Chapter 3
ANDROGEN PHYSIOLOGY: RECEPTOR AND METABOLIC DISORDERS

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INTRODUCTION

Androgens are important hormones for expression of the male phenotype. They have characteristic roles during male sexual differentiation, but also during development and maintenance of secondary male characteristics and during initiation and maintenance of spermatogenesis (1). The two most important androgens in this respect are testosterone and 5α-dihydrotestosterone [Figure 1]. While acting through the same androgen receptor, each androgen has its own specific role during male sexual differentiation: testosterone is directly involved in development and differentiation of wolffian duct derived structures (epididymides, vasa deferentia, seminal vesicles and ejaculatory ducts) [Figure 2A], whereas 5α-dihydrotestosterone, a metabolite of testosterone, is the active ligand in a number of other androgen target tissues, like urogenital sinus and tubercle and their derived structures (prostate gland, scrotum, urethra, penis) [Figure 2B] (2,3). The interaction of both androgens with the androgen receptor is different. Testosterone has a two fold lower affinity than 5α-dihydrotestosterone for the androgen receptor, while the dissociation rate of testosterone from the receptor is five-fold faster than of 5α-dihydrotestosterone (4). However, testosterone can compensate for this "weaker" androgenic potency during sexual differentiation and development of wolffian duct structures via high local concentrations due to diffusion from the nearby positioned testis. In more distally located structures, like the urogenital sinus and urogenital tubercle the testosterone signal is amplified via conversion to 5α-dihydrotestosterone.

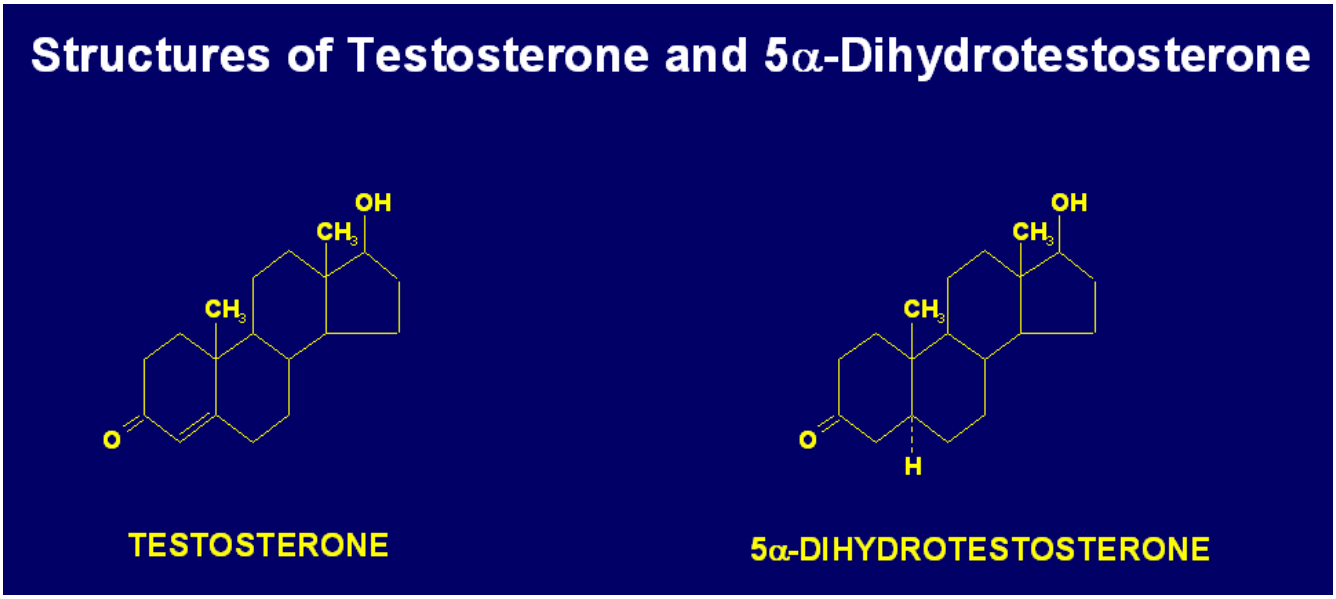

Figure 1. Structures of Testosterone and 5α-Dihydrotestosterone.

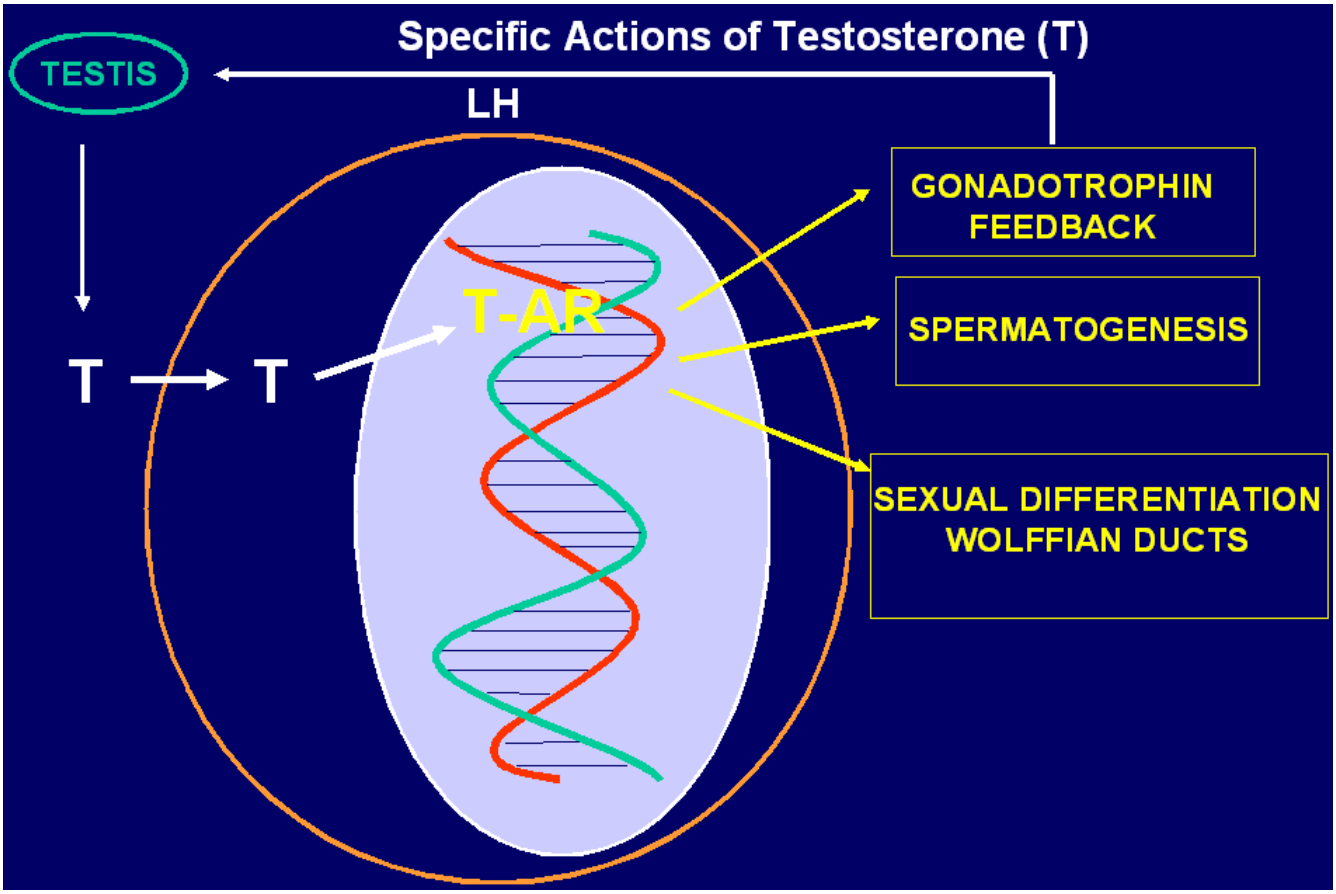

Figure 2a. Specific actions of Testosterone (T). T is synthesized in the testis under control of luteinizing hormone (LH) from the pituitary . After entering the target cells (in the hypothalamus, pituitary, testis and wolffian duct) T is directly bound to the androgen receptor (AR) and the complex T-AR binds to specific DNA sequences and regulates gene transcription, which can result in negative feedback regulation of gonadotrophin synthesis and secretion, in initiation and regulation of spermatogenesis and in differentiation and development of the wolffian duct.

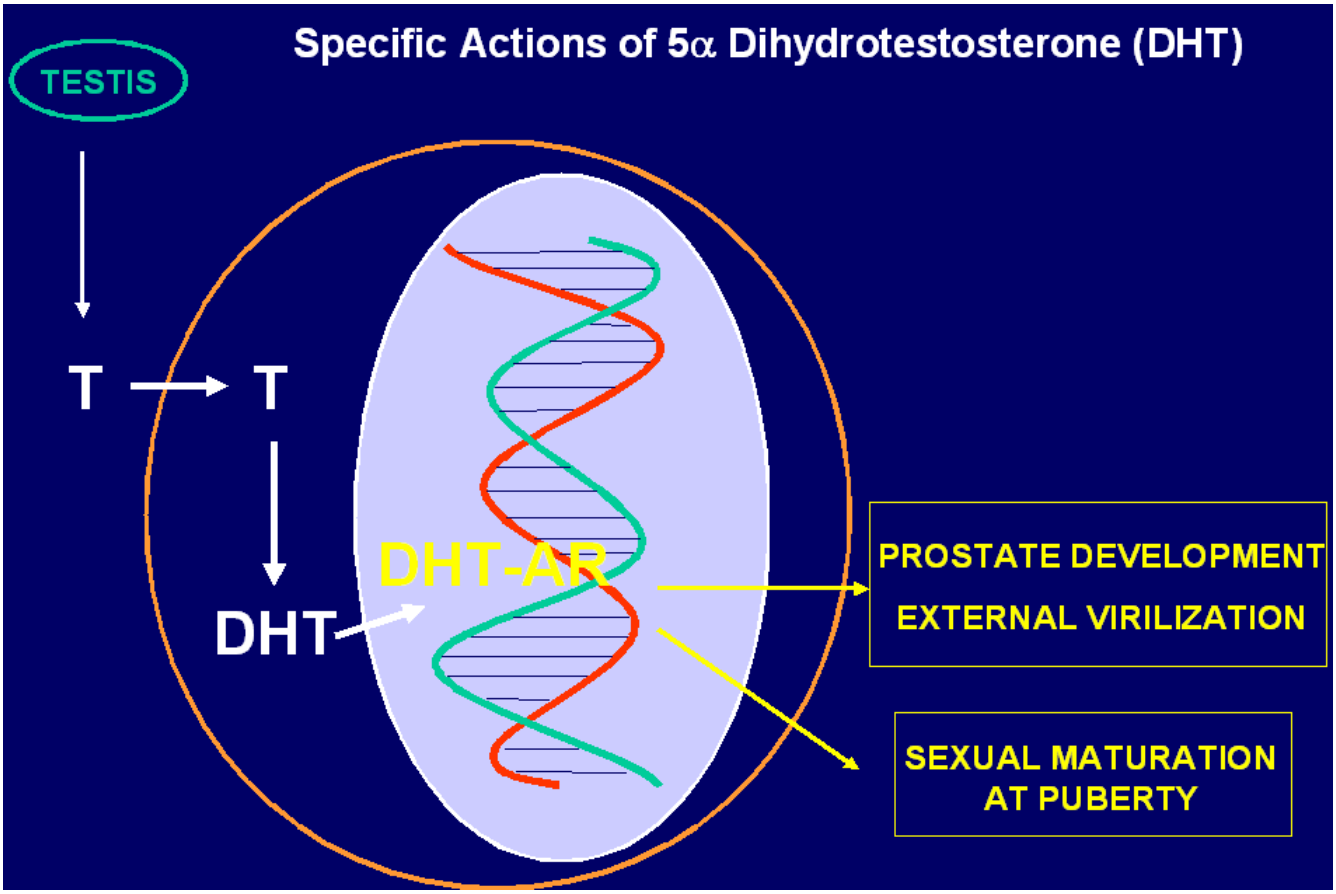

Figure 2b. Specific actions of 5α-Dihydrotestosterone (DHT). T is synthesized in the testis under control of luteinizing hormone (LH) from the pituitary. After entering the target cells (in the urogenital sinus, urogenital tubercle, and several additional androgen target tissues) T is metablized to 5α-Dihydrotestosterone (DHT) by the enzyme 5α-Reductase type 2. DHT binds directly to the androgen receptor (AR) and the complex DHT-AR interacts to specific DNA sequences and regulates gene transcription, which can result in differentiation and development of the prostate, the external genitalia and at puberty in several secondary male characteristics.

