Complete Androgen Insensitivity Due to Deletion of Exon C of the Androgen Receptor Gene Highlights the Functional Importance of the Second Zinc Finger of the Androgen Receptor *in Vivo*

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Androgen-dependent gene transcription is mediated by the androgen receptor (AR) through interaction of its central zinc finger region with specific DNA sequences on target genes. Failure of this receptor-mediated gene transcription results in end organ resistance to androgens—the androgen insensitivity syndromes. In a pair of siblings with complete androgen insensitivity who had supranormal levels of androgen binding in genital skin fibroblasts, polymerase chain reaction and Southern blot analysis of the androgen receptor gene confirmed by polymerase chain reaction and sequence analysis of AR cDNA, revealed an in-frame deletion of exon C encoding the second zinc finger of the receptor. The mutant receptor in cultured genital skin fibroblasts had normal androgen binding affinity and was localized in the nucleus but had markedly reduced DNA-binding affinity. When recreated *in vitro* and tested in a cotransfection assay system the mutant receptor failed to activate transcription of an androgen-responsive reporter gene. This naturally occurring mutation highlights the functional dependence of the AR upon its second zinc finger *in vivo* and explains the complete insensitivity to androgen manifest by the affected individuals despite increased androgen binding. The elevated AR levels in the subjects' genital skin fibroblasts further suggests a possible role for the second zinc finger in autoregulation of receptor levels *in vivo*. (Molecular Endocrinology 6: 1103–1112, 1992)

0888-8809/92/1103-1112$03.00/0
Molecular Endocrinology

INTRODUCTION

Human male sexual differentiation and development are critically dependent upon androgen-driven activation of maleness-determining genes of unknown identity. Failure of normal embryonic induction of such genes in karyotypically 46,XY individuals can result either from inadequate androgen levels or from inadequate response to normal androgen levels and leads to defective fetal masculinization. The major cause of impaired response to androgen is defects of the androgen receptor (AR)—the androgen insensitivity syndromes (AIS). This heterogeneous group of receptor abnormalities produces varying degrees of AR dysfunction accompanied by a constellation of phenotypic abnormalities ranging from mild hypospadias through significant genital ambiguity to complete failure of masculinization resulting in a female external phenotype.

Quantitative and qualitative abnormalities of androgen binding in genital skin fibroblasts have implicated

the AR in the pathogenesis of AIS for many years (1–3). AIS has traditionally been classified as receptor-negative or receptor-positive depending upon the absence or presence of specific high affinity androgen binding in cultured genital skin fibroblasts (4–7). Up to 35% of individuals with complete AIS have normal androgen binding capacity in genital skin fibroblasts (4–8) often associated with qualitative binding abnormalities such as reduced affinity (5, 7), rapid ligand dissociation (7, 9), thermolability (7, 9, 10), and altered binding specificity (7).

The AR is comprised of distinct functional domains (11) encoded by an X-chromosomal gene containing eight exons [designated A–H (12) or 1–8 (13)]: the most 5′ exon (exon A or 1) encodes the N-terminal transcription-regulating domain of the receptor (14); exons B and C (2 and 3) encode the central DNA-binding zinc finger domain (12); exon D (or 4) encodes the hinge region that contains a signal for nuclear translocation of the receptor (14); the 3′ portion of exon D and exons E–H (5 to 8) encode the androgen binding domain (12). Examination of the AR gene in AIS-affected subjects has revealed that underlying the wide spectrum of androgen-binding abnormalities is an equal diversity of molecular defects in exons encoding the androgen-binding domain (12, 15–22). The majority are single base pair mutations that alter an amino acid (12, 16–19), introduce a premature termination codon (20, 21), or cause aberrant mRNA splicing (22).

In a small number of cases of AIS (4, 6) androgen binding in cultured genital skin fibroblasts is apparently qualitatively as well as quantitatively normal, indicating an abnormality in some facet of AR target gene activation distal to androgen binding. The lack of androgen binding abnormality in these cases prompted their designation as postreceptor defects (9, 23); however, the discovery of point mutations in exons B and C (24–26), encoding the receptor zinc fingers, indicates that this form of AIS can result from abnormalities in the DNA binding domain. To further examine the basis for androgen resistance in the presence of normal androgen binding, we studied a pair of siblings with complete AIS who had supranormal levels of high affinity androgen binding in cultured genital skin fibroblasts (27). Analysis of the AR gene defect in these subjects provides an explanation for the functional failure of the AR in the presence of elevated androgen binding; it highlights the importance of the second zinc finger of the AR in mediating receptor-DNA-binding, and thus androgen-dependent transcriptional activation *in vivo*, and suggests a possible role for the second zinc finger in autoregulation of receptor levels.

