Genetic and Functional Analysis
of Androgen Receptor Gene Mutations

Genetische en Functionele Analyse
van Mutaties in het Androgeen Receptor Gen

Proefschrift

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Contents

Abbreviations

AIS	androgen insensitivity syndrome	P-box	proximal box
AF	activation function	pCAF	p300/CBP-associated factor
AMH	anti-müllerian hormone	PCR	polymerase chain reaction
(h)AR	(human) androgen receptor	PMSF	phenylmethylsulfonylfluoride
ARE	androgen response element	poly[dIdC]-poly[dIdC]	
bp	base pair		polydeoxyionosinic-deoxycitidylic acid
Bmax	maximal number of binding sites	(h)PR	(human) progesterone receptor
BMD	bone mineral density	RAR	all-trans retinoic acid receptor
BMI	body mass index	RNA Pol-II	RNA polymerase II
BPS	branch point site	RXR	9-cis retinoic acid receptor
BW	body weight	R1881	methyltrienolone
CAIS	complete androgen insensitivity syndrome	SBMA	spinal and bulbar muscular atrophy
		SCA	spinocerebellar ataxia
CAT	chloramphenicol acetyl transferase	SF-1	steroidogenic factor 1
CBP	CREB-binding protein	SHBG	sex hormone-binding globulin
CHO	Chinese hamster ovary	SHR	steroid hormone receptor
CMV	cytomegalo virus	SSCP	single-strand conformation polymorphism
COS-1	monkey kidney cell line	StAR-protein	
DBD	DNA-binding domain		steroidogenic acute regulatory protein
D-box	distal box	TAF	TBP-associated factor
DHT	5α-dihydrotestosterone	TAT	tyrosine aminotransferase
DTT	dithiothreitol	TAU	transcription activation unit
(h)ER	(human) estrogen receptor	TBP	TATA-binding protein
ERE	estrogen response element	Tfm	testicular feminization
FSH	follicle-stimulating hormone	TIC	transcription initiation complex
GTF	general transcription factor	TIS	transcription initiation site
(h)GR	(human) glucocorticoid receptor	TR	thyroid hormone receptor
GRE	glucocorticoid response element	UTR	untranslated region
HAT	histone acetyl transferase	VDR	1,25-dihydroxy-vitamin D3 receptor
hCG	human chorionic gonadotrophin	3-D	three-dimensional
HD	Huntington's disease		
HDAC1	histone deacetylase 1		
HRE	hormone response element		
17β-HSD	17β-hydroxysteroid dehydrogenase		
hsp	heat-shock protein		
kb	kilo base		
kDa	kilo Dalton		
Kd	equilibrium dissociation constant		
LBD	ligand-binding domain		
LH	luteinizing hormone		
Luc	luciferase		
MAIS	mild androgen insensitivity syndrome		
MMTV	mouse mammary tumor virus		
(h)MR	(human) mineralocorticoid receptor		
NHR	nuclear hormone receptor		
NLS	nuclear localization signal		
ORF	open reading frame		
PAGE	polyacrylamide-gel electrophoresis		
PAIS	partial androgen insensitivity syndrome		

General Introduction

Chapter I
General Introduction

I.1 Steroid hormone receptors

I.1.1 Family members of the androgen receptor

Nuclear hormone receptors (NHRs) are intermediary factors through which extracellular signals regulate expression of genes that are involved in homeostasis, development, and differentiation (Beato *et al.* 1995, Mangelsdorf and Evans 1995). These receptors are characterized by a modular structure, with domains involved in transcription activation, DNA binding, hormone binding, and dimerization. The nuclear receptor super-family comprises three subfamilies of receptors, which might have emerged early during evolution (Laudet *et al.* 1992).
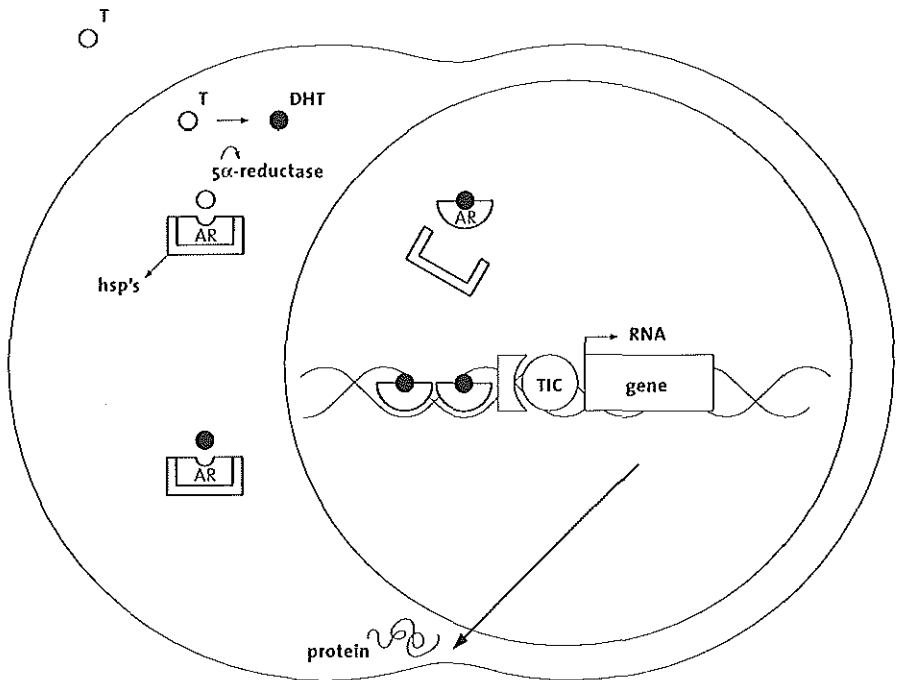
Figure I.1 **Mechanism of action of androgens.** The steroid hormone testosterone (T) enters the cell via passive diffusion. In the cytoplasm, testosterone can be converted to the more potent androgen 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase type II. Both androgens can bind to the AR, which is complexed with heat-shock proteins (hsps). Upon androgen binding the receptor dissociates from the complex, and the AR binds to DNA as a homodimer. Transcription activation takes place, resulting in a cellular response. TIC: transcription initiation complex.

The first subfamily contains so-called orphan receptors, which either have an unknown ligand, or are constitutively active. The second subfamily is formed by receptors for thyroid hormone, vitamin D and retinoids. The third subfamily is formed by steroid hormone receptors (SHRs). This subfamily can be further divided, as the glucocorticoid receptor (GR), progesterone receptor (PR), mineralocorticoid receptor (MR), and androgen receptor (AR) are more closely related to each other than to the estrogen receptor (ER) (Evans 1988). Two isoforms of both the hPR (PR-A and PR-B) (Krett *et al.* 1988), and the GR (GR-α and GR-β) (Bamberger *et al.* 1995) have been identified. These isoforms, which are highly homologous but functionally different, are encoded by single genes. Recently, a second estrogen receptor (ER-β) encoded by a second gene was cloned (rat ER-β: Kuiper *et al.* 1996, hER-β: Mosselman *et al.* 1996), which can heterodimerize with the ER-α (Cowley *et al.* 1997, Pettersson *et al.* 1997), and responds differently to certain ligands (Paech *et al.* 1997).

In the absence of ligand, the receptors for thyroid hormone, vitamin D, and all-trans retinoic acid (TR, VDR, and RAR, respectively) can be involved in silencing of promoter activity through their ligand-binding domain (LBD). In contrast, SHRs are bound by a complex of proteins that among others contains the heat-shock proteins hsp90, hsp70, and hsp56. The SHR in the complex is directly bound to hsp90, which not only keeps the receptor in an inactive state but is also essential for proper folding of the receptor protein (reviewed by Pratt and Toft

Figure I.2 **Human *AR* gene; structural organization and protein.** The *AR* gene was mapped to the long arm of the X chromosome. The hAR prote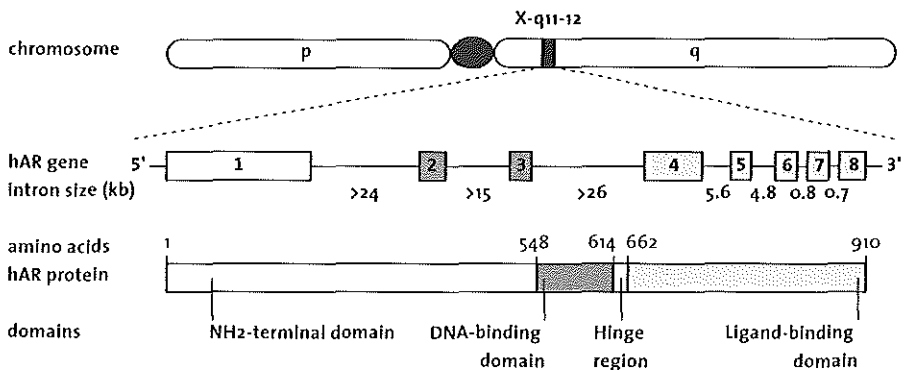in is encoded by 8 exons. Analogous to other nuclear receptors, the protein consists of several distinct functional domains: the NH$_2$-terminal domain containing two polymorphic stretches, the DNA-binding domain, the hinge region, and the ligand-binding domain.

1997). Upon ligand binding, the SHR dissociates from the complex, forms a (homo-)dimer that binds to specific hormone response elements (HREs) to modulate chromatin structure, finally resulting in regulation of gene expression (Figure I.1). Apart from gene activation, SHRs are also involved in repression of genes. This can be accomplished either by competition for overlapping HREs with other SHRs, or by competition for basal transcription factors or coactivators, or by protein-protein interactions that result in transcriptional interference (inhibition of gene expression, regulated by AP-1) (Beato *et al.* 1995).

I.1.2 Organization of the androgen receptor gene, promoter structure, androgen receptor mRNA, and androgen receptor protein

The gene encoding the h*AR* was cloned 10 years ago by several groups (Chang *et al.* 1988a, Lubahn *et al.* 1988a, Trapman *et al.* 1988, Tilley *et al.* 1989) and mapped to the q11-q12 region of the X chromosome (Brown *et al.* 1989). The *AR* gene, which is spanning more than 90 kilo base (kb) genomic DNA, comprises 8 exons (Kuiper *et al.* 1989, Lubahn *et al.* 1989) (Figure I.2).

Expression of the gene is regulated by a single promoter region that contains 2 transcription initiation sites (TIS), located in a 13-base pair (bp) region (Tilley *et al.* 1990, Faber *et al.* 1991a). The AR promoter region does not contain a TATA-box or a CCAAT-box, but other regulatory sequences like a GC-box (recognition site for Sp1), a purine-rich region and a CRE element (AR-CRE, only present in the hAR promoter) are present (Baarends *et al.* 1990, Faber *et al.* 1993, Mizokami *et al.* 1994). In several androgen-responsive cell lines two mRNA species of different length were detected, a 10.6 kb and an 8.5 kb species (Lubahn *et al.* 1988b, Trapman *et al.* 1988, Tilley *et al.* 1990). The transcript of 10.6 kb consists of a 1.1 kb 5' untranslated region (UTR), a 2.7 kb open reading frame (ORF) (Kuiper *et al.* 1989, Lubahn *et al.* 1989), and a relatively long 3' UTR of 6.8 kb (Faber *et al.* 1991a). The shorter mRNA species that contains the normal ORF results from differential splicing in the 3' UTR (Faber *et al.* 1991a).

The number of amino acid residues in the AR is variable, due to the presence of 2 polymorphic stretches (Faber *et al.* 1989). Throughout this thesis, numbering of amino acid residues is based on an AR consisting of 910 amino acid residues, as described by Brinkmann *et al.* (1989). Analogous to other NHRs, the AR is composed of distinct functional domains. Apart from a ligand-binding domain (LBD) and a DNA-binding domain (DBD), a hinge region, and an NH$_2$-terminal domain which is involved in transcription activation, can be distinguished (reviewed by Beato and Sanchéz-Pacheco 1996). The AR protein has an apparent molecular

mass of 110 kilo Dalton (kDa). Like other SHRs, the AR is post-translationally modified by phosphorylation (Kuiper and Brinkmann 1994). Phosphorylation is known to be involved in functional control of many proteins, probably by induction of conformational changes.

Androgen receptor protein is widely expressed in male reproductive tissues, but also in many other tissues in both males and females (Ruizeveld de Winter *et al.* 1991, reviewed by Quigley *et al.* 1995). Regulation of AR transcript and protein expression is complicated, and known to be age- and tissue-dependent (Supakar *et al.* 1993, Wolf *et al.* 1993). Analogous to other SHRs, autoregulation has been implicated (Wolf *et al.* 1993). Apart from androgens, follicle-stimulating hormone (FSH) is also involved in regulation of *AR* gene expression (Blok *et al.* 1989).

In addition to the full-length AR protein (which has an apparent molecular mass of 110 kDa), an NH_2-terminally truncated 87-kDa AR isoform was detected in human genital skin fibroblasts (Wilson and McPhaul 1994). Previously, two functionally different PR isoforms, encoded by a single gene, were identified (Krett *et al.* 1988, Kastner *et al.* 1990). For both isoforms a different promoter specificity was observed (Kastner *et al.* 1990) Under certain conditions, the NH_2-terminally truncated isoform (hPR-A) becomes a dominant inhibitor of gene activation by the full-length PR (hPR-B), and even inhibition of activation by the GR, MR and AR was observed (Tung *et al.* 1993, Vegeto *et al.* 1993). These effects have to be ascribed to a third autonomous activation function (AF-3) that was mapped to the NH_2-terminal part of the hPR, being unique to hPR-B (Satorius *et al.* 1994). Analogous to hPR-A, the NH_2-terminally truncated AR isoform may arise from translation initiation from an alternative translation start site. So far, a possible *in vivo* function of the shortened putative AR isoform is not known. In Chapters II and III reinitiation of translation was studied in genital skin fibroblasts from androgen insensitive patients, having a mutation in their *AR* gene which leads to a premature stop codon upstream of the first internal methionine residue.

I.2 Structure and function of steroid hormone receptors, in particular the androgen receptor

I.2.1 The NH$_2$-terminal domain

In contrast to the relatively high homology observed for other domains, the NH$_2$-terminus is not markedly conserved between SHRs (hGR, hER-α, hER-β, hMR, hPR, and hAR; Hollenberg *et al.* 1985, Green *et al.* 1986, Mosselman *et al.* 1996, Arriza *et al.* 1987, Misrahi *et al.* 1987, Faber *et al.* 1989, respectively). With the exception of ER-α and ER-β, the NH2-terminus constitutes a relatively large part of these receptors.

The NH$_2$-terminal part of the hAR is encoded by one large exon (Faber *et al.* 1989). A highly polymorphic poly-glutamine stretch encoded by (CAG)nCAA, is located between amino acid residues 58 and 77 (Sleddens *et al.* 1992). Two stretches of 6 and 5 glutamine residues, respectively, are located in a more COOH-terminal region (Faber *et al.* 1989). In addition, a poly-alanine stretch, a poly-proline stretch, and a polymorphic poly-glycine stretch, encoded by (GGN)n, are present (Faber *et al.* 1989, Sleddens *et al.* 1993). A polymorphic poly-glutamine repeat, although located in a different region, has also been identified in the mouse AR (He *et al.* 1990, Charest *et al.* 1991, Faber *et al.* 1991b) and rat AR (Chang *et al.* 1988b). In these murine species, other homopolymeric stretches were absent. NH$_2$-terminal domains of the other members of the SHR family do not contain homopolymeric stretches, except for the hMR, which harbors a significant poly-proline stretch (Arriza *et al.* 1987). It is believed that acidic, poly-proline rich, and poly-glutamine sequence motifs confer transcriptional activity (Tjian and Maniatis 1994). Apart from the poly-glutamine stretches and a poly-proline stretch, the AR NH$_2$-terminus contains a relatively high amount of acidic amino acid residues (Faber *et al.* 1989). There are recent findings, indicating that the glutamine stretch is a modifier of AR activity that causes phenotypic variation (see Paragraph I.5.1).

Transcription activation of target genes depends on subdomains with an activation function (AF). Deletion mapping led to the discovery of 2 AFs, located in the NH$_2$-terminal domain (AF-1) and the LBD (AF-2) of various SHRs (GR, Hollenberg and Evans 1988; PR, Meyer *et al.* 1990; ER-α, Tora *et al.* 1989; AR, Simental *et al.* 1991, Jenster *et al.* 1991). Chamberlain *et al.* (1996) identified two smaller activation functions in AF-1 of the rat AR, which mediate transcription activation together. Only recently, a functional AF was identified in the LBD of the hAR (Moilanen *et al.* 1997). The full-length PR (PR-B) contains an additional NH$_2$-

terminal AF (AF-3), mapped to the NH$_2$-terminal region, that is unique to this receptor (Satorius *et al.* 1994). Neither the location of AF-1 nor the amino acid composition is conserved among SHRs, indicating that the NH$_2$-terminus is involved in receptor-specific regulation of target genes. It is generally believed that the NH$_2$-terminal domain of SHRs interacts with basal transcription factors and specific cofactors (Simental *et al.* 1991, Adler *et al.* 1992, Jacq *et al.* 1994, Schwerk *et al.* 1995, Ford *et al.* 1997, McEwan and Gustafsson 1997). Upon deletion of the LBD, SHRs become constitutively active to a variable degree, depending on the type of receptor. This points to a repressive function of the LBD in the absence of hormone. In the NH$_2$-terminal part of the AR, besides AF-1 (Simental *et al.* 1991, Jenster *et al.* 1991), a second transcription activation unit (TAU) was identified that becomes activated after deletion of the LBD (Jenster *et al.* 1995).

First Zn Cluster   **Second Zn Cluster**
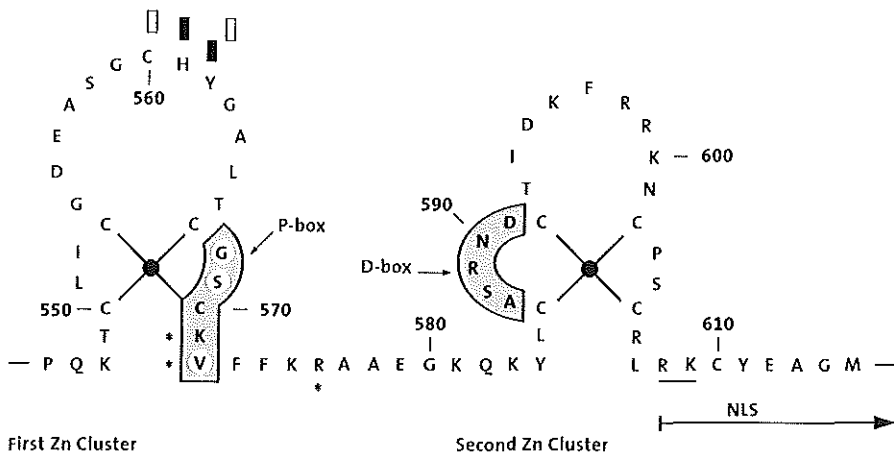
Figure I.3A **The AR DNA-binding domain.** The AR-DBD is defined as a domain, consisting of 65 amino acid residues (K548 - E612) (Trapman *et al.* 1988). In the first zinc cluster the P-box is indicated. Circled amino acid residues in the P-box are involved in ARE half-site recognition (Danielsen *et al.* 1989). At the corresponding position in the ER the amino acid residues EGckA are present (residues involved in HRE recognition are capitalized). The residues marked with an asterisk might also be involved in contacts with the ARE half-site. Residues, marked with boxes are involved in contacts with the phosphate backbone of DNA at specific (black boxes) or at non-specific sites (open boxes) (Luisi *et al.* 1991). In the second zinc cluster, the D-box which is involved in dimerization, is indicated (Umesono and Evans 1989). The second zinc cluster contains also the first part of the nuclear localition signal (NLS).

I.2.2 The DNA-binding domain

Regulation of gene expression by SHRs often involves binding to HREs, which are specific sequences located in the promoter region of regulated genes. In this way, either transcription activation or repression of genes (occlusion mechanism) can be accomplished. However, interaction of SHRs with other regulatory proteins bound to response elements in promoter regions, might contribute to control of gene activation or repression (AR: Kallio *et al.* 1995, Heckert *et al.* 1997; ER: Yang *et al.* 1996; reviewed by Katzenellenbogen *et al.* 1996). Most genomic actions however, involve direct binding of the receptor to DNA, which is accomplished by the DBD.

Structure of the DBD

The DBD, the region that is most conserved among NHRs, contains 9 conserved cysteine residues. Eight of these conserved cysteines are involved in formation of 2 zinc clusters. Four cysteine residues in each of the zinc clusters coordinate

| Consensus GRE | AGAACA | nnn | TGTTCT |
| Consensus ERE | AGGTCA | nnn | TGACCT |

Figure I.3B **Mechanism of DNA binding by SHRs.** Homodimeric receptor binds to 2 half-sites of the HRE, which are organized as an imperfect palindrome, resulting in so-called head-to-head binding. The HRE is located in the major groove of DNA. The consensus HRE for GR-like receptors and for the ER are indicated. The half-sites are spaced by 3 nucleotides (nnn).

zinc in a tetrahedral array (Freedman *et al.* 1988). Nuclear magnetic resonance and crystallographic studies, performed for several NHRs (GR: Härd *et al.* 1990, Luisi *et al.* 1991; ER: Schwabe 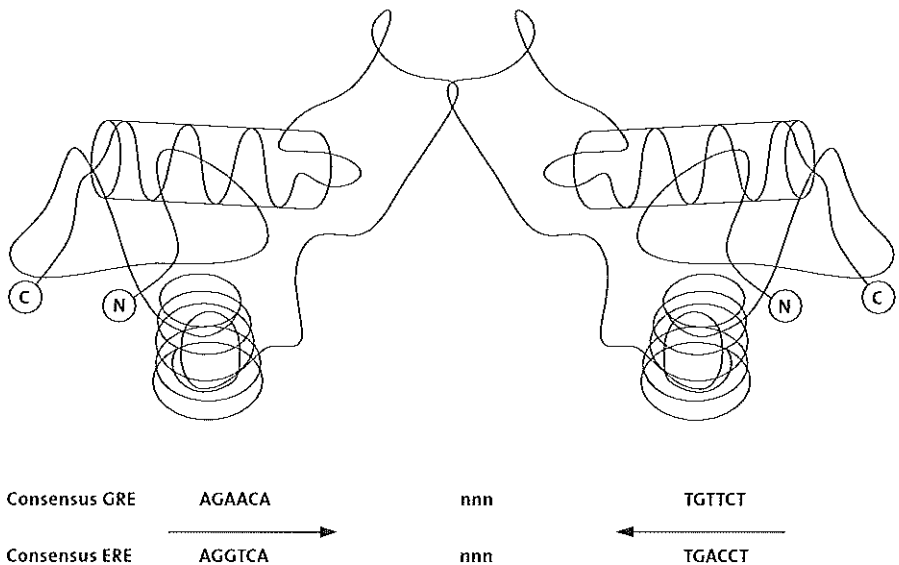*et al.* 1990, Schwabe *et al.* 1993, RAR: Knegtel *et al.* 1993; 9-cis retinoic acid receptor (RXR): Lee *et al.* 1993), revealed similar structures for the distinct receptors. Between the third and fourth cysteine residues in the NH_2-terminal and the COOH-terminal zinc clusters, α-helices are present. Upon DNA binding, these α-helices are packed perpendicularly to each other (see Figure I.3B) In the COOH-terminal part of the DBD of the RXR an additional α-helix that mediates DNA binding of RXR homodimers was detected (Lee *et al.* 1993). This helix is not present in the DBDs of SHRs. So far the three-dimensional (3-D) structure of the AR-DBD has not been solved. However, as is discussed in Chapter V, the AR-DBD is closely related to the GR-DBD (Hollenberg *et al.* 1985, Trapman *et al.* 1988), and therefore it can be attempted to build a 3-D model of the AR-DBD, based on the crystal structure of the GR-DBD.

The ability to discriminate between response elements resides in 3 amino acid residues, two of which are located between the 2 distal cysteines of the NH_2-terminal zinc cluster, in the so-called proximal box (P-box) (Danielsen *et al.* 1989, Zilliacus *et al.* 1991) (Figure I.3A). The α-helix, containing the P-box, is positioned in the major groove of DNA, where the three amino acid residues are making base-specific contacts with DNA. The P-box of receptors that bind to glucocorticoid response element (GRE)-like half-sites contains a glycine, a serine, and a valine residue (Danielsen *et al.* 1989). In the P-box of the ER, which binds to estrogen response elements (EREs) a glutamic acid, a glycine and an alanine residue are present at the corresponding position (Figure I.3A) (Umesono and Evans 1989). In the COOH-terminal zinc cluster, a dimerization function was identified in the so-called distal box (D-box) (Figure 1.3A) (Umesono and Evans 1989). Upon binding to a palindromic HRE, a symmetric dimerization interface is formed (Figure I.3B) (Luisi *et al.* 1991). Other domains of SHRs might as well be involved in dimerization. The LBD of the ER is involved in hormone dependent dimerization (Fawell *et al.* 1990). Both the LBD and the NH_2-terminus contribute to dimerization of the GR, AR, and PR (Dahlman-Wright *et al.* 1992, Wong *et al.* 1993, Tetel *et al.* 1997). There are indications that solution dimerization promotes high-affinity DNA binding, probably by stabilization of the dimer (Kumar and Chambon 1988, Dahlman-Wright *et al.* 1992, Wong *et al.* 1993).

Hormone response elements

Nuclear hormone receptors can bind to HREs in several different modes. Apart from monomeric binding (for orphan receptors, including NGF1-B and SF-1), homodimeric binding and heterodimeric binding can be observed (reviewed by Glass 1994, and by Mangelsdorf *et al.* 1995). Hormone response elements minimally contain a core recognition motif of 6 bp, but most HREs consist of 2 core motifs separated by a spacer of variable length. Three different characteristics of HREs, which determine response element recognition, can be distinguished. First, the sequence of the core motif, which is specific for subgroups of receptors. The GR, MR, PR and AR all bind to GRE-like half-sites (TGTTCT), whereas the ER and several non-steroid hormone nuclear receptors recognize TGACCT (Figure I.3B) (Danielsen *et al.* 1989, Mader *et al.* 1989, Forman and Samuels 1990, reviewed by Glass 1994). The GRE and ERE differ at positions 3 and 4 in both half-sites, which are critical for receptor specific recognition (Nordeen *et al.* 1990). The second characteristic involves the arrangement of the core region, which is organized either as a direct repeat, a palindromic, or an inverted repeat. Third, the HRE is characterized by the spacing of the two half-sites.

In general, SHRs bind as homodimers to (imperfect) palindromic HREs with a spacing of three nucleotides (Figure I.3B) (Umesono and Evans 1989, reviewed by Zilliacus *et al.* 1995). These HREs typically contain a high-affinity binding half-site, and a second half-site of lower affinity. Binding of the first monomer to DNA increases affinity of a second receptor molecule, a mechanism that is named cooperativity of binding (Dahlman-Wright *et al.* 1991). The TR, RAR, VDR and PPAR are fundamentally different from SHRs with respect to DNA binding. These receptors predominantly heterodimerize with the RXR (Kliewer *et al.* 1992, reviewed by Mangelsdorf *et al.* 1995), and bind to HREs, which are organized as direct repeats with a different spacing, or to (inverted) palindromic motifs. Ligand binding might control the formation of either homodimers or heterodimers. In the LBD of the RXR, RAR, and TR, a domain involved in formation of solution heterodimers, confering high-affinity DNA binding, was identified (Perlmann *et al.* 1996). After formation of heterodimers between these distinctive receptors and the RXR, a second dimerization interface can be formed within the DBD, which restricts heterodimer binding to direct repeats (Perlmann *et al.* 1996). The spacing between the direct repeats can vary, and is important with respect to specificity, and determines the polarity of binding of the heterodimer as well (Glass 1994).

Cell specificity

An intriguing question is in what way cell specific effects are exerted when more than one SHR is present in a particular cell. Specificity might be introduced by tissue specific metabolism of hormones, expression level of the receptor, the presence of specific cofactors that modulate DNA binding and/or transcription activation, or interaction with another nuclear receptor (Forman and Samuels 1990, Glass 1994, Rundlett *et al.* 1995). Adler *et al.* (1992) observed that androgen response elements (AREs) in the mouse sex limited protein gene promoter prefer activation by androgens. They showed that DNA sequences adjacent to the ARE were critical for inhibition of regulation by the GR, suggesting that other proteins are involved in AR specific gene activation (Adler *et al.* 1993). Receptor specific response elements might exist as well, as was suggested by Claessens *et al.* (1996). Several groups postulated that high-mobility group protein HMG-1 enhances DNA binding of steroid hormone receptors. HMG-1 causes DNA bending, which probably results in a more stable interaction of the receptor with DNA (Onate *et al.* 1994, Verrier *et al.* 1997). Although it is generally believed that SHRs form homodimers on palindromic HREs, Zhou *et al.* (1997) observed gene activation by the AR through an HRE that was organized as a direct repeat (Zhou *et al.* 1997). In addition, several groups presented *in vitro* and *in vivo* evidence for formation of heterodimers by the GR and MR (Trapp *et al.* 1994, Liu *et al.* 1995).

I.2.3 The hinge region

The DBD and LBD are coupled by a flexible domain, the so-called hinge region. Among members of the SHR-family the hinge region is poorly conserved (Evans 1988). Naturally occurring mutations in the AR that are associated with the androgen insensitivity syndrome (AIS) were not identified in the N-terminal half of the hinge region. Nevertheless, several functions are ascribed to this region. It not only contains a nuclear localization signal (NLS), as was observed by several groups for different SHRs (PR, Guiochon-Mantel *et al.* 1989; ER, Ylikomi *et al.* 1992; AR, Simental *et al.* 1991, Jenster *et al.* 1993), but the hinge region is also involved in interaction with corepressors for NHRs (Chen and Evans 1995, Hörlein *et al.* 1995). In addition,Tetel *et al.* (1997) observed that solution dimerization of the PR involves the hinge region.

I.2.4 The ligand-binding domain

The LBDs of the distinct NHRs are multifunctional; apart from ligand binding, the LBD is involved both in hetero- and/or homo-dimerization and in transcription activation. In the absence of ligand, some members of the NHR family are involved in silencing of promoter activity through their LBD (Horwitz *et al.* 1996), whereas SHRs are bound by a complex of heat-shock proteins. Activity of SHRs is affected by interaction with hsp90, which is critical for proper folding of the receptors (reviewed by Pratt and Toft 1997). Upon deletion of the COOH-terminus, SHRs become constitutively active.

Transcription activation function

While the AFs that were identified in the NH_2-terminal domain of SHRs are constitutively active, in the LBD of these receptors a weak autonomous hormone-dependent AF (AF-2) has been identified (GR, Hollenberg and Evans 1988; ER, Tora *et al.* 1989; PR, Meyer *et al.* 1990). Activation function-2 may be formed by several elements, dispersed over the LBD, which are brought together upon ligand binding, as was found for the ER-α and TR-β (Webster *et al.* 1988, Baniahmad *et al.* 1995). A highly conserved region located near the COOH-terminal end of NHRs was found to be essential for hormone-dependent activation in the GR and ER-α (Danielian *et al.* 1992), and for both hormone-dependent transcription activation and relief of silencing by the TR-β (Baniahmad *et al.* 1995). Interestingly, this particular region is lacking in v-*erbA*, a constitutive silencer (Baniahmad *et al.* 1995), and in some orphan receptors that lack AF-2 activity (Danielian *et al.* 1992). Squelching experiments indicated that AF-2 is likely to be involved in interaction with specific coactivators (Baniahmad *et al.* 1995), a supposition, which was consolidated after the cloning of transcriptional coactivators (Horwitz *et al.* 1996). Although the conserved AF-2 region is also present in the AR-LBD, it was previously thought that AF-2 was not active in the AR (Jenster *et al.* 1995). However, TIF2 and GRIP1 (the mouse homologue of TIF2), two transcriptional mediators of ligand-dependent activation of NHRs, were able to induce AR activity (Hong *et al.* 1996, Voegel et al 1996). In addition, Moilanen *et al.* (1997) recently established a ligand-dependent activation function in the AR-LBD, which was weak in mammalian cells, and relatively active when the AR was expressed in yeast. AR AF-2 activity was impaired by presence of the neighbouring hinge region, suggesting that repressor proteins that bind to the hinge region modulate AF-2 activity. The proteins involved, possibly well conserved from yeast to mammals, might be present in relatively high amounts in mammalian cells.
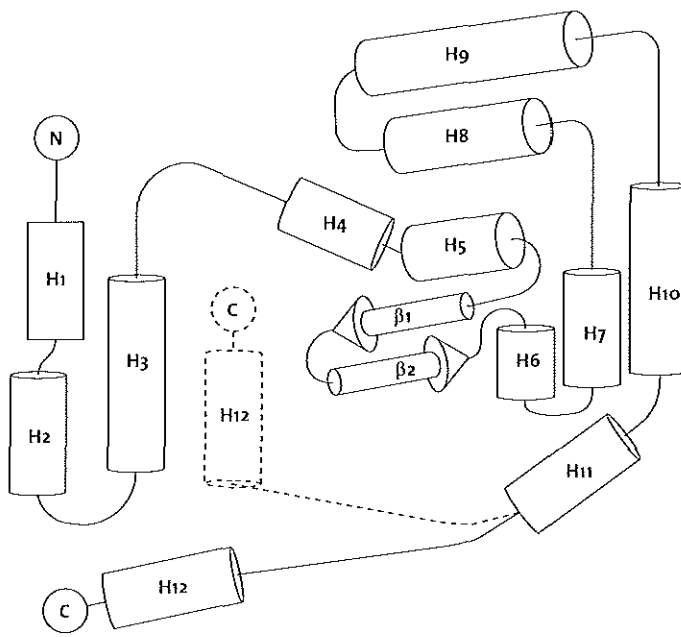
Figure I.4 **Schematic view of the ligand binding domain structure, as predicted for the AR.** An anti-parallel 3-layered α-helical structure, in which helices 4, 5, 6, 8, and 9 are sandwiched between H1 and H3 on one side and helices 7, 10, and 11 on the other side, is predicted. The 12 α-helices are represented by cylinders and the 2 β-strands by arrows. NH2- and COOH-terminal amino acid residues of the α-helices and β-strands, as predicted for the AR, are: H1:Y658-V675 ; H2:? ; H3:A689-K711 ; H4:V721-Y730 ; H5:S731-W742 ; β1:M752-P757 ; β2:P758-N762 ; H6:E763-K768 ; H7:C775-W787 ; H8:P792-L803 ; H9:Q815-K836 ; H10:R846-E863 ; H11:L864-K874 ; H12:E884-S891 (Wurtz *et al.* 1996). Upon ligand binding helix 12 folds back to the ligand-binding pocket.

Structure of the LBD

The structure of the AR-LBD and that of LBDs from other SHRs was probed by limited proteolytic digestion analysis (Allen *et al.* 1992, Beekman *et al.* 1993, Kallio *et al.* 1994, Kuil *et al.* 1994, Lazannec *et al.* 1997). Ligand binding resulted in increased stability towards proteases, indicative of a more compact structure. Hormone-induced conformational changes were also observed in gel-retardation assays. Receptor-HRE complexes showed a faster migration pattern in the presence of an agonist. From both assays it could be concluded that anti-hormones induce different conformations (Allen *et al.* 1992, Kallio *et al.* 1994, Kuil *et al.* 1994).

In 1995, the crystal structure of the unliganded hRXR-α LBD (apo-LBD) and the structures of the hRAR-γ and rat TR-α LBDs, liganded with agonists (holo-LBDs), were resolved (Bourguet *et al.* 1995, Renaud *et al.* 1995, Wagner *et al.* 1995). The LBDs of these receptors contain 12 α-helices and 2 β-strands, which are folded in an α-helical structure of 3 anti-parallel layers, composed of a core region

that is sandwiched between two additional layers (Figure I.4) (Bourguet *et al.* 1995, Renaud *et al.* 1995). Comparison of the structures established for the holo-LBDs of hRAR-γ and rTR-α, and the apo-LBD of hRXR-α, revealed one major difference; the holo-LBDs displayed a more compact structure, as was previously observed in limited proteolytic digestion assays. Ligand binding induces conformational changes mainly affecting helix 12. The AF-2 core region, located in helix 12, folds back to the ligand-binding pocket and comes in close contact with the ligand and helices 3 and 4, thereby forming the 'lid' of the ligand-binding cavity (Wurtz *et al.* 1996). Due to this conformational change, an AF-2 is generated that can interact with coactivators (Renaud *et al.* 1995, Wagner *et al.* 1995). Subsequently, Henttu *et al.* (1997) showed that substitution of lysine residue 366 in the ER - an amino acid residue that is conserved in most NHRs and located in helix 3 - by an alanine residue, resulted in disturbed ligand-induced transcription activation. Interaction with coactivator SRC-1 was disturbed, indicating involvement of helix 3 in formation of the interacting surface (Henttu *et al.* 1997). Although the RXR and the RAR belong to different subfamilies (Laudet *et al.* 1992), their LBDs are folded quite similar. Moreover, the AF-2 core region in helix 12 is conserved in many NHRs involved in ligand-dependent activation. Wurtz *et al.* (1996) therefore proposed that LBDs of other NHR members might display a common structure. Recently, the structure of the ER-α, bound to 17β-estradiol and the tissue specific antagonist raloxifene was solved (Brzozowski *et al.* 1997). The overall structure was indeed similar to that observed for the other holo-LBDs. The binding cavity for 17β-estradiol is formed by parts of H3, H6, H8 with its preceding loop, H11 and H12, and the hairpin that is formed by the β-strands. The selective antagonist raloxifene induced a different position of helix 12, which prevents activation of the AF-2 region. The liganded ER-LBD formed homodimers, mainly through contacts between H11 helices, resulting in a 'head-to-head' arrangement (Brzozowski *et al.* 1997).

Interaction between the NH₂-terminus and the COOH-terminus of the androgen receptor

The COOH-terminal domain of SHRs is involved in dimerization (Kumar and Chambon 1988, Fawell *et al.* 1990, Dahlman-Wright *et al.* 1992, Tetel *et al.* 1997). In addition, upon binding of an agonist, a stable interaction with the NH₂-terminal domain can be established (Kraus *et al.* 1995, Langley *et al.* 1995, Doesburg *et al.* 1997). Indications for such an interaction came initially from several studies. Wong *et al.* (1993) observed that AR dimerization and DNA binding were inhibi-

ted by the NH$_2$-terminal part of the AR in the absence of hormone. From these findings it was concluded that molecular interactions between the NH$_2$- and COOH-terminal parts of the receptor might be involved in DNA binding. In another study, it was observed that the NH$_2$-terminus of the AR lowers the dissociation rate of androgens (Zhou *et al.* 1995). Moreover, the NH$_2$-terminus of the AR contains two TAUs; i.e. TAU-1 and TAU-5 (Jenster *et al.* 1995). TAU-5 becomes activated upon deletion of the AR LBD (Jenster *et al.* 1995), again indicating an interaction between the NH$_2$- and COOH-terminal domain. From deletion mapping studies on the NH$_2$-terminal domain of the AR it was concluded that amino acid residues 141-337 were not involved in the interaction, whereas the regions comprising amino acid residues 14-149 and 338-490, respectively, were indispensable (Langley *et al.* 1995). Apparently, the TAU-1 region (amino acid residues 100-370) is not involved in the NH$_2$- COOH-terminal interaction, whereas TAU-5 (amino acid residues 360-485) is essential (Langley *et al.* 1995, Doesburg *et al.* 1997). Synergism of AF-1 and AF-2 was observed in the ER (Tora *et al.* 1989). Interaction between the NH$_2$-terminal and the COOH-terminal domains might be involved in integration of both AFs. McInerney *et al.* (1997) studied the role of coactivator SRC-1 in the interaction between the NH$_2$-terminus and COOH-terminus of the ER, and concluded that enhancement of ER transcription activation by SRC-1 requires an NH$_2$-terminus that contains an intact AF-1. Interestingly, a functional interaction between the respective domains was established also in the presence of an anti-estrogen, using an expression plasmid encoding the COOH-terminus of the ER fused to the VP16-activation unit (McInerney *et al.* 1997). In addition, coactivators that bind to the AF-2 core region in the COOH-terminus of the AR might be involved in establishment of the interaction. So far, it is not known whether this functional interaction is intra- or inter-molecular, and whether it concerns a direct or indirect interaction. The fact that Doesburg *et al.* (1997) were not able to detect *in vitro* interactions suggests that adapter proteins might be involved.

I.2.5 Transcription activation
Transcription initiation complex
Sequence specific transcription factors, such as SHRs, interact with general transcription factors (GTFs) in the control of gene activation. The GTFs interact with the core promotor elements to induce basal transcription (Beato and Sànchez-Pacheco 1996).

RNA polymerase II (RNA Pol-II) interacts with promoters that contain a TATA-box. To start basal transcription, the GTFs (TFIID, TFIIA, TFIIE, TFIIH, TFIIB) and RNA Pol-II assemble in a transcription initiation complex (TIC). TFIID, composed of TATA-binding protein (TBP) and TBP-associated factors ($TAF_{II}s$), binds to the TATA-box. Subsequently, TFIIB, RNA Pol-II, and TFIIF are recruited. Although these factors can form a minimal TIC, the complex is completed upon binding of TFIIA, TFIIE and TFIIH (Beato and Sànchez-Pacheco 1996). Evidence has been obtained, indicative of the presence of a preformed complex that contains RNA Pol-II and GTFs in cells (Ossipow *et al.* 1995).

Steroid hormone receptors enhance basal transcription activation, either by directly interacting with GTFs or with $TAF_{II}s$. McEwan *et al.* (1997) recently showed that transactivation by the AR involves recruitment of the transcription machinery via TFIIF. *In vitro* and *in vivo* interaction of the NH_2-terminus of the AR with TFIIF was observed. Ford *et al.* (1997) observed interaction between TAU-1 (τ1) of the GR and the TBP-subunit of TFIID. *In vitro* interaction between the PR and $TAF_{II}10$, involving the PR-DBD, was reported (Schwerk *et al.* 1995), and $TAF_{II}30$ interacts with the AF-2 region in the ER (Jacq *et al.* 1994). Apart from these direct interactions between SHRs and TIC, indirect interactions, mediated by transcription intermediary factors or coactivators, are likely to be involved (discussed below).

Interaction of SHRs with other sequence specific transcription factors has been described. Hormone response elements were shown to synergize with response elements for other factors (Schüle *et al.* 1988). Interaction of SHRs themselves with other transcription factors has also been observed; protein-protein interactions between the GR and AP-1 result in mutual modulation of their gene regulatory activities (Beato *et al.* 1995).

Chromatin structure
In eucaryotic cells DNA is wrapped around histones, forming a nucleosome. In this way a nucleosomal structure is obtained, called chromatin. The structure of chromatin is an important determinant of gene regulation (reviewed by Beato and Sànchez-Pacheco 1997, and by Wolffe 1997). Nucleosomes can be destabilized by histone acetylation, causing relaxation of the DNA structure, which facilitates entry of core transcription factors to recognition elements (reviewed by Wolffe 1997). On the other hand, access of these transcription factors can be restricted by histone deacetylation, causing a 'closed' DNA structure (reviewed by Pazin and Kadonaga 1997, and Wolffe 1997). Recently, evidence

was obtained for a role of coactivators and corepressors for NHRs in remodeling of the chromatin structure via recruitment of histon acetylase and histon deacetylase activities, respectively.

