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Functional Analysis of the Human Androgen Receptor Using Synthetic and Naturally Occurring Mutations

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To My Parents,
Hossein and Rouhangiz

ABSTRACT

The human androgen receptor (hAR) is a ligand-activated transcription factor, and like other nuclear receptors, consists of a N-terminal modulatory domain, a central DNA-binding domain, and a C-terminal ligand-binding domain (LBD). Several missense mutations in the LBD cause androgen insensitivity syndrome (AI), a condition in XY individuals with absent or subnormal male primary and secondary sexual characteristics. On the other hand, abnormal expansion of a polyglutamine tract in the N-terminal domain of the hAR causes spinal and bulbar muscular atrophy (SBMA) which also affects males and causes milder forms of AI, in addition to adult-onset motor neuron degeneration and gradual wasting and weakening of the muscles of the limbs, face, throat, and tongue. However, it was not clear how and to what extent these mutations contribute to the clinical phenotype of the affected individuals. In order to investigate this matter, I used PCR site-directed mutagenesis to create plasmids expressing hARs with two pairs of missense mutations in the LBD (Val865Leu and Val865Met, and Arg839His and Arg839Cys), discovered in AI individuals with varying severity of the phenotype, and two abnormal expansions of the polyglutamine repeat discovered in SBMA patients (40 and 50 glutamines). I also synthesized plasmids expressing no glutamines (0 glutamines), 12 glutamines, or 20 glutamines in the same N-terminal region of the hAR. These plasmids were transiently expressed in heterologous cells (COS-1 and PC-3), and the mutant hARs were assayed for ligand binding, stability, and transactivational capacity.

In contrast to the findings by others (McPhaul et al., 1992; Marcelli et al., 1994), in some instances involving identical mutations, I consistently observed a correlation between the biochemical phenotype of the mutant hARs and the clinical phenotype of AI individuals; that is, the more severe receptor phenotype was associated with the more severe AI. These results support the hypothesis that hAR phenotype is the dominant factor in the development of the secondary sexual characteristics in normal and affected individuals.

I also observed a tight negative correlation between polyglutamine tract length and transactivational capacity. This suggests that polyglutamine modulates the activity of the hAR, and that hAR activity might be suppressed in various androgen-sensitive tissues (including motor neurons) in SBMA individuals, thereby contributing to the age of onset and/or progression of the disease, even if it cannot be the primary pathogenic agent of the disease.

ABSTRACT (Français)

Le récepteur de l'androgène humain (hAR) est un facteur de transcription activé par ligand, et comme d'autre récepteurs nucléaires, possède un domaine de modulation N-terminal, un domaine central de liaison d'ADN et un domaine de liaison du ligand en C-terminal (LBD). Plusieurs mutations non-sens dans le domaine C-terminal sont à l'origine du syndrome d'insensibilité androgénique, une condition observe chez des individus XY présentant une absence ou une déficience de caractéristiques sexuelles males primaires et secondaires. D'autre part, l'expansion anormale d'une chaîne de polyglutamine dans le domaine N-terminal de hAR est la cause d'une autre condition, l'atrophie spinobulbaire (SBMA) qui affecte également les mâles et crée, en plus d'une forme modérée d'insensibilité à l'androgène, une dégénération des neurones moteurs chez l'adulte suivie d'une faiblesse et dégénéresceance des muscles des jambes, du visage, de la gorge, et de la langue. Cependant le mécanisme d'action ainsi que la contribution actuelle de ces mutations au phénotype des individus affectés sont encore peu définis. Afin d'étudier le mécanisme d'action de ces mutations, j'ai utilisé la technique de mutagénèse dirigée par PCR pour construire des vecteurs plasmidiques exprimant le gène hAR contenant deux paires de mutations non-sens dans le domaine LBD (Val 865Leu et Val865Met, et Arg839His et Arg839Cys) identifiées chez des patients souffrants de différents niveaux d'insensibilité androgénique. J'ai également construit une série de vecteurs contenants une varieté de chaîne de polyglutamine (0, 12, 40, and 50 répétitions) dont les deux longues chaînes (40 et 50) furent identifiées chez des patients atteints de SBMA. Ces vecteurs furent exprimés de façon transitoire dans dans cultures cellulaires hétérologues (COS-1 and PC-3)et les protéines mutantes furent testées pour leur stabilité, leur capacité à lier un ligand et leur proprieté transactivatrice.

En contraste avec les résultats provenant d'autres laboratoires (McPhaul et al., 1992; Marcelli et al., 1994), et dans plusieurs cas sur des mutations identiques, j'ai observé une corrélation entre le phénotype biochimique des protéines mutantes hAR et le phénotype clinique des individus souffrant d'insensibilité androgénique; entre d'autres mots qu'une déficience sévère du récepteur était associée avec un phénotype clinique sévère. Ces résultats supportent l'hypothèse que le phénotype du récepteur hAR est le facteur dominant dans le développement des caractéristiques sexuelles secondaires chez les individus normaux de même que chez les individus atteints d'insensibilité androgénique.

J'ai également observé une forte corrélation inverse entre la longueur de la chaîne de polyglutamine et les propriétés transactivatrices de hAR. Ceci suggère que la chaîne de polyglutamine détermine le niveau d'activité de hAR et que l'activité de hAR pourrait être supprimée dans différents tissus androgéniques (incluant les neurones moteurs) chez les

individus atteints de SBMA contribuant ainsi à la manifestation clinique tardive et/ou la progression de la maladie; ceci même si hAR ne constitue pas l'agent pathogène primaire de la maladie.

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Paper #2. Substitution of arginine-839 by cysteine or histidine in the androgen receptor causes different receptor phenotypes in the cultured cells and coordinate degrees of clinical androgen resistance. Beitel LK, Kazemi-Esfarjani P, Kaufman M, Lumbroso R, DiGeorge AM, Killinger DW, Trifiro M, and Pinsky L (1994) *J Clin Invest* 94, 546-554

CHAPTER II

Paper #3. Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the $(CAG)_n$-expanded neuronopathies. Kazemi-Esfarjani P, Trifiro MA, and Pinsky L (1995) *Hum Mol Genet* 4, 523-527

MANUSCRIPTS AND AUTHORSHIP

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The thesis must still conform to all other requirements of the guidelines concerning thesis preparation and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: 1) a table of contents, 2) a general abstract in English and French, 3) an introduction which clearly states the rationale and objectives of the study, 4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and 5) a final overall conclusion and/or summary.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

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CONTRIBUTIONS TO THIS THESIS

The authors on papers #1, #2, and #3 of this thesis contributed to this work in the following ways:

Paper #1.

Parsa Kazemi-Esfarjani:

1) Site-directed mutagenesis of Val865Met;
2) compilation of Tables 1 and 2, related statistical analyses, and experiments related to COS-1 dissociation rates and COS-1 dissociation constants;
3) Figure 4, Scatchard analysis and the plots;
4) Figures 5 and 8 and Table 3, transactivation assays, statistical analyses and the plots;
5) Table 4, Figures 7 and 9, MB-binding assays, Western blots, transactivities, related mathematical and stastistical analyses, and the plots and art work;
6) participation in writing and revising the abstract, methodology, results, and discussion of the manuscript.

Lenore K. Beitel:

1) Discovery and site-directed mutagenesis of Val865Leu;
2) Figure 1, sequencing of genomic DNA;
3) participation in writing and revising of the abstract, methodology, results, and discussion of the manuscript.

Mark Trifiro:

Co-supervisor

Morris Kaufman:

1) Tables 1 and 2, GSF assays;
2) Figure 3, dissociation rates and mathematical calculations and the plots.

Patricia Sheppard: (collaborator)

1) Figure 6, PC-3 transactivation assays.

Paul Rennie: (collaborator)

Patricia Sheppard's supervisor.

Robert Matusik: (collaborator)

1) Construction of the probasin promoter-CAT plasmid.

Leonard Pinsky:

1) My supervisor
2) participation in, and directing the writing of the manuscript.

Paper #2.

Parsa Kazemi-Esfarjani:

1) Site-directed mutagenesis of Arg839His;
2) compilation of Table 2, related statistical analyses, and experiments related to COS-1 dissociation rates;
3) Figure 6, thermolability assays and the plots;
4) Figures 7A and 7B, transactivation assays, statistical analyses and the plots;
5) Figures 8 and 9, MB-binding assays, Western blots, transactivities, related mathematical and stastistical analyses, and the plots and art work;
6) participation in writing and revising of the abstract, methodology, results, and discussion of the manuscript.

Lenore K. Beitel:

1) Discovery and site-directed mutagenesis of Arg839Cys;
2) Figure 2, sequencing of genomic DNA;
3) Figure 3, PCR/restriction enzyme digest/PAGE analyses;
4) participation in writing and revising of the abstract, methodology, results, and discussion of the manuscript.

Morris Kaufman and Rose Lumbroso:

1) Figure 4 and 5, Scatchard and thermolability analysis in GSF;
1) compilation of Tables I, GSF offrates;
3) Table II. GSF offrates.

Angelo M. DiGeorge and Donald W. Killinger: (collaborators)

Clinical co-investigators

Mark Trifiro:

Co-supervisor

Leonard Pinsky:

1) My supervisor;
2) participation in, and directing the writing of the manuscript.

Paper #3.

Parsa Kazemi-Esfarjani:

1) recloning of the human androgen receptor (hAR) cDNA fragments with 40 and 50 glutamines (from pCMVhAR.Gln40 and pCMVhAR.Gln50) into the SVhAR.BHEXE expression vector, and PCR-synthesis of hAR plasmids with 0 and 12 glutamines;
2) Figure 1a and 1b, transactivation assays and the plots;

3) Table 1, Scatchard and offrate analyses in COS-1 cells and related mathematical calculations;

4) Western blots (data not shown);

5) Figure 2, full involvement in the construction of the models and the hypotheses regarding mechanisms of pathogenesis (gain-of-function versus loss-of-function);

6) participation in writing of the abstract, introduction, methodology, results, and discussion of the manuscript.

Mark Trifiro:

Co-supervisor

Leonard Pinsky:

1) My supervisor

2) participation in, and directing the writing of the manuscript.

ABBREVIATIONS

5'-UTR	5'-untranslated region
ADF	Age-dependent factor
AF	Associated factor
AI	Androgen insensitivity
AIS	Androgen insensitivity syndrome
Ala	Alanine
AR	Androgen receptor
ARE	Androgen response element
Arg	Arginine
Asn	Asparagine
ASTP	ATP-stimulated translocation promoter
bp	Base pair
C-terminal	Carboxyl-terminal
CA	Cyproterone acetate
CAI	Complete androgen insensitivity
CAT	Chloramphenicol acetyl transferase
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
Cys	Cysteine
D	Dalton
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
DHT-R	5α-dihydrotestosterone-receptor
EMSA	Electrophoretic mobility shift assay
ER	Estrogen receptor
ERE	Estrogen response element
FKBP	FK binding protein
fmol	Femtomole
FSH	Follicle stimulating hormone
GH	Growth hormone
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GR	Glucocorticoid receptor

GRE	Glucocorticoid response element
GSF	Genital skin fibroblast
hAR	Human androgen receptor
hER	Human estrogen receptor
hGR	Human glucocorticoid receptor
His	Histidine
hMR	Human mineralocorticoid receptor
hPR	Human progesterone receptor
HRE	Hormone response element
hsp	Heat shock protein
Ile	Isoleucine
kb	Kilo base pair
kD	Kilodalton
LBD	Ligand-binding domain
Leu	Leucine
LTR	Long terminal repeat
Lys	Lysine
MAI	Mild androgen insensitivity
mAR	Mouse androgen receptor
MB	Mibolerone
mER	Mouse estrogen receptor
Met	Methionine
MMTV	Mouse mammary tumor virus
MT	Methyltrienolone or R1881
N-terminal	Amino-terminal
NF-1	Nuclear factor-1
NF-kB	Nuclear factor-kB
NLS	Nuclear localization signal
nM	Nanomolar
NMR	Nuclear magnetic resonance
Oct1	Octamer-binding factor 1
ORF	Open-reading frame
PCR	Polymerase chain reaction
Poly(A)	Polyadenylation
PR	Progesterone receptor
PRE	Progesterone response element

PSA	Prostate specific antigen
R	Receptor
RAF	Receptor accessory factor
RAP	Receptor-associated proteins
rAR	Rat androgen receptor
RE	Response element
rGR	Rat glucocorticoid receptor
SBMA	Spinal and bulbar muscular atrophy
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel Electrophoresis
Ser	Serine
SNB	Spinal nucleus of the bulbocavernosus
T	Testosterone
T-R	Testosterone-receptor
TAU	Transcription activation unit
Thr	Threonine
TIS	Transcription initiation site
tk	Thymidine kinase
Tyr	Tyrosine
Val	Valine
VP16	Viral protein 16
Zn	Zinc

PREFACE

Hormones are components of a highly complex signal transduction pathway. They affect numerous aspects of our body's physiology: our immune system; our appetite for food; our desire for reproduction. They communicate their message to the target cells via receptors on their cytoplasmic membrane, in their cytoplasm, and/or nuclear matrix. In some instances, after the hormone binds its receptor, the activated hormone-receptor complex initiates a cascade of activation/inactivation of a series of interdependent proteins which culminates in the activation or suppression of a gene or a network of genes. In other instances, the activated hormone-receptor complex activates or represses the target gene(s) directly by interacting with regulatory elements of those gene(s). This latter class of receptors, known as nuclear receptor superfamily, offers a simple model for studying signal transduction and gene regulation.

Our laboratory has elected to study the structural and functional aspects of the human androgen receptor (hAR), a member of the nuclear receptor superfamily. As a model, the hAR offers several advantages over other nuclear receptors. First, the hAR locus is X-linked. Therefore, males are hemizygous for this gene and the analysis of genotype-phenotype is simplified. Second, although a fully functional hAR is essential for genetic viability and reproduction, hence species survival, most of the mutations of the hAR do not appear to affect the mortality or morbidity of the affected individuals. As a result, we and others have been able to compile a large number of naturally occurring mutations with their phenotypic consequences in the affected subjects. These mutations occur throughout the gene and result in amino acid substitutions and partial to complete deletions of the gene product. Finally, because of high homology among members of the nuclear receptor superfamily, especially among those of the steroid receptor subfamily, many of the structural and functional data obtained by studying the hAR can be extrapolated to other members of this group.

This thesis will begin with a review of the relevant literature regarding the AR in human, and to a minor extent in other animals. Whenever necessary, studies on other nuclear receptors have been used to compliment those of the AR. The review is followed by three original research articles to which I made a major contribution.

INTRODUCTION

The androgen receptor and its relatives

The androgen receptor (AR) and other nuclear receptors are ligand-activated transcription factors, and form a large family of proteins known as the nuclear receptor superfamily. The AR, like other steroid and thyroid receptors, is present in trace amounts (10^3 to 10^4 per cell) (Evans, 1988). Their ligands vary in size and structure: steroids are cholesterol-based; thyroid hormones are aromatic amino acids; and retinoids are isoprenoids (Forman and Samuels, 1990). Steroid hormones are further subdivided into three major classes: the adrenal steroids (e.g. cortisol and aldosterone), sex steroids (e.g. progesterone, estrogen, and androgens), and vitamin D_3. Usually, in order to regulate the expression of their target genes, they have to form a complex with their respective receptor. The activated ligand-receptor complexes will then bind, as dimers, to short stretches of regulatory DNA sequences known as hormone-response elements (HRE) to activate or suppress the transcription of the target gene.

The first suggestion of a domain structure came from analysis of limited proteolysis of the purified glucocorticoid receptor (GR) which revealed functionally independent DNA-binding and hormone-binding domains in a single GR molecule (Evans, 1988 and refs. therein). Furthermore, a mutant GR that had lost a portion of its hormone-binding domain constitutively transactivated a reporter gene showing that the full hormone-binding domain and the hormone itself were not essential for DNA binding or transactivation by the receptor.

The origin of the nuclear receptors

Two models have been proposed for the origin and evolution of the nuclear receptors. The first model proposes that the substrate-binding domains in metabolic enzymes and other molecules were joined to DNA-binding motifs to produce ligand-binding transcription factors. The second model proposes that a precurser protein with multiple domains has given rise to other multi-domain nuclear receptors. Sequence comparison and evolutionary analysis has suggested that perhaps the second model is true (Amero et al., 1992). Although the so-called precusor receptor might have been formed by an initial domain shuffling involving other molecules, it seems there has not been any other subsequent shuffling in the generation of nuclear receptors. This presumption is based on the greater sequence homology between the members of the nuclear receptor superfamily than between the individual nuclear receptors and other non-member molecules.

ANDROGEN RECEPTOR GENE STRUCTURE

Genomic organization

The human AR (hAR) locus is mapped to Xq11-12 region on the long arm of the X chromosome (Brown et al., 1989). The hAR is encoded by a single gene with 8 exons, spread over more than 90kb of genomic DNA (Kuiper et al., 1989). The intronic sequences vary in size from 0.7kb to 26kb. The first exon codes for an exceptionally long 5'-untranslated region (5'-UTR) 1.1kb in size, and all of the N-terminal region (1586bp). The smaller exons 2 (152bp) and 3 (117bp) code for the two DNA-binding zinc fingers located centrally in the protein. The ligand-binding domain in the C-terminus is encoded by exons 4 to 7 (288, 145, 131, and 158bp) and part of exon 8 (153bp). The remainder of exon 8 codes for a very long 3'-UTR 6.8kb in length with two functional polyadenylation (poly(A)) signals, ATTAAA and CATAAA, 221bp apart. Poly(A) addition occurs 14-24bp downstream of the poly(A) signal (Faber et al., 1991).

The hAR gene produces two mRNA species of 8 and 11kb (Faber et al., 1991). The 8kb message is the result of alternate splicing of the primary RNA, shortening the 3'-UTR by 3kb. The AR is expressed at low to moderate levels in many tissues and at high levels in the male urogenital system (Mooradian et al., 1987). The same mRNA species are also expressed in LNCaP (human prostate carcinoma) and T47D and MCF-7 (human breast carcinoma) cell lines (Tilly et al., 1990). In the mouse, AR mRNA was detected in the testis, epididymis, kidney, brain and submandibular glands, but not in the spleen (Grossman et al., 1994).

The AR expression is regulated by various small molecules such as follicle stimulating hormone (FSH), cAMP and androgens. Androgens reversibly down-regulate the AR mRNA in such tissues as prostate, kidney, brain, and epididymis. In contrast, FSH and cAMP up-regulate AR mRNA in Sertoli cells (Lindzey et al., 1993). Nevertheless, when androgen down-regulates AR mRNA, it may still up-regulate AR protein and, therefore, androgen-binding activity.

Both messages have an open-reading frame (ORF) of 2751bp (Brinkmann et al., 1989). This ORF codes for a protein that may vary from 897 to 936 amino acid residues. The variability of the protein size is due to two polymorphic, homopolymeric stretches of amino acids in the N-terminal region: a polyglutamine tract of 11-33 residues coded by $(CAG)_nCAA$, and a polyglycine tract of 12-29 residues coded by $(GGN)_n$ (Pinsky et al., 1995). There are other non-polymorphic homopolymeric stretches in the N-terminal region:

two short polyglutamine tracts (n=6 and n=5), a polyproline tract (n=8), and a polyalanine tract (n=5) (Brinkmann et al., 1989) (Fig. 1).

The DNA-binding domain (DBD) is located centrally between amino acids 557 and 623 (67 residues) and the ligand-binding domain (LBD) in the C-terminus consist of residues 669 to 917 (248 residues) (numbering according to Brinkmann et al., 1989). The DBD and LBD are separated by 46 residues that constitute the hinge region (Fig. 1). The calculated molecular weight of a 917-amino acid residue is 98,845D, but on a SDS-PAGE gel the protein appears as a ≈110kD band (Brinkmann et al., 1989). In human genital skin fibroblast, in addition to the 110-kD hAR or the B isoform (AR-B), there is a 87-kD hAR or the A isoform (AR-A) that is expressed at 1/10 of the level of AR-B (Wilson and McPhaul, 1994). AR-A appears to be the result of translation initiation at a more C-terminal ATG.

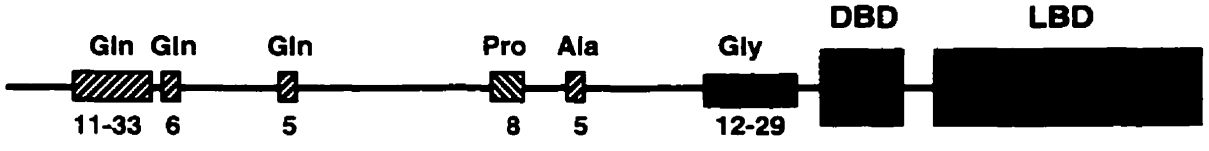

Figure 1. Schematic structure of the hAR and the relative locations of the homopolymeric tracts. The size and amino acid composition of each tract is indicated.

AR 5'-regulatory region

Two transcription initiation site (TIS) of the hAR gene were found 13bp apart, at 1114 and 1126bp upstream of ATG translation start site (Faber et al., 1991). The hAR promoter is unusual in that it lacks common features such as TATA and CCAAT boxes, an initiator sequence, or a HIP1 sequence (Faber et al., 1991). The prominent features in the hAR promoter are a short stretch of G+C-rich sequence and 60bps containing multiple repeats of the motifs GAAA or GAAAA. The former includes a binding site for Sp1, a "housekeeping" transcription factor, but the latter motifs are of unknown function (Faber et al., 1991; Tilly et al., 1990). Band-shift and mutagenesis analyses have shown that utilization of only the proximal site (TISII at 1114bp) is dependent on Sp1-binding, and TISI utilization is dependent on the sequences between -5 and +57 (Faber et al., 1993). This may be an additional level of regulation of the rate and/or tissue specificity of AR expression.

The mouse and rat AR promoters are highly homologous to that of hAR. Nonetheless, one difference stands out and that is the number of GGGGA motifs: 4 in

human, 6 in mouse, and 8 in rat (Faber et al., 1991). Perhaps, this repeat motif fine tunes the expression of AR in each organism.

In the mouse AR (mAR) gene , a second promoter has been described (Grossman et al., 1994). Transcription initiation sites of this promoter are 162 and 170bp downstream from the 5'-most transcription initiation site of the first promoter. The transcripts initiated from the second promoter seem to be more abundant in several tissues and cell lines that have been examined. In addition, it appears that these two promoters might activate the AR gene in a synergistic fashion at least in some cell lines. Also, transcripts from both promoters were down-regulated by DHT.

Another regulatory element that affects the expression of the AR is in the 5'-UTR (Mizokami and Chang, 1994). This element is present between +21 to +149bp of the hAR 5'-UTR and promotes translation without a comparable effect on transcription. Based on its nucleotide sequence, it potentially forms a stem-loop structure in the hAR mRNA.

There are also enhancer elements that regulate the expression of AR in an age-dependent (-329 to -311bp) and tissue-specific (-310 to -286bp) manner (Supakar et al., 1993). These elements of rat AR (rAR) promoter seem to interact with two novel transcription factors in the rat liver. The first, the age-dependent factor (ADF), is ubiquitously expressed and evolutionarily conserved and its activity in the liver nuclear extract decreases about 7-fold with aging between 3 to 26 months of age (Supakar et al., 1995). The second is termed associated factor (AF) and its activity may be restricted to tissues such as liver or liver-derived cell lines.

In addition to transcription enhancer sequences, there are two repressor elements in the AR promoter. The first element was characterized in the mouse and localized to a region between -451 and -418bp of the promoter (Kumar et al., 1994). It is not known how this element exerts its repressor function. The second element has been characterized in the rat AR (rAR) and has been localized to -574 and -554bp of the promoter (Supakar et al., 1995). This region is responsible for the age-dependent decline in the androgen sensitivity of the rat liver, and includes a binding site for the nuclear factor kB (NF-kB).

NF-kB belongs to a family of dimeric transregulatory proteins, critical in immune response, inflammation, oxidative stress and embryonic development (Supakar et al., 1995 and refs. therein). In the rAR, two forms of NF-kB interact with the repressor element: a heterodimer of p50/p65 and a homodimer of p50/p50. The expression of the latter in the rat liver gradually increases to 10-fold in the aged rats exhibiting hepatic desensitization.

Finally, as mentioned above, androgens down-regulate and cAMP up-regulates the AR mRNA (Lindzey et al., 1993). cAMP seems to increase the transcription rate through sequences in the 5'-flanking region and 5'-UTR (-546 to +971bp). However, multiple

effector elements spread across this region might be responsible for the cumulative effect of cAMP. Similarly, although cAMP induction by this region was counteracted in an androgen and AR-dependent manner, the putative ARE at the 5'-end of this sequence did not appear to be responsible for this effect. Therefore, the AR might influence the transcription rate through AREs that deviate from the consensus ARE, or it might exert its effect independent of DNA-binding (e.g. protein-protein interaction).

AR MUTATIONS AND THEIR CLINICAL MANIFESTATIONS

Androgen insensitivity syndrome

In the past 20 years, numerous cases of androgen insensitivity syndrome (AI) have been reported. AI affects 1 in 20 000 to 60 000 XY newborns (MacLean et al., 1995), but the frequency of X-linked AI might be higher if milder forms such as male infertility are included. These individuals have normal to slightly elevated levels of testosterone and luteinizing hormone. However, various androgen target-tissues in their body seem to either not respond to the androgens or do so at suboptimal levels. Although other genes and their products may be involved, so far only mutations in the gene for the AR have been described as the cause in the pathogenesis of this syndrome. Due to the fact that even complete loss of the AR function is not lethal, and the AR gene is X-linked with only one allele active in every cell, the carrier females are usually spared and the affected males are otherwise healthy individuals with variable degrees of androgen insensitivity. The AR is expressed in almost all tissues many of which show sexual dimorphism. AI diagnosis is usually made based on the degree of the severity of the external phenotype, especially the genitalia. Thus, individuals with AI are classified as follows: those with female external genitalia represent complete AI (CAI); those with ambiguous external genitalia represent partial AI (PAI); and those with male-like genitalia represent mild AI (MAI) (Pinsky et al., 1995).

