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Molecular defects of the androgen receptor ☆

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Abstract

Defects of the androgen receptor cause a wide spectrum of abnormalities of phenotypic male development, ranging from individuals with mild defects of virilization to those with complete female phenotypes. In parallel with this phenotypic spectrum, a large number of different mutations have been identified that alter the synthesis or functional activity of the receptor protein. In many instances, the genetic mutations identified lead to an absence of the intact, full-length receptor protein. Such defects (splicing defects, termination codons, partial or complete gene deletions) invariably result in the phenotype of complete androgen insensitivity (complete testicular feminization). By contrast, single amino acid substitutions in the androgen receptor protein can result in the entire phenotypic spectrum of androgen resistant phenotypes and provide far more information on the functional organization of the receptor protein. Amino acid substitutions in different segments of the AR open-reading frame disturb AR function by distinct mechanisms. Substitutions in the DNA binding domain of the receptor appear to comprise a relatively homogeneous group. These substitutions impair the capacity of the receptor to bind to specific DNA sequence elements and to modulate the function of responsive genes. Amino acid substitutions in the hormone-binding domain of the receptor have a more varied effect on receptor function. In some instances, the resulting defect is obvious and causes an inability of the receptor to bind hormone. In other instances, the effect is subtler, and may result in the production of a receptor protein that displays qualitative abnormalities of hormone binding or from which hormone dissociates more rapidly. Often it is not possible to correlate the type of binding defect with the phenotype that is observed. Instead, it is necessary to measure the capacity of the receptor that is synthesized in functional assays in order to discern any type of correlation with phenotype. Finally, two types of androgen receptor mutation do not fit such a categorization. The first of these—the glutamine repeat expansion that is observed in spinal and bulbar muscular atrophy—leads to a reduction of receptor function that can be measured in heterologous cells or in fibroblasts established from such patients. The expression of ARs containing such expanded repeats in men is associated with a degeneration of motor neurons in the spinal cords of affected patients. Likewise, the alterations of androgen receptor structure that have been detected in advanced forms of prostate cancer also behave as gain-of-function mutations. In this latter type of mutation, the exquisite specificity of the normal androgen receptor is relaxed and the mutant receptors can be activated by a variety of steroidal and non-steroidal ligands. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

The androgen receptor mediates a wide range of biological processes. These include events occurring early in embryogenesis, such as the virilization of the Wolffian duct structures and external genitalia, as well as homeostatic processes in the adult, such as the regulation of spermatogenesis [1,2]. Two distinctive androgens mediate this range of processes: testosterone, and its 5 alpha reduced metabolite, 5α-dihydrotestosterone.

Despite the diversity of processes controlled by androgens, it appears that their effects are mediated by a single intranuclear receptor, the androgen receptor (AR), that is encoded on the X-chromosome. Among the members of the steroid receptor gene family, the androgen receptor is unusual in the large number of different mutations that have been defined which result in clinical syndromes of androgen resistance. The relatively high frequency of such lesions has been attribu-

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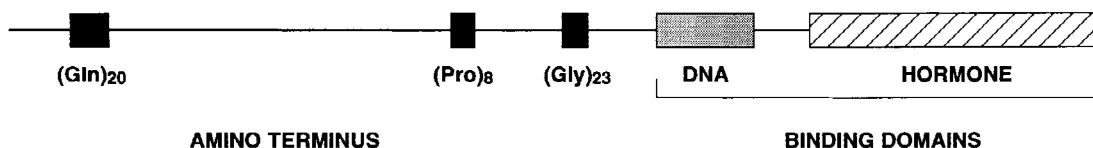

Fig. 1. Schematic structure of the human androgen receptor. The androgen receptor is composed of distinct domains that mediate the binding of the receptor to target DNA sequences (DBD) and the binding of androgens with high affinity (HBD). In addition, the amino terminus is required for the full activity of the androgen receptor in modulating activity of responsive genes. The lengths of the glutamine, glycine, and proline repeats within the amino terminus that are shown in this diagram correspond to those predicted by the cDNA isolated by Tilley et al [8].

ted to two important biological features unique to the androgen receptor. First, as noted, the androgen receptor gene is located on the X-chromosome. As such, males—in which androgens exert their most profound effects—have only a single copy of this gene. For this reason, lesions within the single androgen receptor gene of affected 46, XY males will be discernible owing to the dysfunction of the single mutant allele. Secondly, it appears that androgens are not required for life. Although patients with defects of androgen receptor function may display abnormalities of virilization, these individuals do not appear to exhibit altered viability.

