Functional Characterization of Naturally Occurring Mutant Androgen Receptors from Subjects with Complete Androgen Insensitivity

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Mutations in the androgen receptor (AR) are thought to cause complete androgen insensitivity (CAIS) in 46,XY human subjects who have a female phenotype despite normal adult male concentrations of plasma testosterone. Assays of AR binding in cultured skin fibroblasts from subjects with CAIS show either an apparent absence of AR (AR−) or normal levels of AR (AR+) binding. In several subjects with CAIS, AR−, no gross AR mutation was detected by Southern blot analyses of genomic DNA and normal sized 10 kilobase mRNA was present on Northern blots of poly(A^+) RNA from cultured genital skin fibroblasts. We have used the polymerase chain reaction to amplify individual exons within the human AR gene of subjects with CAIS and have identified point mutations in three subjects. In one AR− subject (R774C), amino acid 774 was changed from arginine (CGC) to cysteine (TGC), in another AR− subject (R831Q), arginine (CGA) was changed to glutamine (CAA) at position 831, and in an AR+ subject (V866M) a methionine (ATG) was substituted for valine (GTG) at position 866.

Transfection of wild type and mutant AR cDNA clones into COS cells results in detection of AR protein by immunoblotting. AR ligand binding activity is absent in cells transfected with AR mutants R774C and R831Q, but present with AR mutant V866M. Androgen binding in cells transfected with AR mutant V866M has a 6-fold lower apparent binding affinity than that of wild-type AR. Transcriptional activation of the MMTV-CAT reporter gene was androgen dependent and specific and nearly maximal at physiological concentrations (10^{-10} M) of androgen when wild-type AR was transfected into cells, whereas neither AR mutants R774C nor R831Q were able to stimulate CAT activity even at 10^{-8} M androgen. AR mutant V866M was able to stimulate CAT activity but the androgen dose dependency was shifted toward pharmacological concentrations of steroid that exceed *in vivo* levels.

The molecular basis of CAIS in humans exhibits genetic heterogeneity. Our study shows that some cases of CAIS are explained by an inability to form a functional AR-steroid complex and hence, the AR is unable to activate transcription of genes essential for male sex differentiation during fetal development. (Molecular Endocrinology 4: 1759–1772, 1990)

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INTRODUCTION

The androgen insensitivity syndrome is an X-chromosome linked disorder expressed in 46,XY human subjects (1). Variable phenotypic expression has been observed, ranging from a complete female phenotype to male genitalia with mild hypospadias (1). Evidence suggests also that the syndrome includes some rare cases of phenotypically normal males with azoospermia (2, 3). In the complete form of androgen insensitivity syndrome (CAIS), affected subjects present a female external genitalia but with a short vagina and absent Mullerian ducts. Gonads are normal-size testes located in the abdominal or inguinal area with hypoplastic Wolffian derivatives. At puberty, normal female secondary sex characteristics occur, but pubic hair is usually sparse or absent. In adulthood, serum testosterone and LH

concentrations are at or above the normal adult male range.

Multiple studies have demonstrated that this syndrome is caused by target organ insensitivity to the actions of testosterone and its more potent 5α-reduced metabolite, dihydrotestosterone (DHT) (1, 4). Although testosterone is able to induce fetal differentiation and development of the Wolffian duct system, the biologically more active androgen, DHT, is required for differentiation and development of the prostate gland and for the male external genitalia (5). Androgenic effects during fetal development require the formation of a biologically active androgen receptor-steroid complex (6). An impairment in androgen receptor (AR) function can account for target organ insensitivity and evidence for such impairment has been reported for numerous families with androgen insensitivity (AIS) (1, 4).

The AR belongs to the subfamily of steroid hormone receptors within a larger family of nuclear proteins that likely evolved from a common ancestral gene (7, 8). Structurally, the 110 kilodalton AR contains an amino terminal region that is variable in length and may have a role in transcriptional activation, a central, cysteine-rich hydrophilic DNA-binding domain, as well as a carboxyl terminal hydrophobic hormone-binding domain. After hormone binding, the DHT-AR complex, probably as a dimer, interacts with hormone response elements on specific target genes to modulate the transcriptional activity of their promoters (9, 10).

The heterogeneity in phenotypic expression of AIS is due to a variety of AR defects, some of which are detectable by biochemical methods (1, 4). For example, AR binding in cultured genital skin fibroblasts of subjects with CAIS may be undetectable, in the so-called receptor negative (AR−) form (11), whereas others have quantitatively normal binding, termed the receptor positive (AR+) form (12). Cloning of human cDNA encoding the AR has been reported (13–16). Using cDNA probes for the human AR gene, we have previously detected a partial deletion in the steroid-binding domain in one family with CAIS, AR− (17). However, five other families with CAIS, AR− did not have any detectable gross molecular lesion in their AR gene, suggesting a point mutation or small undetected deletion or insertion. In one other family with the AR+ form of CAIS (18), we have reported a missense point mutation in the steroid-binding domain of the AR gene that predicted a single amino acid substitution at position 866 of the AR protein (19).

In this report, we demonstrate that mutant ARs resulting from point mutations causing single amino acid substitutions in the steroid binding domain of the protein are functionally defective. In two unrelated families with the AR− form of CAIS, the two different mutant ARs are expressed but do not bind androgen and are defective in transcription activation. In the family with CAIS, AR+, the mutant AR binds androgen with significantly lower affinity and its ability to activate transcription is dependent upon higher concentrations of hormone.

RESULTS

Identification of AR Mutations

Previous studies from our laboratory using various human AR cDNA probes to screen for AR mutations on Southern blots prepared by restriction endonuclease digestion of genomic DNA from human subjects with CAIS showed a partial gene deletion in only one affected family (17). Seven other unrelated families showed no deletion, suggesting that point mutations or small, undetectable deletions or insertions are responsible for the androgen insensitivity. Poly(A^+) RNA was prepared from genital skin fibroblasts of eight affected human subjects with complete AIS, seven with the AR− form and one with the AR+ form. Except for the one subject with a partial gene deletion (subject B), a normal size 10 kilobase (kb) AR mRNA is detected in all subjects (Fig. 1). These results demonstrate that the AR gene is transcribed normally in subjects with CAIS, even in subjects D-I with no detectable AR binding activity in cultured fibroblasts.

In order to identify the AR mutations present in subjects with CAIS, we analyzed individual exons of the human AR gene by denaturing gradient gel electrophoresis (DGGE) after amplification of genomic DNA by the polymerase chain reaction. The 5′-oligonucleotide primers in each amplification reaction contained a 40-base pair (bp) G-C sequence which functioned as a highly stable DNA duplex clamp in the DGGE. For two subjects with CAIS, AR−, we observe an altered mobility for exons 6 and 7 (Fig. 2, subject 5). We also observe altered mobility on DGGE of the amplified exon 7 DNA fragment from the subject with CAIS, AR+ (Fig. 2, subject 4). Two additional amplifications of either exon 6 or 7 from the genomic DNA of these subjects were performed. Each exon was sequenced in both 5′- and 3′-directions by the dideoxynucleotide chain termination method. A single nucleotide base substitution is detected in each of the three AR gene sequences (Fig. 3). For one subject with CAIS, AR−, a C → T base change occurs in exon 6 resulting in the amino acid substitution of cysteine (TGC) for the wild-type arginine (CGC) at position 774 (AR R774C). In exon 7 of the other subject with CAIS, AR−, a G → A base substitution occurred to alter the codon for amino acid 831 from CGA (arginine) in the wild-type sequence to CAA (glutamine) (AR R831Q). We also confirmed our previously published observation (19) by our current methodology, the detection of a G → A base change in exon 7 for the subject with the AR+ form of CAIS, resulting in the substitution of methionine (ATG) for the wild-type valine (GTG) at position 866 (AR V866M).

