Am. J. Hum. Genet. 46:1095–1100, 1990

An Exonic Point Mutation of the Androgen Receptor Gene in a Family with Complete Androgen Insensitivity

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Summary

We have discovered in the X-linked androgen receptor gene a single exonic nucleotide substitution that causes complete androgen insensitivity (resistance) in a sibship with three affected individuals. The mutation, a guanine-to-adenine transition, occurs at nucleotide number 2682 and changes the sense of codon 717 from tryptophan to a translation stop signal. Codon 717 is in exon 4, so the mutation predicts the synthesis of a truncated receptor that lacks most of its androgen-binding domain. The substitution abolishes a recognition sequence for the restriction endonuclease *Hae*III. Amplification of exon 4 by the polymerase chain reaction followed by double digestion with *Hinf*I and *Hae*III permits facile recognition of hemizygotes and heterozygous carriers of the mutation.

Introduction

The androgen receptor is a DNA-binding, transcription-regulating protein whose properties are induced on androgen binding (Pinsky and Kaufman 1987). Absent or seriously defective androgen receptor-binding activity causes complete androgen insensitivity and leads to the birth of a 46,XY individual who has the external phenotype of a female.

The gene that encodes the androgen receptor has been regionally localized to Xq11-12 (Migeon et al. 1981; Brown et al. 1989). It is >90 kb, but only ~3% of it, divided into eight exons, is translated (Kuiper et al. 1989). Exon 1 (or A) encodes the entire amino-terminal portion of the androgen receptor (Faber et al. 1989); its role, probably modulatory, remains to be defined. Exons 2 and 3 (or B and C) encode the central, 66-amino-acid DNA-binding domain, and exons 4–8 (or D–H) encode the carboxy-terminal, ~252-amino-acid androgen-binding domain. Somatic cell clone analysis of X inactivation (Meyer et al. 1975; Elawady et al. 1983), as well as linkage analysis (Wieacker et al. 1987), have incriminated mutations at the locus as the cause of complete androgen insensitivity in some families.

The recent availability of cDNA probes that span the entire coding sequence of the androgen receptor gene (Chang et al. 1988; Lubahn et al. 1989; Trapman et al. 1988; Faber et al. 1989; Tilley et al. 1989) has revealed that gross, but submicroscopic, alterations at the locus are uncommon (Brown et al. 1988; Marcelli et al. 1989; Pinsky et al. 1989; Ris-Stalpers et al. 1989). One report (Lubahn et al. 1989) has described in exon 7 (or G) a missense mutation that seriously impairs the quality of androgen receptor-binding activity in a family with complete androgen insensitivity (Brown et al. 1982). Another preliminary report (Trifiro et al. 1989) has described in exon 6 a missense mutation that eliminates androgen receptor-binding activity in a family with complete androgen insensitivity. In addition, a point mutation in the exon 4–intron 4 donor splice site has been identified (Ris-Stalpers et al. 1989) in a patient with complete androgen insensitivity. In the present paper we describe a third exonic point mutation

Received December 21, 1989; revision received February 15, 1990.

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0002-9297/90/4606-0010$02.00

in the androgen receptor gene: a guanine-to-adenine transition that creates a translation stop signal in exon 4, the most 5′ exon of the androgen-binding domain. The mutation abolishes an *Hae*III restriction site and thereby permits a simple test for hemizygous or heterozygous carriers of the mutation. It was found in one of 10 families with absent or markedly deficient androgen receptor-binding activity. Of the other nine families, two are now known to have the exon 6 mutation noted above, and one has a missense mutation in exon 4 (authors' unpublished results).

Subjects and Methods

Subjects

The mother of the three affected siblings is French-Canadian. Her menarche occurred at 17 years of age, contrary to a statement in a previous description (Pinsky et al. 1977). She has one normal daughter, whose menarche occurred at 11 years of age, and one normal sister, whose four daughters and two sons are unremarkable. Each of the three affected siblings is 46,XY, presented with primary amenorrhea, and has the classic clinical/endocrine phenotype of complete androgen insensitivity. Their cultured genital and nongenital skin fibroblasts have negligible androgen-binding activity when incubated with as much as 7 nM 5α-dihydrotestosterone (Pinsky et al. 1977). This concentration is 35–70-fold more than the equilibrium dissociation constant of the normal androgen receptor for this androgen.

