Molecular Analysis of Human Androgen Receptor Mutations Causing Motor Neuronopathy or Infertility

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TO THE LAND AND
THE GENEROUS PEOPLE OF BAHRAIN,
AND TO
MY WIFE & SON AHMED

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PREFACE

The androgen receptor (AR) is a ligand-activated, DNA-binding, transcription factor that belongs to the steroid receptor family of the nuclear receptor superfamily. It is widely expressed in the body and regulates various biological systems including male sexual differentiation and androgenic sexual dimorphism, neuronal growth and survival, etc.. . Upon ligand binding, AR is translocated to the nucleus and the androgen-AR complex binds to an androgen response element (ARE). This binding, in association with other nuclear factors, stabilizes the preinitiation complex of the basic RNA polymerase II transcription machinery.

Our laboratory has been devoted to the identification, and characterization of natural, germline AR mutations that cause various degrees of androgen insensitivity (AI), and we maintain a computerized database of all such mutations reported in the world. None of these mutations, including complete *AR* deletion, causes any neuronopathy. In contrast, expansion of the polymorphic $(CAG)_nCAA$ tract, coding for polyglutamine (polyGln), in exon 1 from the normal range (11-33) to a range of 40-62, must cause a motor-neuron degenerative disorder known as spinobulbar muscular atrophy (SBMA) by a gain of function.

PolyGln-expanded proteins have been discovered to cause seven other mendelian neurodegenerative diseases, presumably by a similar gain-of-function.

The hypothesis in this thesis was polyGln-expanded AR causes a conformational alteration that makes the mutant AR or a polyGln-expanded fragment of it neurotoxic.

The first part of my experiments tried to answer several questions: Does the polyGln-expanded AR show different transactivation/binding activity compared to the wild type? Does it have a conformational alteration (gain of structure) *in vitro* that facilitates the creation of potentially pathogenic derivative(s)? Does the expanded AR cDNA yield (an) apoptotically pathogenic derivative(s) in transfected cells? Finally, is this derivative (if any) associated with increased cell death? The last section in this part deals with the effect of the polyGln-expanded AR on the T-type Ca^{2+} channel of motor neuron-like cells as a possible proximate mechanism for neuronopathy in SBMA.

The second part of my experiments characterizes two substitution mutations in the AR ligand-binding domain (LBD) that cause impaired spermatogenesis. I determined the effect of the two mutations on the ability of the mutant ARs to bind 5α-DHT and two synthetic androgenic ligands and to

transactivate. Another natural androgen, mesterolone, was apparently therapeutic. I tested its effect on the ability of one LBD mutant to restore conformational normality and to improve transactivation. Finally, an A-R-ARE binding test was performed to assess whether the hypotransactivational activity of the two mutants was due to impaired DNA binding.

A re-revised manuscript submitted to Human Molecular Genetics on November 18, 1997 has been accepted on November 21, 1997. It's title is [Spinobulbar muscular atrophy: polyGln-expanded androgen receptor is proteolytically-resistant *in vitro* and processed abnormally in transfected cells]; (Abdullah, A. A. R. *et al.*, 1997). It was accepted for the March 1998 issue. It includes some of the results of the partial tryptic digestions and COS-1 cell transfection studies shown on pages 87-100 of this thesis.

Abstract

hAR mutations cause, or predispose to, androgen insensitivity syndrome (AIS), spinobulbar muscular atrophy (SBMA, a motor neuronodegenerative disease), and prostate cancer. I investigated the pathogenic mechanism of three AR mutations: expansion of the trinucleotide repeat $(CAG)_n$ coding for the polymorphic polyglutamine (polyGln) tract in exon 1 of the *AR* which causes SBMA, and mutations in exon 5 (Asn727Lys) and exon 8 (Met886Val) of the AR ligand-binding domain (LBD) each of which causes oligospermic infertility. In the transiently transfected motor neuron hybrid cell line (NSC34), the polyGln-expanded AR is ~40% less transactive than the wild-type receptor despite a higher AR protein content. The polyGln-expanded AR has normal androgen-binding kinetics. A polyGln-expanded AR made from mixed CAG and CAA codons is also hypotransactive, suggesting protein dysfunction. The polyGln–expanded AR is more resistant than normal to short, cell-free trypsinolysis, and yields a distinct set of Gln–expanded fragments even after extended exposure with a denaturant, suggesting conformational changes. COS cells transfected with (CAG)– or (CAG/CAA)-expanded AR cDNA generate a unique 75 kDa fragment containing the Gln tract, and are twice as likely to die apoptotically.

Androgen caused a negative shift in the voltage dependence of activation resulting in an increase of the T-type Ca^{2+}-channel window current in two motor neuron cell lines expressing polyGln-expanded AR.

Accumulation of an aberrant or protease-resistant polyGln-expanded fragment of AR may be the proximate motor neuronopathic agent in SBMA. This agent may cause Ca^{2+} overload and motor-neuron apoptosis in SBMA by abnormal activation of voltage-gated Ca^{2+} channel.

Neither the Met886Val nor the Asn727Lys mutation affects androgen binding in genital skin fibroblasts or transfected COS cells. However, both reduce transactivation by ~40%, a result paralleled by A-R binding to a steroid response element. To my knowledge, these are the first AR LBD mutations that impair DNA binding. Mesterolone normalized spermatogenesis in Asn727Lys subject. Interestingly, the Asn727Lys AR is 50% hypertransactive with mesterolone at 37°C but not at 34°C, and trypsinolysis indicates that it is conformationally normal with mesterolone but not with 5α-DHT.

Résumé

Les mutations dans le récepteur androgène humain (hAR) causent, ou du moins prédisposent, à différentes maladies telles que le syndrome d'insensibilité aux androgènes (AIS), l' atrophie musculaire spinobulbaire (SBMA), une maladie neurodégénérative ainsi que le cancer de la prostate. Ma recherche a porté sur le mécanisme de pathogenèse de trois mutations dans le hAR. La première mutation étant une expansion de l'insert poly-glutamine (polyQ) dans le premier exon qui cause le SBMA; les 2 autres mutations sont dans l'exon 5 (Asn727Lys) et dans l'exon 8 (Met886Val) du domaine de liaison du ligand (LBD) qui causent l'infertilité d'origine oligospermique.

Dans les neurones moteurs NSC34 transfectés avec le récepteur androgène contenant une expansion de l'insert polyQ, on a démontré que malgré une plus grande quantité de protéines, il y avait une baisse de 40% de la transactivation. Les essai de liaisons avec l'hAR contenant les expansions polyQ ont démontré des cinétiques normales. En plus, le hAR avec une expansion de l'insert, formé par un mélange de codons CAG et CAA (les deux codent pour l'acide aminé glutamine), démontre aussi moins de transactivation ce qui suggère une protéine disfonctionnelle.

Un protocole de traitement à la trypsine a démontré que le hAR avec l'expansion polyQ est plus résistant que l'hAR normal et que le traitement produit un groupe de fragments distincts contenant l'expansion de l'insert polyQ indiquant ainsi un changement de conformation.

Les cellules COS transfectées avec le hAR contenant l'expansion polyQ produisent un seul fragment de 75kDa contenant l'insert polyQ. Ces mêmes cellules sont deux fois plus susceptibles de mourir d'apoptose.

L' androgène augmente le courant calcique de type T dans deux lignées différentes de neurones moteurs qui expriment le hAR contenant l'expansion polyQ. Il fut suggéré que l'accumulation du fragment de 75 kDa contenant l'expansion de l'insert polyQ résistant aux protéases soit la cause de la neuropathie retrouvée dans le SBMA. Cette accumulation du fragment de 75 kDa pourrait induire une surcharge calcique dans les neurones moteurs via une activation anormale des canaux calciques voltage-dépendant et pourrait ainsi contribuer à l'apoptose des neurones moteurs.

Ni la mutation Met886Val, ni la mutation Asn727Lys ne réduisent la liaison de l' androgène dans les cellules COS transfectées ou dans les fibroblastes cutanées d'origine génitale. Cependant, les deux mutations réduisent le taux de

transactivation de 40% ce qui se reflète dans la réduction de la liaison de l'hAR avec l'élément de réponse dépendant des stéroides. A notre connaissance, ces deux mutations sont les premières dans le AR LBD qui affectent la liaison avec l'ADN et c'est probablement à travers un changement dans l'activité du récepteur d'androgène que ces deux mutations causent une diminution de la fertilitité chez les mâles. Un traitement au mestorolone normalise la spermatogenèse chez les sujets possédant la mutation Asn727Lys. De plus, le Asn727Lys AR démontre 50% de plus de transactivation avec l'hormone mesterolone à 37°C mais pas à 34°C. Le traitement à la trypsine indique également que Asn727Lys a une conformation normale en présence de mesterolone mais pas avec 5α-DHT.

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completing this research and writing this thesis. And last, but not the least, I would like to thank my parents for their continuous support and prayers to achieve my goal.

ABBREVIATIONS

5'-UTR	5'-untranslated region
ADF	Age-dependent factor
AF	Associated factor
AF-2 AD	Activation function-2 activating domain
AI	Androgen insensitivity
AIS	Androgen insensitivity syndrome
Ala	Alanine
AR	Androgen receptor
ARE	Androgen response element
Arg	Arginine
Asn	Asparagine
Asp	Asparatic acid
C-terminal	Carboxyl-terminal
CA	Cyproterone acetate
CAI	Complete androgen insensitivity
CAT	Chloramphenicol acetyle transferase
CBP	CREB-binding protein
COUP-TF	Chicken ovalbumin upstream promoter-transcription factor
CRE	cAMP response element
CREB	CRE-binding protein
Cys	Cysteine
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
EGF	Epidermal growth factor
ERAP	Estrogen receptor associated protein
ER	Estrogen receptor
ERE	Estrogen response element
fmol	Femtomole
FSH	Follicle stimulating hormone
GH	Growth hormone
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine

GR	Glucocorticoid receptor
GRE	Glococorticoid response element
GRIP1	Glucocorticoid receptor interacting protein 1
GSF	Genital skin fibroblast
hAR	Human androgen receptor
hER	Human estrogen receptor
hGR	Human glucocorticoid receptor
His	Histidine
hMR	Human Mineralocorticoid receptor
hPR	Human progesterone receptor
HRE	Hormone response element
hsp	Heat shock protein
IGF	Insuline-like growth factor
Ile	Isoleucine
kbp	Kilo base pair
kDa	Kilo Dalton
LBD	Ligand-binding domain
Leu	Leucine
LNCaP	Lymph node carcinoma of prostate
LTR	Long terminal repeat
Lys	Lysine
MAI	Mild androgen insensitivity
mAR	Mouse androgen receptor
MB	Mibolerone
mER	Mouse estrogen receptor
Met	Methionine
MMTV	Mouse mammary tumor virus
MT	Methyltrienolone or R1881
N-terminal	Amino-terminal
NF-κB	Nuclear factor-kappa B
NLS	Nuclear localization signal
NMR	Nuclear magnetic resonance
ORF	Open reading frame
PCR	Polymerase chain reaction
Phe	Phenylalanine
PAI	Partial androgen insensitivity

Poly(A)	Polyadenylation
PR	Progesterone receptor
PRE	Progesterone response element
PSA	Prostate-specific antigen
R	Receptor
RAF	Receptor accessory factor
RAP	Receptor-associated protein
rAR	Rat androgen receptor
RE	Response element
rGR	Rat glucocorticoid receptor
RT	Room temperature
SBMA	Spinobulbar muscular atrophy
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine
SR	Steroid receptor
SRC-1	Steroid receptor coactivator-1
T	Testosterone
TAU	Transcription activation unit
TBP	TATA-binding protein
Thr	Threonine
TIS	Transcription initiation site
TR	Thyroid receptor
Tyr	Tyrosine
Val	Valine
VDR	Vitamin D receptor
VP16	Viral protein 16
Zn	Zinc

PART I: INTRODUCTION

THE ANDROGEN RECEPTOR AND ITS RELATIVES

The androgen receptor (AR) is one of four closely related steroid receptors: the glucocorticoid (GR), mineralocorticoid (MR), and progesterone (PR) receptors. This subfamily is part of the superfamily of nuclear receptors (Beato *et al.*, 1995; Mangelsdorf *et al.*, 1995a). This includes receptors for thyroid hormone (TRα and TRβ), vitamin D (VDR) and estrogen receptors (ERα, ERβ), (Katzenellenbogen and Korach, 1997; and references therein); retinoic acid (RAR), and a number of orphan receptors for which the ligands are not yet identified (Mangelsdorf *et al.*, 1995b; da Silva and Burbach, 1995).

Nuclear receptors are ligand-activated transcription factors that exist in trace amounts (10^3-10^4 per cell). Development of synthetic, high affinity analogs of steroid ligands overcame the purification difficulties and allowed further clinical and biochemical studies of these receptors (Evans, 1988).

Ligands vary in their size and structure: thyroid hormone, T_3, is an aromatic amino acid, retinoic acid is an isoprenoid, 1 ,25-dihydroxyvitamin D_3 is a secosteroid, and steroids are cholesterol metabolites. The latter are subdivided into three classes of steroid hormones: the adrenal steroids (e.g. cortisol and aldosterone), the sex steroids (e.g. testosterone, progesterone, and estrogen), and vitamin D_3 (Forman and Samuel, 1990). The adrenal steroids play wide roles in controlling body homeostasis, glycogen and mineral metabolism and mediating the stress. They also have several effects on the immune and nervous systems as well as cellular growth and differentiation. The sex steroids trigger the development and determination of the embryonic reproductive system, masculinize or feminize the brain perinatally, and play a role in the development of the secondary sexual characteristics. Vitamin D plays an essential role in normal bone development and differentiation and calcium metabolism. Aberrant synthesis or production of these hormones leads to serious consequences and clinical diseases including cancer (Evans, 1988).

In order to regulate the expression of their responsive genes, hormones usually bind to their receptors and form a complex that is translocated to the nucleus (Wagner *et al.*, 1995). Binding of the hormone to its receptor causes conformational transformation which activates the complex. Once in the nucleus, the complex dimerizes and binds to specific DNA regulatory sequences, known as hormone-response elements (HRE), in order to activate or suppress the transcription of the target gene. In the absence of androgens (male sex steroids

like testosterone and 5α-dihydrotestosterone), AR remains untransformed, and bound to cellular chaperones or heat-shock proteins (hsp); e.g. 90-kDa (Fig. 1), (Smith and Toft; 1993). The activated (transformed) form of the receptor acquires its functions via its structural conformation change.

Steroid receptors have common regions or "domains" that can function independently from each other. These domains (N-terminal transactivation domain, DNA-binding domain or DBD, and ligand-binding domain or LBD) normally exert their function upon hormone-receptor complex formation resulting in modulation of gene transcriptional activation (transactivation). Partial proteolysis of purified glucocorticoid receptor gave rise to separate regions of the receptor that independently bound DNA or ligand, and hence the first suggestion of the term "domain" (Evans; 1988, and references therein). Moreover, C-terminal deletion of GR's LBD left the remainder constitutively active, revealing that neither LBD nor the ligand was needed for DNA binding or transactivation by GR (Evans, 1988 and references therein). Similarly, truncation of the hAR's LBD C-terminus by 258 residues left the receptor constitutively active (Jenster *et al.*, 1991). This suggests that LBD represses the DBD and transactivation domains in the absence of the hormone.

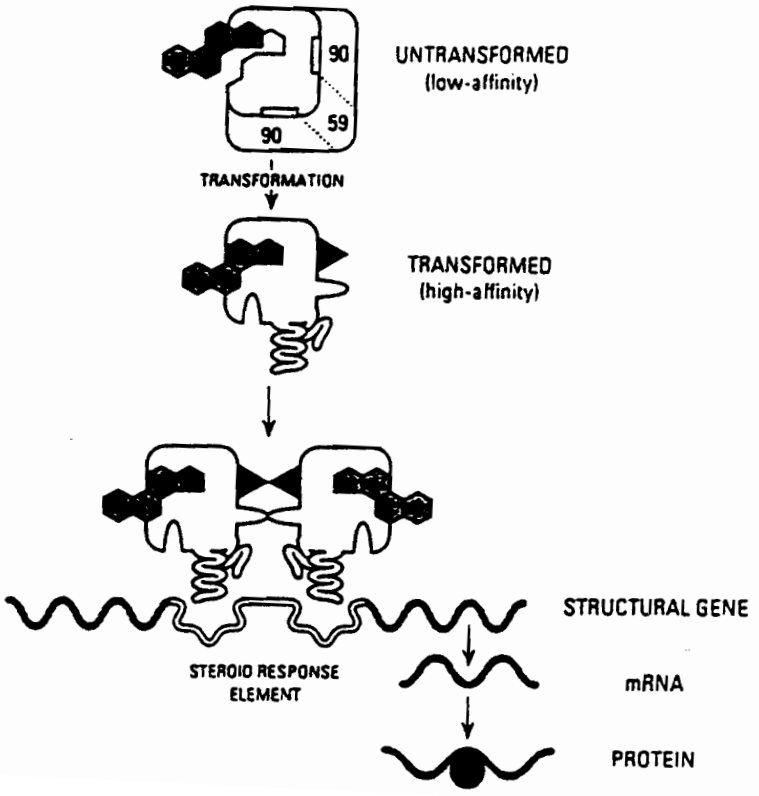
THE MOLECULAR STRUCTURE AND EXPRESSION OF THE ANDROGEN RECEPTOR GENE

The genomic structure of *AR*

The human *AR* (*hAR*) gene is a single copy gene that is located in the pericentromeric region of the long arm of X-chromosome at Xq11-12 (Brown *et al.*, 1989). It and its encoded protein are similar in structure to the rest of the steroid receptor subfamily (Lubhan at al., 1988). Human *AR* gene size spans 75-90 kilo bases (kb) of the genomic DNA (Kuiper *et al.*, 1989; Lubhan *et al.*, 1989; Brinkmann *et al.*, 1989) with a protein coding region of ~2757 base pairs (bp) comprising 8 exons designated A-H (Lubhan *et al.*, 1989) or 1-8 (Marcelli *et al.*, 1990) and 7 introns varying in size from 0.7 kb to more than 26 kbp (kilo base pairs) (Kuiper *et al.*, 1989). Starting at codon 58 of exon 1, there is a polymorphic CAG repeat region that contains an average of 21±2 repeats (La Spada *et al.*, 1991). This region ranges from 11-33 in a normal mixed-sex population (Edwards *et al.*, 1991; Edwards *et al.*, 1992) and shows ethnic polymorphism. In a study

Figure 1. Steroid receptor action

Before ligand binding, the DNA-binding and dimerization domains are concealed. After binding hormone, the receptor is transformed, dimerization occurs, and the homodimer acquires greater affinity for DNA. Binding at a steroid response element can then occur. This process influences transcription from the target gene located near the response element. (From Trifiro *et al.*, 1994).


90
90
59
UNTRANSFORMED
(low-affinity)
TRANSFORMATION
TRANSFORMED
(high-affinity)
STRUCTURAL GENE
STEROID RESPONSE
ELEMENT
mRNA
PROTEIN

that included Hispanic females the mode of the repeat number was 19 (Legro *et al.*, 1994). Another study showed that the repeat average is 22 in Caucasian males (Hardy *et al.*, 1996). This tract expands to a range of 40-62 repeats in patients with spinobulbar muscular atrophy (SBMA or Kennedy disease; La Spada *et al.*, 1991). Towards the 3' end of exon 1, there is a polymorphic polyglycine tract that ranges from 12-29 residues and is encoded by $(GGN)_n$ (Pinsky *et al.*, 1995; Lumbroso *et al.*, 1997). There are several non-polymorphic, homopolymeric stretches in exon 1: two polyglutamine tracts (n=6 and n=5), a polyproline tract (n=8), and a polyalanine tract (n=5) (Brinkmann *et al.*, 1989) (Fig. 2). Exons 2 (152 bp) and 3 (117 bp) code for the DBD, and exons 4-7 (288, 145, 131, and 158 bp; respectively) and part of exon 8 (155 bp) code for the LBD. The remainder of exon 8 is a very long 3'-UTR (6.8 kb) with two functional polyadenylation (poly(A)) signals: ATTAAA and CATAAA, 221 bp apart. Polyadenylation occurs 14-24 bp downstream of these poly(A) signals (Faber *et al.*, 1991a).

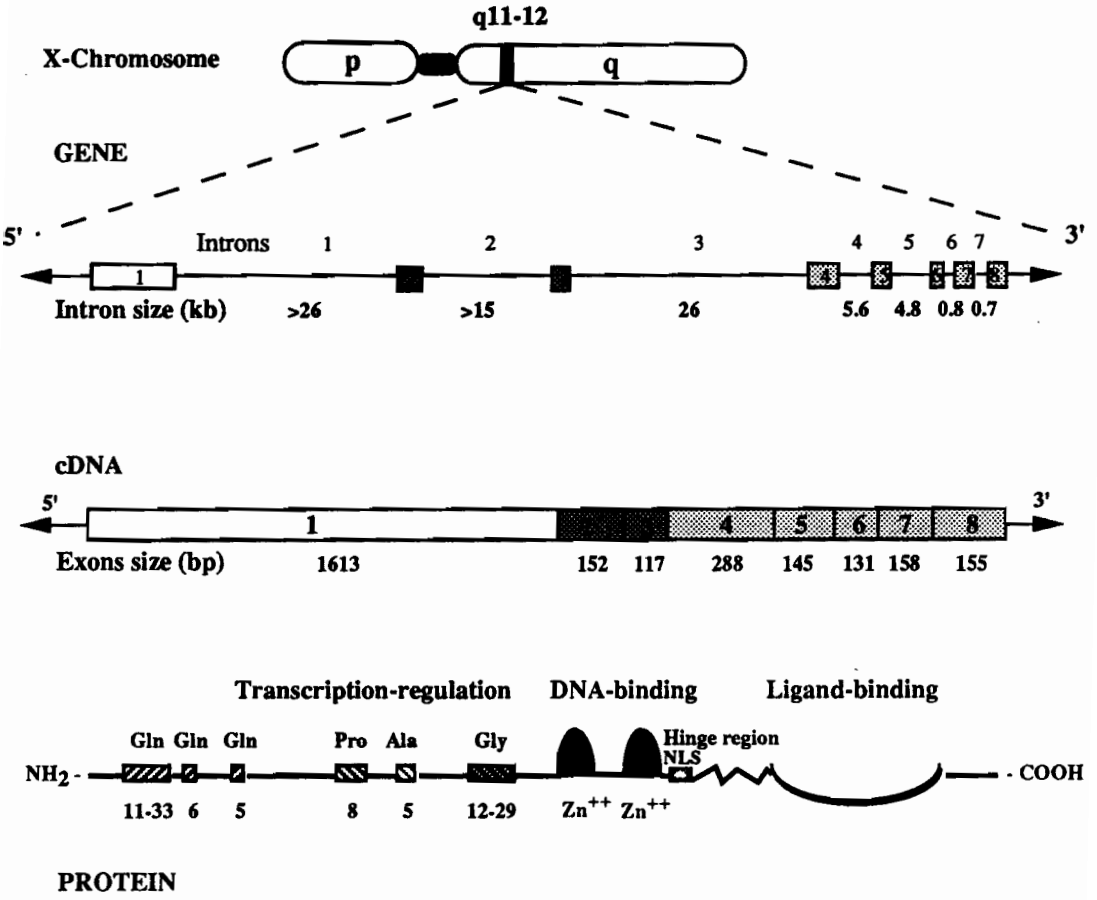
Regulation of AR expression

The *hAR* promoter revealed that it contains two sites of transcription initiation (TIS I and TIS II). TIS I is an adenosine residue and located 1126 bp 5' of the translation initiation methionine codon; the second (TIS II) is 13 bp downstream of TIS I. These two sites are believed to be regulated by different promoter sequences (Tilley *et al.*, 1990; Faber *et al.*, 1991b; Faber *et al.*, 1993). The TIS I is used in all tissues and cell lines studied to date (Tilley *et al.*, 1990); the role of TIS II is unclear.

The promoter of the *hAR* gene lacks a typical TATA or CAAT box; instead, it contains GC-rich elements, including that of the ubiquitous mammalian transcription factor Sp1 (Tilley *et al.*, 1990; Song *et al.*, 1993), a homopurine/homopyrimidine stretch (Baarends *et al.*, 1990; Mizokami *et al.*, 1994; Chen *et al.*, 1997), and a cAMP response element (Mizokami *et al.*, 1994; Lindzey *et al.*, 1993). The mAR and rAR promoters are highly homologous to that of hAR, one difference that could be observed among these promoters is the number of GGGGA homopurines: 4 in hAR, 6 in mAR, and 8 in rAR (Faber *et al.*, 1991b). This might contribute to the regulation of AR expression in each of these species. The homopurine/homopyrimidine (pur/pyr) stretch (~90-bp) is located immediately upstream of the Sp1-binding GC box and it is conserved at the same proximal position in the rat, mouse and human AR gene promoter (Chen *et al.*,

Figure 2. Schematic illustration of the X-chromosomal locus of the *hAR* and structural organization of the gene and protein.

Top, The *hAR* is located in the long arm of X-chromosome at Xq11-12. *Middle*, structural organization of *hAR* showing the eight exons (numbered within boxes) separated by introns (numbered above the diagram) ranging from 0.7 kb to more than 26 kb in size. Intron sizes (kb) are beneath the diagram. The AR complementary DNA (cDNA) comprises a coding region of about 2760 base pairs (bp). The size of each exon is given below the diagram in bp. Exon 1 encodes the amino-terminal (N-terminal) domain; exons 2 and 3 encode the DNA-binding domain; the 5' region of exon 4 encodes the hinge region including the nuclear localization signal (NLS); the 3' portion of exon 4 and exons 5-8 encode the ligand-binding domain. *Bottom*, schematic diagram of AR protein. The main functional domains of the AR are, from left to right: N-terminal (Transcription-regulation) domain including the polyamino acid tracts with the numbers below boxes indicating the size of each tract (aa); DNA-binding domain, comprising the two zinc fingers; hinge region, containing the NLS; and ligand-binding domain (modified from Quigley *et al.*, 1995).


q11-12
X-Chromosome
p
q
GENE
5'
Introns
1
2
3
4
5
6
7
3'
Intron size (kb)
>26
>15
26
5.6
4.8
0.8
0.7
cDNA
5'
3'
Exons size (bp)
1613
152
117
288
145
131
158
155
Transcription-regulation
DNA-binding
Ligand-binding
Gln Gln Gln
Pro Ala
Gly
Hinge region
NLS
NH2 -
- COOH
11-33
6
5
8
5
12-29
Zn++ Zn++
PROTEIN

1997). This stretch exhibits multiple, but weaker, Sp1-binding compared to the conventional adjacent Sp1-binding site (GC box). The deletion of this region resulted in a 3-fold decrease in promoter activity, indicating a regulatory function. The binding of a novel pyrimidine single-strand-specific protein (ssPyrBF) changes the conformation of double-stranded B-DNA preferred by Sp1 and the resulting stabilization of a non-B DNA conformation may prevent its interaction with Sp1. In the TATA-less AR gene promoter, this region may assist transcription initiation by multiple binding of Sp1(Chen *et al.*, 1997).

The minimal *hAR* promoter comprises nucleotides -74 to +84 (Takane and McPhaul, 1994). On the other hand, the rat *AR* promoter/enhancer region contains a more distal 5' region located at -96 to -940 that binds a novel transcription factor (Song *et al.*, 1993). The rAR promoter also has a regulatory element that binds a pair of protein factors apparently responsible for the age dependency of hepatic rAR (Supakar *et al.*, 1993). Interestingly, the region from -574 to -554 in the r*AR* promoter acts as a repressor element by binding nuclear factor κB (NF-κB), the activity of which increases during age-dependent androgen desensitization of the liver (Supakar *et al.*, 1995). NF-κB is a member of the dimeric transregulatory protein family that is critical in immune response, inflammation, oxidative stress and embryonic development. There are two forms of NF-κB interacting with the repressor element of r*AR* promoter: a heterodimer of p50/p60 and a homodimer of p50/p50. The expression of the homodimer gradually increases 10-fold in the aged rats with hepatic desensitization. Also, it was suggested that the expression of rAR mRNA could be regulated by steroid hormones after the delineation of a number of half-palindromic potential HRE-binding sites for AR, PR, and GR, in addition to an ER half-site (Song *et al.*, 1993; Baarends *et al.*, 1990). Another repressor element was found in the mouse AR (mAR) promoter in a region between -451 and -418 bp of the promoter (Kumar *et al.*, 1994). The repressing function of this element is not yet known. A second promoter has been described in the mAR with transcription initiation sites at 162 and 170 bp downstream from the most 5' transcription initiation sites of the first promoter. While the transcripts initiated from the second promoter seem to be more abundant in several tissues and cell lines examined, the two promoters appear to be down regulated by the androgen 5α–dihydroxytestosterone (5α-DHT) and also appear to activate AR gene transcription in a synergistic pattern in some cell lines (Grossman *et al.*, 1994).

In addition to the repressor elements in the AR promoter, there are also enhancer elements which regulate rAR expression in age-dependent (-329 to -311 bp) and tissue-specific manners (-310 to -286 bp) (Supakar *et al.*, 1993). These elements interact with two novel transcription factors in the rat liver. The first, the age-dependent factor (ADF), is widely expressed and evolutionarily conserved. Its activity has been shown to decrease about 7-fold between 3-26 months of age (Supakar *et al.*, 1995). The second, associated factor (AF), is tissue specific and is found in liver or liver cell lines.

While hAR expression is down regulated by androgens in some cell lines (eg. LNCaP cells, lymph node carcinoma of prostate), it is up-regulated in others (eg. bone-forming cells or osteoblasts), (Wiren *et al.*, 1997). AR mRNA steady state increased when the osteoblastic cell line SaOS-2 was treated with the nonaromatizable 5α-DHT. Unlike LNCaP cells, SaOS-2 transiently expressing hAR had increased hAR mRNA in a time- and dose-dependent manner as assayed by RNase protection assay. This up-regulation was antagonized by hydroxyflutamide (an antiandrogen) indicating that mRNA up-regulation is controlled by AR.

Recently, two *cis*-acting AREs in the AR coding region were isolated and found to be required for cell-specific androgenic up-regulation of receptor mRNA (Dai and Burnstein, 1997). The two AREs (182 pb apart) reside in a 350 bp-fragment spanning nucleotides +2004 to +2359 in exons 4 and 5, and encoding portions of the hinge region and LBD. Both AREs were required for maximal androgen induction of a reporter gene cotransfected with AR cDNA in PC3 cells (prostate cancer cell line not expressing AR). In transiently or stably transfected PC3 cells, actinomycin D (mRNA transcription inhibitor) blocked androgenic up-regulation of AR mRNA but not cycloheximide (protein translation inhibitor), indicating that AR is directly involved in transcriptional up-regulation of AR cDNA expression. This was further supported by nuclear run-on assays which showed an increased mRNA upon androgen treatment. Finally, band shift assays revealed specific AR binding to these AREs (Dai and Burnstein, 1997).

A functional ARE in the 5' far upstream flanking region of the human prostate-specific antigen gene was defined (Zhang *et al.*, 1997) in addition to a previously defined ARE at the proximal region (Riegman *et al.*, 1991; Cleutjens *et al.*, 1997). The latter ARE (promoter or pARE) has been identified in the proximal region of the PSA gene's promoter that encompasses sites -177 to -163 (AGAACAgcaAGTGCT), and had a low androgen inducibility in the androgen

responsive cell line, LNCaP, which produces PSA gene product (Young *et al.*, 1995; Warrier *et al.*, 1994). Recently, Zhang *et al.* (1997) identified a second ARE (upstream ARE or uARE) in the 5' far upstream region of the PSA gene that encompasses sites -4145 to -4136 (GGAACAtatTGTATC). Both pARE and uARE together, but not pARE alone, maximized androgen induction of the expression of PSA reporter gene in LNCaP cell line suggesting an important role by the uARE in PSA androgenic up-regulation.

hAR up-regulation can also be achieved translationally. This is controlled by an element of the hAR 5'-UTR located between +21 to +149 bp. The nucleotide sequence of this element suggests the formation of a stem-loop structure in the hAR mRNA (Mizokami and Chang, 1994b). This mechanism may explain the apparent up-regulation of androgen binding after pre-incubation of a fibroblast monolayers with androgen even in serum-free medium (Pinsky *et al.*, 1983; Evans and Hughes 1985; Gad *et al.*, 1988).

It is notable that AR expression is regulated by different molecules including follicle stimulating hormone (FSH), cAMP and androgens. The AR mRNA is down-regulated by androgens in various tissues such as prostate, kidney, brain and epdidymis. Other molecules like FSH and cAMP up-regulate AR mRNA in Sertoli cells (Lindzey *et al.*, 1993). In LNCaP cell line, the hAR mRNA is down-regulated by the epidermal growth factor or EGF (Hinttu and Vinko, 1993) and androgen (Burnstein *et al.*, 1995). In the rat, androgen withdrawal by castration results in the increase of rAR message in ventral prostate and seminal vesicles which is reversed by androgen replacement (Quarmby *et al.*, 1990; Shan *et al.*, 1990).

The hAR mRNA and its tissue distribution

The poly(A^+) RNA isolated from different human tissues has two AR mRNAs: a major one of ~ 10 kb and a less abundant one of ~ 7 kb. The difference in the size is due to the differential processing of precursor mRNA, yielding a deletion of ~ 3 kb from the 3'-UTR (Lubhan *et al.*, 1988; Faber *et al.*, 1991a; Wolf *et al.*, 1993). Both messages have an open reading frame (ORF) of 2751 bp (Brinkmann *et al.*, 1989). The encoded protein varies from 897 to 936 amino acids (aa), due to variation in the two polymorphic, polyglutamine (polyGln) and polyglycine (polyGly) stretches (Pinsky *et al.*, 1995). The difference in size between the mRNA species and the translational ORF is due to the presence of the long 5'- and 3'-UTRs. AR protein and/or mRNA expression are detectable in

various types of genital and nongenital tissues and cell lines in species ranging from human to frog. AR message is also detectable in human testis, prostate and genital skin fibroblast (GSF) and also in some human-derived cell lines such as LNCaP and the human breast cancer cell lines T47D and MCF-7, human liver, kidney, brain, epididymis, anterior pituitary gland (Quigley *et al.*, 1995), and other neural tissues, including motor neurons (Sar and Stumpf, 1977). The expression of AR in many tissues is lower than in male urogenital system (Moordian *et al.*, 1987). In addition, the AR protein is detectable in tissues and cell lines using Western blot, immunohisto- or immunocytochemistry techniques (Quigley *et al.*, 1995 and references therein).

AR PROTEIN STRUCTURE AND FUNCTIONAL DOMAINS

The AR protein ranges from 110 to 114 kDa (Wilson *et al.*, 1992; Quarmby *et al.*, 1990; Jenster *et al.*, 1991) comprising 897-936 aa. This variation is due to the variability of the polyGln and polyGly tracts, as mentioned earlier. A truncated hAR, identified in GSF, lacks approximately 190 aa from the amino terminus (N-terminus) and has M.W. of 87 kDa (Wilson & McPhaul, 1994). Termed AR-A, it represents about 10% of the total AR and is believed to be due to an alternative translation-initiation methionine codon in exon 1. The full-length hAR (or AR-B) is a single polypeptide composed of several domains: the N-terminal (transactivational) domain, the DBD, the hinge region, and the LBD (Fig. 2). The DBD and LBD of the AR are highly homologous to the corresponding domains in other members of the steroid receptor subfamily, indicating descent from a common ancestral molecule (Evans; 1988).

N-terminal domain

The N-terminal domain is hypervariable even among ARs of different species (Landers and Spelsberg, 1992). It is the longest domain in the receptor comprising more than half of the AR protein (aa 1-537); nucleotide and aa numeration is according to Lubhan *et al.*, (1988). To delineate N-terminal sequences involved in transcriptional activation, a series of AR deletion mutants was constructed. Using heterologous promoter in transient transfection studies in COS cells, Simental and coworkers (1991) studied the domain requirements for transcriptional activation. The N-terminal domain plays a role in modulating promoter activity even in the absence of the LBD. The deletion of N-terminal domain up to aa 141 still retains the full inductive AR activity. However, a

complete loss of activity was caused by deletion of 338 aa or more indicating that a region of AR between 141 and 338 is essential for transactivation. This region, like GR and yeast transcription factors GCN4 and GAL4, is highly acidic and its activity is inhibited by the LBD in the absence of hormone. To test whether the N-terminal transactivational signals interact with nuclear proteins involved in transcription, an expression vector with the AR N-terminus only was cotransfected with wild-type AR and the MMTV-CAT reporter. AR activity was inhibited by 17% when equimolar amounts of vectors were used but it increased to 55% when 10- or 20-fold higher level of truncated AR was cotransfected relative to wild type AR. This suggests competition by the N-terminus for the nuclear cofactors required for transcriptional regulation (Simental *et al.*, 1991).

Similar analysis was done on the rAR to delineate sequences involved in transcriptional activation (Palvimo *et al.*, 1993). Interestingly, a region in the N-terminal domain (147-296) was found to be mandatory for transactivation and overlapped the homologous sequence in the hAR (141-336). The homology lies in residues 162 to 294 of hAR (Chang *et al.*, 1988). This region is interrupted by the glutamine tract in the rAR while it lies downstream to the glutamine tract in hAR. A later study by Jenster *et al* (1995) contradicted these results. The deletion of the first 100 aa in the latter study showed a 40% loss of transactivation, whereas in the former study, the deletion of the first 140 aa retained the full hAR transactivation. Moreover, the latter study showed that the deletion of the first 360 aa decreased the receptor's activity to 20% whereas in the former study, the deletion of the first 337 aa inactivates the receptor . Such differences might be due to the different transfectant cell lines or reporter vectors used in the two studies. Jenster *et al.* (1995) suggest the presence of two overlapping transcription activating units (TAU) designated TAU-1 and TAU-5. TAU-1 includes aa 1-485 with a core region located between aa 101-370. TAU-5 includes residues 360-528 with a core region located between residues 360-485. TAU-1 contains higher number of acidic residues, constituting 12.6% of the region; it also has three polyGln tracts, including the polymorphic one and potential phosphorylation sites. The TAU-5 core is not acidic, but it contains three different homopolymeric aa stretches; a proline stretch (aa 371-378); an alanine stretch (aa 397-401); and a glycine stretch (aa 448-468; Jenster *et al.*, 1995).

The homopolymeric tracts

Deletion analysis has identified four distinct types of transcriptional activation domains: 1) acidic; 2) proline-rich; 3) glutamine-rich; and 4) serine / threonine-rich. In addition to regions rich in certain amino acids, transcription factors are associated with polyPro and polyGln tracts. They are encoded by repeated codons which may evolve rapidly, lead to allelic variation and become highly unstable (Caskey *et al.*, 1992). The first to be characterized were homopolymeric glutamine repeats in the *Antennapedia* transcription factor of *Drosophila* (McGinnis *et al.*, 1984). Data from SwissProt demonstrated 82% of proteins containing polymeric stretches of at least 20 Gln or 78% of at least 10 prolines were transcription factors (Gerber *et al.*, 1994). The longest naturally occurring polyGln tract (n=38) is found in the human transcription factor TATA-binding protein (TBP) (Peterson *et al.*, 1990). Expansion of this tract in hAR from 11-33 to ≥ 40 residues alters the function of the receptor in two ways, leading to a motor neuron degenerative disease (spinobulbar muscular atrophy; SBMA) associated with partial androgen insensitivity syndrome (PAIS) known as Kennedy syndrome (La Spada *et al.*, 1991). The exact function of the polyGln tract and the other tracts is not known. One of the first attempts to analyze the contribution of this tract to transcriptional activation by the hAR was made by Mhatre *et al.* (1993). The results showed that hAR with Gln tracts of 40 or 50 residues were less competent than the wild type with residues of 20 Gln. Further experiments included hAR constructs with zero or 12 Gln residues. An inverse correlation was found between tract length and transactivational competence, with the longer tract giving lower transactivation (Chamberlain *et al.*, 1994; Gerber *et al.*, 1994; Kazemi-Esfarjani *et al.*, 1995). Other than the altered transcriptional activation, there is no detectable effect of the polyGln tract on the hAR in transfected COS-1 cells. The transactivational competence of tracts containing homopolyglutamine depends on the context of the flanking sequence: Chimeric proteins with the expanded homopolyglutamine flanked by GAL4 DBD and the activation domain of herpes virus-derived VP16 protein had increased transactivation; while those lacking VP16 showed the opposite (Gerber *et al.*, 1994). Like homopolyglutamine, homopolyproline length is inversely correlated with activation as shown by chimeric proteins of GAL4 DBD and polyPro tracts of zero to 50 residues (Gerber *et al.*, 1994). Unlike polyGln, activation by polyprolines peaks at 10 prolines and declines with longer tract. Naturally occurring stretches of proline in transcription factors are usually short which might be the consequence of strong selection against long polyproline stretches

(Gerber *et al.*, 1994). This suggests that the residues comprising the stretch are important, not just the context of the flanking sequence. Unlike the longer polyPro tracts which were selected against, the normal range of polyGln tract that varies from 10 to 34 residues (in Gerber's work) might indicate the evolutionary importance of this tract in transcription factors to maintain cell activity . In similar series of experiments, the deletion of the N-terminal region of rAR and hAR containing the polyGln tract showed increased transactivation and the expansion of the tract in hAR up to 77 Gln residues progressively decreased transactivation. This effect was reproducible using different promoters with variable HREs (Chamberlain *et al.*, 1994). Although these experiments were more quantitative than those of Mhatre *et al.* (1993), it is noteworthy that the deleted N-termini of the rAR and hAR included aa sequence flanking the Gln-tract (Chang *et al.*, 1988).