Inheritance of AR Mutations

If these point mutations in the human AR gene are associated with CAIS, they should conform to an X-linked recessive pattern of inheritance in affected pedigrees. Point mutations affecting amino acids 774 and

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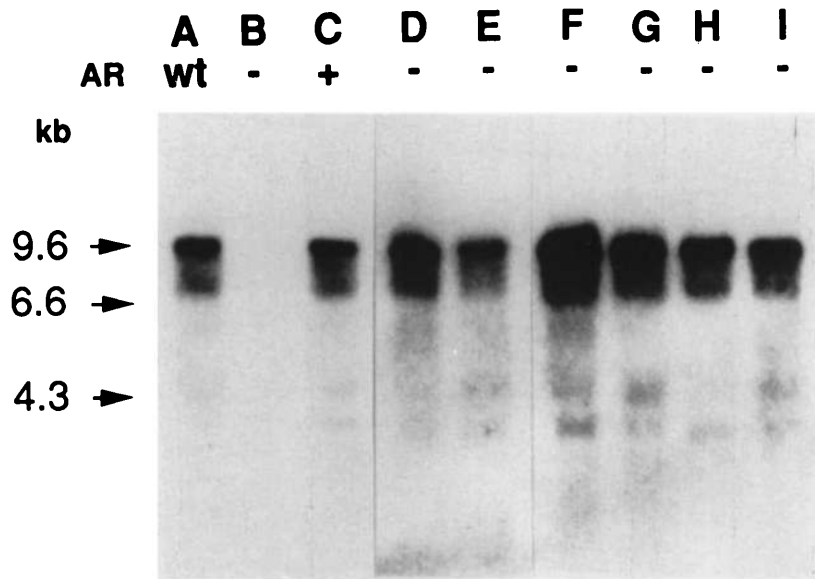

Fig. 1. Northern Blot Analysis of AR mRNA from Cultured Genital Skin Fibroblasts

Poly(A^+) RNA (2–3 μg) isolated from cultured cells was electrophoresed on 1% agarose gels and transferred to nitrocellulose filters. After hybridization with ^{32}P-labeled human AR-1 cDNA (13), autoradiography was carried out for 16 h. RNA was from the cells of human subjects: A, wild-type control; B, subject with CAIS, AR−, and a partial deletion of the AR gene; C, subject with CAIS, AR+ determined to have mutation V866M; D-I, subjects with CAIS, AR−; subjects D and H were determined to have mutations R774C and R831Q, respectively. The *arrows* and size designations in Kb at the left were derived from *Hin*dIII-digested λ-DNA, end-labeled with ^{32}P, and run on the same gel.

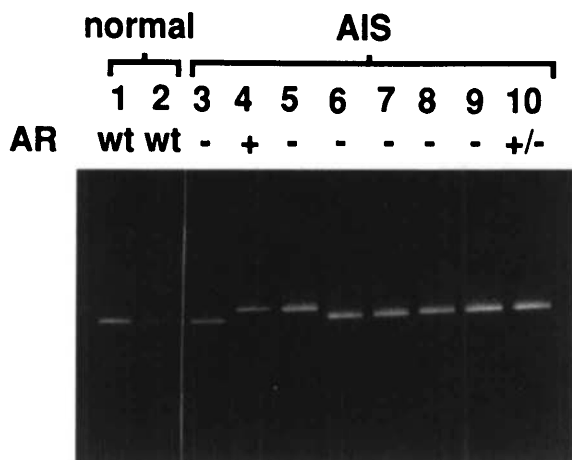

Fig. 2. Denaturing Gradient Gel Electrophoresis of Human AR Exon 7

GC-clamped PCR fragments of exon 7 were electrophoresed in a denaturing gradient gel with 20–80% denaturant. PCR amplified DNA fragments were from genomic DNA of normal subjects (wt, wild-type) and those with CAIS of the AR− or AR+ form or the partial form of AIS (subject 10), with reduced receptor binding, AR±. CAIS subject 4 (AR+) has mutation V866M and subject 5 (AR−) has mutation R831Q.

831 were confirmed by restriction enzyme analysis of the amplified DNA fragments from exons 6 and 7, respectively. The AR-R831Q nucleotide substitution abolishes a restriction endonuclease cleavage site shared by *Bst*BI and *Taq*I within the 417-bp DNA fragment containing exon 7. *Bst*BI digestion of amplified DNA fragments from exon 7 of wild-type, two affected 46,XY family members and the obligate heterozygous 46,XX females from two generations demonstrates the expected restriction fragments from the mutant or wild-type genes (Fig. 4). In addition, the AR-R774C mutation abolishes the *Kpn*I site in genomic DNA or the PCR amplified DNA fragment in the 5′-portion of exon 6 from the affected subject (data not shown).

In other experiments, amplified DNA fragments containing exon 7 from hemizygous 46,XY affected subjects and heterozygous 46,XX carrier subjects with the AR R831Q and V866M mutations all display the expected pattern of electrophoretic mobility upon DGGE (data not shown). The two affected 46,XY family members with the R831Q mutation and three affected 46,XY siblings with the V866M mutation, each show a single DNA fragment with altered electrophoretic mobility similar to that shown in Fig. 2 for subjects 5 (AR−) and 4 (AR+), respectively. The two heterozygous 46,XX subjects carrying the R831Q mutation and the obligate heterozygous mother of the three siblings with the V866M mutation, each exhibit four ethidium bromide stained bands upon DGGE, representing the four combinations of DNA duplex molecules after denaturation/reannealing of the polymerase chain reaction amplified products of the sense and antisense strands of the wild-type and mutant alleles. Taken together, these results demonstrate a pattern of X-linked inheritance and provide evidence for a strong association between the AR gene point mutations and CAIS.

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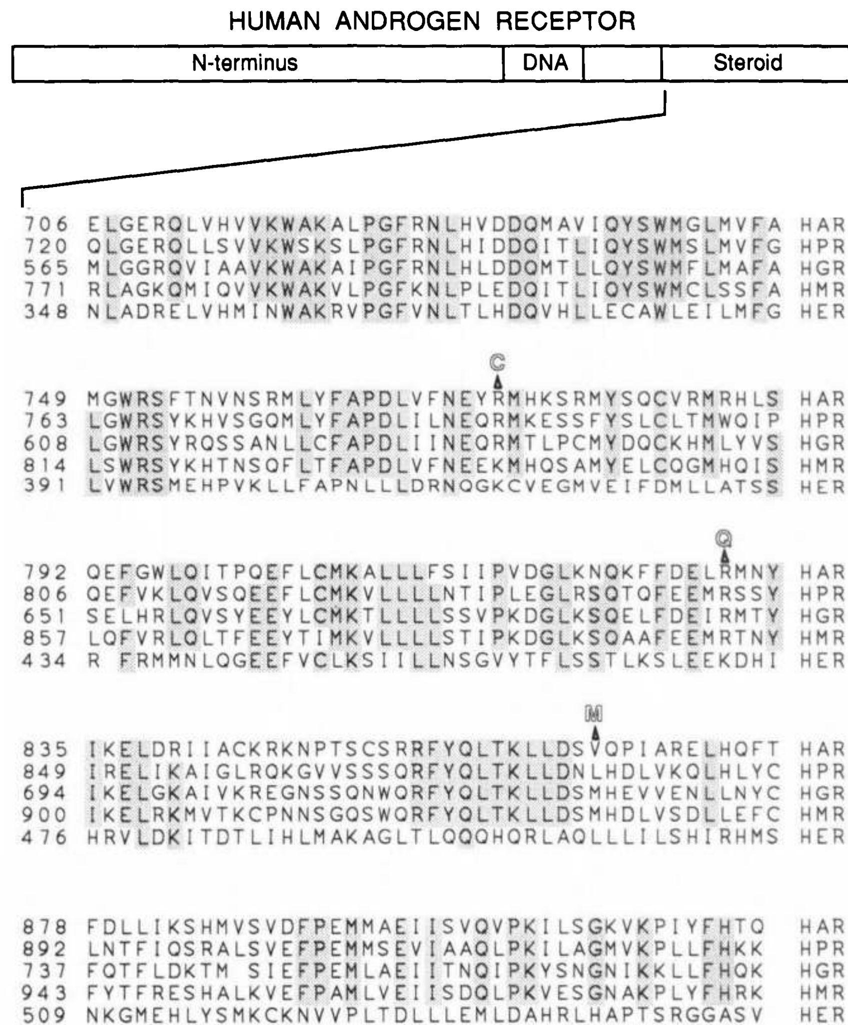

Fig. 3. Partial Amino Acid Sequence Comparisons of Nuclear Steroid Receptors within the Coding Region Containing Single Amino Acid Substitutions Identified in the AR Steroid-Binding Domain of Subjects with CAIS

The single letter code for the amino acid sequences of human AR (HAR; aa 706-919) (13), human PR (HPR; aa 720-933) (71), human GR (HGR; aa 565-778) (72), human mineralocorticoid receptor (HMR; aa 771-984) (73), and human ER (HER; aa 348-550) (74) are shown in alignment. Identical amino acids (*shaded areas*) are indicated relative to HAR. The amino acid substitutions at positions 774, 831, and 866 of the human AR mutants presented in this study are shown as an outlined single letter amino acid code at the appropriate positions.