RESULTS

Deletion of Exon C of the AR Gene in AIS-Affected Siblings

Elevated levels of qualitatively normal androgen binding were demonstrated in previous studies of cultured genital skin fibroblasts of the two 46,XY siblings with complete AIS who were the subjects of this study (27) (see *Materials and Methods*). Thus we postulated an abnormality outside the receptor androgen-binding domain. Analysis of genomic DNA of the two affected siblings revealed a deletion of exon C of the AR gene, encoding the second zinc finger of the receptor DNA-binding domain. This exon failed to amplify from genomic DNA of the subjects after 30 cycles of polymerase chain reaction (PCR) using oligonucleotide primers flanking exon C (Fig. 1A, lanes 1–4). Relatives and unrelated control individuals demonstrated the expected PCR product of 413 base pairs (bp). Amplification of exons A, B, and D-H resulted in the expected sized products in the two AIS subjects and in relatives

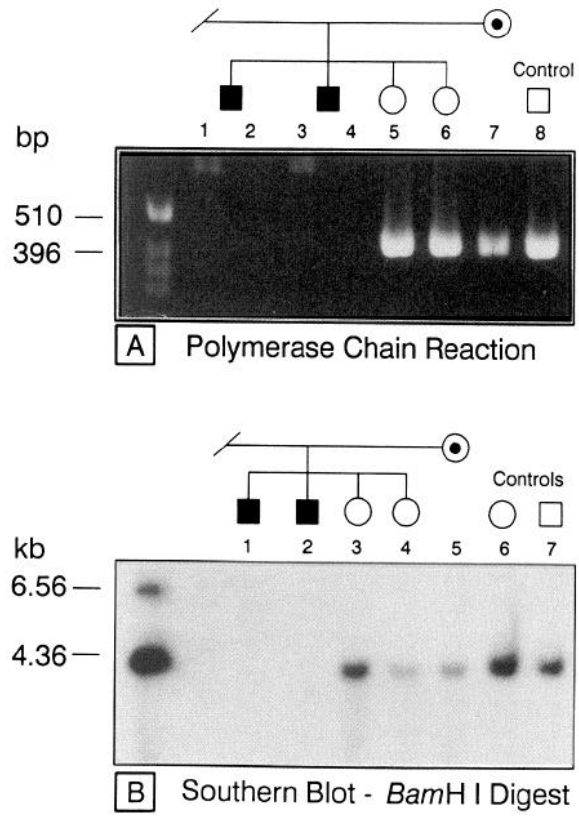

Fig. 1. Evidence of Deletion of Exon C of the Androgen Receptor Gene

■, 46,XY AIS-affected; □, 46,XY normal male; ○ 46,XX female; ⊙, obligate carrier mother. A, PCR amplification of genomic DNA using intron-located primers specific for amplification of exon C. Products of the PCR were electrophoresed through 1.4% agarose and stained with ethidium bromide. Lanes 1–4 are duplicate PCR reactions for each of the affected children, showing absence of the expected 413-bp PCR product. This product is present in lanes 5 and 6 (46,XX siblings), 7 (carrier mother), and 8 (unrelated control). B, Autoradiograph of Southern blot of *Bam*HI-digested genomic DNA (10 μg), hybridized with ^{32}P-labeled exon C-specific probe. The expected band at about 4.4 kb is absent from lanes 1 and 2 (the affected children) but present in lanes 3–7 (siblings, mother, and unrelated controls).

and controls (data not shown). Autoradiographs of Southern blots of *Bam*HI-digested genomic DNA hybridized with a ^{32}P-labeled probe encompassing exon C showed a band at about 4.4 kilobases (kb) in digests of genomic DNA from relatives and controls. This band was absent from digests of DNA from the two affected children (Fig. 1B, lanes 1 and 2). Similarly, the expected 9.4-kb band produced by hybridization of *Eco*RI-digested genomic DNA with the exon C probe was absent from the genomic digests of both affected children (data not shown). When blots were rehybridized with a factor IX cDNA probe (28), all subjects displayed the expected bands at 25 kb on *Bam*HI digestion and 12.5 kb on *Eco*RI digestion, indicating equivalence of DNA digestion, loading, and transfer (not shown).

Complementary DNA produced by reverse transcription of poly(A)$^+$ RNA obtained from genital skin fibroblasts of one of the affected siblings and an unrelated control was amplified by PCR using primers with sequence from exons B and E (Fig. 2). Amplification of control cDNA resulted in a PCR product of the predicted size of about 660 bp. In contrast, a product of about 540 bp resulted from amplification of the subject's cDNA. The size difference between control and subject PCR product reflected the absence of the 117-bp exon C (Fig. 2). The same size difference between subject and control PCR product was seen when cDNA was amplified with primers from exons A and G (not shown). Dideoxy sequencing of the subject's amplified cDNA demonstrated direct continuity of sequence from exon B to exon D, indicating that correct splicing had occurred between these exons with no disturbance of the translational reading frame.