ANDROGEN BIOSYNTHESIS

Androgens (testosterone and 5α-dihydrotestosterone) belong to the group of steroid hormones. The major circulating androgen is testosterone, which is synthesized from cholesterol in the Leydig cells in the testis. Testosterone production in the fetal human testis starts during the sixth week of pregnancy. Leydig cell differentiation and the initial early testosterone biosynthesis in the fetal testis are independent of luteinizing hormone (LH) (5, 6, 7). During testis development production of testosterone comes under the control of LH which is produced by the pituitary gland. Synthesis and release of LH is under control of the hypothalamus through gonadotropin-releasing hormone (GnRH) and inhibited by testosterone via a negative feedback mechanism (8).The biosynthetic conversion of cholesterol to testosterone involves several discrete steps, of which the first one includes the transfer of cholesterol from the outer to the inner mitochondrial membrane by the steroidogenic acute regulatory protein (StAR) and the subsequent side chain cleavage of cholesterol by the enzyme P450scc (9). This conversion, resulting in the synthesis of pregnenolone, is the rate-limiting step in testosterone biosynthesis. Subsequent steps require several enzymes including, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase/C17-20-lyase and 17β-hydroxysteroid dehydrogenase type 3[Figure 3] (10).

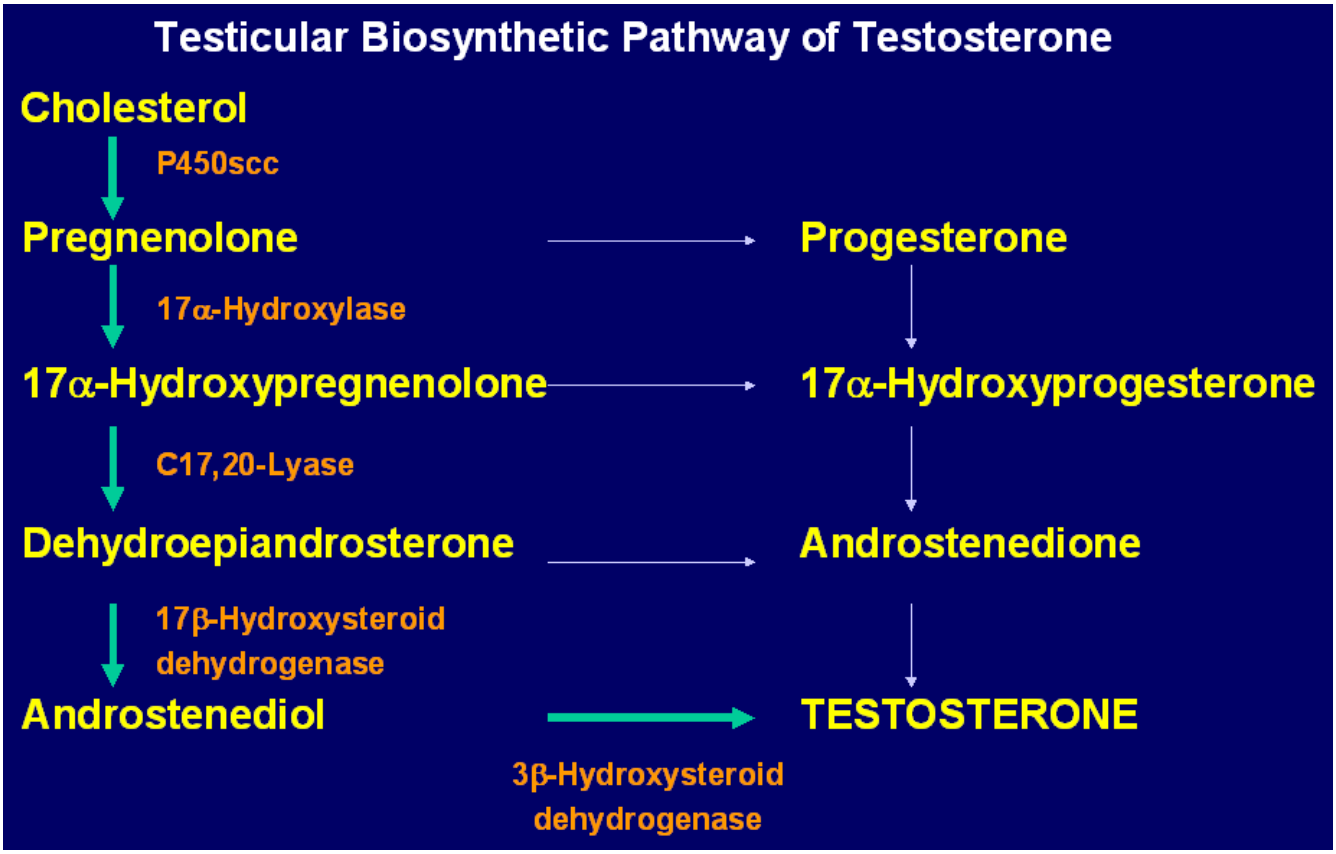

Figure 3. Biosynthetic pathway of testicular Testosterone synthesis.

METABOLISM OF TESTOSTERONE TO 5α-DIHYDRO-TESTOSTERONE

Metabolism of testosterone to 5α-dihydrotestosterone is essential for initiation of the differentiation and development of the urogenital sinus into the prostate. Differentiation of male external genitalia (penis, scrotum and urethra) also strongly depends on the conversion of testosterone to 5α-dihydrotestosterone in the urogenital tubercle, labioscrotal swellings and urogenital folds (1). The irreversible conversion of testosterone to 5α-dihydrotestosterone is catalyzed by the microsomal enzyme 5α-reductase type 2 (SRD5A2) and NADPH dependent.[Figure 4] (11). The cDNA of the gene for 5α-reductase type 2 codes for a protein of 254 amino acid residues with a predicted molecular mass of 28,398 Dalton (12, 13). The NH2-terminal part of the protein contains a subdomain supposedly involved in testosterone binding, while the COOH-terminal region is involved in NADPH-binding (2).

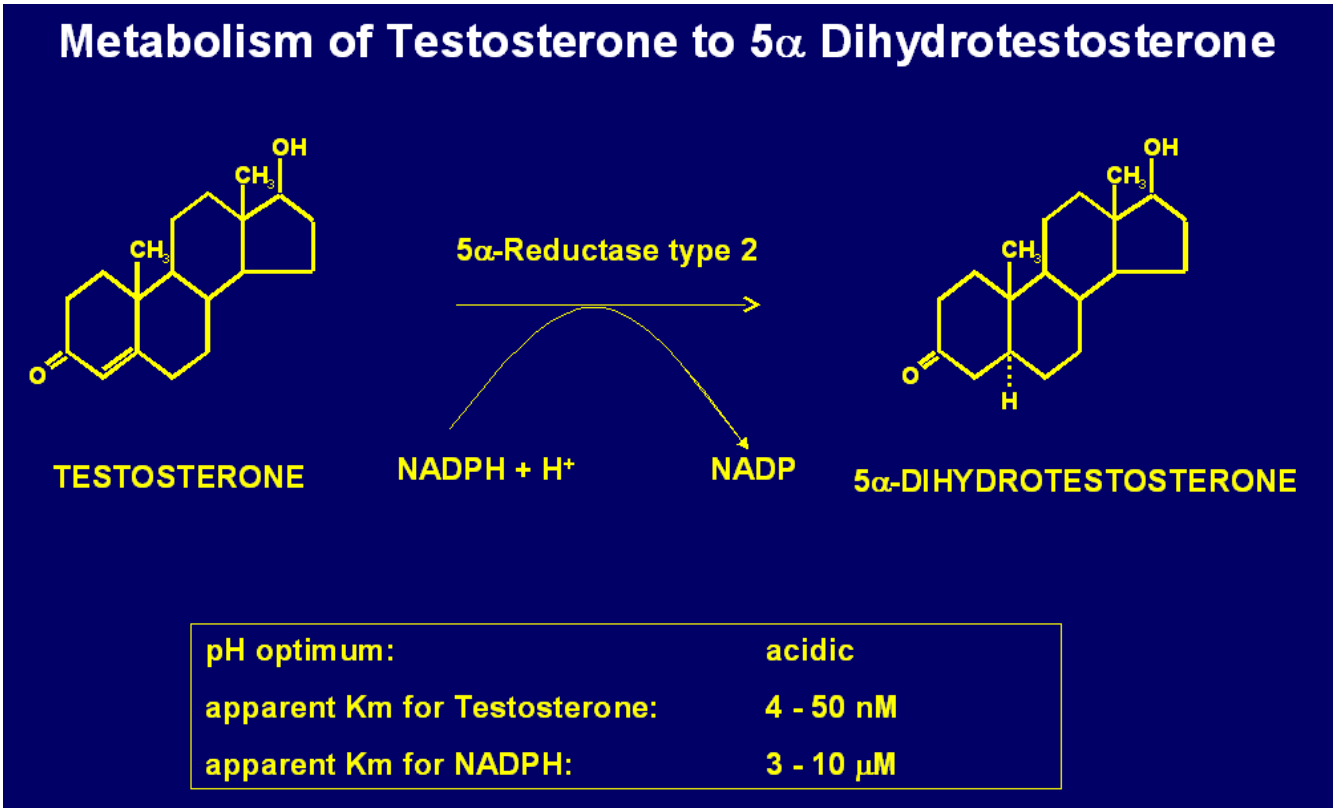

Figure 4. Metabolism of Testosterone to 5α-Dihydrotestosterone by the enzyme 5α-Reductase type 2 (SDR5A2).

ANDROGEN ACTION

The androgen receptor and the nuclear receptor family

Actions of androgens are mediated by the androgen receptor (NR3C4; Nuclear Receptor subfamily 3, group C, gene 4).This ligand dependent transcription factor belongs to the superfamily of 48 known nuclear receptors (14). This family includes receptors for steroid hormones, thyroid hormones, all-trans and 9-cis retinoic acid, 1,25 dihydroxy-vitamin D, ecdysone and peroxisome proliferator-activated receptors (15, 16, 17). In addition an increasing number of nuclear proteins have been identified with a protein structure homologous with that of nuclear receptors, but without a known ligand. These so-called "orphan" receptors form an important subfamily of transcription factors acting either in the absence of any ligand or with as yet unknown endogenous ligands (18). Comparative structural and functional analysis of nuclear hormone receptors has revealed a common structural organization in 4 different functional domains: a NH2-Terminal Domain, a DNA-Binding Domain, a Hinge Region and a Ligand Binding Domain [Figure 5].

