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CHARACTERIZATION OF FOUR POINT MUTATIONS IN THE ANDROGEN RECEPTOR GENE OF SUBJECTS WITH VARYING DEGREES OF ANDROGEN INSENSITIVITY SYNDROME

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July, 1995.

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science.

Dana Shkolny
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THESIS TITLE:

Characterization of four point mutations in the androgen receptor gene of subjects with varying degrees of androgen insensitivity syndrome

SHORTENED VERSION:

Characterization of four mutations involved in androgen insensitivity

This thesis carries a credit weight of 39 credits, from a total of 45 credits required for the Master's degree. Graduate credits are a measure of the time assigned to a given task in the graduate program. They are based on the consideration that a term of full-time graduate work is equivalent to 12 to 16 credits, depending on the intensity of the program.

This work is dedicated to the late Mrs. Marlene Franke
whose endless enthusiasm for biology was my inspiration.

ABSTRACT

This work proves the pathogenicity of four substitution mutations in the androgen-binding domain of the human androgen receptor (hAR) gene of four subjects with varying degrees of androgen insensitivity syndrome (AIS): complete (CAIS), partial (PAIS), or mild (MAIS). Of three unrelated CAIS subjects, two have Arg830Leu, the third has Arg830Gln. Their genital skin fibroblasts (GSF) have negligible androgen binding, but in overexpressing transfectants, the mutant androgen-binding activities have increased dissociation rates and decreased affinity for androgen. Owing to the instability of AR-androgen complexes, both mutants fail to transactivate a reporter gene. Glu771Ala and Arg870Gly caused PAIS and MAIS, respectively. Their normal levels of GSF androgen-binding activity have normal androgen affinity but increased dissociation rates. In transfectants, rates of dissociation resemble those in GSF, but the androgen affinities are questionably abnormal. Instability of Glu771Ala and Arg870Gly AR-androgen complexes caused subnormal transactivation of a reporter gene.

Résumé

Ce travail démontre la pathogénicité de quatre mutations punctuelles dans le domaine fixateur du récepteur humain d'androgène (hAR) de quatre individus souffrant des degrés divers du syndrome d'insensibilité aux androgènes (AIS): insensibilité complète (CAIS), partielle (PAIS), ou légère (MAIS). Chez trois individus CAIS, non apparentés, deux ont une substitution Arg830Leu et l'autre une Arg830Gln. Leurs fibroblastes de peau génitale (GSF) fixent à peine les androgènes, mais après transfection pour surexprimer les récepteurs mutants, on voit que la constante cinétique de dissociation et la constante de dissociation à l'équilibre sont suréleveés. A cause de l'instabilité des complexes androgène-AR, les deux mutants ne peuvent pas activer la transcription d'un gène reporter. Les mutations Glu771Ala et Arg870Gly causent respectivement le PAIS et le MAIS. Leurs GSF fixent normalement les androgènes, la constante de dissociation à l'équilibre est normale, mais la constante cinétique de dissociation est suréleveé. Dans des transfectants, les taux de dissociation sont comme dans les GSF, mais la constante de dissociation à l'équilibre semble souséleveé. L'instabilité des complexes androgène-AR Glu771Ala et Arg870Gly ne permet qu'une faible transactivation d'un gène reporter.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Leonard Pinsky, for his direction in this research project. I would also like to thank Drs. Mark Trifiro and Morris Kaufman for their advice and lively discussions that kept the lab atmosphere interesting and entertaining. Dr. Lenore Beitel, Carlos Alvarado, Parsa Kazemi-Esfarjani, Nelly Sabbagian, Abdullah Abdullah, Marie Vasiliou, Rose Lumbroso, Anand Mahtre, Annie Janvier, and Mark Benisz helped to maintain a progressive working environment, and I thank them for their friendship and their camaraderie. I also would like to thank Chia Chia Sun and Pardeep Kaurah for the PCR reactions of exon 1, and Rose Lumbroso for the sequencing of exon 1. Special thanks are extended to Carlos Alvarado, Nelly Sabbagian, Abdullah Abdullah and Dr. Roseline Godbout for encouragement when challenges seemed imsurmountable.

Finally, I would like to acknowledge my family for their patience, understanding and moral support.

LIST OF ABBREVIATIONS

AAD	Acidic Activating Domain
A-AR	Androgen-Androgen Receptor Complex
ABD	Androgen-Binding Domain
AIS	Androgen Insensitivity Syndrome
AMH	Anti-Müllerian Hormone
AR	Androgen Receptor
ARE	Androgen Response Element
β-gal	β-galactosidase
CAIS	Complete Androgen Insensitivity Syndrome
cDNA	complementary Deoxyribonucleic Acid
DBD	DNA-Binding Domain
DHT	5α-Dihydrotestosterone
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
E771A	Glutamic Acid-771-Alanine
ER	Estrogen Receptor
ERE	Estrogen Response Element
FBS	Fetal Bovine Serum
FSH	Follicle-Stimulating Hormone
GR	Glucocorticoid Receptor
GSF	Genital Skin Fibroblasts
hAR	human Androgen Receptor
HBD	Hormone Binding Domain
hHG	human Growth Hormone
HRE	Hormone Response Element
hsp	heat shock protein
k	Dissociation Rate Constant
kb	kilobase
K_d	Apparent Equilibrium Rate Constant
kDa	Kilodalton
LH	Lutenizing Hormone
LTR	Long Terminal Repeat
MAIS	Mild Androgen Insensitivity Syndrome
MB	Mibolerone

MMTV	Mouse Mammary Tumor Virus
MR	Mineralocorticoid Receptor
mRNA	messanger Ribonucleic Acid
NLS	Nuclear Localization Signal
OD	Optical Density
PAIS	Partial Androgen Insensitivity Syndrome
PCR	Polymerase Chain Reaction
PR	Progesterone Receptor
R^+	Receptor positive
R^-	Receptor negative
R^{def}	Receptor deficient
R830L	Arginine-830-Leucine
R830Q	Arginine-830-Glutamine
R870G	Arginine-870-Glycine
RAR	Retinoic Acid Receptor
RRE	Retinoic Acid Response Element
RXR	Retinoid X Receptor
SBMA	Spinal Bulbar Muscular Atrophy
SDS	Sodium Dodecyl Sulfate
SHBG	Sex Hormone Binding Globulin
SR	Steroid Receptor
SRY	Sex Determining Region of the Y Chromosome
SV40	Simian Virus 40
T	Testosterone
T_m	melting temperature
TR	Thyroid Receptor
TRE	Thyroid-Hormone Response Element
µF	Micro-Faraday
V	Volts
VDR	Vitamin D Receptor

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I. INTRODUCTION

For centuries, the processes of growth and development have been of inherent interest to those who study any aspect of biology. Now, the molecular mechanisms behind these processes are being uncovered. Growth and development require the appropriate cellular interpretation of an interplay of many regulatory molecules acting as morphogens. A complex cascade of gene products must be present in the proper temporal and spatial frame for cell types and tissues to be intricately arranged. A zygote must establish its polarity, which in turn contributes to axes and tissue patterns during embryogenesis. In the embryo, subcellular components, the local environment of surrounding cells, and the external environment, provide target cells with cues for the proper pathway for development. The appropriate developmental response relies not only on the appropriate presentation of signal molecules themselves, but also on the ability of the cell to respond to such signals. If there is a perturbation of the carefully orchestrated permutations of regulatory molecules, maldevelopment or disease is the consequence.

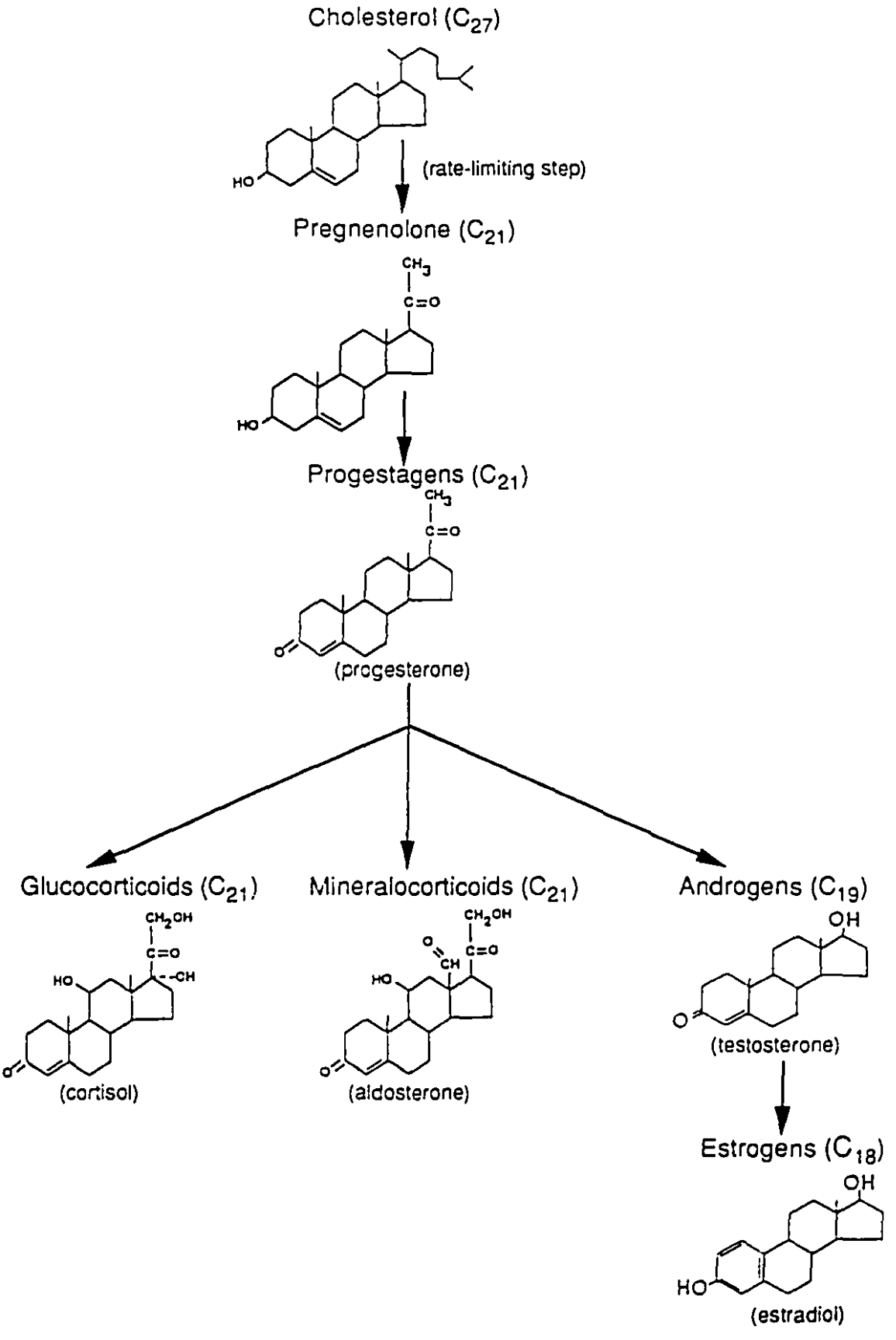
Steroid and thyroid hormones constitute a group of regulatory molecules that can exert their effect over a distance, being carried to the site of action by the circulatory system. These hormones function as important intracellular messengers that mediate physiological responses not only for growth and development, but for reproduction and overall homeostasis as well. For instance, retinoic acid is a well known limb morphogen in vertebrates; thyroid hormone plays a homeostatic role in metabolism; vitamin D plays a role in bone development and calcium metabolism; the adrenal steroid hormones are involved in general homeostasis and stress response; sex steroids are responsible for development of embryonic reproductive systems, secondary sexual characteristics, and have a role in reproduction in the adult.

Steroid hormones in animal cells arise from a common precursor, cholesterol, that can be made in the liver or obtained from the diet. Cholesterol can enter a pathway that results in the irreversible formation of the steroid hormones: glucocorticoids, mineralocorticoids and the sex hormones - androgens, progesterones, and estrogens (Figure 1).

Specialized intracellular receptor proteins in target cells mediate the action of steroid hormones (Jensen and Jacobson, 1962). Studies on the effects of ecdysone, a steroid hormone in insects, provided a clue to downstream events influenced by steroid hormones. In 1960, ecdysone was found to cause chromosomal 'puff' formation in polytene chromosomes of flies (Clever and Karlson, 1960), indicative of accumulation of

Figure 1. A Simplified Biosynthetic Pathway showing the Conversion of Cholesterol to Steroid Hormones.

The synthesis of steroid hormones from cholesterol showing the molecular structure of a representative in each group; C_n refers to the number of carbon atoms in each molecule. Note that the rate-limiting step in the conversion of cholesterol to steroid hormones is the formation of pregnenolone (adapted from Stryer, 1988a, p. 565).


Cholesterol (C_{27})
HO
(rate-limiting step)
Pregnenolone (C_{21})
CH_3
C=O
HO
Progestagens (C_{21})
CH_3
C=O
O
(progesterone)
Glucocorticoids (C_{21})
CH_2OH
C=O
HO
-CH
O
(cortisol)
Mineralocorticoids (C_{21})
CH_2OH
O
CH
C=O
HO
O
(aldosterone)
Androgens (C_{19})
OH
O
(testosterone)
Estrogens (C_{18})
OH
HO
(estradiol)

RNA and protein from transcriptionally active genes (Beermann, 1956). Jensen *et al.* (1968) and Gorski *et al.* (1968) proposed a "two-step mechanism" where steroid hormones combine with their receptors, and then regulate target genes. In the early 1970's, the use of radiolabeled ligands suggested the existence of these specialized receptors that bind to high affinity sites on the DNA (Jensen and DeSombre, 1972).

Steroid hormones are present in minute amounts in the circulation, so their corresponding receptors are highly sensitive and bind their ligands with high affinity [K_d are typically between 10^{-10} to 10^{-8} M (Landers and Spelsberg, 1992)]. Sex hormones characteristically circulate bound to sex-hormone-binding globulin, except for one to three percent that circulates as free hormone. The receptors for steroids (SRs) are present at approximately 10^4 to 10^5 molecules per cell (Landers and Spelsberg, 1992) and are tissue- and steroid-specific.

A. STEROID RECEPTOR SUPERFAMILY

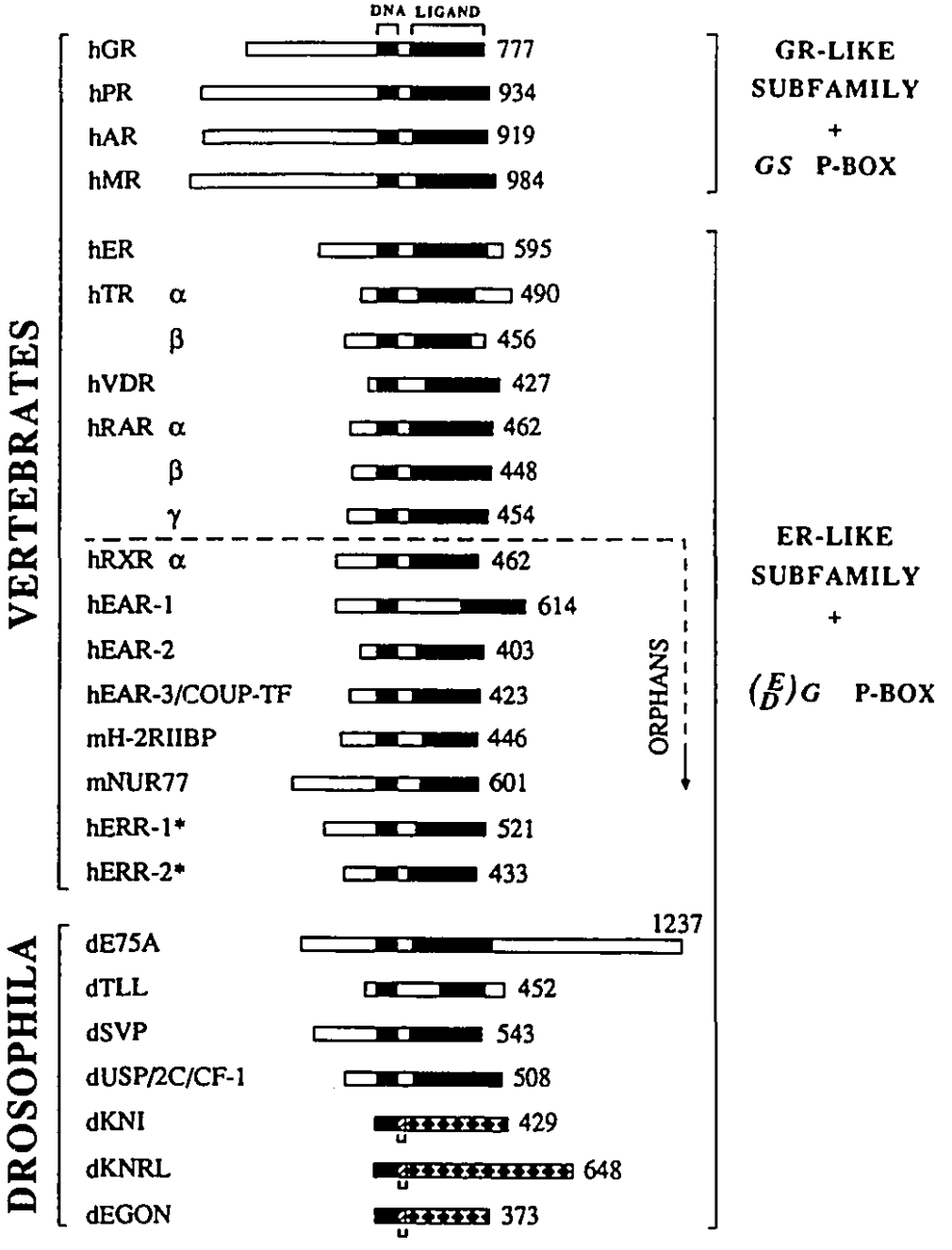
Based on their striking structural and functional homology, nuclear receptor proteins for steroid hormones (SR), thyroid hormones (TR), vitamin D (VDR), and retinoic acid (RAR) are grouped into a superfamily (Evans, 1988; Beato, 1989). Although their ligands are diverse, this superfamily of intracellular receptors controls a variety of a physiological functions through a common mode of action: ligand-initiated, DNA-binding mediated, transcriptional activation. Additional members in the superfamily have been isolated by virtue of their homology to the superfamily, but because their ligands remain unknown, they are termed 'orphan' receptors (Evans, 1988; O'Malley, 1990; Segraves, 1991).

The superfamily of SRs is thought to have arisen from a common ancestor gene more than 500 million years ago, before the divergence of arthropod and vertebrate lineages (Laudet *et al.*, 1992). Through evolution, the ancestral gene was duplicated, subsequently acquired different functions (Amero *et al.*, 1992; Laudet *et al.*, 1992; Evans, 1988; Picard *et al.*, 1990), and now is represented by several subfamilies of nuclear receptor proteins. SRs are divided into two subfamilies: the GR-like subfamily and the ER-like subfamily (Figure 2). Compared to other members of the superfamily of steroid hormone receptors, hormone receptors for androgen (AR), glucocorticoid (GR), mineralocorticoid (MR), and progesterone (PR) share closer amino acid homology, structure, and function.

1. Structure-Function Relationships

Figure 2. Linear Representation of Steroid Receptor Superfamily Members

Abbreviations (relevant here) of schematic representations are: hGR, hPR, hAR, hMR - human glucocorticoid, progesterone, and mineralocorticoid receptors, respectively; hER - human estrogen receptor; hTRα, hTRβ - human thyroid receptors; hVDR - vitamin D receptor; hRARα, hRARβ, hRARγ - human retinoic acid receptors; *Drosophila* gene products: dTLL, d-SVP, dUSP, dKNI, dKNRL correspond to *tailless*, *seven-up*, *ultraspiracle*, *knirps* and *knirps-related.* Numbers to the right of the schematics indicate the amino acid length of the receptors. (Wahli and Martinez, 1991, p. 2245). Note that the inclusion of *Drosophila* gene products in the superfamily indicate the existence of a common ancestral gene.


DNA
LIGAND
VERTEBRATES
DROSOPHILA
hGR
hPR
hAR
hMR
777
934
919
984
GR-LIKE
SUBFAMILY
+
GS P-BOX
hER
hTR α
β
hVDR
hRAR α
β
γ
595
490
456
427
462
448
454
hRXR α
hEAR-1
hEAR-2
hEAR-3/COUP-TF
mH-2RIIBP
mNUR77
hERR-1*
hERR-2*
462
614
403
423
446
601
521
433
ORPHANS
ER-LIKE
SUBFAMILY
+
(E/D)G P-BOX
dE75A
dTLL
dSVP
dUSP/2C/CF-1
dKNI
dKNRL
dEGON
1237
452
543
508
429
648
373

All receptors belonging to the superfamily have three discernable structure-function domains encoded by eight exons: a variable N-terminal or A/B region encoded by exon one, a DNA-binding domain (DBD) encoded in exons two and three, and a hormone-binding domain (HBD) encoded by exons four to eight. The modular arrangement of receptor domains was determined in experiments where the the DNA- and hormone-binding properties were separated by limited proteolysis (de Boer *et al.*, 1987; Evans, 1988) and in deletion experiments where receptors lacking the HBD were still able to bind DNA, and receptors deficient in the DBD were still capable of binding hormone (Kumar *et al.*, 1986; Hollenberg *et al.*, 1987). In their domain swapping experiments, Green and Chambon (1987) proved that domains are compartmentalized: when the DBD of the ER was exchanged for that of the GR, the resulting chimera regulated glucocorticoid-responsive genes upon addition of estradiol. Furthermore, attachment of HBD of SRs to unrelated proteins made them hormone responsive (Picard *et al.*, 1988). Although some functions of the receptor can be performed independently by its specific domain, like DNA binding by the DBD and ligand binding by the HBD, recent experiments have shown that other functions localize to more than one domain; dimerization, nuclear localization and transactivation are examples (Figure 3). Proper juxtaposition of all domains is required to create the three-dimensional structure necessary for normal receptor function.

The mode of SR action has been intensely studied in relation to the SRs specific domains. SRs are ligand-activated and thus remain inactive in the absence of hormone. The mechanism by which the receptor remains inert may involve the interaction of heat shock proteins (hsps) with the HBD. It is known that upon addition of hormone, the receptor undergoes an "activation" or "transformation" step, which may involve release of hsps from the HBD. Depending on the species of the receptor, it may be transported into the nucleus either pre- or post-activation via signals contained within conserved regions in the HBD. SRs homo- or hetero-dimerize through sequences embodied within the DBD and HBD, bind DNA at specific sites through their DBD, and influence transcription of target genes through the N-terminal region (Figure 4).

2. STEROID RECEPTOR DOMAINS

(a) The Hormone-Binding Domain (HBD)

The HBD is the second-best conserved and most C-terminal domain of the SRs. It spans about 250 amino acids (Jänne *et al.*, 1993) and is approximately 25 kilodaltons (kDa) in size (Green and Chambon, 1987). The conserved residues in the HBD among members

Figure 3. Structural and Functional Organization of Steroid Receptors.

Region A/B in this figure corresponds to the N-terminal region of the receptor, region C corresponds to the DNA-binding domain, region D to the hinge region (hatched box), and region E to the ligand-binding domain. Note that some functions of the receptor localize to more than one domain. (Beato, 1989 p. 336).

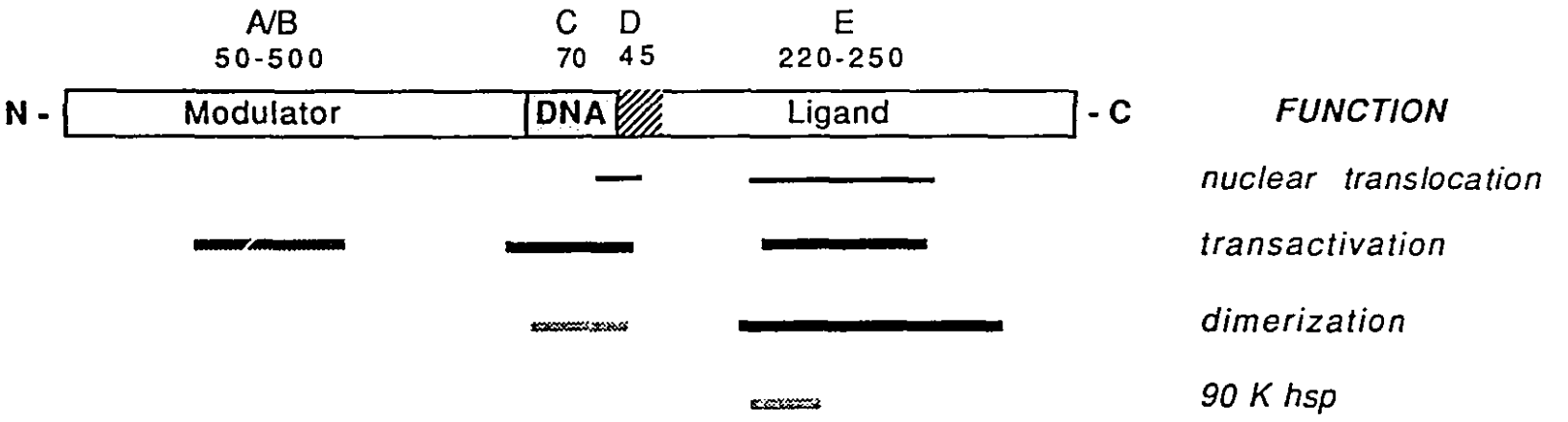

A/B
50-500
C
70
D
45
E
220-250
N -
Modulator
DNA
Ligand
- C
FUNCTION
nuclear translocation
transactivation
dimerization
90 K hsp

Figure 4. Steroid Receptor Action.