Coactivators

Apart from SHRs and basal transcription factors, other factors, called coactivators, are involved in gene activation by steroid hormones. Evidence for the existence of these factors came from squelching experiments (squelching is the phenomenon that a very high expression level of a receptor in transfected cells results in inhibition of the transcription activation function of that receptor or other receptors), indicating that coactivators are present in limiting amounts. Coactivators were defined as factors which, upon ligand binding, interact directly with nuclear receptors. They possess an autonomic activation function, are able to relieve autosquelching of NHRs, and enhance NHR-activity upon overexpression (Horwitz *et al.* 1996, Voegel *et al.* 1996). Potential NHR coactivators have been cloned, and it appeared that several of these have a high level of amino acid sequence identity. Some coactivators, interacting with NHRs, were found to have intrinsic histone acetyl transferase (HAT) activity, whereas others might recruit proteins with HAT-activity. Different (groups of) coactivators, interacting with SHRs, will be discussed below.

A factor which might function as bridging factor, is TRIP1 (TR interacting protein), a homologue of Sug1, a component of the RNA Pol-II holoenzyme (Lee *et al.* 1995). Interestingly, Sug1 was shown to interact with TBP (Swaffield *et al.* 1995) and $TAF_{II}30$ (Vom Baur *et al.* 1996). Mouse Sug1 binds to the AF2-domain of the liganded ER (Vom Baur *et al.* 1996).

Several coactivators are, either directly or indirectly, involved in chromatin remodeling. TIF1 (transcriptional intermediary factor 1) interacts with the ligand activated AF-2 of the ER and PR (Le Douarin *et al.* 1995, Vom Baur *et al.* 1996), and might interact with proteins that influence chromatin structure (Le Douarin *et al.* 1996). SRC-1 (steroid receptor coactivator-1) (Onate *et al.* 1995) and ERAP160 (ER-associated protein) (Halachmi *et al.* 1994) interact with the ER, GR, and PR in the presence of their respective ligands, and enhance transcription activation. SRC-1 was also found to interact with p300/CBP (CREB binding protein) (Kamei *et al.* 1996) and pCAF (p300/CBP-associated factor) (Yang *et al.* 1996), which are proteins that possess intrinsic HAT activity. Recently, SRC-1 was found to have intrinsic HAT activity as well (Spencer *et al.* 1997). TIF2 (transcriptional intermediary factor 2) and GRIP1 (GR-interacting protein 1); (mouse homologue of TIF2)

(Voegel *et al.* 1996, Hong *et al.* 1996) are partially homologous with SRC-1, suggesting the existence of a gene family of nuclear receptor transcriptional mediators (Voegel *et al.* 1996). TIF2 and GRIP1 interact with the AF-2 region in the LBD of the ER, GR, PR, and AR, and contain an activation domain. Another factor related to SRC-1 and TIF2 is RAC3 (receptor-associated coactivator 3) (Li *et al.* 1997) that was shown to interact in a ligand-dependent way with the AF-2 region of non-steroidal NHRs, resulting in enhanced transcription activation.

The protein p300/CBP interacts directly with the LBD of the GR (Kamei *et al.* 1996, Yao *et al.* 1996), and with the coactivators SRC-1 and ERAP160 (Hanstein *et al.* 1996, Yao *et al.* 1996, Spencer *et al.* 1997). It was observed by Smith *et al.* (1996) that SRC-1 and CBP synergize in their stimulation of ER- and PR-dependent transcription. Interestingly, p300/CBP appears to bind to many other transcription factors, such as CREB, c-Fos, v-Jun, c-Jun, and the viral oncoprotein E1a. In addition, interaction with the GTFs TBP and TFIIB was observed (reviewed by Shikama *et al.* 1997). The protein p300/CBP may function as an integrator, which enables regulatory molecules to be recruited and assembled at sites of transcriptional activity (Shikama *et al.* 1997)). Mutations in the *CBP* gene were associated
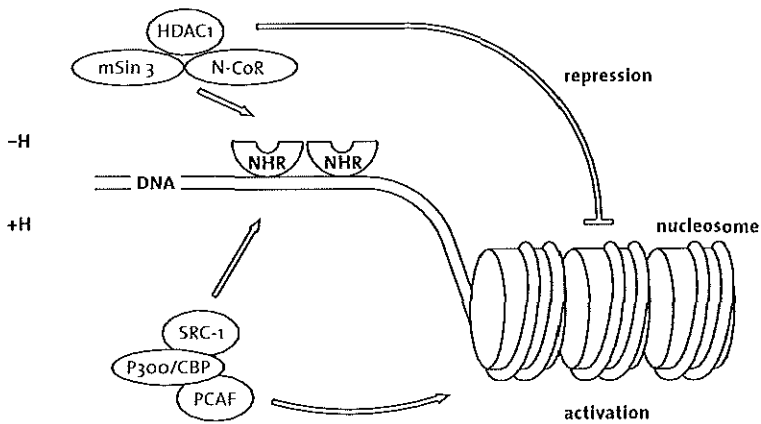
Figure I.5 **Mechanisms of chromatin remodeling.** Upper part: corepressors [for example nuclear receptor corepressor, (N-CoR)] interact with factors such as mSin3, causing histone deacetylation. As a result, basal transcription is repressed. Upon ligand binding (+H), the NHR-corepressor complex dissociates, and coactivators are allowed to bind. Lower part: both non-steroidal NHRs and SHRs tether coactivators to DNA, which possess intrinsic HAT activity (SRC-1, p300/CBP), or interact with other factors, found to have HAT activity (p300/CBP, pCAF) (Based on a model proposed by Heinzel *et al.* 1997).

with Rubinstein-Taybi syndrome, an autosomal-dominant disease, characterized by mental retardation, physical abnormalities, and increased incidence of neoplasia (Petry *et al.* 1995). A general dysregulation of gene expression may underly this syndrome.

As summarized in Figure I.5, SHRs can recruit HAT activity via interaction with coactivators, which can even possess HAT activity by themselves. Although acetylation of histones is an important step with respect to gene activation, it is not sufficient. According to Jenster *et al.* (1997) gene activation by SHRs involves a two-step model. Apart from relaxing the DNA structure, the function of receptors and coactivators is the recruitment of basal transcription factors.

The target protein(s) of several other candidate coactivators is at present unknown. One of them is RIP140 (receptor-interacting protein 140), which enhances transcription activation upon binding to the AF-2 region of the liganded ER (Cavaillès *et al.* 1994, Cavaillès *et al.* 1995). Another example is ARA70 (AR-associated protein), cloned as an androgen-binding protein. Ligand dependent interaction of ARA70 with the LBD of the AR results in 10-fold increased transcription activation (Yeh and Chang 1996). Activation of the PR, GR and ER is only weakly increased.

Corepressors
Some non-steroidal NHRs bind to DNA in the absence of hormone and repress basal promoter activity. The mechanism underlying this way of gene expression was not understood until 1995, when two corepressor proteins were isolated; i.e. N-CoR (nuclear receptor corepressor) (Hörlein *et al.* 1995, Kurokawa *et al.* 1995) and SMRT (silencing mediator for retinoids and thyroid hormone) (Chen and Evans 1995). Corepressor proteins, which are present in the cell in limiting amounts, are tethered to DNA by interaction with non-steroidal NHRs. Upon ligand binding, the corepressor is released, allowing interaction of the receptor with coactivator proteins (reviewed by Horwitz *et al.* 1996). N-CoR was shown to interact with a region in the hinge region of TR, RAR, and TR-RXR, and RAR-RXR heterodimers (Hörlein *et al.* 1995, Kurokawa *et al.* 1995). Interestingly, the type of response element determines the affinity of N-CoR binding (Kurokawa *et al.* 1995). The second corepressor that has been identified is SMRT, a factor interacting with TR, RAR, and RXR (Chen and Evans 1995). Repression results from tethering of histone deacetylases to the DNA at sites close to the responsive element. Both N-CoR and SMRT interact with mSin3A (a homologue of the yeast global transcriptional repressor), which in turn interacts with HDAC1 (histone

deacetylase 1) (Heinzel *et al.* 1997, Nagy *et al.* 1997). Thus, a three-step mechanism can be proposed with respect to transcription regulation by these non-steroidal NHRs. Step one involves the establishment of a repressive chromatin structure, and step two the release of corepressors upon ligand binding, subsequently leading to recruitment of acetyl transferases which disrupt the chromatin template. Finally, step three, the interaction of the AFs of both the receptors and the recruited coactivators with basal transcription factors results in gene activation (Wolffe 1997). Steroid hormone receptors are probably not involved in tethering of histon deacetylase activity to DNA, as they are bound by a hsp complex in the absence of ligand. However, SHRs are inhibitory when they are occupied by antagonists, and Horwitz *et al.* (1996) suggested that corepressors might play a role in this repression. Recently, dominant negative mutations in the *TR*-β gene were associated with persistent SMRT binding in the presence of ligand, while ligand binding was hardly affected (Yoh *et al.* 1997).

A factor that bound to the LBD of the TR, named TRUP (TR-uncoupling protein) inhibited transcription activation by TR and RAR in the presence of ligand. TRUP was shown to inhibit binding of TR-RXR and RAR-RXR heterodimers to DNA (Burris *et al.* 1995).

In Paragraph I.1, a simple model explaining the mechanism of action of SHRs was shown. The picture, however, is getting more and more complicated, which is reflected in the statement by Beato and Sànchez-Pacheco (1996): "SHRs might act as signal integrator; sensing a variety of other signaling systems and at the same time influencing the function of these other pathways".

I.3 Sex differentiation

I.3.1 Mammalian sex differentiation

Chromosomal sex is established at the time of fertilization, when the ovum, containing 22 autosomes and 1 sex chromosome (X) in the female pronucleus, is complemented with 22 autosomes and 1 sex chromosome (X or Y) in the male pronucleus, which is derived from the sperm nucleus. Upon DNA replication and fusion of the female and male pronuclei, a 46,XX or 46,XY zygote is formed. In the presence of a Y chromosome, male gonadal sex is established around day 43-50 of gestation in the human. Analyses of sex chromosomes of 46,XY females and 46,XX males led to the discovery of the *SRY* gene, which could be mapped to

the testis-determining region of the Y chromosome (Sinclair *et al.* 1990, Berta *et al.* 1990). This *SRY* gene triggers the development of the indifferent gonad into a testis (Figure I.6), and mutations in the DNA-binding domain of SRY protein are associated with gonadal dysgenesis in 46,XY males (Berta *et al.* 1990, Jäger *et al.* 1990). On the other hand, overexpression of the mouse homologue of SRY (Sry) in transgenic mice with an XX constitution caused also sex reversal (Koopman *et*

Figure I.6 Sex differentiation cascade
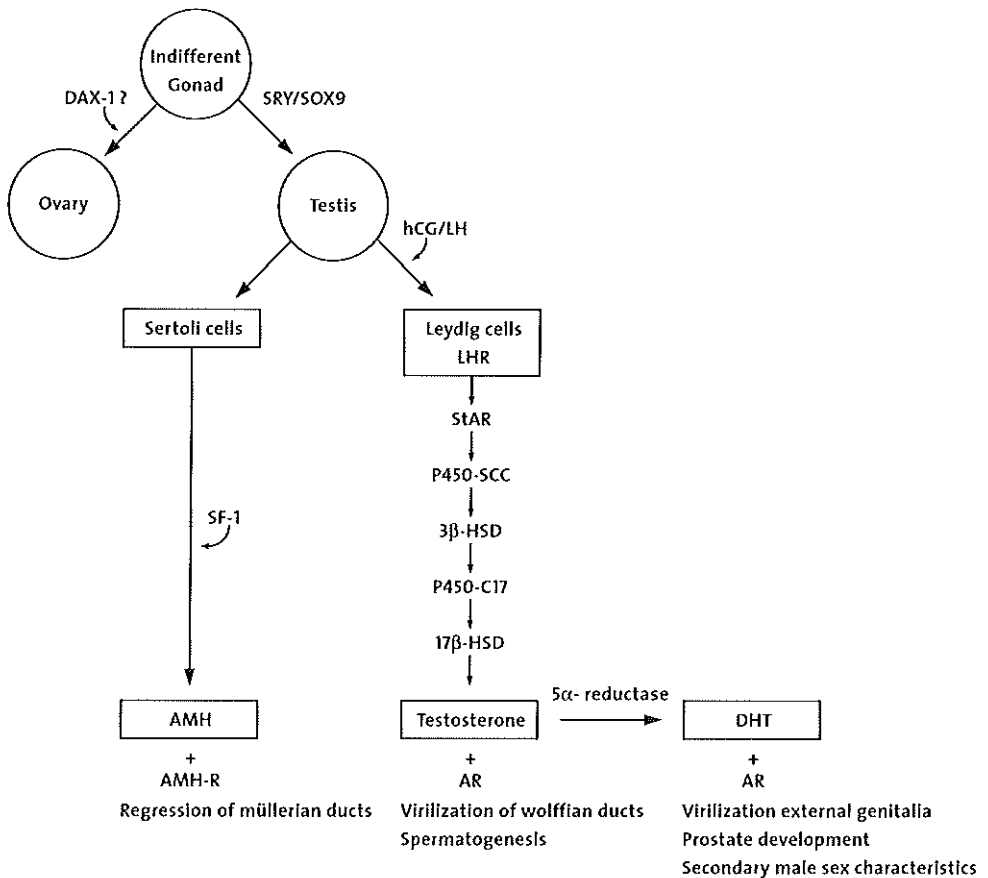DAX-1 : DSS-AHC-critical region on the X gene ; SRY : Sex determining region Y ; SOX 9 : SRY-like HMG box 9 ; LHR : Luteinizing hormone receptor ; AMHR : Anti-müllerian hormone receptor ; StAR : Steroido-genic acute regulatory protein ; P450SCC : Cholesterol side-chain cleavage ; 3b-HSD : 3b-Hydroxysteroid dehydrogenase ; P450-C17 : 17a-Hydroxylase ; 17b-HSD : 17b-Hydroxysteroid dehydrogenase.

al. 1991). The *SRY* gene encodes a DNA-binding protein containing an HMG-box, which is also present in several other transcription factors. On DNA-binding, downstream genes which are involved in sex differentiation are activated. One of the candidate downstream genes is *steroidogenic factor 1* (*SF-1*), encoding an orphan nuclear-receptor protein. The *SF-1* gene product is, among other tissues, expressed in the urogenital ridge prior to differentiation of the gonad (Ikeda *et al.* 1994, Luo *et al.* 1994). Inactivation of the gene encoding the mouse homologue of *SF-1* (*Ftz-F1*) resulted in mice in which adrenals and gonads were lacking (Luo *et al.* 1994). Apart from being involved in formation of steroidogenic organs, SF-1 also plays a more direct role in steroidogenesis (Luo *et al.* 1994). Expression of P450 steroidogenic enzymes is regulated by SF-1 (Clemens *et al.* 1994, Bakke and Lund 1995).

Mutations in several other genes cause various syndromes, with gonadal dysgenesis, indicating that these genes may play a role in development of the gonads. One of them is *SOX9*, an *SRY*-related gene. Mutations in the *SOX9* gene are associated with an autosomal dominant syndrome characterized by skeletal abnormalities (campomelic dysplasia) and gonadal dysgenesis, causing sex reversal in 46,XY individuals (Wagner *et al.* 1994). Mutations in the gene encoding *DAX-1*, a member of the NHR family, result in congenital adrenal hypoplasia and hypogonadotropic hypogonadism (Foster *et al.* 1994, Muscatelli *et al.* 1994). In mice, the gene is expressed at early stages of development of adrenals and gonads. In fetal testes a downregulation is observed, whereas expression continues in developing ovaries (Swain *et al.* 1996). Recently, Swain et al. (1997) suggested that DAX-1 functions as an anti-testis gene.

The fetal testis produces two hormones, indispensable for establishment of phenotypic male sex characteristics (Figure I.6). Sertoli cells produce anti-müllerian hormone (AMH), a member of the TGF-β family of peptide growth/differentiation factors, which is responsible for müllerian duct regression in the male embryo (Josso *et al.* 1977). Expression of AMH is possibly regulated also by SF-1 (Shen *et al.* 1994). In the female embryo, where the ovaries lack production of AMH, müllerian ducts are allowed to develop into female internal genitalia (fallopian tubes, uterus, and the upper part of the vagina) (Conte and Grumbach 1997). AMH overexpression in female transgenic mice caused inhibition of müllerian duct differentiation (Behringer *et al.* 1990). AMH exerts its effects through the AMH type II receptor (Baarends *et al.* 1994, di Clemente *et al.* 1994). Mutations in the gene encoding AMH or the gene encoding the AMH-receptor are associated with persistent müllerian duct syndrome, characterized by the pre-

sence of müllerian derivatives in normal virilized males (Josso *et al.* 1993, Grootegoed *et al.* 1994, Imbeaud *et al.* 1996). Sertoli cells are not only involved in production of AMH, but also stimulate differentiation of Leydig cells from testicular interstitial cells. These Leydig cells, which appear around day 60 of human gestation, start to secrete testosterone. First autonomously, and later under influence of maternal hCG (human chorionic gonadotropin). In the male embryo, the internal genital system (epididymis, vas deferens, seminal vesicles, and ejaculatory ducts) subsequently develops from the wolffian ducts in the presence of testosterone. The male and female internal genital systems are formed from different primordia. In contrast, external genitalia, and prostate and vagina, respectively, develop from the same primordia. In the human, male masculinization of the external genitalia is observed, starting around days 65-77 of gestation. Conversion of testosterone to the more potent androgen DHT by the enzyme 5α-reductase type II in the target tissues is essential (Andersson *et al.* 1991). In the female embryo, the clitoris, labia minora and labia majora (female external genitalia) develop from the genital tubercle, folds and swellings, respectively, and the urogenital sinus becomes the vagina. In the male embryo the presence of DHT results in formation of glans penis, shaft penis and scrotum from genital tubercle, folds and swellings, respectively. The prostate develops from the urogenital sinus under influence of DHT.

The development of secondary sex characteristics is regulated by increased production of sex hormones during puberty. At that time, the pituitary gland is stimulated by gonadotropin-releasing hormone, secreted by the hypothalamus. The pituitary starts in turn to secrete the gonadotropins LH (luteinizing hormone) and FSH, resulting in increased production of sex hormones. In boys, secondary sex characteristics start to develop between 9,5 years and 13,5 years (Marshall and Tanner 1970). Deepening of the voice, appearance of body and pubic hair and spermatogenesis are induced by elevated concentrations of androgens. Usually, the maximum rate of growth is reached when the genitalia are already well-developed (Marshall and Tanner 1970). In girls, puberty starts around the age of 11 years. Breast development, growth of pubic hair (under influence of adrenal androgens), and a growth spurt are observed. The first menarche represents one of the physical changes observed in pubertal girls (Marshall and Tanner 1969). During adult life, gonadotropins and sex hormones are responsible for maintenance and functioning of sex organs in males and females.

I.3.2 Abnormal male sex differentiation

As discussed in the previous section, sex differentiation results from a cascade of events, involving many proteins that are encoded by autosomal- and sex-chromosomal genes. Mutation of these genes can result in aberrant sex differentiation. In general, disorders of sex differentiation can be divided in three categories: true hermaphroditism, female pseudohermaphroditism, and male pseudohermaphroditism. These categories are related to abnormalities in gonadal development and/or gonadal dysgenesis.

Many different forms of gonadal dysgenesis can be observed. Mutations in the *SRY* gene are associated with gonadal dysplasia in both 46,XX and 46,XY individuals. *SOX9* gene mutations can cause, apart from campomelic dysplasia, gonadal dysplasia (reviewed by Conte and Grumbach 1997). True hermaphroditism is seen in individuals presenting with both ovarian and testicular tissue in one or both gonads. The differentiation of the internal genital system and external genitalia is highly variable and depends on the condition and composition of the gonads.

Female pseudohermaphroditism: individuals with a 46,XX constitution presenting with masculinization of internal genital system and external genitalia, although ovaries are present. Congenital adrenal hyperplasia, due to mutations in genes involved in cortisol synthesis [21α-hydroxylase gene *(P450c21)*, *11β-hydroxylase* gene *(P450c11)*], is frequently observed. A high ACTH (adrenocorticotropic hormone) level results in increased adrenal androgen production (Morel and Miller 1991). The degree of masculinization depends on the androgen level and the period of gestation during which the fetus was first exposed to the increased androgen level.

Mutation of the *P450 aromatase* gene, encoding an enzyme involved in conversion of C19 steroids to estrogens, is another cause of female pseudohermaphroditism. An excessive amount of testosterone, resulting from inactive placental P450 aromatase, can cause masculinization of the external fetal genitalia. At the time of puberty, a high testosterone level may cause mild virilization (hirsutism and acne). In addition, absence of breast development, polycystic ovaries, abnormal menstruation, tall stature, and osteoporosis can be observed, because production of estrogens is blocked (Conte *et al.* 1994, Simpson *et al.* 1994).

Male pseudohermaphroditism: 46,XY individuals with testes, showing incomplete masculinization of internal and external genitalia. Several causes, such as Leydig cell hypoplasia, defects in enzymes involved in testosterone synthesis, 5α-reductase type II deficiency, and androgen resistance due to mutation of the *AR* gene, are discussed below.

Male pseudohermaphroditism

46,XY individuals with the persistent müllerian duct syndrome have a rare form of male pseudohermaphroditism. Persistent müllerian duct syndrome is caused either by mutation of the *AMH* gene, or by a defect in the gene encoding the AMH type II receptor, both indispensable for müllerian duct regression (Josso *et al.* 1993, Grootegoed *et al.* 1994, Imbeaud *et al.* 1996). Characteristic for this syndrome, is the presence of a uterus and of fallopian tubes in otherwise normally virilized males.

Leydig cell hypoplasia results in a low rate of production or even absence of testosterone. During the critical period of sex differentiation, testosterone secretion by the Leydig cells is initially autonomous, and thereafter controlled by maternal hCG. After establishment of the pituitary-gonadal axis, this task is accomplished by LH. In some individuals with Leydig cell hypoplasia, mutations were detected in the gene which encodes the LH receptor, through which both hCG and LH are signaling (reviewed by Themmen *et al.* 1997). An inactivating LH receptor mutation can result in different phenotypes, ranging from 46,XY individuals with completely female external genitalia, to individuals who are mildly affected (hypergonadotropic hypogonadism and micropenis). Evidence for autonomously produced testosterone by the fetal Leydig cells came from the observation that androgen dependent structures, epididymides and vasa deferentia, are present in individuals carrying an LH receptor mutation that completely abolished receptor function (Kremer *et al.* 1995, Themmen and Brunner 1996). A different type of mutation of the *LH receptor* gene is associated with male-limited precocious puberty. A mutation of the *LH receptor* gene resulting in constitutive activation of the encoded receptor protein of these individuals, results in continuous stimulation of Leydig cells in the absence of LH (Themmen and Brunner 1996).

Defects in enzymes involved in testosterone biosynthesis are another cause of genital ambiguity in males (Figure I.6). The recently cloned steroidogenic acute regulatory (StAR) protein facilitates transport of cholesterol from the outer to the inner membrane of mitochondria, a rate limiting step in steroidogenesis (Lin *et al.* 1995). Both LH and SF-1 (Clark *et al.* 1994), regulate StAR expression. Mutation of the gene encoding the StAR protein, or one of the genes encoding the downstream enzymes 3β-hydroxysteroid dehydrogenase type II (3β-HSD) and 17α-hydroxylase (P450c17), affect both testosterone and cortisol synthesis (Lin *et al.* 1995, Simard *et al.* 1995, Yanase *et al.* 1991, respectively). As a result, either male or female pseudohermaphroditism, and adrenal insufficiency, are

observed. The enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD) type III is exclusively expressed in the testis (Geissler *et al.* 1994). Mutation of the gene encoding this isotype was identified in 46,XY individuals with unambiguous female external genitalia at the time of birth. During puberty, virilization of the external genitalia is observed. This phenotype closely resembles an incomplete androgen insensitivity phenotype.

The enzyme 5α-reductase is essential for conversion of testosterone to DHT. Both testosterone and DHT activate transcription through the AR. However, DHT binds the AR with greater affinity than testosterone, and is a more potent activator of androgen-responsive genes *in vivo* (Wilbert *et al.* 1983, Deslypere *et al.* 1992). Conversion to DHT and thus amplification of the androgenic response may be essential in peripheral tissues, where testosterone concentrations are lower (Wilbert *et al.* 1983). Alternatively, testosterone and DHT may be involved in regulation of specific genes (Randall 1994a). Two 5α-reductase isoforms have been identified, each showing a tissue specific expression pattern. 5α-Reductase type II, the isoform involved in development of male external genitalia, is expressed in many androgen target tissues (Andersson *et al.* 1991, Wilson *et al.* 1993). Male individuals with 5α-reductase deficiency, caused by mutation of the type II gene, have normal absence of müllerian duct development and normal virilized wolffian duct structures. However, differentiation of the urogenital sinus and genital tubercle is not observed, resulting in absence of the prostate and ambiguous or female external genitalia at birth. Affected individuals are therefore often raised as girls (Imperato-McGinley *et al.* 1974). At puberty, additional virilization results in deepening of the voice, an increased muscle mass, growth of the penis, scrotal development, testicular descend, and affected individuals may even ejaculate. A typical female pubic hair pattern develops (Imperato-McGinley *et al.* 1974). The additional virilization may result from the action of testosterone, because testosterone is available at a high level during puberty. In addition, some testosterone may be converted to DHT by residual 5α-reductase activity and/or by other 5α-reductase isotypes (Randall 1994a).

Inactivating mutation of the *AR* gene resulting in partially or non-responsive androgen target tissues, is the main cause of AIS. The clinical phenotypes and the underlying molecular basis of AIS will be discussed in Paragraphs I.4.1 and I.4.2.

I.4 Androgen insensitivity syndrome

I.4.1 Clinical characteristics

In 1947, Reifenstein reported about families presenting with hypospadias, infertility, and gynecomastia, a syndrome that showed an X-linked pattern of inheritance. A few years later, Morris (1953) described 82 individuals with a female appearance and with testes, a syndrome that he named 'testicular feminization'. Wilson finally proposed that both testicular feminization and Reifenstein syndrome result from end-organ resistance to androgens (Wilson *et al.* 1974). Once the *AR* gene was cloned by several groups, it turned out that mutation of the *AR* gene formed the molecular basis of these forms of male pseudohermaphroditism, called androgen insensitivity syndrome (AIS). AIS is an X-linked disorder (the AR was mapped to Xq11-12), and only 46,XY individuals seem to be affected (Brown *et al.* 1989). Female carriers are unaffected although some delay in puberty has been observed (Quigley *et al.* 1995). Women carrying two affected alleles have never been described, and, as affected males are infertile, this would involve a rare genetic event. The large spectrum of clinical phenotypes observed in AIS will be discussed below.

In individuals with complete androgen insensitivity syndrome (CAIS) due to full inactivation of the AR, testes are present and wolffian ducts are absent. In general, the external genitalia show a female phenotype, and müllerian ducts are usually absent as AMH action in the fetus is normal. However, histopathological studies revealed that in some CAIS patients müllerian duct structures are present (Rutgers and Scully 1991). This may be explained by a high estrogen level in the AIS fetus, which may interfere with AMH action (Ulloa-Aguirre *et al.* 1990). Alternatively, complete AMH action may require androgen action (Quigley *et al.* 1995). At puberty, androgen resistance results in a high LH level and subsequently in an increased testosterone level. Testosterone is in turn aromatized to estradiol, which is responsible for the breast development and the typical female body contour seen in CAIS. Pubic and axillary hair is absent or sparse. In a 46,XY individual with a complete *AR* gene deletion, sparse pubic hair was observed (Quigley *et al.* 1992b); possibly, such sparse hair growth can also occur in the absence of androgens. Individuals with CAIS usually come under medical attention for an inguinal hernia containing a testis, in infancy, or at puberty because of primary amenorrhea.

Partial androgen insensitivity syndrome (PAIS) includes several phenotypes ranging from individuals with a predominantly female appearance (cases with external female genitalia and pubic hair at puberty, or with mild cliteromegaly, and some fusion of the labia), to cases with ambiguous genitalia, or individuals with a predominantly male phenotype (also called Reifenstein syndrome). Patients from this latter group present with a micropenis, perineal hypospadias, and cryptorchidism (non-descended testes). Wolffian duct derived structures may be fully developed or rudimentary in PAIS, dependent on residual androgen activity. At puberty, elevated LH, testosterone and estradiol levels are observed, but in general, the degree of feminization is less as compared to individuals with CAIS. In some publications, it is suggested that the spectrum of phenotypes in AIS should be broadened to include individuals with mild symptoms of undervirilization [mild androgen insensitivity syndrome (MAIS)].

Although severe forms of hypospadias have been described as a feature of AIS (Batch *et al.* 1993b, Ris-Stalpers *et al.* 1994b, Kaspar *et al.* 1993), AR mutations do not seem to be a frequent cause of isolated hypospadias (Hiort *et al.* 1994a, Alléra *et al.* 1995, Sutherland *et al.* 1996).

In several AIS patients having the same AR mutation, genotype-phenotype variation has been observed (Batch *et al.* 1993a, Imasaki *et al.* 1994, Evans *et al.* 1997; see also Table I.1). There are indications that receptor activity is influenced by length of the polymorphic poly-glutamine stretch (Mhatre *et al.* 1993, Chamberlain *et al.* 1994, Jenster *et al.* 1994, Kazemi-Esfarjani *et al.* 1995). In addition, receptor functioning is modulated by additional factors (*e.g.* androgen level, or the expression level of coactivators). It is evident that genotype-phenotype variation is one of the complicating factors with respect to genetic counseling (Morel *et al.* 1994).

I.4.2 Clinical diagnosis, mutation detection, and functional studies

Since the cloning of the AR, molecular biology has greatly improved the diagnostics of AIS. However, as these techniques are very time consuming, clinical endocrinological tests are still indispensable, to exclude beforehand mutation of the *LH receptor* gene, of the genes encoding enzymes involved in testosterone synthesis (*P450c17* gene, *17β-HSD* gene), or of the *5α-reductase type II* gene. These defects may underlie a phenotype resembling AIS, especially in young children. In some individuals with AIS, an elevated testosterone/DHT ratio is measured, although the ratio is still lower than in cases with 5α-reductase type II deficiency. This phenomenon is ascribed to decreased peripheral 5α-reductase

Table I.1 Mutations observed at least 3 or more times in unrelated individuals with AIS.

Mutation	Number of times reported		CpG-site
	CAIS	PAIS	
Arg 598 Gln	-	3	yes
Arg 606 His	4	1	yes
Asn 696 Ser	3	-	no
Asp 723 Tyr	3	-	no
Phe 755 Leu	3	-	no
Ala 756 Thr	4	-	yes
Arg 765 Cys	6	-	yes
Arg 765 His	4	1	yes
Arg 770 Trp	3	-	yes
Met 771 Ile	2	3	no
Gln 789 Glu	-	3	no
Arg 822 Stop	3	-	yes
Arg 831 Cys	-	3	yes
Arg 831 His	-	10	yes
Arg 846 Cys	9	-	yes
Arg 846 His	3	6	yes
Val 857 Leu	-	4	no
Val 857 Met	3	2	yes

According to the *AR* gene mutation database of Gottlieb *et al.* 1997 (release September 1997).
Numbering of the amino acid residues is based on an AR with a poly-glutamine stretch of
20 glutamines, and a polymorphic glycine stretch of 16 residues (Brinkmann *et al.* 1989).

activity, secondary to AIS (Imperato-McGinley *et al.* 1982, Wilson *et al.* 1993). An important clinical test, which can easily be performed and discriminates between CAIS and PAIS, is the SHBG (sex hormone-binding globulin) suppression test (Sinnecker *et al.* 1997). Testosterone in blood is bound (98%) by SHBG (60%) and albumin (38%). Sex hormone-binding globulin is produced in the liver, and in normal males the production is downregulated by androgens. Measuring the SHBG level before and after administration of the anabolic steroid stanozolol, which does not have virilizing potency, provides an indication of AR functioning *in vivo* (Sinnecker *et al.* 1997). This test may be very useful with

respect to sex assignment in children with partial AIS. Holterhus *et al.* 1997 (see also Chapter III) reported an exceptional case presenting with PAIS, in whom the outcome of the SHBG-test and the phenotype did not match, due to somatic mosaicism.

Molecular analysis of the androgen receptor
Most of the mutations that have been found for the AR, are caused by point mutations or small deletions and insertions. Although some mutations were found several times in unrelated individuals (Table I.1) no major hotspots for mutations, as for example is observed in cystic fibrosis (Macek *et al.* 1997), exist in the *AR* gene. This implies that exon- and intron-flanking sequences have to be determined to find mutations (Gottlieb *et al.* 1997). Screening methods like polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (Sheffield *et al.* 1989, De Bellis *et al.* 1994), and PCR single-strand conformation polymorphism (SSCP) analysis (Orita *et al.* 1989, Brüggenwirth *et al.* 1996; see also Chapter II) followed by sequencing, are frequently used for mutation detection in the AR. Both methods allow fast screening, and only small amounts of genomic DNA are needed. Once a mutation has been established, expression plasmids containing cDNA of a mutated receptor can be created, and subsequently functional studies can be performed. DNA binding can be studied *in vitro* by the use of a gel-shift assay, or *ex vivo*, in a promoter-interference assay (Kuil *et al.* 1996). Cotransfection of an AR expression plasmid and a reporter plasmid to Chinese hamster ovary (CHO) cells, or monkey kidney cells (COS-1) provides information about transcription activation capacities of a specific AR. Reporter plasmids carry a gene of which expression can easily be measured, such as the *chloramphenicol acetyl transferase* (CAT) gene, the *luciferase* (Luc) gene, or the *growth hormone* gene. Expression of these genes is regulated by simple or complex androgen-responsive promoter sequences that are cloned in front of the reporter gene. Recently, McPhaul *et al.* (1993a) described an adenovirus-mediated transcription activation assay. Genital skin fibroblasts derived from AIS patients and controls were infected with reporter plasmids, allowing measurement of transcription activation capacities of the endogenous AR in a patient-specific cellular background (McPhaul *et al.* 1993a, McPhaul *et al.* 1997). Although genital skin fibroblasts are no androgen target cells, this assay may be more sensitive than cotransfection studies performed in mammalian cells.

Biochemical studies

Before the cloning of the *AR* gene, only hormone-binding studies were available to establish the diagnosis AIS. Nowadays binding studies are still performed to study receptor abnormalities. Cultured genital skin fibroblasts, a suitable model, are often used. These cells can be obtained relatively easy, and do not contain PRs and ERs (Brown and Migeon 1981). For AR-binding assays, genital skin fibroblasts are cultured in monolayers and incubated with an increasing amount of tritiated hormone. The data obtained can be transformed (Scatchard analysis) to calculate the maximal number of specific binding sites (Bmax) and the equilibrium dissociation constant (Kd). In addition, the hormone-dissociation rate (k) and AR- thermolability can be measured. Based on the results of Scatchard analysis, patients can be classified as being receptor positive or receptor negative. In this latter situation, hormone binding cannot be detected, whereas in receptor-positive AIS either normal, or qualitatively (aberrant Kd) and/or quantitatively (low Bmax) abnormal binding can be observed.

Western blotting followed by immunostaining, is performed to study AR protein expression in transfected cells and in genital skin fibroblasts. The wild type AR appears normally as a 110-112 kDa doublet, although the calculated molecular mass is 98.4 kDa. It is known that the glutamine stretch in the NH$_2$-terminal part of the receptor retards migration of the AR during SDS-polyacrylamide-gel electrophoresis (PAGE); *i.e.* AR protein is more retarded when it contains a longer glutamine stretch. In addition, lengthening of the glutamine stretch causes an increased spacing between the isoforms, indicating that the observed retardation not only results from an increased molecular mass (Jenster *et al.* 1994). The 112-kDa isoform represents a hormone-independently-phosphorylated isoform. Serine 80 and Serine 93, which are in two Ser-Pro directed kinase consensus sites, are essential for the appearance of the 112-kDa isoform, as was shown by amino acid substitution studies (Jenster *et al.* 1994, Zhou *et al.* 1995). Upon incubation of transiently transfected cells or genital skin fibroblasts with androgen (R1881), a third isoform of 114 kDa of which the appearance is correlated with DNA binding, is induced (Jenster *et al.* 1994). This phenomenon was also observed for the PR and ER (Takimoto *et al.* 1992, Lahooti *et al.* 1994). In genital skin fibroblasts of a patient with PAIS, an aberrant hormone-induced upshift was observed (Brüggenwirth *et al.* 1997; see also Chapter VI).

Subcellular localization studies can be performed by immunohistochemistry (Simental *et al.* 1991, Quigley *et al.* 1992a, Jenster *et al.* 1993). Although immunohistochemistry works fine on genital skin sections, it is difficult to perform on

cultured genital skin fibroblasts. Brüggenwirth *et al.* (1997) (see also Chapter VI) therefore studied subcellular localization of the AR by preparing cytosols and tight nuclear-bound fractions which were analyzed by Western-immunoblotting. Recently, Georget *et al.* (1997) studied AR trafficking in living cells using green fluorescent protein derived from the jellyfish *Aequoria victoria* and fused to the AR protein.

Genetic counseling

AIS is not a life-treathening disease, however, because PAIS may lead to severe physical abnormalities that are often causing psychological problems, genetic counseling is offered to AIS families. Once the causative mutation has been
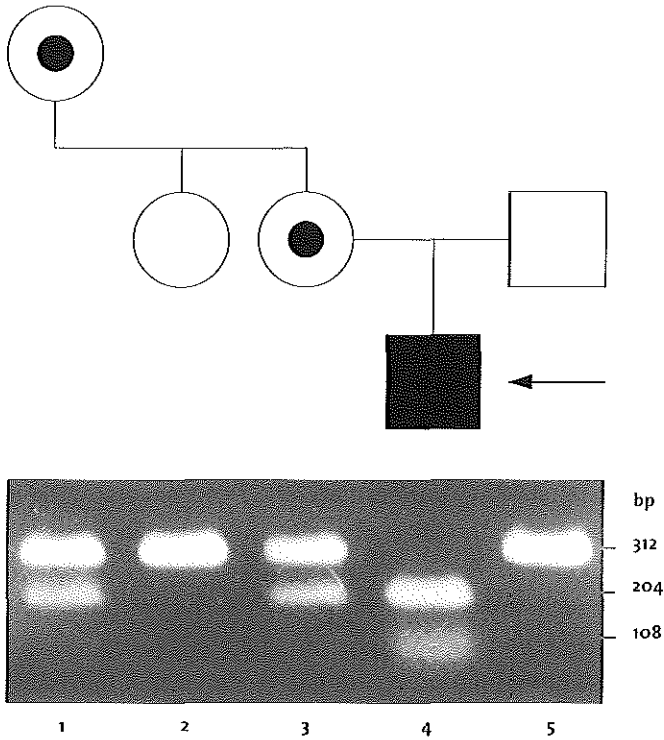
Figure I.7 **Restriction site analysis in a family with CAIS.** Genomic DNA was amplified by the use of exon 2 primers. PCR products were completely digested with *BglII*. Gel-electrophoresis was performed on a 1% agarose gel. In case of a wild type *AR* gene, a 312 bp PCR fragment is visible, whereas in the presence of a mutation the PCR product is digested, and yields 2 PCR fragments of 204 and 108 bp. Details about the patient are described in Chapter V. Lane 1: 46,XX grandmother of the index patient who is a carrier of the mutated gene; lane 2: 46,XX aunt of the index patient; lane 3: 46,XX mother, who is a carrier of the mutated allele; lane 4: 46,XY index patient; lane 5: 46,XY father of the index patient. The index patient is indicated with an arrow.

traced, pedigree analysis can be performed. Some mutations create or destroy a recognition site for a restriction enzyme, allowing rapid screening (for an example, see Figure I.7). Alternatively, intragenic polymorphisms like the highly polymorphic (CAG)nCAA-repeat encoding a glutamine stretch (Sleddens *et al.* 1992), the polymorphic (GGC)n-repeat encoding a glycine stretch (Sleddens *et al.* 1993), and the *Hind*III polymorphism (Brown *et al.* 1989) or *Stu*I polymorphism (Lu and Danielsen 1996), can be used (in combination) as an X-chromosomal marker (Ris-Stalpers *et al.* 1994a, Davies *et al.* 1995). The *Hind*III polymorphism was even applied for prenatal diagnosis in a family with PAIS while the causative mutation had not been identified (Lobaccaro *et al.* 1994). This is not without risk in PAIS families, as ambiguous genitalia can result from mutations in other genes as well (Hughes *et al.* 1994). Therefore, Lobaccaro *et al.* (1994) confirmed the diagnosis by sonography and androgen-binding studies, performed on trophoblastic cells. Boehmer *et al.* (1997) recently described a family, in which the length of the (CAG)nCAA-repeat could not be consistently associated with the disease phenotype, as germ-line mosaicism appeared in the mother. In addition, in X-linked diseases like AIS, a relatively large number of *de novo* mutations are found. This is a complicating factor for pedigree analysis based on polymorphisms, in families with only one affected member (Davies *et al.* 1995).

I.4.3 Mutations found in individuals with androgen insensitivity syndrome

After the cloning of the *AR* gene, many different *AR* gene mutations were detected, especially when compared to other NHRs. There are several explanations for this observation. First, other nuclear hormone receptors are encoded by genes located on autosomes, so the effect of a mutated allele may be compensated by the other allele. However, some mutations, which confer a dominant negative phenotype, may form an exception, as was shown by generating mutations in the ER and PR (Ince *et al.* 1993, Gong *et al.* 1997). Besides, in the majority of individuals with thyroid hormone resistance due to a mutation in one of the alleles encoding the TR-β, an autosomal pattern of inheritance is observed, while subjects with a deletion of one allele do not show clinical abnormalities (Refetoff *et al.* 1993). Dominant-negative receptors may form inactive heterodimers with a wild type receptor. Moreover, mutant homodimers and heterodimers block DNA binding of wild type homodimers. Secondly, mutations in both alleles of many of the other SHRs, causing complete hormone resistance, may be incompatible with life. However, *ER* gene disruption is not lethal, as was

shown by the construction of ER knockout mice which survived to adulthood, and by the fact that a male patient with estrogen insensitivity was found (Lubahn *et al.* 1993, Smith *et al.* 1994). The recently cloned ER-β may compensate for the mutated ER-α, and thereby mask a developmental defect caused by lack of ER-α activity (Kuiper *et al.* 1996, Mosselman *et al.* 1996).

Complete gene deletions or large deletions are a rare cause of AIS; only three individuals with a complete gene deletion have been described, and in two of these cases AIS was accompanied by mental retardation (Trifiro *et al.* 1991a, Quigley *et al.* 1992b, Davies *et al.* 1997a). Amino acid substitutions are most frequently found, and, in general, conservative mutations were less deleterious than nonconservative mutations. The majority of mutations were found in the exons encoding the DNA-binding zinc clusters, and in the 3' half of exon 4 and the exons 5-8 encoding the LBD. This should in part be ascribed to bias caused by the fact that originally mainly patients with abnormal androgen binding were selected and investigated.