2. Clinical Spectrum

Defects of the androgen receptor result in a wide range of clinical phenotypes [3,4]. In the complete absence of androgen receptor function, both the internal and external male structures fail to develop. This clinical picture has been referred to using a variety of terms, including complete androgen insensitivity or complete testicular feminization. Such patients are externally normally developed females with developed breasts and normal external female genitalia. In some instances, decreased axillary and pubic hair may provide a diagnostic clue to the underlying disorder. Careful examination of such individuals will detect testes within the abdominal cavity or within the labia majora. As the testes produce quantities of Muellerian inhibiting substance adequate to effect the involution of the Muellerian-derived duct structures, the uterus and fallopian tubes are absent and the vagina is blind-ending.

Less severely affected individuals can display a range of intermediate phenotypes. Such syndromes have been referred to by a number of terms including partial androgen insensitivity, incomplete testicular feminization, and Reifenstein syndrome. Affected individuals exhibit varying degrees of virilization and may display either a predominantly female phenotype (incomplete androgen insensitivity, incomplete testicular feminization) or may display a predominantly male phenotype with severe urogenital abnormalities (Reifenstein phenotype). In recent years, some authors have suggested a more detailed system by which to categorize patients with incomplete forms of androgen resistance [4].

3. Characterization by the use of binding assays

In parallel with this clinical phenotype, a wide range of defects of the androgen receptor have been defined using ligand binding assays performed in monolayer cultures of fibroblasts established from individual genital skin biopsy specimens. Using such assays, a variety of patterns have been described. In some samples, specific androgen binding is undetectable. In others, qualitative abnormalities of ligand binding are detected or reduced amounts of qualitatively normal receptor are present [3]. Even when the most sensitive assays of ligand binding are performed, a significant proportion of patients are found to exhibit no defect of androgen binding, though endocrine testing and the family history support the existence of a genetic defect of the androgen receptor.

While the information derived from such binding studies has proven to be extraordinarily valuable, the studies provided little information as to the molecular defects causing such androgen resistant phenotypes. The cloning of the androgen receptor provided the tools necessary to analyze such patients at the molecular level [5–8]. Progress in this area has been rapid, and a large number of different mutations have now been described within the androgen receptor that result in the defects of androgen receptor function [3,4]. [1]

4. Structural features of the human AR

The androgen receptor (Fig. 1) shares many features of the steroid hormone-thyroid hormone-retinoic acid receptor family [9,10]. In common with other members of this family, the androgen receptor shares a modular structure, with specific segments that participate principally in the binding to specific DNA sequences and

[1] A listing of androgen receptor mutations can be found at the Androgen Receptor Mutation Database maintained at McGill University at http://www.mcgill.ca/androgendb

those responsible for the high affinity binding of androgens. In addition to these features, the androgen receptor open reading frame also encodes a large amino terminal segment that is required for maximal activation of responsive genes. The androgen receptor is unusual in that it contains three motifs composed of direct repeats of three amino acid residues. One of these is composed of a direct repeat of 20–25 glutamine residues, the second contains 8 proline residues, and the third encodes approximately 23 glycine residues. While such repeats are not unique to the androgen receptor and have been observed in other members of the steroid receptor and other transcription factor families, it is notable that in the case of the androgen receptor one of these elements—the CAG triplet repeat encoding the glutamine homopolymeric segment–has been implicated in the pathogenesis of a human disease. The expansion of this glutamine repeat beyond that observed in the normal human population has been implicated in the pathogenesis of spinal and bulbar muscular atrophy (Kennedy disease, Ref. [11]). Contraction of the same element has been reported as a risk factor of the development of prostate carcinoma [12–14].

Table 1
Changes in the hormone and pattern of addition can have profound effects on the levels of AR activity in functional assays[a]

Hormone	Additions	Normal AR	Mutant 851 (P766 S)[b]
DHT	1	115 (36)	4 (1)
DHT	4	316 (100)	114 (36)
Mb	1	202 (64)	160 (51)
Mb	4	319 (101)	245 (78)

[a] CV1 cells were transfected with cDNAs encoding either normal androgen receptor or a mutant receptor containing an acid substitution (proline to serine) at residue 764. The activity of the MMTV luciferase reporter gene was measured following stimulation with the indicated hormones for 48 h. During this period of time, the additions of medium containing DHT or mibolerone were added either one time or four times (every 12 h). At the end of the incubation time, all the samples were assayed in parallel and the activity of the reporter gene (expressed as fold induction) were normalized to the activity of the androgen receptor measured in cells stimulated with four separate additions of DHT. The data presented are expressed as either fold induction or as the percentage of the activity measured for the normal androgen receptor stimulated with four doses of DHT (in parentheses). Fold induction is defined as the activity following stimulation with hormone divided by the activity of parallel samples in which no hormone was added (adapted from Ref. [21]).