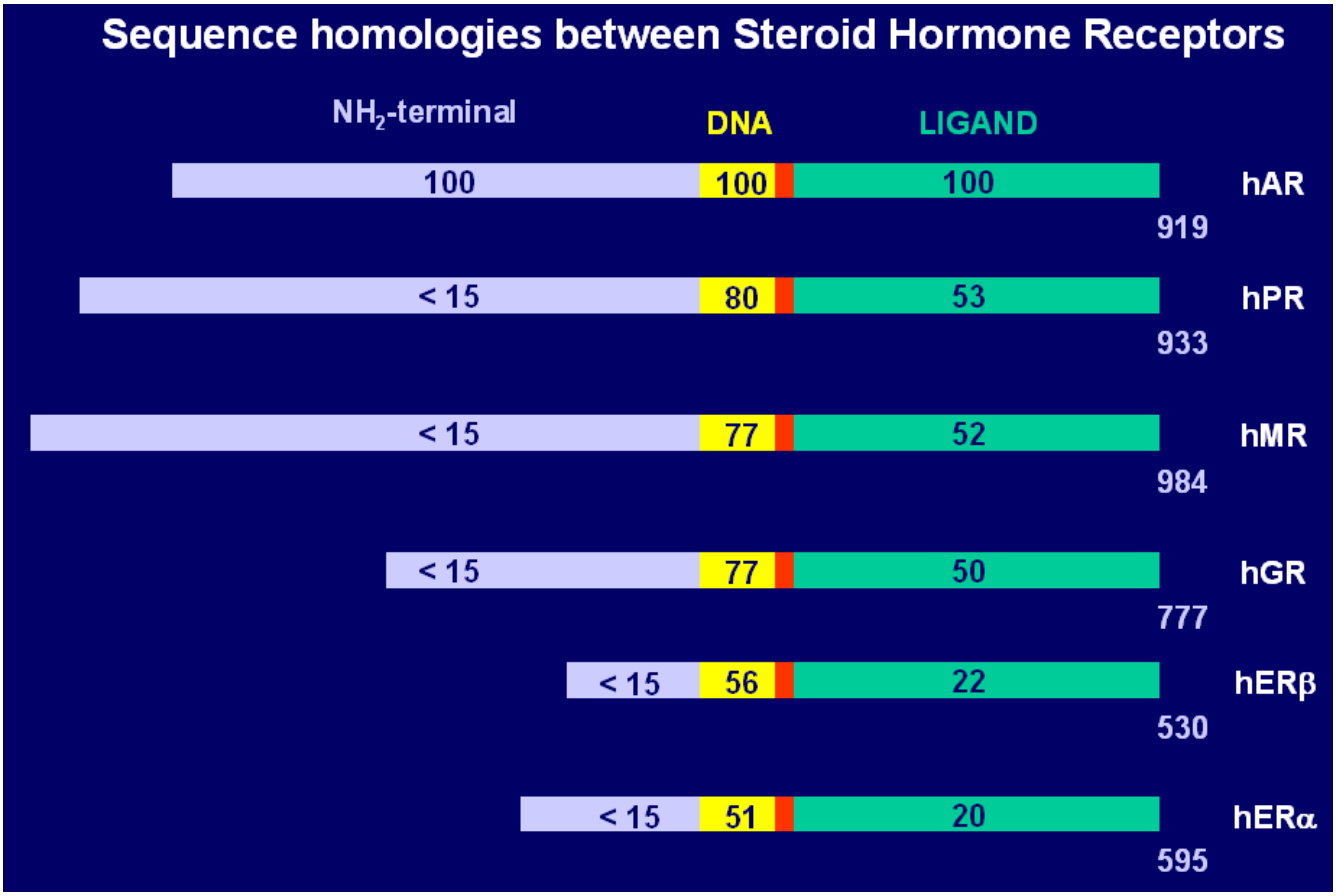

Figure 5. Sequence homologies between the human Androgen Receptor (hAR), human Progesterone Receptor (hPR), human Glucocorticoid Receptor (hGR), human Mineralocorticoid Receptor (hMR) and the human Estrogen Receptors α (hERα) and β (hERβ).

The current model for androgen action involves a multi step mechanism as depicted in Figure 6. Upon entry of testosterone into the androgen target cell, binding occurs to the androgen receptor either directly or after its conversion to 5α-dihydrotestosterone. Binding to the receptor is followed by dissociation of heat shock proteins in the cytoplasm, simultaneously accompanied by a conformational change of the receptor protein resulting in a transformation and a translocation to the nucleus. Upon binding in the nucleus to specific DNA-sequences the receptor dimerizes with a second molecule and the homodimer entity recruits further additional proteins (e.g. coactivators, general transcription factors, RNA-polymerase II) resulting in specific activation of transcription at discrete sites on the chromatin.

Interestingly androgen signaling via the androgen receptor can also occur in a non-genomic, rapid and sex-nonspecific way by crosstalk with the Scr, Raf-1, Erk-2 pathway [Figure 6] (19, 20). The classical androgen receptor is also involved in androgen-mediated induction of Xenopus oocyte maturation via the (MAPK)-signalling cascade in a transcription independent way (21).

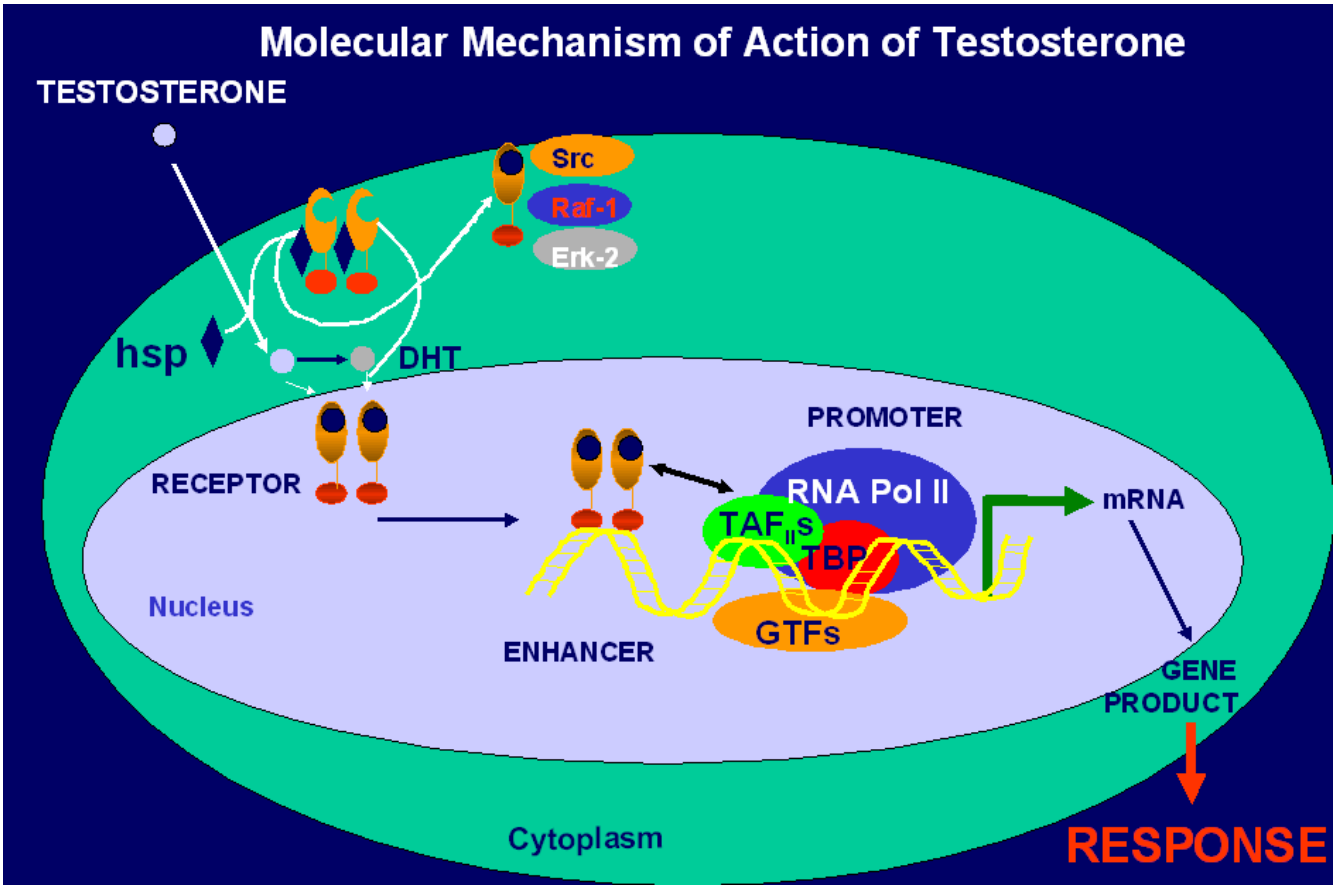

Figure 6. Simplified model of androgen action in an androgen target cell. The key protein is the androgen receptor, which binds testosterone directly or its active metabolite 5α-dihydrotestosterone (DHT). After dissociation of heat shock proteins (hsp) the receptor enters the nucleus via an intrinsic nuclear localization signal. Upon steroid hormone binding, which may occur either in the cytoplasm or in the nucleus, the androgen receptor binds as homodimer to specific DNA elements present as enhancers in upstream promoter sequences of androgen target genes. The next step is recruitment of coactivators, which can form the communication bridge between receptor and several components of the transcription machinery. The direct and indirect communication of the androgen receptor complex with several components of the transcription machinery (e.g. RNA-polymerase II [RNA-Pol II], TATA box binding protein [TBP], TBP associating factors [TAF's], general transcription factors [GTF's]) are key events in nuclear signaling. This communication triggers subsequently mRNA synthesis and consequently protein synthesis, which finally results in an androgen response. A non-genomic pathway involving the classical androgen receptor via cross-talk with the Src/Raf-1/Erk-2 pathway is also known.

Cloning and structural organization of androgen receptor gene

Since cloning of the human androgen receptor cDNA our insights into the mechanism of androgen action have been increased tremendously. Only one androgen receptor cDNA has been identified and cloned, despite the two different ligands (22, 23, 24, 25). The concept of two hormones and one receptor to explain the different actions of androgens has been generally accepted and, according to the information available from the human genome project, it is very unlikely that additional genes exist coding for a functional nuclear receptor with androgen receptor-like properties (17).

The androgen receptor gene is located on the X-chromosome at Xq11.2 -12 [approx. 186 kb] and codes for a protein with a molecular mass of approx. 110 kDa [Figure 7] (26, 27). The gene consists of

8 coding exons and the structural organization of the coding exons is essentially identical to those of the genes coding for the other steroid hormone receptors (e.g. exon/intron boundaries are highly conserved) [Figure 7] (28, 29). As a result of differential splicing in the 3' - untranslated region two androgen receptor mRNA species (8.5 and 11 kb, respectively) have been identified in several cell lines (30). There is no structural indication in the androgen receptor mRNA for any preferential use of either of the two transcripts and neither for a specific function, but it can be speculated that tissue specific factors may determine which transcript is present in which androgen target tissue. In the human prostate and in genital skin fibroblasts predominantly the 11 kb size mRNA is expressed.