CpG dinucleotides
Some mutations were frequently observed in unrelated individuals (for overview see Table I.1). According to Quigley *et al.* (1995) this is not due to a founder effect, as many of the AIS families concerned are of different genetic background. CpG dinucleotides, subject to high mutation rate, have been recognized as a cause of hot spot mutations in hemophilia A (Youssoufian *et al.* 1986). In AIS, a strong association between CpG-dinucleotides and recurrent mutations was also observed (Gottlieb *et al.* 1997).

I.5 Other pathologies related to the androgen receptor

I.5.1 Spinal and bulbar muscular atrophy
Molecular basis of spinal and bulbar muscular atrophy (SBMA)
Several polymorphic repeats are located in exon 1, which encodes the NH2-terminal part of the hAR (Faber *et al.* 1989). A polymorphic poly-glutamine stretch, encoded by (CAG)nCAA, is located between amino acid residues 58 and 77 (numbering of residues is based on an AR consisting of 910 amino acid residues; Brinkmann *et al.* 1989, Sleddens *et al.* 1992). An expanded poly-glutamine stretch is the molecular basis of spinal and bulbar muscular atrophy (SBMA) (or Kennedy's disease) (La Spada *et al.* 1991). In normal individuals the (CAG)nCAA-

repeat contains 9-33 CAGs, whereas 38-75 CAGs are associated with SBMA (Nance 1997). Disease severity is inversely correlated with length of the (CAG)nCAA-repeat (Doyu *et al.* 1993, Igarashi *et al.* 1992, La Spada *et al.* 1992).

Clinical characteristics
Spinal and bulbar muscular atrophy is characterized by progressive muscle weakness and atrophy, and these symptoms are often preceded by cramps. Clinical symptoms usually manifest in the third to fifth decade (reviewed by Nance 1997), and result from severe depletion of lower motornuclei in spinal cord and brainstem, and distal axonopathy of the dorsal root ganglion cells can be observed (reviewed by Robitaille *et al.* 1997). In addition, SBMA patients frequently exhibit endocrinological abnormalities including testicular atrophy, reduced or absent fertility, gynecomastia, and elevated LH, FSH and estradiol levels; these symptoms are also observed in MAIS. Sex differentiation proceeds normally and characteristics of mild androgen insensitivity appear later in life. This may be related to a reduced AR expression level and a lower testosterone level observed in elderly men (Ono *et al.* 1988, Swerdloff and Wang 1993 and references therein). Spinal and bulbar muscular atrophy is an X-linked disease, and only men are affected. In heterozygous women mild symptoms can be observed (Sobue *et al.* 1993, Ferlini *et al.* 1995), indicating that female carriers are protected by (non-) random X-inactivation. Women might as well be protected by a lower androgen level. At present it is not known whether liganded or unliganded receptors are involved in the development of SBMA. However, in two brothers with SBMA, who underwent testosterone therapy for more than 18 months, neither positive nor negative effects of testosterone were observed (Goldenberg and Bradley 1996).

Molecular mechanism underlying the disease
Bingham *et al.* (1995) developed transgenic mouse lines with normal and expanded (CAG)nCAA-repeats in the *AR* gene, as a model to study SBMA. No diseased phenotype was observed, which was ascribed to a low expression level of the mutant protein. From the clinical signs, observed in SBMA, one can conclude that SBMA is likely to result from a combination of a gain-of-function mechanism in motorneurons and a loss-of-function mechanism, causing partial loss of receptor function in androgen target tissues. The fact that neurological symptoms of SBMA are not observed in cases with AIS, which is caused by inactivating mutation of the *AR* gene, points to a gain-of-function mechanism.

However, endocrine abnormalities were also observed in SBMA, indicative of impaired receptor functioning.

Gain-of-function mechanism (1)

Apart from SBMA, six other progressive neurodegenerative diseases are caused by an expanded CAG-repeat that is located in the coding region of the respective gene; *i.e.* Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), the spinocerebellar ataxias (SCA) type 1, SCA type 2, SCA type 3 (Machado-Joseph disease), and SCA type 6 (reviewed by Nance 1997). Although the proteins encoded by the mutated genes [AR, huntingtin (protein product involved in HD), ataxin-3 (protein product involved in SCA type 3), and probably also the other proteins] are widely expressed throughout the body, neuronal tissue is specifically affected (Doyu *et al.* 1994, Schilling *et al.* 1995, Paulson *et al.* 1997). This might be related to the fact that neuronal cells are differentiated and post-mitotic cells, which can not regenerate. Analogous to inactivating mutation of the *AR* gene, targeted disruption of the *HD* gene in the mouse caused a phenotype that was different from a HD phenotype (Duyao *et al.* 1995, Nasir *et al.* 1995). In addition, an individual heterozygous for a deletion in the *HD* gene did not develop a typical HD phenotype (Ambrose *et al.* 1994). Thus, HD is also likely to result from a gain-of-function mechanism.

Theoretically, intragenic expanded CAG-repeats could be pathogenic at DNA, RNA, or protein level. McLaughlin *et al.* (1996) observed increased binding of RNA-binding proteins to RNAs containing expanded CAG-repeats. They proposed that these RNAs might disrupt normal transport in the cell, or titrate away cytoplasmatic proteins that bind to CAGs. Absence of a disease phenotype was observed in transgenic mice that expressed a high level of *HD* transcript, but no huntingtin protein, because protein expression was prevented by a frameshift mutation (Goldberg *et al.* 1996). This proves that DNAs and RNAs containing expanded CAG-repeats are not directly involved in pathogenic interactions. Additional evidence came from Onodera *et al.* (1996), who studied toxicity of fusion proteins in *E.-coli* and observed that only expanded poly-glutamine stretches and not expanded alanine stretches (encoded by GCA, frameshifted CAG) were toxic. Recently, transgenic mice were created that expressed exon 1 of a human *HD* gene, containing an expanded GAG-repeat under control of the huntingtin promoter (Mangiarini *et al.* 1996). Strikingly, this was sufficient to cause a HD-like phenotype. Thus, it is very likely that the underlying mechanism involved an expanded poly-glutamine stretch (Figure I.8).
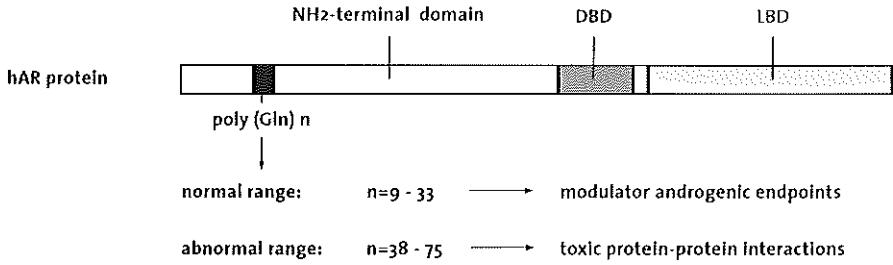
Figure I.8 **Location of the poly-glutamine stretch within the NH$_2$-terminal part of the AR. In normal individuals the stretch is a modulator of receptor activity. Receptor activity is inversely correlated with length of the stretch. An expanded repeat, encoding a longer stretch triggers toxic protein-protein interactions, resulting in SBMA. Mild symptoms of AIS can also be observed in SBMA patients.**

Green *et al.* (1993) proposed that proteins with an expanded poly-glutamine stretch could be a better substrate for transglutaminase, an ubiquitously expressed enzyme that catalyses coupling of glutamine and lysine residues. Kahlem *et al.* (1996) showed that peptides that contained longer glutamine stretches indeed are better substrates (*in vitro*). Accumulation of protein aggregates might be toxic for cells. According to Perutz *et al.* (1994) peptides, containing poly-glutamine stretches might function as polar zippers. Amyloid-like protein aggregates formed *in vitro*, after proteolytic cleavage of GST-huntingtin fusion proteins that contained poly-glutamine stretches of a critical length (Scherzinger *et al.* 1997). Formation of β-sheets by polar zippers may be involved in aggregate formation. Interestingly, the nuclear aggregates were also detectable in neuronal cells of the transgenic mice, which expressed the NH$_2$-terminal part of huntingtin that contained the poly-glutamine stretch (Scherzinger *et al.* 1997). In these transgenic mice, neuronal intranuclear inclusions, containing huntingtin and ubiquitin, were detected (Davies *et al.* 1997b). Intranuclear inclusions and dystrophic neurites, containing huntingtin aggregates, were also present in affected brain regions of patients with HD (DiFiglia *et al.* 1997). The finding that those neuronal intranuclear inclusions were present in symptomatic patients, and absent in presymptomatic patients, indicates that this phenomenon is closely linked to the onset of disease. The presence of ubiquitin, a small protein that tags proteins for destruction (Varshavsky 1997), in these aggregates suggests that they are targets for ubiquitin-mediated proteolysis. Proteolysis seems to be incomplete, and thus the aggregates might interfere with normal neuronal functioning, finally causing HD. It would be interesting to know whether such

aggregates can be found in the affected brain regions of SBMA patients as well. Because many similarities were observed between neurodegenerative diseases, associated with CAG-repeat expansion, a common mechanism might be involved. This disease model does however not explain the different phenotypes.

Loss-of-function mechanism (2)

Endocrine abnormalities observed in SBMA patients were indicative of reduced receptor functioning. Because many transcription factors contain polymorphic glutamine stretches, the question was raised whether the poly-glutamine stretch might be a modulator of transcription activation (Courey *et al.* 1989, Gerber *et al.* 1994). Cotransfection studies demonstrated that the polymorphic poly-glutamine stretch influences transcription activation capacities, although promoter dependent differences were observed (Mhatre *et al.* 1993, Chamberlain *et al.* 1994, Jenster *et al.* 1994, Kazemi-Esfarjani *et al.* 1995). Length of the (CAG)nCAA-repeat was inversely related to transcription activation capacities. Ligand binding (Kd) was not affected (Mhatre *et al.* 1993, Chamberlain *et al.* 1994, Kazemi-Esfarjani *et al.* 1995). From these studies, it was concluded that length of the (CAG)nCAA-repeat might influence intrinsic properties of the receptor. In contrast to these *in vitro* findings, androgen-binding abnormalities were detected in cells derived from SBMA patients. MacLean *et al.* (1995) established abnormal binding affinity in 5 out of 6 cases that were studied. Zhou *et al.* (1995) postulated that the AR NH_2-terminal domain might be involved in receptor stabilization by lowering the dissociation rate of bound hormone and AR degradation. In this way an expanded poly-glutamine stretch might affect the Kd or Bmax. Recently, a functional interaction between the NH_2-terminal and COOH-terminal domains of the AR was established (Langley *et al.* 1995, Doesburg *et al.* 1997). However, Langley *et al.* (1995) showed that expansion of the poly-glutamine stretch from 21 to 66 residues did not interfere with the NH_2-/COOH-interaction. Other groups observed a reduced Bmax in genital skin fibroblasts derived from SBMA patients (Warner *et al.* 1992, Danek *et al.* 1994, Lumbroso *et al.* 1997). AR mRNA expression in the spinal cord of an SBMA patient was decreased as well, in contrast to spinal cord of controls (respectively three subjects with lung cancer and three subjects with amyotrophic lateral sclerosis) (Nakamura *et al.* 1994). These results are difficult to interpret; as quite variable AR expression levels are observed in normal individuals. Transient transfection studies in COS cells and motor-neuron hybrid cells revealed that ARs containing expanded poly-glutamine stretches were expressed at lower levels (Choong *et al.* 1996a,

Brooks *et al.* 1997). However, Tut *et al.* (1997) did not observe these different expression levels. Lower protein expression levels were accompanied by lower mRNA levels (Choong *et al.* 1996a). Therefore it was proposed that the (CAG)nCAA-repeat might interfere with transcription of the *AR* gene, indicating that the mutation also plays a role at the DNA level. On the other hand, expanded poly-glutamine stretches (59-81) caused toxicity in bacteria and COS-cells (Onodera *et al.* 1996), so that a lowered AR expression level probably has to be attributed to increased cell death.

Interestingly, Nasir *et al.* (1995) observed behavioral and morphological changes in heterozygous huntingtin knockout mice. Some of these characteristics were also observed in HD patients, suggesting that a partial loss-of-function mechanism may also be involved in HD.

I.5.2 Prostate cancer
Prostate cancer is the second most frequently observed malignancy in men (Brinkmann and Trapman 1995). Androgens, predominantly DHT, are involved in growth and development of the normal prostate, and also play a role in the initiation of prostate cancer (Coetzee and Ross 1994). Because prostate cancer is often diagnosed at an advanced stage, radical surgery is no longer possible. However, the tumor is initially androgen dependent, and therefore endocrine deprivation therapy can be given. About 80% of the patients initially respond to endocrine deprivation therapy, but ultimately the majority of them show tumor recurrence. Failure of endocrine therapy can be explained by several molecular mechanisms, which are either androgen dependent or independent.

The first androgen independent mechanism involves somatic mutation of the *AR* gene, leading to activation of the receptor by other steroids and anti-androgens. The first mutation was detected in the human prostate carcinoma cell line LNCaP that only expresses the AR but surprisingly showed increased growth rate after culture with estrogens or progestagens (Veldscholte *et al.* 1990). This phenomenon was explained following identification of a mutation in the *AR* gene causing substitution of the threonine residue at position 868 by an alanine residue, resulting in increased binding affinity for estradiol and for progestagens (Veldscholte *et al.* 1990, Ris-Stalpers *et al.* 1993). Even antiandrogens (cyproterone acetate and hydroxyflutamide) were able to induce transcription activation (Veldscholte *et al.* 1992). The discovery of this LNCaP mutation initiated screening of the *AR* gene in tumor specimens. Mutations were rarely observed in primary tumors (Newmark *et al.* 1992, Ruizeveld de Winter *et al.*

1994, Elo *et al.* 1995, Evans *et al.* 1996), with the exception of the study reported by Tilley *et al.* (1996). The majority of mutations were identified in hormone refractory tumor samples (Culig *et al.* 1993b, Castagnaro *et al.* 1993, Gaddipati *et al.* 1994, Suzuki *et al.* 1996) and metastatic lesions (Taplin *et al.* 1995, Suzuki *et al.* 1996). Most mutations mapped to the LBD, which may be related to the fact that in most studies only the DBD and LBD were screened. Tilley *et al.* (1996) reported that 25% of the mutations they identified were located in the NH_2-terminal domain. Only a small part of the mutations were functionally investigated (see Table I.2). Interestingly, some defects were repeatedly identified in unrelated individuals: *i.e.* Val-706-Met (reported twice) (Culig *et al.* 1993b, database Gottlieb *et al.* 1997), His-865-Tyr (reported twice) (Taplin *et al.* 1995, database

Table I.2 **AR Mutations identified in prostate cancer which were functionally characterized**

Exon	Mutation	Remarks	Reference
4	Gly 674 Ala	Normal transcription activation	Koivisto *et al.* 1997
4	Val 706 Met	Broadened steroid-binding specificity	Culig *et al.* 1993a
			Gottlieb *et al.* 1997
5	Arg 717 Leu	Broadened steroid-binding specificity	Elo *et al.* 1995
5	Val 721 Met	Broadened steroid-binding specificity	Newmark *et al.* 1992
			Peterziel *et al.* 1995
5	Val 748 Ala	Normal binding of R1881, but no transcription activation	Gottlieb *et al.* 1997
6	Gln 789 Glu	Inactivating mutation (also associated with PAIS)	Evans *et al.* 1996
8	His 865 Tyr	Broadened steroid-binding specificity	Taplin *et al.* 1995
			Gottlieb *et al.* 1997
8	Thr 868 Ala	Broadened steroid-binding specificity	Veldscholte *et al.*1990
			Gaddipati *et al.* 1994
			Suzuki *et al.* 1996
			Gottlieb *et al.* 1997
8	Thr 868 Ser	Broadened steroid-binding specificity	Taplin *et al.* 1995

Numbering of the amino acid residues is based on an AR with a poly-glutamine stretch of 20 glutamines, and a polymorphic glycine stretch of 16 residues (Brinkmann *et al.* 1989).

Gottlieb *et al.* 1997), Thr-868-Ala (reported 12 times) (Veldscholte *et al.* 1990, Gaddipati *et al.* 1994, Suzuki *et al.* 1996, database Gottlieb *et al.* 1997), and strikingly all of them caused a broadened steroid-binding spectrum (Veldscholte *et al.* 1990, Culig *et al.* 1993a, Taplin *et al.* 1995), indicating that these mutations may play a role in the cancer process (Table I.2) (reviewed by Brinkmann and Trapman 1995). AR mutations in prostate cancer might also reflect genetic instability. Recently, two germ-line mutations were identified in the 5' UTR of the *AR* gene in two individuals with prostate carcinoma (Crocitto *et al.* 1997). One of the point mutations was identified in AR-TIS I [AR-transcription-initiation site I (Faber *et al.* 1993)] and the other mutation in the GC-rich region. The patient with the AR-TIS I mutation had a father and brother with prostate cancer. The authors proposed that these mutations could cause a higher AR expression level. Although a large number of tumors were investigated, AR mutations were rarely observed, indicating that they do not play an important role in development of prostate cancer.

The recent discovery of *AR* gene amplification offers a second, androgen dependent mechanism (Visakorpi *et al.* 1995). Amplification was observed in a substantial part of hormone recurrent tumors but not in primary tumors (Koivisto *et al.* 1997). *Androgen receptor* gene amplification resulted in a higher AR mRNA level, a finding that corresponds with the observation that the majority of androgen independent tumors express a high level of AR protein (van der Kwast *et al.* 1991, Ruizeveld de Winter *et al.* 1994). Increased expression of AR may result in increased sensitivity to low androgen concentrations during therapy, finally causing tumor recurrence (Visakorpi *et al.* 1995, Koivisto *et al.* 1997). Amplification of a gene encoding AIB 1, a SHR coactivator, was observed in breast and ovarian cancer (Anzick *et al.* 1997). Higher expression levels of the usually limiting protein may affect expression of genes, regulated by SHRs, finally resulting in a growth advantage. Amplification of steroid-receptor specific coactivators might be involved in development of prostate cancer as well.

Steroid hormone receptor target genes can be activated through the receptor in the absence of ligand, by cross-talk between SHR pathways and other signal transduction pathways (reviewed by Weigel 1996). Ligand-independent activation was also observed for the AR (Culig *et al.* 1994, Nazareth and Weigel 1996). In contrast, Ikonen *et al.* (1994) and de Ruiter *et al.* (1995) observed potentiation of androgen-dependent activity. Pietras *et al.* (1995) proposed that acquired defects in these signal transduction pathways might contribute to development of hormone independent tumors. Phosphorylation might induce conformational

changes in the LBD of the SHRs, which are also induced upon ligand binding, and activate the receptor through AF-2 (White *et al.* 1997). Ignar-Trowbridge *et al.* (1993) proposed that the estrogen-like effects of epidermal growth factor, observed in the mouse uterus in the absence of estrogens, are due to activation of the AF-1 in the ER NH_2-terminus. It might also be possible that factors, interacting with receptors, are activated by phosphorylation (de Ruiter *et al.* 1995, Bai *et al.* 1997).

Length of the (CAG)nCAA-repeat, a risk factor for prostate cancer

In one prostate tumor, somatic variation of the polymorphic (CAG)nCAA-repeat in exon 1 of the *AR* gene was observed ($CAG_{24}\rightarrow_{18}$) (Schoenberg *et al.* 1994). It is not known whether this mutation was involved in a growth advantage. Recently, evidence was obtained that the (CAG)nCAA-repeat might be a modulator of several androgenic endpoints, even when its length falls within the normal range (9-33 CAGs). Obviously, expanded poly-glutamine stretches in the AR are associated with a partial loss of receptor function in patients with SBMA. Irvine *et al.* (1995) suggested that (CAG)nCAA-repeat length could be a determinant of prostate cancer. In addition, in a small case-control study, a possible positive correlation was found between (CAG)nCAA-repeat length and the age at diagnosis of prostate cancer (Hardy *et al.* 1996). Two large case-control studies were published recently; Giovannucci *et al.* (1997) observed an inverse correlation between (CAG)nCAA-repeat length and a more aggressive form of prostate cancer (587 cases versus 588 controls, mainly Caucasians). A study involving 301 prostate cancer cases and 277 controls (Caucasians) consolidated that length of the (CAG)nCAA-repeat is one of the risk factors associated with development of prostate cancer (Stanford *et al.* 1997).

I.5.3 Breast cancer in males

Breast cancer in males is very uncommon. Clinical conditions involving hypogonadism, which in turn can cause gynecomastia, form an increased risk (Thomas *et al.* 1992). There is evidence supporting a protective role of androgens in breast cancer cells. Androgens inhibit proliferation of the human breast cancer cell line MFM-223, and this effect could be antagonized with antiandrogens, thus indicating that the protective action is through the AR (Hackenberg *et al.* 1991). By increasing the oxidative activity of 17β-HSD in ZR-75-1 human breast cancer cells, androgens induce degradation of estradiol (potent estrogen) to its precursor estrone, which may in part explain the anti-proliferative effects of andro-

gens in these cells. The effect of androgens on 17β-HSD was almost completely reversed by the antiandrogen hydroxyflutamide, indicating that the AR is involved (Couture *et al.* 1993). It is also known that ER levels are downregulated by androgens in ZR-75-1 cells (Poulin *et al.* 1989). Mutations in the *AR* gene, impairing androgen action, might therefore be involved in breast cancer. Indeed, the arginine residue at position 598 (DBD) was found to be substituted by a glutamine residue (Arg-598-Gln) in two brothers with PAIS and carcinoma of the breast (Wooster *et al.* 1992). Lobaccaro *et al.* (1993) screened the AR-DBD of 13 men with breast cancer for germ-line mutations. Surprisingly, the arginine residue at position 599 (adjacent to arginine 598) was substituted by a lysine residue (Arg-599-Lys) in a case with PAIS and carcinoma of the left breast. Both mutations were also found in cases presenting only with partial androgen resistance. The Arg-599-Lys mutation was reported to cause PAIS in a man who had undergone bilateral mastectomy at the age of 13 because of gynecomastia (Saunders *et al.* 1992). This might have protected him from developing breast cancer. The Arg-598-Gln mutation was recently detected in a case with PAIS who has a predominantly female phenotype and is probably at increased risk of developing breast cancer (Hiort *et al.* 1996a). Although both mutations associated with breast carcinoma were germ-line, Hiort *et al.* (1996a) reasoned that somatic mutations in males without AIS might as well be involved in development of male breast cancer. However, after screening tumor material from 11 individuals, no mutations were found.

Arginine 598 is conserved in SHRs and arginine 599 in all members of the NHR-family, indicating that these residues are important for proper receptor functioning. Functional studies revealed that ARs harboring either the Arg-598-Gln or the Arg-599-Lys mutation displayed normal ligand-binding characteristics. Decreased binding to a consensus GRE was observed in a gel-retardation assay, and consequently transcription activation capacities on androgen responsive genes were reduced (Poujol *et al.* 1997). Functional studies were completed with molecular modeling studies, which revealed that the interaction between monomers was disturbed [Poujol *et al.* 1997, for details about modeling, see also Brüggenwirth *et al.* 1998 (Chapter V)]. Large structural alterations of the AR-DBD were not observed. The receptor mutant did not bind to an ERE, which is not surprising because response element specificity is determined by the P-box, located in the first zinc cluster (Green and Chambon 1987, and Freedman *et al.* 1988). Molecular modeling revealed that both affected arginine residues are surface exposed, indicating that they may be involved in protein-protein inter-

actions. Poujol *et al.* (1997) therefore hypothesized that the mutations might confer a gain of function, probably by disturbing interactions with accessory factors. Loss of the protective effect of androgens might also play a role (Lobaccaro *et al.* 1993). However, although a large number of mutations were found in patients with AIS, only two AR mutations were associated with male breast cancer. This implies that mutation of the AR does not play a main role with respect to male breast cancer. On the other hand, patients with a male phenotype who develop gynecomastia will undergo mastectomy, which might protect them.

I.5.4 Other clinical abnormalities related to the androgen receptor
Hirsutism
The condition characterized by excessive hair growth in women in areas other than public and axillary hair is called hirsutism (Rittmaster 1997). Androgens are important with respect to hair growth. This is clearly illustrated by the absence of pubic and axillary hair and absence of beard growth in individuals with CAIS. In addition AIS patients do not develop male type baldness (Randall 1994b). It is known that testosterone is only active in hair follicles after conversion to DHT (Mauvais-Jarvis 1986). In general, hirsutism points to an underlying excessive androgen production, such as can occur in congenital adrenal hyperplasia, Cushing's syndrome, or androgen secreting neoplasms. However, also androgen-independent forms of hirsutism exist. In most cases of hirsutism, a combination of a mildly increased androgen production and increased skin sensitivity to androgens is responsible for the phenotype (idiopathic hirsutism) (Rittmaster 1997). The majority of hirsute women can thus be treated with antiandrogens and/or with 5α-reductase inhibitors.

Increased skin sensitivity might result from increased 5α-reductase activity or from an increased AR expression level (Mauvais-Jarvis 1986, Randall *et al.* 1994b). Mutations that confer a gain of function to the AR might be involved in hirsutism as well. It could be postulated that increased gene activation results from a mutation that renders the AR more sensitive to other steroid hormones, or a mutation that improves the interaction with a coactivator. In this latter case tissue-specific factors might be involved.

Male infertility

A reduced AR level was found in genital skin fibroblasts derived from azoospermic and oligospermic, but otherwise normally virilized males (Aiman and Griffin 1982, Morrow *et al.* 1987). However, in these studies the molecular basis underlying AR impairment was not sufficiently established. A deletion of exon 4 of the *AR* gene was detected in an infertile man (Akin *et al.* 1991). Detailed molecular studies were not performed with this mutant receptor. This finding, however, is very controversial, because two deletions of each 3 nucleotides in exon 4, encoding either an aspartic acid residue at position 681 or an asparagine residue at position 683, respectively, caused PAIS and CAIS. In another study, the coding parts of the *AR* gene of 16 idiopathic oligospermic or azoospermic men were analyzed. No gross deletions and insertions were detected; point mutations were probably missed, as no sequencing analysis or SSCP analysis was performed (Puscheck *et al.* 1994). Interestingly, a mutation was found in exon 5 of the *AR* gene, from a man with severe oligospermia (Yong *et al.* 1994). The asparagine residue at position 718 was changed into a lysine residue. Treatment for 4 months with the androgen analogue Mesterolone resulted in a rise in sperm density, indicating that this ligand could rescue the effect of the mutation. Recently, a relatively long (CAG)nCAA-repeat in the *AR* gene was associated with impaired spermatogenesis (Tut *et al.* 1997).

Osteoporosis in men

Osteoporosis, characterized by low bone mass and microarchitectural deterioration of bone tissue (Consensus Development Conference on Osteoporosis 1993) has a strong genetic component (Christian *et al.* 1989, Pocock *et al.* 1987). Polymorphisms in genes, which encode proteins that might be involved in bone metabolism, were analyzed and related to bone mineral density (BMD). The AR is expressed in osteoblasts (Colvard *et al.* 1989) and in the presence of androgens AR mRNA was upregulated through the AR (Wiren *et al.* 1997). In view of a possible role of the AR in bone metabolism, Brüggenwirth *et al.* (unpublished data) studied the relationship between bone mineral density and length of the polymorphic (CAG)nCAA-repeat in a population of men at the age of 55 years and higher from the Rotterdam study (Hofman *et al.* 1991) (see also Chapter IV). Effects were not observed in the overall population. After stratification to body mass index (BMI) (median) a positive association between (CAG)nCAA-repeat length and bone mineral density was observed in the low BMI group (Brüggenwirth *et al.* unpublished results).

I.6 Aim of the study and scope of the thesis

The AR is a ligand-dependent transcription factor that regulates expression of genes involved in sex differentiation of the male fetus. Postnatal development of male secondary sex characteristics and functioning of the male sex organs depends on androgen action. In the female, androgen action is apparent from axillary and public hair growth, and the AR may also have a role in other processes. Mutation of the gene encoding the AR, is the cause of androgen insensitivity. In addition, expansion of the polymorphic poly-glutamine stretch, encoded by a $(CAG)nCAA$-repeat located in exon 1, is associated with spinal bulbar muscular atrophy, a progressive neuromuscular disease. These defects in AR action are not life-treathening, and subjects with androgen insensitivity or with other pathologies related to the AR provide an opportunity to study the role of the AR in several processes related to sex differentiation and development. Experiments described in this thesis were performed to study structure-function relationship of the AR. Because of the large homology between members of the steroid hormone receptor family, the study of different mutations in the *AR* gene might provide new information relevant for other members as well. Moreover, mutation detection followed by functional analysis is important with respect to counseling and treatment of families with AIS. The present study was focussed on mutations found in the NH_2-terminal part and the DBD of the AR. In the past, these domains were not always extensively investigated. On the one hand because, initially, mainly *AR* genes from individuals with androgen-binding abnormalities were screened. On the other hand, exon 1 is relatively large and GC-rich, which has hampered large-scale screening. Mutations in the NH_2-terminal part and DBD may provide information about transcription activation and/or DNA binding, processes known to be important with respect to normal receptor function. In addition, many potential phosphorylation-sites, which might influence receptor functioning, are located in exon 1.

In Chapters II and III mutations associated with AIS that were identified in the NH_2-terminal domain of the AR are described. Only a small number of mutations was found in this particular domain (Chapter II). All of these mutations resulted in the introduction of a premature stop codon and were the cause of CAIS. Single amino acid substitutions are probably not sufficient to cause a phenotype and can thus be tolerated. In Chapter III a subject in whom genotype and phenotype did not match is described. It turned out that this individual was a somatic mosaic for a mutation in the AR. There are some indications that the

polymorphic poly-glutamine stretch in the NH2-terminal part of the hAR is a modifier of AR activity. In addition, lines of evidence exist, indicating that the AR might be directly involved in bone metabolism. Therefore, length of the poly-glutamine stretch (functional polymorphism) was analyzed in relation to bone mineral density in a sample of elderly men (from the Rotterdam Elderly Study) with either low or high bone mineral density (chapter IV).

In Chapters V and VI, two different mutations, causing CAIS and PAIS, respectively, which both affect DNA binding, are presented. The mutation that caused CAIS was studied by functional assays and 3-D modeling, as reported in Chapter V. The results obtained with both methods were compared. In Chapter VI a very unusual splice acceptor site mutation is discussed. Two receptor variants that were functionally inactive due to disturbed DNA binding were detected. One of them was associated with aberrant androgen-dependent receptor phosphorylation.

In the General Discussion (Chapter VII), the different *AR* gene mutations described in this thesis are discussed, and compared with mutations reported by other groups. The possibility of post-receptor defects, causing an androgen insensitivity-like phenotype, is considered. Finally, the possible influence of the length of the poly-glutamine stretch in the NH_2-terminus of the AR on androgenic end points is discussed.

Molecular Basis of Androgen Insensitivity

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Chapter II
Molecular Basis of Androgen Insensitivity

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Summary

Mutations in the *androgen receptor* gene in 46,XY individuals can be associated with the androgen insensitivity syndrome of which the phenotype can vary from a female phenotype to an undervirilized or infertile male phenotype. We have studied the *androgen receptor* gene of androgen insensitivity patients to get information about amino acid residues or regions involved in deoxyribonucleic acid binding and transcription activation. Genomic DNA was analyzed by PCR-single strand conformation polymorphism analysis under two different conditions. Three new mutations were found in exon 1 of three patients with a female phenotype. A cytosine insertion at codon 42 resulted in a frameshift and consequently in the introduction of a premature stop at codon 171. Deletion of an adenine at codon 263 gave rise to a premature stop at codon 292. In both these cases, receptor protein was not detectable and hormone binding was not measurable. In a third patient, a guanine to adenine transition at codon 493 converted a tryptophan codon into a stop codon. Genital skin fibroblasts from this patient were not available. In exon 2 of the *androgen receptor* gene of a patient with receptor positive androgen insensitivity, a cytosine to adenine transition, converting alanine 564 into an aspartic acid residue, resulted in defective DNA binding and transactivation. In three other receptor-positive androgen insensitivity patients no mutations were found with PCR-single strand conformation polymorphism analysis.

Introduction

Androgens play a major role in male sexual differentiation and development. The actions of androgens are exerted through the AR, which modulates transcription of androgen responsive genes. The AR belongs to a superfamily of receptors for steroid hormones, thyroid hormones and retinoids. Characteristic for the members of this family are the distinct functional domains; the NH_2-terminal domain involved in transcription regulation, a DBD, composed of two zincclusters, a hinge region and the C-terminal LBD (Evans 1988). Mutations in the *AR* gene in 46,XY individuals are associated with AIS, a disorder with a wide spectrum of phenotypes. Subjects with CAIS exhibit a female phenotype, whereas other AIS subjects show a phenotype with ambiguous genitalia, called PAIS. The majority of the mutations reported so far are point mutations, located in the LBD (Brinkmann *et al.* 1992, Sultan *et al.* 1993). AIS subjects with an AR of normal molecular mass and no abnormalities in ligand binding are an interesting group, because they may provide information about essential amino acid resi-

dues or regions, directly involved in transcription activation. Deletion mapping revealed that almost the entire NH$_2$-terminal domain is necessary for full AR transactivating activity (Jenster *et al.* 1995). Therefore, AR mutations interfering with correct receptor functioning may be expected in this domain. However, except for the expanded glutamine stretch, associated with Kennedy's disease, only 6 mutations in exon 1 of the AR have been reported (La Spada *et al.* 1991, McPhaul *et al.* 1991a, McPhaul *et al.* 1991b, Batch *et al.* 1992, Zoppi *et al.* 1992, Hiort *et al.* 1994b). Five of them resulted either directly or indirectly in the introduction of a premature stop codon.

In this study we describe three new exon 1 mutations, all resulting in the introduction of premature stop codons. Zoppi *et al.* (1992) reported a patient in which a single nucleotide substitution introduced a premature stop at codon 60. Synthesis of AR protein was found to be initiated downstream of the termination codon. Therefore, we have investigated whether truncated AR forms were present in genital skin fibroblasts from the patients with an exon 1 mutation. A new mutation was also detected in the first zinc cluster of the DBD of a patient with receptor-positive AIS. Exon 1 mutations, resulting in receptor-positive AIS, were not found. In three other patients with receptor-positive AIS, no mutation was detected by PCR-SSCP analysis.

Subjects and methods
Clinical subjects
- Subject A: 46,XY index patient with CAIS was admitted at the age of 1 yr. because of a bilaterally inguinal hernia. She has a younger 46,XY sister with the same phenotype.

- Subject B: 46,XY patient, diagnosed as having CAIS. The sister of the patient's mother was known with primary amenorrhea.

- Subject C: 46,XY patient, with a complete female phenotype and from a family with more affected members.

- Subject D: 46,XY patient who was diagnosed as having CAIS at birth in the absence of a positive family history. She came to medical attention because of suspected dysmorphology. After further clinical examination the diagnosis AIS was made.

Table II.1 Summary of phenotypes and *AR* gene mutations of index subjects

Subject:	Phenotype:	Mutation:	Position change:
A	cAIS	C insertion	codon 42
B	cAIS	A deletion	codon 263
C	cAIS	TGG → TGA	Trp493Stop
D	cAIS	GCT → GAT	Ala564Asp
E	pAIS	no mutation *	
F	cAIS	no mutation *	
G	cAIS	no mutation *	

The amino acid numbering is based on 910 residues, corresponding with a glutamine stretch of 20 residues and a glycine stretch of 16 residues. * Only screened by PCR-SSCP.

- Subjects without a mutation: all patients displayed a 46,XY chromosome pattern, had no müllerian duct remnants and male hormonal levels were present in the serum. Two of them had the typical phenotype of CAIS and one case has a phenotype with ambiguous genitalia. The phenotype and the *AR* gene mutation of each subject are summarized in Table II.1.

Mutation detection

Genomic DNA, isolated from blood lymphocytes, was screened by PCR-SSCP. Seventeen primer sets for overlapping fragments were used to amplify the coding region and the exon flanking intronic regions of the human AR (hAR). A 15 µl PCR reaction mixture was used, containing 100 ng genomic DNA, 70 ng of each oligonucleotide, 40 µM of each dNTP, 10 mM Tris HCl (pH 9.0), 1.5 mM MgCl2, 5 mM KCl, 10% DMSO (in case of amplifying exon 1), 1.0 µCi [α^{32}P] dATP (Amersham, Little Chalfont, UK) and 0.1 unit Supertaq DNA polymerase (HT Bio-technology LTD). For PCR fragments covering the glycine stretch, 50% deaza dGTP was used with 50% dGTP. Reactions were denatured at 95 °C and subjected to 30 cycles of denaturation at 95 °C for 1 minute, annealing for 2 minutes at different temperatures (Table II.2) and elongation at 72 °C for 1 minute. In Table II.2, oligonucleotides used for PCR amplification of the human AR gene and for direct sequencing are indicated. Samples consisting of 1 µl of PCR product and 9 µl sample buffer (95% formamide, 5% glycerol, 20 mM EDTA, 0.02% bromophenol blue and 0.02% xylene cyanol FF) were denatured before loading on a gel,

Table II.2 **Sequence of oligonucleotides used for PCR-SSCP screening of the human *AR* gene**

Oligo	Location	PCR Fragment	Annealing Temperature °C	Sequence
-70A	5'-UTR, exon1	Exon 1 A*	55	GCCTGTTGAACTCTTCTGAGC
95B	Exon 1			CTTGGGGAGAACCATCCTCA
35A	Exon 1	Exon 1 B	58	TcCGCGAAGTGATCCAGAAC
95B	Exon 1			CTTGGGGAGAACCATCCTCA
80A	Exon 1	Exon 1 C	64	AGCAAGAGACTAGCCCCAGGCAGC
172B	Exon 1			CGGAGCAGCTGCTTAAGCCGGGG
160A	Exon 1	Exon 1 D	62	GCTGCCCCATCCACGTTGTCCCTGCT
250B	Exon 1			ACTCAGATGCTCCAACGCCTCCAC
240A	Exon 1	Exon 1 E	62	TGTGTAAGGCAGTGTCGGTGTCCAT
320B	Exon 1			CGCCTTCTAGaCCTTTGGTGTAAC
305A	Exon 1	Exon 1 F	64	CAGGCAAGAGCACTGAAGATACTGC
385B	Exon 1			GGTTCTCCAGCTTGATGCGAGCGTG
361A	Exon 1	Exon 1 G	58	CGCGACTACTACAACTTTCCACTGG
445B	Exon 1			CACACGGTCCATACAACTG
1A	Exon 1	Exon 1 H	55	TCCTGGCACACTCTCTTCAC
490B	Exon 1			GCCAGGGTACCACACATCAGGT
470A	Exon 1	Exon 1 I	57	GTAGCCCCCTACGGCTACA
1B	Intron 1			CAGAACACAGAGTGACTCTGC
2A	Intron 1	Exon 2	55	GTCATTTATGCCTGCAGGTT
2B	Intron 2			TCTCTCTCTGGAAGGTAAAG
3A	Intron 2	Exon 3	55	TCAGGTCTATCAACTCTTG
3B	Intron 3			GGAGAGAGGAAGGAGGAGGA
4A	Intron 3	Exon 4 A	55	ATTCAAGTCTCTCTTCCTTC
14NB	Exon 4			TGCAAAGGAGTtGGGCTGGTTG
4AA	Exon 4	Exon 4 B	55	CAGAAGCTtACAGTGTCACACA
4B	Intron 4			GCGTTCACTAAATATGATCC
5A	Intron 4	Exon 5	55	GACTCAGACTTAGCTCAACC
5B	Intron 5			ATCACCACCAACCAGGTCTG
6A	Intron 5	Exon 6	55	CAATCAGAGACATTCCTCTGG
6B	Intron 6			AGTGGTCCTCTCTGAATCTC
7A	Intron 6	Exon 7	55	TGCTCCTTCGTGGGCATGCT
7B	Intron 7			TGGCTCTATCAGGCTGTTCTC
8LA	Intron 7	Exon 8	55	AGGCCACCTCCTTGTCAAC
8B	3' UTR, exon 8			AAGGCACTGCAGAGGAGTA

*This PCR product is relatively large and was therefore digested with the restriction enzyme *Pst*I, prior to PCR-SSCP analysis. The CAG(n)CAA-repeat length was studied with primer pair 35A and 95B. Mismatches are indicated with a small letter.

containing 7% acrylamide, 1 x TBE buffer (Tris-borate, pH 8.2, 2.5 mM EDTA) and 5% or 10 % glycerol. Gels were run for 16 hours at 6 Watt either with 0.5 x TBE buffer (5% glycerol gels) or 1 x TBE (10% glycerol gels) at room temperature. Direct sequencing was performed in case an aberrant SSCP pattern was detected. Amplification took place in 100 μl reaction mixtures containing 100 ng genomic DNA, 400 ng of each primer, 200 μM of each dNTP, 10 mM Tris HCl, 1.5 mM MgCl2, 5 mM KCl, 10% DMSO (in case of amplifying exon 1) and 2.5 units Ampli-taq DNA polymerase (Perkin Elmer, Roche Molecular Systems Inc. Branchburg, NY, USA). PCR products were purified from Seakem agarose with Spin-X columns (Costar, Badhoevedorp, The Netherlands) and about 100 ng PCR-product was used as template in the cycle sequencing reaction (Sequitherm kit, Epicenter, Biozyme, Landgraaf, the Netherlands). Primers, developed for PCR-SSCP, were end-labeled with T4 polynucleotide kinase in the presence of $[\gamma^{-33}P]$ dATP (Amersham, Little Chalfont, UK) and used in the cycle sequencing reaction.

Cell culture conditions

Genital skin fibroblasts were cultured in Modified Eagle's medium (MEM, containing 10% fetal calf serum, minimal essential amino acids (Gibco, Life Technologies, Breda, The Netherlands) and antibiotics at 37 °C and 5% CO_2. Fibroblasts were grown to confluence, washed two times with PBS-buffer and cytosols or whole cell lysates were prepared.

Preparation of whole cell lysates

Genital skin fibroblasts were grown to confluence and scraped in 1 ml lysis-buffer [40 mM Tris, 1 mM EDTA pH 7.4, 10 % glycerol, 10 mM dithiothreitol (DTT), 1% (vol/vol) Triton, 0.08% SDS, 0.5% sodium-deoxycholate, 600 μM phenyl-methylsulfonylfluoride (PMSF), 500 μM bacitracin]. After 5 minutes the cell extract was centrifuged for 10 minutes at 4000 RPM at 5 °C. The supernatant was stored at - 80 °C.