[b] As noted in the text and in the Fig. 1 legend, the amino acid coordinates derived from each of the different AR cDNAs reported differ, owing to the length of the CAG (glutamine) repeat in the amino terminus. As the database maintained at McGill University employs the coordinates of Lubahn et al [6], the same numbering system has been employed for the amino acid positions referred to in this discussion.

5. Methodologies employed to characterize mutations in the AR

The methodologies that have been used to analyze the structure and function of the AR or consequences of mutations in the androgen receptor are straightforward. Analysis of the gene structure is performed using the polymerase chain reaction to amplify small segments of the androgen receptor gene [15,16]. Sequence analysis of the resulting fragments are compared in the sequence of the normal androgen receptor. Deviations from this normal sequence are inserted into the androgen receptor cDNA by site directed mutagenesis. The functional consequences that result from alterations of the AR coding sequence are assessed by introduction of a mutant AR cDNA into heterologous cells to examine the effect of such alterations on assays of ligand binding, expression of the immunoreactive receptor, and the capacity to activate model androgen responsive reporter genes.

The effect of a mutation on AR function is the principal determinant of how severely androgen action will be altered (see below). For this reason, it has been necessary to develop methods to accurately assess the functional activity of normal and mutant androgen receptors in a way that is not dependent upon the capacity to bind ligand. A number of different reporter genes have been employed in such measurements, including the MMTV promoter [17,18], the PSA promoter [19], and the probasin promoter [20], as well as artificial constructions based on the thymidine kinase promoter (e.g. the PRE_2-TK promoter construct). Experiments employing these plasmids, cDNAs encoding and the normal or mutant receptor are introduced into recipient cells, incubations are performed with or without the ligand being tested, and the activities of the reporter gene (most commonly chloramphenicol acetyl transferase or luciferase) are measured. By comparing the activity of mutant receptors in such assays to those in which aliquots of the normal androgen receptor cDNA are introduced, it is possible to assess the functional activities of individual mutant receptors.

In experiments designed to measure receptor function, it is possible to employ a variety of different androgens to stimulate the AR. In most instances, physiological ligands such as testosterone or 5α-dihydrotestosterone are employed. It should be noted that enzymes which metabolize the 17-hydroxyl groups of these physiological androgens are abundant in most cell types, except those cell types derived from steroidogenic tissue lineages. For this reason, considerable differences may be observed in assays performed following stimulation with such physiological ligands—in which the half life of the ligand employed is relatively brief—compared to experiments in which non-metabo-

lizable ligands, such as mibolerone (7,17-dimethyl-19-nortestosterone) are employed. In some instances, such considerations are crucial to the interpretation of the results that are obtained [21]. An example of the effect of stimulation with different ligands on the activities of normal and mutant ARs in functional assays is shown in Table 1.

6. Termination codons, frame shifts, and alterations of mRNA splicing

Each of these mechanisms results in the production of an androgen receptor gene product that differs from that of the native protein in its primary amino acid sequence. In the case of premature termination codons, single nucleotide substitutions alter an individual codon to one that specifies the termination of translation. While mechanistically distinct, deletions or insertions of segments of the androgen receptor open reading frame result in similar alterations of androgen receptor structure. While somewhat less common, alterations of mRNA splicing that disrupt the integrity of the AR open reading frame have similar effects. In each instance, the mutation causes the synthesis of an AR protein that is structurally distinct from that of the native protein. In addition, such alterations may also result in the decreased synthesis of AR mRNA or protein [22,23].

As the synthesis of the androgen receptor protein proceeds from the amino terminal transactivation domain toward the carboxy terminal hormone binding domain, the resulting molecules are predicted to progressively lack the hormone binding and DNA binding domains, essential elements of receptor function. As such, each of the mutant receptors produced as the result of such mutations is defective in its ability to bind ligand, as well as its capacity to respond to androgen agonists. It should be noted that the distribution of such mutations appears to be relatively random, as insertions, deletions, and single nucleotide substitutions that result in truncations of the receptor protein have been identified in each exon of the receptor.