Before ligand binding, the DNA-binding and dimerization domains are concealed (horizontal and vertical stipled bars). After binding hormone, the receptor is transformed, dimerization occurs, and the homodimer acquires greater affinity for DNA. Binding at an steroid response element can then occur. This process influences transcription from the target gene located near the response element. (Pinsky *et al.*, 1992 p. 458).

of the subfamily are thought to form the structural components of the domain, and the non-conserved residues are thought to confer ligand specificity of the receptor. The HBD forms a specialized hydrophobic pocket into which the ligand binds, and mutational and chemical analyses have identified specific residues that participate in forming the pocket for ligand binding (Carlstedt-Duke *et al.*, 1988; Smith *et al.*, 1988).

It was postulated that the only function of the HBD was to "mask" the DBD and the transcriptional activation domain (Godowski *et al.*, 1987; Hollenberg *et al.*, 1987). However, Picard *et al.* (1988) found when they positioned the HBD of the GR to the N-terminus of the receptor, neither DNA-binding activity nor transcriptional regulation was affected, suggesting that the HBD functioned somewhat independently of the structure of the rest of the protein. Through the analysis of C-terminal deletions of the GR (Hollenberg *et al.*, 1987), PR (Carson *et al.*, 1987), and AR (Rundlett *et al.*, 1990; Simental *et al.*, 1991; Jenster *et al.*, 1992) it was found that receptors missing a significant C-terminal portion of the HBD (~200-300 amino acids) activated a reporter gene constitutively. Thus the HBD is thought to embody a repressor function for transcriptional activation.

It has been suggested by Hollenberg *et al.* (1987) that ligand-independent, constitutively active receptors could be involved in oncogenesis as has been implicated by a ligand-independent viral oncogene *v-erbA*, a cellular analogue of a thyroid receptor (Weinberger *et al.*, 1986; Zenke *et al.*, 1990). Mutations and internal deletions in the HBD of SRs causing resistance to ligand can also have clinical consequences due to deficient or defective transcriptional activation of target genes. Many point mutations and some small deletions have been documented within the HBD of the AR (amino acids 657 to 918) that cause poor response to androgen (Pinsky *et al.*, 1992). A point mutation in the HBD of the GR gene is responsible for familial glucocorticoid resistance (Hurley *et al.*, 1991).

Overall, the ligand-binding process facilitates receptor functions including transformation [discussed in (i) of this section], nuclear localization [(ii) of this section]; dimerization (Laudet *et al.*, 1992), DNA-binding (Smith and Toft, 1993; Veldscholte *et al.*, 1992a) [discussed in the DBD section]; and utimately transcriptional regulation of target genes (Klein-Hitpass *et al.*, 1991; Tsai *et al.*, 1990; Elliston *et al.*, 1990) [addressed in the section on the N-terminal region].

(i) Transformation

SRs undergo ligand-induced transformation, a conformational change that induces the receptor to adopt a DNA-binding state allowing other functional regions of the receptor to be exposed, or permitting other inactivation factors to be dissociated from the receptor. Before ligand-mediated transformation, SRs sediment in sucrose density gradients as a

heterocomplex of 8S to 10S[1] (Baulieu *et al.*, 1971) but sediment as 4S-6S[2] complexes after binding ligand, representing a dissociation of the heterocomplex (Pratt, 1992a). A conserved sequence in the HBD is speculated to bind a dimer of heat shock protein 90 (hsp90) (Denis and Gustafsson, 1989) in untransformed receptors (Schuh *et al.*, 1985; Catelli *et al.*, 1985; Housley *et al.*, 1985), but not in receptors induced to the DNA-binding state (Sanchez *et al.*, 1985; Mendel *et al.*, 1986; Sanchez *et al.*, 1987). Stabilization of the SR-hsp90 association inhibits the 8S to 4S transformation of SRs (Pratt, 1987); therefore, transformation was thought to be associated with hsp90 release (Catelli *et al.*, 1985; Denis *et al.*, 1988; Chambraud *et al.*, 1990). Moreover, steroid-inducible mutant SRs but not constitutively active mutant receptors are bound to hsp90 (Pratt *et al.*, 1988; Carson-Jurica *et al.*, 1989; Chambraud *et al.*, 1990; Cadepond *et al.*, 1991). Ligand-induced activation was discovered to be more than simply hsp removal; when the PR was isolated free of hsps, transcriptional activation remained hormone dependent (Bagchi *et al.*, 1991; DeMarzo *et al.*, 1991). Futhermore, limited protease digestion experiments of ER (Beekman *et al.*, 1993), PR (Allan *et al.*, 1992a), and AR (Kuil and Mulder, 1994) have shown that ligand binding, but not hsp90 removal (Allan *et al.*, 1992b), induces an allosteric change in the SR that results in more compact structure of the receptors. Several functions for the SR-hsp90 interaction have been proposed; it may increase competence for ligand binding while simultaneous masking domains of an active receptor (Bresnick *et al.*, 1989) possibly by blocking dimerization (DeMarzo *et al.*, 1991), it may provide protection against receptor proteolysis (Danielsen *et al.*, 1986), or it may facilitate nuclear transport of the receptor (Smith and Toft, 1993).

It has since been found that inactive receptors are "docked" to a large multimeric structure consisting of hsp90, hsp70, hsp 56-59[3], and p23 (Pratt *et al.*, 1992b). The role of hsp70 in receptor transformation is unclear as it has been isolated from both active and inactive PR receptors (Kost *et al.*, 1989; Estes *et al.*, 1987). By virtue of its unfoldase activity known to be involved in protein transport across membranes, hsp70 may have a role in transport of the SR across the nuclear membrane (Sanchez *et al.*, 1990a; Shi and Thomas, 1992). It is known that hsp56 is associated with inactive SRs and interacts directly with hsp90 (Renoir *et al.*, 1990). Hsp56 may have a role in protein trafficking in the cell as deduced from its microtubular localization, or have a role in protein folding, or both (Czar *et al.*, 1994). It was postulated that the hsp 'holo-complex' may function as a

---

[1] henceforth referred to as 8S for simplicity
[2] referred to as 4S
[3] referred to as hsp56

chaperone in protein folding (Weich *et al.*, 1992) or may act as a transport particle (Pratt, 1992).

(ii) Nuclear localization

In general, proteins can enter the nucleus by passive diffusion if they are less than 60kDa (kilodaltons; Peters 1986); larger proteins such as the SRs ranging in size from 48 kDa in the VDR to 107 kDa in the MR utilize a nuclear localization signal (NLS) to be actively transported into the nucleus. Active transport of SRs is indicated by failure of nuclear transport of the PR in the presence of energy synthesis inhibitors (Guiochon-Mantel *et al.*, 1991). It has been shown that nuclear translocation involves two distinct steps: translocation to the nuclear pore, and the energy-dependent translocation through the pore (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). The first step is assisted by NLSs and the second step may be assisted by hsp70 which contains an ATPase activity (Shi and Thomas, 1992).

Two NLSs were identified in the GR by *in vitro* mutagenesis (Picard and Yamamoto, 1987): one similar to the NLS of the SV40 large T-antigen (Kalderton *et al.*, 1984a; Kalderton *et al.*, 1984b) that is constitutively active (Figure 5); the other is hormone-induced. The consitutively active sequence of SRs is found in a small subdomain between the HBD and the DBD called the hinge region (Ylikomi *et al.*, 1992; Picard and Yamamoto, 1987). Deletion of the constitutive signal in the PR caused the receptors to remain cytoplasmic, but to become nuclear upon the addition of progesterone (Guiochon-Mantel *et al.*, 1989), suggesting that ligand binding may unmask the second NLS. The ligand-induced NLS for PR (Guiochon-Mantel, 1988) and GR (Picard and Yamamoto, 1987) is situated in the DBD and the HBD, respectively. Like the GR and PR, the AR has a bipartite nuclear localization signal. It is comprised of basic amino acids with an intervening spacer of ten amino acids: RKCYEAGMTLGARKLKK. Mutagenesis experiments show that disruption of either motif results in deficient transport of the receptor into the nucleus. Deletion or insertion of amino acids in the spacer region only partially disrupted nuclear transport, arguing against a strict structural requirement for the intervening sequence (Zhou *et al.*, 1994).

Originally, the intracellular localization of all SRs was thought to be cytoplasmic, becoming nuclear only after ligand binding (Gorski *et al.*, 1968). However, the development of better immunocytochemical techniques revealed that the ER (Welshons *et al.*, 1984; King and Greene, 1984), PR (Perrot-Applanat *et al.*, 1985; Elashry-Stowers *et al.*, 1988) and AR (Husmann *et al.*, 1990) are nuclear even in the absence of ligand. GR

Figure 5. Comparison of Nuclear Localization Signals of Various Steroid Receptors with SV40 Large T Antigen.

The postion of the first amino acid in the nuclear localization signal is indicated by the numbers listed; bracketed numbers refer to the distance from the first amino acid in the nuclear localization signal to last conserved cysteine in the DNA-binding domain. (Guichon-Mantel *et al.*, 1989, p.1152).

Protein	Species	Sequence
SV40 large T antigen		126 P K K K R K V
		P K K K R K V
Progesterone receptor	rabbit	638 (+10) R K F K K F N K
	human	637 (+10) · · · · · · · ·
	chicken	491 (+10) · · · · · L · ·
Glucocorticoid receptor	human	491 (+10) R K T K K K I K
	mouse	498 (+10) · · · · · · · ·
	rat	510 (+10) · · · · · · · ·
Androgen receptor	human	628 (+10) R K L K K L G N
	rat	612 (+10) · · · · · · · ·
Mineralocorticoid receptor	human	673 (+10) R K S K K L G K
Estrogen receptor	human	256 (+11) R K D R R G G R
	rat	261 (+11) · · · · · · · ·
	mouse	260 (+11) · · · · · · · ·
	chicken	250 (+11) · · · · · · · E
	Xenopus	251 (+11) · · · · · · · ·
Consensus sequence		R R R
		R K · · ·
		K K K

(Wikström *et al.*, 1987) and MR (Lombès *et al.*, 1990) were found to be cytoplasmic or both nuclear and cytoplamic in the absence of ligand. On the basis of the similar constitutive nuclear localization of the PR, it may be speculated that the AR also has a ligand-induced NLS in the DBD.

(b) The DNA-Binding Domain

The DNA-binding domain (DBD) consists of 66-68 amino acids and is the most conserved domain among members of the superfamily (Jänne, 1993). Originally, the DNA-binding specificity of the DBD was demonstrated by replacing this domain in the ER with the corresponding one of the GR; the chimeric receptor was able to activate glucocorticoid-responsive genes upon induction with estradiol (Green and Chambon, 1987). The DBD is responsible for the binding of the SR to hormone response elements (HREs), specific regulatory DNA sequences of responsive genes [discussed in (i) of this section]. Another portion of the DBD is partly responsible for forming a dimerization interface (Umesono and Evans, 1989) [discussed in (ii) of this section].

The DBD of SRs binds two ions of zinc (Zn^{++}) (Freedman *et al.*, 1988) in a zinc-(Zn-) finger-motif similar to those originally described in *Xenopus laevis* transcription factor TFIIIA (Miller *et al.*, 1985; Brown *et al.*, 1985). Further investigation showed that the Zn fingers of SRs and TFIIIA comprise two distinct classes based on their respective amino acid sequences and probable 3-dimensional structures (Berg, 1989): Zn^{++} is coordinated by a pair of cysteines and histidines in TFIIIA (Miller *et al.*, 1985), but is tetrahedrally coordinated by cysteines in the SRs (Lee *et al.*, 1989) (Figure 6a). Using NMR studies, the Zn fingers of the DBD of the GR (Härd *et al.*, 1990) and ER (Schwabe *et al.* 1990) were confirmed.

The Zn fingers of the SRs have the consensus sequence Cys-X_2-Cys-X_{13}-Cys-X_2-Cys-$X_{15\text{-}17}$-Cys-X_5-Cys-X_9-Cys-X_2-Cys-X_4-Cys, where X_n is the number of intervening amino acids (Berg, 1989). The more N-terminal finger (CI) contains several hydrophobic amino acids and four conserved cysteines, while the more C-terminal finger (CII) contains a higher number of basic amino acids than CI and has five conserved cysteines. The most distal cysteine, although strictly conserved, is not involved in coordinating zinc (Severne *et al.*, 1988). Through mutational analysis and deletion experiments, it was found that both Zn fingers are essential for DNA-binding activity of the SR (Danielsen *et al.*, 1987; Hollenberg *et al.*, 1987; Miesfeld *et al.*, 1987; Hollenberg and Evans, 1988), but are not sufficient on their own. CI and CII, encoded by a separate exons (Ponglikimongkol *et al.*, 1988), are each followed by an α-helical region and an extended region. CII stabilizes the

Figure 6a. Zinc-Finger Motifs in the DNA-Binding Domain of a Steroid Receptor.

The DNA-binding domain of the ER showing the two zinc-finger motifs and invariant cysteines that coordinate the zinc ion. The proximal (P) box interacts with the DNA and the distal (D) box is responsible for receptor dimerization. (Rhodes and Klug, 1993, p. 63).

Figure 6b. Schematic Model of the Protein-DNA Complex of Steroid Receptors.

Steroid receptors bind as dimers to specific palindromic sequences in DNA that constitute their response elements. Zinc ions are shown as black circles, and the C- and N- termini of each subunit are indicated. The orientations of subunits allow each recognition helix to be antiparallel and interact in the major groove of the DNA. (Schwabe and Rhodes, 1991, p.295)

entire domain, forms a dimerization interface called the "D" box (Umesono and Evans, 1989), and exposes an α-helix that is perpendicular to the α-helix following CI. CI exposes the α-helix of the SR protein that functions as a DNA-recognition unit and that interacts with bases of the major groove of the DNA (Figure 6b). Mutational analysis determined that three amino acids at the base of CI, the proximal (P) box, are critical for HRE-binding specificity (Green *et al.*, 1988; Mader *et al.*, 1989). In fact, the GR-like and ER-like subfamilies of steroid receptors can be distinguished on the basis of their P box sequence (Figure 7, Figure 2). Changing the Gly-Ser sequence between the distal pair of cysteines in CI of the GR to Glu-Gly, located at the homologous positions of the ER, rendered the chimeric receptor transcriptionally active through an estrogen response element (ERE) (Umesono and Evans, 1989; Danielsen *et al.*, 1989). The crystal structure of the GR DBD complexed with DNA showed that dimerization enables the subunits to contact adjacent major grooves in the DNA and X-ray crystallography showed the phosphates on either side of the major groove are the sites of interaction (Luisi *et al.*, 1991). Dimerization allows the recognition helix to fit into the major groove in the DNA and to form bonds with the base pairs of the HRE.

Mutations in the DBD affect SRs' ability to bind DNA or to discriminate appropriate half sites for DNA binding. This can cause deficient or inappropriate transcriptional activation of target genes. Single amino acid substitutions have been found in the DBD of the hAR that cause a total resistance to androgen *in vivo* and show lack of DNA-binding activity *in vitro* (Beitel *et al.*, 1994). A point mutation in the "P" box of the v-*erb*A oncogene alters the DNA recognition properties such that it binds inappropriate half sites (Smit-McBride *et al.*, 1994; Nelson *et al.*, 1994). Mutations in the Zn fingers have also been found to inhibit receptor function in the VDR, causing vitamin D-resistant rickets (Hughes *et al.*, 1988).

(i) Hormone Response Elements

HREs function as classical enhancer elements (Ptashne,1988). They operate in *cis* in either orientation at locations distant (but usually located upstream) from the transcription start site of target genes. The DNA sequence of HREs comprise dyadic half sites usually separated by 3 base pairs (bp); the palindromic organization is in accord with SRs binding to their HREs as dimers (Kumar and Chambon, 1988; Tsai *et al.*, 1988; Fawell *et al.*, 1990a). The consensus glucocorticoid response element (GRE), first described in the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Payvar *et al.*, 1981), is an imperfect palindrome of 15 bp (Scheidereit *et al.*, 1986; Jantzen *et al.*, 1987) of the

Figure 7. Comparison of P and D Boxes of Various Steroid Receptors.

The P box is responsible for hormone reponse element recognition, and is highly conserved within the GR-like and ER-like subfamilies. The D box is involved in receptor dimerization, and is less well conserved among superfamily members (Umesono and Evans, 1989, p.1142).

Receptor	P Box	D Box
GR subfamily		
GR, MR, PR	GSCKV	AGRND
AR	GSCKV	ASRND
ER/TR subfamily		
ER	EGCKA	PATNQ
TRα	EGCKG	KYDSC
TRβ	EGCKG	KYEGK
RARα, RARβ	EGCKG	HRDKN
VD_3R	EGCKG	PFNGD
NGFI-B	EGCKG	LANKD
TR-2	EGCKG	RGSKD
v-*erbA*	EGCKS	TYDGC
ear2	EGCKS	RSNRD
ear3	EGCKS	RANRN
knirps	EGCKS	KNEGK
knirps-related	EGCKS	KNNGE
ERR1	EACKA	PASNE
ERR2	EACKA	PATNE

sequence 5' GGTACANNNTGT(T/C)CT 3' (where N represents any of the four nucleotides). This sequence also confers response from the AR, MR, and PR (Cato *et al.*, 1986; Arriza *et al.*, 1987; Strähle *et al.*, 1987; Ham *et al.*, 1988). Although purified GR and PR yield the same exonuclease III footprints from the MMTV-LTR (von der Ahe *et al.*, 1985), each SR contacts different bases in the regulatory element suggesting that the molecular mechanism for HRE recognition may be different for each receptor (von der Ahe *et al.*, 1986). DNase I footprinting and methylation techniques showed a difference between the length of each of individual DNase I footprints and in the purine residues contacted by GR and PR in the HRE in the MMTV-LTR (Chalepakis *et al.*, 1988). The apparent disparity in the transactivational ability of different receptors at an identical HRE will be described in section 3.

HREs recognized by more divergent members of the superfamily differ from the GRE by sequence of the half sites, their orientation, or their spacing (Figure 8). For instance, the estrogen response element (ERE) binds at similar but not identical sequence of the GRE (Klock *et al.*, 1987; Ryfell *et al.*, 1989), recognition half-sites for the VDR can be directly repeated (Glass, 1994), and half-site spacers of 3, 4, and 5 bp specify transcriptional responses to vitamin D, thyroid hormone, and retinoic acid, respectively (Umesono *et al.*, 1991; Glass, 1994). It is known that negative response elements exist for the GR (nGRE; Figure 8). GRs binding at nGREs have been shown to inhibit regulation of gene transcription of the genes for proopiomelanocortin (Drouin *et al.*, 1987; Drouin *et al.*, 1989), prolactin (Camper *et al.*, 1985) and α-fetoprotein (Huang *et al.*, 1985) by blocking the binding of an essential transcription factor to an element neigbouring the nGRE.

The high level of expression from hormone responsive genes in the presence of hormone may result from synergistically acting HREs (Tsai *et al.* 1989; Schmid *et al.*, 1989); when one of a pair of HREs is deleted from the aminotransferase gene, its inducibility decreases greatly (Jantzen *et al.*, 1987). HREs also act synergistically with DNA binding elements of transcription factors CAAT box factor, NF-1, Sp-1 and octamer transcription factors (Schüle *et al.*, 1988; Strähle *et al.*, 1988; Schatt *et al.*, 1990). When these sites are placed in close proximity to HREs, stimulatory activity is increased.

Response elements do not always conform to the consensus HREs; thus SRs may have different intrinsic affinities for non-consensus HREs (Wahli and Martinez, 1991). For instance, AREs have been found upstream of the probasin (McQueen *et al.*, 1991; Rennie *et al.*, 1993), and prostate specific antigen (Roche *et al.*, 1992; Riegman et al., 1991) genes that confer response preferentially by the AR. Recent evidence suggests that

Figure 8. Consensus Response Elements for Nuclear Receptors.

Response elements for nuclear receptors differ in the sequence of their half sites and/or in the intervening nucleotide spacer. The consensus sequences for the half sites of the GRE, PRE, ARE, and MRE are identical with an nucleotide spacer of three; n may be any nucleotide. The ERE and retinoic acid response element (RRE) differ in nucleotide sequence and have no intervening spacer as indicated by the dashed lines. The negative GRE differs in nucleotide sequence from the positive GRE; Y and W indicate a base that is not conserved. (Beato, 1989, p. 336)

CONSENSUS RESPONSIVE ELEMENTS FOR NUCLEAR RECEPTORS

	1 2 3 4 5 6 10 11 12 13 14 15
1. GRE (+)	GGTACAnnnTGTTCT
2. PRE	"
3. ARE	"
4. MRE	"
5. ERE	AGGTCAnnnTGACCT
6. EcRE	AGGGTTnnnTGCACT
6. TRE	TCAGGTCA---TGACCTGA
7. RRE	"
8. GRE (-)	ATYACNnnnTGATCW
	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

the sequence surrounding the HRE may play a role in the differential use of the element by different hormone receptors (Adler *et al.*, 1992; Rennie *et al.*, 1993).

(ii) Receptor Dimerization

The palindromic arrangement of HREs provided a clue that SRs bind to DNA as dimers, and binding of receptors to HREs was confirmed by exonuclease III protection experiments (von der Ahe *et al.*, 1985). Stoichiometry has shown that two receptor molecules bind to one HRE (Tsai *et al.*, 1988; Wrange *et al.*, 1989) and DeMarzo *et al.* (1991) have suggested that dimerization is a critical factor in receptor recognition of their HREs. The GR-like subfamily of SRs bind to their HREs as homodimers, but the RAR, VDR, and TR preferentially bind their HREs as heterodimers with RXR (retinoid X receptors; Yu *et al.*, 1991; Marks *et al.*, 1992).

The dimerization domain of steroid/thyroid hormone receptors localizes to two regions: the D box of the DBD (Figure 7; Dahlman-Wright *et al.*, 1991; Luisi *et al.*, 1991), and a conserved heptad of hydrophobic residues in the HBD (Kumar and Chambon, 1988; Tsai *et al.*, 1988; Fawell *et al.* 1990a; Forman *et al.*, 1990b). Deletion mutants in the AR have shown that a dimerization region within the HBD may interact with a region outside the HBD in the other dimer component (Wong *et al.*, 1993).

The D box is important for determination of half-site spacing of HREs (Umesono and Evans, 1989) through dimerization. By generating chimeric molecules, it was determined that the D box of the TR is necessary for TRE activation and also for dimer formation (Hirst *et al.*, 1992). The DBD of the TR binds the TRE (no nucleotide spacer) as a dimer, but binds the ERE (3 bp nucleotide spacer) as a monomer. The reverse is true of the DBD of the ER, the difference due to steric hindrance introduced by the presence or absence of a nucleotide spacer in the HRE (Fawell *et al.*, 1990b). A mutation found within the D box of the AR impaired the receptor-DNA interaction by inhibiting the formation of active dimers (Kaspar *et al.*, 1993).

The colocalization of the HBD and a dimerization domain is coordinate with *in vivo* data that shows dimerization is promoted by hormone binding (Kumar and Chambon, 1988; Guiochon-Mantel *et al.*, 1989). Disruption of ER's conserved residues in the dimerization domain of the HBD decreased DNA binding (Fawell *et al.*, 1990a). The conserved sequences in the HBD have been implicated in the heterodimerization that occurs between the RXR and RAR, VDR, and TR (Glass *et al.*, 1989). The dimerization domain in the HBD has features in common with the leucine zipper (Landschulz *et al.*, 1988) and

helix-loop-helix motif (Murre *et al.*, 1989) reported to be involved in dimerization of other proteins (Kouzarides and Ziff, 1988; Landschulz *et al.*, 1988; Ransone *et al.*, 1989).