Deletion of the pentaglutamine tract that lies downstream to the polymorphic Gln tract of hAR showed a similar increase in transactivation to that provided by the deletion of N-terminal polyGln tract (Kazemi-Esfarjani *et al.*, abstract 1995). Interestingly, the location of the pentaglutamine tract is the same as the 22-Gln tract of the rAR (Chang *et al.*, 1988). Together these results demonstrate that the polyGln tracts serve as important modulatory subdomains that, together with the flanking sequences, serve to orchestrate the function of the N-terminus in the receptor, yet its presence is rather irrelevant to essential AR function. Finally, deletion of other tracts in the N-terminus, including the hexaGln, octaproline, or polyGly tracts diminished slightly and inconsistently (Kazemi-Esfarjani *et al.*, abstract 1993).

Depending on the reading frame of the message, poly(CAG) yields three different homopolymeric tracts (polyGln, polySer, or polyAla). Sequence databases indicate a clear bias in the size and frequency of these homopolymeric tracts as follows: $(Gln)_n > (Ser)_n >> (Ala)_n$. Changing the reading frame of the rGR polyGln tract to polySer or polyAla in the N-terminus showed that the mutant receptor was incapable of fully transactivating the reporter vector containing rabbit β-globin gene under the control of MMTV promoter (Lanz *et al.*, 1995). Prolongation of polyAla tract from 23 to 25 Ala resulted in a remarkable decrease in transactivation, whereas polyGln expansion had a minor consequence on GR activity compared to the progressive transactivation reduction caused by polySer expansion (Lanz *et al.*, 1995). Although the construction of other reading frames of polySer and polyAla had altered the flanking sequence of the tract, there

appears to be a selective defect caused by changing the triplet repeat. This defect varies depending on the nature and the position of the tract although the insertion of the polyAla tracts at different sites in the receptor showed an almost consistent pattern.

Why do so many transcription factors contain polyamino acid stretches? Many eukaryotic transcription factors are functionally robust compared to the prokaryotic transcription factors. Eukaryotic transcriptional regulatory machinery contains more than 50 proteins and depends on multiple, and redundant interactions. Even though the interaction between transcription factor partners is specific, changes in protein dimensions may be tolerated by some eukaryotic transcription factors (Kunzler *et al.*, 1995). It could be that a polyamino acid stretch helps a transcription factor to tolerate major structural changes without affecting function, which might be advantageous for a rapid intermolecular interactions in the eukaryotic transcription machinery.

The DNA-binding domain

The DNA-binding domain (DBD), the central domain of the AR, is encoded by exons 2 and 3 and contains 66 aa (residues 559-624). There is 75-80% homology in this domain among the AR subfamily, which includes GR, MR, and PR; the ER shares ~55% of this domain with the AR. The domain is rich in cysteine, lysine and arginine, which are the most conserved residues among steroid receptors (Evans, 1988). Eight of the nine cysteine residues contributes to two finger-like structures. Each is centered by a zinc ion coordinating with four cysteine residues arranged in the tetrahedral geometry necessary for the formation of a "zinc finger" (Fig. 3). Each of these fingers lies N-terminal to an α-helix structure; these α-helices (based on GR-DBD structural similarity to AR-DBD) would extend N-terminally from Gly576 to Glu 587 or Gly 588. Similarly, the C-terminal α-helices would extend from Pro 611 to Gly 622 (Pinsky *et al.*, 1994). This configuration enables the receptor to bind specifically to the androgen-response element (ARE). The first zinc finger (residues 559-579) is encoded by exon 2 and the second zinc finger (residues 595-619) is encoded by exon 3. At the base of the first zinc finger exist 3 aa (Gly 577, Ser 578, Val 581), which are identical in GR, PR and MR, that interact with the ARE sequence. The second zinc finger, which is highly basic, helps stabilize DNA-receptor interaction by contact with the DNA phosphate backbone and mediates receptor

dimerization (Quigley *et al.*, 1995, and references therein; Forman and Samuel, 1990).

Recognition of hormone-response element by DBD

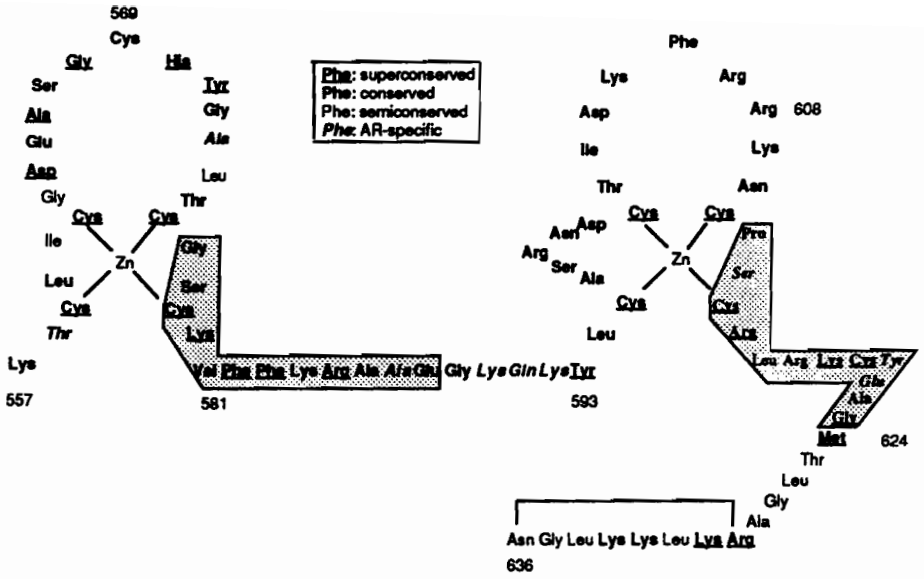
The discrimination among specific binding sites by the steroid receptors is partly determined by the three aa at the base of the first zinc finger. It is this discriminatory position that classifies the nuclear superfamily members into 4 groups. The first group includes the GR, MR, PR, and AR. These receptors contain Gly, Ser, and Val at the discriminatory position at the base of the first zinc finger as mentioned earlier. All of these recognize the same DNA sequence, consensus, that makes an inverted repeat with a three-nucleotide gap (AGAACAnnnTGTTCT). The second group includes receptors for thyroid hormones, retinoids, vitamin D, and orphan receptors. The discriminatory position of these receptors contains Glu, Gly, and Gly and recognize the HRE consensus AGGTCATGACCT. The third group includes receptors with members like the chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and the *Drosophila* knirps transcription factor (*kni*) which contains Glu, Gly and Ser at a similar position. It has been shown so far that only COUP-TF interacts with the consensus sequence GTGTCAAAGGTCA. The fourth group contains ER with Glu, Gly, and Ala at the discriminatory positions and binds to AGGTCAnnnTGACCT. This inverted repeat is identical to the second group's HRE except for a central three-nucleotide spacer (Forman and Samuel, 1990).

The tertiary structure of the hAR DBD is not known, but due to the high homology of hAR to the rest of the steroid receptors subfamily, especially at the DBD, data on the two- and three- dimensional structure of the GR could be extrapolated to the hAR DBD. 2-D nuclear magnetic resonance (NMR) spectroscopy (Hard *et al*, 1990) and crystallography studies (Luisi *et al.*, 1991) on the GR showed that the N-terminal α-helix of each monomer fits into the major grooves on one surface of the DNA and interacts with GRE half-site. The recognition of the DNA is achieved by the residues Gly 458, Ser 459, and Val 462. These residues are conserved and correspond to the hAR residues Gly 577, Ser 578, and Val 581. Substitution of these residues in the GR with those from the ER (Glu, Gly, and Ala) which makes the mutated GR to recognize ERE and vice versa (Luisi *et al.*, 1991).

In addition to its DNA-binding function, the DBD makes contact with other components of the transcriptional machinery. This region of GR includes

Figure 3. Schematic illustration of the DNA-binding domain of hAR.

(Top) The underlined bolded letters denote residues that are invariant in the AR subfamily and are highly conserved in the ER, and in the receptors for thyroxine, vitamine D, and retinoic acid. The bolded letters identify residues that are invariant in the AR subfamily. The regular letters identify residues that are conservatively substituted among members of AR subfamily. The italicized bolded letters refer to residues that are unique to AR. Certain residues are numbered for landmark purposes. The hatched sequence would represent the recognition α-helix. The bracket that overlies residues 628 to 636 identifies a nuclear localization signal (NLS). (Bottom) A model of the dimeric DNA-binding domain of the GR showing the arrangement of α-helices (cylinders) and zinc fingers in relation to GRE. The AR would be expected to have similar features.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Phe: conserved
Phe: semiconserved
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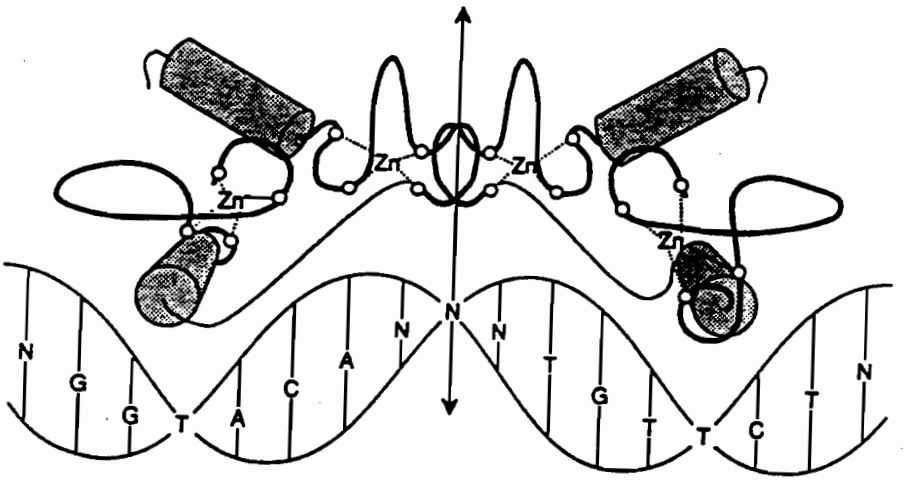
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Arg 488, Arg 489, and Asn 491. Subsitution of each of these residues results in a receptor that binds GRE normally but is defective in *in-vivo* transactivation (Hard *et al.*, 1990). Such substitution has been recognized in two natural hAR mutations (Arg 607Gln and Arg 608Lys) which correspond to Arg 488 and Arg 489 positions of the GR. These mutations in hAR are associated with rare cases of male breast cancer combined with PAI (Wooster *et al.*, 1992; Lobaccaro *et al.*, 1993). Recent functional and structural analyses revealed that the mutant ARs had normal androgen-binding affinities and weaker DNA binding to an isolated androgen-responsive element. In cotransfection assays, the mutant ARs displayed a reduced transactivation efficiency. Neither binding to an estrogen-responsive element nor transactivation efficiency of an ERE reporter gene was observed. Molecular modeling revealed that Arg607 and Arg608 were partially surface-exposed and located in adjacent areas in the AR-DBD complex with DNA. The investigators suggested that this is in favor of a protein-protein interaction and it is conceivable that such an interaction could be affected by mutation of one of these two arginines (Poujol *et al.*, 1997).

In fact, DBD integrity is important not only for DNA binding and transregulation, but also for receptor stability and activity, and, perhaps, message stability (Beitel *et al.*, 1994a).

The hinge region

Encoded by the 5'-end of exon 4 (residues 628-666), and lying C-terminal to the DBD, there exists a region that contains the major part of the hAR nuclear localization signal (NLS) at position 629-636. It consists of the residues: Arg, Lys, Leu, Lys, Lys, Leu, Gly, and Asn and is highly homologous with the NLS of the simian virus (SV40) large T antigen (Guiochon-Mantel *et al.*, 1989) and GR. A later study by Zhou *et al.* (1994) showed that NLS is a bipartite signal and includes a nuclear targeting sequence in the DBD and the hinge region composed of aa 617-633. This sequence consists of two clusters of basic aa (underlined) separated by 10 aa (RKCYEAGMTLGARKLKK). A full-length hAR lacking down to 32 residues in the hinge region of exon 4 (NLS) was mainly cytoplasmic in the presence of androgen (Simental *et al.*, 1991; Jenster *et al.*, 1993). However, a truncated hAR consisting of residues 1-503 is localized to the nucleus in the absence of androgen (Simental *et al.*, 1991). The latter event could be due to protein-protein interaction with other nuclear factors. Meanwhile, a truncated hAR consisting of the DBD and the flanking sequence including the NLS

(residues 507-660) is localized to the nucleus in the absence of androgen. The same study revealed that within the LBD there exists a transactivational-inhibitory region the deletion of which yields a constitutively active hAR in the absence of androgen (Simental *et al.*, 1991). Also, the interaction of other cellular factors to the LBD (hsp90) might inhibit the process of nuclear localization (Scherrer *et al.*, 1993). This suggests that the NLS is somehow masked by a region in the LBD via a direct intramolecular interaction or indirectly either through a conformational alteration or interaction with other proteins, and addition of hormone renders this region's "masking" function.

The ligand-binding domain

The LBD, the C-terminal region encoded by exons 4-8 of hAR, encompasses approximately residues 670-919. There is about 50% identity with the corresponding residues in GR, MR, and PR. There is 100% homology between hAR, rAR, and mAR LBD (Choong *et al.*, 1996). The evolutionary conservation of this region predicts its functional importance. No direct studies about the three-dimensional structure of the hAR LBD are available , but from the available mutations at this domain one can infer three of its structure-function features. First, for normal ligand binding to occur, the entire length of this domain must be intact. For example, truncation of the C-terminus by 12 residues abolishes androgen-binding activity (Jenster *et al.*, 1991). Second, the unliganded LBD exerts a dominant inhibition on all of the receptor's functions. Third that liganded LBD has a number of subfunctions that complement those of other domains of the receptor: dimerization, transactivation, and translocation. This domain, due to its conserved feature among other members of the steroid receptors, serves analogous functions in PR and ER (Landers and Spelsberg, 1992, and references therein). Studies on the GR LBD showed its key role in transactivational activity and this activity is independent of the rest of the receptor's structure. Interestingly, repositioning the GR's LBD from the C-terminus to the N-terminus does not alter its DNA-binding activity or transcriptional regulation (Picard *et al.*, 1988). Moreover, the fusion of the GR LBD with the adenovirus E1A gene product places that protein's activity under hormonal control (Picard *et al.*, 1988). It has been shown that fusing the LBD with other transcription factors, in chimeric proteins, appears to have inhibitory function on the DNA-binding of that protein, a process that is reversed upon hormonal addition. This characteristic is not universal with the LBD of all receptors however (Landers and Spelsberg,

1992, and references therein). Many models that discuss the action of steroid receptors assume that the steroid binding to the receptor transforms the receptor to its activated form which is capable of high-affinity binding to HRE and regulating target gene. However, *in vitro* studies showed that receptor-DNA interactions can occur in the absence of hormones (William and Beato, 1986; Kuiper *et al.*, 1993a). This is not necessarily what occurs *in vivo,* especially as these experiments were done using purified or cell-free translated receptors which may have lost associated factors that normally act together with the receptor in a heteromeric complex to inhibit binding to DNA. The addition of hormone, or purification, induces the release of such factors as hsp90, hsp70, and hsp56 which are associated with steroid-receptor complexes. Hormone binding increases off-rate of the receptor from HRE by as much as 30-fold over the unbound form, whereas the on-rate is affected to a lesser extent (5-fold increase) (Landers and Spelsberg, 1992). Similarly, ER chimeras were created by replacing the ER DBD with that of GAL4, and the N-terminal AF-1 domain with the strong constitutive activation domain of VP16 (Lees *et al.*, 1996). These chimeras bind DNA in a ligand-independent manner, but are ligand-dependent transactivators, unlike VP16-GAL4 which is constitutively active (Lees *et al.*, 1996). However, it is not only the binding of a ligand to receptor and consequently shedding of other molecules that brings the receptor to its functional form; the binding of an androgen antagonist can promote the binding of a ligand-receptor complex to ARE, and yet not transactivate an androgen-regulated gene (Pinsky *et al.*, 1994, and references therein).

The LBD appears to contribute to different functions depending on the form of the receptor. In the absence of the natural ligand, it has an inhibitory function, e.g. on receptor-DNA binding or the regulatory function of the N-terminus. Androgen-receptor binding enables transactivation of a target gene by promoting release of the AR from its associated inhibitory factors and by permitting conformational alterations to the transformed (activated) state. The interaction between N-terminus and LBD was shown to occur *in vitro* using the yeast two-hybrid system (Fields and Song, 1989) for protein-protein interactions (Langley *et al.*, 1995). The interaction occurs only in the presence of hormone and it is inhibited by antiandrogen binding (Wong *et al.*, 1993).

Defining the LBD's essential regions

Data on naturally occurring mutations (either missense or nonsense mutations) in the LBD of hAR revealed two regions which have the highest density of mutations causing AI. These two regions lie in exons 5 and 7 between residues 726 and 772, and between 829 and 853, respectively (McPhaul *et al.*, 1992). Data analysis on hAR mutations showed that exon-5 region or hot spot 1 contains 37% of the 87 LBD mutations studied and these mutations affect 68% of the available codons in that region. Hot spot 2 in exon 7 contains 18% of the studied mutations with a mutation density of 39%. Forty-four per cent of mutations in hot spot 2 occur at CpG sites; in hot spot 1 these represent 22%. Thus, the frequency of mutations in exon 5 exceeds its CpG-dependent susceptibility to mutagenesis (Gottlieb *et al.*, 1996). These data indicate the importance of these two regions for functional conformation and structure of the LBD.

There are no direct data on the three-dimensional structure of the hAR's LBD; however, predictions have been derived from RXRα, RARγ and GR LBD-3-D structure (Wurtz *et al.*, 1996), and an earlier model was based on data from natural and synthetic mutations and from binding and partial proteolysis studies with various ligands (Goldstein *et al.*, 1993) (Fig. 4). The latter model was generated by sequence alignment of the LBD of steroid receptors with thermitase, a member of the substilisin-like serine proteases. The overall fold is a doubly wound β-sheet consisting of a core of hydrophobic parallel β-strands surrounded by five α-helices. The inner helices are hydrophobic, whereas the three outer helices are amphipathic in the serine protease: these features are well preserved in the steroid receptors.

The 3-D model of the generic nuclear receptor LBD by Wurtz *et al.* (1996) suggests that a twenty amino-acid region, a "nuclear receptor-specific signature", contains most of the conserved residues that stabilize the core of the canonical fold of a nuclear receptor LBD. This model also suggests a common ligand-binding pocket of predominantly hydrophobic residues. The RAR holo-LBD (liganded) is more compact than the RAR apo-LBD (unliganded); this was attributed to: a) the folding back of helix 12 (H12), which encompasses the AF-2 AD, towards the main body of the LBD, b) the flipping of the Ω-loop underneath H6 in the holo-structure, c) the bending of H3 towards the core of the holo-LBD, d) the shifting of the β-turn towards the surface of the holo-LBD, and e) the tilting of H11 away from the hydrophobic core. AR mutations occurring at the predicted LBD pocket or AF-2 region impair the receptor's activity (Gottlieb *et*

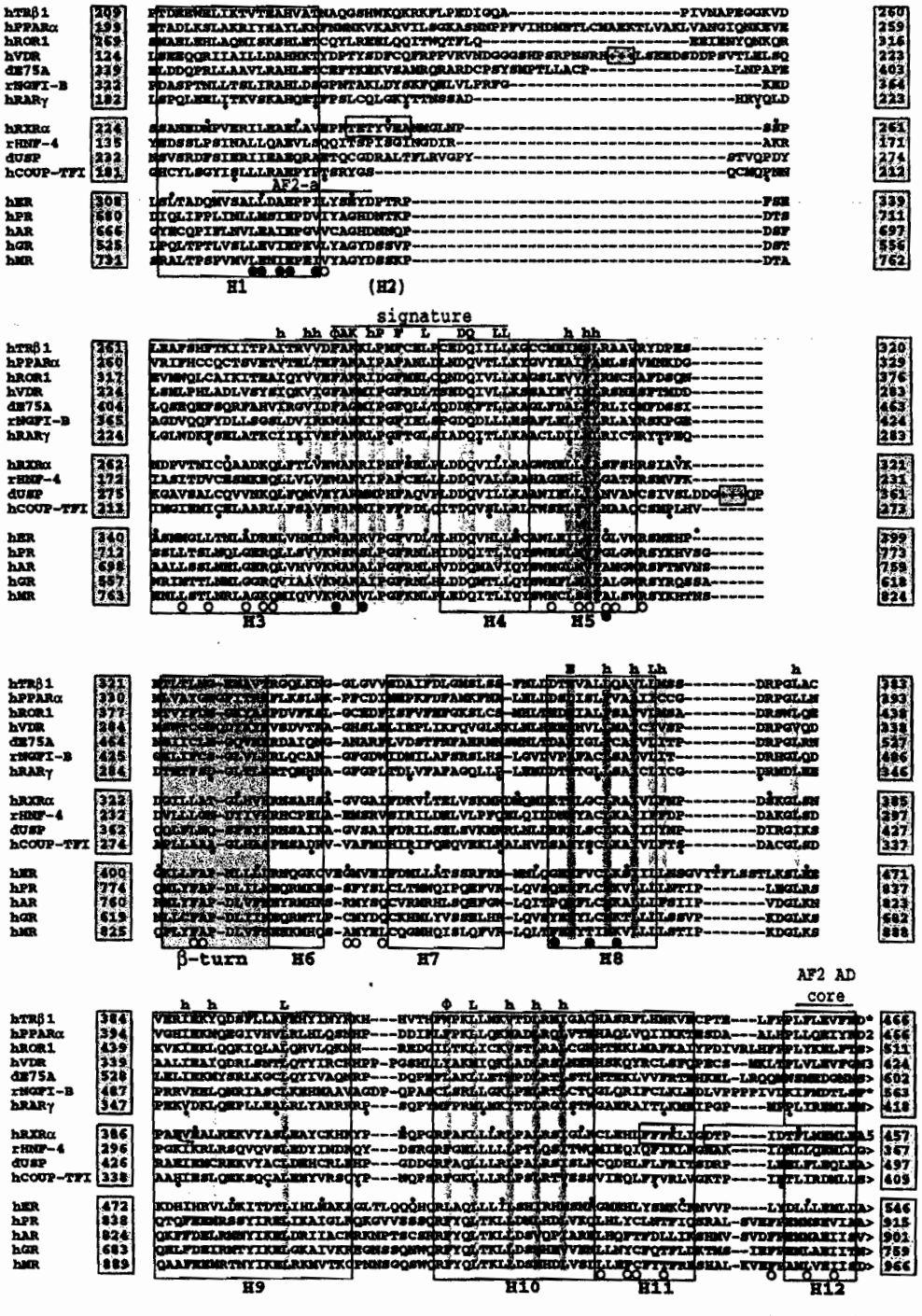
Figure 4. Sequence alignment of nuclear receptor LBDs.

(a) Sequences corresponding to the secondary structure elements found in the hRARγ LBD are boxed (helices H1 and H3 to H12); a shaded box indicates the β;-turn. Separate boxes shown only in the hRXRα sequence represent helices H2, H11 and H12. The Box with "+++" indicates long insertions in loop regions for VDR and USP. Conserved residues are indicated above the alignment by the single-letter code for invariant or highly conserved amino acids (h, hydrophobic; F, aromatic). Open circles below the alignment denote residues in the immediate vicinity of the ligand in RARγ; filled circle represent amino acids involved in key contacts between H1 and the LBD core. The ER activation domain AF-2α, the AF-2 AD core and the sequence containing the LBD-specific signature are indicated by the top lines. At the end of each sequence, asterisks denote the C-terminus, the number of residues to follow is indicated by a digit if below ten or by > if greater (Wurtz *et al.*, 1996).

(b) Alignment of the GR subfamily with thermitase. Also shown the multiple alignment of portions of other members of nuclear receptor superfamily with the GR subfamily. The receptor sequences are given their Swiss-Prot Database designations and are listed in the order rGR, hPR, mAR, hMR, hER, hRAR1, and mTRα1. Thermitase is identified as 1TEC(E). Residues identified biochemically are shaded. α-Helices and β-sheets are enclosed in rounded and squared boxes, respectively. Core secondary structural elements are notated in the last line, with the strands of the central b sheets designated A-G, the surrounding α-helices numbered 1-5, and an exposed β-bend designated X and Y (Goldstein *et al.*, 1993).

(c) Schematic representation of a nuclear receptor ligand-binding domain (adapted from Parker and White, 1996). The amino (N) and carboxy (C) termini of the LBD are indicated. The α helices (H1 and H3-H12) are represented by boxes and the β strands (s1 and s2) forming the β-turn by shaded arrows. "L" denotes ligand in the right hand model.

(a)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H1
(H2)
signature
H3
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β-turn
H6
H7
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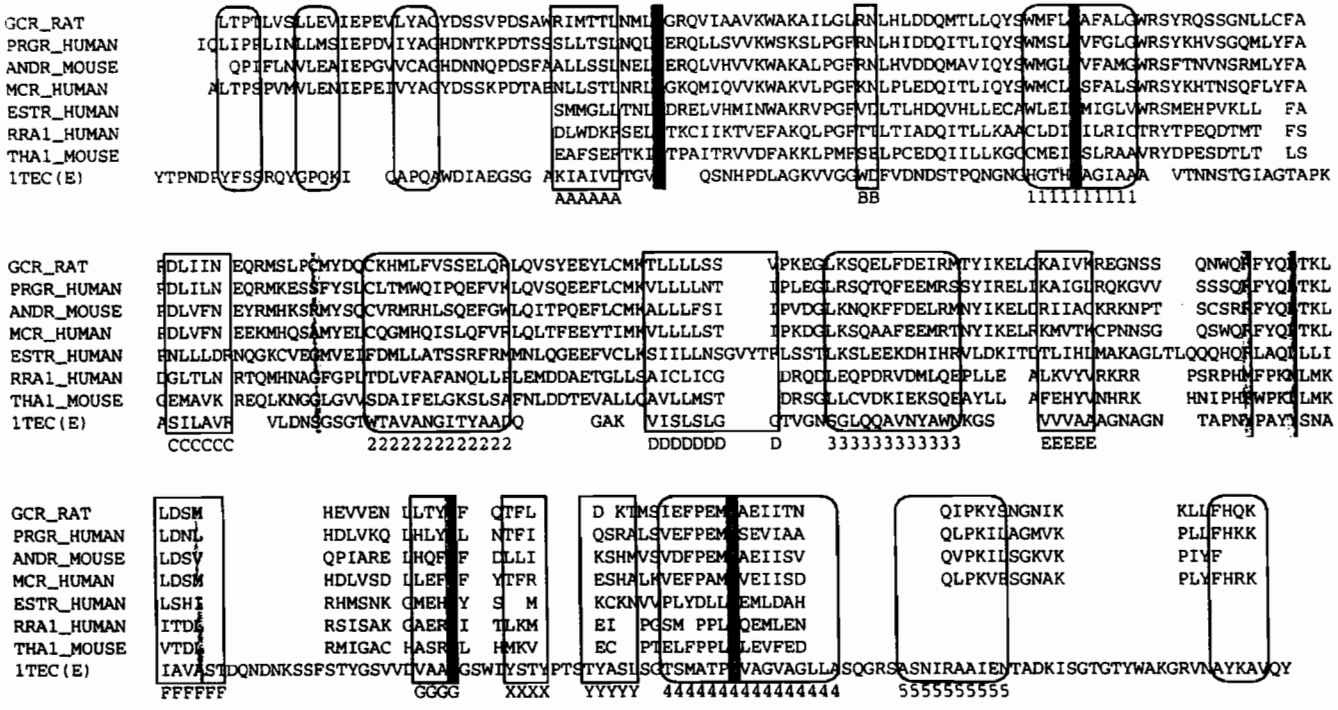
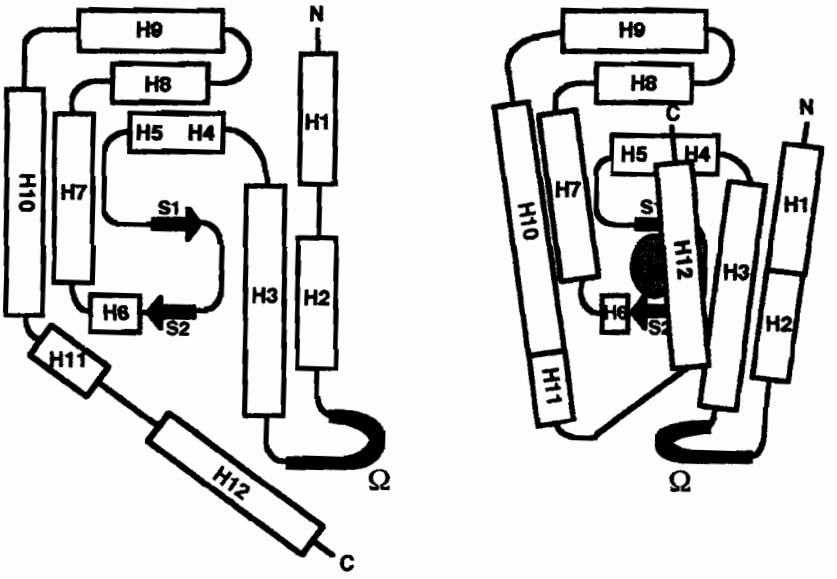
H10
H11
H12

(b)


GCR_RAT
PRGR_HUMAN
ANDR_MOUSE
MCR_HUMAN
ESTR_HUMAN
RRA1_HUMAN
THA1_MOUSE
1TEC(E)
AAAAAA
BB
1111111111
2222222222222
DDDDDDD
333333333333
EEEEE
FFFFFF
GGGG
XXXX
YYYYY
4444444444444444
5555555555

(c)


H9
H8
H5
H4
N
H1
H10
H7
S1
H3
H2
H6
S2
H11
H12
C
Ω
H9
H8
C
N
H5
H4
H10
H7
S
H12
H3
H1
H6
S2
H2
H11
Ω

al., 1997; Wurtz *et al.*, 1996). The model indicates that the integration of H12 acts as a lid to stabilize ligand binding in the LBD pocket. This process is also essential for both the transactivation by AF-2 and the ligand-induced interaction between the LBDs and putative transcriptional mediators of AF-2 (Wurtz *et al.*, 1996, and references therein).

Missense mutations at either end of the LBD estimates its functional N- and C-terminal boundaries. A single base mutation (T ---> C) creates Leu677Pro substitution in the N-terminal region of the LBD (Belsham *et al.*, 1995). This site is highly conserved among the steroid receptor family (Wurtz *et al.*, 1996). Transfection studies showed that the mutation abolished receptor binding activity. Two N-terminal missense mutations at residue 695 that change Asp to His or Asn cause CAI associated with abnormal ligand binding either quantitatively or qualitatively, respectively (Ris-Stalpers *et al.*, 1991). This residue is conserved in other steroid receptors like hGR, hMR, and hPR. The most C-terminal natural mutation is at residue 909, a Gly909Arg substitution that shows 4- to 5-fold less affinity for the natural androgen DHT, about half normal ligand-binding capacity, and impaired transactivation (Choong *et al.*, 1996). Moreover, the artificial deletion of the last 12 C-terminal residues of the hAR leaves the receptor defective in both binding and transactivation (Jenster *et al.*, 1991).

Another critical region in the LBD is the dimerization subdomain which was first discovered in the mouse ER (mER) and shows high homology among members of nuclear receptors (White *et al.*, 1991). This subdomain contains several elements like the hydrophobic heptad repeat which begins at Leu 859 in the hAR and includes Val 866, Leu 873, and Leu 880. Mutagenesis of Arg in mER at the position homologous to Leu 859 of hAR abolishes ER dimerization and specific ER-ERE binding, but not estrogen binding. Another substitution mutation (Met886Val), which is described in this thesis, occurs 6-residues C-terminal to the dimerization subdomain (this subdomain is discussed below), leaves ligand binding normal but leads to oligospermia by reducing transactivation (50%), causing a ~ 40% loss in ARE binding. In contrast, substitution of Arg at the mER position homologous to Val 866 in hAR yields a receptor defective in ligand binding, dimerization and ERE binding. This region, again, lies in the context of α-helix/β-turn units model proposed by Goldstein *et al.* (1993), and also lies in the LBD functional region AF-2 AD, described by Wurtz *et al.* (1996).

Ligands and conformational alteration of hAR

The AR adopts a ligand-specific conformation when bound to androgen. This conformation is altered when androgens are replaced by antagonists (Kallio *et al.*, 1994). Partial proteolytic digestion with trypsin or chymotrypsin of the unliganded AR or AR liganded to natural (T, DHT) or synthetic (MB) androgens, or the antiandrogens cyproterone acetate (CA) or casodex, (Kallio *et al.*, 1994) showed that all androgens generated a 30-kDa protease-resistant fragment. This fragment was absent in samples from the unliganded receptor or the receptor bound to antagonists. This fragment was not of N-terminal origin as the tryptic digestion of N-terminally deleted AR lacking residues 48 to 408 still produced the 30-kDa fragment when bound to androgen. A Met807Arg substitution mutation at the LBD altered the conformation of the androgen-bound AR as predicted by the disappearance of the 30-kDa fragment up on trypsinolysis (Kallio *et al.*, 1994).

In a similar study, the partial proteolytic digestion of hAR bound to the synthetic androgen methyltrienolone (MT or R1881) generated a protected fragment of 29-kD (Kuil *et al.*, 1994). When the antiandrogen ICI 176.334 was used, a larger fragment of 35-kDa was generated. The incorporation of both ligands in the same reaction generated both fragments in a competitive concentration-dependent manner. In the same study, the N-terminus deletion of androgen-bound hAR did not affect the protection of the 29-kDa fragment.

The LNCaP cell line grows faster in response to steroid hormones including androgens, estrogens, progesterones and several antiandrogens like CA and hydroxyflutamide but not ICI 176.334. This response is attributed to AR as ER and PR are not detected in this cell line. LNCaP cells have a single base substitution in the LBD of hAR (Thr877Ala) (Veldscholte *et al.*, 1992). The wild-type AR, when bound to MT or DHT, generated a protected 29-kDa fragment upon partial proteolysis; it generated a 35-kDa fragment when bound to the antagonist ICI 176.334. The mutant AR-MT and -DHT complexes also protected the 29-kDa fragment; but, surprisingly, complexes of the antiandrogens CA or hydroxyflutamide with the mutant AR also protected the 29-kDa fragment (Kuil and Mulder, 1994). Interestingly, those two antiandrogens act as agonists with this mutant, a fact that could explain the promiscuous binding of several steroid ligands to this mutant AR. It is likely that agonist- and antagonist-complexes with wild-type receptor have different conformations that dictate their differential transactivational activity. The LBD mutation in the LNCaP cells lets the receptor

acquire an activated conformation, similar to the wild type, even when bound to the two antagonists.

Dimerization

Most nuclear receptors, including all steroid receptors, need to dimerize for efficient regulation of a target gene. As discussed earlier, one subdomain of the LBD is involved in receptor dimerization and hence activation. DBDs and LBDs of AR, GR, and MR were expressed separately as fusion proteins with glutathione-*S*-transferase (GST) in *Escherichia coli* and analysed for their dimerization and DNA-binding properties (Nemoto *et al.*, 1994). The GST moiety has the property of self-interaction and therefore forms dimers of GST-DBD via GST, not DBD, as proved by thrombin cleavage of the recombinant proteins. Consequently, GST-DBD can specifically bind HRE as a dimer, and this binding is abolished when GST portion is cleaved. On the other hand, GST-LBD fusion proteins formed a high-molecular weight protein oligomer, apparently by two separate interactions (GST-GST and ARLBD-ARLBD) between the fusion molecules, and this interaction was hormone-independent (Nemoto *et al.*, 1994). These findings suggests that ARLBD has a potent ability to initiate homodimerization whereas ARDBD does not. This is followed by cooperative binding of DBD-DNA. Hard *et al.* (1990) showed that GR possessed a strong dimerization function in the C-terminal domain whereas the isolated DBD is monomeric in solution.

There is a region in the DBD where the C-terminal modules (second zinc fingers) of the two monomers of the GR interact without contacting the minor groove (Luisi *et al.*, 1991). The contacts occur between the C-terminal module and the DNA-phosphate backbone. These stabilize the dimer, and may explain the requirement of two DBD monomers at the GRE-half sites. Crystallographic analysis of GR dimer bound to GRE indicated that the dimer-GRE binding is more stable than monomer-GRE half-site binding even when the spacer nucleotides are increased from 3 to 4 nucleotides. This suggests tight and specific binding of one monomer, and variable, nonspecific binding of the other one (Luisi *et al.*, 1991). This suggestion is further supported by DNAse I footprinting analysis (De Vos *et al.*, 1993) in which fusion proteins of rAR and hGR DBDs were compared in the context of various natural REs from MMTV-LTR and the ARE in the promoter of the C3 (1) gene [rat prostatic steroid-binding protein, Parker *et al.*, (1983)]. It was suggested that by dimerization, both AR and GR DBDs equally protected the

REs similar to the GRE/PRE consensus. In contrast, when only one consensus half-site was used in the RE, the DNAse I footprint pattern showed that AR and GR are bound as monomers. The protection of some nucleotides downstream of the TGTTCT half-site might suggest dimer formation with one subunit bound tightly to the consensus half-site and the other more loosely as discussed earlier.

Studies done on baculovirus-expressed recombinant AR in insect cells of *Spodoptera frugiperda* (Sf9 cells) demonstrated by mobility shift assays that the full-length AR required intracellular androgen exposure to dimerize and bind to ARE (Wong *et al.*, 1993). This androgen dependence was essential only when N-terminus domain was present; as the truncated AR (Δ 1-506) could dimerize and bind ARE in the absence of androgen. Moreover, this dimer bound only to full ARE and not half-site ARE. This indicates that the N-terminal domain acts to inhibit AR dimerization and DNA binding in the unliganded receptor (Wong *et al.*, 1993).

RECEPTOR-ASSOCIATED PROTEINS (RAP)

The transactivational activity of the nuclear receptors involves multiple interactions with other nuclear protein (co-factors and intermediatory factors) in order to mediate their function. These interacting molecules are specific and serve several function: stabilize the untransformed receptor, assist receptor-ligand binding, mediate receptor-DNA binding, and/or stabilize the receptor's interaction with the basic transcriptional regulatory machinery required for gene expression (Beato *et al.*, 1995).

The preparation of monoclonal antibodies and their use for immunoaffinity chromatography has revolutionized the study of steroid hormone receptor complexes and revealed that steroid hormone receptors are associated with other proteins (Landers and Spelsberg, 1992 and references therein). Steroid receptors exist as part of a large heteromeric complex in their untransformed state within the cells as 8 to 10S complexes in the cytoplasm, while the activated form of the receptor exists as a 4S complex. Earlier studies have shown that GR exists as 300-kD (8S/9S) form; the actual monomer size is ~94-kD, and it is localized in the cytoplasm.