7. Alterations that impair receptor function, without affecting ligand binding

The analysis of the androgen receptor gene of affected individuals from pedigrees of patients who are predicted to have defects of androgen receptor function (on the basis of family history and endocrine testing) has been quite revealing. Such receptor defects are traced almost uniformly to mutations localized to the DNA binding domain of the receptor. In most instances, such defects are single amino acid substitutions within the receptor protein. Less frequently, however, small deletions or insertions have been identified within this segment that preserve the open reading frame of the remainder of the receptor.

The mutations within this category appear to comprise a relatively homogeneous functional class. Insertion of the mutations identified in such pedigrees into androgen receptor cDNAs and the analysis of these cDNAs in functional assays performed in eukaryotic cells reveals no substantial alterations of ligand binding, consistent with observations made using cultures of patients' fibroblasts. Despite the absence of effects on ligand binding, these receptors are found to exhibit a range of defects in functional assays using androgen-responsive reporter genes. When such mutant ARs are studied in vitro, the disturbances of function that are evident reflect the degree of impairment that the mutant receptors exhibit in assays capable of measuring the capacity of the receptor to bind to target DNA sequences [24–26].

8. Amino acid substitutions that result in absent ligand binding in GSF cultures

When examined in detail in heterologous cells, the amino acid substitution mutations that cause absent binding in genital skin fibroblasts fall into two general classes. The first is relatively infrequent and results in the synthesis of a receptor protein that is completely unable to bind ligand. It is presumed that such mutations occur at critical sites within the receptor protein and alter the conformation of the ligand-binding pocket in such a manner that its interaction with ligand is completely prevented. It is interesting to note that one such mutation is a substitution of a charged residue for a hydrophobic residue that is predicted to be a part of the ligand binding pocket [21].

When analyzed in heterologous cells, however, many such amino acid substitution mutations are found to be capable of binding androgen. The binding measured is often found to be qualitatively abnormal and often displays substantial instability. This apparent discordance between the results of binding studies performed in genital skin fibroblasts and in heterologous cells likely is simply a reflection of the levels of androgen receptor expressed and the sensitivity of the assays employed.

9. Mutations causing qualitative abnormalities of ligand binding

The comparison of results describing qualitative abnormalities from different research groups is diffi-

cult, as the methods that are employed differ between laboratories. In general, however, such tests center on measurements of the affinity of the receptor for its ligand, the stability of the AR protein expressed (e.g., to thermal denaturation), and the stability of the hormone-receptor complexes that are formed.

Analyses in a number of laboratories have identified a host of different amino acid substitutions that result in mutant ARs that exhibit qualitative abnormalities of androgen receptor function. Virtually all of these mutations are localized to the hormone binding domain and their distribution within the HBD is similar compared to the distribution of the mutations that result in the lack of detectable ligand binding [27]. This finding suggests that the degree of disruption of the structure of the ligand binding is related to the type of binding abnormality that is identified. Those substitutions that more radically disrupt the hormone-binding domain structure lead to absent ligand binding, while less severe alterations lead to qualitative defects of the AR. Such conclusions have been reinforced most dramatically by studies in which distinct mutations of the androgen receptor have been localized to the same amino acid residue of the AR open-reading frame. In one instance, Prior et al found that replacement of an arginine residue by cysteine residue (R774C) led to absent ligand binding in genital skin fibroblast cultures [28]. Replacement of this same arginine residue by a histidine, by contrast, led to normal levels of androgen binding that displayed marked thermal instability. Other investigations have identified other mutant receptors in which substitution of different amino acid residues at a single position in the AR open-reading frame led to different effects on ligand binding and on receptor function [29–31]. In those instances in which substitution with different amino acids has resulted in discernibly different phenotypes, the level of AR function measured for the mutant receptors has paralleled the clinical phenotype.