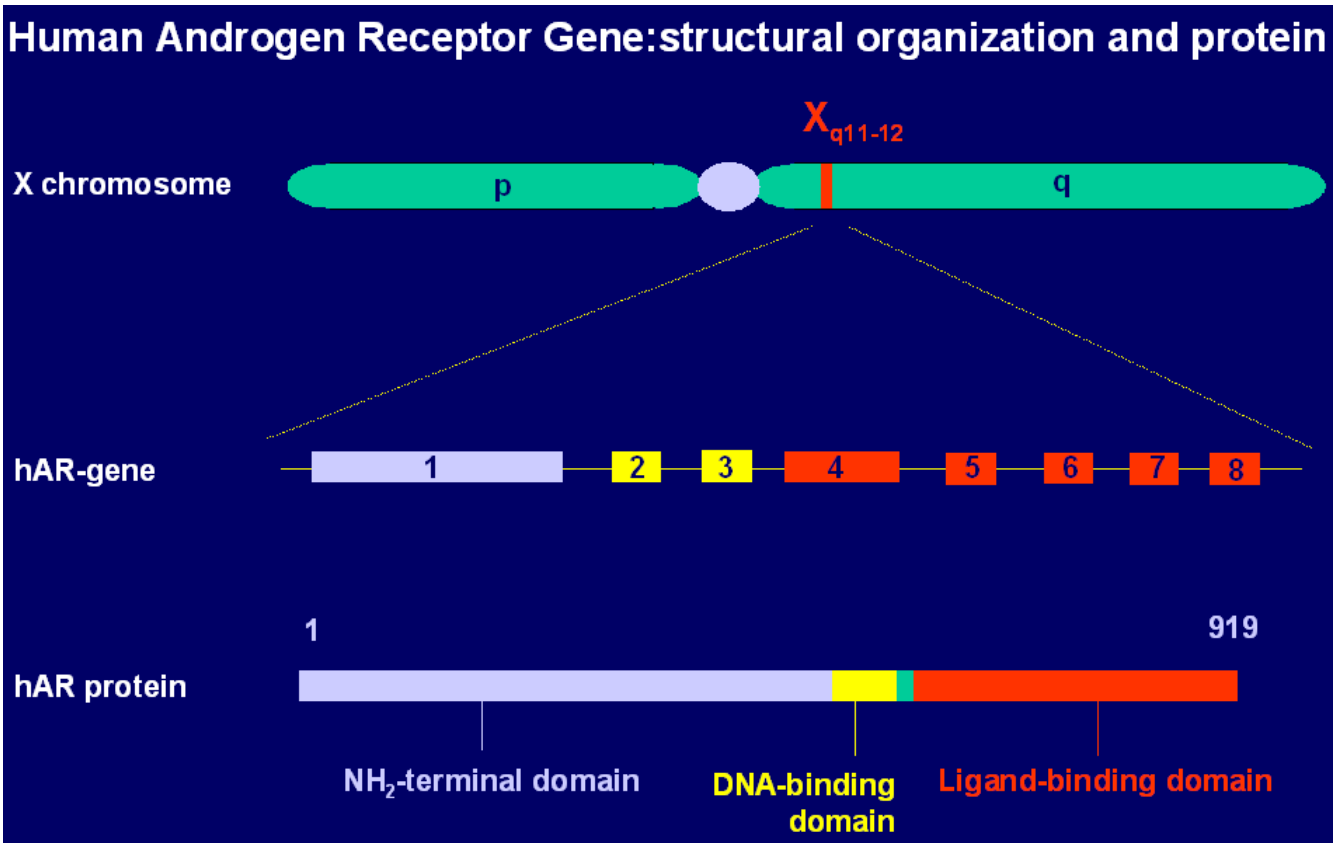

Figure 7. Human androgen receptor gene was mapped to the long arm of the X-chromosome. The human androgen receptor protein (919 amino acid residues) is encoded by 8 exons. Analogous to other nuclear receptors the protein consists of several distinct functional domains: the NH2-terminal domain containing two polymorphic stretches, the DNA-binding domain, the hinge region and the ligand binding domain.

Androgen receptor polymorphisms

The androgen receptor DNA - and ligand-binding domains have a high homology with the corresponding domains of the other members of the steroid receptor subfamily.

There is a remarkably low homology between the androgen receptor NH2-terminal domain and that of the other steroid receptors [Figure 5, see above] (31, 32, 33, 34, 35, 36). A poly-glutamine stretch, encoded by a polymorphic (CAG)nCAA - repeat is present in the NH2-terminal domain [Figure 8] (37). The length of the repeat has been used for identification of X-chromosomes for carrier detection in pedigree analyses (38, 39).

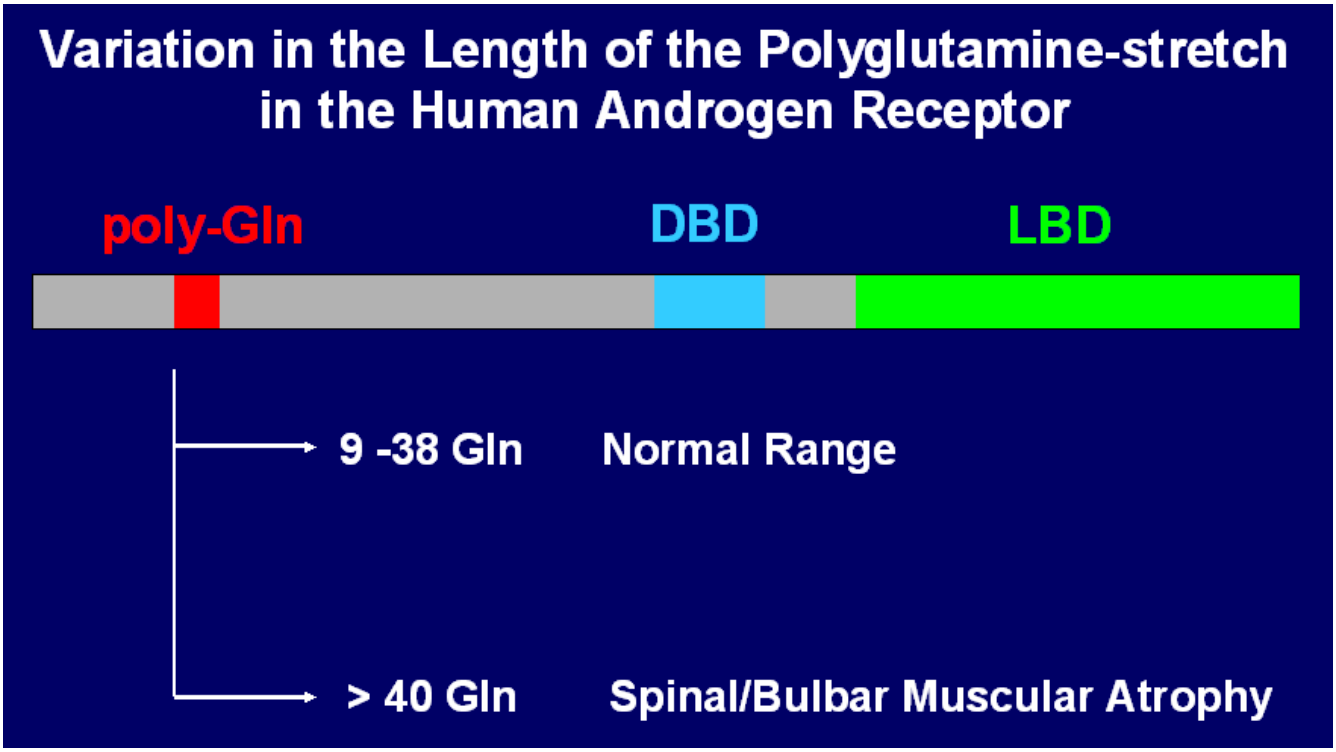

Figure 8. Variation in the polyglutamine stretch encoded by the (CAG)nCAA repeat in exon 1. The normal range of this stretch is 9 - 38 glutamine residues, while in the motor neuron disease spinal/bulbar muscular atrophy (also called Kennedy's disease) the (CAG)nCAA repeat in exon 1 is expanded and codes for more than 40 Glutamine residues.

Variation in length (9 - 38 glutamine residues) is observed in the normal population and has been suggested to be associated with a very mild modulation of androgen receptor activity (40). This assumption is based on in vitro experiments after transient transfection of androgen receptor cDNA's containing (CAG)nCAA - repeats of different lengths (41, 42). Translating this finding to the in vivo situation it can be envisaged that either shorter or longer repeat lengths can result in a relevant biologic effect during lifetime. This concept has been explored in epidemiological studies of men with prostate cancer or infertility. With respect to prostate cancer, a clear picture has not emerged and controversy persists. In several studies, shortening of the (CAG)nCAA repeat length in exon 1 of the androgen receptor gene was found to correlate with an earlier age of onset of prostate cancer, and a higher tumor grade and aggressiveness (43, 44, 45). However, in other epidemiological studies in prostate cancer patients these associations were not confirmed (46, 47).

In several investigations in male infertile patients an association was found between a longer (CAG)nCAA repeat and the risk of defective spermatogenesis (48, 49, 50). This suggests that a less active androgen receptor, due to a moderate expanded repeat length, may be a factor in the etiology of male infertility.

The (CAG)nCAA - repeat in exon 1 of the androgen receptor gene is expanded in patients with spinal and bulbar muscular atrophy (SBMA) and varies between 38 and 75 repeat units [Figure 8] (40, 51). Spinal and bulbar muscular atrophy is characterized by progressive muscle weakness and atrophy and is associated with nuclear accumulation of androgen receptor protein with the expanded polyglutamine stretch in motor neurons. Clinical symptoms usually manifest in the third to fifth decade and result from severe depletion of lower motornuclei in the spinal cord and brainstem (40, 52, 53). Furthermore overexpression of wildtype androgen receptor in skeletal muscle fibers in a mouse model results in the same androgen-dependent disease phenotype as when mutant androgen receptor is broadly expressed, suggesting an alternative model for the role of the AR in SBMA (54). The neurotoxity of the polyglutamine androgen receptor may involve generation of NH_2-terminal truncation fragments, as such peptides occur in SBMA patients (55). Therapeutic approaches in SBMA are

focussing on reducing nuclear localized mutant androgen receptor via enhanced mutant androgen receptor degradation or by disrupting the interaction with androgen receptor coregulators (56, 57). In addition, SBMA patients frequently exhibit endocrinological abnormalities including testicular atrophy, infertility, gynecomastia, and elevated LH, FSH and estradiol levels. Sex differentiation proceeds normally and characteristics of mild androgen insensitivity appear later in life.

ANDROGEN RECEPTOR: FUNCTIONAL DOMAIN STRUCTURE

The NH2-terminal domain

The androgen receptor NH2-terminal domain harbors the major transcription activation functions and several structural subdomains. Within its 538 amino acids, two independent activation domains have been identified: activation function 1 (AF-1) (located between residues 101 and 370) that is essential for transactivity of full length AR, and activation function 5 (AF-5) (located between residues 360-485) that is required for transactivity of a constitutively active androgen receptor, which lacks its LBD [Figure 9] (58). Evidence is available now that the AF-5 region in the AR NH_2-terminal domain interacts with a glutamine rich domain in p160 cofactors like SRC-1 and TIF2/GRIP1 and not with their LxxLL-like protein interacting motifs (59). The NH_2-terminal domain is highly flexible. It has a structure between a fully folded state and a structured folded conformation: a molten-globule conformation (60). Only large deletions and/or multiple amino acid substitutions within the androgen receptor NH2-terminal domain will affect transcription activity. Single amino acid substitutions within the androgen receptor NH2-terminus have not been observed to significantly impair androgen receptor function, with the exception of a very few amino acid substitutions found in patients with the androgen insensitivity syndrome (61).