The clinical diagnoses also include other features of the AI. For example, CAI is often associated with gynecomastia (breast development), sparse, or absence of, pubic and axillary hair, and female body habitus. PAI may also be associated with gynecomastia and defective axillary and pubic hair growth. MAI may be associated with cryptorchidism, micropenis, and azoospermia or oligospermia resulting in weak, or lack of, fertility.

To find the molecular and biochemical bases of the patients' clinical phenotype, measurements of AR function were initially performed on fibroblast cell strains derived from genital skin biopsies. Genital skin fibroblasts (GSF) have three-fold higher androgen

binding compared to fibroblasts from nongenital skin (Kaufman et al., 1977). Patients were classified based on whether their GSFs were positive for androgen-binding (≥15 fmol/mg protein), intermediate (between 5-15 fmol/mg protein), or negative (<5 fmol/mg protein).

The mutations that cause AI cover the whole spectrum of molecular defects. Complete AR gene deletions have been reported in two unrelated families by our group and others (Trifiro et al., 1991; Quigley et al., 1992). Although in both families the affected individuals had CAI, the single affected individual in our report also suffered from muscle weakness and neurological and psychological problems (Trifiro et al., 1991) while the two affected siblings in the other report were otherwise healthy. There are also several nonsense, frame-shift, and splice-junction mutations; almost invariably, the outcome is an inactive AR causing CAI in the affected individual (MacLean et al., 1995; Gottlieb et al., 1996), so far with two exceptions. In one case, the deletion of part of intron 2 and exon 3 gave rise to an AR that yielded PAI (Ris-Stalpers et al., abstract 1992). In a second case, the in-frame deletion of exon 4 (the "hinge region" and a small portion of the LBD) only caused azoospermia in the affected subject (Akin et al., 1991).

In one case, the nonsense mutation resulted in a premature stop codon in the N-terminal region of the AR. However, in spite of this mutation, the AR mRNA was translated into a N-terminally truncated AR using a downstream initiation site (Zoppi et al., 1993). Under transfection conditions, this mutant AR could have up to 30% wild-type AR transactivity at very high protein expression levels. Despite this residual activity, it caused CAI in the patient.

Some missense mutations such as Gly907Arg (Choong et al., 1996) or Arg773Cys (Marcelli et al., 1991) causing PAI or CAI, respectively, are also associated with decrease in AR mRNA levels by S1 nuclease protection assay. In contrast, genital skin (GSF) from a patient with an identical mutation at position 773 had normal mRNA levels by Northern blot analysis (Prior et al., 1992). Either this difference is due to the sensitivity of the assays or due to cell culture and/or GSF biopsy procedures. The stability of the AR mRNA was also reduced 10- to 20-fold by a frame-shift mutation in the Tfm mouse AR gene (Charest et al., 1991). Cycloheximide injection of the Tfm mouse resulted in a 17-fold increase in the AR mRNA isolated from the kidney and liver, with an identical size to that of the normal mRNA. This increase was about 70% of the wild-type AR mRNA levels.

In contrast to gross AR rearrangements, there are many missense mutations that result in receptors with normal androgen-binding capacity under saturating conditions. Most of these receptors, however, have other qualitative defects. Some show androgen-selectivity associated with PAI by having a normal androgen dissociation rate with one

ligand but not with another (Normal DHT rates and abnormal MT rates (Val865Leu in Jukier et al., 1984 and Kazemi-Esfarjani et al., 1993) or vice versa, normal MT rates and abnormal DHT rates (Ser813Asn in Pinsky et al., 1984)). Some ARs with missense mutations causing CAI have dissociation constants that are normal under equilibrium conditions (K_d) but abnormal under nonequilibrium conditions (Val889Met in Zhou et al., 1995), and some with opposite androgen-binding characteristics causing MAI (Arg840Cys in Beitel et al., 1994). Interestingly, the deficiency associated with Asn727Lys mutation in a case of an infertile male could be compensated by a high dose of a synthetic androgen, mesterolone (Yong et al., 1994). This evidence indicates that perhaps more subtle defects in the LBD might affect particular steps in the ligand-binding process while sparing others.

There are also those mutations that affect DNA-binding, usually causing CAI with (Beitel et al., 1994) or without (Marcelli et al., 1991) deficiencies in ligand-binding. DNA-binding mutations may also affect the steady-state level of AR mRNA (Beitel et al., 1994). On the other hand, so far ~~no~~ few detrimental missense mutations have been discovered in the N-terminal region in AI, or for that matter, in any other disease. Thus, the principal functional components of this domain ~~must~~ may be regional and modular rather than specific amino acid sequences.

Prostate and breast cancers

Aside from the missense mutations in the AR that cause AI, there are those that are associated with cancer of classical endocrine target tissues such as the prostate and the breast. These mutations may (Wooster et al., 1992; Lobaccaro et al., 1993) or may not (Elo et al., 1995) be associated with AI. Male breast cancer is a very rare condition accounting for about 1% of all breast cancers (Sasco et al., 1993). So far, two cases of male breast cancer with PAI have been reported. They are associated with missense mutations of two consecutive codons in the DBD-coding segment causing Arg607Gln or Arg608Lys substitutions (Wooster et al., 1992; Lobaccaro et al., 1993). It has been suggested that abnormal DNA-binding specificity of the mutant AR might allow it to bind estrogen response elements (EREs) promoting estrogen receptor (ER) target genes hence increasing the risk of breast cancer (Wooster et al., 1992; Sultan et al., 1993). Our results have shown that ARs with single codon deletions or an amino acid substitution in different regions of the DBD can superactivate the wild-type hAR, hGR, and human progesterone receptor (hPR) in a transient cotransfection assay (Kazemi-Esfarjani et al., abstract 1994). Our results with hER were inconclusive. Whether Arg607Gln or Arg608Lys can bind ERE or superactivate other steroid receptors including ER is not known.

Prostate cancer is a very common male form of cancer (Carter et al., 1990). The progress of most primary prostate cancers is androgen dependent, and about 75% initially respond to androgen ablation therapy (MacLean et al., 1995). However, more than half of the responding carcinomas gradually become resistant (Suzuki et al., 1993). In the recent years, it has become evident that at least in some of the prostatic carcinomas, the AR is still expressed. Several groups have screened the AR gene in the prostatic carcinomas for mutations. As a result, several missense mutations have been reported (Suzuki et al., 1993; Veldscholte et al., 1992; Peterziel et al., 1995). The common theme among these mutations is an AR with a wider range of ligand specificity while maintaining its wild-type affinity for androgens. This allows the mutant AR to bind to other steroids, androgen metabolites, and even some antiandrogens in a productive fashion, and perhaps activate androgen-responsive genes.

Higher risk of prostate cancer appears also to be linked to shorter N-terminal polyCAG tract (<22 CAGs) and longer polyGGC tract (>16 GGCs) in a population of control subjects from three racial backgrounds in the United States: African-American, non-Hispanic white, and Asian (Japanese and Chinese) (Irvine et al., 1995). Furthermore, the same study showed a significant negative correlation between the length of polyCAG and polyGGC tracts among the prostate cancer patients under study. These data support our cell transfection findings that shorter polyCAG in the hAR is associated with higher transactivity, and perhaps precise deletion of polyGGC is associated with lower transactivity (and by inference, the longer polyGGC is associated with higher transactivity) (discussed in more detail in the section on the N-terminal domain). In turn, higher transactivity of the hAR may result in increased androgen-induced initiation and/or progression of the prostate cancer. This was also further substantiated by a more recent study showing that the length of the polyglutamine tract directly correlated with the age of onset of the prostate cancer (Hardy et al., 1996). This possible increase in transactivity may also explain the negative correlation between the polyCAG tract length and Ferriman Gallwey score (i.e., shorter polyCAG was associated with more hirsutism or virilization) in a Hispanic subpopulation of normoandrogenic women with idiopathic hirsutism (Legro et al., 1994).

Spinal and bulbar muscular atrophy

X-linked spinal and bulbar muscular atrophy (SBMA) or Kennedy syndrome is a motor neuron degenerative disease often preceded by mild to partial androgen insensitivity. The neurological symptoms usually appear between the third and fifth decade of life and gradually progress until death (Harding et al., 1982). The average life span may be reduced

only slightly and most patients continue working for several years after the onset of disease. The neurological findings include cramps, fasciculation, weakness, and wasting of the face, tongue, and proximal limb muscles. Similar abnormalities in distal limb muscles, hand tremor, and less frequently, dysarthria and dysphagia are also observed (Harding et al., 1982). Loss or weakness of sensory nerve pathways have also been reported. The endocrinological findings include gynecomastia (over 50% of the cases) and perhaps less frequently testicular atrophy and infertility.

SBMA was originally linked to the same chromosomal region (Xq12-21) as the gene for the hAR (Xq11-12). Therefore, the AR gene in SBMA patients was screened for mutations by Southern blot, PCR amplification and sequencing (LaSpada et al., 1991). The only difference in the AR gene between the 35 SBMA patients from 23 unrelated families and 75 control subjects was an enlarged CAG repeat sequence that encodes a polyglutamine tract in the N-terminal portion of the hAR. This CAG repeat is expanded from the normal 11-33 repeat to over 40 in SBMA (LaSpada et al., 1991; Pinsky et al., 1995).

After this discovery in SBMA, similar expansion of coding CAG repeats were found in four other neurodegenerative disorders (including Huntington disease) affecting various regions of the central nervous system, especially the brain (Ross, 1995). In SBMA (and more strongly in the other four disorders), there is a correlation between the length of the CAG repeat and the severity and age of onset of the disease: the longer the repeat, the earlier the age of onset and more severe the disease (LaSpada et al., 1992; Doyu et al., 1992; Igarashi et al., 1992). However, this was not confirmed by another group (MacLean et al., 1995).

The androgen insensitivity associated with SBMA may be explained by the fact that the hARs containing the expanded polyglutamine tract have decreased ligand affinity in GSF from SBMA individuals (MacLean et al., 1995) and decreased transactivation capacity in heterologous cell culture (Kazemi-Esfarjani et al., 1995). However, the biochemical mechanism underlying neurodegeneration in SBMA is still unknown.

In order to begin to explain the pathogenic role of the expanded polyglutamine tract in neurodegeneration of motor neurons, a few observations deserve attention. First, the AR is expressed in many neuronal tissues including motor neurons in the anterior horn of the spinal cord where degeneration occurs, and the levels of expression are comparable to those of other non-neuronal androgen target tissues such as GSF (≈50 fmol/mg protein) (Sarrieau et al., 1990; Kerr et al., 1995). Second, androgens prevent normally occurring motor neuron death in the spinal nucleus of the bulbocavernosus (SNB) during late perinatal and early postnatal development in male rats (Nordeen et al., 1985). The same level of protection was achieved for androgen-treated female rats. Finally, the most

important observation is that the subjects with mild, partial, or complete AI, including those with AR truncations or complete gene deletions, do not have SBMA, and for that matter any other neurological disorders [except for a CAI individual who had complete gene deletion with non-SBMA neurological findings (Trifiro et al., 1991)]. Therefore, the AR appears to contribute to the normal development and function of the motor neurons, but its absence is not sufficient to cause motor neuron degeneration. This fact invoked the hypothesis that the polyglutamine-expanded hAR has gained a new function that results in degeneration of motor neurons, and this effect is perhaps exacerbated by the loss of hAR's usual function in motor neurons (Mhatre et al., 1994; Kazemi-Esfarjani et al., 1995). As all other disorders of polyglutamine-expansion are autosomal dominant disorders, this provided a paradigm for further experiments that could explain neurodegenerartion not only in SBMA, but also in other aforementioned disorders.

Two models have attempted to explain the mechanism of gain-of-function associated with the expansion of polyglutamines. The first model suggests covalent isopeptide bond formations between the glutamines of the expanded polyglutamine tract and a lysine residue of another protein, catalyzed by a particular transglutaminase, result in abnormal accumulation of the protein complexes, hence neurotoxicity (Green, 1993). The second proposes that polyglutamines form "polar zippers" mediated by the hydrogen bonds between the main- and side-chain amides of adjacent proteins (Perutz et al., 1994). This model suggests that abnormally expanded polyglutamine tracts form excessively stable protein complexes that accumulate and cause neurotoxicity. Experimental data using chimeric proteins support the latter model (Scott et al., 1995). There is also evidence that peptides composed mainly of large polyglutamine tracts accumulate in heterologous cultured cells (COS cell) (Ikeda et al., 1996). This effect appears to be restricted to polyglutamine tract size range observed in CAG-expansion associated neurodegenerative disorders.

AR PROTEIN STRUCTURE AND ITS FUNCTIONAL DOMAINS

The ligand-binding domain

Similar to other nuclear receptors, the LBD is encompassed in the ≈250 amino acid residues of the C-terminal region of the AR forming a hydrophobic pocket, encoded by exons 4 to 8. In addition to its ligand binding function, various portions of the LBD are involved in transactivation, nuclear localization, dimerization and hsp binding. These will be dicussed in the subsequent sections. There is 100% homology between human, rat and mouse AR LBDs and about 50% homology among the LBDs of the hAR and the human receptors for mineralocorticoids, glucocorticoids and progesterones (Choong et al., 1996). The three-dimensional structure of the AR LBD is not known. However, there is considerable functional and structural data derived from naturally occurring and synthetic mutations, and from binding and partial proteolysis studies with various agonist and antagonist ligands. To facilitate the integration of data gathered from different sources, a computer-generated three-dimensional model has been put forth (Goldstein et al., 1993).

This model is based on homology of the LBD with a protease called thermitase which is a member of the subtilisin-like serine proteases. It was generated by protein sequence alignment and other experimental data such as photoaffinity labelling and the effect of substitution of the amino acid residues in the LBD of various nuclear receptors, especially those of the steroid receptors. The model suggests a doubly wound β-sheet made of a core of hydrophobic parallel β-strands covered by α-helices. The ligand-binding pocket is lined with mainly hydrophobic residues and the entrance to it corresponds to the catalytic site of thermitase.

Definition of the LBD by natural and synthetic mutations

The wealth of naturally occurring mutations in the hAR, most of which result in amino acid substitutions, with the corresponding phenotype of the receptor and the patient allows investigators to gradually discover the role of each residue in the LBD. The mutations in the hAR LBD that cause AI loosely cluster to two regions in the LBD: between residues 726 and 772 and between residues 829 and 853 (McPhaul et al., 1992). This observation has been confirmed by compilation of a larger number of natural mutations of the hAR (Gottlieb et al., 1996). This indicates that some segments of the LBD are more critical for the integrity of the three-dimensional structure and/or function of the LBD.

The N-terminal boundary of the hAR LBD is provisionally defined by two missense mutations at residue 686 that change an aspartic acid to histidine or asparagine,

both causing CAI associated with quantitative or qualitative ligand binding abnormalities, respectively (Ris-Stalpers et al., 1991). This residue is also shared with other members of the subfamily of steroid receptors (i.e., hGR, human mineralocorticoid receptor (hMR), and hPR). The most C-terminal natural mutation is a glycine to arginine substitution at residue 907, only 10 amino acids from the C-terminal end (Choong et al., 1996). The receptor with Gly907Arg substitution has 4- to 5-fold less affinity for the natural androgen DHT and about half the normal ligand-binding capacity, and has impaired transactivation. In addition to the natural mutations, there is a synthetic mutant hAR that lacks the most C-terminal 12 amino acids (Jenster et al., 1991). This hAR cannot bind ligand and is inactive.

Gross conformations of the agonist and antagonist receptor complexes

Further evidence regarding the definition of the boundaries and three-dimensional structure of the LBD comes from partial proteolytic digestion of the AR bound to various agonist and anatagonist ligands. In one study, the unliganded hAR and hARs bound to the natural (DHT or T) or synthetic (MB) androgens, or antiandrogen cyproterone acetate (CA) or casodex, were partially digested with trypsin or chymotrypsin (Kallio et al., 1994). The analysis of the digestion reaction products by SDS-PAGE showed that all three androgens protected a 30-kD fragment against protease digestions. This fragment was absent in reactions where the hAR was unliganded or bound to antiandrogens. Furthermore, an N-terminal internal deletion mutant that lacks residues 48 to 408 when bound to androgens protected the same 30-kD fragment hence confirming that the protected fragment does not originate from the N-terminus. An intact LBD with normal ligand binding function was necessary for protection of the 30-kD fragment as a Met807Arg LBD mutant AR could not protect this fragment against proteolysis in the presence of androgen.

In another study, partial proteolytic digestion of the hAR bound to a synthetic androgen (MT or R1881) resulted in a similar protected fragment of 29kD (Kuil and Mulder, 1994). However, under the reaction conditions of this study, the antiandrogen (ICI 176.334) protected a larger fragment of 35kD. In addition, the androgen and antiandrogen, when present together in the same reaction, could compete in producing their respective fragment in a concentration-dependent manner. It was also confirmed that the protected fragments originate from the LBD by using a deletion AR construct that lacked the DBD and the N-terminal region and only contained residues 615 to 910 of the LBD. This truncated AR could also protect a 29-kD fragment.

In similar studies, when the in vitro-translated PR bound to progesterone was partially digested by proteases and subjected to polyacrylamide gel electrophoresis (PAGE) analysis, a 30kD band was evident. However, PR complexed with the anti-progesterone

RU486 and subjected to the same analysis gave a smaller band of 27kD (Baniahmad and Tsai, 1993). In addition, while the binding of RU486 was not affected when 42 amino acids from the C-terminus of the human PR was deleted, it resulted in complete loss of progesterone-binding.

LNCaP is a tumor cell line derived from a metastatic lesion of human prostatic carcinoma (Veldscholte et al., 1992 and refs. therein). The growth of this cell line accelerates in response not only to androgens but also to estrogens, progesterones and several antiandrogens (such as cyproterone acetate and hydroxyflutamide but not ICI 176.334). The hormone response is presumably mediated through the AR, as ER and PR are not detected in this cell line. The cause of this promiscuity in hormone response was eventually attributed to a single base substitution that changes a threonine at position 868 of the hAR LBD to alanine (Thr868Ala) (Veldscholte et al., 1992).

Thr868Ala mutant AR bound to MT, DHT, or the antiandrogen ICI 176.334 was subjected to partial proteolysis and SDS-PAGE analysis as above (Kuil and Mulder, 1994). As with the wild type AR, MT- and DHT-receptor complexes protected a 29-kD fragment while the ICI 176.334-receptor complexes protected a fragment of 35kD. It was intriguing that antiandrogens cyproterone acetate and hydroxyflutamide that act as agonists with this mutant AR also produced agonist-like fragments of 29kD. Therefore, it appears that agonist- and antagonist-receptor complexes have distinct conformations regardless of prior events (such as functional groups of the ligands or AR mutations) leading to their formation.

Subtle conformational differences versus transactivational competence

The missense mutation Thr868Ala in LNCaP is not the only amino acid substitution in the LBD that alters one property (in this case, ligand-binding specificity) without affecting other functions (e.g., its usual ligand affinity). These mutations are very helpful in predicting the residues or regions of the LBD that contact particular parts or functional groups of a ligand. Also due to their selective effects, they allow investigators to tease out critical residues that are involved in other functions of the LBD such as transactivation.

Two other such mutations have been described in the AR of human prostate carcinomas where in each case a valine to methionine substitution has occurred: one at residue 715 (Val715Met) and another at residue 730 (Val730Met) (Peterziel et al., 1995). The two amino acid substitutions do not affect the affinity of the AR for the androgen MT or the natural androgen metabolites androstanedione, androsterone, or androstanediol. However, they are more efficient transactivators of a synthetic reporter gene (ARE_2-tkCAT) in the presence of the androgen metabolites mentioned above. Moreover, because

transactivities by androsterone- and androstanediol-mutant receptor complexes were equally greater than that of androstanedione-mutant receptor complexes, and that the former two ligands have a hydroxyl group (OH) at position C3 while the latter, as with T and DHT, has a keto group (O=) at this position, the authors proposed that the Val715 and Val730 might be in the vicinity of the C3 position of the cholesterol A ring of the ligand. This is an example of the power of site-directed mutagenesis in the analysis of the structure and function of the steroid receptors.

Surprisingly, despite the fact that in comparison with the wild-type AR, Val715Met and Val730Met mutant ARs are stronger transactivators in the presence of the antiandrogen hydroxyflutamide, the wild-type AR in the presence of similar concentrations of hydroxyflutamide forms the 29-kD trypsin-resistant fragment as well as the mutant ARs do (Peterziel et al., 1995). Therefore, perhaps more subtle conformational differences or electrostatic interactions dictate quantitative transactivational differences among various ligand-receptor complexes.

Differential effects of T versus DHT

There are two naturally occurring potent androgens, T and its 5α-reduced form DHT, that take part in the development and virilization of the male reproductive system. T is responsible for the development of the male internal reproductive accessory organs such as wolffian duct structures (epididymis, vas deferens, seminal vesicles) whereas DHT promotes the virilization and growth of the prostate and the external genitalia: penis and scrotum. The distinct roles of T and DHT were especially evident in a Dominican Republic kindred carrying a mutation in the gene for the type 2 isozyme of 5α-reductase that is responsible for the conversion of T to DHT in the prostate and many other androgen-sensitive tissues (Andersson et al., 1991). The affected individuals in this large family have apparently female phenotype till the onset of puberty when their female external genitalia are masculinized by a surge in their plasma testosterone levels (Thigpen et al., 1992).

A study on differential effects of T and DHT on the activation of a heterologous reporter gene (MMTV-CAT) found that under saturating conditions both hormones can activate MMTV-CAT to the same extent (Deslypere et al., 1992). On the other hand, under subsaturating concentrations of either hormone, DHT was 10 times more potent than T; that is, DHT-R complexes reached half-maximal activity at 10 times lower hormone concentrations than that for T-R complexes. Therefore, in contrast to the mutant A-R complexes in the previous section, both T and DHT upon binding to the AR produce the same conformation (Kallio et al., 1994), but the binding, and perhaps subsequent conformational changes, occur at different rates. This is partly because T dissociates from

the AR three (Zhou et al., 1995) to four times (Kaufman et al., 1990) faster than DHT, and is less effective in stabilizing the AR (Zhou et al., 1995). Under saturating conditions where receptors are constantly occupied by either ligand, T-R and DHT-R complexes have the same Bmax (Grino et al., 1990).

Factors that affect ligand binding of the AR

In general, the binding characteristics of the AR do not vary among diverse tissues and species. For example, androgen-binding sites with K_d values in the nanomolar range have been described in goldfish brain, the skin of brown trout, testes of dogfish, the calf uteri, and monkey ovaries (Fitzpatrick et al., 1994 and refs. therein), in addition to those in the tissues of rodents and human. Nevertheless, slight variation of K_d from 0.22 to 0.45nM has been noted among different inbred strains of mice (Kemp and Drinkwater, 1989).

Although the sequence spanning the LBD of the AR is sufficient for ligand-binding, it appears that other regions of the AR may modulate its activity. The effect of the N-terminal domain on ligand-binding was analyzed by internal deletions of the hAR and domain-swapping between the rAR or hAR and the rGR (Zhou et al., 1995). The analysis of the dissociation half-time revealed that the N-terminal domain of the hAR, but not that of the rGR, stabilizes androgen-receptor complexes by 2 to 3 fold. Moreover, the AR N-terminal domain could also stabilize dexamethasone-binding of AR/GR chimeric receptor composed of the complete AR N-terminal region and the DBD and LBD of the GR. In the same study, more detailed analysis suggested that the residues between 14 and 28 of the N-terminus of the hAR stabilize the A-R complexes.

The DNA-binding domain

The DNA-binding domain is located in the central region of the nuclear receptors. Among the nuclear receptors, there is high homology in the amino acid sequence of the DNA-binding domain. Out of 67 residues, 20 are invariant (Evans, 1988). Nine of the invariant residues are cysteine. The first 4 cysteines coordinate a zinc ion to form the first subdomain or "finger" of the DNA-binding domain. The other 5 cysteines also coordinate a zinc ion to form the second finger. These structures are known as zinc fingers and were originally observed in other transcription factors such as TFIIIA associated with RNA polymerase III and transcription of ribosomal RNA 5S. The two zinc fingers are encoded by separate exons, and are distinct based on their structure and function. The first zinc finger is more highly conserved among the nuclear receptors and is more hydrophilic with a few basic residues. The second zinc finger is highly basic due to lysine and arginine residues (Evans,

1988). The invariant and conserved residues form a general DNA-binding domain. On the other hand, discrimination among different binding sites is achieved by three residues at the base of the first zinc finger (Forman and Samuels, 1990 and refs therein).

Hormone-response elements and their discrimination by the DBD

Hormone-response elements (HREs) are enhancer-like, palindromic DNA sequences located in the regulatory regions of hormone responsive genes (Beato, 1991). They consist of two unequal half-sites separated by 3 to 5 variable nucleotides. As with the RE for vitamin D receptor, they also sometimes occur as direct repeats. Based on homology of the right-half palindrome sequence and the type of nuclear receptor they bind to, they are divided into two subgroups: GRE/PRE subgroup with the half palindrome consensus sequence TGTYCT binding to the receptors of group I, and ERE subgroup with the half palindrome consensus sequence TGACC binding to other nuclear receptors (groups II, III, and IV).

There is considerable variation around the consensus sequences. For example, although the last base of the GRE/PRE right-half site is highly conserved, it is not essenial for binding (Beato, 1991). Since in 40% of cases there is a C at the fourth position, the distinguishing feature between GRE/PRE and ERE is the nucleotide at the third position. The flexibility of RE-binding by the nuclear receptors is also demonstrated by the observation that GR, PR, as well as ER, can bind REs containing a pair of half-sites from both GRE/PRE and ERE subgroups (Beato, 1991).

Based on the amino acid residues of the discriminatory positions, the members of the nuclear receptor superfamily can be divided into four groups. The AR along with GR, MR, and PR comprise group I (Forman and Samuels, 1990). The discriminatory residues in this group are Gly, Ser, and Val. The DNA-binding domains of this group recognize the consensus RE, AGAACAnnnTGTTCT.