Construction and Expression of Mutant AR cDNAs

Having demonstrated the association of AR gene point mutations with CAIS, we went on to show the effect of each mutation on the biological function of AR. Complementary DNA fragments containing each of the three nucleotide base changes from the two AR− and one AR+ subjects, were ligated into the *Hin*dIII/*Bam*HI sites of the parent wild-type pCMV expression vector so as to construct a human AR cDNA with the representative mutations (Fig. 5). The presence of each nucleotide base substitution at the appropriate position and the absence of other base changes was verified by sequencing the complete *Hin*dIII/*Bam*HI fragment from each plasmid.

The pCMV human AR expression vector containing the wild-type AR sequence or mutations AR-R774C, -R831Q and -V866M was co-transfected into COS-1 monkey kidney cells along with the plasmid pCH110, containing the lac Z gene driven by the SV40 promoter. Transfection efficiency was determined by comparing AR to β-galactosidase activity.

Immunoblots of human AR protein expression in transfected COS-1 cells were performed with each of the five following transfections: mock COS, AR-wt, AR-R774C, AR-R831Q, and AR-V866M. For the wild-type and each of the three mutants, a protein of 110 kDa reacted specifically with hAR52 antiserum which recognizes an epitope immediately 5′ to the DNA binding domain (Fig. 6).

To determine whether the expressed AR proteins could bind androgen, COS-1 cells were transfected and

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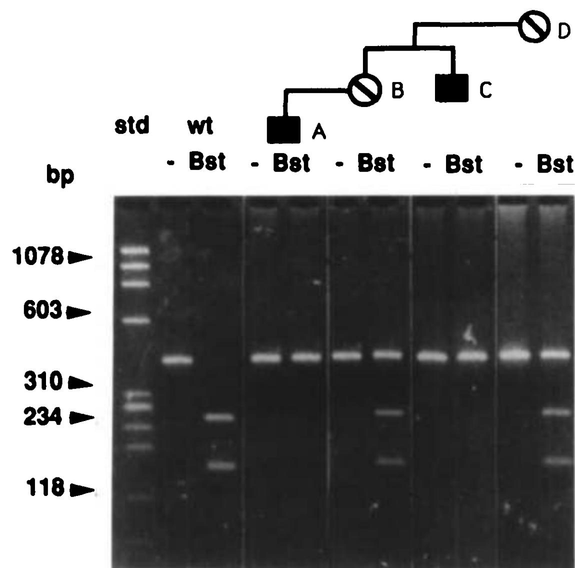

Fig. 4. *Bst*BI Restriction Endonuclease Digestion of DNA from Exon 7 of Human AR Gene with Mutation R831Q

PCR amplified DNA fragments of exon 7 were incubated in the absence (−) or presence (*Bst*) of *Bst*BI for 1 h at 65 C. The digested DNA was electrophoresed on a 4% NuSieve GTG agarose gel with ØX 174-*Hae*III digested DNA as a standard. Genomic DNA was from one normal subject (wt) and four members (A–D) of a pedigree with CAIS, AR− determined to have mutation R831Q: A, 46,XY subject with CAIS, AR−; B, 46,XX obligate heterozygous mother of subject A; C, sibling of subject B with CAIS, AR−; and D, 46,XX obligate heterozygous mother of subjects B and C.

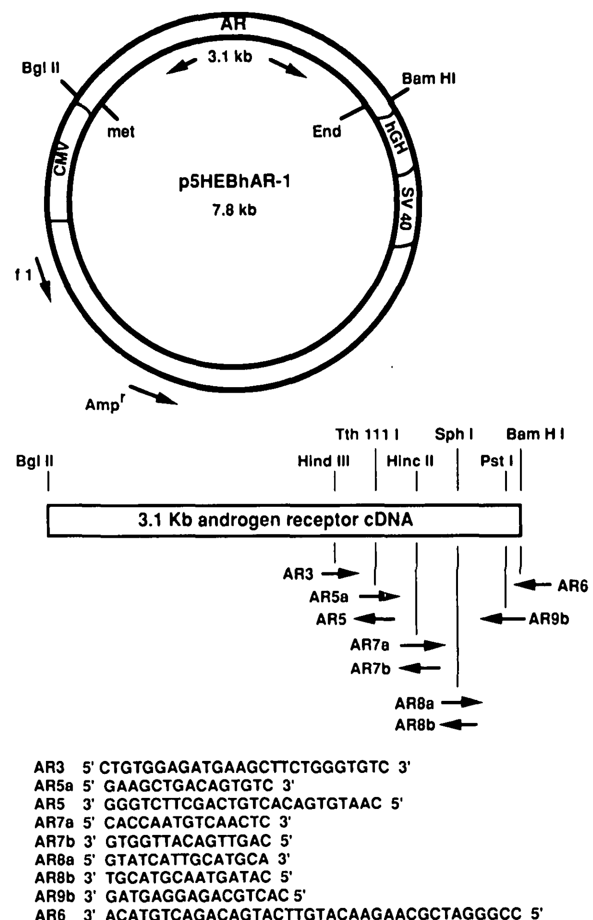

Fig. 5. Map of Expression Vector and cDNA Subcloning Strategy

A 3.1 kb human AR cDNA was cloned into the pCMV5 expression vector as shown (*top*). A series of AR specific oligonucleotides (*bottom*) was synthesized to overlap restriction endonuclease sites within the 3′-region of the AR cDNA (*middle*) to facilitate both subcloning and sequencing of various AR constructs.

incubated with a saturating concentration of ^{3}H-R1881 (5 nM) for 2 h and assayed for the level of specific androgen receptor binding activity. In cells transfected with either AR-wt or AR-V866M, a high level of ^{3}H-R1881 binding is observed (Table 1). However, when cells are mock-transfected or transfected with AR-R774C and AR-R831Q, specific ^{3}H-R1881 binding is absent. Scatchard analyses of ^{3}H-R1881 binding in replicate plates of COS cells transfected with AR-wt (0.05-1.5 nM ^{3}H-R1881) or AR-V866M (0.2-5.0 nM ^{3}H-R1881) shows a 6.5-fold lower apparent binding affinity (K_d) for the mutant receptor (2.46 nM) than for the wild-type receptor (0.38 nM) (Fig. 7). This result is similar to the difference in binding affinity reported earlier for cultured genital skin fibroblasts (18) from normal subjects and three affected siblings of this pedigree with complete AIS, AR+ (AR-V866M) (Table 1). Furthermore, the absence of R1881 binding in COS-1 cells transfected with AR-R774C and AR-R831Q is not due to the lack of protein translation nor to a truncated or significantly unstable AR protein created by any of the mutations.