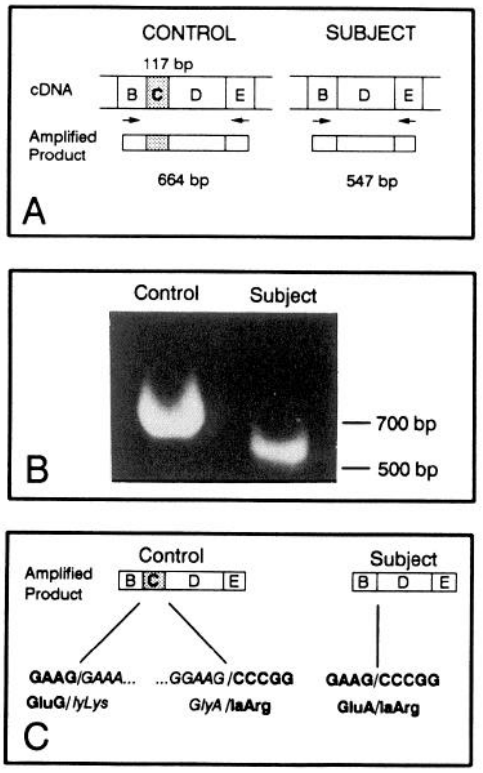

Fig. 2. PCR and Sequence Analysis of cDNA

A, PCR was carried out using as template either control (*left*) or subject (*right*) cDNA. PCR primers designed from sequences in exons B and E produce a 664-bp product when used with control cDNA. A 547-bp product was predicted for amplification of subject cDNA (reflecting absence of the 117-bp exon C). B, Result of 30 cycles of PCR using 250 ng control (*left*) and subject (*right*) cDNA. Amplification products were electrophoresed through 1.2% agarose and stained with ethidium bromide. The expected sized products are seen for control (at around 660 bp) and subject (around 540 bp). C, Dideoxy sequencing of control (*left*) and subject (*right*) PCR products cloned into pGEM 3Zf(+). There was direct continuity of sequence between exons B and D (*bold type*) in the subject's cDNA, confirming the in-frame deletion of exon C. The resulting amino acid sequence in the vicinity of the splice junction is shown below the nucleotide sequence for control and subject.

The Zinc Finger-Deficient Receptor Localizes in the Nucleus But Has Reduced DNA Binding and is Transcriptionally Inactive

Deletion of the second zinc finger of the AR predicts a number of possible consequences for receptor function. Subcellular localization of receptor could potentially be disturbed either by the structural alteration of the receptor or by a change in its DNA-binding characteristics. However, as demonstrated in Fig. 3, immunoperoxidase staining using the primary immunoglobulin G (IgG) fraction of antibody AR 32 [directed to the N-terminal region of the AR (29)], demonstrated predominant nuclear localization of AR in both control (30) and subject genital skin fibroblasts in the presence and absence (not shown) of 50 nM R1881.

Second, it was expected that deletion of the second zinc finger would disrupt receptor-DNA binding and consequently target gene transcriptional activation. AR-DNA binding was examined by partition sedimentation analysis (31–33) of binding of [^{3}H]dihydrotestosterone (DHT)-AR complexes to the mouse mammary tumor virus-long terminal repeat sequence (MMTV-LTR) that contains hormone response element (HRE) nucleotide sequences previously shown to be androgen responsive (34). AR extracted from control fibroblasts demonstrated high affinity [dissociation constant (K_d) = 0.1 nM] and low affinity (K_d = 2.7 nM) components of DNA binding. Subject AR showed only low affinity DNA binding (K_d = 5.0 nM) (Fig. 4).

Transcriptional activation by the zinc finger-deficient AR was tested by transient transfection of the mutant AR cDNA recreated in the eucaryotic expression vector pCMVhAR (34) using PCR mutagenesis methods (14, 35). CV1 cells were cotransfected with the reporter plasmid pC29GtkCAT (36) that contains two glucocorticoid response elements [shown to be androgen responsive (14)] spaced 29 bp apart and positioned 5′ of the thymidine kinase promoter and the chloramphenicol acetyl transferase (CAT) gene, together with either the normal (pCMVhAR)- or mutant (pCMVhARdelC) AR

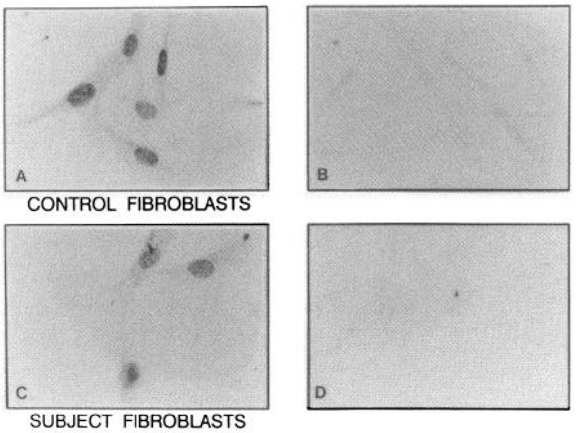

Fig. 3. Immunocytochemical Localization of AR in Cultured Genital Skin Fibroblasts

Cultured genital skin fibroblasts plated into chamber slides were incubated for 24 h in 50 nM R1881, fixed, treated with 2 μg/ml primary IgG fraction of a polyclonal antibody raised against a sequence in the N-terminal region of the AR (AR 32), and stained by the avidin-biotin-peroxidase method. Antibody-bound AR is dark-staining material concentrated in the nuclei of control (A) and subject (C) cells. This staining is abolished by incubation with immune IgG preadsorbed with AR peptide (B and D).