Group II includes receptors for thyroid hormones, retinoids, and vitamin D. This group also includes several members known as orphan receptors which do not bind a known ligand. These receptors contain Glu, Gly, and Gly at their discriminatory position and interact with the consensus RE AGGTCATGACCT. Group III receptors with members such as chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and Drosophila's Knirps have Glu, Gly, and Ser at a similar position. So far, only COUP-TF is shown to interact with the RE GTGTCAAAGGTCA.

Group IV contains the ER with Glu, Gly, and Ala at the discriminatory position which allows it to bind to AGGTCAnnnTGACCT.

Three-dimensional structure of the DBD

The 67 amino acids of the DBD that include the two zinc fingers are encoded by exons 2 and 3 (Pinsky et al., 1995). The tertiary structure of the hAR DBD is not known. On the other hand, there are nuclear magnetic resonance (NMR) and crystallographic data on the GR DBD which can be extrapolated to AR because of very high homology in the DBD among the members of the steroid receptor subfamily.

Two-dimensional NMR and distance geometry analysis of a 71-residue stretch of the rGR DBD has detected several secondary structures including two α-helical regions (Härd et al., 1990). The two α-helices are perpendicular to each other, exposing their hydrophilic surfaces to the solvent. In a model of the GR DBD-GRE complex that accomodates these NMR observations, the α-helix of the N-terminal finger forms the "recognition helix" placed in the DNA major groove with residues Gly458, Ser459, and Val462 very near the middle AT base pairs of the GRE. According to the this model, Lys461, Lys465, and Arg466 contact other bases within the GRE or the DNA phosphate backbone.

This model is also consistent with the role of the DBD in transactivation through contacts with other transcription factors. The region of the second finger that extends away from the DNA, and is potentially accessable for contact by the transcriptional machinary, includes residues Arg488, Arg489, and Asn491. Substitution of each of these residues results in a receptor that can bind GRE normally but cannot transactivate in vivo (Härd et al., 1990). Interestingly, Arg607Gln and Arg608Lys mutations in the hAR, that correspond to Arg488 and Arg489 positions of the GR, are associated with rare cases of male breast cancer combined with PAI (Wooster et al., 1992; Lobaccaro et al., 1993).

The crystallographic analysis is in good agreement with the NMR study especially in the tertiary structure of the N-terminal zinc finger (Luisi et al., 1991). In the crystal model of the residues 440-525 of the GR bound to GRE, the DBD has a compact and globular tertiary structure which can be divided into two modules, each with a zinc tetrahedral centre followed by an amphipathic α-helix. The intact α-helical motifs of both modules are essential for normal DNA-binding (Beitel et al., 1994). The two modules differ structurally and functionally. The β sheet of the N-terminal finger helps to orient residues involved in phosphate contact and the residues in its α-helix contact the bases in the major groove of the GRE. The C-terminal module is involved in phosphate backbone contact, dimerization on the GRE, and transactivation.

Cellular factors that enhance DNA binding

Beyond the interaction between the AR monomers, there are other protein-protein interactions that stabilize DNA binding. One such factor, known as receptor accessory factor (RAF), was first detected in the rabbit reticulocyte lysate (Kupfer et al., 1993). In a electrophoretic mobility shift assay (EMSA) with a truncated rAR (residues 460-704) containing the DBD and portions of its N-terminal and C-terminal flanking sequences, and a 28-bp ARE from the first intron of the C3 gene, RAF enhanced the efficiency of binding by 25-fold. By gel filtration assay, RAF was estimated to be a 130-kD protein. The RAF-like activity was also detected in extracts of several cell lines such as HeLa and mouse Leydig tumor cells, but this activity could not be detected in the cell lines of kidney origin from monkey (CV1) and human (293). Therefore, its expression might be involved in the tissue-specific activity of the AR. The interaction between RAF and the truncated AR required the N-terminal segment flanking the DBD (residues 460-521). Interestingly, an antibody that recognizes the same region of the AR also enhanced DNA binding by 30-fold.

The antibody, and apparently RAF, enhance DNA-binding through protein-protein interaction with the AR, but the exact nature of this interaction is not known. Also, it is unknown whether the RAF activity is associated with all steroid receptors and for that matter the intact AR; under the same experimental conditions, RAF activity was detected with the truncated and full-length GR but not with the intact, full-length AR (Kupfer et al., 1993).

A factor with similar activity was also detected in the nuclear extracts from several different cell types such as a rat hepatoma cell line (FTO-2B), HeLa cells, and rat prostate and kidney cells (De Vos et al., 1994). These experiments again involved EMSA with truncated rAR or hGR. However, unlike the aforementioned experiments, the REs were in a relatively natural context of MMTV-LTR (141bp) and C3(1) first intron (158bp) sequences. This system allowed the identification of a 24-bp segment of the C3(1) fragment that was necessary for the DNA-binding enhancement of the truncated AR. This segment contains the half-sites for consensus GRE and nuclear factor 1 (NF-1), but the mechanism of enhancement does not seem to be due to NF-1 or AR DNA-binding.

Dimerization

Dimerization by the DBD

Like many transcription factors, all steroid receptors seem to dimerize for efficient transregulation of their target genes. In a dimer configuration on the GRE, the interacting C-terminal modules of the two GR subunits are positioned over the minor groove without contacting it (Luisi et al., 1991). The contacts between the DNA phosphate backbone and the C-terminal module apparently stabilize the dimer, hence the cooperative binding of the two DBD monomers to the GRE half-sites. In addition, the analysis of the crystal of a GR dimer bound to GRE with four spacer nucleotides between its half-sites, as opposed to the normal three, indicated that the stability of the dimer interface is greater than that of a monomer and a GRE half-site. This suggests that differences and similarities in specificity and strength of RE-binding by different steroid receptors may be accommodated by the tight, specific binding of one subunit of a dimer and variable, non-specific binding by the other (Luisi et al., 1991).

In a different study, the role of various REs in dimer formation by steroid receptors was analyzed by DNAse I footprinting experiments (De Vos et al., 1993). In this experiment, the footprinting pattern of fusion proteins of rAR and hGR DBDs were compared in the context of various natural REs from the MMTV-LTR and the first intron of the androgen-regulated C3(1) gene. The results suggested that, apparently by dimer formation, both AR and GR DBDs equally protected the REs that closely resemble the consensus GRE/PRE. On the other hand, where only one consensus half-site is present in the RE, the DNAse I footprint pattern suggested that AR and GR are bound as monomers (De Vos et al., 1993). However, because some nucleotides downstream of TGTTCT half-site were also protected against DNAse I, the footprint might indeed correspond to a dimer with one subunit bound tightly and the other more loosely as discussed in the previous paragraph. If this is the case, it might explain the similarity in the efficiency of protection between full and half-site GREs in the MMTV-LTR.

Evidence for the physiological role of AR dimerization came from five patients with Reifenstein syndrome from two unrelated families (a form of PAI) (Klocker et al., 1992). In the patients, a missense mutation in the hAR gene has resulted in the substitution of a threonine residue at position 596 for an alanine (Ala596Thr), in the conserved D-box of the subfamily of steroid receptors (Kaspar et al., 1993). This mutant AR was unable to bind an ARE in a band-shift assay, and a N-terminally truncated version of this mutant AR was unable to form heterodimers with a wild-type AR (Gast et al., 1995).

It was intriguing, however, that Ala596Thr AR could activate transcription from a promoters with multiple AREs, or with an ERE followed by an ARE (Kaspar et al., 1993). The authors concluded that this property might explain PAI (as opposed to CAI) in these pateints.

Dimerization by other functional domains of the AR

There is also evidence that the LBD of the AR might be involved in dimerization. A conserved heptad repeat of hydrophobic residues similar to a leucine zipper or a coiled coil motif in the ER is responsible for its in vitro homodimerization (Fawell et al., 1990; White et al., 1991). Amino acid substitutions in a 26-amino acid segment of the mER suggested that the residues involved in dimerization might be distinct but overlap the residues involved in ligand-binding (White et al., 1991). When a 30-amino acid segment of this region of mER was replaced by the corresponding region of the hAR, the chimeric receptor retained DNA-binding (and by inference dimerization), albeit at subnormal levels. A dimerization mutant Ile518Arg ER (Fawell et al., 1990) could not bind DNA in the same band-shift assay (White et al., 1991). This indicated that dimerization of the mER LBD is important for DNA-binding, and that hAR has a similar domain that can allow the chimeric receptor to dimerize and bind DNA.

The N-terminal domain of the AR may also contribute to its dimerization. The interaction of the AR N-terminal region with the LBD was demonstrated by a series of chimeric proteins (Langley et al., 1995). These chimera were made of the strong transactivation domain of the VP16 (herpes simplex viral protein 16 acidic domain) or the yeast protein GAL4 DNA-binding domain fused to either the LBD or the N-terminal domain of the hAR. The induction of the reporter gene by these constructs indicated that perhaps the N-terminal domain can both homodimerize and heterodimerize with the LBD. Androgen was necessary for the interaction between the N-terminus and the LBD, and the extent of this interaction seemed to depend on the type of ligand. Consistent with the higher stability (Zhou et al., 1995) and half-maximal transactivation capacity (Deslypere et al., 1992) of DHT-R compared to T-R complexes, DHT was more potent than T in inducing this heterodimerization. The anti-androgen hydroxyflutamide inhibited this interaction.

The interaction between the N-terminus and the LBD was also reduced by various internal deletions of the N-terminus (including one spanning the polyglutamine tract), and was abolished by a deletion near the DBD that spans the polyproline and polyglycine tracts. These deletions also reduced homodimerization of the N-terminus, but in a pattern unlike that of its heterodimerization with the LBD. It is interesting to note that the longer

polyglutamine tract (66 glutamines) of a N-terminal construct increased its dimerization with the wild-type N-terminal construct (21 glutamines).

The N-terminal domain

The N-terminal domain is the least conserved region among the nuclear receptors. Even in the subfamily of steroid receptors, this region is extremely variable in both size and primary structure among the members. Its functional importance comes from deletion experiments. Deletions of this region of the GR result in 10- to 20-fold decrease in transactivation of the reporter gene (Evans, 1988). Similarly, although the ER with an amino-terminal deletion activates a vitellogenin promoter normally, it is 10-fold less active on the estrogen responsive-promoter p52. One of the prominent features of this region in the AR is the numerous tracts of homopolymers of amino acids. Even more astonishing is the fact that the position, the length and/or composition of the homopolymeric tracts varies among species according to evolutionary distance. For instance, there is a polyglutamine tract near the N-terminal end of the hAR that is polymorphic (11-33 glutamines) among individuals (Pinsky et al., 1995; Kazemi-Esfarjani et al., 1995). A similar polyglutamine tract exists in the mouse (Faber et al., 1991) and rat ARs (Chang et al., 1988), but it is further from the N-terminus, is apparently fixed among the individuals at 20 (mAR) or 22 (rAR) residues, and interrupted by three histidine residues in the mAR (Fig. 2). Furthermore, at the nucleotide level, the hAR polyglutamine tract is coded by CAG repeats except for its most C-terminal glutamine residue that is coded by a CAA. The polyglutamine tracts of the mAR and rAR, on the other hand, are encoded by a presumably random mixture of CAG and CAA. A polyglutamine tract similar in length and position to the one in the hAR exists in the rGR (Miesfeld et al., 1986), whereas its counterpart located in the same region in the hGR consists of only two glutamine residues (Hollenberg et al., 1985).

The other polymorphic tract in the hAR is a polyglycine tract (12-29 glycines) (Pinsky et al., 1995). Again, the polyglycine tract in the mAR and rAR is fixed at 5 residues (Faber et al., 1991; Chang et al., 1988). Similar to the polyglutamine tract, the nucleotide composition of the polyglycine tract is different among species. In the human, there is a relatively long stretch of glycines encoded by the trinucleotide GGC, flanked at its N-terminal side by other codons for glycine. In the mAR and rAR, the nucleotide composition of the polyglycine is identical, coded by GGA GGC GGG GGC GGC. Perhaps, lack of codon reiterations in the polyglutamine and polyglycine of the mAR and rAR prevent the expansions and contractions that occur in the hAR polymorphic tracts.

There are other homopolymeric tracts in the N-terminal region of the AR. The longest in the hAR, after polyglutamine and polyglycine, is an octaproline tract (8 prolines). There is a comparable polyproline tract in the mAR and rAR that consist of 7 residues and includes an interruption by a histidine. Interestingly, an octamer of prolines is also present in the hMR, but it is located in the region between its DBD and LBD (Arriza et al., 1987).

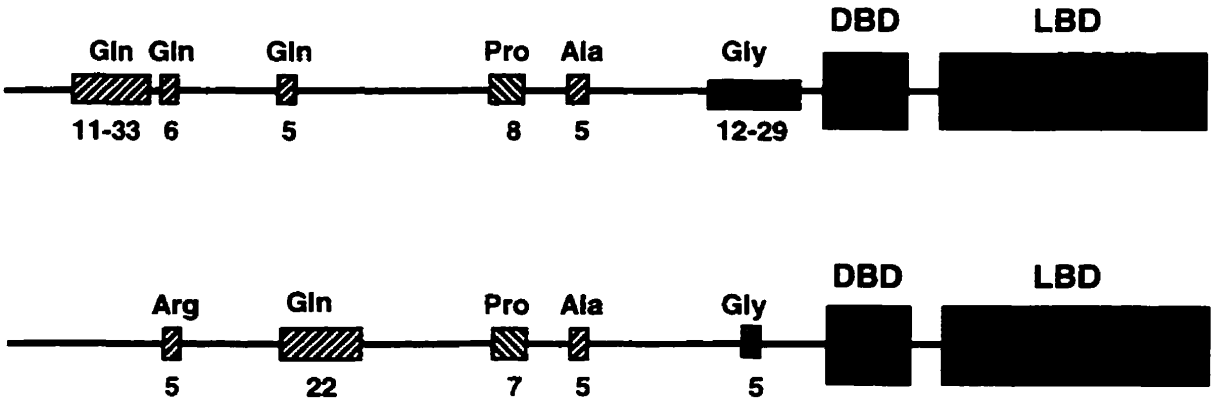

Figure 2. Comparison of the homopolymeric tracts in the human (top) and rat (bottom) androgen receptors.

Functional subdomains of the N-terminal region

Although the LBD and DBD are involved in AR transactivity, the transcriptional activity of the AR is mainly attributed to the N-terminal region. In contrast to DNA- and ligand-binding functional domains of the steroid receptors, the transcription activation units (TAUs) of the N-terminal region varies tremendously among the members in primary structure, location and size (Jenster et al., 1995).

The functional significance of various regions of the N-terminal domain has entirely relied on deletion analyses of the AR on heterologous reporter genes in cell cultures. Although the results from these experiments have been very informative, in some instances they have been contradictory. Earlier internal and N-terminal deletions of the hAR in COS cells with MMTV-CAT as reporter gene indicated that approximately a quarter of the N-terminal region (residues 1-140) is dispensable for full transcriptional activity (Simental et al., 1991). In a more refined series of experiments, this has recently been contradicted by other N-terminal deletion hAR constructs (Jenster et al., 1995), although the differences in the results may partly be due to the differences in the cell lines (HeLa cells) and/or reporter genes ($(GRE)_2$tkCAT). In the latter study, deletion of the first 100 amino acids from the N-

terminus of an otherwise wild-type hAR resulted in 40% decrease in transactivity. On the other hand, a deletion hAR in the former study lacking the first 337 residues was completely inactive (less than 1% wild-type activity) whereas its counterpart deletion hAR missing the first 359 residues retained 20% transactivity.

Nevertheless, other constructs suggested that residues 1-485 are necessary for full transactivity of the wild-type hAR. Noticeably, the deletion of residues 485-528 flanking the DBD did not affect wild-type activity. A similar region (460-520) was implicated in the enhancement of DNA-binding of hAR by a novel protein termed receptor accessory factor (RAF) (Kupfer et al., 1993).

In the context of a hAR construct lacking the entire LBD and the hinge region, the same N-terminal deletions behaved differently (Jenster et al., 1995). In this case, deletion of the DBD-flanking region 485-528 did decrease transactivation by about 40% (consistent with the loss of RAF interaction). Furthermore, unlike the results with the deletion AR construct containing the LBD region, the residues 360-528 were sufficient for full transactivity.

In conclusion, there seem to be two separate but overlapping TAUs in the N-terminal region of the hAR: one, designated TAU-1, includes residues 1-485 with a core of activity around residues 101-370, and has relatively larger number of acidic residues (34/270 residues or 12.6%), three polyglutamine repeats including the polymorphic one, and putative phosphorylation sites; and the second, designated TAU-5, includes residues 360-528 with a core of activity around residues 360-485, and has lower number of acidic residues (10/126 residues or 7.9%), a polyproline, a polyalanine and a polyglycine tracts (Jenster et al., 1995). Perhaps to further substantiate these results, similar experiments should be performed using natural androgen-responsive promoters such as those of prostatic specific antigen (PSA) (Riegman et al., 1991) and probasin genes (Kasper et al., 1994).

The role of the homopolymeric tracts

Recently, several reports, including this thesis, have attempted to understand the role of the homopolyamino acid tracts in protein activity. In a SwissProt database search, the majority of high-scoring proteins with polyglutamine tracts of at least 20 glutamines (82%) and polyproline tracts of at least 10 proline (78%) were, or were thought to be, transcription factors (Gerber et al., 1994). Several other transcription factors such as Sp1 and CTF/NF-I are rich in glutamine or proline residues, respectively, with short stretches of homopolymeric regions (Mitchell and Tjian, 1989). Further evidence for the functional importance of the homopolymeric tracts came from the discovery of hAR polyglutamine

expansion from the normal 11-33 residues to over 40 in a motorneuron degenerative disease combined with mild androgen insensitivity known as spinobulbar muscular atrophy or Kennedy syndrome (LaSpada et al., 1991). Also, in transfection studies, it was shown that a short polyglutamine of 12 residues exacerbated the effect of a natural Tyr761Cys substitution in the hAR LBD (McPhaul et al., 1991).

As a first step to determine the role of the homopolyamino acids in the hAR, plasmids were created that expressed hARs with 0, 12, 20, 40, or 50 glutamines in their polyglutamine tract (Mhatre et al., 1993; Kazemi-Esfarjani et al., 1995). Earlier results showed hARs with polyglutamine tracts of 40 or 50 residues are equally less competent than the hAR with a polyglutamine of 20 residues in transactivating the reporter gene MMTV-growth hormone (MMTV-GH) in COS-1 cells (Mhatre et al., 1993). With slightly different cotransfection parameters providing a more sensitive transactivation assay, and by including plasmids expressing 0 or 12 glutamines, in addition to ones with 20, 40 or 50 glutamines, it was discovered that there is an inverse correlation between the length of the polyglutamine tract and transactivational competence: the longer the length of the polyglutamine, the lower the transactivation (Kazemi-Esfarjani et al., 1995). The length of the polyglutamine tract had no detectable effect on other functions of the hAR or its stability. Therefore, it appears that the function of the polyglutamine tract is the modulation of transactivation function.

In a similar series of experiments, the long polyglutamine tracts of the hAR and rAR showed the same modulatory effect on transactivation, despite the fact that the deletions and insertions included sequences that flank the polyglutamine tract (Chamberlain et al., 1994). Although interpretations of the results were rendered less reliable by the alteration of the flanking sequences, these results suggested that the deletion of the polyglutamine tract of the rAR has a more dramatic effect on transactivation than the deletion of the hAR N-terminal polyglutamine tract. This might be due to the more C-terminal location of the polyglutamine tract in the rAR. Indeed, in the hAR, there is a pentaglutamine tract in the same location as the 22-glutamine tract of the rAR (Chang et al., 1988). The deletion of this tract in the hAR also resulted in a significant increase in transactivation (Kazemi-Esfarjani et al.; abstract 1995). Moreover, this increase was similar to the one provided by the deletion of the N-terminal polyglutamine tract. These results further confirmed the modulatory role of the polyglutamine tracts in the AR. It is important to note that increase in polyglutamine length may either decrease or increase transactivation depending on the context of the surrounding sequences. Chimeric proteins containing longer polyglutamines, flanked by GAL4 DBD and the acidic activation domain of VP16

had increased transactivation, in contrast to the ones without the VP16 fragment (Gerber et al., 1994).

The results of similar transactivation studies with hARs containing precise deletions of the hexaglutamine, octaproline, and polyglycine tracts were less dramatic (Kazemi-Esfarjani et al.; abstract 1993). There appeared to be a slight diminution in transactivity with all three mutant hARs, but the results were not consistent as the differences between the mutants and the wild-type hARs were small under the same experimental conditions. Moreover, similar inverse correlation between polyproline length and transactivity was suggested by chimeric proteins composed of GAL4 DBD and polyproline tracts of 0-50 residues (Gerber et al., 1994); the transactivity peaked at 10 prolines and declined thereafter with larger number of prolines.

Cellular factors that modulate transregulation

Several cellular factors may affect transregulation by the steroid receptors such as: 1) metabolic conversion of the ligand to active or inactive forms; 2) receptor levels; 3) receptor-specific REs; and 4) receptor-specific interaction with other cellular proteins or components (Gordon et al., 1995). Recently, Miesfeld and his colleagues attempted to address the issue of the effect of cellular environment on transactivation by taking advantage of several cell lines including a panel of T-antigen-transformed rat prostate epithelial cells (Gordon et al., 1995). Cotransfection of rAR and MMTV-CAT showed that there was up to 60-fold difference in the expression of CAT between various cell lines. The results were as dramatic (21-fold difference) in the stably-expressing AR cell lines: those from a prostate cell line had the highest AR-dependent expression of CAT while a rat hepatoma cell line had the lowest CAT expression.

Using two other reporter genes containing different promoter sequences (thymidine kinase vs alcohol dehydrogenase) and variable number of REs (2 GREs from MMTV vs 3 GREs from TAT gene), it appeared that the only cis-elements mediating the observed effects were the REs, as similar cell-specific differences were evident with both reporter genes. This effect could not be easily explained by differences in AR DNA-binding in the two cell lines. In fact, the nuclear extract from the cell line with lower activity had more GRE-binding by its endogenous AR than its counterpart. After further analysis, the effect was attributed to the N-terminal domain of the AR using AR/GR and GAL4/AR chimeric proteins. Moreover, similar, but much attenuated, cell-specific differences were observed for the rGR with identical stable cell lines and reporter genes. Thus, these effects are AR-specific (Gordon et al., 1995).

Interaction with other transcription and chromosomal factors plays a major role in transregulation by steroid receptors. The mouse mammary tumor virus (MMTV) hormone response system is one of the well-characterized HRE systems. In the MMTV long terminal repeat (LTR), there are four copies of the hexanucleotide TGTTCT (GRE/PRE right half-site) between -190 and -75 base pairs (bp) relative to the transcription initiation site. The most distal of the four is part of an imperfect palindrome between -190 and -160 bp while the other three stand alone as half-sites (Beato, 1991). Within the chromatin of yeast and mammalian cells, this region of the MMTV promoter is packaged into nucleosomes in a fashion that allows binding of the steroid receptors, but prevents the binding of at least two other transcription factors, nuclear factor I (NFI) and octamer-binding factor 1 (Oct1), in the absence of hormone-receptor complexes (Truss et al., 1995). In vivo, hormone treatment induces NFI binding to its cognate site at -75 to -63 bp. It appears that receptor binding in that region in vivo results in partial nucleosomal rearrangement, rather than shifting or removal, and simultaneous accessibility of the NFI and Oct1 binding-site. Furthermore, Oct1 seems to be the major mediator of transcription enhancement by hormone-receptor complexes. There are two binding sites for Oct1 located between the NFI binding site and TATA box in the MMTV-LTR. The interaction of Oct1 and the receptor appears to be by direct protein-protein interaction.

The transcriptional activity of the AR may also be modulated by other steroid receptors. For instance, cotransfection of equal amounts of mAR and hER together with MMTV-CAT into quail fibrosarcoma cells (QT6) results in a 60% reduction in transactivation compared to a cotransfection lacking hER (Kumar et al., 1994). This repression effect was ligand-dependent and could be inhibited by anti-estrogen ICI 164384. It has also been observed that ARs with internal deletions in the N-terminal domain can inhibit the transactivity of the wild-type AR in cell culture, perhaps through heterodimerization (Palvimo et al., 1993). We have observed enhancement of the hAR transactivity in the presence of DBD mutant hARs in COS-1 cells with MMTV-GH reporter gene (Kazemi-Esfarjani et al., Abstract, 1994). This effect was seen with three different natural mutations affecting the putative α-helical regions of the DBD (Beitel et al., 1994).

Recently, several groups using yeast and mammalian versions of the two-hybrid system have discovered several coactivators and corepressors. ARA_{70} is an AR-specific coactivator with 619 amino acids and a calculated molecular mass of 70kD isolated from a human brain library by the yeast two-hybrid system (Yeh and Chang, 1996). Its mRNA is expressed at high levels in prostate, testis, heart, preputial gland and thymus, but interestingly, it is undetectable in the brain cortex. ARA_{70} enhances the transcription by the AR approximately 10-fold in transfected human prostate cancer cells DU145.

Similarly, c-Jun, a component of the AP-1 transcription factor was shown to enhance the transactivity of the AR in a dose-dependent manner (Bubulya et al., 1996). The effect appeared to be through protein-protein interaction requiring the hAR DBD, and perhaps its hinge region. Futhermore, this interaction was inhibited by c-Fos, most likely by sequestering c-Jun.

ERM, PEA3, and c-est-1 are three members of the ets-related transcription factors that were shown to down-regulate the expression of the matrix metalloproteinases, such as collagenase I. This down-regulation appeared to be specifically through the AR, and not GR or MR (Schneikert et al., 1996). In addition, the ERM-mediated repression of the collagenase I promoter appeared to act through the N-terminal domain of the AR.