There is evidence that the binding of SRs to HREs is a cooperative event (Dahlman-Wright *et al.*, 1990; Tsai *et al.*, 1988). Binding of one PR dimer to the first of two tamdemly linked PRE/GREs increased the binding affinity of the second response element by 100-fold (Tsai *et al.* 1989) and resulted in their observed synergistic effects on transcription. Replacing the D box in the GR with the corresponding region of a TR abolished cooperative binding of GR monomers to an HRE (Dahlman-Wright *et al.*, 1991). The cooperativity may result from protein-protein contacts of the receptors or altered DNA conformation in the region, allowing the second dimer easier access to DNA.

(c) The N-terminal Region

The N-terminal domain or A/B region is the most variable in length and amino acid sequence, and consequently is the most immunogenic region of nuclear receptor proteins. Lengths range from 25 amino acids in the VDR to 600 amino acids in the MR (Jänne *et al.*, 1993). This structural divergence may be a reflection of the functional individuality of the receptors.

Hollenberg *et al.* (1987) and Miesfeld *et al.* (1987) used deletion mutants to demonstrate that the N-terminal region of the GR is necessary for activation from MMTV-LTR HREs. Other experiments have also shown that the N-terminal region embodies the transactivation functions of SRs (Tora *et al.*, 1988; Conneely *et al.*, 1989; Krust *et al.*, 1986; Kumar *et al.*, 1986; Jenster *et al.*, 1991; Simental *et al.*, 1991). Analysis of deletion mutants of the AR established that the presence of the N-terminal region blocks hormone-independent DNA binding (Wong *et al.*, 1994).

The N-terminal region of SRs contains a high amount of acidic amino acid residues (Danielsen *et al.*, 1987), the net negative charge of which permits the necessary protein-protein contact for transcriptional activation (Sigler, 1988). Similar acidic activation domains (AADs) were found to be responsible for the transactivation function of the yeast transcription factors GAL4 and GCN4. In GAL4 and GCN4, acidic residues interact with the pentapeptide repeated "tail" of RNA polymerase II to increase transcription initiation frequencies (Allison *et al.*, 1988). The AADs of SRs may also interact with a general component of the transcriptional initiation machinery, possibly with the TATA-binding protein TFIID or perhaps RNA polymerase II itself (Ptashne, 1988). Transcriptional activation could occur either by protein-protein interactions that favor a stable initiation

complex, or by modifying chromatin structure to allow DNA access for transcription machinery (Green and Chambon, 1987).

The N-terminus of the AR is distinct from other SRs in that it contains three polymorphic regions: a polyglutamine (poly-gln) stretch of 15-31 residues (Edwards *et al.*, 1992), a polyglycine stretch of 16-24 amino acids (Sleddens *et al.*, 1993) and a polyproline stretch of 8 amino acids (Faber *et al.*, 1989). AADs, and regions rich in proline and glutamine are thought to interact with other proteins (Mitchell and Tijan, 1989). Polyglutamine tracts have been found in transcription factors Sp1 (Courey *et al.*, 1988), Jun (Bohmann *et al.*, 1987), TFIID (Kao *et al.*, 1990) and the Drosophila gene *Antennapedia*. A proline-rich segment has been identified in many mammalian transcription factors including CTF/NF-1 (Mermod *et al*, 1989), AP-2 (Imagawa *et al.*, 1987), Jun (Strähl *et al.*, 1988), OCT-2 (Müller *et al.*, 1988) and SRF (Norman *et al.*, 1988).

(i) Transcriptional Regulation

Transactivation is thought to be controlled by the N-terminal region; however, there also is evidence that there is a transactivation function in the HBD. Deletion of the N-terminal domain in the GR (Miesfeld *et al.*, 1987; Hollenberg *et al.*, 1987; Danielsen *et al.*, 1987; O'Malley, 1990), PR (O'Malley, 1990; Gronemeyer *et al.*, 1987), and ER (Kumar *et al.*, 1987), decreases but does not abolish transcriptional activation. Webster *et al.* (1988) localized a transcriptional activation function in the HBDs of ER and GR using chimeric receptors consisting of the DBD of GAL-4 and the HBD of ER or GR. Transcriptional response from a GAL-4 promoter was hormone dependent, indicating that a transactivating function was indeed present in the HBD.

Two AADs were found in the GR: one in the N-terminus designated τ1, and one in the C-terminus that localizes between the DBD and the HBD (the hinge region) designated τ2 (Hollenberg and Evans, 1988). In the ER, two hormone inducible transcriptional functions (TAF1 and TAF2) were identified that are nonacidic in nature and thus are distinct from τ1 and τ2 (Tora *et al.*, 1989) suggesting that they activate transcription in mechanically different ways (Tasset *et al.*, 1990). Using transient transfection studies with hER mutants, Tzukerman *et al.* (1994) found that function of TAF1 or TAF2 in the hER depends on promoter context, and experiments showed that TAF1 may be the major transcriptional activatior and that TAF2 may function as a transcriptional facilitator.

Interestingly, C-terminal truncations and internal deletions of SRs have revealed that the unoccupied HBD contains a transactivational repressor function. Receptors with

C-terminal deletions have been isolated with up to wild-type transcriptional activity in the absence of hormone (Giguére *et al.*, 1986; Godowski *et al.*, 1987; Danielsen *et al.*, 1987; Miesfeld *et al.*, 1987; Bocquel *et al.*, 1989). When the HBD of the GR was fused to an unrelated protein, E1A, the resulting chimeric protein was hormone-inducible. This confirmed that the HBD confers both repression and steroid-mediated derepression of transactivational function (Picard *et al.*, 1988).

3. Discrimination of Transcriptional Response by SRs

The structural and functional similarity of the GR-like subfamily of SRs bestow a degree of overlap in transcriptional response. What is the mechanism of discrimination of transcriptional response employed by various SRs that can act upon an identical HRE? The pattern of gene transcription may be achieved, either positively or negatively, through the interaction of *trans*-acting proteins with *cis*-acting DNA promoter elements (Ptashne, 1986).

The cellular context of the SR is a strong determinant of SRs' transcriptional activation. Where SRs have the ability to recognize identical HREs, tissue-specific expression of SRs can prevent transactivational promiscuity. This is illustrated by an experiment using rat hepatoma cells that normally contain only GR. When PR is transiently transfected into these cells there is PR-directed induction of glucocorticoid-responsive genes (Strähle *et al.*, 1989).

Each cell type has a uniquely organized chromatin structure and consequently, a different assortment of response elements may be accessible for hormone-receptor complexes to bind (Evans, 1988). Aside from physical availability of HRE sites, there is evidence that HRE sequence itself is not the sole determinant of positive and negative transcriptional regulation (Mordacq and Linzer, 1989). Transcriptional regulation may function through cooperative interaction with other appropriate transcription factors (Day *et al.*, 1990; Rhodes *et al.*, 1993) limiting transcriptional response to cells with these factors. *In vitro*, a composite GRE found upstream of the mouse proliferin gene can confer positive and negative glucocorticoid regulation depending on cellular factors. This composite GRE has overlapping binding sites for both SRs and the AP-1 subunits, c-Jun and c-Fos (Diamond *et al.*, 1990). Pearce and Yamamoto (1993) identified a difference between GR and MR action on a composite GRE; when equal amounts of c-Jun and c-Fos were present, repression of expression was maintained with both receptors. When the amount of c-Jun was increased, MR failed to repress transcriptional activation but GR did not. Other

experiments have shown that transcriptional activation from a GRE can occur in some cellular contexts but not others (Diamond *et al.*, 1990; Yoshinaga and Yamamoto, 1991)

Aside from cell-specfic nonreceptor proteins, the receptors themelves may differ in their structure or modifications. Gronemeyer *et al.* (1987) showed that there are two forms of PR: A and B, where A corresponds to a N-terminally truncated version of B. Tora *et al.* (1988) reported that there is functional difference between A and B forms when comparing the transcriptional activation from an MMTV- or ovalbumin-linked reporter gene. Post-translational modifications may occur in some SRs. Hypo- or hyper-phosphorylation in different regions of SRs may provide a mechanism for transcriptional regulation of certain genes (Kuiper *et al.*, 1993) by increasing the negative charge and acidity of a region (Kuiper and Brinkmann, 1994). Phosphorylation is known to play an important role in PR activation (Denner *et al.*, 1990).

B. Human Sexual Development

Three sequential events are required for sexual differentiation of the embryo: establishment of genetic sex, gonadal sex, and phenotypic sex (Griffin and Wilson, 1980). Genetic sex is defined by the sex chromosome harboured by the fertilizing spermatozoa, resulting in a homogametic (XX; female) or heterogametic (XY; male) combination in the zygote. The principal genetic determinant on the Y chromosome is the *SRY* gene (sex-determining region of the Y chromosome), and the presence or absence of its corresponding gene product determines gonadal sex. The *SRY* gene product initiates undifferentiated gonads to develop into testes and its absence results in establishment of ovaries (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). This is coordinate with the presence of *SRY* in XX males (Abbas *et al.*, 1993) and its absence or mutation in XY females in humans (Jager *et al.*, 1990; Muller *et al.*, 1992). In an experiment with transgenic mice, *SRY* was able to direct testes development in chromosomally female mouse embyos (Koopman *et al.*, 1991). The last event in embryonic sexual differentiation, induced by hormones secreted from ovaries or testes, is phenotypic sex determination. Phenotypic sex of the embryo normally conforms to the genetic sex.

For two months of gestation, human embryos develop identically regardless of genetic sex (Wilson *et al.*, 1981). The internal genitalia of males and females develop from two different primordial structures both present in the embryo: the Wolffian and Müllarian ducts, respectively. In the male, Wolffian ducts develop into epididymis, vas deferens, the ejaculatory duct, and the seminal vesicle, while the Müllarian ducts regress. In the female, Müllarian ducts develop into fallopian tubes, the uterus, and the vagina, while the Wolffian

ducts regress (Figure 9). In contrast to the internal genitalia that orginate from different embryonic structures, the external genitalia originate from bipotential structures. The genital tubercle and urogenital swellings develop into the penis glans and scrotum in the male, or into the clitoris and labia majora in the female (Figure 10).

1. Male Sexual Development

The development of the testes from the embryonic undifferentiated gonad, brought about by the expression of *SRY*, is the paramount event in male sexual development. Once established, the testes begin to secrete androgens. Jost (1972) deduced that hormonal signals from the testes were directing male sexual development by observing that castrated rabbits developed as phenotypic females. Two hormones secreted from the fetal testes are testosterone (T) and a glycoprotein, anti-Müllarian hormone (AMH). T is secreted from the Leydig cells of the testes and directs the differentiation of the Wolffian ducts, while AMH, secreted by the fetal Sertoli cells of the testes induces regression of the Müllarian ducts (Josso *et al.*, 1991). The cellular response to T causes the upper portion of the Wolffian ducts to connect to the testes to form the epididymis, the mid-portion to develop into the vas deferens and the terminal portion to form the ejaculatory duct and seminal vesicle. At this time, T can be converted to another androgen, 5α-dihydrotestosterone (DHT), (Figure 11) in the urogenital sinus and urogenital tubercles (Siiteri *et al.*, 1974) by the action of the 5α-reductase enzyme. DHT is responsible for the differentiation of the prostate and external genitalia (Griffin and Wilson, 1989). The anatomical development of the external and internal genitalia of the embryo is mostly completed by the end of the first trimester of gestation.

At puberty, androgens are responsible for development of secondary male sexual characteristics and for initiation and maintenance of spermatogenesis (Griffin and Wilson, 1980). The effects of T can be inferred from males with 5α-reductase defeciency; T is responsible for vocal cord thickening, upper skeletal muscularity, spermatogenesis and libido. DHT induces the maturation of external genitalia, growth of facial and sexual hair and, later in life, recession of temporal hair.

The principal androgen in the circulation is T, which is under control of lutenizing hormone (LH) via the pituitary. The androgen testosterone (T) is synthesized via five enzymatic reactions where the rate-limiting step is the conversion of pregnenolone from cholesterol (Figure 1). In the adult male, approximately 6 mg of testosterone is synthesized daily in response to LH. Most of the circulating testosterone is bound to sex hormone-binding globulin (SHBG), leaving only 2-3% to circulate in the free form. T can function

Figure 9. Formation of the Internal Genital Tract of Male and Female Embryos. (Wilson *et al.*, 1981 p.4)

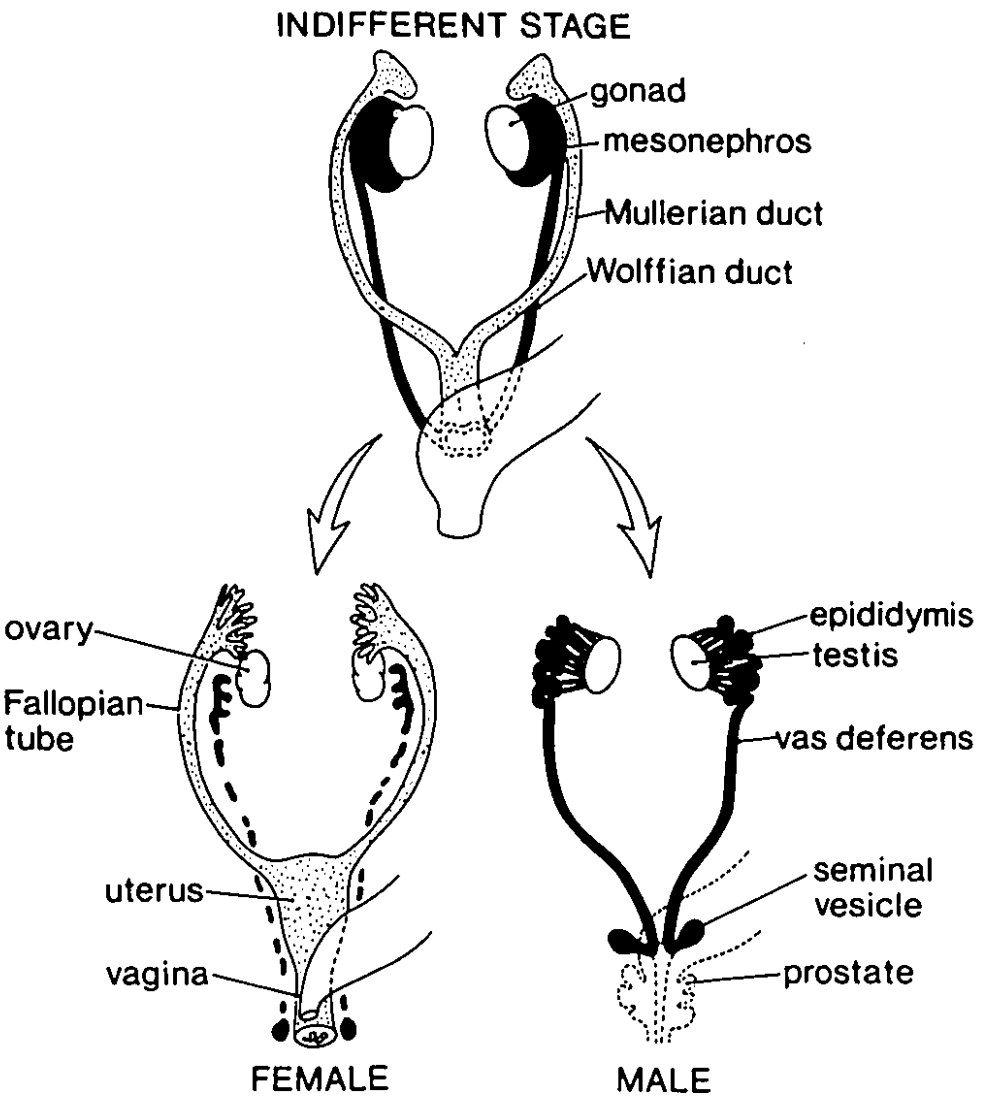

INDIFFERENT STAGE
gonad
mesonephros
Mullerian duct
Wolffian duct
ovary
Fallopian tube
uterus
vagina
epididymis
testis
vas deferens
seminal vesicle
prostate
FEMALE
MALE

Figure 10. Formation of the External Genital Tract of Male and Female Embryos. (Wilson *et al.*, 1981 p.5)

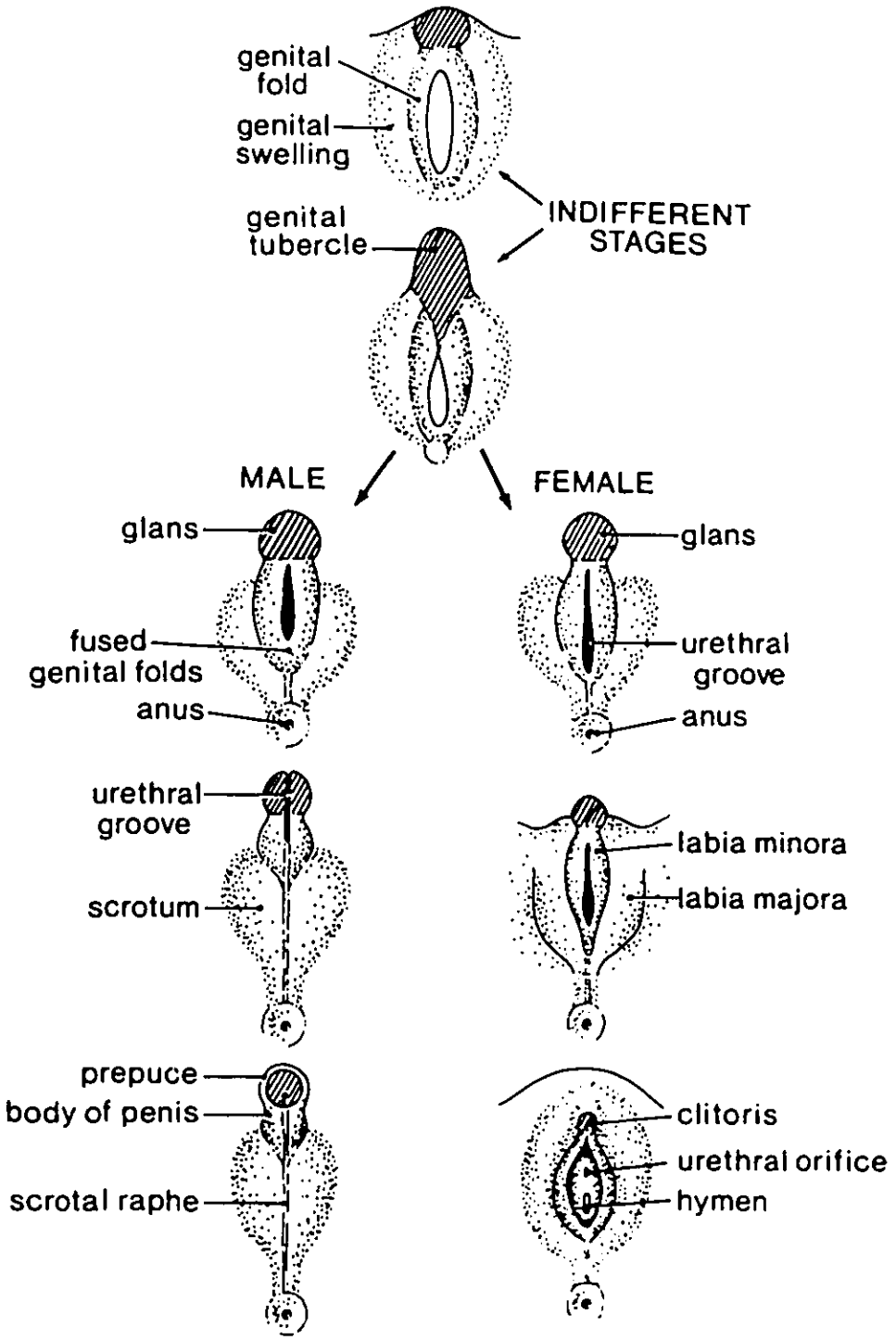
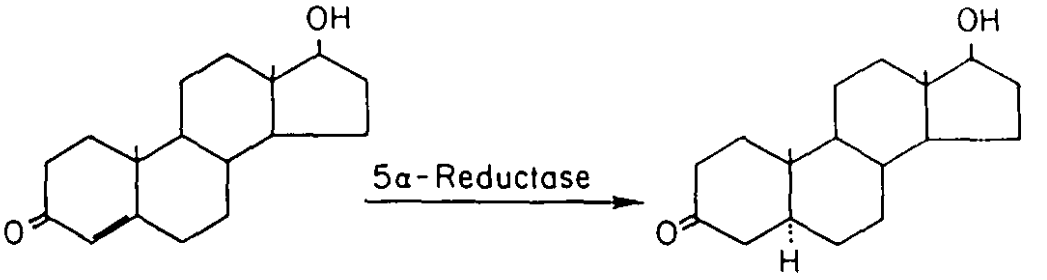

genital fold
genital swelling
INDIFFERENT STAGES
genital tubercle
MALE
FEMALE
glans
fused genital folds
anus
glans
urethral groove
anus
urethral groove
scrotum
labia minora
labia majora
prepuce
body of penis
scrotal raphe
clitoris
urethral orifice
hymen

Figure 11. Formation of 5α-dihydrotestosterone from Testosterone by the Action of the 5α-reductase Enzyme. (Wilson *et al.*, 1981 p.9)



TESTOSTERONE

DIHYDROTESTOSTERONE

as a hormone, or as a prohormone before conversion to DHT or estrogen in peripheral tissues.

(a) Aberrant Male Sexual Development

Defects in the sexual development of the embryo can manifest themselves in phenotypic variations pre- or post-natally, or may even be apparently absent until adulthood. Phenotypic variations can be accounted for by inappropriate levels of androgen or inadequate tissue response to androgens.

A defect caused by inappropriate levels of androgen is represented by an autosomal recessive disorder formerly known as pseudovaginal perineoscrotal hypospadias. Here, 46,XY individuals are unable to synthesize DHT because of a deficiency of 5α-reductase, and they develop predominantly as phenotypic females. Since DHT is responsible for differentiation of the male external genitalia, it is not surprising that these individuals have characteristically female external genitalia, while the internal structures, which are dependent on T, are male. Subjects are characterized by inguinal testes, epididyme, vasa deferentia, seminal vesicles, and ejaculatory ducts that empty into a vagina. Increased T levels at puberty are sometimes sufficient enough to cause some of these individuals to experience external genital masculinization (Imperato-McGinley *et al.*, 1974).

Inadequate response to androgens in 46,XY individuals is commonly due to defects in the AR (Griffin and Wilson, 1989). Deficient or defective AR protein causes a lack of androgen response in target organs and manifests as an array of clinical phenotypes collectively called androgen insensitivity syndromes (AIS) (French *et al.*, 1966). Affected individuals can be classified into one of three types: complete androgen insensitivity (CAIS), partial androgen insensitivity (PAIS) or mild androgen insensitivity (MAIS). There is a high rate of ascertainment of diagnosis of these syndromes due to the presence of anatomical or functional abnormalities of genitalia; individuals with AIS are usually infertile.

2. CAIS, PAIS, and MAIS - Clinical Phenotypes

CAIS, also known as testicular feminization (Morris, 1953), has an incidence of 1/20 000 to 1/64 000 male births (Griffin and Wilson, 1989). Subjects are characterized by a 46,XY karyotype with unambiguous female external genitalia. Normally, a prepubertal inguinal hernia or the postpubertal symptom of primary amenorrhea prompts individuals to seek a clinical diagnosis. Closer examination of these subjects shows an absence of Müllerian duct derivatives, normal size testes in inguinal or labia majora areas, and a short

blind-ending vagina. Later, testicular tissue of these individuals has a propensity to become malignant; gonadectomy is performed and replacement female sex hormones are then administered to onset puberty and/or maintain a feminizing effect. At the expected time of puberty, these individuals have normal breast development and a subcutaneous fat distribution that is typically female. Pubic or axillary hair, which is androgen dependent, may be sparse or absent (Kaufman *et al.*, 1976).

PAIS, also called Reifenstein syndrome, is about one tenth as common as CAIS (Griffin and Wilson, 1989). Affected 46,XY individuals are characterized by having external genitalia that are not distinctly male nor female; consequently, this type of AIS has the widest spectrum of phenotypes. Abnormalities may range from isolated clitoromegaly with rugosity of the labial folds which may be partially fused, to severe perineoscrotal hypospadias (penile malformation where the urethral opening is located ventrally, not at the tip of the penis). Müllerian duct derivatives are absent, but Wolffian ducts are present. Severely affected individuals may have underdeveloped epididymides, vas deferentia, seminal vesicles, and ejaculatory ducts that empty into a vagina. Incomplete virilization at puberty may be accompanied by gynecomastia. Axillary and pubic hair are normal but facial and chest hair tend to be scant. Within the same family, affected individuals may rarely exhibit phenotypes with considerable variation (Wilson *et al.*, 1974). Normally the clinical administration of androgen in PAIS subjects does not yield further virilization and is not usually recommended due to side effects. In one case, family history suggested that androgen therapy may work, and with parental insistance to raise the child as a male, high dose androgen therapy was administered with enough success to allow reconstructive surgery (Grino *et al.*, 1989). The fact that PAIS subjects have some undergone degree of virilization indicates that the subject's AR has residual function.