Methods

RNA Isolation.—A total of 40–60 million confluent genital skin fibroblasts were washed, collected by scraping, and pelleted at room temperature in 20 mM Tris-HCl, pH 7.4. The pellet was vortexed gently in 4 ml of 4 M guanidinium isothiocyanate, 50 mM Tris-HCl, pH 7.5, 0.5 M EDTA (GMC), and the suspension was placed on a 7-ml cushion of 5.6 M CsCl, 0.1 M EDTA in a 12-ml polyallomer tube. RNA was pelleted in an SW 40ti rotor (16–24 h, 21°C, 25,000 rpm). The pellet was dissolved in 400 μl of 10 mM Tris-HCl, pH 7.5, and RNA was precipitated (overnight at −20°C) by 40 μl sodium acetate (2 M, pH 5.5) and 1 ml of 95% ethanol, before it was dissolved in water treated with diethylpyrocarbonate.

Reverse transcription (RT).—Ten micrograms of total RNA were heat-denatured at 85°C for 3 min. RT was carried out in a final volume of 20 μl at 37°C for 30 min, with 500 μM each dNTP, 1 mM DTT, 25 units RNAsin, and 200 units of MMLV reverse transcriptase in polymerase-chain-reaction (PCR) buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg BSA/ml) containing 10 pmol primer A (5′-GTGGAAATAGATGGGCTTGA), a 20-mer complementary to a sequence near the 3′ terminus of the androgen-binding domain in the androgen receptor mRNA. The reaction was terminated by heating to 95°C for 5 min.

PCR amplification of androgen receptor cDNA.—Reverse-transcribed cDNA was amplified in a final volume of 100 μl PCR buffer with 2.5 units of *Taq* polymerase, 200 μM each dNTP, 50 pmol primer A, and 50 pmol of primer B (5′-TCACACATTGAAGGCTATGA). The latter is a 20-mer corresponding to a sequence that overlaps the 5′-terminus of the androgen-binding region of the androgen receptor cDNA. The reaction was carried out in a DNA Thermal Cycler (Perkin-Elmer) programmed to denature at 94°C for 1 min, to anneal at 52°C for 2 min, and to extend at 72°C for 1.5 min. After 40 cycles, the products were extracted with chloroform and were analyzed on a 1.5% Electran agarose gel (BDH, Montreal).

DNA isolation.—A total of 40–60 million confluent genital skin fibroblasts were washed, collected by scraping, and pelleted in 0.85% NaCl. After being washed and repelleted in the same solution, they were resuspended in 4 ml of High TE (100 mM Tris-HCl, 40 mM EDTA, pH 8) and were lysed by adding 4 ml of 100 mM Tris-HCl, pH 8, 40 mM EDTA, 0.2% SDS, 1 M NaCl, and by passing the suspension repeatedly through an 18-gauge needle attached to a 10-ml syringe. After extraction with phenol and back-extraction with High TE, the aqueous phases were combined and extracted with 1:1 chloroform:isoamyl alcohol. DNA was precipitated by the addition of 0.02 vol 5 M NaCl and an equal volume of isopropanol. The DNA pellet was resuspended in Low TE (10 mM Tris-HCl, pH 8, 1 mM EDTA). DNA was isolated from peripheral blood lymphocytes according to the method of Greenberg et al. (1987).

PCR amplification of genomic exons 4–8 for sequencing.—This was performed essentially as described by Saiki et al. (1988) by using sets of primers complementary to intronic sequences flanking each exon. For exon 4, the primers were 5′-AAGTCTCTCTTCCTTCCCAA and 3′-TCACTTGCGAGGATACCTGG. Each 100-μl reaction mixture contained PCR buffer, 0.01% gelatin, 20 nmol each dNTP, 100 pmol each primer, 1 μg DNA,

and 2.5 units of *Taq* polymerase and was covered with 100 µl of mineral oil. The reaction was carried out cyclically, as above, with denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. At the end of the 25th cycle, the reaction was incubated at 72°C for 7 min. The PCR products were extracted with chloroform, analyzed on a 2% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME), blunt-ended with T_4 polymerase, subcloned into the *Sma*I site of M13 mp19, and sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1980) using ^{35}S-dATP and a universal primer.