Nuclear localization signals

The final step in the classical androgen induction pathway takes place in the cell nucleus. Therefore, after its synthesis on the rough endoplasmic reticulum, the AR has to travel back into the nucleus. There is still controversy over the cellular distribution of the wild-type AR in the absence of androgens. Transient transfection experiments suggest that the unliganded AR might be predominantly in the cytoplasm or in the nucleus, or evenly distributed between the cytoplasm and nucleus depending on the cell type (Jenster et al., 1993). In the presence of ligand, on the other hand, the intact AR is always nuclear independent of cell type. Similarly, immunohistochemical analysis of tissues from various anatomical regions of the prostate from intact and castrated rats has shown that AR-specific signal was confined to the nuclei in intact rats and gradually became cytoplasmic postcastration (Prins and Birch, 1993).

Amino acid sequence comparison between steroid receptors and nucleoplasmin, the major nuclear protein of the Xenopus oocyte, has revealed a bipartite nuclear localization signal (NLS) composed of two basic segments separated by ten amino acid residues (Jenster et al., 1993). The simian virus large T antigen also contains a NLS that resembles the C-terminal half of this bipartite motif. NLS might be the site of interaction with a novel 57-kD protein known as ATP-stimulated translocation promoter (ASTP) that increases the binding of glucocorticoid-receptor complexes to nuclei in the presence of ATP (Okamoto et al., 1993). Deletion and site-directed mutagenesis experiments have suggested that intact N-terminal or DNA-binding domains are not necessary for normal nuclear localization. In contrast, the function of NLS seem to be regulated by the LBD (Jenster et al., 1993). These data suggest that the LBD blocks NLS activity, and this block is removed either by binding of ligand or by deletion of a large segment of the LBD, hence the possibility of a steric

hindrance mechanism. However, in contrast to transactivation function, the conformational requirements for nuclear localization are not ligand-sensitive as non-androgenic ligands such as estradiol, R5020 (a synthetic progestagen), dexamethasone, and anti-androgens such as hydroxyflutamide could also promote nuclear localization of the AR.

RECEPTOR-ASSOCIATED PROTEINS

Generally, in the absence of ligand, the inactive AR and other steroid receptors are located in the cytoplasm as 8 to 10S multimeric complexes (Baniahmad and Tsai, 1993). Two of the receptor-associated proteins (RAPs) in the complex belong to a family of stress-induced proteins known as heat-shock proteins (hsp). Based on their size, they are called hsp90 (90kD) and hsp70 (70kD) (Smith and Toft, 1993). Hsp90 is an abundant and ubiquitously expressed protein. Several experiments have suggested that it might be a chaperone for protein folding, and maintain the receptor in the high-affinity ligand-binding configuration. It might also mask the functional domains of the receptor and keep it in the inactive form in the cytoplasm. Hsp70 also appears to function as a chaperone in protein folding and unfolding, disassembly of complexes, and protein import through cellular membranes.

Other RAPs include p60, p59, p54, p50, and p23. As with hsp90 and hsp70, the common theme among them is broad tissue and species distribution. The 59kD protein, p59 or hsp59, is induced by heat shock and binds to the immunosuppressant drug FK506. The proteins that bind FK506 and rapamycin comprise a family of proteins known as immunophilins. For this reason, p59 is also called FK binding protein (FKBP). The functions of the other four RAPs is not clearly known (Smith and Toft, 1993).

In addition, steroid receptors may vary in their RAP requirement. For instance, GR seem to be associated with hsp90 and p59, but not with hsp70. On the other hand, human AR and PR associate with hsp90 and hsp70, but not with p59 (Smith and Toft, 1993).

Binding of the ligand to the receptor results in dissociation of RAPs and appearance of a smaller 4S species. This dissociation step is believed to be important for the activation of the receptor. However, in the absence of hormone, purified PR devoid of RAPs was unable to bind to its RE and activate transcription. Therefore, RAP dissociation does not constitute complete receptor activation (Baniahmad and Tsai, 1993). Similarly, yeast genetic manipulations have suggested that the presence of another molecular chaperone known as dnaJ may be necessary for full, ligand-dependent hAR transactivational capacity (Caplan et al., 1995).

STEROID RECEPTORS AND OTHER SIGNALING PATHWAYS

There are three mechanisms by which steroid receptors interact with other signaling pathways: 1) influence of phosphorylation/dephosphorylation on receptor activity; 2) direct or indirect interaction between steroid receptors and other transcription factors; and 3) regulation of the level of the steroid receptors by factors in other signaling pathways (Burnstein and Cidlowski, 1993). Evidence for the first mechanism in the hAR comes from pulse-labeling and alkaline phosphatase treatment of the newly synthesized AR from LNCaP or COS cells. The results show that the hAR is synthesized as a 110-kD protein that is rapidly phosphorylated resulting in a shift to 112-kD size on SDS-PAGE (Kuiper et al., 1991). Moreover, separation of the tryptic phosphopeptides show that the hAR is phosphorylated at several sites, including an androgen-induced site (Kuiper and Brinkmann, 1995). These sites appear to be exclusively comprised of serine residues. Three serine phosphorylation sites have been analyzed by site-directed mutagenesis: two in the N-terminal region (Ser81 and Ser94) and one in the hinge region (Ser650) (Zhou et al., 1995). Substitution of Ser650 by an alanine residue reduced transactivity by about 30%. Ser81Ala and Ser94Ala, on the other hand, did not have appreciable effect on hAR transactivity.

Further evidence for the significance of phosphorylation in AR function was provided by the effects of modulators of protein phosphorylation on AR transactivation in transient cotransfection of HeLa or CV-1 cells. Activators of protein kinase-A (8-bromo-cAMP) or protein kinase-C (phorbol 12-myristate 13-acetate), or an inhibitor of protein phosphatase-1 and 2A (okadaic acid) increased testosterone-dependent transcription of MMTV-CAT cotransfected with the rAR (Ikonen et al., 1994). In the absence of androgens, these compounds had little effect on MMTV-CAT activation. It appeared that at least for the activators of protein kinase-A, 8-bromo-cAMP and forskolin, the observed increased in transactivation is not due to changes in cellular AR levels or its ligand- or DNA-binding properties.

Evidence for the second mechanism is provided by the proliferin gene promoter where direct interaction between a steroid receptor and a transcription factor has been shown (Diamond et al., 1990). Within this promoter, there is a composite regulatory sequence that can bind the components of the AP-1 transcription factor, Jun and Fos, as well as GR. When AP-1 (Jun/Fos heterodimer) is bound to this promoter, binding of GR represses the transactivation. On the other hand, if the homodimer of Jun/Jun is bound to the composite site, binding of GR activates transcription. In vitro sudies localized the DNA-binding inhibitory activities of GR/AP-1 interaction to the GR zinc-finger DBD, and to regions outside of the leucine-zipper (dimerization domain) and basic region (DBD) of

the Fos and Jun (Kerppola et al., 1993). For Jun, it was the N-terminal region, and for Fos it was the C-terminal region.

Finally, the third mechanism is exemplified by the presence of an apparently functional cAMP response element (CRE) in the hAR promoter. Deletion analysis of the hAR promoter localized cAMP induction effect to a region between -530 and -380, upstream the transcription initiation site which includes a potential CRE (Mizokami et al., 1994). These results suggest that the level of hAR expression is regulated by other signaling pathways.

INTRODUCTION TO CHAPTER I

Missense mutations have been very useful in delineating the functional domains of the AR as they provide a mutational map of much higher resolution than internal deletions or truncations. Missense mutations have also been very helpful in answering fundamental questions regarding the extent of influence of the AR phenotype on the outcome of the clinical phenotype in androgen insensitivity. In other words, is the severity of the clinical phenotype solely attributable to the AR phenotype caused by the missense mutation, or there are other non-receptor factors that play a role as well? So far, evidence has been at best contradictory. This is partly due to the experimental design and partly due to the fact that only particular amino acid residues in the AR may indeed be involved in interacting with other cellular factors. In turn, polymorphism in the levels or structure of these cellular factors may influence the clinical outcome of a particular mutation.

In this chapter, I present two original articles that support the view that at least in some cases, the clinical phenotype is correlated with the phenotype of the mutant AR. Each article presents the results of functional assays on a pair of missense mutations affecting the same amino acid residue, discovered in unrelated families with one or more androgen insensitive members. The mutations in each pair result in the substitution of the same amino acid residue by different amino acids, with one causing a more severe AR and clinical phenotype. In order to confirm that the amino acid substitutions are responsible for the AR phenotypes, they were recreated by site-directed mutagenesis and tested in a heterologous cell culture.

CHAPTER I

Paper #1. Substitution of valine-865 by methionine or leucine in the human androgen receptor causes complete or partial androgen insensitivity, respectively with distinct androgen receptor phenotypes.

Paper #2. Substitution of arginine-839 by cysteine or histidine in the androgen receptor causes different receptor phenotypes in the cultured cells and coordinate degrees of clinical androgen resistance.

(Papers 1 and 2 appear at the end of the thesis.)

INTRODUCTION TO CHAPTER II

While there have been numerous cases of androgen insensitivity due to mutations of the DNA- or ligand-binding domains, only two reports have implicated the naturally occurring mutations of the N-terminal domain associated with a clinical manifestation. Both of these mutations affect the same polymorphic stretch of polyglutamines: in one case, the shortened polyglutamine exacerbates the effect of a missense mutation in the LBD that causes androgen insensitivity (McPhaul et al., 1991), whereas in the other case abnormal expansion of the same polyglutamine tract results in spinal and bulbar muscular atrophy (SBMA) (LaSpada et al., 1991), a motor neuron degenerative disease associated with mild androgen insensitivity (MAI).

Other groups had used N-terminal and internal deletions to search for functional domains within this region. In this article, we began our search with the polyglutamine tract that is affected in SBMA. Concurrently, we synthesized plasmids that expressed ARs containing deletions or abnormal expansions of the polyglutamine, and tested their function by biochemical assays in a heterologous cell culture system. The results presented in this article not only allowed us to speculate on the possible mechanism of pathogenesis in SBMA and its associated MAI, but also provided evidence for a possible modulatory role for polyglutamine tracts in hundreds of other proteins discovered in human and other organisms.

CHAPTER II

Paper #3. Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the $(CAG)_n$-expanded neuronopathies.

(Paper 3 appears at the end of the thesis.)

SUMMARY

I investigated the role of particular amino acid residues in the N-terminal and ligand-binding domains of the human androgen receptor (hAR) by studying their pathogenic modifications and their consequences. I used PCR site-directed mutagenesis to create naturally occurring and synthetic mutations in plasmids expressing the hAR. I then used heterologous cell cultures to examine the functional consequences of these mutations in the hAR. Our data indicate that at least in several instances the phenotypes of the recombinant and endogenous (genital skin fibroblast culture) mutant ARs correlate with the phenotypes of the patients carrying those mutations. This supports the hypothesis that AR is a key determinant of virilization in normal and androgen insensitive (AI) individuals.

Our results on the hAR N-terminal polyglutamine tract suggest that there is an inverse correlation between the polyglutamine length and its transactivity, hence its role in modulation of AR transactivity. Therefore, the expanded polyglutamine tracts in the spinal and bulbar muscular atrophy (SBMA) may contribute to the degeneration of the affected motor neurons by the androgen insensitivity they incur on those motor neurons. This modulatory effect of the polyglutamine is supported by the mild AI observed in SBMA patients, and by the observations made by other investigators with respect to the occurrence and/or severity of prostate cancer and hirsutism in particular subpopulations of control and affected individuals.

PERSPECTIVES

We discovered and recreated the alternative amino acid substitutions Val865Met and Val865Leu, and Arg839His and Arg839Cys, in the hAR. We also showed that the more severe biochemical phenotype of the mutant hARs is correlated with the more severe clinical phenotype of the affected AI individuals. Furthermore, we found that the primary defect in the transactivation of our reporter gene (and perhaps the natural target genes of the hAR) by the mutant hARs is the instability of the mutant androgen-receptor complexes (A-R) rather than an intrinsic transactivational defect.

In addition, we observed that in the presence of the androgen (mibolerone) during the experimental time course, the immunoreactivity of the normal hAR increased, and more so than its androgen-binding activity. Initially, we speculated that the A-R complexes were protected against degradation in the nucleus. We thought perhaps the nuclear environment was devoid of the proteases that break down the hAR. Thus, as long as the AR remains bound to the hormone and in the nucleus, it remained immunoreactively intact. Presently, there is evidence that this scenario may be false, as a hAR lacking its nuclear localization signal (NLS), hence remaining mainly or exclusively in the cytoplasm accumulates in a similar way to that of the normal hAR. It seems paradoxical that the presumably "naked" or transformed hAR bound to its ligand is more resistant to degradation than the hAR that is bound to several large and medium-size proteins (e.g., hsp90 and hsp70) in the cytoplasm.

Therefore, the question of why the liganded hAR protein accumulates remains unanswered. The crystal structure of the DBDs of the GR and ER, the unliganded RXRα LBD, and the liganded TRα LBD have been determined (Luisi et al., 1991; Mangelsdorf and Evans, 1995 and refs. therein). Perhaps, the crystal structure of the liganded and unliganded hAR functional domains, in the context of the intact hAR, will shed light on how the hAR becomes resistant to degradation upon ligand binding.

I also defined the function of the N-terminal polyglutamine tract in the hAR by synthesizing and transfecting hAR plasmids that express hARs with various polyglutamine lengths. I showed that this polyglutamine tract modulates the transcriptional activity of the hAR. Presently, we do not know how this modulatory function is mediated. Structural studies are necessary to extend our knowledge on how the transcriptional regulatory domains in the hAR, and in particular the polyglutamine and polyproline tracts, interact with the transcriptional machinery. Such studies might show how the hAR polyglutamine tract exerts its gradient modulatory function. Furthermore, these studies will be crucial in defining the gain-of-function mechanism by which the expanded polyglutamine tracts cause neuronal damage.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

In this thesis, I have described the following novel and original findings.

1) The functional assays of hARs with alternative amino acid substitutions Val865Met or Val865Leu, and Arg839His or Arg839Cys, showed that the more severe biochemical phenotype of the mutant hARs correlated with the more severe clinical phenotype of the affected AI individuals.

2) In the mutations described above, the defect in the androgen response pathway was demonstrated not to be due to an intrinsic transcriptional activation deficiency in the mutant hARs. Rather, it was hypothesized to be caused by a defect in the stability of the mutant hARs, once they have been exposed to the hormone. The combined immunoreactivity and hormone-binding experiments suggested a novel model in which the mutant hARs remained in the non-binding form longer, hence they were exposed to protein degradation pathways (e.g., proteases) more than the normal hAR was.

3) A gradient modulatory function for the long polyglutamine tract of the hAR was defined. It was found that the length of the polyglutamine tract inversely correlated with the transcriptional enhancement capacity of the hAR. Therefore, the term "gradient modulatory domain" was coined, to describe the functional nature of this homopolymeric amino acid stretch. This is the first solid example where such function is described for the polyglutamine tract in the hAR, or any other protein.

4) Based on the observation regarding the inverse correlation between the modulation of transcriptional activity and the length of the polyglutamine tract, and the fact that the motor neurons that are affected in SBMA patients are normally androgen sensitive, we were the first to propose that the defect in the SBMA may also have a loss-of-function component that aggravates its gain-of-function component.

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CHAPTER I

Substitution of Valine-865 by Methionine or Leucine in the Human Androgen Receptor Causes Complete or Partial Androgen Insensitivity, Respectively with Distinct Androgen Receptor Phenotypes

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We have identified two different single nucleotide missense substitutions at valine-865 in exon 7 of the human androgen receptor (AR) gene in two families with androgen resistance. Val → methionine is associated with the complete syndrome; Val → leucine is associated with the partial form. In genital skin fibroblasts, both alterations yield a normal maximum binding capacity, but an increased apparent equilibrium dissociation constant for all androgens tested. In genital skin fibroblasts, Val^{865}-Met A-R complexes have increased rate constants of dissociation with 5α-dihydrotestosterone, and the nonmetabolized ligands methyltrienolone or mibolerone (MB); their Val^{865}-Leu counterparts have increased rates with methyltrienolone and MB, but not with 5α-dihydrotestosterone. In transiently transfected COS-1 or PC-3 cells, Met^{865} AR is more severely impaired than Leu^{865} AR in transactivating two different androgen-responsive reporter constructs, thereby correlating with clinical phenotype. In COS-1 cells exposed to MB for 74 h, this relative impairment correlates with the relative instability of the MB-binding activity of each mutant AR, suggesting that their respective intrinsic transcriptional regulatory competence is normal. Notably, these mutant ARs lose significantly more MB-binding activity than immunoreactivity, suggesting that prolonged MB exposure induces them to adopt a nonbinding state. The position homologous to Val^{865} in the AR is occupied by Leu or Met in the three steroid receptors closely related to the AR. This indicates the structural subtlety that underlies the steroid-binding activity of different steroid receptors. (Molecular Endocrinology 7: 37–46, 1993)

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INTRODUCTION

Prenatal masculinization and pubertal virilization depend upon a normal androgen response system. A principal component of that system is the androgen receptor (AR), an androgen-modulated DNA-binding protein that regulates the transcription of androgen target genes (1, 2) and may also affect their expression posttranscriptionally (3, 4). The AR's androgen-binding function is vested in a 250-amino acid C-terminal domain encoded by exons 4–8 (or D-H) (1, 2). By homology with other members of the nuclear hormone receptor superfamily, portions of this domain are also very likely concerned with dimerization of the AR during its specific DNA binding to androgen response elements (AREs) of target genes and with its capacity to trans-

regulate after dimerization (5). Genetically deficient or defective androgen-binding activity produces a spectrum of somatosexual phenotypes in 46,XY persons, extending from apparently normal females (formerly, complete testicular feminization) to men with minimal undervirilization (6). The cloning of AR cDNA (7–10) and the application of derivative molecular biological techniques have allowed us (11–15) and others (16–21) to identify several point mutations in the AR gene that cause various degrees and types of human androgen insensitivity associated with impaired androgen-binding activity. The ultimate purpose of such studies is to generate a fine structure-function map of the several overlapping functions in the androgen-binding domain of the AR. In this paper, we identify two mutations at a single codon in this domain and correlate them with the different clinical and receptor phenotypes produced by each. The codon is numbered 865 in the report of Chang *et al.* (7), 866 in that by Lubahn *et al.* (8), and 864 in that by Tilley *et al.* (10).

RESULTS

Androgen Binding in Genital Skin Fibroblasts (GSF)

We had previously reported (22) that the GSF of subjects 4578 and 90055 (brothers 1 and 4, respectively, of the sibship with partial androgen resistance shown in Fig. 1) had normal specific 5α-dihydrotestosterone (DHT)-binding activities (22 and 25 fmol/mg protein, respectively), and normal rates of dissociation (k values) of their DHT-receptor complexes at 37 C. We also noted, however, that the apparent equilibrium dissociation constant (K_d) for DHT in the fibroblasts of subject 4578 was elevated (1.3 nM; normal, <0.3 nM).

We are now able to summarize previous and new ligand-specific dissociation rates and apparent K_d values for the GSF of subjects 4578 and 90055. Table 1 reveals that both have normal 37 C k values for DHT, but moderately elevated values for methyltrienolone (MT) and mibolerone (MB) as well (as judged by subject 90055). In contrast, Table 2 reveals that the GSF of both brothers have increased K_d values not only for MT, but also for DHT and MB (as judged by subject 90055). In aggregate, therefore, the mutant AR in the GSF of this family with partial androgen resistance has increased apparent K_d and k values for MT and MB; for DHT, its K_d is increased, but not its k. In contrast, as reported previously (23–27) and extended here, in the GSF of KIL, the unrelated subject with complete androgen resistance, the mutant AR not only has severely increased k values for all three androgens (Table 1), but also K_d values for all three androgens that are more abnormally elevated than those of subject 4578 or 90055 (Table 2).

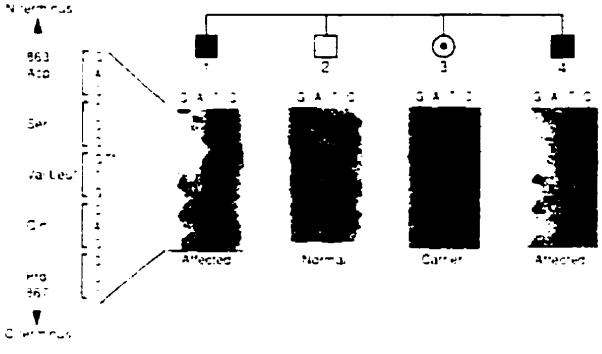

Fig. 1. Partial Sequence of AR Exon 7 Showing the G/T Transversion That Changes the Sense of Codon 865 from Val to Leu in the Family with Partial Androgen Resistance

Subjects 4578 and 90055, affected brothers, are individuals 1 and 4, respectively, in the sibship.

Identification of the DNA Sequence Alterations

The abnormal androgen-binding kinetics observed in GSF from the two families implied that each had a distinct alteration in its androgen-binding domain. For this reason, we sequenced each of the five exons (no. 4–8, and flanking intronic segments) that encode this domain and found a unique aberration in each family. The G-T transversion responsible for the family with partial androgen resistance changes GTG (Val) to TTG (Leu) at position 865, as depicted in Fig. 2; Fig. 1 shows it hemizygously in the affected brothers, 4578 and 90055 and heterozygously in their 46,XX sister. The G-A transition that changes codon 865 to ATG (Met) in KIL, the unrelated subject with complete androgen resistance, is also depicted in Fig. 2, but is not shown, nor have we had access to her mother to ascertain whether she is heterozygous. We also sequenced exon 1 that encodes the transcriptional modulatory domain, and exons 2 and 3 that encode the DNA-binding domain. There were no concurrent alterations. The mutant ARs have 20–23 glutamines in their poly-Gln tracts and at least 16 glycines in their poly-Gly tracts.

Transfection and Transient Expression Studies

Since Leu occupies the position in the human progesterone receptor that is homologous to human (h) AR 865, and Met occupies it in the human glucocorticoid receptor and human mineralocorticoid receptor (7), it was important to prove that Val^{865}-Leu and Val^{865}-Met represented pathogenic, not benign, sequence alterations in the AR gene. For this purpose and to generate sufficient amounts of each mutant AR for better characterization, we cotransfected COS-1 or PC-3 cells with AR cDNAs carrying each mutation and one or another androgen-responsive reporter constructs.

First, we assessed the androgen-binding properties of the mutant ARs in COS-1 cells. Val^{865}-Met had increased k values with all three androgens (Table 1), but they were only modestly elevated with DHT (Fig. 3). Strikingly, Val^{865}-Leu had normal k values with MB and MT, as well as with DHT (Table 1). Both mutants had abnormally high K_d values, as measured with MB,

Table 1. Ligand-Specific Nonequilibrium Dissociation Rates (k) of the Normal and Mutant hARs Measured at 37 C in GSF and Transiently Transfected COS-1 Cells

Ligand	Dissociation Rate ($\times 10^{-3}$/min)						
	Normal		Val^{865}-Leu			Val^{865}-Met	
	GSF	COS-1	GSF (90055)	GSF (4578)	COS-1	GSF (KIL)	COS-1
MB	3.0 ± 0.10	3.0 ± 0.00	6.0 ± 0.58		4.0 ± 0.30	8.7 ± 1.20	5.5 ± 0.50
DHT	6.3 ± 0.25	5.0 ± 1.41	7.0 ± 1.00	6.7 ± 0.88	6.0	14.0 ± 2.00	9.0 ± 1.00
MT	12.0 ± 0.50	10.2 ± 1.20	20.0 ± 1.46	17.0 ± 0.58	12.5 ± 0.50	31.5 ± 3.50	30.0 ± 2.00

The values are the mean ± SEM where the particular experiment was performed at least twice.

Table 2. Apparent Equilibrium Dissociation Constants (K_d) of the Normal and Mutant hARs Measured at 37 C in GSF and Transiently Transfected COS-1 Cells

Ligand	K_d (nM)						
	Normal		Val^{865}-Leu			Val^{865}-Met	
	GSF	COS-1	GSF (90055)	GSF (4578)	COS-1	GSF (KIL)	COS-1
MB	0.12 ± 0.02	0.44 ± 0.07	0.52 ± 0.08		1.16 ± 0.19	2.50	1.47 ± 0.10
DHT	0.10 ± 0.00		1.03 ± 0.43	1.09 ± 0.17		1.95 ± 0.95	
MT	0.18 ± 0.02	0.84	0.79 ± 0.19	1.18 ± 0.22	1.06	5.80	2.13

The values are the mean ± SEM where the particular experiment was performed at least twice.

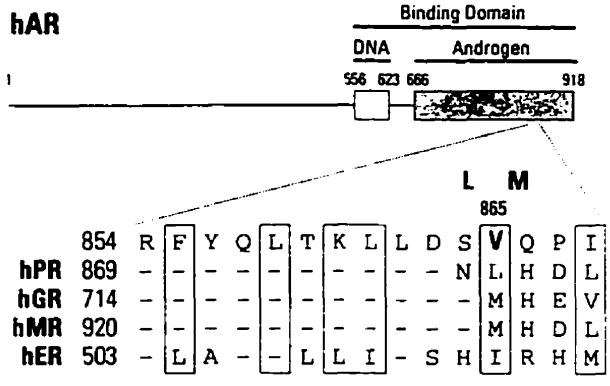

Fig. 2. Location and Context of Val^{865}-Leu (Subjects 4578 and 90055 with Partial Androgen Resistance) or Val^{865}-Met (Subject KIL with Complete Androgen Resistance) in a Region of the Androgen-Binding Domain of the hAR That Is Encoded by Exon 7

Dashes indicate amino acid identity in the homologous positions of the human receptors for progesterone (hPR), glucocorticoid (hGR), mineralocorticoid (hMR), and estrogen (hER). A homologous region is essential for dimerization of the mouse ER (5). *Boxes* indicate positions occupied by hydrophobic amino acids.

and for Val^{865}-Met, with MT as well (Fig. 4 and Table 2). We could not get a valid measure of K_d for DHT because COS-1 cells catabolize it so quickly.