Among the receptor-associated proteins (RAP) is the group of hsp that includes hsp90, hsp70, and hsp56. Hsp90 is one of the well-studied hsp associated with steroid receptors. It is a 90-kDa, highly conserved, ubiquitous, and abundant phosphoprotein that is associated with steroid receptors in cytosol

extracts. It exists as homodimer with at least 2:1 ratio to the GR. Hsp90 binding is required for strong ligand-binding activity (Brensick *et al.*, 1989); but once liganded, the hormone-binding receptor can be sustained in the absence of hsp90. Regulation of hAR by hsp90 was investigated in the yeast *Saccharomyces cerevasiae* in a mutant strains expressing hsp90 under temperature-sensitive conditions (Fang *et al.*, 1996). At restrictive temperature, there is a decrease in hormone affinity and hormone-dependent transactivation by hAR which was not due to a lower steady state level of AR protein. Not all RAPs appear to associate with the steroid receptors equally. For instance, GR associates with hsp90 and p59, whereas chicken PR (cPR) associates with hsp70 but not p59 (Smith and Toft, 1993). Other RAPs include p60, p59, p54, p50 and p23 which are widely expressed in human tissues.

Most of these associated proteins are complexed with the receptor in its inactivated form and serves to maintain its stability as mentioned earlier. Binding to the ligand causes the dissociation of RAPs as reflected by the decrease in the sedimentation factor (S) to 4S. This dissociation is important in activating the receptor. On the other hand, RAPs association is important for PR pre-liganding and binding to its RE, which suggests the contribution of RAPs in maintaining the authentic conformation of the unliganded receptor (Baniahmed and Tsai, 1993). Yeast mutants of abnormal hsp90 have been shown to be insufficient for the expression of functional GR (Smith and Toft, 1993). Similarly, yeast mutants defected in dnaJ chaperone failed to express full ligand-dependent hAR transactivational capacity (Caplan *et al.*, 1995). Another example of a RAP is receptor accessory factor (RAF). This factor enhances specific DNA-binding of RAR and GR by 25- and 6-fold, respectively, by heteromeric complex formation. RAF enhancement of AR-DNA binding is optimal with ARE. It interacts directly with AR and that interaction is enhanced in the presence of intact N-terminus suggesting that RAF might influence the ability of steroid receptors to transactivate (Kupfer *et al.*, 1993). In some cases, the association of RAP is ligand dependent as in ER (ERAP). An ERAP of 160-kD that exhibits estradiol-dependent binding to LBD of the receptor was identified. Mutational analysis of ER shows that its ability to transactivate parallels its ability to bind ERAP 160. This association is inhibited when antiestrogens are bound to the receptor (Halachmi *et al.*, 1994). It is more likely that antiestrogens change ER conformation so that ERAP 160 is impaired, and ER transactivation is reduced.

Recent studies revealed that ERAP 160 belongs to the steroid receptor coactivator 1 (SRC-1) protein family (reviewed by Horwitz *et al.*, 1996).

Glucocorticoid receptor interacting protein 1 (GRIP1) is another member of the SRC-1 protein family, which was isolated from a mouse brain cDNA library, that serves as transcriptional coactivator in yeast for steroid receptors LBDs including that of AR (Hong *et al.*, 1996). Recently, it has been found that proteins belonging to the SRC-1 family interact with the AF2 in the LBD of SRs in a ligand-dependent manner (Howitz *et al.*, 1996; and references therein). In addition to LBD binding, GRIP1 can act as a bridging protein between the nuclear receptors and the basic transcription machinery. This interaction may contribute to the formation of the preinitiation complex and/or its stabilization (Hong *et al.*, 1996). Further studies revealed a signature motif that exists in most of these coactivators which is composed of LeuXXLeuLeu (where X is any amino acid). The integrity of this motif is essential to interact with the LBD H12 of steroid receptors like ER and RAR (Heery *et al.*, 1997). In addition to that SRC-1 protein family of coactivators, there are at least six other protein families that serves as nuclear receptor coactivators (Horwitz *et al.*, 1996).

These coactivators can interact directly with the nuclear receptors in a ligand-dependent manner and form ternary complexes to enhance transactivation (Horwitz *et al.*, 1996). This interaction between the nuclear receptors and coactivators is usually associated with other interacting proteins that contribute to the stabilization of the preinitiation complex of transcription (Horwitz et al., 1996; and references therein).

Other coactivator proteins belong to different protein families which interact with nuclear receptors and enhance their transcription. In the yeast two-hybrid system using AR LBD as a bait, an androgen receptor-associated protein of 70 kDa (ARA_{70}) was isolated from human prostate cells (Yeh and Chang, 1996). This interaction with AR was in an androgen-dependent manner *in vitro*. ARA_{70} enhances AR-dependent transcription 10-folds compared to 2-fold with GR, PR or ER indicating AR specificity.

In addition to their binding to nuclear receptors, the SRC-1 protein family like cAMP response element-binding protein (CREB)-binding protein (CBP) and its associated protein p300 (p300/CBP-associated factor, PCAF) possess HAT (histone acetyltransferase) activity (Spencer *et al.*, 1997; Perlman and Evans, 1997).

The suggested model by Jenster *et al.* (1997) involves two steps of transcription induction by steroid receptor. The first step involves remodeling of transcriptionally repressed chromatin by loosening the packed histone complexes of nucleosomes via HAT activity of the coactivator. The second step is to enhance the stabilization of the preinitiation complex by the activation of functions of coactivators and the liganded receptor itself.

On the other hand, histone deacetylase 1 (HD1) was directed to a promoter using the GAL4 DNA binding domain fused to a C-terminal truncated hPR LBD ($PR_{\Delta 42}$) and HD1 (Jenster *et al.*, 1997). This construct can be activated by the PR antagonist RU486 but not by PR agonist R5020. In cotransfection studies using HeLa cells, this chimeric construct did not affect the basal levels of reporter activity in the absence of the antagonist. However, in the presence of the antagonist, the fused protein bound DNA and repressed transcription. Increasing amounts of the transfected construct decreased transcription proportionally, indicating that HD1 is a potent repressor of transcription and the repression is achieved presumably by the deacetylation of histones.

Other repressor proteins repress transcription by binding to DNA and blocking the receptor's binding, or by binding directly to the DBD of the receptor. For example, calreticulin can block the transcription induction of AR, GR and RAR by interacting with their DBDs (reviewed in Horwitz et al., 1996).

Interestingly, several reports showed that AR interacts directly *in vitro* with TFIIB and TFIIF (basic transcription factors) (McEwan and Gustafsson, 1997, and references therein), suggesting that the steroid receptor might be the first step to initiate the transcription process by RNA polymerase II.

THE PHYSIOLOGICAL EFFECTS OF THE NATURAL ANDROGENS AND AR ON MASCULINIZATION

Sex determination and differentiation is a multistage process that begins at fertilization, proceeds through reproductive morphogenesis, and culminates in sexual maturation and fertility at puberty. Human embryos with either a 46, XX or 46, XY karyotype are sexually undifferentiated until the sixth week of gestation when testes start to differentiate from the undifferent gonadal ridge under the direction of the testis determinating factor, a DNA-binding transcriptional protein encoded by a gene on the short arm of Y chromosome (SRY) gene (Sinclair *et al.*, 1990). Testicular differentiation is androgen-independent; however, androgen and AR are crucial for subsequent events in male sexual differentiation and

development. Male sex differentiation involves two processes: one inhibitory, the other stimulatory. Male sexual differentiation begins with regression of the Mullarian ducts, thereby repressing the development of female internal genitalia: Fallopian tubes, uterus, and upper vagina. This process occurs between weeks 6 and 8 of gestation and is mediated by a glycoprotein , antiMullarian hormone, secreted by the testicular Sertoli cells (Quigley *et al.*, 1995).

The stimulatory process of male sex differentiation is an androgen/AR-dependent process in which the Wolffian ducts differentiate into epididymes, vasa deferentia and seminal vesicles. The stimulatory events occur between 9 and 13 weeks of gestation and are induced by the action of testosterone (T). T secretion begins at week 8 by fetal Leydig cells and peaks between 11 and 18 weeks gestation. This process of internal masculinization does not involve the potent T metabolite DHT since the enzyme required to convert T to DHT, 5-α reductase 2, is not expressed in these tissues until about 13 week gestation, at which time the process of internal masculinization is complete. On the other hand, development of prostatic urethra and masculinization of external genitalia into the penis, penial urethra and scrotum which also occurs between 9 and 13 weeks gestation appears to require DHT, and the necessary enzyme, 5-α reductase 2 is expressed in these tissues at the appropriate time.

These ordered events of sexual differentiation and masulinization require the production and action of androgens. Disturbance of either event will interrupt the expression of androgen-responsive genes and result in the failure of complete masculinization (Quigley *et al.*, 1995).

STEROID RECEPTORS AND SIGNAL TRANSDUCTION

The contribution of steroid receptors to the process of signal transduction is not completely understood but there are several ways that the receptors may be involved.

The androgen receptor, as well as the rest of its subfamily members, are phosphorylated. AR phosphorylation occurs in the presence and absence of androgen. The first indication that phosphorylation might play a role in SR structure and function was the observation that specific glucocorticoid-binding activity in rat thymus lymphocytes was dependent on glucose and oxygen. The concept was further supported by the fact that the ability of GR to bind hormone paralleled intracellular ATP levels. It was found that GR existed in two forms in intact cells and that ATP was required for the generation of a steroid-binding form

of the receptor (Landers and Spelsberg, 1992). Evidence for hAR phosphorylation comes from in vitro analysis of hAR expressed in LNCaP or COS cells. hAR exists in two phosphorylated forms, a 110 kDa, and 112 kDa, size on SDS-PAGE analysis (Kuiper *et al.*, 1991). Phosphotryptic peptide analysis of hAR protein by two-dimensional peptide mapping and reverse phase HPLC showed phosphorylation at multiple serine residues. LNCaP cells incubated with the synthetic androgen R1881 had a new tryptic phosphopeptide (Kuiper and Brinkmann, 1995). Partial proteolysis of hAR protein labelled with ^{32}Pi revealed that phosphorylation occurs mainly in the N-terminal transactivation domain (Kuiper *et al.*, 1993b). Interestingly, lengthening of the glutamine tract caused an increase in the spacing between the two isotypes (110 and 112 kDa), with an extra isotype, 114 kDa, formed upon hormonal treatment (Jenster *et al.*, 1994). In addition, mutations in the DBD or deleting the N-terminal domain resulted in a reduced amount of the slowest migrating isotype. This affirmed that phosphorylation is mainly N-terminal (Kuiper *et al.*, 1993b). There are two N-terminal phosphorylated sites (Ser 81 and Ser 94) in hAR and one in the hinge region (Ser 650). Only Ser 650 was involved in transactivational regulation; activity was 30% reduced when it was substituted by alanine (Zhou *et al.*, 1995a).

Thus, phosphorylation of the AR N-terminus may be one mechanism for modulating its transcriptional regulatory activity. Interestingly, the PR, GR, and ER are also predominantly phosphorylated N-terminally. In other words, phosphorylation may be a generic regulation of nuclear receptor transcriptional activity (Kuiper *et al.*, 1995 and references therein).

Modulators of protein phosphorylation participate in regulating AR transactivation in transient cotransfection of HeLa or CV-1 cells. The activation of two key phosphorylation enzymes and the inhibition of a major dephosphorylation enzyme in these experiments increased testosterone-dependent transactivation of MMTV-CAT cotransfected with rAR. In the absence of androgen, activators of protein kinase-A (8-bromo-cAMP) or protein kinase-C (phorbol 12-myristate 13-acetate), or an inhibitor of protein phosphatase-1 2A (okadaic acid) showed a slight effect on reporter transactivation. The increase in transactivation in the presence of the hormone did not appear to be due to change in AR level or to altered binding of ligand or DNA (Ikonen *et al.*, 1994).

In addition to the process of phosphorylation, AR may interact with other factors involved in the process of signal transduction as has been shown by GR and AP-1 components (Fos and Jun) (Diamond *et al.*, 1990). The promoter of the proliferin gene does contain elements for AP-1 and GR. When AP-1 (Jun/Fos heterodimer) is bound to the promoter, GR binding to the site represses the transactivation. On the other hand, when the homodimer Jun/Jun binds to the site, binding of GR activates transcription. Interestingly, the promoter of human tissue transglutaminase also contains sites for AP-1 and GR binding (Lu *et al.*, 1995). Data on possible interaction(s) are not yet available.

The pS2 gene expression in the breast cancer cell line MCF-7 is controlled by estrogens but it is estrogen-independent in other tissues like stomach mucosa (Nunez *et al.*, 1989). The pS2 5'-flanking region contains a complex enhancer region that responsive to estrogens, epidermal growth factor, the c-Ha-ras oncoprotein and the c-jun protein, suggesting a cross-talk signalling between estrogen and other transcription factors involved in the regulation of cell growth. In rat, estrogens negatively regulates LHα and β-subunit and FSHβ, whereas androgens have no effect on FSHβ (Gharib *et al.*, 1990; and references therein). The effect of sex steroid on gonadotropin subunit synthesis was examined directly at he level of the pituitary gland using cultured rat pituitary gland (Ghraib *et al.*, 1990).Testosterone increased FSHβ mRNA 3-fold in females and males in a dose-dependent manner, but had no effect on α or LHβ mRNA levels in either sex. This suggests that, unlike estrogen, androgens have a direct effect on pituitary cells in regulating gonadotropin mRNA and hence the differential action of sex steroids on gonadotropin regulation. The growth and differentiation of neuroblastoma cell line SK-ER3 stably transfected with ER was controlled by the insulin and insulin-like growth factors (IGFs) (Ma *et al.*, 1994). This control was absent in the ER-minus parental cell line. The effects elicited by the growth factors was blocked by antiestrogen. Transient transfection studies showed that IGFs modulated ER-activated promoters indicating the existence of cross-talk between membrane and intracellular receptors, and demonstrating the physiological consequences of such regulatory communication between systems classically considered to be independent.

Another mechanism by which AR might be linked to the signal transduction pathway is via other factors that are directly involved in transcription. Human AR promoter contains an apparent functional cAMP response element (CRE) in a region between -530 and -380 (Mizokami *et al.*,

1994b). This suggests that hAR expression is regulated by components of other signaling pathways.

ANDROGEN RECEPTOR MUTATIONS AND THEIR CLINICAL MANIFESTATION

AR gene defects lead to a well recognized phenotype in human males. This genotype-phenotype relationship has facilitated the analysis of numerous number of hAR gene mutations. There are more than 220 hAR mutations in the database (Gottlieb *et al.*, 1997) and this number is increasing steadily. Affected individuals are of 46, XY karyotype, and their clinical phenotype behaves as an X-linked recessive trait. Their AR gene mutations can be classified as follows: 1) major structural defects due to complete or partial gene deletion; 2) minor structural defects due to one to four bp deletions or insertions; 3) point mutations that cause premature termination or alter mRNA splicing; 4) point mutations that cause amino acid substitution (missense mutations); and 5) expansion of (CAG)n repeat in exon 1.

Androgen insensitivity syndrome (AIS)

There were several case reports of individuals with probable androgen insensitivity syndrome (AIS) during the 1800's and 1900's. However, in 1953, John Morris from Yale University reported a series of 82 individuals with female phenotype described as "testicular feminization" (Morris, 1953). Since then, studies of the endocrinology, pathophysiology, biochemistry, and molecular biology of AIS have contributed to analyzing the structure-function properties of the AR in androgen action, and genotype/phenotype relations for various AR mutations. In AIS 46, XY individuals, androgen secretion by the testes is normal or increased; however, due to defective or deficient AR function, there is absent or reduced response by target tissues to androgen (for a review see Quigley *et al.*, 1995). Affected individuals, karyotypically males, are healthy with variable degrees of androgen insensitivity, whereas carrier females appear normal. Some carriers have delayed, sparse or asymmetrical sexual hair (Pinsky *et al.*, 1994). AIS classification is based on severity of external phenotype, especially the genitalia, and is classified as follows: individuals with complete AI (CAI) have female external genitalia; individuals with partial AI (PAI) have ambiguous external genitalia; individuals with mild AI (MAI) have male, or near-male genitalia (Pinsky *et al.*, 1995). Other clinical features of AI may include gynaecomastia (breast

development), sparse or absent pubic and axillary hair, and female body habitus seen in CAI. PAI may be associated with gynaecomastia, and defective axillary and pubic hair growth. MAI may be associated with azoospermia or oligospermia resulting in reduced fertility, and with cryptorchidism, impotence micropenis, and gynecomastia. However, impaired spermatogenesis does not appear to be an essential expression of the phenotype.

The biochemical kinetics of mutant AR of AI patients was initially studied on genital skin fibroblasts (GSF) to find the molecular and biochemical bases of the syndrome. GSF have about 3-fold higher androgen binding than nongenital skin fibroblasts (Kaufman *et al.*, 1977). Based on such measurement, patients are classified as positive (≥15 fmol/mg protein), deficient (5-15 fmol/mg protein), or negative (< 5 fmol/mg protein) for androgen binding.

AR gene mutations reported in AIS can be divided into two main groups: the first contributes to ~8% of AIS and includes complete or partial gene deletion and 1-4 bp deletion and insertion mutations; the second contributes to ~92% of AIS and includes single base mutations that change the sense of codons, or impair RNA splicing (Quigley *et al.*, 1995; Gottlieb *et al.*, 1997). The CAI patients are usually healthy apart from sexual maldevelopment; however, in two unrelated families with CAI due to complete AR deletion, two affected siblings in the first family were otherwise healthy (Quigley *et al.*, 1992); the single CAI patient in the second family also suffered from muscular atrophy and neurological and physiological problems (Trifiro *et al.*, 1991).

The severity of AI is determined by the type, degree, and site of mutation. Therefore, some mutations occur in essential sites that are important for AR structure and function like those occurring at the α-helix motif of the DBD. The deletion of Phe 582 in the N-terminal α-helical region of the DBD, and the deletion of Arg 615 in the C-terminal α-helix of DBD or its substitution to His all result in CAI, since both helices contribute importantly to the configuration of the DBD (Beitel *et al.*, 1994a). There are several kinds of nonsense, frame-shift, and splice-junction mutations that yield an inactive AR causing CAI in the affected individuals (Gottlieb *et al.*, 1997). The N-terminal premature termination mutation at Gln 60 which causes CAI and resulting in high ligand dissociation rate (k), low B_{max}, and normal binding affinity (K_d) of the truncated hAR initiated at an internal methionine codon. When overexpressed under transfection conditions, the mutant had 30% of the normal transactivation activity (Zoppi *et al.*, 1993). Interestingly, this naturally occurring mutation is in agreement with the artificial

deletion mutation of the entire N-terminal domain (residues 1-506) analyzed by Zhou *et al.* (1995b). In this study, N-terminally deleted AR transiently expressed in COS cells resulted in dissociation half-time of 0.8, 1.0, and 0.4 hr for MT, DHT, and T, respectively, at 37°C which is 3- to 5-fold shorter than that of full-length AR. In addition, the N-terminal deletion lead to a higher rate of degradation (up to 7-fold) despite higher concentrations of androgen incubations.

Other missense mutations result in a decrease in AR mRNA stability when assayed by S1 nuclease protection method. This was found in the PAI-causing mutation Gly909Arg (Choong *et al.*, 1996) or in the CAI-causing mutation Arg774Cys (Marcelli *et al.*, 1991). On the other hand, an identical mutation at position 774 in an unrelated individual yielded normal levels of mRNA as extracted from patient's GSF and subjected to Northern analysis (Prior *et al.*, 1992). This discordance may be due to the different sensitivities of the techniques applied and/or GSF biopsy procedure. AR mRNA level in the Tfm mouse was reduced 10- to 20-fold due to a frame-shift mutation caused by a cytosine deletion at codon 371 leading to a stop codon at position Val 412. Cycloheximide treatment of the mutated mice caused a significant increase in AR message in kidneys (17-fold). This suggests that mRNA instability might be due to an increase in AR mRNA degradation (Charest *et al.*, 1991).

Other AR missense mutations that result in other receptor qualitative defects. For example, some mutated hAR receptors show selectively normal binding to certain androgens but defective with other androgens. Such phenomenon was found in the PAI-causing mutation (Val866Leu) (Jukier *et al.*, 1984; Kazemi-Esfarjani *et al.*, 1993) which has normal DHT rate of dissociation (off-rates) and abnormal MT off-rates of dissociation. The opposite is observed in CAI-causing mutation (Ser814Asn) that displays normal MT off-rates but abnormal DHT off-rates (Pinsky *et al.*, 1984). The missense mutation that causes CAI (Val889Met) has a normal dissociation constant under equilibrium conditions (K_d) but an increased dissociation rate under nonequilibrium conditions (Zhou *et al.*, 1995b). Moreover, the mutation at position 727 in exon 5 that changes asparagine to lysine in a case of an infertile male could be compensated by a high dose of a testosterone metabolite, mesterolone (Yong *et al.*, 1994a). As reported before, this compensation is obvious in transient hAR expression in COS-1 cells using MMTV-GH reporter and mesterolone in transactivational analysis of this mutation. Mesterolone, but not other androgens could bring transactivation to normal level at 37°C (this thesis).

Prostate and breast cancer

Mutations in tumor suppresser or transcription-regulating genes such as AR gene could disturb the balance between cell proliferation or cell death in a single clone of cells. Because of their important roles in the development of the prostate cancer, androgens and the AR have lately become the focus of breast and prostate cancer research. One of the features of prostate carcinoma is the initial remission of cancer growth after medical or surgical castration; the tumor then becomes androgen-independent. Mutations in hAR could induce tumor progression by altering the orthodox receptor-ligand specificity (Kuil and Mudler, 1994) and subsequently may result in a loss of transcriptional control. Mutations in the LBD could render the receptor constitutively active due to the loss of the normal repression of AR transactivation in the absence of androgen, or by becoming promiscuous and binding to different ligands in addition to T and DHT. So far, most of the prostate-cancer causing mutations of hAR are somatic. There is one substitution mutation in exon 5 (Arg726Leu) that was detected in germ line and passed to the offspring in which the hAR is qualitatively normal (Elo *et al.*, 1995). The Androgen Receptor Mutations Database (curated by Dr. B. Gottlieb at the Lady Davis Institute of Medical Research, McGill University, Montreal) lists more than 220 mutations, 24 of which are associated with prostate cancer. There are 9 different mutations occurring in the N-terminus in exon 1; two of these mutations occurred in the polyCAG tract. The first is a contraction in the CAG repeat; the prostate tumor had two alleles, one of the wild-type CAG length (n=24), and the other n was 18 CAG long. However, this somatic mutation does not give a strong explanation for the cause of the cancer since 18 repeats lies within the normal range of polyGln tract. Possibly, it might increase transactivation of target gene responsible for cell growth and development as discussed earlier (Kazemi-Esfarjani *et al.*, 1995). The second mutation that occurs in the (CAG)n tract is Gln64Arg substitution, which caused prostate cancer; functional characterization was not done (Tilley *et al.*, 1996). This reflects the importance of the N-terminus in modulating transactivational ability of hAR and the involvement of the polyGln tract in fine-tuning this activity.

hAR gene mutations in prostate cancer have been found in exons 4-8. Three different mutations occurred in the hinge region, which includes the NLS subregion, (Lys630Thr, Ser647Asn, and Gln670Arg). The rest of the mutations cover most of the LBD. The most frequent mutation is found in exon 8 (Thr877Ala); this mutation is the same one in the LNCaP cell line. Obviously, Thr

877 plays an important role in ligand specificity for the AR, especially as this site is unique for hAR and lies in the H11 of the predicted 3-D model of the nuclear receptor LBD and also predicted to interact directly to the ligand (Wurtz *et al.*, 1996).

The only two mutations of hAR that have been detected in male breast cancer have occurred at sites in the DBD, encoded by exon 3. These are missense mutations of consecutive sites, Arg607Gln and Arg608Lys, and are associated with PAI (Wooster *et al.*, 1992; Lobaccaro *et al.*, 1993). It is suggested that these mutations may disturb protein-protein interaction between AR and other nuclear factors (Poujol *et al.*, 1997)

Spinobulbar muscular atrophy

This disease is another example of a distinctive genotype/phenotype relation caused by a different type of hAR mutation. SBMA or Kennedy disease is an adult-onset disease caused by the expansion of the CAG repeat coding for the homopolymorphic Gln tract of the N-terminus. The expansion from the normal range of 11-33 Gln to ≥ 40 was first discovered in SBMA patients in 1991 (La Spada *et al.*, 1991). This disease was originally described in men by Kennedy *et al.* (1968) as a slowly progressive syndrome of muscle cramps of 4th or 5th decade fasciculations, wasting and weakness, usually with bulbar involvement affecting swallowing and speech. The disease affects males only and is inherited as an X-linked recessive. More than half of the affected individuals develop gynaecomastia, testicular atrophy, and progressive infertility due to testicular failure (Arbizu *et al.*, 1983). These latter expressions of PAI occurs in adulthood, but before the onset of motor neuron degeneration in the spinal cord, some loss of sensory neurons in the dorsal root ganglia, and brain stem motor nuclei (Brooks and Fischbeck, 1995).

Transactivational analysis of SBMA mutant AR was done in vitro using COS-1 cells and cDNAs constructed with either 40 or 50 CAG repeats in comparison to the wild type hAR with 20 CAG repeats. The polyGln-expanded receptor was hypotransactive on the reporter gene (GH) driven by the androgen-responsive promoter in the context of MMTV-LTR (Mhatre *et al.*, 1993). Moreover, the same group showed that the expression of the reporter gene was inversely correlated with the length of the CAG tract when zero, 12, 20, 40, and 50 repeats were included in the analysis (Kazemi-Esfarjani *et al.*, 1995). This

indicates that the expansion of the repeats impairs normal transactivation level of hAR.

After discovery of the SBMA mutation, expansion of CAG repeats in the coding region was detected in the genes for six other neurodegenerative diseases. These include Huntington disease (HD) (Huntington's Disease Collaborative Research Group, 1993), spinocerebellar ataxia type-1 (SCA 1) (Orr *et al.*, 1993), dentato-rubral pallidoluysian atrophy (DRPLA) (Nagafuchi *et al.*, 1994) also described as Haw River disease (Burke *et al.*, 1994), Machado-Joseph disease (MJD1 or SCA3), spinocerebellar ataxia type 2 (SCA2) ((Pulst *et al.*, 1996; Sanpei *et al.*, 1996; Imbert *et al.*, 1996), and spinocerebellar ataxia type 6 (SCA6) (Zhuchenco *et al.*, 1997). Each of these diseases involves neuronodegeneration in different regions of the central nervous system (Ross, 1995), and shows some degree of inverse relationship between expanded repeat length, age of onset, and severity; the longer the repeat, the earlier the age-of-onset and more severe the disease. These phenomena have not been confirmed by others studying SBMA (MacLean *et al.*, 1995a; MacLean *et al.*, 1996).

The expanded hAR transiently expressed in COS cells is not unstable and binds androgen normally (Mhatre *et al.*, 1993; Kazemi-Esfarjani *et al.*, 1995). In contrast, three studies have investigated the Gln-expanded hAR binding to androgens in cultured GSF. One group found moderately lower AR binding (B_{max}) in scrotal skin fibroblasts from 3 SBMA patients from one family but not of 5 other patients from 3 other families(Warner *et al.*, 1992). Danek *et al.* (1994) described one case with a decreased B_{max} in GSF, and a third group found that in 5 out 6 SBMA patients, the AR had a normal Bmax but a significant decrease in affinity of AR for androgen (K_d) in cultured suprapubic skin fibroblasts (MacLean *et al.*, 1995b). The different results for androgen binding in the two systems might be due to the different cells used in the experiments, suggesting a possible tissue-selective abnormality. Interestingly, postmortem quantitative analysis of hAR mRNA extracted from the spinal cord of one SBMA individual using reverse transcriptase-PCR (RT-PCR) showed a decreased amount of mRNA compared to controls from subjects with amyotrophic lateral sclerosis (ALS) and lung cancer. This decrease in message was accompanied by a decrease in the amount of AR protein as analyzed by Western blot and compared to the same set of controls used for RT-PCR (Nakamura *et al.*, 1994). Although these data were obtained from analysis of one case, and may not apply to the rest of SBMA patients, it may be a possible mechanism contributing to PAI associated with

SBMA in addition to hypotransactivation. To assess whether the reduction of AR transactivation could be detected in genital skin fibroblast strains established from SBMA patients, McPhaul *et al.* (1997) infected the monolayers with adenovirus vector carrying the MMTV-luciferase reporter gene. The GSF of all four SBMA cases had normal androgen binding. Nevertheless, the level of AR function was reduced in all four instances compared to the normal fibroblasts. In three cases, the level transactivation was comparable to patients with Reifenstein syndrome. In one case, the AR transactivation was markedly reduced like in CAI patients.

Moreover, Gln-expanded hAR transiently expressed in the NSC34 cell line (a hybrid cell line generated by fusing mouse embryonic motor neuron-enriched spinal cord tissue with neuroblastoma cell line; Cashman *et al.*, 1992) had increased androgen binding, increased AR protein level as tested by Western blot analysis, and yet was hypotransactive on a GH-reporter gene driven by MMTV-LTR (this thesis). On the other hand, two studies polyGln-expanded AR were recently reported by others. One used a hybrid cell line similar to NSC34, MN (Salazar-Grueso *et al*, 1991), stably transfected with the wild-type or the polyGln-expanded AR (Brooks *et al.*, 1997). In their work, Brooks *et al.* found that both forms of AR were able to bind ligand and transactivate a reporter gene equally well. There was no distinguishable phenotype caused by the two forms even after differentiation. The polyGln-expanded form, though, was expressed less than the wild type as judged by Western analysis. The authors attributed the lower AR level to lower mRNA expression caused by the expanded CAG repeat. The second study showed that the size of the CAG repeat related inversely with expressed AR mRNA and protein levels in transiently transfected COS-1 cells, but did not alter ligand binding nor transactivation activity (in CV-1 cells) (Choong *et al.*, 1996). The authors suggested that the expansion of CAG repeat does not alter AR function, but rather reduces AR mRNA expression which might contribute to the androgen resistance observed in SBMA. In this second study, AR ligand-binding and mRNA analysis was done in COS-1 cells, whereas transactivational analysis was done in the parental cells of COS-1 (CV-1). In addition, the transactivation data were not expressed per unit of A-R complex. This is the ultimate way to quantitate transactivation competence. In our laboratory, we always find a decrease in ligand-binding activity of the wild-type AR transiently expressed in CV-1 cells compared to COS-1 (unpublished data). From the data reported on AR expression and binding activity, it is apparent that

there is appreciable variation in AR expression even among different cell types of patients with SBMA (Jenster *et al.*, 1993; Mhatre *et al.*, 1993; Kazemi-Esfarjani *et al.*, 1995; Warner *et al.*, 1992; Danek *et al.*, 1994; MacLean *et al.*, 1995b; Nakamura *et al.*, 1994; McPhaul *et al.*; 1997). AR expression and transactivational analysis using the same vector and method of transfection will help to explain inconsistent AR expression by different cell lines.

Subjects with CAIS, including those with complete deletion of the gene, have no consistent neurological symptoms. SBMA patients, on the other hand, are phenotypically males with neurologic symptoms that are not seen in cases of CAI or loss-of-function mutations; expansion of CAG repeat, therefore, cannot cause the neuronopathy of SBMA by a simple loss of AR activity. Rather, it must gain a function either in degree or quality, and also it must lose part of its native function. The latter mechanism might contribute to MAI since the expanded AR is hypotransactive. The gain of function that renders the SBMA AR toxic to motor neurons in the brain stem and spinal cord is not yet known.

There are several proposals to explain the neuropathogenicity of genes with expanded CAG repeats in their coding regions. One is that the expanded polyGln tract becomes a substrate for the enzyme transglutaminase which forms an irreversible, covalent bond (isodipeptide bond) between a glutamine and lysine residues, the resulting complex being selectively neuronotoxic (Green, 1993). A recent study by Kahlem *et al.* (1996) showed that synthetic peptides with variable lengths of polyglutamine flanked by the adjacent amino acid residues of SCA1, SCA3 or DRPLA were found to be good substrates for rat liver transglutaminase in vitro, and the reaction was improved upon lengthening the Gln tract. The peptide $R_5Q_{18}R_5$ generated insoluble aggregates when mixed with brain extracts and rat liver transglutaminase. In vivo affirmation of this hypothesis is needed.

Another proposal involves the formation of a glutamine polar zipper. Perutz *et al.* (1994) showed by molecular modeling followed by optical, electron, and x-ray diffraction studies of a synthetic poly(L-glutamine) that it forms β-sheets strongly held together by hydrogen bonds. This model raises the possibility that binding of polyGln-expanded proteins with other transcription factors may sequester such factors or lead to their gradual precipitation in neurons.

A third model hypothesized that polyGln-expanded proteins resist the endogeneous proteolysis and, hence, accumulate as abnormal large polyGln

moieties that may disturb the cellular and mitochondrial milieu and impair energy metabolism, rendering specific regions of the nervous system susceptible to these hypothesized neuroexcitotoxic molecules (Cha and Dure, 1994).

Transgenic mice

A transgenic animal model will be of great value in understanding the pathogenetic events of the expanded (CAG)n repeat neurodegenerative diseases. Unlike the rest of the genes linked to the other five diseases, normal AR function in many tissues is known, as well as SCA6-linked gene, α_{1A}-voltage-dependent Ca^{2+} channel. This may facilitate investigation of pathogenesis by the expanded AR. A transgenic mouse line expressing a hAR cDNA with 45 repeats has been constructed, however, it does not show a neurological phenotype (Bingham *et al.*, 1995). These cDNAs, driven by either the metallothionein or neuron-specific enolase promoters, inserted randomly in the genome. However, the expression of the mutant hAR in the brain and spinal cord was very low. Also, unlike in humans, the repeat length was stable in these mice across at least four generations and 76 meioses. Low expression of mutant hAR and expression of the endogenous AR might explain the failure of these mice to develop a neuronopathy. To construct a better transgenic model, the endogenous background of normal AR of the animal must be abolished by, for example, homologous recombination to replace the normal-size CAG tract by an expanded one in exon 1 with the aim of expressing the mutated hAR gene at or near its usual tissue-specific level. Alternatively, the Tfm animals would be a good model to avoid the endogenous AR expression and might also answer the question whether heterozygousity or species specificity plays any role in suppressing expression of the disease.

Animal models have been generated for three other (CAG)n-expanded neurodegenerative diseases: SCA1, MJD (SCA3) and HD. Ataxin-1 encoded by full-length SCA1 cDNA carrying a polyGln repeat expansion with $(CAG)_{82}$ was overexpressed in mouse cerebellum under the control of a Purkinje cell specific promoter (pcp) (Burright *et al.*, 1995). Three heterozygous and two homozygous lines showed a progressive ataxic phenotype between 12 and 26 weeks of age. Pathology revealed a significant loss of Purkinje cells.

Transgenic mice were generated with a $(CAG)_{79}$ version of full-length SCA3 gene and with an isolated $(CAG)_{79}$ polyGln tract under the control of a Purkinje-specific (L7) promoter (Ikeda *et al.*, 1996). Transgenic mice with the

isolated polyGln-expanded tract showed severe ataxic symptoms with Purkinje cell death. Surprisingly, no phenotype was observed in the transgenics with the full-length expanded SCA3 gene. Again, this might be due to lower expression of the full-length expanded gene, especially since the authors did not mention the expression level of the full-length gene in the transgenic mice compared to those expressing the polyGln-expanded tract. Nonetheless, it is possible that the full-length Gln-expanded mutant must be processed to a smaller form in order to be pathogenic.

Lately, HD transgenic mice were established with a 1.9 kb human genomic fragment including the endogenous promoter and expanded exon 1 carrying $(CAG)_{115}$-$(CAG)_{150}$ repeats (Mangiarini *et al.*, 1996). Again, this fragment was sufficient to generate a progressive neurological phenotype that exhibits many features of HD including choreiform-like movements, tremor, involuntary stereotypic movement disorder and epileptic seizures. No morphological abnormalities or neurodegeneration were observed in these transgenics; however, sections of the brains showed a consistent size reduction which appeared to be uniform throughout all central nervous system structures. This might suggest that localized atrophy may be secondary to the primary HD clinical symptoms.

It is noteworthy that hAR transgenic mice carried poly$(CAG)_{45}$ tract of which is significantly shorter than the other tracts used in the other three animal models, this might attribute to a delayed symptoms of neuronodegeneration that is longer than the mouse life span. Also, the expression of the hAR in the transgenic mice was low compared to the other models. Thus polyGln-expanded neuronopathies may be concentration-dependent. Therefore, the length of the CAG repeat, the protein expression level and stability, and promoter are likely to contribute to the earlier onset of the clinical symptoms of the disease.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

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

1) The polyGln-expanded hAR was less transactive than the normal hAR when transiently expressed in NSC-34 motor neuron-like cell line despite higher ligand-receptor complex formation. This higher complexes formation is apparently due to more AR protein content. This finding is relevant to the hypothesis that loss of function is necessary to cause SBMA. This indicates a cellular specificity in the behavior of the poly(CAG)n-expanded *hAR* and/or (Gln)n-expanded hAR protein.

2) Partial proteolytic analyses in a cell-free system revealed that the polyGln-expanded hAR is conformationally altered and it is relatively resistant to protease activity presumably because it is conformationally altered.

3) In the cell-free system, protease resistance is exemplified not only by slower degradation of the parental polyGln-expanded hAR, abut also by the accumulation of polyGln-expanded-containing fragments. COS-1 cells transiently transfected with the (CAG)n-expanded AR accumulated an unorthodox 75-kDa polyGln-expanded polypeptide for at least 4 days, and were twice as likely to die apoptotically, suggesting the likely contribution of the polyGln-expanded fragment in cytotoxicity and hence SBMA pathogenicity. To my knowledge, this is the first study to show cytotoxicity caused by the intact full-length hAR containing a polyGln-expanded fragment.

4) The polyGln-expanded hAR transiently or stably expressed in two different motor neuron hybrid cell lines caused a significant androgen-dependent negative shift of the steady-state activation of a T-type voltage-gated Ca^{2+} channel. The observed negative shift (>-10 mV) in steady-state activation resulted in a large increase in the T-type Ca-channel window current and, hence, Ca^{2+} influx, possibly simulating motor-neuron apoptosis in SBMA.

5) The pathogenetic analysis of two hAR missense mutations causing oligospermic infertility revealed, for the first time, impaired AR binding to ARE due to alterations in the LBD, perhaps by impaired LBD-mediated dimerization

(Met886Val) and/or conformational changes (Asn727Lys). This impairment resulted into a reduced transactivation capacity of the mutant hAR without a detectable ligand-binding abnormality.

PART II. MATERIAL AND METHODS

SUBJECTS AND DIAGNOSIS

Two mutations were identified by Dr. E. L. Yong's laboratory, The National University of Singapore. One, previously reported (Yong *et al.*, 1994a), an Asn727Lys substitution in exon 5 of hAR, was presumed to cause severe oligospermia. In a 51-year-old infertile man with no other signs of AI. Sexual hair distribution and external genitalia were normal, apart from reduced testicular volume of 12 ml, bilaterally. Normal serum gonadotrophins and testosterone were found. However, semen analysis indicated severe oligospermia with sperm counts ranging from 2.3 to 3.7 million/ml (normal:>20 million/ml). This patient was treated with 25 mg mesterolone daily. Four months later, his sperm count rose to 6.5 million/ml; it reached a peak of 28 million/ml six months after beginning mesterolone therapy. His wife conceived during this period and delivered a normal infant. Sperm counts fell to 5 million/ml after he stopped mesterolone therapy.

The other mutation, found in two unrelated subjects (CML and KLH), was a Met886Val substitution. CML presented at 31 years after four years of infertility. He needed to shave infrequently, about once a week. Right-sided cryptorchidism was corrected at 7 years. Testicular volume was 10 ml bilaterally and his sperm count about 0.5 million/ml. KLH presented at 40 years after 7 years of infertility. He had Tanner grade 2 persistent postpubertal gynaecomastia. Testicular volume was 6 ml bilaterally with sperm counts around 0.3 million/ml. Secondary sexual development was otherwise normal in both subjects. They had a normal male karyotype and normal serum levels of gonadotrophins, androgens, sex-hormone binding globulin, prolactin and estradiol.