Mutant ARs are Defective in Transcriptional Activation

The relative abilities of the wild-type and mutant human AR proteins to function as activators of gene transcription were studied in CV-1 cells by their ability to induce chloramphenicol acetyltransferase (CAT) activity in the presence of various steroid hormones. The mouse mammary tumor virus-long terminal repeat (MMTV-LTR) contains four partial inverted repeats of the sequence, TGTTCT, which function as response elements for AR, as well as for glucocorticoid and progestin receptors. Therefore, we cotransfected cells with the MMTV-LTR-CAT reporter gene, with pCH110 containing the lac Z gene, and with the AR expression vector constructs. CAT activity is absent in CV-1 cells if steroid is not added, regardless of the transfection of wild-type or mutant human ARs (Fig. 8). In the presence of 10 nM androgen (DHT or R1881), AR-wt or AR-V866M, both of which bind androgen, significantly induce CAT activity when expressed in CV-1 cells. By contrast, the AR− mutants, AR-R774C and AR-R831Q, are totally ineffective in stimulating CAT activity when androgen is added. The addition of 10 nM progesterone or 25 nM

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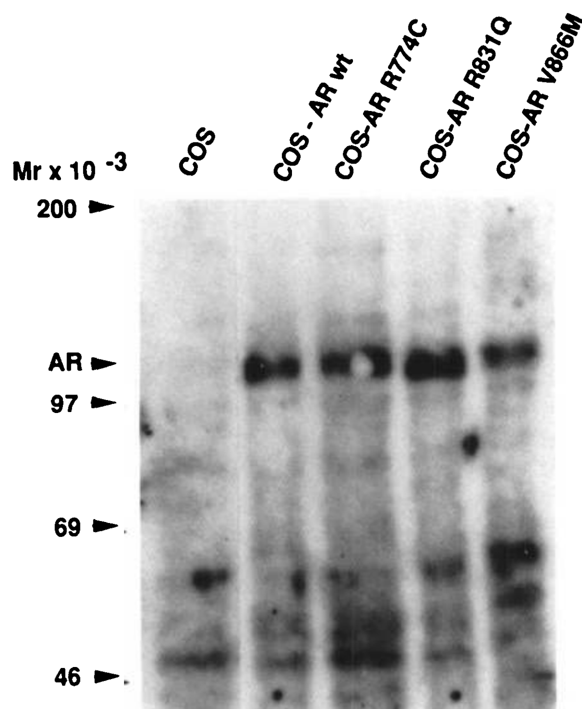

Fig. 6. Immunoblot of Wild-Type and Mutant ARs Expressed in COS Cells

COS-1 cells were mock transfected (COS) or cotransfected with AR-wt or mutant AR-R774C, -R831Q, or -V866M and pCH110 plasmids by the calcium phosphate co-precipitation method. Aliquots (50–75 μl) of cell extracts normalized for β-galactosidase activity were run on 7.5% polyacrylamide-SDS gels, transferred to nitrocellulose filters, and incubated with antipeptide specific AR antiserum (AR52), diluted 1:250 (11 μg protein/ml). Antigen-antibody complexes were labeled with ^{125}I-protein A and visualized by autoradiography. The relative position of the protein molecular weight markers ($M_r \times 10^{-3}$) and of AR are indicated on the *left*.

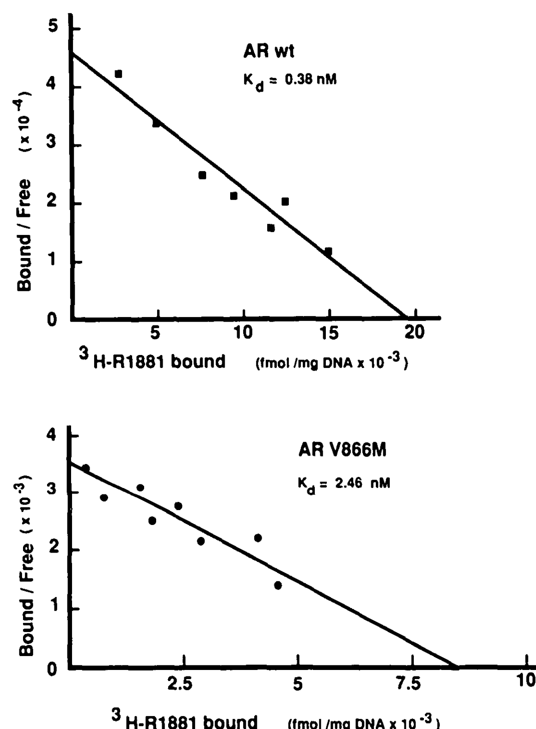

Fig. 7. Scatchard Analyses of R1881 Binding to AR in COS-1 Cells Transfected with Wild-Type and Mutant AR Expression Vector

COS-1 cells were cotransfected by the calcium phosphate coprecipitation method with AR-wt or AR-V866M and pCH110. Replicate plates of cells containing AR-wt were incubated with 0.05–1.5 nM ^{3}H-R1881 or AR-V866M were incubated with 0.2–5.0 nM ^{3}H-R1881 for 2 h at 37 C. Specific AR binding of R1881 was assayed in cell extracts normalized for DNA content and for the relative efficiency of transfection according to the β-galactosidase activity.

Table 1. Androgen Binding by Normal and Mutant Androgen receptors

AR	GSF		COS-1
	B_{max} (fmol DHT/mg DNA)	K_d(nM)	Specific Binding (fmol R1881/mg DNA)
Wild-Type	544	0.53	16182
R774C	<10	ND	196
R831Q	<10	ND	128
V866M	720	1.50	9081

AR binding of DHT, analyzed by Scatchard plots of data from the incubation of cultured genital skin fibroblasts (GSF) from normal and CAIS subjects with radiolabeled steroid as previously reported (11, 18), were used to determine the number of binding sites (B_{max}) and the apparent binding affinity (K_d). AR binding of R1881 was assayed in COS-1 cells transfected with the wild-type or mutant AR expression vector and incubated with a saturating concentration (5 nM) of radiolabeled R1881 (n = 3). ND, Not determined due to absence of steroid binding.

dexamethasone fails to stimulate CAT activity by any of the expressed AR proteins. AR-wt is, however, able to induce CAT activity upon the addition of 10 nM estradiol, whereas none of the mutant AR proteins, including AR-V866M, is able to stimulate CAT activity in the presence of estrogen.

When the level of CAT activity is quantified by a two-phase partitioning radioactive scintillation assay, distinct differences are observed in the ability of the various androgen ligands to induce enzyme activity with the wild-type receptor, AR-wt, and the AR+ mutant, AR-V866M (Fig. 9). Using a range of steroid concentrations from 0.1–10 nM, DHT, R1881, and testosterone cause a dose-dependent increase in CAT activity. However, distinct differences are observed between the ability of AR-wt and AR-V866M to stimulate CAT activity at a given concentration of DHT, R1881, or testosterone. At the highest concentration of steroid (10 nM), little difference is seen between AR-wt and AR-V866M, the increase being about 100-fold above the baseline level observed in the absence of steroid. However, at 0.1 nM DHT or R1881, a major difference in the magnitude of CAT stimulation by the same two receptor contructs is

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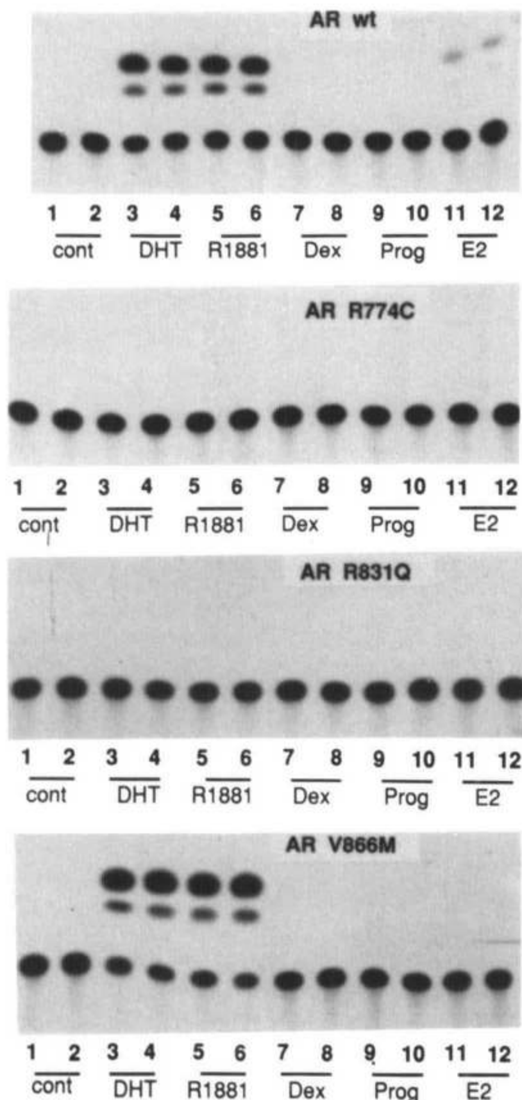