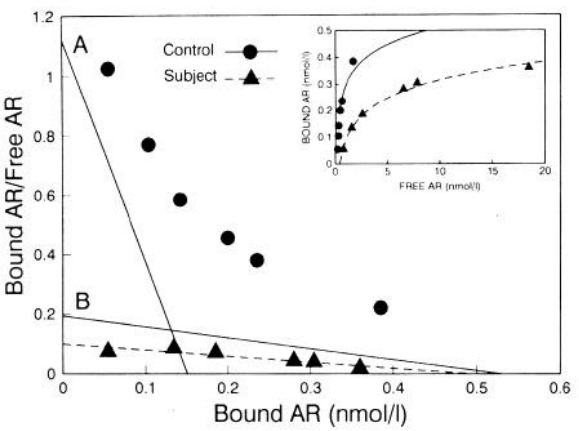

Fig. 4. AR Binding to MMTV-LTR Sequence

Crude nuclear extracts from control and subject fibroblasts were incubated with [^{3}H]DHT and [^{3}H]DHT-AR complexes obtained by elution from an affinity matrix. The labeled complexes were incubated with linearized pMSG-CAT, and [^{3}H]AR bound to DNA was separated from unbound [^{3}H]AR by fractionation in 5–20% sucrose gradients. Binding data are shown as Scatchard plots (*main graph*) and saturation curves (*inset*). Vectoral analysis of control data (●) by the method of Rosenthal (73) suggests high affinity (A, K_d = 0.1 nM) and low affinity (B, K_d = 2.7 nM) components of DNA binding. The subject AR shows only low affinity DNA binding (▲, *dashed line*, K_d = 5.0 nM).

expression vector. Expression of normal human AR cDNA in the presence of 50 nM R1881 (methyltrienolone), a saturating concentration of androgen, produced greater than 30-fold androgen-dependent activation of CAT activity. In contrast, expression of the second zinc finger-deletion mutant under the same conditions failed to induce activation of the CAT gene (Fig. 5). Control transfection with the parent vector [pCMV5 (37)], without insert, produced no CAT activation. The recreated mutant AR expressed in COS 7 cells displayed high affinity binding of [^{3}H]R1881 (K_d = 0.16 nM), similar to that of the normal AR (K_d = 0.18 nM). Furthermore, immunoblots of extracts from COS 7 cells transiently expressing the normal or mutant AR demonstrated

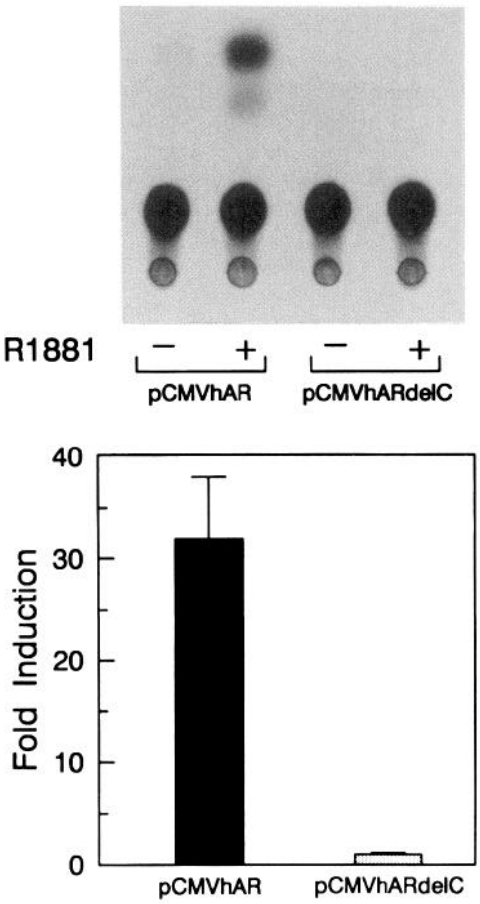

Fig. 5. Transcriptional Activation Function of Zinc Finger-Deficient AR Recreated by PCR Mutagenesis

Normal (pCMVhAR) or exon C-deleted (pCMVhARdelC) AR cDNAs cloned into the pCMV5 expression vector were used in cotransfection assays to determine the androgen-dependent transcriptional activation function of the expressed ARs. The androgen-responsive reporter plasmid pC29GtkCAT was co-transfected into CV1 cells with the AR vector of interest. *Top*, Autoradiograph of a representative TLC plate. Cells were incubated in the absence (−) or presence (+) of 50 nM R1881. The pCMVhAR vector, containing the normal AR cDNA (lanes 1 and 2) shows marked androgen-dependent CAT activation, while the pCMVhARdelC vector, containing the exon C-deleted AR cDNA (lanes 3 and 4) shows no CAT activity. *Bottom*, Bar graph displaying fold induction of CAT activity by AR vectors. The normal AR (pCMVhAR) induces CAT by 32- ± 6-fold, while exon C-deleted AR (pCMVhARdelC) shows no induction (1.0 ± 0.1). Results represent the mean + SD of three experiments.

equivalent levels of receptor expression, indicating that the failure of AR-mediated *trans*-activation was not a function of reduced expression of the mutant AR compared with the normal AR (data not shown).