MOLECULAR ANALYSES

Identification of mutations

Screening for mutations in oligospermic cases was done in Yong's laboratory at the National University of Singapore. DNA was extracted from the peripheral blood of both oligospermic and control subjects, and subjected to PCR and then screened by single-stranded conformation polymorphism (SSCP) (Yong *et al.*, 1994b). This sensitive method relies on the fact that a point mutation can alter the secondary structure of single stranded DNA which migrates differently from a corresponding wild type on non-denaturing polyacrylamide gel

electrophoresis. Exons 2-8 were first amplified using flanking intronic primers. Ten µl of the PCR product were analysed on a 8% nondenaturing polyacrylamide gel containing 5% glycerol at 12°C. Gels were silver stained and searched for fragments with mobilities different from normal. Positive controls representing previously characterized mutations in the DBD and LBD were included. Suspect fragments were sequenced using a Taq dideoxy terminator cycle sequencing kit with [^{33}P] as the radioactive label. Exon 1 was screened using specific primers and the poly(CAG)$_n$ and poly(GGN)$_n$ tracts were sequenced directly.

PCR-amplified genomic fragments corresponding to exons 1-8 from subjects KLH and CML were re-sequenced in our laboratory to reconfirm the mutations.

The expansion mutation in the trinucleotide repeat of the receptor was previously subcloned in our laboratory and in Dr. K. Fischbeck's laboratory at the University of Pennsylvania, Philadelphia.

Oligonucleotide primers

Oligonucleotide primers were chosen for optimum melting temperature (T_m) and synthesized by the Sheldon Biotechnology Centre, McGill University (Montreal).

PCR amplification

DNA amplification was performed using a thermal cycler (Perkin-Elmer Cetus, Connecticut) and *Pfu* (Stratagen, La Jolla, CA) or *Pwo* DNA polymerase (Boehringer Mannheim, Germany). The reaction generally consisted of one denaturation step at 97°C for 3 minutes, followed by 25-30 three-step amplification cycles (denaturation , 30 sec, 97°C; annealing, 1 minute, 57°C; extension, 1.5 minutes, 72°C. PCR conditions were optimized whenever necessary depending on the primers T_m. Each reaction mixture consisted of 20 nmol of each dNTP (Pharmacia Biotech, Sweden), 25 pmol of each primer, 1 µg of genomic DNA (50 ng in the case of plasmid templates), 10 µl of the 10X reaction buffer and 2.5 units of the DNA polymerase to a final volume of 100 µl in a 500 µl PCR tube. Amplification of exons 2-8 involved intronic primers for the exon to be amplified (Trifiro *et al.*, 1997). For exon 1, deaza-GTP was used instead of dGTP and the reaction consisted of a 1min denaturation at 98°C, 1 min annealing at 55°C, and a 1.5 min extension at 72°C. Negative controls were prepared by

omitting the DNA template in order to detect contaminating DNA. Generally, each reaction mixture consisted of 100 µl final volume overlaid by 100 µl of mineral oil to prevent evaporation.

PCR mutagenesis of the expression vector

The mutated hAR cDNA was recreated by the overlap extension method of Higuchi (1990).

(i) Recreation of exon-5 and exon-8 mutations in the pSVhAR.BHEX and pcDNA3 expression vectors

The mutant cDNA hAR was generated by two PCR reactions using the pSVhAR.BHEX (Beitel *et al.*, 1994b) expression vector as the template DNA. For each site-directed mutation, each primary PCR reactions involved the use of one mutant primer (a, the sense strand; or b, the antisense strand) with an outside antisense wild-type primer (a 3' flanking primer , or a 5' flanking primer). For the exon 5 and exon 8 mutations, the external primers were Primer-B and P-3' (see Table I for primer list). The latter primer is located in the β-globin poly-A tail in vector sequence. The mutated primers for site-directed mutation were: PR5a, PR5b, PR8a, and PR8b (see Table I for primers sequences). The choice of the primer was made with regard to incorporation of a flanking restriction site that was unique in the expression vector. The final PCR product was gel purified by excising the amplified DNA band from 1% low-melt (LMT) agarose prestained with ethidium bromide and examined under UV light. Purification of the band was done by the QN-butanol method by (Langridge *et al.*, 1980). The DNA was measured spectrophotometrically at OD_{260}, digested with the desired restriction enzymes and the double-digested fragment containing the mutated site ligated into the corresponding sites of the similarly treated pSVhAR.BHEX or pSVhAR.BHEXE expression vectors (Fig. 5). For the exon 5 mutation, a *Xho*I/*Eco*RI double digest was done; for exon 8 mutation, *Eco*RI/*Bam*HI double-digestion was used .

After recreating both the exon 5 and exon 8 mutations in pSVhAR.BHEX, the mutated cDNA was first sequenced (Fig. 6) and then subcloned into the pcDNA3 expression vector (Invitrogene,) to replace the expanded hAR cDNA (pcDNA3hAR44Q) using *Nhe*I/*Bam*HI double digestion.

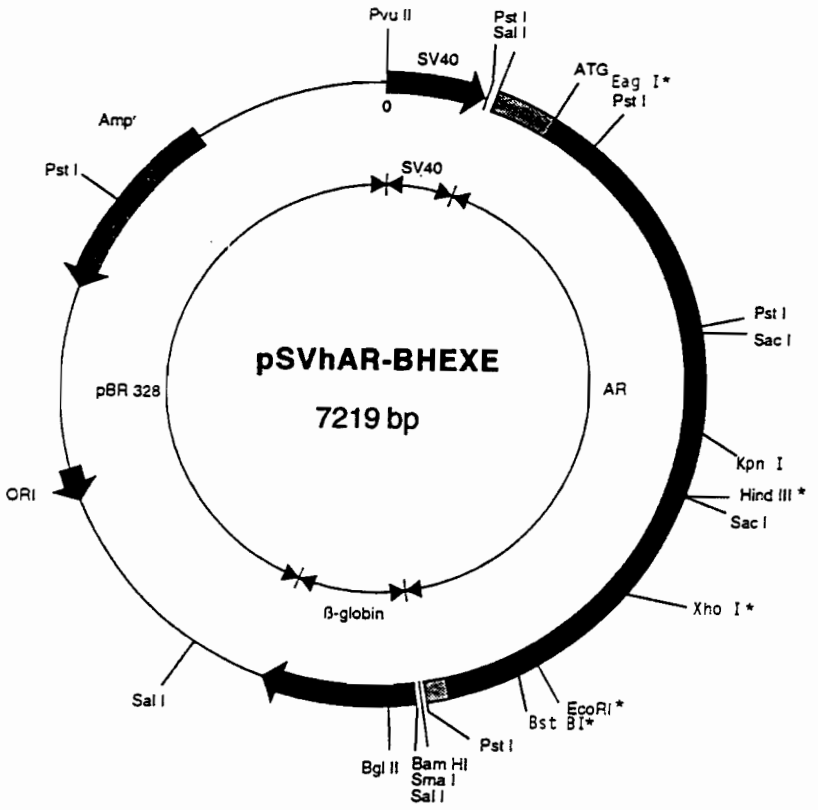
(ii) Creation of the mixed $(CAG/CAA)_n$ trinucleotide repeat

Figure 5. pSVhAR.BHEXE expression vector and hAR cDNA sites used for PCR amplification and cloning.

(a) pSVhAR.BHEXE vector is a pBR328-based vector containing the SV40 early promoter, the rabbit β-globin polyadenylation signal and the ampicillin resistance gene indicated as SV40, β-globin and Ampr, respectively. To facilitate cloning, the vector was modified to include five unique restriction sites (*Bst* BI, *Hin* DIII, *Eco* RI, *Xho* I and *Eag* I) within the hAR cDNA that are absent from the rest of the vector. These sites are marked with asterisk.

(b) hAR cDNA restriction and primer map showing sites used for cloning or PCR amplification. Numbering is based on the first adenine of translation initiation site (+1). Arrow dirtection indicates the preintation of the primer; UTR, untranslated region; MCS, multiple cloning site.

(a)


Pvu II
Pst I
Sal I
SV40
0
ATG
Eag I*
Pst I
Amp'
Pst I
SV40
pSVhAR-BHEXE
7219 bp
pBR 328
AR
Pst I
Sac I
Kpn I
Hind III *
Sac I
Xho I *
ORI
ß-globin
Sal I
Bst BI*
EcoRI *
Pst I
Bgl II
Bam HI
Sma I
Sal I

(b)

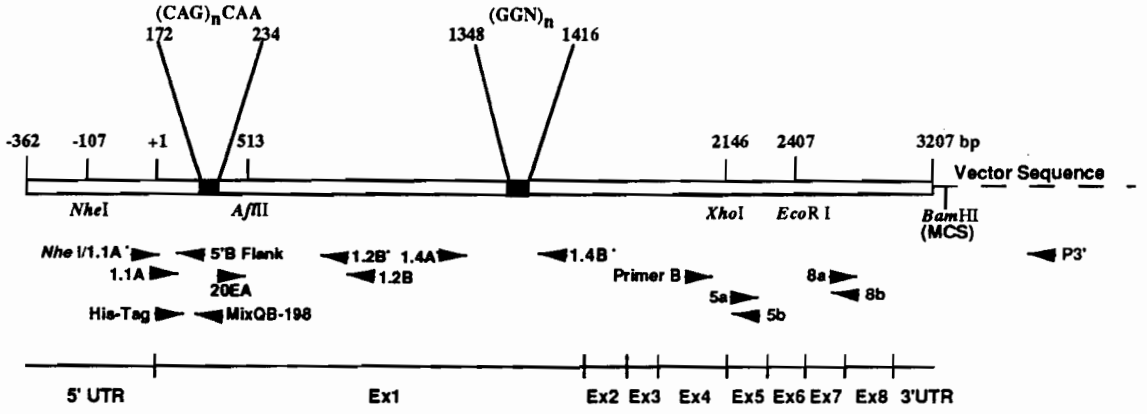

(CAG)nCAA
172
234
(GGN)n
1348
1416
-362
-107
+1
513
2146
2407
3207 bp
Vector Sequence
NheI
AflII
XhoI
EcoR I
BamHI
(MCS)
Nhe I/1.1A '
5'B Flank
1.2B'
1.4A'
1.4B '
P3'
1.1A
20EA
1.2B
Primer B
8a
5a
8b
His-Tag
MixQB-198
5b
5' UTR
Ex1
Ex2 Ex3 Ex4 Ex5 Ex6 Ex7 Ex8 3'UTR

Figure 6. DNA sequence of EX-5, EX-8, and (CAG)n tract from hAR cDNAs with variable tracts size and type.
(a and b) DNA sequence of the normal and EX-5 mutation site, respectively. (c and d) DNA sequence of the normal and EX-8 mutation site, respectively.
DNA sequence of the CAG tract showing the standard normal (e); the standard expanded (f); mixed normal (g) and mixed expanded (f) of hAR cDNA clones used. The primer MixQB-198 primer was originally designed to generate a $(CAG/CAA)_{50}$ tract. All clones isolated contained tracts ranging from 17-35 repeats. This could be either due to nonhomogeneous primer product or due to the secondary structure of the long primer.

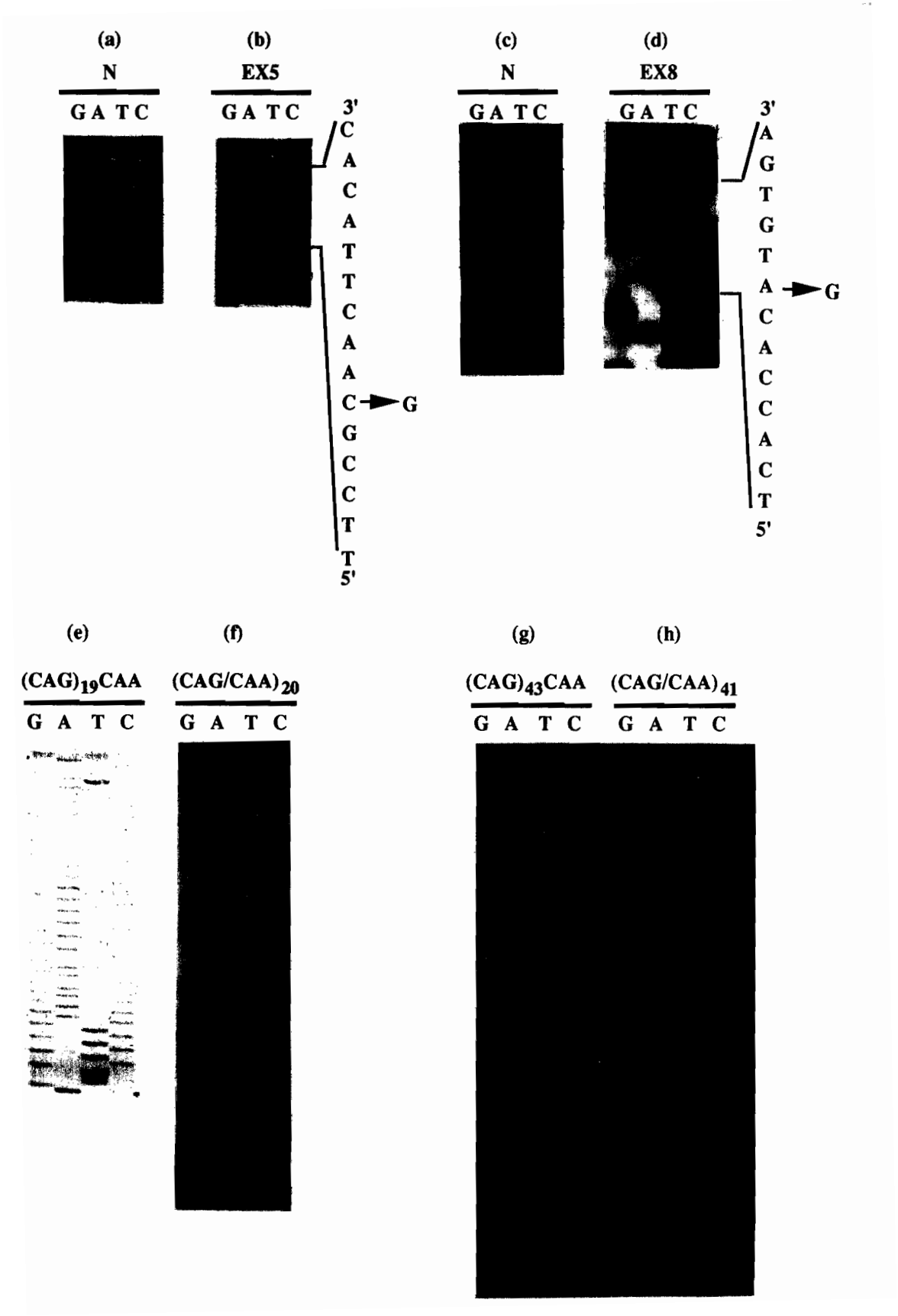

(a)
N
G A T C
(b)
EX5
G A T C
3'
C
A
C
A
T
T
C
A
A
C → G
G
C
C
T
T
5'
(c)
N
G A T C
(d)
EX8
G A T C
3'
A
G
T
G
T
A → G
C
A
C
C
A
C
T
5'
(e)
(CAG)19CAA
G A T C
(f)
(CAG/CAA)20
G A T C
(g)
(CAG)43CAA
G A T C
(h)
(CAG/CAA)41
G A T C

Table I. List of primers used in PCR amplification and DNA sequencing analysis.

Table I. List of primers used for PCR amplification and sequencing

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PR 1.1A° (5'-AGCCTGTTGAACTCTTCTGAGC-3')

PR 1.1A (5'-GTGGAAGATTCAGCCAAGCT-3')

PR 1.2B (5'-GGTGGAACTCCCAAAAGTGG-3')

PR 1.4A° (5'-CCAGAGTCGCGACTACTACAACTTTCC-3')

PR 1.4B° (GGACTGGGATAGGGCACTCTGCTCACC-3')

PR Primer B (a sense) 5'-CACACATTGAAGGCTATGA-3')

P3' (3' antisense primer) 5'-CACCAACCTTCTCGATAGGCAGC-3')

P5a (5'-GGCTTCCGCAAGTTACACGTG-3')

P5b (5'-GTCCACGTGTAACTTGCGGAA-3')

P8a (5'-CAAGTCACACGTGGTGAGCGTG-3')

P8b (5'-CACGCTCACCACGTGTGACTT-3')

PR MixQB-198 (5'-
CTGCTGCTGCCTGGGGCTAGTCTCCTGCTGCTGTTGTTGCTGCTGTTGTT
GTTGTTGCTGCTGCTGTTGTTGCTGCTGTTGCTGCTGCTGCTGTTGTTGTTGCTGC
TGCTGCTGCTGTTGTTGTTGCTGCTGCTGTTGTTGCTGTTGCTGCTGCTGCTGTTG
TTGCTGTTGTTGCAGCAGCAGCAAACTGGCGCCGGG-3')

PR 20EA (5'-GAGACTAGCCCCAGGCAGCAGCAG-3')

PR 1.2B° (5'-GGTGGAACTCCCAAAAGTGG-3')

PR 5'B flank (5'-CCCGGCGCCAGTTTGCTGCTG-3')

PR NheI 1.1A° (5'-GGCGTGCTAGCAGCCTGTTGAACTCTTCTGAGC-3')

PR His-Tag (5'-GGCGTGCTAGCATGCACCACCATCACCATCAC
GACGATGACGATAAGATGGAAGTGCAGTTAGGG-3')

To replace the conventional tract of the $(CAG)_nCAA$ trinucleotide repeat in exon 1 by the mixed $(CAG/CAA)_n$ trinucleotide repeat, an antisense primer (PRMixQB-198; with 50 trinucleotide repeats) flanked by 24 (Table I, underlined) nucleotides of the original sequence was synthesized. In the primary reaction, two fragments flanking and overlapping with MixQB-198 were amplified. The downstream fragment was amplified by using a sense complementary primer (Primer 20EA) for the flanking 5'-end of the MixQB-198 together with a downstream primer (Primer 1.2B°). The upstream fragment was amplified by using an overlapping antisense primer (Primer 5'B flank) for the flanking 3' end of MixQ-198 and an upstream primer designed to contain a *Nhe*I restriction site, (Primer-NheI 1.1A°). The secondary amplification was accomplished by combing the two fragments generated by the primary reaction and the bridging primer MixQB-198. The reaction was done in two stages, primer extension and amplification. In the first stage, the mixture contained the flanking fragments, MixQB-198 and the remaining PCR cocktail. Extension was for two cycles; each consisted of 3 min denaturation at 98°C; 2 min annealing at 57°C (ramping time of 2 min) and 10 min extension at 72°C. After the end of the second cycle, the external flanking primers (*Nhe*I 1.1A° and 1.2B°) were added and the reaction was continued for 30 cycles. The final product was gel purified, digested with *Nhe*I/*Afl*II and the double-digested fragment generated was ligated into the pcDNA3hARΔQ expression vector to replace the corresponding fragment lacking the $(CAG)_n$-CAA tract.

(iii) Introducing the His-Tag sequence in the hAR cDNA

To generate the His-Tag expanded $(CAG)_{49}CAA$ tract at the 5' end of the hAR cDNA (pcDNA3hAR-His50Q), a sense 65-mer oligonucleotide primer (PR His-Tag) containing $(His)_6$-coding repeats (underlined), restriction sites for *Nhe*I and *Sph*I and a Met start codon was used as an upstream primer with the downstream Primer 1.2B°. The expression vector, pSVhAR50Q (Mhatre *et al.*, 1993), was used as a template and the amplification reaction was performed as described above. The product was purified from the gel and the DNA fragment was double-digested with *Nhe*I/*Afl*II. The final double-digested fragment was detected on LMT agarose, excised, melted and ligated into pcDNA3hARΔQ predigested with the same enzymes in order to replace the ΔQ fragment of the cDNA. Positive clones were confirmed by digestion with *Nhe*I/*Afl*II or *Sph*I, followed by sequencing.

pcDNA3hAR-His20Q was prepared by enzymatic digestion of the baculovirus expression vector [(pETLhAR-His20Q), Beitel *et al.*, (1996)]. The 5' end of the insert including the His-Tag and the $(CAG)_{19}CAA$ were excised by using a *Nhe*I/*Afl*II double digest and subcloned into pcDNA3hARΔQ to replace the corresponding fragment.

pETLhARHis50Q and pETLhAR50Q were prepared by subcloning the *Nhe*I/*Afl*II fragments of pcDNA3hAR-His50Q and pSVhAR50Q, respectively, into pETLhARHis20Q to replace the His-Tag20Q fragment of the expression vector.

(iv) QN-butanol procedure

This method uses the ability of hexacetyltrimethylammonium bromide (QN) (Sigma Chemicals, St. Louis, MI) to bind DNA in low salt concentration. The QN-butanol two phases were added to the molten gel slice (65°C) containing DNA complex (1X volume of each layer), vortexed and spun for 2 minutes in a microcentrifuge at maximum speed. The upper butanol layer was transferred to a clean microfuge tube and 1/4 volume of 0.2 M NaCl solution was added, vortexed and spun as before. The bottom layer was saved, diluted with 1X volume ddH_2O and 1X volume of chloroform (1 isoamyl alcohol:24 chloroform) and vortexed. After standing on ice for 5 min, it was spun for 4 minas above. The pellet was washed with 75% ethanol, dried and dissolved in ddH_2O before its OD_{260} was measured.

Subcloning into the expression vectors

The expression vectors used in these studies were pSVhAR.BHEX and pcDNA3hAR. To generate a convenient cloning site, pSVhARo (Brinkmann *et al.*, 1989) was previously modified in our laboratory to produce pSVhAR.BHEXE, Fig. 5 (Beitel *et al.*, 1994b). The other expression vector (pcDNA3, Invitrogene) was a gift from Dr. Fischbeck's laboratory and contained the hAR cDNA with the expanded $(CAG)_{43}CAA$ trinucleotide repeat (Fig. 6) cloned into the *Bam*HI site of the vector's multiple cloning site. This vector contains the prokaryotic promoters T7 and Sp6 for *in vitro* transcription purposes.

After PCR mutagenesis of the desired sites in the hAR cDNA, the purified secondary PCR products were digested with restriction enzymes (New England Biolabs; BRL; Pharmacia Biotech) according to the sites flanking the product. For the Met886Val mutation, the restriction enzymes used were *Eco*RI and *Bam*HI; for Asn727Lys, *Xho*I and *Eco*RI. For replacing the expanded $(CAG)_{43}CAA$ tract

of pcDNA3hAR44Q with other variable tracts (the mixed $[CAG/CAA]_{20/41}$ generated by PCR (Fig. 6); the expanded His-Tag $[CAG)_{49}CAA]$ generated by PCR, the ΔQ enzymatically excised from pSVhAR50Q, and the 20Q enzymatically excised from pSVhAR.BHEX), the restriction enzymes *Nhe*I and *Afl*II (New England Biolabs) were used. The expression vector and fragments were digested with the same enzymes for compatible sticky ends, QN-butanol-purified and added, in a 1:3 molar concentration to a mixture of T4 DNA ligase buffer and T4 DNA ligase (BRL-Canadian Life Technologies, Montreal, PQ). Ligation took place for 4 hrs at room temperature or overnight in a 16°C circulating water bath. Usually, the ligation sets included a negative control of the digested vector without added fragment. The ligation reaction was terminated by heat inactivation at 65°C for 15 minutes, 5 µl of the mixture were added to the XL-1 Blue cells (Davis *et al.*, 1989) and transformation was effected by heat shock (Sambrook 1989). Bacteria were plated on LB-ampicillin (50 µg/ml; Boehringer, Mannheim, Laval, PQ) plates and the recombinant colonies were selected as colonies on plates. Ampicillin-resistant colonies were grown up in a 3 ml LB-Amp overnight. Minipreps were done on the resulting culture using the alkaline lysis method of Sambrook *et al.* (1989) and positives verified by restriction enzyme digests and agarose gel electrophoresis. Maxi-preps were done on recombinant clones before sequencing.

Preparation of XL-1 Blue competent cells

An overnight bacterial culture was prepared by inoculating a colony of XL-1 Blue cells (Stratagene, La Jolla, CA) into 50 ml of LB medium. The culture was allowed to grow overnight on a shaker at 37°C. The next day, 1 ml of the overnight culture was transferred into 50 ml of prewarmed LB medium and shaken until it reached $OD_{590} = 0.3$. Cells were pelleted at 2000 rpm and resuspended in 20 ml of 50 mM $CaCl_2$, iced for 30 min, pelleted at 4°C and resuspended in 4 ml of ice-cold 15% glycerol in 50 mM $CaCl_2$. Cells were immediately aliquoted into 200 µl fractions, frozen in dry ice and stored at -70°C.

Heat-shock transformation method

DNA solution (10-50 ng/transformation) was added to 100 µl of competent cells and incubated on ice for 30 minutes. Cells were then shocked at 42°C for 90 sec and immediately chilled on ice for a further 2 min. Four hundred µl of LB medium was added and cells shaken for 1 hr at 37°C. One hundred-200

µl of the transformed cells were transferred to an LB-ampicillin plate and incubated overnight at 37°C (Sambrook *et al.*, 1989).

Maxi-preparation of plasmid DNA

Five ml of LB medium with 50 µg/ml ampicillin was inoculated with a clone of interest and shaken at 37°C for 6-8 hrs. The culture was transferred to 500 ml of LB with 50 µg/ml ampicillin and left to shake overnight at 37°C at 250 rpm. Plasmid DNA was purified by the Qiagen Plasmid Purification System (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions. DNA yield was quantitated at OD_{260} and quality was assessed by a scan from 230-320 nm.

Preparation of chimeric constructs

DNA fragments encoding aa 11-341(*Acc* I-*Acc* I sites) or 38-341 (*Sma* I-*Acc* I sites) of the normal 20-Gln or the expanded 50-Gln AR (Mhatre *et al.*, 1993) were blunt-ended and cloned into the *Sma*I site of pAS2-1, a high-level yeast expression vector containing GAL4-DBD, to produce AR/GAL4-DNA-binding domain (BD) chimeric proteins. Transformation was done by lithium acetate/heat shock at 42°C. Yeast were grown and lysates for western analysis prepared as described (Dunn and Wobbe, 1988).

DNA sequencing

(i) Plasmid DNA sequencing

After PCR mutagenesis, both strands of the insert were completely sequenced to beyond the ligation points by the dideoxy termination method using with Sequenase enzyme following the manufacturer's instructions. Radiolabelled [α–^{32}P] dATP or [α–^{35}S] dATP (Amersham, Mississauga, ON) was used as the internal labeling nucleotide in these sequencing reactions. The sequencing reactions were resolved on a 5% denaturing (8M urea) polyacrylamide gel at 64 Watts for 2.5 hrs.

(ii) Sequencing of PCR-amplified genomic DNA

The dideoxy method was used in these reactions by end labeling the sequencing primer using [γ–^{32}P] ATP and T4 polynucleotide transferase (PNK). Seven to ten µl of the molten gel slice containing PCR product (~ 100-200 ng/60 µl) and 2-3 pmoles of the sequencing primer were used per reaction. This was mainly done to confirm the mutations of subjects CML and KLH using genomic

DNA.

***In vitro* protein synthesis and proteolysis using reticulocytes cell-free lysate**

(i) Protein synthesis

A coupled rabbit reticulocyte lysate-based transcription/translation system kit, (Promega) and [^{35}S]Met and [^{14}C]Gln (Amersham) were used in these experiments. The reaction consisted of 1 μg of supercoiled plasmid (pcDNA3; Invitrogene Corporation) containing hAR cDNA, 25 μl of reticulocyte lysate, 2 μl of the reaction buffer, 1 μl of 1 mM cold amino acids mixture minus methionine or glutamine, or 1 μl of complete cold amino acids mixture, 2 μl (200 fM final concentration) of [^{35}S]Met (1000Ci/mmol) alone, or 100 μl (0.34 nM final concentration) of the dried [^{14}C]Gln solution (290Ci/mmol) with 1 mM radioinert complete amino acid mixture, 1 μl T7 RNA polymerase and 1 μl of RNAsin (40 units/μl; Promega) in a final volume of 50 μl. Transcription-translation was done at 30°C for two hrs in a circulating water bath.

(ii) Partial proteolytic digestion

Radiolabelled hAR (normal 20-Gln, expanded 44-Gln) was partially digested by adding 40 ng of trypsin (Sigma Chemicals, St. Louise, MI) in 1 μl of water to 10 μl of *in vitro* product at room temperature for 0 - 10 minutes. The reaction was terminated by adding one volume of 2X SDS/sample buffer [3% SDS; 10% glycerol; 5% 2-mercaptoethanol; 0.062 M Tris-Cl, pH 6.8; and 0.01% bromophenol blue]. For more vigorous digestion, 15 μl of *in vitro* product were exposed to 80 ng of trypsin in the presence or absence of 2 M urea (Fisher Scientific) for 60 minutes at room temperature. These reactions were terminated by boiling for 5 minutes.

TISSUE CULTURE

COS-1 cells (SV40 transformed CV-1, monkey kidney 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, Mississuaga, ON), and 5-10% fetal calf serum (ICN Biomedicals Canada, Mississauaga, ON) in a 37°C humidified incubator with 5% carbon dioxide.

GSF (genital skin fibroblasts) obtained from patients and controls were maintained in the same manner as the COS-1 cells.

The motor neuron-like cell, NSC34, (a hybrid of a neuroblastoma cell line x fresh mouse embryonic spinal cord cells), established by Dr. Cashman *et al.* (1992), was maintained in DMEM (Gibco/BRL Life Technologies) with antibiotics as in Opti-MEM and 10% fetal calf serum, and incubated as above.

TRANSFECTION EXPERIMENTS

Transfection parameters

(i) COS-1 cells

Confluent cells were harvested from T-170 flasks (FALCON) with 0.1% trypsin (Difco) for 10 min at 37°C. Cells were spun at 1800 rpm for 1 min and resuspended in the above medium to a concentration of 20 x 10^6 per ml. Ten million cells were used per electroporation cuvette. The amount of plasmid used varied with the type of the experiment; for androgen-binding experiments (off-rate and Scatchard analysis), 1μg of hAR cDNA expression vector with the wild type or mutant sequence was used. For transactivational analysis, 20 μg pMMTV-GH (human growth hormone reporter driven by the LTR sequence of MMTV which contains four full steroid response elements or SRE; Ham *et al.*, 1988) or 10 μg pMMTV-Luciferase reporters and 2 μg pCMV-βgal (the β-galactosidase gene driven by cytomegalovirus promoter, as a control for transfection efficiency) were used. In experiments for protein analysis and apoptosis, 5 μg of hAR cDNA expression vector were used and pMMTV-GH plasmid omitted. Cells were mixed well with the DNA in an eppendorf tube and transferred to 0.4 cm electroporation cuvette, left to settle for 5 minutes and then gently mixed and electroporated at room temperature using a Gene Pulser (BioRad, Richmond) at 250V, and 960 μFaradays to yield a time constant of 20-25 millisec. Cells were then diluted with media, pooled with those from identical transfection tubes and plated at 3 x 10^5 cells per 35-mm plate (Becton Dickson Canada Inc.). Transfectants were assayed for androgen-binding activity 48 hrs later, or for growth hormone secreted into the medium 72 hrs later.

(ii) NSC34 cells

Cells were dislodged by trypsinazation for 3 minutes at room temperature, pelleted for 1 min at 1800 rpm, and resuspended in 10% DMEM medium. Cells were preconditioned for lipofection by seeding 2 x 10^6 cells per T-25 flask and

overnight incubation. The next day, the lipofectamine-DNA complex was prepared by mixing 1 μg of hAR cDNA expression vector, 3 μg pMMTV-GH and 0.2 μg pCMV-βgal in 500 μl serum-free DMEM. This mixture was added to a mixture of 25 μl of lipofectamine (2 mg/ml; Gibco/BRL Life Technologies, Burlington, ON) in 500 μl of serum-free DMEM. The combined mixture was left to precipitate at room temperature for 45 minutes before being diluted to 3 ml final volume with the same medium. Finally, the mixture was added to the cell monolayer prewashed with the serum-free DMEM and incubated as above for 6 - 8 hrs. At the end of the incubation period, the lipofection mixture was replaced with a fresh 5 ml of 10% DMEM and incubated overnight as above. The cells were dislodged the next day, dispensed in culture plates and incubated for 48-72 hrs.

Androgen-binding assays

In transactivation experiments, replicate wells or dishes of cell monolayers were incubated in the desired growth medium and serum supplemented with [^{3}H]-androgen alone (to measure total androgen binding), while other plates were incubated with [^{3}H]- androgen plus 200-fold excess radioinert androgen to measure nonspecific androgen-binding. Hormone was added the day the cells were dispensed and the cells were incubated for 48-72 hrs as above. For off- rate, Scatchard and thermolability analyses, transfected COS cells or fibroblasts cell monolayers were grown in the specified media supplemented with serum for 48 hrs as above. The media were then replaced with serum-free MEM (Minimal Essential Medium, Gibco) supplemented with Hank's salt and HEPES buffer (ICN Biomedicals Canada) and incubated at various stated conditions of temperature, time, and hormone concentrations. To assay for binding activity, the plates were washed twice on ice, 5 minutes each, followed by a 7-minute wash at room temperature with 0.02 M Tris-HCl (pH 7.4). Finally, 1-1.5 ml of 0.5 M NaOH was added directly to the wells or dishes to lyse the cells. Aliquots of the lysates were mixed with Beta Max ES (ICN Biomedicals Canada) and radioactivity was quantitated in a TriCarb 1500 liquid scintillation counter (Canberra-Packard Canada). Additional aliquots were used to quantitate protein and androgen-binding expressed per mg total protein. Specific androgen-binding activity was calculated by subtracting the nonspecific fraction from the total (Kaufman and Pinsky, 1989). Data were analysed using the computer programs Microsoft Excel and Cricket Graph ™.

Four 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 (T) (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, MT (Dupont, Mississuaga, ON) and MES (mesterolone; Sigma Chemicals).

Off-rate experiments (nonequilibrium dissociation constants; k)

Forty-eight hrs after transfection, triplicate monolayers expressing hAR were labelled with 3 nM of [^{3}H]-androgen and duplicate wells were labelled with 3 nM [^{3}H]-androgen plus 200-fold excess of radioinert androgen and the plates were incubated in the presence of 100 μM cycloheximide for 2 hrs at 37°C or 42°C. The incubation media were then replaced by ones containing only 200-fold radioinert androgen acting as a "chase" for radiolabelled androgen binding. Periodically, cells were washed and lysed as above, and assayed for androgen-binding activity.

Androgen-binding activity at zero min represented 100% of binding activity of the particular transfectant or strain. Androgen-binding activities were measured at 0, 30, 60, 90, and 120 min. Results were plotted semilogarithmically as percent of androgen-binding activity remaining at each time point vs. time. The k value was determined by the equation: $k = 1/t_{1/2} \times \ln 2$, where $t_{1/2}$ is the time at which 50% of A-R complexes remain.

Scatchard analysis (apparent equilibrium dissociation rate constants; K_d)

Triplicates monolayers cells were labelled in MEM supplemented with 100 μM cycloheximide (Sigma Chemicals) and 3 nM, 1.5 nM, 0.75 nM, 0.38 nM, 0.19 nM or 0 nM [^{3}H]-androgen, and duplicate wells were labelled with the corresponding concentration of hormone plus 200-fold radioinert androgen. Cells were incubated at 37°C for 2 hrs followed by washing and lysis as above.

Results were plotted as bound androgen versus bound/free androgen and K_d values were calculated from the slopes of Scatchard plots (Scatchard, 1949).

Thermolability of A-R complexes

Triplicate 60-mm dishes containing CML or KLH fobroblast monolayers were labelled with 3 nM [^{3}H]-MB or [^{3}H]-MT and duplicate dishes were labelled with 3 nM [^{3}H]-MB, or [^{3}H]-MT plus 200-fold excess radioinert MB or MT at

37°C for 2 hrs to allow the formation A-R complexes. 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 containing 100 μM cycloheximide. One series of plates was left at 37°C, and another series of dishes was placed at 42°C (KLH MT-binding was done at 41°C). Binding assays were performed at 2, 4, 6 hrs of incubation.

Androgen-binding activity after the initial incubation time (time zero) represented 100% of binding activity of the particular strain. Androgen-binding activity at other points were expressed as a percentage of the activity of the respective strain at time zero. Results were plotted semilogrithmically as percent of androgen-binding activity remaining at each time point.

Reporter gene assays

(i) Growth hormone assay

Cells were co-transfected as described above and incubated with 3 nM, 2 nM, 1 nM, 0.1 nM, 0.05 nM or 0 nM of [^{3}H]-MB or [^{3}H]-MT, or the corresponding concentrations of [^{3}H]-androgens plus 200-fold their radioinert form in duplicate wells of a 24-well plate. When mesterolone (MES) or 5α-DHT were used, only radioinert form was used at 300 nM, 200 nM, 100 nM, 50 nM, 25 nM or 0 nM. Upon the addition of androgen, the A-R complex that translocates to the nucleus binds to the GRE in the MMTV-GH and activates transcription of the human growth hormone (hGH) reporter gene. hAR transactivation is measured by the amount of secreted growth hormone in the media. Seventy-two hours post-incubation, media were collected from the wells to measure secreted hGH and cells used to determine the amount of A-R complexes, in the case of labelled androgen. This assay is based on biotin/avidin affinity. The assay kit uses two antibodies that recognize specific epitopes of the hGH molecule. One of the antibodies is labelled with ^{125}I, the other is coupled to biotin. The addition of an avidin-coated bead to the assay mixture allows specific binding of the antibodies-antigen complex to the beads while shaking for 1 hr at 27°C.

Twenty-50 μl of media diluted with 150 μl ddH_2O were incubated as mentioned above with 50 μl of antibody solution and an avidin-coated bead supplied with the growth hormone kit (Nichols Institute Diagnostics, San Juan Capistrano, CA). The beads were then washed twice with growth hormone-washing buffer supplied by the manufacturer to eliminate any unbound components in the mixture and the radioactivity bound to the beads was

quantitated in a gamma counter. A standard curve was established from control hGH samples in the kit. Results are reported as growth hormone activity versus androgen concentration or as percentage of normal.

(ii) Luciferase assay

Two days after transfection, cells were washed and scraped with sodium phosphate buffer (PBS; pH 7.4). Following a 1 min spin in the microcentrifuge the cells were lysed in 100 µl of the lysis buffer supplied in the luciferase kit (BioOrbit). Dilutions of the samples were prepared to optimise the range of detectable luciferase; peaks were obtained by injecting the mixture of the sample and substrate mixture into the luminometer in the presence of ATP solution supplied with the kit. Standards of positive controls were run for each experiment. Units of luciferase were determined by calculating the total area (mm^2) under the curve or by weighing (µg) the paper area under the curve. Luciferase activity was expressed as unit per mg of total protein.

Preparation of COS-1 nuclear extract

Nuclear extract was prepared according to Dignam *et al.* (1983). COS-1 monolayer cultures transiently transfected with 5-10 µg of hAR expression vector/10 million cells were incubated as mentioned for 72h in the presence of 10 nM radio-inert MB. Cells were washed with PBS, scraped into the same buffer and centrifuged for 10 min at 2000 rpm. The pellet was resuspended in 5X volumes of 4°C PBS and collected as above; the remaining steps were done at 4°C. The cells were resuspended in 5X volume of suspension buffer [10 mM HEPES (pH 7.9 at 4°C), 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT], left for 10 min before pelleting, resuspended in 2X volume of the same buffer, and lysed by 10 strokes of Dounce homogenizer (B type pestle). The homogenate was centrifuged for 10 min at 2000 rpm in a Sorvall SS34 rotor to pellet nuclei. The supernatant was decanted and the pellet recentrifuged for 20 min at 25,000 g to remove the residual cytoplasmic material. The nuclei were resuspended in 3 ml of nuclear lysis buffer [20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 50 µg/ml of each of the protease inhibitors (AEPSF, leupeptin, apoprotinin, Boehringer Anneheim, Germany), and 0.5 mM DTT] per 10^9 cells and lysed by 10 strokes of a Dounce homogenizer (B type pestle). The suspension was stirred with a magnetic stirring bar for 30 min and then centrifuged for 30 min at 25,000 g using a Sorvall SS34 rotor. The nuclear

supernatant was dialyzed overnight with gentle stirring at 4°C with 50 volumes of dialysis buffer [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 50 µg/ml of the above protease inhibitors, and 0.5 mM DTT; DTT and protease inhibitors were added fresh to the buffers just before use]. The dialysate was centrifuged at 25,000 g for 20 min to remove the precipitate. The resulting nuclear extract was aliquoted and frozen in liquid nitrogen and stored at -80°C.