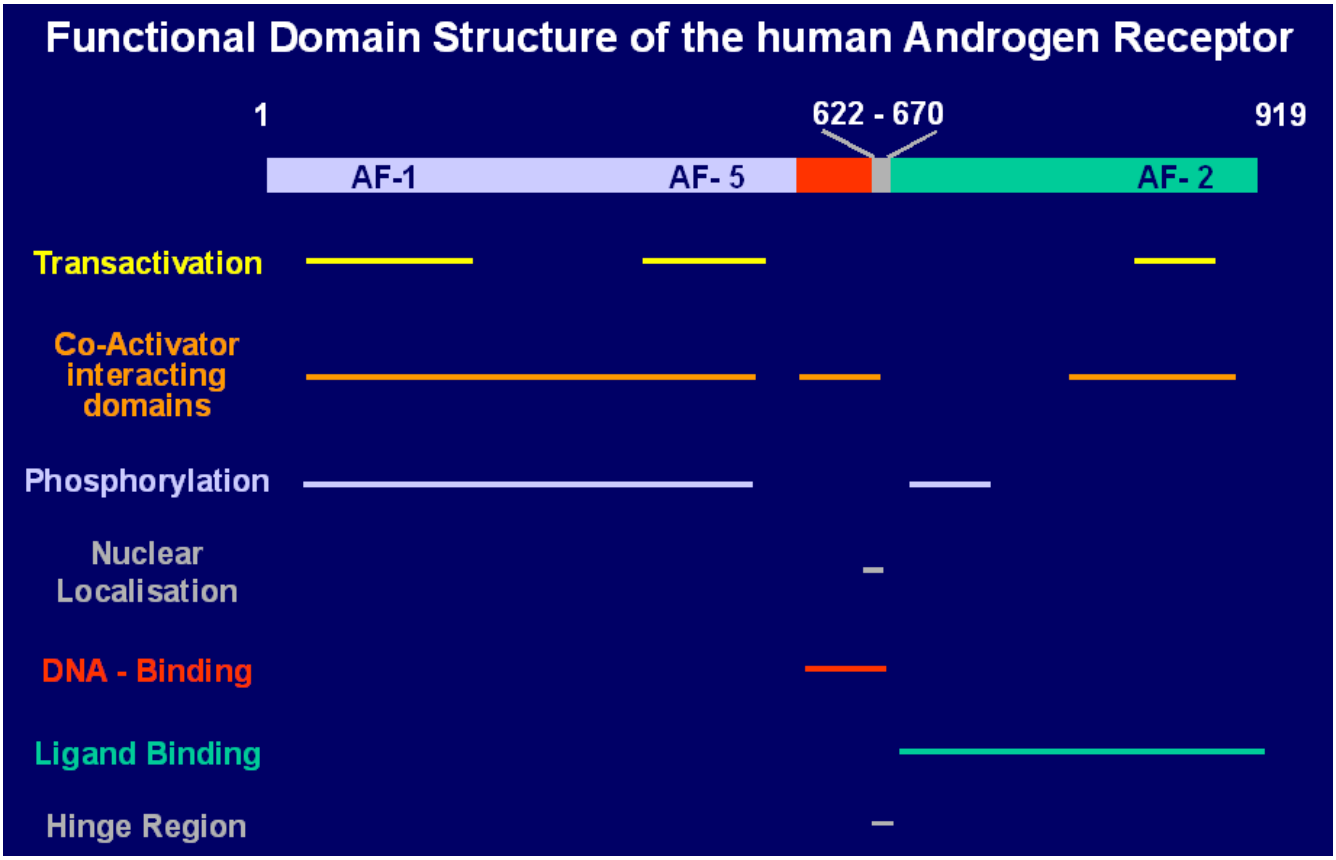

Figure 9. Functional domain structure of the human androgen receptor. The protein consists of several distinct functional domains: transcription Activation Functions (AF-1; AF-5; in the NH2-

terminal domain; and AF-2 in the ligand binding domain); Coactivator interacting domains, Phosphorylation sites, a nuclear localisation signal, the DNA-binding domain, the hinge region and the ligand binding domain.

Another function of the androgen receptor NH2-terminal domain is its binding to the COOH-terminal LBD (N/C interaction) (62, 63). The NH2-terminal regions required for the binding of the LBD have been mapped to two essential units: the first 36 amino acids and residues 370-494 (64).

The hormone dependent interaction of the NH2-terminal domain with the ligand binding domain can play a role in stabilization of the androgen receptor dimer complex and in stabilization of the ligand receptor complex by slowing the rate of ligand dissociation and decreasing receptor degradation (65, 66). Agonists like T and DHT, but not antagonists like hydroxyflutamide or bicalutamide induce the N/C interaction in full length AR. In a FRET (Fluorescence resonance energy transfer) study it was clearly shown that the AR N/C interaction is rapidly initiated in the cytoplasm after hormone binding as an intramolecular interaction and is followed by an intermolecular N/C interaction in the nucleus, contributing to AR dimerization (67). The N/C interaction occurs preferentially in the mobile AR, where it protects the coactivator binding groove for ultimitely and unfavourable protein-protein interactions . Specifically bound to DNA, the N/C interaction is lost allowing cofactor binding (68). Several mutations in the ligand binding domain, detected in patients with the syndrome of androgen insensitivity, affect negatively the interaction of the NH2-terminal domain with the ligand binding domain, while androgen binding was impaired, indicating the importance of this interaction (69).

The DNA-binding domain

The DNA-binding domain is the best conserved among the members of the receptor superfamily [Figure 5, see above]. It is characterized by a high content of basic amino acids and by nine conserved cysteine residues [Figure 10]. Detailed structural information has been published on the crystal structure of the DNA-binding domain of the glucocorticoid receptor complexed with DNA (70). 3D-information became available for AR-DNA interaction at different types of response elements (71).

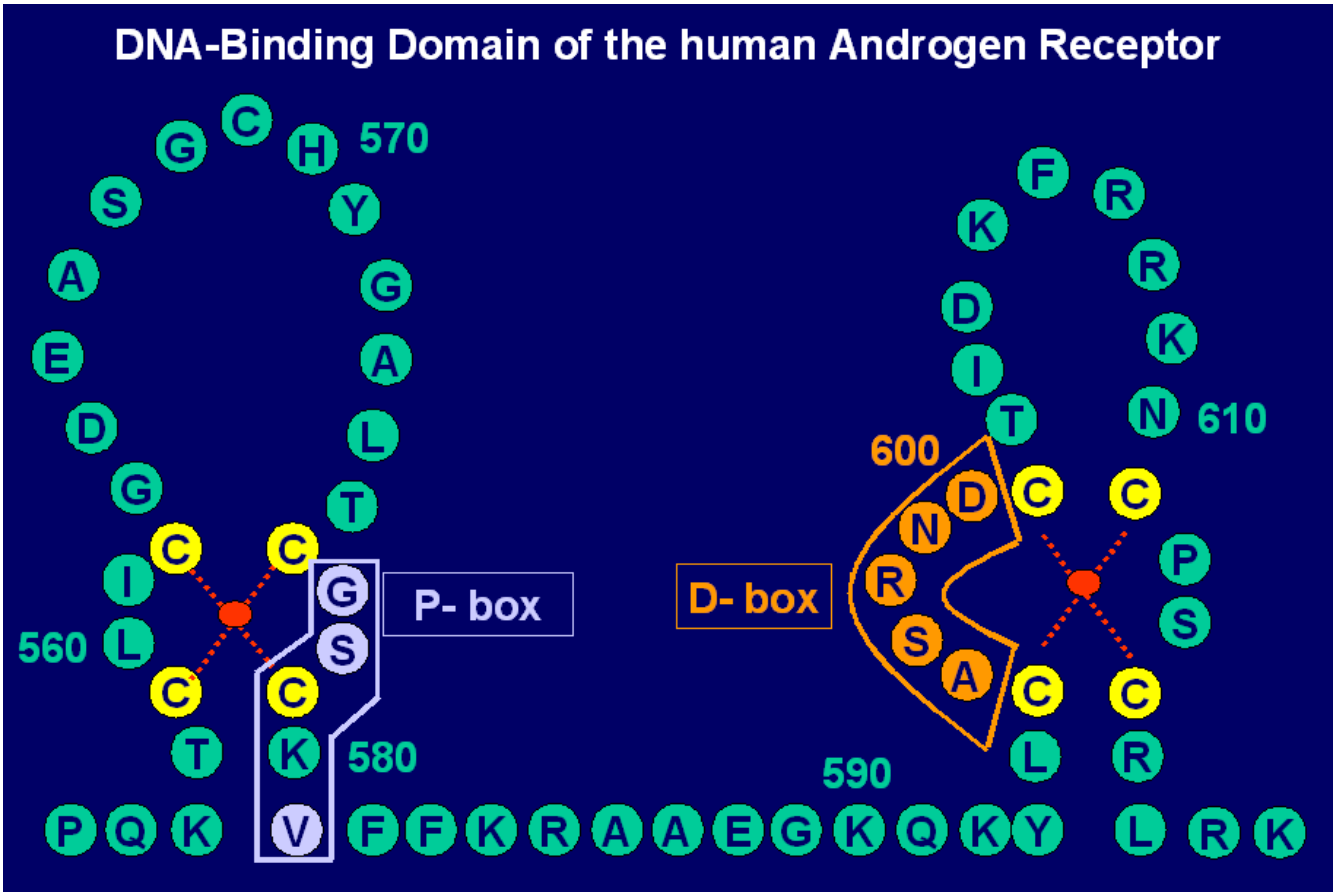

Figure 10. Structure of the DNA binding domain of the human androgen receptor. The protein structure is represented in the one-letter code. The domain consists of two zinc cluster modules, which are stabilized by the coordination binding of a zinc atom (red dot) by 4 cysteine residues (yellow). The first zinc cluster contains the P-box (proximal box), of which three residues determine androgen response element recognition. The second zinc cluster contains the D-box (distal box) in which amino acids are located that are involved in protein-protein interactions with a second receptor molecule in the homodimer complex.

Briefly, the DNA-binding domain has a compact, globular structure in which three substructures can be distinguished: two zinc clusters and a more loosely structured carboxy terminal extension (CTE) (72). Both zinc substructures contain centrally one zinc atom which interacts via coordination bonds with four cysteine residues [Figure 10]. The two zinc coordination centers are both C-terminally flanked by an α-helix (70). The two zinc clusters are structurally and functionally different and are encoded by two different exons. The α-helix of the most N-terminal located zinc cluster interacts directly with nucleotides of the hormone response element in the major groove of the DNA. Three amino acid residues at the N-terminus of this α-helix are responsible for the specific recognition of the DNA-sequence of the responsive element [Figure 10]. These three amino acid residues, the so-called P(roximal)-box [Gly; Ser; Val;] are identical in the androgen, progesterone, glucocorticoid and mineralocorticoid receptors, and differ from the residues at homologous positions in the oestradiol receptor. It is not surprising therefore, that the androgen, progesterone, glucocorticoid and mineralocorticoid receptors can recognize the same response element. The AR DNA binding domain requires a CTE of minimally four residues (AR 625 – TLGA – 628) for proper binding to an ARE (androgen response element) with an inverted repeat of high affinity ARE-half sites and a CTE of at least twelve residues (AR 625 – TLGARKLKKLGN – 636) for binding to an ARE with one high and one low affinity half site (73). For the hormone and tissue specific responses of the different receptors additional determinants are needed. Important in this respect are DNA-sequences flanking the hormone response element, receptor interactions with other proteins and receptor concentrations. The

second zinc cluster motif is supposed to be involved in protein-protein interactions such as receptor dimerization via the so-called D(istal)-box [Figure 10] (70).

