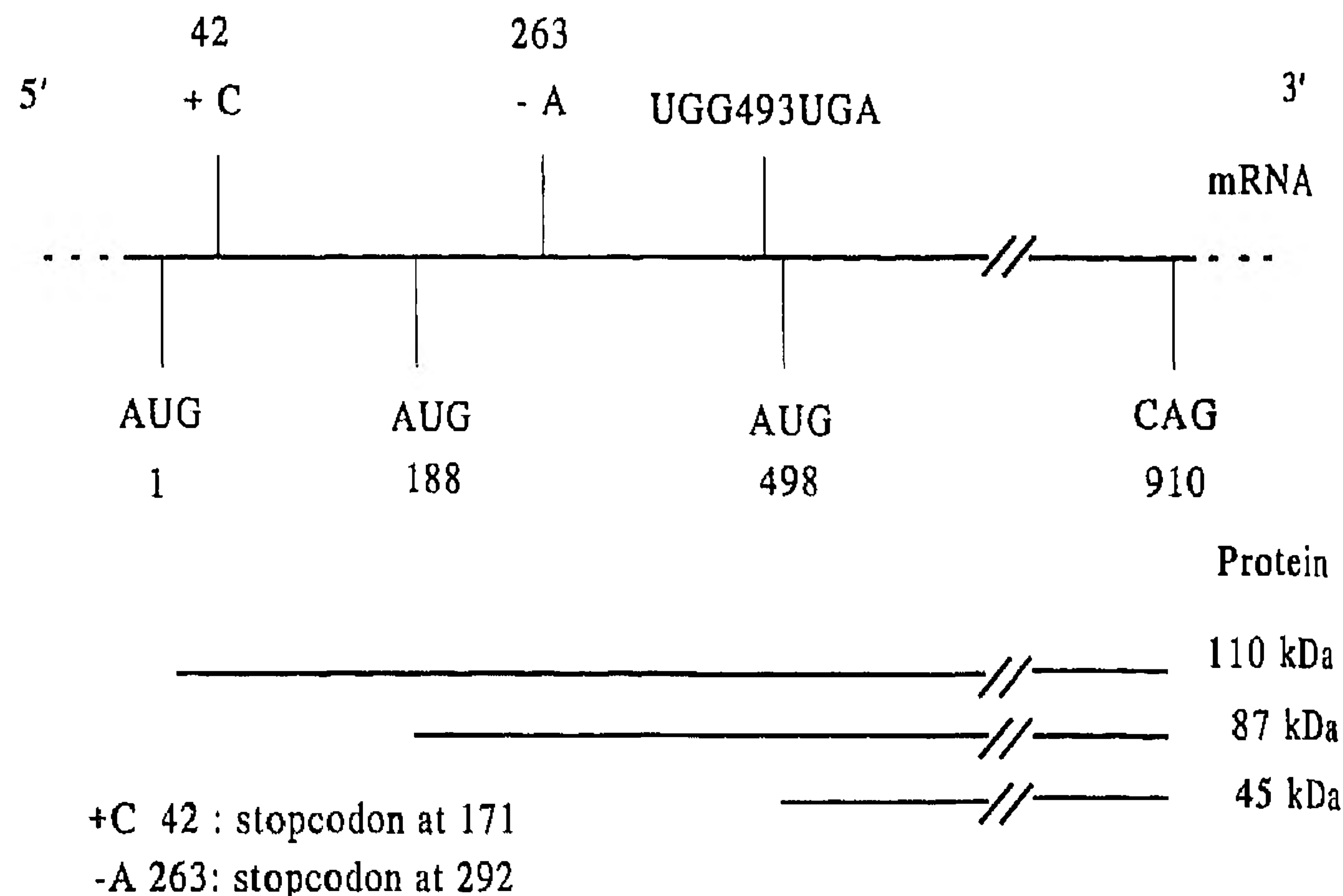

Fig. 2. Localization of mutations and internal AUGs in exon 1 of the androgen receptor gene. The mutations found in exon 1 of the AR gene are shown; + C: insertion of a cytosine at codon 42, resulting in a premature stop at codon 171; − A: deletion of an adenine at codon 263, resulting in a premature stop at codon 292. At codon 493 a transition of a guanine to an adenine was found, directly resulting in a premature stop codon. Translation start sites and the molecular mass of proteins, resulting from translation initiation, governed by these AUGs, are depicted.