Fig. 8. Steroid-Specific Transcriptional Activation of CAT in CV-1 Cells Transfected with Wild-Type and Mutant ARs

CV-1 cells were cotransfected with AR-wt or mutant AR-R774C, -R831Q, or -V866M, the MMTV-LTR-CAT reporter gene and the pCH110 lac Z gene plasmids by the calcium phosphate coprecipitation method. Replicate plates of cells were subsequently incubated for 36 h in the absence or presence of 10 nM DHT, methyltrienolone (R1881), progesterone (Prog) or estradiol (E_2), or 25 nM dexamethasone (Dex). The acetylation of ^{14}C-chloramphenicol by extracts (normalized for transfection efficiency based upon β-galactosidase activity) of transfected steroid-treated CV-1 cells was determined by autoradiography after TLC resolution of the reaction products.

observed; CAT induction being 60-fold above baseline for AR-wt but only 3-fold higher for AR-V866M. In the presence of 1 nM DHT, R1881, or testosterone, the CAT activity is 75- to 110-fold over baseline with AR-wt but with the AR+ mutant, it is 33-, 60-, and 4-fold for the three steroids, respectively. When 10 nM estradiol is added to the cells, an 11-fold increase of CAT activity over baseline occurs in cells with AR-wt but no effect is observed for AR-V866M. When DHT (1.0 nM) and estradiol (10 nM) are added in combination, there is no evidence for synergism or antagonism, the stimulation of CAT activity for AR-wt by the two steroids is additive and for AR-V866M is equivalent to 1.0 nM DHT alone.

DISCUSSION

The AR is a member of the steroid, thyroid, and retinoic acid hormone receptor superfamily of proteins that mediate the actions of their respective cognate ligands through the regulation of gene transcription (7–10). The proteins are modular in nature, comprised of a highly variable N-terminal domain, thought to function in transcriptional activation, and well-characterized DNA- and hormone-binding domains (20, 21). The latter regulates, in unknown fashion, the ability of the DNA-binding domain of the protein to recognize DNA sequence elements of responsive genes and modulates the transcription of those genes.

Several pathophysiological conditions in humans result not from a lack of the hormone but rather from an impairment of the mechanism which activates hormone-responsive genes. These conditions of hormone insensitivity in humans have been described for thyroid (22), vitamin D (21), glucocorticoid (24), mineralocorticoid (25), and androgen (1, 4). In androgen insensitivity, biochemical studies have shown that the impairment in hormonal responsiveness correlates with defects in binding of androgen to its receptor (1, 4, 11, 12, 18). This study shows that ligand binding and transcriptional activation are impaired due to naturally occurring mutations in the androgen receptor gene and establishes the conclusion that AR defects underlie the condition of CAIS in humans.

The heterogeneous nature of human CAIS became apparent from studies of AR binding in cultured genital skin fibroblasts from affected subjects (1, 11, 12). In some cases of CAIS, AR binding was undetectable (AR−), whereas in others quantitatively normal binding (AR+) was observed. More recently using cDNA probes for the human AR gene, we reported that a partial deletion was responsible for complete androgen insensitivity in one family (17). While this finding provided an explanation for the physiological insensitivity and the absence of AR binding activity in cultured genital skin fibroblasts from affected members of this family, other families with absent AR binding had no apparent structural defect in the AR gene (17). In fact, AR gene transcription also appeared normal on Northern blots of poly(A^+) RNA isolated from cultured genital skin fibroblasts of six subjects with CAIS, AR−, and one patient with CAIS, AR+. On this basis, we suggested that point mutations in the AR gene might be responsible for the expression of androgen insensitivity in these subjects. Appropriately, we identified a single nucleotide substitution within the open reading frame of the gene in the AR+ family who had quantitatively

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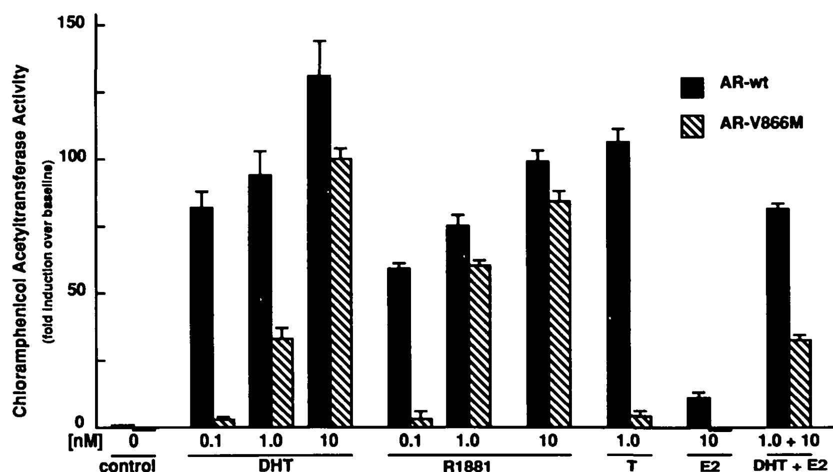

Fig. 9. Concentration-Dependent Transcriptional Activation of CAT by Androgens for Wild-Type AR and AR Mutant V866M

CV-1 cells were cotransfected with AR-wt or AR-V866M, MMTV-CAT reporter gene, and pCH110 lac Z gene plasmids by the calcium phosphate coprecipitation method. Three replicate plates of cells were subsequently incubated for 36 h with 0.1–10 nM DHT or R1881, 1.0 nM testosterone (T), 10 nM E_2 or a combination of 1 nM DHT and 10 nM E_2. CAT activity was measured by incubation of extracts from transfected, steroid-treated cells with chloramphenicol, and ^{3}H-acetyl CoA. The partitioning of the ^{3}H-acetylated chloramphenicol reaction products into an organic scintillation cocktail was monitored over time by repetitive scintillation spectrophotometric counting. All reactions were linear for greater than 3 h at room temperature with a correlation coefficient (r) of greater than or equal to 0.95 determined by linear regression analysis. Fold induction of CAT activity over baseline was calculated from the linear slope of each kinetic reaction curve using the following formula:

$$\frac{\text{CAT(AR-transfected + steroid)}}{\beta\text{-gal(AR-transfected + steroid)}} - \frac{\text{[CAT(mock transfected) − CAT(no cell extract)]}}{\beta\text{-gal(mock transfected)}}$$

The *bars* represent the mean ± SEM from the three replicate plates of cells per group assayed in duplicate.

normal but qualitatively abnormal AR binding activity (18). The nucleotide change (G → A) resulted in the substitution of methionine for valine in amino acid position 866 (19). Although this amino acid substitution was correlated with CAIS in three affected members of this family and was transmitted as an X-linked trait, functional studies of the putative mutant AR were not obtained.

In this study, we report point mutations in the AR genes of two unrelated families with CAIS, AR−. Because their receptors did not bind DHT in cultured genital skin fibroblasts (1), we amplified in each family, exons 4 to 8 of the AR gene which encode the steroid-binding domain (13). In one family, the point mutation occurs in exon 7 with a G → A change resulting in an amino acid substitution of glutamine for arginine at position 831 (13). Based upon restriction endonuclease digestion of DNA with *Bst*BI, we showed the X-linked inheritance of this mutation. In a second family, we identify within exon 6, a single nucleotide substitution (C → T) that results in the substitution of a cysteine for an arginine at amino acid position 774 (13). This substitution eliminates a *Kpn*I restriction endonuclease cleavage site near the 5′-border of exon 6, a finding recently reported to occur in three other unrelated families (26–28). We cloned each of these point mutations into an AR expression vector (13) and transfected them into COS cells. The presence of the expressed proteins was verified by immunoblotting with an AR specific antiserum (13). The fact that both proteins are unable to bind R1881 with high affinity or to transactivate the MMTV-CAT reporter gene in an *in vitro* transfection assay for biological function, provides definitive proof linking these single amino acid substitutions with the condition of CAIS.