An unambiguously male phenotype with some degree of undervirilization, with or without some degree of infertility, is characteristic of MAIS. The incidence of MAIS is unknown but may account for a proportion of male infertility resulting from azoospermia or oligospermia. These individuals may present with mild hypospadias, normally formed but small phallus, or a male phenotype accompanied by sperm deficiency. Some individuals may have gynecomastia resulting from increased testosterone levels and its conversion to estrogen. Some subjects are undervirilized and have gynecomastia, but are fertile (Grino *et al.*, 1988). In one subject with MAIS, administration of pharmacologic doses of androgen improved virilization: facial and pubic hair development, lowered voice pitch (McPhaul *et al.*, 1991). In another MAIS subject, androgen was successfully administered to correct infertility (Yong *et al.*, 1994).

CAIS, PAIS, and MAIS subjects classically present with high plasma LH levels despite high plasma concentrations of T (Boyar *et al.*, 1978). This indicates inability of the hypothalamic-pituitary axis to respond to circulating androgens in the feedback regulation of LH secretion (Faiman and Winter, 1974).

3. CAIS, PAIS, and MAIS - Biochemical Phenotypes

Before the cloning of the AR gene, the diagnosis of AIS relied on the clinical phenotype, the ratios of circulating hormones (Hughes and Evans, 1988; Griffin and Wilson, 1989) and androgen-binding studies on patients' genital skin fibroblasts (GSF; Pinsky and Kaufman, 1987). GSF were studied because they have approximately three times more androgen-binding activity than nongenital skin fibroblasts (Kaufman *et al.*, 1976; Kaufman *et al.*, 1977). Androgen-binding levels in GSF are determined using tritiated androgens. There are three categories of receptor status based on maximal binding capacity of the patients' GSF: receptor-negative (R^-) when maximal androgen-binding is $\leq$ 5 fmol bound hormone/mg protein; receptor-deficient (R^{def}) when it is between 5-15 fmol bound hormone/mg protein; and receptor-positive (R^+) when levels are between 15-40 fmol bound hormone/mg protein (normal levels).

Subjects with varying degrees of AIS may have quantitatively or qualitatively defective androgen binding that is not distinctive to any one particular type of AIS. In 1976, Amerhein *et al.* reported that CAIS subjects can have GSF that are classified as R^+ or R^-. Where GSF of CAIS subjects are classified as R^+, there is a qualitative defect in the receptor such as increased thermolability of the AR when complexed to androgen (A-AR; Griffin, 1979), low affinity for androgens (Kaufman *et al.*, 1979) or defective upregulation of AR protein (Kaufman *et al.*, 1981). AR defects in GSF of PAIS subjects have been related to qualitative defects in the AR such as thermolability of A-AR (Grino *et al.*, 1989) which may be ligand-specific (Kaufman *et al.*, 1986). Other PAIS subjects had lower affinity for androgen with faster dissociation of A-AR (Kaufman *et al.*, 1984), or the inability to upregulate androgen receptor protein in the prolonged presence of ligand (Pinsky *et al.*, 1985). Some MAIS subjects had androgen-binding defects including defective upregulation of receptor levels, and increased dissociation rates of A-AR (Grino *et al.*, 1988). In phenotypically normal men (Pinsky *et al.*, 1989) with idiopathic azoospermia or oligospermia (Aiman and Griffin, 1982), low androgen-binding affinity in subjects' GSF was found.

4. Molecular Biology of the Androgen Receptor and its Gene

The human androgen receptor protein (hAR) is an androgen-dependent, DNA-binding, transcription factor that influences expression of target genes (Brinkmann *et al.*, 1989; Simental *et al.*,1991; Jenster *et al.*, 1992). The hAR gene is located on the X chromosome (Lubahn *et al.*, 1988a) at position q11-12 (Brown *et al.*, 1989). The gene spans more that 90kb of genomic DNA but only 3% (Kuiper *et al.*, 1989) is translated into a protein of 110 kDa that has a calculated molecular weight of 98 500 Da (Brinkmann *et al.*, 1992a). The hAR has been described to consist of 917 (Brinkmann *et al.*, 1989), 918 (Chang *et al.*, 1988), or 919 (Lubahn *et al.*, 1988b) amino acids[4]. This apparent discrepency in amino acid length is due to polymorphic regions in the poly-gln and/or poly-glycine stretches encoded by exon one (as mentioned in the section on the N-terminal region). These polymorphisms in the AR have been used successfully as a marker in prenatal diagnosis for two high-risk families with CAIS or PAIS (Lobaccaro, 1992).

Encoded by 2730 nucleotides and 8 exons, the hAR has 3 main functional domains like other members of the steroid receptor subfamily (Figure 3). Exon 1 exclusively encodes the amino-terminal (N-terminal) domain (Faber *et al.*, 1989) which is responsible for transactivation of the hAR (Jenster *et al.*, 1991; Simental *et al.*, 1991). The DNA-binding domain (DBD) is encoded by exons 2-3, and the androgen-binding domain (ABD) is encoded by exons 4-8.

The promoter region of hAR gene does not contain a TATA or CAAT box. It does contain a GC box which binds Sp1, a long homopurine stretch (Faber *et al.*, 1993), and a potential cAMP response element (Mizokami *et al.*, 1994). Recently, Grossmann *et al.* (1994a) characterized a second functional promoter in the mouse AR gene, 3' to the previously characterized promoter. It does not possess a TATA or CAAT box, but does contain a GC box, an AP2 binding site, and a PU box. Two different AR transcripts of eight and eleven kb have been isolated; the difference is due to differential splicing within the 3' untranslated region (Tilley *et al.*, 1990; Faber *et al.*, 1991) the significance of which is unknown. Regions in the 5' untranslated portion of the mouse AR have a negative regulatory function on transcription. These data suggest there are protein-DNA contacts within this region that may act by halting transcription elongation machinery (Grossmann *et al.*, 1994b). Other evidence shows that the 5' untranslated region of the hAR may play an essential role in induction of AR translation (Mizokami and Chang, 1994).

The effect of androgen at the level of androgen receptor gene expression remains somewhat controversial. The expression of the hAR gene is thought to be androgen-

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[4] amino acid number designations are consistent throughout this thesis and conform to Chang *et al.*, 1988.

regulated at the level of transcription initation. Upon addition of androgen, mRNA levels decrease drastically in androgen target tissues (Quarmby *et al.*, 1990). LNCaP cells (a prostate tumor cell line) had a decrease in AR mRNA in the presence of androgen but normal GSF did not (Wolf et al., 1993). In the rat prostate, determination of AR expression with use of *in situ* hybridization and immunohistochemical procedures confirmed that AR decreased at the protein and mRNA level upon androgen-withdrawl (Takeda *et al.*, 1991). Another study showed that there was an increase in AR mRNA in rat prostate after castration, and suppression of receptor mRNA after restimulation (Tan *et al.*, 1988). Still others have found upregulation at the protein level upon hormone addition (Kaufman *et al.*, 1981; Rossini, 1991).

5. Molecular Genetics of AIS due to *AR* Mutation

CAIS is predominantly caused by point mutations affecting premature protein termination (Zoppi *et al* 1993, Marcelli *et al* 1990a, Marcelli *et al* 1990b, Sai *et al.*, 1990, Trifiro *et al.*, 1991), or the integrity of the DBD or the HBD. When the truncated receptors were studied, the abnormal biochemical phenotype was due to reduced amount of truncated protein, as well as functional impairment of the receptor. In the DBD, a mutation of a conserved cysteine in either zinc finger of the AR and resulted in CAIS (Zoppi *et al.*, 1992), as did other point mutations in the DBD (Marcelli *et al.* 1991a; Mowszowicz *et al.*, 1993). These defective ARs were unable to activate a reporter gene due to their inability to bind DNA. Many point mutations have been found in the AR's HBD that caused functional impairment of the receptor: decreased androgen-binding activity (De Bellis *et al.*, 1992; McPhaul *et al.*, 1992; De Bellis *et al.*, 1992; Shkolny *et al.*, 1995), decreased AR affinity for androgen (Lubahn *et al.*, 1989; Brown *et al.*, 1990), increased AR-androgen dissociation rates (Ris-Stalpers *et al.*, 1991; Kazemi-Esfarjani *et al.*, 1993), and increased thermolability of AR-androgen complexes (Prior *et al.*, 1992; Kasumi *et al.*, 1993).

CAIS is infrequently caused by deletions, insertions and splice site mutations in the AR gene. The null phenotype for AR was described in families with CAIS due to a complete deletion of the AR gene (Trifiro *et al.*, 1991b; Quigley *et al.*, 1992). Brown *et al.* (1988) identified a partial deletion in the HBD that rendered R^- CAIS. Interestingly, another study showed different members of the same family had AR deletions in different exons that could have been the result of an unequal crossing-over event in the germ cells (MacLean *et al.*, 1993). Ris-Stalpers *et al.* (1990) reported single amino acid substitution that disrupted the splice donor site of exon 4 in the AR. This mutation ablated normal

mRNA splicing and activates a cryptic donor site, resulting in a deletion of 41 amino acids from the AR protein.

PAIS is also attributable to point mutations within the AR gene. Mutations in the DNA-binding domain (DBD) that disrupt the Zn fingers cause defective DNA binding in PAIS subjects (Zoppi *et al.*, 1992; Wooster *et al.*, 1992; De Bellis *et al.*, 1994). Mutations in the HBD have also been shown to be a pathogenic factor in PAIS causing lower androgen-binding capacity (McPhaul *et al.*, 1992; De Bellis *et al.*, 1994), decreased affinity for androgen and increased A-AR dissociation rates (Kazemi-Esfarjani *et al.*, 1993; Beitel *et al.*, 1994), and increased A-AR thermolability (Nakao *et al.*, 1993; McPhaul *et al.*, 1992; work presented in this thesis).

Reduced potency of the AR in MAIS subjects has been shown to be caused by mutations in the HBD (work presented in this thesis). Still others report, based on biochemical and androgen-binding data in GSF, that MAIS is caused by a qualitative defect in the AR (Aiman *et al.*, 1982; Grino *et al.*; 1988, Pinsky *et al.*, 1989)

Mutations causing CAIS, PAIS, or MAIS are not localized to any specific portions of the gene. There may be a correlation, however, between the severity of AIS and the extent of conservation of amino acid polarity and acidity of the given substitution. For instance, replacement of an arginine (a basic amino acid) with cysteine (a neutral, nonpolar amino acid) at codon 854 causes CAIS, whereas replacement with histidine (a basic amino acid) causes PAIS (Batch *et al.*, 1992; De Bellis *et al.*, 1992). This is not always the case however, since there is also a report of the identical arginine to histidine substitution at codon 854 that has been classified as CAIS (McPhaul *et al.*, 1992). A study of two substitutions at arginine-839 revealed that a of histidine at this location caused a more severe PAIS phenotype than cysteine (Beitel *et al.* 1994).

Amino acid conservation within the steroid receptor subfamily can indicate the importance of a specific residue at its specific location in the AR. Amino acids conserved in their corresponding positions in the GR, PR, MR and AR are likely structurally important for receptor function. For instance, disruption of highly conserved amino acids in the AR usually causes CAIS (Brown *et al.*, 1990; Ris-Stalpers *et al.*, 1991; De Bellis *al.*, 1992; Kasumi *et al.* 1993; Beitel *et al.*, 1994; work presented in this thesis) and sometimes PAIS (work presented in this thesis). In two studies where alternative amino acid substitutions at the same codon have been examined (Beitel *et al.*, 1994; Kazemi-Esfarjani *et al.*, 1993), AR biochemical phenotype measured in GSF is the coordinate with severity of AIS. Based only on sequence information, it is difficult to predict the severity of AIS.

Kennedy's disease or Spinal Bulbar Muscular Atrophy (SBMA) (La Spada *et al.*, 1991), an adult-onset motoneuron disease that is sometimes associated with MAIS (Warner *et al.*, 1991), is caused by expansion of the AR's poly-gln tract from around 20 to 40-62 (La Spada *et al.*, 1992; 45-68, Patterson *et al.*, 1994a; Patterson et al., 1994b). The more the poly-gln stretch is expanded, the earlier the onset of SBMA (Doyu *et al.*, 1992; La Spada *et al.*, 1992). The expansion could bring about a structural change in the receptor that promotes a loss of transcriptional activation at HREs, by binding increased quantities of a protein normally bound, or by erroneously binding another protein. Another alternative may be that that expanded AR may super-repress a gene necessary for motor neuron longevity (Kazemi-Esfarjani *et al.*, 1995) thus gaining a new transcriptional regulatory function (Mhatre et al., 1993) .

Mutations in the AR gene are thought to also play a role in the progression of prostate cancer. Although AR mutations are rare in the early and intermediate stages of human prostatic carcinoma, (Newmark *et al.*, 1992; Culig *et al.*, 1993a) they may be prevalent in the advanced stages and progression of the disease (Newmark *et al.*, 1992). LNCaP cells, a prostate tumor cell line, have a mutation in the HBD (Thr868Ala) that allows the receptor to bind not only androgen, but progestins, estradiols and some antiandrogens as well (Veldscholte *et al.*, 1990; Veldscholte *et al.*, 1992b). The same Thr868Ala mutation was also found in a number of advanced-stage prostatic carcinoma tumours (Gaddipati *et al.*, 1994). A different mutation in the AR's HBD was found in another prostatic tumour. In this case, the mutant AR was able to transactivate an androgen reporter gene in response to adrenal androgens and progesterone (Culig *et al.*, 1993b).

Since it is known that SR hyperactivity plays a role in some endocrine disorders, antihormones that bind the SR and block transactivation have been used in the treatment of hormone-dependent cancer of the breast and prostate, treatment of high blood pressure, and even to interrupt early pregnancy. The mechanism of antihormone interference of SR action has been partly elucidated by limited proteolytic digestion experiments. Protease-resistant fragments induced in the SRs by antihormones are indicative of aberrant SR conformations (Beekman *et al.*, 1993; Allan *et al.*, 1992a; Allan *et al.*, 1992b). Other studies have shown that antihormone-receptor complexes can impair receptor dimerization (Fawell *et al.*, 1990b; Klein-Hitpass *et al.*, 1991), restrict DNA binding (Fawell *et al.*, 1990b; Klein-Hitpass *et al.*, 1991; Allan *et al.*, 1992b), or weaken transcriptional activity (Webster *et al.*, 1988; Bagchi *et al*, 1990) possibly due to the distorted conformation of the receptor complex.

C. OBJECTIVES

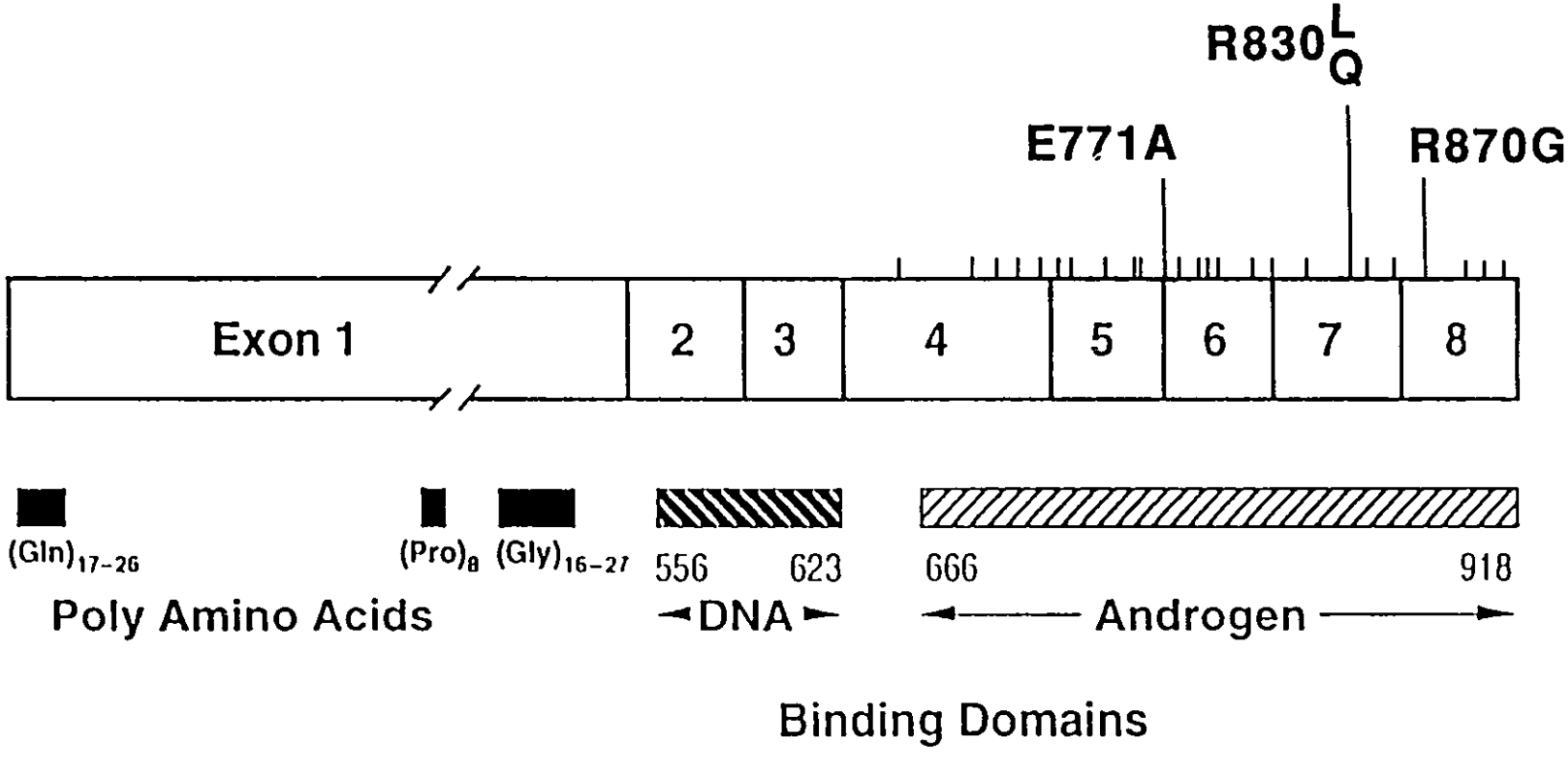
The aim of this study was to prove the pathogenicity of four point mutations in the androgen-binding domain of the hAR in four individuals with varying degrees of AIS and to explore correlations among molecular genotype, receptor phenotype, and patient phenotype. Two point mutations in two CAIS subjects occurred at an identical nucleotide in exon seven. These mutations allowed the replacement of arginine with leucine or glutamine at codon 830. Characterization of two amino acid substitutions of a single residue was performed to elucidate any subtle differences in biochemical phenotype of these ARs. A point mutation in exon five at codon 771 caused a glutamic acid to alanine amino acid substitution occurred in an individual with PAIS. An individual with MAIS had a point mutation in exon eight at codon 870 replaced arginine by glycine (Figure 12).

This study will help to further delineate the fine-structure map of a member of the SR subfamily.

Figure 12. Four Amino Acid Substitutions in the Androgen-Binding Domain of the Human Androgen Receptor.

A schematic representation of the human androgen receptor showing the location of amino acid substitutions E771A, R830L, R830Q and R870G in the androgen-binding domain.

Human Androgen Receptor (hAR)



II. MATERIALS AND METHODS

A. The Five Subjects and Their Families

Subjects REL, 2222 and 31393 have a 46,XY karyotype, unambiguous external genitalia and positive family histories for CAIS. REL has a maternal aunt who experienced delayed menarche (age 21) indicative of the aunt's probable carrier status (Kaufman *et al.*, 1976). Another maternal aunt is an obligate heterozygote by virtue of her three affected daughters. By inference, REL's maternal grandmother and great grandmother are also obligate carriers (Figure 13a). At age 15, REL had her inguinal testes removed and histological analysis revealed that one had a Seritoli tumor. Subject 2222 has an affected sister (646845) and maternal aunt, and two affected maternal great-aunts; probably another great-aunt was similarly affected and she is shown as affected in Figure 13b. 31393 has a positive family history (Figure 13c) as reported earlier (Brown *et al.*, 1990).

Subject 3287 has PAIS with no known family history. His karyotype is 46,XY. He was born with undescended testes, microphallus, and severe perineal hypospadias that required surgical correction. He does not have any Müllarian duct derivatives.

Subject 4007 (Figure 14) has MAIS with no other affected family members. He has a small, nonhypospadic penis. He has masculine skeletal muscularity, normal axillary and pubic hair, but scant facial, leg and chest hair, and a high-pitched voice. At puberty he developed persistent gynecomastia that required reduction mammoplasty. Semen analysis revealed a low volume with 40% living sperm, 13% tapered forms, and 11% amorphous forms. Administration of testosterone did not enhance his secondary sex characteristics. Subject 4008, the sister of 4007, is a carrier of her brother's mutation as determined by DNA sequencing, thus their mother is obligate heterozygote of undetermined origin.

GSFs of these subjects and controls were derived from skin biopsies that were obtained with informed consent according to approved protocols.

B. Molecular Biology

1. Direct Sequencing of PCR Products to Identify Mutations

All mutations were identified previously by other laboratory personnel except for the one in 4008. DNA was extracted from peripheral blood lymphocytes or GSF by the method of Greenberg *et al.* (1987) or Trifiro *et al.* (1991a), respectively. Exons two to

Figure 13a. Pedigree of Subject REL.

Subject REL has an extensive family history of CAIS. REL has three affected cousins and an aunt who had delayed menarche (indicated *). Diamond-shaped symbols indicate an unaffected individual of either gender.

Figure 13b. Pedigree of Subjects 2222 and 646845.

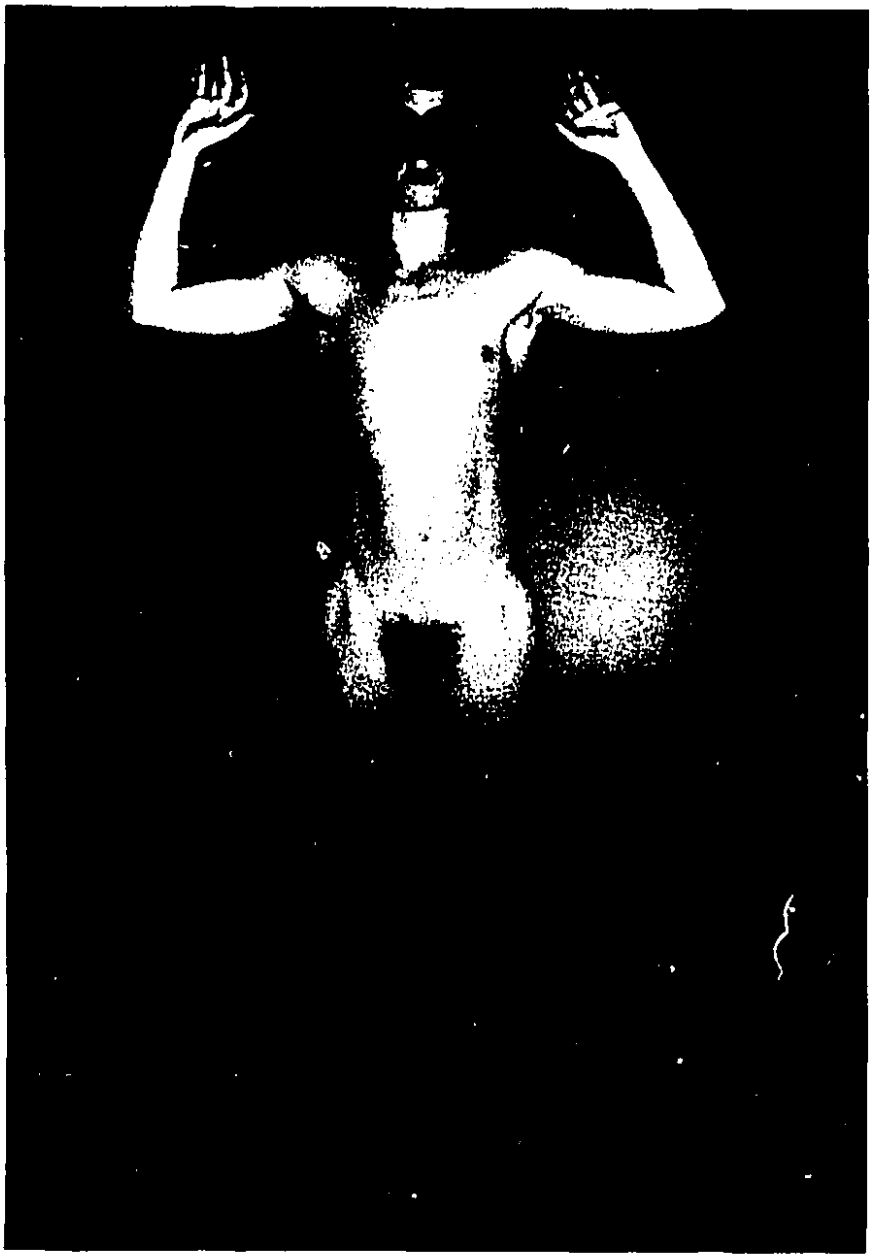
Subjects 2222 and 646845 have one affected maternal aunt and three affected great-aunts. Obligate heterozygotes are shown.