Western-immunoblotting was performed to investigate if a truncated AR form was expressed in genital skin fibroblasts of subjects A and B. Truncated forms might result from internal reinitiation of translation,

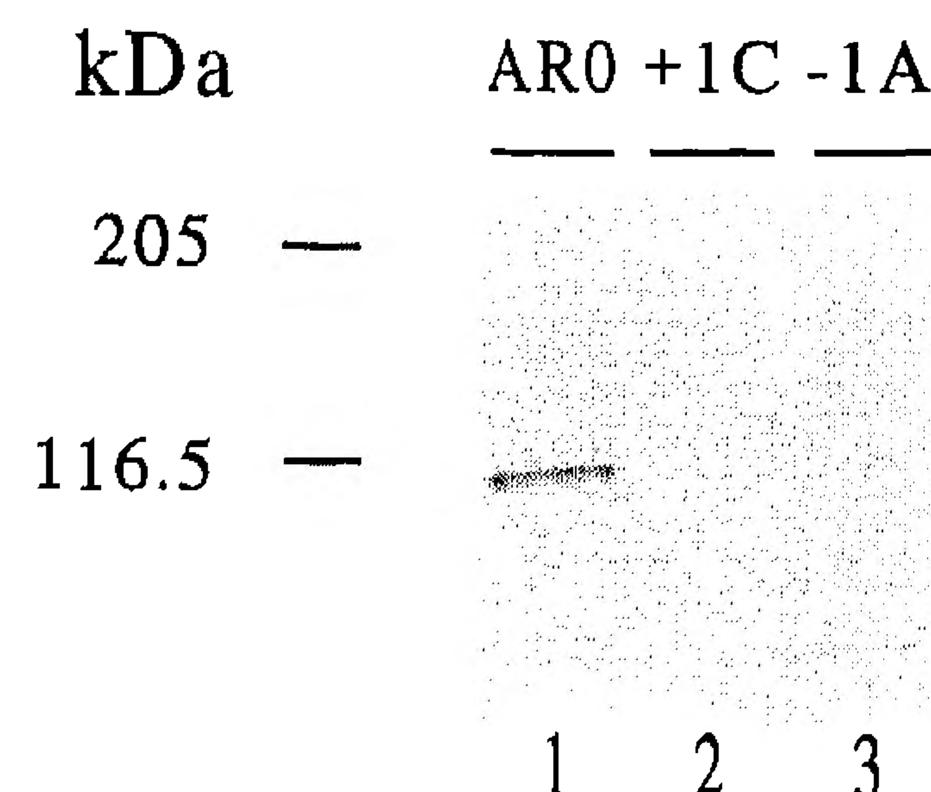

Fig. 3. Western blot analysis of androgen receptor expression. AR protein was immunoprecipitated with monoclonal antibody F39.4.1 from genital skin fibroblast lysates, and separated on a 7% SDS-PAGE gel. After electrophoresis the proteins were transferred to nitrocellulose and immunostained with the polyclonal antibody SP061. The AR protein was visualized with a peroxidase-coupled goat-anti-rabbit antibody. Lane 1: Wild-type (Wt) AR from control genital skin fibroblasts (GSF); lane 2: AR with insertion of cytosine (+1C) from GSF of subject A; lane 3: AR with deletion of adenine (−1A) from GSF of subject B. Molecular mass markers are indicated on the left.