The hinge region

Between the DNA-binding domain and the ligand binding domain a non-conserved hinge region is located, which is also variable in size in different steroid receptors [Figure 9, see above]. The hinge region can be considered as a flexible linker between the ligand binding domain and the rest of the receptor molecule. The hinge region is important for nuclear localization and contains a bipartite nuclear localization signal. Also co-repressor binding can occur via the hinge region. In some nuclear receptors, including the AR, an acetylation can occur in the hinge region at a highly conserved acetylation consensus site [KLLKK] (74).

The ligand binding domain

Finally the second-best conserved region is the hormone binding domain. This domain is encoded by approximately 250 amino acid residues in the C-terminal end of the molecule [Figure 5, see above] (24, 31, 32, 33, 34, 75). The crystal structure of the human androgen receptor ligand binding in complex with the synthetic ligand methyltrienolone (R1881) and 5α-dihydrotestosterone, respectively, have been determined (76, 77). The 3-dimensional structure has the typical nuclear receptor ligand binding domain fold. Interestingly the ligand binding pocket consists of 18 amino acid residues interacting more or less directly with the bound ligand (76). The ligand binding pocket is somewhat flexible and can accommodate ligands with different structures. The structural data are being used in designing optimized selective androgen receptor modulators (SARMs) (78). Several AR mutations found in prostate tumors have been investigated functionally, including T877S, T877A, H874T, V715M, W741C, and L701H as a single mutation or in combination with T877A. Similarly to T877A these AR mutations have a broadened ligand specificity and are activated by different low affinity ligands like oestradiol, progesterone, glucocorticoids and different partial and full antagonists (79, 80, 81, 82, 83, 84, 85).
Crystallographic data on the ligand binding domain complexed with agonist predict 11 helices (no helix 2) with two anti-parallel β-sheets arranged in a so-called helical sandwich pattern. In the agonist-bound conformation the carboxy-terminal helix 12 is positioned in an orientation allowing a closure of the ligand binding pocket. The fold of the ligand binding domain upon hormone binding results in a globular structure with an interaction surface for binding of interacting proteins like co-activators. In this way the androgen receptor recruits selectively a number of proteins and can communicate with other partners of the transcription initiation complex. Crystallization studies of wild type AR ligand binding domain with antagonists were up till now unsuccessful (86).
The androgen receptor can use different transactivation domains (AF1 and AF5, respectively, in the NH2-terminal domain and AF2 in the COOH-terminal domain) depending on the "form" of the receptor protein [Figure 9, see above] (58). The AF2 function in the ligand binding domain is strongly dependent on the presence of nuclear receptor coactivators. In vivo experiments favour a ligand dependent functional interaction between the AF-2 region in the ligand-binding domain with the NH2-terminal domain (62, 64).
Deletions in the ligand binding domain abolish hormone binding completely (87). Deletions in the N-terminal domain and DNA-binding domain do not affect hormone binding. Deletion of the ligand binding domain leads to a constitutively active androgen receptor protein with trans-activation capacity comparable to the full length androgen receptor (87). Thus it appears that the hormone binding domain acts as a repressor of the trans-activation function in the absence of hormone. This regulatory function of the androgen receptor ligand binding domain in the absence of hormone, is not unique for the androgen receptor and has been reported also for the glucocorticoid receptor (88).
Interestingly, a previously unknown regulatory surface cleft, named BF-3, has been recently identified

in the AR LBD (86). BF-3 comprises of Ile-672, Phe-673, Pro-723, Gly-724, Asn-727, Phe-826, Glu-829, Asn-833, Glu-837 and Arg-840. The AR transcriptional activity and co-activator binding can be decreased by binding to the BF-3 of thyroid hormones triiodothyronine (T3) and TRIAC and three non-steroidal anti-inflammatory drugs. In addition, several mutations of the amino acid residues of BF-3 have been found in subjects with either androgen insensitivity syndrome (AIS, loss of function mutation) or in prostate cancer (gain of function mutation) (89). Mutational analyses have shown the requirement of several of these amino acid residues for AR transactivity. However, these analyses have been performed only in the presence of DHT (86). The influence of each of these residues in the presence of T3, TRIAC or other nonsteroidal anti-inflammatory drugs is therefore unknown.

Androgen Receptor Phosphorylation

Immediately after translation, the androgen receptor becomes phosphorylated resulting in the appearance of two isoforms separable by SDS-polyacrylamide gel electrophoresis (90). The non-phosphorylated faster migrating 110 kDa isoform is converted into a 112 kDa phospho-isoform. Mutational analysis of serine 81 or serine 94 in the androgen receptor NH2-terminal domain abolishes this up-shift indicating that phosphorylation of these serine residues likely contributes to the phosphorylation of the 112 kDa androgen receptor isoform (41, 91). Three other androgen receptor phosphorylation sites have been identified using mutational analysis and trypsin-digestion of 32P-labelled androgen receptor followed by HPLC analysis and Edman degradation (91, 92, 93). These include the serine residues at position 515, 650, and 662. Substitution of serine 650 reduced androgen receptor activity by up to 30%. Mutation of serines 81 and 94 had little or no effect on androgen receptor function (41, 91). Several other sites have been identified in the NH_2-terminal domain at positions S16, S213, S256, S308, and S424 (94, 95, 96, 97). The function of phosphorylation of these sites is in the majority of the cases unknown or controversial. Additional two sites (S578 and S791) have been identified and characterized in the DNA-binding and ligand binding domains, respectively (98, 99). Not until recently, tyrosine phosphorylation was thought not to occur in the AR. However, this has been disapproved by the use of phospho-tyrosine specific antibodies. It has been shown that AR tyrosine residue 534 is highly phosphorylated (100, 101). This phosphorylation is induced by EGF via activation of Src. Like tyrosine phosphorylation, it was thought that threonine phosphorylation does not occur for the AR. Recently it was reported that threonine phosphorylation of the AR in human sperm cells could be demonstrated with the use of a phosphothreonine specific antibody (102). However, the exact location of the site was not determined.

The role of AR phosphorylation still remains unclear. Mutagenesis of individual phosphosites or of five phosphosites in one AR protein, did not reveal an indication for function, nor does the phosphorylation mimicking mutation of six serine residues to aspartic acid (94). In many studies the trancriptional activity of the AR has not been studied with androgens in a dose-dependent way, whereas it has been shown that a high dose of androgens can result in a normal AR activity (100).

Besides the basal phosphorylation resulting in the 110-112 kDa doublet, addition of androgen induces another shift and the generation of a 110-112-114 kDa androgen receptor triplet (41). This triplet is the result of both an addition and a redistribution of phosphorylated sites, however, it is unknown which exact residues are involved (103). Interestingly, mutations that inactivate androgen receptor function, such as mutations resulting in loss of DNA binding or transactivation, inhibit the formation of the 114 kDa isoform. This suggests that part of the androgen - induced phosphorylation occurs during or after androgen receptor transcription regulation (41). In conclusion phosphorylation of the androgen receptor can be linked to activation of hormone binding and to modulation of DNA binding (92, 104). Furthermore phosphorylation of the androgen receptor can play an essential role in the hormone independent activation of the androgen receptor by protein kinases in the MAPK and AKT (protein kinase B) signaling pathways, activated either through HER-2/neu or growth factors (105, 106).

Anti-androgens

Androgen receptor antagonists are compounds that interfere in some way in the biological effects of androgens and are frequently used in the treatment of androgen-based pathologies. The steroidal anti-androgens, cyproterone acetate (CPA) and RU38486 (RU486; mifepristone), have partial agonistic and antagonistic actions. Interestingly both compounds display also partial progestational and glucocorticoid action and are therefore considered not to be pure anti-androgens. The non-steroidal anti-androgens hydroxyflutamide, nilutamide and bicalutamide are pure antiandrogens (107, 108, 109).

Mechanism of action of antiandrogens

In contrast to the full antagonists hydroxyflutamide and bicalutamide, CPA and RU486 can partially activate the androgen receptor with respect to transcription activation (110, 111). With a limited proteolytic protection assay, it was demonstrated that binding of androgens by the androgen receptor results in two consecutive conformational changes of the receptor molecule. Initially, a fragment of 35 kDa, spanning the complete ligand binding domain and part of the hinge region, is protected from digestion by the ligand. After prolonged incubation times with the ligand a second conformational change occurs resulting in protection of a smaller fragment of 29 kDa (110, 111). In the presence of several anti-androgens (e.g. cyproterone acetate, hydroxyflutamide and bicalutamide) only the 35 kDa fragment is protected from proteolytic digestion, and no smaller fragments are detectable upon longer incubations. Obviously, the 35 kDa fragment can be associated with an inactive conformation, whereas the second conformational change, only inducible by agonists and considered as the necessary step for transcription activation, is lacking upon binding of anti-androgens.

Selective Androgen Receptor Modulation

The term SARM (= Selective Androgen Receptor Modulator) was introduced in 1999 in analogy of the term SERM (Selective Estrogen Receptor Modulator) (112). A SARM can be defined as a molecule that targets the androgen receptor, and elicits a biological response, in a tissue specific way. In a sense, anti-androgens (molecules that target specifically the androgen receptor pathway resulting in inhibition of the biological effects of androgens) can be considered as a special subtype of SARMs. Information became available on androgen signalling via the androgen receptor in a non-genomic, rapid and sex-nonspecific way by crosstalk with the Scr, Raf-1, Erk-2 pathway [Figure 6, see above] (19, 20). The anti-apoptotic action via the androgen receptor in bone cells (osteocytes, osteoblasts), and also in HeLa cells, could be induced by androgens and estrogens and inhibited by antiandrogens as well as anti-estrogens. The anti-apoptotic action appeared to be dissociated from the genomic action of the androgen receptor. Also the progesterone induced oocyte maturation in Xenopus laevis appeared to be mediated in a non-genomic way by androgens and the androgen receptor via activating the MAPK pathway after the rapid conversion of progesterone to androstenedione and testosterone (21). These findings stimulated the development of new compounds which can selectively activate the androgen receptor either in a non-genomic pathway or in a genotropic transcriptional activation pathway. In an extensive overview the current standing of clinical trials with newly developed SARMs by several different pharmaceutical companies is presented and discussed (113).
Based on the conformational changes of the AR ligand binding domain, induced by androgens or anti-androgens, it can be concluded that the different transcriptional activities displayed by either full agonists (testosterone, 5α-dihydrotestosterone, methyltrienolone), partial agonists (RU486 and CPA) or full antagonists (hydroxyflutamide, bicalutamide) are the result of recruitment of a different repertoire of co-regulators (coactivators or corepressors) as a consequence of these conformational changes. The differential recruitment of co-regulators can be considered as a special form of ligand selective modulation of the androgen receptor ligand binding domain and can be applied in a broader sense also to the tissue selective modulation of androgen action, where levels of co-activators and co-repressors may ultimately determine the final activity (114).