Figure 13c. Pedigree of Subject 31393.

Subject 31393 has an affected aunt. Obligate heterozygotes are shown.

Figure 14. Photograph of Subject 4007.

Note normal skeletal muscularity, axillary and pubic hair, a small nonhypospadic penis and scant facial, leg and chest hair. Gynecomastia required reduction mammoplasty.



eight were amplified by polymerase chain reaction (PCR) using intronic primers; because of its length, exon one was amplified in segments using exonic primers.

PCR products were excised from a 0.7% low-temperature agarose (ICN, Mississauga, ON) gel and stored at -20°C until sequencing was performed. For sequencing, intronic (Ris-Stalpers *et al.*, 1990) or exonic primers were end-labeled with γ-dATP ^{32}P (ICN, Biomedicals Canada, Mississauga, ON) and reactions were performed as described (Trifiro *et al.*, 1991a; Prior *et al.*, 1992) using sequenase enzyme (US Biochemicals, Cleveland, OH) using dideoxyribonucleotide triphosphates (ddNTPs), and deoxyribonucleotide triphosphates (dNTPs) from Boehringer Mannheim (Laval, PQ). For sequencing exon 1, deaza-GTP (Pharmacia Biotechnologies Inc., Baie D'Urfé, Quebec) was substituted for dGTP to alleviate compression bands due to high GC content. The sequencing reactions were analyzed in a 5% denaturing polyacrylamide gel on a Base Runner sequencing gel apparatus (IBI, Terochem, Dorval, PQ) at 64 Watts for 2.5 hours (hrs).

2. Primers and Primer Synthesis

Chosen for their melting temperature (T_m) and their position relative to the segment to be amplified, primers were synthesized using a Pharmacia Gene Assembler at the Lady Davis Institute or by Sheldon Biotechnologies (Montreal). The primers were eluted from the solid support by incubation in concentrated NH_4OH at 55°C overnight. The resulting solution was concentrated in a Savant Speed-Vac, resuspended in 200ml ddH_2O and purified on a Sephadex column. The primer DNA was quantitated spectrophotometrically at optical density (OD) 260nm.

3. PCR Amplification, and PCR Mutagenesis

DNA amplification was accomplished using a thermal cycler (Perkin-Elmer Cetus, Connecticut) using Taq, Vent (New England Biolabs, Mississauga, ON), or Pfu polymerase (Stratagene, La Jolla, CA). The thermal cycles generally consisted of a 45 second (sec) denaturation step at 95°C, a 45 sec annealation step at 55°C and a 1 minute (min) 45 sec extension step at 72°C for 30 to 40 cycles. Where problems in amplification existed, modifications were made in the time or temperature of the annealation or extension step with attention to the T_m of the primers used. Each analysis included a control reaction lacking template DNA to detect contaminating DNA. Three drops of mineral oil were added to each reaction tube to prevent evaporation.

(a) PCR Amplification of Genomic DNA

In each 100 µl reaction, 20 nmol of each dNTP (Boehringer Mannheim, Laval, PQ), 25 pmol of intronic primer for the exon to be amplified (exons two to eight) or exonic primers for exon one, 1 µg of genomic DNA and 2.5 units of Taq, Vent or Pfu polymerase were added and amplified at the temperatures mentioned above. For exon one, dGTP was replaced by deaza-GTP and the reaction was amplified by a 1 min denaturation at 98°C, 1 min annealing at 55°C, and a 1.5 min extension at 72°C.

(b) PCR Mutagenesis of the Expression Vector

The hAR cDNAs containing each mutation were recreated by the overlap extension method (Figure 15) of Higuchi (1990). PCR mutagenesis was performed in two PCR reactions where the pSVhAR.BHEX expression vector served as the template DNA. For each mutation, primary PCR reactions involved the use of one mutant primer (A, the sense strand; B, the antisense strand) with an outside primer (a 3' flanking primer with mutant primer A, or a 5' flanking primer with the mutant primer B). The choice of outside primer was made with regard to incorporation of a flanking restriction enzyme site convenient for cloning into the expression vector. The PCR reaction included 50 pmol of either mutant primer (A or B) and its appropriate outside primer, 1µg of SVhAR.BHEX plasmid, 20 nM of each dNTP and 1 unit of Pfu or Vent polymerase enzyme, the appropriate buffer for the polymerase used, in each of five 100 µl reactions for 30 to 40 cycles. The two primary PCR products, one with the mutation and upstream sequences and the other with the mutation and downstream sequences, were visualized under UV in a 1% agarose gel by ethidium bromide staining. The bands were excised from the gel, purified by QN-butanol procedure devised by Langridge *et al.* (1980), and measured spectrophotometrically at OD_{260}. The secondary PCR reaction amplified the full-length DNA fragment containing the mutation, from one outside primer to the other. The reaction used 50 pmol each of the outside and mutant primers, with equimolar quantities of each of the purified fragments from the primary PCR reaction; concentrations of dNTPs, and polymerase, and number of cycles remained the same as in the primary PCR reactions. For PCR mutagenesis of E771A, the annealing temperature needed to be lowered to 52°C.

The mismatched nucleotide corresponding to the point mutation in the primer is underlined and the outside primers used for incorporation of each mutant are indicated.

Figure 15. PCR Mutagenesis.

A schematic of the overlap-extension method of PCR mutagenesis is shown. The incorporated mutation is indicated as △. (Higuchi *et al.*, 1990, pp. 177-183).

E771A:

E771A-A	5' CTG GTT TTC AAT G<u>C</u>G TAC CGC A 3'	T_m=76°C
E771A-B	3' GAC CAA AAG TTA C<u>G</u>C ATG GCG T 5'	

Outside primers to incorporate E771A:

4A4	5' CAG GAG GAA GGA GAG GCT 3'	T_m=58°C
KIL-B	3' GAC CTG AGG TAC GTC GGA TA 5'	T_m=62°C

R830L:

R830L-A	5' GAA CTT C<u>T</u>A ATG AAC TAC ATC AAG 3'	T_m=64°C
R830L-B	3' CTT GAA G<u>A</u>T TAC TTG ATG TAG TTC 5'	

R830Q:

R830Q-A	5' GAA CTT C<u>A</u>A ATG AAC TAC ATC AAG 3'	T_m=64°C
R830Q-B	3' CTT GAA G<u>T</u>T TAC TTG ATG TAG TTC 5'	

Outside primers to incorporate R830L and R830Q:

30285-A	5' AAT GAG TAC CAC ATG CAC AA 3'	T_m=56°C
P3'	3' GTG GTT GGA AGA CTA TCC GTC G 5'	T_m=68°C

R870G:

R870G-A	5' GCA GCC TAT TGC G<u>G</u>G AGA GCT GC 3'	T_m=66°C
R870G-B	3' CGT CGG ATA ACG C<u>C</u>C TCT CGA CG 5'	

Outside primers to incorporate R870G:

TCF-A	5' GTC CCG GAT ATA CAG CCA G 3'	T_m=60°C
P3'	3' GTG GTT GGA AGA CTA TCC GTC G 5'	T_m=68°C

The resulting fragments from the secondary PCR reaction were purified by the QN-butanol procedure, quantitated with a spectrophotometer at OD_{260}, and were prepared for subcloning into the expression vector as follows.

(i) QN-Butanol procedure

This procedure uses the ability of hexadecyltrimethylammonium bromide (QN) (Sigma Chemicals, St. Louis, MI) to bind DNA in low salt concentrations. The QN-butanol solution was added to the molten gel slice containing the fragment of interest. The upper butanol phase containing the eluted DNA complexed to QN was removed and the salt concentration of the solution was increased allowing the DNA-QN complex to dissociate. The DNA is then eluted into the bottom aqueous phase, the DNA was purifed with chloroform, and precipitated with ethanol.

4. Subcloning into pSVhAR.BHEX

The expression plasmid used for these studies was a variation of $pSVhAR_0$ (Figure 16; Brinkmann *et al.*, 1989). The hAR cDNA consisting of 7219 bp was originally cloned as a *SalI-PstI* fragment into pBR328 which contains the SV40 early promoter, the rabbit β-globin polyadenylation signal, and an ampillicin resistance gene for positive selection of clones. For cloning purposes, the cDNA was modified further to create other restriction sites and is referred to as pSVhAR.BHEX (Figure 17).

After incorporating a mutation into the hAR cDNA by PCR amplification, the purified secondary PCR product was digested with restriction enzymes (Promega, Montreal, PQ; BRL, Bethesda, MD) that flank the product. For E771A the restriction enzymes used were *EcoRI* and *XhoI*, for R830L and R830Q they were *EcoRI* and *BamHI*, and for R870G they were *BstBI* (New England Biolabs, Mississauga, ON) and *BamHI*. The pSVhAR.BHEX plasmid was digested with the identical enzymes in order to create a vector with compatible sticky ends. Purified vector and fragment were added together in a 1:3 molar concentration, along with T_4 DNA ligase buffer and T_4 DNA ligase (BRL-Canadian Life Technologies, Montreal, PQ), and allowed to ligate at room temperature for 4 hrs or overnight at 14°C. The negative control for the ligation utilized the cut vector alone in another reaction. Five µl of ligation mixture was added to competent XL-1 Blue cells (Davis et al., 1986) and transformation was done by heat shock. Bacteria were plated out on LB-ampillicin (50µg/ml; Boehringer Mannheim, Laval, PQ) plates and the recombinants were selected as colonies on the plate. Ampillicin-resistant colonies were grown up as a 5 ml overnight culture. Minipreps were done on resulting cultures and the correct insertion

Figure 16. hAR Expression Vector $pSVhAR_0$.

The hAR expression vector $pSVhAR_0$ containing the complete cDNA of hAR within a Sal I - Pst I fragment, the SV40 early promoter, the rabbit β-globin polyadenylation signal, and sequences from the pBR328 plasmid containing the ampillicin resistance gene. Hatched boxes represent the 5' and 3' untranslated regions. (Brinkmann *et al.*, 1989, p. 308)

Figure 17. hAR Expression Vector pSVhAR.BHEX.

The hAR expression vector pSVhAR.BHEX is a variation of $pSVhAR_0$ where *Bst BI*, *HindIII* and *EcoRI* sites in $pSVhAR_0$ were eliminated and unique restriction sites were created in the hAR cDNA (*BstBI*, *EcoRI*, *XhoI*, and *HindIII*; shown in bold) to facilitate cloning. The SV40 early promoter, the rabbit β-globin polyadenylation signal, and sequences from the pBR328 plasmid containing the ampillicin resistance gene are indicated.

of the fragment was validated by restriction enzyme digests and agarose gel electrophoresis. A maxi-prep was done on recombinant clones before sequencing.

(a) Preparation of XL-1 Blue competent cells

Forty ml of Luria Broth (LB) medium were incubated with XL-1 Blue cells (Stratagene, La Jolla, CA). The culture was allowed to proceed to log phase on a shaker at 37°C. Cells were pelleted and resuspended in 20 ml of 50mM $CaCl_2$, iced 30 min, pelleted at 4°C and resuspended in 4 ml of 50mM $CaCl_2$.

(b) Transformation via the heat-shock method

The ligation mixture was added to 100µl of XL-1 Blue competent cells and incubated on ice for 30 min. Cells were then shocked at 42°C for 90s and immediately incubated on ice for a further 1 to 2 min. 800 µl of SOC media was then added and cells were allowed to recover at 37°C for 45 min on a shaker. Appropriate volume was transferred to an LB-ampicillin plate and incubated overnight at 37°C (Sambrook *et al.*, 1989a).

(c) Mini-preparation of plasmid DNA

Plasmid DNA was isolated from a 5 ml overnight culture using the alkaline-lysis procedure of Sambrook *et al.* (1989b).

(d) Maxi-preparation of plasmid DNA

Five ml of LB with 50 µg/ml ampillicin (Boehringer Mannheim, Laval, Quebec) was inoculated with clone of interest as overnight culture. This was used as the inoculum for 500 ml of LB with 50 µg/ml ampicillin. Plasmid DNA was purified by the Qiagen Plasmid Purification System (Qiagen Inc., Chatsworth CA) according to manufacturer's instructions. Yield was quantitated by optical density at 260nm and quality was assessed by a scan from 230-320nm.

5. Plasmid sequencing

To verify that the appropriate mutations were incorporated and that extraneous mutations were not, the insert was sequenced beyond the ligation points. Dideoxy sequencing was performed with Sequenase enzyme according to manufacturer's protocol. Primers were used such that each mutation was sequenced in the sense and anti-sense directions. Radiolabeled α^{32}P-dATP or ^{35}S-dATP (Amersham, Mississauga, ON) was

used in these sequencing reactions. The sequencing reactions were run on a 5% denaturing polyacrylamide gel at 64 Watts for 2.5 hrs.

C. Tissue Culture

COS-1 cells used for transfection experiments were originally purchased from American Type Culture Collection (ATCC) (Rockville, MD). They were maintained in Opti-MEM (Gibco/BRL Life Technologies, Burlington, ON) with 1 mM pyruvate, 10 mg/l gentamycin sulfate (Schering Canada, Point Claire, PQ), 60 mg/l penicillin G (Ayerst, St. Laurent, PQ), 60 mg/l streptomycin sulfate (ICN Biomedicals Canada, Mississauga, ON), and 5-10% fetal calf serum (ICN Biomedicals Canada, Mississauga, ON) in a 37°C humidified incubator with 5% carbon dioxide.

GSF obtained from patients and controls were maintained in the same manner as the COS-1 cells.

D. Transfection Experiments

1. Transfection Parameters

Confluent cells COS-1 were dislodged from T-150 flasks (Becton Dickson Canada Inc., Saint-Laurent, Quebec) with 0.1% trypsin (Difco, Detroit, MI) for 5 minutes at 37°C. Cells were spun down at 1800 rpm for 2 minutes and were resuspended in the above media to a concentration of 20×10^6 cells per ml. Ten million cells were added to a cuvette (0.4 cm electrode gap; Bio-Rad Laboratories, Mississauga, ON) and the appropriate amount of plasmid is added (1 or 2 µg of plasmid for normal, E771A, or R870G; and 1µg of plasmid for normal or 10 µg of plasmid for R830L or R830Q; unless stated otherwise). For transactivation experiments, 2 µg of pMMTV-GH was transfected along with 1 µg of each of the mutants or normal. For all experiments, 2 µg of pCMV-βgal (the β–galactosidase gene driven by the cytomegalovirus promoter) was transfected to control for transfection efficiency. The suspension was then shocked at room temperature using a Gene Pulser (Bio-Rad, Richmond, VA) at 250 Volts, and 960 µFaradays to yield a time constant of 20-25 milliseconds. Cells were then diluted with media, with identical transfections being pooled, to obtain 3×10^5 cells per well for a 24-well plate (Becton Dickson Canada Inc., Saint-Laurent, Quebec), and 6×10^5 cells per 35 mm plate (Becton Dickson Canada Inc., Saint-Laurent, Quebec). Often, the normal plasmid was transfected and plated out in duplicate serving as an internal control for the experiment. Transfectants were assayed for

androgen-binding activity 48 hrs later, or 76 hrs later in the case of the growth hormone assay as described.

2. Androgen-Binding Assays

Replicate wells or dishes of cell monolayers were incubated in MEM (Minimal Essential Media, Gibco, Mississauga, ON) supplemented with Hank's salt and HEPES buffer (ICN Biomedicals Canada, Mississauga, ON) with tritiated (^{3}H) androgen, while other plates were incubated with ^{3}H-androgen plus 200-fold excess radioinert androgen in MEM to measure background androgen-binding activity. The cells were allowed to incubate for various stated conditions of temperature, time, and hormone concentrations. The plates were washed twice on ice, and washed once at room temperature for 7 min with 0.02 M Tris-HCl (pH 7.4). 0.5 M NaOH was then added directly to the wells or dishes to lyse the cells. Aliquots of the lysate were added to Beta Max ES (ICN Biomedicals Canada, Mississauga, ON) and radioactivity was quantitated in a TriCarb 1500 liquid scintillation counter (Canberra-Packard Canada). Additional aliquots were used to quantitate protein and androgen-binding activity was expressed per mg protein. Specific androgen-binding activity was calculated by subtracting the nonspecific fraction from the total (Kaufman and Pinsky, 1989).

Three tritiated androgens were used. They were: DHT, [1,2,4,5,6,7-^{3}H] 5α-dihydrotestosterone (120 Ci/mmol); [17α-methyl-^{3}H] mibolerone (MB), 7α,17α-dimethyl-19-nortestosterone (80.6 Ci/mmol); [17α-methyl-^{3}H] methyltrienolone (MT), 17β-hydroxy-17α-methyl-estra-4,9,11,-triene-3-one (83 Ci/mmol) (Amersham, Oakville, ON). Radioinert androgens used were DHT, MB, and MT (Dupont, Mississauga, ON).

Data were graphed using the computer program Cricket Graph™ and where appropriate, linear regression was used for curve fit.

(a) Other Hormone Binding Assays

These experiments were carried out on mutant R870G and normal hARs. Triplicate wells of transfected COS-1 cells were labeled with 5nM ^{3}H-MB or 5nM ^{3}H-MB plus 10-, 100-, or 1 000-fold radioinert hormone at 37°C for 2 hrs. The radioinert hormones used were: progesterone, estradiol, dexamethasone, spironolactone (an anti-androgen), and MB (Dupont, Mississauga, ON) as a control.

3. Offrate Experiments (nonequilibrium dissociation constants; k)

Forty-eight hrs after transfection, quadruplicate wells of transfected COS-1 cells were labeled with 3nM of ^{3}H-androgen and duplicate wells were labeled with 3nM ^{3}H-androgen plus 200-fold excess of radioinert androgen. The plates were incubated for 2 hrs at the appropriate temperature (37°C for E771A and R870G; and 22°C or 37°C for R830L and R830Q). The incubation media were replaced by ones containing only 200-fold radioinert androgen acting as a chase. Cells were harvested at 0, 30, 60, 90, and 120 min and assayed for androgen-binding activity.

Androgen-binding activity after two hrs of incubation (time zero) represented 100% of binding activity of the particular transfectant. Androgen-binding activities of transfectants at other time points were expressed as a percentage of their respective activities at time zero. Results for each transfectant were plotted semilogrithmically as percent of androgen-binding activity remaining at each time point. The k values were determined directly from the slopes of the lines.

4. Scatchard Analyses (apparent equilibrium dissociation rate constants; K_d)

Quadruplicate wells of transfected COS-1 cells were labeled in MEM supplemented with 100 µM cycloheximide (Sigma Chemicals, St. Louis, MI) and 3nM, 1.5nM, 0.75nM, 0.38nM, 0.19nM or 0nM ^{3}H-androgen, and duplicate wells were labeled with the corresponding amount of hormone plus 200-fold radioinert androgen. Cells were labeled at the appropriate temperature (37°C for E771A and R870G; and 22°C or 37°C for R830L and R830Q) for the appropriate time to saturate the receptors (2 hrs for E771A and R870G; and 4 hrs for R830L and R830Q).

Results were plotted as bound/free androgen versus bound androgen and K_ds were calculated from the slope of the Scatchard plots.

5. Thermolability of A-ARs

Quadruplicate wells of transfected COS-1 cells were labeled with 3nM ^{3}H-MB and duplicate wells were labeled with 3nM ^{3}H-MB plus 200 fold radioinert MB at temperature "1" (37°C for E771A and R870G; and 22°C for R830L and R830Q) for the appropriate time (2 hrs for E771A and R870G; and 4 hrs for R830L and R830Q) to allow the formation of A-ARs. One set of plates was assayed after the initial incubation time for

androgen-binding activity. For other time points, media in each well is replaced by fresh media differing only in that it contained 100 mM cycloheximide. One series of plates was left at temperature 1 as a control, and another series of plates were placed at temperature "2" (42°C for E771A and R870G; and 37°C for R830L and R830Q) and binding assays were performed at 2, 4, and 6 hrs of incubation.

Androgen-binding activity after the initial incubation time (time zero) represented 100% of binding activity of the particular transfectant. Androgen-binding activity at other time points were expressed as a percentage of the activity of the respective transfectant at time zero. Results were represented in a bar graph.

(a) Recovery of Androgen-Binding Activity After a Temperature Challenge

These experiments were carried out with mutant E771A and R870G hARs and compared to normal hAR. Quadruplicate wells of transfected COS-1 cells were labeled with 3nM ^{3}H-MB and duplicate wells were labelled with 3nM ^{3}H-MB plus 200-fold radioinert MB at 37°C for one hr to allow the formation of complexes between androgen and the AR (A-AR). Transfectants were assayed for initial MB-binding activity that was designated as 100%. The media was removed from the remaining plates and replaced with one containing the same [illegible] concentrations plus 100 mM cycloheximide. After one hr at 42°C, or one hr at 42°C plus one or two hrs at 37°C, transfectants were assayed for MB-binding activity.

6. Thermolability of Free Receptor

Transfected COS-1 cells were incubated in MEM containing 100 mM cycloheximide and were allowed to stabilize for 30 min at temperature "1" (37°C for E771A and R870G; and 22°C for R830L and R830Q). Initial MB-binding activity for each transfectant was determined by incubating quadruplicate wells with MEM containing 3nM ^{3}H-MB with 100 µM cycloheximide, and duplicate wells in the identical labeled media plus 200-fold radioinert MB for one hr at temperature 1. After the initial 30 min stabilization, other plates were shifted to temperature "2" (42°C for E771A and R870G; and 37°C for R830L and R830Q) for 2, 4, or 6 hrs preceding a one hr incubation with labeled media at temperature 2. As a control, androgen-binding activity was determined from a series of plates that remained at temperature 1 (for 2, 4, or 6 hrs).

Results are plotted as percent of androgen-complexes remaining over time and represented in a bar graph.

7. Growth Hormone Assay

COS-1 cells were co-transfected with 1μg of mutant or normal pSVhAR.BHEX plasmid, 2 μg of pCMV-β-gal, and 10 μg of the reporter construct pMMTV-GH (Figure 18; Prior *et al.*, 1992). Four hrs later, MEM plus 10% fetal calf serum (FCS) with 5 nM, 2.5 nM, 1.25 nM, 0.6 nM, 0.3 nM, 0.15 nM or 0 nM of ^{3}H-MB, or the corresponding concentration of ^{3}H-MB plus 200-fold radioinert MB is added to duplicate wells of a 24-well plate. Upon addition of androgen, the AR-androgen complex binds to GREs in the MMTV-GH and activates transcription of the human growth hormone (hGH) reporter gene. Growth hormone secreted into the media is a measure of transactivation by the hAR. Seventy-two hrs after adding androgen, media from the wells was harvested to quantitate secreted hGH and cells were used to determine androgen-binding activity as described. 50 μl of media collected from transfected cells was added to 100 μl of antibody solution supplied with the growth hormone kit (see below).

Growth hormone was measured using a immunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) according to manufacturer's instructions. This kit uses two antibodies that recognize distinct epitopes of the hGH molecule. One molecule is labeled with ^{125}I, the other is coupled to biotin. The addition of an avidin-coated bead to the reaction mixture allows specific binding of the sandwich complex to the solid phase. The beads were then washed to eliminate any unbound components in the mixture; radioactivity bound to the solid phase was quantitated in a gamma counter. A standard curve is established by control hGH samples in the kit.

Results are plotted as growth hormone activity versus MB concentration.

8. Western Blotting

Forty-eight hrs after transfection, transfectants in duplicate 60 mm dishes (Becton Dickson Canada Inc., Saint-Laurent, Quebec) were incubated in MEM containing ^{3}H-MB and 10% FCS or in the same media without hormone for 2, 4, 8, and 18 hrs at 37°C. Plates were then placed on ice and cells washed three times with 0.02 M Tris-HCl (pH 7.4). Cells were dislodged in 0.02 M Tris-HCl using a rubber policeman, duplicate 35 mm dishes were pooled and the cells were collected by microfuging at 14 000 rpm for 20 sec. 140 μl of cell lysis buffer (50 mM Tris-HCl, pH 6.8), containing 10% glycerol, 2% SDS, 100 mM DTT, and 1ml/mg of the following protease inhibitors (ICN, Mississauga, ON): APMSF [(4-aminophenyl)-methanesulfonylfloride], Aprotinin, Bestatin, E-64, Leupeptin, and Pepstatin) were added before passage through a 26G needle 5 times. The lysis mixture

Figure 18. Human Growth Hormone Expression Vector pMMTV-GH.