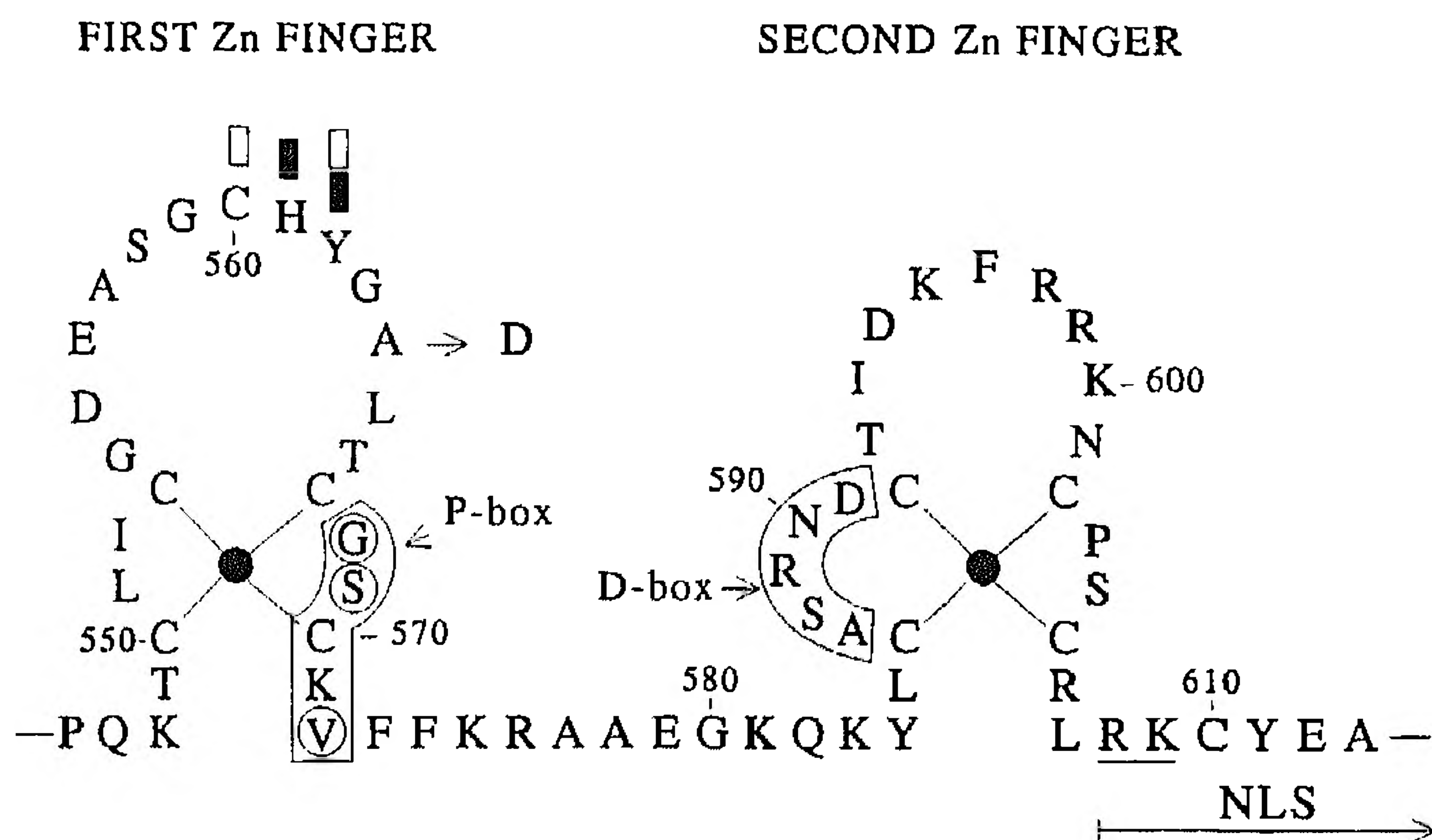

Fig. 4. Position of the Ala→Asp mutation in the first zinc finger of the androgen receptor DNA binding domain. The amino acid sequence of the DNA binding domain of the AR, consisting of two zinc fingers, is shown in one letter code. The first zinc finger contains the P-box in which the three circled amino acid residues determine hormone-responsive element specificity. Boxes indicate residues that make contacts with the phosphate backbone of the DNA, either at specific sites (black boxes) or at non-specific sites (open boxes) [14]. The mutation is depicted at position 564. In the second zinc finger the D-box and part of the nuclear localization signal (NLS) are located.

proceeding from internal AUG codons (Fig. 2). In case of patient A no AR protein of 87 kDa could be detected (Fig. 3, lane 2). This corresponds with the observation that ligand binding was not measurable in cytosols, prepared from genital skin fibroblasts of patient A. The stop codon, introduced in the AR of patient B is also located upstream of an internal in frame AUG. However, a shortened AR protein could not be detected on a Western blot (Fig. 3, lane 3). Furthermore ligand binding studies were negative. Genital skin fibroblasts were not available from patient C.

A new mutation was found in exon 2, which encodes the first zinc finger of the DNA binding domain of the AR. A transition of cytosine to adenine converts an alanine residue at position 564 into an aspartic acid residue (position depicted in Fig. 4). This mutation resulted in defective DNA binding and transactivation, and will be described in more detail elsewhere.

In two of the patients, diagnosed as having complete AIS, and in one patient with partial AIS, no mutation was found after screening all the exons and their flanking intronic sequences with PCR–SSCP. All three patients were clinically well diagnosed. AR protein was isolated from genital skin fibroblasts, obtained from these subjects, and molecular mass was established after SDS–PAGE and Western blotting. In all cases the AR protein migrated as a normal 110–112 kDa doublet. Receptor characteristics were further studied by Scatchard analysis, from which it became clear that the AR of these three patients displayed normal K_d and B_{max} values.