DISCUSSION

Because hormone-binding is the primary event in AR-mediated target gene activation, it is not surprising that abnormalities of AR androgen binding result in defective target gene expression. In keeping with the high frequency of androgen-binding abnormalities in AIS, the great majority of cases in which a molecular defect has been determined are associated with abnormalities in exons encoding the androgen-binding domain of the receptor (12, 15–22). Definition of the molecular defect in cases of AIS in which all characteristics of androgen binding are normal has been more elusive. This report illustrates such an abnormality—a defect outside the hormone-binding domain that functionally incapacitates the receptor.

The affected siblings described in this report displayed complete androgen resistance despite supranormal concentrations of qualitatively normal androgen binding (27) and normal nuclear localization of the AR in genital skin fibroblasts. Examination of the AR gene and cDNA revealed a deletion of exon C, resulting in splicing of exon B to exon D, without disturbance of the translational reading frame. The deletion of exon C predicts a receptor protein that lacks 39 amino acids encompassing the second zinc finger, but retains the hinge region and androgen-binding domain; the retention of these regions explains the normal nuclear localization and high affinity androgen binding of this receptor. This mutant receptor is, however, defective in target gene activation, evidenced *in vivo* by the complete absence of response to androgen—the total failure of masculinization of the karyotypically 46,XY affected siblings—and *in vitro* by the failure of the recreated zinc finger-deficient AR to activate transcription of an androgen-responsive reporter gene in cotransfection assays. The loss of *trans*-activating ability of this naturally occurring mutant receptor probably results from severe impairment of receptor interaction with HRE nucleotide sequences at target genes, as evidenced by the decreased affinity of the mutant AR extracted from genital skin fibroblasts for the HRE-containing MMTV-LTR sequence.

This study highlights the importance of the second zinc finger of the AR in target gene activation *in vivo* and provides some insight into other functional roles of this region of the receptor. Although no similar natural deletion has previously been reported, single amino acid alterations in the zinc fingers of the AR (24–26) and of the vitamin D receptor (38, 39) have been described in association with hormone resistance *in vivo* and diminished transcriptional activation when recreated *in vitro*. Transcriptional activation by the AR and many other steroid receptors follows a series of coordinated events. Hormone binding, receptor activation, and nuclear translocation are followed by interaction of the hormone-receptor complex, apparently as a dimer, with specific enhancer-like nucleotide sequences—HREs—in the vicinity of target genes to modulate activity of target gene promoters (40).

Both zinc fingers of the DNA-binding domain are absolutely required for transcriptional regulation *in vitro* (41, 42). Mutagenesis (41–44) and crystallographic (45) studies of other steroid receptors have revealed that the two zinc fingers are structurally and functionally distinct subdomains. The first finger is responsible for recognition of the specific target DNA sequence (42–44). The second finger orients the receptor for DNA binding (43), stabilizes DNA-protein interaction (42, 43), contains a site directly involved in transcription activation (46), and provides the dimerization interface (45). Dimerization and DNA binding appear to be intimately related processes. Crystallographic analysis of the DNA-binding domain of the glucocorticoid receptor (GR) has revealed that DNA binding induces dimerization, placing the dimer subunits in adjacent major grooves on the DNA (45). Other studies in GR, however, have shown that dimerization of receptor is the primary event, essential for specific high affinity receptor-DNA binding (47), and that the GR exists as a dimer even in the absence of DNA (48). The progesterone (49) and estrogen receptors (50) have also been shown to interact with their HREs as dimers, and the AR presumably functions similarly. Although one study (51) has defined a region within the steroid-binding domain as critical for dimerization, the major determinant of dimerization is probably the second zinc finger (47). Thus it is likely that the second zinc finger-deficient AR described in this report is incapable of dimerization and thus of forming the stable high affinity interactions with target DNA necessary for transcriptional activation; the absence of high affinity DNA binding demonstrated in this mutant receptor is consistent with this speculation.