Next, we assessed the ability of both mutant ARs to transactivate the mouse mammary tumor virus-GH (MMTV-GH) reporter in COS-1 cells. Figure 5, a representative of several experiments (Table 3), shows that both mutant ARs performed less well than normal at various concentrations of MB up to 3.8 nM. The performance of the Val^{865}-Leu receptor fell between that of the normal receptor and that of the Val^{865}-Met mutant; however, at 3.80 nM, Val^{865}-Leu equaled that of the normal receptor, and Val^{865}-Met approached 80% of normal. Figure 6, comprising three experiments, illustrates the same hierarchy of results using the p(−286)probasin (PB)-chloramphenicol acetyltransferase (CAT) reporter in DHT-tested PC-3 cells. Notably, this assay appears to discriminate well between the two mutant ARs even at 10 nM DHT. Another striking difference between the normal and mutant ARs was the marked instability of the mutant androgen-binding activities in COS-1 cells during prolonged 74-h exposure to MB (Table 4 and Fig. 7). The Val^{865}-Met activity was more unstable than that of Val^{865}-Leu. Indeed, when the averages of the 46–48 h and 46–120 h values for specific MB-binding activity in Fig. 7 were plotted against the concentration of GH produced by cells in the same experiment, there was no difference in the transactivational activity between the normal and the mutant ARs (Fig. 8).

Finally, we determined the extent to which the loss of mutant androgen-binding activity during prolonged androgen exposure was correlated with the loss of AR immunoreactivity. Comparison of the *top* with the *middle* and *bottom panels* of Fig. 9 shows that, compared to normal AR, both mutant ARs lost androgen-binding activity, they did so to a greater extent than they lost immunoreactivity, and Val^{865}-Met was more abnormal than Val^{865}-Leu in these latter respects. In contrast, the normal AR increased its immunoreactivity in response to prolonged androgen exposure, while its androgen-

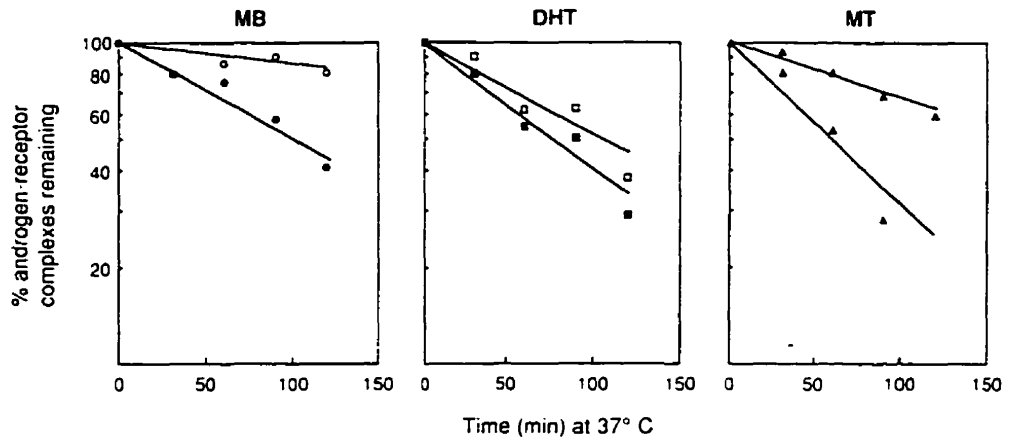

Fig. 3. Dissociation Rates of Various A-R Complexes at 37 C in COS-1 Cells Transiently Expressing Normal (○, □, and △) or Val^{865}-Met Mutant AR (●, ■, and ▲)

The cells were incubated with 3 nM [^{3}H]androgen for 2 h, washed, and chased with 0.6 μM of the same radioinert androgen for up to 120 min. Specific binding (the mean of total binding in triplicate minus nonspecific binding in duplicate) was plotted as the percentage of that remaining at indicated times after initiating the chase.

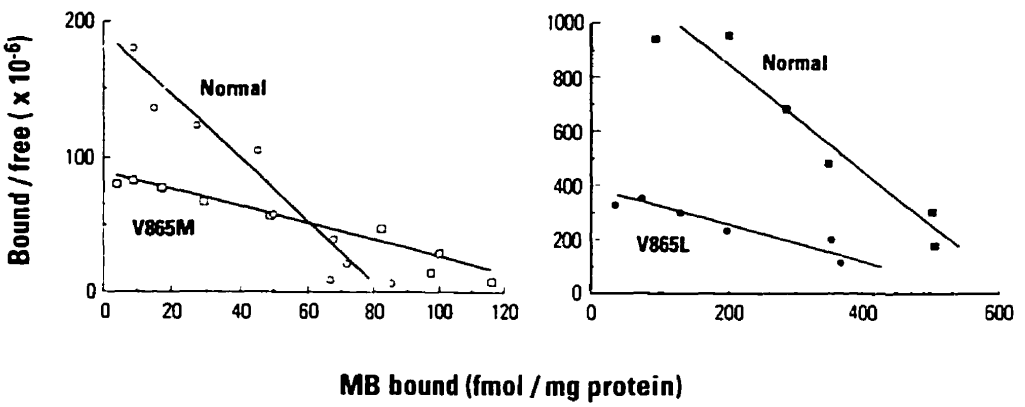

Fig. 4. Scatchard Analysis Using MB on COS-1 Cells Transfected by Normal, Val^{865}-Met, or Val^{865}-Leu hAR cDNA

Left, Normal K_d, 0.4 nM; Val^{865}-Met, 1.5 nM. *Right*, Normal K_d, 0.5 nM; Val^{865}-Leu, 1.7 nM.

binding activity remained essentially unchanged. As an internal control for the chronobiology of the transfections, it is important to note that for all three, the androgen-binding activity determined between 118–120 h after transfection was about 40% of the corresponding activity between 46–48 h after transfection.

DISCUSSION

Valine-865 of the AR is near the C-terminal end of a region in its androgen-binding domain whose N-terminal two thirds shares complete sequence identity with the AR subfamily of steroid receptors (Fig. 2). By homology with the ER and other members of the nuclear hormone receptor superfamily, this region may also contribute to AR dimerization (5) and, therefore, to its DNA-binding and transregulating functions. Hydrophobic amino acids, considered crucial for dimerization (5), occupy 6 of 15 homologous positions in the region; Val^{865} in the AR is one of them.

The finding of two families with mutations leading to methionine or leucine substitutions of valine-865 allowed us to evaluate genotype in respect to comparative clinical and receptor phenotype for each mutation. This evaluation was particularly tantalizing because the other three members of the AR subfamily of steroid receptors have leucine or methionine in the position homologous to Val^{865}, while the human estrogen receptor has isoleucine in that position.

The Val^{865}-Met mutation has been reported once before (16), in a family with complete androgen insensitivity in whose GSF the AR had several-fold lower than normal binding affinity for DHT (28). Our discovery of subject KIL thus confirms the association of Val^{865}-Met with complete androgen insensitivity, and our ligand-binding data reveal that this mutant AR has diminished affinity for MB and MT as well as for DHT not only in GSF (23–27), but also in transfected COS-1

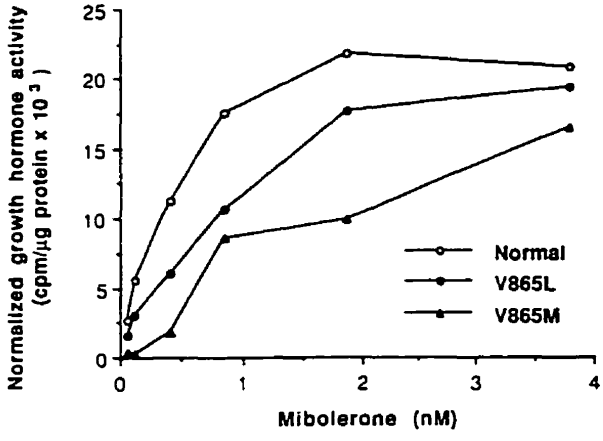

Fig. 5. MB-Dependent Production of GH in the Medium of COS-1 Cells Transiently Expressing Normal or Either Type of Mutant AR

After cotransfection with expression plasmids containing normal, Val^{865}-Leu, or Val^{865}-Met cDNA and the MMTV-GH reporter, the cells were exposed to indicated concentrations of MB. GH activity was adjusted for cell protein and transfection efficiency.

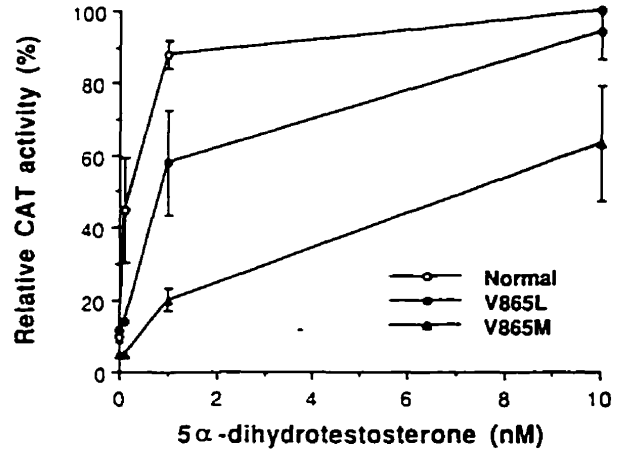

Fig. 6. DHT-Dependent Production of CAT Activity, under the Influence of the PB AREs, in PC-3 Cells Transiently Expressing Normal or Either Type of Mutant AR: Val^{865}-Leu or Val^{865}-Met

CAT activity, adjusted for transfection efficiency, is expressed as a percentage of the response of normal AR in cells exposed to 10 nM DHT (4177 ± 182 dpm/min·mg protein). The *bars* indicate ±SEM.

Table 3. Normalized GH Activity in Relation to Hormone Concentration as a Percentage of Normal

MB (nM)	% Activity		
	Normal	Val^{865}-Leu	Val^{865}-Met
0.05	9	6	5
0.09	20	8	4
0.35	46	25	10
0.72	70	39	28
1.43	90	58	42
2.84	100[a]	84	61

The difference between the normal and each mutant hAR is significant ($P < 0.01$, by paired sample t test). The MB concentrations and GH activities in the normal and Val^{865}-Leu groups are the averages from four independent experiments. The values for Val^{865}-Met are the averages from two of the four independent experiments that included V865M.

[a] 100% corresponds to the GH activity of the normal receptor at the highest MB concentration in each experiment.

cells. When these COS-1 cells were cotransfected with the MMTV-GH reporter, their response to MB was concentration dependent; below 1 nM MB, it was about 40% of normal, and near 4 nM MB, it approached 80% of normal. Likewise, the response to DHT of PC-3 cells cotransfected with the Val^{865}-Met receptor and the (−285)PB-CAT reporter was about 20% of normal at 1 nM and about 50% at 10 nM. Our results for DHT and MB (in two cotransfection systems using different AREs) are thus much like those of Brown *et al.* (16) for the same mutation using DHT and MT. We also showed, however, that the Val^{865}-Met receptor (KIL) was much less stable than normal in COS-1 cells during the 74-h period when GH production was being stimulated by MB. Indeed, when we estimated the steady state levels of MB-binding activity in COS-1 cells transfected by normal AR or Val^{865}-Met AR (by averaging the values determined for these activities 2 and 74 h after exposure to MB), there was no appreciable difference between Val^{865}-Met and normal AR in apparent transactivational competence.

In relation to the association of the Val^{865}-Met AR with complete androgen resistance, several properties of the Val^{865}-Leu AR are in accord with its less severe clinical phenotype of partial androgen resistance. First, in GSF, the Val^{865}-Leu AR has a normal off-rate (k value) for DHT, and its rates for MB and MT are barely double normal, while those of the Val^{865}-Met AR are 2.5-fold normal for all three androgens. Second, in COS-1 cells, the Val^{865}-Leu AR has normal off-rates with all three androgens, while the rates of Val^{865}-Met AR are increased for all three androgens, albeit less so than in GSF for MB and DHT. Third, when transfected with Val^{865}-Leu AR, GH production in MB-stimulated COS-1 cells or CAT expression in DHT-stimulated PC-3 cells is intermediate between those of normal AR and Val^{865}-Met AR. Fourth, during prolonged androgen exposure, the Val^{865}-Leu mutant receptor is less stable than normal, but more stable than the Val^{865}-Met mutant.

Hence, as discussed for the Val^{865}-Met AR, the relative transactivational deficiency of the Val^{865}-Leu AR appears to be coordinate with its relative instability during prolonged androgen stimulation, as measured in COS-1 cells. This suggests that Val^{865}-Leu or -Met AR has a normal intrinsic ability to transactivate MMTV-GH in COS-1 cells (when this ability is measured per U estimated steady state androgen-binding activity) and predicts that both mutant ARs dimerize normally. Pending direct testing, this prediction is eminently compatible with the fact that in both mutant ARs, Val^{865} is substituted by a hydrophobic amino acid, thereby preserving the highly hydrophobic character of the strongly con-

Table 4. Androgen-Binding Activity (Femtomoles per mg Protein) of the COS-1 Cells Transiently Transfected with Normal and Mutant hAR cDNAs in the Presence of Hormone from the 46th to 48th and from the 46th to 120th h Posttransfection

Exp	MB (nM)	Specific MB-Binding Activity (fmol/mg Protein)					
		Normal		Val^{865}-Leu		Val^{865}-Met	
		46–48 h	46–120 h	46–48 h	46–120 h	46–48 h	46–120 h
1	3.38	707	689	825	312	652	103
2	2.58	395	379	323	92	—[a]	—
3	2.84	459	415	439	69	328	18
4	2.54	505	773	409	110	—	—
46–120 h:46–48 h (%)		109 ± 15[b]		27 ± 4		11 ± 6	

These data correspond to those in Table 3.
[a] V865M was not included in Exp 2 and 4.
[b] Mean ± SEM.

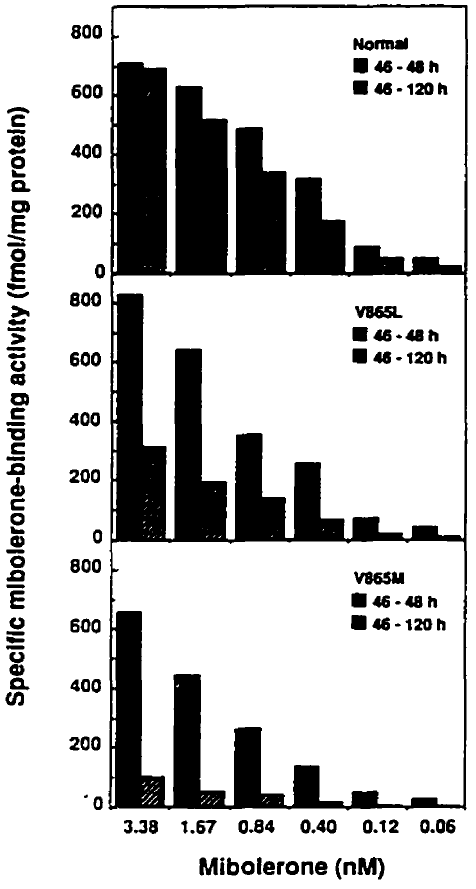

Fig. 7. Specific MB-Binding Activity 2 (46–48 h) and 74 (46–120 h) h after the Addition of the Indicated Concentrations of MB (46 h after Transfection) to the Medium of COS-1 Cells Transiently Expressing Normal or Either Type of Mutant AR: Val^{865}-Leu and Val^{865}-Met.

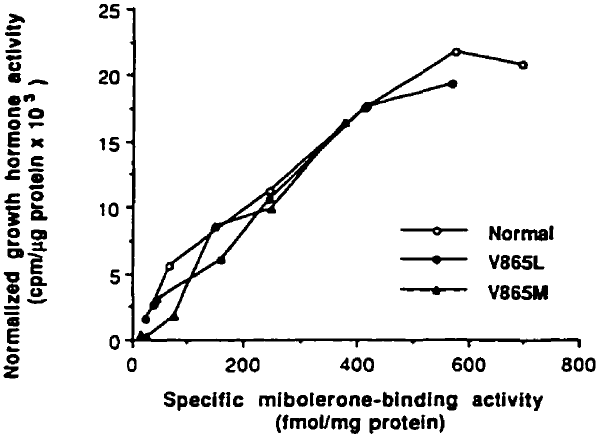

Fig. 8. MB-Dependent Production of GH by COS-1 Cells Transiently Expressing Normal or Either Type of Mutant AR: Val^{865}-Leu and Val^{865}-Met

GH activity, adjusted for cell protein and transfection efficiency, is plotted against the mean of the specific MB-binding activity 2 (46–48 h) and 74 (46–120 h) h after exposure of the same cells to the concentrations of MB indicated in Fig. 5.

served, putative dimerization domain (Fig. 2). Contrarily, the apparently selective impaired androgen-binding affinity of both mutant ARs is a measure of the stereochemical stringency that underlies the steroid-binding specificity of different steroid receptors.

It is remarkable that in COS-1 cells exposed to androgen for a prolonged period, both mutant receptors lost androgen-binding activity to a much greater extent than the normal receptor, and for each mutant receptor, the loss of binding activity greatly exceeded the loss of immunoreactivity. This indicates that prolonged androgen exposure induces many of the mutant receptors to adopt a nonbinding state. In contrast, under the same conditions, the normal receptor not only maintained its androgen-binding activity, but also increased its immunoreactivity. We postulate that this behavior reflects protection against degradation resulting from its greater affinity for androgen. In any event, since prolonged androgen exposure caused immunoreactivity to increase relative to androgen-binding activity for the normal receptor as well, the nonbinding state of the receptor may be a normal transitional one in the intracellular cycling of the AR in COS-1 cells, to which the mutant receptors are abnormally disposed.

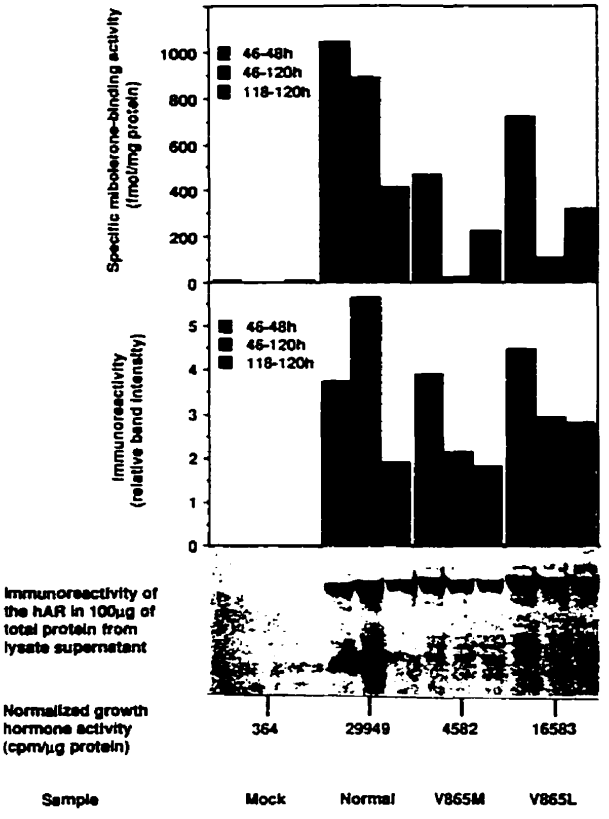

Fig. 9. Androgen-Binding Activity (*Top Panel*; not Corrected for Transfection Efficiency) and Immunoreactivity (*Middle and Bottom Panels*) Were Measured Concurrently after the Addition of 3 nM MB for Various Intervals to Replicate Wells of COS-1 Cells That Had Been Cotransfected with pMMTV-GH and pCMV-βGAL Alone (Mock) or Additionally with $pSVhAR_o$ (Normal) or One of Its Two Mutant Versions: V[865]-Met or Val[865]-Leu

Relative band intensity remained constant from at least 20–80 sec of film exposure to the chemiluminescence reaction. Exposure time was 60 sec.

The normal k (but abnormal apparent K_d) value for DHT of the Val[865]-Leu AR in GSF is superficially paradoxical, particularly in view of the moderately increased values demonstrable for MB and MT; however, the observation is supported strongly by data gathered nearly 8 yr apart on cell lines developed independently from two affected brothers. We reported a different form of androgen-selective androgen affinity defect in the GSF of two other families with partial androgen insensitivity (29, 30). Furthermore, we observed the association of increased apparent K_d values with normal k values for DHT, MB, and MT in the GSF of another family with partial androgen insensitivity (unpublished).

Finally, there is a growing body of evidence that receptor-associated factors can modify the classical behavior of steroid receptors (31). We propose that such factors account for normalization of the k values of the two mutant ARs for various androgens in COS-1 cells compared to those in GSF.

THE FAMILIES

The Val[865]-Leu alteration was first recognized heterozygously in the sister of three brothers of Irish origin born with markedly ambiguous external genitalia due to partial androgen insensitivity. She had delayed appearance of pubic hair as an expression of heterozygosity. She is individual 3 in Fig. 1 of this paper and was identified as individual II-4 in Fig. 1 of the study of Jukier *et al.* (22), which reported the clinical-endocrine features and the qualitative AR defect in this family. She has had an affected son (Holland, F. J., personal communication). Retrieval of the misplaced GSF line from one of her affected brothers [coded 90055; individual 4 in Fig. 1 of this paper; individual II-5 in Fig. 1 of the study of Jukier *et al.* (22)] permitted us to perform studies that confirm and extend the androgen-selective nature of the androgen affinity defect in the family's mutant AR, as originally reported for the GSF of another brother, coded 4578 (22).

The Val[865]-Met alteration was discovered in KIL, an isolated subject of Scottish origin, whose clinical-endocrine phenotype of complete androgen insensitivity and qualitatively impaired AR have been previously described in detail (23–27).

MATERIALS AND METHODS

Specific androgen-binding activity, maximum androgen-binding capacity, the apparent equilibrium dissociation constant (K_d), and the rate constant of dissociation (k) of various A-R complexes were determined on monolayer cultures, as described previously (32). The androgens used were: [17α-*methyl*-^{3}H]MB (7α,17α-dimethyl-19-nortestosterone; 80 Ci/mmol), [1,2,4,5,6,7-^{3}H]DHT (120 Ci/mmol), and [17α-*methyl*-^{3}H]MT (17β-hydroxyl-17α-methyl-4,9,11-estriene-3-one; 87 Ci/mmol).

DNA isolation from cell monolayers or peripheral blood lymphocytes (13) and polymerase chain reaction (PCR) amplification, followed by direct sequencing of exons 1–8 (13, 14), were performed as described previously.

Site-Directed Mutagenesis

We used the overlap extension method of Higuchi (33) to construct hAR cDNAs containing each mutation. We generated *Eco*RI fragments extending from the *Eco*RI site in exon 6 to the termination codon in exon 8, and substituted them for the fragment between the *Eco*RI sites in exon 6 and in the $pSVAR_o$ expression vector [kindly donated by Brinkmann *et al.* (1)]. Primary PCR products were produced in two separate reactions: 1) primers CA-A (5′-GGACTCC*T*TGCAGCCTAT-TGCG-3′) or KIL-A (5′-CTGG-ACTCCATGCAGCCTAT-3′) with primer SVEco (5′-GGAATTCCTCACTGGGTGTGGAAA-TAGA-3′) to introduce an *Eco*RI site at the end of the hAR-coding sequence; and 2) primers CA-B (5′-CGCAATAGGCT-GACA*A*GGAGTCC-3′) or KIL-B (5′-ATAGGCTGCA*T*GGAGTC-CAG-3′) with primer B (5′-TCACACATTGAAGGCTATGA-3′) up-stream of the exon 6 *Eco*RI site. The primary PCR reactions were carried out in the presence of 2 μg $pSVAR_o$, using VENT polymerase (New England Biolabs, Beverley, MA) with a final Mg^{2+} concentration of 5 mM for 20 cycles with denaturation at 95 C for 30 sec, annealing at 55 C for 45 sec, and extension at 75 C for 1 min. The primary PCR products were gel purified and used to initiate the secondary PCR reactions. Five cycles were performed, as described above, to allow the overlapping products to be extended. Then, an additional 19 cycles were used with 50 pmol each of primers B and SVEco to generate an extended fragment carrying the desired mutation. The resulting product was digested with *Eco*RI, gel purified, and ligated to previously purified, *Eco*RI-digested, and phosphatased $pSVAR_o$. The resulting clones were checked for insert

orientation, and large scale plasmid preparations were made using Qiagen columns (Qiagen, Inc., Chatsworth, CA). The amplified region was sequenced to ascertain the presence of the desired mutation and the absence of extraneous ones.

Transfections

COS-1 cells, maintained in OptiMEM (Gibco, Grand Island, NY) containing 5% fetal bovine serum (FBS), were transfected by electroporation. The cells were trypsinized, washed with ice-cold PBS, counted, and resuspended in cold PBS at a final concentration of 20 million cells/ml. Ten million cells were transferred to an electroporation cuvette (0.4-cm gap; Bio-Rad, Richmond, VA), and 5–10 μg mutant or normal SVhAR plasmid DNA were added. After 5 min on ice, the cells were shocked at 0.25 kV at 960 μF (time constant between 30–38 msec), iced for a further 10 min, then diluted in Minimum Essential Medium (MEM) supplemented with 10% FBS for plating in 16-mm multiwell petri plates. Various concentrations of [^{3}H]MB were added 48 h after transfection, and specific androgen binding, K_d, and k were determined 2 and/or 72 h later. To assess transactivational efficiency, we cotransfected 10 μg pMMTV-GH [hGH gene under the control of the AREs (34) in the MMTV-LTR and its promoter] and measured GH in the medium 72 h after adding androgen.