The human growth hormone expression vector pMMTV-LTR was created by inserting the MMTV-LTR as a *HindIII - NheI* fragment inserted into the human growth hormone cDNA contruct pØGH. (Prior *et al.*, 1992, p.145)

was boiled for 10 mins and cellular debris was pelleted by microfuging at 10 000 rpm for 5 min. The supernatant was then frozen at -20°C overnight. After protein assays were done, 100 µg of protein was loaded on an 8% SDS-PAGE gel exposed to 90 volts for 10 hrs and electroblotted to a nitrocellulose filter (Xymotec Biosystems, Montreal, PQ) for 3.5 hrs at 325 milliamps. The filter was blocked for 1 hr with Tween/TBS buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.5% Tween 20, BDH, St. Laurent, PQ). The primary antibody used was a monoclonal antibody (F39.4.1) to a peptide (Sf61) corresponding to amino acids 301-320 in the hAR (Zegers *et al.*, 1991). After dilution to 1:10 000 in TBS/Tween buffer this antibody was added to the blot and allowed to rock for 1 h. After 6 final wash steps with TBS/Tween the blot was probed with a secondary antibody of horseradish peroxidase-goat anti-mouse immunoglobulin G (Professional Diagnostics, Edmonton, AB) at a dilution of 1:5 000. All incubations with antibody and wash steps were performed at 27°C. Six more wash steps follow before incubation with ECL Western blotting chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Blots were exposed to X-OMAT-AR X-ray film (Eastman Kodak, Rochester NY) for approximately 1 min. The control for protein loading was the monoclonal anti-α tubulin antibody (Amersham Corp., Arlington Heights, IL) diluted 1:1 000 in Tween/TBS buffer and used as described above. Densitometry was performed with an LKB Ultroscan XL densitometer (LKB, Rockville, MD) using the Gel Scan XL Program (Pharmacia, Pitcataway, NJ)

9. Protein Assays

To quantitate protein for androgen-binding and β-galactosidase assays, a Lowry (1951) procedure was followed, using purchased phenol reagent (American Chemicals, Montreal). Twenty to 40 µl of cell lysate, ddH_20 up to 70 µl, 250 µl protein C (Fisher Scientific, Nepean, ON) and 25 µl phenol reagent was added to each well in a 96-well plate (Becton Dickson Canada Inc., Saint Laurent, Quebec). The reaction was allowed to proceed for 30 min. before reading at OD_{650} in a microtitre reader (Titertek Multiscan, ICN, Missassauga, ON).

For protein quantitation of samples to be used in Western blots, the Lowry assay (*DC* Protein Assay, Bio-Rad, Mississauga, ON) was modified to surmount overestimates of protein due to high SDS in the cell lysis mixture. These reactions were also done in a 96-well plate and read by a microtitre reader.

In both cases, a standard curve for protein was prepared using bovine serum albumin (BDH, Montreal).

10. β-galactosidase Assay

Transfected COS-1 cells in 35 mm dishes were washed on ice 3 times with 0.02M Tris-HCl (pH 7.4). The cells in each dish were dislodged into 1 ml of wash buffer, centrifuged at 14 000 rpm for 20 sec, resuspended in wash buffer and recentrifuged. The pellet was resuspended in 100 µl of 0.25 M Tris-HCl (pH 7.8) and the cells were lysed by three freeze-thaw cycles in an ethanol dry ice bath. Cellular debris was removed by microfuging at 14 000 rpm for 5 mins at 4°C. Thirty µl of supernatant was used in the β-galactosidase assay as described by Sambrook *et al.* (1989c). β-galactosidase in the supernatant hydrolyzes a o-nitrophenyl β-D galactopyranoside (ONPG; Boehringer Mannheim, Laval, PQ) substrate to produce a color change that can be quantitated with a spectrophotometer. Samples were read at 420nm for absorbance and β-galactosidase is calculated using the following equation:

$$\text{units/ml} = \frac{A_{420}/0.0045}{(\text{reaction time})(\text{volume of cell extract})}$$

Protein concentration in the cell lysate was measured as above. The β-galactosidase activity was then expressed as units per mg protein.

E. The Baculovirus System

1. Subcloning into pETL.BHEX

Using *NheI* and *BamHI*, hAR cDNAs were excised from mutant pSVhAR.BHEX plasmids and ligated into pETL.BHEX plasmid cut with the same restriction enzymes. The method of Sambrook *et al.* (1989d) was used to ligate the vector and fragments without purification from low-melt agarose gel slices. Competent XL1-Blue cells were transformed by heat shock or electroporation. Prior to electroporation, XL1-Blue cells were grown in LB to an OD_{600} of 0.5 to 0.6, harvested and washed with 5% in double-distilled water glycerol to eliminate salts and prevent sparking. Cells were shocked in pre-chilled 0.1 cm cuvettes (Bio-Rad, Laboratories, Mississauga, ON) with a Bio-Rad Gene Pulser set at 220Ω, 25µF and 1.75 kV with a resulting time constant of 4-5 milliseconds.

(a) Obtaining Recombinant Virus by Cotransfection

Sf9 cells (Invitrogen, San Diego, CA) were harvested, counted and 1 X 10^6 cells were allowed to attach to a 35 mm dish for 1 hr. A lipofection mixture of 1µg AcNPV viral DNA, 2 µg pETL.BHEX mutant plasmid DNA, 20 µl Lipofectin (Canadian Life Technologies, Burlington, ON), in 100µl of 1X TMN-FH serum-free media [containing Graces's Antheraea medium (Gibco/BRL, Mississauga, ON), yeastolate (Difco Laboratories, Detroit, MI), lactalbumin hydrolysate (ICN, Montreal, PQ) Garamycin, (Schering Canada Inc., Pointe-Claire, PQ), Fungizone, (Squibb Canada, Montreal, PQ)] was prepared and allowed to sit at room temperature for 10 mins. Media was removed from the dishes and 300 µl of 1X TMN-FH serum-free media was added to the plates. 100 µl of lipofection mixture was added dropwise. Dishes were left for 4 hrs at room temperature and 3 ml of media containing 10% FCS was added. The plates were sealed with parafilm and left at 27°C for 3-6 days. The medium containing recombinant viruses was harvested and stored at 4°C.

(b) Baculovirus Plaque Assay

Three and a half million Sf9 cells were added to a 60 mm dish and were allowed to attach 1 hr. Dilutions of transfection supernatants containing virus were made from 10^{-3} to 10^{-5} in 1 ml of 1X TMN-FH. Media was aspirated from the plates and 1 ml of dilution was added to each plate. The plates were incubated at room temperature for one hr, rocking back and forth several times. Viral media was then aspirated and 4 ml of agarose-media [1.5% agarose, 1X TMN-FH with 10% FCS, 0.74% X-gal (Boehringer Mannheim, Laval, PQ)] at 37°C was added to each plate. The plates were wrapped in Parafilm and incubated for 3 to 6 days at 27°C. Appearance of blue plaques indicated presence of recombinant virus.

(c) Purification of Recombinant virus

Recombinant plaques were picked by stabbing through the agarose with a sterile pasteur pipet. The agarose plug was placed in 1 ml of serum-free media. Virus was allowed to diffuse from the agarose for one hr, the tube was vortexed and serial dilutions of 10^{-1} to 10^{-3} were made in serum-free media. Three rounds of purification were needed to obtain recombinant virus that was free of contaminating wild-type virus. Viral titre was then determined (Summers and Smith, 1987).

III. RESULTS

A. Identification of Mutations

Sequencing of exons two to eight of the androgen-binding domain (ABD) of the AR gene revealed a point mutation at nucleotide 2489 in exon seven of subjects REL (Kaufman *et al.*, 1981), 2222 (sequencing performed previously in our lab by other personnel), and 31393 (Brown *et al.*, 1990). The mutations correspond to a substitution of a conserved arginine at codon 830 for leucine or glutamine: a G to T transversion caused Arg830Leu (R830L) in subjects REL and 2222, and a G to A transition caused Arg830Gln (R830Q) in subject 31393 (Figure 19). These mutations ablate a *Bst BI* restriction site.

An A to C transversion at nucleotide 2312 (identified in our lab by other personnel) in exon five of the ABD in subject 3287 corresponds to a substitution of a conserved glutamic acid to alanine in codon 771: Glu771Ala (E771A; Figure 20).

An A to G transition was found in exon eight at nucleotide 2608 (identified in our lab by other personnel) that causes a unconserved arginine residue to be substituted with a glycine residue (R870G; Figure 21). This mutation was found in subject 4007 and his heterozygous sister 4008.

The conservation of amino acids throughout the subfamily in the region of the three affected codons described here are shown in Figure 22. Note that codon 771 falls within a region of considerable amino acid conservation within the subfamily, codon 830 falls between two such regions, and codon 870 is in a unconserved region. The structures of the amino acids involved in the E771A, R830L, R830Q and R870G substitutions are shown in Figure 23.

Sequencing of the glycine and glutamine tracts of exon 1 was carried out in the lab by Rose Lumbroso and revealed a glycine tract of 13 for REL, 17 for 31393 and 3287, and 18 for 4007. The glutamine tracts were 21 for REL and 26 for 646845. The length of these homopolymeric stretches fall within the normal range and establish that REL and 2222 (by inference from the 646845 data) are likely from unrelated families.

B. Site-Directed Mutagenesis

All four mutations were separately incorporated into the pSVhAR.BHEX expression vector by PCR mutagenesis (Higuchi, 1990). For E771A, the upstream primary PCR reaction used primers E771A-B and A4A to create a 406-bp fragment, and the downstream PCR reaction used primers E771A-A and KIL-B to create a 305-bp

Figure 19. Point Mutations and Corresponding Amino Acid Substitutions of REL and 31393.

The DNA sequencing ladders of REL and 31393 are compared to normal. The nucleotide changes are indicated with an * and corresponding amino acid substitutions are shown. A guanine to thymine transversion in exon 7 causes an arginine to leucine amino acid substitution in REL, and 2222 (not shown), and a guanine to adenine transition causes an arginine to glutamine amino acid substitution in 31393. This sequencing data was generated previously by other laboratory personnel.

Figure 20. Point Mutations and Corresponding Amino Acid Substitutions of 3287.

The DNA sequencing ladder of 3287 is compared to normal. The nucleotide change is indicated with an * and corresponding amino acid substitution is shown. An adenine to cytosine transversion in exon 5 causes an glutamic acid to alanine amino acid substitution. This sequencing data was generated previously by other laboratory personnel.

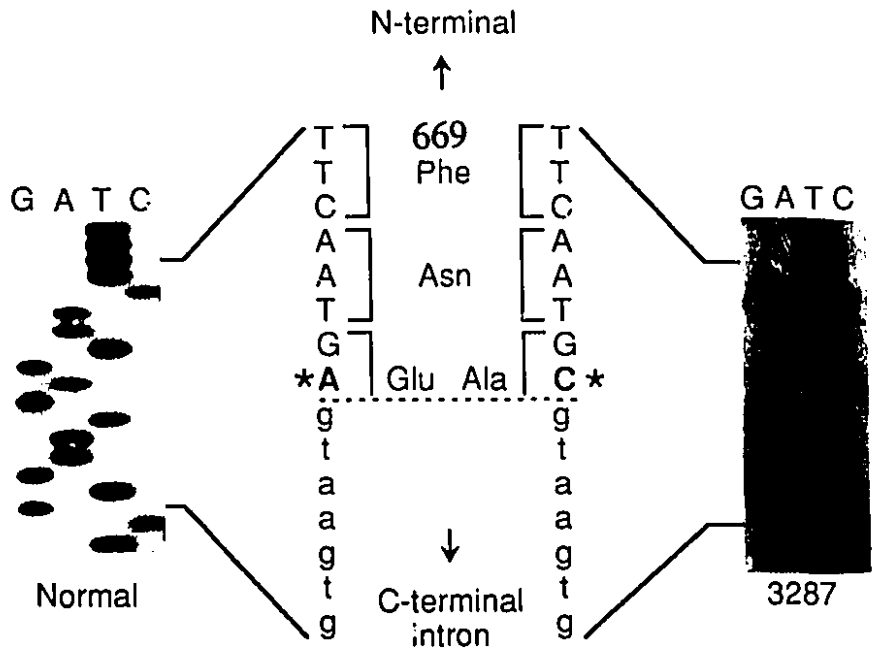

N-terminal
669
Phe
Asn
Glu
Ala
G A T C
G A T C
Normal
3287
C-terminal
intron

Figure 21. Point Mutations and Corresponding Amino Acid Substitutions of 4007 and 4008.

The DNA sequencing ladders of 4007 and 4008 are compared to normal. The nucleotide change is indicated with an * and corresponding amino acid substitution is shown. An adenine to guanine transition in exon 8 causes an arginine to glycine amino acid substitution. Subject 4008 is a carrier of 4007's mutation, thus, at the position of the mutation both the normal and mutant base is present. This sequencing data was generated previously by other laboratory personnel except for that of 4008 which was generated in this study.

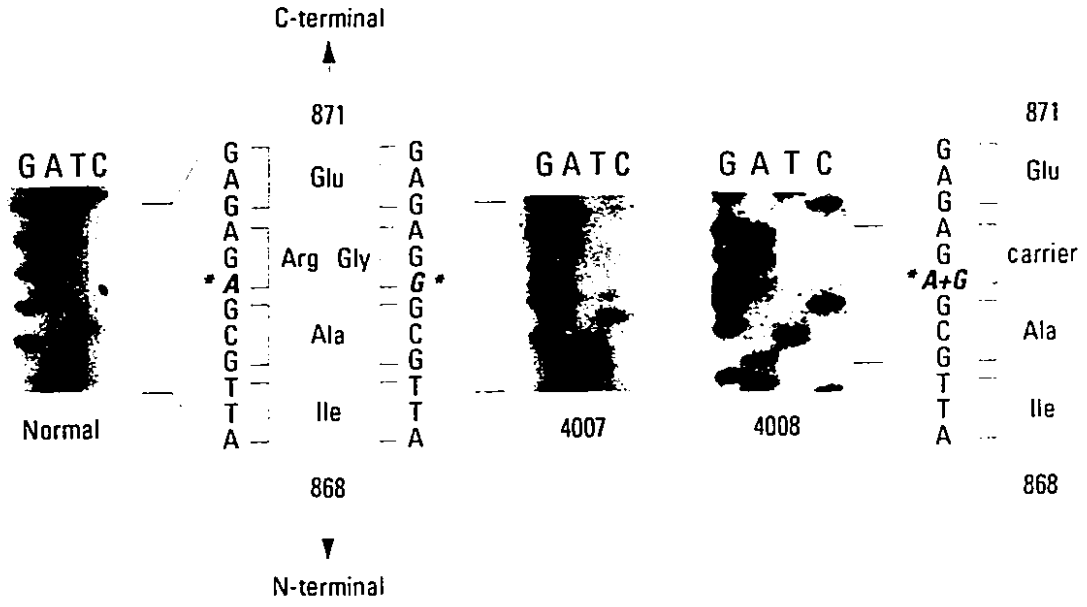

C-terminal
871
GATC
G A G A G *A G C G T T A
Glu
Arg Gly
Ala
Ile
G A G A G *G G C G T T A
868
N-terminal
Normal
GATC
4007
GATC
4008
G A G A G *A+G G C G T T A
Glu
carrier
Ala
Ile
871
868

Figure 22. The Amino Acid Composition of the Androgen-Binding Domain of the Human Androgen Receptor Compared to Other Members of the Steroid Receptor Subfamily.

The sequence of amino acid residues in the androgen-binding domain of the human androgen receptor (hAR) is compared to the ligand-binding domain of the human progesterone receptor (hPR), the human glucocorticoid receptor (hGR), and the human mineralocorticoid receptor (hMR). The dashes indicate identical amino acids and the bottom brackets indicate the regions of conservation within the steroid receptor subfamily. Exon boundaries are shown by vertical lines with the corresponding adjoining exon numbers listed. Amino acids substitutions isolated in our lab from AIS subjects are shown in bold. Codons 771, 830 and 870 and their corresponding amino acid substitutions are emphasized. The + and - superscripts denote receptor -positive or -negative phenotypes as measured in the genital skin fibroblasts. Note that glutamic acid at codon 771 and arginine at codon 830 are conserved throughout all members of the steroid receptor subfamily, but that the arginine at codon 870 is not conserved. Codon 771 falls in a highly conserved region, denoted by the bottom bracket, and codon 830 falls between two such regions. Codon 870 is situated in a unconserved region.

hAR Androgen-Binding Domain: Amino Acid Substitutions at Three Codons

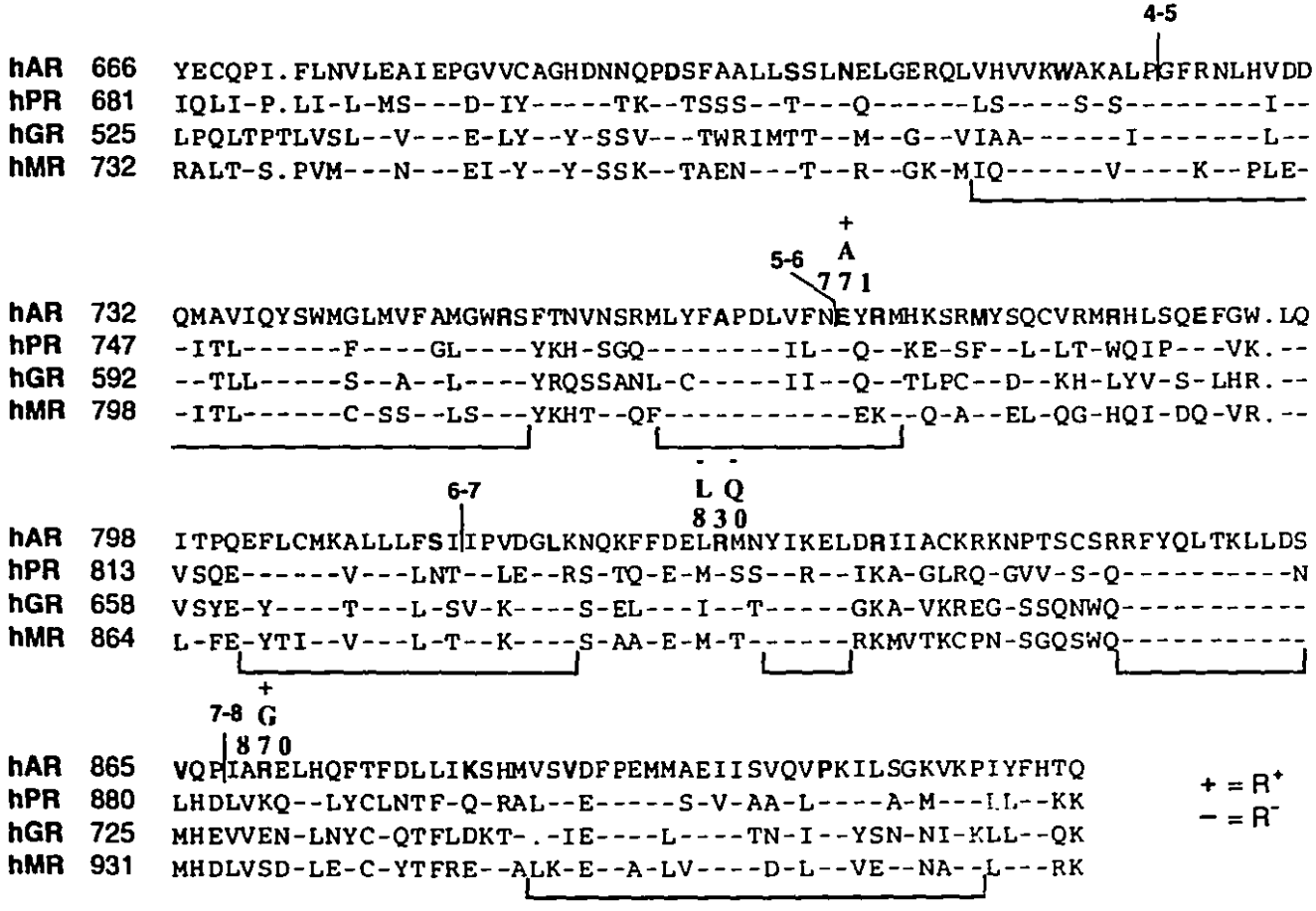
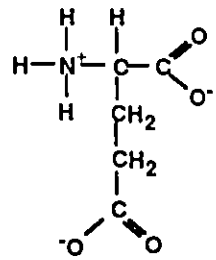
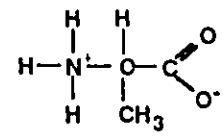


Figure 23. The Structures of the Residues Involved in the E771A, R830L, R830Q and R870G Amino Acid Substitutions.

E771A:

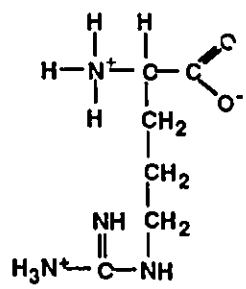


Glutamic Acid
(Acidic)

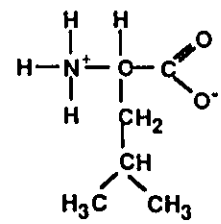


Alanine
(Neutral - Polar)

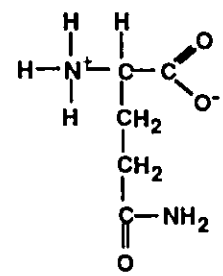
R830$^{L}_{Q}$:



Arginine
(Basic)

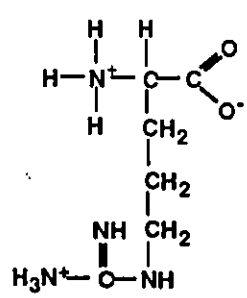


Leucine
(Neutral - Polar)

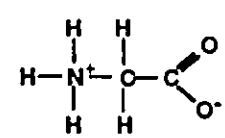


Glutamine
(Neutral - Nonpolar)

R870G:



Arginine
(Basic)



Glycine
(Neutral - Polar)

fragment. The secondary PCR reaction with outside primers 4A4 and KIL-B created a 689-bp fragment which was subsequently digested with *EcoRI* and *Xho I* to yeild a product of 378 bp. This fragment replaced the corresponding normal sequence in pSVhAR.BHEX.

For R830L and R830Q, the upstream PCR reactions used primers R830L-B or R830Q-B with 30285-A to create a 198-bp fragment, and the downtream PCR reaction using primers R830L-A or R830Q-A with P3' created a 433-bp fragment. The secondary PCR reaction produced a fragment of 607 bp. This fragment was digested with *EcoRI* and *BamHI* to liberate a 519-bp fragment that replaced the normal sequence in pSVhAR.BHEX.

The upstream PCR reaction for R870G used primers R870G-B and TCF-A to create a 290-bp fragment; the downstream PCR reaction using primers R870G-A and P3' created a 317-bp fragment, respectively. The secondary PCR product of 584 bp was digested with *BstBI* and *BamHI* to yield a fragment of 352 bp that replaced the corresponding normal sequence in pSVhAR.BHEX.

These new mutant clones of pSVhAR.BHEX were confirmed by DNA sequencing prior to their use in functional studies.

C. Androgen-Binding Studies of hARs in GSFs

Kinetic studies of the mutant hAR in GSFs of subjects REL (Kaufman *et al.*, 1981), 31391 (Brown *et al.*, 1990), and 3287 and 4007 (Kaufman *et al.*, 1990a) were done previously. REL and 31393 have R^- GSF were able to bind < 5 fmol of androgen per mg protein, too little to characterize further. Since GSF strains 3287 and 4007 had normal androgen-binding capacity (66 and 61 fmol/mg protein, respectively); further kinetic studies were possible. The A-ARs formed in strains 3287 and 4007 have an unusual characteristic: a normal apparent equilibrium dissociation rate constant (K_d; 0.1nM to 0.3 nM; Table 1) with concomitant increased non-equilibrium dissociation rates for most ligands tested (k; Table 2). The mutant hAR in the GSF of strain 4007 have a normal k value for MT and T at 37°C, a slightly elevated k for MB at 37°C and a clearly increased k for DHT at 37°C and MB at 42°C. Strains 3287 and 4007 are able to upregulate their androgen-binding activity normally (Kaufman *et al.*, 1990a). When labeled at 37°C in the presence of 100mM cycloheximide followed by incubation at 42°C, A-ARs were thermolabile as described by Kaufman *et al.* (1990a). Previously unliganded receptors were not thermolabile under similar conditions.

Table 1. Apparent Equilibrium Dissociation Constants (K_d; nM) for GSF of Subjects 3287 and 4007.