Preparation of cytosols

Cells were scraped in 1 ml cytosol-buffer (40 mM Tris, 1 mM EDTA pH 7.4, 10 % glycerol, 10 mM DTT, 10 mM molybdate, and freshly added: 600 μM PMSF, 500 μM bacitracin and 500 mM leupeptin), and homogenized with a Teflon potter. The derived cell homogenates were centrifuged for 10 minutes at 100,000xg, and the supernatants stored at -80 °C until use.
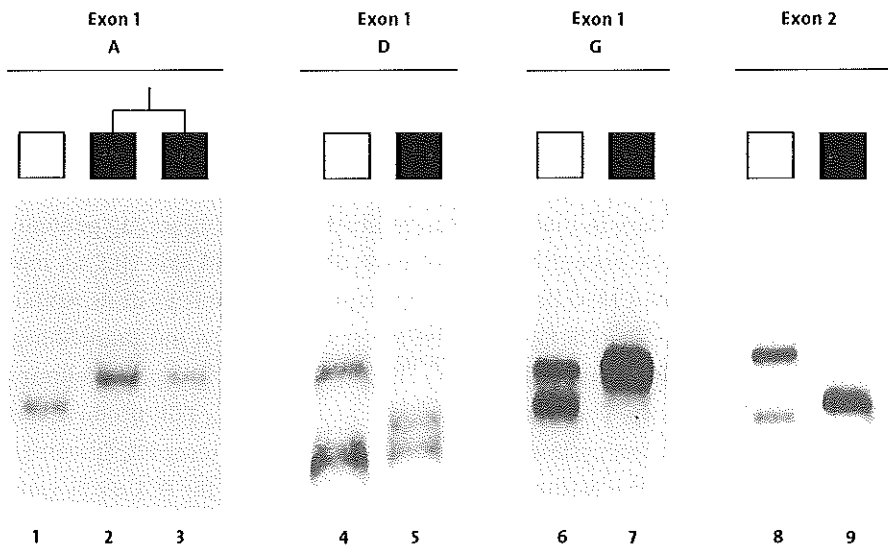
Figure II.1 **PCR-SSCP analysis of AR gene mutations.** SSCP analysis of different parts of exon 1 and of exon 2. Lane 1, wild type AR pattern of fragment A [nucleotides (-70)-(+286)]; lanes 2 and 3, patterns of fragment A of index patient A (2) and her 46,XY sister (3); lane 4, wild type pattern of fragment D (nucleotides 707-958); lane 5, pattern of fragment D of patient B; lane 6, wild type pattern of fragment G, starting at nucleotide 1402 and ending in intron 1; lane 7, SSCP pattern of fragment G of patient C; lane 8, wild type pattern of exon 2 fragment starts in intron 1 and ends in intron 2; lane 9, exon 2 pattern of patient D. Numbers are based upon an open reading frame of 2730 nucleotides (Brinkmann et al. 1989).

Characterization of the AR protein by SDS-PAGE and immunostaining

The AR protein was immunoprecipitated from genital skin fibroblast lysates, with monoclonal antibody F39.4.1, as described before (Ris-Stalpers et al. 1991). After electrophoresis on a 7% SDS-PAGE gel, the proteins were transferred to nitrocellulose and immunostained with polyclonal antibody Sp061. After washing, the membrane was incubated with a second, peroxidase-coupled antibody, to visualize the protein.

Scatchard analysis

For studying the binding characteristics two different assays were used: either a binding assay in which genital skin fibroblast cytosols were used, or a whole cell assay. Cytosols were incubated overnight at 4 °C with increasing concentrations (0 - 0.1 - 0.25 - 0.5 - 1.0 - 2.5 - 5.0 - 10.0 nM) of 17β-hydroxy-17α-[^{3}H]-methyl-4,9,11-estratrien-3-one ([^{3}H]R1881) (NEN-DuPont de Nemours, 's Hertogenbosch, The Netherlands), in the absence or presence of a 100 fold molar excess of non-

labeled R1881 to determine non-specific binding. After protamine precipitation to remove the free steroid, specifically bound [^{3}HR1881] was measured. The total amount of protein was assayed according to the method of Bradford (Bradford 1976).

Genital skin fibroblasts were incubated with increasing concentrations of [^{3}H]R1881 (0.02, 0.05, 0.3, 1.0, 3.0 nM) to obtain a saturation state. The non-specific binding was determined after incubation with a 100 fold molar excess of non-radioactive steroid. After a one-hour incubation period at 37 °C, cells were washed four times with ice-cold 20 nM Tris, 0.15 mM NaCl, pH 7.4. The cells were scraped in 1 ml lysis-buffer (TEG pH 7.4: 20 mM Tris, 15 mM EDTA, 10 % (vol/vol) glycerol, 600 µM PMSF and 500 µM bacitracin). After 10 minutes centrifugation at 800xg the pellet was lysed by adding 1 ml 0.5 N NaOH and a 30 minutes incubation at 56 °C. Scintillation cocktail (5 ml) Clumin (Packard) was added to 500 µl of each sample and ^{3}H-activity was measured in a scintillation counter. The amount of protein was quantified by the method of Bradford. Scatchard analysis was carried out to determine the Kd and the Bmax.

Results

The coding part of the h*AR* gene was screened by amplification of genomic DNA with a set of 17 primer pairs, followed by SSCP analysis, performed under two different conditions. In case of an aberrant PCR-SSCP profile, direct sequencing was performed. Three new exon 1 mutations, all resulting in the introduction of a premature stop codon were detected in the hAR of 3 CAIS patients (Figure II.1). In one receptor-positive patient a mutation was found in exon 2 (Figure II.1). In three other patients, diagnosed as having a receptor-positive form of AIS, no aberrant PCR-SSCP pattern was detected.

Sequence analysis indicated a cytosine insertion at codon 42 of the *AR* gene of patient A resulting in a frameshift and consequently in a premature stop at codon 171 (Figure II.2). In a second CAIS subject (B) a deletion of an adenine at codon 263 of the *AR* gene gave rise to a stop codon at position 292 (Figure II.2). A guanine to adenine transition at codon 493 changed a tryptophan codon (TGG) into a stop codon (TGA) in the *AR* gene of patient C (Figure II.2). The *Kpn*I restriction site, a unique site in AR cDNA was destroyed by the mutation and is informative with respect to carrier detection. Western immunoblotting was performed to investigate if a truncated AR form was expressed in genital skin fibroblasts of subjects A and B. Truncated forms might result from internal reinitiation of translation, proceeding from internal AUG codons (Figure II.2). In case
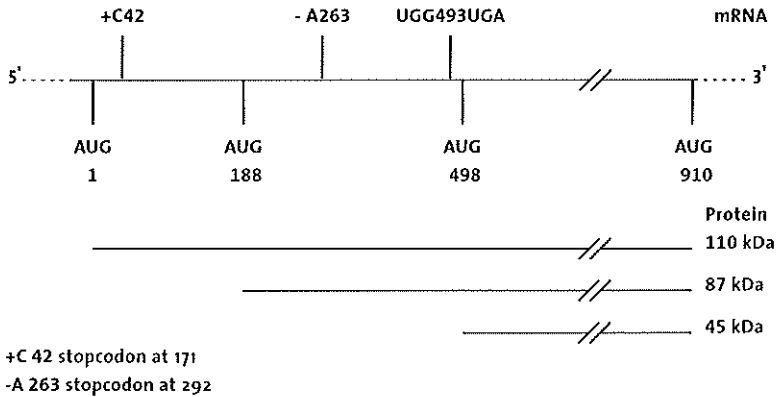
Figure II.2 **Localization of mutations and internal AUGs in exon 1 of the AR gene.** The mutations found in exon 1 of the AR gene are shown; + C: insertion of a cytosine at codon 42, resulting in a premature stop at codon 171; -A: deletion of an adenine at codon 263, resulting in a premature stop at codon 292. At codon 493 a transition of a guanine to an adenine was found, directly resulting in a premature stop codon. Translation start sites and the molecular mass of proteins, resulting from translation initiation, governed by these AUGs, are depicted.

of patient A an AR protein of 87 kDa could not be detected (Figure II.3, lane 2). This corresponds with the observation that ligand binding was not measurable in cytosols, prepared from genital skin fibroblasts of patient A. The stop codon, introduced in the AR of patient B is also located upstream of an internal in frame AUG. However, a shortened AR protein could not be detected on a Western blot, because the epitope is missing (Figure II.3, lane 3). Furthermore ligand-binding studies were negative. Genital skin fibroblasts from patient C were not available.

A new mutation was found in exon 2, which encodes the first zinc cluster of the DBD of the AR. A transition of cytosine to adenine converts an alanine residue at position 564 into an aspartic acid residue. This mutation resulted in defective DNA binding and transactivation, and will be described in more detail elsewhere (see Chapter V).
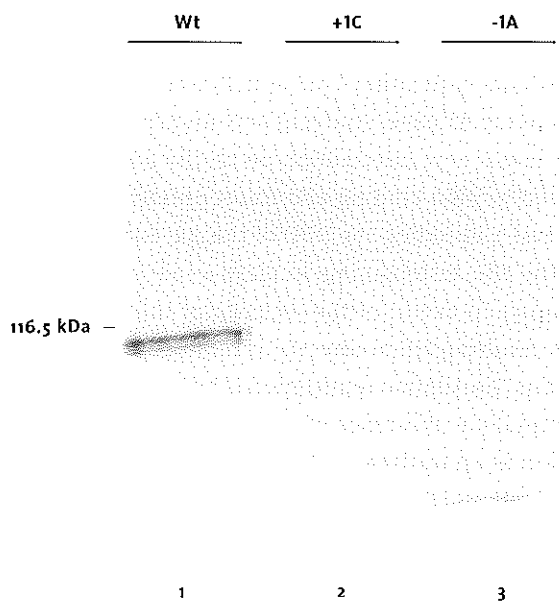
Figure II.3 Western blot analysis of AR expression. AR protein was immunoprecipitated with mono-clonal antibody F39.4.1 from genital skin fibroblast lysates, and separated on a 7% SDS-PAGE gel. After electrophoresis the proteins were transferred to nitrocellulose and immunostained with the polyclonal antibody SP061. The AR protein was visualized with a peroxidase-coupled goat anti-rabbit antibody. Lane 1, Wild type (Wt) AR from control genital skin fibroblasts; lane 2, AR with insertion of cytosine (+1C) from genital skin fibroblasts of subject A; lane 3, AR with deletion of adenine (-1A) from genital skin fibroblasts of subject B. Position of molecular mass markers are indicated on the left.

In two of the patients, diagnosed as having CAIS and in 1 patient with PAIS no mutation was found after screening all the exons and their flanking intronic sequences with PCR-SSCP. All 3 patients were clinically well diagnosed. AR protein was isolated from genital skin fibroblasts, obtained from these subjects, and molecular mass was established after SDS-PAGE and Western blotting. In all cases the AR protein migrated as a normal 110-112 kDa doublet. Receptor characteristics were further studied by Scatchard analysis, from which it became clear that the AR of these three patients displayed normal Kd and Bmax values.

Discussion

Genomic DNA from patients with either CAIS or PAIS was screened by PCR-SSCP analysis for mutations in the *AR* gene. Studying new mutations in receptor-positive AIS patients may reveal new information on amino acid residues in functional domains, involved in DNA binding and transcription regulation. Besides mutations in exons 2 and 3, encoding the DBD of the AR, exon 1 mutations may be expected, because the NH_2-terminal region plays a role in transcription activation (Jenster *et al.* 1995). In exon 1 of the *AR* gene of three CAIS patients we have found mutations resulting either directly or indirectly in the introduction of premature stop codons. Translation reinitiation is known to occur in mammalian cell mRNAs. Mammalian ribosomes can reinitiate translation at an AUG codon, after termination at an upstream site (Liu *et al.* 1984, Peabody and Berg 1986). According to Peabody and Berg (1986) it may be possible that the 40 S ribosomal subunit remains associated with the mRNA, and regains the capacity to scan along the mRNA, until it encounters an initiation codon. At this point, the loose ribosomal subunit becomes associated and synthesis of the polypeptide chain starts again. In the Tfm (testicular feminization) mouse a single nucleotide in a hexacytidine stretch (nucleotides 1107-1112) is deleted in the *AR* gene, resulting in a frame shift and the introduction of a premature stop at codon 412. Downstream of this premature stop codon three AUGs are located, in frame with the premature termination codon. A low level of high affinity binding to androgens and also to DNA could still be measured, which is indicative of reinitiation of translation (Charest *et al.* 1991, Gaspar *et al.* 1991, He *et al.* 1994). The low protein level resulted from instability of the mRNA (Charest *et al.* 1991, Gaspar *et al.* 1991). Zoppi *et al.* (1992) described an AIS patient in which a cytosine to thymine transition, converting a glutamine (CAG) residue at position 60 in the polymorphic glutamine stretch, into a premature stop (TAG). In addition, *in vitro* experiments suggested that internal initiation occurs from the first in frame AUG codon at position 188, resulting in an 87-kDa protein. In genital skin fibroblasts a low, but detectable level of specific androgen binding with an accelerated dissociation rate was measured. In patient A, the premature stop codon was present at codon 171. A shortened protein of approximately 87 kDa, however, could not be detected in genital skin fibroblasts. These results correspond with the observation that specific androgen binding was not measurable in cytosol from the patient's genital skin fibroblasts. In the other two mutant *AR* genes we described, the stop codon occurred after the first internal AUG. No truncated receptor proteins could be detected in genital skin fibroblasts derived from patient B. It is

unclear which factors dictate the efficiency with which such translation reinitiation occurs. To some extent the efficiency of reinitiation may be governed by the position of the termination codon, relative to the subsequent downstream AUG codons (Liu et al 1984, Peabody and Berg 1986, Kozak 1987). In patient A, the new start is located 10 codons downstream of the premature stop codon, whereas in the patient described by Zoppi *et al.* (1992) and in the Tfm mouse, this distance was 128 and 132-134 codons, respectively. This might be the reason why in genital skin fibroblasts from this patient A, no 87 kDa AR and ligand binding were detected.

Two isoforms of the PR have been described, a protein of normal structure (PR-B) and a shortened form (PR-A), lacking 164 amino acids at the NH_2-terminus (Kastner *et al.* 1990). Depending on cell type and promoter context, functional differences between the two forms have been found. PR-A can act as an activator of transcription and as a repressor of transcription by PR-B and even as an inhibitor of transcription, mediated by the glucocorticoid receptor, the mineralocorticoid receptor and the AR (Tung *et al.* 1993, Vegeto *et al.* 1993). Wilson and McPhaul (1994) reported that two forms of human AR, comparable to the PR isoforms, are present in human genital skin fibroblasts. An 87-kDa isoform was postulated, which constitutes 7-15% of the total AR, arising from alternative initiation of translation. We were, however, unable to detect such a 87-kDa protein in genital skin fibroblasts of a control subject.

So far, amino acid substitutions in exon 1, resulting in a receptor-positive form of AIS have not been found. However, recently a proline to leucine substitution at position 339 was found, in DNA isolated from a prostate tumor (Castagnaro *et al.* 1993). No functional studies have been performed as yet to study the significance of this mutation.

The cytosine to adenine transition at codon 564, converting an alanine residue into an aspartic acid residue in the first zinc cluster of the DBD of the AR, resulted in a CAIS phenotype. The phenotype was caused by the defective DNA binding and transactivation capacities of the mutant receptor. Although the correct 3-D structure of this mutant AR is not available, the effect of the mutation can be deduced from the 3-D structure of the glucocorticoid-receptor DBD (Luisi *et al.* 1991). Cysteine 560, histidine 561, and tyrosine 562 are involved either in specific or non-specific contacts with the phosphate backbone of the DNA (Figure I.3A). The amino acid substitution in the mutant receptor might interfere with these contacts, resulting in defective DNA binding.

In the *AR* gene of 3 subjects with an AIS-like phenotype, no mutations were found with PCR-SSCP. Although receptor characteristics appear to be normal, the presence of a mutation, not influencing ligand binding, cannot be excluded. There is still a possibility that some mutations are being missed by the screening procedure we used. However, in our laboratory so far all mutations, that were previously found by direct sequencing, could be confirmed by PCR-SSCP afterwards. AR proteins appeared as normal 110-112 kDa doublets in genital skin fibroblasts and furthermore normal Kd and Bmax values were measured. This does not rule out abnormal protein expression in other target tissues. Another explanation for the androgen resistance syndrome might be the presence of a mutation in a gene, encoding a factor specifically involved in regulation of transcription of AR target genes.

Mosaicism Due to a Somatic Mutation of the Androgen Receptor Gene Determines Phenotype in Androgen Insensitivity Syndrome

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Chapter III

Mosaicism Due to a Somatic Mutation of the Androgen Receptor Gene Determines Phenotype in Androgen Insensitivity Syndrome

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Summary

Premature stop codons of the human *androgen receptor* gene are usually associated with a complete androgen insensitivity syndrome. We, however, identified an adult patient with a 46,XY karyotype, carrying a premature stop codon in exon 1 of the *androgen receptor* gene presenting with signs of partial virilization: pubic hair Tanner stage 4 and clitoral enlargement. No other family members were affected. A point mutation at codon position 171 of the *androgen receptor* gene was detected that replaced the original TTA (Leu) with a premature stop codon TGA (opal). After careful examination of the sequencing gel, however, also a wild type allele was identified, indicating a mosaicism. In addition, elimination of the unique *Afl*II recognition site induced by the mutation was incomplete, thus confirming the coexistence of mutant and wild type androgen receptor alleles in the patient. Normal R1881 binding and a normal 110-112 kDa androgen receptor doublet in Western immunoblots consolidated the molecular genetic data by demonstrating the expression of the wild type receptor in the patient's genital skin fibroblasts. Transfection analysis revealed that only a relatively high concentration of plasmids carrying the mutated androgen receptor complementary DNA lead to expression of a shortened androgen receptor due to downstream reinitiation at methionine 188. Thus, reinitiation does not play a role in the presentation of the phenotype; rather the partial virilization is caused by the expression of the wild type androgen receptor due to a somatic mosaic. We conclude that somatic mosaicism of the *androgen receptor* gene can represent a substantial factor for the individual phenotype by shifting it to a higher degree of virilization than expected from the genotype of the mutant allele alone.

Introduction

The androgen insensitivity syndrome probably represents the most common cause of male pseudohermaphroditism (Quigley *et al.* 1995). Mutations of the *AR* gene are responsible for a variable degree of impaired androgen action. The AIS phenotypes extend over a broad clinical spectrum ranging from external female genitalia through a large group of subjects with incomplete masculinization and ambiguous genitalia to minimal forms with only slightly diminished virilization and/or infertility (McPhaul *et al.* 1993b, Hiort *et al.* 1996b). However, reliable genotype-phenotype correlations do not exist, and the phenotype can vary even in different patients carrying the same mutation McPhaul *et al.* 1993b, Rodien *et al.* 1996, Sinneker *et al.* 1997).

Single base mutations resulting in amino acid substitutions (missense mutations) represent the most common structural defects of the *AR* gene in either PAIS or CAIS (Sultan *et al.* 1993, Brinkmann *et al.* 1995, Quigley *et al.* 1995, Hiort *et al.* 1996b). More extensive structural alterations at the protein level can be due to nonsense mutations inducing the formation of a premature translation termination (stop) codon. This may be the result of either the direct conversion of amino acid codons into stop codons through point mutations (Trifiro *et al.* 1991b, Zoppi *et al.* 1992, Hiort *et al.* 1994b) or the indirect formation after disarrangements of the translational reading frame by frame shift mutations (He *et al.* 1991, Batch *et al.* 1992, Hiort *et al.* 1994b, Brinkmann *et al.* 1995). Based on events on the molecular level [truncation of the AR, reduced AR mRNA level (Marcelli *et al.* 1990, Charest *et al.* 1991), and physiologically insufficient downstream initiation (Zoppi *et al.* 1992)] androgen action is seemingly completely abolished *in vivo*. As expected, in the current literature premature stop codon mutations have exclusively been associated with the CAIS phenotype (Trifiro *et al.* 1991b, Batch *et al.* 1992, Zoppi *et al.* 1992, Hiort *et al.* 1994b, for a review see Quigley *et al.* 1995 and references therein).

To characterize causes for incongruent genotype-phenotype correlations in patients with androgen insensitivity, we investigated an AIS patient with partial virilization of the external genitalia despite the presence of a premature stop codon within the *AR* gene. Functional studies were performed on the DNA level and on the protein level, leading to the elucidation of the underlying molecular mechanism. We discuss the clinical significance of an almost unrecognized and probably underestimated phenomenon for the clinical treatment and the genetic counseling of patients with androgen resistance.

Subjects and methods
Patient
A 23-yr.-old woman of Polish origin contacted her physician because of primary amenorrhea. Her external genitalia were predominantly female. However, clitoral enlargement to 2.0 cm and pubic hair Tanner stage 4 indicated partial virilization (Figure III.1). Orificium urethrae externum and introitus vaginae were separated. No fusion of the posterior labial folds was present. In addition, bilateral axillary hair was observed, and the voice was normal female. Breast development corresponded to Tanner stage 5. Two different karyotype analyses, on blood lymphocytes and on genital skin fibroblasts, displayed a 46,XY karyotype. Serum testosterone (31.2 nmol/l) was in the upper male range, LH (11.0 U/l)
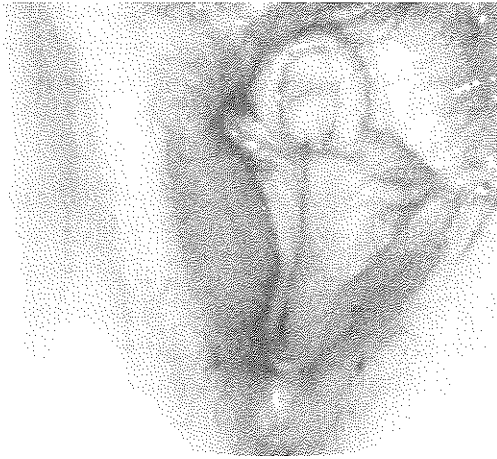
Figure III.1 **External genitalia of the patient, showing pubic hair Tanner stage 4 and clitoral enlargement**

and the LH x testosterone product (343) were elevated (normal LH x testosterone product, < 170). As an estimation of *in vivo* function of the AR, serum SHBG was measured before and after 3 days of oral administration of 0.2 mg/kg body weight/day of the anabolic steroid stanozolol according to a standardized test protocol (Sinnecker *et al.* 1997). Basal SHBG was low but still normal for age (27.8 nmol/l). No decrease in SHBG, but, rather an increase was observed in response to stanozolol. The lowest value corresponded to 112.7% (31.3 nmol/l) of the initial concentration [normal response: 51.4% ± 2.1% (±SE); range, 35.6-62.1%] (Sinnecker *et al.* 1997). On laparoscopy, bilateral ivory-colored testis-like structures each measuring 2-3 cm in length, were demonstrated. No müllerian remnants were detected. To prevent the patient from further virilization, the gonads were removed. Histological examination revealed testis-specific tissue differentiation with atrophic germinal epithelium and Leydig-cell hyperplasia. After gonadectomy, hormone substitution therapy was started with estradiol valerate.

Cultering conditions of cells
All cells were cultured at 37 °C and 5% CO_2. Genital skin fibroblasts of the patient, obtained during gonadectomy, and male control fibroblasts, derived from fore-skin specimens, were maintained in MEM (Life Technologies, Grand Island, NY), supplemented with 10% (vol/vol) FCS, 1% (vol/vol) MEM non essential amino acids (Life Technologies) and penicillin (200IE/ml)/streptomycin (0.2 mg/ml).

COS-1 and CHO cells were cultured in DMEM with the nutrient mix F12 (Life Technologies), 5% (vol/vol) FCS, and antibiotics. For transcription activation studies, CHO cells were plated in medium containing 5% dextran-charcoal-treated FCS.

Androgen binding studies
Androgen binding studies were performed as previously described (Brüggenwirth *et al.* 1996). In brief, confluent cultures of genital skin fibroblasts were incubated with medium containing increasing concentrations of [³H]R1881 (0.02-3.0 nM) in either the presence or absence of a 200-fold molar excess of unlabeled ligand. All incubations were performed in duplicate. After 1 hour at 37 °C, 50 μl culture medium was taken from each dish for determination of total counts. Cell monolayers were washed with 2 ml Tris-saline (20 mM Tris, 0.15 M NaCl, pH 7.4) and scraped in 1 ml TEG buffer [20 mM Tris, 1.5 mM EDTA, 10% (vol/vol) glycerol, 600 μM PMSF, 500 μM bacitracin (pH 7.4)]. After centrifugation of the cell suspensions (10 minutes, 800x*g*), pellets were washed in TEG without protein inhibitors and dissolved in 1 ml 0.5 N NaOH (30 minutes, 56 °C). Five hundred microliters were taken for liquid scintillation counting, 100 μl were used in duplicate for protein determination. Scatchard calculations were performed using Microsoft Excel Personal Computer software (Microsoft Corp., Richmond, WA).

Immunoblot analysis
Western immunoblot analysis was done as described previously (van Laar *et al.* 1989) with only minor changes. Briefly, confluent cultures of genital skin fibroblasts (175 cm²) or transfected COS-1 cells (75 cm²) were lysed in a buffer containing 40 mM Tris-HCL (pH 7.4), 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, 1% Triton X-100, 0.08% SDS, 0.5% sodium deoxycholate, 600 μM PMSF, and 500 μM bacitracin. AR was immunoprecipitated from whole cell lysates by the monoclonal antibody F39.4.1 (Zegers *et al.* 1991) coupled to goat antimouse agarose beads (Sigma Chemical Co., St. Louis, MO). Samples were separated by SDS-PAGE (4% stacking gel, 7% separating gel) for 1 h. at 200 V. Electroblotting on cellulose nitrate membranes was performed for 1 h. at 100 V. After drying and rinsing several times with PBS, 0.1% Tween-20 (Bio-rad, Richmond, CA), the membranes were blocked with PBS, 0.1% Tween-20, and 5% nonfat dry milk (blocking buffer) and subsequently incubated for 1 h. in a moist chamber with primary antibody Sp197 or Sp061 (each diluted 1:1000 in blocking buffer), directed against amino acids 1-20 or 301-320 of the AR, respectively (van Laar *et al.* 1989, Zegers *et al.*

1991). As second antibody, an antirabbit peroxidase conjugate (Sigma) was used in a 1:4000 dilution. Protein detection was performed by chemoluminescence using the Renaissance Western Blot Chemoluminescence Reagent (DuPont-New England Nuclear, Boston, MA).

DNA studies

Genomic DNA was extracted from peripheral blood leukocytes according to standard procedures and served as template for the PCR. Exons 1-8 of the *AR* gene were individually amplified, followed by mutation screening using nonisotopic SSCP analysis as reported previously (Hiort *et al.* 1993, Hiort *et al.* 1994a, Hiort *et al.* 1994b). After detection of an aberrant migration pattern on SSCP, a 414 bp PCR fragment representing segment 2 of exon 1 was purified employing the Qiaquick extraction kit (Qiagen, Hilden, Germany), and directly sequenced using $[\gamma^{-32}P]$-ATP end-labeled primers with the Sequenase sequencing kit (Amersham Buchler, Braunschweig, Germany) (Hiort *et al.* 1993). *Afl*II (New England Biolabs, Beverley, MA) restriction site analysis of the 414 bp exon 1 PCR fragment was done on genomic DNA derived from either blood leukocytes or genital skin fibroblasts according to instructions of the manufacturer. Several controls for wild type AR DNA contamination of the PCR reactions were performed by repeating separate experiments in different laboratories and by using different genomic DNA preparations. Template-free conditions were always included. The samples were analyzed on a 5% glycerol polyacrylamide gel followed by silver staining.

AR expression vectors and transfections

The human AR expression vector pSVAR0 (Brinkmann *et al.* 1989) served as a starting point to create a mutant cDNA construct containing the stop codon TGA at codon position 171. After *Afl*II digestion of pSVAR0, the overhanging 5'- ends were blunted by a 15 min. incubation with 0.06 U/μl S1 nuclease (Pharmacia). This was followed by *Bam*HI digestion, thus removing a 2318 bp *Afl*II/S1-*Bam*HI fragment from the vector. Two oligonucleotides (5'- TGAACTAGTCGATG-3' and 5'- GATCCATCGACTAGTTCA-3') were hybridized to construct a linker containing the TGA stop codon, an additional *Spe*I-site, and a *Bam*HI 5' overhanging end. Subsequently, the linker fragment was cloned into the prepared vector. In-frame ligation was verified by plasmid sequencing. In a second cloning step, the above mentioned 2318 bp fragment was recloned into the first step product. The latter was pretreated before ligation by *Spe*I digestion followed by blunting of the 5' overhanging *Spe*I end (S1 nuclease) and an additional *Bam*HI digestion. The final
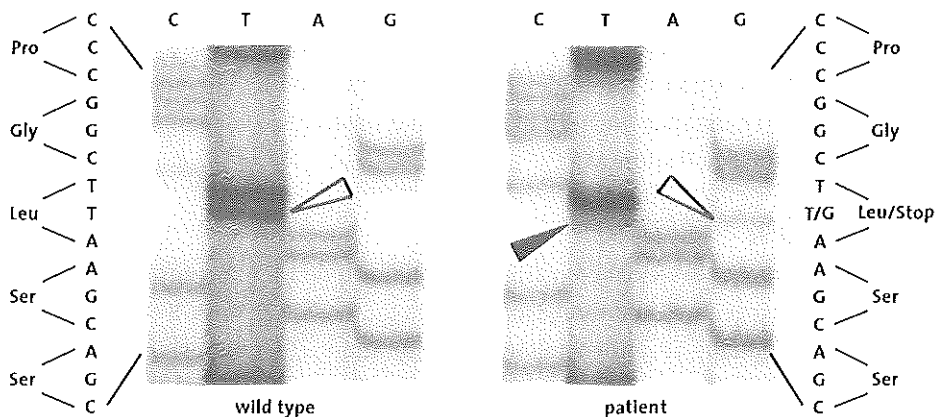
Figure III.2 **DNA-sequencing of genomic DNA PCR-amplified fragments encompassing codons 169 to 173 of the AR gene.** The left panel represents a male control DNA; the right panel belongs to the index patient. In codon 171, a point mutation leads to a substitution of a T by a G, thus creating a premature stop codon (marked by open arrows). The black arrow indicates the additional presence of the wild type T-band in the patient's DNA.

construct pSVAR171-stop was verified for the correct sequence by plasmid sequencing. The construction of the expression vector pSVAR121, representing an NH$_2$-terminal-deleted AR cDNA encompassing codons 188 to 910, was published before by Jenster *et al.* (1995). COS-1 and CHO cells were maintained as described above and transiently transfected by the calcium-phosphate precipitation method (Chen and Okayama 1987). For transcription activation studies, CHO cells were cultured in 10-cm^2 six-well multidishes, using 12.5-2,500 ng AR expresion vector/dish (50-10,000 ng/ml precipitate), 0.5 µg (2 µg/ml) of the reporter plasmid Luc, regulated by the mouse mammary tumor virus promoter (MMTV) (Organon, Oss, The Netherlands) (de Ruiter *et al.* 1995) adjusting to a final amount of 5 µg (20 µg/ml) plasmid DNA with the pTZ19 carrier plasmid. Transfections were performed in triplicate in three independent experiments (concentrations 100 ng-10,000 ng/ml precipitate: two experiments, each performed in triplicate). Twenty-four hours before cell lysis, cells were incubated with medium containing either no hormone or 1 nM R1881 (DuPont-New England Nuclear). Luciferase activity was determined as previously described (de Ruiter *et al.* 1995). For studying expression of the AR, 75 cm^2 subconfluent cultures of COS-1 cells were transfected with 1.5 µg (1µg/ml) expression plasmid (pSVAR0, pSVAR121 or pSVAR171-stop, respectively), adjusted to a final amount of 30 µg (20 µg/ml) with the carrier plasmid pTZ19. Twenty-four hours after transfection,

cells were glycerol shocked by a 1.5 min.-incubation with 15% glycerol-MEM without FCS. Whole cell lysates were generated another 24 h. later preceding immunoprecipitation of the AR.

Results
Mutation detection analysis
All eight exons of the *AR* gene of the patient's genomic DNA were successfully amplified by PCR and resulted in PCR fragments of the predicted length (Hiort *et al.* 1994b). Exons 2-8 were individually amplified in a single step, exon 1 was divided into seven overlapping segments. Because of an abnormal migration pattern on SSCP analysis, the 414-bp segment of exon 1 of the gene was sequenced. At codon position 171 (numbering according to Brinkmann *et al.* 1989), a point mutation TTA to TGA, was detected that replaced the original leucine residue and created a premature stop codon (opal; Figure III.2), thus eliminating a restriction recognition site for the enzyme *Afl*II. In addition to the mutant DNA sequence, also the wild type DNA sequence was observed (Figure III.2). To confirm these data, an *Afl*II restriction recognition site analysis of the respective 414-bp PCR fragment was performed. Restriction of PCR products from normal controls lead to formation of two fragments, 205 bp and 209 bp in length, respectively. In separate DNA preparations from peripheral blood leukocytes and genital skin fibroblasts of the patient, however, the PCR products were partially digested (Figure III.3, lanes 2 and 4, respectively). Hence, sequencing data and restriction site analysis both demonstrate the presence of mutant and wild type AR DNA sequences in the patient's blood leukocytes and genital skin fibroblasts. Because karyotype analyses excluded chromosomal mosaicism, and sequencing of the CAG-repeat in exon 1 supplemented the presence of only one X-chromosome, mosaicism due to a somatic mutation of the *AR* gene was considered.

AR binding characteristics
Androgen binding properties were studied in a whole cell assay of genital skin fibroblasts using the synthetic androgen methyltrienolone (R1881). In Figure III.4, a Scatchard plot is presented that demonstrates specific R1881 binding of genital skin fibroblasts of the patient. The calculation of the Bmax revealed a value of 22.56 fmol/mg protein, which is below the normal male range (39.0-169.0 fmol/mg protein). A normal Kd of 0.063 nM was found (normal male range, 0.03-0.13 nM). This result indicates the expression of an AR containing a functionally intact androgen-binding domain.
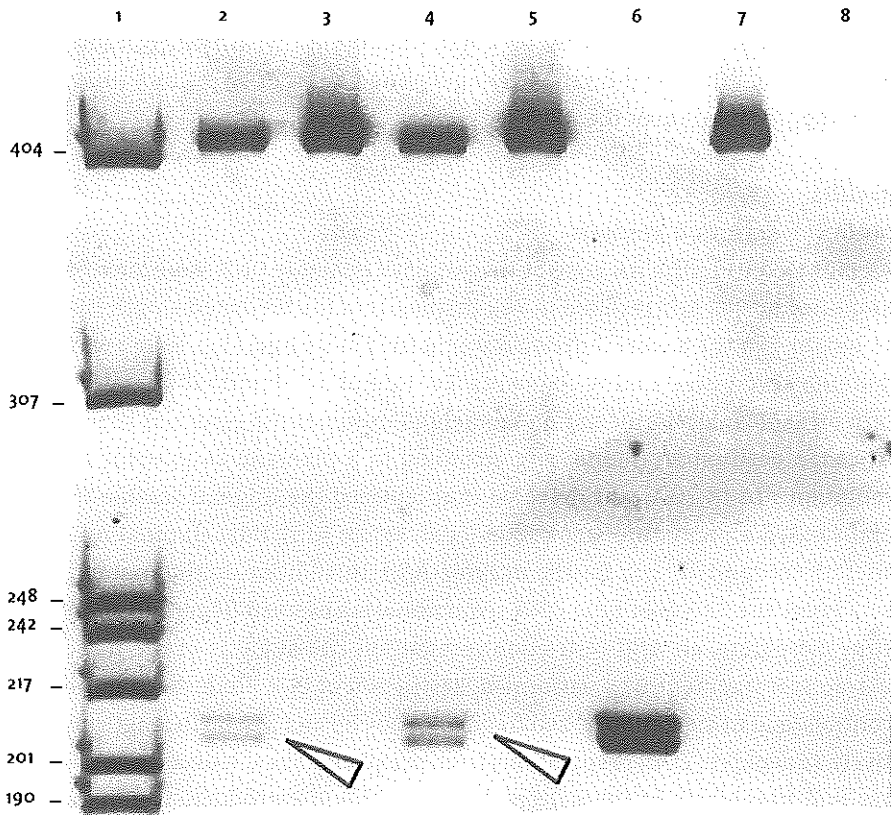
Figure III.3 AflII restriction site analysis of 414-bp genomic DNA PCR fragments derived from the patient (blood leukocytes, genital skin fibroblasts) and a male control (blood leukocytes), respectively. Lane 1, marker (base pair numbers are indicated on the left); lane 2, blood leukocyte DNA (patient), *AflII*-digest; lane 3, blood leukocyte DNA (patient) undigested; lane 4, genital skin fibroblast DNA (patient), *AflII*-digest; lane 5, genital skin fibroblast DNA (patient), undigested; lane 6, blood leukocyte DNA (control), *AflII*-digest; lane 7, blood leukocyte DNA (control), undigested; lane 8, no template. Partial digestion of the patient's DNA is indicated by open arrows and confirms the coexistence of wild type and mutant AR gene sequences.

Western immunoblot analysis on genital skin fibroblasts

Investigation of AR expression in the patient's tissue was performed by immunoprecipitation of the AR from whole cell lysates of equally grown confluent cultures of genital skin fibroblasts preceding Western immunoblot analysis. Using monoclonal antibody F39.4.1 for immunoprecipitation and polyclonal antibody Sp061 for immunodetection on cellulose nitrate membranes, a normal 110/112-kDa AR doublet was found in the genital skin fibroblasts of the patient
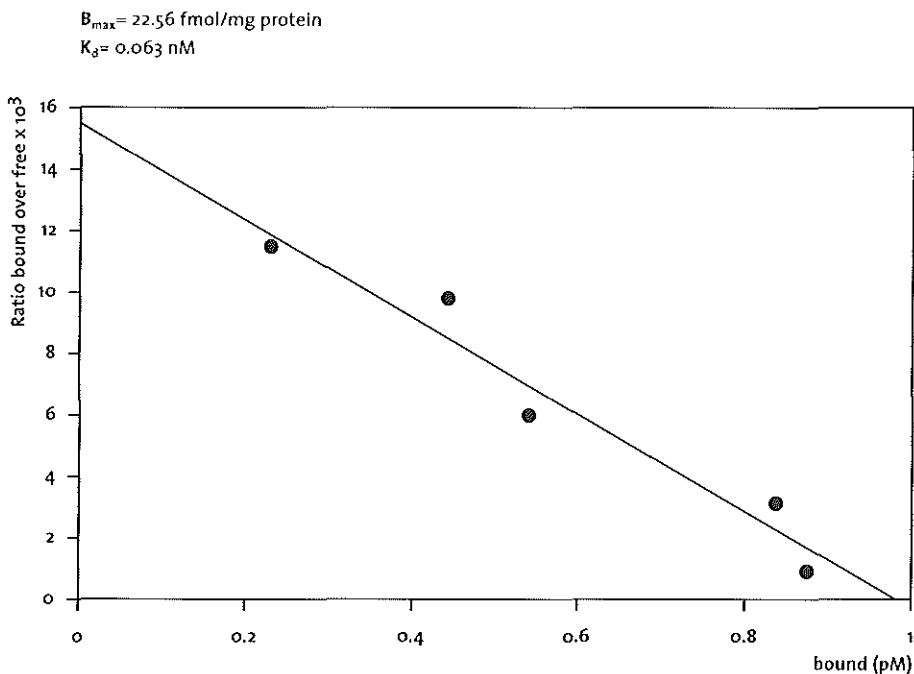
$B_{max} = 22.56$ fmol/mg protein
$K_d = 0.063$ nM

Figure III.4 Scatchard plot representing R1881 binding on genital skin fibroblasts of the patient. Specific androgen binding, normal Kd and low Bmax.

(Figure III.5, lane 3), indicating the expression of the wild type AR. The signal intensity of the 110/112-kDa band in the patient was always severely reduced in comparison to the male control genital skin fibroblast strain (Figure III.5, lane 2 vs. lane 3). This is in accordance with the low Bmax observed in the Scatchard analysis and could be an indication of a reduced amount of wild type AR expressed in a given population of genital skin fibroblasts of the patient. However, any quantitative interpretation should be restrained because the ratio of fibroblasts in tissue culture containing either the mutant or the wild type AR allele does not necessarily reflect *in vivo* conditions.

Expression study of pSVAR171-stop, pSVAR121, and pSVAR0

AR expression using pSVAR171-stop, pSVAR121 or pSVAR0 expression plasmids has been investigated in transiently transfected COS-1 cells. Antibody F39.4.1 has been used for immunoprecipitation, whereas either Sp197 or Sp061 served for immunodetection on Western blots. Figure III.5, lane 1, illustrates the formation of a normal 110/112-kDa AR after transfection of the wild type AR construct pSVAR0. Construct pSVAR121, encoding amino acids 188-910 (Jenster *et al.* 1995), demonstrated the expression of the predicted NH_2-terminal-deleted 87-kDa AR
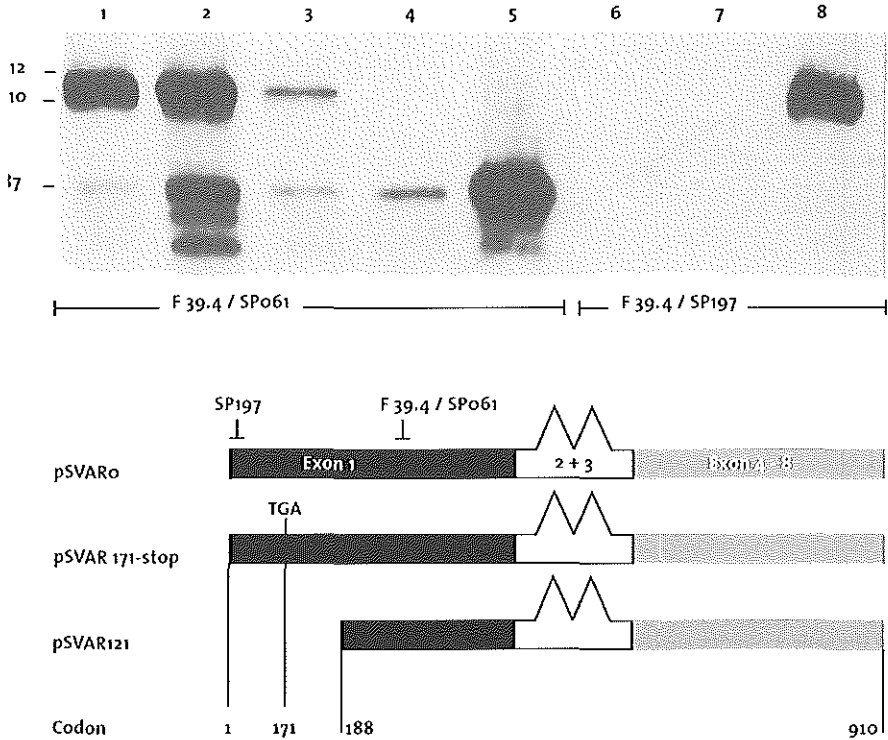
Figure III.5 **Western immunoblot analysis.** AR was immunoprecipitated from whole cell lysates from either genital skin fibroblasts or transfected COS-1 cells using the monoclonal antibody F39.4.1. Immunodetection on Western blot was performed by Spo61 (lanes 1-5), and Sp197 (lanes 6-8), respectively. Epitopes for antibodies and transfected constructs are illustrated in the scheme (see also in the text). Lane 1, COS-1 cells transfected with pSVARo [1/20th lysate from one 75-cm² culture flask (cf)]; lane 2, genital skin fibroblasts, control male (entire lysate from one 175-cm² cf); lane 3, genital skin fibroblasts (patient) (entire lysate from one 175-cm² cf); lane 4, COS-1 cells transfected with pSVAR171-stop (1/20th lysate from one 75-cm² cf); lane 5, COS-1 cells transfected with pSVAR121 (1/20th lysate from one 75-cm² cf); lane 6, COS-1 cells transfected with pSVAR171-stop (1/20th lysate from one 75-cm² cf); lane 7, COS-1 cells transfected with pSVAR121 (1/20th lysate from one 75-cm² cf); lane 8, COS-1 cells transfected with pSVARo (1/20th lysate from one 75-cm² cf). Lane 3 demonstrates the expression of 110/112-kDa wild type AR in the patient. The signal intensity is considerably reduced compared with that in the control male cell line in lane 2. Lane 5 shows the expression of an 87-kDa AR fragment by pSVAR121 that cannot be recognized by Sp197 (lane 7). The same pattern is seen in case of pSVAR171-stop (lanes 4 and 6, respectively), and is consistent with downstream initiation of translation of an 87-kDa NH2-terminally truncated AR using Methionine 188 as start codon.

fragment using Sp061 in immunodetection (Figure III.5, lane 5). In accordance, no signal could be obtained using antibody Sp197 (Figure III.5, lane 7). Remarkably, a weaker band following the same pattern in migration (87 kDa) and immunodetection (Sp061, signal; Sp197, no signal) as pSVAR121 was found for pSVAR171-stop (Figure III.5, lanes 4 and 6, respectively), indicating the expression of an 87-kDa AR fragment, most likely caused by downstream initiation of translation at Methionine 188. The 87-kDa band that is visible in lane 3 representing the patient's genital skin fibroblasts, is also consistent with downstream initiation, because no 87-kDa signal could be obtained in a different blot using antibody Sp197 (data not shown). Whether the smaller bands in lane 2 representing the control fibroblast strain are also caused by the use of alternative start sites or by proteolysis has not been investigated.