In contrast to its critical role in the DNA binding essential for transcriptional activation, the second zinc finger does not appear to be required for nuclear localization of the receptor; fibroblasts from these AIS-affected siblings showed normal nuclear retention of [^{3}H]DHT (27) and normal nuclear localization of the AR in immunocytochemical studies. Although a hormone-dependent nuclear localization signal has been described within the second zinc finger of the progesterone receptor (52), our data from this mutant AR indicate that neither nuclear transport nor nuclear retention of AR are qualitatively dependent upon sequences within or functions of the second zinc finger. This is consistent with *in vitro* data indicating that a major nuclear localization signal in the AR resides in the hinge region downstream of the second zinc finger (14) and parallels the observation in the progesterone receptor and estrogen receptor (52, 53) that the hinge-region signal plays the dominant role in mediating receptor nuclear transport.

An intriguing feature of this mutant AR is its supranormal concentration in cultured genital skin fibroblasts of the affected siblings (27). One possible explanation for this finding is failure of autologous down-regulation, a normal feature of AR (54, 55) and other steroid receptors (56–59). Receptor down-regulation is thought to be mediated by a decrease in the rate of transcription or the half-life of receptor mRNA (55, 57, 59). The mechanism involved is unknown, however it may be analogous to receptor-mediated inhibition of target gene transcription. This has been shown to be a function of the DNA-binding domain in the GR (46, 60) with which the AR shares high sequence homology. The testicular feminized (*Tfm*) rat, an animal model of androgen insensitivity in which a single amino acid alteration in the androgen-binding domain severely disturbs AR function (61), does not show the normal androgen-dependent down-regulation of AR mRNA seen in the wild type (54). Similarly, although we have not quantified AR mRNA in this study, the elevated receptor levels found in our subjects' genital skin fibroblasts could perhaps reflect an inability of the zinc finger-deficient receptor to mediate down-regulation of AR mRNA.

No other natural intragenic exon deletion has been described in the AR gene or other steroid receptor genes; however, this type of mutation is common in the huge X-chromosomal gene encoding the structural muscle protein, dystrophin. Deletions that disturb the translational reading frame of this gene produce the severe Duchenne form of muscular dystrophy, while in-frame deletions that presumably result in semifunctional internally deleted proteins cause the milder Becker form (62). Analysis of deletions in the dystrophin gene has revealed that the disruption or maintenance of the reading frame by an intragenic exon deletion depends upon the nature of the exon-intron borders of the exons flanking the deletion: only deletions that juxtapose two exons with borders at the same position in the triplet will maintain the reading frame. In the AR gene only exons C and D are flanked by exons with borders which, when spliced, produce a complete triplet (*i.e.* B/D or C/E). Consequently, deletion of exons other than these will produce a frameshift mutation and thus a severely truncated protein. In the dystrophin gene the deletion breakpoints have been shown to cluster within the larger introns of the gene (63). Analogous to this, the potential for deletion of exons C or D of the AR gene may be increased by the fact that both are flanked by the largest introns in the gene, approximately 10–15 kb each (13; Lubahn, D. B., and J. A. Simental, unpublished observations). Because of this large size of the introns flanking exon C, we have not attempted to map the deletion breakpoints.

With the definition of the molecular basis of many AR defects, the traditional receptor-positive *vs.* receptor-negative terminology has become inaccurate. The term receptor-negative, which has been liberally applied to AIS cases characterized by absence of androgen binding, now accurately applies only to instances in which no AR protein is produced—complete deletion of the AR gene (64, 65), or other defects such as premature termination codons (13, 20, 21) or aberrant splicing (22) that may disrupt mRNA stability and thus receptor protein production. The term postreceptor defect is equally unenlightening. Mutations within the DNA binding domain such as described here and elsewhere (24–26), although having no effect on androgen binding, nonetheless produce a severely dysfunctional receptor. Another class of mutations as yet undefined could include abnormality of the promoter region of a critical AR target gene or dysfunction of some other factor specifically required for efficient target gene transcription by the AR. These, if discovered, would represent the true postreceptor defects.

MATERIALS AND METHODS

Subjects

The clinical details of the affected children and their unaffected relatives have been previously described (27). The index subject is a phenotypic female with 46,XY karyotype. She presented at 10 days of age with bilateral inguinal herniae, each of which contained a testis. There was no clitoromegaly or other clinical evidence of androgen effect. One of three older sisters was subsequently found to also have a 46,XY karyotype. She entered puberty at 12.8 yr and progressed to full feminization but developed no pubic hair. The testes were removed at the completion of puberty. The younger child is currently prepubertal at 12 yr of age. Both children underwent genital skin biopsy for androgen binding studies on cultured fibroblasts.

Previous analysis demonstrated a 2.5-fold elevation of total cellular AR concentration in genital skin fibroblasts of these children compared with controls. The [^{3}H]DHT binding affinity, thermostability, and dissociation rate of the DHT-receptor complex were normal, and the proportion of whole cell [^{3}H]DHT binding located in the nuclear fraction was 60% both for the subjects' cells and a normal fibroblast strain (27).

Methods

Polymerase Chain Reaction Approximately 0.5–1.0 μg genomic DNA extracted from leukocytes of subjects, female relatives, and unrelated controls by standard methods were used in each 50- or 100-μl PCR reaction. Twelve sets of oligonucleotide primers 23–29 bp in length were used in PCR to amplify the eight exons of the AR gene as previously described (12, 66). Products of the PCR (285–455 bp in length) were examined by ethidium bromide staining after electrophoresis in 1.2–1.4% (wt/vol) agarose gels.