PC-3 cells, grown in 100-mm plates with MEM plus 10% FBS, were transfected by 5 μg normal or mutant plasmid AR cDNA and 5 μg p(−286)PB-CAT (35, 36), using calcium phosphate precipitation. Probasin is an androgen-regulated nuclear protein isolated originally from the dorsolateral rat prostate (37). The p(−285)PB reporter plasmid contains two AREs and the endogenous promoter in the first 285 basepairs of flanking sequence of the *PB* gene. Six hours after transfection, the cells were treated with 20% glycerol-MEM for 2 min, and 24 h before harvest, they were fed MEM with 5% charcoal-stripped FBS plus the indicated concentrations of DHT.

GH and CAT Assays

The Allegro Human Growth Hormone Kit (Nichols Institute, San Juan Capistrano, CA) was used according to the manufacturer's instructions to assay duplicate 100-μl samples of culture medium. GH values were normalized for cell protein and transfectional efficiency using 7 μg pCMV-βGAL (CMV, cytomegalovirus; βGAL, β-galactosidase). β-Galactosidase activity was determined according to the method of Sambrook *et al.* (38) and expressed per μg cell protein. CAT activity was determined by a two-phase fluor diffusion assay (39) on supernates of PC-3 cells that were harvested with PBS-EDTA, lysed with 0.1 M Tris-HCl and 0.1% Triton X-100 (pH 7.8), and centrifuged (14,500 × *g*; 15 min; 4 C). Transfection efficiency was measured on 1- and 0.5-μg DNA samples isolated from the insoluble material that were slot blotted and probed with a ^{32}P-labeled probe for the inserted AR cDNA. Autoradiograms were analyzed by two-dimensional laser densitometry. CAT activities were normalized for transfection efficiency.

Western Analysis of the AR in Transfected COS-1 Cells

Confluent monolayers (~1 million cells) in 30-mm multiwell petri dishes (Falcon, Oxnard, CA) were washed twice with 20 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl, and the scraped cells from duplicate dishes were combined in 1 ml of the same buffer. After microfuging, the cells were resuspended in 160 μl 0.25 M Tris-HCl (pH 7.8) containing 73 μg/ml Aprotinin (Sigma, St. Louis, MO), lysed by three freeze-thaw cycles, and microfuged (12,000 × *g*; 5 min; 4 C). Fifty microliters of the supernatant were used for the Lowry (40) protein assay, and 100 μg supernate protein (50–100 fmol MB-binding activity) from each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western transfer by electroblotting according to the methods described by Sambrook *et al.* (41). The nitrocellulose filters were blocked by immersion in TBS [20 mM Tris-HCl (pH 7.4) and 500 mM NaCl] with 0.5% Tween-20 for 1 h at room temperature and incubated overnight at 4 C with a monoclonal antibody (F39.4.1) to a peptide (Sf61) corresponding to amino acids 301–320 in the N-terminal portion of the hAR (42). The antibody was diluted 1:10,000 in TBS with 0.05% Tween-20. After three washes in TBS with 0.5% Tween-20, the filters were incubated with a 1:5,000 dilution of horseradish peroxidase-goat anti-mouse immunoglobulin G for 1 h at room temperature. After five more washes in TBS with 0.5% Tween-20, the blot was developed using the ECL Western blotting chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Densitometry was performed with an LKB Ultroscan XL densitometer (LKB, Rockville, MD) and the Gel Scan XL Program (Pharmacia, Piscataway, NJ).

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Substitution of Arginine-839 by Cysteine or Histidine in the Androgen Receptor Causes Different Receptor Phenotypes in Cultured Cells and Coordinate Degrees of Clinical Androgen Resistance

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Abstract

We aim to correlate point mutations in the androgen receptor gene with receptor phenotypes and with clinical phenotypes of androgen resistance. In two families, the external genitalia were predominantly female at birth, and sex-of-rearing has been female. Their androgen receptor mutation changed arginine-839 to histidine. In a third family, the external genitalia were predominantly male at birth, and sex-of-rearing has been male: their codon 839 has mutated to cysteine. In genital skin fibroblasts, both mutant receptors have a normal androgen-binding capacity, but they differ in selected indices of decreased affinity for 5α-dihydrotestosterone or two synthetic androgens. In transiently cotransfected androgen-treated COS-1 cells, both mutant receptors transactivate a reporter gene subnormally. The His-839 mutant is less active than its partner, primarily because its androgen-binding activity is more unstable during prolonged exposure to androgen. Adoption of a nonbinding state explains a part of this instability. In four other steroid receptors, another dibasic amino acid, lysine, occupies the position of arginine-839 in the androgen receptor. Androgen receptors with histidine or cysteine at position 839 are distinctively dysfunctional and appear to cause different clinical degrees of androgen resistance. (*J. Clin. Invest.* 1994. 94:546–554.) Key words: androgen • receptor • mutation • steroid • resistance

Introduction

The androgen receptor (AR)[1] protein is an androgen-sensitive transcription factor. Androgen binding enables its DNA-binding domain to recognize particular regulatory sequences of DNA (androgen-response elements) and thereby to increase or decrease the expression of certain target genes. In 1974 (1) several laboratories (2, 3) began to characterize the androgen-binding activities in the genital skin fibroblasts (GSF) of 46,XY humans with various clinical expressions of androgen resistance (insensitivity), on the presumption that they had mutant ARs. More recently, the cloning of the AR cDNA (4–7) and the application of derivative molecular techniques have permitted us (8–14) and others (15–27) to try to correlate specific germline lesions in the androgen-binding region of X-linked *AR* gene with various dysfunctional behaviors of the AR and with their clinical consequences for the affected subjects. The ultimate aim of such correlative studies is to elucidate the stereochemistry that imparts androgen-binding specificity to an AR and the contribution of an androgenic ligand to the transcriptional regulatory attributes of an A–R complex. In this paper, we describe three families that have alternative, single-nucleotide, missense mutations at the Arg-839[2] codon in exon 7 of the androgen-binding domain of the *AR* gene (Fig. 1).

Methods

Subjects and their families. 4308, the index subject in a black-American family, was described previously (28). She was born with a single perineal urogenital orifice flanked by labioscrotal folds. With the aid of corrective surgery, she and an affected maternal aunt were reared as females. The properties of the specific androgen-binding activity in 4308's GSF (28) are recalled, as appropriate, below.

A.E.L. and K.E.L. are maternal half-sisters in a second black-American family, historically unrelated to the one above. A.E.L. was brought to the St. Christopher's Hospital for Children at age 2 wk because of a right groin mass. There was posterior labial fusion and questionable clitoromegly. The karyotype was 46,XY. At age 4 mo, vaginography revealed a short, blind vagina, and extirpative surgery revealed histologically normal testes, epididymes, and proximal vasa deferentia. K.E.L. was born 6 yr later with a 1.5-cm clitoris, posterior labial fusion, and masses in the right labium majus and left groin. Vaginography revealed a short, blind vagina. The karyotype was 46,XY. At age 8 mo, basal serum testosterone was 197 ng/dl; after 4,000 IU of human chorionic gonadotropin daily for 3 d, it rose to 980 ng/dl. Bilateral orchiectomy revealed normal testicular histology. The abnormalities of the specific androgen-binding activity in the GSF of these two half-sister subjects are presented here for the first time.

335002 and 333203 are brothers in a white-Canadian family. 335002 was born with cryptorchidism and penile hypospadias; each required

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1. *Abbreviations used in this paper:* AI, androgen insensitivity; AR, androgen receptor; DHT, [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone; GSF, genital skin fibroblasts; h, human; k, nonequilibrium rate constant of dissociation; K_d, (apparent) equilibrium dissociation constant; MB, [17α-methyl-^{3}H]mibolerone; MMTV.GH, mouse mammary tumor virus.growth hormone; MT, [17α-methyl-^{3}H]methyltrienolone; pCMV.β-gal, cytomegalovirus promoter, β-galactosidase reporter gene construct; T, testosterone.

2. Number according to reference 4; the same codon is numbered 840 in reference 5 and 838 in reference 7.

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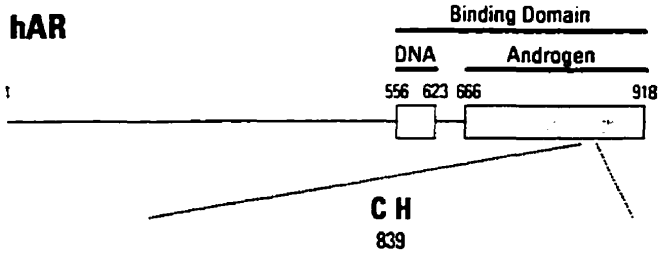

	830	R M N Y I K E L D **R** I I A C K R K N P
hPR	845	- S S - - R - - I K A - G L R Q - G V
hGR	690	- - T - - - - - G K A - V K R E G - S
hMR	896	- T - - - - - - R K M V T K C P N - S
hER	472	K D H I H R V - - K - T D T L I H L M

Figure 1. (*Top*) A linear version of the DNA- and androgen-binding domains of the human AR, indicating their terminal amino acids. (*Bottom*) An expanded portion of exon 7 indicating the location of residue 839 and its mutant amino acid substitutions. For comparison, the homologous residues are given for the human forms of the receptors for progesterone (P), glucocoticoid (G), mineralocorticoid (M), and estrogen (E). The single letter code for amino acids is: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; and Y, tyrosine.

staged surgical correction. Bilateral gynecomastia appeared at 12 yr and required reduction mammoplasty. Pubic and axillary hair appeared at age 14. At 22 yr, he had no facial or chest hair; pubic hair was Tanner stage 3. The right testis was 25 ml; the left was 10 ml. He is potent. Between 20 and 23 yr of age, repeated serum total testosterone (T) determinations varied from 72 to 180 nM (normal: 10–35) while his non—sex hormone–binding globulin-bound (free T) levels varied from 8.3 to 31 nM (normal: 3–13). On these occasions, his free T varied from 12 to 17% of the total serum T. In normal males, the fraction of total T that is not bound to sex hormone–binding globulin is ~ 30%. The values for luteinizing hormone (LH) varied from 16 to 23 U/liter (normal: 2–15), and those for follicle-stimulating hormone (FSH) have been 15 on two occasions (normal: 4–18). After 3 d of abstinence, a 0.5-ml sample of semen contained 11.8 million sperm/ml; 10% had normal motility, but 90% had normal morphology. 333203 was born 3.5 yr later with a nonhypospadic micropenis and bilateral cryptorchidism that needed several orchidopexy procedures. Bilateral gynecomastia required mammoplasty at age 14. At 19 yr he had no facial or chest hair; axillary hair was plentiful; pubic hair was Tanner stage 4. The penis was only 3 cm long. The testes were 8 ml bilaterally. At 20 yr, when his total serum T was 74 nM, the free was 12 nM, 17% of the total; the LH level was 21 U/liter (normal: 5–25), and the FSH was 29 U/liter (normal: 4–18). The affected brothers have three affected maternal first cousins once removed, two of whom are siblings. Two were born with penile chordee; one had micropenis with hypospadias at the penile base. All three had a nonbifid scrotum and have been reared as males with supportive surgery.

Androgen-binding activity in cultured cells. For GSF, specific androgen-binding activity, maximum androgen-binding capacity, the apparent equilibrium dissociation constant (K_d), the rate constant of dissociation (k), and theromstability of various A–R complexes or of the free AR were determined on monolayer cultures as described previously (29). The same methods were used for COS-1 cells except that they were washed and lysed in 0.5 N NaOH directly in the culture dishes. The androgens used were: [17α-methyl-^{3}H]mibolerone (MB; 7α, 17α-dimethyl-19-nortestosterone; 80 Ci/mmol); [1,2,4,5,6,7-^{3}H]5α-dihydrotestosterone (DHT; 120 Ci/mmol); and [17α-methyl-^{3}H]methyltrienolone (MT; 17β-hydroxyl-17α-methyl-4,9,11-estriene-3-one; 87 Ci/mmol). The synthetic androgens are nonmetabolizable.

PCR amplification and direct DNA sequencing of AR genomic exons. These were modified (30) from methods described in reference 12.

Construction of cDNA expression vectors with R839H or R839C. The germline sequence alterations identified in the DNA of representative affected subjects were reproduced using a modification of Higuchi's (31) site-directed PCR mutagenesis method. Four separate primary PCR reactions were performed using 2 μg of the human (h) AR cDNA expression vector pSVhAR.BHEX[3] as template and 50 pmol each of the following primers (mutated bases are underlined and bold faced):

1a. inside, AEL-A (5′-CAAGGAACTCGATC**A**TATCATTGC-3′)
 b. outside, SVEco (5′-GGAATTCCTCAC**T**GGGTGTGGAAAT-AGA-3′)
2a. inside, AEL-B (5′-GCATGCAATGATA**T**GATCGAG-3′)
 b. outside, Primer B (5′-TCACACATTGAAGGCTATGA-3′)
3a. inside, 335002-A (5′-CAAGGAACTCGAT**T**GTATCATTGC-3′)
 b. outside, Primer P3′ (5′-GCTGCCTATCAGAAGGTTGGTG-3′)
4a. inside, 335002-B (5′-GCAATGATAC**A**ATCGAGTTCCTTG-3′)
 b. outside, Primer B (see 2b above).

The primary fragments were amplified for 25 cycles by Pfu DNA polymerase (Stratagene, La Jolla, CA) with the following parameters: denaturation at 95°C for 1 min, annealing at 55°C for 75 s, and extension at 75°C for 90 s. The fragments from the four primary PCR reactions were gel-purified and resuspended in 20 μl H_2O, and 1–2 μl of the latter was used for the secondary PCR in the following combination

3. pSVhAR.BHEX is a version of pSVhAR$_o$ (32) that we have made more useful by making three of its restriction sites unique and by introducing a fourth site at codons 676–677 in the AR, numbered according to reference 4.

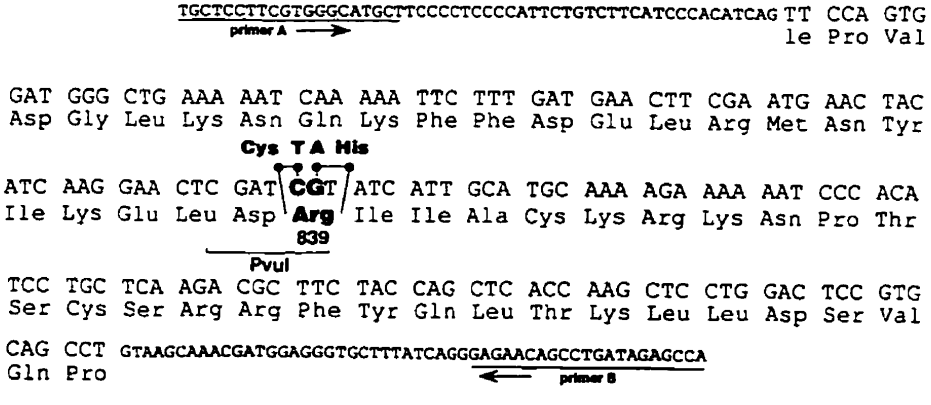

Figure 2. The nucleotide, amino acid, and flanking intronic sequences of exon 7 in the *AR* gene. The PCR primers are underlined. Arg-839, the cytosine (C) → thymine (T) transition leading to Cys-839, and the guanine (G) → adenine (A) transition leading to His-839 are in bold. The PvuI recognition sequence that is altered by each mutation is bracketed.

reactions: for A.E.L. (R839H) fragments from reactions 1 and 2; for 335002 (R839C) fragments from reactions 3 and 4. The secondary PCR reactions contained 0.5 pmol of each of the respective internal primers and 50 pmol of each of the outside primers: for 335002, P3′ and Primer B; for A.E.L., SVEco and Primer B. Except for the greater number of cycles (35 cycles), the cycle parameters were the same as the primary PCR reactions. The fragments from the four secondary PCR reactions were gel-purified. R839C (335002) fragments were double-digested with restriction enzymes EcoRI and BamHI, and R839H (A.E.L.) fragments were digested with EcoRI. The digested fragments were ligated into pSVhAR.BHEX[3] (R839C) or pSVhAR$_o$ (R839H), each deleted for the corresponding restriction fragments. XL1 Blue competent bacteria were transformed with the ligation products, and the colonies were screened by PvuI restriction enzyme digestion (both mutations abolish a PvuI site in the hAR cDNA). The appropriate clones were grown in 500 ml of liquid cultures, and the plasmid vectors were purified by QIAGEN columns (QIAGEN Inc., Chatsworth, CA). The inserted fragments were sequenced by the dideoxy method to confirm exclusivity of the site-directed nucleotide substitutions.

Assessment of transactivational efficiency of mutant ARs with R839H or R839C. The construction of the mouse mammary tumor virus.human growth hormone (MMTV.hGH) reporter plasmid and its cotransfection with the pCMV.β-gal vector to assess transfection efficiency have been reported (12, 13). To achieve consistent relative transactivational activities among replicate experiments, the prior transfection protocol (13) was modified as follows: we electroporated confluent COS-1 cells at 350 V/960 μF (~ 20 ms) in the presence of 1 μg normal or mutant pSVhAR and 20 μg pMMTV.GH, and 6 h later we exposed the cells to various concentrations of mibolerone for an additional 72 h.

Western analysis of the AR in transfected COS-1 cells. Confluent monolayers (~ 1 million cells) in 30-mm multiwell petri dishes were washed twice with 20 mM Tris/HCL (pH 7.4) containing 0.15 M NaCl, and the scraped cells from duplicate dishes were combined in 1 ml of the same buffer. After microfuging, the cells were resuspended in 160 μl of 0.25 M Tris/HCl (pH 7.8) containing 3.5% (vol/vol) aprotinin (Sigma Immunochemicals, St. Louis, MO), lysed by three freeze-thaw cycles, and microfuged (12,000 *g*, 5 min, 4°C). 50 μl of the supernatant was used for protein assay (33), and 100 μg of supernatant protein from each sample was subjected to SDS-PAGE and Western transfer by electroblotting according to standard methods (34). The nitrocellulose filters were blocked by immersion in TBS (20 mM Tris/HCl [pH 7.4] and 500 mM NaCl) with 0.5% Tween 20 for 1 h at room temperature and incubated overnight at 4°C with a monoclonal antibody (F39.4.1) to a peptide (Sf61) corresponding to amino acids 301–320 in the NH_2-terminal portion of the hAR (35). The antibody was diluted 1:10,000 in TBS with 0.05% Tween 20. After three washes in TBS with 0.5% Tween 20, the filters were incubated with a 1:5,000 dilution of horseradish peroxidase goat anti-mouse IgG for 1 h at room temperature. After five more washes in TBS with 0.5% Tween 20, the blot was developed using the ECL chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Using the LKB Ultroscan XL densitometer and the Pharmacia Gel Scan XL program (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), we validated the chemiluminescence method by demonstrating linearity with 10–300 fmol of androgen-binding activity in the standard Western blotting procedure and by exposing film to the Luminol reaction for various times (30 s–20 min) to define a window during which relative band intensity remained constant densitometrically.

Results

Identification and restriction-site analysis of the mutations. By PCR amplification and direct sequencing of exons 1–8, we found the guanine to adenine transition responsible for Arg839His first in the GSF of subject 4308 and then in those of the half-sisters A.E.L./K.E.L. (Fig. 2). In the same way, we later found the cysteine to thymine transition responsible for Arg839Cys in the brothers 335002/333203 (Fig. 2). The ablation of a PvuI site by either substitution allowed us to prove maternal heterozygosity for the half-sisters A.E.L./K.E.L. (Fig. 3, *top*) and for the brothers 335002/333203 (Fig. 3, *bottom*). The families of 4308 and A.E.L./K.E.L. are historically unrelated. They also differ in the highly polymorphic length (36) of the poly-glutamine tract in exon 1 of the *AR* gene: 4308 has

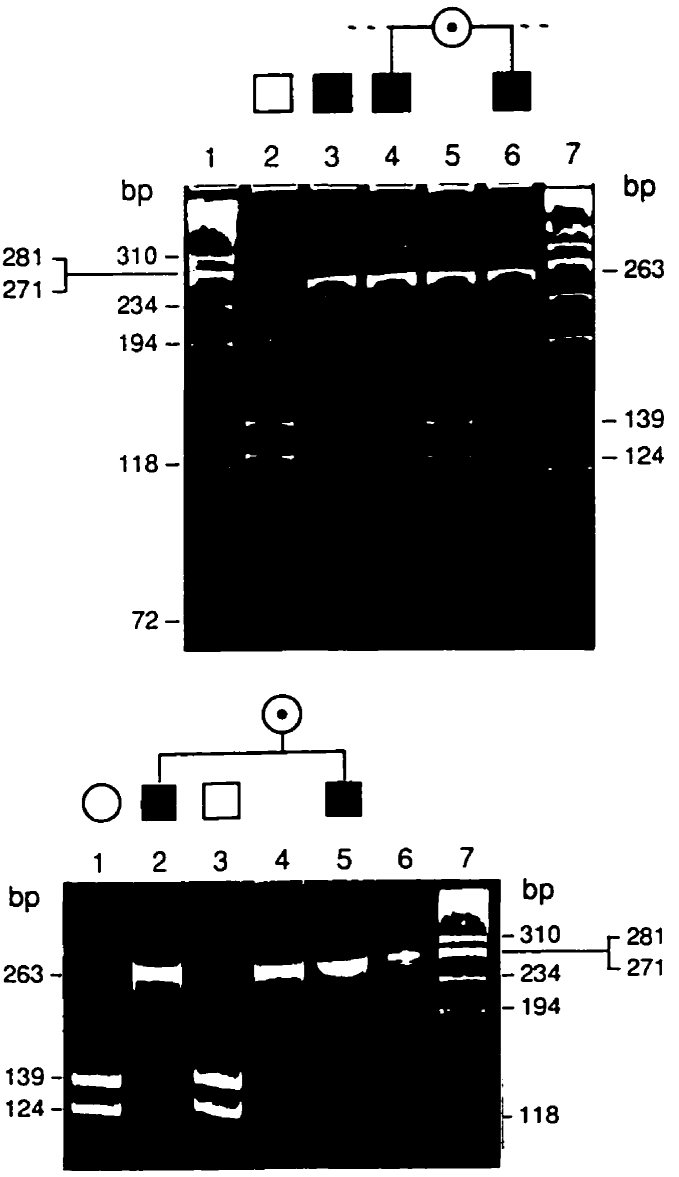

Figure 3. Ethidium bromide–stained, 8% PAGE analysis of PvuI-treated PCR-amplified normal and mutant exon 7. (*Top*) Lanes *1* and *7*, size standards generated by HaeIII digestion of ϕX 174 RF. Lane *2*, a normal person; lane *3*, subject 4308; lanes *4* and *6*, the half-sister subjects, A.E.L. and K.E.L., respectively; lane *5*, their mother. When exon 7 of the hAR is amplified between the intronic primers indicated in Fig. 2 it is within a 263-bp product. A normal allele is cleaved by PvuI into 139- and 124-bp fragments. The mutant allele resists cleavage. Hence, the heterozygous mother has three bands. (*Bottom*) Lanes *1* and *3*, unrelated normal persons; lanes *2* and *5*, the brothers 335002/333203; lane *4*, their mother; lane *6*, same as lane *5* but not exposed to PvuI; lane *7*, size standards as above.

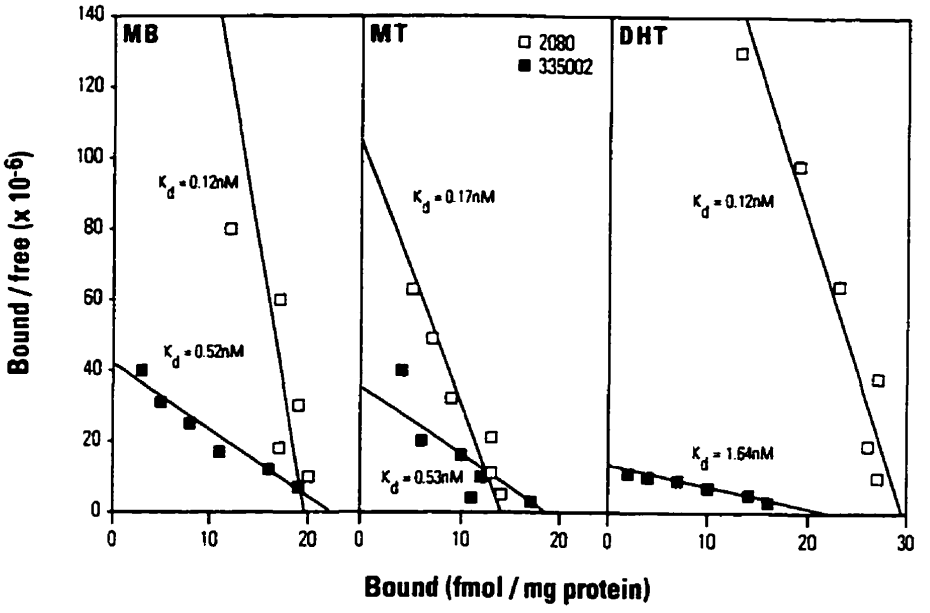

Figure 4. Scatchard plots representing saturation analysis performed on the specific MB-, MT-, and DHT-binding activities in whole GSF. 2080, normal; 335002, mutant AR Arg839Cys. The K_d was computed from the slope of a line; maximum androgen-binding capacity was estimated by its intercept on the horizontal axis.

19 repeats, A.E.L./K.E.L. have 18, their mother has ones of 18 and 20; and 335002 has 20.

Androgen-binding activity in GSF. We first determined various androgen-binding parameters of the mutant AR in the GSF of the half-sisters A.E.L./K.E.L. and the brothers 335002/333203. Fig. 4 shows that 335002's AR has modestly increased K_d values for MB, MT, and DHT that are comparable with those published previously (28) for 4308 (0.57, 2.2, and 1.0 nM, respectively; normal < 0.3 nM) and to those of A.E.L./K.E.L. (0.35, 1.0, and 0.56 nM, respectively).