PCR amplification of exon 4 for double digestion with HinfI and HaeIII.—Exon 4 was amplified as above, with the following minor modifications: (1) the primers used differed from but overlapped those defined above; (2) the reaction mixture contained 1 unit of *Taq* polymerase, 50 pmol of each primer, and 100 µg BSA/ml in place of 0.01% gelatin; (3) the annealing phase was carried out at 56°C; and (4) 35 amplification cycles were used. A portion of the reaction products (~100 ng DNA) was exposed to 10 × each of *Hinf*I and *Hae*III, and the digestion products were analyze on an 8% polyacrylamide gel.

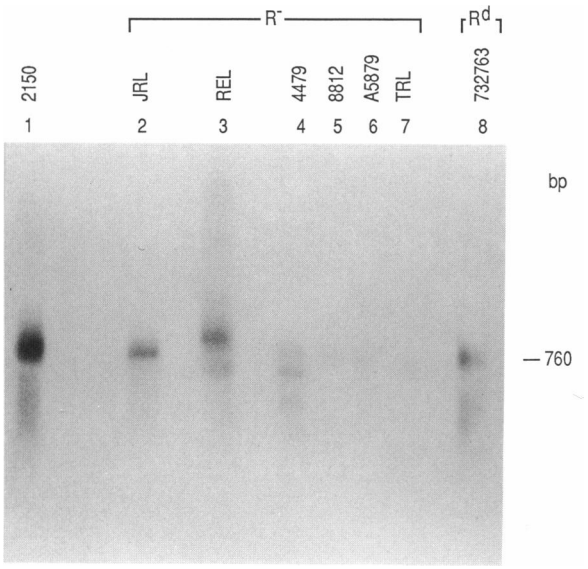

Figure 1 Southern blot analysis of the PCR-amplified androgen receptor cDNA products reverse-transcribed from total RNA in the genital skin fibroblasts of 2150 (a control), JRL (an affected sibling), and a variety of unrelated subjects with androgen resistance. R^- = receptor negative; R^d = receptor deficient. Among the former, 8812 has a deletion of the androgen receptor gene (Pinsky et al. 1989), a deletion that will be described in detail in a forthcomong paper.

Results

Analysis of Androgen Receptor mRNA Expression

To determine whether the mutant androgen receptor gene was expressed, RNA was extracted from genital skin fibroblasts of one affected sibling (JRL), reverse transcribed, and amplified by PCR using primers A and B. Subsequently, the PCR product was analyzed by Southern blotting using a piece of androgen receptor cDNA designated as probe hAR-1 by Brown et al. (1988). As can be seen in figure 1, the size of the amplified product of JRL (lane 2) was identical to that derived from a control RNA sample (lane 1). This indicated that gross alterations in the gene, or major alterations of its transcription, could not be the cause of the absent receptor activity and the consequent androgen insensitivity.

Analysis of Exon 4

In the belief that JRL's lack of androgen receptor-binding activity is the result of a minor sequence alteration in one or another of the five exons that encode the androgen-binding domain of the androgen receptor, each of these exons was amplified between specific intronic primers and sequenced. Figure 2 shows the only sequence alteration found in the sense strand: a guanine-to-adenine transition, at nucleotide (nt) 2682 of exon 4, that changes the sense of codon 717 from TGG (Trp) to TGA, a translation stop signal. The position numbers are those given by Chang et al. (1988). A confirmatory cytosine-to-thymine substitution was observed in the antisense strand.

The sequence alteration predicted the abolition of a recognition site for *Hae*III (Fig. 3). To confirm the

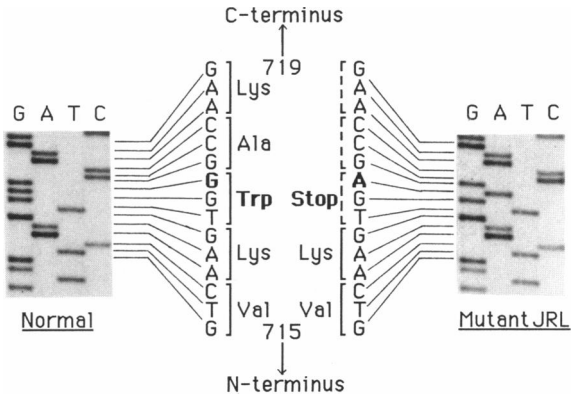

Figure 2 Partial sequence of androgen receptor exon 4 showing the guanine-to-adenine substitution that changes the sense of codon 717 from Trp (TGG) to a translation stop signal (TGA).