ANDROGEN RECEPTOR DISORDERS

Androgen insensitivity syndrome

It has been known for quite some time that defects in male sexual differentiation in 46, XY individuals have an X-linked pattern of inheritance. It was Reifenstein who reported in 1947 on families with severe hypospadias, infertility and gynecomastia (115). The end-organ resistance to androgens has been designated as androgen insensitivity syndrome (AIS) and is distinct from other XY disorders of sex development (XY, DSD; formerly named : male pseudohermaphroditism) like 17β-hydroxy-steroiddehydrogenase type 3 deficiency or 5α-reductase type 2 deficiency (2, 116, 117, 118). It is generally accepted that defects in the androgen receptor gene can prevent the normal development of both internal and external male structures in 46, XY individuals and information on the molecular structure of the human androgen receptor gene has facilitated the study of molecular defects associated with androgen insensitivity. Due to the X-linked character of the syndrome, only 46, XY individuals are affected, while in female carriers only sporadic reports are available on delayed menarche (119). Naturally occurring mutations in the androgen receptor gene are an interesting source for the investigation of receptor structure-function relationships. In addition, the variation in clinical phenotypes provides the opportunity to correlate a mutation in the androgen receptor structure with the impairment of a specific physiological function.

Clinical features of the Complete Androgen Insensitivity Syndrome (CAIS)

The main phenotypic characteristics of individuals with the complete androgen insensitivity syndrome (CAIS) are: female external genitalia, a short, blind ending vagina, absence of wolffian duct derived structures like epididymides, vasa deferentia and seminal vesicles, the absence of a prostate, the absence of pubic and axillary hair and the development of gynecomastia (120, 121). Müllerian duct derived structures are usually absent because anti-müllerian hormone action is normal due to the presence of both testes in the abdomen or in the inguinal canals. Usually, testosterone levels are within the normal range (10 - 40 nmol/L) or elevated, while elevated luteinizing hormone (LH) levels (> 10 IU/L) are also found indicating androgen resistance at the hypothalamic-pituitary level. The high testosterone levels are also substrate for aromatase activity, resulting in substantial amounts of estrogens, which are responsible for further feminisation in CAIS individuals.

Clinical features of the Partial Androgen Insensitivity Syndrome (PAIS)

In the partial androgen insensitivity syndrome (PAIS) several phenotypes ranging from individuals with predominantly a female appearance (e.g. external female genitalia and pubic hair at puberty, or with mild cliteromegaly, and some fusion of the labia) to persons with ambiguous genitalia or individuals with a predominantly male phenotype (also called Reifenstein syndrome) (120, 121). Patients from this latter group can present with a micropenis, perineal hypospadias, and cryptorchidism. In the group of PAIS individuals, wolffian duct derived structures can be partially to fully developed, depending on the biochemical phenotype of the androgen receptor mutation. At puberty, elevated luteinizing hormone, testosterone, and estradiol levels are observed, but in general, the degree of feminization is less as compared to individuals with CAIS. Individuals with mild symptoms of undervirilization (mild androgen insensitivity syndrome) and infertility have been described as well. Phenotypic variation between individuals in different families has been described for several mutations (121, 122, 123, 124). However, in cases of CAIS no phenotypic variation has been described within one single family, in contrast to families with individuals with PAIS (125).

Genetics of Androgen Insensitivity Syndrome

Since the cloning of the androgen receptor cDNA in 1988 and the subsequent elucidation of the genomic organization of the androgen receptor gene, molecular biology tools have been available for the molecular analysis of the androgen receptor gene in individuals with AIS [Figure 7, see above] (28, 29). In addition to endocrinological data, such as levels of testosterone, luteinizing hormone, androstenedione, and 5α-dihydrotestosterone, which can vary widely in AIS individuals, the most reliable approach is the sequencing of each individual androgen receptor exon and the flanking intron sequences. In general, AIS can be routinely analyzed and separated from entirely different syndromes presenting with similar phenotypes including testicular enzyme deficiencies, 5α-reductase type 2 deficiency, and Leydig cell hypoplasia due to inactivating luteinizing hormone receptor mutations. Furthermore, in pedigree analysis intragenic polymorphisms like the highly polymorphic (CAG)nCAA repeat encoding a poly-glutamine stretch, the polymorphic GGN repeat encoding a poly-glycine stretch, the HindIII polymorphism [Figure 8, see above] (26) and the StuI polymorphism (126), can be used as X-chromosomal markers (38, 127, 128). Extensive general information can be obtained at the internet site: www.genecards.org on the AR (NR3C4) gene and on the 233 identified single nucleotide polymorphisms (SNP's).

Mutations in the Androgen Receptor gene

In the androgen receptor gene, 4 different types of mutations have been detected in 46, XY individuals with AIS: single point mutations resulting in amino acid substitutions or premature stop codons, nucleotide insertions or deletions most often leading to a frame shift and premature termination, complete or partial gene deletions (>10 nucleotides), and intronic mutations in either splice donor or splice acceptor sites which affect the splicing of androgen receptor RNA (89). In general in 70% of the cases, AR gene mutations are transmitted in an X-linked recessive manner, but in 30% the mutations arise de novo. When de novo mutations occur after the zygotic stage, they result in somatic mosaicisms (129). The most recent update on androgen receptor gene mutations is available at http://www.mcgill.ca/androgendb/(89).

Mutations in the NH2-terminal domain

Mutations in the NH2-terminal domain (exon 1 of the gene) do not occur frequently and the vast majority of the mutations result directly in a stop codon or in premature termination due to frameshifts caused by nucleotide insertions or deletions. Mutations in 72 different codons have been reported in the NH2-terminal domain, which is in approx. 13 % of all codons in exon 1 (http://www.mcgill.ca/androgendb/) (89, 130, 131, 132).
An interesting mutation is described in the fourth nucleotide, which results in a decreased translational efficiency of the androgen receptor mRNA in an individual with PAIS (133). Three other missense mutations were reported in combination with mosaicism or with a mutation in another region of the gene. In a family with PAIS associated with severe hypospadias, the length of the androgen receptor NH2-terminal poly-glutamine repeat has been reported to be shortened to only 12 glutamine residues (134). The shortened glutamine stretch as such, is not the cause for the androgen resistance but seems to increase the thermolability of the androgen receptor in combination with a point mutation in exon 5 (Y763C) in the ligand binding domain. This point mutation causes rapid dissociation but no thermolability. These data support a functional interaction of the two separated regions in the androgen receptor and indicates further that the defect becomes critical in only part of the androgen target tissues because of the partial character of the androgen resistance found in this family (134).

Mutations in the DNA-binding domain

In general, mutations in the DNA binding domain (e.g. single nucleotide substitutions) result in a normal hormone-binding protein, which is defective in DNA-binding/dimerization and consequently in transcription activation. In total 50 different mutations have been reported in 34 different codons in the

DNA-binding domain, which is in approx. 30% of all codons in exons 2 and 3 (http://www.mcgill.ca/androgendb/) (89, 135). Twenty-five mutations were observed in the first zinc cluster and twenty-one in the second zinc cluster. Since the 3D structures of the DNA-binding domain of several nuclear receptors have been published, the consequence of mutations in the androgen receptor DNA-binding domain can predicted on basis of the structure of the glucocorticoid receptor DNA-binding domain (70). This is illustrated in two studies in which 3D-modelling of the mutated DNA binding domain of the androgen receptor predicts the functional activity of mutant receptors (136, 137). A mutation (G577R) in the so-called P-box [Figure 10, see above], which is involved in androgen response element recognition, was found in a PAIS individual. This mutation affected differentially transactivation of several natural and synthetic promoters, suggesting that androgen target genes may be differentially affected by this mutation (138). An interesting observation was made with respect to the second zinc cluster in which either one of two adjacent arginine residues (Arg607 & Arg608) were found to be mutated in PAIS individuals who developed breast cancer [Figure 10, see above] (139, 140). It is speculated that a decrease in androgen action within the breast cells could account for the development of male breast cancer by the loss of a protective effect of androgens. However, the same mutations in several other PAIS individuals did not result in breast cancer development.
The mutation Ala596Thr in the second zinc cluster in the so-called D-box resulted in abolishment of dimerization in a PAIS individual [Figure 10, see above] (141). A similar mutation at an identical position in the second zinc cluster of the glucocorticoid receptor DNA-binding domain has been created to discriminate between dimerization/DNA binding of the glucocorticoid receptor and protein-protein interactions with other transcription factors such as the AP-1 transcription complex (142). It appeared that the dimerization mutant did not affect the cross-talk with other transcription factors. In this way, a tissue specific response can be influenced by a single amino acid change and if this is also true for the mutant androgen receptor then the partial phenotype can be explained. Interestingly a Ser579Arg , also located in the D-box can cause significantly different phenotypes ranging from under-virilisation to a normal male phenotype (143).

Mutations in the hinge region

In the so-called hinge region, located between amino acid residues 622 and 670 [Figure 9, see above], only seven mutations have been reported. The relatively low number of mutations in the hinge region (only in 8% of all codons) indicates that this region might be very flexible and that some variation in composition and length of this region is not detrimental for androgen receptor function (http://www.mcgill.ca/androgendb/) (89).
Two amino acid substitutions within the hinge region have been described that resulted in CAIS, three in PAIS and one in MAIS. The I664N substitution on the border of the hinge region and ligand-binding domain, resulted in a decreased hormone binding (144).

Mutations in the ligand-binding domain

It can be expected that mutations in the ligand binding domain might affect different functional aspects (eg. loss of ligand binding, changes in ligand binding affinity and specificity, changes in co-activator receptor interactions, changes in receptor stability and thermolability). A large number of mutations (247 different mutations in 148 codons, which is in 59 % of all codons of the ligand binding domain) in the ligand binding domain have been reported in all 5 exons in individuals with either CAIS, PAIS or MAIS (http://www.mcgill.ca/androgendb/) (89, 145, 146). However, it appears that most mutations are located in exon 4 (31 mutations in helix 3), in exon 5 (39 mutations in helices 4 and 5) and in exon 7 (39 mutations in helices 9 and 10; and β strands). Interestingly mutations are found in 13 of the 18 amino acid residues considered to interact with the ligand directly (76). For some mutations (in total 14, distributed over the whole ligand binding domain) both a complete and a partial phenotype has been described, indicating that phenotype does not always match with genotype. In the AF-2 core region (893-EMMAEIIS-900) of the androgen receptor ligand-binding domain a relatively low number

of mutations have been reported [see Figure 9 for location of AF-2]. Only at positions M895 and Ile898 substitutions have been described in individuals with the complete syndrome (147, 148). It can be speculated that in this part of helix 12 mutations in the androgen receptor ligand-binding domain are less deleterious for androgen receptor function as compared to those in helix 5 and in the β-turn, where almost every amino acid residue has been found to be mutated in AIS individuals. However, functional analysis of a novel AR mutation, Q902K in helix 12, in an individual with partial androgen insensitivity, indicated that this residue is important for modulation of NH2/COOH terminal interaction and TIF-2 activation (149). Interestingly a mutation, F826L, found in a PAIS patient, displayed an unexpected increased N/C interaction and TIF2 coactivation (146). An explanation for the phenotype of the patient could be that the AR mutant recruits a different repertoire of co-activators absent in genital tissues. Alternatively an altered conformation of the ligand binding domain may enhance preferential recruitment of co-repressors.