The MB- and MT-R complexes preformed in the GSF of 333203 or A.E.L. at 37°C were much more thermolabile than normal when the cells were postincubated at 41°C with 100 μM cycloheximide in the presence of 3 nM of the appropriate [^{3}H]androgen (Fig. 5). Furthermore, the Arg839His MB-receptor complexes in A.E.L. were more thermolabile than the Arg839Cys ones in 333203. However, when the GSF of subject 333203 or A.E.L. were incubated at 37°C in serum-free medium overnight and then exposed to 100 μM cycloheximide at 41°C, they lost their androgen-binding activity during the next 2 h at the same rate as control GSF (t = 1.5 h; data not shown). These disparate responses of A.E.L.'s GSF to thermal stress closely resembled those in 4308's GSF under slightly different conditions (28).

Table I shows the rates at which various [^{3}H]A-R complexes dissociated in GSF chased by a great excess of the corresponding radioinert androgen at specific temperatures. A.E.L.'s DHT-R complexes dissociated moderately faster than normal not only at 37°C, as suggested by those of 4308, but also at 40 and 42°C. In contrast, 335002/333203 had normal DHT k values ("off-rates") at 37 and 42°C. Likewise, these brothers had a normal mean MT k value at 37°C, whereas the corresponding value for A.E.L. and 4308 was suspiciously high. Notably, A.E.L., 4308, and the brothers 333203/335002 all had normal MB k values at 37°C; yet, all three tested had clearly increased MB k values at 40 and/or 42°C. We have reported such patterns of ligand-specific, temperature-restricted departures from normal k values in the GSF of other subjects with partial androgen resistance (37).

Androgen-binding and transactivational activity of trans-

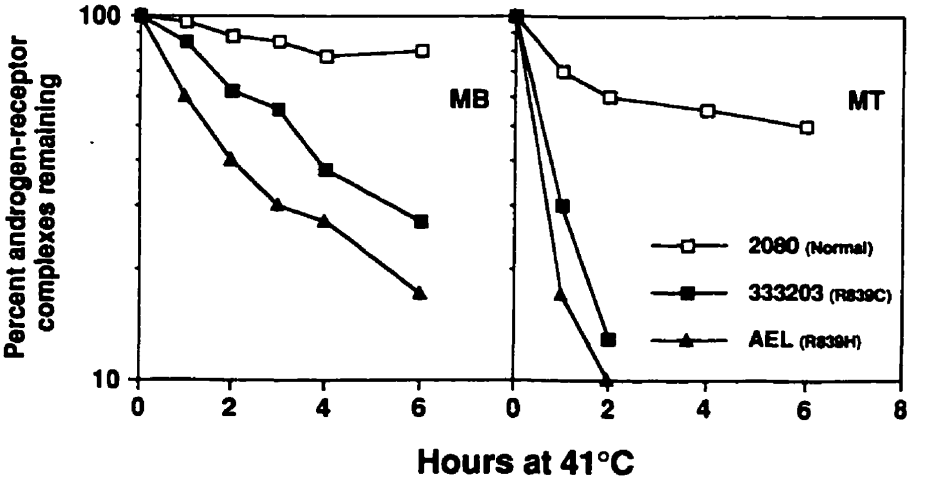

Figure 5. MB- and MT-R complexes were formed in GSF incubated with 3 nM [^{3}H]MB or MT at 37°C (0 time). The cells were then postincubated at 41°C with 100 μM cycloheximide and 3 nM of the same [^{3}H]-androgen. A-R complexes remaining were measured at intervals up to 6 h. 2080, normal; 333202, Arg839Cys; A.E.L., Arg839His.

Table I. Rate Constants of Dissociation (k: 10^{-1} min^{-1}) of Control and Mutant A–R Complexes in GSF Monolayers at Various Temperatures

	DHT			MT		MB		
	°C			°C		°C		
	37	40	42	32	37	37	40	42
4308*	9‡				30‡	4		16‡
	8‡				15			14‡
A.E.L.*	10	15	28	8	17	4	15	18
	10					5		
335002§	6		17		12	3		15
333203§	5				16	3		
Control(s)	6	10	17	6	12	3	6	9
±SD (n)	±1 (30)				±2 (26)	±0.5 (25)		±0.7 (12)

The monolayers were incubated in serum-free medium at 37°C with 3 nM [^{3}M]androgen alone or together with 0.6 μM radioinert androgen. Specific androgen-binding activity was measured at 1 h, then the cells were washed and chased with the same medium containing only radioinert androgen, and the rate of decline of the activity was measured at intervals for 2 h. * 4308 and A.E.L. (unrelated) share Arg839His. ‡ From Table I in reference 22. § 335002/333203, brothers, share Arg839Cys.

fected ARs. To prove that Arg839His or Cys are pathogenic substitutions, we created each mutation in an otherwise normal hAR cDNA expression vector and transiently transfected it into COS-1 cells. Table II shows that the cells with the Arg839His AR, but not those with the Arg839Cys AR, had higher 37°C *k* values for all three androgens than those with the normal AR. These results conserved the pattern of 37°C *k* values in normal and either type of mutant GSF.

Likewise, the MB–Arg839His receptor complexes produced in COS-1 cells at 37°C were more thermolabile than their Arg839Cys counterparts when shifted to 42.5°C (Fig. 6 *A*). In contrast, as previously shown for GSF, when the transfected COS-1 cells were first exposed to a temperature of 42.5°C in the presence of 100 μM cycloheximide and then measured for androgen-binding activity at various intervals, there was no appreciable difference between the thermostability of the normal receptor and either of the two mutant ARs (Fig. 6 *B*).

Fig. 7 *A* shows that in COS-1 cells treated with MB 6–80 h after cotransfection the Arg839His AR had a clear tendency to be less active in promoting transcription of the GH gene than the Arg839Cys AR and that both of them were less active than the normal hAR.

We also assessed stability of the normal and either type of mutant AR in COS-1 cells by measuring their androgen-binding activities during various intervals after transfection. Fig. 8 shows that the normal binding activity was stable, or relatively so, during prolonged (46–120 h) incubation at all MB concentrations tested, whereas both types of mutant activities were clearly unstable at the same MB concentrations. Again, the Arg839His mutant was inferior to the Arg839Cys. The approximate 50% difference between the 2-h binding activities at the beginning and the end of the prolonged exposure to MB reflects the transience of the transfections. In this respect, all three transfections behaved very similarly.

In another transfection experiment using only 3 nM MB (Fig. 9), the normal and mutant androgen-binding activities were subjected to immunoblot analysis. As above, the 118–120-h binding activities were 40–50% of those in their 46–48-h counterparts, and their respective blot intensities were appropriate. As before (Fig. 8), prolonged (46–120 h) incubation with MB caused cells with Arg839His to lose more (> 75%) of their starting binding activity than cells with Arg839Cys (50%). Under the same conditions, the cells with the wild-type hAR essentially maintained their initial androgen-binding activity. After prolonged MB incubation, the immunoreactivities of the wild-type hAR and both mutant forms increased relative to their respective androgen-binding activities. This implies that fractions of their respective pools of ARs were in a

Table II. Ligand-specific k of the Normal and Mutant hARs in GSF and COS-1 Cells at 37°C

	Dissociation rate ($\times 10^{-1}$ min^{-1})					
	Normal		Arg839Cys		Arg839His	
Ligand	GSF	COS-1	GSF*	COS-1	GSF*	COS-1
MB	3.0±0.1‡	3.1±0.10	3.0±0.00	3.2±0.30	4.3±0.33	4.9±0.35
DHT	6.3±0.25	7.1±0.35	5.3±0.75	6.9±0.65	9.3±0.48	9.2±0.70
MT	12±0.5	12.3±0.30	14.3±2.25	10.9±0.70	20.7±4.70	15.6±0.20

* The GSF values have been combined for affected members of the same family or unrelated subjects with the same mutation. ‡ The values are the mean±SE.

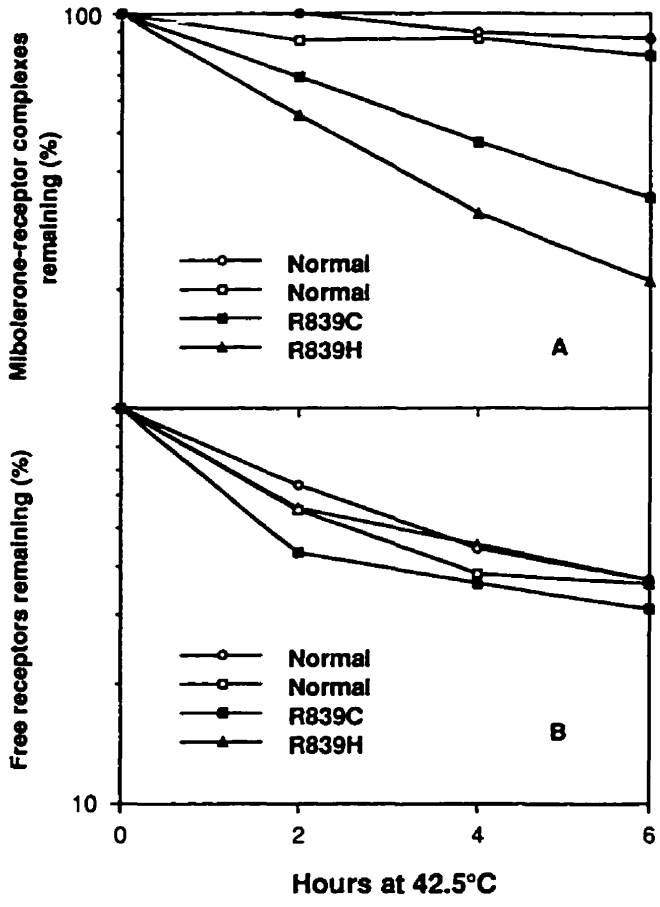

Figure 6. (*A*) MB–receptor complexes were formed in COS-1 cells incubated with 2.4 nM MB at 37°C for 2 h (46–48 h after transfection; 0 time). The cells were then postincubated at 42.5°C under the conditions defined in the legend to Fig. 5, and residual MB–receptor complexes were measured at intervals up to 6 h. ○, Two samples of normal hAR cDNA expression plasmid transfected separately. (*B*) 48 h after transfection, COS-1 cells at 37°C were incubated at 42.5°C in the presence of 100 μM cycloheximide. Thereafter, at intervals up to 6 h, the decline of mibolerone-binding activity was measured by incubating with 3 nM MB for 1 h at 37°C.

non–androgen-binding state; it is evident that these fractions were greater for the mutants.

In Fig. 7 *B*, for each of three experiments, we replotted GH production (shown in aggregate in Fig. 7 *A*) against relative MB-binding activity during prolonged (6–78 h) MB incubation. In two experiments, the normal and mutant receptors were indistinguishable; in the third, the differences were slight.

Discussion

In our initial report (28) on the partial androgen insensitivity syndrome in 4308, we noted that her GSF AR phenotype was distinctive; for instance, her DHT–receptor complexes had questionably increased 37°C *k* values, yet the 42°C *k* values for her MB–receptor complexes were clearly high. Many years later, we realized that 4308 and the unrelated half-sisters, A.E.L./K.E.L., shared not only the Arg839His AR but also a very similar GSF AR phenotype and predominantly female external genitalia. The broad phenotypic similarity in the two

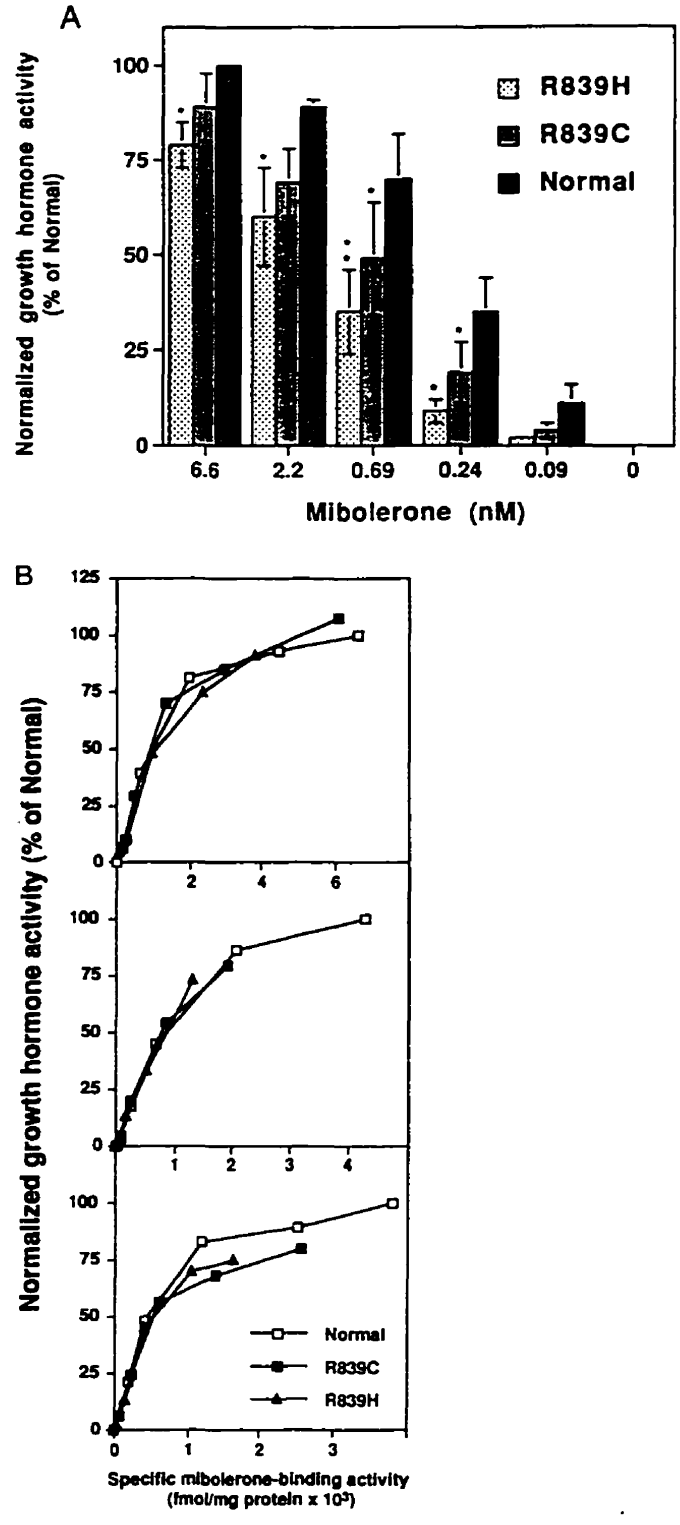

Figure 7. (*A*) COS-1 cells were transfected with normal hAR expression plasmid, or with a plasmid containing either mutant AR, and also with both the MMTV.GH and CMV.β-gal plasmids. 6 h after transfection, different concentrations of MB were added to the medium, and 72 h later samples of medium and cells were taken to measure GH and β-gal activity, respectively. The latter activity was used to assess transfection efficiency, and the GH values were normalized for variation in that efficiency. $*P < 0.05$ versus normal; $**P < 0.05$ versus normal or R839C. (*B*) The GH data above are replotted against specific MB-binding activities at a given concentration of MB in the 6–78-h intervals after transfection.

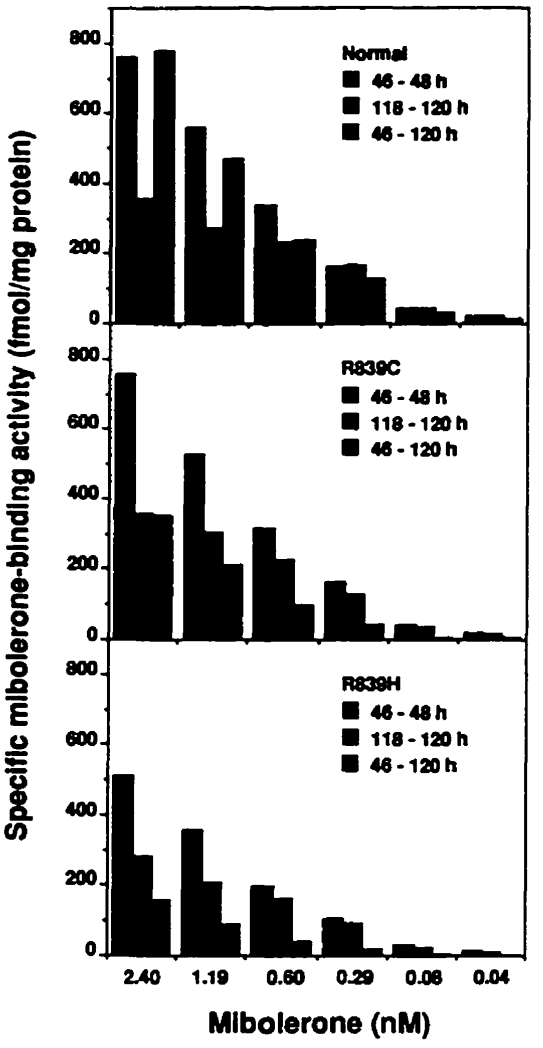

Figure 8. COS-1 cells were transfected with normal hAR expression plasmid or with plasmid containing either mutant AR. Specific MB-binding activity at various concentrations of MB was measured for 2 h, at 46 and 118 h after transfection, and for 74 h, from 46 to 120 h after transfection.

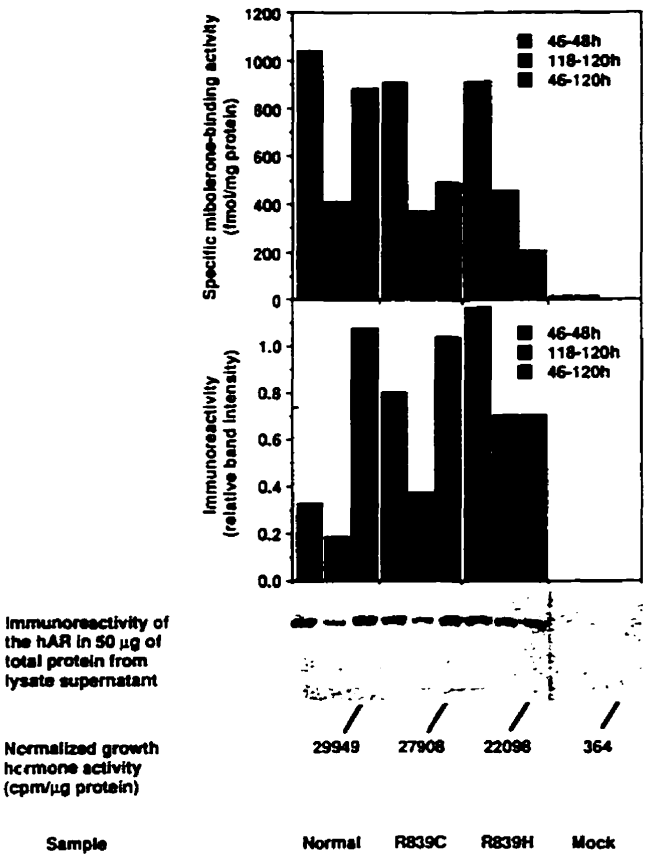

Figure 9. Androgen-binding activity (*top*, not corrected for transfection efficiency) and immunoreactivity (*middle* and *bottom*) were measured concurrently after the addition of 3 nM MB for various intervals to replicate wells of COS-1 cells that had been cotransfected with pMMTV.GH and pCMV.β-gal alone (*Mock*) or additionally with pSV.hAR.BHEX (*Normal*) or one of its two mutant versions, R839H or R839C.

families with Arg839His AR appreciated in value both by similarity and by contrast with the Arg839Cys AR in the brothers (335002/333203) with partial androgen insensitivity (AI). For example, in GSF both mutant ARs had variably elevated but overlapping K_d values for all three androgens, and their respective MB k values were normal at 37°C but elevated at 40–42°C. By contrast, the GSF with Arg839His had slightly to moderately elevated DHT k values at 37–42°C, while the corresponding values for those with Arg839Cys were normal.

The fact that the GSF with Arg839His had abnormal DHT k values while those with Arg839Cys did not corresponded with the greater undermasculinization of the subjects with Arg839His than those with Arg839Cys. This was borne out by the relative transregulatory dysfunction of each mutant receptor within transfected COS-1 cells: the Arg839His AR was less active than the Arg839Cys AR when GH production was plotted against MB concentration. Importantly, however, the difference disappeared when GH production was plotted against specific MB-binding activity. This suggested that the mutant MB–receptor complexes had normal intrinsic transactivational competence, but that they were unstable during prolonged exposure to MB. An immunoblot analysis revealed that some of the mutant AR instability reflected conversion of the mutant AR to a non–androgen-binding state. Such a state may be a part of normal intracellular AR recycling (38), but the mutant ARs may be abnormally disposed to occupy it. Despite these observations, it is important to appreciate that there is strong theoretical and experimental (39) reason to believe that the transregulatory activity of normal or mutant (40) steroid receptors is influenced by cell type and by the promoter context of a particular target gene. Hence, the disparity we observed among the normal and mutant ARs using the MMTV.hGH reporter gene in COS-1 cells might well change in degree and/or quality, if we were to use a reporter gene with a different androgen-responsive promoter context and a transfectant cell type with a different set of cofactors that affect the transregulatory activity (or stability) of the normal and mutant ARs. The ideal candidates would be physiologic target cells rendered AR-free and reporter genes whose products are essential for male sexual differentiation.

Our data and the fact that all the other steroid receptors have lysine in the position homologous to Arg-839 in the AR

(Fig. 1) imply that the hAR must have Arg or Lys at position 839 to function normally. Our data also suggest that His-839 tends to disrupt the AR more than Cys-839 does: the former is likely to cause predominantly female external genitalia and a female sex-of-rearing; the latter is likely to cause predominantly male external genitalia and a male sex-of-rearing. The two unrelated individuals with Arg839Cys reported by others (25) also had partial AI with a male phenotype (Reifenstein syndrome) and, as we have found, in the GSF of the one tested, MB–receptor complexes had normal 37°C k values. Others have found the Arg839His mutation in four unrelated individuals. All four had partial AI: two with a female phenotype (25, 27), one with a male phenotype (25), and one ambiguous (41). Importantly, as we found, in the GSF from the one subject with a female phenotype tested, the AR had an increased k value for MB at 37°C (25). Remarkably, the GSF of the one with a male phenotype had almost unmeasurable androgen-binding activity, typical of subjects with complete AI (25). This is a striking indication that "background" factors can mitigate the typical clinical expression of an *AR* mutation.

The fact that neither the His nor the Cys substitution of Arg-839 disrupts the AR sufficiently to cause complete AI is provocative, in light of information available on the same set of substitutions at two other Arg codons in the AR's androgen-binding domain. For instance, Arg773Cys disrupts the AR phenotype more than Arg773His (12), yet both cause complete AI exclusively (12, 24, 26), possibly because Arg-773[4] is within an ultraconserved region of the androgen-binding domain. Contrarily, Arg854Cys has caused complete AI in three out of three subjects (25, 26, 42), while Arg854His has caused partial AI in three out of four subjects (24, 25, 43), yet Arg-854[5] is at the NH_2-terminal limit of an 11–amino acid stretch that is absolutely identical among the members of the AR subfamily. The facts that Arg-839 is not within a highly conserved region of the androgen-binding domain and that neither Cys-839 nor His-839 causes complete AI indicate that Arg-839 is less important than Arg-773 or Arg-854 to the AR's androgen-binding properties. It is also noteworthy that Cys-839 appears to be less disruptive than His-839, whereas Cys-773 and Cys-854 appear to be more disruptive than His-773 and His-854, respectively. Perhaps this is because Cys-839 does not provoke a harmful disulphide bridge, whereas Cys-773 and Cys-854 do. This hypothesis is strengthened by the ligand- and temperature-restricted character of the moderately abnormal nonequilibrium rate constants of dissociation that we have observed in GSF carrying either of the present mutations. In the GSF of most subjects with complete AI due to missense mutations in their androgen-binding domains, these rate constants of dissociation are more abnormal absolutely and seldom ligand- or temperature-restricted (14).

Arg839Cys and Arg839His represent G:C → A:T transitions. This reflects the tendency for cytosines at CpG dinucleotides to become thymines by methylation at carbon 5 and spontaneous deamination at carbon 4. Indeed, 9 out of 28 point mutations that we have identified in the *hAR* gene fall into this category (14).

4. Number according to reference 4: the same codon is numbered 774 in reference 24 and 772 in reference 25.

5. Number according to reference 4: the same codon is numbered 855 in reference 24 and 853 in reference 25.

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CHAPTER II

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Evidence for a repressive function of the long polyglutamine tract in the human androgen receptor: possible pathogenetic relevance for the $(CAG)_n$-expanded neuronopathies

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We have reported that polyglutamine (polyGln)-expanded human androgen receptors (hAR) have reduced transactivational competence in transfected cells. We presumed that maximal hAR transactivation requires a normal-size polyGln tract. Here we report, however, that hAR transactivity and polyGln-tract length are related inversely: n = 0>12>20>40>50. Thus, a normal-size polyGln tract represses the transactivational competence of a polyGln-free hAR, and polyGln expansion increases that negative effect. This observation has pathogenetic implications for X-linked spinobulbar muscular atrophy (Kennedy syndrome), and possibly for the autosomal dominant central neuronopathies associated with $(CAG)_n$ expansion in the translated portion of four different genes.