Transcription activation studies
To exclude the possibility that partial virilization of the patient could have been significantly influenced by the NH_2-terminal-truncated AR caused by the mutant AR allele, transcription activation properties of pSVAR171-stop compared with those of pSVAR121 and pSVAR0 were investigated. Induction of the MMTV-Luc reporter plasmid using transiently transfected CHO cells was measured in either the presence or absence of 1 nM R1881. Maximum reporter gene induction by the wild type AR plasmid pSVAR0 was found using 12.5 ng expression plasmid in transfections of 10-cm^2 cultures of CHO cell; a 46-fold induction (range, 45.2-46.8) relative to basal activity in the absence of R1881 was observed (Figure III.6). Under these conditions, no significant transcription activation of the reporter gene by pSVAR171-stop was present (1.4-fold; range, 0.4-2.6; Figure III.6). Only at highly elevated concentrations of pSVAR171-stop, partial transcription activation of MMTV-Luc could be observed, with a maximum induction of 13-fold (range, 10.3-15.7) using 1250 ng expression plasmid (5 µg/ml precipitate; Figure III.6). With respect to the AR expression studies (Figure III.5), this activity is most likely due to the NH_2-terminal- truncated 87-kDa AR caused by downstream initiation. The pSVAR121 expression plasmid represents the same NH_2-terminal-truncated AR as the downstream initiation product caused by pSVAR171-stop. Partial transcription activation of the reporter plasmid was observed using pSVAR121 (6.8-fold; range, 5-8.4), corresponding to previously published data (Figure III.6) (Jenster *et al.* 1995).
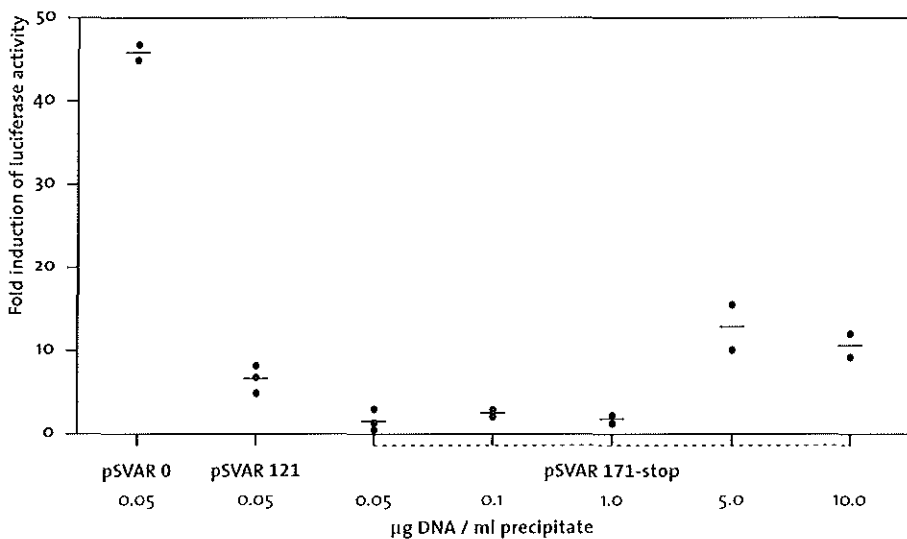
Figure III.6 **Transcription activation study of wild type and mutant AR expression plasmids.** The y-axis represents the fold induction of MMTV-Luc reporter gene in the presence of 1 nM R1881 in relation to basal activity in the absence of hormone. Results of transfections (each done in triplicate) are indicated by black points (— = mean). On the x-axis, the amount of DNA per ml precipitate is indicated.

Discussion

In order to determine factors influencing the genotype-phenotype correlation in patients with AIS, we investigated a PAIS subject with discrepancies among phenotype, genotype, and the result of the SHBG response to stanozolol. The phenotypic appearance with obvious signs of virilization demonstrated significant androgen action in the patient. In contrast, the absent SHBG decrease in response to stanozolol would predict a CAIS phenotype with total abolishment of AR function (Sinnecker *et al.* 1997), a striking discrepancy with the clinical observations. Mutation detection analysis revealed a premature stop codon within the NH_2-terminus of the *AR* gene. Again, this finding was not consistent with the PAIS phenotype of the patient, as in the current literature premature stop codons within the *AR* gene have always been associated with CAIS (Trifiro *et al.* 1991b, Batch *et al.* 1992, Zoppi *et al.* 1992, Hiort *et al.* 1994b, for review see also Quigley *et al.* 1995 and Gottlieb *et al.* 1996).

DNA sequencing and restriction site analysis demonstrated the presence of a somatic mosaic of mutant and wild type AR alleles in the patient. This was confirmed by the presence of normal androgen binding properties in cultured genital skin fibroblasts and the detection of a normal 110/112-kDa AR doublet in Western immunoblots, both indicating the expression of the wild type AR. A physiological significance of downstream initiation of translation at Methionine 188 resulting in an 87-kDa AR, as demonstrated in Figure III.5, seems unlikely, because only relatively high plasmid concentrations of the pSVAR171-stop plasmid lead to relevant reporter gene induction in cotransfection assays. Different reports by others on premature stop codons of the *AR* gene in the human (Zoppi *et al.* 1992) as well as in the mouse (He *et al.* 1994) being associated with partially active NH_2-terminal-truncated AR fragments caused by downstream initiation support our findings. The respective phenotypes have always been CAIS, despite the occurrence of downstream initiation (Zoppi *et al.* 1992, He *et al.* 1994). Thus, we conclude that the partial virilization of our patient is most likely due to the expression of the wild type AR based on somatic mosaicism. The absent SHBG decrease in response to stanozolol may be due to variations in the tissue distribution of mutant and wild type AR alleles. One would expect, with respect to this consideration, that liver parenchymatous cells predominantly, if not exclusively, contain the mutant form of AR alleles.

The occurrence of somatic mosaicism in genetic diseases is not a rare event. For example, the McCune-Albright syndrome is due to somatic mutations of the gene coding for the α-subunit of the G protein ($G_s\alpha$) (Weinstein *et al.* 1991, Shenker *et al.* 1994). The variability in the severity of the clinical manifestations in this disease is consistent with the presence of different ratios of mutated and wild type alleles in tissues of individual patients (Weinstein *et al.* 1991, Shenker *et al.* 1994). Concerning AIS, the possibility of somatic mosaicism hardly received attention in the current literature. Publications dealing with somatic mutations of the *AR* gene have mostly been restricted to malignant disease (Culig *et al.* 1993a, Suzuki *et al.* 1993, Schoenberg *et al.* 1994). To date, only one case of a somatic mutation of the *AR* gene in AIS has been published (Hiort *et al.* 1993). Partial virilization of that patient has been suggested to be most likely due to the expression of the wild type AR based on the somatic mosaic. A comparable animal model has been studied with the XX^{Tfm}-Sxr mouse in detail (Cattanach *et al.* 1971, Drews 1975). Different ratios of androgen responsive and unresponsive cells

in the somatic mosaics of these mice are responsible for phenotypes ranging from predominantly male with hypospadias to severely impaired masculinization with predominantly female appearance (Drews 1975).

Somatic mosaicism of the *AR* gene represents the first clearly defined mechanism influencing significantly the genotype-phenotype correlation in patients with AIS. Expression of the wild type AR plays the crucial role in molecular genetic constellation by shifting the AIS subtype to a higher degree of virilization than expected from the mutant allele alone. For clinical purposes, knowledge about somatic mosaicism of the *AR* gene in a particular patient with AIS provides important information for further management. First, somatic mosaicism can elucidate possible discrepancies among phenotype, genotype, and the SHBG androgen sensitivity test and therefore be of help in the interpretation of the data obtained. Second, it provides the basis for genetic counseling of these families as the risk for another child with AIS is low if somatic mosaicism is present in the index patient. Thirdly, early gonadectomy is prudent in all patients rendered female to prevent undesired virilization during puberty (cliteromegaly deepening of the voice) because of presumed partial androgen action.

Acknowledgements

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Association Analysis of the Length of the (CAG)nCAA-repeat in the Human Androgen Receptor Gene with Bone Mineral Density and Anthropometric Variables

Chapter IV

Association Analysis of the Length of the (CAG)nCAA-repeat in the Human Androgen Receptor Gene with Bone Mineral Density and Anthropometric Variables.

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Summary

Length of the polymorphic (CAG)nCAA-repeat in exon 1 of the human *androgen receptor* gene is inversely related to transcription activation capacity. Lower androgens, and androgen receptor levels are observed in elderly men. Under such conditions, the length of the (CAG)nCAA-repeat might become critical in its effect on physiological end points. In view of a possible role of androgens in bone metabolism, we hypothesized that the length of the (CAG)nCAA-repeat might be correlated with bone mass. This was investigated in 365 elderly men of 55 yr. and older from the Rotterdam Study (Hofman *et al.* 1991). Individuals represented the upper and lower quintiles (20%) of the population with respect to bone mineral density at the femoral neck. Allele typing was performed, using a PCR-based assay and automated sequencing, and genotypes were correlated with bone density and anthropometric variables. Interestingly, alleles were unequally distributed among the low and high BMD groups. Individuals with low BMD were overrepresented among those with shorter (CAG)nCAA-repeats. We observed a positive association between length of the (CAG)nCAA-repeat and bone mineral density at the femoral neck and the Ward's triangle in individuals with low body mass index (< 25.3 kg/m^2).

Introduction

Osteoporosis, the most common bone disease, especially in old age, is characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture risk (Consensus Development Conference on Osteoporosis 1993). Bone mineral density (BMD), an important predictor for osteoporotic fractures, is influenced by many factors. Up to 75% of variation in BMD might be determined by genetic factors (Christian *et al.* 1989, Pocock *et al.* 1993). Allelic differences in genes playing a role in bone metabolism have been studied in order to identify protective or risk factors. So far several candidate genes have been analyzed in relation to BMD and/or osteoporotic fractures, including the *collagen Iα1* gene (Grant *et al.* 1996) and the *interleukin-6* gene (Murray *et al.* 1997). Genes encoding the vitamin D receptor (Morrison *et al.* 1994, Morrison *et al.* 1997, Uitterlinden *et al.* 1996) and the estrogen receptor (Kobayashi *et al.* 1996) were analyzed as well. Although polymorphisms in these genes have been found associated with BMD, controversy remains, since others could not replicate these findings (Han *et al.* 1997, Nelson *et al.* 1997).

Another steroid hormone receptor with effects on bone metabolism is the androgen receptor (AR). The AR is present in physiologic concentrations in human osteoblast-like cells (Colvard *et al.* 1989). Expression of the AR in normal developing human bone and in adult osteophytic bone was studied *in situ* by Abu *et al.* (1997). They showed that the AR was highly expressed by osteoblasts and chondrocytes in both males and females. In addition, hypogonadism in men is associated with reduced BMD (reviewed by Orwoll and Klein 1995), and testosterone administration resulted in increased BMD and lean body mass in men with acquired hypogonadism (Katznelson *et al.* 1996). There are several lines of evidence for a direct role of androgens in bone formation. 5α-Dihydrotestosterone which cannot be converted to 17β-estradiol, stimulated proliferation of bone cells *in vitro*. This effect was inhibited by antiandrogens, indicating that the AR was involved (Kasperk *et al.* 1989). In addition, 46,XY complete androgen insensitivity patients achieve a typically female bone mass (Orwoll and Klein 1995, Soule *et al.* 1995). Moreover, in an adolescent complete androgen insensitivity patient reduced BMD was observed before gonadectomy, although no lack of estrogens was found (Munoz-Torres *et al.* 1995).

The AR consists of several distinct functional domains: an NH_2-terminal domain involved in transcription activation, a DNA-binding domain, and a ligand-binding domain (Beato *et al.* 1995). In the NH_2-terminal domain two polymorphic stretches are located: a GGC-repeat (encoding 16-24 glycine residues), and a highly polymorphic (CAG)nCAA-repeat with a length ranging from 9-33 CAGs in normal individuals (Sleddens *et al.* 1992, Sleddens *et al.* 1993, Nance 1997). Poly-glutamine stretches, encoded by CAG-repeats, are present in activation domains of many transcription factors, and stretches of variable length can modulate transcriptional activity of these factors (Courey *et al.* 1989, Gerber *et al.* 1994). The function of the transcriptional glycine stretch is unknown.

Influence of the length of the (CAG)nCAA-repeat on the transcription activation capacity of the AR was studied *in vitro*. Relatively long (CAG)nCAA-repeats ($n \geq 40$) were inhibitory to transcription activation, although promoter dependent differences were observed (Mhatre *et al.* 1993, Chamberlain *et al.* 1994, Jenster *et al.* 1994, Kazemi-Esfarjani *et al.* 1995). An extremely expanded (CAG)nCAA-repeat ($n = 48-75$) is associated with Spinal and Bulbar Muscular Atrophy (SBMA), characterized by progressive muscle weakness of upper and lower extremities (La Spada *et al.* 1991). In affected males, symptoms of mild androgen insensitivity such as gynecomastia and reduced fertility are frequently observed, indicating that repeat length influences receptor activity.

Recently Tut *et al.* (1997) associated relatively long (CAG)nCAA-repeats with an increased risk of impaired spermatogenesis. Moreover, in two large case-control studies it was shown that shorter (CAG)nCAA-repeats were associated with a higher risk of prostate cancer (Stanford *et al.* 1997) and with a more aggressive phenotype of prostate cancer (Giovannucci *et al.* 1997).

Although conflicting reports have appeared, many studies suggest that the bio-available testosterone level declines with age (Swerdloff and Wang 1993). Aging also changes target cell sensitivity for reproductive hormones (Supakar *et al.* 1993). In pubic skin fibroblasts from elderly men a lower AR expression level was observed when compared to the AR expression level in pubic skin fibroblasts derived from younger men (Ono *et al.* 1988). In rats, an age dependent decreased AR level was observed in the liver (Song *et al.* 1991). In aging individuals the length of the (CAG)nCAA-repeat might become critical in its effect on physiological end points. We investigated whether the length of the (CAG)nCAA-repeat in the *AR* gene was associated with BMD. Allele typing was performed in a sample of 365 men of 55 yr. and over from the Rotterdam Study (Hofman *et al.* 1991).

Subjects and methods

Population

The Rotterdam Study is a prospective cohort study of determinants of disease and disability in elderly people, mainly of Caucasian extraction. At base line participants were 55 yr. and over, and living in Ommoord, a suburb of the city Rotterdam in The Netherlands. A total of 10,275 persons of whom 9161 (89%) were living independently were invited for the study. In the independently living population the overall response rate was 77% for the home interview and 71% for the examination in the research center. Written informed consent was obtained from each participant. The Rotterdam Study has been approved by the Medical Ethics Committee of Rotterdam University Medical School. From all 5931 independently living participants with bone density measurements, 1453 subjects were excluded according to the following criteria: older than 80 yr., use of a walking aid, known diabetes, or use of cytostatics, thyroid hormone, or diuretics. From the 4478 remaining subjects 179 and 186 men were selected, representing a random sample of the upper and lower quintile (20%) of the BMD (at the femoral neck) (cut off values 0.987 g/cm^2 and 0.764 g/cm^2, respectively).

Bone mineral density measurements and anthropometry

BMD at the femoral neck, the trochanter, and Ward's triangle (g/cm²) was measured by dual-energy X-ray-absorptiometry (DXA, Lunar Corporation, Madison, WI, USA) as described previously (Burger *et al.* 1994). The *in vivo* coefficient of variation was 3.2% in femoral neck, 2.5 % in the trochanter, and 3.1 % in the Ward's triangle. Lumbar spine BMD measurements were not included in the analysis since lumbar spine values might be biased due to arthritis (Burger *et al.* 1994). Height and body weight (BW) was determined in a standing position without shoes, and body mass index (BMI) (kg/m²) was calculated as a measure of obesity.

Determination of (CAG)nCAA-repeat length

Genomic DNA was isolated from blood lymphocytes by standard procedures. Labeled PCR-products were generated and analyzed as described previously (Sleddens *et al.* 1992). Six randomly chosen samples were directly sequenced by automated sequencing, to confirm allele typing. Template was made with primer -70A and 320B, and sequenced with reverse primer 172B (Brüggenwirth *et al.* 1996).

Statistical analysis

Data are presented as means ± standard deviations (SD). Mean BMD, BMI and BW were compared for different genotypes by analysis of covariance (ANACOVA) and by Student t-test. Frequencies of alleles among the low and high BMD group were compared by the $\chi 2$ test. The relation between (CAG)nCAA-repeat length and BMD, BMI, and BW, respectively, was quantified with linear regression analysis. All analyses were carried out after adjustment for age, which is a known determinant of bone density. Repeat length was used both as a continuous variable and as a categorical variable. Alleles were therefore divided in 2 and 3 arbitrarily chosen groups of increasing repeat length (*i.e.* ≤ 22 compared to > 22, and groups containing 14-19, 20-23, and 24-31 CAGs). Logistic regression analysis was used to calculate the Odds ratio as an estimate of the relative risk with 95% confidence intervals (CI).

Results

General characteristics of individuals in both study groups, *i.e.* upper and lower quintiles of BMD, are presented in Table IV.1. Body weight, height, and BMI were significantly lower in the low BMD group.

The frequency distribution of AR (CAG)nCAA alleles in the groups with low and high BMD is presented in Figure IV.1. In both groups allele (CAG)21CAA was most frequently observed. Alleles that were observed in > 1% of the population showed a different distribution among both groups (χ^2 test 36.93, *d.f.* = 12, p = 0.0002) (the expected values for the low BMD group are based on the allele frequencies that were established in the high BMD group). Repeat alleles containing ≤ 22 repeat units were slightly overrepresented in the low BMD group (Figure IV.1 and Table IV.2).

Table IV.1 **Characteristics of the population of elderly men studied**

	Low BMD < 0.764 g / cm²	High BMD > 0.987 g / cm²	*p*-value
Number	186	179	–
Age (yr.)	67.3 ± 7.1	67.4 ± 7.2	0. 96
BMD (g / cm²)	0.7 ± 0.06	1.06 ± 0.091	< 0.001
BMI (kg / m²)	24.3 ± 2.6	26.7 ± 2.8	< 0.001
BW (kg)	73.0 ±8.9	83.1 ± 10.9	< 0.001
Height (cm)	173.4 ± 6.9	176.4 ± 6.1	< 0.001

Values presented are the mean ± SD, *p*-values were calculated by the Student *t*-test.

By stratifying high vs. low BMD according to BMD (cut off point = 0.90 g/cm²) and number of CAGs (cut off point 22 repeats) the relative risk of having a low BMD was estimated for individuals carrying less than 22 CAGs, by applying logistic regression analysis (Table IV.3). Although there was a 1.3 increased risk when all individuals were analyzed this was not significant. However, the analysis was also stratified according to the median BMI in this population (25.3 ± 2.7 kg/m²) because BMI is a major determinant of BMD. A significantly increased risk of 1.9 was observed for individuals with a BMI lower than 25.3. Age- and
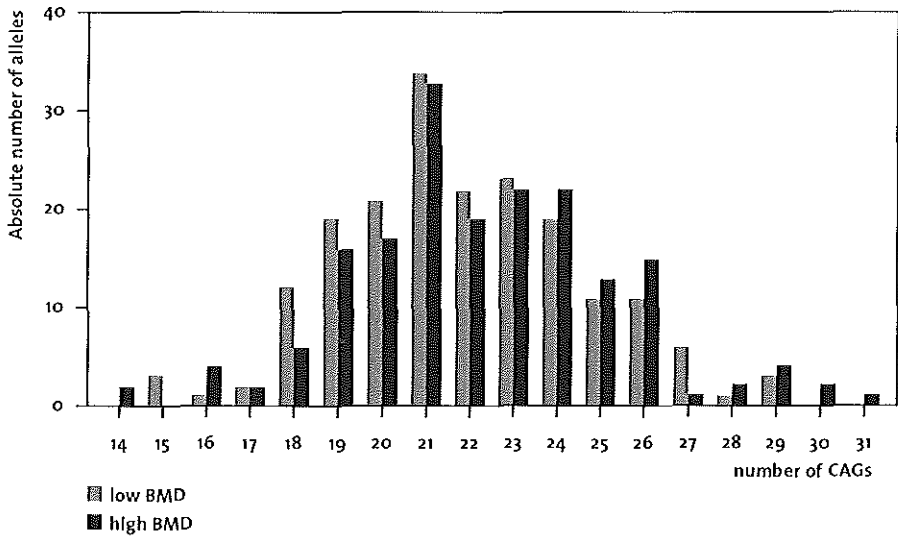
Figure IV.1 **Distribution of AR (CAG)nCAA-repeat allele sizes.** The open bars represent the population with the 20 % lowest BMD values, the black bars individuals with the 20% highest BMD values. Alleles with frequency > 1%: χ^2 = 36.93, *d.f.* = 12, *p* = 0.0002.

weight-adjustment did not change these point estimates (Table IV.3). To further explore this association, mean BMD was compared between (CAG)nCAA-repeat groups (Table IV.4). This showed that individuals with a BMI < 25.3 and carrying longer (CAG)nCAA-repeats have a significantly higher bone mass at the femoral neck and the Ward's triangle. Similar differences were observed at the trochanter although these did not reach significance (data not shown). For men with BMI ≥ 25.3 no significant differences in BMD among genotype groups were observed. By applying linear regression analysis it was calculated that the increase in BMD per increase in (CAG)nCAA-repeat group between genotype groups corresponds to 0.045 ± 0.019 (SE) (*p* = 0.02). When (CAG)nCAA-repeat alleles were analyzed individually this corresponded to 0.009 ± 0.005 (SE) (*p* = 0.05) increase in bone mass per CAG copy. When adjustment for age was included in the regression analysis, point estimates did not change.

Table IV.2 Frequency distribution of (CAG)nCAA-repeat allele sizes

(CAG)nCAA	Low BMD		High BMD	
14	0	(0)	2	(1.1)
15	3	(1.6)	0	(0)
16	1	(0.5)	4	(2.2)
17	2	(1.1)	2	(1.1)
18	12	(6.4)	6	(3.3)
19	19	(10.1)	16	(8.8)
20	21	(11.2)	17	(9.4)
21	34	(18.1)	33	(18.2)
22	22	(11.7)	19	(10.5)
23	23	(12.2)	22	(12.2)
24	19	(10.1)	22	(12.2)
25	11	(5.9)	13	(7.2)
26	11	(5.9)	15	(8.3)
27	6	(3.2)	1	(0.6)
28	1	(0.5)	2	(1.1)
29	3	(1.6)	4	(2.2)
30	0	(0)	2	(1.1)
31	0	(0)	1	(0.6)
Total	**188**	(100%)	**181**	(100%)

Alleles with frequency > 1%: $\chi^2 = 36.93$, $d.f. = 12$, $p = 0.0002$.
Percentages are presented between brackets.

Discussion

Many genetic and environmental factors play a role in bone metabolism. Poly-morphisms in genes involved in bone metabolism, have been analyzed in order to find risk factors for osteoporosis. The AR might be directly involved in bone metabolism (Kasperk *et al.* 1989, Orwoll and Klein 1995, Soule *et al.* 1995, Vander-schueren *et al.* 1995). In the NH_2-terminal part of the AR a polymorphic (CAG)nCAA-repeat is present, which influences receptor activity, as was shown *in vitro* (Mhatre *et al.* 1993, Chamberlain *et al.* 1994, Jenster *et al.* 1994, Kazemi-Esfarjani *et al.* 1995). Expansion of the (CAG)nCAA-repeat (>38-75 CAGs) in the AR

Table IV.3 Relative risk of low BMD by (CAG)nCAA-repeat length

	(CAG)nCAA	Nr. of cases Low BMD	Controls High BMD	OR* Crude	OR* Age-adjusted
All	> 22	73	81	1 (reference value)	1
	≤ 22	113	98	1.28 [0.84-1.94]	1.28 [0.84-1.94]
	Total 365				
BMI < 25.3	> 22	46	30	1 (reference value)	1
	≤ 22	79	27	1.91 [1.01-3.61]	1.91 [1.01-3.62]
	Total 182				
BMI ≥ 25.3	> 22	27	51	1 (reference value)	1
	≤ 22	34	71	0.91 [0.48-1.69]	0.90 [0.48-1.69]
	Total 183				

* OR, Odd's ratio; the 95% confidence interval is presented between brackets.

Table IV.4 Mean BMD by (CAG)nCAA-repeat group stratified by BMI

	(CAG)nCAA	Cases n	Femoral neck vs. 14-19	p	Ward's triangle vs. 14-19	p
BMI < 25.3	14-19	33	0.76 ± 0.18	-	0.60 ± 0.20	-
	20-23	95	0.80 ± 0.18	0.26	0.65 ± 0.19	0.24
	24-31	54	0.85 ± 0.20	0.02	0.70 ± 0.20	0.03
	Total	182				
BMI ≥ 25.3	14-19	34	0.97 ± 0.19	-	0.82 ± 0.21	-
	20-23	94	0.94 ± 0.18	0.50	0.80 ± 0.21	0.49
	24-31	55	0.93 ± 0.21	0.40	0.78 ± 0.21	0.27
	Total	183				

IV (CAG)nCAA-repeat in the AR and BMD

is associated with SBMA, a neuromuscular disease, which is often accompanied with mild symptoms of androgen insensitivity (La Spada *et al.* 1991). Therefore, length of the (CAG)nCAA-repeat in the AR was associated with possible androgenic end-points. Recently, two reports appeared in which shorter (CAG)nCAA-repeats were associated with a higher risk for prostate cancer (Ingles *et al.* 1997, Stanford *et al.* 1997). A significant association between (CAG)nCAA-repeat length and age at onset of prostate cancer was descibed by Hardy *et al.* (1996). Giovannucci *et al.* (1997) found an inverse relation between (CAG)nCAA-repeat length and a more aggressive phenotype of prostate cancer. Moreover, Tut *et al.* (1997) showed that a relatively long (CAG)nCAA-repeat might be a risk factor for impaired spermatogenesis.

In the present study, it was investigated whether length of the polymorphic (CAG)nCAA-repeat in the NH_2-terminus of the *AR* gene was associated with BMD, in a population of healthy elderly men representing either low or high BMD. In the first instance an association between (CAG)nCAA-repeat length and BMD was not detected. Interestingly, alleles were unequally distributed among the low and high BMD group; *i.e.* individuals with low BMD were overrepresented among those with shorter (CAG)nCAA-repeats. Because weight and, consequently, BMI (a measure of obesity), is a known determinant of BMD, the population was stratified according to median BMI (25.3 kg/m^2). In the group with BMI < 25.3 a positive association was observed between repeat length and BMD. This observation might be explained by the fact that androgens inhibit the release of LH, an action that is mediated by the AR (Schwarz and McCormack 1972). Sobue *et al.* (1994) measured plasma testosterone, LH, and FSH levels in SBMA patients and in controls after administration of fluoxymesterone (androgenic hormone). Their findings indicated a less strong negative feedback mechanism in SBMA patients compared to controls. In addition, Legro *et al.* (1994) determined length of the (CAG)nCAA-repeat of female Hispanics, and found a positive association among waist-hip ratio and repeat length. This result might as well be explained by an effect of (CAG)nCAA-repeat length on the testosterone level. In a study by Elbers *et al.* (1997) it was shown that testosterone administration in female to male transsexuals caused an increased amount of visceral fat, suggesting that under influence of testosterone the waist-hip ratio increases. However, because these women were gonadectomized, it might also be possible that a lower estrogen level is involved.

We postulate that an AR which harbors a relatively long repeat is less active, and thus exerts less strong negative feedback on the pituitary gland. A less strong negative feedback mechanism will result in a higher testosterone level, which might be beneficial with respect to BMD. An aspect that should be taken into account is that testosterone can be aromatized to estrogens (Purohit *et al.* 1992). Estrogens are important for controlling bone turnover rate and for achieving peak bone mass, not only in women but also in men. This is illustrated by disturbed skeletal maturation, lower bone density, and higher bone turnover rate in male patients with aromatase deficiency, resulting from mutations in the *CYP19* gene (Conte *et al.* 1994, Morishima *et al.* 1995). In a man with estrogen resistance due to ER mutation, the same features were observed (Smith *et al.* 1994). In a recent study by Slemenda *et al.* (1997) bone density in healthy elderly men was associated with serum sex steroids. A positive association between BMD and estrogen levels was established, whereas a weak negative association was observed between serum testosterone level and BMD. According to Slemenda *et al.* (1997) this negative association can be explained in part by the influence of BW on BMD. Weight is an important determinant of BMD, and testosterone level is inversely related with BW. No association was established between (CAG)nCAA-repeat length and BMD in the group with a BMI $\geq$ 25.3. The effect of the (CAG)nCAA-repeat length on BMD might be overruled by BW.
To find out whether length of the (CAG)nCAA-repeat in the normal population affects the negative gonadal-pituitary feedback mechanism, and whether this results in higher testosterone and/or 17β-estradiol levels, free testosterone and free 17β-estradiol levels, and/or a change in the SHBG level, will be determined in sera of the men under study. Moreover, the study population will be expanded with elderly men, in whom intermediate BMD was established, to study in more detail a possible relation between length of the (CAG)nCAA-repeat in the *AR* gene and BMD and/or anthropometric variables.

Substitution of Ala-564 in the First Zinc Cluster of the DNA-binding Domain of the Androgen Receptor by Asp, Asn, or Leu Exerts Differential Effects on DNA Binding

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Chapter V

Substitution of Ala-564 in the First Zinc Cluster of the DNA-binding Domain of the Androgen Receptor by Asp, Asn, or Leu Exerts Differential Effects on DNA Binding

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Summary

In the androgen receptor of a patient with androgen insensitivity the alanine residue at position 564 in the first zinc cluster of the DNA-binding domain was substituted by aspartic acid. In other members of the steroid-receptor family, either valine or alanine is present at the corresponding position, suggesting the importance of a neutral amino acid residue at this site. The mutant receptor was transcriptionally inactive, which corresponded to the absence of specific DNA binding in gel-retardation assays and its inactivity in a promoter-interference assay. Two other receptor mutants with a mutation at this same position were created to study the role of position 564 in the human androgen receptor on DNA binding in more detail. Introduction of asparagine at position 564 resulted in transcription activation of a mouse mammary tumor virus promoter, although at a lower level compared to the wild type receptor. Transcription activation of an (ARE)$_2$-TATA promoter was low, and binding to different hormone response elements could not be visualized. The receptor with a leucine residue at position 564 was as active as the wild type receptor on a mouse mammary tumor virus promoter and an (ARE)$_2$-TATA promoter, but interacted differentially with several hormone response elements in a gel-retardation assay. The results of the transcription activation and DNA-binding studies could partially be predicted from three-dimensional modeling data. The phenotype of the patient was explained by the negative charge, introduced at position 564.

Introduction

The *AR* gene is composed of eight exons and encodes a protein of 910 amino acids with an apparent molecular mass of 110 kDa (Brinkmann *et al.* 1989). The AR belongs to a superfamily of nuclear receptors for steroid hormones, thyroid hormones, vitamin D, and retinoids. These receptors are characterized by distinct functional domains: an NH$_2$-terminal part, involved in transcription activation, a DBD, a hinge region, and a C-terminal part involved in ligand binding, dimerization and transcription activation (Beato *et al.* 1995, Mangelsdorf *et al.* 1995). The DBD of steroid receptors is encoded by two exons and consists of two functionally different DNA-binding zinc clusters (Freedman *et al.* 1988). Steroid receptors bind to HREs as homodimers, in contrast to several other nuclear receptors that can heterodimerize with the retinoid X receptor (Freedman *et al.* 1988). Although the structure of the DBD is well conserved between nuclear receptors, several groups of receptors bind to specific DNA sequences (Forman and Samuels 1990). The GR and ER DBDs interact with distinct, although related

HREs (Green *et al.* 1986, Freedman *et al.* 1988). Three amino acid residues located in the so-called P-box (proximal box) are essential for specific interaction with base pairs from the HRE, located in the major groove of DNA (Danielsen *et al.* 1989). The GR, the AR, the MR, and the PR recognize the same HRE (AGAACAnn-nTGTTCT) (Forman and Samuels 1990). Specificity with respect to transcription activation is probably introduced by auxiliary factors, which can change the affinity and specificity of binding sites (Freedman *et al.* 1988). However, recently Claessens *et al.* (1996) reported an ARE in the probasin promoter that is AR specific. The consensus HRE for steroid receptors is an imperfect palindromic sequence, consisting of two half-sites, spaced by three nucleotides (Dahlman-Wright *et al.* 1991). Binding of the first receptor molecule enhances binding of the second molecule. Important determinants for this so-called cooperativity of binding are the spacing between the two half-sites of the HRE and protein-protein contacts (Dahlman-Wright *et al.* 1991).

Male sex differentiation and development proceed under direct control of the male sex hormones testosterone and 5α-dihydrotestosterone, and actions of both androgens are mediated by the AR. Mutations in the *AR* gene of 46,XY individuals are associated with AIS, a disorder of sex differentiation. Many abnormalities have been described, causing a wide spectrum of phenotypes, ranging from subjects with an external female phenotype and the absence of müllerian and wolffian duct derivatives, designated as CAIS, to a phenotype with ambiguous genitalia, called PAIS (Quigley *et al.* 1995). The most frequently reported defects are point mutations in the ligand- and DNA-binding domains of the AR (Quigley *et al.* 1995, Gottlieb *et al.* 1997).

In the present study a mutation in exon 2 of a subject with CAIS is reported. The alanine residue at position 564 in the DBD was substituted into aspartic acid (mutant A564D). The effect of the A564D mutation on AR function was investigated, as was the effect of an asparagine substitution (mutant A564N) and a leucine substitution (mutant A564L) at this same position. These studies were completed with molecular modeling.

Subjects and methods
Materials
Primers were obtained from Pharmacia Biotech Benelux (Roosendaal, The Netherlands). [γ-^{32}P]ATP (specific activity: 3000 Ci/mmol) was obtained from Amersham (Little Chalfont, UK). 17β-Hydroxy-17α-[3H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881; specific activity 85 Ci/mmol) and unlabeled R1881 were purcha-

sed from New England Nuclear-DuPont de Nemours ('s Hertogenbosch, the Netherlands). The double stranded probe, containing an ARE, derived from the tyrosine aminotransferase (TAT) promoter (half-sites in *italics*) was obtained from Promega (Woerden, the Netherlands). The 27 bp oligonucleotides, used to produce two other double stranded probes, (half-sites in *italics*), containing, respectively, the strongest ARE from the MMTV promoter (Ham *et al.* 1988) and a consensus ARE, (Roche *et al.* 1992), were obtained from Pharmacia Biotech Benelux.

ARE TAT :   5' TCGAC*TGTACA*GGA*TGTTC*TAGCTACT 3'
ARE MMTV: 5' TCGAC*GTTACA*AAC*TGTTC*TAGCTACT 3'
ARE consensus: 5' TCGAC*GGTACA*GTTT*GTTC*TAGCTACT 3'

Clinical data

The patient exhibiting female external genitalia, atrophic epididymides and vasa deferentia, was diagnosed as having AIS at the age of 1 yr. in the absence of a positive family history. A blind ending vagina was present, the uterus was absent and testes with a normal histology for a boy of this age were present in the inguinal canal. Testosterone synthesis disorders were excluded as a cause of the 46,XY sex reversal. Genital skin fibroblasts were obtained from the index patient for Scatchard analysis and structural analysis of the *AR* gene. AR sequence analysis of relatives was performed on white blood cell genomic DNA.

Mutation detection

PCR-SSCP analysis and direct sequencing were performed as described previously (Brüggenwirth *et al.* 1996).

Ligand-binding study

For determination of ligand-binding characteristics of the AR of the AIS subject, Genital skin fibroblasts were incubated with serial dilutions [^{3}H]R1881 (0.02, 0.05, 0.3, 1.0, 3.0 nM, respectively) in serum free medium. The binding assay was performed as described previously (Brüggenwirth *et al.* 1996).

Western blot analysis

AR protein, derived from genital skin fibroblasts or transiently transfected CHO cells was immunoprecipitated and analyzed by Western immunoblotting according to the method of Ris-Stalpers *et al.* (1991).

Construction of AR expression vectors

pAR(0), a human wild type AR complementary DNA expression plasmid was described previously (Brinkmann *et al.* 1989). Expression plasmids encoding the various mutants, pAR(A564D), pAR(A564N) and pAR(A564L) respectively, were constructed by site directed mutagenesis. The *KpnI-AspI* digested fragment of pAR(0) was exchanged with mutated *KpnI-AspI* fragments generated in two separate PCR amplifications (Higuchi *et al.* 1988). Sense primer 470A (Brüggenwirth *et al.* 1996), located upstream of the *KpnI* site in exon 1, was combined with an antisense primer, containing the mutation (antisense primers: construct A564D, 5'CATGTGAGAtCTCCATAGTGACAC 3'; construct A564N, 5'CATGTGAGAttTCCATAGTGACACCC 3'; construct A564L, 5' CATGTGAGAagTCCATAGTGACACCC 3'). A sense primer, introducing the mutation (sense primers: construct A564D, 5' GTGTCACTATGGAGaTCTCACATG 3'; construct A564N, 5' GGTGTCACTATGGAaaTCTCACATGTGG 3'; construct A564L, 5' GGTGTCACTATGGActTCTCACATGTGG 3'), was used in combination with an antisense primer 14NB (Brüggenwirth *et al.* 1996), located downstream of a unique *AspI* site in exon 4. One microliter of both PCR products was used as template in a second PCR reaction using primers 470A and 14NB. The resulting PCR fragment was digested with *KpnI* and *AspI*, and exchanged for the corresponding wild type fragment in pAR0.

pSG5AR(0), a human wild type AR cDNA expression vector (provided by Dr. A.C.B. Cato, Karlsruhe, Germany) was used for transient transfection of COS-1 cells. pSG5AR(A564D), pSG5AR(A564N) and pSG5AR(A564L) were constructed by exchanging the 472 bp *KpnI-AspI* insert from pSG5AR(0) and the *KpnI-AspI* fragment from the pSVAR plasmids, encoding the variant ARs. MMTV-Luc reporter plasmid, cytomegalovirus (CMV)-Luc and the CMV-(ARE)^3-Luc reporter plasmids and pJH4-(ARE)$_2$-TATA-Luc, containing the TATA-box and an Sp1-site derived from the Oct 6-gene promoter have been described previously (Blok *et al.* 1992, de Ruiter *et al.* 1995, Kuil and Mulder 1996).

Cell culture conditions and transfections

Genital skin fibroblasts and COS-1 cells were cultured as previously described (Ris-Stalpers *et al.* 1990). CHO cells were cultured according to the COS-1 cell culture protocol. The CHO cells, used for transcription activation studies and promoter-interference assays were plated in 7 or 11 cm^2 (promoter-interference assay) wells and grown for 24 hours. Cells were cotransfected, using the calcium-phosphate method (Chen and Okayama 1987), with AR expression plasmid (10

ng/ml precipitate) and reporter plasmid (2 μg/ml precipitate). Carrier DNA (pTZ19) was added to a total of 20 μg DNA/ml precipitate, and 90 μl precipitate were added per well. In the promoter-interference assay, 300 ng AR expression plasmid and 30 ng reporter plasmid (CMV-Luc or CMV-(ARE)$_3$-Luc), respectively, were added per ml precipitate. pTZ19 was added to a total of 20 μg DNA/ml precipitate, and 250 μl precipitate were added to 11 cm^2 wells. The transfection and luciferase assay were performed as described before (Kuil *et al.* 1995). Both transcription activation studies and the promoter-interference assay were performed at least three independent times in triplicate, using three independent isolates of expression plasmid. In case of transcription activation studies, luciferase activity was expressed, relative to basal activity measured after culturing in the absence of hormone. For promoter interference studies, luciferase activity in cells, transfected with CMV-(ARE)$_3$-Luc and AR expression plasmid and cultured in the absence of hormone was set at 100%. Inhibition of promoter activity in the presence of hormone was expressed relative to this 100% activity. CHO cells used for expression studies by Western blotting were also transiently transfected by the calcium-phosphate method. To this end, cells were plated in 175 cm^2 culture flasks and transiently transfected with 20 μg expression plasmid. COS-1 cells were plated in 80 cm^2 culture flasks and transfected with 9.4 μg expression plasmid, using the diethylaminoethyl-dextran method (Gerster *et al.* 1987). Twenty-four hours before harvesting, CHO and COS-1 cells were washed and incubated with medium containing 1 nM R1881.