An additional interesting aspect of the studies of mutant ARs harboring amino acid substitutions in the HBD are those described by Marcelli et al [21]. This study examined the functional responsiveness of a number of the androgen receptor mutants that exhibited different types of qualitative abnormalities. When assayed in cells capable of metabolizing the androgens testosterone and 5α-dihydrotestosterone, it was observed that the presentation and type of androgen used in functional assay experiments had a dramatic effect on the levels of androgen receptor function that were observed. These findings were relatively consistent for all the mutant receptors that were capable of binding androgen. In each case, testosterone was the least potent, while dihydrotestosterone and mibolerone exhibited higher potencies. Furthermore, repetitive pulsing with metabolizable androgens, such as testosterone and 5α-dihydrotestosterone, augmented the activity of mutant ARs that displayed an instability of the hormone-receptor complex (Table 1). These experiments permitted three important conclusions. First, these results suggested that mutant receptors that were able to bind hormone—however weakly—could be manipulated pharmacologically to exhibit near normal levels of androgen receptor function. This possibility has been tested in only a limited number of circumstances [32–34]. Second, these experiments demonstrated the importance of the stability of the hormone-androgen receptor complex. Conditions that favored the formation and stability of these complexes could be shown to have major effects on the function of the mutant receptors in functional assays. Finally, these experiments demonstrate that extreme caution must be used in attempting to correlate the results of functional assays performed using transfected cells with the phenotype observed in vivo, as minor alterations in protocol can lead to major differences in the levels of receptor function that are measured.

10. Development of assays to corroborate the results of cell transfection assays and to diagnose androgen resistance

The identification of mutations that cause complete androgen resistance and the demonstration of the effects that such mutations have on receptor function do not usually pose any significant methodological problems, as such mutant ARs display severely compromised function. Thus, distinguishing the activity of an AR devoid of function from that of a normally active AR is not difficult. By contrast, quantitating the differences between receptors that exhibit only partial defects of function can be considerably more difficult. This is made even clearer when considering the studies referred to above in which subtle differences in the way that androgen receptor function is measured in transfection assays can have a profound effect on the results of such measurements [21].

In parallel with these difficulties, it is clear that establishing that a clinical abnormality of virilization is caused by a defect of androgen action can also be quite challenging. Thus, even when information from endocrine evaluations and the family history are available, the identification of patients in which defects of virilization are caused by abnormalities of the AR can be quite difficult (particularly when compared to the larger group of patients in whom such defects cannot be traced to an AR mutation). These concerns are further heightened in those instances in which the endocrine data or family history are not available or are not clear-cut. For this reason, developing methods by which subtle defects of androgen receptor function