INTRODUCTION

As in many transcriptional regulatory proteins of eukaryotes (1), the transcriptional modulatory domains of the X-linked human, rat and mouse androgen receptor proteins have long polyGln tracts that are presumed to be functionally equivalent (2). In humans the tract varies polymorphically from n = 11–33 (3,4), its modal length is about 20 (3), and its location is more NH_2-terminal than in the rodents (2). Expansion of the long $(CAG)_nCAA$ tract in the h*AR* of males to $n \geq 40$ causes spinobulbar muscular atrophy (SBMA), an adult-onset selective motor neuronopathy that is often associated with a mild, selective form of androgen insensitivity (Kennedy syndrome) (5). No other *AR* mutation, including a complete deletion (6), causes SBMA. Hence, $(CAG)_n$ expansion must endow the hemizygous mutant *AR* gene (or one of its products) with a gain-of-function that is neuronopathic (7). This pathogenetic paradigm is also applicable to four mendelian-dominant central neuronopathies caused by expansion of 5′ translated $(CAG)_n$ repeats: Huntington disease (HD), spinocerebellar ataxia type 1 (SCA1) and dentatorubropallidoluysial atrophy (DRPLA) (8), and Machado–Joseph disease (MJD) (9). The gain-of-function is most probably expressed by the polyGln-expanded proteins (10,11) encoded at each of the five loci. These mutant proteins may become neuronopathic by acquiring a property that is entirely foreign to their respective normal proteins, or simply by expressing a pathogenically increased amount of a usual property whose functional effect is negative or positive. Green (10) suggested that the property gained by polyGln-expanded proteins is the ability to become conjugated to other proteins by virtue of transglutaminase-catalysed isopeptide crosslinks. Perutz *et al.* (11) proposed that expanded polyGln tracts act as 'polar zippers' by virtue of hydrogen bonds that form between the main-chain and side-chain amides of adjacent proteins.

The normal functions of the deduced proteins encoded at the loci for HD, SCA1, DRPLA and MJD are unknown. Hence, it has been impossible to determine whether polyGln expansion causes any loss of function in one or more of these proteins and, if so, to consider whether this loss may be necessary, but not sufficient, to cause a given neuronopathy or to influence its severity. On the other hand, the function of the AR protein is known; thus, it is proper to presume that polyGln expansion causes the mild androgen insensitivity associated with SBMA by impairing AR function. Furthermore, with previous supportive data from two laboratories (1,7), it has been tacitly accepted that this loss is mediated by a decrease in a positive function that a polyGln tract contributes to the transactivational competence of an AR. Our present data support a contrary notion: that polyGln expansion increases the

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negative contribution of a polyGln tract to the transactivational competence of an AR. This notion is also relevant to the still unknown pathogenesis of HD, SCA1, DRPLA and MJD, as we discuss below.

In our earlier study (7), we cotransfected AR-free COS-1 cells with a cytomegalovirus-promoted hAR expression vector (pCMVhAR), and with a reporter plasmid composed of the human growth hormone gene connected to the long terminal repeat of the mouse mammary tumor virus (pMMTVGH) that contains several steroid (androgen) response elements. We found that the transactivational competence of ARs with 40 or 50 Gln tracts were equally inferior compared with that of an AR with 20 glutamines (7) and, in preliminary experiments, that a precise deletion of the long polyGln tract either did not impair hAR transactivity, or improved it slightly, compared with that of a 20-Gln AR. These results suggested that basal transregulatory function of an AR may not require a polyGln tract, despite its evolutionary conservation in the human compared with the rodent ARs, and despite several lines of evidence that its presence (12), or its size (13), are critical for the function of other transregulatory proteins. Finally, we knew that hARs constructed to have NH_2-terminal (14) or internal (15) deletions that included their polyGln tracts had been reported to be transcriptionally normal or subnormal, respectively. This disparity could have arisen from concomitant deletion of different adjacent amino acid sequences.

On the background of the foregoing information, the present study had two specific objectives: (a) to determine whether an hAR with 12 glutamines, or one with a clean deletion of its polyGln tract, is just as transactive as one with a typically normal number of 20 glutamines, or even more transactive; and (b) to discern a different degree of impaired transactivity between hARs with 40 or 50 glutamines. In the present experiments, we cotransfected COS-1 cells with the same reporter plasmid as above, but with a set of SV40-promoted AR expression vectors. We also changed the electroporation conditions, the amounts of AR expression and reporter vectors transfected, and the post-transfection times at which specific androgen-binding activity and growth hormone concentration were measured.

RESULTS

Effect of polyGln-tract length on the transregulatory function of a hAR

The results of three independent experiments are shown in Figure 1 as paired panels (experiment 1: a and b; experiment 2: c and d; experiment 3: e and f). In each experiment we found that complexes formed between mibolerone [MB, (7α, 17α-^{3}H-dimethyl-19-nortestosterone), a synthetic, nonmetabolizable androgen] and a mutant hAR with 50 glutamines were less able to transactivate growth hormone (GH) gene expression than a mutant 40-Gln hAR. This difference was apparent even when the cells were incubated with only 0.4 nM MB, but it was prominent at both higher concentrations (Fig. 1: a,c,e). These results were the first to indicate a hierarchical relation between the performance of a hAR as a transactivator, and the length of its expanded polyGln tract.

Our most provocative observation was the clear transactivational superiority of an unnatural 0-Gln *AR* compared with a typically natural 20-Gln h*AR*. Furthermore, a 12-Gln h*AR*

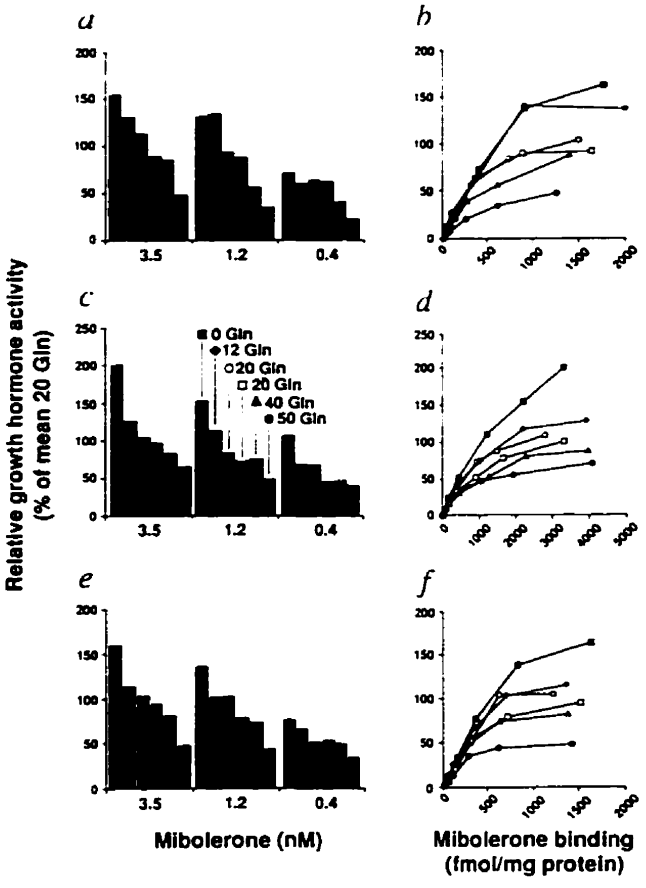

Figure 1. Growth hormone secretion by mibolerone (MB)-stimulated COS-1 cells cotransfected with pMMTVGH, pCMVβgal and pSVh*AR*-0, -12, -20, -40, or -50 Gln. The pairs of adjacent panels (a, b; c, d; e, f) show the results of three independent experiments. a, c, and e relate GH secretion to the various concentrations of [^{3}H]MB added to the media. b, d, and f relate the same GH secretion data to specific MB-binding activities in the COS-1 cells at various [^{3}H]MB concentrations, including those indicated in a, c, and e, respectively. Three to 6 h after transfection various concentrations of [^{3}H]MB were added to replicate wells of 16 mm multiwell plates, either with or without 0.6 μM radioinert MB, to measure nonspecific or total MB-binding activity, respectively. Specific MB-binding activity was measured 24 and 96 h later and the values were averaged. GH activity, assayed at 96 h, was normalized for transfection efficiency by simultaneous measurement of βgal activity in the replicate wells. After subtraction of background GH secreted into the medium of replicate cotransfected cells not exposed to MB, GH activity was expressed as a percent of mean GH activity stimulated by two independent samples of pSVh*AR*-20 Gln.

performed better than its 20-Gln partners, especially at both higher concentrations of MB, and it was better than its 40- or 50-Gln partners, even at 0.4 nM MB (Fig. 1: a, c, e). Thus, overall, there was an inverse relation between a hAR's polyGln-tract length and its transregulatory competence. (For all data sets in Figure 1, r and P values vary from 0.85 to 0.96 and 0.034 to 0.002, respectively. At 3.5 nM MB, panels, a, c and e, r values are 0.95, 0.89 and 0.95; P values are 0.004, 0.018 and 0.004, respectively).

To discern whether polyGln-tract size modulated GH expression by virtue of corresponding changes in androgen binding,

Table 1. Apparent equilibrium dissociation constant and rate constant of dissociation at 37°C of 0-Gln and 20-Gln hARs in transfected COS-1 cells

Plasmid	$k(10^{-1}min^{-1})$			K_D (nM)	
	MB	DHT	MT	Exp 1	Exp 2
0 Gln	2.3	6.9	13.3	0.39	0.45
20 Gln	3.1	5.9	12.2	0.34	0.44
Normal	2.9 ± 0.2	6.2 ± 0.5	12.3 ± 0.1		0.42 ± 0.05
		k ± SEM (n = 4)			K_D ± SEM (n = 6)

k, rate constant of dissociation; K_D, apparent equilibrium dissociation constant with MB.

we replotted the GH data against specific androgen-binding activity for various MB concentrations, including those shown in Figure 1a, c and e. The parallel panels (Fig. 1b, d and f) display the expected functional hierarchy for different polyGln-tract sizes. This indicated that the transactivational differences observed between the polyGln-deleted and polyGln-expanded hARs, or hARs with normal-size polyGln tracts, were intrinsic and not due to differences in their androgen-binding properties; for the 0 Gln hAR, we affirmed this indication directly by finding its nonequilibrium rate constant of dissociation and its apparent equilibrium dissociation constant, at 37°C, to be normal (Table 1). We had previously reported normal androgen-binding properties for 40- or 50-Gln hARs in COS cells (7), as had others for a 12-Gln hAR in CHO cells (16). However, inconsistent androgen-binding abnormalities have been found for polyGln-expanded ARs in cultured scrotal (17) or suprapubic (18) skin fibroblasts.

DISCUSSION

Our experiments have exposed an inverse correlation between the size of a $(CAG)_n$ trinucleotide repeat in an hAR cDNA expression vector, and the degree of expression of an androgen-regulated reporter gene. This relation very likely reflects the action of corresponding hAR proteins with different polyGln-tract sizes. If so, several statements are applicable to our results. First, a polyGln tract with the quantitative character we have observed may be called a 'gradient modulatory domain'. Second, a polyGln tract may not be critical to each transregulatory function of a hAR, as Stallings recently implied (19). Third, a polyGln tract may contribute negatively to the net transregulatory activity of a hAR. Fourth, polyGln tracts of different normal sizes may be responsible for more-or-less subtle differences in the physiologic action of their respective hARs; these differences may have selective value individually (20) or evolutionarily (1).

There have been very few intracellular studies on the quantitative structure–function relations of polyGln tracts (1,12,13) or Gln-dense domains (21) in transregulatory proteins. There is a close experimental association between polyGln-tract size and the testis-determining competence of the putative transcriptional regulatory protein encoded by the *Sry* mouse gene (13). When a Y chromosome bearing an *Sry* encoding 12 glutamines is backcrossed into C57BL/6J males, testis determination is normal; with 11 glutamines, it is impaired severely; with 13 glutamines partially. The importance of context is raised by evidence that the association might be stronger for *Sry* alleles that use an early stop codon (22). Gerber *et al.* (1) confirmed our earlier results (7) using transfected Hela cells to show that transactivation by a chimeric protein containing the GAL4 DNA-binding domain depended on the length of the polyGln tract to which it was fused: when n was 6–26, the effect was maximal; when it was 34–98, there

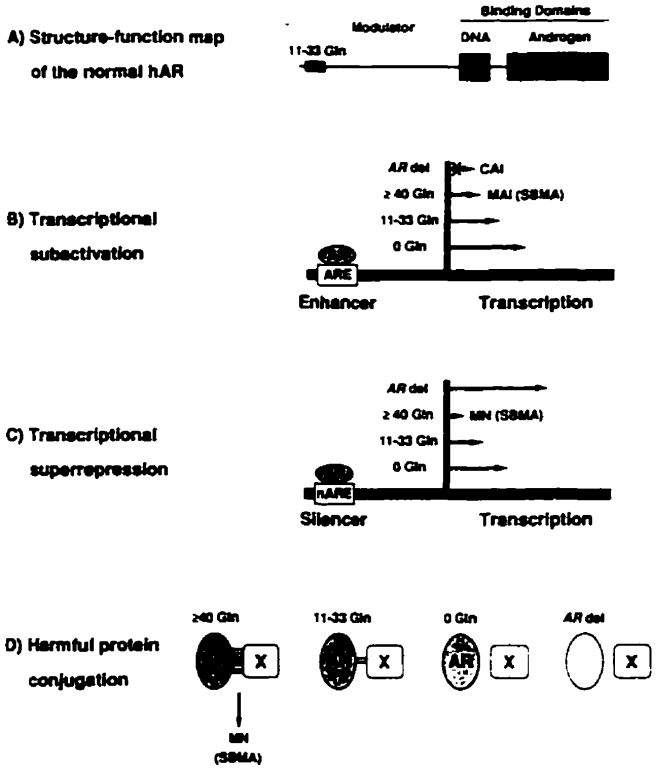

Figure 2. Pathogenetic schemes for the mild androgen insensitivity (MAI) and motor neuronopathy (MN) of SBMA (Kennedy syndrome). (**A**) Structure–function organization of the androgen receptor (AR) and location of its long polyglutamine (polyGln) tract. (**B**) Transcriptional subactivation: compared to a normal AR (11–33 Gln), transcriptional activation is increased by a polyGln-deleted AR (0 Gln), is decreased by a polyGln-expanded AR (≥40 Gln) resulting in the MAI of SBMA, and is eliminated by a deleted AR (*AR* del) resulting in complete AI (CAI). (**C**) Transcriptional super-repression: compared with a normal AR (11–33 Gln), transcriptional repression is decreased by a 0-Gln AR or eliminated by an *AR* del, both without harm, but it is increased by a ≥40 Gln AR resulting in the MN of SBMA. (**D**) Harmful protein conjugation: compared with a normal AR (11–33 Gln), a ≥40 Gln AR is able to conjugate with protein X excessively. The conjugate itself is directly motor neuronotoxic (MN), or the consequent concentration of free protein X falls below a physiologic threshold that is necessary for normal survival of certain motor neurons (not shown).

was a progressive decline. Again, context was important: different results were obtained when the polyGln tracts were flanked by the activation domain of herpesvirus-derived VP16 protein, or when the experiments were conducted *in vitro*.

While this manuscript was in preparation, Chamberlain *et al.* (23) reported cotransfection experiments very similar to ours. They found that an internally deleted polyGln-free hAR or rat AR had greater transactivational potency than its respective normal version (containing 25- or 22-Gln tracts, respectively), and that a polyGln-expanded hAR had progressively less transactivational competence as n rose from 25 to 77. It is noteworthy, however, that their polyGln-free ARs also lacked some flanking amino acids. In that particular regard, our results supercede theirs.

The orthodox postulate for the pathogenesis of SBMA is that a polyGln-expanded AR loses something that it needs to sustain full androgen sensitivity of classical targets of androgen action, and gains something that is harmful to certain motor neurons. Our results are compatible with two different versions of the postulate in regard to its gain-of-function component: each version has possible pathogenetic relevance for the four other $(CAG)_n$-expanded neuronopathies. First, our data indicate that the loss of function in a polyGln-expanded AR (that is, reduced transcriptional activation of certain androgen target genes resulting in mild androgen insensitivity) may originate not from a decrease in the positive contribution of a polyGln tract to the transactivational potency of a hAR, but rather from an increase in its negative contribution to that ability (Fig. 2B). This loss of transactivational function of a polyGln-expanded AR might be mediated by an illegitimate or exaggerated ability to bind to another protein (10,11). This protein might be the same one (protein X) that conjugates strongly with a polyGln-expanded AR to yield a motor neuronopathic gain of function (Fig. 2D). In either case, the conjugate itself might be directly neuronopathic (Fig. 2D), or the process of conjugation might deplete the level of unbound protein X below a limit that is necessary for the normal lifespan of selected motor neurons.

In the second version, the neuronopathic gain of function would derive from the supposition that the normal AR partially represses the transcription of an androgen target gene whose product is necessary for normal longevity of certain motor neurons. If a polyGln-expanded AR super-represses such a target gene, its product may be chronically depleted to a level that is gradually neuronopathic (Fig. 2C). Notice that the propensity of polyGln-expanded proteins to conjugate with other proteins, or with themselves (10,11), would promote transcriptional repression (24). In fact, the same propensity might promote derepression of an androgen target gene that is normally repressed; however, this cannot be the neuronopathic mechanism because SBMA does not occur with traditional loss-of-function *AR* mutations, including gross gene deletions. Furthermore, a steroid receptor may exert a transcriptional repressive effect by protein−protein interaction even when it does not bind to a steroid response element (25,26). Hence, a polyGln-expanded AR could mediate super-repression of a target gene without necessarily binding to an androgen response element. Likewise, the normal products encoded at the HD, SCA1, DRPLA and MJD loci do not appear to be DNA-binding proteins, but they may still have transregulatory functions (1), and their mutant polyGln-expanded versions may become transcriptional super-repressors by virtue of their predisposition to illegitimate or excessive protein−protein interaction. According to this unifying hypothesis, the neuronopathic differences among the four diseases caused by these mutant proteins would stem from the identity of the interacting proteins, and/or of the genes that are consequently super-repressed.

Finally, androgens are known to be motor neuronotrophic in various experimental systems (27–29). Hence, we are obliged to entertain the hypothesis that polyGln expansion causes the AR to lose a motor neuronotrophic function (partly or completely), and that such a loss is a necessary, but insufficient, factor in the causation and/or severity of SBMA. Likewise, it is reasonable to consider whether SCA1, DRPLA or MJD may not be pure gain-of-function diseases. For HD this seems unlikely now that two homozygous individuals with undistinguished phenotypes have been reported (30). Obviously, to test this consideration rigorously we must know the identity and function of the products normally encoded at each of these loci.

MATERIALS AND METHODS

Construction of mutant expression vectors

These vectors were ultimately derived from pSV*hARo* (31). To improve it as a cloning vehicle, we modified it as follows: *Bst*B1 and *Hin*dIII in the 5′ polylinker, *Eco*RI in the 3′ polylinker, and the *Eag*I site in the pBR328 portion were eliminated; a *Xho*I site was created (32) by introducing two silent base changes at positions 2557–2562 (numbered as in ref. 33). We called the resultant vector pSVh*AR*.BHEXE. We used the overlap extension method (32) of PCR site-directed mutagenesis to construct the 0 and 12 Gln plasmids with Pfu DNA polymerase (Stratagene) and the following internal primers: 21QΔ21Q A (sense) (5′ GGCGCC AGTTTG CTGCTG CTGGAG ACTAGC CCCAGG CAGCAG-3′) and 21QΔ21Q B (antisense) (5′-CTGCTG CCTGGG GCTAGT CTCCAG CAGCAG CAAACT GGCGCC-3′) for the 0 Gln sequence, and 20E A (sense) (5′-GAGACT AGCCCC AGGCAG CAGCAG-3′) and 12Q B2 (antisense) (5′-CTGCTG CTGCCT GGGGCT AGTCTC TTGCTG CTGCTG CTGCTG CTGCTG CTGCTG CTGCTG CAGCAG CAGCAA ACTGGC GCCGGG-3′) for the 12 Gln sequence. Outside primers for both PCRs were: 1.1 A′ (sense) (5′-AGCCTG TTGAAC TCTTCT GAGC-3′) at the 5′ end, and 1.2 B′ (antisense) (5′-CTGCCT TACACA ACTCCT TGGC-3′) at the 3′ end. The PCR products and pCMVh*AR*.Gln40 and pCMVh*AR*.Gln50 (7) were *EagI*/*Bfr*I-restricted, and then were ligated into the *EagI*/*Bfr*I-digested pSVh*AR*.BHEXE. Correct sequence and expression of the vectors were verified by dideoxy sequencing and Western analysis (34).

Cotransfection

The cells were cotransfected as previously described (7), with the following modifications: (a) electroporation was performed at room temperature; (b) we used 0.1 μg normal or mutant pSVh*AR*.BHEXE, 20 μg pMMTVGH as the reporter, and 0.5 μg pCMV-βgal to monitor transfection efficiency; (c) the cells were shocked at 350 V/960 μF (time constant ~20 ms); and (d) medium containing MB was added 3–6 h after electroporation.

βgal, GH and androgen-binding assays

βgal activity was determined according to the method of Sambrook *et al.* (35). GH activity was assayed at 96 h using the Allegro Human Growth Hormone Kit (Nichols Institute, San Juan Capistrano, CA) according to the manufacturer's instructions with following modifications: we used 5–10 μl of sample medium, 150 μl H_2O, and 50 μl GH antibody. After correcting the GH values for the amount of protein present in each well and transfection efficiency, they were expressed as a percent of the mean GH activity of two *AR* expression vectors, each with 20 Gln, that were handled separately. Specific androgen-binding activity, maximum androgen-binding capacity, the apparent equilibrium dissociation constant (K_D), and the rate constant of dissociation (k) of various A−R complexes were determined on monolayer cultures as described previously (36) with the following modifications: (a) the plates containing the cells were transferred on ice and washed once with 20 mM Tris−HCl (pH 7.4) containing 0.15 M NaCl; the cells were washed again at room temperature for 7 min, and then lysed in plates by addition of 1 ml

0.5 M NaOH solution; (b) 50 µl and 0.7 ml of the lysate were used for Lowry protein assay in microplates and scintillation counting, respectively; and (c) MB-binding activity was expressed as the mean of results obtained 24 and 96 h after electroporation.

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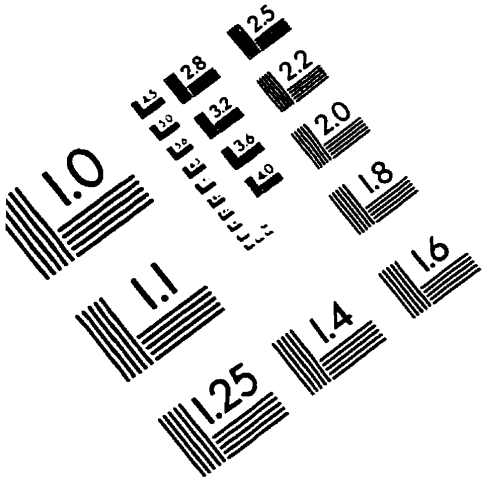
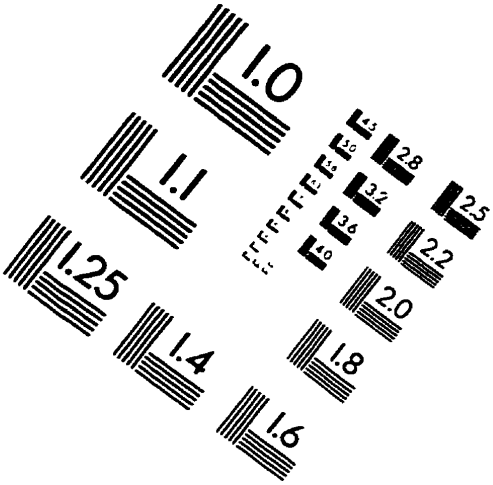
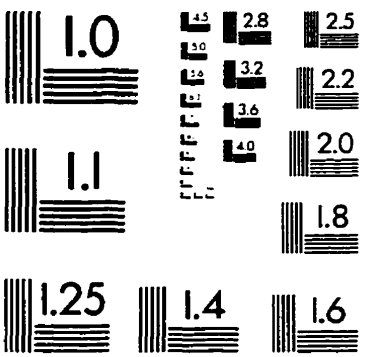
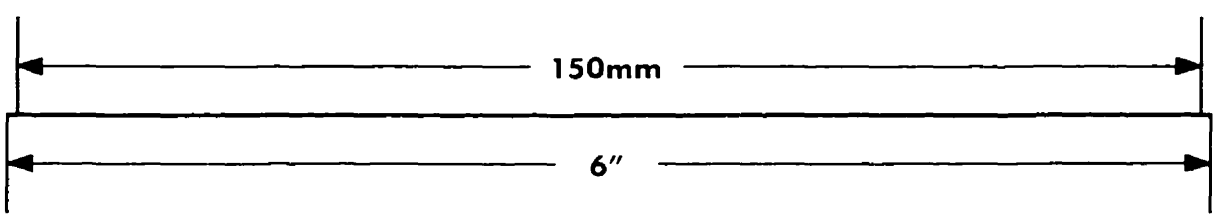
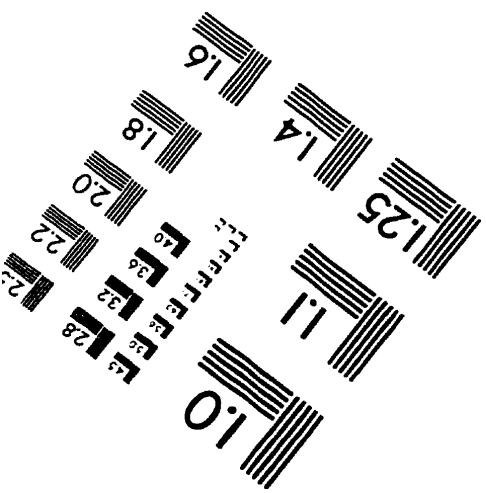
IMAGE EVALUATION TEST TARGET (QA-3)

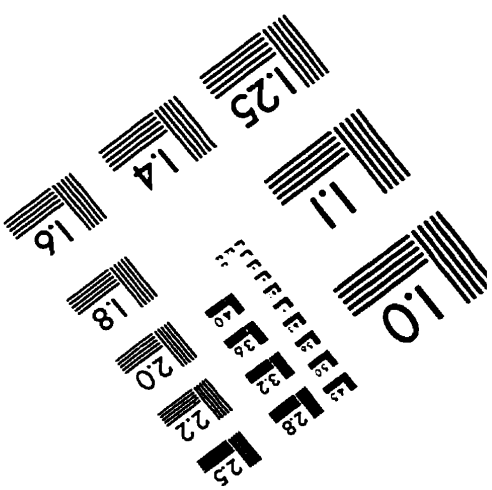












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