Deletions and duplications of the Androgen Receptor gene

Only a few cases have been reported on partial or complete androgen receptor gene deletions, indicating the relatively low frequency of this type of androgen receptor defect (http://www.mcgill.ca/androgendb/) (89, 150, 151, 152, 153, 154, 155, 156). All cases reported are found in CAIS individuals, with the exception of two cases, one in which an exon 4 deletion was found in a person with azoospermia (151) and another one in which a large intron 2 deletion of at least 6 kb was reported involving a branch point site, which resulted in a partial exon 3 skipping during the splicing process (155).
Deletion of either exon 3 or exon 4 occur both in-frame and result in a non-functional protein lacking either the second zinc cluster or the hinge region and the NH2-terminal part of the ligand-binding domain [see Figure 7 for genomic organization of AR gene]. In case of an exon 3 deletion, an intact and functional ligand-binding domain is present [Figure 7]. So far, functionally significant mutations in the androgen receptor promoter region or in the 5'- and 3'- untranslated regions of the gene have not been reported.

Splice site mutations affecting Androgen Receptor RNA splicing

A special group of interesting, but rare mutations are the splice donor and splice acceptor site mutations in the androgen receptor gene in AIS individuals (http://www.mcgill.ca/androgendb/) (89). For all splice donor sites in the gene, the consensus splice donor site sequence GTAAG/A is present. The 9 reported mutations in donor splice sites are all substitutions either at position +1 (G --> A or G --> T), position +3 (A --> T), position + 4 (A --> T) or position + 5 (G --> A) and result in defective splicing with the consequence of one or more exons spliced out, or the use of a cryptic splice donor site within the preceding exon (157, 158, 159, 160, 161). In 8 of the reported cases, the phenotype is complete androgen insensitivity. In one case, an insertion of one nucleotide (T) at position + 4 in the splice donor site of intron 6 has been reported, resulting in a partial androgen insensitive phenotype (161). Only 4 mutations have been reported in splice acceptor sites, which all affect the splicing of the androgen receptor RNA. Interestingly, a substitution at position -11 (T>G) has been found in the pyrimidine-rich region of the splice acceptor site of intron 2, resulting in the activation of a cryptic splice acceptor site at position -70/-69 and consequently in the insertion of 69 nucleotides (corresponding to 23 additional amino acid residues) in the mRNA between exons 2 and 3 (104). The corresponding protein is defective in DNA-binding because the insertion has occurred between the first and second zinc cluster [Figure 7, see above]. In another CAIS patient a splice junction mutation at the intron2/exon3 splice acceptor site resulted in the utilisation of the same cryptic splice acceptor site and also in the insertion of 69 bp in the mRNA, predicting the insertion of 23 amino acid residues in frame between the two zinc clusters (162).

ANDROGEN METABOLISM DISORDERS

The metabolism of testosterone to 5α-dihydrotestosterone by the enzyme 5α-reductase type 2 (SRD5A2) is essential for the initiation of the differentiation and development of the urogenital sinus into the prostate. The differentiation of the male external genitalia (penis, scrotum and urethra) also strongly depends on the conversion of testosterone to 5α-dihydrotestosterone in the urogenital tubercle, labioscrotal swellings and urogenital folds, respectively [Figure 2B, see above] (2, 3).

Clinical features of the syndrome of 5α-reductase type 2 deficiency

46, XY individuals with impairment of 5α-reductase type 2 have normally virilized wolffian duct derived structures, with seminal vesicles (although small seminal vesicles have been reported as well), with vasa deferentia, epididymides and ejaculatory ducts and no müllerian duct derived structures (2, 163, 164). However, differentiation of the urogenital sinus and genital tubercle is not observed, resulting in absence of the prostate and in ambiguous or in female external genitalia at birth (2, 163, 164). Affected 46, XY individuals are therefore often raised as girls. At puberty all affected individuals show some or a severe degree of virilization often resulting in deepening of the voice, an increased muscle mass, growth of the penis, scrotal development, testicular descent and sometimes leading to a gender change (2, 165).
Gynecomastia in adulthood does not occur. The additional virilization may result from the action of testosterone, because testosterone is available at high levels during puberty. In addition, some testosterone may be converted to 5α-dihydrotestosterone by some residual 5α-reductase activity and by the action of 5α-reductase type 1, which is expressed in non-genital skin, pubic skin, liver and certain brain regions. In the affected 46, XY individuals a typical female pubic hair pattern develops, while the facial and body hair amount is absent or reduced (3). This last observation points to a role for 5α-reductase type 2 in the normal development of this type of body hair. Male pattern baldness has never been observed. 5α-reductase type 2 deficient patients are usually infertile due to the absence or underdevelopment of the prostate and seminal vesicles, in addition to oligospermia or azoospermia due to maldescent of the testes. However, fertile patients have also been reported (2, 163). 46, XX female carriers have normal fertility, decreased body hair and delayed menarche, normal sebum production but no history of acne (2, 163). This suggests a role of 5α-reductase type 2 enzyme in females in the physiology and pathophysiology of body hair growth, menarche and follicular development (163).

Molecular basis for the syndrome of 5α-reductase type 2 deficiency

A reflection of defective or absence 5α-reductase type 2 enzyme activity can be obtained in patients serum and urine samples by measuring testosterone levels (elevated), 5α-dihydrotestosterone levels (decreased) and by measuring the ratio of testosterone/5α-dihydrotestosterone (increased after hCG stimulation) (2). Furthermore in cultured genital skin fibroblasts (if available) the conversion of testosterone to 5α-dihydrotestosterone can be assessed and is an option for establishing a defective enzyme. In broken cell preparations at pH 5.5 the type 2 isozyme activity is measured more specifically and can be compared with a preparation from a normal person (2).
Genetic analysis of 5α-reductase type 2 deficiency has become possible since the cloning of the cDNA (13). The gene is located on chromosome 2 at locus 2p23. The enzyme is encoded by 5 exons and the most reliable approach to detect gene mutations is the sequencing of each individual exon and the flanking intron sequences [Figure 11].

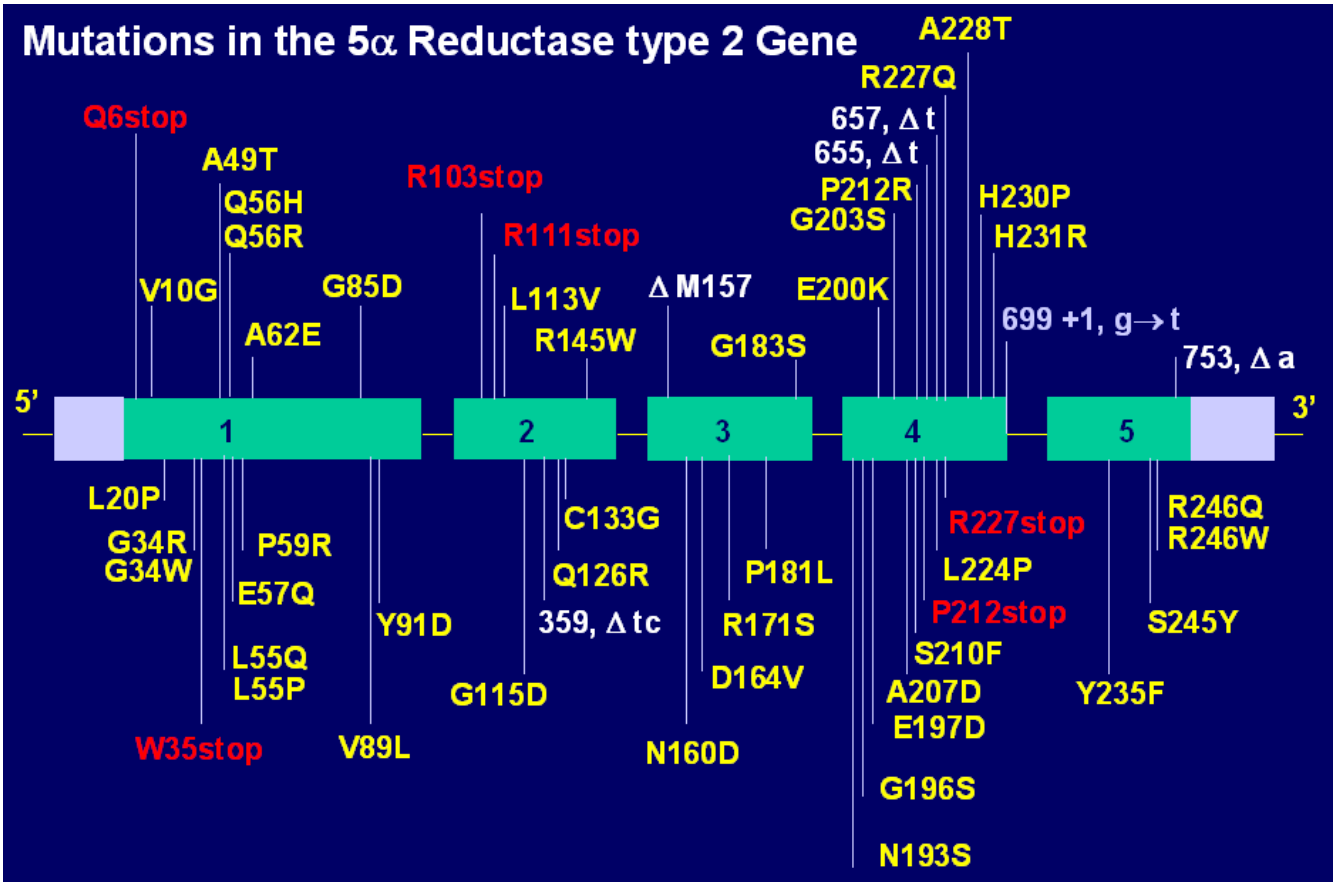

Figure 11. Mutations in the human 5α-reductase type 2 gene (SDR5A2) reported in patients with the syndrome of 5α-reductase deficiency. The 5α-reductase type 2 enzyme is encoded by 5 different exons and mutations have been reported in all 5 exons, as well as a complete gene deletion, small deletions of nucleotides and splice site mutations.

Interestingly worldwide 54 different mutations have been detected in the 5α-reductase type 2 gene in patients with the syndrome of 5α-reductase type 2 deficiency in several different ethnic groups [Figure 11] (2, 3, 163-184). Identical mutations have been reported in different ethnic groups and some of them can be considered to be due to a founder effect and some to have occurred de novo (2, 163). The mutations were found in all five exons of the gene and comprise of 42 amino acid substitutions (77.4%), one complete gene deletion (12), five small deletions resulting in either a premature stop codon or in an in-frame amino acid residue deletion, 6 nonsense mutations and one splice donor site mutation in intron 4, resulting in aberrant splicing [Figure 11]. The majority of the reported patients are homozygous for one of the mutations. A smaller number of patients appeared to be compound heterozygous, while also a small group of patients are heterozygous (2, 163).

In general male carriers of a single mutant allele have a normal fertility as is the case for female carriers. The largest investigated kindreds were found in the Dominican Republic, in Turkey and in New Guinea (2, 163). In all three kindreds the high incidence can be directly related to a founder affect in geographical isolated populations with a high degree of inbreeding. A relatively large number loss of function mutations in the Type II steroid 5α-reductase has been identified in XY disorders of sex development (XY, DSD). This rare autosomal recessive disorder is characterized by impaired enzymatic activity. Also de novo mutations in prostate cancer were reported, resulting in increased 5α-reductase activity (185). This finding indicates a role for increased 5α-dihydrotestosterone levels in the prostate, during prostate cancer progression in a subset of patients. The V89L mutant significantly reduced SRD5A2 enzymatic activity by almost 30% (186). The rare allele frequency of the V89L

variant is 22%, 23,5%, and 46,1% for African Americans, Caucasians, and Asians, respectively, paralleling a substantial racial/ethnic variation in prostate cancer risk, indicating that this polymorphism might be implicated in prostate cancer carcinogenesis (187, 188).

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