Western blotting

Cells expressing androgen receptor were incubated at 37°C for a specified period of time with or without androgen using 5% serum-OptiMEM (COS-1) or 10% serum-DMEM (NSC34). Cells were grown in 35 or 60 mm dishes (FALCON). Cells were dislodged in 0.02 M Tris-HCl (pH 7.4) using a rubber policeman and collected by centrifugation at 13 000 rpm for 30 sec. 100-200 µl of lysis buffer (20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA. 0.5 mM DTT; from Dignam *et al.*, 1983) 4% SDS, and 20 µg/ml of protease inhibitors (AEBSF, Apoprotinin, Bestatin, E-64, Leupeptin, and Pepstatin) were added before 5 passages through a 25G needle. The lysis mixture was boiled for 5 minutes and cellular debris pelleted by microcentrifuging at 14 000 for 2 min. The supernatant was frozen at -70°C. After protein assay was done, 100 µg of protein was loaded on an 8% SDS-PAGE using a Bio-Rad minigel apparatus or standard 20 x 20 cm gels according to Okajima's method (Okajima *et al.*, 1993) along with Rainbow molecular weight markers (Amersham Life Sciences). The gel was run at 100-250 V for 1-2 hours and electroblotted to nitrocellulose membrane (Xymotec Biosystems) overnight at 165 mAmp. The filter was blocked for 1 hr in a solution containing 5% skim dried milk, 0.5% Tween (BDH, St-Laurent, Que.) in 1X TBS (20mM Tris-HCl pH 7.4, 50 mM NaCl) or in 1X PBS buffer. The primary antibodies used were the monoclonal antibody (F39.4.1), raised against a peptide (Sf61) corresponding to amino acids 301-320 in the hAR (Zegers *et al.*, 1991), or the monoclonal antibody 1C2, raised against polyGln tract (38 rsidues) in the human TATA-Binding Protein (TBP) (Trottier *et al.*, 1995; Lescure *et al.*, 1994). The former antibody was diluted 1:10 000 in TBS/Tween (20 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.05% Tween; BDH); the latter was diluted 1:1000 in 0.5% dried milk in PBS. Diluted antibody was added to the blot and shaken for 1 hr at room temperature followed by 3 washes with the corresponding buffers for 10 min each. The secondary antibody (horseradish peroxidase-conjugated goat anti-mouse immunoglobin G; Professional Diagnostics) was added at a dilution of

1:5000 and was shaken for 1 hr at room temperature. The blot was washed as before and then incubated with reagents from the ECL Western blotting chemiluminescence detection system (Amersham). Blots were exposed to X-OMAT-AR X-ray film (Eastman Kodak) for 30 sec to 20 min. Monoclonal anti-hsp70 was diluted 1:10 000 in Tween/TBS buffer and used as a control for protein loading and integration.

Protein assays

To quantitate protein levels for androgen-binding and β-galactosidase assays, a Lowry (1951) procedure was followed using phenol reagent (American Chemicals). Analysis was done using either large scale or small scale assays. For the small scale assay, 10-70 µl of cell lysate plus ddH_2O up to 70 µl, 250 µl protein C (Fisher Scientific, Nepean, ON) and 25 µl of phenol reagent (diluted 1:1 with ddH_2O) was added to each well in a 96-well plate (Becton Dickson Canada Inc., St. Laurent, Quebec). The reaction was allowed to proceed for 30 minutes before reading at OD_{650} in a microtitre reader (Titertek Multiscan, ICN). For samples containing SDS or NP-40, the protein-assay kit was used (DC Protein Assay, Bio-Rad). These reactions were also done in a 96-well plate and read by a microtitre reader. Large scale assay was done in a final volume of 3 ml with a 10-fold upscaled volume of the reagents. Reading for large scale assay was done at OD_{650} using micro-sample spectrophotometer 300-N (Gilford Instrument Laboratories INC.)

In both cases, bovine serum albumin (BDH, Montreal) was used to produce the protein standard curve.

β-galactosidase assay

This assay is usually done to estimate the transfection efficiency difference due to the molar variation of DNA entering the transfected sets of cells. Corrections for transfection are made by calculating the β-galactosidase activity for each transfected group and comparing it to (usually) normal.

Cells were washed once with 0.02 M Tris-HCl (pH 7.4), dislodged into 1 ml of the same buffer, and centrifuged at 14 000 rpm for 20-30 sec. Cell pellets were resuspended in 150 µl of 0.25 M Tris-HCl (pH 7.8) buffer and freeze-thawed thrice in dry ice/37°C water bath followed by centrifugation at 14,000 rpm for 4 min at 4°C. Thirty µl of lysate were used for each assay that includes 201 µl 0.1M sodium phosphate (pH7.4), 66 µl of o-nitrophenyl β-D galactopyranoside (ONPG,

20 mg/ml in the same buffer; Boehringer Mannheim) and 3 µl of 100X Mg^{2+} buffer (Sambrook *et al.*, 1989). β-galactosidase in the supernatant hydrolyzes ONPG substrate and color appears that can be quantitated with a spectrophotometer at 450 nm absorbance.

Binding of androgen-receptor complexes to ARE

A modified binding assay was developed to measure A-R complex-ARE binding. This assay gave similar results when the same experiment was repeated using a modified assay (Beitel *et al.*, 1994a) of Yu *et al.*, (1991). Ten million COS-1 cells were transfected with 5 µg of normal or mutant pSVhAR expression plasmid including the negative control DBD mutant (ΔF582), or mock transfected, and incubated for 48-72 h at 37°C. The medium was replaced by one containing 3 nM [^{3}H]MB, and incubation was continued for 2h. Cell monolayers were washed three times at room temperature for 5 min each with Tris-Cl buffer [0.02 M Tris-HCl (pH 7.4)], the cells were scraped into the same buffer and spun for 10 min at 2000 rpm. This and all the following steps were done at 4°C. MB-receptor complexes were extracted from the cells by 3 freeze/thaw cycles in 1 ml of binding buffer [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, and 50 µg/ ml of protease inhibitors (AEPSF, Leupetin, Apoprotin)]. The lysate was cleared by centrifugation at 12,000 g for 30 min and 10 µl samples were measured for radioactivity. Each assay mixture contained 10 µg of poly (dI-dC) (Pharmacia), a sample of supernatant with 50,000 dpm of MB-receptor complexes, and binding buffer to a final volume of 500 µl. 150 pmol of 3'-biotinylated double-stranded oligonucleotide sequence of the synthetic ARE: (5'-CTAGAAGTCTGGTACAGGGTGTTCTTTTTGCA-biotin); (Roche *et al.*, 1992), or MMTV-ARE (5'-TATGGTTACAAACTGTTCTT-biotin); (Ham *et al.*, 1988), and 50 µl of streptavidin-agarose beads (GIBCO BRL) or streptavidin-magnetic beads (Promega) were added before incubation for 2 hr at 4°C. The beads were collected either by microcentrifugation or a magnetic bar at RT, washed thrice with binding buffer, and resuspended either in 0.5 ml ethyl alcohol to measure bound radioactivity by liquid scintillation, or in protein-SDS sample buffer for protein analysis (in the case of polyGln-expanded hAR analysis). The beads were shown to bind negligible amounts of A-R complexes in the absence of ARE. Experiments were repeated at least twice and each data point is the mean of duplicate experiments calculated as the percentage of binding observed with wild type.

CELL DEATH ANALYSIS

COS-1 cells were electroporated at 250V, 960 μF and were transfected with 5 μg of hAR cDNA (normal 20-Gln, expanded 44-Gln) and 1 μg of pCMV·βgal per $1x10^7$ cells. Controls included separate sets of COS cells transfected with pCMV·βgal, pcDNA3, or nothing. Each set of cotransfections was done in triplicate; the cells were pooled and diluted with 5% serum-OptiMEM. Cells were seeded in 4-well growth chambers ($1.25x10^5$ cells/well; Lab-Tek) and incubated at 37°C for 4-6 hours to let the cells attach to the surface. Dead cells resulting from electroporation were removed by 3 washes with the same medium and then the cells were incubated overnight in a 37°C incubator in the same medium with or without 3 nM MB.

The monolayers were washed twice with PBS and then stained with 10 μg/ml ethidium monoazide bromide (EMA; Molecular Probes) in PBS for 10 minutes at RT under UV light exposure for cross-linking. The cells were subsequently washed twice in PBS and fixed in 4% paraformaldehyde for 20 min at RT. Unlike standard ethidium bromide, EMA covalently binds to the DNA of dead cells thereby preventing dye leakage post-fixation (Martin *et al.*, 1994). Total cell numbers were determined in 25X fields under phase microscopy. Dead cells were identified by the presence of bright red nuclear staining under epifluorescence microscopy (Leits Diaplan Microscope) using a rhodamine filter. The appearance of shrunken, condensed nuclei and presence of small round apoptotic bodies constituted morphological evidence of apoptosis in these cultures. Eight sister cultures were evaluated for each experimental group. A minimum of 140 total cells was assessed per culture. The extent of cell death was expressed as the ratio of EMA-positive to total cells in monolayer. Statistical analysis was performed using Student's paired t-test (2-tailed) with $p< 0.05$ indicating significance.

ELECTROPHYSIOLOGICAL ANALYSIS

Electrophysiological analysis was done on two different motor-neurons hybrid cell lines. The NSC34 cell line was used for transient expression of hAR, whereas the MN (neuroblstoma-motor neuron) cell line (Salazar-Grueso *et al.*, 1991) is stably transfected with hAR (Brooks *et al.*, 1997).

Non-confluent cells were trypsinized, spun, resuspended in 10% serum-DMEM medium and dispensed into petri dishes as $2x10^5$ cells/dish. NSC34 cells analysis included: untransfected cells, and cells transfected with hAR-20Q or

hAR-44Q. Each of these sets was analysed in the presence or absence of 5 nM mibolerone in a period 1-4 days posttransfection with the medium replaced every two days.

Three types of stably transfected MN cells expressing hAR-24Q (clone AR24-1) hAR-65Q (clone AR65-8) or the vector (pcDNA3) alone with no hAR (clone Neo-7) were previously selected for their G-418 (neomycin analog) resistance (Brooks *et al.*, 1997). For electrophysiological analysis, the cells were seeded as above and incubated overnight at 37°C. On the next day, each cell type was divided into two groups: undifferentiated or differentiated (see below); and each of these groups was incubated either in the presence or absence of mibolerone. To prepare the cell groups, two types of DMEM medium were made: a) undifferentiating medium containing 2% serum-DMEM with or without 5 nM mibolerone; the cells were analysed the following day; b) differentiating medium that consisted of 2% serum-DMEM, 1 mM butyric Acid (Sigma), and 0.4 µg/ml aphidicholin antibiotic (Sigma Chemicals); the cells were differentiated for 3 days in the presence or absence of 5 nM mibolerone with a daily change of medium before being subjected to analysis.

Recording of Ca^{2+} channel currents

For whole-cell recordings, solutions were used to suppress Na^+, K^+, and Cl^- channel currents. The pipette (intracellular) solution consisted of (mM), 130 N-methyl-D-glucamine, 20 EGTA (free acid) and 5 BAPTA [bis(2-aminophenoxy) ethane-*N, N, N', N'*-tetraacetate] (Ca^{2+} chelators), 10 HEPES, 6 $Mg(OH)_2$, 4 $Ca(OH)_2$, pH buffered to 7.3 with methanesulfonic acid. The external solution contained (mM), 20 $Ba(OH)_2$, 60 tetraethylammonium (OH), 5 4-aminopyridine, 10 HEPES, pH buffered to 7.4 with methanesulfonic acid. Whole-cell currents were recorded using an Axopatch 200A patch clamp amplifier. Currents were digitized after filtering at 2 kHz, with capacitative currents nulled and up to 80% of the series resistance compensated. Pulse generation, current recording, and data analysis were performed using software based on the Fastlab system (Indec Systems). The pure (+) stereoisomer of the dihydropyridine (DHP) antagonist PN200-110 (isradipine) and SDZ 202-791 were generous gifts from Sandoz Canada. A 10 mM stock solution was prepared in 98% ethanol and stored at -20°C. The final concentrations of solvent were <0.1% and had no effect on the calcium channel currents. All experiments were performed at room temperature (20° to 23° C).

PART III. RESULTS

ANALYSES DONE ON THE EXPANDED-POLY(Gln) hAR

AR expression and transactivational analysis in NSC34 and COS-1 cells

To compare the transcription induction by the wild type versus the polyGln-expanded AR, cotransfection of NSC34 cells was done using the AR cDNA and the MMTV-GH reporter gene and A-R complexes and the transactivational competence were assayed. Specific A-R binding activity analysis showed that the polyGln-expanded hAR had higher binding activity than the wild-type AR at different hormone concentrations (Fig. 7). This remarkable observation seems to be cell line specific as it is not exhibited by COS-1. Western analysis of expressed hAR in NSC34 for up to 6 days shows higher protein signals of the expanded form versus the normal as detected by mAb F39.4.1. which is accompanied with higher hormone binding as well (Fig. 7).

Transactivational analysis were done by transiently cotransfecting NSC34 cells. There is a decrease of about 50% of the expanded AR transactivational capacity in the presence of hormone (MB) using pMMTV-hGH reporter, which shows a dose-dependent pattern (Fig. 7). This decrease in transactivation was consistently observed in NSC34 cells which is similar to those results previously done on COS-1 in our laboratory (Mhatre *et al.*, 1993; Kazemi-Esfarjani *et al.*, 1995).

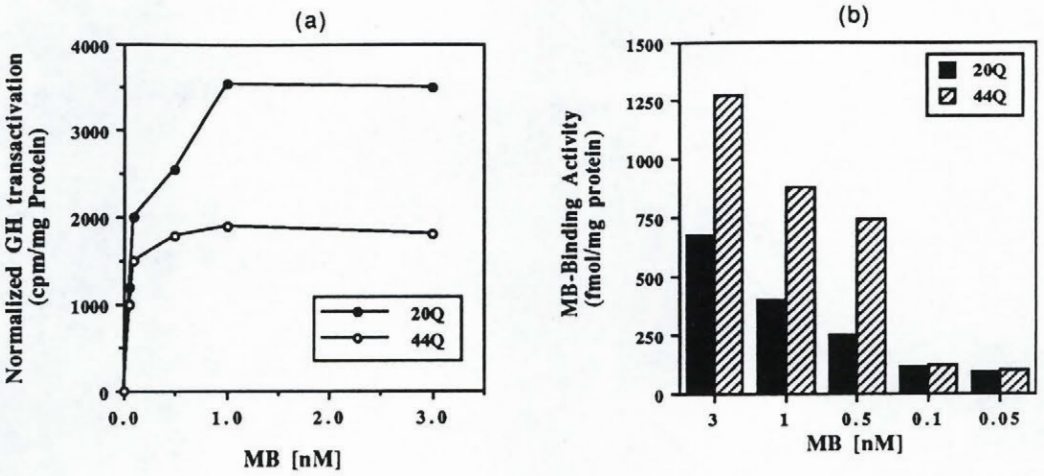
To test whether the codon type plays any role in the pathogenicity of the expanded hAR, COS-1 cells were transiently transfected with constructs of 20 and 41 mixed codons (CAG/CAA). The results revealed that the longer tracts had less transactivation capacity regardless of codon type (Fig. 8). Any difference in cell death rate between wild type and polyGln-expanded AR was considered to be insignificant, as justified below (Table II of Cell death analysis), where the difference in cell death rate between normal and polyGln-expanded AR was only about 5% despite the use of 5 to 10-fold more plasmid.

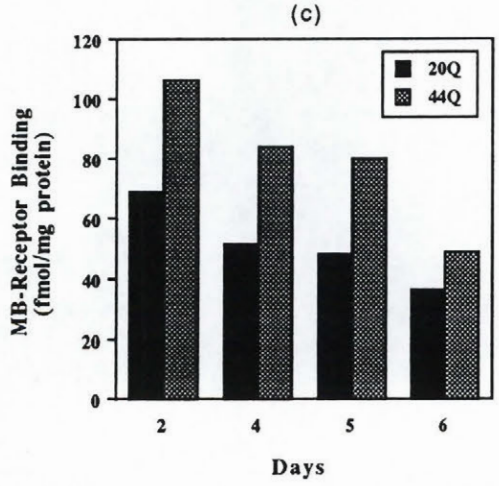
Androgen-binding analysis

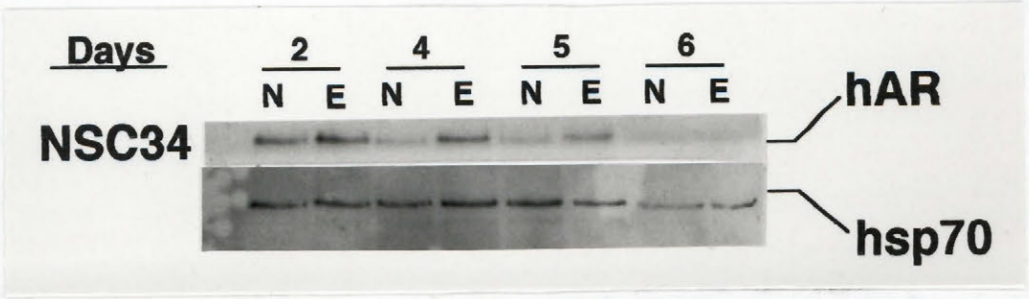
Off-rate and Scatchard analyses of the expanded versus wild type androgen receptors were done with NSC34 cells using MB or DHT. No difference was detected in the dissociation rates or the apparent equilibrium

Figure 7. Analysis of transactivational competence, androgen binding, and receptor stability of the normal versus polyGln-expanded hAR in NSC34 cell line.

(a) MB-dependent production of GH in the medium of NSC34 cells transiently expressing normal hAR with a 20-Gln tract (20Q) or polyGln-expanded hAR (44Q). After cotransfection with expression plasmids containing normal or expanded forms of hAR cDNA and MMTV-GH reporter, the cells were exposed to indicated concentrations of MB for 72 h. GH activity was adjusted for cell protein and transfection efficiency using pCMV.βgal. (b) Specific MB-binding activity of 20Q versus 44Q hAR analysed in (a) after the addition of the indicated MB concentrations (72 h) to the medium of NSC34 cells transiently expressing hAR; binding activity was corrected for transfection efficiency. (c) Specific MB-binding activity of 20Q versus 44Q hAR transiently expressed in NSC34 cells and incubated with 3 nM MB for the indicated time. Below is the corresponding immunoblot using anti-hAR mAb F39.4.1 and anti-human-hsp70 mAb as internal control.


(a)
Normalized GH transactivation (cpm/mg Protein)
4000
3000
2000
1000
0
0.0
1.0
2.0
3.0
MB [nM]
20Q
44Q
(b)
MB-Binding Activity (fmol/mg protein)
1500
1250
1000
750
500
250
0
3
1
0.5
0.1
0.05
MB [nM]
20Q
44Q


(c)
MB-Receptor Binding (fmol/mg protein)
120
100
80
60
40
20
0
2
4
5
6
Days
20Q
44Q


Days
2
4
5
6
N E N E N E N E
NSC34
hAR
hsp70

(d)

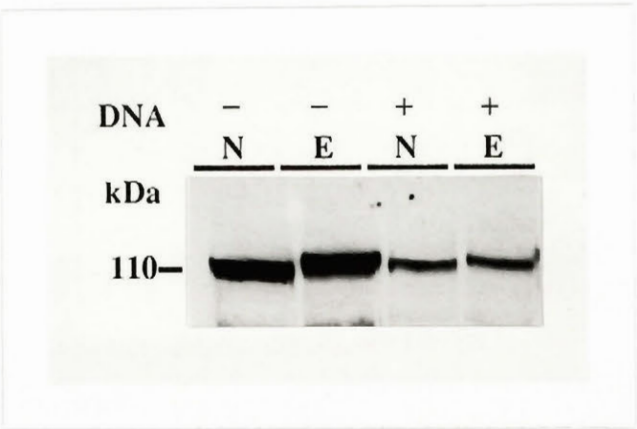


Figure 8. Analysis of transactivational competence and specific androgen-binding activity of the normal versus polyGln-expanded hAR in COS-1 cells using hAR cDNAs with tracts made of mixed glutamine codons.

(a) Transactivational analysis of hAR made of the normal [$(CAG)_nCAA$] or mixed (CAG/CAA)n glutamine codons in the normal or expanded forms. COS-1 cells were cotransfected with hAR cDNA and MMTV-GH and incubated with 1.5 nM MB for 72h at 37°C. GH activity was measured as the percentage of the normal-length hAR cDNA with the normal codon [$(CAG)_{19}CAA$], 20Q. The rest of the hAR cDNAs were: 20QMix $(CAG/CAA)_{20}$; 41QMix $(CAG/CAA)_{41}$; and 44Q [$(CAG)_{43}CAA$]. (b) The specific MB-binding activity of MB at 1.5 nM the hAR transiently expressed in COS-1 cells of part (a). Transactivational analysis and specific binding activity were corrected for transfection efficiency.

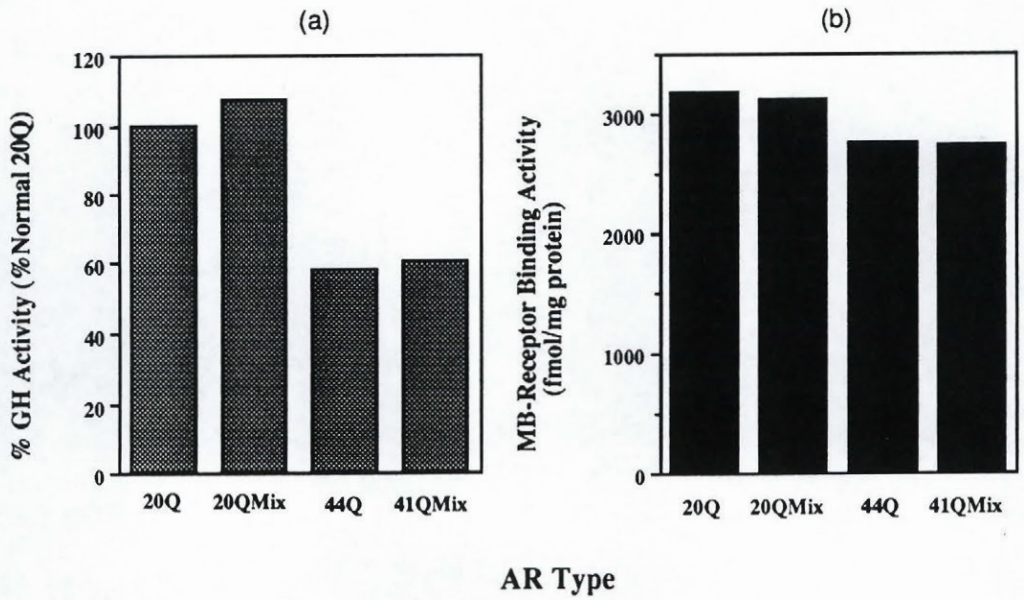
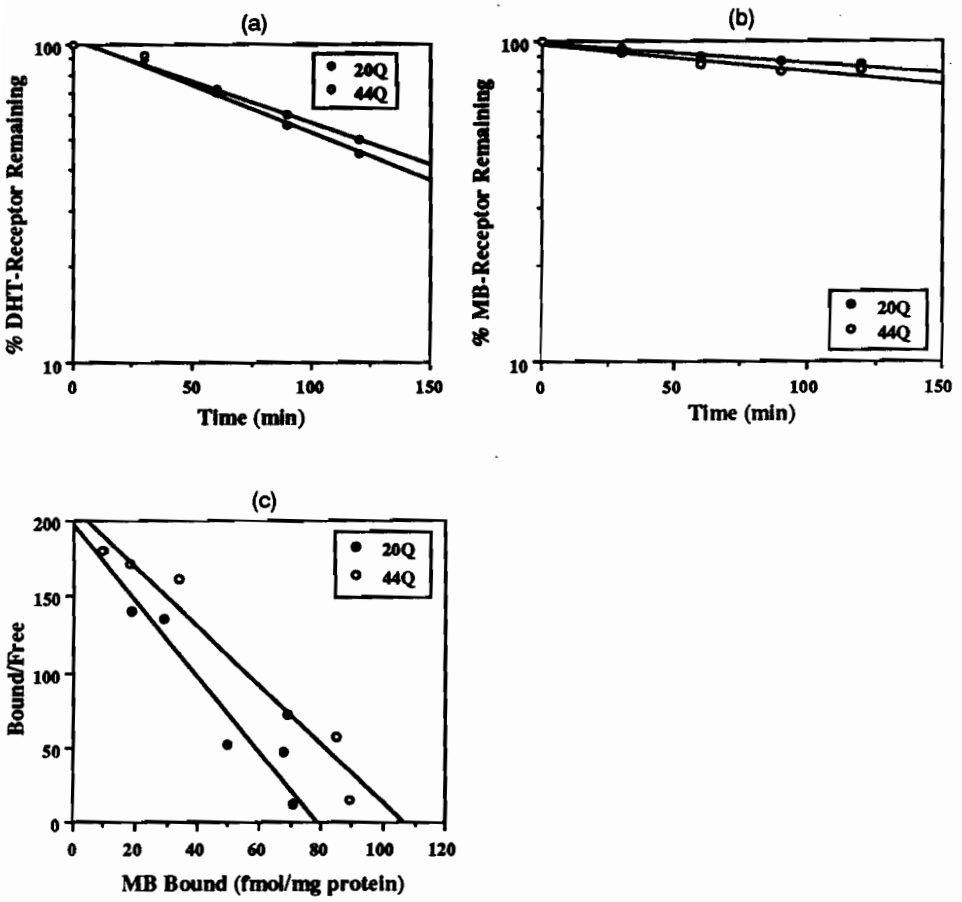

(a)
% GH Activity (%Normal 20Q)
120
100
80
60
40
20
0
20Q
20QMix
44Q
41QMix
(b)
MB-Receptor Binding Activity (fmol/mg protein)
3000
2000
1000
0
20Q
20QMix
44Q
41QMix
AR Type

Figure 9. Analysis of binding kinetics of the normal versus polyGln-expanded hAR transiently expressed in NSC34.

Androgen-receptor dissociation-rate analysis of the normal (20Q) versus the polyGln-expanded (44Q) hAR with DHT (a) or MB (b). NSC34 cells were transfected with hAR cDNA and incuabted at 37°C. After 48 h, triplicate wells were incubated with 3 nM [^{3}H]androgen and 100 μM cycloheximide for 2 h at 37°C, washed, and chased with 200-fold of the same radioinert androgen for up to 120 min. Specific binding (the mean of total binding in triplicate minus nonspecific binding in duplicate) was plotted as the percentage of that remaining at indicated times after initiating the chase. (c) Scatchard analysis of NSC34 transfected with 20Q or 44Q and incubated for 48 h at 37°C. [^{3}H]MB at 0-3 nM concentrations was added in triplicate wells and 200-fold excess of radioinert MB corresponding to each concentration was added in duplicates and incubated for 2 h at 37°C in the presence of 100 μM cycloheximide. Specific binding (the mean of total binding in triplicate minus nonspecific binding in duplicate) was plotted as fmol of bound MB per mg of total protein versus bound/free MB.


(a)
100
10
% DHT-Receptor Remaining
20Q
44Q
0
50
100
150
Time (min)
(b)
100
10
% MB-Receptor Remaining
20Q
44Q
0
50
100
150
Time (min)
(c)
200
150
100
50
0
Bound/Free
20Q
44Q
0
20
40
60
80
100
120
MB Bound (fmol/mg protein)

dissociation constants. This indicated that the higher ligand binding of the polyGln-expanded receptor is not due to higher receptor-ligand affinity (Fig. 9).

Western blotting of ARE-bound androgen-receptor complexes

To test whether impaired ARE binding by polyGln-expanded hAR might contribute to hypotransactivation, the ability of the expanded AR-MB complexes to bind a synthetic ARE (Roche *et al.*, 1992) was investigated using COS-1 cells nuclear extract expressing either normal or expanded poly(Gln) hAR. Immunoblots of ARE/MB-R complexes detected by mAb F39.4.1 were not different for the normal and the expanded hARs. This suggested that impaired ARE binding does not contribute to reduced transactivation by the polyGln-expanded hAR.

***In vitro* and cellular proteolytic analysis in COS-1 cells**

This analysis was performed to investigate whether the neuronotoxic gain-of-function of the Gln-expanded AR reflects the gain of a new structure or conformation. The rationale for the investigation was that any conformational differences between the normal and mutant proteins could provide clues as to the molecular pathogenesis of SBMA. This conformational analysis was done by partial tryptic digestion of the radiolabelled, *in vitro* synthesized ARs.

I chose to use trypsin for several reasons. Trypsin has been used successfully to analyse the conformational alterations of several proteins including AR (Kallio *et al.*, 1994; Kuil and Mulder, 1994, and references therein). Partial tryptic digestions assays are rapid and easy to perform; trypsin can react at pH 7.6-8.8, 25°C, in the presence of protein denaturants like SDS and urea (Beynon and Bond, 1990). Previous reports on AR partial tryptic digestions revealed conformational changes upon ligand binding (Kallio *et al.*, 1994; Kuil and Mulder, 1994) and LBD trypsin-resistant fragment(s) were generated using different ligands (Kallio *et al.*, 1994).

In these reactions, equal volumes of reaction mixtures were used on the assumption that the rates of synthesis of the normal and expanded ARs are equal. If, in fact, the expanded AR was synthesized at lower rate, then its concentration would be lower and it would be more sensitive to partial proteolysis.

Partial tryptic digest of [^{35}S]Met-labelled normal (Gln-20) versus expanded (Gln-44) hAR

The *in vitro* proteolytic experiments were designed to answer the following question: Does the expanded AR *in vitro* have a conformational alteration (gain of structure) that facilitates the creation of potentially pathogenic derivative (s)?

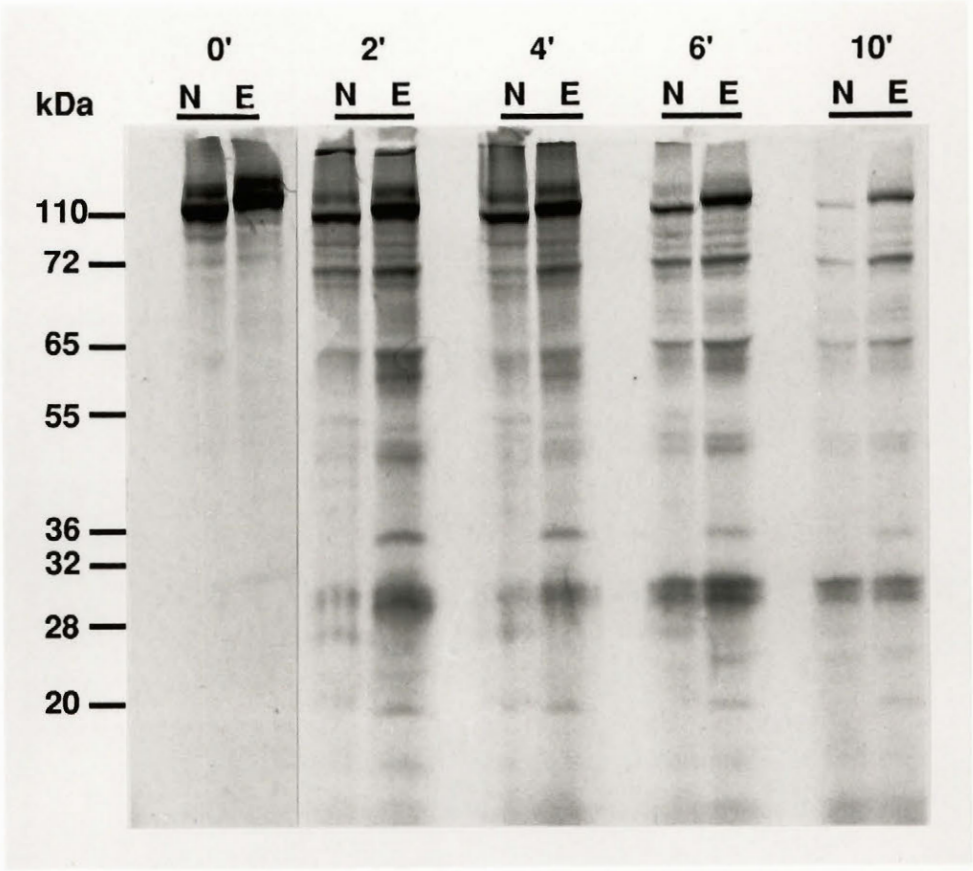
[^{35}S]Met-labelled, *in vitro*-translated normal and Gln-expanded hAR were subjected to partial tryptic digestion for 2-10 min and then analyzed by SDS-PAGE (Fig. 10). Expectedly, the normal and expanded parental hARs had slightly different mobilities. By densitometry, over 80% of the normal full-length protein was digested at 10 min compared to 50% of the Gln-expanded full-length hAR. The relative resistance to trypsin of the Gln-expanded hAR indicates that it is conformationally different from the wild-type hAR. Nonetheless, most of the fragments in both digests migrate identically. This indicates that an N-terminal portion containing the polyGln tract is cleaved from both the normal and expanded parental hARs soon after the start of tryptic digestion. Thus, a trypsin-cleavage site C-terminal to the normal or expanded tract is highly exposed. Contrarily, the Gln-expanded hAR and its normal counterpart yield several distinct tryptic fragments: most notable are the 65-, 36-, and 20-kDa products in the expanded hAR digest, and those of 55- and 28-kDa in the normal AR digest. The migration differences of the possible pairs of cognate fragments (apparent kDa of 65 versus 55, and 36 versus 28, respectively) far exceed those expected from the value of 3.1 kDa computed fro the 24 extra residues in the expanded polyGln tract.

Partial tryptic digest of [^{14}C]Gln-labelled normal (Gln-20) versus expanded (Gln-44) hAR

To enhance recognition of fragments containing the Gln tract, partial tryptic digests were performed on [^{14}C]Gln-labelled normal and Gln-expanded hAR (Fig. 11). By densitometry, about 90% of the normal AR was digested at 6 min, compared to about 50% of the expanded AR, affirming a similar observation in Fig. 10. The Gln-expanded hAR digest has several major bands, most notably those of 36 and 28 kDa compared to the less intense bands of 27 and 25 kDa in the normal hAR digest. Again, assuming homology, the mobility difference between the 36- and 27-kDa pair greatly exceeds the MW difference of 3.1 kDa between the 44- and 20-Gln species. A [^{14}C]Gln-labelled fragment of 36 kDa

Figure 10. Partial tryptic digests of [^{35}S]Met-labelled normal and polyGln-expanded hAR.

Zero to 10 min trypsinolysis of *in vitro*-translated [^{35}S]Met-labelled normal (N) or Gln-expanded (E) hAR was done at room temperature (RT), fractionated by SDS-PAGE, and radioautographed. The heavier member of parental doublets probably represents extended translation. The 0' lanes were derived from the same experiment as the 2'-10' lanes; they were transposed from the right to the left side of the figure simply for visual purposes.


0'
2'
4'
6'
10'
kDa
N E
N E
N E
N E
N E
110
72
65
55
36
32
28
20

very likely contains several Met residues; hence, it probably corresponds to the 36-kDa fragment seen in the digest of the [^{35}S]Met-labelled Gln-expanded hAR.

Western blot analysis of partial tryptic digests of the normal and the expanded hAR

The epitope for the monoclonal antibody F39.4.1 is amino acids 301-320 of the wild type hAR. Aside from the set of bands representing the respective full-length proteins, this mAb only detects a pair of ~72-kDa derivatives that display the mobility difference expected from disparate Gln-tract length (Fig. 12). In this experiment, the apparent sensitivity of the expanded AR to trypsin is due to the unequal AR amounts loaded. This mobility difference is not seen with the 72-kDa fragment derived from both parental proteins after [^{35}S]Met labeling (Fig. 10). The surprising absence of any immunoreactive bands smaller ~72 kDa indicates that removal of a fragment containing the intact epitope, and its own subsequent rapid digestion, or cleavage through the epitope itself, is an early event in trypsinolysis of the wt and expanded AR.

Glutamine tract-size effects on protein mobility

AR fragments containing normal or expanded Gln tracts may migrate differently than expected from size alone. To assess this, chimeric cDNAs coding for the GAL4-DBD fused to various N-terminal fragments of either a normal 20-Gln hAR or a 50-Gln-expanded hAR were constructed. Four different chimeric proteins were expressed in yeast cells: GAL4-DBD/AR 20-Gln/aa 38-341/52.2 kDa; GAL4-DBD/AR 50-Gln/aa 38-341/55.9 kDa; GAL4-DBD/AR 20-Gln/aa 11-341/55.4 kDa; and GAL4-DBD/AR 50-Gln/aa 11-341/59.1 kDa were made. Western blot analysis was done with AR antibody F39.4.1 after standard SDS-PAGE. In Figure 13, the proteins in the two middle lanes are predicted to have the same MW (~ 52.4, 55.9 kDa), and are seen to have migrated essentially equally, although their Gln tracts differ by 30 residues. The proteins in the outer lanes differ by 30 Gln residues. The proteins in the outer lanes also differ by 30 Gln residues but, in addition, they differ in MW (52.2, 59.1 kDa), and this difference is expressed appropriately in their respective mobilities. Thus, by 8% SDS-PAGE, apart from size, Gln-tract lengths only a slight influence on the protein mobility of half-normal size AR fragments. Therefore, in addition to slightly disparate mobility attributable to Gln-tract expansion, it is more likely that the distinct trypsinolytic

patterns of the normal and Gln-expanded AR primarily reflect conformational properties that provide differential access to trypsin-cleavage sites.

Immunoanalysis following progressive proteolysis in the presence of denaturant

To estimate the relative trypsin resistance of certain expanded-hAR fragments, the trypsin concentration was increased by 50% and digestion was extended to 1 hr in the presence or absence 2 M urea (Fig. 14). These harsher conditions highlight the disparate fragments observed earlier (Figs. 10 and 11) to distinguish the normal from the Gln-expanded hAR. The prominence of the [^{14}C]Gln-labelled 20-kDa fragment in the Gln-expanded, trypsin-treated lanes of Figure 13, is particularly striking.

Figure 15 is a Western analysis performed with mAb1C2 after blotting the same [^{14}C]Gln-labelled gel described in Figure 14. It documents and illustrates dramatically that four of the fragments that resist aggressive proteolysis of the Gln-expanded hAR, themselves contain the Gln-expanded tract. Appropriately, these four fragments also yield the most intense radioautographic signals in Figure 14.

Transfection studies

To assess whether the expanded AR cDNA can yield apoptotically pathogenic derivative in transfected cells, cell transfection and protein analysis studies were pursued.

In these experiments, only the COS-1 cells were used to analyse both AR expression and processing as they are easy to transfect and express higher amounts of AR compared to the motor neuron-like cells. COS cells were transfected with normal or Gln-expanded hAR to determine whether they show increased cell death in association with the production of unique Gln-expanded fragments of hAR. Western blots using mAb1C2 and F39.4.1 antibodies were performed on cell extracts obtained 1-4 days later (Fig. 16). Slightly less stringent conditions allowed the mAb1C2 to detect both the parental normal hAR and the Gln-expanded parent. Monoclonal Ab1C2 clearly exposed a novel band of approximately 75 kDa only in COS cells transfected with the $(CAG)_n$-expanded hAR cDNA. No counterpart was obtained from COS cells transfected with wild type hAR cDNA, even after prolonged exposure to the chemiluminescence detection system. Western blots using the F39.4.1 antibodies revealed no

Figure 11. 0- to 6-min partial tryptic digests at RT of *in vitro*-translated [^{14}C]Gln-labelled normal (N) hAR or Gln-expanded (E) hAR. The reduced rate of digestion in the E lanes compared to the N lanes is obvious. At 6 min, the N digest has faint 27-, 25- and 30-kDa fragments, whereas the E digest has stronger 36-, 28- and 20-kDa fragments. The more intense E fragments predicts the presence of a polyGln-tract.