The exon C-specific probe for Southern analysis was prepared by PCR of exon C using control genomic DNA as template. The product, purified by electroelution from an agarose gel slice, was ^{32}P-labeled using a random prime kit (Boehringer Mannheim, Indianapolis, IN).

Southern Blot Analysis Genomic DNA (10 μg) of subjects, female relatives, and controls was digested separately with restriction endonucleases *Eco*RI and *Bam*HI (BRL, Gaithersburg, MD) for 16 h under reaction conditions specified by the manufacturers. DNA was separated on 1% (wt/vol) agarose gels and transferred to GeneScreen Plus (NEN, Boston, MA) in 0.4 M NaOH buffer. Prehybridization, hybridization, and washing of the membranes were as previously described (15). The ^{32}P-labeled probe was used at 10^6 cpm/ml solution in hybridizations. Membranes were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with intensifying

screens at −70 C for up to 10 days. To control for equivalence of DNA digestion, loading, and transfer, the blots were washed free of probe according to the manufacturer's specifications and rehybridized with a ^{32}P-labeled *Xba*I fragment encompassing exon 1 of the factor IX gene (28).

Analysis of cDNA Genital skin fibroblasts obtained at biopsy were grown to confluence in Medium 199 (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.0 mM L-glutamine, and 20 mM HEPES. For total RNA extraction, cells were harvested in guanidine thiocyanate (67) and centrifuged through a cesium chloride gradient. Polyadenylated RNA was isolated by chromatography with oligo(dT) cellulose (Collaborative Research, Bedford, MA) (68). cDNA was produced by reverse transcription from 5 μg poly(A)$^+$ RNA using avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). PCR was performed on 50 ng subject and control cDNA [obtained from a control fibroblast cell strain (16)] using primers designed from sequences in exons B and E (nucleotides 1991–2028 and 2628–2653, respectively) or exons A and G (nucleotides 1844–1869 and 2847–2870, respectively) (numeration according to Ref. 12). The PCR products were electrophoresed through 1.2% agarose and examined for the 117-bp size difference corresponding to exon C. The amplified cDNA from the latter PCR was subsequently purified, cloned into pGEM −3Zf(+) (Promega, Madison, WI), and sequenced by the dideoxy chain termination method (69) using the exon A and G primers detailed above and Sequenase DNA polymerase (United States Biochemical, Cleveland, OH).

Site-Directed Mutagenesis To examine the functional characteristics of the zinc finger-deficient receptor, the mutation was recreated by site-directed mutagenesis using a modification (14) of the PCR technique described by Higuchi (35). The AR expression vector pCMVhAR (34) containing the full-length human AR cDNA cloned into the eukaryotic vector pCMV5 (37) was used as the starting DNA in these experiments. A pair of complementary mutagenic oligonucleotide primers was designed comprising the last 20 bp of 3′-exon B sequence in continuity with the initial 18 bp 5′-exon D sequence, and containing none of the intervening exon C sequence. Two separate primary PCR reactions were performed, each using one of the mutagenic primers and an appropriate upstream or downstream primer with sequence from exon A or H (the external primers). The products of the primary PCR were purified, combined, denatured, and annealed and used as template in the secondary PCR using the two external primers. This produced the large amplified product with the intended deletion. This final PCR product and the starting vector, pCMVhAR, were digested with *Hin*dIII (BRL) and *Csp*45 I (Promega) and the mutant cDNA fragment cloned back into the starting vector to create the exon C-deleted mutant AR vector, pCMVhARdelC, for subsequent use in functional studies. The PCR-generated region of this construct—bounded by the 5′ *Hin*dIII (exon B) and 3′ *Csp*45 I (exon G) cloning sites—was sequenced to exclude PCR errors.

Transient DNA Transfections and Transcription Activation by ARs To examine transcriptional activating ability of the mutant AR, 10-cm dishes containing 1.3×10^6 monkey kidney CV1 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's Modified Essential Medium-High Glucose (DMEM-H; GIBCO BRL) supplemented as above except that fetal calf serum was at 5%. Cells were transfected with 1 μg of either the normal AR expression vector, pCMVhAR, or the exon C deletion mutant AR vector, pCMVhARdelC, using the calcium phosphate precipitation method (61). The reporter plasmid pC29GtkCAT (10 μg) (36), containing two glucocorticoid response elements separated by 29 bp positioned 5′ of the thymidine kinase promoter and the CAT gene (70), was cotransfected. Cells were incubated for 48 h in medium containing 0.2% serum with or without 50 nM R1881, harvested, and assayed as previously described (34) for activity of the CAT enzyme by reaction with D-threo-[1,2-^{14}C]chloramphenicol (ICN Biomedicals, Costa Mesa, CA). The reaction products were separated by TLC and autoradiographs examined for the presence of the faster migrating acetylated products of chloramphenicol. To quantitate the stimulation of CAT activity, regions of the TLC plates containing the acetylated and nonacetylated chloramphenicol were cut out and counted in scintillation liquid (Scintiverse BD, Fisher Scientific, Raleigh, NC) in a liquid scintillation counter. The percent acetylated [^{14}C]chloramphenicol was calculated as (cpm acetylated ÷ cpm total) × 100, for each reaction. The percent acetylated [^{14}C]chloramphenicol in the absence of R1881 was defined as baseline for each vector tested. Fold induction of CAT activity was calculated as percent acetylated in the presence of R1881 ÷ baseline, for each vector. The results are given as mean + SD for three independent experiments, using three separate clones of the pCMVhARdelC construct.