A multifunctional role in hormone action has been proposed for the steroid binding domain. In addition to the well-documented role of this C-terminal domain in steroid binding, it also functions in transcriptional activation and receptor dimerization (29–37). Our results demonstrate that a single amino acid substitution within the steroid binding domain from a basic residue, arginine, at positions 774 or 831, to a polar amino acid like cysteine or to glutamine, respectively, can eliminate the hormone binding capacity of the protein. The normal function of the steroid is to bind AR and to release the inhibitory influence of the unoccupied receptor on transcription activation; a biological activity that is defective in mutants R774 and R831Q. Therefore, these naturally occurring inactive mutant ARs are differentiated from the genetically engineered AR deletion mutants of the steroid binding domain which are constitutive activators of transcription (38).

Interestingly, the mutations in AR, R774C, and R831Q, occur in arginine residues that are not contained within blocks of conserved amino acid homology among the various steroid receptors shown in Fig. 3. However, the wild-type AR amino acid residue arginine

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is conserved among the most highly homologous neighbors of the human AR, namely glycocorticoid receptor (GR) and progesterone receptor (PR) (Fig. 3). The fact that the wild-type arginine residues at positions 774 and 831 are neither unique nor completely conserved among all steroid receptors suggests that they do not directly contact the steroid molecule. Instead, the lack of steroid binding and consequent lack of transcription activation in these mutants is likely to be the result of an altered conformation in the AR protein that prevents hormone binding, and thus transcription activation, but does not significantly influence its turnover as shown by immunoblotting. Interestingly, a human AR cDNA clone isolated from a human testis cDNA library contains four amino acid substitutions in the region immediately adjacent to R831Q (39). This sequence alteration results in an AR protein which does not bind steroid with high affinity and also fails to activate gene transcription *in vitro*.

Although many cases of CAIS result from the absence of ligand binding by AR, relatively fewer cases are characterized by quantitatively normal but qualitatively abnormal androgen binding activity (1, 4, 18). The mutation V866M (valine → methionine) represents such a case, where a normal to elevated number of ARs were measured in cultured genital skin fibroblasts but the apparent binding affinity for DHT was decreased by severalfold (18). Expression of this mutant in the AR expression vector resulted in an AR protein with over 6-fold reduced affinity for binding of R1881. When the mutant AR cDNA expression vector was cotransfected with the MMTV-CAT reporter gene, a dose-dependent androgen stimulation of CAT activity by R1881 or DHT was observed. At physiological steroid concentrations of 0.1 nM, the mutant AR-V866M stimulated CAT activity only minimally, much less than AR-wt. At high concentrations of steroid (10 nM), mutant AR-V866M stimulated gene transcription *in vitro*, an effect that would not occur *in vivo* at physiological concentrations of free androgen available to the target cells. The relatively low induction of CAT activity by mutant AR-V866M was steroid concentration dependent and the potency of effect followed the relative binding affinities for R1881 > DHT > testosterone to AR (40). Interestingly, the V866M and wild-type AR are also distinguished by the induction of CAT activity by estradiol. AR-wt stimulates CAT activity in the presence of 10 nM estradiol but V866M does not, suggesting that sufficiently high concentrations of estradiol may actually activate gene transcription by binding to the wild-type AR (41, 42).

The mechanism by which the point mutation V866M results in only a modest change in steroid binding properties of the AR but causes total loss of *in vivo* AR functional activity is not yet clear. It appears that complete transformation of the receptor subsequent to steroid binding requires precise structural interaction between receptor and ligand (37). Steroid antagonists, for example, have recently been shown to bind estrogen receptor (ER) and GR and promote specific binding to hormone response elements without enhancing gene transcription as would a normal agonist. Hormone binding domains of nuclear receptors are now recognized to have transcription activation function (31, 33–37). Ligand binding to AR and other steroid hormone receptors initiates a transformation process that results in enhanced binding of receptors to their hormone response elements (29–32). This process may involve a conformational change in the receptor protein or separation from a nonsteroid-binding component that unmasks the DNA-binding domain and a nuclear transfer sequence located in the C-terminal region downstream from the DNA-binding domain. The process may also activate within the steroid binding domain, a dimerization sequence shown to be required for strong interaction of the steroid receptor with its response element and for transactivation of gene expression (34–36). Recently, Parker and co-workers (33, 34) have shown that a C-terminal region, which includes the mutation at amino acid 866, contains hydrophobic residues arranged in a heptad and conserved in all nuclear receptors. *In vitro* mutations in this region in the mouse ER blocked receptor dimerization, decreased DNA binding affinity and eliminated steroid binding. Thus, V866M may be defective in any one of a number of regulatory steps involved in steroid hormone action.

Whereas the decreased but not absolute absence of biological function of AR mutant V866M may not appear to be consistent with the phenotype of complete AIS, one must take into account several additional factors such as: 1) whether the concentration of endogenous androgen in the developing fetus is sufficient to induce androgen-dependent musculinization if the AR mutation is present; 2) whether the presence of the mutation interfers in some way with the actions of other factors involved in androgen-stimulated gene transcription; and 3) whether the hormone response element within the MMTV-LTR promoter, which is promiscuous in the presence of AR, GR, or PR, is an accurate androgen response element to determine the specificity of androgen action (41–46).

Whereas the direct action of testosterone or its more potent 5α-reduced metabolite, DHT, on transcription involves multiple nuclear proteins, the specificity of transcriptional activation by androgens most probably lies in the AR itself. Thus, it is not surprising that insensitivity to androgens arises from mutations in the AR, analogous to those recently shown to be responsible for vitamin D (47–49) and thyroid (50) resistant states. In this study, point mutations in the human AR gene steroid-binding domain have been definitively associated with functional defects in the AR proteins from human subjects with AIS. Whereas complete insensitivity to androgen is readily apparent in 46,XY subjects with a completely female phenotype as seen in this study, it is likely that other mutations within the AR gene lead to partial insensitivity with ambiguity of the external genitalia (1) and possibly even less severely affected phenotypes as in phenotypic men with azoospermia (2, 3). Finally, the occurrence of AIS might also arise from changes in other elements of the andro-

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gen-inducible transcription pathway, either in specific transcriptional factors (51, 52) or in the important target genes themselves. Presumably, each might have a unique clinical presentation that is not currently appreciated.

MATERIALS AND METHODS

Experimental Subjects

Informed written consent for genital skin biopsy or blood sample was obtained from each individual according to protocols approved by the Johns Hopkins Joint Committee for Clinical Investigation. Each affected subject with CAIS had a normal 46,XY karyotype with normal female external genitalia and absence of uterus and fallopian tubes. A short vagina ended in a blind vaginal pouch. Bilateral testes were located in the abdomen or in the labia majora but there were little or no Wolffian duct derivatives. Breast development occurred spontaneously at puberty and there was sparse to absent pubic and axillary hair despite blood testosterone concentrations in the normal adult male range. Androgen receptor binding assays in cultured human genital skin fibroblasts have been reported previously for all subjects included in this study (1, 11, 12, 18).

Cell Culture

Human genital skin fibroblasts were propagated in tissue culture from explants of normal male foreskin (46,XY control subjects) or a biopsy specimen from the labia major of 46,XY subjects with CAIS. Genital skin fibroblasts as well as CV-1 and COS-1 monkey kidney cells for DNA transfection experiments were maintained in minimal essential minimum (MEM) with Earle's salts and supplemented with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and 10% fetal bovine serum.