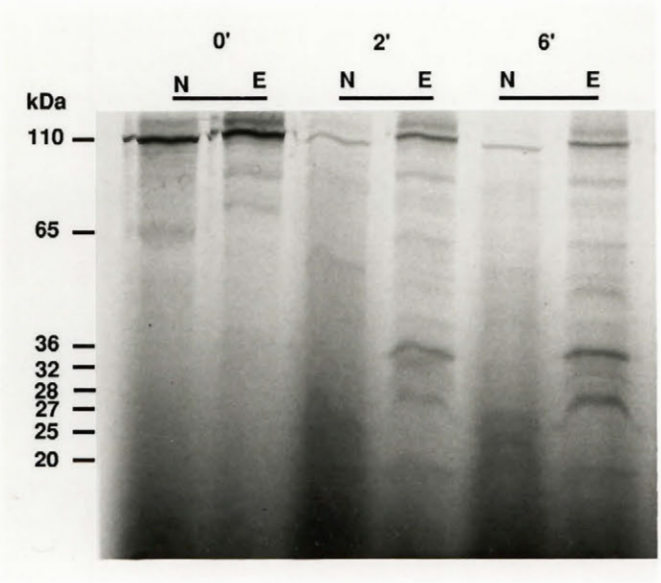

0'
2'
6'
N
E
N
E
N
E
kDa
110
65
36
32
28
27
25
20

Figure 12. Western-blot analysis of partially digested *in vitro*-translated normal (N) hAR or Gln-expanded (E) hAR.

Samples were fractionated by SDS-PAGE, electrotransferred to nitrocellulose membrane, and immunoreacted with mAb F39.4.1. Each panel included N or E hAR partially digested 0-6 min.

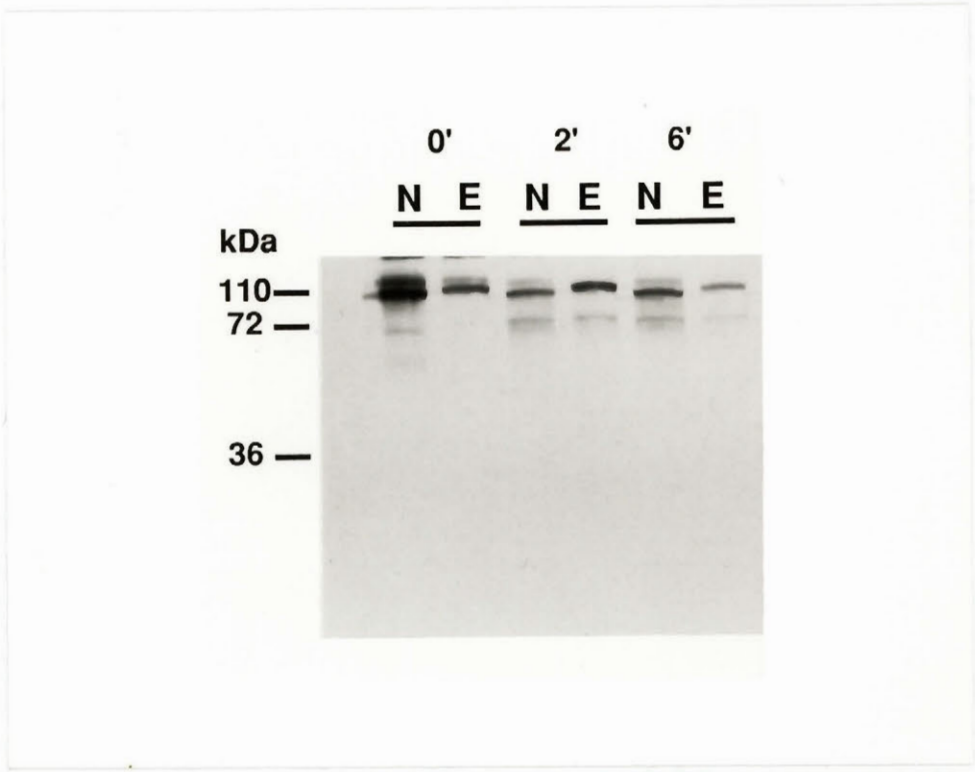
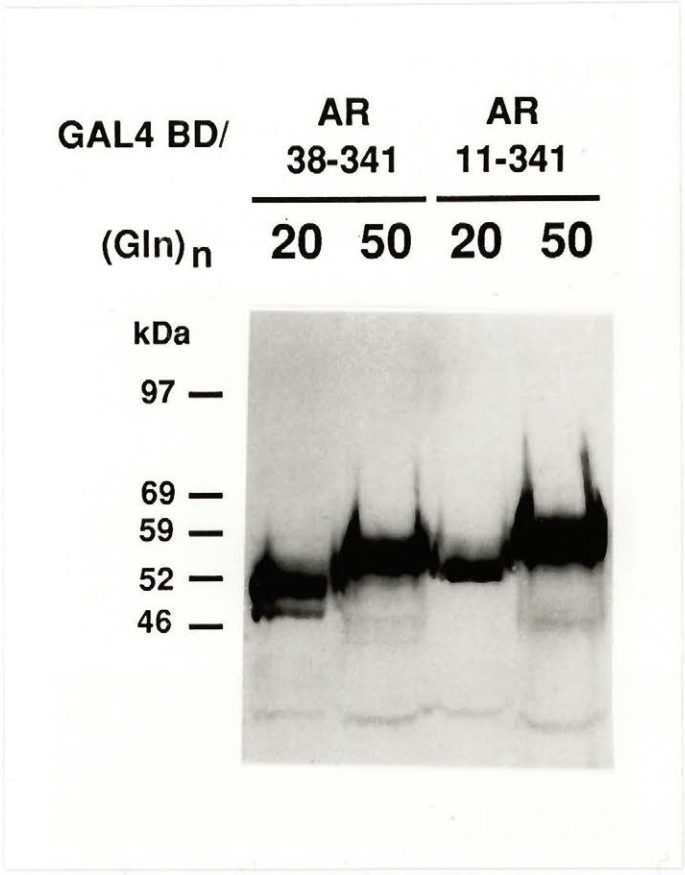

0'
2'
6'
N E N E N E
kDa
110
72
36

Figure 13. Western analysis of chimeric proteins expressed in yeast.

(a) Yeast extracts were fractionated by SDS-PAGE, electroblotted and subjected to Western analysis using F39.4.1 (epitope: amino acids 301-320 of hAR). Expected MW for the various chimeric proteins are given in the text. (b) Schematic diagram of DNA fragments encoding the above peptides; amino acids 11-341 (*Acc* I-*Acc* I sites) and 38-341 (*Sma* I-*Acc* I sites) of the normal (Gln-20) and a Gln-expanded hAR (Gln-50) were cloned into pAS2-1 to produce GAL4BD-AR chimeric proteins in yeast strain CG-1945.

(a)


GAL4 BD/
AR 38-341
AR 11-341
(Gln)n
20 50 20 50
kDa
97
69
59
52
46

(b)

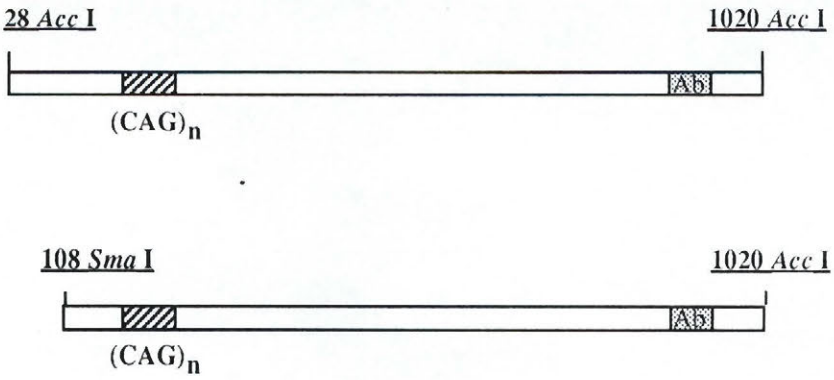

28 Acc I
1020 Acc I
AB
(CAG)n
108 Sma I
1020 Acc I
AB
(CAG)n

Figure 14. Extended Partial tryptic digestion of [^{14}C]Gln-labelled hAR in the presence of urea.

One-h tryptic digest of [^{14}C]Gln-labelled normal (N) or Gln-expanded (E) hAR in the presence (+) or absence (-) of 2 M urea. Notice the persistence of 65-, 36-, 32- and 20-kDa fragments in the E lane, even in the presence of 2 M urea. In contrast, the N lane has predominant fragments of 55, 27, 25 and 22 kDa.

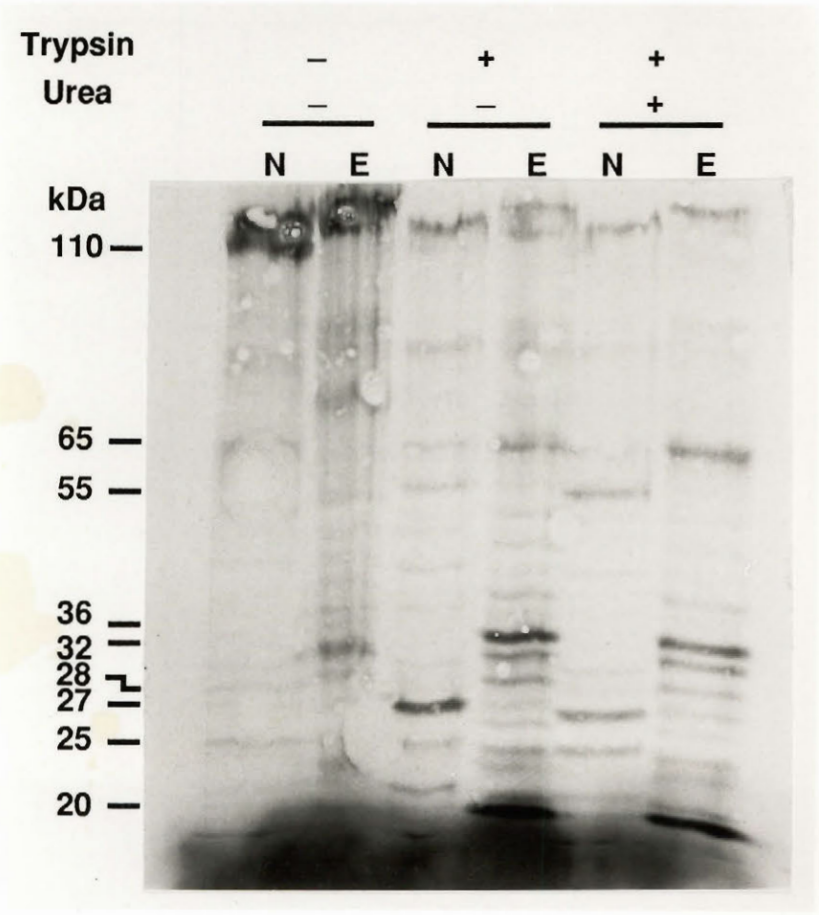

Trypsin
Urea
–
–
+
–
+
+
N
E
N
E
N
E
kDa
110
65
55
36
32
28
27
25
20

Figure 15. Western analysis of the extended trypsinolysis of hAR.

Western analysis of the same blot shown in Fig. 14 using the polyGln-specific mAb1C2. Only the full-length expanded (E) AR and its 65-, 36-, 32- and 20-kDa derivatives react with 1C2 indicating that they have an expanded polyGln-tract.

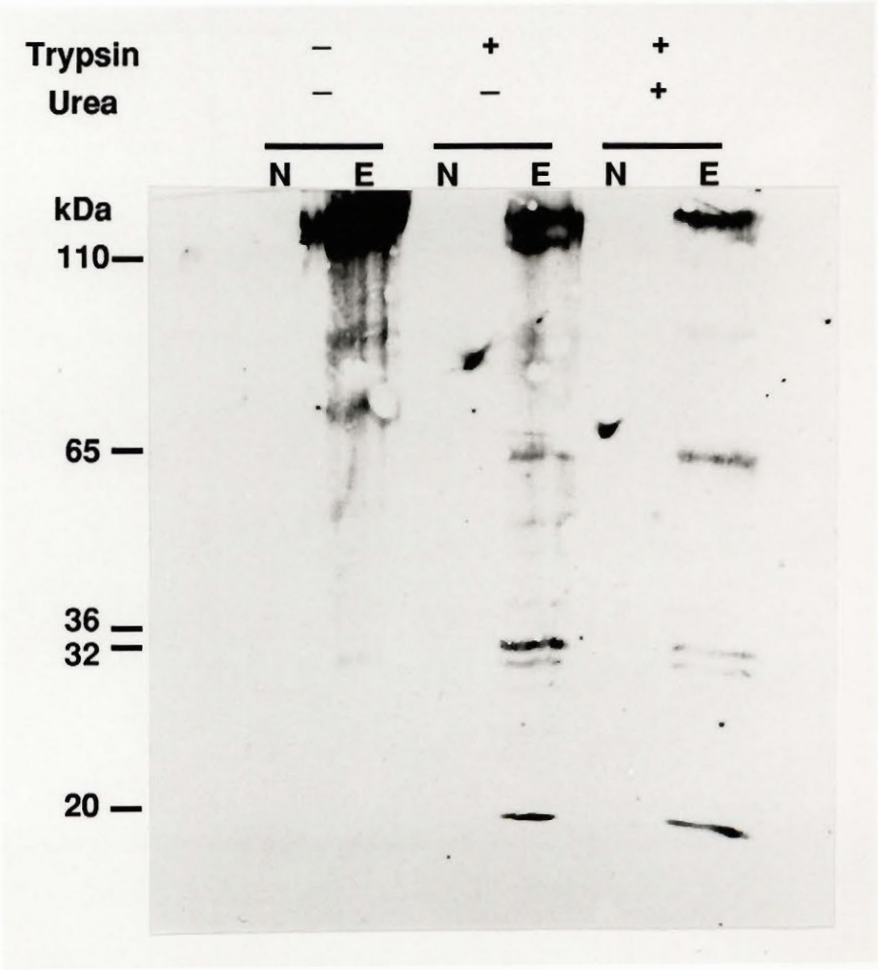
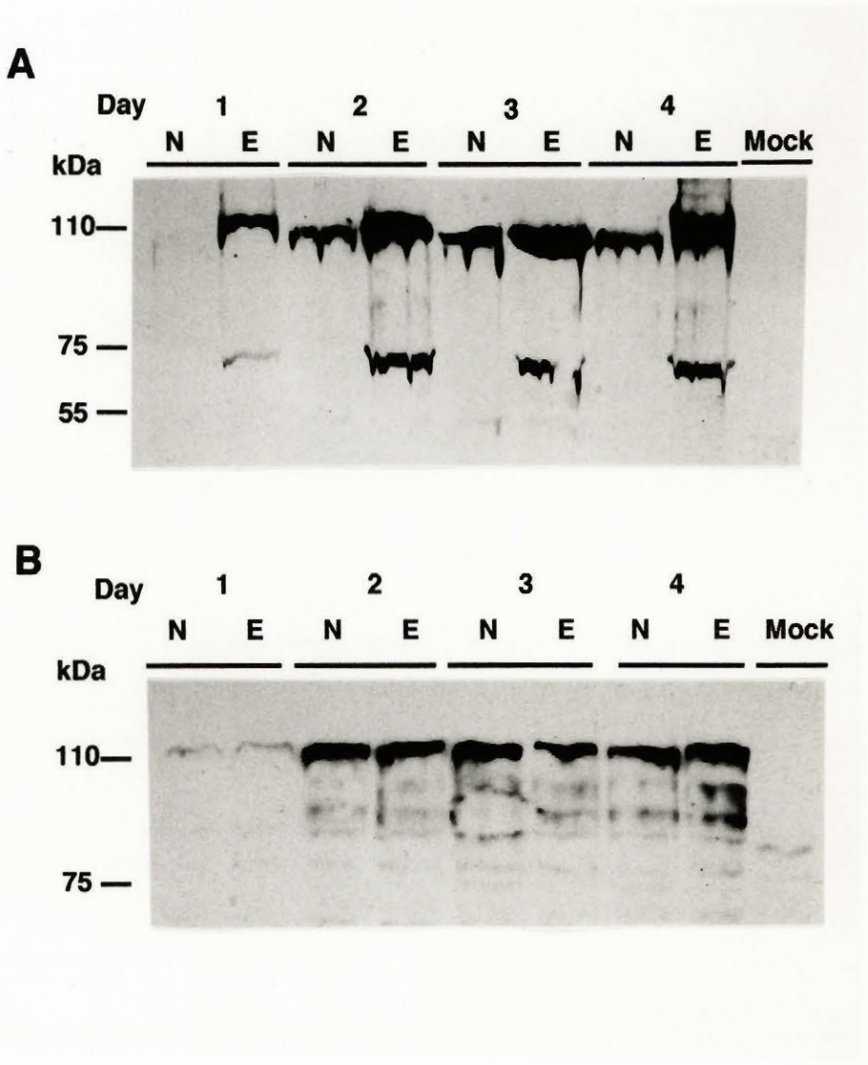

Trypsin
Urea
–
–
+
–
+
+
N
E
N
E
N
E
kDa
110
65
36
32
20

Figure 16. Western analysis of hAR transiently expressed in COS-1 using mAb1C2.

Western analysis. Panel A shows a Western blot using mAb1C2; a 75-kDa fragment can be seen in the lanes containing the poly-Gln expanded hAR on all four days, and it is absent from the normal AR lanes. Panel B shows a Western blot using the F39.4.1 antibody; the AR, (110 kDa) is clearly visible in both the normal and expanded AR. Notice the absence of the 75-kDa fragment.


A
Day 1 2 3 4
N E N E N E N E Mock
kDa
110
75
55
B
Day 1 2 3 4
N E N E N E N E Mock
kDa
110
75

differences between extracts from cells transfected with the normal or the Gln-expanded hAR. Thus, the ~75-kDa fragment is uniquely associated with expression of the Gln-expanded hAR, and it contains a polyGln tract, but apparently not the epitope for mAb F39.4.1 (aa 301-320). A conventional 75-kDa fragment of the AR that includes the polyGln tract should also contain the F39.4.1 epitope. The apparent absence of that epitope suggests unconventional proteolysis or some form of misprocessing of the AR, among other explanations, as discussed below.

To assess whether this aberrant fragment is due to a post-trnslational misprocessing or nucleic acid misbehavior, AR cDNA constructs containing two types of the Gln-coding repeats: the standard $(CAG)_nCAA$ or a mixed Gln-coding tract $(CAG/CAA)_n$ were made. Both types of tracts were made in the normal or the expanded form. Western analysis using the mAb1C2 showed that this novel 75-kDa fragment was shown to exist in cells transfected with the mixed polyGln-expanded AR as well as the standard-expanded AR (lanes 4 and 6, Fig. 17) indicating that both proteins may be identical in conformation. The novel band was missing in the mixed-normal 20-Gln AR (lane 5) and in the standard-20 Gln AR (lane 3) indicating that the appearance of the 75 kDa fragment is likely due to post-translational misprocessing. To assess whether this fragment is associated with increased cell death, cell analysis was done using epiconfocal microscopy.

Cell death analysis

In this analysis, the COS-1 cell line was used as a model for cytotoxicity studies. It was used as a convenient cell type for preliminary studies of cytotoxicity and proteolysis of other polyGln-expanded proteins (Ikeda *et al.*, 1996). COS cells transfected with normal or (CAG)n-expanded hAR cDNA were stained with ethidium monoazide bromide, which detects the DNA of dead cells only, and examined by epifluorescence microscopy to assess cell toxicity and the rate of cell death.Table II demonstrates a 2-fold increase in death with distinct apoptotic morphology in cells transfected with Gln-expanded versus normal AR. The AR transfected cells tended to become round but remained attached. This effect was observed in the presence or absence of androgen which is compatible with the idea that a polyGln-expanded fragment is a prerequisite for neuronopathy to occur.

Figure 17. Western analysis of AR made from variable types/sizes of glutamine codon and tract transiently expressed in COS-1 cells.

Lane 1 shows extract of the mock-transfected cells . Lane 2 is extract from cells with Δ Gln-AR protein. Lane 3 contains extract from cells with the standard-codon 20 Gln AR protein. Lane 4 contains extract from cells with standard-44 Gln AR. Lane 5 contains extract from cells with mixed-codon 20-Gln AR and lane 6 contains mixed-codon 41-Gln AR. The 75 kDa fragments present in cells from both in both the standard- and mixed-codon-encoded polyGln-expanded AR.

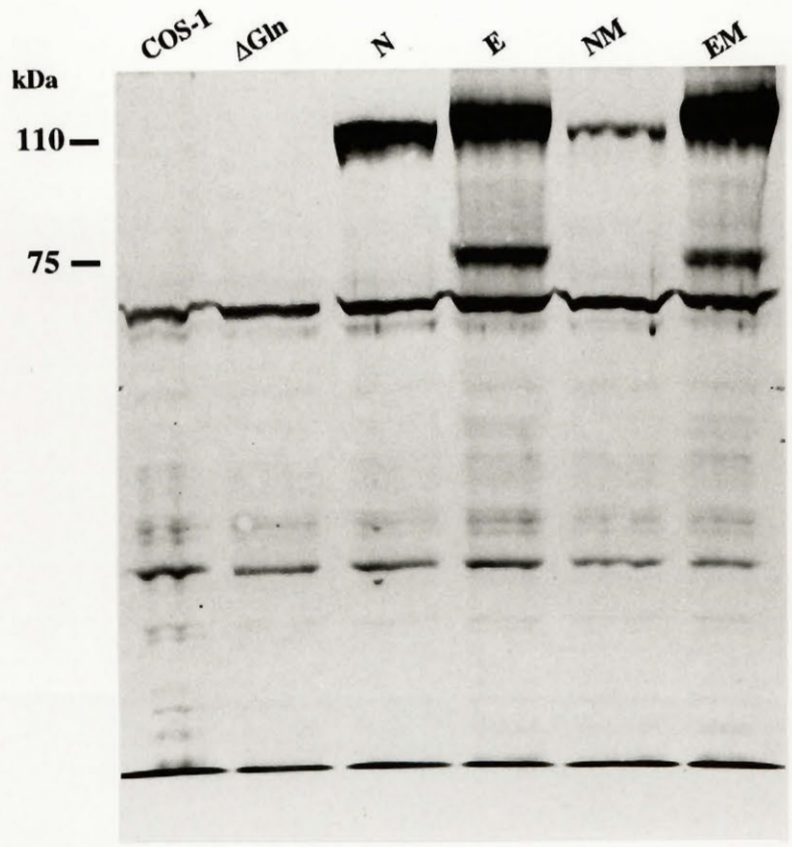

COS-1
ΔGln
N
E
NM
EM
kDa
110
75

Table II. Percent death of COS-1 cells transfected with normal or polyGln-expanded hAR.

Death of transfected COS cells in the presence or absence of 3 nM Mibolerone was determined by ethidium bromide monoazide (EMA) staining. Exent of cell death is expressed as percent EMA-positives. The data were corrected for transfection efficiency by cotransfection of pCMV.β-gal. Mock cells were cotransfected with pcDNA3 vector and pCMV.β-gal and routinely showed 0.5-1.0% cell death. By Student's paired T-test, $p<0.001$ for expanded versus normal hAR, with or without Mibolerone.

Table II. Percent (%) cell death in cell toxicity studies.

	- MB		+ MB	
	Normal hAR	Gln-Expanded hAR	Normal hAR	Gln-Expanded hAR
Experiment 1	5.4	9.0	5.5	11.0
Experiment 2	4.2	12.4	8.6	13.2
Experiment 3	8.1	12.7	8.3	16.8
Experiment 4	3.6	9.8	5.3	9.6
AVERAGE	5.3	11.0	6.9	12.7

ELECTROPHYSIOLOGICAL ANALYSIS OF MOTOR NEURONS EXPRESSING hAR

Electrophysiological studies on neurons revealed several types of Ca^{2+} channels that are classified according to their activation threshold, persistence, and pharmacological properties (Akaike, 1991). These include T-type channels (low-threshold), N-type and L-type channels (high-threshold) in addition to other types (P/Q-type). These channels exist in many neurons and are co-expressed in several neuronal tissues like the hypothalamic neurons or hippocampal neurons.The activation (peak of current) and inactivation (decay of current) of the current are strongly potential-dependent (voltage-dependent). Both kinetics become more rapid with increasing depolarization. A similar phenomenon is observed in dissected hippocampal, amygloid and cerebellar neurons (Akaike, 1991; and references therein).

To assess the effect of AR on the function of Ca^{2+} channels, electrophysiological studies were done using two motor neuron-like cells. The choice of this cell line as well as another cell line that was stably transfected with AR (MN, Brooks et al., 1997) was based on several factors. Primary cultures from mouse embryo spinal cords are short lived (< 6 weeks), and cannot be easily transfected, except by adenovirus (Dr. Heather Durham, personal communication) therefore long-term AR expression cannot be assessed using primary culture. Transgenic SBMA mice generated so far are clinically normal and their AR expression level is lower than normal (Bingham *et al.*, 1995). The production of transgenic mice expressing polyGln-expanded AR is therefore a long-term project. The NSC34 (neuroblastoma x spinal cord) hybrid cell line was developed by fusing the aminopterin-sensitive mouse neuroblastoma N18TG2 with motor neuron-enriched embryonic day 12-14 mouse spinal cord cells. NSC34 cells display a multipolar neuron-like phenotype. They were found to express choline acetyl-transferase and induce acetylcholine receptor clusters, and twitching, in co-cultured mouse myotubules (Cashman *et al.*, 1992). This cell line also possesses additional properties expected of motor neurons, including generation of action potentials, expression of neurofilament triplet proteins, acetylcholine synthesis, storage, and release, and a vimentin-neurofilament switch with maturation in culture, similar to that occurring in neuronal development. NSC34 cells therefore appear to model selected aspects of motor neurons in an immortalized clonal system. In culture, nearly 100% of NSC34 cells form processes, 100% synthesize neurofilament proteins and >50% generate action

potentials (Dr. Neil Cashman, personal communication). They are transfectable using lipofectamine, in my experiments transient transfection efficiencies approach 50%.

The other motor neuron-like hybrid cell line (MN) was prepared earlier (Salazar-Grueso *et al.*, 1991) in a similar way to that of NSC34 and exhibits similar characteristics of those observed in motor neurons and described for NSC34. In addition, MN cells were stably transfected with AR cDNA coding for a normal polyGln tract of 24 residues, polyGln-expanded tract of 65 residues, or mock stables (Neo-7) transfected with pcDNA3 plasmid only (Brooks *et al.*, 1997). The clones used in this thesis are AR24-1 (wild type AR), AR65-8 (expanded AR), and Neo-7 which were received from Dr. Fischbeck's laboratory (University of Pennsylvania, Ph). cytotoxicity studies done on these cells showed no signs of cell death induced by AR expression (Brooks *et al.*, 1997). Androgen binding of these cells was done using 3 nM [^{3}H]MB for 2 hrs at 37°C. Binding activity was 430, 150, and 5 fmol/mg protein for AR24-1, AR65-8 and Neo-7 cells, respectively.

Motor neuron-neuroblastoma hybrid cell lines transiently or stably transfected with wild-type or expanded ARs were studied to compare the effect of exposure to androgen agonist (5 nM mibolerone) on Ca^{2+} currents in the three groups (mock, wild-type AR transfected, expanded AR transfected) of each cell line (NSC34 and MN) described in the method section. Both types of cell line expressed a unique low threshold, transient type of Ca^{2+} current that was not affected by L-type blocker (PN 200-110), N-type Ca^{2+} channel blocker (ω-conotoxin GVIA) or P-type Ca^{2+} channel blocker (Agatoxin) but was blocked by either 200 μM Cd^{2+} or Ni^{2+} (Fig 20). Exposure to androgen agonist had no effect in control cell lines or cells transfected with normal (Fig. 18 A,B; (Fig. 19 A-E) androgen receptor but significantly changed the steady-state activation in cells transfected with expanded androgen receptor. The observed negative shift (> - 10 mV) in steady-state activation would result in a large increase in the transient Ca-channel window current.

Ca^{2+} channel currents recorded during depolarizations of NSC-34 cells activated around -50 mV (Fig. 18). The current/voltage relationship of normalized peak currents had a maximum around -20 mV (Fig. 18 A-B). Analysis of tail currents at the end of depolarization pulses suggests a biphasic voltage dependence of activation with a half activation voltage of the first Boltzmann fit

($V_{1/2}$) around -30 mV (-27 to -30 mV, Fig 18, D-F). The $V_{1/2}$ was unchanged by exposure to androgen agonist (5 nM, mibolerone) in the mock transfected NSC-34 cells (Fig. 18, A, D), or cells transfected with normal AR (Fig. 18, B, E). However, in NSC-34 cells transfected with the polyGln-expanded AR, this concentration of mibolerone induced a remarkable negative shift in the $V_{1/2}$ of the first Boltzmann (from -30 mV to -44 mV) (Fig. 18 F). The steady-state inactivation was unchanged with the polyGln-expanded AR after the addition of the agonist (Fig. 18 I). The negative shift in the voltage dependence of activation of Ca^{2+} channel currents in the NSC34 cells transfected with polyGln-Expanded AR in response to androgen agonist resulted in a significant increase in the window current (Fig. 18 C).

Ca^{2+} channel currents recorded in a stably transfected cell line were similarly affected by androgen agonist in cells expressing polyGln-expanded AR (Fig 19). As with NSC34 cell line, the peak i/v had thresholds around -50 mV and the current voltage relationship of normalized peak currents had a maximum around -20 mV (Fig. 19 A-F). The negative shift in the voltage dependence of activation of Ca^{2+} channel currents in the cell line stably transfected with polyGln-expanded hAR resulted in an increase in the window current after differentiation (Fig. 19 C, F). The addition of agonist to the differentiated cells with the polyGln-expanded AR significantly increased the window current (Fig. 19F). These data suggests that in NCS34 cell line as well as the stably transfected cell line, an increase in the window of the Ca^{2+} current in response to androgen may be in part responsible for the neurotoxicity of the SBMA gene. To identify the Ca^{2+} channel type(s) expressed in NSC34 cell line specific Ca^{2+} channel blockers were tested (Fig. 20). The effect of these agents on Ca^{2+} channel currents in neuroblastoma x motor neuron hybrid cell lines were compared to the effect of the drugs on Ca^{2+} channel currents in dorsal root ganglia neurons. These neurons were chosen as they are known to express all major neuronal type Ca^{2+} channel currents (Sculptoreanu *et al.*, 1995). Figure 20 shows that the Ca^{2+} currents in these cell lines were unresponsive to L-type Ca^{2+} channel dihydropyridine agonist (SDZ 202-791) and antagonist (PN 200-110), N-type Ca^{2+} channel antagonist ω- conotoxin (CTX) or P-type channel blocker Agatoxin GVIA (ATX). However, Cd^{2+} and Ni^{2+} (T-type Ca^{2+} channel blockers) effectively blocked the Ca^{2+} currents in the hybrid cell lines at 200 mM. This suggests that the only Ca^{2+} channel current experessed in both cell lines used in these experiments was T-type.

Figure 18. Current-voltage relationship of peak Ca^{2+} currents in NSC-34 cell lines before (empty symbols) and after (filled symbols) exposure to androgen in control (A, mock transfection, NSC34), normal androgen receptor (B, 20 Gln) and cells expressing SBMA androgen gene (C, 44 Gln). Note the marked shift toward negative voltages in the latter. Currents were normalized to individual cell membrane capacitances and results from several cells (recorded in the interval of 4 month) averaged. Results are shown as mean ± SEM (n=no. of cells). Current-voltage relationship of tail Ca^{2+} currents at the end of a 50 ms test pulse in NSC-34 cell lines before (empty symbols) and after (filled symbols) exposure to androgen in control (D, mock transfection, NSC34), normal androgen receptor (E, 20 Gln) and cells expressing polyGln-expanded AR (F, 44 Gln). Note the marked shift toward negative voltages in the abnormal androgen receptor transfected cell line after exposure to androgen. Currents were normalized to individual cell membrane capacitances. Smooth curves were fitted with the sum of two Boltzmann's equations. D-F The half voltage of activation of the first Boltzmann ($V_{1/2}$) are shown as insets. G-I. Current-voltage relationship of peak Ca currents and steady-state inactivation curves obtained in the same cells as in A-F (pre-pulse 800 ms long followed by a 5 ms interval at -60 mV and a 100 ms to 0 mV). Note the marked shift toward negative voltages in the abnormal androgen receptor transfected cell line after exposure to androgen. Peak currents were normalized to individual cell membrane capacitances.

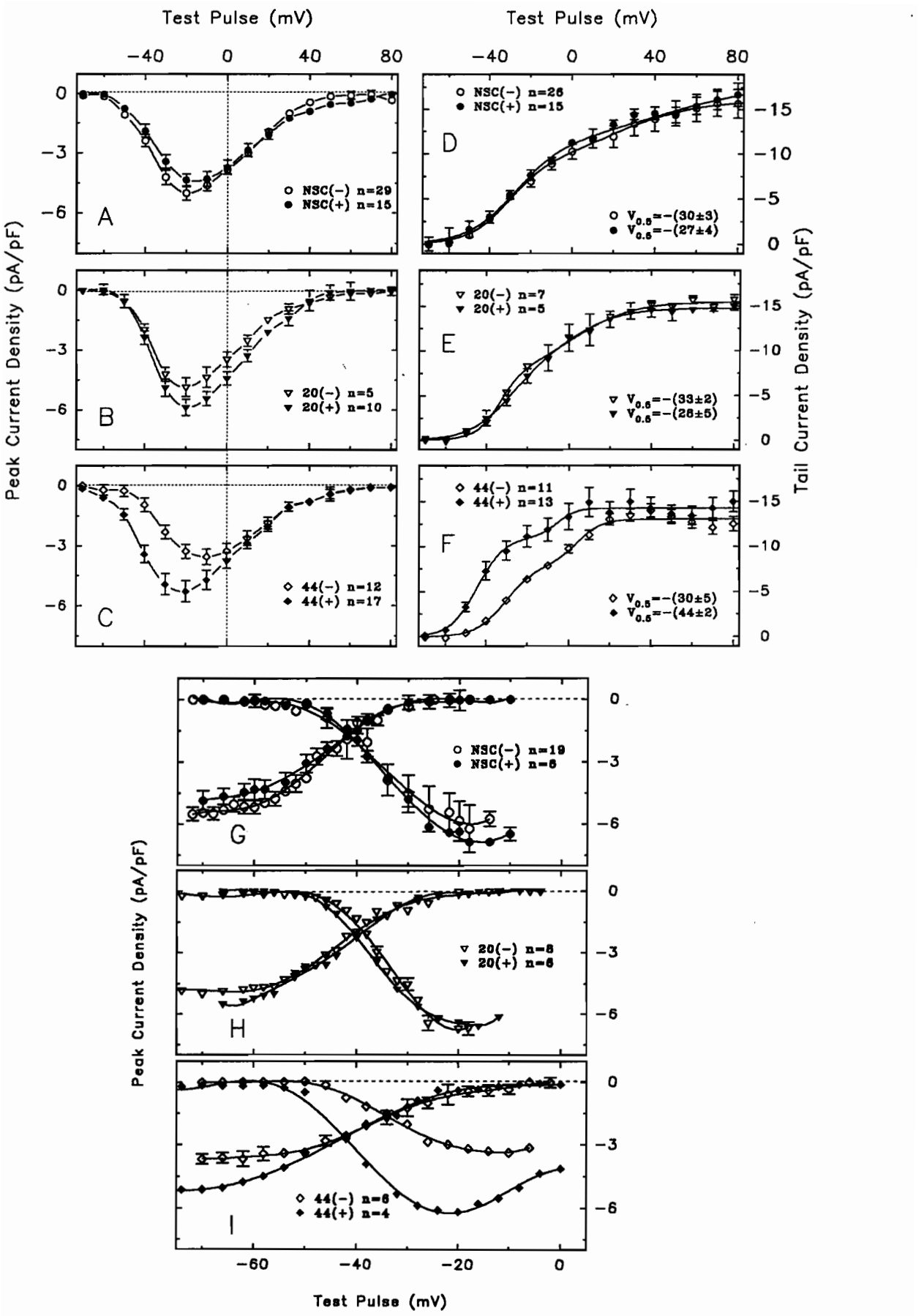

Test Pulse (mV)
−40
0
40
80
Peak Current Density (pA/pF)
Tail Current Density (pA/pF)
A
NSC(−) n=29
NSC(+) n=15
B
20(−) n=5
20(+) n=10
C
44(−) n=12
44(+) n=17
D
NSC(−) n=26
NSC(+) n=15
V0.5=−(30±3)
V0.5=−(27±4)
E
20(−) n=7
20(+) n=5
V0.5=−(33±2)
V0.5=−(26±5)
F
44(−) n=11
44(+) n=13
V0.5=−(30±5)
V0.5=−(44±2)
G
NSC(−) n=19
NSC(+) n=6
H
20(−) n=6
20(+) n=6
I
44(−) n=6
44(+) n=4
−60
−20
Test Pulse (mV)

Figure 19. Current-voltage relationship of peak Ca^{2+} currents in a stably transfected cell line (MN).

(A-F) Bar graph of mean peak i/v current density before (A-C) and after differentiation (D-F). Mock cells (A, D) and cells transfected with normal (B, E) or polyGln-expanded hAR (C, F). Note the marked enhancement of the current densities in differentiated cells expressing polyGln-expanded AR before (empty symbols) and after (filled symbols) exposure to androgen (F) and the marked shift toward negative voltages.

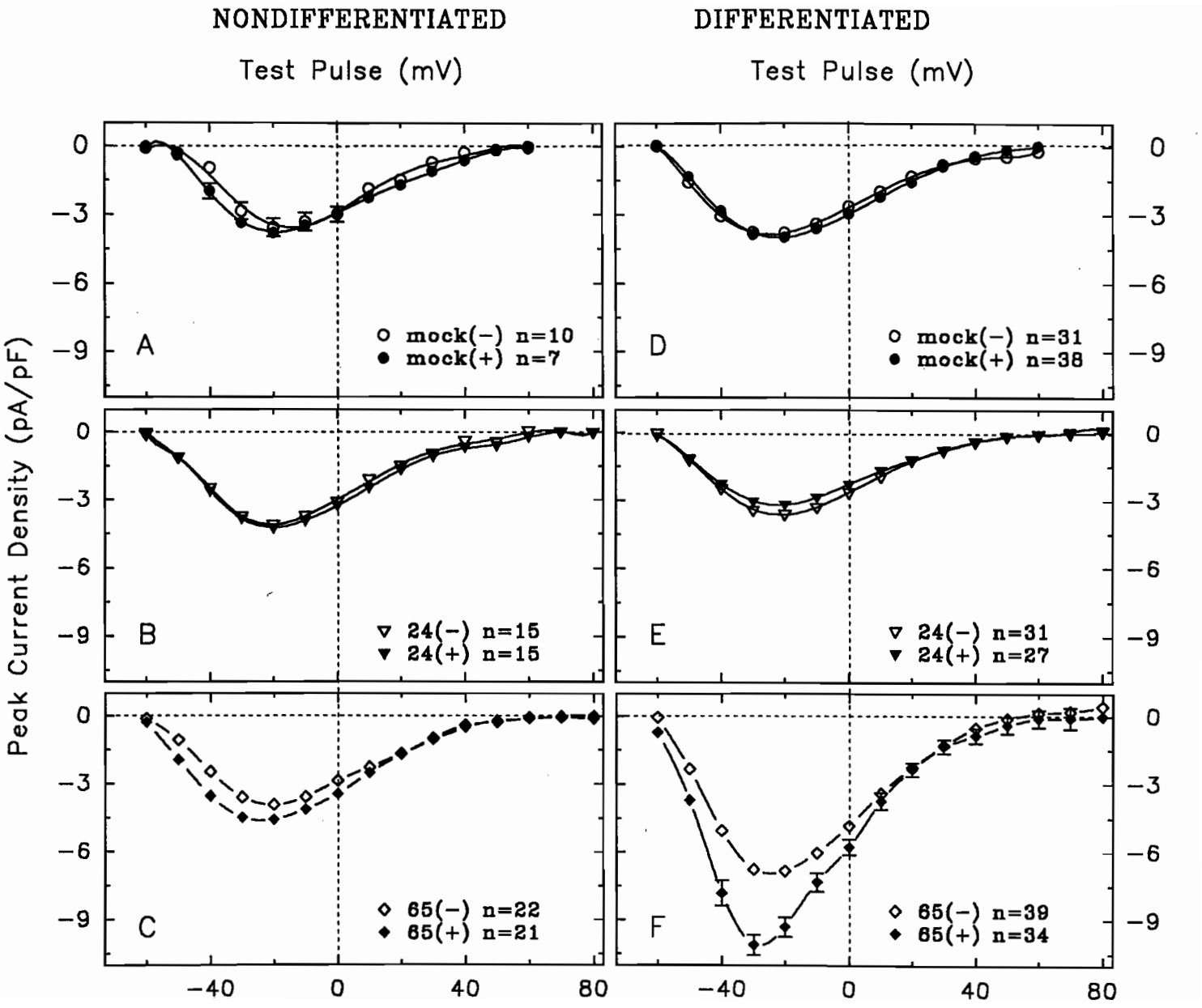
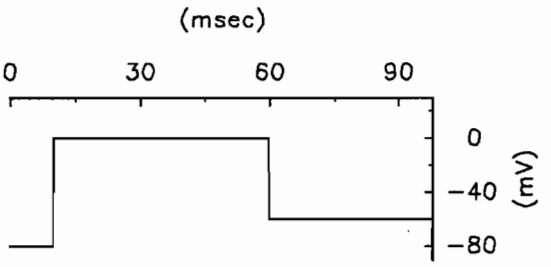
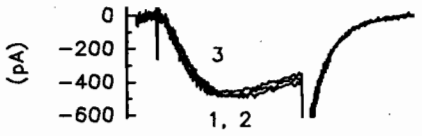

NONDIFFERENTIATED
DIFFERENTIATED
Test Pulse (mV)
Test Pulse (mV)
Peak Current Density (pA/pF)
A
mock(−) n=10
mock(+) n=7
B
24(−) n=15
24(+) n=15
C
65(−) n=22
65(+) n=21
D
mock(−) n=31
mock(+) n=38
E
24(−) n=31
24(+) n=27
F
65(−) n=39
65(+) n=34
0
−3
−6
−9
−40
0
40
80

Figure 20. Responsiveness of Ca^{2+} currents to different Ca^{2+} channel blockers.