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ATTCAAGTCTCTCTTCCTTCCCAATAG CC  CGG AAG CTG AAG AAA CCT GGT AAT CTG
                            la  Arg Lys Leu Lys Lys Leu Gly Asn Leu

AAA CTA CAG GAG GAA GGA GAG GCT TCC AGC ACC ACC AGC CCC ACT
Lys Leu Gln Glu Glu Gly Glu Ala Ser Ser Thr Thr Ser Pro Thr

GAG GAG ACA ACC CAG AAG CTG ACA GTG TCA CAC ATT GAA GGC TAT
Glu Glu Thr Thr Gln Lys Leu Thr Val Ser His Ile Glu Gly Tyr

GAA TGT CAG CCC ATC TTT CTG ATT GTC CTG GAA GCC ATT GAG CCA
Glu Cys Gln Pro Ile Phe Leu Asn Val Leu Glu Ala Ile Glu Pro
                                                *
GGT GTA GTG TGT GCT GGA CAC GAC AAC AAC CAG CCC GAC TCC TTT
Gly Val Val Cys Ala Gly His Asp Asn Asn Gln Pro Asp Ser Phe

GCA GCC TTG CTC TCT AGC CTC AAT GAA CTG GGA GAG AGA CAG CTT
Ala Ala Leu Leu Ser Ser Leu Asn Glu Leu Gly Glu Arg Gln Leu

                      A      Hae III

GTA CAC GTG GTC AAG TGG GCC AAG GCC TTG CCT G GTAAGGAAAAGGGAAGTGGG
Val His Val Val Lys Trp Ala Lys Ala Leu Pro G
                    717    STOP                       * Hinf I

AGCATGAGATAAGGGGGATCATATTTAGTGGAACGC
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Figure 3 Nucleotide and amino acid sequences of exon 4 of the androgen receptor gene, with portions of its flanking introns. The guanine-to-adenine substitution at codon 717 is highlighted in boldface type. The underlines identify the set of neighboring *Hae*III recognition sequences. The arrows delimit the 6 nt that separate the cleavage sites in each *Hae*III sequence. The asterisk identifies the unique *Hinf*I site. The mutation eliminates the 5′ *Hae*III sequence and predicts a *Hinf*I-*Hae*III fragment of 78 nt.

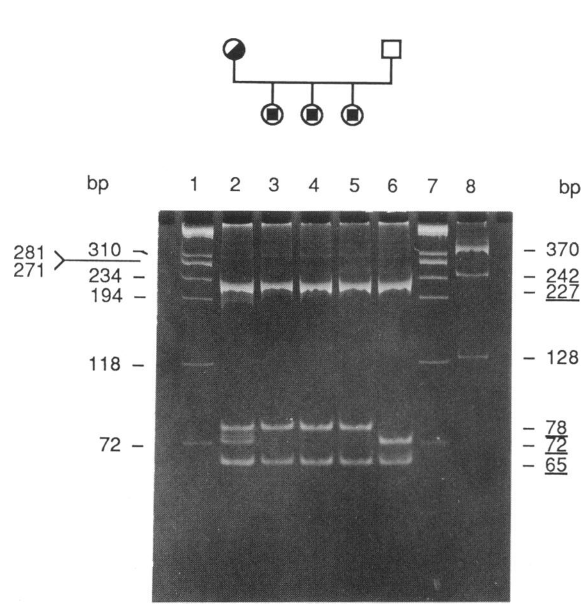

Figure 4 Polyacrylamide gel analysis of PCR-amplified exon 4 double-digested with *Hinf*I and *Hae*III. The three affected siblings (lanes 3–5) only have the mutant 78-nt fragment. Their father (lane 6) only has the normal 72-nt fragment. Their mother (lane 2), an obligate heterozygote, has both fragments. All members of the family have the 227-nt and 65-nt flanking fragments. The 6-nt *Hae*III-*Hae*III fragment generated by the normal allele is not demonstrable. The size markers in lanes 1 and 7 were generated by *Hae*III digestion of ΦX174RF. The size markers in lane 8 represent both the undigested PCR product containing exon 4 (370 nt) and two fragments of it (242 nt and 128 nt) generated by digestion at a unique exonic restriction site.

alteration detected by sequencing, exon 4 from genomic DNA of JRL, of her two affected siblings, and of their parents was amplified and digested with *Hinf*I and *Hae*III. Exon 4 has a single *Hinf*I site (fig. 3), and the sequence alteration eliminates an *Hae*III site 6 nt upstream of a second *Hae*III site (fig. 3). Hence, double-digested mutant exon 4 should yield a *Hinf*I-*Hae*III fragment that is 78 nt long instead of 72 nt long.