AR Binding Assay

AR binding activity was determined in replicate culture dishes of DNA-transfected COS-1 cells by the procedure described previously for cultured human genital skin fibroblasts (40). For total AR binding, cells were incubated for 2 h at 37 C in the presence of 5 nM ^{3}H-R1881 (87 Ci/mmol; methyltrienolone; New England Nuclear, Wilmington, DE) in MEM. For Scatchard analyses (53), COS-1 cells were incubated in an identical manner with a range of ^{3}H-R1881 concentrations from 0.05–5.0 nM. The level of specific radiolabeled steroid binding was normalized for DNA content as measured by the method of Burton (54) and corrected for efficiency of DNA transfection according to the β-galactosidase activity (55) in replicate plates of cells.

Construction of Human AR Expression Vector

The full-length coding sequence of the human AR contained within a 3.1 kb *Bgl*II-*Bsp*HI (blunt) cDNA insert was cloned into the pCMV5 parent vector (56) at the *Bgl*II-*Hin*dIII (blunt) sites (13). The complete human AR expression vector, termed p5HBhAR-A, contains the immediate early promoter region of the human cytomegalovirus promoter, human GH transcription termination and polyadenylation signal, the SV40 origin of DNA replication and early region enhancer sequences, the bacteriophage FI origin of replication, an *Escherichia coli* gene encoding ampicillin resistance and a polylinker region (56). The p5HBhAR-A vector was subsequently modified to eliminate the *Eco*RI site (nucleotide 911) in the 5′-polylinker region thus retaining a now unique *Eco*RI site (nucleotide 3542) within the human AR cDNA sequence. We then subcloned a 1206-bp human AR cDNA fragment by insertion at its natural 5′-*Hin*dIII site (nucleotide 2829) and ligated it to the *Bam*HI site (nucleotide 4066) within the 3′-polylinker region of the vector, thus retaining unique internal *Hin*dIII and *Eco*RI sites within the AR cDNA and an external 3′-*Bam*HI site, these sites being convenient for subsequent subcloning of cDNA fragments containing specific point mutations in the human AR. We termed this new expression vector, p5HEBhAR-1. Preparation of these mutant ARs is described below and in Fig. 5.

RNA Preparation, Northern Blots, and cDNA Synthesis

Total RNA was prepared from human genital skin fibroblasts by solubilization in 4 M guanidinium isothiocyanate and centrifugation through a 5.7 M cesium chloride cushion, estraction with phenol and chloroform, and repeated precipitation with ethanol in the presence of potassium acetate (57). The poly(A^{+}) RNA fraction was obtained by passing the crude RNA over two successive oligo-dT columns and ethanol precipitation (58).

For Northern blot analyses (59), 2–3 μg poly(A^{+}) RNA was electrophoresed on a 1% agarose-1.13% formaldehyde gel in 20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA at 5 V/cm, transferred to nitrocellulose overnight in 20× SSC, and baked at 80 C for 2 h. The filter was wet briefly in deionized formamide and prehybridized at 42 C in a solution of 25 mM KPO_4, pH 7.4, 5× SSC, 5× Denhart's, 50 μg/ml yeast tRNA, 50% formamide, and 10% dextran sulfate. Hybridization of RNA to human AR or chicken β-actin cDNA probes was performed in the presence of 2-4 × 10^{6} cpm random-primed ^{32}P-labeled probe/ml hybridization solution at 42 C. The filters were washed twice for 15 min in 1× SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature and twice for 15 min in 0.25× SSC/0.1% SDS at room temperature and exposed to Kodak XOMAT film at −70 C with an intensifying screen.

Poly (A^{+}) RNA (1 μg) was used to prepare single-stranded cDNA (60) with random hexamer priming following the protocol supplied with the Amersham cDNA Synthesis System Plus (Arlington Heights, IL). The 20-μl reaction volume was diluted 25-fold in TE, pH 7.5, and stored at 4 C. An aliquot of the cDNA pool (1 μl) was amplified by the polymerase chain reaction (PCR) using 25 pmol each human AR cDNA oligonucleotide primer designed to overlap the *Hin*dIII and *Bam*HI restriction sites (AR3 and AR6 of Fig. 4) in a 50 μl total reaction volume containing 200 μM each dNTP in reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.01% gelatin). The reaction mixture was denatured at 94 C for 5 min and cooled to 72 C. Taq DNA polymerase (Perkin-Elmer/Cetus, Emeryville, CA) (2.5 U) was added and a first cycle of amplification (94 C, 1 min; 56 C, 2 min; 72 C, 40 min) was followed by additional cycles of amplification (94 C, 40 sec; 56 C, 2 min; 72 C, 3 min) and a final extension at 72 C for 15 min.

The amplified product was digested with *Hin*dIII and *Bam*HI and purified on a 4% NuSieve GTG agarose (FMC Bioproducts, Rockland, ME) gel. The purified 1.2 kb AR cDNA fragment was ligated directly from the melted agarose into the *Hin*dIII/*Bam*HI sites of the p5HEBhAR-1 expression vector for transfection into competent HB101 cells. Colonies containing plasmid DNA were selected for growth on LB-ampicillin (200 μg/ml) agar plates. Five to 10 colonies were selected, inoculated into 5 ml liquid cultures of LB containing 50 μg/ml ampicillin and grown overnight at 37 C. Plasmid DNA was prepared by a modification of the alkaline miniprep method described by Maniatis *et al.* (61).

For sequencing of double-stranded plasmid DNA, 2–3 μg purified DNA were denatured in 0.2 N NaOH at room temperature for 5 min and precipitated in the presence of 1.5 M ammonium acetate by addition of ethanol at −80 C for 15 min. The DNA was collected by centrifugation, washed with 70% ethanol, dried, and resuspended in 7 μl water. The DNA was

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initially sequenced following the protocol supplied by the manufacturer of the Sequenase kit (United States Biochemicals, Cleveland, OH) using human AR cDNA specific oligonucleotide primers (Fig. 5) to verify the presence in each selected plasmid of the altered base pair for the three affected human subjects. After identification of appropriate plasmid containing colonies, the entire *Hin*dIII-*Bam*HI human AR cDNA insert region was sequenced to eliminate the possibility that PCR induced nucleotide errors might be contained in the expression vector AR cDNA insert sequence or that more than one mutation might be contained within the AR gene of subjects with CAIS.

Density Gradient Polyacrylamide Gel Electrophoresis

Genomic DNA was prepared from cultured genital skin fibroblasts or blood leukocytes, as previously described (17). DNA (500 ng) was amplified by PCR as performed by Lubahn *et al.* (19) except that oligonucleotide primers for exons 4-8 were used at a final concentration of 1 μM and each 5′-oligonucleotide primer contained a 40-bp G-C clamp (5′ GCCGCCCGCC GCCGCCCGCC GCCGCCGCCC GCCGCCCGCC 3′) before the human AR specific 5′-primer sequence (62). Amplification conditions consisted of an initial denaturing step at 94 C for 5 min before addition of 2.5 U Taq DNA polymerase followed by one cycle of 94 C, 2 min; 56-60 C, 2 min; and 72 C, 6 min; and 30 cycles of 94 C, 1 min; 56–60, 2 min; and 72 C, 3 min; with a final extension step at 72 C for 10 min. DNA products of the correct size were verified by electrophoresis on a 2% agarose gel at 100 V for 4 h.

Amplified DNA fragments (10-μl reaction aliquot) were electrophoresed on a 6.5% polyacrylamide gel in TAE buffer with a 20–80% denaturant gradient (100% denaturant is 40% formamide/7 M urea) (62–64). Electrophoresis was carried out using a system designed by Green Mountain Lab Supply (Waltham, MA) with a 60 C recirculating TAE buffer bath for a total of 1300 V·h. The gel was stained with ethidium bromide and photographed under UV light to determine the relative mobility of each DNA fragment.