Apparent equilibrium dissociation rate constants were averaged, and ± values represent the standard deviation of the mean; where only two experiments were performed, ± values represent the range of the values. ND denotes that the value was not determined. These data generated previously by other laboratory personnel.

Table 2. Non-equilibrium Dissociation Rate Constants (k; X 10^{-3} min^{-1}) for GSF of Subjects 3287 and 4007.

Non-equilibrium dissociation rate constants were averaged, and ± values represent the standard deviation of the mean; where only two experiments were performed, ± values represent the range of the values. ND denotes that the value was not determined. These data generated previously by other laboratory personnel.

Apparent equilibrium dissociation rate constants at 37°C
(K_d; nM)

Ligand	Normal	3287	4007
MB	0.1 to 0.3	0.1±0.04 (n=8)	0.25 (n=1)
MT	0.1 to 0.3*	0.15±0.05 (n=2)	ND
DHT	0.1 to 0.3*	0.15±0.05 (n=2)	0.25 (n=1)

*values from Pinsky *et al.*, 1985

Nonequilibrium dissociation rate constants at 37°C and 42°C
(k; X 10^{-3} min^{-1})

Ligand	Normal	3287	4007
MB (37°C)	3±0.1 (n=15)	9±0 (n=2)	5±1.3 (n=4)
DHT (37°C)	6±1.2 (n=15)*	13±3.8 (n=3)	16 (n=1)
MT (37°C)	12±3 (n=26)*	35±0 (n=2)	13±3 (n=2)
T (37°C)	24 (n=1)**	ND	24 (n=1)
MB (42°C)	8±0.5 (n=10)*	17±2 (n=2)	12±2 (n=2)

*values from Pinsky *et al.*, 1985
**results from 5α-reductase-deficient cells (Kaufman *et al.*, 1983)

D. Androgen-Binding Studies of the mutant hARs in Transfected COS-1 Cells

Expression plasmids for each mutation were transfected in AR-free heterologous cells and pathologic properties of each mutant AR were monitored. Androgen-binding assays similar to those conducted with GSF were performed to determine: androgen-binding capacity, non-equilibrium dissociation constants in offrate experiments, apparent equilibrium dissociation rate constants in Scatchard analyses (Scatchard, 1949), thermolability of previously unliganded receptors as well as A-ARs, transactivation potential, and hAR protein stability by Western blotting experiments.

1. R830L and R830Q:

To confirm that R830L and R830Q are pathogenic mutations and to distinguish their residual androgen-binding activities, transfection experiments were carried out with the SVhAR.BHEX expression vector carrying the individual mutation. Not surprisingly in view of the R^- GSF of strains REL, 2222, and 31393, mutant hARs R830L and R830Q had much lower than normal androgen-binding capacity in transfected COS-1 cells. To obtain sufficient androgen-binding activity for reliable measurement, 10μg of mutant plasmid were transfected. The androgen-binding activity was higher for the mutant hARs at 22°C than at 37°C or 4°C with all androgens tested, except for R830Q with DHT (Figure 24). The highest androgen-binding activity for normal hAR occurred at 37°C with all ligands. Three experiments with MB (Figure 24a) showed that at 37°C, the R830Q mutant hAR had about 40% and the R830L mutant hAR had about 10% of normal androgen-binding activity. Relative to their respective androgen-binding activities at 37°C, R830Q and R830L mutant hARs had increased androgen-binding activity at 22°C while that of the normal decreased by approximately 20%. At 37°C and 22°C, all three genotypes were distinguishable. At 4°C, the androgen-binding activities of the R830Q mutant hAR and normal hAR decreased to a similar extent based on their respective androgen binding activities at 37°C, while the R830L mutant hAR remained approximately the same. Essentially, the differences in androgen-binding ability between mutants R830L and R830Q was diminished at 4°C.

(a) Offrate Experiments (Non-equilibrium dissociation rates; k)

The dissociation rate (k) of various androgens from the mutant and normal hARs were determined. Both R830L and R830Q exhibited similarly increased non-equilibrium dissociation rates compared to normal hAR (Table 3). The R830Q mutant hAR had

Figure 24. The Effect of Temperature on Androgen-Binding Activities of Normal, R830L and R830Q COS-1 Transfectants.

Androgen-binding activity for the normal at 37°C was designated as 100% for the particular ligand tested, and androgen-binding activities for mutant and normal were presented as a fraction of this number. MB-binding activities for normal, R830L and R830Q hARs at 4°C, 22°C and 37°C are shown in Figure 24a. The bars represent the mean MB-binding activity from three experiments for each strain; error bars indicate the standard error of the mean. With all ligands tested, the androgen-binding activity was the greatest for the mutant hARs when measured at 22°C, while the normal hAR had its highest androgen-binding activity when measured at 37°C. A parallel experiment is shown for MT and DHT (Figures 24b and 24c, respectively).

Table 3. Non-equilibrium Dissociation Rates at 37°C and 22°C of Androgen-Receptor Complexes Formed with Various Androgens in Normal, R830L, and R830Q COS-1 Transfectants.

Non-equilibrium dissociation rates are listed for experiments performed at 37°C and 22°C. The k values were averaged and the ± values represent the standard error of the mean, or where only two experiments were perfomed ± values represent the range of the data. Note that Normal#1 and Normal#2 were averaged together. Both R830Q and R830L have increased k values at both temperatures with all ligands tested. ND denotes that this value was not determined.

Nonequilibrium Dissociation Rate Constants at 37°C

(k; X 10^{-3} min^{-1})

Ligand/Experiment	Normal#1	Normal#2	R830L	R830Q
MB				
Experiment 1	3	ND	4	3
Experiment 2	3	3	5	6
Average	3±0		4.5±0.5	4.5±1.5
MT				
Experiment 1	6	7	14	17
Experiment 2	7	7	21	23
Experiment 3	7	7	16	20
Average	6.8±0.2		17±2	20±1.7
DHT				
Experiment 1	3	3	13	9
Experiment 2	4	5	11	13
Average	3±0.8		12±1	11±2

Nonequilibrium Dissociation Rate Constants at 22°C

(k; X 10^{-3} min^{-1})

Ligand/Experiment	Normal#1	Normal#2	R830L	R830Q
MB				
Experiment 1	2	2	3	ND
MT				
Experiment 1	1	2	4	3

increased non-equilibrium dissociation rates that were at least double the normal value with DHT and MT at 37°C, MT at 22°C, and in one of two experiments with MB at 37°C. The R830Q mutant hAR results with MB could be made more decisive with a third experiment showing an increased k value. The R830L mutant hAR had increased non-equilibrium dissociation rates with all ligands tested at 37°C and 22°C: for MT and DHT, the values were at least twice normal.

(b) Scatchard Analyses (Apparent equilibrium dissociation rate constants; K_d)

Apparent equilibrium dissociation rate constants (K_ds) were measured to quantitate the receptors' affinity for various androgens. R830L and R830Q mutant hARs had elevated K_ds with MB and MT at 37°C and 22°C that were at least ten-fold higher than that of the normal hAR in most experiments (Figure 25, Table 4). Scatchard analyses using DHT revealed that the DHT-binding activity was too low to yield interpretable Scatchard plots.

(c) Thermolability of A-ARs

To obtain maximal MB-binding activity, transfectants were exposed to MB at 22°C for four hrs. When shifted to 37°C in the presence of MB and 100 mM cycloheximide for six hrs, the R830Q and R830L mutant hARs lost 69% and 67% of their MB-binding activity, respectively; normal hARs lost only 14% of their MB-binding activity (Figure 26b).

Calculations of androgen-binding activity for R830L and R830Q mutant hARs were made as follows. The MB-binding activity of R830L and R830Q mutant hARs increased over four hrs at 22°C (Figure 26a; the control experiment). For this reason, the loss of MB-binding activity measured at 37°C was underestimated when compared to the 100% value at time zero. For more accurate representation of the data, the MB-binding activity at 37°C was calculated as a function of the MB-binding activity present for the same time point at 22°C in the control experiment.

(d) Thermolability of Previously Unliganded Receptor

Transfectants were incubated in media containing 100 mM cycloheximide to inhibit new synthesis of hARs and allowed to stabilize for 30 minutes at 22°C. One series of plates was incubated at 22°C (Figure 27a; the control experiment) and one series was incubated at 37°C. After 0, 2, 4, or 6 hrs of incubation, the cells were incubated for one

Figure 25. Representative Scatchard Analyses for COS-1 Cells Transfected with Normal, R830L and R830Q Expression Vectors.

Representative Scatchard Analyses for normal, R830L, and R830Q transfectants in the presence of MB or MT. Both R830L and R830Q have decreased affinity for MB and MT, R830L having lower affinity than R830Q. The corresponding K_d values can be found in Table 4.

Table 4. Apparent Equilibrium Dissociation Rate Constants at 37°C and 22°C of A-ARs Formed with MB or MT in Normal, R830L, and R830Q COS-1 Transfectants.

Apparent equilibrium dissociation rate constants for experiments performed at 37°C and 22°C are shown. R830L and R830Q have decreased affinity for MB and MT at both temperatures, R830L having lower affinity than R830Q. K_d values were computed from the slope of the line in the Scatchard plot.

Apparent Equilibrium Dissociation Rate Constants at 37°C
(K_d, nM)

Ligand/Experiment	Normal#1	Normal#2	R830L	R830Q
MB				
Experiment 1	0.20	0.21	>30	3.96
Experiment 2*	0.18	0.13	25.7	3.24
Experiment 3	0.32	0.37	>30	6.67
MT				
Experiment 1	0.27	0.25	8.01	3.17
Experiment 2*	0.29	0.30	4.3	2.35
Experiment 3	0.58	0.79	4.78	3.34

*denotes the experiment represented in Figure 26. Note that the control is is Normal #1 in this figure.

Apparent Equilibrium Dissociation Rate Constants at 22°C
(K_d, nM)

Ligand/Experiment	Normal#1	Normal#2	R830L	R830Q
MB				
Experiment 1	0.11	0.15	0.83	0.51
MT				
Experiment 1	0.17	0.13	0.80	0.57

Figure 26. Thermolability of A-ARs Formed with MB in Normal, R830L, and R830Q COS-1 Transfectants.

Normal, R830L or R830Q mutant hARs were allowed to form complexes with MB at 22°C; the MB-binding activity after four hours preincubation was specified as 100% for each transfectant. Cells were then challenged at 37°C for various lengths of time with MB in the presence of cycloheximide. In the control experiment, cells were continuously incubated at 22°C with MB in the presence of cycloheximide (Figure 26a). MB-AR complexes formed in both R830L and R830Q transfectants were more thermolabile than normal at 37°C (Figure 26b). Bars are the mean of MB-binding activity for three experiments for normal and R830L hARs and the mean for two experiments for the R830Q hAR; error bars represent the standard error of the mean for normal and R830L and the range for R830Q.

Figure 27. Thermolability of Previously Unliganded Androgen Receptor in Normal, R830L, and R830Q COS-1 Transfectants.

Normal or R830L or R830Q mutant hARs were incubated at 22°C in media containing cycloheximide for 30 minutes. Cells were then challenged at 37°C for various lengths of time and then allowed to bind MB for one hour; MB-binding activity before the temperature shift is specified as 100% for each transfectant. In the control experiment, cells underwent a continued incubation at 22°C for various lengths of time before MB-binding activity was determined (Figure 27a). MB complexes formed at 37°C in R830L and R830Q transfectants were not more thermolabile than normal, and may even be slightly more thermostabile than normal (Figure 27b). Bars are the mean of MB-binding activity for four experiments for normal and R830L hARs and the mean for three experiments for the R830Q hAR; error bars represent the standard error of the mean.

hour with media containing ^{3}H-MB in the presence of cycloheximide. When MB-binding activity was measured, the mutant hARs did not exhibit increased thermolability compared to normal hAR; if anything, they appeared to be more thermostabile than normal hAR. After 6 hours, the normal hAR retained 59% of the original binding activity while R830L and R830Q mutant hARs retained 78% and 65%, respectively (Figure 27b).

(e) Transactivation Potential

When transfectants were exposed to various concentrations of ^{3}H-MB for 72 hours, the R830Q mutant hAR was able to transactivate the growth hormone reporter gene better than the R830L mutant AR: 16-31% of normal versus 1-8% of normal respectively. At the end of 72 hours of incubation, there was no detectable specific MB-binding activity in the cells transfected with either mutant hAR. The expression of the growth hormone reporter gene directed by the R830L and R830Q mutant AR-androgen complexes is shown in Figure 28.

(f) Western Blotting Analysis

Transfectants were labeled with ^{3}H-MB over 18 hours. At various times, MB-binding activity was determined, and cellular protein was harvested for Western analysis. Co-migration of R830L and R830Q mutant hAR protein with the normal hAR protein revealed that these mutant hARs were of normal size (~110kDa; Figure 29a). After binding MB for 18 hours at 37°C, R830L and R830Q had lost 100% and 82% of the initial MB-binding activity, respectively, while the normal gained 10 to 20%. Despite a dramatic concurrent loss of MB-binding activity over 18 hours, there was no visually detectable loss of mutant hAR protein. Under the same conditions, less immunoreactive normal hAR gave higher MB-binding activity that remained constant or increased very slightly over 18 hours. Densitometric analysis was hindered by the high levels of protein expressed from the increased amounts of normal or mutant SVhAR.BHEX plasmid that were transfected to obtain reliable androgen-binding activity. Transfectants that were incubated in the absence of ligand also had hARs of normal size (Figure 29b). The amount of immunoreactive protein in transfectants incubated with MB was greater than those incubated without MB. hAR protein expressed in transfectants was driven by a constitutive promoter (SV40 large T antigen), so the latter increase reflects protein stability in the presence of ligand rather than increased transcription from the promoter.

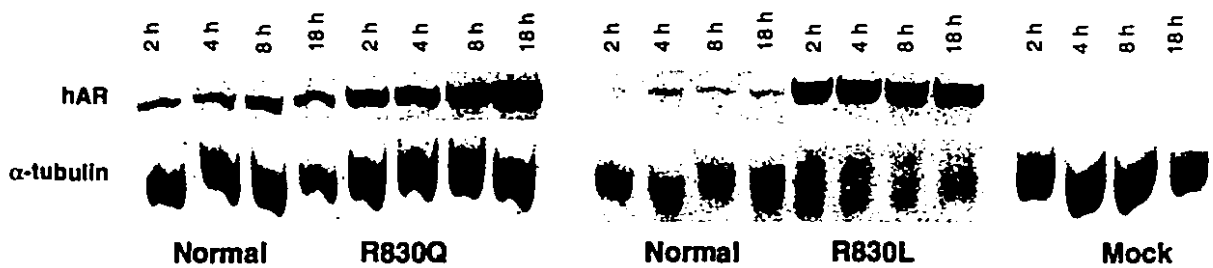
Figure 28. Transactivation Potential for Normal or Mutant hARs in Normal, R830L, and R830Q COS-1 Transfectants.

Transactivation potential for Normal, R830L, and R830Q mutant hARs was measured as the ability of the MB-receptor complex to activate transcription of growth hormone from a cotransfected MMTV-GH reporter gene. Both R830L and R830Q mutant hARs were dramatically inferior at transactivating the reporter gene compared to normal.

Figure 29. Immunoblotting and MB-Binding Activity for Normal, R830L, and R830Q hARs in COS-1 Transfectants.

Cellular lysate was harvested from normal, R830L and R830Q transfectants at various times after prolonged incubation with MB. MB-binding activity was also measured at each time point (Figure 29a). More immunoreactive hAR protein was present for R830L and R830Q compared to normal hAR because ten-fold more mutant plasmid was transfected to achieve reliable MB-binding activity. Blots were incubated with the monoclonal anti-α tubulin antibody (yielding a band of ~55 kDa) to control for equal protein loading. Immunoreactive hAR protein in cells that were incubated in media without MB is shown in Figure 29b. Mock-transfected cells incubated in the presence of MB were included as a negative control for immunoreactive hAR protein in COS-1 cells.

B.



2. E771A and R870G:

To confirm the pathogenicity of the E771A and R870G mutations, transfection experiments were carried out with the SVhAR.BHEX expression vector carrying the individual mutations. Normal levels of androgen-binding activity was obtained with both E771A and R870G, in accord with the data on GSF of 3287 and 4007, respectively.

Other hormone binding assays used different concentrations of radioinert MB, progesterone, estradiol, dexamethasone, or spironolactone (an anti-androgen) to displace bound ^{3}H-MB for normal and mutant R870G hAR (Figures 30a-e, respectively). These studies revealed that progesterone, estradiol, dexamethasone, and spironolactone at concentrations of 10X, 100X or 1000X the concentration of ^{3}H-MB used in the experiment did not successfully displace ^{3}H-MB binding until the ligand was increased to 1000X the concentration of ^{3}H-MB. Radioinert MB, however, was a successful at displacing the ^{3}H-MB even at 10X concentration, reducing the ^{3}H-MB-binding activity to less than 20% of the activity with 5 nM ^{3}H-MB alone. In all displacement experiments except with dexamethasone at 100X concentration, the radioinert hormones displaced ^{3}H-MB more easily from the mutant hAR compared to the normal hAR.

(a) Offrate Experiments (Non-equilibrium dissociation rates; k)

The non-equilibrium dissociation rates of various androgens from normal, E771A and R870G mutant hARs were determined (Table 5). The E771A mutant hAR exhibited increased k values with MT and DHT measured at 37°C that were at least twice as fast as normal. With MB, k values for E771A were clearly increased over normal at 37°C and 42°C. Non-equilibrium dissociation rates for the R870G mutant hAR remained in the range of normal with all ligands tested, even with MB measured at 42°C.

It is noteworthy that the R870G mutant did not show an increased k value with DHT. Since DHT had an increased k value in 4007's GSFs, which contain much less androgen receptor protein, I performed a transfection experiment with reduced amounts (0.05 μg) of normal and mutant expression vector. This experiment also revealed a normal result (Table 6).

(b) Scatchard Analyses (Apparent equilibrium dissociation rate constants; K_d)

E771A and R870G mutant hARs showed increased apparent equilibrium dissociation rate constants with MB and MT at 37°C (Figure 31, Table 7). Both E771A and R870G mutant hARs showed K_ds for MB that were approximately one and a half to

Figure 30. MB-Binding Activity of R870G and Normal COS-1 Transfectants when Incubated in the Presence of Excess Concentrations of Various Radioinert Hormones.

Normal and R870G transfectants were incubated in 5nM ^{3}H-MB with excess concentrations of various radioinert hormones to determine if displacement of bound ^{3}H-MB would occur. The radioinert hormones used were: MB (Figure 30a; control), progesterone (Figure 30b), estradiol (Figure 30c), dexamethasone (Figure 30d), or spironolactone (Figure 30e). The experiments show that the R370G amino acid substitution does not confer increased binding activity of mutant hAR for other steroid hormones tested.

Table 5. Non-equilibrium Dissociation Rates at 37°C and 42°C of A-ARs Formed with Various Androgens in Normal, E771A, and R870G COS-1 Transfectants.

Non-equilibrium dissociation rates are listed for experiments performed at 37°C and 42°C. The mutant E771A hAR had increased k values at both temperatures with all ligands tested. The mutant R870G hAR k values fell in the range of normal at both temperatures with all ligands tested. Values represent the mean k values for the experiments; ± indicates the range for MB at 37°C and 42°C and the standard error of the mean for MT at 37°C. Normal#1 and Normal#2 were averaged together.

Nonequilibrium Dissociation Rate Constants at 37°C
($k; X 10^{-3} min^{-1}$)

Ligand/Experiment	Normal#1	Normal#2	E771A	R870G
MB				
Experiment 1	3	3	4	3
Experiment 2	3	4	5	3
Average	3.3±0.3		4.5±0.5	3.0±0
MT				
Experiment 1	8	7	17	7
Experiment 2	6	4	13	6
Experiment 3	5	6	12	6
Average	6.0±0.6		14±1.5	6.3±0.3
DHT				
Experiment 1	3	4	6	5

Nonequilibrium Dissociation Rate Constants at 42°C
($k; X 10^{-3} min^{-1}$)

Ligand/Experiment	Normal#1	Normal#2	E771A	R870G
MB				
Experiment 1	4	3	11	4
Experiment 2	4	3	10	3
Average	3.5 ±0.3		10 ± 0.5	3.5 ± 0.5

Table 6. Non-equilibrium Dissociation Rates at 37°C for DHT when Reduced Amounts of Normal or R870G hAR Expression Vectors were Transfected into COS-1 Cells.

Non-equilibrium dissociation rates are listed for experiments performed at 37°C with DHT. Mutant R870G hAR did not exhibit elevated k values over normal when 0.5 or 0.05 μg of plasmid was transfected.

Nonequilibrium Dissociation Rate Constants at 37°C

(k; X 10 $^{-3}$ min $^{-1}$)

Ligand/Experiment	Normal	R870G
DHT - 0.5 µg plasmid		
Experiment 1	6	6
DHT - 0.05 µg plasmid		
Experiment 1	8	7

Figure 31. Representative Scatchard Analyses for COS-1 Cells Transfected with Normal, E771A, and R870G Expression Vectors.

Representative Scatchard Analyses for Normal, E771A , and R870G transfectants in the presence of MB, MT or DHT are shown. Both E771A and R870G mutant hARs have decreased affinity for MB, MT, and DHT compared to the normal hAR. The corresponding K_d values can be found in Table 7.

Table 7. Apparent Equilibrium Dissociation Rate Constants at 37°C of A-ARs Formed with MB, MT or DHT in Normal, E771A, and R870G COS-1 Transfectants.

Apparent equilibrium dissociation rate constants for experiments performed at 37°C with MB, MT, and DHT. E771A and R870G mutant hARs had decreased affinity for all three ligands compared to the normal hAR. K_d values were computed from the slope of the line in the Scatchard plot. The * denotes the experiment represented in Figure 31.

Apparent Equilibrium Dissociation Rate Constants
(K_d, nM)

Ligand/Experiment	Normal#1	Normal#2	E771A	R870G
MB				
Experiment 1*	0.28	0.28	0.83	0.8
Experiment 2	0.20	0.20	0.33	0.36
Experiment 3	0.71	ND	0.78	0.96
MT				
Experiment 1*	0.51	0.51	0.78	1.0
Experiment 2	0.35	0.30	0.64	0.56
Experiment 3	0.88	0.96	0.96	0.79
DHT				
Experiment 1*	4.7	5.3	7.4	6.4

* denotes the experiments represented in Figure 32. Note that the control is Normal#1 in this figure.

three times higher than normal hAR, with the exception of E771A in experiment 3. With MT the difference was similar, with increased K_ds of approximately one and a half to two times higher than the normal hAR.

(c) Thermolability of A-ARs

To obtain maximal MB-binding activity, transfectants were exposed to MB at 37°C for four hrs. When shifted to 42°C in the presence of MB and 100 mM cycloheximide for six hrs, the E771A and R870G mutant hARs lost 84% and 72% of their MB-binding activity, respectively; normal hARs lost only 33% of their MB-binding activity (Figure 32b). In the control experiment, where the cells were only incubated at 37°C, neither mutant receptor lost MB-binding activity at a greater rate than the normal receptor (Figure 32a).

(d) Recovery of Androgen-Binding Activity After a Temperature Challenge

Cells were allowed to bind MB at 37°C for one hour. Cycloheximide was added to the media containing hormone for subsequent time points. After one hour at 42°C, normal and mutant R870G hARs lost approximately the same amount of MB-binding activity, 37% and 40% respectively, while E771A lost 75%. Subsequent incubation at 37°C for two hrs allowed R870G to recover its androgen-binding activity even better than the normal, 95% compared to 78% of pre-temperature challenge levels, respectively, while E771A performed more poorly recovering only to 50% pre-temperature challenge levels (Figure 33).

(e) Thermolability of Previously Unliganded Receptor

New synthesis of normal and mutant hARs was inhibited by the addition of 100 mM cycloheximide to the media. One series of plates was incubated at 37°C and one series was incubated at 42°C. After 0, 2, 4, or 6 hrs of incubation, the cells are incubated with media containing ^{3}H-MB and 100 mM cycloheximide for one hr. When MB-binding activity was measured, the mutant hARs were not more thermolabile than normal hAR; E771A lost 56% and R870G lost 47% of MB-binding activity while the normal hAR lost 52% (Figure 34b). The control experiment was done at 37°C without a shift to 42°C (Figure 34a).

(f) Transactivation Potential

After 72 hours in the presence of various concentrations of ^{3}H-MB, the E771A and R870G mutant hARs transactivated the growth hormone reporter gene less that the normal

Figure 32. Thermolability of A-ARs Formed with MB in Normal, E771A, and R870G COS-1 Transfectants.