Figure 4 shows that exon 4 of JRL and of her affected siblings yielded only the mutant fragment. That of their father yielded only the normal fragment; their mother's DNA yielded the mutant and the normal fragment, indicating that she is heterozygous for the mutant allele.

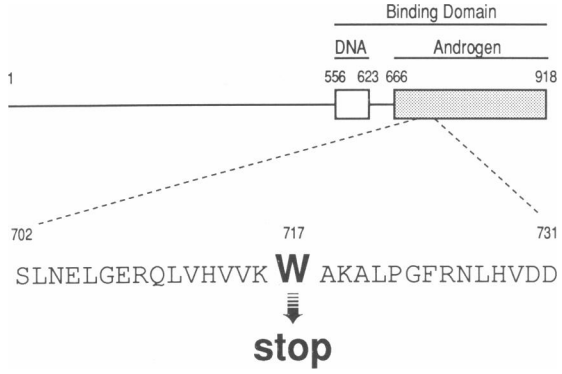

Figure 5 Location and content of the Trp_{717}→stop mutation in exon 4 of the androgen receptor gene in the family.

Discussion

The applicaiton of molecular-genetic technology has permitted us to define in the androgen receptor gene a point mutation that is the cause of complete androgen insensitivity in three 46,XY siblings of a French-Canadian family. The mutation, TGG(Trp)→TGA (stop), occurred at codon 717 in exon 4; its location and context in the structural organization of the androgen receptor are summarized in figure 5.

It is noteworthy (see fig. 6) that the homologous position is conserved as Trp in the receptors for progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and estrogen and that the mutation is found in a region of the androgen receptor that is ultra-conserved in respect to its amino acid homology with the PR, GR, and MR (Chang et al. 1988 and references therein).

Constitutive transcription-regulating activity has been observed in in-vitro expression experiments on laboratory-generated carboxy-terminal-deleted glucocorticoid receptors that lack all or most of their steroid-binding domains (Godowski et al. 1987; Hollenberg et al. 1987). Comparable experiments would have to be performed on the present mutant receptor to determine whether it is constitutively active in vitro. However, the mutant phenotype of the hemizygotes we have studied clearly indicates that their truncated androgen receptor is not constitutively active in vivo, at least at a level necessary for even a minimal degree of male sexual differentiation.

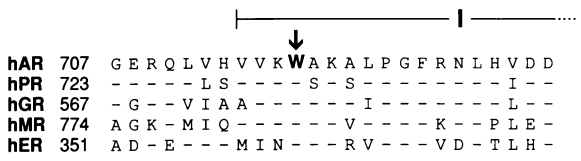

Figure 6 Location of the Trp_{717}→stop mutation in exon 4 of the androgen receptor gene in relation to its amino acid homology with other members of the steroid receptor family. The top bar indicates a region of high homology among the AR, PR, GR, and MR that compose a subfamily.

The fortuitous positions of the unique *Hinf*I recognition sequence in exon 4, and of the *Hae*III recognition sequence that is abolished by the mutation, combine to yield a mutant *Hinf*I-*Hae*III fragment that readily identifies the PCR-amplified mutant allele. Thus, all three allelic combinations at the locus are detectable.

We wish to emphasize a clinical/endocrine sign of the heterozygous carrier state that was present in the mother of this family: delayed menarche. It is observed only in a minority of female heterozygotes, presumably a reflection of random X-chromosome inactivation, but it is a pathognomonic indicator of receptor-defective androgen resistance that we have recorded in four of 26 families (Kaufman et al. 1976; M. Kaufman, C. Straisfield, and L. Pinsky, unpublished data). Furthermore, it strongly indicates that androgens have a role in resetting the normal hypothalamic-pituitary gonadostat of pubertal females. Such a role is not mentioned in a recent review (Odell 1989).

Acknowledgments

We thank Nelly Sabbaghian and Francine Busschaert for faithful assistance. This work was supported by NIH grants DK37694 (S.L.) and HD06308 (R.L.R.), by the Medical Research Council of Canada Group Grant in Genetics (L.P.), by the Dutch Organization for Scientific Research (Medigon-NWO), and by the Dutch Cancer Foundation.

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