In cases where exon-specific amplified DNA fragments from subjects with CAIS exhibited differential mobility on DGGE as compared to wild-type DNA fragments, the genomic DNA was reamplified by the PCR reaction but without the 40-bp G-C clamp oligonucleotide primer. Amplified DNA products were separated from oligonucleotide primers by centrifugation through a Centricon 30 microfiltration unit and directly sequenced (65, 66) with T7 DNA polymerase (Sequenase, United States Biochemicals). Sequencing primers, the same as those used for the amplification reactions, were end-labeled with γ-^{32}P ATP (3000 Ci/mmol; New England Nuclear) using T_4 DNA polynucleotide kinase (Bethesda Research Laboratories, Gaithersburg, MD). Approximately 80 ng PCR product were annealed with 10 ng appropriate ^{32}P-labeled sequencing primer in a volume of 11 μl on ice for 10 min. After heat denaturing for 5 min at 95 C, 2.5 μl annealed sample were added to four tubes with 3 μl sequencing mixture containing nonradioactive dNTPs at a concentration of 64 μM, ddNTPs at a concentration of 6.2 μM and 2 U T7 DNA polymerase in buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 70 mM NaCl, and 7 mM dithiothreitol). The mixture was incubated for 15 min at 37 C followed by the addition of 3 μl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Samples were boiled for 3 min before loading 3 μl onto a 7% polyacrylamide/8 M urea gel. After electrophoresis at 60 W for 2–2.5 h, gels were dried and exposed to Kodak XOMAT film for 16 h at −70 C with an intensifying screen. Each sequence was verified by sequencing both the sense and antisense strands from two independent amplification reactions for each subject with AIS.

Transient DNA Transfection

On the day before transfection, CV-1 or COS-1 monkey kidney cells were transferred to 100-mm culture dishes at a density of 1 × 10^6 cells per dish. Two to 4 h before DNA transfection, 5 ml fresh MEM containing 20% charcoal-treated fetal bovine serum were added to each culture dish. Cells were transfected with 30 μg total plasmid DNA consisting of 10 μg pCH110 (gift from M. West, Johns Hopkins University), 10 μg MMTV-CAT (gift from L. Sanders, Johns Hopkins University), and 10 μg human wt or mutant AR in p5HEBhAR-1 or 10 μg control vector in 0.5 ml HBS (19 mM HEPES, pH 7.05, 0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, and 5.5 mM glucose) and 0.125 M $CaCl_2$ by the calcium phosphate co-precipitation method (67). Cells were incubated in the presence of DNA for 4 h at 37 C after which the medium was removed. The cells were washed twice with 5 ml serum-free MEM, incubated for 1 min in HBS containing 15% glycerol (COS-1 cells) or 20% glycerol (CV-1 cells) and rinsed with 1 × HBS. Cells were incubated overnight in MEM supplemented with 5% charcoal-treated fetal bovine serum. The next day, transfected cells from each group were detached from plates in PBS (0.15 M NaCl, 1.15 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl)-EDTA (0.53 mM)-trpysin (0.05%) and suspended in MEM-5% charcoal treated FBS and passaged to 100-mm culture dishes. These replicate culture dishes were incubated for 4 h at 37 C to allow cells to attach to the dishes before the addition of steroids in ethanol (<0.1%) to the medium.

Immunoblotting

COS-1 cells transiently transfected with the human AR cDNA expression vector were washed three times on ice with 5 ml PBS. Cells were directly solubilized by the addition of 300 μl hot (95 C) 2× electrophoresis sample buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 1.4 M 2-mercaptoethanol). The viscosity of this cell lysate was reduced by the addition of 0.1 μg each of DNase and RNase. Samples were stored frozen at −70 C until use.

Electrophoresis was carried out in 7.5% polyacrylamide gels containing 3.5 mM SDS according to the method of Laemmli (68), using a vertical polyacrylamide gel (1.5 mm thick, 14 cm wide, and 12.5 cm long) with a 1 cm stacking gel. Samples were boiled for 5 min before application of 50- to 75-μl aliquots in gels. Proteins were transferred from polyacrylamide gels to nitrocellulose membrane filters by electrophoresis at 500 mA in 2.5 mM Tris, 0.19 M glycine at room temperature for 1.5 h. Nitrocellulose filters were preincubated (30 min) in 0.01 M Na_2HPO_4, pH 7.4, 0.15 M NaCl, 0.5% Triton X-100 containing 20 μg/ml BSA before incubation for 1 h with the affinity purified anti-peptide antibody (AR1-52) diluted 1:250 (11 μg/ml) in 0.01 M Na_2HPO_4, pH 7.4, 0.15 M NaCl, 0.5% Triton X-100 (13). Filters were washed several times with buffer in the absence of additional protein and then incubated with buffer containing 20 μg/ml BSA and 1 μCi ^{125}I-labeled protein A (8.29 μCi/μg; New England Nuclear) for 1 h. Finally, the filter was washed with several changes of protein-free buffer. The washed filters were exposed to Kodak XOMAT film for 40–64 h at −70 C with an intensifying screen.

Enzyme Assays

Cells transfected with plasmid DNA were washed three times with 5 ml ice-cold PBS and scraped on ice into 1 ml 0.04 M Tris-HCl, pH 7.4, 1 mM EDTA, and 0.15 M NaCl. Cells were collected by centrifugation (4 C, 2 min) in a microfuge and stored frozen at −70 C or immediately resuspended in 150 μl 0.25 M Tris-HCl, pH 7.8. The cell suspension was subjected to three cycles of freeze-thawing in dry-ice/ethanol for 5 min and 37 C for 5 min. The cell debris was pelleted in a microfuge (4 C, 5 min) and the supernatant was transferred to a new tube. The cellular extract was frozen at −70 C and used for assay of β-galactosidase or CAT activities.

For β-galactosidase (54), 30 μl cell extract were mixed with 1 ml reaction buffer (60 mM Na_2PO_4/40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.05 M β-mercaptoethanol, pH 7.0), and 0.2 ml substrate (2 mg/ml *O*-nitro-β-D-galactopyranoside in 60

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mM Na_2PO_4/40 mM NaH_2 PO_4). The reaction was allowed to proceed at 37 C until a faint yellow color was observed (1 h or less for COS-1 cells and 2–3 h for CV-1 cells). The reaction was stopped by the addition of 0.5 ml 1 M Na_2CO_3 and the difference between the absorbance peak at 420 nm and the baseline at 550 nm was recorded. The β-galactosidase activity was used as a measure of DNA transfection efficiency and to normalize CAT and AR binding activities between culture dishes and treatment groups.

For CAT activity, two separate assays were used. The first assay used ^{14}C-chloramphenicol (dichloroacetyl-1,2-^{14}C; 58.2 mCi/mmol; New England Nuclear) as substrate and resolved the acetylated ^{14}-C products by TLC and subsequent autoradiography (69). The second assay used ^{3}H-acetyl coenzyme A (acetyl-^{3}H; 2.5 Ci/mmol; New England Nuclear) as the cofactor for acetylation of chloramphenicol in a two-phase partition liquid scintillation spectrophotometric method (70). In the former assay, 30 μl cell extract were combined with 0.1 μCi (1.7 μmol) ^{14}C-chloramphenicol, 0.5 M Tris-HCl, pH 7.8, and 0.53 mM acetyl coenzyme A (CoA) in a total volume of 150 μl and incubated at 37 C for 3 h. The reaction mixture was extracted with 500 μl ethyl acetate. A 400-μl aliquot of the organic phase was dried, resuspended, and chromatographed on silica gel thin-layer plates in $CHCl_3$:methanol (90:10). The chromatogram was exposed to Kodak XOMAT film overnight at room temperature to visualize the acetylated products.

For the quantitative assay of CAT activity by scintillation spectrophotometry, 30 μl cell extract were mixed in a scintillation counting vial with 1.25 mM chloramphenicol, 0.1 M Tris-HCl, pH 7.8, and 0.1 μCi (40 pmol) ^{3}H-acetyl CoA in a total volume of 0.2 ml. The reaction mixture was overlaid with a 5 ml Betafluor (National Diagnostics, Sommerville, NJ) and placed into a scintillation spectrophotometer set for 0.5 min/sample and automatic recycling for 6–10 cycles. The linear rate of CAT enzymatic activity was detected as the diffusion of ^{3}H-acetylated chloramphenicol into the organic scintillation cocktail. All samples generated a linear curve of enzymatic activity ($r > 0.95$) in this assay for at least 3 h (*i.e.* six or more counting cycles).

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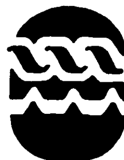

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