Gel-retardation assays
Transfected COS-1 cells were collected in 5 ml PBS and the pellet was resuspended in extraction buffer [10 mM NaH$_2$PO$_4$ (pH 7.4), 0.4 M KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM bacitracin, 0.5 mM leupeptin and 0.6 mM PMSF, 10 mM DTT] and subjected to four freeze- thaw cycles, followed by 10 minutes centrifugation at 400,000x*g* at 4 °C in a TLA120.2 rotor (Beckman, Fullerton, CA) in a Beckman Optima TLX ultracentrifuge. The TAT ARE containing probe (5'-TCGAC*TGTACAGGATGTTCT*AGCTACT-3') (half-sites in italics) was obtained from Promega. Two other probes were produced by annealing a 27-bp oligonucleotide with an oligonucleotide of complementary sequence. One of them (5'-TCGAC*GTTACAAACTGTTCT*AGCTACT-3') (half-sites in *italics*) contains the strongest ARE from the MMTV promoter (Ham *et al.* 1988), and the other probe (5'-TCGAC*GGTACAGTTTGTTCT*AGCTACT-3') (half-sites in *italics*) contains a consensus ARE (Roche *et al.* 1992). The ARE containing probes were end-labeled using T4 polynu-
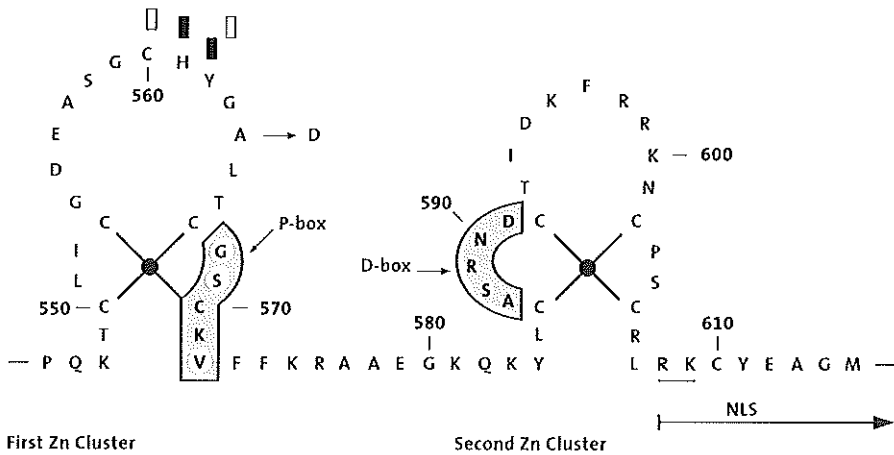
G C H

S G C Y

A 560 G

E A → D

D L

G T P-box

C C

I G

L S

550 — C C — 570

T K

— P Q K V F F K R A A E G K Q K Y

First Zn Cluster

K F R

D R

I K — 600

T N

590 D C C

N P

D-box → R S

S A C C

580 L R

L R K C Y E A G M —

610

Second Zn Cluster

|— NLS ——————▶

Figure V.1 **Sequence of the AR DBD with the A→D mutation, located in the first zinc cluster.** The mutation found in the index patient's AR is present at position 564 of the AR DBD, located near the P-box of which the circled amino acid residues are involved in ARE recognition. The boxes indicate amino acid residues that interact with the phosphate backbone of DNA, either at specific (black boxes) or at non-specific sites (open boxes) (Luisi et al. 1991, Lobaccaro et al. 1996). The second zinc cluster contains the D-box, which is involved in dimerization with an other AR molecule. The first part of the nuclear localization signal (NLS) is also shown (underlined). The numbering of the various codons is based on a total of 910 amino acid residues in the human AR (Brinkmann et al. 1989).

cleotide kinase and double stranded probe was purified from a 4% acrylamide gel, in 0.5 x TBE (1 x TBE= 50 mM Tris base, 50 mM boric acid, 1 mM EDTA, pH 8.6). Cellular extracts were incubated in binding buffer [10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM DTT, 1 mM EDTA, 4% ficoll], 1 μg polydeoxyinosinic-deoxycitidylic acid (poly[dI-dC]-poly[dI-dC]) in the absence or presence of the polyclonal AR antibody Sp197 (10-fold diluted) (Kuiper et al. 1993a). After an incubation period of 10 minutes on ice, 2 μl purified DNA probe (50,000 cpm/μl) were added and incubation was continued for 20 minutes at room temperature. The 20-μl sample was separated on a 4% polyacrylamide gel in 0.5 x TBE. Gels were fixed for 10 minutes in 10% acetic acid-10 % methanol, and subsequently dried and exposed.

Molecular modeling

The crystal structure of the rat GR DBD, bound to a GRE was used as a basis upon which the 3-D AR models were built. The 3-D model of AR bound to a GRE has previously been described (Lobaccaro et al. 1996). The A564D, A564N and A564L mutants were built according to the same strategy as that used to build the wild type model. Briefly, the side-chains of the AR mutants that were substituted in

the GR model were placed in energetically favorable conformations, using the SMD program (Tufferey *et al.* 1991). The whole system was then energy minimized with the AMBER program (Pearlman *et al.* 1991, University of California, San Francisco, CA). During the optimization process, the oligonucleotide was kept frozen to prevent unrealistic deviation from the initial crystal structure. Moreover, positional restraints on the backbone and on side-chains of conserved residues were applied and gradually released during the optimization. Figure V.5A was generated with the Insight II viewer (Biosym Technologies, San Diego, CA) and Figure V.5B was generated using the program MOLSCRIPT (Kraulis 1991).

Results

Mutation detection

Genomic DNA of the index patient was used to amplify the coding part and intronic sequences flanking the exons of the *AR* gene, followed by SSCP analysis performed under two different conditions. An aberrant banding pattern was found for exon 2, which encodes the first zinc cluster of the DBD. Direct sequencing showed a single nucleotide substitution at codon 564 (C to A) that resulted in substitution of alanine to aspartic acid (Figure V.1). The numbering of amino acid residues throughout the text is based on a total number of 910 amino acid residues in the human AR (Brinkmann *et al.* 1989). The mutation created a *BglII* site, which was used to investigate the segregation of this mutation in the family of the index patient. The mother and grandmother of the index patient were heterozygous carriers of this AR mutation (results not shown).

Functional properties of the mutant receptor

The AR protein was isolated from genital skin fibroblasts, obtained from the index subject. Molecular mass was checked by immunoblotting after immunoprecipitation. The AR protein migrated as a normal 110-112 kDa doublet on SDS-PAGE (data not shown). For Scatchard analysis, cultured genital skin fibroblasts were incubated for 1 hour with increasing concentrations of [^{3}H]R1881 either in the presence or absence of a 200-fold molar excess of non-radioactive R1881. Both the Kd (0.07 nM) and Bmax (58 fmol/mg protein) values were within the normal range (Kd: 0.03-0.13 nM, Bmax: 39-169 fmol/mg protein), indicating that ligand binding was not affected by the mutation.
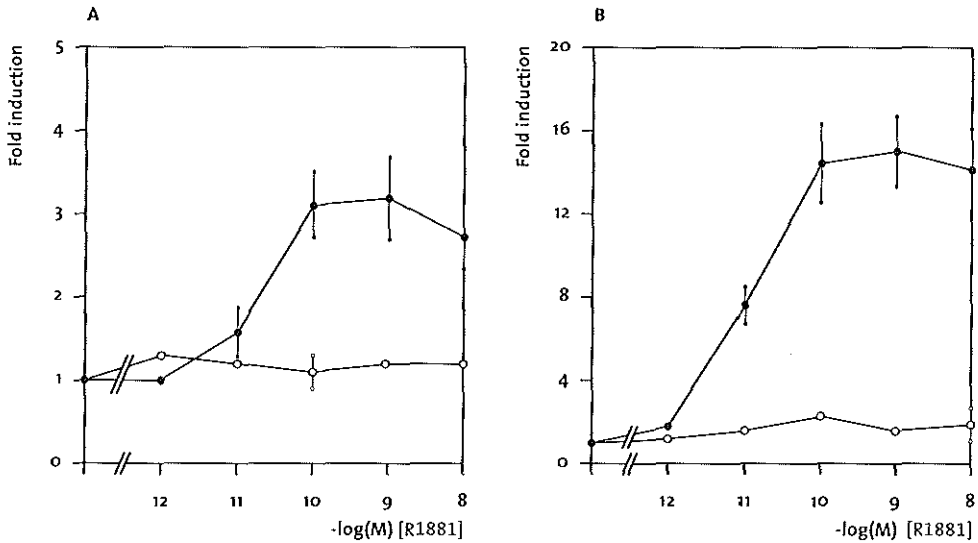
Figures V.2A and B **Transcriptional activity of AR A564D at different promoters.** CHO cells were cotransfected with a reporter plasmid [(ARE)$_2$-TATA-Luc or MMTV-Luc] and the wild type or mutant expression plasmid. After 24 hours cells were cultured with medium containing increasing concentrations of R1881 for another 24 hours before a luciferase assay was performed. Each data point was tested in triplicate. A Induction of luciferase activity was calculated from five different experiments, in which (ARE)2-TATA-Luc was used as a reporter gene. Symbols represent the mean ± SEM (● : wild type, o : A564D). Transcription activation by AR A564D was significantly different from that by the wild type AR (by Student's *t* test, *p* = 0.05). B Induction of luciferase activity was calculated from three different experiments, in which MMTV-Luc was used as a reporter gene. Symbols represent the mean ± SEM (● :wild type; o :A564D). Transcription activation by AR A564D was significantly different from that by wild type AR (by Student's *t* test, *p* = 0.05).

Transcriptional activity of AR A564D

AR A564D was inactive in CHO cells cotransfected with (ARE)$_2$-TATA-Luc, in contrast to the wild type AR (Figure V.2A). The promoter of this reporter construct contains a TATA-box and an Sp1 site derived from the Oct-6 gene promoter, and two AREs (Blok *et al.* 1992). In general, comparable levels of wild type and mutant receptor protein were expressed in transiently transfected CHO cells, as verified by SDS-PAGE and immunoblotting (for example, see Figure V.3C). AR A564D was also unable to activate transcription from the complex MMTV promoter in CHO cells (Figure V.2B).
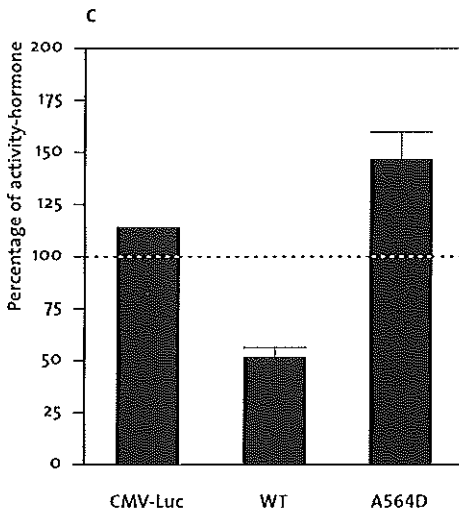
Figure V.2C **Promoter interference of wild type AR and AR A564Dz.**CHO cells were transiently transfec-
ted with expression plasmid and CMV-(ARE)$_2$-Luc reporter plasmid. Twenty-four hours after transfec-
tion, cells were cultured in the absence or presence of 1 nM R1881 and incubated for another 24 hours
before performing a luciferase assay. Each data point was tested in triplicate. CMV-Luc reporter con-
struct cotransfected with wild type AR was taken as a control (n=2). The luciferase signal, measured in
the absence of R1881 was arbitrarily set at 100%, and activities, measured in the presence of 1 nM R1881
were related to these values. The mean promoter activity ± SEM in the presence of hormone is repre-
sented.

In vivo DNA binding of AR A564D

In vivo DNA binding was studied with a promoter-interference assay. CHO cells
were cotransfected with CMV-(ARE)$_3$-Luc. Three consensus AREs are inserted
between the TATA-box of the constitutively active CMV promoter and the trans-
cription start site of the *luciferase* gene (Roche *et al.* 1992). Binding of the AR hin-
ders the assembly of a transcription initiation complex and, therefore, also
interferes with the expression of the *luciferase* gene (Kuil and Mulder 1996). The
level of inhibition is taken as a measure of specific DNA binding. In the presence
of 1 nM R1881, the wild type AR showed a 48% reduction of luciferase activity
whereas no reduction was seen in cells cotransfected with AR A564D (Figure
V.2C). The AR could sequester factors that are essential for transcriptional acti-
vity of the CMV promoter (squelching). However, no reduction of luciferase
expression was seen in cells cotransfected with CMV-Luc (Figure V.2C).
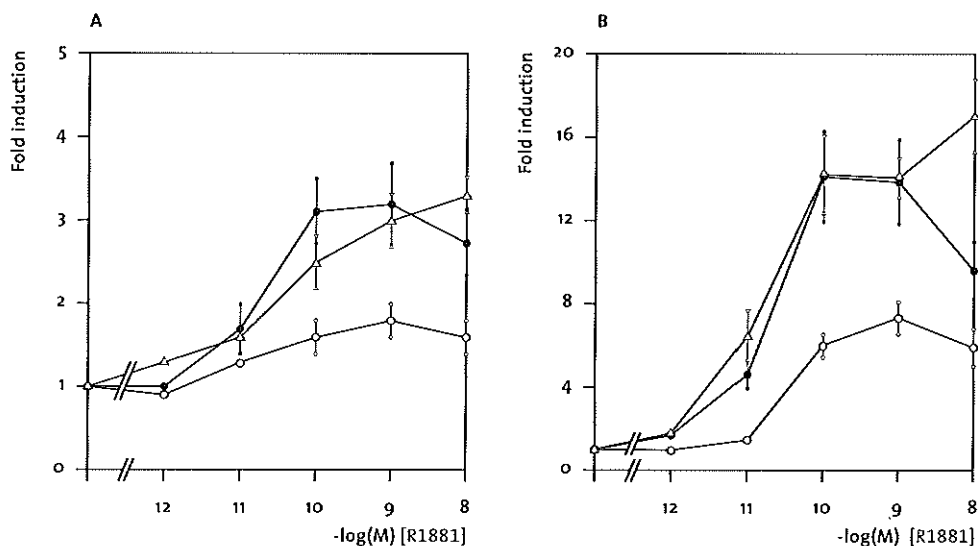
Figures V.3A and B Transcription activation by the wild type receptor and A564N and A564L using different reporter genes. CHO cells were cotransfected with a reporter plasmid [(ARE)₂-TATA-Luc or MMTV-Luc] and either the wild type or one of the mutant expression plasmids. Twenty-four hours after transfection, cells were cultured in medium containing increasing concentrations of R1881 for another 24 hours. Each data point was tested in triplicate. A Induction of luciferase activity was calculated from five different experiments in which (ARE)₂-TATA-Luc was used as a reporter gene. Symbols represent the mean ± SEM (● : wild type, ○: A564N, Δ: A564L). Transcription activation by AR A564N was significantly different from that displayed by the wild type AR (by Student's t test, p = 0.05). B Induction of luciferase activity was calculated from five different experiments in which MMTV-Luc was used as a reporter gene. Symbols represent the mean ± SEM (● : wild type, ○: A564N, Δ: A564L). Transcription activation by AR A564N was significantly different from activation, displayed by the wild type receptor (Student's t-test, p = 0.05).

Transcriptional activities of AR A564N and AR A564L

To investigate whether the inactivity of AR A564D was caused by steric hindrance or by a conformational change due to the introduction of a negative charged amino acid residue, the alanine residue was replaced by either a leucine residue (A564L) or an asparagine residue (A564N). Leucine has a larger side chain, like the aspartic acid residue. However, leucine is a neutral amino acid as is the alanine residue present in the wild type receptor. Asparagine has also a larger side chain, but is a polar amino acid residue. CHO cells were transiently cotransfected with AR expression plasmids and (ARE)₂-TATA-Luc. Wild type AR
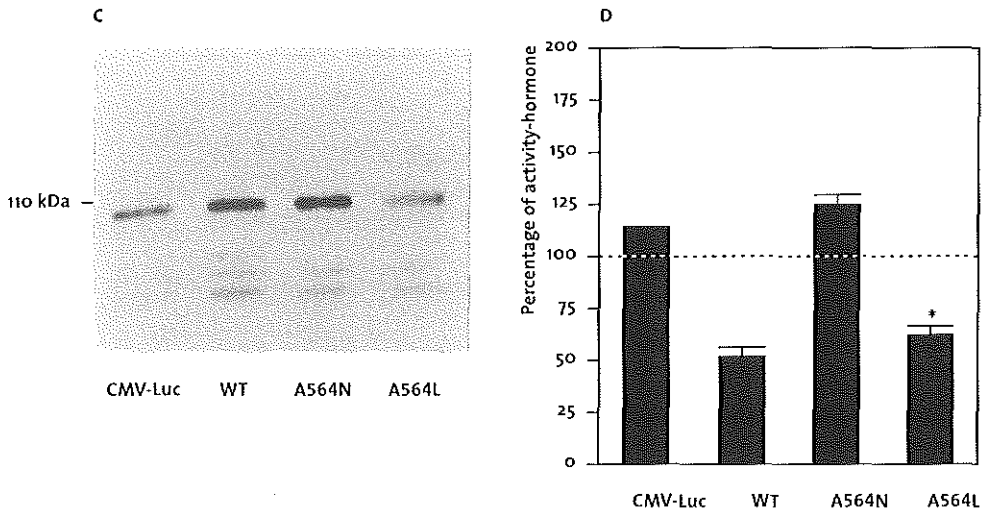
Figure V.3C **Western blot analysis of wild type and mutated ARs after transient transfection in CHO cells.** Culture flasks with CHO cells were transfected with expression plasmid and after 48 hours cell lysates were prepared. From these lysates, the receptor was immunoprecipitated with monoclonal antibody F39.4.1 and separated on a SDS-PAGE gel. After blotting, receptor protein was detected with polyclonal antibody Spo61 and an alkaline phosphatase-coupled goat anti-rabbit antibody. Lane 1, wild type AR; lane 2, AR A564D; lane 3, AR A564N; lane 4, AR A564L.

Figure V.3D **Promoter-interference assay of AR A564N and AR A564L. The DNA-binding properties of the mutated receptors were also tested in intact cells.** CHO cells were transiently transfected with expression plasmid and CMV-(ARE)2-Luc reporter plasmid. Twenty-four hours after transfection, cells were cultured in the absence or presence of 1 nM R1881 and incubated for another 24 hours before performing a luciferase assay. Each data point was tested in triplicate. The CMV-Luc reporter construct, cotransfected with wild type AR was taken as a control (n=2). The luciferase signal, measured in the absence of R1881 was arbitrarily set at 100%, and activities, measured in the presence of 1 nM R1881 were calculated relative to these values. The mean promoter activity ± SEM in the presence of hormone is represented (n=4). *, Significantly different from inhibition shown by the wild type receptor (by Student's t test, $p < 0.05$).

and AR A564L showed comparable activation of the minimal promoter at increasing amounts of R1881, whereas AR A564N showed strongly reduced transcription activation compared to the wild type AR (Figure V.3A). On the more complex MMTV promoter AR A564L showed activity comparable to that of the wild type receptor and AR A564N displayed a low level of hormone induced transcription activation (Figure V.3B). All proteins were expressed, and in general, expression levels were comparable (Figure V.3C).

A

WT   A564D   A564N   A564L

FP

	1	2	3	4	5	6	7	8	9	10
Sp197	--	-	+	-	+	-	+	-	+	+
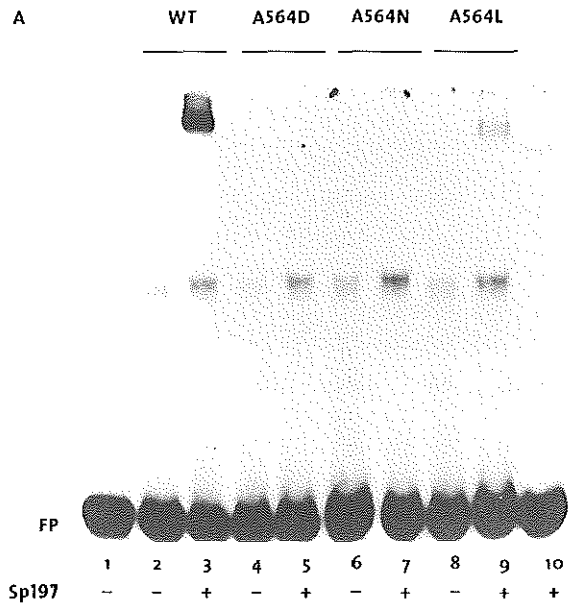
In vivo DNA binding of AR A564N and AR A564L

DNA binding was studied in CHO cells, cotransfected with CMV-(ARE)$_3$-Luc (Figure V.3D). In contrast to the wild type AR, luciferase expression was not lowered after cotransfection of cells with AR A564N and culture in the presence of hormone. AR A564L showed 38% inhibition, which was significantly different ($p<0.05$) from the 48% inhibition observed for the wild type receptor. Protein expression levels were identical for all mutant receptors (see also Figure V.3C).

In vitro DNA binding, comparing different AREs

The DNA-binding capacities of the wild type and the different AR mutants were tested in vitro in gel-retardation assays, using probes containing various AREs. AR was produced in transfected COS-1 cells. The amount of receptor protein was checked by Western blotting, followed by immunostaining. Comparable amounts of AR in COS-1 cellular extracts were incubated with a ^{32}P-labeled probe in either the absence or presence of polyclonal antibody Sp197, which stabilizes AR-dimers bound to the DNA (Kuiper et al. 1993a). Wild type AR and AR A564L did bind to the ARE, derived from the TAT promoter (Figure V.4A: lanes 3 and 9, respectively) whereas binding of AR A564D and AR A564N could not be detected (Figure V.4A: lanes 5 and 7). Gel-retardation assays were also performed with two
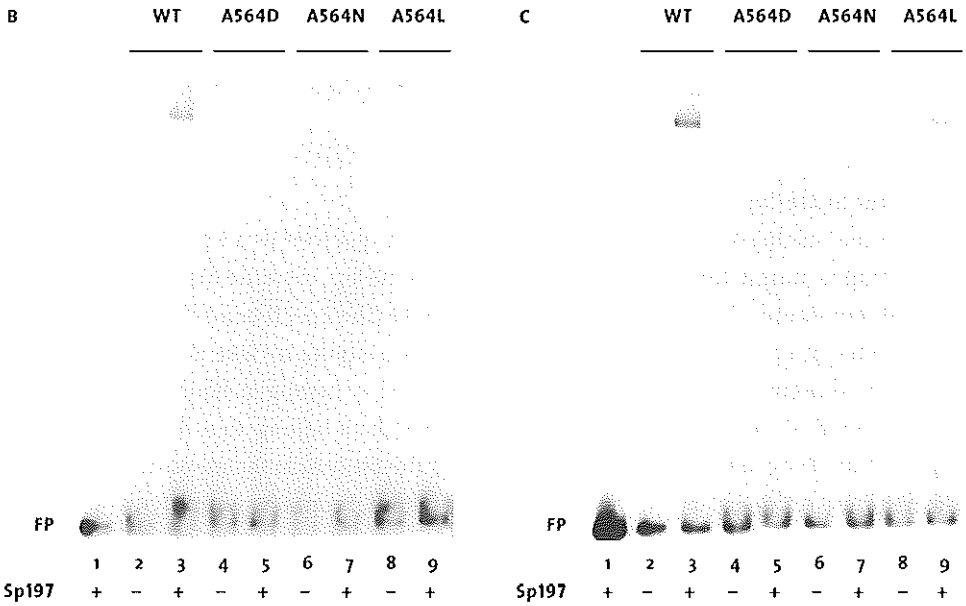
Figure V.4A, B and C **Gel-shift assay with three different ARE-probes.** Each labeled ARE-probe (50,000 cpm) was incubated with nuclear extracts, prepared from transiently transfected COS-1 cells. Incubations were performed in the absence (-) or presence (+) of the polyclonal antibody Sp197. The complexes were analyzed by polyacrylamide gel electrophoresis as described in the methods section. The position of the shifted complex is indicated by an arrow, FP indicates the position of the free ^{32}P-probe. A The probe contained an ARE, derived from the TAT promoter. Lane 1, No receptor protein; lanes 2 and 3, wild type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L; lane 10, free probe. After incubation of the probe with the antibody, no specific shifted band could be seen. B The probe contained the strongest ARE, derived from the MMTV promoter (Ham et al. 1988). Lane 1, No receptor protein; lanes 2 and 3, wild type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L. C The probe contained an consensus ARE (Roche et al. 1992). Lane 1: No receptor protein; lanes 2 and 3, wild type AR; lanes 4 and 5, AR A564D; lanes 6 and 7, AR A564N; lanes 8 and 9, AR A564L.

other probes. One of the probes contained the strongest ARE from the MMTV promoter (Ham et al. 1988), which was also present in (ARE)2-TATA-Luc. The other probe contained a consensus ARE (Roche et al. 1992), which was also cloned behind the constitutively active CMV promoter that was used for promoter interference studies. Wild type AR was able to shift the probes in the presence of antibody, indicative of specific DNA binding (Figure V.4B: lane 3, and Figure V.4C: lane 3). Neither probe could be shifted with AR A564D (Figure V.4B, lane 5, and Figure V.4C, lane 5) or AR A564N (Figure V.4B, lane 7, and Figure V.4C, lane 7), although A564N showed transcription activation on a complex MMTV promoter

and even on a minimal ARE promoter. AR A564L interacted with both probes, although less efficiently than the wild type AR, which is in agreement with the results of the promoter-interference assay (Figure V.4B, lane 9, and Figure V.4C, lane 9).

Molecular modeling

The alanine residue at position 564 is buried, as it is involved in a hydrophobic cluster that is mainly formed by leucine 551, isoleucine 552, cysteine 610, alanine 613, and methionine 615 (Figures V.1 and V.5, A and B). The Cα-Cβ bond of the alanine residue at position 564 is directed towards the cysteine residue at position 610 in the protein core. The backbone of residue 564 is hydrogen-bonded with the backbone of histidine residue 561, as both residues belong to a β-hairpin (Figure V.5B). They are located at the same side of the hairpin at facing positions. The histidine residue at position 561 is involved in direct contacts with DNA and participates in ARE recognition (Figure V.5B). Molecular modeling showed that in the A564D mutant the aspartic acid residue is still buried. However, burying of charged residues is unfavorable, unless a compensatory charge forms a salt
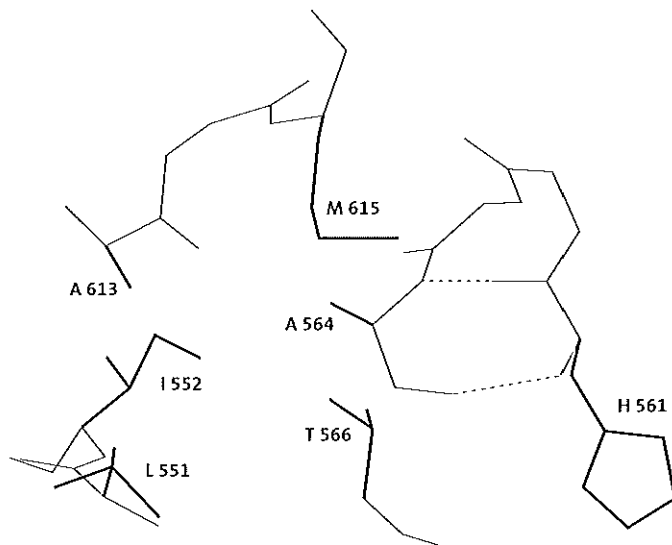
Figure V.5A **View of the hydrogen bonding between the alanine residue at position 564 and the histidine residue at position 561.** The hydrogen bonding is shown by the dashed lines. Also shown are residues constituting the hydrophobic pocket in which the alanine residue at position 564 is buried (i.e. leucine 551, isoleucine 552, threonine 566, alanine 613, and methionine 615). The backbone is shown by thin lines, and the side chains are shown as thick lines.
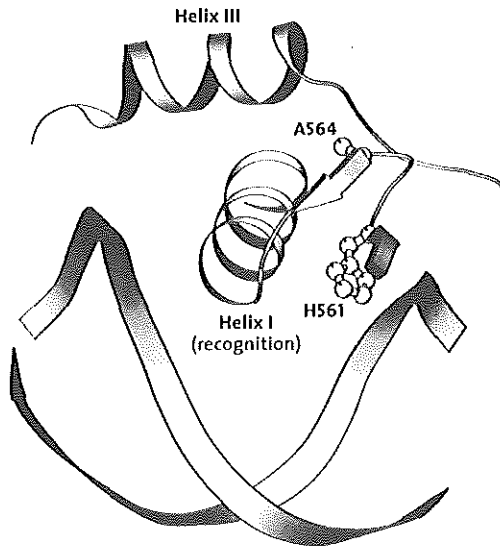
Figure V.5B Schematic view of part of the ARE and the wild type AR. Helix I, helix III, and the side chains of the histidine residue at position 561 and the alanine residue at position 564 (ball and stick) are shown. The histidine residue and the alanine residue belong to a small β-hairpin (strands shown as thick arrows) and the histidine residue makes direct contacts with DNA. Part of the ARE is displayed as long thin antiparallel arrows.

bridge and is also buried at the same site. The only way AR A564D can take a more favorable conformation, is by breaking of the β-hairpin and the hydrogen bonding with the histidine residue at position 561. This most likely affects the conformation of the histidine residue 561, resulting in disturbed ARE recognition. The asparagine residue in A564N should lead to smaller perturbations because it will remain buried, although asparagine is a polar residue. The modeling showed that hydrogen bonding of asparagine with threonine 566 may roughly compensate for the unfavorable burying of polar atoms. Modeling of the A564L mutant showed that the larger leucine side-chain could be accommodated without difficulties in the hydrophobic pocket. Burying of the larger hydrophobic surface may even provide additional stability to the AR. Therefore, the leucine mutant was not expected to significantly perturb DNA recognition.

Discussion

The DBD is the most conserved region within the nuclear-receptor family. Characteristic are the eight cysteines in this domain, forming the two zinc clusters. Zinc ion-coordinated binding is essential for proper folding and DNA binding. The structure of the DBDs of the GR and ER respectively, in complex with their response element on the DNA, was solved by X-ray crystallography. Almost similar conformations were displayed (Luisi *et al.* 1991, Schwabe *et al.* 1993). The AR DBD is more closely related to the GR DBD sequence than to the ER DBD sequence. Fifteen amino acid residues in the AR DBD, which is defined as a 65 amino acid domain starting at lysine 548, are different from the GR DBD (Hollenberg *et al.* 1985, Trapman *et al.* 1988). Only 5 of them are located in the first zinc cluster, and 1 of these should be considered as a conservative change according to the chemical properties of its side-chain (Trapman *et al.* 1988). Therefore, it seems reasonable to deduce the structural consequences of mutations in the AR DBD from the 3-D structure of the GR DBD.

In the present paper the characterization of a mutation at position 564 in the first zinc cluster of the DBD of the human AR is reported. Mutations in the first zinc cluster of the AR have been described previously (Gottlieb *et al.* 1997). Some of them disrupt the zinc cluster structure because one of the cysteines is substituted, resulting in a CAIS phenotype (Gottlieb *et al.* 1997). Alanine 564 is partially conserved in other members of the receptor family. At the corresponding position in the vitamin D receptor (VDR) an alanine residue is present as well (Baker *et al.* 1988). In the human ER, human GR, human PR, and human MR a valine residue is located at the corresponding position, implying that thepresence of a neutral amino acid residue at this position is critical for proper interaction of the receptor with DNA (Hollenberg *et al.* 1985, Green *et al.* 1986, Arriza *et al.* 1987, Misrahi *et al.* 1987). In the AR of the patient, described in this report, alanine was substituted by the negatively charged aspartic acid residue. The aspartic acid residue is located upstream of an α-helical region that is expsed to the major groove of DNA. and downstream of cysteine 560, histidine 561 and tyrosine 562, which are involved in specific as well as non-specific contacts with the phosphate backbone of the DNA (Luisi *et al.* 1991). Substitution of the latter residues by non-conservative amino acids in the GR resulted in loss of function (*in vivo*) and *in vitro* reduced DNA-binding affinity was seen (Schena *et al.* 1989). Warriar *et al.* (1994) substituted the cysteine residue at position 560 in the human AR by a serine residue. Although this is a relatively conservative change, decreased DNA binding and transcription activation were observed, which was attributed to the instability of the AR mutant-DNA complex.

We showed that AR A564D displayed defective transcription activation. Specific binding to DNA, which was studied *in vitro* by gel-shift assays with oligonucleotide probes containing different AREs, and *in vivo* with a promoter-interference assay, could not be detected. From 3-D modeling studies it became clear that the alanine residue at position 564 is buried in a hydrophobic cluster (Figures V.1 and V.5A). A hydrogen bond is formed between the backbone of alanine 564 and the backbone of histidine 561. This latter residue is involved in direct interaction with the phosphate backbone of the DNA and is conserved in other nuclear receptors. Yagi *et al.* (1993) reported a patient with hereditary 1,25-dihydroxyvitamin D-resistant rickets caused by substitution of the conserved histidine at position 35 of the VDR, comparable to histidine 561 in the AR. The phenotype of the patient was caused by perturbation of the conserved site that contacts the phosphate backbone of DNA. For AR A564D, the computer model displayed almost the exact conformation as that seen for the wild type, showing that there is no steric hindrance due to the mutation. However, buried charged residues are only observed in proteins when they can form salt bridges with residues of opposite charge. Therefore, the modeled conformation appears unlikely. Probably, aspartic acid 564 adopts a more favorable conformation, and as a result, the main chain hydrogen bond between aspartic acid 564 and histidine 561 will be broken, which has consequences with respect to DNA binding.

To investigate the role of the alanine residue at position 564 in more detail, the residue was also replaced by an asparagine or a leucine residue. 3-D modeling predicted that introduction of an asparagine residue should have intermediate effects with respect to transcription activation. Hydrogen bonding between asparagine 564 and threonine 566 might compensate for unfavorable burying of the polar amino acid residue. Substitution by an asparagine residue resulted in a less stable AR-DNA complex which had clear consequences for transcription activation on a minimal $(ARE)_2$-TATA promoter and to a lesser extent on a complex MMTV promoter. DNA binding was not observed in vitro by gel-shift analysis. DNA-binding capacity remained undetectable, even when studied in whole cells. However, the functionality of AREs is determined by additional transcription factor binding sites in the vicinity of AREs. Interaction with other proteins might stabilize the AR-DNA complex. This might explain as well the stronger activation of AR A564N on the MMTV promoter, compared to its activity on the minimal $(ARE)_2$-TATA promoter (Schüle *et al.* 1988, Rundlett and Miesfeld 1995, Claessens *et al.* 1996).

Modeling showed that no particular constraint resulted from the larger size of the leucine residue. It was predicted that the leucine residue, because of its larger hydrophobic surface, provides even more favorable stability to the AR. However, reduced DNA-binding affinity, *in vitro* as well as *in vivo*, was observed for AR A564L. Transcription activation was comparable with activation displayed by the wild type AR. Apparently, molecular modeling has some limitations, with respect to the prediction of complex interactions which might be explained by the fact that the model was based upon the crystal structure, which was solved for the GR DBD. In addition, functional studies were performed with the intact receptor and not only the DBD.

In conclusion, the negative charge, introduced by the aspartic acid residue, destabilizes the normal conformation of the AR DBD, resulting in disturbed ARE recognition, in agreement with the phenotype of the patient expressing this mutant receptor. Results from the functional assays were partially supported by predictions, made by 3-D modeling. Although not predicted by molecular modeling, steric hindrance might have an impact on the DNA-binding capacities of AR A564N and AR A564L.

Molecular Analysis of the Androgen Receptor Gene in a Family with Receptor-Positive Partial Androgen Insensitivity; An Unusual Type of Intronic Mutation

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Chapter VI

Molecular Analysis of the Androgen Receptor Gene in a Family with Receptor-Positive Partial Androgen Insensitivity; An Unusual Type of Intronic Mutation

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Summary

In the coding part and the intron-exon boundaries of the *androgen receptor* gene of a patient with partial androgen insensitivity, no mutation was found. The androgen receptor of this patient displayed normal ligand-binding parameters, and migrated as a 110-112 kDa doublet on SDS-PAGE in the absence of hormone. However, after culturing of the patient's genital skin fibroblasts in the presence of hormone, the slower migrating 114-kDa protein, which reflects hormone-dependent phosphorylation, was hardly detectable. Furthermore, receptor protein was undetectable in the nuclear fraction of the fibroblasts, after treatment with hormone, which is indicative of defective DNA binding. By sequencing part of intron 2, a T→A mutation was found 11 base pairs upstream of exon 3. In our screening of 102 chromosomes from unrelated individuals this base pair substitution was not found, indicating that it was not a polymorphism. mRNA analysis revealed that splicing involved a cryptic splice site located 71/70 base pairs upstream of exon 3, resulting in generation of mRNA with an insert of 69 nucleotides. In addition, a small amount of a transcript with a deleted exon 3 and a very low level of wild type transcript were detected. Translation of the extended transcript resulted in an androgen receptor protein with 23 amino acid residues inserted in between the two zinc clusters, displaying defective DNA binding and defective transcription activation.

Introduction

Expression of a number of genes involved in male sex differentiation and development is regulated by the AR. The AR belongs to the family of steroid hormone-activated transcription modulators (Evans 1988). Like the other steroid hormone receptors, the AR consists of distinct functional domains. The NH_2-terminal part is involved in transcription activation and is encoded by exon 1 (Faber *et al.* 1989). Two highly conserved DNA-binding zinc clusters are encoded by exons 2 and 3. The NH_2-terminal zinc cluster recognizes specific consensus DNA sequences, whereas the C-terminal zinc cluster is involved in dimerization (Dahlman-Wright *et al.* 1991, Luisi *et al.* 1991). Parts of exons 3 and 4 encode the hinge region, which contains a NLS that is involved in nuclear import, and exons 4-8 encode the LBD.

On ligand binding, the AR undergoes conformational changes and binds to AREs in the promoter regions of androgen-regulated target genes (Beato and Sánchez-Pacheco 1996). Recently, coactivators, interacting with the LBD of

steroid hormone receptors have been cloned (reviewed by Horwitz *et al.* 1996). One of these, ARA70, appears to be involved in transcription activation by the AR (Yeh and Chang 1996).

Defects in the hAR cause disturbed virilization in 46,XY individuals, which is called AIS (reviewed by Quigley *et al.* 1995). Many qualitative and quantitative AR abnormalities, causing a broad range of AIS phenotypes, have been described (Gottlieb *et al.* 1997). The spectrum of phenotypes ranges from individuals with completely female external genitalia and absence of müllerian and wolffian duct derivatives (complete AIS) to patients with ambiguous genitalia (partial AIS) or with mild hypospadias (Quigley *et al.* 1995). The mutations that are most frequently observed, are nonsense or missense point mutations. Mutations resulting in aberrant splicing are much less common, only six of them have been reported (Gottlieb *et al.* 1997). In five of these, a consensus splice donor site was mutated, resulting in complete AIS.

PCR-SSCP analysis is a screening method, often used for mutation detection in the hAR. Using this method, we and others have reported that in some individuals, clinically diagnosed as having AIS, no mutation was found in the coding region and exon flanking intronic sequences of the *AR* gene (Morel *et al.* 1994, Brüggenwirth *et al.* 1996, Weidemann *et al.* 1996). In the present study, we investigated and characterized the *AR* gene of a patient with partial AIS. Initially no mutation was detected in the coding region of the *AR* gene in this patient, although several biochemical and cell biological assays revealed that the encoded AR of this patient was unable to bind to DNA.

Subjects and methods
Clinical subjects
A family with three individuals clinically suspect for AIS (II-4, III-1 and III-2; for pedigree, see Figure VI.1), was referred for further diagnosis, treatment and genetic counseling. All affected individuals were 46,XY and had a female habitus with normal female external genitalia, and normal but underdeveloped testes with epididymides and vasa deferentia were present. No müllerian remnants were found. One postpubertal patient (II-4) was Tanner P3, M5 and had axillary hair. Testosterone synthesis disorders, which could be another cause for such a 46,XY phenotype, were excluded by analysis of the circulating levels of steroid hormones and their precursors in this patient. At the age of 15.5 yrs., she had a normal male level of testosterone (21 nmol/liter) (normal range in adults: 10-30 nmol/liter) combined with high levels of LH (13 IU/liter) (normal range in adult
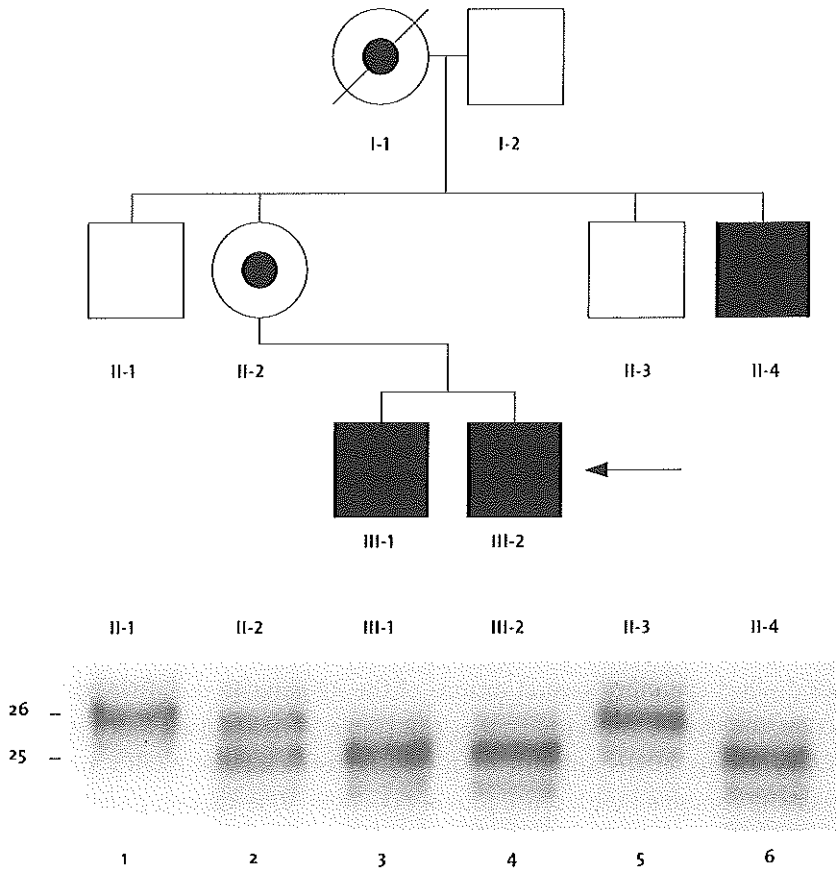
Figure VI.1 Pedigree and sizing of the poly-glutamine stretch of the present family with AIS. PCR products, obtained by amplification of genomic DNA by primers flanking the (CAG)nCAA-repeat, were resolved on a 7% denaturating polyacrylamide gel. Lane 1, normal male; lane 2, mother of the index subject; lane 3, 46,XY sister of the index subject; lane 4, 46,XY index subject; lane 5, normal male; lane 6, 46,XY affected aunt. The index subject (III-2) is indicated with an arrow. Numbers in the left margin of the lower part of the figure represent the numbers of glutamine residues encoded by the (CAG)nCAA-repeat.

males: 1.5-8 IU/liter). For final proof of the diagnosis and for the purpose of genetic counseling of this family, DNA analysis of the *AR* gene was started. Blood cells were obtained from the three 46,XY patients, and genital skin fibroblasts from III-1 and III-2. Genital skin fibroblasts containing wild type AR protein and AR protein with an exon 3 deletion, derived from a patient with Reifenstein syndrome, previously described by Ris-Stalpers *et al.* (1994b), were used for comparison. Genomic DNA from 74 unrelated individuals was used for intron 2 screen-ing. Informed consent was obtained from all individuals.

Mutation detection

Genomic DNA was isolated from blood cells or genital skin fibroblasts, according to standard procedures (Sambrook *et al.* 1989). Single strand conformation polymorphism analysis and direct sequencing were performed as described previously (Brüggenwirth *et al.* 1996). A total of 102 normal chromosomes from unrelated individuals were analyzed by automated sequencing. Template was made using intron 2 sense primer C1 and intron 3 antisense primer C2 (Lubahn *et al.* 1989), and purified by use of the Boehringer High Pure PCR Product Purification Kit (Boehringer Mannheim). Sequencing was performed with antisense primer 3BB (Table VI.1). Determination of the length of the polymorphic CAG-repeat in exon 1, used as an intragenic polymorphic marker, was performed according to Sleddens *et al.* (1992).