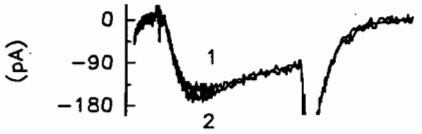
The effect of Ca^{2+} channel blockers on Ca^{2+} currents in NSC34 cells (left panel) was compared to in dorsal root ganglia (DRG), (left panel), which were chosen as they are known to express all major neuronal type Ca^{2+} channel currents (Sculptoreanu *et al.*, 1995). The Ca^{2+} currents in the NSC34 cells were unresponsive to L-type Ca^{2+} channel dihydropyridine agonist (SDZ 202-791) and antagonist (PN 200-110), N-type Ca^{2+} channel antagonist ω- conotoxin GVIA (CTX) or P-type channel blocker Agatoxin GVIA (ATX). However, Cd^{2+} (a T-type Ca^{2+} channel blocker) effectively blocked the Ca^{2+} currents in NSC34 cells at 200 mM. Similar results were obtained with Ni^{2+}. The same effect was observed in the other cell line, MN. This suggests that the only Ca^{2+} channel current expressed in both cell lines used in these experiments was T-type.



CELL LINE



1. control
2. SDZ (5 μM)
3. CTX (10 μM)



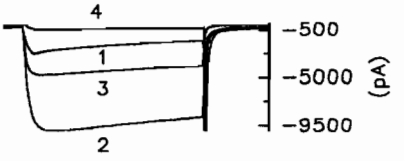
1. control
2. PN (25 μM)

1. control 3. Cd^{2+} (0.5mM)
2. PN200 (100 μM) +AGA−Tx (500 nM)

NEURONS

1. control
2. SDZ (5 μM)

1. control
2. SDZ (5 μM)
3. CTX (10 μM)



1. control 3. CTX (10 μM)
2. SDZ (5 μM) 4. AGA−Tx (500nM)

ANALYSIS OF LBD MUTATIONS IN THE *hAR* OF OLIGOSPERMIC INFERTILE PATIENTS

Germline mutations detected in the AR of oligospermic patients were not present in normal fertile controls

Two mutations were identified in three severely oligospermic, infertile patients by the laboratory of E. L. Yong. One of these mutations occurs in exon 5 and causes Asn727Lys substitution (AAC to AAG) creating a new Mae III restriction site and was previously reported by Yong *et al.*, (1994a). In summary, the patient, aged 51 years, was infertile with no signs of AI. Sexual hair distribution and external genitalia were normal apart from reduced testicular volume of 12 ml. He had normal serum gonadotrophins and testosterone. However, semen analysis indicated severe oligospermia with sperm counts ranging from 2.3 to 3.7 million/ml. The other mutation was detected by the same laboratory using SSCP analysis of exons 2-8 of *AR* from 153 subjects with defective spermatogenesis and male infertility (Yong *et al.*, 1996). Two unrelated patients, CML and KLH, showed differential migration of exon 8 PCR fragments compared to fertile controls. The suspected fragments were sequenced and both patients had the same mutation in exon 8 involving amino acid 886, an A to G transition resulting in substitution of valine for methionine. This mutation resulted in the creation of a new *Bbr* P1 restriction site. Both CML and KLH had severe oligospermia with repeated sperm counts of less than 5 million per ml. No other coexisting mutations were detected in exon 1 to 8 when genomic DNA of the two subjects were re-examined by SSCP. The two polymorphic triplet repeat tracts (coding for polyGln and polyGly) in exon 1 were directly sequenced (in our laboratory) using PCR-amplified product from KLH and CML genomic DNA. The 1.1A° and 1.2B° primers were used to amplify and sequence (CAG)n tract, while 1.4A° and 1.4B° were used to amplify and sequence $(GGN)_n$ tract. CML had 23 codons in each tract, while KLH had 21 Gln and 24 Gly codons, confirming that the two subjects were not genetically related. Both subjects inherited the mutation from their mothers who were proven to be heterozygous carriers.

Met886Val in the LBD had no effect on androgen-binding characteristics in genital skin fibroblasts

Genital skin fibroblasts were obtained from both subjects with the exon 8

mutation (CML and KLH); but GSF from the exon-5 mutation were not accessible.

Androgen-binding properties of the mutant receptors were examined in fibroblast monolayers cultured from scrotal skin biopsies. The fibroblasts were exposed to radiolabelled androgens in increasing concentrations and specific androgen binding was compared to that obtained from normal controls. Normal Scatchard analyses were obtained using DHT at 37°C (Fig. 21); with a K_d of 0.31 and 0.52 nM, and a B_{max} of 36 and 37 fmol/mg protein for CML and KLH, respectively (Normal values for DHT: K_d, 0.3-0.6 nM; B_{max}, 26-43 fmol/mg protein). Scatchard analyses were repeated on the GSF of KLH using the synthetic, unmetabolizable androgens MB and MT. The results show normal values of K_d and B_{max} for both androgens (Table III). To determine whether the mutant receptor was thermolabile, binding studies were performed at higher temperatures for a short or prolonged time. When exposed to DHT at 42°C, the K_d and B_{max} values of the mutant AR in CML fibroblasts, 0.27 nM and 35 fmol/mg protein respectively, were no different from the corresponding values at 37°C (Fig. 22). Prolonged labelling for 15h at 37°C with 2 nM MT or MB, followed by a shift to 41°C or 42°C for up to 6h in the presence of 100 μM cycloheximide failed to demonstrate any difference between mutant and normal cells (Fig. 23).

Chase experiments were performed to examine the dissociation kinetics of mutant androgen-receptor complexes compared to normal controls. These experiments were repeated multiply. When chased at 37°C and 42°C, the dissociation rates for DHT and MB were similar for both normal and mutant fibroblasts (Fig. 22). Thus, the rate constants of dissociation, k, for DHT at 37°C were 5.6 and 5.8 ($10^{-3}min^{-1}$) for CML and KLH compared to 5.1 and 5.0 ($10^{-3}min^{-1}$) for two normal controls. Similarly, there were no differences when "off-rates" were measured with MB at 37°C [k; 4.1, 3.7 versus 4.1, 4.4 (10^{-3} min^{-1})]. After an 18-h incubation with testosterone, the remaining androgen-receptor complexes dissociated linearly with a k value of 5 ($10^{-3}min^{-1}$) at 37°C. This value is typical of DHT-receptor complexes, and indicates normal conversion of T to DHT. If the same experiment was repeated in the presence of 5α-reductase inhibitor, the k value is ~ 20 (10^{-3} min^{-1}), typical of T-R complexes. Receptor upregulation was normal as shown by the values of 48 and 104 fmol/mg protein when incubated with MB for 2 and 20 hrs, respectively. In aggregate, these

experiments indicate that the Met886Val mutation of LBD does not change any androgen-binding properties of the receptors.

Impaired transactivation capacity of the mutant receptors

To investigate the transactivational activity of the mutant receptors, the mutant DNA sequence for both exon 5 (EX5) and exon 8 (EX8) was recreated in a mammalian expression plasmid containing the AR cDNA driven by a SV40 promoter (pSVhAR$_o$). Wild-type or mutant plasmids were cotransfected into COS-1 cells together with hGH or luciferase reporter vectors (pMMTV-hGH or pMMTV-LUC). These reporters contain hormone-inducible AREs in the LTR portion of MMTV promoter. The first series of experiments (Fig. 24) indicated that the mutants had only ~ 30 to 55% of the transactivation capacity of the wild-type receptor in COS-1 cells when exposed to various concentrations of DHT, MB and MT. Growth hormone activities, corrected for protein content and transfection efficiency, were consistently lower for the mutants compared to the wild-type in at least three independent experiments. The ARs encoded by the normal and mutant plasmids each demonstrated dose-dependent increases in transactivation capacity with DHT, but at all doses from 12.5 to 300 nM, the transcriptional capacity of both Asn727Lys and Met886Val ARs was only about half that of the wild-type. The transactivation capacity of the mutant receptors was saturated at about 100 nM with DHT, 1 nM with MB (using pMMTV-hGH reporter) and 1 nM with MT (using pMMTV-LUC reporter). The wild-type receptor gave about 12.5-fold, 18-fold and 11.2-fold increases in reporter activity when saturated with DHT, MB and MT, respectively. In contrast, the two mutants were able to induce only 6-fold with DHT and 10-fold with MB, while Asn727Lys induced 8.4-fold with MT compared to 6.0-fold by Met886Val mutant. The difference observed between the mutants (Fig. 24c) might be due to the sensitivity of the LUC reporter and/or the effect of MT. The hypotransactivation by the mutant receptors was not associated with differential ligand-binding activity at different dose concentrations. Thus the mutant ARs had only about half-normal transactivation capability for all three androgens examined. The increasing concentration of MB/MT complexes with increasing hormone (Fig. 24 a, c) probably represent stabilization, and saturation at 0.25-1.0 nM probably represent squelching of coregulatory molecules.

Figure 21. Scatchard analysis of ARs in genital skin fibroblasts.

Fibroblasts monolayer cultures of two normal (1 and 2) and the two patients (CML, KLH) were exposed to increasing doses (0-3.0 nM) of [^{3}H]DHT alone or with 200-fold excess of radioinert DHT at the corresponding concentration. The specific binding (bound fmol/mg protein) was plotted against bound/free hormone at each DHT concentration. Each data point was the mean of four replicates.

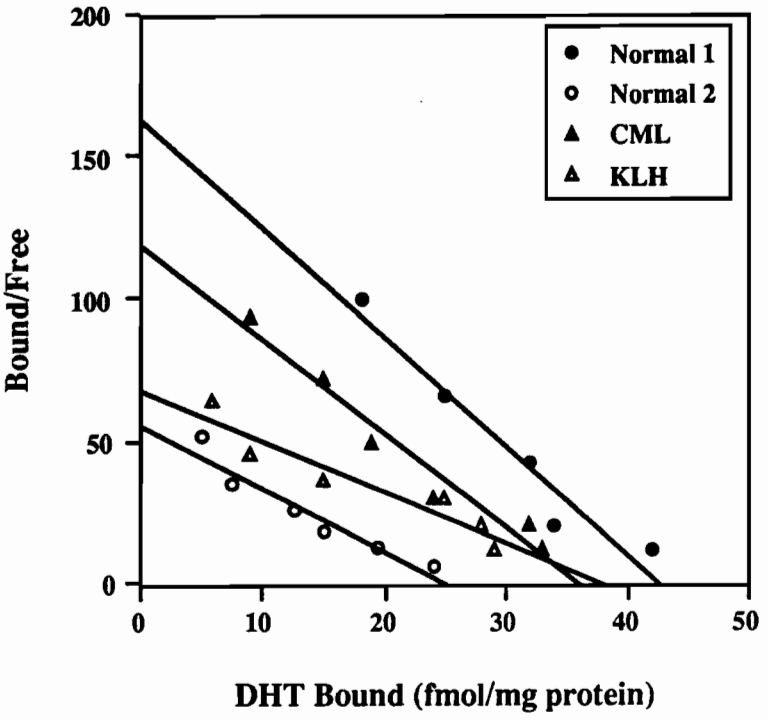

200
150
100
50
0
Bound/Free
0
10
20
30
40
50
DHT Bound (fmol/mg protein)
Normal 1
Normal 2
CML
KLH

Table III. K_d values of Scatchard analysis on genital skin fibroblasts

Table III. Mutations and genital skin fibroblasts androgen binding

Hormone	Subject	B_{max} (fmol/mg protein)	K_d (nM)
	CML	36	0.31
DHT	KLH	37	0.52
	Normal	26-43	
MB	KLH	52	0.10
MT	KLH	52	0.12
	Normal	15-50	0.1-0.3

Figure 22. Dissociation kinetics of ARs in genital skin fibroblasts.

Normal (closed circles and solid lines) and mutant (CML, open circles, dotted lines; KLH, closed triangles, dashed lines) fibroblast monolayers were exposed to 3 nM [^{3}H]DHT (upper panels) or 2 nM [^{3}H]MB (lower panels) at 37°C (left panels) or 42°C (right panels) for 2h. The radiolabelled medium was discarded, replaced with 200-fold excess unlabelled androgen and replicates samples were removed at the indicated times to be assayed for remaining [^{3}H]androgen bound to the ARs. Each data point is the mean of four replicates expressed as a percentage of maximum binding at zero time.

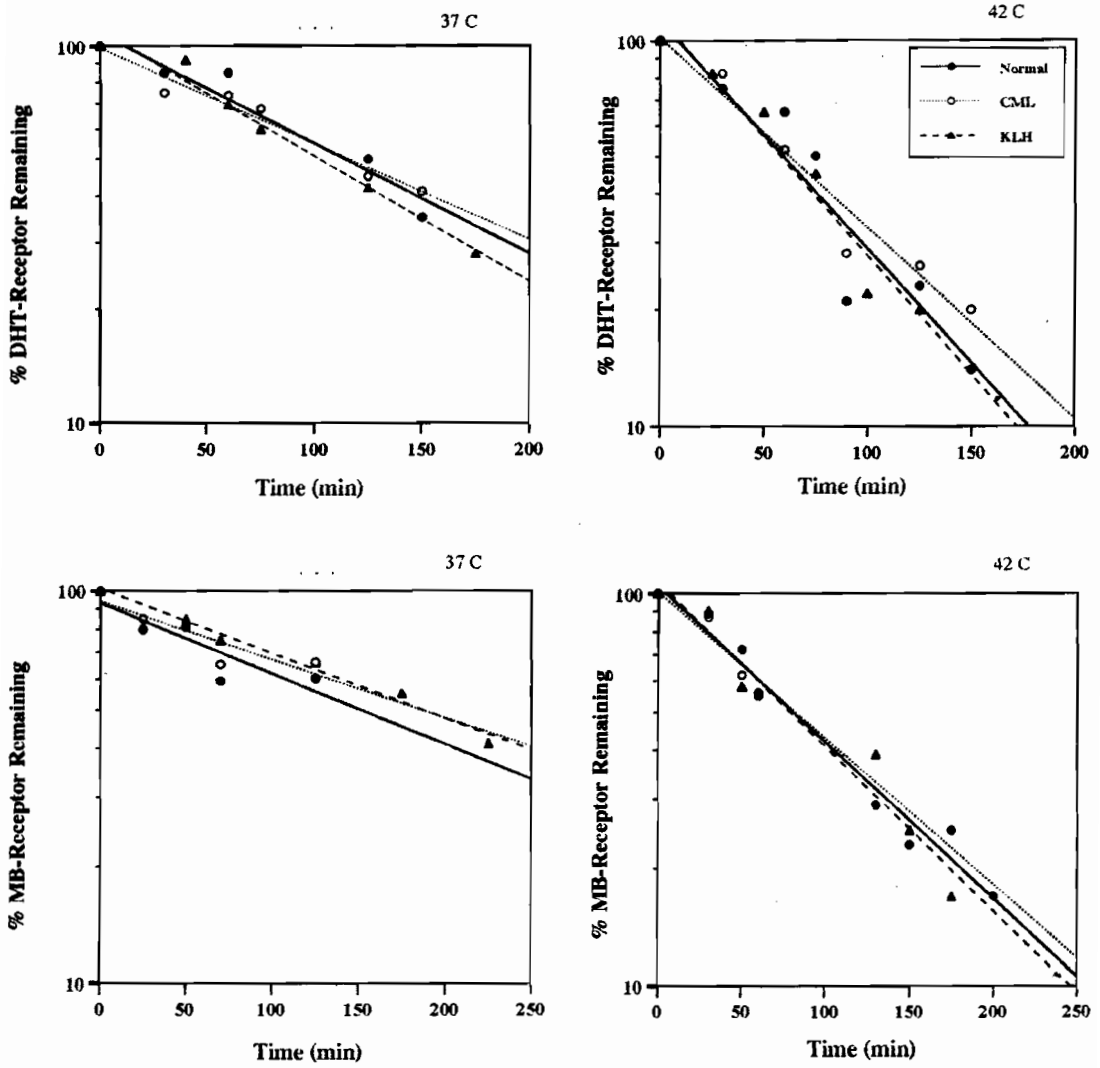

37 C
42 C
% DHT-Receptor Remaining
% MB-Receptor Remaining
Time (min)
Normal
CML
KLH
100
10
0
50
100
150
200
250

Figure 23. Thermolability of receptors in genital skin fibroblasts.

MB- and MT- complexes were formed in GSF incubated with 2 nM [^{3}H]MB or [^{3}H]MT at 37°C (0 time). The cells were then incubated at 42°C or 41°C with 100μM cycloheximide and 2 nM of the same [^{3}H]-androgen. A-R complexes were measured at intervals up to 6h. 2080, normal; CML and KLH, two unrelated mutants with Met886Val.

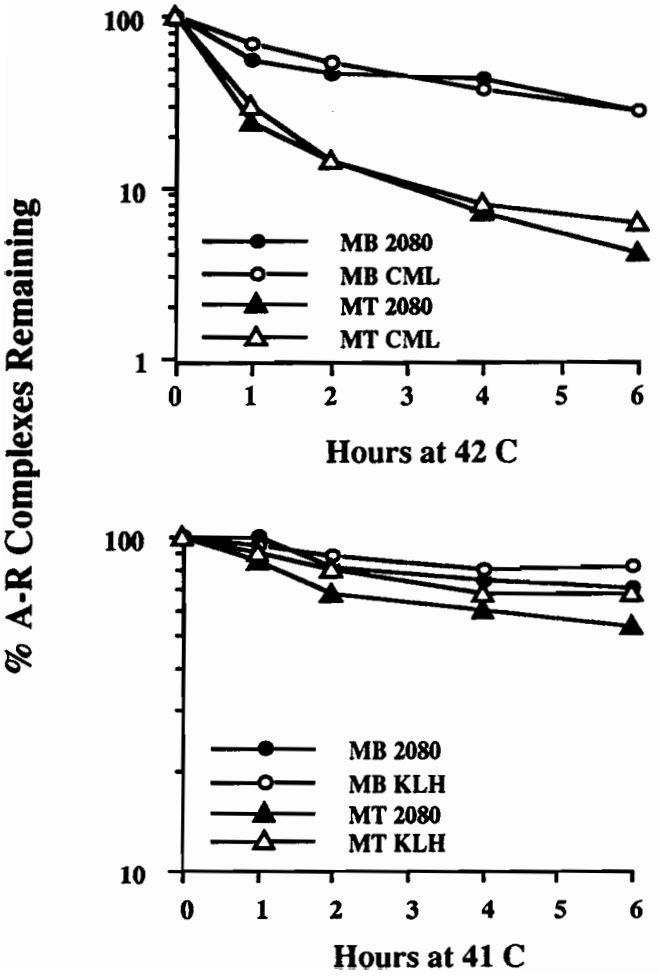
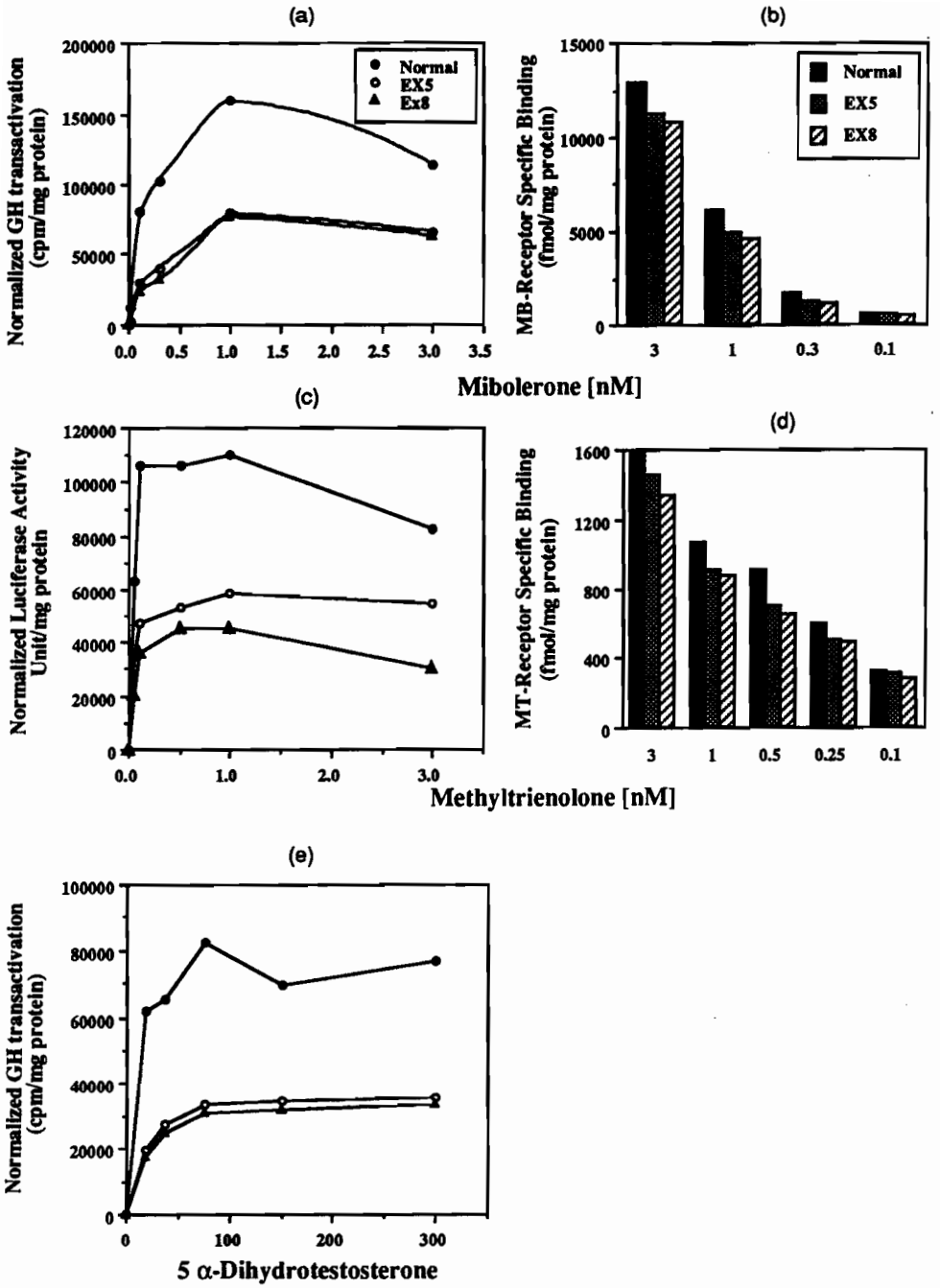

% A-R Complexes Remaining
100
10
1
0
1
2
3
4
5
6
MB 2080
MB CML
MT 2080
MT CML
Hours at 42 C
100
10
0
1
2
3
4
5
6
MB 2080
MB KLH
MT 2080
MT KLH
Hours at 41 C

Figure 24. Transactivation and binding activities of Met886Val and Asn727Lys with increasing doses of MB, MT (0-3 nM), or DHT (0-300 nM) using two reporter (MMTV-GH, MMTV-LUC).

Wild type (Normal, closed circles, filled bars), Asn727Lys (EX5, open circles, dotted bars) and Met886Val (EX8, filled triangles, diagonal bars) were transiently expressed in COS-1 cells and exposed to [^{3}H]MB and [^{3}H]MT, or radioinert 5α-DHT. In the upper panel (a and b), COS-1 cells were exposed to 0-3 nM [^{3}H]MB post-transfection and incubated at 37°C for 72h. (a) is the transactivational activity of the various ARs at different androgen-receptor concentartions (b). The same experiment was repeated (c and d) using the reporter (MMTV-LUC) and [^{3}H]MT. Transactivation was done using 0-300 nM radioinert 5α-DHT (e). β–gal activity and protein concentration were used to normalise for transfection efficiency and cell numbers, respectively.


(a)
Normalized GH transactivation (cpm/mg protein)
200000
150000
100000
50000
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Normal
EX5
Ex8
(b)
MB-Receptor Specific Binding (fmol/mg protein)
15000
10000
5000
0
3
1
0.3
0.1
Normal
EX5
EX8
Mibolerone [nM]
(c)
Normalized Luciferase Activity Unit/mg protein
120000
100000
80000
60000
40000
20000
0
0.0
1.0
2.0
3.0
(d)
MT-Receptor Specific Binding (fmol/mg protein)
1600
1200
800
400
0
3
1
0.5
0.25
0.1
Methyltrienolone [nM]
(e)
Normalized GH transactivation (cpm/mg protein)
100000
80000
60000
40000
20000
0
0
100
200
300
5 α-Dihydrotestosterone

Asn727Lys, but not Met886Val, is thermo- and hypertransactive with mesterolone

To explain the therapeutic impact of mesterolone on the infertile subject with the Asn727Lys mutation, I used the heterologous transient expression system of COS-1 cells to test whether this hormone was able to enhance transactivation by the patient's mutant AR. Dosage-dependent transactivation of wild-type AR and both mutants was observed when mesterolone ranging from 12.5 to 300 nM was used to induce the pMMTV-hGH reporter (Fig. 25). At 34°C, the Met886Val mutant induced the reporter to about 44% of the wild-type AR activity compared to about 67% at 37°C. In contrast, the Asn727Lys mutant could not only transactivate the reporter to about 70% of the wild-type activity at 34°C, but it peaked to about 150% of the wild-type at 37°C. This result provides an experimental basis for the therapeutic effect of mesterolone on the infertile subject with Asn727Lys. It also suggests that mesterolone is somehow able to overcome the transactivational incompetence of this mutant AR when it is liganded with other androgens.

Androgen-binding characteristics of mutant receptors in COS cells are normal despite the transactivation defect

The binding properties of mutant receptors expressed in COS-1 cells were examined. Scatchard analyses showed that the Met886Val and Asn727Lys mutations did not affect the K_d values for MB, DHT and MT. It is 1.1 nM for MB for both receptors, when compared to the wild-type, 0.90 nM (Fig. 26). The K_d for DHT was 1.23 nM for Asn727Lys, 1.72 nM for Met886Val, and 1.75 nM for wild-type. Similarly, the MT K_d was 0.71 nM for Asn727Lys , 0.60 nM for Met886Val , and 0.83 nM for the wild-type.

In the chase experiments there were no differences in the dissociation rates of both mutants and the wild-type receptors when bound to DHT, MB, or MT. Thus, the k values for Asn727Lys, Met886Val and wild-type receptors were 11.5, 10.1 and 10.1 (10^{-3} min^{-1}) respectively for DHT at 42°C; 5.5, 5.5, and 5.0 (10^{-3} min^{-1}) for MB at 42°C; and 7.8 (10^{-3} min^{-1}) for the three receptors with MT at 37°C (Fig. 27). Thus the reduction in transactivation capacity observed above was not due to abnormal ligand-binding properties of the recreated mutant AR expressed in COS-1 cells.

Impaired binding of mutant AR-ligand complexes to androgen response elements

To assess whether the hypotransactivational activity of the two mutations is due to impaired ARE binding, the post-androgen binding effects of the two mutations were investigated by the ability of the mutant AR-MB complexes to bind two different androgen response elements (Fig. 28). The MMTV-ARE used centers around position -177 of the MMTV-LTR (Ham *et al.*, 1988), and can function as a response element for AR, GR and PR. The synthetic ARE was based on the consensus DNA-binding site for the AR that emerged from DNA-binding site selection assays using a pool of random oligonucleotide sequences (Roche *et al.*, 1992). In two independent experiments using ARE-MMTV, the ligand -activated mutant receptors displays a mean 58% (Asn727Lys) and 63.5% (Met886Val) of the ARE binding observed with the wild-type AR . In two more independent experiments, similar results were obtained when the same assay was repeated with the synthetic ARE: Asn727Lys AR had a mean 47.5%, whereas Met886Val had 52% of normal ARE binding. In comparison, an AR mutant with severely impaired DNA binding (ΔPhe582 or strain 12474, Beitel *et al.*, 1994, with an in-frame deletion of phenylalanine in codon 582 in the DBD) exhibited about 19.0% of normal binding. Lysates from mock-transfected cells showed less than 3% ARE binding. Overall, in four independent experiments using two different AREs, the mutant receptor had only 55.25% (SE, ± 5.17) of the wild-type DNA-binding capacity. This defect in DNA-binding was proportionate to the degree of impaired transactivation observed above.

Partial tryptic digestion of Asn727Lys mutant revealed differential conformation with DHT but not MES

To test the possibility of conformational alteration caused by the Asn727Lys substitution mutation , [^{35}S]Met-labelled mutant and wild-type ARs were subjected to partial tryptic digestion in the presence or absence of DHT and MES (Fig. 29). The mutant and wild-type receptors exhibited an identical pattern of digestion fragments in the absence of ligand. In presence of DHT, trypsinolysis generated ~ 31-, 30-, 21-, 18-, and 14-kDa fragments in both receptors. A fragment of 25 kDa was detected in the wild type only. The 21-, 18- and 14-kDa fragments of the mutant were more trypsin sensitive than their wild type counterpart. Surprisingly, the addition of MES did not cause any apparent differential conformation between the mutant and wild-type; rather. Although the

mutation in exon 5 (Asn727Lys) creates a new trypsin site, the trypsin-digestion pattern of the mutant did not change remarkably as would have been expected if the created site was fully exposed to trypsin.

Figure 25. Transactivation activity of Asn727Lys and Met886Val mutant ARs using mesterolone.

Wild type (Normal, filled circles), Asn727Lys (EX5, open circles) and Met886Val (EX8, filled triangles) receptors were transiently cotransfected with the MMTV-GH reporter in COS-1 cells. The cells were exposed to high doses of radioinert mesterolone (0 to 300 nM) and incubated either at 34°C (upper graph) or 37°C (lower graph) for 72h. After the period of incubation, the transactivation function of the receptor-ligand complexes was assessed by assaying for growth hormone secreted into the medium. β–gal activity and protein content were used to correct for differences in transfection efficiency and cell numbers, respectively.

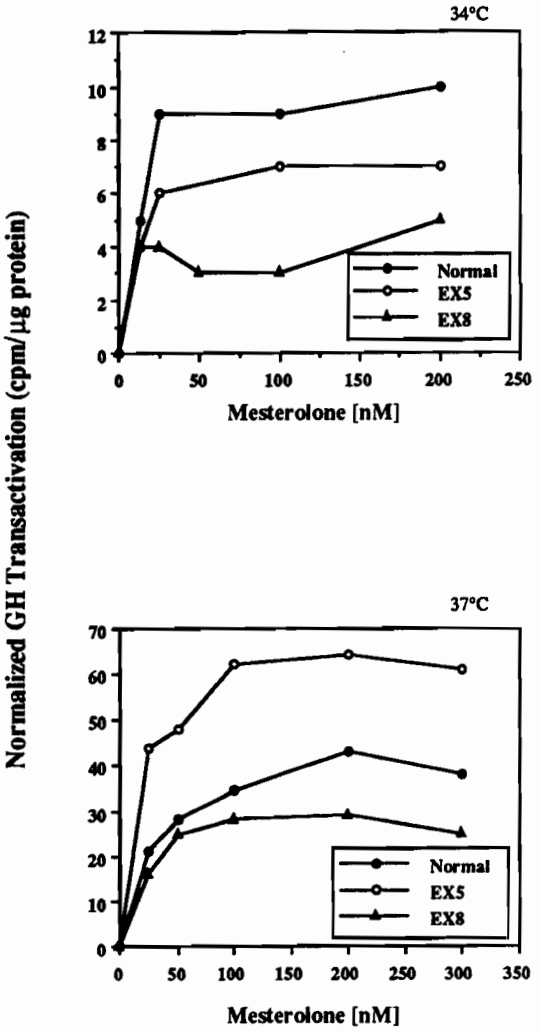

34°C
12
10
8
6
4
2
0
0
50
100
150
200
250
Mesterolone [nM]
Normal
EX5
EX8
37°C
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
Mesterolone [nM]
Normal
EX5
EX8
Normalized GH Transactivation (cpm/μg protein)

Figure 26. Scatchard analyses of ARs in COS-1 cells.

Wild type (Normal, filled circles), Asn727Lys (EX5, open circles) and Met886Val (EX8, filled triangles) mutant ARs were transiently expressed in COS-1 cells and incubated in MEM medium containing 100 μM cycloheximide and increasing amounts (0.09 to 3 nM) of [^{3}H]DHT (a), [^{3}H]MB (b), or [^{3}H]MT (c) in triplicate alone or with 200-fold excess of corresponding radioinert androgen in duplicate to determine non-specific androgen binding. The cells were incubated for 2h at 37°C, washed and lysed to assess the amount of radiolebelled androgen specifically bound.

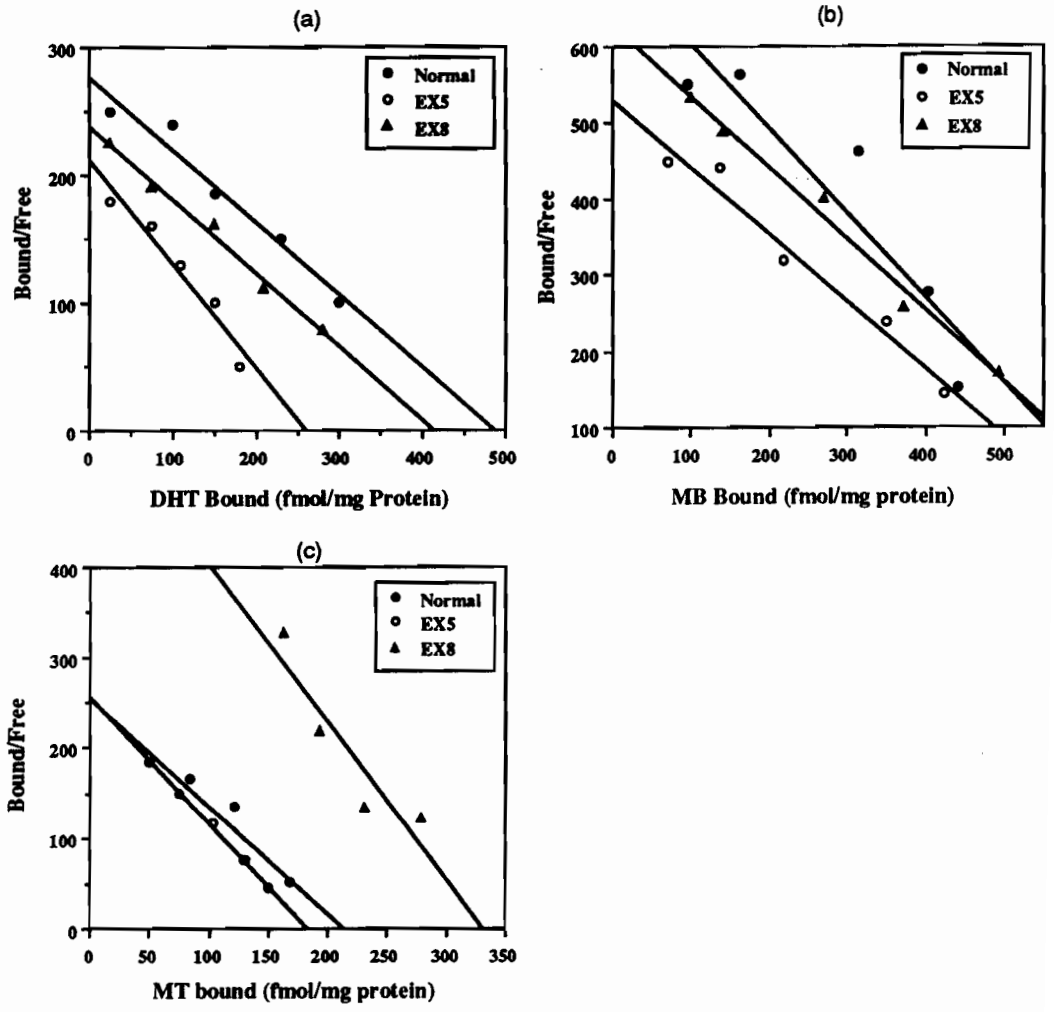
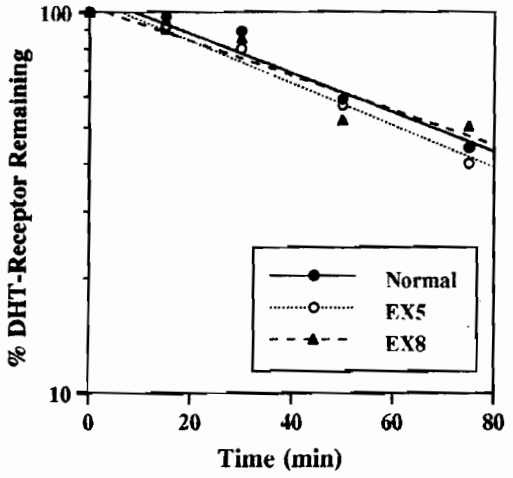
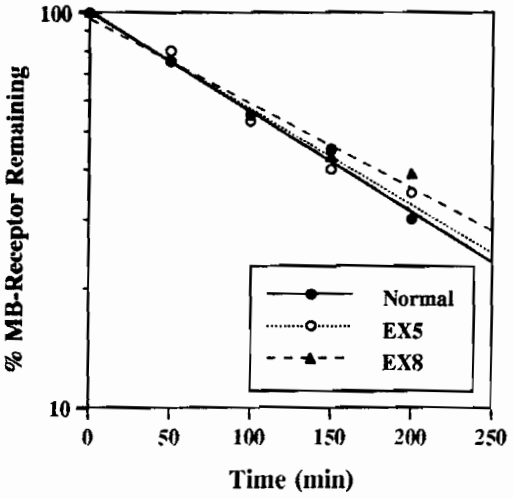

(a)
Normal
EX5
EX8
Bound/Free
DHT Bound (fmol/mg Protein)
(b)
Normal
EX5
EX8
Bound/Free
MB Bound (fmol/mg protein)
(c)
Normal
EX5
EX8
Bound/Free
MT bound (fmol/mg protein)

Figure 27. Dissociation kinetics of Asn727Lys and Met886Val mutant ARs in COS-1 cells.