Immunoblots and Androgen Binding of Expressed AR For analysis of mutant AR protein expression and androgen binding, COS 7 cells (American Type Culture Collection) were plated in 10-cm dishes at 1.2×10^6 cells per dish or in 48-well culture plates plated at 50,000 cells per well, respectively, and grown in DMEM-H with 5% fetal calf serum, antibiotics, and 15 mM HEPES (pH 7.2). After 24 h, the cells were transfected with 10 μg/dish or 0.5 μg/well normal (pCMVhAR) or mutant (pCMVhARdelC) AR expression vector using the diethylaminoethyl-dextran method (71). Whole cell androgen binding assays over the range 0.05–5.0 nM R1881 and immunoblots using antibody AR 52, an antibody to the N-terminal region of AR, were performed as previously described (34, 61).

Immunocytochemical Localization of the AR Cultured genital skin fibroblasts from one of the affected siblings and a normal foreskin fibroblast cell strain (30) were plated into two-chamber glass slides in standard supplemented medium as above. After 48 h the medium was changed to serum-free, phenol red-free DMEM-H, with or without the addition of 50 nM R1881. Cells were grown for up to a further 24 h, washed in PBS, and fixed in 2% paraformaldehyde, 10% sucrose, and 0.1 M sodium phosphate, pH 7.2. They were treated with 2 μg/ml of the primary IgG fraction of AR 32 (29) and stained by the avidin biotin peroxidase complex method as previously described (29, 72). To control for specificity of the antibody reaction, duplicate wells were incubated with preimmune IgG or with antibody preadsorbed with the free peptide antigen.

Partition Sedimentation Analysis of AR Binding to DNA The interaction of androgen-AR complexes with the MMTV-LTR sequence was assayed using a zonal sedimentation velocity technique (31–33). Crude nuclear preparations of subject and control genital skin fibroblasts were extracted with 20 mM HEPES pH 7.4, 1 mM EDTA, 1 mM EGTA, 600 mM KCl, 1 mM phenylmethylsulfonyl fluoride, and 20 nM [^{3}H]DHT, centrifuged at 50,000 rpm for 30 min at 4 C and desalted using a Sephadex G25 column. [^{3}H]DHT-AR complexes were prepared by incubation of crude nuclear extracts of subject and control cultured genital skin fibroblasts with [^{3}H]DHT followed by elution from a DHT-17-hemisuccinate affinity matrix (as described in Ref. 31). Plasmid pMSG-CAT (Pharmacia, Knowlhill, Great Britain) that contains androgen response element sequences within the MMTV-LTR sequence was linearized by digestion with *Pvu*I and incubated (approximately 2 nM) with [^{3}H]DHT-receptor complexes (0–20 nM) in reaction mixtures containing 1 mM Na_xPO_4, pH 7.0, 0.1 mM EDTA and 50 mM NaCl. After 60 min incubation on ice, 140-μl aliquots were layered over 5–20% (wt/vol) linear sucrose gradients (5 ml) in buffer as above and centrifuged in a Sorvall TV865 vertical rotor (Dupont UK Ltd., Hertfordshire, UK; r_{av} = 7.83 cm) for 35 min at 65,000 rpm (370,000 × G_{av}). Since [^{3}H]DHT-AR-DNA complexes sediment to the bottom of the gradient at high concentrations of normal AR (20 nM), gradients were fractionated from the bottom, and DNA-bound AR was concentrated in fractions 2–7. AR-DNA binding data were plotted

according to Scatchard and the binding affinities obtained by vectoral analysis of Scatchard plots using the method of Rosenthal (73).

Acknowledgments

We are very grateful to Malcolm V. Lane for excellent and good-natured technical assistance.

Received December 13, 1991. Revision received April 6, 1992. Accepted April 22, 1992.

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This work was supported in part by NIH Grants P30-HD-18968 (Recombinant DNA and Histochemistry Cores), R01-HD-04466, and FY91-0195 from the March of Dimes Birth Defects Foundation.

* Recipient of a Medical Foundation Medical Research Fellowship from the Postgraduate Committee in Medicine, University of Sydney, Australia, and holds the Genentech Clinical Scholar Award, 1991-1993.

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