DISCUSSION

Genomic DNA from patients with either complete or partial AIS were screened by PCR–SSCP analysis for mutations in the AR gene. Studying new mutations in receptor-positive AIS patients may reveal new information on amino acid residues in functional domains involved in DNA binding and transcription regulation. Besides mutations in exons 2 and 3, encoding the DNA binding domain of the AR, exon 1 mutations may be expected, because the N-terminal region plays a role in transcription activation [4]. In exon 1 of the AR gene of three complete AIS patients we have found mutations resulting either directly or indirectly in the introduction of premature stop codons. Translation reinitiation is known to occur in mammalian cell mRNAs. Mammalian ribosomes can reinitiate translation at an AUG codon, after termination at an upstream site [15, 16]. According to Peabody *et al.* [16] it may be possible that the 40 S ribosomal subunit remains associated with the mRNA, and regains the capacity to scan along the mRNA until it encounters an initiation codon. At this point, the loose ribosomal subunit becomes associated and synthesis of the polypeptide chain starts again. In the Tfm mouse a single nucleotide in a hexacytidine stretch (nucleotides 1107–1112) is deleted in the AR gene, resulting in a frame shift and the introduction of a premature stop at codon 412. Downstream of this premature stop codon three AUGs are located, in frame with the premature termination codon. A low level of high affinity binding to androgens and also to DNA could still be measured, which indicates reinitiation of translation [17–19]. The low protein level resulted from instability of the mRNA [17, 18]. Zoppi *et al.* [10] described an AIS patient in which a cytosine-to-thymine transition, converting a glutamine (CAG) residue at position 60 in the polymorphic glutamine stretch, into a premature stop (TAG). *In vitro* experiments suggested that internal initiation occurs from the first in-frame AUG codon at position 188, resulting in a 87 kDa protein. In genital skin fibroblasts, a low but detectable level of specific androgen binding with an accelerated dissociation rate was measured. In patient A, the premature stop codon was present at codon 171. A shortened protein of approximately 87 kDa, however, could not be detected in genital skin fibroblasts. These results correspond with the observation that binding was not measurable in cytosol from the patient's genital skin fibroblasts. In the other two mutant AR genes we described, the stop codon occurred after the first internal AUG. No truncated receptor proteins could be detected in genital skin fibroblasts derived from patient B. It is unclear which factors dictate the efficiency with which such translation reinitiation occurs. To some extent the efficiency of reinitiation may be governed by the position of the termination codon, relative to the subsequent downstream AUG codons [15, 16, 20]. In patient A, the new start is located 10 codons downstream of the premature stop codon, whereas in the patient described by Zoppi *et al.* [10] and in the Tfm mouse, this distance was 128 and 132–134 codons,

respectively. This may be the reason why, in genital skin fibroblasts from this patient A, no 87 kDa AR and ligand binding were detected.

Two isoforms of the progesterone receptor (PR) have been described, a protein of normal structure (PR-B) and a shortened form (PR-A), lacking 164 amino acids at the N-terminus [21]. Depending on cell type and promoter context, functional differences between the two forms have been found. PR-A can act as an activator of transcription and as a repressor of transcription by PR-B and even as an inhibitor of transcription, mediated by the glucocorticoid receptor, the mineralocorticoid receptor and the AR [22]. Wilson *et al.* [23] reported that two forms of human AR, comparable to PR isoforms, are present in human genital skin fibroblasts. A 87 kDa isoform was postulated, which constitutes 7–15% of the total AR, arising from alternative initiation of translation. We were, however, unable to detect such a 87 kDa protein in genital skin fibroblasts of a control subject.

Up to now, amino acid substitutions in exon 1, resulting in a receptor-positive form of AIS have not been found. However, recently a proline-to-leucine substitution at position 339 was found, in DNA isolated from a prostate tumour [24]. No functional studies have been performed as yet to study the significance of this mutation.

The cytosine-to-adenine transition at codon 564, converting an alanine residue into an aspartic acid residue in the first zinc finger of the DNA binding domain of the AR, resulted in a complete AIS phenotype. The phenotype was caused by the defective DNA binding and transactivation capacities of the mutant receptor. Although the correct 3-D structure of this mutant AR is not available, the effect of the mutation can be deduced from the 3-D structure of the glucocorticoid DNA binding domain [14]. Cysteine 560, histidine 561, and tyrosine 562 are involved either in specific or aspecific contacts with the phosphate backbone of the DNA (Fig. 4). The amino acid substitution in the mutant receptor might interfere with these contacts, resulting in defective DNA binding.

In the AR gene of three subjects with an AIS-like phenotype, no mutations were found with PCR–SSCP. Although receptor characteristics appear to be normal, the presence of a mutation, not influencing ligand binding, cannot be excluded. There is still a possibility that some mutations are being missed by the screening procedure we used. However, in our laboratory so far all mutations which were previously found by direct sequencing, could be confirmed by PCR–SSCP afterwards. AR proteins appeared as normal 110–112 kDa doublets in genital skin fibroblasts and furthermore normal K_d and B_{max} values were measured. This does not rule out abnormal protein expression in other target tissues. Another explanation for the androgen resistance syndrome might be the presence of a mutation in a gene, encoding a factor specifically involved in transcriptional regulation of AR target genes.

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