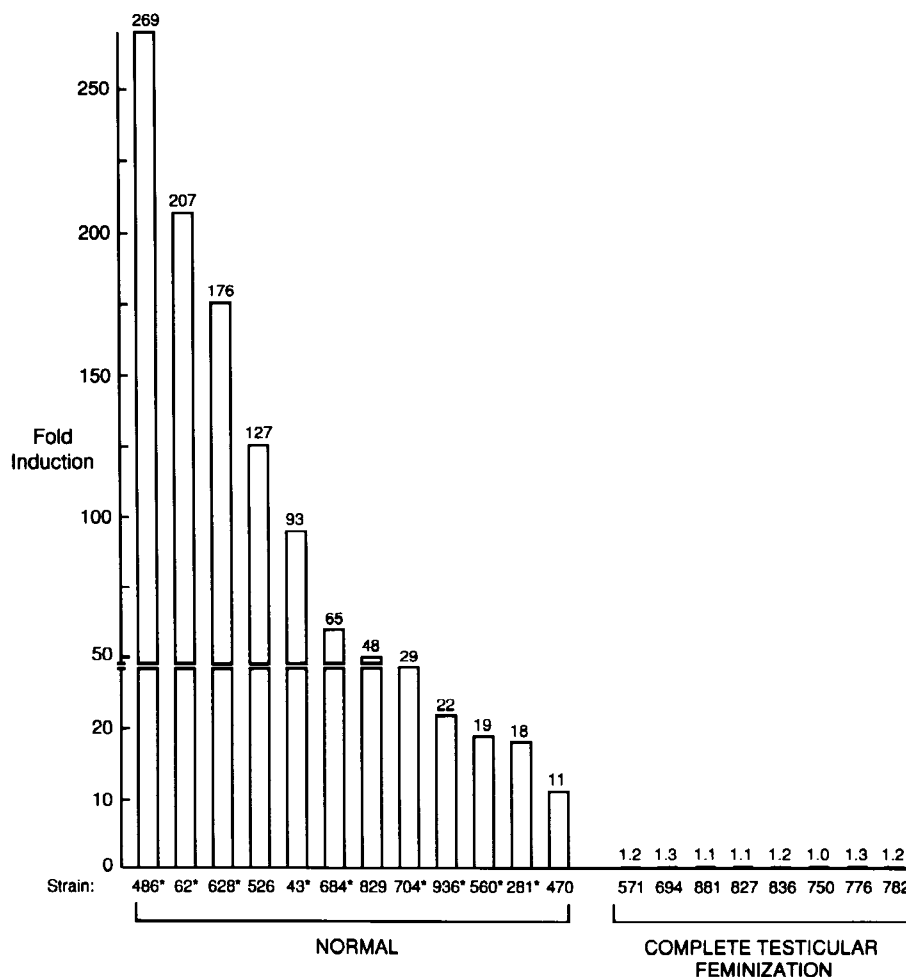

Fig. 2. *An androgen-responsive reporter gene can distinguish the level of AR activity assayed in genital skin fibroblasts from patients with complete forms of androgen resistance caused by mutations in the AR gene*
Genital skin fibroblasts strains established from normal subjects and from patients with complete testicular feminization (complete androgen insensitivity) were infected with an adenovirus carrying the MMTV-luciferase reporter gene. Twenty-four hours after infection, the medium containing either no hormone or containing saturating doses of mibolerone was added and the incubations continued for an additional 72 h. At the end of the incubations, the cells were lysed and assayed to measure the level of luciferase reporter gene activity. Fold induction is defined as the level of reporter gene activity following incubation with mibolerone divided by the basal activity (i.e., with no hormonal stimulation). A ratio of 1.0 denotes an absence of stimulation in response to androgen. Reprinted with permission from [35].

could be quantitated in a fashion that minimizes potential artifacts derived from the cotransfection assay itself would also be valuable.

To address these problems, we established techniques to measure the levels of receptor function of genital skin fibroblasts established from individual patients. While this technique employs the same model androgen responsive gene that we have used in our transfection assays (MMTV luciferase), these experiments circumvent some of the potential artifacts by directly measuring the level of receptor function in the fibroblast cultures [35]. When this technique was used to quantitate the level of AR function in genital skin fibroblasts established from biopsies of normal subjects and patients with complete testicular feminization, it was evident that this method was capable of distinguishing samples expressing normal and inactive ARs (Fig. 2).

In subsequent studies, it appears that this test is capable of distinguishing more subtle defects of AR function. This has been explored by measuring the level of receptor function in genital skin fibroblasts established from a patient with a range of defects of the AR, including patients with the Reifenstein phenotype and spinal and bulbar muscular atrophy [36]. These findings suggest that defects of androgen receptor function can be measured in genital skin fibroblasts established from individual patients. Furthermore, the alterations of AR function that are measured in such patients agree well with the results of transfection assays that have been performed in heterologous cells.

Finally, when this method is applied to the measure-

ment of androgen receptor function in genital skin fibroblasts, infrequent patients are identified that have normal androgen receptor gene structure, normal androgen receptor expression, yet exhibit defects of androgen receptor function in fibroblasts cultures [36]. The genesis of such defects has not been determined, but it is possible that such individuals might harbor lesions that impair the normal function of the androgen receptor in target cells (e.g., in the level or function of coactivators required for normal AR function).

11. Lessons from the study of AR mutations

Considerable information has been gleaned from the analyses of the different types of AR mutation. The behaviors of most, if not all, of the mutations encountered in patients presenting with disorders of virilization are consistent with a 'loss of function' mechanism. In this context, it appears that there is general agreement between the phenotype that is observed and measurements of AR function in transfection assays or in assays performed in genital skin fibroblast cultures.

In recent years, an increasing number of investigations have been conducted that have examined the role of alterations of AR structure in diseases other than those that are associated with classic forms of androgen resistance. In spinal and bulbar muscular atrophy and in prostate cancer, alterations in the length of triplet repeat encoding the glutamine homopolymeric domain within the amino terminus have been implicated as contributing to the pathogenesis or progression of these diseases. While the mechanisms by which these changes in AR structure contribute to the biology of these diseases have not been elucidated, it appears that in both instances the genetic alteration confers novel activity upon the mutant receptor (i.e., 'gain-of-function' mutations). In the case of the CAG triplet expansion characteristic of patients with SBMA, this may be related to toxicity of the AR caused by changes in processing or aggregation of mutant receptors containing the expanded glutamine repeat [37–39]. In the same vein, several of the somatic mutations of the androgen receptor that have been identified in advanced prostatic malignancies display altered ligand responsiveness, suggesting that the appearance of such genetic alterations in the tumor cells may play a role in the progression of this disorder as well [40–42].

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