Reverse-transcriptase-PCR (RT-PCR) reaction

Total RNA was extracted from genital skin fibroblasts using TRIzol reagent (Gibco BRL) and quantified by absorption at 260 nm. Amplification of genomic DNA was prevented because the primers used in the RT-PCR reaction resulted in a PCR product spanning several introns of the *AR* gene. In the cDNA synthesis reaction, the exon 5 antisense primer 5BB (Table VI.1) was used for first strand cDNA synthesis. cDNA amplification was performed by PCR with antisense primer 5BB combined with sense primer J3A (Table VI.1), spanning the 3' end of exon 1 and the 5' end of exon 2. To obtain sufficient quantities of template DNA spanning exon 2 and 3 sequences, for direct sequencing, a nested-PCR reaction was performed, by use of sense primer J3A and exon 4 antisense primer 14NB (Table VI.1).

Ligand-binding study

For determination of ligand-binding characteristics of the AR of the AIS patients, a whole cell assay was performed on genital skin fibroblasts as described elsewhere (Brüggenwirth *et al.* 1996).

Western blot analysis

AR protein, obtained from cultured genital skin fibroblasts or transiently transfected CHO and COS-1 cells, was immunoprecipitated and analyzed by Western immunoblotting according to Ris-Stalpers *et al.* (1991).

Table VI.1 **Oligonucleotides used for cDNA synthesis and allele specific hybridization**

Oligonucleotide	Location	Sequence
3BB	intron 3	5' AGAGAAAGAAAAGTATCTTAC 3'
5BB	exon 5	5' CGAAGTAGAGgATCCTGGAGTT 3'
J3A	exon 1 - exon 2	5' gAtGGatcCATGCGTTTGGAGACTGC 3'
14NB	exon 4	5' TGCAAAGGAGTtGGGCTGGTTG 3'
470A	exon 1	5' GTAGCCCCCTACGGCTACA 3'
wild type	exon 2 - exon 3	5' GCTGAAGGGAAACAG 3'
69 bp insertion	exon 2 - intron 2	5' CTGAAGAAATACCCG 3'
exon 3 deletion	exon 2 - exon 4	5' CTGAAGCCCGGAAGC 3'
2AA	exon 2	5' CAGAAGACCTGCCTGATCTGT 3'

Lower case lettering indicates mismatches

Construction of expression vectors

Human wild type AR cDNA expression plasmid pSVAR0 (Brinkmann *et al.* 1989) was used to construct pSVAR129, encoding an AR with 23 additional amino acid residues between the two zinc clusters. To this end, the 472-bp *KpnI-AspI* fragment from pSVAR0 was exchanged with the 541 bp *KpnI-AspI* fragment, generated by RT-PCR from the AR mRNA of patient III-2. cDNA was synthesized as described in *Mutation detection* above. In the PCR reaction, following first strand cDNA synthesis, exon 1 sense primer 470A (Table VI.1) was used, allowing digestion with *KpnI*. A nested PCR was performed by use of sense primer 470A and exon 4 antisense primer 14NB (Table VI.1). All PCR products were checked by sequencing. Expression plasmid BHEX-AR33, an expression plasmid with an inframe deletion of exon 3, was constructed as described elsewhere (Ris-Stalpers *et al.* 1994b). To generate pSG5AR129 and pSG5AR33, the 541 bp *KpnI-AspI* fragment containing the 69 additional bp, and the 355 bp fragment from which exon 3 had been deleted were exchanged with the 472 bp *KpnI-AspI* fragment of wild type AR expression vector pSG5AR0 (provided by Dr. A.C.B. Cato, Karlsruhe, Germany). pSG5 plasmids were used to obtain AR protein for gel-shift assays.

Cell culture and transfections

Genital skin fibroblasts and COS-1 cells were cultured as described by Ris-Stalpers *et al.* (1990). CHO cells were treated like COS-1 cells. CHO cells were plated at 7 cm, wells and grown for 24 hours before they were transiently transfected, by the use of the calcium phosphate method (Chen and Okayama 1987), with AR expression plasmid (10 ng DNA/ml precipitate suspension) and the MMTV-Luc reporter plasmid (2 µg DNA/ml precipitate suspension) (de Ruiter *et al.* 1995). Carrier DNA (pTZ19) was added to 20 µg DNA/ml precipitate suspension, and 90 µl precipitate suspension was added per well. Twenty-four hours after transfection, cells were incubated with increasing concentrations of R1881. Luciferase assays were performed as described before, after 24 hours incubation (Kuil *et al.* 1995). Each receptor mutant was assayed three times in triplicate, by use of three independently isolated expression-plasmid preparations. Luciferase activity was related to basal activity, measured in the absence of hormone. CHO cells, used for expression studies, were plated in 175 cm² culture flasks and transfected with 200 µl DNA precipitate, as described above, by use of the calcium phosphate method. COS-1 cells were transiently transfected in 80 cm² culture flasks with 9.4 µg expression plasmid, by use of the diethylaminoethyldextran method (Gerster *et al.* 1987). Cells were shocked with 80 µM chloroquine for 2 hours. Transfected CHO and COS-1 cells were washed after 24 hours and cultured for another 24 hours in either the presence or absence of 10 nM R1881.

Preparation of cytosolic fractions and nuclear extracts

Genital skin fibroblasts were grown until confluence in 175 cm² culture flasks, incubated for 24 h in culture medium (Ris-Stalpers *et al.* 1990) containing 10% hormone-depleted fetal calf serum, and were cultured for another 24 hours with medium either with or without 10 nM R1881. Next, cells were washed twice in PBS, were collected in 1 ml lysis buffer A [40 mM Tris, 1 mM EDTA, 10% (vol/vol) glycerol, 10 mM DTT, 10 mM Na_2MoO_4, 0.5 mM bacitracin, 0.5 leupeptin, 0.6 mM PMSF], and subjected to four freeze-thaw cycles, followed by 10-min centrifugation at 800x*g* in a Biofuge (Heraeus) at 4 °C. The supernatant was centrifuged for 10 min. at 400,000x*g* at 4 °C (TLA120.2 rotor; Beckman, Fullerton, CA). The cytosol fraction (supernatant) was stored at -80 °C until use. The pellet, remaining after the first 800x*g* centrifugation step was resuspended in buffer B (buffer A with 0.2 % Triton X-100) and incubated for 5 min at 4 °C, followed by 10 min centrifugation at 800x*g* in a Biofuge 13. The resulting pellet (nuclear fraction) was washed with buffer C (buffer A without leupeptin), and was resuspended in equal

volumes of 1 M NaCl and 0.5 M NaCl and incubated for 1 hour at 0 °C. Nuclear extract (supernatant) was obtained by centrifugation for 10 min at 400,000xg at 4 °C (TLA120.2 rotor). The total nuclear extract and 250 μl cytosol fraction were taken separately for immunoprecipitation.

Gel-shift assay

After transfection with pSG5AR0, pSG5AR33 or pSG5AR129, COS-1 cells were collected in 5 ml PBS. The pellet was resuspended in extraction buffer [10 mM NaH$_2$PO$_4$ (pH 7.4), 0.4 M KCl, 1 mM EDTA, 10% (vol/vol) glycerol, 0.5 mM bacitracin, 0.5 mM leupeptin and 0.6 mM PMSF, 10 mM DTT] and was frozen and thawed four times, followed by 10 min centrifugation at 400,000xg (TLA120.2 rotor) at 4 °C. The double-stranded DNA probe, containing the ARE from the TAT promoter (5'-TCGAC*TGTACAGGATGTTCT*AGCTACT 3') (half-sites in *italics*), was obtained from Promega (Woerden, the Netherlands). Labeling and purification of the DNA probe and the gel-shift assay were performed as previously described by Brüggenwirth *et al.* (1998) (see also Chapter V).

Allele-specific oligonucleotide hybridization

RT-PCR and nested-PCR reactions were performed as described above (*see the Construction of expression vectors subsection above*). The resulting PCR product was amplified once more in a PCR reaction of 30 cycles, by use of exon 1 sense primer 470A and exon 4 antisense primer 14NB. Plasmid (pSVAR0, BHEX-AR33, and pSVAR129) fragments were amplified once under identical conditions, by use of the same primers. Dot blots were prepared in a Schleicher & Schuell apparatus according to the manufacturer's protocol. In the case of PCR product obtained by plasmid amplification, an equivalent amount of DNA was spotted. Membranes were preincubated for 10 min with hybridization mix [50 mM NaH2PO4, 0.75 M NaCl, 5 mM EDTA (= 5xSSPE); 1% SDS; 0.05 mg/ml herring sperm DNA]. For each of the splice variants, specific oligonucleotides were designed: a wild type probe, an exon 3 deletion probe, and a 69-bp insertion probe (Table VI.1) (Pharmacia Biotech Benelux). The filters were subsequently hybridized and rinsed as described by Boehmer et al. (1997), but at a temperature of 35 °C instead of 37 °C. The membranes probed with wild type probe or exon 3 deletion probe were washed for an additional 10 and 15 min, respectively, in 0.1 x SSC/0.1 % SDS at 38 °C, before exposure. After autoradiography, the membranes were stripped and the procedure was repeated with probe 2AA (Table VI.1) as a control.

Results

Screening for mutations

PCR-SSCP analysis was performed under two conditions to screen for mutations in the *AR* gene of an index subject with partial AIS. In the coding part and the exon-flanking intronic sequences, no mutation was found. To study segregation of a presumed AR defect, the length of the *AR* gene CAG-repeat was determined in DNA from several family members. Results are presented in Figure VI.1. The mother (II-2) of the two 46,XY sisters (III-1 and III-2) had two different alleles, one with a (CAG)24CAA unit coding for 25 glutamine residues and one with a (CAG)25CAA unit coding for 26 glutamine residues. The three affected 46,XY individuals (II-4, III-1 and III-2) had the (CAG)24CAA allele. Therefore, X-linked inheritance could not be excluded (Figure VI.1). The marker was informative, since two brothers of the 46,XX carrier (II-1 and II-3) had the (CAG)25CAA allele.

Receptor characteristics

AR protein isolated from genital skin fibroblasts from the index patient (III-2) appeared as a normal 110-112 kDa doublet after SDS-PAGE and immunoblotting. Hormone-binding parameters were determined in a whole cell-binding assay of genital skin fibroblasts. The receptor displayed a Kd of 0.08 nM and a Bmax of 64 fmol/mg protein, both within the normal range (Kd: 0.03-0.13 nM; Bmax: 39-169 fmol/mg protein). The AR of patient III-1 displayed a Kd of 0.07 nM and a Bmax of 63 fmol/mg protein. Therefore, the 46,XY individuals (III-1 and III-2) in this AIS family were classified as having receptor-positive AIS.

Hormone-dependent receptor phosphorylation

The AR is phosphorylated and many potential phospho-sites are located in the part encoded by exon 1. In preparations from cells cultured in the absence of hormone, usually two receptor isotypes are present, which are visible as a 110-112 kDa doublet on a SDS-PAGE immunoblot (Kuiper *et al.* 1991). AR protein was isolated from control genital skin fibroblasts and from genital skin fibroblasts of the index patient (III-2). In both genital skin fibroblast preparations, cultured in the absence of androgens, the 110 and the 112 kDa AR isotypes were present (Figure VI.2A, lanes 1 and 2). The AR from patient III-2 contains a relatively long glutamine stretch (25 glutamines compared with 19 in the control AR), resulting in a slower migration pattern. On hormone binding, the AR undergoes additional phosphorylation, reflected by a 114-kDa isoform. The appearance of this isoform is dependent on DNA binding and/or transcription activation (Jenster *et*

Figure VI.2A and B **Western blot analysis of wild type and mutant androgen receptor proteins.**
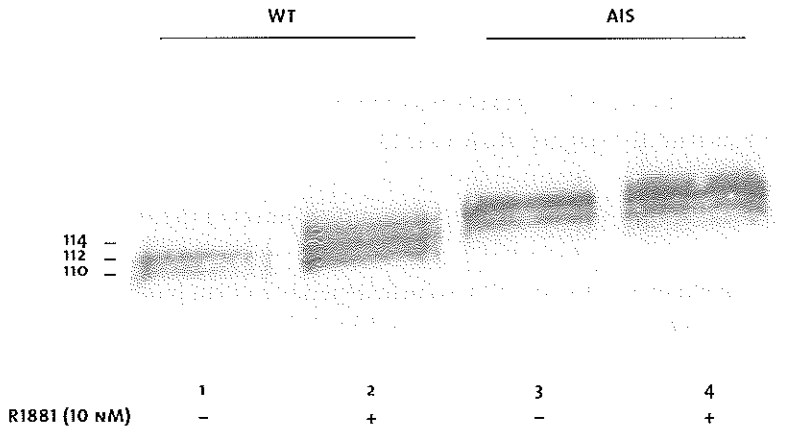
A Western blot, showing receptor isotypes of the wild type AR and the mutant AR. AR protein was prepared from genital skin fibroblasts, that had been cultured in the absence (-) or presence (+) of 10 nM R1881. After immunoprecipitation with monoclonal anti-AR antibody F39.4.1, receptor protein was separated on a 7% SDS-PAGE gel. After immunoblotting the blot was incubated with Sp061, a polyclonal anti-AR antibody. The blot was washed and then incubated with an alkaline phosphatase-coupled goat-anti-rabbit antibody to visualize the AR. Lanes 1 and 2, wild type AR; lanes 3 and 4, mutant AR.
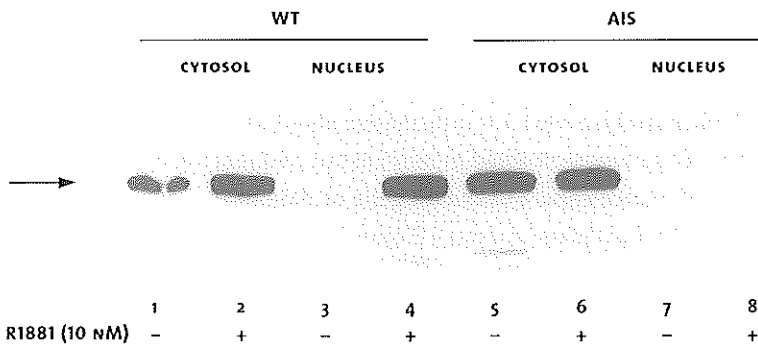
B Subcellular localization of the wild type AR and the mutant receptor in genital skin fibroblasts of patient III-2, after culturing of genital skin fibroblasts in the absence or presence of R1881. Genital skin fibroblasts were cultured for 24 hours either in the absence (-) or presence (+) of 10 nM R1881. Nuclear and cytosol fractions were prepared, and the AR was immunoprecipitated with monoclonal antibody F39.4.1 from both the total nuclear extract and one-fourth of the cytosol fraction, Western blotting and immunostaining were performed as described in the Legend to Figure VI.2A. AR was visualized with a peroxidase-coupled goat-anti-rabbit antibody. Lanes 1 and 2, cytosol fraction from wild type genital skin fibroblasts; lanes 3 and 4, nuclear extract from wild type genital skin fibroblasts; lanes 5 and 6, cytosol fraction from patient III-2 genital skin fibroblasts; lanes 7 and 8, nuclear extract from patient III-2 genital skin fibroblasts.

al. 1994). This hormone-induced phosphorylation was used as a marker for proper receptor functioning. Control cells cultured in the presence of hormone displayed the expected, slower migrating, third isoform (114 kDa) (Figure VI.2A, lane 2). However, the 114-kDa isoform was hardly detectable in preparations derived from patient III-2 (Figure VI.2A, lane 4).

Subcellular localization studies in genital skin fibroblasts of the index subject

Because the appearance of the 114-kDa isoform depends on DNA binding (Jenster *et al.* 1994), we investigated whether AR was detectable in the tightly nuclear bound AR fraction in genital skin fibroblasts from the patient. Control genital skin fibroblasts and genital skin fibroblasts from the index patient were cultured in either the absence or presence of 10 nM R1881, and cytosol and nuclear fractions were prepared. When cells were cultured in the absence of hormone, AR was found in the cytosol fraction (Figure VI.2B, lanes 1 and 5), but not in the nuclear fraction (lanes 3 and 7). After culture in the presence of hormone, although AR was still detectable in the cytosol fractions (lanes 2 and 6), wild type AR was clearly present in the nuclear extract (lane 4), whereas AR protein was not observed in the nuclear extract of the patient's genital skin fibro-blasts (lane 8). This suggests a defect in DNA binding of the AR in the index patient (III-2).

Mutation detection and the effect of the mutation on the splicing process

Although PCR-SSCP analysis was not informative about an *AR* gene mutation in patient III-2, the *AR* gene was studied in more detail. Sequencing of the flanking intronic regions of exons 2 and 3 of the *AR* gene of patient III-2 demonstrated a mutation (T→A) 11 bp upstream of exon 3 (Figure VI.3A). The same mutation was found in the AR gene of patients II-4 and III-1. Because the mutation is located in the region where forward primer (primer 3A) anneals, the mutation remained undetected in PCR-SSCP analysis. In RT-PCR experiments, two different receptor variants were found. Predominantly, a transcript containing 69 additional nucleotides between the sequences of exons 2 and 3 (mutant transcript I), and a smaller amount of a transcript from which exon 3 was deleted (mutant transcript II) (Figure VI.3B) were detected. Translation of transcript I will result in an AR with a 23 amino acid insertion between the two zinc clusters, and transcript II encodes a protein that lacks the second zinc cluster. Wild type transcript was not detected by RT-PCR. SDS-PAGE and immunoblot analysis revealed only a protein with an increased molecular mass in genital skin fibroblasts from patient

Figures VI.3A and B **Characterization of the point mutation**
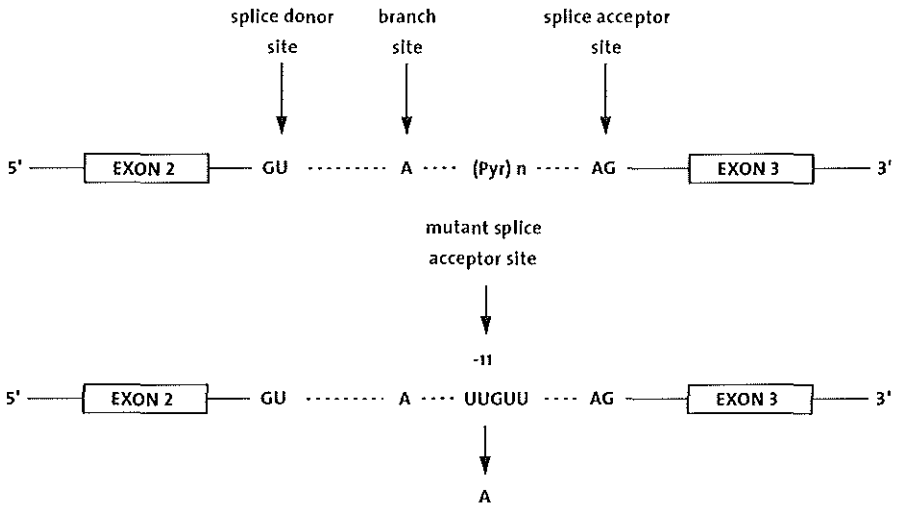
A Position of the point mutation, found in intron 2. Represented are exons 2 and 3 and significant flanking intron 2 sequences of both wild type and the mutant AR pre-mRNAs. The positions of the splice donor site, the branch site, and the splice acceptor site are indicated. The mutation is located at position -11 in intron 2 in the conserved pyrimidine-rich region 5' upstream of the splice acceptor site of intron 2.
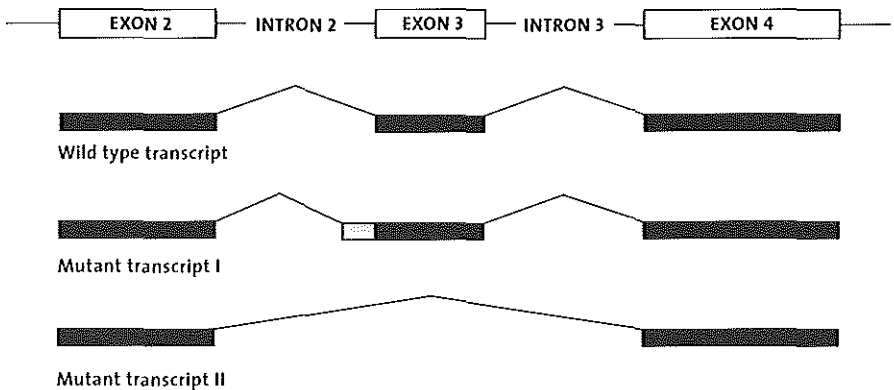
B Illustration of the wild type and the aberrant splicing process, resulting from the intron 2 mutation. Black bars represent exons 2, 3, and 4; gray bar represents 69 additional nucleotides. Mutant transcript I and mutant transcript II were found by RT-PCR studies on genital skin fibroblast mRNA from the index subject.

III-2 (Figure VI.2A, lanes 3 and 4). The 23 additional amino acid residues, as well as the relatively long glutamine stretch, contributed to the slower migration pattern seen with SDS-PAGE analysis, as compared with the control AR. To prove that the mutation at position -11 is not a common polymorphism, 102 normal chromosomes from unrelated individuals were screened for the presence of this mutation in intron 2 of the *AR* gene. The mutation was not detected in these control individuals. In addition, a larger part of intron 2 of the *AR* gene of the index patient was sequenced to exclude the presence of an additional mutation that could have induced the preferential use of the cryptic splice site. No other alterations were found in intron 2 up to position -137.

Immunoblot analysis of the AR protein

The expression plasmids pSG5AR0, pSG5AR129, and pSG5AR33, encoding the wild type AR, AR TI, and AR TII, respectively, were transiently expressed in COS-1 cells. In the cells transfected with pSG5AR0, the 114-kDa isoform was detected after culturing in the presence of hormone. However cells, transiently transfected with either one of the receptor mutants almost lacked this third isoform (Figure VI.4A, lanes 4 and 6).

Gel-retardation assay

In order to establish whether DNA binding by the mutant AR was affected, *in vitro* binding to a consensus ARE was studied. In the presence of polyclonal antibody Sp197, which stabilizes the protein-DNA complex (Kuiper *et al.* 1993a), the wild type receptor was able to bind to a consensus ARE, resulting in a shifted probe (Figure VI.4B, lane 5). No shifted probe was detected for the mutant AR TI and TII (Figure VI.4B, lanes 7 and 9) or in the control lane (Figure VI.4B, lane 3). The amount of receptor protein was checked by Western blotting and immunostaining. Comparable amounts of AR protein were incubated.

Transcription-activation assay

The wild type AR showed transcription activation on the MMTV promoter in CHO cells (Figure VI.4C). However, AR TI and TII were unable to activate transcription on an MMTV-Luc promoter (Figure VI.4C). SDS-PAGE, followed by immunoblotting, showed that AR protein expression was identical for the three different receptor molecules (results not shown).

	WT		T II		T I	
114 —						
112 —						
110 —						

	1	2	3	4	5	6
R1881 (10 nM)	–	+	–	+	–	+
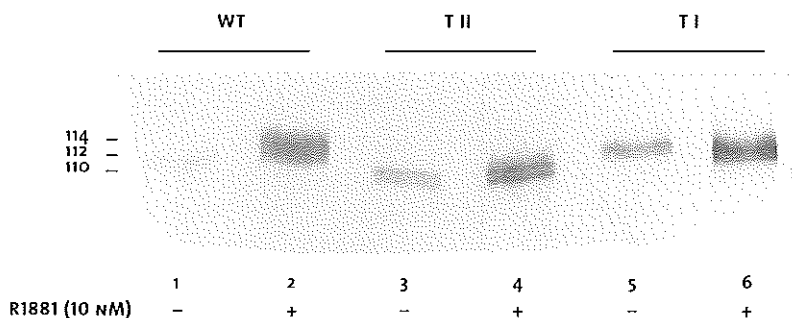
Figure VI.4A **Functional analysis of wild type and mutant androgen receptors** Hormone-induced ups-hift of the AR proteins. AR protein variants were expressed in COS-1 cells after transfection with pSG5AR0 (WT), pSG5AR33 (TII), or pSG5AR129 (TI). The cells were cultured for 24 hours in the absence (-) or presence (+) of 10 nM R1881. After immunoprecipitation using monoclonal antibody F39.4.1, SDS-PAGE was performed followed by immunoblotting. The AR was detected by immunostaining using polyclonal antibody Sp061 and an alkaline phosphatase-coupled anti-rabbit-antibody. Lanes 1 and 2, wild type AR (WT); lanes 3 and 4, AR deletion mutant (TII); lanes 5 and 6, AR insertion mutant (TI).

	COS -1	WT	T I	T II

	1	2	3	4	5	6	7	8	9
Sp197	+	–	+	–	+	–	+	–	+
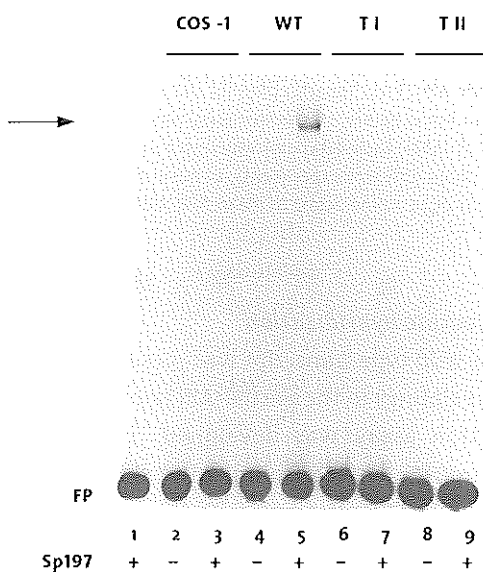
Figure VI.4B **Functional analysis of wild type and mutant androgen receptors** Gel-shift assay. Cell extracts, prepared from COS-1 cells were incubated for 10 min. on ice in binding buffer with poly[dI-dC]-poly[dI-dC], either in the presence (+) or absence (-) of polyclonal anti-AR antibody Sp197. Labeled ARE probe (50,000 cpm) was added and the samples were incubated for 20 min. at room temperature. Com-plexes were analyzed by polyacrylamide gel electrophoresis, as described in Subjects and Methods. The position of the shifted complexes is indicated by an arrow, and FP indicates the position of the free probe. Lane 1, free probe; lanes 2 and 3, COS-1 extract; lanes 4 and 5, wild type AR (WT); lanes 6 and 7, mutant AR, encoded by transcript I (TI); lanes 8 and 9, mutant AR, encoded by transcript II (TII).
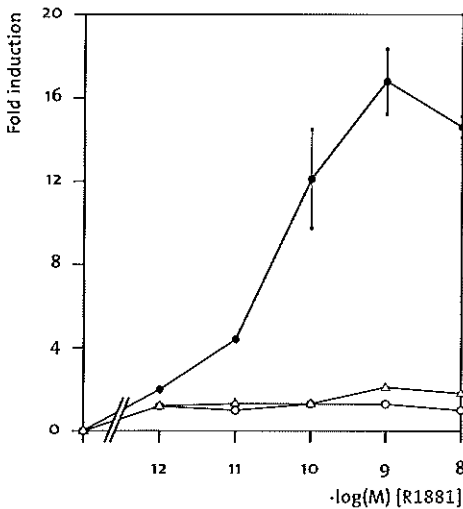
Figure VI.4C **Functional analysis of wild type and mutant androgen receptors** Transcriptional activity. CHO cells were cotransfected with MMTV-Luc reporter plasmid and with either wild type or mutant AR expression plasmids. Twenty-four hours before a luciferase assay was performed, cells were incubated with increasing concentrations of R1881. Each datapoint was tested in triplicate. ● : mean-fold induction ± SEM of wild type AR, for three different experiments; o : mean-fold induction ± SEM calculated for AR mutant with the insertion of 23 amino acids; Δ: mean-fold induction ± SEM of the AR mutant with the exon 3 deletion.

Allele-specific oligonucleotide hybridization

To investigate whether a wild type transcript was present in genital skin fibroblasts of the two affected siblings in the partial-AIS family, an allele specific oligonucleotide-hybridization assay was developed. First-strand cDNA was synthesized from total mRNA, derived from genital skin fibroblasts, and was amplified in three consecutive PCR reactions. mRNA was isolated from wild type genital skin fibroblasts, from genital skin fibroblasts from patients III-1 and III-2, and as a control from genital skin fibroblasts derived from a patient with partial AIS who has been described previously by Ris-Stalpers *et al.* (1994b). The mutation, found in this latter patient causes differential splicing, resulting in 10 % wild type mRNA and 90% mRNA of a splice variant with a deletion of exon 3. The PCR products were spotted on membranes and subsequently hybridized with oligonucleotides specific for the splice variants and the wild type AR (Table VI.1). Hybridization with the wild type probe revealed wild type mRNA in genital skin fibroblasts from a normal control male, in genital skin fibroblasts from the par-

tial- AIS patient (positive control), and in genital skin fibroblasts from one of the 46,XY patients (III-1) (Figure VI.5, WT-1b, 1c, and 1d, respectively). In genital skin fibroblasts from patient III-2, wild type transcript was either not present or below the detection limit of the assay (Figure VI.5, WT-1e). TI was only present in genital skin fibroblasts from patients III-1 and III-2 and not in wild type genital skin fibroblasts (Figure VI.5, TI-3d and 3e, respectively). TII was detected in genital skin fibroblasts of the positive control and patient III-1 (Figure VI.5, TII-5c and 5d, respectively). The signal at position 5e in Figure VI.5 (patient III-2), which was comparable to the a-specific signal at position 5b in Figure VI.5, resulted from
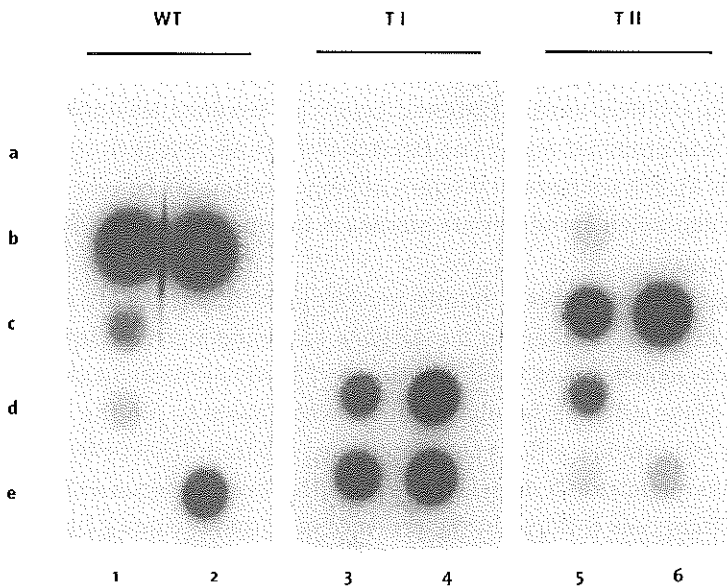
Figure VI.5 **Allele specific oligonucleotide hybridization. DNAs were amplified using exon 1 sense primer 470A and exon 4 antisense primer 14 NB in two consecutive PCR reactions.** The expression plasmids (pSVAR0, BHEX-AR33, and pSVAR129), used as controls, were amplified in one PCR reaction. The resulting PCR products were spotted on three separate membranes, which were subsequently hybridized with allele specific oligonucleotides as described in Subjects and Methods. Membrane WT: wild type probe; membrane TI: 69 bp insertion mutant; membrane TII: exon 3 deletion mutant. For lanes 1 (WT), 3 (TI), and 5 (TII), a = blank; b = amplified cDNA of normal control; c = amplified cDNA of a patient with Reifenstein syndrome; d = amplified cDNA of patient III-1; e = amplified cDNA of patient III-2. For lanes 2 (WT), 4 (TI), and 6 (TII), a = denaturation buffer; b = amplified wild type expression plasmid; c = amplified expression plasmid (BHEX-AR33); d = amplified expression plasmid (pSVAR129); e = mixture of amplified expression plasmids pSVAR0, BHEX-AR33, and pSVAR129 (1:1:8).

cross-hybridization of the probe with wild type DNA and was considered as background. DNA, amplified from expression plasmids, which was used as a positive control, is visible in rows 2, 4 and 6 of Figure VI.5. Comparable amounts of DNA were spotted, as was assessed by hybridization with an exon 2 probe (results not shown).

Discussion

It is well established that AIS is caused by mutations in the *AR* gene. However, reports have appeared about patients with an AIS phenotype in which no mutation was detected by use of PCR-SSCP analysis (Brüggenwirth *et al.* 1996; Weidemann *et al.* 1996) and DNA sequencing (Morel *et al.* 1994), in spite of clear phenotypic, endocrinological, and biochemical evidence for AIS. Mutations might be missed because PCR-SSCP is not 100% sensitive. Often, only the exonic sequences and their flanking intronic regions are screened, leaving mutations in intronic and promoter regions undetected. For the AR, if cells from patients are available, RT-PCR studies and Western immunoblotting can be very informative, in particular when mutations are present in intronic regions (Ris-Stalpers *et al.* 1990, 1994b).

In the present report, a family presenting with partial AIS is described. No mutation was detected in the *AR* gene of the index patient after PCR-SSCP analysis or sequencing of the exons. However, biochemical studies revealed that the AR of the affected family members was unable to bind to DNA. In the absence of hormone, wild type receptor derived from transiently transfected COS-1 cells migrates as a 110-112 kDa doublet (Kuiper *et al.* 1993a). AR preparations derived from cells cultured in the presence of hormone contain, in addition to the 110-112 kDa doublet, a slower-migrating, 114-kDa isoform, representing hormone-dependent phosphorylation (Jenster *et al.* 1994). It has been shown that the appearance of the 114-kDa isoform is correlated with DNA binding and/or transcription activation (Jenster *et al.* 1994). In the present report, hormone-dependent phosphorylation of the AR protein was used as an indicator of receptor dysfunctioning. Since the 114-kDa isoform was hardly detectable in genital skin fibroblasts derived from the index subject (III-2) and her sister (III-1), cellular distribution of the AR from the index subject was studied. AR was undetectable in the tight nuclear-bound fraction of hormone-exposed genital skin fibroblasts from the index subject, which points to disturbed AR-DNA binding and, consequently, to a defect in transcription activation. Sequencing analysis revealed a mutation at position -11 in intron 2, in the *AR* gene of both siblings,

their 46,XY aunt, and the index patient's mother (carrier of the mutation). Since this mutation was not found in 102 chromosomes derived from unrelated controls, we have concluded that it is not a common polymorphism.

The effect of the mutation on the splicing process was studied. Splicing of pre-mRNA begins with the cleavage at the 5' splice donor site, which is attacked by the 2-OH group of the branch-site adenosine. The 3' splice site is attacked by the newly formed 3'-OH of the upstream exon, the intron is released in the form of a lariat, and the exons are joined (Sharp 1985). Around the splice acceptor site a consensus sequence, (T/C, T/C, T, T, T/C, T/C, T/C, T/C, T/C, T/C, N, C, A, G, G) important for proper splicing, is present (Shapiro and Senapathy 1987). The present AR mutation was located in this pyrimidine-rich consensus sequence preceding the splice acceptor site. Aberrant splicing took place, resulting in different transcripts encoding defective ARs, which might explain the observed phenotype. The cryptic splice site, that was used is located at position -71/-70 in intron 2, thus resulting in a mRNA variant containing an insertion of 69 nucleotides. We determined that sequences in and around this cryptic splice site did not contain any mutation, thereby not enhancing preferable usage of this site.

According to Nakai and Sakamoto (1994), almost all major cryptic splice sites, which are activated by mutations, are located within 100 nucleotides from the original splice site. On the basis of comparison with a consensus sequence, the cryptic splice site located at position -71/-70 in the AR shows more homology with the consensus sequence than does the wild type splice acceptor site (the cryptic splice site is T, C, T, T, T, T, C, T, G, T, T, C, T, A, G, A; the wild type intron 2 splice site: T, A, T, T, T, G, T, T, C, T, C, C, C, A, G, C). However, RT-PCR studies performed on wild type mRNA demonstrated that this cryptic splice site is normally not used. Watakabe *et al.* (1992) proposed that suboptimal 3' splice site sequences require stimulation by downstream sequences. Thus, exon 3 sequences of the AR may play a role in splice acceptor site selection. The newly created splice acceptor site at position -11/-10 was not used, probably because, compared with the novel cryptic site at position -71/-70, the sequence was less favorable for splicing. A small amount of a transcript, from which exon 3 was deleted, was detected. wild type transcript was not observed, although this could be expected on the basis of the partial-AIS phenotype of the affected individuals. We concluded that the wild type splice acceptor site has become weaker because of the base pair substitution at position -11. Alternatively, closely spaced splice sites can inactivate each other because of sterical hindrance of bound splicing factors (Black 1991). Nelson and Green (1988) showed with *in vitro* splicing experiments

that, in a β-globin intron, insertion of a new splice donor site close to the wild type splice donor site prevented splicing at both sites. Therefore, aberrant splicing in our patients could also be the result of interference by two closely spaced splice acceptor sequences.

Quigley *et al.* (1992b) have previously described the so-called null phenotype of AIS. A deletion of the X chromosome spanning the complete *AR* gene caused complete AIS. The patient showed inguinal or abdominal testes, no wolffian-duct development, and absence of masculinization of the external genitalia. Sparse pubic and axillary hair was detected. Also, a complete external female phenotype and absence of secondary hair were seen in a 46,XY individual with a complete deletion of the AR gene, as reported by Hiort *et al.* (1996). All AIS subjects in the family reported in this paper showed remnants of vasa deferentia and epididymides. The aunt (II-4), clinically investigated postpubertally, has pubic and axillary hair. These phenotypic characteristics suggest that some residual AR activity is present. Therefore, DNA binding and transcription-activation capacities of the splice variants were investigated. The AR, expressed in genital skin fibroblasts from the index subject has 23 additional amino acids between the first zinc cluster and the second zinc cluster. Ducouret *et al.* (1995) cloned a teleost-fish glucocorticoid receptor (GR) with 9 additional amino acid residues between the two zinc clusters. This fish GR, however, was still capable of activating a reporter gene, from which it was concluded that the folding of the GR could compensate for separation of the two zinc clusters by an extra 9-amino-acid stretch. The AR mutant with the insertion of 23 amino acid residues did not bind to a consensus ARE (on the basis of a gel-retardation assay). Consequently, there was absence of transcription activation. The splice variant with a deletion of the second zinc cluster was also unable to bind specifically to DNA, corresponding to the results reported by Quigley *et al.* (1992a) and Ris-Stalpers *et al.* (1994b). On the basis of these results and in view of the phenotype of the affected family members, we concluded that splicing was not completely aberrant. Indeed, a very small amount of wild type transcript was detected in genital skin fibroblasts of patient III-1, by use of the allele-specific oligonucleotide-hybridization method. The transcript with the deletion of exon 3 (i.e., AR TII) was detected in genital skin fibroblasts of patient III-1 and not in genital skin fibroblasts of patient III-2. However, this was not reproduced in all experiments, since AR TII had previously been detected by RT-PCR studies using RNA from patient III-2.

The intronic mutation, discussed in this paper was missed by PCR-SSCP analysis. There are several other explanations for the apparent absence of AR

mutations in several cases of AIS. Neutral mutations, for example, have to be interpreted with caution. Richard and Beckmann (1995) found a synonymous-codon mutation (GGC→GGT) in the cDNA of the *calpain* (CANP3) gene, which turned out to be pathogenic, because a splice donor site was created. Kallio *et al.* (1996) suggested that in 46,XY subjects without a mutation in the *AR* gene and a typical AIS phenotype, post-receptor defects might be the cause of the disease. This may involve receptor-specific cofactors or corepressors. Recently, a family with dominant inheritance of thyroid hormone resistance was reported, which could not be linked to defects in the *thyroid hormone-receptor* α or β genes (Weiss *et al.* 1996). It was postulated that an abnormal cofactor, playing a role in regulation of thyroid-hormone action, might be involved. Most cofactors, reported to date are not AR specific, so mutations in these factors will probably be lethal or give rise to complex phenotypes. One coactivator, ARA70, which binds to the ligand-bound AR, has been reported by Yeh and Chang (1996).

In conclusion: the mutation presented in this paper remained undetected with PCR-SSCP analysis. *AR* genes from AIS subjects for whom the clinical diagnosis is well established, and in whom no mutation can be detected after PCR-SSCP screening and additional sequencing of the coding parts of the gene should be analyzed for intronic mutations. RT-PCR studies and Western immunoblotting, using cultured genital skin fibroblasts, can provide important information.

General Discussion

Chapter VII
General Discussion

Table 7.1 **Mutations observed in unrelated individuals with AIS.** Based on the *AR* gene mutation database (Gottlieb *et al.* 1997)

	CAIS	CAIS/PAIS	PAIS	MAIS	Total
Structural gene defects					
partial deletions	10	-	-	1	11
complete gene deletion	3	-	-	-	3
1-4 bp deletion / insertion	11	-	1	-	12
intronic deletion	-	-	1	-	1
Single base mutation					
premature termination codon	21	-	-	-	21
splice junction abnormality	6	-	1	-	7
amino acid substitution	59 (92)	9 (37)	57 (73)	6 (7)	131 (209)
multiple amino acid substitution	3	-	3	-	6
Total number	113 (146)	9 (37)	63 (79)	7 (8)	192 (270)

The total numbers of different mutations, identified in unrelated patients with AIS are summarized. The total number of unrelated individuals, in whom a particular type of mutation was detected, are indicated between brackets.

VII.1 Mutations in the androgen receptor gene

During the first years after the cloning of the AR gene in 1988, mainly mutations in the LBD were studied. However, later on many mutations were identified also in the DBD. An overview of the type of mutations, reported worldwide until September 1997, is given in Table VII.1 (Gottlieb *et al.* 1997). Missense mutations were most frequently reported; in total, 131 different missense mutation have been identified. In 92 unrelated individuals with CAIS due to a missense mutation, 59 different mutations were found. Nine different missense mutations caused either CAIS or PAIS (genotype-phenotype variation) in 37 individuals. Fifty-seven different missense mutations caused PAIS in 73 individuals, and 6 different missense mutations caused MAIS in 7 individuals.