Wild type (Normal, filled circles and solid lines), Asn727Lys (EX5, open circles and dotted lines) and Met886Val (filled triangles and dashed lines) androgen receptors were transiently expressed in COS-1 cells and incubated with 3 nM of [^{3}H]DHT (a), [^{3}H]MB (b), or [^{3}H]MT (c) for 2h at 37°C. Cells were then exposed to 600 nM of the respective unlabelled androgen for increasing time intervals at 42°C before being assayed for specific ligand binding. Each point (the mean of triplicates) was expressed as percentage of maximum binding at zero minutes.


100
10
% DHT-Receptor Remaining
0
20
40
60
80
Time (min)
Normal
EX5
EX8


100
10
% MB-Receptor Remaining
0
50
100
150
200
250
Time (min)
Normal
EX5
EX8

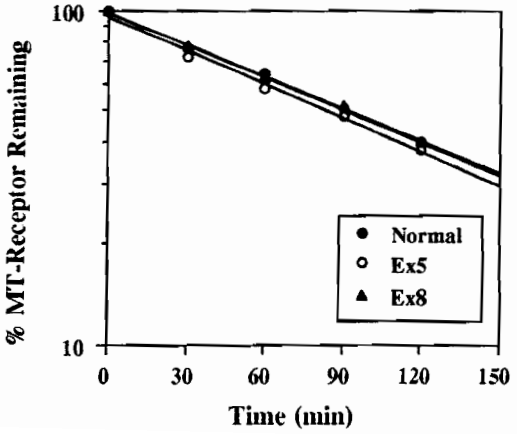
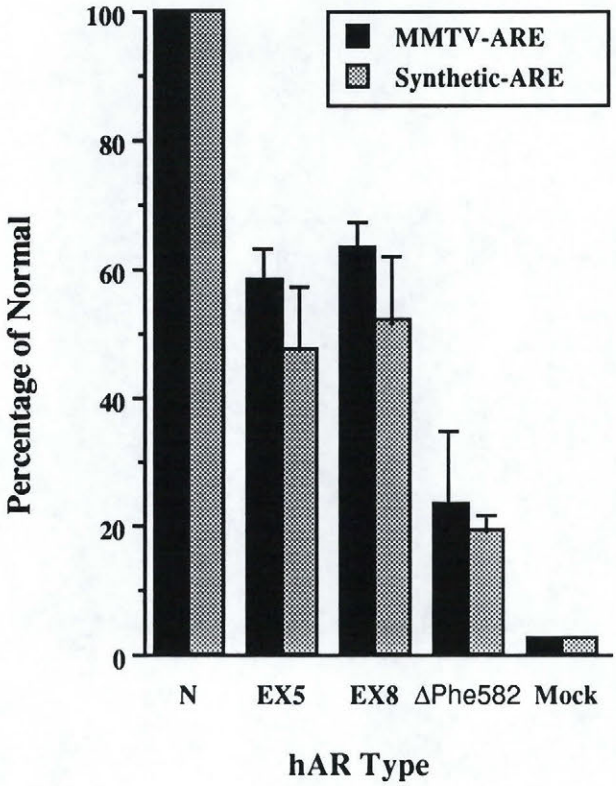

100
10
% MT-Receptor Remaining
0
30
60
90
120
150
Time (min)
Normal
Ex5
Ex8

Figure 28. Binding of mutant receptors to androgen response elements.

(A) Wild type (N), Asn727Lys (EX5), Met886Val (EX8) ARs and a negative control AR mutant known for its severe impairment of DNA binding due to Phe582 deletion (ΔPhe582) were expressed in COS-1 cells, exposed to 3 nM [^{3}H]MB and 200 fM of MB-R complexes were incubated with 50 pmol of either biotin-tagged MMTV- (filled bars) or synthetic-ARE (dotted bars). Streptavidin-biotin bound AREs were quantitated by scintillation counting. Assays using mock-transfected cells showed minimal background activity. Each data point was the mean of two experiments and bars indicate their range. (B) Western blot showing the corresponding AR transiently expressed in COS-1 cells; equal amounts of cell extracts were loaded (20 μg total protein) in each lane; (strain 12474) is AR cDNA for ΔPhe 582.

(A)

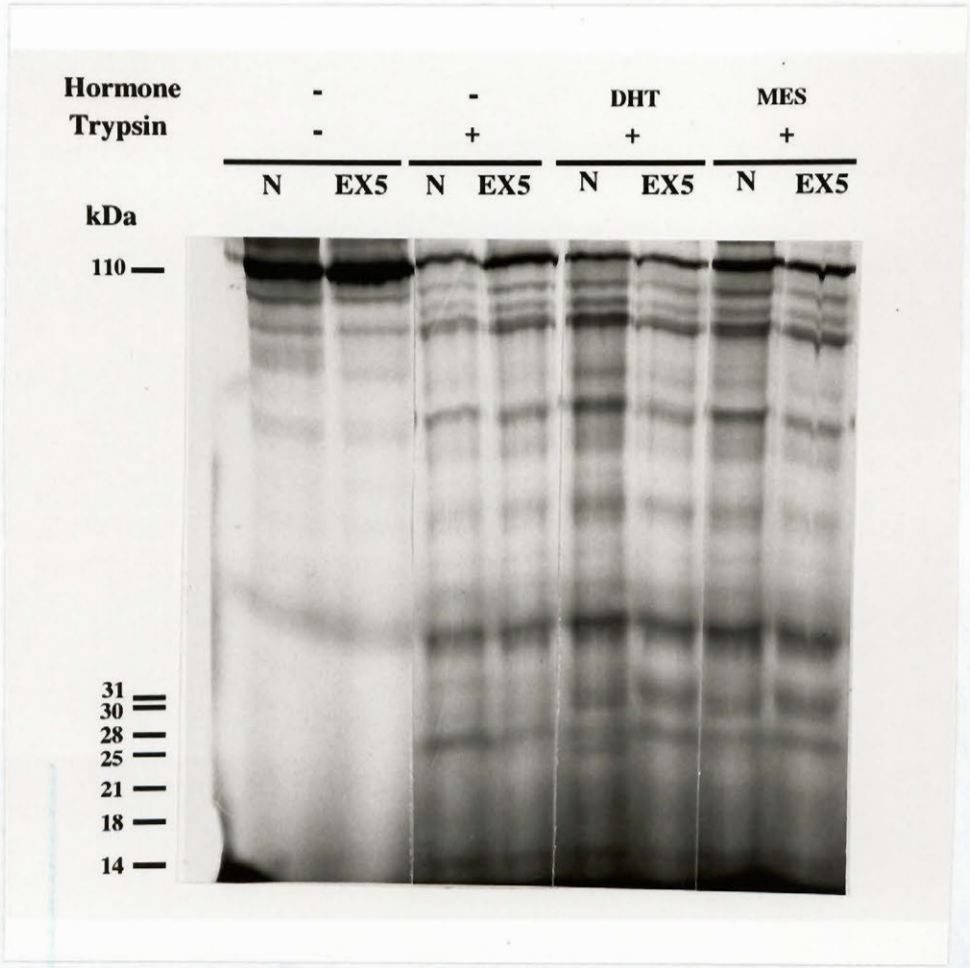

MMTV-ARE
Synthetic-ARE
Percentage of Normal
100
80
60
40
20
0
N
EX5
EX8
ΔPhe582
Mock
hAR Type

(B)


N
EX5
EX8
12474

Figure 29. Partial tryptic digests of [^{35}S]Met-labelled *in vitro*-translated Asn727Lys AR mutant.

A 10 min trypsinolysis of *in vitro*-translated [^{35}S]Met-labelled wild type (N) or Asn727Lys (EX5) hARs was done at room temperature (RT), fractionated by SDS-PAGE, and radioautographed. Reactions were done with or without DHT or MES.


Hormone
Trypsin
-
-
-
DHT
MES
-
+
+
+
N
EX5
N
EX5
N
EX5
N
EX5
kDa
110
31
30
28
25
21
18
14

PART III. DISCUSSION

PATHOGENETIC INVESTIGATION OF POLYGln-EXPANDED hAR THAT CAUSES SBMA

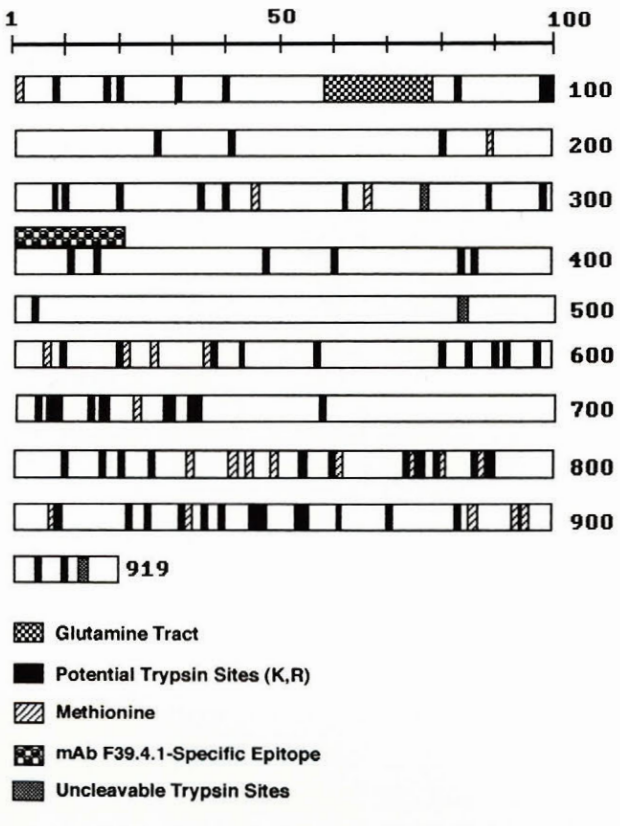
The androgen receptor plays important roles in sex differentiation and secondary sexual development. It is widely expressed in human tissues and is also involved in functions not usually considered to be sexually dimorphic. Mutations in h*AR* usually cause AIS varying in severity from MAI to CAI depending on the site and the type of mutation. Most of these mutations are detected in the LBD and concentrated in two hot spots; one in exon 5 and the other in exon 7, indicating the functional importance of these two regions for AR activity.

Another type of mutation that occurs in h*AR* is expansion of the trinucleotide repeat $(CAG)_n$ coding for glutamine in the N-terminus of exon 1. Expansion of the CAG repeat from a normal polymorphic number of 11-34 to a pathogenic range of 40-62 causes SBMA. Over 140 different loss-of-function mutations of the h*AR*, including complete gene deletions, cause various degrees of androgen resistance (Gottlieb *et al.*, 1997); yet, none of these mutations is selectively neuronopathic. Therefore, amplification of the CAG repeat in the *AR* must render the polyGln-expanded AR protein neuronotoxic by a gain-of-function. In this thesis, I questioned whether the neuronotoxic "gain-of-function" of a polyGln-expanded AR reflects the gain of a "new" structure or conformation. Conformational differences between the normal and mutant proteins could provide clues to the molecular pathogenesis of SBMA.

[^{14}C]Gln or [^{35}S]Met labelling during *in vitro* synthesis, partial-progressive trypsinolysis with and without denaturant, and monoclonal antibodies to two different portions of the N-terminal region of the hAR were used to demonstrate that normal and polyGln-expanded versions of hAR share certain conformational properties and differ in others. For instance, most of the [^{35}S]Met-labelled fragments in early partial digests of both parental forms have identical mobilities. Since the parental forms themselves do have distinguishable mobilities, this suggests that respective portions containing the Gln tract are removed relatively early in their digestion, presumably because they share a very exposed cleavage site C-terminal of the tract (Fig. 30). Likewise, each parental form yields only a single ~ 72 kDa derivative early on in digestion that is immunoreactive with mAb F39.4.1 (Fig. 12). Again, this suggests that both parental forms have a highly

Figure 30. hAR diagram for tryptic sites.

A diagram of the 919-aa hAR showing a normal 20-Gln tract; K, lysine and R, arginine residues indicating cleavable trypsin sites; methionines; the epitope for F39.4.1, aa 301-320; and three uncleavable trypsin sites.


1
50
100
100
200
300
400
500
600
700
800
900
919
Glutamine Tract
Potential Trypsin Sites (K,R)
Methionine
mAb F39.4.1-Specific Epitope
Uncleavable Trypsin Sites

exposed trypsin-cleavage site in a region near the N-terminal border of the relevant epitope, residues 301-320, and possibly in the epitope itself (which has two possible trypsin-cleavage sites; shown in Fig. 30).

Of greater relevance to the possible pathogenesis of SBMA are the different patterns of proteolytic resistance and susceptibility observed during partial-progressive trypsin digestion of the normal and Gln-expanded AR (Fig. 10, 11, 14, 15). For instance, a greater difference in SDS-PAGE mobility, than expected from size alone, of a pair of apparently homologous polypeptides could stem from abnormal conformations conferred by the expanded Gln tracts themselves and/or by amino acid sequences affected by them, whether adjacent or not. The greater-than-expected difference in SDS-PAGE mobility of the full -length normal and Gln-expanded forms of Huntington has been explained this way (Trottier *et al.*, 1995); one of its theoretical bases, intermolecular polar zippering, has been preferred by others (Perutz *et al.*, 1994). It is also possible that a particular pair of fragments is incompletely homologous because conformational differences between the normal and expanded AR dictate the use of different nearby trypsin-cleavage sites. The result of my effort to evaluate the two possibilities at least implicates the second one because only a minimal mobility difference is discerned between two chimeric AR molecules , both predicted to be ~55-56 kDa, despite the presence of 30 extra residues in the Gln tract of one of them.

The most striking mobility differences observed are the disparate sets of [^{14}C]Gln-labelled trypsin- and trypsin-2 M urea-resistant fragments derived from the mutant compared to the normal hAR (Fig. 14, 15). As revealed by their immunoreactivity with mAb1C2 (Fig. 15), four of the residual fragments in the mutant digest have an expanded polyGln tract that contains 24 more Gln residues than the normal AR. These four fragments do not appear to be the counterparts of otherwise homologous fragments, each with a normal-size Gln tract, that are derived from the normal full-length AR. Alternatively, these data suggest that conformational differences are induced by Gln-tract expansion, and are in the vicinity of the tract itself.

Finally, DNA transfection studies performed in this work clearly demonstrate a unique ~75 kDa polyglutamine-expanded polypeptide that is generated only by cells transfected with the Gln-expanded hAR cDNA, but does not react with F39.4.1 epitope. The most likely explanation for such a molecule is that it is derived, at least in part, from hAR cDNA. Presumably, some form of

misprocessing is responsible, whether transcriptionally, translationally or post-translationally. One particularly provocative possibility is that a proteolytic portion of the Gln-expanded AR, lacking the epitope for F39.4.1, forms a covalent compound of ~75 kDa by a transglutaminase-catalysed reaction, as recently described by Kahlem *et al* (1996). Such a foreign molecule might well be motor neuronotoxic, as exemplified, provisionally, by the greater apoptotic death rates in COS-1 cells transfected with polyGln-expanded hAR cDNA. A second possibility, postulated by Cha and Dure (1994), is that Gln-expanded fragments from a variety of polyGln-expanded proteins may be neuronotoxic, particularly if they accumulate, in part because of reduced susceptibility to endopeptidases. The spongiform encephalopathies, other prion-related diseases (Kocisko *et al*, 1996; Brown *et al.*, 1996; Forloni *et al.*, 1993), and Alzheimer disease (Nordstedt *et al.*, 1994) offer precedent for presumably neuronotoxic peptide fragments that are resistant to proteolysis and denaturation. In summary, Cha and Dure (1994), postulated that "abnormal post-translational cleavage products" (polyglutamine-expanded) might be neuronotoxic. Ikeda *et al.* (1996) predicted the existence of such products in order to account for the fact that a portion of the CAG-expanded MJD gene is cytotoxic, while a full-length version is not. Finally, the full-length, polyGln-expanded AR generates a smaller, aberrant product that may be responsible for increased apoptosis in COS cells. This observation thus provides a link between the postulate and the prediction of previous investigators. Interestingly, mice transgenic for exon 1 HD carrying $(CAG)_{115}$ to $(CAG)_{156}$ repeat expansion developed neuronal intracellular inclusions, containing the proteins huntingtin and ubiquitin, prior to developing a neurological phenotypes which are characteristically similar to nuclear abnormalities observed in biopsy material from HD patients (Davies *et al.*, 1997). These inclusions were insoluble and formed amyloid-like protein aggregates in vitro and in transgenic mice (Scherzinger *et al.*, 1997).

Monoclonal Ab1C2 has previously been shown to react with the poly-Gln-expanded proteins of HD, SCA1 and SCA3 (Trottier *et al.*, 1995). Experiments in this thesis have shown that this antibody can react with the Gln-expanded AR (Fig. 16, 17). Indeed, the DNA transfection studies in COS-1 cells clearly demonstrate a Gln-repeat containing fragment of hAR that is unique to the Gln-expanded hAR. The specificity of this fragment to the polyGln-expanded AR was assessed by the observations that this fragment was absent in mock cells using mAb1C2, and under lower stringency of mAb1C2 washing it detected both

the wild type and the polyGln-exapnded parents (Fig. 17), but only extracts from cells transfected with polyGln-expanded AR showed the 75 kD fragment even after longer exposure of the blot to the x-ray film.

Changing the codon degeneracy of the glutamine tract does not alter expression of the AR protein or the nature of its protein stability (Fig. 9, 19). This supports the view that the pathogenesis of SBMA begins with the behavior of glutamine-expanded protein synthesized, not with the DNA or RNA responsible for its transcription and translation.

Although experimentat data are lacking regarding Western analyses done in COS-1 cells with mAb1C2, transfectional analysis of both types of hAR in the motor neuron-like cell line (NSC34) provided evidence of the polyGln-expanded hAR is probably more stable as judged by the higher protein stability up to 6 days in cultured cells, in the presence of cycloheximide, and further confirmed by Western analysis (Fig. 8). Such data, as well as the COS-1 cell toxicity studies, suggest that Gln-expanded fragments resistant to proteolysis may also occur *in vivo* and cause apoptosis.

In transient transfections of COS-1 cells, a truncated AR containing only the N-terminus (residues 1-503, ~ 70 kDa) was localized to the nucleus in the absence of hormone and was able to inhibit wild-type hAR from transactivating the reporter gene in a level proportional to its molar concentration (Simental *et al.*, 1991). This suggests competition for nuclear factors required for transcriptional regulation. Accumulation of proteolytic-resistant polyGln-expanded fragments may be cytotoxic by a similar mechanism.

Besides gaining a new function, the polyGln-expanded AR becomes hypotransactive in transiently transfected COS-1 and NSC34 cells. These data might reflect the MAI associated with SBMA.

The NSC-34 cell line transiently transfected with the normal AR, and another neuroblastoma-motor neuron hybrid cell line (MN cells) stably transfected with the normal AR express a homogeneous and unique type of Ca^{2+} channel, that has a kinetic voltage-dependence of activation and inactivation and a pharmacological profile which most closely resembles that of T-type channels.

A striking observation was the marked shift in the voltage dependency of activation seen in NSC-34 and MN cells after transfection with polyGln-expanded AR and exposure to androgen. I suggest that the resulting doubling in size of window currents and the consequent added Ca^{2+} load to neurons could be one mechanism responsible for neuronal degeneration in Spinobulbar

Muscular Atrophy. An increase in T-type Ca^{2+} channel window current has also been proposed to account for the pathophysiology of genetic cardiomyopathy (Bkaily *et al.*, 1997), and Ca^{2+} overload may be a common apoptotic mechanism (Mariggio *et al.*, 1994; Santos-Argumedo *et al.*, 1993; Gong *et al.*, 1995). An androgen-mediated increase in the cytosolic Ca^{2+} of Sertoli cells has also been reported (Gorczynska and Handelsman, 1995). It is even possible that a degradative byproduct of the polyGn-expanded AR may interact directly with the channel molecule in the presence of androgen.

Historically, the gain-of-function hypothesis for pathogenesis of autosomal dominant $(CAG)_n$-expanded hereditary neuronopathies was based on three facts. First, a loss-of-function could not explain SBMA, since subjects with CAI do not get SBMA, even if they have a complete *AR* deletion (Trifiro *et al.*, 1991). Second, individuals with heterozygous deletions of $4p^-$, including *HD*, do not get HD. Third, individuals with homozygous HD are not more severely affected than heterozygotes.

The possibility that a loss-of-function may contribute a necessary, but insufficient, component to the pathogenesis of these gain-of-function diseases is also based on three facts. In historical order, they are, first, that a polyGln-expanded AR does suffer a loss of function and, at least experimentally, androgen is both neuronotropic and neuronotrophic (Sar and Stumpf, 1977; Breedlove, 1986;Yu, 1989; Goldstein and Sengelaub, 1992). Second, heterozygous knock-out HD mice do have some neuropathology and neurosymptomatology reminiscent of HD (Nasir *et al.*, 1995). Third, and most strikingly, the pathogenic $(CAG)_n$ expansion in *SCA6* does not exceed 27 (Zhuchenko *et al.*, 1996); this is well below n ~40 that applies to all the other CAG-expanded neuronopathies, and point mutations in *SCA6* yield phenotypes that overlap those produced by (CAG)n-expanded *SCA6* (Zhuchenko *et al.*, 1996; and references therein; Matsuyama *et al.*, 1997; Riess *et al.*, 1997). In other words, there is strong circumstantial evidence, at this point, that (CAG)n-expanded *SCA6* is primarily a loss-of-function disease with, perhaps, a gain-of-function component that is, or is not, necessary but insufficient.

The nature of its involvement and the extent to which the AR contributes to Ca^{2+} channel regulation in various cell types is not yet clear. In one study, previously mentioned, (Gorczynska and Handelsman, 1995), it was found that testosterone (T) rapidly increased (20-40 sec) the cytosolic concentration of Ca^{2+} in Sertoli cells from about 80 to 150 nM. This effect of T was inhibited by

the removal of extracellular calcium or pharmacological blockade of voltage-activated or voltage-independent membrane calcium channels. Immobilizing T by conjugation to BSA resulted in a rapid increase in cytosolic calcium similar to the unconjugated T, suggesting a plasma membrane site of action. Yet this nongenomic hormonal regulation of Sertoli cells requires AR, since it is inhibited by antiandrogens, in addition to the slower genomic response. Moreover, testosterone induced Ca^{2+} influx via non-genomic surface receptors in activated T cells (Benten *et al.*, 1997). The Ca^{2+} influx was inhibited by Ni^{2+}, suggesting a T-type Ca^{2+} channel. This form of Ca^{2+} induction is not inhibited by cyproterone, a blocker of the classical AR, indicating that it is not AR-mediated.

It is noteworthy that the cytotoxicity data on COS-1 cells showed that androgen (mibolerone) had no effect in inducing cell death (Table II). Similarly, electrophysiological analysis of motor neurons showed that Ca^{2+} influx was not increased by androgen alone. In both cases, the AR expression was necessary to induced cell death in COS cells and increase Ca^{2+} influx in motor neurons. In the latter case, both androgen and the polyGln-expanded AR were necessary to cause a negative shift in the activation potential, but it is not clear whether this mechanism is achieved by a direct AR action (genomic effect) or through a membrane receptor like those discussed above especially that antiandrogens were not used in these analyses. It would be possible to test between a genomic and epigenomic effect by using a DBD-mutated AR. Patients with the inherited autosomal dominant SCA6 had the CAG expansion in the coding region of the α_{1A}-voltage-dependent calcium channel subunit gene (Zhuchenko *et al.*, 1996; Matsuyama *et al.*, 1997; Riess *et al.*, 1997). This supports the suggestion of this thesis that abnormal function of a voltage-dependent calcium channel is involved in the pathogenesis of SBMA. It is possible, in the case of motor neurons, that androgens predispose the cells to degenerate by binding to a membrane receptor involved Ca^{2+} influx mechanism, and AR contributes, at least through its polyGln-expanded derivative, to an earlier activation of a T-type voltage-gated calcium channel.

MALE INFERTILITY DUE TO SUBSTITUTION MUTATIONS IN THE LBD

Earlier studies have provided endocrine-biochemical evidence linking oligo/azoospermia, with or without other signs of undervirilization, to quantitative and/or qualitative abnormalities of the AR (Aiman and Griffin, 1982; Bouchard *et*

al., 1986; Morrow *et al.*, 1987; Migeon *et al.*, 1984; and Smallridge *et al.*, 1994). There have been only two case reports (Tsukada *et al.*, 1994; Yong *et al.*, 1994) of *AR* point mutations in men with different degrees of impaired spermatogenesis as one expression of MAI. Yong *et al.* (1997 in press) studied a series of 153 men with varying degrees of impaired spermatogenesis to look for constitutional *AR* mutations. They had discovered an exon-5 mutation, an Asn727Lys substitution, in one man (Yong *et al.*, 1994). Among the subset of 70 men with severe, idiopathic oligospermia, Yong *et al.* (1996) discovered two, unrelated men each of whom inherited from his mother the same novel *AR* mutation: Met886Val. The fact that the variant was not found in 100 normal fertile males indicated that it was not a benign polymorphism. One of the men shaves infrequently; the other has Tanner II persistent postpubertal gynaecomastia. These two facts strengthened the idea that their *AR* variant was pathogenic. To prove its pathogenicity, and that of Asn727Lys mutant, the studies whose results are discussed below were undertaken.

Novel mutations in the LBD that do not affect ligand binding

The location of the two LBD mutations Asn727Lys and Met886Val, in exons 5 and 8, respectively, led me to expect of some degree of androgen-binding abnormality since a nearby mutation Val889Met, just 3 residues downstream of Met 886, had been reported to cause CAI due to defective androgen-binding capacity (De Bellis *et al.*, 1994); and two mutations, Leu728Ser and Val730Met, downstream to Asn727 cause PAI (lower ligand affinity, thermolability) or prostate cancer (promiscuous ligand binding and hypertransactivation), respectively (Newmark *et al.*, 1992; Peterziel *et al.*, 1995). Furthermore, all mutations so far described in exon 8 of *AR* manifest some abnormality of androgen binding (Quigley *et al.*, 1995). Surprisingly, all tests for androgen-binding properties of the Met886Val AR GSF of both probands with exon 8 mutation were within normal limits. GSF from exon 5 subject were not accessible. The mutant AR of CML and KLH demonstrated normal apparent dissociation equilibrium constants (K_d), normal androgen binding capacity (B_{max}), normal rate constants of dissociation, normal thermolability when tested with the physiological androgens, T and DHT as well as with two synthetic nonmetabolisable ones, MB and MT. Similarly, the androgen-binding properties of the mutant AR were normal for both mutations in the transfected mammalian cell line, COS-1. Thus, the two mutations reside in the hormone-binding domain of

AR, but do not affect the conformation required for normal function of the ligand-binding pocket of the LBD.

Abnormal transactivation by the mutant receptors

When recreated in an expression plasmid and cotransfected into COS cells with an androgen-inducible reporter gene, the mutant receptors were able to transactivate only 50 to 70% as well as the wild type with all androgens tested except MES. With the Asn727Lys mutation, the latter had different transactivational capability at different temperatures. At 34°C, it was still hypotransactive, but at 37°C it became hypertransactive to about 150% the activity of the wild type. Observation suggests that MES could be therapeutic in the infertile subject with Asn727Lys mutation. When bound to DHT, partial proteolysis of the mutant Asn727Lys indicated that it had a different conformation than the wild-type AR; with MES, it had, apparently, identical conformation, indicating a ligand-specific conformational alteration caused by the mutation that contributes to the impaired transactivation at competence with one ligand but not with another. In the absence of ligand, the digestion pattern was identical for both types of receptor. However, the DHT-liganded wild-type AR generated four extra fragments that are more resistant to proteolysis , ~ 23, 20, 18, and 14 kDa. Since the Asn727Lys mutation creates a new trypsin site, it is likely that these fragments contain the Asn727Lys site and disappeared by digestion. Conformational alteration leading to inaccessibility of trypsin to Asn727Lys site would have been detected as a heavier band(s) in the mutant lane. The latter hypothesis is further supported by the disappearance of the four fragments from the digestion of the wild-type liganded with MES, without an effect on the rest of the higher-molecular-weight fragments in both receptors.

All transactivation-defective LBD mutations reviewed to date (Quigley *et al.*, 1995; Gottlieb *et al.*, 1997) have been associated with androgen-binding abnormalities as measured by Scatchard analyses (K_d, B_{max}), non-equilibrium dissociation kinetics (Beitel *et al.*, 1994b) and/or thermolability (Nakao *et al.*, 1993). One possible exception, recently described (Bevan *et al.*, 1996), is a Gln798Glu mutation in a patient with PAI: it was transactivation-deficient without apparent androgen-binding abnormality based on measurement of equilibrium androgen-binding affinity with one analog. Importantly, non-equilibrium androgen dissociation kinetics were, however, not examined in that case. In this regard, it is striking to note that nearby mutations like Val889Met

caused CAI with increased androgen dissociation kinetics despite normal androgen-binding affinity (K_d) (Zhou *et al.*, 1995b). Similarly, two pathogenic mutations flanking Asn727 site showed differential binding activity. One, Arg726Leu, one residue upstream, caused prostate cancer with normal binding kinetics, whereas a downstream mutation, Leu728Ser, leads to PAI with low binding. I expected that the Asn727Lys substitution would alter A-R binding, since this site is conserved as Asn among hAR, hPR, and hGR (Wurtz *et al.*, 1996; Quigley *et al.*, 1995) (Fig. 4), and it lies in one of two hot spots of hAR's frequent sites of mutation (hot spot 1; residues 726 to 772; Gottlieb *et al.*, 1996) which is a highly conserved region among these receptors. This suggests that neither this site (Asn727) nor the substituting Lys residue are directly involved in ligand binding as supported by the alignment data for the superfamily of nuclear receptors (Wurtz *et al.*, 1996).

Transactivation defect due to decreased ability of mutant receptor-ligand complexes to bind ARE

Since H12 is a subdomain necessary for transactivation, Met886Val substitution might have caused MAI by hypotransactivation due to interaction of the LBD with intermediary transcription co-factors that are tissue-specific. However, both activated mutant receptors studied had impaired ability to bind oligonucleotides containing two different AREs: the first, a naturally occurring steroid response element found in MMTV; the second, an artificial ARE based on the consensus DNA-binding site for androgen receptor. Binding to both AREs in repeated experiments was only about 60% of the wild type, a value proportionate to the degree of disrupted transactivation, suggesting that the transactivation defect was the proximate result of abnormal DNA binding. Unfortunately, the commercial unavailability of labelled mesterolone limited the further experimentation to test whether the normalized transactivation competence of Asn727Lys by mesterolone is in fact a result of normal DNA binding. The androgen receptor undergoes dimerization in association with ARE binding and as a monomer fails to bind ARE (Wong *et al.*, 1993). Evidence from the expression of deletion mutants (Langley *et al.*, 1995) and fusion proteins (Nemato *et al.*, 1994) indicates that a dimerization function resides in the LBD of the AR. One of the mutations (Met886Val) is in a subdomain of the LBD, conserved among the members of steroid receptor family, that is thought to mediate dimerization. A heptad repeat of hydrophobic residues in this subdomain

resembles the leucine zipper or coiled-coil structure implicated in the dimerization of a number of DNA-binding proteins. Crystallographic analyses have also indicated that this heptad repeat forms α-helical structures that are involved in homodimerization of the hRXR (Bourguet *et al.*, 1995) and RARγ (Wurtz *et al.*, 1996). Similarly, this region in the estrogen (Kumar and Chambon, 1988; Fawell *et al.*, 1990) and the human vitamin D (Jin *et al.*, 1996) receptors has been implicated in receptor dimerization, suggesting a conservation of function in this domain. Moreover, data obtained from 3-D structure of RAR and its alignment with other nuclear receptors including the hAR subfamily showed that Met 886 lies in a loop between helices H11 and H12 that contributes importantly to ligand binding and, by sequence homology to the predicted heptad region of mER (Fawell *et al.*, 1990; White *et al.*, 1991). This region is predicted to encompass residues from 853-887 of hAR (Fig. 4). Furthermore, two regions flanking the heptad region of TRβ were shown to affect its dimerization (Nagaya and Jameson, 1993). The C-terminal flanking region (TRβ residues 443-466) includes H11 and H12, the loop between them. The latter would include Met 886 of AR (Fig. 4).

Although there is evidence that the LBD of the AR can interact with the N-terminal domain in an antiparallel orientation in protein interaction assays (Langley *et al.*, 1995), the precise dimerization interfaces of the LBD in the AR have not been defined.

The other mutation studied in this thesis (Asn727Lys) occurs in a highly conserved region in the nuclear receptors superfamily, and the site itself is absolutely conserved in all four members of the AR subfamily receptors of androgen, progesterone, glucocorticoid, and mineralocorticoid. Although this novel mutation occurs in a conserved region of nuclear receptors that contains most of the residues that stabilize the core of the LBD (Wurtz *et al.*, 1996), unexpectedly, it did not alter the receptor's function remarkably. Only spermatogenesis was defective. This indicated that the hAR Asn727 site is not in the immediate vicinity of the ligand and/or is not involved in the interaction of the LBD helices that form the ligand-binding pocket. Indeed, alignment data generated from x-ray crystallography of RAR LBD (Wurtz *et al.*, 1996) show that hAR Asn 727 site and its homologues in at least 16 nuclear receptors are not in the immediate vicinity of the ligand nor among the conserved residues that are considered to contribute to interactions among LBD's helices.

Recently, a transcriptional coactivator for AF-2 transactivation domain (the predicted H12 in nuclear receptors) of GR has been cloned (Hong *et al.*, 1997).

This GR-interacting protein 1 (GRIP1), isolated from a mouse brain cDNA library, is the probable ortholog of the human transcription intermediary factor 2 (TIF2) and is also partially homologous to steroid receptor coactivator 1 (SRC-1) (Onate *et al.*, 1995). Full-length GRIP1 interacted with the LBDs of steroid receptors in a hormone-dependent manner and also with LBDs of TRα, VDR, RARα, and RXRα (Hong *et al.*, 1997). GR interaction with GRIP1 was inhibited by glucocorticoid antagonists indicating that it is conformation-dependent. In the yeast two-hybrid system, GRIP1 enhanced transactivation of a reporter gene by interacting with fusion proteins made from the aforementioned LBDs and the GAL4-DBD. It also enhanced the hormone-dependent transactivation activity of full-length GR, ER, and MR. GR deletions and mutants indicated that GRIP1 interacted with and enhanced the activity of the C-terminal AF-2 but not the N-terminal AF-1 transactivation domain of GR (Hong *et al.*, 1997). These results indicate that AF-2- enhanced transactivation activity is accomplished through GRIP1 as a coactivator such as GRIP1.

This may be a possible explanation for, at least, the impaired transactivation by hAR Met886Val mutation since this mutation occurred 7 residues upstream to the predicted AF-2 region (fig. 4). It is possible that the mutation altered the A-R complex conformation and hence abrogated the interaction with such coactivators.

Interestingly, these two LBD mutations (Asn727Lys and Met886Val) are the first discovered to cause impaired hAR-DNA binding, yet they are not the first such mutations to be observed among steroid receptors. In fact, two artificial mutations in hER (Cys447Ser and Cys530Ser) were shown to cause defective DNA-binding and subcellular distribution (Neff *et al.*, 1994). Cysteine 530 in hER is a position homologous to Val 889 site of hAR which, as mentioned above, is 3 residues downstream of Met886Val mutation studied in this thesis (Fig. 4).

In view of the data documented in this thesis, it is very likely that the DNA-binding defect of the Asn727Lys and Met886Val mutations and their hypotransactivation are very likely due to impaired AR dimerization. These data provide the first evidence from naturally occurring point mutation that the dimerization/ARE-binding and ligand-binding functions of the AR's LBD do not overlap completely.

To my knowledge, this study of Asn727Lys and Met886Val, is the first to indicate the diagnostic value of screening for *AR* mutations in patients with severe idiopathic oligospermia. Further, there is evidence that some of these

abnormalities are correctable by hormonal manipulation (Yong *et al.*, 1994). The association of these two *AR* mutations with defective sperm production, but with almost no other phenotypic features of CAI or PAI, indicates that Asn727 and Met886 of the hAR have important roles in the transactivation of genes required for spermatogenesis.

SUMMARY

Pathogenetic investigation of the SBMA-causing polyGln-expanded hAR transiently expressed in NSC34 motor neurons revealed its hypotransactivational competency despite its higher ligand-binding and stability compared to the wild-type AR. Changing the standard (CAG) tract to a mixed (CAG/CAA) did not alter AR transactivational properties when expressed in COS-1 cells supporting the hypothesis that the pathogenesis occurs at a protein level. Partial tryptic digestion of *in vitro* translated AR revealed conformational alterations in the polyGln-expanded AR and generated Gln-expanded derivatives that are trypsin-resistant even during prolonged digestion in the presence of denaturant. PolyGln-expanded AR transiently expressed in COS-1 cells generated a unique 75-kDa polyGln-expanded product that is probably AR derivative. Cells expressing the polyGln-expanded AR are twice as likely to die apoptotically. Electro-physiological analysis of motor neurons transiently or stably expressing polyGln-expanded AR in the presence of androgen revealed a negative shift in the voltage dependence of activation in a T-type Ca^{2+}-channel. This increased the size of window currents, and consequently, the Ca^{2+} load to neurons. This calcium influx could be one mechanism responsible for neuronal degeneration in SBMA. "Misprocessing" of the polyGln-expanded AR, exemplified by protease-resistance or accumulation of toxic polyGln-containing fragments, may abrogate the function of Ca^{2+} channel in motor neurons leading to elevation of Ca^{2+} influx and cell death.

Pathogenetic investigation of the Asn727Lys and Met886Val mutations causing oligospermia revealed that their hypotransactivation was not due to impaired androgen binding, but to impaired DNA binding. This impaired DNA-binding is likely due to a ligand-induced conformational alteration that impairs dimerization. The impairment of dimerization appears to differentially affect a target gene (or genes) that is important for spermatogenesis. Asn727Lys loss of normal transactivation was restored using mesterolone at 37C. This means that defect in spermatogenesis was caused by the failure of the AR to bind its response element and transactivate target gene(s) involved in spermatogenesis. These impairments could be restored using different androgens.

REMARKS

1- The electrophysiological analysis of motor neurons was done by Dr. Adrian Sculptoreanu and his summer student Hanan Abramovici at the Lady Davis Institute for Medical research. Cell culture, transfection and differentiation was done by me. The patch clamp technique and Ca^{2+} channel recording analysis were performed in my presence and clearly explained to me.

2- Confocal microscopy and cell death analysis was done by Dr. Schipper's graduate student Dov Frankel at the Lady Davis Institute for Medical Research.

3- Chimeric GAL4-DBD/AR construction and expression analysis were done by Dr. Lenore Beitel and Carlos Alvarado in our laboratory

SUGGESTED EXPERIMENTS

1) To assess the higher polyGln-expanded AR protein content in NSC34, RT-PCR analysis was initiated using total RNA extracted from motor neurons transiently expressing wild-type or polyGln-expanded ARs. Preliminary results showed more message of the poly(CAG)-expanded AR cDNA. This analysis will be repeated using the transcription inhibitor actinomycin-D to compare mRNA stability of both types of AR.

2) Analyse the NSC34 cells transiently expressing AR for a possible misprocessing/proteolytic resistance polyGln-expanded AR and its derivatives using mAb1C2 and F39.4.1. To test whether such derivative fragments may arise from proteolysis, I suggest the use of different protease inhibitors and proapoptotic proteases to track the generation of such fragments in COS-1 and motor neurons.

3) To correlate the length of polyGln tract to the appearance of the 75 kD fragment and ceall death rate, I suggest transfection experiment using AR cDNAs with different sizes of the $(CAG)_n$ tract in COS-1 cells and motor neuron hybrid cells at different type points.

4) Using the nuclear extract I prepared from COS-1 cells transiently transfected with either type of AR, another student in the laboratory has demonstrated the strong presence of the 75 kDa polyGln-expanded fragment in the nuclear extract from cells treated with hormone. The nuclear residence of this fragment is in keeping with the very recent publication of papers in Cell and Neuron demonstrating the nuclear residence of polyGln-containing products of Huntingtin and, reportedly, of the AR in transgenic mice. I suggest the purification of this fragment and partial sequencing to identify the nature of this product.

5) Perform androgen receptor dimerization assay on Asn727Lys and Met886Val to demonstrate that lower binding to ARE reflects decreased dimerization.

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