Normal, E771A or R870G mutant hARs were allowed to form complexes with MB at 37°C; the MB-binding activity after four hours pre-incubation was specified as 100% for each transfectant. Cells were then challenged at 42°C for various lengths of time with MB in the presence of cycloheximide. In the control experiment, cells underwent a continued incubation at 37°C with MB in the presence of cycloheximide (Figure 33a). MB complexes formed in both E771A and R870G transfectants were more thermolabile than normal at 42°C (Figure 33b). Bars are the mean of MB-binding activity for four experiments for normal, E771A and R870G hARs; error bars represent the standard error of the mean.

Figure 33. Recovery of Androgen-Binding Activity After a One Hour Temperature Challenge at 42°C for Normal, E771A, and R870G COS-1 Transfectants.

Normal, E771A or R870G mutant hARs were allowed to form complexes with MB at 37°C for one hour and the MB-binding activity at this time was specified as 100% for each transfectant. Transfectants were then challenged at 42°C for one hour and MB-binding activity was determined. Subsequently, cells were allowed to recoup their MB-binding activity for one and two hours at 37°C. MB-AR complexes formed in normal and R870G transfectants behaved similarly, recovering most of their MB-binding activity after two hours at 37°C, however, E771A was not as successful, recovering only half of its MB-binding activity after the same time period. Bars are the mean of MB-binding activity for two experiments for normal, E771A and R870G hARs; error bars represent the range of the mean values.

Figure 34. Thermolability of Previously Unliganded Androgen Receptor in Normal, E771A, and R870G COS-1 Transfectants.

Normal or E771A or R870G mutant hARs were incubated at 37°C in media containing cycloheximide for 30 mins. Cells were then challenged at 42°C for various lengths of time and then allowed to bind MB for one hr; MB-binding activity before the temperature shift is specified as 100% for each transfectant. In the control experiment, cells were a continuously incubated at 37°C for various lengths of time before MB-binding activity was determined (Figure 34a). MB-AR complexes formed at 37°C in E771A and R870G transfectants were not more thermolabile than normal, and may even be slightly more thermostable than normal (Figure 34b). Bars are the mean of MB-binding activity for four experiments for the normal hAR, and two experiments for E771A and R870G hARs; error bars represent the standard error of the mean for normal and the range of the mean values for E771A and R870G.

hAR: 63-73% and 45-61% of normal, respectively (Figure 35). In one experiment the E771A hAR performed better than the R870G hAR, in another experiment, they were equivalent in transactivating the reporter gene.

(g) Western Blotting Analysis

Normal, E771A and R870G transfectants were labeled with ^{3}H-MB over 18 hrs. At various times MB-binding activity was determined, and cellular protein was harvested for Western analysis. Co-migration of E771A and R870G mutant hAR protein with the normal hAR protein revealed that these mutant hARs were of normal size (~110kDa; Figure 36a). After binding MB for 18 hrs at 37°C, E771A and R870G hARs had lost 40% and 22% of the initial MB-binding activity respectively, while the normal had gained 30%. Despite the concurrent loss of MB-binding activity over 18 hrs, there was no visually detectable loss of mutant hAR protein. Under the same conditions, less immunoreactive normal hAR gave MB-binding activity that increased over 18 hrs. Densitometric analysis was hindered by the high levels of protein expressed from the amounts of normal or mutant SVhAR.BHEX plasmid transfected to obtain reliable androgen-binding activity. The amount of immunoreactive protein in transfectants incubated with MB was greater than those incubated in media without MB (Figure 36b) reflecting increased protein stability in the presence of ligand.

E. Baculovirus Expression System

Due to the large size of the PETL.BHEX plasmid, transformation of bacteria by heat-shock was not always dependable, although PETL.BHEX.E771A and PETL.BHEX.R830Q were obtained through this procedure. The other two mutants, PETL.BHEX.R830L and PETL.BHEX.R870G, were not obtained, despite numerous attempts at transforming competent XL1-Blue cells through heat shock and electroporation procedures.

Sf9 cells were infected and recombinant virus was harvested for PETL.BHEX.E771A and PETL.BHEX.R830Q. Viral titre was determined by in our lab by Dr. Lenore Beitel who will conduct further studies with these recombinant clones.

Figure 35. Transactivation Potential for Normal or Mutant hARs in Normal, E771A, and R870G COS-1 Transfectants.

Transactivation potential for Normal, E771A, and R870G mutant hARs was measured as the ability of the MB-AR complexes to activate transcription of growth hormone from a cotransfected MMTV-GH reporter gene. Both E771A and R870G mutant hARs were inferior at transactivating the reporter gene compared to normal.

Figure 36. Immunoblotting and MB-Binding Activity for Normal, E771A, and R870G hARs in COS-1 Transfectants.

Cellular lysate was harvested from normal, E771A and R870G transfectants at various times after prolonged incubation with MB. Concurrent MB-binding activity was determined. Blots were incubated with the monoclonal anti-α tubulin antibody (yielding a band of ~55 kDa) to control for equal protein loading. Immunoreactive hAR protein in cells that were incubated in media without MB is shown in Figure 36b. Mock-transfected cells incubated in the presence of MB were included as a negative control for immunoreactive hAR protein in COS-1 cells.

IV. DISCUSSION

The four mutations in the hAR gene identified in our lab were found to cause varying degrees of AIS. Sequencing of exons two to eight revealed point mutations that caused amino acid substitutions in each case. A transversion of A to C at amino acid position 771 in exon five causes a substitution of alanine for a conserved glutamic acid in subject 3287; this subject has PAIS. A transversion of G to T, and a transition of G to A at amino acid position 830 in exon 7 cause a substitution of a conserved arginine by leucine or glutamine, respectively. These substitutions cause CAIS in three unrelated subjects, each with a positive family history: REL, 2222, 31393. A transition of A to G at amino acid position 870 in exon eight causes an arginine to glycine substitution in subject 4007 and results in MAIS. Subject 4008, the sister of 4007, was found to be a carrier of her brother's mutation.

Each of the four mutations replace amino acids in the ABD. Presumably their altered conformations cause the mutant hARs exhibit distinctive impairment in androgen-binding. The four mutant hARs, previously studied in the GSF, were further characterized in transfected COS-1 cells. Where no androgen-binding activity was detected in GSF for the mutations at codon 830, transfectants proved their pathogenicity and revealed the functional disadvantage of the mutant hARs. The results in the GSF and COS-1 transfectants of the mutations at codon 771 and 870 were compared in order to prove the pathogenicity of each mutation and to characterize the nature of their respective androgen-binding defects.

The mutations affecting codon 830 render the GSF devoid of androgen-binding activity; thus, further kinetic analyses of these receptors were not possible in this cellular context. It was not surprising that COS-1 cells transfected with R830L or R830Q also had very low androgen-binding activity. In fact, even in this cellular context, their androgen binding was so low that, for most of the transfection studies, 10-fold more mutant plasmid than normal plasmid had to be transfected to achieve sufficient mutant androgen-binding activity for analysis.

Androgen-binding activity for the R830L and R830Q mutant hARs increase at 22°C compared to 37°C, while that of the normal hAR decreases. This augmented androgen-binding of the mutant hARs at 22°C suggests that at that temperature they are able to adopt a conformational state that favors androgen binding. This may represent a shift in the association:dissociation equilibrium thereby allowing an increased rate of AR-androgen association, a decreased rate of AR-androgen dissociation, or a combination of both. Both R830L and R830Q mutant hARs have equivalent increased androgen dissociation rates at

22°C and 37°C indicating that the augmented androgen-binding activity may be attributable to increased androgen association at 22°C compared to that at 37°C.

The R830L and R830Q mutant hARs formed over a four-hour incubation with MB at 22°C were more thermolabile than normal hAR over a subsequent six-hour incubation at 37°C. This thermal misbehavior was not observed in similar experiments with previously unbound mutant receptors: the R830L and R830Q mutant hARs were no more thermolabile than normal hAR. The results from these experiments suggests that the thermolability exhibited by mutant AR-androgen complexes at 37°C is not attributable to thermolability of the portion of the receptor population that has not previously bound ligand.

It was a curious observation that the R830L and R830Q mutant hARs, but not the normal hAR, were able to augment their MB-binding activity over six hours at 22°C in the presence of MB and cycloheximide (Figure 26a). Cycloheximide was added to inhibit further protein translation, so that the same receptor population could be studied for the duration of the experiment. The increase in MB-binding activity of the mutant hARs could not be attributed to increased receptor quantity over the course of the experiment, but was due to another factor or factors. At 22°C the mutant hARs apparently adopt a conformational state that favors androgen binding, and prolonged incubation in the presence of MB and cycloheximide at this temperature allows a larger proportion of the receptors to achieve this ligand-binding state. Conversely, the normal hAR which achieves its highest androgen-binding activity at 37°C, ceased to augment its activity at 22°C to the extent of the mutant hARs. The fact that the normal plasmid, transfected at a quantity of 10 times less than the mutant, does not exhibit similar "upregulative" behavior suggests the possibility that the increase in MB-binding activity of the mutant hARs could also be a function of the increased plasmid transfected for mutant hARs. Interestingly, the mutant hARs incubated at 22°C in the absence of hormone for up to six hours before a one-hour MB-binding assay, did not show an equivalent increase in their respective MB-binding activities (Figure 27a). A larger proportion of the mutant hARs progressively adopted an androgen-binding state over 6 hours in the presence of cycloheximide and MB, than in those incubated with cycloheximide in the absence of MB. This suggests that the presence of MB plays a role in the increased stability of the mutant A-ARs over time.

Western blots of hAR protein from R830L- and R830Q-transfected COS-1 cells revealed that the protein produced in these cells were normal-sized. A single immunoreactive band for the normal hAR and the mutant hARs on the Western blot revealed that neither the normal nor the mutant hARs were prone to proteolysis. Despite the dramatic decrease in MB-binding activity for the mutant hARs measured over 18 hours

at 37°C, there was no coordinate loss of immunoreactive hAR protein. MB-binding activity for the normal hAR was increased over the values for either mutant hAR and remained stable or increased slightly over the course of the experiment, despite much less immunoreactive protein compared to the R830L and R830Q mutant hARs. This indicated that the initial MB-binding activity detected for the mutant hARs involved only a small portion of the total amount of mutant hAR protein expressed in the transfectants. Prolonged incubation at 37°C with MB caused the mutant hARs to progressively adopt a non-binding state.

The transactivational activity of both R830L and R830Q mutant hARs was clearly impaired, R830L being worse that R830Q. This result is in accord with their respective androgen-binding capacity at 37°C. This result is not surprising given that the typical CAIS phenotype of these subjects does not indicate expression of any androgen-responsive genes responsible for supporting male sexual development.

Point mutations have been responsible for 14 amino acid substitutions at arginines within the ABD of the hAR, including the two studied here. Three of the 14 have yielded stop codons, one at codon 830 (DeBellis et al., 1992), all resulting in subjects with CAIS. Interestingly eight of the 14 mutations in the ABD causing substitutions for arginine have occurred in exon 7 (Patterson *et al.*, 1994a), yet exon 7 contains less than 30% of the arginines in the ABD. Nine of 14 cases of arginine substitutions in the ABD result in subjects with CAIS. One results in MAIS and was studied here: R870G.

There have been a few studies comparing alternative amino acid substitutions at the same codon of the AR that give receptor-deficient or R^+ GSF (Kazemi-Esfarjani *et al.*, 1993; Beitel *et al.*, 1994; Ris-Stalpers *et al.*, 1991; Prior *et al.*, 1992). The present work with the R830L and R830Q mutant hARs illustrates how comparative analysis of mutant ARs overexpressed in COS-1 cells can be informative even when the GSF of the affected subjects are R^-. The differences between the two mutants illustrated by these kinetic studies is a reflection of the impact of two aberrant conformations generated by the amino acid change. These studies revealed that arginine at this codon is crucial for appropriate hAR action and that subtle differences result from the placement of leucine or glutamine at codon 830. This is also supported by the fact that it is a conserved amino acid at the homologous position among members of the steroid receptor subfamily, and it is conservatively replaced by lysine in the estrogen receptor. These studies will be helpful in delineating the subtle structure-function relations of the androgen receptor.

The results obtained with transfectants of E771A and R870G mutant hARs can be compared to those obtained with GSFs of subjects 3287 and 4007, respectively. Normal levels of androgen-binding activity were obtained both with the GSF and the transfected

COS-1 cells. This suggested that these mutant hARs have a qualitative androgen-binding defects. The absolute values for k and K_d of the normal or mutant hARs in transfected COS-1 cells were not identical to those obtained from their GSFs. Hence, the mutant hARs were designated as abnormal if they fell outside the range of the normal hAR in a particular transfection experiment. In the GSFs of subject 3287, the non-equilibrium dissociation rate constants were increased with all ligands tested; in the corresponding transfectants, the k values were also elevated over the normal hAR with all ligands tested. In the GSFs of subject 4007, the k values were normal for MB and MT, but were elevated with DHT. The corresponding transfectants had k values in the range of normal hAR for all three ligands. An attempt to closer imitate the hAR concentrations in GSF by transfecting less mutant plasmid did not reveal an increased k value for DHT. The K_d values for both E771A and R870G mutant hARs were questionably abnormal in transfectants; in GSF, however, they remained in the range of the normal hAR.

In terms of the clinical phenotype of subject 4007, the faster dissociation rate with DHT in the GSF is noteworthy. DHT is responsible for development of the external genitalia and subject 4007 has an undervirilized, but normally-formed external genitalia.

The normal K_d values despite increased k values obtained from the GSF of subjects 3287 and 4007 merit further consideration. Usually, increased k values are associated with increased K_d values (Brown *et al.*, 1982; Ris-Stalpers *et al.*, 1991; Prior *et al.*, 1992; Kazemi-Esfarjani *et al.*, 1993; Beitel *et al.*, 1994). For normal K_d values to accompany increased k values, an unusual shift in the association:dissociation ratios would have to occur. In order to maintain a normal equilibrium affinity for androgens, the increased offrate of androgen from the mutant receptor must be compensated by an equally increased on-rate of the androgen to its receptor. Grino *et al.* (1988) found a similar situation between k and K_d values for the hAR in GSF of a family with MAIS. The affected individuals are fertile but have some features of androgen resistance; they are undervirilized, have gynecomastia and elevated serum androgen levels. A mutation causing similar receptor misbehavior in the GR has also been reported (Palmer and Harmon, 1991). It has been shown for the GR that rates of association with various steroid agonists are similar (Bell and Munck, 1973), but increased rates of association of an antagonist from the GR have been described (Lamontagne *et al.*, 1984). Thus, it seems possible that the E771A and R870G mutations cause the mutant hARs to have a faster association rate for androgen with an accompanying increased dissociation rate. The putative increased rate of association of androgen with the mutant receptor is difficult to measure because of its rapidity. These measurements could not be made in our laboratory.

The thermolability data of the E771A and R870G transfectants were in accord with the GSF data from the respective subjects. Both mutant hARs, when complexed to MB either in the GSF or transfected COS-1 cells, exhibited increased thermolability. Previously unbound mutant hAR in either GSF or the transfected COS-1 cells were not thermolablile. This suggests that the altered conformation of the hARs induced by E771A and R870G make the mutant hARs more vulnerable than the normal hAR to increased dissociation or decreased association with MB at 42°C; E771A more so than the R870G mutant hAR. It is interesting to note that the hARs with increased k values and normal K_d values studied by Grino *et al.* (1988, 1989) were also thermolabile.

Another experiment where E771A and R870G mutant hARs illustrated their thermal misbehavior was their recovery of androgen-binding activity after a one-hour incubation at 42°C. The mutant A-ARs experienced a dramatic decrease of MB-binding activity at 42°C compared to the normal A-AR. This was followed by an inability of the mutant hARs to recover the lost androgen-binding activity to the extent of the normal hAR, once shifted back to 37°C. In these experiments E771A mutant hAR performed more poorly than the R870G mutant hAR.

After incubation with MB for 18 hrs, the R870G and E771A mutant hARs lost significant MB-binding activity; the normal hAR was able to augment its MB-binding activity over the same time period. Western blotting experiments revealed one immunoreactive band for normal and the mutant hARs, indicating proteolysis was not a factor in the loss of MB-binding activity over 18 hrs for the mutant hARs. Thus, prolonged incubation with MB caused the mutant hARs to progressively adopt a non-binding state.

Both E771A and R870G mutant hARs were less capable of transactivating a reporter gene than the normal hAR. In COS-1 transfectants, the E771A and R870G mutant hARs displayed increased A-AR thermolability, and in the case of E771A mutant hAR increased rates of dissociation, that translated to impaired transactivation. There was no clear hierarchy of transactivation potential of E771A and R870G mutant hARs despite the hierarchy that existed between them in various androgen-binding assays in both the GSF and COS-1 transfectants. It is possible that any putative differences in transactivation potential did not manifest themselves under these conditions. Transactivation experiments use an artificial reporter gene in heterologous host cells in an attempt to monitor the transcriptional activation ability of the mutant hARs. This artificial environment often can provide a clue to the extent of transactivational impairment of mutant hARs. A reporter gene driven by a true ARE rather than the GREs within the MMTV-LTR may be able to reveal a decreased transactivational ability of mutant E771A hAR as compared to R870G

mutant hAR. The clinical phenotypes of subjects 3287 and 4007 are true *in vivo* evidence that neither mutant hAR was capable of supporting normal male sexual development, and that the E771A mutant hAR was less so. The data from the transactivation assays do not contradict these *in vivo* observations.

The increased k and thermolability of mutant hARs in GSFs from subjects 3287 and 4007 have been analyzed in the context of a model of serial A-AR energy states (Kaufman *et al.*, 1990a). According to this model, the normal hAR is conformationally mobile and undergoes a series of androgen-dependent transitions. The different dissociation rates for various androgens from the normal hAR (Table 2) correspond to the different energy states the receptor can assume. T, MT, DHT, and MB form a hierarchy of dissociation states that correspond to distinct energy states (Kaufman *et al.*, 1990b). According to this model, the mutant hARs are not able to undergo all the transitions of the normal hAR and are "blocked" in androgen-inappropriate states. Thus, mutant hARs attain to obtain androgen dissociation rates that are uncharacteristic for the particular ligand. For instance, the MB-receptor complexes formed within subject 4007's GSFs behave like DHT-AR complexes, and DHT-AR complexes behave like MT-AR complexes. In other words, the dissociation state of A-AR depends on the energy state of the complex and not the type of androgen complexed to the receptor. This model is not absolute, however: subject 3287's hAR complexed to MB dissociates at temperature-dependent rates that fall between two energy states. For a complete review of this model refer to Kaufman *et al.*, 1990a and 1990b.

A mutation in the vicinity of arginine-870 was found in a prostate tumour cell line, LNCaP. It contains an androgen receptor with a mutation in the ABD at codon 876 where neutral-nonpolar amino acid threonine is replaced by the neutral-polar amino acid alanine (Veldscholte, 1990). This mutation is only six amino acids downstream of the R870G mutation. This seemingly conservative substitution at codon 876 allows for a broader range of steroid specificity conferring response from a reporter gene upon the addition of progestins, estradiol, and several antiandrogens. A comparatively less conservative amino acid substitution of R870G did not show any broader range of steroid specificity.

R870G is located very close to the putative dimerization region of the hAR. The conserved amino acids for the dimerization domain are thought to localize to amino acids 852-886 of the hAR (White *et al.*, 1991). Although arginine at codon 870 does not fall within the ultra-conserved hydrophobic residues of this region, it is just two amino acids downstream of the final amino acid in that group. Arginine at codon 870, although not conserved throughout the subfamily, may play an important role in the conformation of the

dimerization interface and thus, the homodimerization of the hAR. No dimerization studies were performed here to test this hypothesis, but the results may prove interesting.

The pathogenicity of E771A, R830L, R830Q, and R870G mutant hARs were proven here through functional studies with each mutant receptor. Arginine at position 830 in the ABD of the hAR and in homologous postions of the GR, MR, and PR, indicate that this residue is structurally important for receptor function. The results of transfection studies with mutant receptors R830L and R830Q in the hAR clearly illustrate the functional impairment of these mutant hARs. The low androgen-binding activity of both mutant hARs at 37°C, as well as the qualitative defects of androgen binding, including increased androgen dissociation, decreased androgen affinity, increased thermolability of A-ARs , and negligible transactivational capability of the hARs substantiate the pathogenicity of both mutations. From my experiments, I can conclude that leucine or glutamine in place of arginine-830 causes significant conformational change within the androgen-binding domain that disallows normal androgen-binding activity. The severe functional impairment of the R830L and R830Q mutant hARs correlates well with the CAIS phenotype in three affected individuals.

Positions homologous to codon 771 of the hAR are occupied by glutamic acid in the GR, MR, and PR implying that this residue may be structurally important for the function of all four receptors. Although arginine at residue 870 of the ABD of the hAR is not conserved in any member of the subfamily, this position in the PR is occupied by another dibasic amino acid, lysine. The phenotype of subject 4007 suggests a relatively mild defect in the function of the mutant R870G hAR. For most experiments, there was a clear hierarchy for mutant E771A and R870G hAR performance; R870G mutant hAR was superior. E771A mutant hAR displayed increaed dissociation rates and thermolability of A-ARs compared to the R870G mutant hAR. These data are in accord with the clinical phenotypes of the two individuals; the hAR of the more affected individual, 3287, is more impaired than the hAR of subject 4007. The results from kinetic studies on androgen binding in the GSF and transfected COS-1 cells for E771A and R870G mutant hARs confirm that these mutations are pathogenic.

With the advent of DNA cloning and transfection techniques, the ability to express genes in heterologous systems became a important tool for analyzing the specific effects of mutations and deducing fine-structure maps of proteins. This strategy has long been used for the analysis of the structure-function relationships of steroid receptors. Another useful tool has been the expression of the androgen receptor by the baculovirus in a heterologous host cell of *Spodoptera frugiperda* (Sf9) (Xie *et al.*, 1992; Wong *et al.*, 1993; Kallio *et al.*,

1994). Mutations E771A and R830Q have been successfully incorporated into the baculovirus. Expression studies will be carried out by others in the laboratory at the L.D.I.

The advantages of the baculovirus expression system are the high yield (up to 30% of total cellular protein) of the protein of interest, with similar immunological and functional properties as those found in mammalian cells. Structural and functional studies with members of the steroid hormone superfamily have already been performed with receptors derived in the baculovirus expression system (Elliston *et al.*, 1990; Srinivasan, 1992; Jänne *et al.*, 1993b; Obourn *et al., 1993*; Ross *et al.*, 1991). For instance, the rat androgen receptor so expressed has the appropriate size, binds androgen and interacts with AREs with high affinity (Xie *et al.*, 1992; Kallio *et al.*, 1994). Footprinting experiments using deletion mutants in this system showed that the receptor binds the DNA as a dimer (Kallio *et al.*, 1994). The hAR has been expressed in this system to investigate nuclear localization, dimerization, and DNA-binding properties (Wong *et al.*, 1993). Expression of hAR in the baculovirus system has use for investigations of its role as a transcription factor. Due to the high amount of hAR protein that can be expressed in these cells, the hAR can be isolated and used in *in vitro* transcription studies. Studies with E771A and R870G mutant hARs in the baculovirus system will be carried out in the lab to further define the mechanism of functional impairment of these receptors.

V. CONCLUSION

The pathogenicity was proven for four point mutations in the ABD of the hAR. Two mutations involve an amino acid change at an identical codon. Of the three codons affected, two involve amino acid substitutions at residues that are strictly conserved among members of the steroid receptor subfamily that includes the AR. Substitutions of leucine or glutamine for the conserved arginine at codon 830 cause deficient and defective androgen-binding activity of the mutant hARs. In the GSF of these subjects, androgen-binding activity is barely detectable. In transfected COS-1 cells, the androgen-binding defect is revealed as the instability of a limited quantity of A-ARs. These mutant hARs had negligible ability to transactivate the reporter gene. These data are in accord with the CAIS phenotype of the corresponding individuals; there was no evidence that the mutant hARs had any ability to generate an androgen response capable of supporting male sexual development. Another amino acid substitution that occurred at a conserved residue is E771A. Subnormal activation of the reporter gene by the E771A mutant hAR was due to instability of A-ARs shown through offrate and thermolability experiments. These data are in accord with what is observed in the clinical phenotype of PAIS in this individual. In other words, the mutant hAR of this subject allowed a degree of androgen-response, enough to support significant masculinization of the individual. The R870G amino acid substitution occurred at a unconserved residue. The R870G mutant hAR conferred increased thermolability of the mutant A-ARs that was not as pronounced as that of the E771A mutant hAR. R870G allowed subnormal activation of the reporter gene due to instability of A-ARs shown through thermolability experiments. These kinetic data correlate well with the subject's clinical phenotype of MAIS, where the mutant R870G hAR supported a mildly deficient androgen response. The degree of conservation of the particular amino acid throughout the subfamily, as well as the degree of biochemical defectiveness of the mutant hAR measured in kinetic assays in GSFs and transfected COS-1 cells, correlates well with the degree of AIS of the subjects studied here.

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