Mutations in the NH$_2$-terminal domain and the hinge region
So far, only three missense mutations have been reported in exon 1 of the *AR* gene. A transition of guanine to adenine in codon 2, which changed the glutamic acid residue at position 2 into a lysine residue, was associated with PAIS (Choong *et al.* 1996b). The bp change disturbed the translation initiation consensus sequence, causing reduced translational efficiency, finally resulting in a lower AR expression level (Choong *et al.* 1996b). In addition, a slightly increased hormone dissociation rate was established, indicating an effect of the AR NH$_2$-terminus on androgen bind-ing kinetics (Choong *et al.* 1996b). In an individual with CAIS, apart from substitution of the proline residue at position 389 by an arginine residue, the glycine residue at position 442 was changed into an arginine residue (Gottlieb *et al.* 1997). Other types of mutations that were identified in exon 1 result in premature stop codons, frameshifts, or extreme expansion of the polymorphic glutamine stretch. The latter mutation is the cause of spinal and bulbar muscular atrophy (SBMA) (La Spada *et al.* 1991). The premature stop codons in exon 1, either resulting from nonsense mutations or introduced by a frameshift mutation, were all associated with CAIS (Gottlieb *et al.* 1997). Most of the individuals having such a mutation were receptor negative, i.e. specific androgen binding was not detected (Gottlieb *et al.* 1997), with the exception of a patient described by Zoppi *et al.* (1993). Fibroblasts of this patient displayed a low amount of androgen binding, which was ascribed to reinitiation of translation. Reinitiation of translation offers also an explanation for the specific androgen binding that was measured in the Tfm mouse. In the *AR* gene of this mouse a single nucleotide insertion was detected that caused a

frameshift, introducing a stop codon at position 412 (Charest *et al.* 1991, Gaspar *et al.* 1991, He *et al.* 1991). Despite the presence of a ligand binding AR, both the Tfm mouse and the AIS subject had a complete phenotype. This should be ascribed to impaired transcription activation capacity of the truncated receptors, and to their low expression levels (Zoppi *et al.* 1993). The low AR expression levels might result from mRNA instability (Charest *et al.*1991) and/or inefficient reinitiation of translation, and/or instability of the NH_2-terminally truncated protein (Zoppi *et al.* 1993).

In the hinge region, which harbors part of the NLS and is encoded by the 5'-half of exon 4, only 2 (missense) mutations have been found that were located out-side the NLS. Both substitutions (Ala-636-Asp, Ile-645-Asp) were underlying PAIS (Pinsky *et al.* 1992, Hiort *et al.* 1996b). However, functional studies have not been performed with these mutant receptors.

The NH_2-terminal domain and the hinge region are the least conserved regions of the AR, and are therefore believed to be important with respect to receptor specificity. It is remarkable that only a low number of amino acid substitutions have been found in the NH_2-terminal domain and in the first part of the hinge region. This seems to suggest that the presence of substitutions in these particular regions do not cause a pathogenic phenotype. Indeed, it was shown that parts of the NH_2-terminus could be deleted without dramatic effect on transcription activation (Simental *et al.* 1991, Jenster *et al.* 1991, Jenster *et al.* 1995). Schena *et al.* (1989) studied mutations in the GR that were obtained by random mutagenesis. Point mutations were not recovered in the part of the gene encoding the NH_2-terminal half of the hinge region. This indicates that also in the GR single amino acid changes in this particular region do not result in a defective receptor with respect to transcription activation.

Mutations in the DBD

Many different mutations have been detected in the DBD, and the majority of these mutations probably affects DNA binding. Some amino acid residues were substituted more than once in unrelated individuals (Gly-559, Cys-579, Phe-573, Arg-606, Leu-607, and Arg-608) (Gottlieb *et al.* 1997). Although it is theoretically possible that mutations in the P-box change DNA-binding specificity, the phenotype of the patient in whom the valine residue at position 572 was substituted by a phenylalanine residue indicates complete androgen insensitivity (Lumbroso *et al.* 1993). As was discussed in Chapter I, mutations that were identified in exon 3 of the AR in cases of male breast cancer did not result in altered DNA-binding specificity (Poujol *et al.* 1997). A mutation, found in the D-box (Ala-587-

Thr) interfered with dimerization, causing PAIS (Gast *et al.* 1995). A mutation identified in the NLS (Leu-607-Pro) resulted in an inactive receptor due to defective DNA binding. Molecular modeling predicted that the amino acid substitution might interfere with protein structure or dimerization (Lobaccaro *et al.* 1996).

The amino acid composition of the GR-DBD is closely related to that of the AR. Therefore it seems reasonable to build a 3-D model of the AR-DBD, based on the crystal structure of the rat GR-DBD. Molecular modeling was applied to predict consequences of mutations in the AR (Lobaccaro *et al.* 1996, Poujol *et al.* 1997, Brüggenwirth *et al.* 1998). As was shown by Brüggenwirth *et al.* (1998) (see also Chapter V), molecular modeling might have its limitations. Because only the DBD of the GR was crystallized (Luisi *et al.* 1991), the influence that the liganded LBD might have on the conformation of the DBD (Glass 1994) was not taken into account. Moreover, the crystal structure is obtained under static conditions, while DNA binding is a very dynamic process. In addition, modeling based on the crystal structure of the AR-DBD, which is at present unknown, would be more precise, as not all amino acid residues are conserved between the AR- and GR-DBD (see also Chapter V).

Mutations in the LBD

Mutations that are the cause of either PAIS or CAIS show a comparable distribution over the length of the AR-LBD, with the exception of β-sheet 1 (see Table VII.2). The majority of mutations in the LBD were found to alter binding affinity. One mutation was reported (Gln-789-Glu) that impaired receptor activity while ligand binding was not affected (Bevan *et al.* 1996). A large part of the mutations that were associated with prostate cancer influence ligand binding specificity, suggesting that these mutations affect the ligand binding pocket in a subtle way. Only one mutation (Gln-789-Glu), found in prostate cancer, was also identified in a patient with AIS (Bevan *et al.* 1996, Evans *et al.* 1996). The mutation was not only present in the tumor, but also in blood cells of the man with prostate cancer, indicating its genomic origin (Evans *et al.* 1996). The prostate cancer patient was married but had no children, whereas the patient described by Bevan *et al.* (1996) had PAIS.

Recently, the crystal structure of the unliganded hRXR-α LBD, and the liganded hRAR-γ, rat TR-α, and hER-α LBDs were solved (Bourguet *et al.* 1995, Renaud *et al.* 1995, Wagner *et al.* 1995, Brzozowski *et al.* 1997). The overall structure of the different LBDs appeared to be quite similar, and therefore the structure of the AR-LBD could be predicted (see Figure I.4) (Wurtz *et al.* 1996).

Table VII.2 Mutations in the AR-LBD

A

Region	CAIS	PAIS	MAIS	Prostate carcinoma
< 658		Ala 636 Asp Ile 655 Asn		Ser 638 Asn
658-675 Helix 1	Leu 668 Pro Glu 672 Lys Val 675 Ile	Pro 662 His		Gln 661 Arg Ile 663 Thr Gly 674 Ala
676-688	Gly 679 Glu Asn 683 del Asp 686 Asn Asp 686 His	Cys 677 Arg Ala 678 Val Asp 681 del Asp 686 Asn		
689-711 Helix 3	Ser 693 Ala Asn 696 Ser (3) Leu 698 Arg Gly 699 Val	Ser 694 Gly Gly 699 Ala Leu 703 Phe		Leu 692 His Val 706 Met (2) Lys 711 Glu
712-720	Leu 713 Phe	Phe 716 Leu Leu 719 Ser	Asn 718 Lys	Ala 712 Thr Arg 717 Leu
721-730 Helix 4	Asp 723 Asn Asp 723 Tyr (3) Tyr 730 Arg	Gln 724 His Ile 728 Thr		Val 721 Met (2)
731-742 Helix 5	Trp 732 Arg Gly 734 Val Met 740 Val (2) Gly 741 Asp (2) Trp 742 Arg	Met 733 Val Met 733 Ile Gly 734 Val Leu 735 Phe Met 736 Thr Val 737 Met (2) Ala 739 Asp		
743-751	Arg 743 Gln (2) Phe 745 Val (2) Ser 750 Phe (2)	Phe 745 Leu (2) Asn 747 Ser		Val 748 Ala

Region	Phenotype			
	CAIS	PAIS	MAIS	Prostate carcinoma
752-757 β-sheet 1	Leu 753 Phe (2) Tyr 754 His Phe 755 Leu (3) Ala 756 Tyr (4) Ala 756 Val Pro 757 Ser	Tyr 754 Cys (2)		
758-762 β-sheet 2	Asp 758 Glu	Asn 762 His		
763-768 Helix 6	Arg 765 Cys (6) Arg 765 His (4)	Glu 763 Ala Glu 763 Gly Arg 765 His		
769-774	Arg 770 Trp (3) Met 771 Ile (2)	Met 771 Ile (4)		Ser 773 Asn
775-787 Helix 7	Met 778 Val Phe 785 Ser (2)		Leu 781 Phe Glu 784 Asp	
788-791		Gln 789 Glu (3)		Gln 789 Glu
792-803 Helix 8	Val 798 Met Met 798 Arg	Cys 797 Thr		
804-814	Gly 811 Ala	Ser 805 Asn (2) Leu 812 Val		
815-836 Helix 9	Arg 822 Gln (2) Arg 822 Leu (2) Tyr 825 Cys Ile 833 Thr	Arg 831 Cys (3) Arg 831 His (11) Ile 832 Ser Ile 833 Thr		
837-845	Asn 839 Lys	Arg 845 Lys		

| c | Phenotype | | | |
Region	CAIS	PAIS	MAIS	Prostate carcinoma
846-863 Helix 10	Arg 846 Cys (9)	Arg 846 His (6)	Arg 862 Gly	
	Arg 846 His (3)	Val 857 Leu (4)		
	Leu 854 Arg	Val 857 Met (2)		
	Asp 855 Asn	Ile 860 Met		
	Asp 855 Gly	Ala 861 Val		
	Val 857 Met (3)			
	Val 857 Glu			
	Ala 861 Gly			
864-874 Helix 11	Leu 872 Val			His 865 Tyr (2)
				Thr 868 Ala (5)
				Thr 868 Ser
875-883	Val 880 Met	Val 880 Met	Met 875 Val (2)	
884-891 Helix 12	Ile 889 Thr			
892-910	Pro 895 Ser	Val 894 Met		Gln 893 Arg
	Pro 895 His	Gly 900 Leu		Gln 910 Arg
	Leu 898 Phe	Pro 904 Ser		

Structural domains are indicated in the first column. The phenotype, associated with the mutations, is specified in columns 2-5. The frequency of mutations, which are identified more than once, is indicated between brackets. Structural domains were derived from homologous locations between AR and hRXR-α (Wurtz et al. 1996). Numbering of amino acid residues is based on an AR with a poly-glutamine stretch of 20 glutamines, and a polymorphic glycine stretch of 16 residues (Brinkmann et al. 1989).

In Table VII.2 the α-helical regions, the 2 β-strands, and the linker regions, as predicted for the AR by Wurtz *et al.* (1996), are indicated. Mutations that are associated with several forms of AIS and/or prostate cancer are categorized according to these structural elements. Except for one amino acid residue, all residues forming the first β-strand were mutated in individuals with AIS. The hydrophobic ligand-binding pocket of the ER-α-LBD is formed by parts of helices 3, 6, 8, the preceding loop of helix 11, helix 12, and the two β-strands (Brzozowski *et al.* 1997), which might be similar in the other NHRs as well (Brzozowski *et al.* 1997). Amino acid residues Glu-353, Arg-394, and His-524 located in helices 3 and 11, respectively, were identified as the ones that are directly contacting 17β-estradiol (Brzozowski *et al.* 1997). Wurtz *et al.* (1996) proposed a model for dexamethasone binding by the hGR, in which the D-ring was bound between helices 3 and 6. Based on the results obtained by Brzozowski *et al.* (1997) this model should be rejected, as the A-ring of 17β-estradiol was bound between helices 3 and 6, by Glu-353 and Arg-394. This result corresponded to the predictions made by Anstead *et al.* 1997. The arginine residue (Arg 394 in hER-α) is conserved in the AR, PR, GR, and MR, whereas the histidine residue is conserved at the corresponding position in the AR and the PR. Instead of the glutamic acid residue at position 353, a glutamine residue is present at the corresponding position in the AR, PR, GR, and MR, which might be involved in hydrogen bonding with the 3-keto group in the A-ring of steroids (Brzozowski *et al.* 1997).

Interestingly, mutations that were identified in prostate tumor specimens, and associated with a broadened ligand-binding specificity, map to regions predicted to form helix 3 (Val-706-Met), helix 4 with its preceding loop (Arg-717-Leu, Val-721- Met), and helix 11 (His-865-Tyr, Thr-868-Ala, Thr-868-Ser) (Table VII.2). The conserved histidine residue (position 865 in the AR) was substituted by a tyrosine residue, in a case of prostate cancer (Taplin *et al.* 1995). Threonine-868 (LNCaP-AR mutation) was substituted by an alanine residue in several prostate tumor specimens, and by a serine residue in another prostate tumor sample (Veldscholte *et al.* 1990, Gaddipati *et al.* 1994, Taplin *et al.* 1995, Suzuki *et al.* 1996, Gottlieb *et al.* 1997). In the ER-α, a serine residue is present at the corresponding position, and, indeed, mutant Thr-868-Ser displayed increased activity after incubation with 17β-estradiol, although this effect was observed also after incubation with R5020 (synthetic progestagen) (Ris-Stalpers *et al.* 1993). Threonine-868 is very likely to be involved in ligand binding. The observation that the equivalent cysteine residue, present in the mouse and

rat GR, could be crosslinked to triamcinolone acetonide (Carlstedt-Duke *et al.* 1988, Byravan *et al.* 1991), is further proof that this residue is positioned very close to the ligand.

Mutations causing aberrant splicing of the AR mRNA

About 15% of the point mutations in different genes, associated with diseases in humans, alter mRNA splicing. Splice donor sites are most frequently affected (Cooper and Krawczak 1993). Mutations causing splicing alterations are a rare cause of AIS (7 out of 270 mutations: 2.6 %) (see also Table VII.1). Seven of such mutations were reported, and 5 of them were affecting splice donor sites (Figure VII.1B). The guanine at position +1 of the splice donor site, which is completely conserved (Figure VII.1A) (Shapiro and Senapathy 1987), was found to be mutated in three unrelated AIS patients. Skipping of exon 3 was observed in an individual with a transition of the conserved guanine at position +1 of intron 3 to adenine (Gottlieb *et al.* 1997). Transition of guanine to adenine at position +1 in intron 7 caused skipping of exon 7. Exons 6 and 8 were joined, which caused a frameshift (Lim *et al.* 1997). In another patient, transition of guanine to thymine at position +1 in intron 4 caused activation of a cryptic splice donor site in the preceding exon, resulting in an in frame deletion (Ris-Stalpers *et al.* 1990). Splice donor site analysis revealed that an adenine at position +3 is most common (60%), followed by guanine (35%) (Shapiro and Senapathy 1987). Due to the insertion of thymine at position +3 (intron 1), only a very small amount of wild type mRNA was detected in an individual with CAIS (Trifiro *et al.* 1997). Transversion of adenine to thymine at position + 3 of the splice donor site in intron 6 caused joining of exons 5 and 7, in turn generating a premature stop (Trifiro *et al.* 1997). A large deletion in intron 2 (> 6 kb), starting from the nucleotide at position -18 and containing the branch point site (BPS), resulted mainly in an exon 3 deleted transcript (Ris-Stalpers *et al.* 1994b). The BPS is involved in assembly of the spliceosome; base paring between the BPS and U2snRNP is observed. Due to usage of a cryptic BPS, a small amount of wild type transcript was found, which explains the Reifenstein phenotype of this case (Ris-Stalpers *et al.* 1994b). Recently, a mutation was detected in the pyrimidine-rich sequence preceding the splice acceptor site in intron 2 of the AR (Brüggenwirth *et al.* 1997; see also Chapter VI). Transversion of thymine to adenine created an AG-sequence at position -11/-10. A cryptic splice site 5' of the wild type splice site was used predominantly, resulting in an in frame insertion of 69 base pairs in between exons 2 and 3.

Figure VII.1A **Location of splice donor and splice acceptor sites.** The consensus sequences are indicated (Shapiro and Senapathy 1987).

Figure VII.1B **Structural organization of the AR gene.** The positions of mutations that are causing aberrant splicing are indicated. Upwards: mutations resulting in PAIS; downwards: mutations resulting in CAIS.

Mutations affecting phosphosites

The function of basal and hormone-dependent phosphorylation of the AR is largely unknown. Kuiper *et al.* (1993b) observed that the majority of potential phosphosites are located in the NH_2-terminal domain, in the region where AF-1 was mapped (Jenster *et al.* 1995), but residues that undergo basal or androgen-dependent phosphorylation have not been identified yet. Phosphosites in both PR-A and PR-B have been mapped, and the effect of phosphorylation on their function was studied by mutagenesis. Only subtle effects were observed in vitro (Takimoto *et al.* 1996). One consensus phosphosite (casein kinase II site) was found to be mutated in an individual with PAIS (Ser-694-Gly). The mutation caused reduced ligand-binding affinity (Kaspar *et al.* 1993), probably due to interference with ligand binding because it is located in the region predicted to form helix 3. Defective hormone-dependent phosphorylation was associated with an intronic mutation in the AR gene. Due to the mutation, 23 additional amino acid residues were inserted between the two zinc clusters of the DBD, which completely destroyed DNA-binding capacity (Brüggenwirth *et al.* 1997).

Tyrosine-537, located NH$_2$-terminally of AF-2 in the hER-α was identified as a tyrosine phosphorylation site (Arnold *et al.* 1995). Substitution of this tyrosine residue (Tyr-541) in the mouse ER-α either by an asparagine or a glutamic acid residue (both negatively charged), or by an alanine residue, caused ligand-independent activation (White *et al.* 1997). Because these ER-α mutants interacted with coactivators RIP140 and SRC-1, it is likely that the above-mentioned amino acid residue changes and probably tyrosine phosphorylation as well, induce a different conformation. Phosphorylation of the tyrosine residue was not required for ligand-dependent activity, suggesting that it renders AF-2 inactive in the absence of an agonist (White *et al.* 1997). Mutations, causing a constitutively active receptor might be involved in steroid hormone-related cancer.

VII.2 Polymorphic glutamine stretch as a modifier of androgenic end points

Kashi *et al.* (1997) proposed that simple sequence repeats might be modulators of gene expression and function, causing quantitative genetic variation. Thus, apart from causing SBMA when expanded, the polymorphic glutamine stretch in the AR might as well be a modifier of several androgenic end points, even when the length of the stretch is within the normal range. Glutamine stretch expansion is associated with a loss of receptor function in individuals with SBMA. Testis atrophy, gynecomastia, infertility, and elevated LH, FSH, and estradiol levels are frequently observed (Danek *et al.* 1994). Overall sex differentiation appears to be normal. The appearance of endocrine abnormalities later in life might be related to a lower AR expression level and a lower testosterone level, as is generally observed in elderly men (Ono *et al.* 1988, Swerdloff and Wang 1993). Evidence supporting the idea that the length of the glutamine stretch in the AR is a modulator of androgenic end points, also without evident signs of AR malfunction, came from epidemiological studies. Results reported by Tut *et al.* (1997) imply that individuals with a relatively long poly-glutamine stretch are at a higher risk of defective spermatogenesis. (CAG)nCAA-repeat length might be a determinant of prostate cancer, as was discussed in Chapter I (Irvine *et al.* 1995, Hardy *et al.* 1996, Giovannucci *et al.* 1997, Stanford *et al.* 1997). In a population of healthy elderly men the possible relation between length of the (CAG)nCAA-repeat and BMD was studied (Chapter IV). However, the data obtained were not conclusive. One intriguing question now remains. It is known that androgens

exert negative feedback through the AR, resulting in a lower LH level, which in turn gives a lower androgen level (Schwartz and McCormack 1972). ARs containing a relatively short glutamine stretch were associated with higher AR activity, and thus it is possible that such receptors exert stronger negative feedback on LH production by the pituitary gland. Sobue *et al.* (1994) indeed observed a less strong negative feedback mechanism in SBMA patients after administration of fluoxymesterone (androgenic synthetic hormone). It would be interesting to study whether (CAG)nCAA-repeat length is associated with free testosterone, free 17β-estradiol, and SHBG levels, in a large population of healthy individuals.

VII.3 An androgen insensitivity-like phenotype without a mutation in the androgen receptor gene

Several reports describe patients with an AIS-like phenotype without a mutation in the *AR* gene (Brüggenwirth *et al.* 1996, Weidemann *et al.* 1997, Morel *et al.* 1994). It can be hypothesized that in these cases hormone resistance might be caused by mutations in genes encoding specific coactivators or corepressors. Weiss *et al.* recently described a family without mutations in the genes encoding TR-α and TR-β, although thyroid hormone resistance has never been associated with TR-α alterations. Far-Western blotting and gel-retardation assays, in which nuclear extracts prepared from cultured skin fibroblasts obtained from family members, revealed a protein that specifically interacted with the TR-β of affected individuals. The molecular mass of the protein did not correspond to that of the RXR or Trip1, a thyroid hormone receptor interacting protein. The interacting protein might be a defective factor, specifically involved in thyroid hormone receptor-mediated gene regulation. Mutations in AR-specific coactivators, which aberrantly interact with the AR-LBD, and therefore result in AIS, can as yet not be excluded.

It is also possible that mutations in the *AR* gene have not been detected, using standard screening procedures. In these cases it might be helpful to screen cDNA, prepared from mRNA, derived from genital skin fibroblasts from the patients. Protein studies could provide important additional information, as was shown in Chapter VI. Special attention should be paid to silent mutations, which might create new splice donor or acceptor sites (Richard and Beckman 1995). As was recently shown by Liu *et al.* (1997) silent mutations which do not directly affect these splice sites may still affect the splicing mechanism, for example by

changing sequences involved in binding of splicing factors. In another study, silent mutations were identified being responsible for enhanced translation (Milland *et al.* 1995).

VII.4 Future directions

Bevan *et al.* (1997) reported that some mutant receptors, identified in individuals with PAIS, were functionally more impaired than mutant receptors associated with CAIS, implying that additional factors which are variant between individuals influence AR activity. In addition, it is to be expected that individual genetic background is underlying genotype-phenotype variation in the expression of AR function. Therefore, better insight in functioning of the AR might be obtained by transfection of cultured fibroblasts, derived from the patients, with a reporter plasmid (McPhaul *et al.* 1993a, and McPhaul *et al.* 1997). Furthermore, important information may be obtained by studying expression levels of endogenous androgen-regulated genes. Genital skin fibroblasts, however, are not androgen target cells, and there are practical and maybe also ethical objections against obtaining real target cells from AIS patients. The SHBG-test (Sinnecker *et al.* 1997, and described in the Introduction and in Chapter III) is therefore a welcome supplementary test.

Development of several (human) androgen target cell lines is necessary to investigate AR structure-function relationship on a particular cellular background. These studies provide insight in tissue-specific androgen action. The development of mouse models would be important to study possible effects of subtle mutations, including those affecting phosphosites. As was suggested by Takimoto *et al.* 1996, cellular transfection systems probably lack sensitivity. In addition, it would be very interesting to create a female AR knockout mouse to study the role of the AR in females. This can be performed by crossing a female mouse, carrying an inactivating AR mutation, with a male mouse, expressing an *androgen receptor* gene of which the expression can be repressed. Mice that are either overexpressing a SHR-coactivator or corepressor, or in which such a gene is inactivated, would also provide interesting data.

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Summary

The androgens testosterone and dihydrotestosterone are important for male sex differentiation, and in addition the androgens are involved in development of male secondary sex characteristics during puberty and functioning of the male sex organs throughout adult life. In females androgens play a much less prominent role. Androgens bind to the androgen receptor (AR), a transcription factor that is expressed in androgen target cells. Upon hormone binding the AR becomes activated, and the hormone-receptor complex translocates to the nucleus where it recognizes and binds to androgen-response elements on the DNA. Androgen-response elements are located in the promoter regions of androgen-regulated genes, and AR binding regulates expression of these genes. Mutations in the *AR* gene are the cause of androgen insensitivity syndrome (AIS). Target organs of 46,XY individuals with AIS show either reduced sensitivity or are completely insensitive to androgens, which results in the development of ambiguous genitalia or female genitalia, respectively. Research described in this thesis was performed to obtain information about the structure-function relationship of the AR. In addition, information that is relevant for other steroid hormone receptors might be obtained, because of the large homology observed between those receptors. Furthermore, identification and subsequent characterization of mutations might be important for patients with AIS. Based on results obtained from functional studies, better treatment can sometimes be given. Moreover, genetic counseling can be offered to families, to identify possible carriers of the mutation. Mutations were detected in individuals with different forms of AIS. The present study was focussed on patients with the receptor positive form of AIS. AR protein is normally expressed in androgen target cells of these individuals, and normal hormone binding characteristics can be observed. This implied that mutations were mainly found in exons 1-3, which respectively encode the NH_2-terminal part of the receptor (exon 1) and the DNA-binding domain (DBD) (exons 2 and 3). These domains were not always intensively screened in the past. In previous studies, predominantly *AR* genes from patients with androgen-binding abnormalities were screened. In addition, exon 1 is relatively large and GC-rich, which has hampered screening. Mutations in the NH_2-terminal domain and DBD can provide information about amino acid residues, or protein regions, involved in DNA binding and/or transcription activation. In addition, many potential phosphosites are located in exon 1.

In the General Introduction (Chapter I) the mechanism of action and functional characteristics of steroid hormone receptors are discussed, with particular emphasis on the AR. Male sex differentiation and the role of the AR therein is

described. Apart from being the cause of AIS, mutations in the AR are also associated with other diseases, such as prostate cancer, and also Kennedy's disease, which is a progressive neuromuscular disease. Kennedy's disease is caused by expansion of a polymorphic poly-glutamine stretch, located in the NH_2-terminal part of the AR. Interestingly, several other neuromuscular diseases, for example Huntington's disease, are associated with expansion of poly-glutamine stretches. Mechanisms that might underlie the role of these poly-glutamine stretches in these diseases are discussed.

In Chapter II, several mutations found in exon 1 of the *AR* gene are described. Amino acid substitutions were not detected. However, we found point mutations that either directly or indirectly caused introduction of a premature stop codon. All these mutations were associated with complete AIS. Although in theory reinitiation of translation could occur from a codon for an internal methionine residue, resulting in expression of an NH_2-terminally truncated AR protein, evidence supporting such a mechanism was not obtained.

In Chapter III a 46,XY individual is described, who was completely insensitive to androgens, according to the sex hormone-binding globulin (SHBG) test, but the phenotype of this patient was typical for partial AIS. The SHBG-test is being used to study the activity of the endogenous AR in the patient's target cells (*in vivo*). SHBG-expression is studied before and after administration of stanozolol, which is a synthetic androgenic anabolic steroid. In normal individuals, treatment with stanozolol results in lower SHBG levels. Normal hormone binding and a low number of binding sites were established in genital skin fibroblasts, derived from this patient. Molecular analysis finally showed that the patient had a somatic mosaicism for a mutation in exon 1 of the *AR* gene. The mutation introduces a premature stop codon, which either results in a truncated, non-functional AR protein, or in absence of the protein, due to mRNA instability. Liver cells, which produce SHBG, probably express only the mutated AR, which gives an explanation for the fact that lower SHBG-levels could not be observed after administration of stanozolol.

AR protein containing an expanded poly-glutamine stretch is less active with respect to transcription activation, a finding which explains the mild symptoms of AIS in patients with Kennedy's disease. Variable length of the poly-glutamine stretch is observed also in the general population without clear signs of AIS, but yet might be related to a minor change in receptor activity. AR protein is expressed in osteoblasts, cells that are involved in bone formation. There is also evidence for a direct role of the AR in bone metabolism. In view of this, length of

the poly-glutamine stretch was analyzed in relation to bone mineral density in a cohort of elderly men from the Rotterdam Elderly Study (Chapter IV). Although a clear association could not be established, individuals with low bone mineral density were over-represented among those with relatively short poly-glutamine stretches.

In Chapter V, a mutation, found in exon 2 of the *AR* gene of a 46,XY individual with complete form of AIS is discussed. This particular exon encodes the first zinc cluster, which is involved in specific DNA binding. The neutral asparagine residue at position 564 is substituted by an aspartic acid residue, introducing a negative charge. To study whether inactivity of the aspartic acid mutant was caused by steric hindrance, or by the introduction of negative charge, the mutant AR and two other receptor mutants were expressed and functionally investigated. In addition, a 3-dimensional (3-D) model was created for the DBDs of the AR and the three AR mutants. The wild type AR model was based upon the 3-D model that was made for the DBD of the glucocorticoid receptor, of which the crystal structure was clarified. Results obtained with both approaches were compared. It was concluded that the phenotype of the patient can be explained by the introduction of an extra negative charge, which destabilized the normal conformation of the AR-DBD.

In Chapter VI a family with partial AIS is described. After molecular analysis of the coding parts and the exon flanking intronic regions of the *AR* gene, no mutation was identified. Testosterone biosynthesis defects, another cause of male pseudohermaphroditism, were excluded. The polymorphic CAG-repeat located in exon 1 of the *AR* gene was used as an X-chromosomal marker. The three affected individuals had the same X chromosome, and X-linked inheritance could not be excluded. Protein studies showed an abnormal electrophoretic mobility pattern of the AR protein during SDS-PAGE, reflecting defective hormone-dependent phosphorylation, which is associated with disturbed DNA binding. Indeed, receptor protein was not detectable in a nuclear fraction of genital skin fibroblasts that were cultured in the presence of hormone, which is indicative of defective DNA binding. By reexamining the gene, finally a mutation was found in intron 2 at position -11 of the *AR* gene. Due to this mutation, a new splice acceptor site was created at position (-10)-(-11) in intron 2, which was not used. However, a cryptic splice site at position (-70)-(-71) in intron 2 and at position (-1)-(-2) in intron 3 were activated. Both splice variants, an AR protein with 23 additional amino acid residues in between the two zinc clusters, and an AR protein from which the second zinc cluster was deleted, were inactive.

Finally, a very small amount of wild type transcript was detected in genital skin fibroblasts, derived from the index patient, by use of an allele-specific oligo-nucleotide-hybridization assay. This explains the phenotype of the affected individuals. The mutation was not detected in 102 chromosomes of unrelated individuals, indicating that it is not a common polymorphism, but a pathogenic mutation.

In the General Discussion (Chapter VII) an overview is presented of mutations detected in AIS. Recently the 3-D structures of the ligand-binding domains (LBDs) of several nuclear hormone receptor family members were clarified. Because the overall structure turned out to be quite similar, a 3-D model for the AR-LBD could be predicted. In Chapter VII, a distribution of mutations identified in the LBDs of the AR of patients with AIS and prostate cancer over the different structural regions is presented and discussed. Furthermore, the possible influence of the length of the poly-glutamine stretch on several androgenic end points is discussed. The possible existence of post-AR defects, causing an AIS-like phenotype in the presence of an intact AR is discussed. However, because cofactors specific for the AR have not been identified yet, post-AR defects are not very likely to occur. Finally, some future directions, concerning AR research are given.

Samenvatting

De androgenen testosteron en dihydrotestosteron zijn belangrijk voor de geslachtsdifferentiatie bij de man, en postnataal voor de ontwikkeling van de mannelijke secundaire geslachtskenmerken en het functioneren van de mannelijke geslachtsorganen. Bij de vrouw spelen androgenen een beperkte rol. Androgenen binden aan de androgeenreceptor (AR), een transcriptiefactor welke aanwezig is in androgeen doelwitcellen. Na binding van het hormoon wordt de AR geactiveerd. Het hormoon-receptor complex verhuist van het cytoplasma naar de celkern, waar het complex androgeen-responsieve elementen van het DNA herkent, waaraan het vervolgens bindt. Deze hormoon-responsieve elementen zijn gelegen in de promotor regio van androgeen-gereguleerde genen. Het binden van de AR reguleert de expressie van deze genen, hetgeen uiteindelijk leidt tot een respons. Mutaties in het gen dat codeert voor de AR zijn de oorzaak van het androgeenongevoeligheidssyndroom (AIS). De doelwitorganen van genetisch mannelijke (46,XY) individuen met AIS zijn verminderd gevoelig of totaal ongevoelig voor androgenen, waardoor deze individuen ambigue genitaliën, dan wel vrouwelijke genitaliën ontwikkelen (mannelijk pseudohermaphroditisme). Het onderzoek, beschreven in dit proefschrift werd verricht om inzicht te verkrijgen in structuur-functie relatie van de AR. Dit onderzoek verschaft tevens informatie omtrent het functioneren van andere steroidhormoonreceptoren, die veel homologie vertonen met de AR. Het vaststellen en vervolgens karakteriseren van mutaties is ook van belang voor patiënten met AIS. Functionele studies kunnen informatie opleveren die in sommige gevallen gebruikt kan worden voor behandeling van patiënten. Indien gewenst kan familieonderzoek worden uitgevoerd, zodat eventuele draagsters van mutaties kunnen worden opgespoord. In patiënten met verschillende vormen van androgeenongevoeligheid werd naar mutaties in het AR gen gezocht. In het bijzonder werden patiënten bestudeerd met de receptor positieve vorm van androgeenongevoeligheid. In de androgeen-doelwitcellen van deze individuen komt het AR eiwit normaal tot expressie. Bovendien vertonen deze receptoren normale hormoonbindings-karakteristieken. Over het algemeen betreft het dan mutaties in de exonen 1, 2 en 3, welke respectievelijk coderen voor het NH_2-terminale gedeelte van de AR (exon 1), en het DNA-bindend domein (DBD) (exonen 2 en 3). In het verleden zijn deze gebieden niet uitgebreid onderzocht, enerzijds omdat de nadruk lag op het screenen van patiënten met afwijkende hormoonbinding, en anderzijds omdat exon 1 relatief groot en CG-rijk is, hetgeen een belemmering vormde voor screening op grote schaal. Mutaties in het NH_2-terminale gedeelte en het DBD kunnen meer informatie opleveren omtrent amino-

zuurresiduen of eiwit regio's, betrokken bij receptor DNA binding en/of transcriptieactivatie, processen die van belang zijn met betrekking tot het normaal functioneren van de AR. Bovendien zijn er in de NH_2-terminus van de AR veel aminozuur residuen gelegen, die mogelijk gefosforyleerd kunnen worden.

In de General Introduction (Chapter I) wordt het functioneren van de AR en andere steroidhormoonreceptoren beschreven. Vervolgens wordt de mannelijke geslachtsdifferentiatie en de specifieke rol die de AR daarin vervult, toegelicht. Mutaties in de AR zijn ook geassocieerd met andere ziekten, zoals prostaatkanker, maar ook de ziekte van Kennedy, een progressieve neuromusculaire aandoening. Deze laatste aandoening wordt veroorzaakt door een extreme expansie van een polymorfe poly-glutamine-keten, gelegen in het NH_2-terminale gedeelte van de AR. Diverse andere neuromusculaire aandoeningen, zoals de ziekte van Huntington, zijn geassocieerd met verlengde poly-glutamine-ketens in andere eiwitten. Mechanismen die mogelijk ten grondslag liggen aan deze neuromusculaire aandoeningen worden besproken.

In Chapter II worden diverse mutaties beschreven die gevonden zijn in het eerste exon van het *AR* gen. Er werden geen aminozuur substituties gevonden, maar wel verschillende puntmutaties die direct of indirect leiden tot de introductie van een prematuur stopcodon. Al deze mutaties veroorzaken complete AIS. Hoewel in theorie een translatie herstart vanaf een codon voor een intern methionine residu zou kunnen leiden tot expressie van een eiwit dat aan NH_2-terminale zijde verkort is, zijn hiervoor geen aanwijzingen gevonden.

De *sex hormone-binding globulin* (SHBG) test wordt gebruikt om de activiteit van de endogene AR in doelwitcellen van patiënten (*in vivo*) te bestuderen. Voor en na behandeling met stanozolol, een anabool steroid, wordt de expressie van SHBG, een androgeen-gereguleerd gen, bestudeerd. Stimulatie met stanozolol leidt normaliter tot verlaging van de SHBG spiegel. In Chapter III wordt een 46,XY individu beschreven waarbij een volledige ongevoeligheid voor androgenen was voorspeld aan de hand van de SHBG-test, terwijl het fenotype van de patiënt duidelijk partieel was. In genitale huidfibroblasten van de patiënt werd een normale hormoonbinding aangetoond, alhoewel het aantal receptoren laag was. Moleculaire analyse van het gen wees uit dat bij deze patiënt sprake is van een somatisch mozaïcisme voor een mutatie in exon 1. Als gevolg van deze mutatie wordt een prematuur stopcodon geïntroduceerd, hetgeen leidt tot de aanwezigheid van een verkort niet-functioneel AR eiwit, of de afwezigheid van AR eiwit als gevolg van instabiel boodschapper RNA. In levercellen, die SHBG produceren, wordt vermoedelijk alleen het gemuteerde AR eiwit tot expressie

gebracht, hetgeen een verklaring is voor het feit dat er geen daling van het SHBG werd waargenomen bij de patiënt.

Een AR eiwit dat een geëxpandeerde poly-glutamine-keten bevat, vertoont verminderde activiteit, hetgeen bij patiënten met de ziekte van Kennedy veelal resulteert in milde verschijnselen van AIS. Ook binnen de normale populatie varieert de lengte van de poly-glutamine-keten, en daarmee mogelijk de receptoractiviteit. De AR is aangetoond in osteoblasten, de botvormende cellen. Er zijn aanwijzingen dat de AR een directe rol speelt met betrekking tot botmetabolisme. Gezien de mogelijke modulerende activiteit van de poly-glutamine-keten met betrekking tot receptor activiteit is bestudeerd of er mogelijk een associatie bestaat tussen botmineraaldichtheid en de lengte van de poly-glutamine-keten binnen een cohort mannen van de Rotterdam Ouderen Studie (Chapter IV). Hoewel een duidelijke associatie niet kon worden aangetoond, waren individuen met een lage botmineraaldichtheid oververtegenwoordigd in de groep met een relatief korte poly-glutamine-keten.

In Chapter V wordt een mutatie besproken welke gevonden werd in exon 2 van het *AR* gen van een 46,XY individu met compleet AIS. Exon 2 codeert voor het eerste zink-cluster, betrokken bij specifieke DNA binding. Als gevolg van de mutatie is het neutrale alanine residu op positie 564 veranderd in een asparaginezuur, waardoor een extra negatieve lading wordt geïntroduceerd. Deze AR mutant en een tweetal andere mutanten werden tot expressie gebracht en functioneel onderzocht. Op deze wijze kon worden nagegaan of de defecte DNA binding, waargenomen in geval van de asparaginezuur mutant, veroorzaakt werd door de introductie van negatieve lading dan wel door sterische hindering. Tevens werd een 3-dimensionaal (3-D) model gecreëerd voor het DBD van de wild type AR en de drie gemuteerde receptoren. Het wild type AR model is gebaseerd op het 3-D model voor het DBD van de glucocorticoid receptor, waarvan de kristal structuur is opgehelderd. De resultaten, verkregen middels beide benaderingen, werden met elkaar vergeleken, en wezen uit dat met name de negatieve lading, geïntroduceerd door het asparaginezuur residu, verantwoordelijk is voor het fenotype van de patiënt.

Een familie met partiële androgeenongevoeligheid wordt beschreven in Chapter VI. Na moleculaire analyse van de coderende gedeelten van het *AR* gen en de flankerende introngedeelten werd geen mutatie gevonden. Testosteronbiosynthesestoornissen, ook een oorzaak voor mannelijk pseudohermaphroditisme, konden worden uitgesloten. Met behulp van de polymorfe *CAG-repeat*, gelegen in exon 1 van het *AR* gen, werd aangetoond dat de 3 aangedane indivi-

duen hetzelfde X chromosoom hadden, en daardoor kon X-gebonden overerving niet worden uitgesloten. Studies van het receptoreiwit lieten een afwijkend fosforylerings-patroon zien, hetgeen nog niet eerder bij AIS patiënten was aangetoond. Dit afwijkende patroon is geassocieerd met verstoorde DNA binding. Na opnieuw analyseren van het *AR* gen werd uiteindelijk toch een mutatie gevonden in intron 2 op positie -11. Als gevolg van de mutatie wordt een nieuwe acceptor splitsingsplaats gecreëerd op positie (-10)-(-11) in intron 2, welke echter niet gebruikt wordt. Daarentegen worden een cryptische acceptor splitsingsplaats op positie (-70)-(-71) in intron 2 en de acceptor splitsingsplaats op positie (-1)-(-2) in intron 3 geactiveerd. Beide splitsingsvarianten, een AR eiwit met 23 additionele aminozuren tussen de twee zink-clusters en een AR eiwit met een in frame deletie van het tweede zink-cluster zijn functioneel onderzocht. Geen van beide vertoonde enige activiteit. Middels een zeer gevoelige dot-blot techniek is een geringe hoeveelheid wild type mRNA aangetoond in genitale huidfibroblasten, hetgeen het partiële AIS fenotype van de patiënten kan verklaren. Het gegeven dat deze mutatie niet werd gevonden na analyse van 102 chromosomen van niet gerelateerde controle personen, vormt additioneel bewijs voor de pathogeniciteit van de intron mutatie.

In de General Discussion (Chapter VII) wordt een overzicht gegeven van alle mutaties, gevonden in AIS. Recentelijk zijn de 3-D structuren van de ligandbindende domeinen (LBDs) van een aantal leden van de kernreceptorfamilie, waartoe ook de AR behoort, opgehelderd. In Chapter VII zijn alle mutaties, gevonden in het AR-LBD van patiënten met AIS en prostaatkanker, onderverdeeld naar de diverse structurele eenheden binnen het LBD. Verder wordt de mogelijke invloed van de lengte van de poly-glutamine-keten op diverse androgeen gereguleerde processen besproken. Vervolgens wordt er gespeculeerd over mogelijke defecten in de door androgenen en AR gereguleerde processen (post-AR defecten), welke zouden kunnen resulteren in een fenotype dat gelijkenis vertoont met AIS. Aangezien er tot op heden geen AR-specifieke co-factoren zijn beschreven is dit niet aannemelijk. De discussie wordt afgesloten met een korte toekomstvisie, betreffende onderzoek aan de AR.

List of publications

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Curriculum Vitae

Hennie Brüggenwirth werd geboren op 25 april 1969 te Helmond.
Het VWO diploma werd behaald in 1987 aan het Peelland College te Deurne.
In hetzelfde jaar begon zij met de studie Gezondheidswetenschappen aan de
toenmalige Rijksuniversiteit Limburg. Na de propaedeuse werd gekozen voor
de studierichting Biologische Gezondheidkunde.

Bij de afdeling Biologische Toxicologie van het onderzoeksinstituut CIVO-TNO
te Zeist (drs. H. Koëter) werd een studie verricht naar effecten van prenatale
blootstelling aan lood op het gedrag en de lichamelijke ontwikkeling van jonge
Wistar ratten. Tijdens de afstudeerstage bij de Afdeling Anthropogenetica van
de Katholieke Universiteit Nijmegen (dr. H. Smeets) werden genotype-fenotype
studies verricht bij patiënten met de erfelijke spierziekte myotone dystrofie.
In januari 1993 werd het doctoraalexamen behaald, waarna zij korte tijd
werkzaam was als projectmedewerker bij het Laboratorium voor Carcinogenese
en Mutagenese van het RIVM te Bilthoven.

In mei 1993 werd aangevangen met het promotieonderzoek waarvan de
resultaten in dit proefschrift beschreven staan, bij de Afdeling Endocrinologie &
Voortplanting van de Erasmus Universiteit in Rotterdam (promotor: prof. dr. J.A.
Grootegoed, co-promotor: dr. A.O. Brinkmann).

Sinds januari 1998 is zij werkzaam als wetenschappelijk medewerker in
tijdelijke dienst bij de Afdeling Celbiologie en Genetica aan de Erasmus
Universiteit te Rotterdam.

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